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Van G. Wilson *Editor*

SUMO Regulation of Cellular Processes

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Preface

Posttranslational modification of proteins has proven to be a critical regulatory mechanism to control protein function, location, and interaction with partners. In 1996 several groups independently identified a new protein modifier moiety that has become known as SUMO (small ubiquitin-like modifier). It was quickly established that SUMO is part of a larger ubiquitin superfamily and that the enzymology of SUMO addition to substrates paralleled the canonical ubiquitin modification pathway. Early studies in the field of sumoylation examined single protein substrates, and the total number of known substrates grew slowly for the first 15 years after the discovery of SUMO. Much of this early work focused on transcription factors as they were among the first identified substrates for sumoylation. A large literature established a critical regulatory role for sumoylation in fine-tuning many transcriptional units that impacted a variety of cellular systems including development and differentiation; cell growth and division; DNA organization, replication, and repair; and defense from pathogens. As would be expected given the involvement of sumoylation in so many fundamental cellular processes, mutations affecting the sumoylation components typically result in lethal or severely dysfunctional phenotypes. Additionally, disruption of SUMO homeostasis was shown to contribute to the development and establishment of some oncogenic malignancies.

In the years since the original edition of this book, there has been an enormous increase in the interest in and understanding of the widespread role of sumoylation in biological processes. The application of proteomics approaches in the mid-2000s rapidly expanded the identification of SUMO substrates along with the mapping of sumoylation sites in these substrates. This enlarged cohort of substrates revealed new pathways and processes modulated by sumoylation and confirmed that sumoylation is a globally significant regulator of cellular biology. It also became clear that the original definition of a SUMO accepter site accounted for only a portion of the mapped sites, leading to the elucidation of several other types of consensus acceptor motifs. Other advances include an increasing appreciation of the role of sumoylation in network coordination (mediated via SUMO-modified proteins binding to proteins with specific SUMO-interacting motifs), of the importance of cross talk between the SUMO and ubiquitin systems, and of the critical contributions of sumoylation to host health and defense against

pathogens. All of these topics are addressed in revised or new chapters of this edition, and the contributors hope that this book will provide both a strong foundation of established information in this field and an introduction to cutting-edge finding and unanswered questions.

Bryan, TX, USA Van G. Wilson

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Introduction to Sumoylation

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Abstract

Reversible post-translational modification is a rapid and efficient system to control the activity of pre-existing proteins. Modifiers range from small chemical moieties, such as phosphate groups, to proteins themselves as the modifier. The patriarch of the protein modifiers is ubiquitin which plays a central role in protein degradation and protein targeting. Over the last 20 years, the ubiquitin family has expanded to include a variety of ubiquitin-related small modifier proteins that are all covalently attached to a lysine residue on target proteins via series of enzymatic reactions. Of these more recently discovered ubiquitin-like proteins, the SUMO family has gained prominence as a major regulatory component that impacts numerous aspects of cell growth, differentiation, and response to stress. Unlike ubiquitinylation which often leads to proteins turn over, sumoylation performs a variety of function such as altering protein stability, modulating protein trafficking, directing protein-protein interactions, and regulating protein activity. This chapter will introduce the basic properties of SUMO proteins and the general tenets of sumoylation.

Keywords

SUMO • Ubc9 • SAE1/2 • SENP • SUMO Ligases

1.1 The Sumo Proteins

Over two decades ago, a small cellular protein of 12 kDa, with 18% homology to the well-known ubiquitin protein, was co-discovered and termed **S**mall **U**biquitin-like **MO**difier or SUMO. SUMO was independently identified by four groups in 1996: Freemont's group found it as a small ubiquitin-like protein associated with PML in an

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interacting complex and called it PIC1 (Boddy et al. 1996), Chen's group identified it in a two-yeast hybrid screen of proteins associated with cellular DNA repair proteins (Shen et al. 1996), Yeh's group identified it as a small modifier associated with Fas which they called sentrin (Okura et al. 1996), and Blobel's group discovered that RanGAP was modified by a small ubiquitin-like protein which they designated GMP1 (Matunis et al. 1996). These modifiers were all the same protein that is now commonly referred to as SUMO.

SUMO is conserved from yeast to mammalian cells though the number of SUMO genes varies greatly (Chen et al. 1998). The budding yeast, *Saccharomyces cerevisiae*, possesses only one SUMO gene, Smt3, whose protein product shares 48% identity and 75% similarity with the mammalian SUMO1 (Huang et al. 2004). Likewise, both *Drosophila melanogaster* (Lehembre et al. 2000) and *Caenorhabditis elegans* (Jones et al. 2002) each have a single SUMO gene. In contrast, plants express 8 SUMOs (Kurepa et al. 2003) and vertebrates have 4 SUMOs. There are four different genes in the human genome coding for the different SUMO modifiers, SUMO1, 2, 3, and 4. SUMO2 and 3 share about 92% identity but they only related to SUMO1 at 48% identity (Kamitani et al. 1998a). While SUMO1, 2, and 3 are expressed in all tissues tested, SUMO4 transcription is restricted primarily to the kidneys, lymph nodes and spleen (Bohren et al. 2004). SUMO4 has been less studied than the others, but seems to play a role in diabetes (see Chap. 18) and stress response (Wei et al. 2008). SUMO1 is a 12 kDa protein of 101 amino acids that is related in structure and in sequence to the 9 kDa ubiquitin protein, as both modifiers share ~18% primary structure identity to each other and have 48% similarity in their three-dimensional structure (Bayer et al. 1998). Ubiquitin is only a 76 amino acid polypeptide, and the difference between those two modifiers mainly resides in the extended N-terminal structure of SUMO as this extension is absent in ubiquitin.

At the tertiary level, the basic structures have been solved for SUMO1 (Bayer et al. 1998), SUMO2 (Huang et al. 2004), and SUMO 3 (Ding et al. 2005). All three SUMOs share a central compact, globular domain with the characteristic ββαββαβ ubiquitin fold. The SUMOs also each have both N- and C-terminal extensions, with the N-terminal extension being much longer than for ubiquitin. Within this extension in SUMO2/3 is a lysine at position 11 that can itself be conjugated with SUMO to yield SUMO2/3 chains (Tatham et al. 2001). SUMO1 lacks a suitable lysine for conjugation and does not appear to form chains *in vivo*, though *in vitro* chain formation has been observed (Yang et al. 2006a). The biological role and function(s) of the N-terminal extension are not well understood, but the C-terminal extension is important for direct contact with the SUMO activating enzyme, SAE1/2 (Lois and Lima 2005).

One of the ongoing questions about the SUMOs is the functional difference between the SUMO1 and SUMO2/3 families. Certain biological variations have already been identified, including different responses to environmental conditions (Saitoh and Hinchey 2000; Manza et al. 2004; Deyrieux et al. 2007), different susceptibilities to various SUMO proteases (Gong and Yeh 2006; Mikolajczyk et al. 2007), and differences in subcellular localization and abundance (Saitoh and Hinchey 2000; Manza et al. 2004; Ayaydin and Dasso 2004). The substrate pool for these two SUMO groups is also different with some substrates capable of being modified by either SUMO1 or SUMO2/3, and other substrates showing a clear preference for one or the other SUMO type (Saitoh and Hinchey 2000; Rosas-Acosta et al. 2005; Vertegaal et al. 2006; Citro and Chiocca 2013). While SUMO preference differences exist for individual substrates, in general for both the SUMO1 and SUMO2/3 modified proteins, the substrates are predominantly nuclear and are often involved in regulation of nucleic acid structure and function. Just how biologically important this demarcation in the substrate preference is remains unclear as SUMO1 knockout mouse studies have suggested that SUMO2/3 can compensate for absent SUMO1 (Evdokimov et al. 2008; Zhang et al. 2008), suggesting considerable redundancy between the SUMO paralogs. However, more

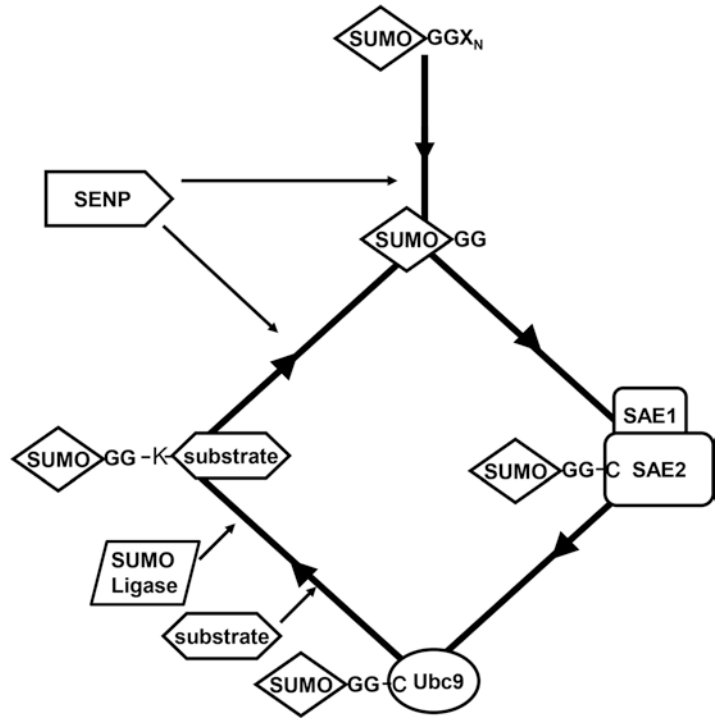

Fig. 1.1 Representation of the enzymatic cascades leading to the covalent attachment of SUMO to a substrate protein. The SUMO enzymes are the SENP isopeptidase, the SAE1/SAE2 activating enzyme, the Ubc9 conjugating enzyme, and the SUMO ligases. Attachment of SUMO to SAE2 and Ubc9 is via a thioester linkage to a cysteine residue in the enzymes. SUMO attached to the substrate is via a lysine residue to form a stable isopeptide bond

recently it was shown that SUMO2 is essential during mouse embryonic development, while SUMO3 was dispensable (Wang et al. 2014), indicating that there are suitable functional differences even between these nearly identical paralogs. Interestingly, it was previously shown that SUMO3 can be phosphorylated at serine 2, while SUMO2 cannot be phosphorylated since it has an alanine at this position (Matic et al. 2008). This observation suggests one basis for functional, regulatory, or substrate preference differences between the highly identical SUMO2 and SUMO3 proteins could be related to differences in their own post-translational modification. Much additional work is needed to clarify the common and distinct roles of the various SUMO proteins.

1.2 The Enzymology of Sumoylation

Sumoylation is the enzymatic activity which results in the covalent attachment of SUMO to a large number of proteins, including cellular and viral proteins. This multi-step enzymatic process (Fig. 1.1) includes a heterodimeric activating enzyme, SAE1/2, a monomeric conjugating enzyme, Ubc9, and multiple ligases and isopeptidases (Wilson 2004). SUMOs are translated as precursor forms which are initially processed by specific isopeptidases (SENPs) to remove C-terminal residues and generate a mature SUMO, terminating with a C-terminal diglycine (Johnson et al. 1997). Interestingly, SUMO4 has a proline residue at position 90 that prevents this processing by the SENPs (Owerbach et al. 2005) and instead it is processed only under stress conditions by a stress-induced hydrolase (Wei et al. 2008). The mature forms of SUMOs then interact with the SUMO E1 E1 activating enzyme, SAE1/2. SAE1 is a 346 amino acid polypeptide while SAE2 is 640 amino acids and contains the catalytic cysteine at residue 173; the SUMOs interact exclusively with the SAE2 subunit. The SAE2 subunit also contains a nuclear localization signal that may contribute to the enrichment of sumoylation components in the nucleus. Together, the SAE1 and 2 proteins form an U-shaped heterodimer complex with a large

groove that has the ATP-binding motif at the base of the groove (Lois and Lima 2005). Binding of SUMOs to SAE2 positions the SUMO diglycine motif for adenylation, then the activated SUMO can be covalently attached to the catalytic cysteine via a thioester linkage.

Subsequent to formation of the SAE1/2-SUMO complex, the activating enzyme transfers SUMO to SUMO E2 conjugating enzyme, Ubc9. Unlike the ubiquitin pathway that contains many E2 enzymes, Ubc9 is the sole conjugating enzyme for SUMO and functions with all 4 SUMOs. Once again, there is a conserved domain motif [αββββββ(ββ)αααα] common to all E2 enzymes known as the ubc superfold (Tong et al. 1997). Within this domain is the catalytic groove that contains the active site cysteine, amino acid 93. Binding of SAE1/2 to Ubc9 allows transfer of the SUMO C-terminus to cysteine 93, again through formation of a thioester linkage, and the structural contexts of the SAE2-Ubc9 interaction are highly conserved across species (Wang et al. 2010). Lastly, Ubc9 transfers SUMO to the substrate protein, where SUMO is covalently linked to a lysine residue through an isopeptide bond between the epsilon amino group of the lysine and the carboxyl group of the C-terminal glycine on SUMO.

The lysine residue utilized for sumoylation commonly falls in the ΨKxE/D motif, where Ψ is a hydrophobic residue (typically Val, Ile, Leu, Met, or Phe), K is the target lysine, x is any amino acid, and the fourth position is an acidic residue (Hay 2005). However, even early studies of sumoylated proteins found that not all were modified at lysines in sequence contexts that match the consensus motif, indicating that alternative sequence features could also specify a particular lysine for SUMO modification (Kamitani et al. 1998b; Rangasamy et al. 2000; Hoege et al. 2002). Subsequently, numerous proteomics approaches have identified hundreds of sumoylated proteins and characterized the SUMO addition sites in many of these substrates, revealing a site selection complexity much greater than the original consensus motif. Zhou et al. used a proteomics approach and found that five of the ten sumoylation sites determined for yeast proteins were in non-canonical sequences (Zhou et al. 2004). Similarly, Chung et al. examined SUMO2 conjugation sites for *in vitro* sumoylated proteins and found that half the identified sumoylation sites (three of six) where in sequences which did not conform to the ΨKxE/D motif (Chung et al. 2004). These and similar studies confirmed that while the ΨKxE/D motif is often associated with SUMO addition, only about half the identified SUMO substrates have the original consensus motif (Matic et al. 2010). In some cases sumoylation appears fairly promiscuous with many lysines in the substrate capable of serving a SUMO acceptors (Eladad et al. 2005; Chymkowitch et al. 2015; Gonzalez-Prieto et al. 2015), especially after mutation of the predominant SUMO target(s). In these cases, the substrate typically has a SUMO-interacting motif (SIM; see below) that recruits the sumoylation machinery (Chang et al. 2011; Meulmeester et al. 2008). However, more commonly these other SUMO acceptor lysines fall within alternative SUMO conjugation motifs, including the inverted (E/DxKΨ) motif (Matic et al. 2010), the hydrophobic (ΨΨΨKxE) motif (Matic et al. 2010), the phosphorylation-dependent (PDSM; ΨKxExxS^{P}P) motif (Hietakangas et al. 2006), the negatively charged amino acid-dependent (ΨKxExxEEEE) motif (Yang et al. 2006b), the phosphorylated (ΨKxS^{P}P) motif (Picard et al. 2012), and the extended phosphorylation (ΨKxS^{P}PS^{P}xxxS^{P}P) motif (Picard et al. 2012). Collectively, this array of motifs helps explain the large number of lysines capable of being sumoylated and may contribute to paralog specific modification differences for individual substrates.

Unlike ubiquitinylation, which absolutely requires an E3 ubiquitin ligase for transfer of ubiquitin to the substrate, sumoylation occurs readily *in vitro* without a ligase requirement (Melchior 2000). Nonetheless, several SUMO ligases have now been identified, including SP-RING type ligases such as the PIAS family (Johnson and Gupta 2001) and MMS21 (Potts and Yu 2005). Members of this family share sequence homology with the RING domain of ubiquitin RING ligases. The SP-RING domain

directly interacts with Ubc9, inducing a conformational change that enhances transfer of SUMO from Ubc9 to the substrate (Rytinki et al. 2009). Additional identified SUMO ligases include RanBP2 (Pichler et al. 2002), Pc2 (Kagey et al. 2003), and TOPORS (Weger et al. 2005), as well as a few other proteins that appear to facilitate sumoylation but whose mechanisms are poorly defined. Given that there are roughly 600 ubiquitin ligase genes in the human genome (Deshaies and Joazeiro 2009), it is quite likely that many more SUMO ligases remain to be identified. Generally, all these SUMO ligases enhance sumoylation both *in vitro* and *in vivo*, and influence substrate selection (Gareau and Lima 2010). For instance, PIAS acts as a SUMO ligase, preferentially targeting the tumor suppressor p53, c-Jun, STAT1, and the nuclear androgen receptor AR (Schmidt and Muller 2002; Ungureanu et al. 2003; Sachdev et al. 2001). RanBP2 stimulates sumoylation of the promyelocytic leukemia protein (PML), the nuclear body SP100 protein, and the histone deacetylase HDAC4 (Pichler et al. 2002), while Pc2 is the unique E3 ligase for the transcriptional factor co-repressor CtBP (Kagey et al. 2003). In addition to enhancing the overall sumoylation reaction and substrate selection, these ligases likely also influence preferential utilization of the SUMO paralogs.

The SENPs, the SUMO isopeptidases, play a dual role; they are involved in the maturation of SUMO and in the de-conjugation of SUMO from its target proteins (Hang and Dasso 2002; Gong et al. 2000). There are 6 SENPs that function with SUMO, 1–3 and 5–7 (there is no SENP 4, and SENP 8 is a Nedd 8 protease). In mammalian cells these enzymes are differentially located, with SENP1 located at the PML bodies, SENP6 in the cytoplasm, SENP3 in the nucleolus, and SENP2 at the nuclear pore complexes (Gong and Yeh 2006). Therefore, it appears that desumoylation of conjugates is possible at different subcellular locations, and access of individual substrates to specific SENPs may provide an additional level of regulation. Additionally, specific functional differences have been observed among the 6 SENPs regarding their maturation and deconjugation activities. While SENP1 and SENP2 can generally process all the SUMOs 1–3 precursors (Nayak and Muller 2014), SENP5 preferentially processes the SUMO3 precursor (Di Bacco et al. 2006). With regard to deconjugation, SENP1 functions primarily with SUMO1 conjugates (Sharma et al. 2013), while the other SENPs strongly prefer SUMO2/3 substrates. Additionally, SENP6 and SENP7 are most adapt at disassembly of SUMO2/3 chains (Lima and Reverter 2008; Drag et al. 2008). Deletion of the SENP genes, like deletion of Ubc9 in yeast, stops cell cycle progression and further highlights that reversible sumoylation is an essential and critical function in the cell life cycle (Li and Hochstrasser 1999). Overall, the diversity and specificity of SENPs undoubtedly helps regulate the dynamic and reversible sumoylation process.

1.3 Sumoylation Functions

Functionally, sumoylation is a more diverse modifier than ubiquitin. Unlike ubiquitinylation, which has a major role of targeting proteins for proteasome degradation, addition of the SUMO moiety does not directly target proteins to the proteasome. Instead, there are examples of substrates where sumoylation blocks proteosomal degradation by competing with ubiquitinylation for a common lysine residue (Desterro et al. 1998; Klenk et al. 2006; Escobar-Ramirez et al. 2015). Since over 25% of the SUMO sites in human proteins are known ubiquitination sites (Hendriks et al. 2014), regulation of degradation through such competition may be more common than anticipated. Intriguingly, lysine residues are also targets for modification by acetylation and methylation, so sumoylation may also be competing with those events to regulate protein activity as has been shown for Iκβα (Desterro et al. 1998), delta-lactoferrin (Escobar-Ramirez et al. 2015), and STAT5 (Van Nguyen et al. 2012).

Further cross-talk between the SUMO and ubiquitin systems is mediated by SUMO-Targeted ubiquitin ligases (STUbls) (Xie et al. 2007; Prudden et al. 2007; Sun et al. 2007; Uzunova et al. 2007). This novel class of ubiquitin ligases functions by specifically interacting

with SUMO moieties on sumoylated proteins, thereby causing ubiquitination and subsequent degradation (Perry et al. 2008). This interaction is depends on SUMO-interacting motifs (SIMs) present on the STUbls. The canonical SIM is a hydrophobic motif with the consensus V/I-x-V/I--V/I (Song et al. 2004; Hecker et al. 2006), and the interaction between the SIM and the SUMO is through a β strand of the SIM and the β2 strand of SUMO (Sekiyama et al. 2008; Namanja et al. 2012). Both of the human STUbls, RNF4, RNF4 and RNF111, contain at least 3 SIM motifs, so they preferentially target proteins with poly-SUMO signals, either multiple SUMO moieties or SUMO chains (Tatham et al. 2008; Erker et al. 2013). Lastly, there is at least one example of a viral protein whose stability is indirectly tied to sumoylation levels (Wu et al. 2009). Through an undefined mechanism, the stability of the human papillomavirus E2 E2 protein is greatly enhanced when overall sumoylation levels increase, suggesting that further examples of cross-talk between the ubiquitin and SUMO pathways await discovery, and that these two systems may have an even richer interplay than currently imagined (see Chap. 6).

In contrast to its modest role in protein stability, it is now clear that SUMO has a major role in transcriptional regulation (see Chaps. 2 and 3), both through direct modification of individual transcription factors and co-factors (Verger et al. 2003; Garcia-Dominguez and Reyes 2009), and through chromatin remodeling (Cubenas-Potts and Matunis 2013). For most transcription factors, sumoylation reduces their transactivation capacity, though enhanced transcriptional activity has also been demonstrated for a few substrates, including heat shock factors (Goodson et al. 2001; Hong et al. 2001), Oct4 (Wei et al. 2007), and Smad4 on some promoters (Long et al. 2004). The negative transcriptional effects can be due to changes in transcription factor stability and/or subcellular localization, particularly the recruitment of sumoylated transcription factors into PML nuclear bodies as has been observed for HIPK2 (Kim et al. 1999), Sp3 (Ross et al. 2002), NACC1 (Tatemichi et al. 2015), and other proteins (Sahin et al. 2014). Alternatively, SUMO modification can have more global effects on transcription by affecting chromatin remodeling. Examples are plentiful of sumoylation facilitating the recruitment and/or modification of various remodeling enzymes including histone deacetylases (HDACs) (Wagner et al. 2015; de la Vega et al. 2012; Citro and Chiocca 2013; Girdwood et al. 2003; Yang et al. 2003), histone demethylases demethylases (Huang et al. 2016; Bueno and Richard 2013), and methyltransferases (Spektor et al. 2011; Lee and Muller 2009; Riising et al. 2008), as well as directly modifying histones (Shiio and Eisenman 2003; Nathan et al. 2006; Zheng et al. 2015; Dhall et al. 2014). Clearly, all of these mechanisms could be reversed by desumoylation with SENPs, leading to dynamic and controllable effects on transcription of individual or groups of genes. Thus, sumoylation effects on transcriptional activity would reflect the overall dynamics of sumoylation/desumoylation that may vary with cell cycle, cell growth conditions, and disease state.

In addition to regulating transcriptional activity, sumoylation also has an important regulatory role for other nuclear functions, including RNA processing (see Chap. 2), genome maintenance and repair (see Chaps. 4 and 5), and nucleocytoplasmic transport (see Chap. 7). More recently, non-nuclear functions of sumoylation have been identified (Wasik and Filipek 2014), and Chaps. 8 and 9 will explore the role of SUMOs in regulating ion channel activity and metabolic pathways, respectively. Because of this pleiotropic ability to modify numerous proteins and affect transcriptional activity or cellular environment on a global scale, sumoylation in now recognized as a regulatory process involved in mitosis (Chap. 10), meiosis (Chap. 11), differentiation and development (Chap. 12), and senescence (Chap. 13). While much of the focus in the sumoylation field is on vertebrates, sumoylation is equally important for plants (Chap. 14) and invertebrates (Chap. 15). Much progress has been made in recent years in understanding the roles of sumoylation in these diverse areas of cell biology, particularly through global proteomics efforts (Tammsalu et al. 2015; Eifler and Vertegaal 2015; Hendriks et al. 2015a; Xiao et al. 2015; Yang and Paschen 2015), but much

work remains, and for most of these processes there still are many more questions than answers.

One recently emerging theme that likely contributes to the ability of sumoylation to control the cellular processes mentioned above is the coordinate modification of functionally related groups of protein in response to specific stimuli (Jentsch and Psakhye 2013; Raman et al. 2013). Overall increases in cellular sumoylation levels have long been seen in response to various kinds of stress (the SUMO stress response, SSR) (Zhou et al. 2004; Manza et al. 2004; Tempe et al. 2008), and more recent studies are now revealing that much of this increased sumoylation is associated with networked proteins (Lewicki et al. 2015; Castro et al. 2012; Hendriks et al. 2015b; Xiao et al. 2015). For example, DNA damage has been shown to elicit the sumoylation of numerous proteins in the homologous recombination system (Psakhye and Jentsch 2012). Many of the proteins in these networks contain SIM motifs, so increased sumoylation would likely contribute to enhanced interactions and stability of these multi-protein complexes. Thus, by subtle interplay of sumoylation and desumoylation these protein complexes and functional pathway could be fine-tuned to produce rapid and appropriate levels of response to changing cellular conditions. Interestingly, at least in vertebrates, it is SUMO2/3 that are mostly involved in SSR, and the intracellular pools of free SUMO2/3 are rapidly lost after exposure to stress-inducing agents as SUMO2 and SUMO3 becomes largely conjugated to their substrates.

Lastly, given the breadth of SUMO modified targets and the critical pathways involved, it is not surprising that dysregulation of the SUMO system can contribute to disease states. Increasing evidence links over or under expression of various sumoylation components to diseases as diverse as neurodegeneration (Chap. 16), cancer (Chap. 17), diabetes (Chap. 18), craniofacial disorders (Chap. 19), and vascular disease (Chap. 20). It is also now apparent that utilization and/or modulation of the host sumoylation system are an important aspect of many infection diseases, both viral (Chap. 21) and bacterial (Chap. 22). This emerging recognition of a role for sumoylation in disease and infection is exciting as it may ultimately offer new insights for diagnosis, therapeutics, and prevention. The next several years should bring exciting new insight into the role of sumoylation, not only in fundamental cellular processes, but also in applications to understanding and managing disease states.

1.4 Conclusion

In the 20 plus years since its discovery, SUMO has gone from an obscure and functionally unknown protein to one that is recognized as a key regulator of multiple nuclear and cytoplasmic events. The principal components of this modification system have been identified, their basic structures elucidated, and the general features of their enzymology understood. Thanks to the combination of individual targeted protein studies and more global proteomics approaches, hundreds of sumoylation targets are now known, providing a rich resource for subsequent functional studies. The sumoylation system has been shown to be an important player in many biological processes, such as cellular differentiation, transcriptional regulation, and cell growth (Deyrieux et al. 2007; Gill 2005; Ihara et al. 2007). Perturbing this biological system changes the cellular response to diverse signaling pathways (Sharrocks 2006) and likely leads to disease. In the chapters that follow, the role of sumoylation in a variety of cellular processes will be explored. The focus will range from effects on molecular targets through cell processes to the organismal level. While many exciting questions remain unanswered, by spanning from molecules to multicellular systems, the full impact and profound significance of the sumoylation system should become apparent. We hope that both newcomers to this field, as well as veterans, will find this comprehensive compilation of state-of-the-art reviews on current sumoylation topics useful and insightful.

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Part I

Molecular Functions

Roles of Sumoylation in mRNA Processing and Metabolism

2

Patricia Richard, Vasupradha Vethantham, and James L. Manley

Abstract

SUMO has gained prominence as a regulator in a number of cellular processes. The roles of sumoylation in RNA metabolism, however, while considerable, remain less well understood. In this chapter we have assembled data from proteomic analyses, localization studies and key functional studies to extend SUMO's role to the area of mRNA processing and metabolism**.** Proteomic analyses have identified multiple putative sumoylation targets in complexes functioning in almost all aspects of mRNA metabolism, including capping, splicing and polyadenylation of mRNA precursors. Possible regulatory roles for SUMO have emerged in pre-mRNA 3′ processing, where SUMO influences the functions of polyadenylation factors and activity of the entire complex. SUMO is also involved in regulating RNA editing and RNA binding by hnRNP proteins, and recent reports have suggested the involvement of the SUMO pathway in mRNA export. Together, these reports suggest that SUMO is involved in regulation of many aspects of mRNA metabolism and hold the promise for exciting future studies.

Keywords

mRNA • Splicing • Capping • 3'-end processing • Transcription • RNA editing

2.1 A Brief Introduction to RNA Processing Events, Interconnections to Transcription and Export

Eukaryotic pre-mRNAs must be processed in order to become fully functional mRNAs. Most mRNA precursors (pre-mRNAs) undergo three

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processing steps; the 5′ end is capped by addition of 7-methylguanosine, introns are removed and exons ligated by splicing and the 3′ end is created by an endonucleolytic cleavage followed by addition of a 100–300 nt long poly(A) tail. It is becoming increasingly clear that these processes are cotranscriptional events rather than posttranscriptional, with the C-terminal domain of the largest subunit of RNA polymerase II (CTD), consisting almost entirely of the heptapeptad repeat (consensus YSPTPS), playing an important role in coupling RNA processing and transcription. The CTD forms a scaffold or platform to recruit processing factors on the pre-mRNA (reviewed in Hirose and Manley 2000; Proudfoot et al. 2002; Maniatis and Reed 2002; Bentley 2005), and phosphorylated CTD plays an integral role as a participant of capping (Shatkin and Manley 2000), splicing (Hirose and Manley 2000; Bentley 2005), chromatin remodeling (Rosonina et al. 2014) as well as 3′ processing (Bentley 2002; Hirose and Manley 1998; Hsin et al. 2014b; Hsin et al. 2011) and expression of upstream antisense RNAs (ua RNAs) (Hsin et al. 2014a; Descostes et al. 2014). RNA processing events in turn are highly interlinked and play important roles in influencing transcriptional elongation and termination (reviewed in Bentley 2005; Rosonina et al. 2006; Pandit et al. 2008). The formation of a transport competent mRNP complex is also closely coordinated and coupled to transcription and all processing events, and quality control mechanisms exist to ensure that only correctly processed mRNAs are exported (reviewed in Rodriguez et al. 2004; Luna et al. 2008).

2.2 RNA Processing Factors as Sumoylation Substrates

The small ubiquitin related modifier (SUMO) has gained prominence as a posttranslational modifier that regulates a large number of biological processes, including transcription, DNA repair, genome stability, chromatin organization, PML body function, nucleocytoplasmic transport, to name a few (Johnson 2004). The pathway by which SUMO is conjugated to substrate proteins and the enzymes of the pathway have already received an excellent introduction in the earlier chapters of this book. SUMO has garnered a great deal of interest primarily due to its ability to variously influence substrate function, through protein stability, subcellular localization and altering interactions with other proteins (reviewed in Johnson 2004; Hay 2005; Geiss-Friedlander and Melchior 2007). While the addition of SUMO to a substrate would by itself change substantially its interaction surfaces, the involvement of SUMO-Interacting Motifs (SIMs) in several proteins that can interact with SUMO or sumoylated proteins noncovalently lends another dimension to SUMO regulated interactions (Minty et al. 2000; Song et al. 2004; Hecker et al. 2006). SUMO substrates have been shown to cluster in macromolecular complexes in proteomic analyses (Wohlschlegel et al. 2004) and the presence of more than one SUMO substrate in a functional complex has often shown to be involved in the assembly of such complexes (reviewed by Matunis et al. 2006).

The number of studies detailing sumoylation of RNA processing factors is by no means comparable to that of say, transcription or DNA repair, but the number keeps growing. Studies describing sumoylation of RNA binding proteins and factors involved in 3′ pre-mRNA processing, transcription termination, RNA editing and mRNA export (Vassileva and Matunis 2004; Desterro et al. 2005; Vethantham et al. 2008, 2007; Xu et al. 2007; Lamoliatte et al. 2014; Richard et al. 2013) have helped to expand the role of this modifier to the field of RNA processing and metabolism (reviewed in Rouviere et al. 2013). Developing large scale mass spectrometry (MS)-based proteomics and affinity purification strategies using for example tagged SUMO peptides, several groups have identified a number of proteins involved in RNA processing events such as capping, splicing, polyadenylation and mRNA export in yeast (Panse et al. 2004; Wohlschlegel et al. 2004; Wykoff and O'Shea 2005; Hannich et al. 2005; Denison et al. 2005), mammals (Zhao et al. 2004; Vertegaal et al. 2004, 2006; Li et al. 2004; Manza et al. 2004; Rosas-Acosta et al. 2005;

Gocke et al. 2005; Guo et al. 2005; Golebiowski et al. 2009; Becker et al. 2013; Lamoliatte et al. 2014; Liu et al. 2015; Bruderer et al. 2011; Hendriks et al. 2014; Matic et al. 2010; Tammsalu et al. 2014; Schimmel et al. 2014; Blomster et al. 2009; Tatham et al. 2011), flies (Nie et al. 2009), worms (Kaminsky et al. 2009) and plants (Miller et al. 2010). In one of these studies, a significant proportion (17%) of the SUMO-modified proteins identified were found to be involved in RNA-related processes (Denison et al. 2005). A non-exhaustive list of RNA processing related proteins identified in yeast and human proteomic analyses is presented in Table 2.1.

Table 2.1 Non-exhaustive list of RNA binding proteins and mRNA processing factors identified in yeast and mammalian proteomic analyses

Mammalian proteins	Yeast proteins	Function	References[a]
–	Abd1	Capping	Panse et al. (2004)
–	Ceg1	Capping	Panse et al. (2004)
–	Cet1	Capping	Panse et al. (2004), Hannich et al. (2005), Denison et al. (2005), and Wohlschlegel et al. (2004)
–	Cft2/Ydh1	Polyadenylation	Panse et al. (2004)
–	Dcp2	Decapping	Panse et al. (2004) and Denison et al. 2005
–	Ecm2	Splicing	Denison et al. (2005)
–	Fir1	Polyadenylation	Hannich et al. (2005)
–	Hrp1 (hnRNP A/B)	Polyadenylation	Hannich et al. (2005)
–	Npl3	mRNA export	Denison et al. (2005)
–	Pab1	Polyadenylation	Panse et al. (2004)
–	Pbp1	Polyadenylation	Hannich et al. (2005)
–	Prp22	Splicing	Wohlschlegel et al. (2004)
–	Rrp6	mRNA turnover	Wohlschlegel et al. (2004)
–	Ref2	Polyadenylation	Panse et al. (2004)
–	Sub2	mRNA export	Hannich et al. (2005), Panse et al. (2004), and Wohlschlegel et al. (2004)
–	Yra1	mRNA export	Wohlschlegel et al. (2004)
–	Ysh1	Polyadenylation	Wykoff and O'Shea (2005), Hannich et al. (2005), and Wohlschlegel et al. (2004)
ADAR/ADAR1		RNA editing	Golebiowski et al. (2009), Matic et al. (2010), Bruderer et al. (2011), Schimmel et al. (2014), and Tammsalu et al. (2014)
ADARB2	–	RNA editing	Vertegaal et al. (2006)
[b]CDC73	–	Polyadenylation	Golebiowski et al. (2009), Lamoliatte et al. (2014), Tammsalu et al. (2014), and Hendriks et al. (2014)
[b]CLP	–	Polyadenylation	Golebiowski et al. (2009)
[b]CPSF2 (CPSF100)	–	Polyadenylation	Golebiowski et al. (2009)
CPSF7 (CFI 59)	–	Polyadenylation	Tammsalu et al. (2014), Liu et al. (2015), and Tammsalu et al. (2014)
DDX3X	–	Splicing	Manza et al. (2004), Blomster et al. (2009), Bruderer et al. (2011), Becker et al. (2013), and Hendriks et al. (2014)
DDX5	–	Splicing/ transcription	Manza et al. (2004), Blomster et al. (2009), Bruderer et al. (2011), Becker et al. (2013), Lamoliatte et al. (2014), Hendriks et al. (2014), and Tammsalu et al. (2014)

(continued)

Table 2.1 (continued)

Mammalian proteins	Yeast proteins	Function	References[a]
DDX17	–	Splicing	Manza et al. (2004), Blomster et al. (2009), Bruderer et al. (2011), Becker et al. (2013), and Hendriks et al. (2014)
DDX42 (SF3b125)	–	Splicing	Rosas-Acosta et al. (2005), Blomster et al. (2009), and Hendriks et al. (2014)
FUS	–	RNA binding	Blomster et al. (2009) and Tammsalu et al. (2014)
hnRNP A1	–	Splicing	Li et al. (2004), Manza et al. (2004), Becker et al. (2013), Lamoliatte et al. (2014), Hendriks et al. (2014), Tammsalu et al. (2014), and Liu et al. (2015)
hnRNP C	–	Splicing/export	Vassileva and Matunis (2004), Li et al. (2004), Manza et al. (2004), Golebiowski et al. (2009), Blomster et al. (2009), Matic et al. (2010), Bruderer et al. (2011), Becker et al. (2013), Lamoliatte et al. (2014), Hendriks et al. (2014), Tammsalu et al. (2014), Liu et al. (2015), and Schimmel et al. (2014)
hnRNP D	–	RNA binding	Guo et al. (2005), Blomster et al. (2009), and Becker et al. (2013)
hnRNP F	–	Splicing/ Transcription elongation	Li et al. (2004), Manza et al. (2004), Guo et al. (2005), Bruderer et al. (2011), Hendriks et al. (2014), and Tammsalu et al. (2014)
hnRNP H1	–	Splicing/RNA binding	Blomster et al. (2009), Lamoliatte et al. (2014), and Hendriks et al. (2014)
hnRNP K	–	Splicing	Li et al. (2004), Guo et al. (2005), Blomster et al. (2009), Bruderer et al. (2011), Hendriks et al. (2014), and Tammsalu et al. (2014)
[b]hnRNP L	–	RNA binding	Guo et al. (2005), Rosas-acosta et al. (2005), Golebiowski et al. (2009), Blomster et al. (2009), Bruderer et al. (2011), Becker et al. (2013), Hendriks et al. (2014), and Tammsalu et al. (2014)
[b]hnRNP M	–	Splicing	Vassileva and Matunis (2004), Vertegaal et al. (2004), Gocke et al. (2005), Golebiowski et al. (2009), Blomster et al. (2009), Matic et al. (2010), Bruderer et al. (2011), Becker et al. (2013), Lamoliatte et al. (2014), Hendriks et al. (2014), Tammsalu et al. (2014), Schimmel et al. (2014), and Liu et al. (2015)
hnRNP U	–	Splicing	Blomster et al. (2009); Bruderer et al. (2011), Lamoliatte et al. (2014), Hendriks et al. (2014), Tammsalu et al. (2014), and Liu et al. (2015)
[b]NONO (p54nrb)	–	Splicing/ transcription/ polyadenylation	Rosas-Acosta et al. (2005), Golebiowski et al. (2009), Blomster et al. (2009), Bruderer et al. (2011), Becker et al. (2013), Lamoliatte et al. (2014), and Hendriks et al. (2014)
Nova2	–	Splicing	Lamoliatte et al. (2014)
NXF1/TAP	–	mRNA export	Bruderer et al. (2011), Lamoliatte et al. (2014), Hendriks et al. (2014), and Tammsalu et al. (2014)
[b]PARP1	–	Polyadenylation	Golebiowski et al. (2009), Blomster et al. (2009), Matic et al. (2010), Golebiowski et al. (2009), Bruderer et al. (2011), Becker et al. (2013), Tammsalu et al. (2014), Lamoliatte et al. (2014), and Hendriks et al. (2014)

(continued)

Table 2.1 (continued)

Mammalian proteins	Yeast proteins	Function	References[a]
PCF11	–	Termination/ polyadenylation	Golebiowski et al. (2009), Tammsalu et al. (2014), Hendriks et al. (2014), and Schimmel et al. (2014)
[b]PSF/SFPQ	–	Splicing/ transcription/ polyadenylation	Manza et al. (2004), Rosas-Acosta et al. (2005), Golebiowski et al. (2009), Blomster et al. (2009), and Bruderer et al. (2011)
[b]PTB/PTBP1(hnRNP I)	–	Splicing/ polyadenylation	Rosas-Acosta et al. (2005), Manza et al. (2004), Golebiowski et al. (2009), Blomster et al. (2009); Bruderer et al. (2011), Becker et al. (2013), and Lamoliatte et al. (2014)
RBBP6	–	Polyadenylation	Hendriks et al. (2014) and Schimmel et al. (2014)
RBM25	–	Splicing	Golebiowski et al. (2009), Matic et al. (2010), Becker et al. (2013), Tammsalu et al. (2014), and Schimmel et al. (2014)
Rrp6 (EXOSC10)	–	mRNA turnover and processing	Golebiowski et al. (2009), Blomster et al. (2009), Becker et al. (2013), Bruderer et al. (2011), Tammsalu et al. (2014), Hendriks et al. (2014), and Schimmel et al. (2014)
[b]Rrp44 (Dis3)	–	mRNA turnover and processing	Golebiowski et al. (2009), Bruderer et al. (2011), Lamoliatte et al. (2014), and Hendriks et al. (2014)
Rrp45 (EXOSC9)	–	mRNA turnover and processing	Golebiowski et al. (2009). Bruderer et al. (2011), Lamoliatte et al. (2014), Tammsalu et al. (2014), and Hendriks et al. (2014)
Rrp46 (EXOSC5)	–	mRNA turnover and processing	Becker et al. (2013)
SAFB1	–	Splicing	Golebiowski et al. (2009), Blomster et al. (2009), Matic et al. (2010), Schimmel et al. (2014), Hendriks et al. (2014), and Liu et al. (2015)
SART1	–	Splicing	Vertegaal et al. (2004), Gocke et al. (2005), Golebiowski et al. (2009), Bruderer et al. (2011), Matic et al. (2010), Becker et al. (2013), Tammsalu et al. (2014), Hendriks et al. (2014), and Schimmel et al. (2014)
SETX (senataxin)	–	Termination	Golebiowski et al. (2009), Bruderer et al. (2011), Hendriks et al. (2014), Schimmel et al. (2014), and Bursomanno et al. (2015)
SF3A1 (SAP114)	–	Splicing	Golebiowski et al. (2009), Hendriks et al. (2014), and Schimmel et al. (2014)
SF3A2 (SAP62)	–	Splicing	Rosas-Acosta et al. (2005) and Hendriks et al. (2014)
SF3B1 (SAP155)	–	Splicing	Manza et al. (2004), Golebiowski et al. (2009), Blomster et al. (2009), Matic et al. (2010), Bruderer et al. (2011), Becker et al. (2013), Hendriks et al. (2014), and Schimmel et al. (2014)
SF3B2 (SAP145)	–	Splicing	Golebiowski et al. (2009), Hendriks et al. (2014), and Schimmel et al. (2014)
SF3B3 (SAP130)	–	Splicing	Blomster et al. (2009) and Becker et al. (2013)
SF3B4 (SAP49)	–	Splicing	Guo et al. (2005) and Rosas-acosta et al. (2005)
SKIV2L2 (hMTR4)	–	mRNA turnover and processing	Bruderer et al. (2011) and Schimmel et al. (2014)
SLU7	–	Splicing	Lamoliatte et al. (2014) and Hendriks et al. (2014)

(continued)

Table 2.1 (continued)

Mammalian proteins	Yeast proteins	Function	References[a]
SYMPK (symplekin)	–	Polyadenylation	Gocke et al. (2005), Golebiowski et al. (2009), Matic et al. (2010), Hendriks et al. (2014), and Schimmel et al. (2014)
[b]TARDBP (TDP-43)	–	Splicing	Golebiowski et al. (2009), Bruderer et al. (2011), Lamoliatte et al. (2014), Tammsalu et al. (2014), and Hendriks et al. (2014)
THOC1	–	Processing/export	Golebiowski et al. (2009), Matic et al. (2010), Lamoliatte et al.(2014), Hendriks et al. (2014), and Schimmel et al. (2014)
THOC2	–	Processing/export	Golebiowski et al. (2009), Becker et al. (2013), and Schimmel et al. (2014)
THOC3	–	Processing/export	Hendriks et al. (2014) and Tammsalu et al. (2014)
U2AF2	–	Splicing	Blomster et al. (2009), Bruderer et al. (2011), Becker et al. (2013), and Hendriks et al. (2014)
U2SURP		Splicing	Lamoliatte et al. (2014) and Schimmel et al. (2014)
U2 snRNPA	–	Splicing	Golebiowski et al. (2009)
U5 snRNP 200 kda helicase (SNRNP200)	–	Splicing	Vertegaal et al. (2004) and Blomster et al. (2009)
U5 snRNP specific protein, 116 kda (EFTUD2)	–	Splicing	Manza et al. (2004), Golebiowski et al. (2009), and Blomster et al. (2009)
WDR33	–	Polyadenylation	Blomster et al. (2009) and Hendriks et al. (2014)
Xrn2	–	Termination	Blomster et al. (2009), Bruderer et al. (2011), Lamoliatte et al.(2014), and Hendriks et al. (2014)
ZCCHC7	–	mRNA turnover and processing	Golebiowski et al. (2009), Lamoliatte et al. (2014), and Hendriks et al. (2014)

[a]Only the proteomic analyses are cited in this column, please refer to the main body of the text for other pertinent references

[b]Show increased sumoylation after heat shock in Golebiowski et al. (2009)

Enzymes of the SUMO pathway have been found to colocalize in nuclear bodies and substructures together with components of the RNA processing machinery. Members of the PIAS (protein inhibitor of STAT) family of SUMO E3 ligases were found to be localized to nuclear speckles (Tan et al. 2002), which are subnuclear structures that are enriched for pre-mRNA splicing factors (Lamond and Spector 2003; Hall et al. 2006). One study has shown that SUMO-1 and the E2 conjugating enzyme ubc9 are localized to Cajal bodies, which are the sites of maturation of small nuclear ribonucleoproteins (snRNPs) required for pre-mRNA processing (Navascues et al. 2008). However, another study showed that ubc9 localized to nuclear speckles in mouse oocytes along with the splicing factor SRSF2 (previously SC35), one of the main components of nuclear speckles, and overexpression of ubc9 led to an increase in size of nuclear speckles (Ihara et al. 2008). Interestingly, a recent study shows that Ubc9 depletion in U2OS cells affects the cytoplasmic distribution of specific intronless mRNAs and leads to the accumulation of SRSF2 into cytoplasmic foci (Zhang et al. 2014). It will be important to determine in the future the sig-

nificance of these altered localizations, and whether this results in any changes in processing of mRNA precursors.

In the remainder of this chapter, we discuss SUMO targets related to mRNA metabolism along with a brief description of the RNA processing events themselves. Even though several of these putative SUMO targets were identified mainly by proteomic analyses, the clustering of these targets in key complexes involved in processes such as capping, splicing and polyadenylation make them worthwhile for discussion. Together these studies reveal that the involvement of SUMO in mRNA processing events and export is wider than previously thought, and support the possibility that SUMO plays a significant role in these processes.

2.3 5′ Capping

5′ capping is the first of the three processing reactions and takes place on nascent RNA polymerase II (RNAPII) transcripts when they are less than 50 nts long. The cap plays important roles in mRNA stability, maturation and translation. 5′ N-7methylguanosine caps are attached in a three steps reaction involving an RNA triphosphatase (RT) in the first step and guanyltransferase (GT) in the second step to add a guanosine nucleoside at the 5′ end. A methyltransferase (MT) functions in the third step for addition of the methyl group to the guanosine at the N7 position (reviewed in Shatkin and Manley 2000). In mammals, the RT and GT activities are encoded by a multifunctional capping enzyme (CE), however, in yeast these are present in two separate polypeptides, Cetl and Ceg1, respectively (reviewed in Shatkin and Manley 2000). 5′ capping is closely coupled to transcription elongation through the CTD, which recruits the CE to the pre-mRNA. GT binds specifically to the form of the CTD that is phosphorylated at Ser5 residues by a cyclin-dependent kinase associated with the general transcription factor TFIIH (McCracken et al. 1997; Schroeder et al. 2000). The transcription elongation factor Spt5 binds to the CE and both p-CTD and Spt5 stimulate CE or Ceg1/Cet1 to carry out the capping reaction (Wen and Shatkin 1999). At least in yeast, phosphorylated CTD also recruits MT (Abd1) to the cap and stimulates its activity. The capping enzymes also reciprocally function to enhance RNAPII transcription by stimulating promoter clearance (Mandal et al. 2004; Schroeder et al. 2004).

In yeast, all three capping enzymes were identified as putative sumoylation substrates (Panse et al. 2004). Cet1 has in fact been identified in multiple SUMO proteomic screens (Panse et al. 2004; Hannich et al. 2005, Wohlschlegel et al. 2004; Denison et al. 2005), strongly suggesting that it may be a specific SUMO target. That these factors are involved in multiple dynamic interactions with the transcriptional machinery provide possibilities that sumoylation may influence these interactions. In this regard, it is noteworthy that Spt5 was also found in a proteomic screen of SUMO-conjugated proteins (Zhou et al. 2005). It will be of interest in the future to determine precisely how SUMO functions in capping, and whether mammalian capping enzymes are also sumoylated.

2.4 Splicing

The removal of introns and the ligation of exons takes place in an extremely precise manner via two transesterification reactions. The splicing reaction takes place in a megadalton ribonucleoprotein complex called the spliceosome, which is comprised of five snRNP subcomplexes (U1, U2, U4, U5 and U6), each consisting of an snRNA and associated proteins. A host of other proteins such as RNA helicases and SR proteins assist in dynamic assembly of the complex and in enhancing intron recognition (reviewed in Brow 2002; Jurica and Moore 2003). Catalysis of the splicing reaction takes place by the ordered assembly of the various snRNPs on the pre-mRNA and the

formation of several intermediate spliceosomal complexes, finally the catalytic C complex. Intron recognition, and both steps of catalysis, progress through a complex network of RNA-RNA, RNA-protein and protein-protein interactions between components of the snRNPs and intronic RNA sequences (reviewed in Brow 2002; Smith et al. 2008). Although most of the spliceosomal conformational changes are effected by ATP-dependent DExD/H box RNA helicases and unwindases (Cordin et al. 2006), transient protein modifications such as phosphorylation (Shi et al. 2006) and ubiquitination (Bellare et al. 2008) of snRNP-associated proteins have also been shown play a role in changing protein conformation to affect formation of splicing complexes. Ubiquitin-mediated interactions were shown to be important for the assembly of a multi-snRNP complex that joins the spliceosome as a single entity, the U4/U5/U6 tri-snRNP (Bellare et al. 2008).

The evidence linking sumoylation to splicing is, as with capping, almost entirely based upon proteomic reports. However, as opposed to capping, studies with mammalian systems revealed most of the splicing related targets. One splicing-related protein that has been validated as a SUMO target is SART1 (Vertegaal et al. 2004, 2006). SART1 is localized in nuclear speckles, has been shown to be a component of the U4/U5/U6 tri-snRNP, and is important for tethering of the tri-snRNP to the pre-spliceosome (Makarova et al. 2001). Several subunits of protein complexes associated with U2 snRNP, namely SF3A (SAP62, SAP114) and SF3B (SAP49, SF3b125, SAP130, SAP145, SAP155) were also found to be sumoylated (Manza et al. 2004; Guo et al. 2005; Rosas-Acosta et al. 2005; Golebiowski et al. 2009; Becker et al. 2013; Hendriks et al. 2014). These factors are essential for the assembly of the U2 snRNP complex and for proper tethering of the U2 snRNP to its intronic recognition sequence (Das et al. 1999; Will and Luhrmann 2001). In this regard, it is interesting that the SUMO E3 ligase PIAS1, which as mentioned above is found in nuclear speckles (Tan et al. 2002), was also found to co-purify with mammalian spliceosomes (Rappsilber et al. 2002). These results raise the possibility that sumoylation may influence the interactions of these factors and thus the assembly and/or function of spliceosomal complexes.

Another interesting class of splicing-related factors found in SUMO proteomic screens includes proteins that participate in other processing events and have roles in transcription. The polypyrimidine tract binding factor (PTB) associated splicing factor PSF forms a heterodimer with p54nrb and this multifunctional complex has been implicated in splicing, transcription initiation, cleavage and polyadenylation as well as transcription termination (Mathur et al. 2001; Emili et al. 2002; Rosonina et al. 2005; Liang and Lutz 2006; Kaneko et al. 2007). PSF and p54nrb were both identified as putative SUMO targets (see Table 2.1). PSF was validated as a SUMO substrate and sumoylation was found to promote its transcriptional repression properties (Zhong et al. 2006). PTB, which was also identified as a putative sumoylation target (Manza et al. 2004; Rosas-Acosta et al. 2005), is again a multifunctional RNA binding protein originally identified as a splicing repressor but also has roles in cleavage-polyadenylation, mRNA stability and translation initiation (reviewed in Sawicka et al. 2008).

More recently, purification of chromatin-associated proteins sumoylated by SUMO-1 confirmed sumoylation of several splicing factors, such as hnRNP A1, SF3A2 and SNRNP200, during S phase of the cell cycle (Liu et al. 2015). In the same study, Scaffold Associated Factor-B (SAFB), known to interact with the CTD of RNAPII, was shown to be a SUMO-1 substrate that binds promoters of highly expressed genes. SUMO-1 and SAFB depletion resulted in a decrease of the splicing rate of mRNAs encoding ribosomal protein, suggesting a role for sumoylated SAFB in coupling transcription and RNA processing (Liu et al. 2015).

2.5 3′ End Processing

The poly(A) tail, found at the 3′ end of nearly all eukaryotic mRNAs, is important for transcript stability, transport into the cytoplasm and translation initiation. The 3′ ends of pre-mRNAs are formed in a two-step process, with an endonucleolytic cleavage generating a 3′ OH end, followed by synthesis of a poly(A) tail (reviewed by Colgan and Manley 1997; Proudfoot and O'Sullivan 2002). This apparently simple reaction requires a surprisingly complex set of factors (Shi and Manley 2015). The multisubunit cleavage/polyadenylation specificity factor (CPSF) and cleavage stimulatory factor (CstF) complexes define the poly(A) site by binding cooperatively to the conserved AAUAAA and GU-rich sequence elements upstream and downstream, respectively, of the cleavage site (Murthy and Manley 1992; Takagaki and Manley 2000; Kaufmann et al. 2004). Cleavage factors I (CFI) and II (CFII) help in complex assembly and in the first step (Takagaki et al. 1989; de Vries et al. 2000; Brown and Gilmartin 2003). The single-subunit enzyme poly(A) polymerase (PAP) catalyzes poly(A) addition and is also in most cases required in some way for the cleavage reaction (Raabe et al. 1991). Nuclear poly(A) binding protein helps in increasing the processivity of PAP and in elongating the poly(A) tail (reviewed by Kuhn and Wahle 2004).

PAP appears to play a significant role in the regulation of 3′ processing, and is subject to extensive modification. For example, multiple isoforms can be produced by alternative splicing (Zhao and Manley 1996) and the enzyme is post-translationally modified by phosphorylation and acetylation (e.g., Colgan et al. 1996; Shimazu et al. 2007). The cyclin-dependent kinase cdc2/cyclinB hyperphosphorylates PAP during mitosis and meiotic progression, thus downregulating PAP activity, which is important for normal cell growth (Colgan et al. 1996, 1998; Zhao and Manley 1998).

The RNAPII CTD participates in the 3′ end processing reaction and plays a critical stimulatory role (McCracken et al. 1997, Hirose and Manley 1998). 3′ processing factors are recruited from the promoter onwards throughout the length of the gene, dependent on the phosphorylation status of the CTD and a number of 3′ processing factors make direct contacts with the CTD (reviewed by Bentley 2005; Proudfoot 2004). The formation of 3′ ends is also closely coupled to transcription termination (reviewed by Buratowski 2005; Rosonina et al. 2006; Richard and Manley 2009).

Proteomic reports have identified several polyadenylation factors as putative SUMO substrates. A number of yeast polyadenylation factors were identified in independent proteomic screens. These include Ysh1 and Ydh1, the yeast homologs of the CPSF3 (aka CPSF-73) and CPSF2 (CPSF-100) subunits (Wykoff and O'Shea 2005, Panse et al. 2004, Wohlschlegel et al. 2004, Hannich et al. 2005). The poly(A) binding protein Pbp1, and the regulatory yeast factors Fir1 and Ref2, which interact with Pbp1 to regulate poly(A) tail length (Mangus et al. 2004), were also identified as SUMO targets (Panse et al. 2004; Hannich et al. 2005). In mammals, symplekin, a scaffolding protein that bridges CPSF-CstF complexes, was identified using an in vitro expression cloning approach of human cDNA library and validated as a SUMO substrate in vitro (Gocke et al. 2005). Symplekin was later found in several large-scale proteomics analysis to be sumoylated by SUMO-2 (see Table 2.1).

The first evidence that 3′ processing activity could be affected by sumoylation was obtained in yeast in 1996 (as the SUMO pathway was just being discovered). Specifically, the SUMO E1 enzyme uba2 was found to interact with Pap1 (del Olmo et al. 1997). Uba2 depletion from extracts was found to increase polyadenylation activity, suggesting that sumoylation is inhibitory to Pap1 activity. However, SUMO modification of Pap1 was not shown in this study (del Olmo et al. 1997), and a later study by the same group showed evidence of PAP being ubiquitinated but not sumoylated (Mizrahi and Moore 2000).

Subsequent more extensive studies of sumoylation of mammalian 3′ processing factors have provided evidence that SUMO is capable of modulating pre-mRNA 3′ processing and regu-

lating the function of specific polyadenylation factors (Vethantham et al. 2007, 2008). The discovery of mammalian PAP sumoylation in western blots of mouse tissues and cell lines revealed a remarkable accumulation of higher molecular weight forms of PAP, which were found to reflect modification by the SUMO-2/3 isoforms (Vethantham et al. 2008). PAP proved to be an unusual substrate in displaying high levels of modification in specific tissues and cell lines and directly interacting with ubc9, even though PAP lacks any consensus sumoylation sites. The sites of PAP sumoylation mapped to known regulatory region of the protein, and SUMO was indeed found to be crucial for PAP function. Sumoylation was required for correct nuclear localization, as mutating the sites of sumoylation, which overlapped with a nuclear localization signal, or overexpressing the SUMO protease SENP1, led to mislocalization of PAP in the cytoplasm. Depletion of ubc9 or overexpressing a SUMO protease resulted in decreased PAP levels, indicating that sumoylation promotes PAP stability. Finally, in vitro sumoylated PAP displayed lower poly(A) synthesis activity in polyadenylation assays. This study showed a profound effect of sumoylation on PAP function, and implicates SUMO as a major regulator of PAP activity. However, the physiological significance of this regulation, including the role of tissue-specific sumoylation, remains unknown.

A separate study examined sumoylation of the 3′ processing factors CPSF-73, the endonuclease, and symplekin, as well as the effect of sumoylation on 3′ processing activity (Vethantham et al. 2007). CPSF-73 is the most highly conserved of the 3′ processing factors, consistent with its role as the endonuclease that catalyzes the cleavage reaction. This was suggested first by its identification as a member of the metallo-β-lactamase family of Zn-dependent hydrolytic enzymes (Callebaut et al. 2002; Ryan et al. 2004). More conclusively, structural and biochemical studies with purified CPSF-73 provided unequivocal evidence that it indeed possesses endonucleolytic activity (Mandel et al. 2006). Symplekin was uncovered as a protein that bound strongly to both CstF and CPSF, and was proposed to function as a scaffolding factor (Takagaki and Manley 2000). Later studies implicated symplekin in the related processes of histone pre-mRNA 3′ end processing (Kolev and Steitz 2005) and cytoplasmic polyadenylation (Barnard et al. 2004).

Both CPSF-73 and symplekin, like PAP, are specifically modified by the SUMO-2/3 isoform. As with PAP, the sites of sumoylation mapped to potential regulatory regions. A siRNA knockdown/rescue experiment of symplekin revealed that a sumoylation-deficient mutant cannot rescue the cell death phenotype of the knockdown cells, indicating that sumoylation is required for normal function of symplekin. This study also examined the effect of sumoylation on 3′ processing activity in nuclear extracts. Desumoylation of nuclear extracts by SUMO protease or depletion of ubc9 had an inhibitory effect on 3′ processing activity, and the formation of specific 3′ processing complexes was blocked by SUMO protease treatment. This correlated with the specific interaction of the SUMO protease with CPSF-73 and symplekin, suggesting that the desumoylation of CPSF-73 and/or symplekin may be involved in this inhibition.

Other components of the polyadenylation machinery are also modified by SUMO (see Table 2.1). The CFI complex is an essential 3′ processing factor that binds pre-mRNAs upstream of the cleavage site and also functions in regulation of alternative polyadenylation (Kim et al. 2010; Gruber et al. 2012). CPSF7 (CFI-59), a component of CFI, has been identified as a chromatin-associated protein sumoylated by SUMO-1 (Liu et al. 2015) as well as SUMO-2 (Tammsalu et al. 2014). Among the other proteins directly involved in pre-mRNA 3′ processing, large-scale affinity purifications found CPSF-100, and CLP and PCF11, components of the CFII complex, as SUMO substrates that contain polySUMO chains (Golebiowski et al. 2009; Tammsalu et al. 2014; Hendriks et al. 2014; Schimmel et al. 2014; de Vries et al. 2000).

Several other proteins found in the massive, ~80 polypeptide 3′ processing "holo-complex" (Shi et al. 2009) have been identified as sumoylated in large scale proteomics (WDR33, RBBP6, SKIV2L2 (hMTR4), RBM25) (see

Table 2.1). However, the significance and regulation leading to these modifications as well as their roles will need further investigation. The CPSF subunit WDR33 has recently been shown to bind directly the poly(A) signal AAUAAA (Chan et al. 2014; Schonemann et al. 2014). It will be interesting to test whether WDR33 sumoylation plays any role in this function.

The Polymerase II-Associated Factor complex (PAFc) is a conserved complex that plays multiple roles during transcription, including help couple transcription and 3′ processing. The tumor suppressor parafibromin (CDC73) is a PAFc subunit and plays a role in mRNA 3′ processing (Rozenblatt-Rosen et al. 2009). Interestingly, CDC73 sumoylation is upregulated after proteasome inhibition and affects CDC73 cellular localization. A more recent study using a highly sensitive strategy that detects SUMO remnant chains following tryptic digestion identified five sumoylation sites on CDC73, four of them also being ubiquitylation targets (Lamoliatte et al. 2014). Two other independent large scale analysis mapping SUMO-2 and SUMO-3 sites confirmed CDC73 sumoylation at multiple lysines for a total of seven identified sites so far (Tammsalu et al. 2014; Hendriks et al. 2014).

These studies on the role of SUMO in RNA 3′ processing are remarkable for the multiple, distinct effects that SUMO can have on individual factors and on the activity of the complex. The presence of multiple SUMO targets in the same complex and the known ability of SUMO to affect interactions and thus complex assembly raise the possibility that SUMO-mediated noncovalent interactions are necessary for efficient assembly of the polyadenylation complex. That SUMO could promote 3′ processing complex assembly and activity in nuclear extracts and yet inhibit enzymatic activity of purified PAP suggests that PAP activity alone and within the polyadenylation complex are distinct and highlights the complex nature of 3′ processing and its regulation by SUMO.

The effect of sumoylation on 3′ processing in mammals also contrasts with early findings in yeast. This is not altogether surprising considering that regulation of 3′ processing in yeast has not always correlated with that of mammalian systems. Although the SUMO target lysines in homologs of both CPSF-73 and symplekin are conserved in yeast, this is not the case for PAP. In fact, the C-terminal regulatory region of mammalian PAP is completely absent in yeast. Additionally, phosphorylation has different effects on 3′ processing in yeast and in mammals (He and Moore 2005; Ryan 2007; Colgan et al. 1996, 1998) and this seems likely to be the case for sumoylation as well.

2.6 Transcription Termination

Transcription termination is another important aspect of transcription that is coupled to 3′ processing (reviewed in Richard and Manley 2009). The DNA/RNA helicase Senataxin (SETX) has been shown to play an important role in this regulation by resolving DNA/RNA hybrids, known as R loops, which are formed behind elongating RNAPII and downstream of the poly(A) signal and 3′ pause sites of a subset of genes (Skourti-Stathaki et al. 2011, 2014). It is believed that after cleavage at the poly(A) site, unwinding R loops by SETX provides access to the 5′-3′ exonuclease Xrn2 to the downstream 3′ RNA leading to its degradation and subsequent RNAPII release from the DNA template (West et al. 2008). SETX was initially shown to be sumoylated by SUMO-1 and SUMO-2 in a yeast two-hybrid (Y2H) assay (Hecker et al. 2006). Subsequently, proteomic analyses of SUMO substrates revealed that SETX is highly sumoylated after heat shock, forming polySUMO chains, and also identified Xrn2 as another sumoylated protein (see Table 2.1) (Golebiowski et al. 2009; Bruderer et al. 2011). Additionally, two studies using the N-terminus of SETX as bait in an Y2H screen found Ubc9 and the E3 SUMO-protein ligase PIAS1 as SETX-interacting proteins (Richard et al. 2013; Bennett et al. 2013). Both screens also found an interaction with Rrp45 (EXOSC9), a component of the exosome that functions in RNA processing and degradation (Januszyk and Lima 2014). Importantly, interaction with Rrp45 was shown to depend on SETX

sumoylation and SETX and Rrp45 co-localize in R-loop-dependent nuclear foci after induction of replication stress (Richard et al. 2013).

SETX function in R-loop resolution extends beyond its role in termination, and is likely relevant to the DNA damage response and to certain neurodegenerative diseases. Specifically, *SETX* is mutated in two distinct neurological disorders, a form of Amyotrophic Lateral Sclerosis known as ALS4 (Chen et al. 2004) and a form of ataxia named AOA2 (Ataxia with Oculomotor Apraxia type 2) (Moreira et al. 2004). Strikingly, three AOA2 mutations located in the SETX N-terminus abolished SETX sumoylation and Rrp45 interaction, while nearby ALS4 mutations did not (Richard et al. 2013). It has been proposed that replication stress leads to an increase in SETX sumoylation that recruits the exosome through its interaction with Rrp45 to sites of R-loop formation, preventing DNA damage accumulation and degrading unwanted RNAs (Richard and Manley 2014). Indeed, persistence of R loops leads to double strand breaks and genome instability (Santos-Pereira and Aguilera 2015). Consistent with this, a proteomics analysis identifying SUMO-2 targets in response to replication stress showed that indeed SETX sumoylation increases after replication stress (Bursomanno et al. 2015). Additionally, a large-scale quantitative proteomics screen examining sumoylation dynamics during cell cycle progression found SETX highly sumoylated in early S phase, S/G2 and G2/M (Schimmel et al. 2014). Since SETX nuclear foci form during S/G2 phase (Yuce and West 2013), it is very likely that SETX sumoylation helps to regulate its accumulation at stress foci. It is worth noting that a large throughput analysis showing that about 10% of human proteins might be sumoylated identified seven SUMO sites in SETX (Hendriks et al. 2014). Intriguingly, most of those lysines are in proximity of identified AOA2 mutations, consistent with the possibility that disruption of SETX sumoylation is directly linked to the disease. Together, these data indicate that SETX sumoylation plays a significant role in the DNA damage response during replication stress. However, important details of the underlying mechanism remain to be determined, and whether this modification plays a role during normal transcription termination remains unknown.

2.7 Sumoylation of hnRNPs

Heterogeneous nuclear RNA binding proteins (hnRNPs) are a structurally diverse group of RNA binding proteins that associate rapidly with nascent RNAs and contain auxiliary domains that bind other proteins (Krecic and Swanson 1999). While hnRNPs participate in a variety of processes such as mRNA biogenesis, telomere maintenance and initiation of translation, they are best known for their roles in regulation of RNA processing events, especially splicing, stabilization of mRNA and mRNA export. Some hnRNPs, such as hnRNP A1, are nucleocytoplasmic shuttling proteins, while others, such as hnRNP C, remain in the nucleus (reviewed by Dreyfuss et al. 2002, 1993; Martinez-Contreras et al. 2007).

HnRNPs are extensively sumoylated by both SUMO-1 and SUMO-2/3 (see Table 2.1) (Blomster et al. 2009; Hendriks et al. 2014). Vassileva and Matunis (2004) first demonstrated that hnRNPs C and M are targets for modification by SUMO. Moreover, the SUMO E3 ligase nup358/RanBP2 was found to enhance sumoylation of both classes of hnRNPs, indicating that the sumoylation of these hnRNPs very likely occurs at the nuclear pore complex (NPC). The SUMO acceptor lysine was further identified in hnRNP C and it was found that sumoylation can inhibit the RNA binding capacity of hnRNP C, as in vitro SUMO modified hnRNP C displayed a significantly lower affinity for ssDNA. Since both SUMO modification and demodification enzymes are localized at the NPC, the authors proposed a model whereby sumoylation regulates the organization of the mRNP complexes at the NPC and helps to facilitate nucleocytoplasmic transport. The hnRNP C and M proteins also have roles in regulating pre-mRNA splicing (Kafasla et al. 2002; Venables et al. 2008), which were not addressed in this study.

A number of other groups independently identified hnRNPs M, L and I as being sumoylated in proteomic analyses (Rosas-Acosta et al. 2005; Guo et al. 2005; Gocke et al. 2005). A study by Li et al. (2004) identified six hnRNPs, including A1, H1, U, F and K, in proteomic analysis of sumoylated proteins in human cell lines, and confirmed that hnRNPs A1, F and K were indeed sumoylated in vivo. Putative sumoylation consensus sequences were located in the RNA binding domains of these three proteins, raising the possibility that, as with hnRNP C, sumoylation may modulate the RNA binding function of these proteins. A long list of recent large-scale affinity-purifications of sumoylated proteins and high-resolution MS-based mapping of SUMO sites confirmed the sumoylation of most hnRNPs (see Table 2.1) as well as the polySUMOylation of a large number after heat shock (Bruderer et al. 2011).

2.8 Extending the Role of Sumo to mRNA Export

The export of mRNA across the NPC is closely linked to mRNA synthesis and maturation and requires that the mRNA is capped, spliced and polyadenylated. Transport of mRNA also generally requires the highly conserved export factor, Mex67 in yeast and NXF1/TAP in metazoans, which has been found sumoylated in several proteomics analysis (see Table 2.1). The hnRNP-like protein Yra1 or the Aly/REF complex enhances the affinity of mRNA to the export factor and shuttling hnRNP proteins like Npl3 act as additional adaptors by binding to Mex67 (reviewed in Rodriguez et al. 2004; Huang and Steitz 2005). UAP56/Sub2, which also functions in splicing, interacts closely with Aly/Yra1 and helps to couple splicing with export (Strasser and Hurt 2001). Both Sub2 and Yra1 are components of the TREX complex, which is recruited to the mRNA during transcriptional elongation through the THO complex (Tho2, Hpr1, Mft1, Thp2) (Chavez et al. 2000). Absence of the THO complex leads to the retaining of mRNPs in the nucleus (Dominguez-Sanchez et al. 2011; Strasser et al. 2002). It has been hypothesized that interaction of Yra1 with Mex67 displaces Sub2 at the NPC thus facilitating export (Strasser and Hurt 2001; Reed and Hurt 2002). Np13 is also recruited to the pre-mRNA during early elongation via interaction with RNAPII, providing another link of transcription to mRNA export (Lei et al. 2001). In addition, Npl3 has been shown to link 3′ processing with export (Gilbert and Guthrie 2004). The nuclear exosome, which physically interacts with the TREX complex, functions in mRNA surveillance to degrade unadenylated or unprocessed mRNA before export (reviewed in Rodriguez et al. 2004). Mlp1/Mlp2 in yeast functions in mRNA surveillance at the NPC prior to export to retain unspliced RNAs (Green et al. 2003; Galy et al. 2004).

It has recently been shown that sumoylation of the C-terminus of the THO complex component Hpr1 controls the association of the THO complex with mRNPs in a SUMO protease Ulp1-dependant manner (Bretes et al. 2014). While blocking Hrp1 sumoylation does not appear to affect mRNA export, it leads to improper mRNP assembly of a subset of stress-induced transcripts that are normally degraded by the exosome.

As described above, Vassileva and Matunis (2004) suggested a role for hnRNP sumoylation in influencing mRNA export in mammalian cells. Another study in *Arabidopsis thaliana* established an intriguing link between sumoylation and mRNA export involving Nua, the plant homolog of Mlp1/Mlp2, which in yeast serves as the anchor of hnRNPs (Green et al. 2003) and the SUMO protease Ulp1 (Zhang et al. 2002) at the NPC. The *nua* mutant shared striking similarities with another mutant, *esd4*, which encodes the *A. thaliana* homolog of mammalian SENP2 and yeast Ulp1. *nua* or *esd4* single mutants and *nua/esd4* double mutants displayed altered expression of flowering regulators, an accumulation of SUMO conjugates and retention of poly(A) RNA in the nucleus, indicating that these proteins function in the same pathway (Xu et al. 2007).

Proteomic reports have identified key mRNA export and surveillance factors, including multiple subunits of the TREX complex, as putative sumoylation targets in yeast and mammals. These

include Yra1 (Wohlschlegel et al. 2004), Npl3 (Denison et al. 2005), Rrp6 and Sub2, which were identified in multiple proteomic screens (see Table 2.1). The brief descriptions above, together with proteomic data, provide fodder to explore the role of SUMO in mRNA export. In addition, an active sumoylation machinery is known to exist at the NPC, and suggestive evidence that the sumoylation machinery at the NPC may be involved in mRNA export was obtained from data that *ULP1* was identified as a high copy suppressor of a *yra1* temperature sensitive strain (Kashyap et al. 2005). Sub2, Yra1 and Np13 connect export to transcription and processing events (reviewed in Luna et al. 2008), and the possibility that sumoylation may be involved in these interactions remains a very tempting target for future studies.

2.9 Sumo and RNA Editing

ADAR1 is an RNA editing enzyme that binds to double-stranded RNA and converts adenosine to inosine, which results in changes in amino acid coding and thus change in the protein sequence/function. Of the three isoforms of ADAR, ADAR1, 2 and 3, ADAR1 was found to be modified by SUMO-1 (Desterro et al. 2005). While SUMO did not influence the localization of ADAR1 in the nucleolus, it seemed to repress the RNA editing activity of the enzyme, as a sumoylation-deficient mutant was considerably more active in vivo and in vitro. In addition, sumoylation of ADAR1 in vitro resulted in inhibition of nonspecific RNA editing activity. The SUMO acceptor lysine was found to be located in a putative dimerization domain of the protein. ADAR heterodimers and homodimers have been shown to regulate activity and specificity of this enzyme. The authors hypothesized that by inhibiting dimerization, SUMO can regulate the activity and the function of ADAR1, and hence RNA editing. Several more recent MS-based proteomics data revealed that ADAR1 is also sumoylated by SUMO-2 (see Table 2.1) and is in fact a polySUMO-modified protein (Bruderer et al. 2011).

2.10 Conclusions

The events governing the processing of mRNA precursors are closely coupled to transcription, export and other nuclear events. SUMO has been known to be an important regulator of nuclear functions, including transcription, DNA repair and genome stability. The evidence discussed above, from many proteomic analyses and in some cases functional studies, points to an important role for SUMO in essentially all nuclear RNA processing and handling events. This is highlighted by the presence of multiple putative SUMO targets in functional capping, splicing, polyadenylation, termination and mRNA export complexes. So far the study of sumoylation of RNA processing/binding proteins has been largely performed in vitro. Considering the intricate connections of RNA processing to transcription and other events, a study of sumoylation of RNA processing factors remains incomplete until reliable in vivo processing assays are developed. The goal of understanding the roles of SUMO in mRNA metabolism holds a great deal of promise and much excitement, not only for elucidating mechanisms of basic cellular processes, but also for providing novel insights into human disease.

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SUMO and Chromatin Remodeling

3

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Abstract

Many of the known SUMO substrates are nuclear proteins, which regulate gene expression and chromatin dynamics. Sumoylation, in general, appears to correlate with decreased transcriptional activity, and in many cases modulation of the chromatin template is implicated. Sumoylation of the core histones is associated with transcriptional silencing, and transcription factor sumoylation can decrease gene expression by promoting recruitment of chromatin modifying enzymes. Additionally, sumoylation of transcriptional corepressors and chromatin remodeling enzymes can influence interactions with other transcriptional regulators, and alter their enzymatic activity. In some cases, proteins that are components of transcriptional corepressor complexes have been shown to be SUMO E3 ligases, further emphasizing the integration of sumoylation with the regulation of chromatin remodeling. Despite the evidence suggesting that sumoylation is primarily repressive for access to chromatin, recent analyses suggest that protein sumoylation on the chromatin template may play important roles at highly expressed genes. Elucidating the dynamic interplay of sumoylation with other post-translational modifications of histones and chromatin associated proteins will be key to fully understanding the regulation of access to the chromatin template.

Keywords

SUMO • Chromatin • HDAC • Polycomb • Transcription

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Abbreviations

SUMO	small ubiquitin like modifier
Ubc9	ubiquitin-conjugating enzyme 9
HAT	histone acetyl transferase
HDAC	histone deacetylase
HP1	heterochromatin protein 1
MAR	matrix attachment region
PML	promyelocytic leukemia protein
PIAS	protein inhibitor of activated STAT
RING	really interesting new gene (a zinc binding domain)
SP-RING	Siz/PIAS RING
PRC	polycomb repressive complex
CBX	chromobox

3.1 Introduction

A large number of proteins involved in the regulation of transcription and chromatin accessibility are substrates for modification by SUMO. Numerous transcription factors themselves have been shown to be sumoylated, and in general, this results in decreased transcriptional activation (Ouyang and Gill 2009). The nucleosome, which forms the basic repeating unit of chromatin, consists of DNA wrapped around a histone octamer (Luger and Hansen 2005). Arrays of regularly spaced nucleosomes are packaged into chromatin fibers, which include other histone binding proteins, as well as linker histones. Within the eukaryotic nucleus, chromatin is further organized into higher order structures. Transcriptionally silent heterochromatin is often localized to the nuclear periphery, and is interspersed with nuclear domains enriched for active chromatin (Akhtar and Gasser 2007). The complex patterns of histone modifications, such as acetylation, methylation and phosphorylation, have led to the histone code hypothesis (Jenuwein and Allis 2001; Strahl and Allis 2000). Histones act as platforms to which modifications are added, and the combinations of modifications are then read by protein complexes which bind to specifically modified histones (Ruthenburg et al. 2007). In addition to the more extensively studied histone modifications, such as lysine acetylation and methylation, histones can also be ubiquitinated on specific lysine residues, further expanding the complexity of this signaling platform (Robzyk et al. 2000; Zhang 2003). It is also clear that the histones are targeted for sumoylation, and that this can have direct effects on DNA accessibility and gene expression (Nathan et al. 2006; Shiio and Eisenman 2003). Transcription factors, and many other chromatin-associated proteins are also known to be sumoylated, expanding the role of SUMO in governing access to the chromatin template. Genome-wide analysis of SUMO distribution suggests that rather than being simply a repressive mark, SUMO modification of the chromatin template and associated proteins may play a more complex and dynamic role in regulating expression of highly transcribed genes (Liu et al. 2012; Neyret-Kahn et al. 2013; Niskanen et al. 2015; Seifert et al. 2015). Here we discuss the links between the sumoylation machinery and chromatin remodeling, primarily with respect to the regulation of transcription.

3.2 Histone Sumoylation

Direct modification of the histones themselves by SUMO is the simplest model by which sumoylation can modulate chromatin dynamics. Histone sumoylation was first demonstrated in mammalian cells, where sumoylation was detected predominantly on histone H4, but is also found to some degree on all four core histones (Shiio and Eisenman 2003). Sumoylation of H4 increased its interaction with a histone deacetylase (HDAC1) and with HP1γ (heterochromatin protein 1), suggesting a repressive role for histone sumoylation. Somewhat surprisingly, SUMO was detected on acetylated histone H4, and over-expression of the p300 transcriptional coactivator, which has histone acetyl transferase (HAT) activity, increased H4 sumoylation. In

these analyses, acetylation was detected with an antibody which recognizes acetylated lysines 5, 8, 12 and 16 in H4, and it was not known which lysines were sumoylated (Shiio and Eisenman 2003). It is, therefore, possible that sumoylation of H4 at another lysine might override the effects of acetylation at one or more of these lysines. However, despite the apparent contradiction between the activating (acetyl) and repressing (SUMO) modifications, this clearly suggests that other histone modifications can influence H4 sumoylation.

Further evidence for the direct regulation of chromatin dynamics via direct histone sumoylation comes from the budding yeast, *S. cerevisiae* (Nathan et al. 2006). All four yeast core histones are sumoylated, and sumoylation is associated with transcriptional repression. In contrast to the specific modifications that are associated with transcriptional repression in mammalian cells, the major characteristic of transcriptional silencing in budding yeast is histone H3 and H4 hypoacetylation; a lack of modification, rather than the presence of repressive modifications (Berger 2002). Thus, the identification of histone sumoylation as a specific modification, which promotes silencing in *S. cerevisiae* helped fill this apparent gap. Histone sumoylation is enriched at telomeres (Nathan et al. 2006), which are maintained in a transcriptionally silent state, associated with low levels of histone acetylation and ubiquitylation (Rusche et al. 2003). Histone sumoylation was also found at inducible genes in the uninduced state, and the level of histone sumoylation was shown to decrease with transcriptional induction. A reciprocal pattern of H2B sumoylation and acetylation of lysine 16 is seen at inducible genes, again implying a dynamic interplay between sumoylation and other histone modifications. For the sumoylation of mammalian histones, no E3 has been identified. In contrast, in *S. cerevisiae*, histone sumoylation is enhanced by the Siz1 and Siz2 ligases, which are the major E3s in this yeast (Johnson and Gupta 2001; Nathan et al. 2006).

For *S. cerevisiae* H4 and H2B, it appears that one or more of several lysines can be modified. In H4, lysines 5, 8, 12, 16 and 20 are the major SUMO acceptors, and in H2B, two pairs of lysines (either K6/7 or K16/17) within the repeated AEKKPA motif are modified (Nathan et al. 2006). SUMO acceptor lysines have been identified in mammalian histones H3 and H4 via large-scale proteomic approaches (Galisson et al. 2011), but there has not been an extensive analysis of which lysines are modified. In one analysis, histone H4 K12 and H3.1 K24 were found to be modified by SUMO. In addition to the core histones, the linker histone, H1, and the variant histones, H2AX and H2A.Z have been shown to be sumoylated, with the latter two playing a role in the DNA damage response (Chen et al. 2013; Galisson et al. 2011; Kalocsay et al. 2009; Matafora et al. 2009; Shiio and Eisenman 2003). The SUMO acceptor lysines which have been identified in the histones do not conform to the classical SUMO consensus site (KxE; (Melchior 2000)). Interestingly, the best match to this site (lysine 79 in histone H3 [FKTD]), is conserved from yeast to mammals, and has been shown to be a site of modification by the Dot1 family of methyltransferases, so is clearly accessible for modification (Ng et al. 2002; van Leeuwen et al. 2002). As with the majority of other known histone modifications, sumoylation occurs primarily within the flexible amino-terminal tails, favoring a model in which sumoylation and other modifications, such as acetylation, may compete either for individual lysines, or specific histone tails. Alternatively, histone sumoylation might result in the recruitment of other histone modifying proteins, such as HDACs, to further modify the chromatin template. Recent work examining chromatin compaction with nucleosomes that were homogenously modified by SUMO3 at H4K12 suggests an additional level of regulation (Dhall et al. 2014). In this analysis the addition of SUMO3 inhibited the higher order compaction of nucleosome arrays by preventing internucleosome interactions. In this model, the addition of SUMO might then be expected to favor chromatin accessibility by preventing compaction of the chromatin template. However, it should be noted that this work was carried out

with uniformly modified nucleosomes, and it is likely that *in vivo* modifications would be more sporadic. While it is clear that histones are modified by SUMO, the outcomes are less well understood, and it is possible that histone sumoylation plays different roles at different loci or in different physiological settings. The potential interplay of histone sumoylation with other histone modifications suggests that sumoylation contributes to the dynamic mechanism by which combinatorial histone modifications modulate access to the DNA template.

3.3 SUMO and Higher Order Chromatin Structure

While histone sumoylation may affect chromatin packaging at the most basic level, changes in nuclear organization may also alter chromatin structure and accessibility. The sumoylation machinery is important for overall nuclear integrity (Heun 2007), and for the formation of subnuclear structures, such as PML bodies (Muller et al. 1998; Shen et al. 2006). Evidence for a role for SUMO in higher order nuclear structure comes from targeted mutation of the mouse *Ubc9* gene. This mutant results in embryo inviability soon after implantation, and defects in chromosome condensation and segregation in mutant blastocysts cultured *in vitro* (Nacerddine et al. 2005). Additional defects in nuclear structure, including disruption of PML bodies, nucleoli, and the nuclear lamina were also observed in the absence of Ubc9. While some of these effects are consistent with SUMO playing a major role in the regulation of chromatin structure and overall nuclear architecture, it is also possible that the causes could be more indirect. For example, defects in nuclear transport in the absence of Ubc9 might have profound effects on nuclear architecture by altering the import of proteins required to maintain nuclear integrity (Melchior et al. 2003).

In mammalian cells, SUMO and sumoylated proteins have been observed to colocalize with highly heterochromatic regions in the nucleus. During meiosis in male cells, the sex chromosomes are packaged into the XY body, a specialized chromatin domain that is transcriptionally silent and does not undergo recombination. Although the precise function of the XY body is not clear, it may be involved in maintaining gene silencing and preventing potentially deleterious recombination events between the sex chromosomes (Handel 2004). In addition to colocalization of SUMO itself with the XY body, sumoylated proteins including Daxx and PML associate with this specialized chromatin domain (Rogers et al. 2004). SUMO can also be found localized to constitutive heterochromatin, and specifically to the regions of centromeric heterochromatin on human chromosomes 1 and 9, during meiosis (Brown et al. 2008; Metzler-Guillemain et al. 2008).

In *Drosophila* SUMO can be seen both at discrete locations in euchromatic regions of the polytene chromosomes and at the chromocenter (Lehembre et al. 2000). The suppressor of position effect variegation, Su(var)2–10, encodes a *Drosophila* member of the PIAS family of proteins, which are SUMO E3 ligases (Hari et al. 2001). Although PIAS proteins may have functions other than as E3s, this clearly raises the possibility that sumoylation regulates chromatin structure in flies. Additionally, a role has been demonstrated for sumoylation in the regulation of the gypsy insulator in flies (Capelson and Corces 2006). Insulators are thought to act as chromatin organizers, which establish distinct chromosomal domains, such that gene expression can be independently regulated in adjacent domains (Bushey et al. 2008). Two components of the *Drosophila* gypsy insulator can be sumoylated, and on polytene chromosomes SUMO associates with a fraction of the insulators (Capelson and Corces 2006). Interestingly, mutations in the fly genes encoding the SUMO E2 or SUMO itself suppressed the effects of mutations in components of the gypsy insulator. This suggests an antagonistic role for SUMO in insulator function, which may be explained by decreased clustering of insulators when sumoylated. There is also evidence that mamma-

lian CTCF, which has insulator function, is sumoylated dependent on the Pc2/CBX4 E3, suggesting that sumoylation may affect insulator function in mammals (Macpherson et al. 2009).

Matrix attachment regions (MARs) and MAR-binding proteins play a role in integrating global chromatin organization with the regulation of gene expression (Bode et al. 2000). In pre-B cells the MARs of the immunoglobulin μ locus are bound by the special AT-rich sequence binding protein 2 (SATB2), resulting in increased gene expression (Dobreva et al. 2003). SATB2 sumoylation, which is promoted by PIAS1, affects both the sub-nuclear localization of SATB2 and its ability to regulate gene expression. Mutation of the SUMO acceptor lysines within SATB2 decreased its association with the nuclear periphery, a localization that could be restored by fusion to SUMO3 (Dobreva et al. 2003). The T cell specific SATB1 has also been shown to be sumoylated, although in this case sumoylation increased the caspase mediated cleavage of SATB1 during apoptosis, suggesting that multiple regulatory mechanisms may be controlled by SUMO (Tan et al. 2008). Additional MAR associated proteins, including SAFB (scaffold attachment factor B) and SAFB2 have been shown to be sumoylated, and the PIAS1 E3 can promote modification of SAFB1 (Garee et al. 2011; Liu et al. 2015). There is evidence for both positive and negative effects of SAFB sumoylation on gene expression, perhaps consistent with the MAR-binding proteins functioning to modify the effects of other transcriptional regulators.

The effects of SATB2 sumoylation on gene expression and localization may be linked since inactive genes often preferentially localize to the nuclear periphery (Akhtar and Gasser 2007). For example, in *S. cerevisiae* the Siz2 SUMO E3 regulates the perinuclear tethering of telomeres, and this is likely dependent on sumoylation of components of the Sir complex or of Yku70/80 (Ferreira et al. 2011). Thus it appears that sumoylation may regulate the function of insulators and MARs, and likely plays an important role in regulating the partitioning of chromatin domains and of their positioning within the nucleus.

3.4 Telomeres and Centromeres

Centromeres and telomeres are specialized chromatin domains with roles in chromosome structure and maintenance, which have also been extensively studied for effects of chromatin structure on transcriptional regulation. Sumoylated histones are enriched at the telomeres in *S. cerevisiae*, correlating with transcriptional repression at these loci (Nathan et al. 2006). In addition, there is evidence from the fission yeast, *S. pombe*, and from *S. cerevisiae* that SUMO plays a role in maintaining chromatin structure at both centromeres and telomeres. Indeed, the essential *S. cerevisiae* gene, *SMT3*, which encodes the single yeast SUMO was first identified as a high copy suppressor of mutations in the *MIF2* gene, which encodes a centromere binding protein (Meluh and Koshland 1995). In addition to Siz1 and Siz2, Zip3 and Mms21 are also SUMO E3 ligases in *S. cerevisiae* (Cheng et al. 2006; Zhao and Blobel 2005). Zip3 plays a role in the formation of the synaptonemal complex during meiosis. Mms21 was found to copurify with a DNA repair complex, which included the Smc5 and Smc6 (structural maintenance of chromosomes) proteins (Zhao and Blobel 2005). Smc5 and the yeast DNA repair protein, Yku70, were both shown to be sumoylated substrates of Mms21. Mutation of *MMS21* resulted in a number of nuclear phenotypes, including increased DNA damage sensitivity and defects in telomeric silencing and length regulation. Human MMS21 is also a SUMO ligase, which promotes sumoylation of DNA repair proteins including SMC6 and TRAX, and is required for efficient DNA repair (Potts and Yu 2005). In addition to effects on telomeric silencing, there is evidence that sumoylation can regulate telomere length in yeast. SUMO modification of a component of the shelterin complex in *S. Pombe* prevents accumulation of telomerase and maintains normal telomere length regulation (Miyagawa et al. 2014), and sumoylation of Cdc13 is required for telomere length regulation *S. cerevisiae* (Hang et al. 2011). In *S. pombe*, there are two known SP-RING family SUMO E3s, Pli1 and Nse2 (Watts et al. 2007). Deletion of Pli1 does not result in a severe growth defect

(unlike mutations in the genes encoding the *S. pombe* SUMO and Ubc9 homologs), but causes decreases in global sumoylation (Xhemalce et al. 2004). Cells lacking Pli1 showed alterations in both telomere and centromere homeostasis. Telomeres elongated via what appeared to be a Rad51-dependent gene conversion-like mechanism, minichromosome instability was increased, and reporter genes integrated at centromeres were lost by gene conversion (Xhemalce et al. 2004). Additionally, telomerase activity can be increased by inhibition of the SUMO pathway, dependent on Pli1, but not Nse2 (Xhemalce et al. 2007). In cells lacking Pli1, there was also a decrease in transcriptional silencing at centromeres, further pointing to changes in chromatin structure (Xhemalce et al. 2004). As with yeast, a link to the regulation of telomere homeostasis has been uncovered in mammalian cells. In certain cancer cells, which obtain abnormally long telomeres by recombination rather than increased telomerase activity, telomeres have been found clustered at PML bodies (Yeager et al. 1999). In these cells, the SMC5/6-MMS21 complex was found to colocalize with the PML-telomere clusters, and the telomere binding proteins RAP1 and TRF2 were shown to be sumoylated in a MMS21-dependent manner (Potts and Yu 2007). Mutations in TRF2, which abolished its sumoylation led to decreased localization at PML, and experimentally decreasing expression of MMS21 resulted in shorter telomeres. Based on studies from yeast to humans, it appears that sumoylation may play multiple roles at telomeres, directly regulating chromatin structure at the level of the histones themselves, and also regulating higher order telomere structure, via the modification of telomere binding proteins and proteins involved in length regulation and end protection.

In *S. pombe*, centromeric heterochromatin has been extensively studied, and many of the components involved in its maintenance have been identified (Grewal and Jia 2007). Swi6, and the paralogous Chp2, are members of the HP1 family (Lomberk et al. 2006), which bind to methylated lysine residues on histone H3, via their conserved chromodomains (Jacobs and Khorasanizadeh 2002; Nielsen et al. 2002; Bannister et al. 2001). Clr4, which is a relative of the *Drosophila* Su(var)3–9 suppressor of position effect variegation, is a lysine methyl transferase. Clr4 methylates lysine 9 of histone H3, facilitating binding of HP1-like proteins, such as Swi6, and the spreading of heterochromatin (Rea et al. 2000). Mutations in the *pmt3* gene, which encodes the *S. pombe* SUMO, result in decreased silencing of a reporter gene inserted either at the silent *mat3* mating type locus, or at the centromere (Shin et al. 2005). This decreased silencing is associated with an increase in levels of histone H3 methylated at lysine 4, a mark of actively transcribing chromatin. A model for the role of SUMO at heterochromatic regions in *S. pombe* has been suggested, in which it is sumoylation of histone binding proteins, which regulates the association of these proteins with chromatin (Shin et al. 2005). The Ubc9 homolog, encoded by *hus5*, associates with heterochromatic regions, dependent on Clr4 and Swi6. Both Clr4 and Swi6 interact with Hus5 and both proteins can be sumoylated. Importantly, non-sumoylated mutants of Swi6 showed reduced silencing, with some decrease in the amount of Swi6 recruited to the silent loci. Thus it appears that recruitment of the SUMO E2 to silent regions, via interactions with proteins such as Clr4 and Swi6, may allow for their sumoylation, which in turn contributes to the stable maintenance of heterochromatin structure. In this context, it is of interest that in mammalian cells, targeting Ubc9 to DNA via a heterologous DNA binding domain resulted in an increase in the amount of both SUMO and HP1γ at chromatin, together with reduced histone H3 acetylation on lysines 9 and 14 (a mark of actively transcribing genes) (Shiio and Eisenman 2003). Unlike at the *S. pombe* centromeric heterochromatin, in this case it may be that direct sumoylation of the histones was the outcome. In mammalian cells, there is also evidence for recruitment of HP1 proteins to pericentric heterochromatin, dependent on the SUMO pathway. At centromeric and pericentric chromatin, HP1 pro-

teins bind to trimethylated H3K9, which is a mark of repressed chromatin. *De novo* targeting of HP1α was found to require its modification by SUMO1, and this recruitment of modified HP1 was via interactions with a long non-coding RNA derived from the pericentric alpha-satellite DNA (Maison et al. 2011). This requirement for HP1 sumoylation appears to be transient, with SUMO modification being dispensable for maintenance of HP1 at pericentric heterochromatin. Indeed, depletion of a SUMO protease (SENP7) that was able to de-sumoylate HP1α resulted in loss of HP1α from these chromatin domains, suggesting a requirement for transient rather than stable modification (Maison et al. 2011, 2012). However, it is also possible that SENP7 plays a more structural role in maintaining HP1 at heterochromatin, by bridging interactions between adjacent HP1 molecules, with the de-sumoylation activity of SENP7 being less important (Romeo et al. 2015).

There is considerable evidence that sumoylation plays roles in regulating chromatin structure and in the assembly or maintenance of specific chromatin domains. However, the precise functions of sumoylation are not always clear and it appears that, depending on the protein that is modified and the loci under consideration, sumoylation can have what appear to be opposing effects on access to the chromatin template. Such apparently contradictory effects may also reflect differential and transient requirements for sumoylation at different steps of a process.

3.5 SUMO-Dependent Recruitment of General Transcriptional Corepressors

Sumoylation provides an attractive model for modulating protein recruitment, particularly with the identification of non-covalent SUMO-interaction motifs (SIMs), which may facilitate protein interactions dependent on the sumoylation of one partner (Hannich et al. 2005; Minty et al. 2000; Song et al. 2004, 2005). These primarily hydrophobic patches in SUMO-interacting proteins interact with relatively low affinity with SUMO, but when present in multiple copies, or together with other interaction domains, can contribute significantly to protein interactions. Thus sumoylated proteins that are associated with the chromatin template may function as recruitment signals for additional chromatin regulatory proteins.

One clear example of SUMO-dependent recruitment of a chromatin modifying activity operates for the ETS-related transcription factor Elk-1 (Yang and Sharrocks 2004). Sumoylation, primarily of a single site, within the transcriptional repression domain of Elk-1 is required for repression (Yang et al. 2003). Sumoylated Elk-1 is associated *in vivo* with histone deacetylase activity, and recruits HDAC2 via its sumoylated repression domain (Yang and Sharrocks 2004). Interestingly, in the case of Elk-1 the sumoylated repressive complex is thought to be poised at promoters, such that in response to mitogenic signals via the MAP kinase pathway, Elk-1 is desumoylated allowing for rapid activation of gene expression (Yang et al. 2003). This relatively simple model provides an important paradigm for SUMO-dependent repression, in which sumoylation of a transcription factor results in recruitment of a chromatin remodeling activity to specifically repress target gene expression. Additionally, the regulated removal of SUMO allows for the reversibility of this switch, from repression to activation.

A similar model for the inhibition of transcriptional activation by the transcriptional coactivator, p300, has been proposed (Girdwood et al. 2003). In this case it is the class II histone deacetylase, HDAC6, which is recruited in a SUMO-dependent manner resulting in inhibition of transcriptional activation. HDAC6 is recruited to a region in p300, which acts as an independent transcriptional repression domain, and is separate from the HAT domain required for transcriptional activation. Regulation of HDAC recruitment by sumoylation has been identified for an increasing number of transcriptional regulators, including the p68 DEAD box RNA helicase, and Reptin, which is a component of a chromatin remodeling complex with links to tumor progression. The

sumoylated form of p68 represses gene expression likely via the preferential recruitment of HDAC1 (Jacobs et al. 2007). Similarly, Reptin interacts with HDAC1 in a sumoylation dependent manner (Kim et al. 2006). Thus, both transcription factors and transcriptional coregulators can be modified by SUMO to convert them to a more repressive form, via the recruitment of HDACs. However, particularly with histone deacetylases, the possibility exists that it is not just the chromatin template that is being targeted for deacetylation, but the regulatory proteins themselves. HATs have long been known to acetylate other proteins in addition to histones, and in some cases this is known to be reversed by specific HDACs (Glozak et al. 2005).

There is evidence that other histone modifying activities and larger corepressor complexes can be recruited in a SUMO-dependent manner. Sumoylation of Sp3 at a single lysine residue blocks its ability to activate transcription (Ross et al. 2002; Sapetschnig et al. 2002), and this may in part be explained by the SUMO-dependent recruitment of a transcriptional repression complex (Stielow et al. 2008a). At least in some cell types, sumoylation of Sp3 also results in a redistribution of Sp3 to the nuclear periphery and nuclear foci, and it is possible that these two mechanisms may act in concert (Ross et al. 2002). However, mutation of the sumoylated lysine in Sp3 relieves repression, allowing Sp3 to activate transcription. Multiple proteins required for the SUMO-dependent inhibition of Sp3 transcriptional activation were identified in cultured *Drosophila* cells, including components of the sumoylation machinery and proteins with links to transcriptional repression (Stielow et al. 2008a). These included the ATP-dependent chromatin remodeling protein, Mi-2 and the related Chd3. In addition to their ATPase and helicase domains, both proteins also have PHD (plant homeodomain) and chromodomains. Mi-2 is a component of the NuRD (nucleosome remodeling and deacetylase) complex, a general transcriptional repression complex with both histone deacetylase and ATP-dependent nucleosome remodeling activities (Zhang et al. 1999). Mi-2 binds better to sumoylated Sp3 than to unmodified Sp3, and Mi-2 recruitment to chromatin was decreased in the presence of SUMO-mutant Sp3 (Stielow et al. 2008a). Also identified in this screen were MEP-1, a zinc finger containing protein, which in *C. elegans* is associated with Mi-2, and Sfmbt, which binds to methylated histones H3 and H4 (Klymenko et al. 2006; Unhavaithaya et al. 2002). Both were shown to interact with Mi-2 and bind sumoylated Sp3. This led to the model that these proteins form a transcriptional repression complex, which is targeted to sumoylated transcription factors. There is also evidence for a similar SUMO-dependent repression of Sp3 via Mi-2 recruitment in mouse cells, pointing to a conserved mechanism (Stielow et al. 2008a). Recruitment of this SUMO-dependent transcriptional corepressor complex results in local transcriptional repression by the formation of a heterochromatin like state, with increases in methylation of histone H3 on lysine 9, and H4 on lysine 20 (Stielow et al. 2008b). The *in vivo* importance of Sp3 sumoylation was demonstrated by the generation of a mouse line with a single amino-acid change in Sp3 that prevented sumoylation of the Sp3 transcriptional inhibitory domain (Stielow et al. 2010). This resulted in derepression of Sp3 target genes, together with reduced recruitment of corepressors, including Mi-2, and a reduction in repressive chromatin marks at the promoters of derepressed genes. The recruitment of large corepressor complexes to chromatin can be mediated in part by interaction of a SIM-containing component of the complex with a sumoylated transcription factor. For example, a CoREST1 complex that includes LSD1 and HDACs can interact with SUMO2 via a slightly divergent hydrophobic SIM in CoREST1. This results in recruitment of the CoREST1/LSD1/HDAC complex to sumoylated transcription factors and subsequent repression of transcription (Ouyang et al. 2009). Thus transcriptional silencing can be initiated by the sumoylation of a transcription factor, which in turn recruits the machinery to modify the chromatin template, thereby altering accessibility to other factors.

3.6 SUMO-Dependent Modulation of General Coregulator Activity

The recruitment of chromatin modifying complexes via a sumoylated transcriptional regulator is clearly a major way in which SUMO contributes to transcriptional regulation, as evidenced by the simple fact that artificially fusing SUMO to a transcription factor generally results in transcriptional repression (for example see (Ross et al. 2002)). However, other mechanisms have been proposed, such as the sequestration or relocalization of sumoylated proteins, as discussed for Sp3. Additionally, the transcriptional regulators CBP and Daxx have been shown to localize to PML oncogenic domains (PODs) in a SUMO dependent manner (Best et al. 2002). This localization prevents them from regulating transcription, however, once desumoylated by a SUMO protease, CBP and Daxx can be released from PODs allowing them to perform their gene regulatory functions.

The mechanisms discussed so far result in the inhibition of transcriptional activation by sumoylation, either by driving the recruitment of general repressors, or by removing the activator. However, sumoylation of general transcriptional corepressors, including HDACs, may alter their activity. Human HDAC1 can be sumoylated at two carboxyl-terminal lysines (David et al. 2002). Blocking sumoylation of HDAC1 resulted in decreased transcriptional repression, presumably by reducing histone deacetylation. Additionally, there is evidence that sumoylation affects HDAC1 stability, and this appears to be dependent on whether HDAC1 is modified by SUMO1 or SUMO2, suggesting both positive and negative effects of sumoylation (Citro et al. 2013). HDAC4 is a class II HDAC, which has a large amino-terminal domain with a high degree of similarity to the MITR transcription factor, and is known to be present in both nuclear and cytosolic compartments (Grozinger et al. 1999; McKinsey et al. 2001). Sumoylation of HDAC4 occurs at a single lysine close to the amino-terminus of its HDAC domain (Kirsh et al. 2002). Blocking HDAC4 sumoylation results in decreased deacetylase activity, suggesting that sumoylation is important for modulating deacetylase-mediated repression of gene expression. Although it is not clear how sumoylation of HDACs regulates deacetylase activity, it is possible that it has subtle effects on sub-cellular localization or interaction with other proteins that may alter HDAC activity. Support for sumoylation of general corepressors as a targeting mechanism comes from the sumoylation of HP1α driving recruitment to pericentric heterochromatin, as discussed earlier (Maison et al. 2011). Additionally, the lysine demethylase, KDM5B, is sumoylated and this results in increased occupancy at target genes, resulting in the demethylation of trimethylated H3K4 and transcriptional repression (Bueno and Richard 2013).

There is evidence for a more complex interplay of sumoylation and acetylation, which is dependent on HDAC4. MEF2 transcription factors can be sumoylated on a single conserved lysine, and this is increased by HDAC4, suggesting a role for HDAC4 as a SUMO E3 (Gregoire and Yang 2005; Zhao et al. 2005). Sumoylation of MEF2 at this site decreases its ability to activate gene expression. Interestingly, MEF2 can also be acetylated by the coactivator, CBP, on the same lysine at which it is sumoylated. In contrast to sumoylation, acetylation increases MEF2 activity (Zhao et al. 2005). The switch between acetylation and sumoylation of MEF2 is controlled by the class III HDAC, SIRT1, together with HDAC4. Thus, SIRT1 deacetyates MEF2, followed by HDAC4-dependent sumoylation of MEF2, together decreasing its transcriptional activation potential (Zhao et al. 2005). A similar mechanism for deacetylation followed by sumoylation, mediated by SIRT1 and HDAC4 has been demonstrated for HIC1, suggesting that this may be a more general mechanism (Stankovic-Valentin et al. 2007). In this case, sumoylated HIC1 can recruit the NuRD complex via interaction with MTA1, and NuRD complex recruitment can be inhibited by acetylation of HIC1 (Van Rechem et al. 2010). Thus, in addition to SUMO regulating the ability of HDACs to modify chromatin structure via the deacetylation of histones, there appears to be a more intricate

and complex interplay of sumoylation with other protein modifying activities emerging.

3.7 The Role of SUMO E3 Ligases in Chromatin Remodelling

Although sumoylation can occur in the absence of an E3 ligase, their presence can increase specificity and the efficiency of the sumoylation reaction (Johnson 2004). The presence of a SUMO E3 ligase as an integral part of a complex with chromatin modifying activity represents an efficient and specific way for sumoylation to regulate chromatin remodeling. One of the first SUMO E3s to be identified in mammalian cells was PIASy, a member of the SP-RING family of E3s (Sachdev et al. 2001). PIASy was shown to promote sumoylation of the transcription factor, LEF1, sequestering it at nuclear bodies and decreasing its ability to activate gene expression. PIASy was found to be present at MARs, perhaps suggesting a role in modulating higher order chromatin structure, a notion which is also supported by the fact that mutations in the gene encoding a member of the PIAS family have effects on position effect variegation in *Drosophila* (Hari et al. 2001). Indeed PIAS family members in mammalian cells are well characterized as transcriptional coregulators for multiple transcription factors, and they may perform other functions in addition to driving sumoylation of their interacting partners (Rytinki et al. 2009). The polycomb protein, Pc2/CBX4, was shown to be a SUMO E3 for the transcriptional corepressor, CtBP (Kagey et al. 2003). Pc2 was first identified based on its homology to the *Xenopus* homolog of *Drosophila* Pc, and was shown to localize to sub-nuclear foci, or polycomb bodies (Satijn et al. 1997). Polycomb foci have been observed in numerous cell types, and presumably are centers of heterochromatic transcriptional silencing. Indeed, polycomb proteins localize to regions of pericentric heterochromatin on human chromosome 1 (Saurin et al. 1998). Although the domains of Pc2/CBX4 required for E3 activity are well conserved among vertebrates (Kagey et al. 2005; Wotton and Merrill 2007), there is relatively little similarity of these domains to the founding member of the family, *Drosophila* Pc, suggesting that Pc2/CBX4 E3 activity is a vertebrate specific function. Additional SUMO substrates for Pc2 have been identified, including the *de novo* methyl transferase, Dnmt3a, the kinase HIPK2 and the zinc finger proteins, SIP1 and CTCF (Li et al. 2007; Long et al. 2005; Macpherson et al. 2009; Roscic et al. 2006). In most cases Pc2/CBX4 SUMO substrates colocalize at polycomb foci, raising the possibility that colocalization with Pc2 may contribute to maintaining substrate sumoylation. Although Pc2 has *in vitro* E3 activity (Kagey et al. 2003), it remains possible that *in vivo*, it also functions in part by protecting sumoylated proteins from desumoylation. Although relatively little is known about whether sumoylation contributes to polycomb body formation, it is tempting to speculate that SUMO plays a role, as it does with PML domains (Muller et al. 1998; Shen et al. 2006). Recent work has begun to suggest that the different functions of Pc2/CBX4 may regulate separate processes. Analysis of a role for Pc2/CBX4 in the homeostasis of epidermal stem cells and the epithelial identify of keratinocytes provides evidence that the highly conserved chromodomain, which binds methylated H3K9 and H3K27 (Bernstein et al. 2006) is required to limit cellular senescence. In contrast, SUMO mediated functions, dependent on the SIMs limit differentiation, and inactivating either function alone was shown to have differential effects on gene expression programs (Luis et al. 2011; Mardaryev et al. 2016). However, there is also evidence that H3K27Me3 binding and SUMO dependent activities within Pc2/CBX4 may be coordinated. Pc2/CBX4 is known to be sumoylated, and may function as an E3 for its own modification (Merrill et al. 2010). Recruitment of the Pc2/CBX4-containing PRC1 polycomb complex to the promoters of the *Gata4* and *Gata6* genes was shown to require the chromodomain binding to H3K27Me3, but this was modulated by sumoylation of Pc2/CBX4 (Kang et al. 2010). Desumoylation of Pc2/CBX4 driven by SENP2 reduced recruitment to the *Gata4* and *Gata6* genes, suggesting that sumoylated Pc2/CBX4

was better able to bind heterochromatin via its chromodomain.

Further support for the role of SUMO E3s as integral components of chromatin remodeling complexes comes from the analysis of the KAP1 transcriptional corepressor (also known as TIF1β) (Ivanov et al. 2007). KAP1 contains a PHD domain, which has some sequence similarity to the class of RING finger domains found in SUMO E3s (Hochstrasser 2001). In addition, KAP1 has an adjacent bromodomain, which binds acetylated histones H3 and H4. KAP1 is sumoylated within its bromodomain, dependent on the adjacent PHD domain, suggesting that this domain acts as an intra-molecular SUMO E3 (Ivanov et al. 2007). Sumoylation of the KAP1 bromodomain results in increased interaction with SETDB1 and the CHD3-containing NuRD complex. A model emerges in which sumoylation of KAP1 results in histone deacetylation, via the HDAC components of the NuRD complex. This is followed by SETDB1-dependent histone methylation, which facilitates binding of HP1 via its chromodomain to the methylated histone tails. Thus KAP1 SUMO E3 activity initiates a series of protein modifications, which result in the recruitment and spreading of HP1 to generate a locally silenced chromatin domain. One point to note with respect to chromatin associated SUMO E3s, such as KAP1, is that they may have relatively few SUMO substrates. This is in contrast to some members of the SP-RING family of E3s and RanBP2/Nup358, which may play much more general roles in sumoylation. The regulation of chromatin accessibility is modulated by protein complexes, which possess multiple enzymatic activities, including sumoylation. This can result in the modification of both histones and non-histone proteins in a coordinate manner, to regulate the accessibility of the DNA template. In summary, it is clear that sumoylation has joined the longer known protein modifications as a key regulator of chromatin dynamics. However, while much of the initial evidence suggested that sumoylation was repressive, there is emerging evidence that things may be more complex than this.

3.8 Global Analysis of Chromatin Modification by SUMO

In much of what has been discussed so far, the addition of SUMO results in decreased chromatin accessibility and transcriptional silencing. While this is clearly a major role of nuclear SUMO (Gill 2005), other possibilities should be considered. In *S. cerevisiae*, SUMO was found at the promoters of actively transcribed genes, but not at repressed genes (Rosonina et al. 2010). Activation of inducible gene expression was associated with increased SUMO and Ubc9 at their promoters. Inactivation of Ubc9 resulted in less promoter-associated SUMO and increased transcription due to a delay in shutting off gene expression. While this analysis focused on selected genes, it clearly raises the possibility that sumoylation on chromatin may play complex regulatory roles that cannot simply be defined as a repressive mark. This likely comes in part from the fact that sumoylation occurs not only, or even primarily, on histones, but on a large array of other chromatin associated proteins as well. It was later shown that the Gcn4 transcription factor was sumoylated at the promoters of inducible genes and that this facilitated its removal from chromatin following RNA polymerase II recruitment (Rosonina et al. 2012). Interestingly, the removal of Gcn4 was enhanced by the presence of the corepressor Tup1, which is also subject to regulation by sumoylation, suggesting that the coordinated modification of both activators and repressors by SUMO may be required (Ng et al. 2015). Thus unraveling functional consequences of chromatin sumoylation across the genome may require a knowledge of which proteins are conjugated with SUMO at each locus.

Several recent studies have analyzed the genome-wide distribution of SUMO in mammalian cells, and begun to address the consequences of sumoylation (Liu et al. 2012; Neyret-Kahn et al. 2013; Niskanen et al. 2015; Seifert et al. 2015). These analyses reveal some common themes, and also highlight some additional questions. In contrast to what might be expected,

genome-wide analyses found SUMO to be primarily localized to regions of active open chromatin. SUMO-enriched regions were more associated with activating chromatin marks such as H3K4 methylation, rather than with repressive histone modifications. This is perhaps in line with the idea that histone H4 sumoylation has been proposed to reduce nucleosome packing (Dhall et al. 2014). However, analysis of SUMO distribution by ChIP-seq likely reflects a combination of histone sumoylation and SUMO modification of other chromatin bound factors. In one study, the authors identify SAFB1 as one potential factor that is sumoylated at the promoters of highly transcribed genes, and further suggest that sumoylated SAFB1 promotes RNA PolII recruitment (Liu et al. 2012, 2015). An alternative suggestion is that SUMO enrichment at active promoters is due to sumoylation of components of the pre-initiation complex (Neyret-Kahn et al. 2013). Analysis of the effects of cellular stress on SUMO distribution may help explain the perhaps surprising finding that SUMO is primarily present at active genes (Niskanen et al. 2015; Seifert et al. 2015). One proposed function for the accumulation of SUMO2 at the transcriptional start sites of active genes is that sumoylation correlates with polymerase pausing and thereby limits the transcriptional response to heat-shock (Niskanen et al. 2015). In line with this, SUMO was found to be primarily correlated with the promoters of histone genes as well as those involved in protein biogenesis, and at PolI and PolIII transcribed genes, and depletion of SUMO resulted primarily in up-regulation of expression (Neyret-Kahn et al. 2013). The suppressive role of sumoylation suggested by these two studies is in contrast to the apparently activating role of SAFB1 sumoylation (Liu et al. 2015). However, on depletion of SUMO there were both increases and decreases in gene expression, as would be expected when examining transcription on a genome-wide scale. One possible explanation for apparently opposing effects of sumoylation is suggested by a second analysis of the response to stress (Seifert et al. 2015). Here, the authors show that SUMO2 is induced at active nucleosome depleted regions of the genome in response to heat-shock, but does not simply correlate with increased or decreased gene expression. Rather, they suggest that sumoylation acts to maintain the integrity of large chromatin bound protein complexes under conditions of stress, and possibly to some degree under normal cellular conditions. In this scenario, inhibition of sumoylation might activate some genes and repress others, depending on whether the particular gene is under the control of a regulatory complex that requires sumoylation for its integrity.

In summary, these recent analyses suggest that the role of sumoylation on the chromatin template is perhaps more complex than originally thought. However, they clearly point to SUMO as a regulator of highly expressed genes, and suggest that SUMO is primarily at relatively open chromatin regions. Any interpretation of the effects of SUMO depletion on genome-wide chromatin accessibility must be tempered by the potentially opposing effects of this modification on its multiple substrates. In the future it will be of interest to begin to dissect how sumoylation of specific chromatin associated proteins or protein complexes affects accessibility and downstream function.

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Functions of SUMO in the Maintenance of Genome Stability

4

Nicola Zilio, Karolin Eifler-Olivi, and Helle D. Ulrich

Abstract

Like in most other areas of cellular metabolism, the functions of the ubiquitin-like modifier SUMO in the maintenance of genome stability are manifold and varied. Perturbations of global sumoylation causes a wide spectrum of phenotypes associated with defects in DNA maintenance, such as hypersensitivity to DNA-damaging agents, gross chromosomal rearrangements and loss of entire chromosomes. Consistent with these observations, many key factors involved in various DNA repair pathways have been identified as SUMO substrates. However, establishing a functional connection between a given SUMO target, the cognate SUMO ligase and a relevant phenotype has remained a challenge, mainly because of the difficulties involved in identifying important modification sites and downstream effectors that specifically recognize the target in its sumoylated state. This review will give an overview over the major pathways of DNA repair and genome maintenance influenced by the SUMO system and discuss selected examples of SUMO's actions in these pathways where the biological consequences of the modification have been elucidated.

Keywords

SUMO • DNA repair • Genome stability • Homologous recombination • Base excision repair • DNA replication • Telomeres • Chromosome segregation • Poly-SUMO chains • SUMO-targeted ubiquitin ligases

Abbreviations

ALT	alternative lengthening of telomeres
DSB	double-strand break
dsDNA	double-stranded DNA

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E1	SUMO (or ubiquitin) activating enzyme
E2	SUMO (or ubiquitin) conjugating enzyme
E3	SUMO (or ubiquitin) protein ligase
FA	Fanconi anemia
HR	homologous recombination
HU	hydroxyurea
MEF	mouse embryonic fibroblast
MMS	methyl methanesulfonate
NHEJ	non-homologous end joining
ORC	origin recognition complex
PIP	PCNA-interacting protein
pre-RC	pre-replication complex
rDNA	ribosomal DNA
RFC	replication factor C
RPA	replication protein A
SAC	spindle assembly checkpoint
SENP	sentrin-specific protease
SIM	SUMO-interacting motif
SMC	structural maintenance of chromosomes
SP-RING	Siz/PIAS really interesting new gene
ssDNA	single-stranded DNA
STUbL	SUMO-targeted ubiquitin ligase
WT	wild-type

4.1 Introduction

Our cells face the constant challenge of protecting their DNA from spontaneous and exogenous insults that include single- and double-strand breaks (DSBs), various base adducts (Lindahl 1993), replication blocks and topological stress. Dealing with these problems is essential for the maintenance of genome stability because mutations arising from unrepaired DNA can lead to loss or incorrect transmission of genetic information, which in turn can predispose to cancer (Hanahan and Weinberg 2011). Therefore, cells have evolved many mechanistically diverse DNA repair and genome maintenance pathways that are able to respond to damage rapidly, ensuring that mutations do not become fixed in the genome (Hoeijmakers 2001). One way of achieving this responsiveness is the activation or modulation of the properties of key DNA repair factors through post-translational modifications, which usually result in changes in their activities, localization or interactions with other cellular proteins (Huang and D'Andrea 2006).

Like other post-translational modifiers such as phosphate groups and ubiquitin, the ubiquitin-like protein SUMO is important for the maintenance of genome stability (Huang and D'Andrea 2006). On one hand, budding (*Saccharomyces cerevisiae*) and fission yeast (*Schizosaccharomyces cerevisiae*) mutants of the SUMO E1, E2 and some E3 enzymes are hypersensitive to various DNA damaging agents, accumulate gross chromosomal rearrangements, lose mini-chromosomes frequently and fail to maintain telomeres and segregate chromatids properly (Tanaka et al. 1999; Ho and Watts 2003; Maeda et al. 2004; Xhemalce et al. 2004, 2007; Andrews et al. 2005; Zhao and Blobel 2005; Motegi et al. 2006; Takahashi et al. 2006; Watts et al. 2007). Altering the normal regulation of sumoylation is also detrimental to some pathways of DNA repair in human cells (Li et al. 2000; Potts and Yu 2005). On the other hand, proteins involved in many of the main DNA repair pathways have been shown to be sumoylated. These include components of nucleotide excision repair [e.g. XPC and XRCC4 (Wang et al. 2005; Yurchenko et al. 2006)], base excision repair [e.g. thymine DNA glycosylase, (Hardeland et al. 2002)], homologous recombination [HR, e.g. Rad52, PCNA and the RECQ family of DNA helicases (Kawabe et al. 2000; Hoege et al. 2002; Eladad et al. 2005; Sacher et al. 2006)] and non-homologous end joining [NHEJ, e.g. Ku70 (Zhao and Blobel 2005)]. Sumoylation also modulates the functions of proteins that are not directly involved in DNA repair but that nonetheless play a role in preserving genome stability, such as DNA replication factors (Wei and Zhao 2016), topoisomerase II (Bachant et al. 2002) and many proteins essential to protect telomeres (Potts and Yu 2007).

Recent advances in mass spectrometry have revealed so many new sumoylation substrates that our insight into SUMO's mechanism of action is lagging far behind the number of its

known targets (Hendriks et al. 2014; Lamoliatte et al. 2014; Tammsalu et al. 2014). For some of the proteins mentioned above, however, we understand how SUMO alters their functions. Beginning with a brief overview over the components of the SUMO system relevant to genome maintenance, this review will highlight such cases, focusing on DNA replication, homologous recombination, base-excision repair, telomere maintenance, and chromosome segregation pathways. From these examples, it will become clear that whatever the downstream effect of sumoylation may be, it usually involves a change in the affinity of the modified proteins for either other proteins or DNA.

4.2 Components of the SUMO Pathway

Rather than giving a full account of the SUMO system here, the intention of this section is to provide a brief mechanistic overview over those features relevant for understanding SUMO metabolism and highlight those components that play prominent roles in genome maintenance.

4.2.1 SUMO Proteins

SUMO belongs to the family of ubiquitin-like modifiers, which share a common three-dimensional structure and a C-terminal di-glycine motif needed for attachment to a lysine residue via an isopeptide bond (van der Veen and Ploegh 2012). In contrast to other ubiquitin-like modifiers, SUMO possesses a long flexible N-terminal tail. While only one SUMO paralogue is present in budding or fission yeast (Smt3 or Pmt3, respectively), human cells have four different SUMO isoforms, SUMO1–4. All are translated as longer precursors that need to be cleaved to obtain the corresponding mature forms. SUMO1 shares about 48% sequence identity with SUMO2, while SUMO2 and SUMO3 are highly similar with 95% sequence identity (Saitoh and Hinchey 2000). Therefore, these two isoforms, which are most closely related to the fungal proteins, cannot be distinguished via immune-staining and are usually referred to as SUMO2/3. SUMO4 seems to be processed to its mature form only under rare conditions and has so far only been described to be conjugated to other proteins in serum-starved cells (Wei et al. 2008).

Like ubiquitin, SUMO2/3, Smt3 and Pmt3 can form polymeric chains on their substrates (Tatham et al. 2001; Bylebyl et al. 2003; Matic et al. 2008; Windecker and Ulrich 2008). These are predominantly linked via lysine residues in the N-terminal tail. SUMO chains play important roles in genome maintenance, as demonstrated by the fact that budding yeast mutants accumulating them show pleiotropic phenotypes, including hypersensitivity to genotoxins (Bylebyl et al. 2003). While mono-sumoylation can act antagonistically to ubiquitylation (Desterro et al. 1998), poly-SUMO chains can induce ubiquitylation and subsequent proteasome-mediated degradation, with important implications for genome stability (see Sect. 4.2.4).

4.2.2 SUMO Ligases

SUMO ligases boost the efficiency and determine the substrate specificity of sumoylation events mediated by the sole SUMO-specific E2, UBC9. The largest, most conserved category of SUMO E3s is the PIAS/SIZ family of proteins. In mammals it includes PIAS1, PIAS2 (PIASx), PIAS3 and PIAS4 (PIASy), which were initially described as protein inhibitors of the activated JAK-STAT signaling pathway. Fungal PIAS/SIZ proteins are Siz1 and Siz2 in budding yeast, and Pli1 in fission yeast. Both in vitro and in vivo, these enzymes show a significant amount of redundancy (Reindle et al. 2006). Mms21 (also known as Nse2 in fission yeast and NSMCE2 in humans) also contains an SP-RING domain, but does not strictly belong to the PIAS/SIZ family of proteins. This E3 will be discussed in detail in Sect. 4.5.1.

Structurally, PIAS/SIZ proteins share a modular architecture that consists of four domains. The N-terminal SAP domain interacts with DNA but is dispensable for catalytic activity (Okubo et al.

2004; Takahashi et al. 2005; Parker et al. 2008; Suzuki et al. 2009). The PINIT motif directly contacts certain sumoylation substrates and helps determine the selectivity for both the target protein and the target site (Takahashi et al. 2005; Yunus and Lima 2009). The SIZ/PIAS RING (SP-RING) finger harbors the catalytic activity (Kotaja et al. 2002; Takahashi et al. 2005; Yunus and Lima 2009). It resembles the RING finger of ubiquitin E3s but, unlike such folds, which sport two zinc-coordinating loops, the SP-RING domain contains only one. The second loop is instead held together by hydrogen bonds and Van der Waals forces (Duan et al. 2009; Yunus and Lima 2009). Like RING-type ubiquitin ligases, PIAS/SIZ proteins enhance sumoylation likely by facilitating the interaction between the SUMO-loaded E2 and its substrates. At their C-termini, PIAS/SIZ E3s contain a SUMO-interacting motif (SIM). This motif is not essential for catalytic activity but promotes SUMO conjugation, probably by contacting the SUMO appendage of the charged UBC9 (Takahashi et al. 2005; Yunus and Lima 2009).

PIAS1 and PIAS4 play critical roles in the response to DNA DSBs. In human cells, these lesions trigger a cascade of events controlled by different types of post-translational modifications, such as phosphorylation and ubiquitylation, which leads to the formation of microscopically visible repair foci and culminates in the recruitment of the repair factor BRCA1 (Jackson and Bartek 2009). SUMO1, SUMO2/3, UBC9, PIAS1, PIAS4 and MMS21 are all recruited to such DNA repair foci, and depleting PIAS1 or PIAS4, but not MMS21, obstructs their formation. However, PIAS1 and PIAS4 do not act redundantly. While PIAS4 is required for the recruitment of SUMO2/3, PIAS1 is necessary to recruit SUMO1, and each E3 appears to mediate the accumulation of a different set of additional signaling factors (Galanty et al. 2009; Morris et al. 2009). Consistent with a role in controlling DNA repair, depletion of PIAS1 or PIAS4 renders cells sensitive to various genotoxins and reduces their ability to mend DSBs by HR and NHEJ. The relevant substrates remain unclear, but one of them could be BRCA1 itself. Although depletion of PIAS1/4 also affects factors upstream of BRCA1 in the pathway, BRCA1 is modified by SUMO1 and SUMO2/3 in a PIAS1/4-dependent manner following exposure to genotoxic stress (Galanty et al. 2009; Morris et al. 2009). Sumoylation likely enhances BRCA1's ubiquitin ligase activity, as mutating a sumoylation consensus motif within BRCA1 reduces the formation of K6-linked ubiquitin chains in vivo, a chain type characteristic for BRCA1 activity. Consistent with these results, in vitro sumoylation of BRCA1 enhances its activity by an order of magnitude (Morris et al. 2009).

4.2.3 SUMO Proteases

SUMO proteases catalyze both the maturation of SUMO and its deconjugation from target proteins. The largest category of SUMO proteases is the family of sentrin-specific proteases (SENPs). It comprises two members in budding yeast, Ulp1 and Ulp2, and six members in mammalian cells, SENP1, −2, −3, −5, −6 and −7 (Hickey et al. 2012; Nayak and Muller 2014). These proteases have varying preferences for the different SUMO paralogues and chain lengths and exhibit distinctive localizations within the cell, which largely determines their substrate specificity. Ulp1 is targeted to the nuclear pore and processes a broad range of substrates. It is also responsible for the maturation of most of the SUMO translational fusions (Li and Hochstrasser 2003). Ulp2, on the other hand, is nucleoplasmic and has a strong preference for poly-sumoylated target proteins (Li and Hochstrasser 2000; Bylebyl et al. 2003). The mammalian homologue of Ulp2, SENP6, and its closest relative, SENP7, preferentially deconjugate SUMO chains in biochemical assays and are distributed throughout the nucleoplasm (Drag et al. 2008; Lima and Reverter 2008). SENP1 and SENP2 localize to the nuclear pore, and SENP3 and SENP5 show preferential retention at the nucleolus (Hang and Dasso 2002; Gong and Yeh 2006).

4.2.4 SUMO-Targeted Ubiquitin Ligases

SUMO-targeted ubiquitin ligases (STUbLs) are ubiquitin E3s that recognize SUMO moieties via internal SIMs and thereby specifically ubiquitylate sumoylated proteins (Fig. 4.1). Three STUbLs have been described in yeasts. *S. cerevisiae* Uls1 is a large RING finger protein that binds to SUMO via four internal SIMs (Fig. 4.1a). Although *uls1* mutant cells accumulate SUMO conjugates, efficient ubiquitylation of sumoylated proteins by Uls1 has so far not been validated biochemically (Hannich et al. 2005; Uzunova et al. 2007). Budding yeast Rad18 exhibits a highly specific STUbL-like function towards its substrate, PCNA, and will be discussed in detail in Sect. 4.5.4 (Parker and Ulrich 2012). The third and major STUbL, which is present in both budding and fission yeasts, is the RING E3 Slx8 (Mullen et al. 2001; Ii et al. 2007a; Uzunova et al. 2007; Xie et al. 2007). Its RING domain forms an obligatory complex with a SUMO-binding subunit, Slx5 in budding yeast, and Rfp1 (or the redundant Rfp2) in fission yeast (Prudden et al. 2007; Sun et al. 2007; Uzunova et al. 2007; Xie et al. 2007; Mullen and Brill 2008). Slx5's preference for poly-SUMO chains (Uzunova et al. 2007) is likely shared by Rfp1 and Rfp2 because they, like Slx5, possess multiple SIMs (Prudden et al. 2007; Sun et al. 2007). Accordingly, Slx5/8 and Rfp1/2-Slx8 efficiently ubiquitylate a model substrate in vitro only if it is sumoylated (Sun et al. 2007; Mullen and Brill 2008). In vivo, the presence of proteins that are simultaneously sumoylated and ubiquitylated strictly depends on Slx5/8 and the ability of SUMO to form chains (Uzunova et al. 2007).

In budding and fission yeasts, inactivating the Slx8 complex results in increased levels of sumoylated species (Burgess et al. 2007; Ii et al. 2007b; Uzunova et al. 2007; Wang et al. 2006; Xie et al. 2007; Mullen and Brill 2008). A similar phenotype results from defects in ubiquitin conjugation or proteasome activity (Uzunova et al. 2007), indicating that Slx8 generally mediates the ubiquitin-dependent degradation of poly-sumoylated proteins (Uzunova et al. 2007, Fig. 4.1b). The ability of Slx8-like complexes to modify sumoylated proteins and potentially target them for degradation is important for genome stability. In budding and fission yeasts, Slx8-complex mutants are hypersensitive to various genotoxins such as hydroxyurea (HU) and methyl-methanesulfonate (MMS) (Mullen et al. 2001; Zhang et al. 2006; Kosoy et al. 2007; Ii et al. 2007b; Prudden et al. 2007; Sun et al. 2007; Xie et al. 2007; Mullen and Brill 2008). They also show a high incidence of events that may arise from the repair of spontaneous DSBs by HR, such as Rad52 foci during S phase, gross chromosomal rearrangements, gene conversion events and small point mutations (Zhang et al. 2006; Burgess et al. 2007; Prudden et al. 2007; Nagai et al. 2008).

It should be noted that the Slx8 complex also comprises SUMO-independent functions as demonstrated for the transcriptional repressor Matα2, needed to control mating and differentiation. For example, Slx5/8 was shown to ubiquitylate unmodified Matα2 and was able to trigger proteasomal turnover of Matα2 also in the absence of a functional sumoylation system (Xie et al. 2010).

Despite a striking difference in structure and size, the small mammalian RING finger protein RNF4 can rescue the genome stability defects of Slx5/8-deficient yeast cells, clearly demonstrating that the function of STUbLs is evolutionary conserved among eukaryotes (Prudden et al. 2007; Sun et al. 2007). In addition to its RING domain, RNF4 contains four SIMs (SIM1–4), which explains its preference for poly-SUMO chains (Tatham et al. 2008, Fig. 4.1a). SIM2 and SIM3 have been shown to be necessary and sufficient for the binding to chains of at least two SUMO moieties, while SIM4 only contributes to interactions with longer chains. SIM1, on the other hand, is likely irrelevant for SUMO binding, resulting in three functional SIMs in RNF4 (Keusekotten et al. 2014). Binding to SUMO chains induces homodimerization and thereby activation of RNF4, promoting the transfer of ubiquitin to the distal SUMO of the chain (Rojas-Fernandez et al. 2014; Xu et al. 2014).

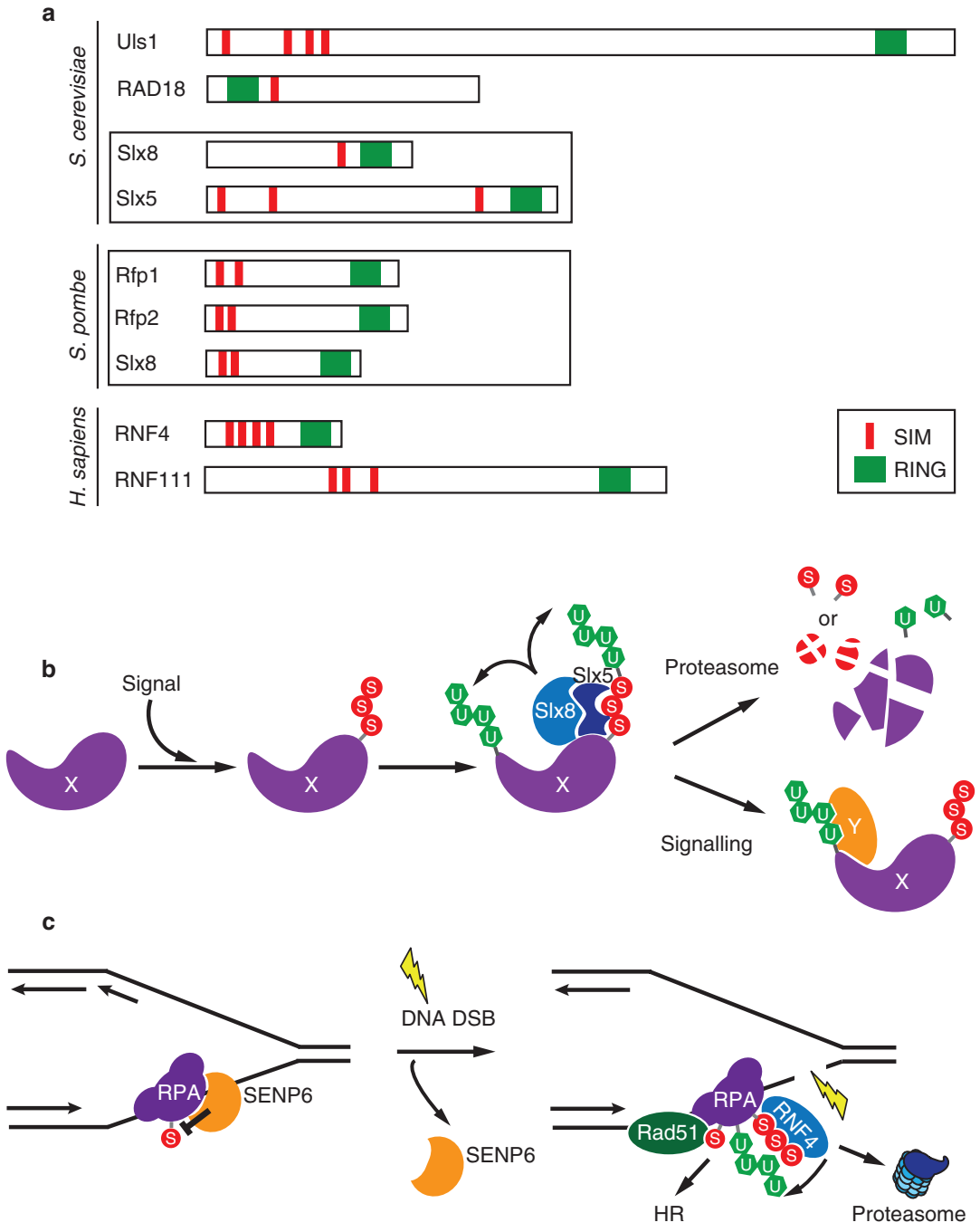

Fig. 4.1 *Ubiquitylation of poly-sumoylated proteins via SUMO-targeted ubiquitin ligases.* (**a**) Domain architecture of the SUMO-targeted ubiquitin ligases (STUbLs) from yeast and humans. (**b**) In response to a signal, a poly-SUMO (S) chain may form on a certain sub-population of protein X. By means of its SIMs, the Slx5/8 complex recognizes such poly-sumoylated substrate and ubiquitylates it (U). Following ubiquitylation, the modified substrate is degraded by the proteasome. Whether the SUMO moieties are degraded together with the substrate or deconjugation occurs before proteolysis is unknown. Adapted from Ulrich (2008). (**c**) RPA binds to ssDNA during replication. Under undisturbed replication conditions, RPA is kept in a hyposumoylated state through associating with the SUMO protease SENP6. After DNA double-strand formation during replication, SENP6 dissociates from the chromatin, allowing poly-sumoylation of RPA. On the one hand, this induces the recruitment of RAD51 to DNA DSBs. On the other hand, it promotes binding of RNF4 to the poly-SUMO chains via its internal SIMs and poly-ubiquitylation of RPA. The poly-ubiquitin chains serve as a signal for proteasomal degradation of RPA, allowing its replacement by Rad51 needed for repair of the DSBs via homologous recombination

RNF4 has been extensively characterized in the context of arsenic-induced degradation of PML and its oncogenic variant PML-RARα, a fusion protein expressed in acute promyelocytic leukemia (Lallemand-Breitenbach et al. 2008; Tatham et al. 2008; Weisshaar et al. 2008). In RNF4-depleted cells, sumoylated PML and mixed poly-ubiquitin and poly-SUMO chains highly accumulate in the nucleus, clearly indicating that RNF4 targets these substrates for degradation (Tatham et al. 2008). Other proteins that co-localize with PML in so-called PML bodies carry poly-SUMO chains, suggesting a more global function for RNF4 in PML body turnover. While these findings suggest that RNF4 mainly catalyzes the attachment of K48-linked ubiquitin chains for proteasomal degradation of target proteins, depletion of RNF4 has also been described to result in a decrease in K63 ubiquitin chains (Yin et al. 2012). In line with this finding, RNF4 can cooperate with the K63-specific ubiquitin E2 UBC13-UEV1 in vitro to poly-ubiquitylate an N-terminally mono-ubiquitylated SUMO2 (Tatham et al. 2013). Rather than leading to degradation, modification with these chains is generally assumed to facilitate complex assembly and signal transduction.

Apart from a striking increase in SUMO chains, RNF4-deficient cells as well as RNF4 knockout mice show increased sensitivity to a variety of DNA damaging agents (Luo et al. 2012; Yin et al. 2012; Vyas et al. 2013). Additionally, *RNF4*$^{-/-}$ mice show impaired spermatogenesis, as described after depletion of other regulators of DSB repair. These studies implicate a key role for RNF4 in the assembly and disassembly of DNA repair complexes at the sites of DNA DSBs. Without RNF4, important DNA damage response factors such as MDC1, RNF8, 53BP1, and BRCA1 are still recruited to DSBs but their removal from repair foci is delayed (Luo et al. 2012; Yin et al. 2012).

A second mammalian STUbL, RNF111/Arkadia, has been identified by computational means (Sun and Hunter 2012). Similar to RNF4, Arkadia contains a RING domain and three clustered SIMs (Erker et al. 2013). While the SIMs do not seem to be required for Arkadia's function in the TGF-β pathway, they are essential for its interaction with sumoylated PML after arsenic treatment. Indeed, sumoylated PML highly accumulates after depletion of Arkadia, suggesting a destabilizing effect of Arkadia on PML bodies similar to RNF4 (Erker et al. 2013). However, Arkadia and RNF4 do not form heterodimers, but seem to act independently on PML. Apart from its proteolytic activity towards PML, Arkadia catalyzes the formation of K63-linked ubiquitin chains on sumoylated XPC, a key regulator of nucleotide excision repair, after UV treatment. Ubiquitylation by Arkadia strongly induces XPC recruitment to UV lesions, revealing an essential non-proteolytic function for this STUbL in DNA repair (Poulsen et al. 2013).

4.2.5 SUMO-Targeted Ubiquitin Proteases

Given the importance of STUbLs, it is not surprising to find a class of proteases that catalyze the reverse reaction, i.e. the removal of ubiquitin from SUMO or sumoylated proteins. One such enzyme is Wss1, a metalloprotease from budding yeast. It was initially linked to SUMO by the observation that its deletion suppresses the phenotypes of a SUMO mutant, *smt3–331* (Biggins et al. 2001). Indeed, Wss1 directly binds to SUMO and efficiently deubiquitylates ubiquitin-SUMO hybrid chains and also ubiquitin-SUMO fusion proteins in vitro. Although Wss1 also interacts with proteasomal subunits, it exhibits direct proteolytic activity towards poly-SUMO chains. In contrast, its activity on ubiquitylated substrates and poly-ubiquitin chains is not as pronounced. Hence, it is not entirely clear whether Wss1 acts as a SUMO protease or a SUMO-targeted ubiquitin protease (Mullen et al. 2010). Wss1 helps the formation of SUMO chains at sites of DNA damage through forming a complex with the ubiquitin-dependent protein segregase Cdc48/p97 and its adaptor Doa1. It then further promotes auto-cleavage and proteolytic degradation of associated proteins, resulting in extraction of sumoylated proteins from the chromatin (Balakirev et al. 2015).

In mammalian cells two potential SUMO-targeted ubiquitin proteases have been described, USP11 and USP7. USP11 is a functional interactor of RNF4 and specifically deubiquitylates ubiquitin-SUMO hybrid chains. It also stabilizes PML bodies through deubiquitylation of sumoylated PML, thereby directly antagonizing RNF4 function (Wu et al. 2014; Hendriks et al. 2015b). Downregulation of USP11 expression confers PML destabilization and several malignant characteristics to a glioma tumor cell line, such as increased proliferation, invasiveness and tumor growth, implying that USP11 might serve as an interesting novel cancer drug target (Wu et al. 2014).

USP7 deubiquitylates SUMO2 in vitro and in vivo and appears to specifically act on SUMO substrates at replication forks (Lecona et al. 2016). Inhibiting USP7 in cells increases the amount of sumoylated and ubiquitylated proteins on newly replicated chromatin, and additional inhibition of the Cdc48/p97 segregase further enhances this effect. This indicates that USP7 limits ubiquitylation of SUMO targets on chromatin, thus preventing their subsequent extraction by Cdc48/p97. In this way, USP7 appears to regulate the balance of post-translational modifications at and around replication forks that leads to a high concentration of SUMO at active forks, while ubiquitin conjugates dominate on mature chromatin (Lopez-Contreras et al. 2013). Although USP7 is clearly important for efficient DNA replication, its exact substrates in this context remain unknown.

4.3 SUMO Proteomics

The number of proteomic screens for SUMO targets has greatly expanded within the last decade. Due to substantial methodological improvements in mass spectrometry, hundreds of SUMO target proteins and thousands of SUMO sites are known to date, many of which imply far-reaching consequences for genome maintenance pathways. One recurring problem in the identification of SUMO targets is the observation that the fraction of sumoylated protein for a given target is usually minute and can strongly vary between different cell cycle stages or cellular responses. Therefore, the sumoylated fraction often has to be enriched via appropriate treatment of the cells and subsequent affinity purification. In addition, SUMO proteases are highly potent and therefore have to be inactivated by working with denaturing buffers or specific protease inhibitors.

To be able to efficiently purify endogenous SUMO targets from a wide range of samples, such as patient material or rare tissues, monoclonal antibodies raised against human SUMO1 and SUMO2 have been used for immunoprecipitation (Barysch et al. 2014). So far, almost 600 human SUMO targets have been identified in this manner (Becker et al. 2013). An issue with this method is that it requires relatively large amounts of starting material. For a much more efficient purification of SUMO targets, several proteomic approaches made use of N-terminally tagged SUMO alleles exogenously expressed in cells. Altogether, more than 3000 human proteins have been reported to be sumoylated in such studies (Vertegaal et al. 2006; Schimmel et al. 2008; Golebiowski et al. 2009; Matic et al. 2010; Tatham et al. 2011; Hendriks et al. 2014; Impens et al. 2014; Lamoliatte et al. 2014; Schimmel et al. 2014; Schou et al. 2014; Bursomanno et al. 2015; Hendriks et al. 2015a; Sohn et al. 2015; Tammsalu et al. 2014, 2015; Xiao et al. 2015). However, this approach is usually restricted to cultured cells, and overexpression of SUMO might lead to false positive results. Therefore, potential SUMO targets identified in this manner should first be validated via purification of the endogenous proteins.

The identification of sumoylation sites is even more challenging, since it relies on the detection of a proteolytic remnant of SUMO's C-terminus on the modified lysine residue, after digestion with a protease specific for basic amino acids. For ubiquitin targets, marked by a di-glycine remnant, this approach has been highly successful; however, the corresponding remnant of SUMO is too large to be efficiently identified via mass spectrometry. To overcome this difficulty, several approaches have made use of an additional proteolytic cleavage site introduced via

point mutations close to SUMO's C-terminus (Matic et al. 2010; Hendriks et al. 2014; Impens et al. 2014; Lamoliatte et al. 2014; Schimmel et al. 2014; Tammsalu et al. 2014, 2015; Bursomanno et al. 2015; Hendriks et al. 2015a; Sohn et al. 2015; Xiao et al. 2015). These mutant alleles can be exogenously expressed as tagged versions in the sample of interest. In this manner, more than 7000 SUMO sites have been reported under various conditions in human cells to date. Proteome-wide identification of SUMO sites under completely endogenous conditions, however, still remains unsolved.

Thanks to these developments, many new general characteristics of sumoylation have been revealed by analyzing the dynamics of the SUMO proteome during different cell cycle stages and in response to specific external stimuli (Golebiowski et al. 2009; Psakhye and Jentsch 2012; Hendriks et al. 2014; Schimmel et al. 2014; Cubenas-Potts et al. 2015; Hendriks et al. 2015c; Xiao et al. 2015). These studies strongly support the concept of SUMO group modification, i.e. the collective modification of an ensemble of functionally related proteins at their site of action (Johnson 2004; Matunis et al. 2006; Jentsch and Psakhye 2013). This concept, according to which SUMO acts as a "molecular glue" that promotes local protein-protein interactions in a relatively redundant manner, was first systematically substantiated for the HR-mediated repair of DSBs in yeast (Psakhye and Jentsch 2012), but appears to apply to other pathways relevant to genome maintenance, such as nucleotide excision repair and DNA replication, and may well turn out to be a common theme in protein sumoylation. In human cells, treatment with MMS not only triggers sumoylation of HR proteins, but also affects many chromatin remodelers and transcriptional regulators, suggesting an important role of sumoylation in changing chromatin dynamics and the transcriptional program in response to MMS (Hendriks et al. 2015c). Similarly, two proteomic screens that analyzed sumoylation after replication stress showed a dynamic sumoylation response on several components of the DNA replication machinery and on factors involved in DNA repair (Bursomanno et al. 2015; Xiao et al. 2015).

In conclusion, the recent advances in SUMO proteomics demonstrate that in contrast to those post-translational modifiers that mostly target specific proteins, SUMO can act on large protein complexes and functional networks to elicit a global cellular response to external stimuli.

4.4 Effects of SUMO on DNA Replication and Replication Stress

Accurate and complete DNA replication is essential for genome maintenance even in the absence of exogenous damage, as both over- and under-replication of the genome will inevitably lead to problems with subsequent chromosome segregation. Moreover, most types of DNA damage strongly interfere with the progression of replication forks. Hence, the response to replication stress appears to be a finely tuned reaction ranging from subtle effects that can be viewed as part of the normal replication process up to a full-blown damage response that follows from replication fork collapse and the emergence of replication-associated DSBs. The SUMO system has been shown to contribute to this process at several levels (reviewed by Garcia-Rodriguez et al. 2016).

4.4.1 SUMO in Replication Initiation

DNA replication initiates at characteristic sequences named origins of replication, which are marked as such by the association of the origin recognition complex (ORC). In preparation for replication, origins are primed for activation by the assembly of the pre-replicative (pre-RC) complex, which includes the hexameric MCM2–7 complex as a precursor of the replicative helicase. Conversion to the active helicase at the entry into S phase requires phosphorylation of the complex and association of additional subunits, Cdc45 and the GINS complex, which then allows DNA unwinding, recruitment of DNA polymerases and other accessory factors, and finally initiation of DNA synthesis (Fragkos et al. 2015). SUMO has

recently been reported to exert a subtle, but measurable, negative influence on this process. In budding yeast, all subunits of the MCM complex are subject to sumoylation (Wei and Zhao 2016). Interestingly, different E3s appear to act on the various subunits, and the modification patterns vary somewhat over the cell cycle (de Albuquerque et al. 2016). Overall, sumoylation was shown to exhibit a pattern complementary to MCM phosphorylation, i.e. it was found highest at the pre-RC stage upon loading of the inactive complex onto DNA, and diminished in the course of S phase. Inhibition of one of the relevant kinases, DDK, or interference with origin firing by other means prevented desumoylation of the MCM complex. In contrast, local enhancement of sumoylation by means of tethering a strong SUMO-binding domain to Mcm6 compromised helicase activation and thus inhibited origin firing, likely via the SUMO-dependent recruitment of a phosphatase, Glc7. This enzyme appears to preferentially interact with the sumoylated form of Mcm6, thereby preventing essential phosphorylation events required for helicase activation (Wei and Zhao 2016). The significance of such a complex sumoylation pattern of the different MCM subunits for replication initiation is not yet understood, and apparently the inhibitory effect of SUMO cannot be ascribed to the modification of an individual subunit.

In vertebrate systems, a similar effect may apply, although it appears to be regulated in a different fashion. In *Xenopus* egg extracts, SUMO exerts a negative influence on replication initiation, as inhibition of sumoylation caused an increase in origin firing (Bonne-Andrea et al. 2013). Here, the sumoylation target responsible for the effect was cyclin E, which was modified upon its recruitment to pre-RCs. The mechanistic details of this phenomenon have not been elucidated, but its recurrence in different organisms suggests that SUMO may contribute to limiting excessive origin firing. In this context, it is interesting that many pre-RC components, including ORC subunits, have been found to be sumoylated (Golebiowski et al. 2009), thus possibly indicating a case of group sumoylation at replication origins.

4.4.2 SUMO at Replication Forks and in Replication Stress

In human cells, SUMO has been shown to be strongly enriched at replication forks (Lopez-Contreras et al. 2013), and in budding yeast, numerous components of the replication machinery are sumoylated, such as subunits of DNA polymerases, the replicative clamp loader and the Rad27 flap endonuclease (Cremona et al. 2012). The relevance of this enrichment is not entirely clear yet, but the maintenance of appropriate sumoylation levels appears to be important for efficient replication, given the actions of the human SUMO-targeted ubiquitin protease USP7 at replication forks (Lecona et al. 2016). As described above (see Sect. 4.2.5), USP7 appears to counteract RNF4, which would otherwise target sumoylated replication factors for ubiquitylation and extraction from chromatin by the ubiquitin-dependent chaperone Cdc48/p97. In budding yeast, the STUbL complex Slx5/8 also appears to influence events at replication forks; however, here it seems more relevant as a response to fork damage. This is particularly important for refractive sequences such as CAG triplet repeats, which are prone to fragility and instability. During replication, these regions tend to localize to the nuclear periphery, where they have been suggested to undergo processing and fork restart by HR in a Slx5/8-dependent manner (Su et al. 2015). This principle of damage relocalization not only applies to damaged replication forks, but also to DSBs (see Sect. 4.5).

A cross-talk between SUMO and ubiquitin is also observed in the Fanconi anemia (FA) pathway, a system for the resolution of replication fork problems as well as DNA interstrand crosslinks (reviewed by Walden and Deans 2014; Coleman and Huang 2016). The FA pathway coordinates the cooperation between components of different repair systems, involving nucleotide excision repair, HR, and translesion synthesis. FA pathway mutations are associated with a rare hereditary disease, Fanconi anemia, which is associated with bone marrow and congenital abnormalities as well as cancer predisposition (reviewed by Kee and D'Andrea 2012). Two

components of this pathway, FANCI and FANCD2, form a heterodimer, the ID complex, which is loaded onto chromatin after stalling of replication forks. This is accompanied by several post-translational modification events, including phosphorylation and ubiquitylation, which facilitates the recruitment of downstream factors. After being loaded onto chromatin, both FANCI and FANCD2 are also sumoylated in a PIAS1/4-dependent manner. This promotes poly-ubiquitylation of the complex by RNF4 and subsequent extraction from the chromatin by Cdc48/p97 (Gibbs-Seymour et al. 2015). SENP6 antagonizes PIAS1/4-dependent sumoylation of FANCI and FANCD2, thus stabilizing the ID complex at stalled replication forks by abolishing RNF4-mediated ubiquitylation (Gibbs-Seymour et al. 2015).

Another sumoylation target within the FA pathway is FANCA, a subunit of the FA core complex, which acts as an ubiquitin ligase on the ID complex at stalled replication forks. A patient-derived point mutation in FANCA abolishes the interaction of this protein with another core complex subunit, FAAP20, and increases FANCA sumoylation (Xie et al. 2015). This in turn induces ubiquitylation of FANCA by RNF4 and subsequent proteasomal degradation, which prevents efficient execution of downstream events. Interestingly, not only the patient-derived mutant, but also wild-type (WT) FANCA, is sumoylated and targeted by RNF4, even though to a lesser extent, possibly suggesting that a regulated release of FANCA from the FA core complex is physiologically relevant. In conclusion, the extensive crosstalk between ubiquitylation and sumoylation fine-tunes the FA pathway at multiple levels.

4.5 Effects of SUMO on Homologous Recombination

HR involves the exchange or replacement of genetic information between homologous DNA regions, which is vital to repair DSBs and damaged replication forks, but also for the correct pairing and segregation of chromosomes during meiosis. When a DSB occurs, its ends are initially clipped by the MRX/MRN complex (Mre11-Rad50-Xrs2/Nbs1) and Sae1/CtIP and subsequently resected further by Exo1 and Sgs1 to produce 3′ single-stranded DNA (ssDNA) overhangs. This DNA is coated by the ssDNA-binding trimeric replication protein A (RPA, Rfa1–3), which is exchanged for Rad51 by means of Rad52 (or BRCA2 in vertebrates). The resulting Rad51-ssDNA filaments search DNA molecules for regions of homology. These are subsequently invaded by displacing the homologous strand. Following strand extension and capture of the second end, four-way DNA structures called Holliday junctions are generated, which migrate along the DNA to create extended heteroduplex regions. The junctions are eventually resolved by specific nucleases to yield two intact DNA molecules (reviewed by San Filippo et al. 2008). In addition to the proteins involved in the core pathway described above, additional factors can control when and where HR takes place. These factors include anti-recombinogenic helicases such as Sgs1, Srs2 and Rrm3 in budding yeast and WRN, BLM and RECQ5 in mammals (Branzei and Foiani 2007; Bachrati and Hickson 2008).

Sumoylation plays important roles in controlling HR at several stages. It affects overall damage-induced recombination rates in mammalian and yeast cells (Li et al. 2000; Maeda et al. 2004), but it also controls the initial resection/clipping of DSBs (Cremona et al. 2012). SUMO targets many proteins with well-established roles in this repair pathway in both budding yeast and human cells, such as the MRN/MRX complex, Sae2, Rad52, Rad59 and many more (Golebiowski et al. 2009; Cremona et al. 2012; Psakhye and Jentsch 2012). In response to DNA damage, several, although not all, of these proteins are synchronously sumoylated (Cremona et al. 2012; Psakhye and Jentsch 2012). This modification "wave" probably occurs due to the coordinated recruitment of multiple HR factors and a suitable SUMO E3 to DNA. On one hand, the process strictly depends on the resection of a DSB to ssDNA, which is necessary for HR proteins to

accrue on damaged DNA. In fact, deleting *mre11*, *exo1* or *sgs1* significantly reduces the sumoylation of recombination factors, while mutations that accumulate unusually high amounts of ssDNA, such as *cdc13*^{ts}, enhance the modification. On the other hand, the coordinated sumoylation of recombination factors requires the SUMO E3 Siz2 and its recruitment to DNA. The latter is likely mediated by a combination of two features: a direct binding of Siz2 to DNA via its SAP domain and a SIM-mediated interaction of Siz2's C-terminus with sumoylated Mre11. Although it remains to be determined whether the interaction between Siz2 and Mre11 actually depends on the sumoylation of Mre11 itself, this model would explain why deletion of *MRE11*, but not an allele encoding a catalytically inactive mutant, abolishes collective sumoylation of HR proteins (Cremona et al. 2012; Psakhye and Jentsch 2012).

Sumoylation apparently also influences where in the nucleus HR takes place. As described above for damaged replication forks, DSBs also re-localize to the nuclear envelope in budding yeast and cannot be efficiently processed in mutants where the integrity of the nuclear pore is compromised (Nagai et al. 2008). Recent findings demonstrate that the relocation of DSBs to the nuclear pore is dependent on poly-sumoylation mediated by the E3s Siz2 and Mms21 in G1 phase, which leads to the recruitment of Slx5/8 to DSBs. This STUbL then promotes the relocation of lesions to the nuclear envelope (Horigome et al. 2016). Accordingly, Slx5 colocalizes with Rad52 and Rad9 at repair foci in a SIM- and Slx8-dependent manner (Cook et al. 2009). Interestingly, when Slx5 is artificially targeted to undamaged DNA, it is sufficient to induce relocalization of these loci to nuclear pores, independently of previous sumoylation. While this essential function of Slx5/8 seems to be specific for repair processes in G1 phase, DSBs arising in S phase appear to trigger Mms21-dependent mono-sumoylation and subsequent relocalization to the nuclear periphery, but not the nuclear pore. In this case, association is mediated by the membrane protein Mps3 and is promoted by, but not dependent on, the presence of Slx5 (Horigome et al. 2016). This finding might also explain why another study found that deletion of *SLX8* does not affect the survival of cells where replication forks are transiently stalled or collapsed (Zhang et al. 2006).

Some aspects of SUMO with particular relevance to HR have been characterized in detail and will be discussed below: (1) the SUMO ligase activity of Mms21, (2) the sumoylation of the ssDNA-binding RPA complex, (3) of the recombinase Rad52, (4) of the eukaryotic DNA polymerase processivity factor PCNA, and (5) of the helicase Sgs1/BLM.

4.5.1 MMS21-Dependent Sumoylation

Mms21 (also called Nse2 or NSMCE2) is part of an essential complex defined by two structural maintenance of chromosome (SMC) proteins, Smc5 and Smc6, and several non-SMC elements, called Nse1–6 in yeast (Stephan et al. 2011). In addition to Smc5/6, eukaryotes possess two additional SMC complexes: cohesin (Smc1/Smc3) and condensin (Smc2/Smc4). SMC proteins share a common structure that consists of a central coiled coil, which brings their globular N- and C-termini together to form an ATPase domain, and a hinge region that mediates heterodimerization. It is generally accepted that SMC heterodimers encircle DNA providing structural support to chromosomes and possibly targeting non-SMC partners to relevant loci (Lehmann et al. 1995; Fousteri and Lehmann 2000; Lehmann 2005; Zhao and Blobel 2005; Taylor et al. 2008; Uhlmann 2016).

Mms21 is essential in almost all species tested so far, except for *Arabidopsis thaliana* and chicken DT40 cells, where SMC5 itself is also dispensable (Giaever et al. 2002; McDonald et al. 2003; Huang et al. 2009; Kliszczak et al. 2012; Jacome et al. 2015). Mutating or removing Mms21's catalytic domain is compatible with viability, but slows growth, sensitizes cells to various genotoxins and leads to increased levels of chromosome mis-segregation in both mitosis and meiosis, thus pointing to a specific role in genome maintenance (McDonald et al. 2003;

Pebernard et al. 2004; Andrews et al. 2005; Potts and Yu 2005; Zhao and Blobel 2005; Behlke-Steinert et al. 2009; Rai et al. 2011; Xaver et al. 2013; Liu et al. 2014; Yuan et al. 2014).

In budding yeast, these phenotypes most probably derive from the formation of toxic sister chromatid junctions at damaged replication forks that likely represent HR intermediates (Branzei et al. 2006). Presently, the Mms21 targets responsible for these phenotypes have not been identified. In fission yeast, the processing of damaged replication forks also seems to involve Mms21-dependent sumoylation (Pebernard et al. 2008). In a mutant where replication forks are induced to irreversibly collapse, the Smc5/6 complex re-localizes to sub-telomeric regions, which are sequences particularly prone to fork stalling. A similar re-localization is observed in MMS-treated WT cells. Additionally, a functional Smc5/6 complex is required for efficient HR and the repair of collapsed replication forks and DSBs (Ampatzidou et al. 2006; Potts et al. 2006). These phenotypes closely resemble those seen for other Smc5/6 complex mutants, which indicates that Mms21, as an integral component of this complex, is required to prevent DNA damage or that its absence creates toxic DNA structures. Accordingly, a budding yeast *mms21* mutant lacking the SP-RING domain not only shows a mild, but constitutive, activation of the DNA damage checkpoint, but it also requires a functional checkpoint to grow properly (Rai et al. 2011). Mms21 itself is phosphorylated upon activation of the S phase checkpoint during DNA replication. Inhibiting this modification causes a mild increase in the rate of chromosome loss after DNA damage and reduces sumoylation of Mms21 targets, suggesting that phosphorylation is required for full activation of this E3 (Carlborg et al. 2015).

Structural studies on budding yeast Mms21 show that it interacts with Smc5's coiled-coil domain via its N-terminus (Duan et al. 2009; Duan et al. 2011), while its C-terminus contains the catalytic SP-RING finger. Disrupting the Mms21-Smc5 interaction recapitulates many of the defects observed in *mms21* or Smc5/6 mutants, such as gross defects in chromosome segregation and reduced sumoylation of Mms21 targets (Bermudez-Lopez et al. 2015). A Smc5 mutant that proficiently binds to Mms21 and chromatin, but is defective in ATP binding, also impairs Mms21 ligase function. Considering that ATP binding seems to change the structure of the Smc5-Mms21 complex in vitro (Bermudez-Lopez et al. 2015), this suggests that an ATP-driven conformational change within the Smc5/6 complex could contribute to activating the E3 function of Mms21 (Bermudez-Lopez et al. 2015). The observation that SMC5 and NSE2 are epistatic in chicken DT40 cells with respect to DNA damage sensitivity supports this idea (Kliszczak et al. 2012).

Mms21 contributes to sumoylation of several proteins with known roles in DNA damage and repair, such as fission yeast Smc6, Nse3 and Nse4 (Andrews et al. 2005; Pebernard et al. 2008) and budding yeast Ku70, Smc5 and Bir1 (Zhao and Blobel 2005; Montpetit et al. 2006; Yong-Gonzales et al. 2012). In human cells, MMS21 also modifies SMC6, and several components of the telomeric shelterin complex (Potts and Yu 2005; Potts et al. 2006; 2007; see Sect. 4.7). Other prominent substrates of Mms21 include yeast and human cohesin subunits (Almedawar et al. 2012; McAleenan et al. 2012; Wu et al. 2012), as described in more detail below (see Sect. 4.7.1).

Although the consequences of Mms21-dependent sumoylation are often poorly understood, it appears that in many instances the sumoylated targets are subject to subsequent STUbL-mediated ubiquitylation and possibly proteasomal degradation. Accordingly, budding yeast Slx5/8 was shown to act on many Mms21-dependent SUMO conjugates (Albuquerque et al. 2013). One of these is Bir1, a component of the chromosome passenger complex, which regulates several key mitotic events, including activation of the spindle assembly checkpoint (SAC; Carmena et al. 2012). Upon mild replicative stress induced by a dysfunctional allele of the replication factor Mcm10, deletion of *SLX5* caused a SAC-mediated mitotic block and accumulation of sumoylated Bir1 (Thu et al. 2016). Moreover, inhibition of the proteasome led to a similar accu-

mulation of Bir1 SUMO conjugates, consistent with a model where the joint action of Mms21 and the Slx5/8 complex suppresses SAC activation via degradation of Bir1, thus allowing progression through mitosis in the presence of tolerable replicative stress (Thu et al. 2016).

4.5.2 Sumoylation of RPA

RPA serves as a platform for various ssDNA-associated protein complexes during a multitude of DNA transactions, including HR. The largest subunit of the human complex, RPA1 (RPA70), is sumoylated, but is kept in a hyposumoylated state during unperturbed S phase by means of a tight interaction with the SUMO protease SENP6 (Dou et al. 2010). In response to DSBs, SENP6 dissociates from RPA1, which thus becomes sumoylated at K449 and K577. This in turn leads to an increase in the number of HR events (Dou et al. 2010). On one hand, sumoylation of RPA1 boosts the interaction with RAD51 in vitro, suggesting that the modification could promote the assembly of the recombinogenic filament by means of enhancing the RPA1-RAD51 interaction (Dou et al. 2010). On the other hand, some of the consequences of RPA sumoylation may be mediated by RNF4. This STUbL is essential for the removal of RPA1 from resected DNA to allow the subsequent loading of RAD51. Hence, formation of RAD51 repair foci is abolished and RPA1 association is prolonged in cells depleted of RNF4. Similarly, an unsumoylatable mutant of RPA1 remains associated with chromatin after DSB formation (Galanty et al. 2012). RPA1 interacts with RNF4 in a SIM-dependent manner, and association with the proteasomal subunit PSMD4 is observed after DNA damage in the presence of RNF4. Although biochemical evidence for a preferential action of RNF4 on sumoylated RPA1 is still needed, these results strongly suggest that the extraction of RPA1 from the chromatin is mediated by sumoylation-induced, RNF4-dependent, ubiquitylation and subsequent proteasomal degradation (Galanty et al. 2012). Taken together, both mechanisms, i.e. RPA's induced binding to RAD51 and its extraction from damaged DNA after sumoylation, likely promote the formation of RAD51 filaments and might jointly facilitate DSB repair by HR (Fig. 4.1c).

4.5.3 Sumoylation of RAD52

Sumoylation of Rad52 is a widely conserved phenomenon observable in both budding and fission yeasts, *Xenopus laevis* egg extracts and human cells (Ho and Watts 2003; Leach and Michael 2005; Sacher et al. 2006; Ohuchi et al. 2008). However, the process is best understood in *S. cerevisiae*. Budding yeast Rad52 is sumoylated at K10, K11 and K220 both in vivo (via Siz2) and in vitro (in the absence of any E3). Whereas in vitro K220 is the predominant target and K10 and K11 appear to be modified as a consequence of K220 sumoylation, in vivo all three lysine residues are required for efficient sumoylation. This phenomenon may reflect the actions of an E3 in cells (Sacher et al. 2006). In vitro, sumoylation of Rad52 requires its C-terminal DNA-binding domain and is stimulated by naked or RPA-covered ssDNA, but not by Rad51 filaments (Altmannova et al. 2010). In vivo, the modification is boosted by DSBs induced during meiotic recombination or by DNA-damaging agents (Sacher et al. 2006). It also requires the presence of Mre11, but not its nuclease activity, and is enhanced by deleting *RAD51*, but not other factors involved in later steps of HR (Ohuchi et al. 2008). This suggests that Rad52 sumoylation may occur just before or at the time of Rad51 recruitment to a DSB. Artificially tethering Rad52 to DNA, via a sequence-specific DNA-binding domain, also promotes its sumoylation, even in the absence of exogenous DNA damage.

Functionally, sumoylation appears to mildly modulate the known properties of Rad52. In vitro, SUMO does not affect Rad52's oligomerization state or its interaction with Rad51 or RPA, but it reduces its affinity for both ssDNA and double-stranded DNA (dsDNA), and it slightly impairs its ssDNA annealing activity. In vivo, cells that carry an unsumoylatable *rad52* mutant (*rad52*^{K10,11,220R}) are not sensitive to DNA-

damaging agents (Sacher et al. 2006; Silva et al. 2016) and are proficient in forming Rad52 foci as a mark of ongoing HR, albeit with a slightly reduced half-life and an altered distribution (Altmannova et al. 2010; Yong-Gonzales et al. 2012). Overall, Rad52 sumoylation appears to influence not so much the efficiency of HR, but rather the type of recombination pathway that is used for repair, i.e. the balance between single-stranded annealing, gene conversion and break-induced replication events. However, not all studies agree on the direction or magnitude of these phenotypes (Sacher et al. 2006; Ohuchi et al. 2008; Altmannova et al. 2010).

Sumoylation also appears to affect the stability of Rad52, but different studies report contrasting results. Sacher et al. (2006) report that SUMO protects Rad52 from accelerated proteasomal degradation. In contrast, Su et al. (2015) show that the STUbL Slx5/8, which preferentially targets sumoylated Rad52 in vitro (Xie et al. 2007), promotes the degradation of a Rad52-SUMO fusion upon DNA damage in vivo. Moreover, *slx8Δ* is epistatic with the non-sumoylatable *rad52*^{K10,11,220R} mutant with respect to recombination rates at sequences that interfere with DNA replication (Su et al. 2015).

Although the majority of the phenotypes caused by preventing Rad52 sumoylation are minor, some are more obvious, and these relate to how Rad52 interacts with the anti-recombinogenic helicases Rrm3 and Srs2. While Srs2 acts globally, Rrm3 specifically prevents recombination and facilitates replication fork restart within the rDNA (Veaute et al. 2003; Torres et al. 2004a; Torres et al. 2004b). An *rrm3*Δ *srs2*Δ double mutant is inviable, but can be rescued by deleting *RAD52*, indicating that unrestrained recombination at the rDNA locus causes the lethality (Torres et al. 2004a; Sacher et al. 2006). Inhibiting Rad52 sumoylation also suppresses the inviability of *rrm3*Δ *srs2*Δ cells, suggesting that the modification may selectively affect the role of Rad52 in rDNA recombination (Sacher et al. 2006). Although the *rad52*^{K10,11,220R} mutant is proficient in rDNA recombination, it causes Rad52 foci to form within the nucleolus, while in WT cells Rad52 foci assemble outside of this compartment, possibly due to a transient re-localization of the break. These observations therefore suggest that Rad52 sumoylation could be critical to exclude the core HR machinery from the nucleolus. Hence, the rescue of the *rrm3*Δ *srs2*Δ mutant lethality by *rad52*^{K10,11,220R} may be due to a facilitated access of HR factors to the nucleolus, which might allow replication fork restart in the rDNA even in the absence of Rrm3 and Srs2 (Torres-Rosell et al. 2007). Presently, it is unknown how sumoylation affects Rad52's accessibility to the nucleolus, but it may involve a SUMO-dependent change in interactions between Rad52 and its partners. Surprisingly, the consequences of altering Rad52 sumoylation for the single *srs2Δ* mutant are strikingly different from those observed for *rrm3*Δ *srs2*Δ cells: preventing Rad52 sumoylation slightly aggravates the damage sensitivity of the *srs2Δ* mutant, while a Rad52-SUMO fusion fully rescues it (Esta et al. 2013). Further evidence for a direct role of Rad52 sumoylation in controlling Srs2 functions is that overexpressing *SIZ2*, encoding Rad52's cognate E3, also rescues the *srs2Δ* mutant phenotypes as long as Rad52 can be sumoylated (Esta et al. 2013). Given the well-established role of Srs2 in disassembling Rad51-ssDNA complexes, it is therefore likely that Rad52 sumoylation prevents the formation of excessive or defective nucleoprotein filaments by modulating the interactions of Rad52 with Rad51. The observations that Rad51 contains a SIM within its C-terminus that enhances its interaction with Rad52, and a Rad52-SUMO fusion protein binds to Rad51 somewhat better than unmodified Rad52 support this hypothesis (Bergink et al. 2013).

Rad52 sumoylation may not just control the properties of Rad51 filaments directly, but it could recruit other proteins to do so, such as Cdc48/p97 with its cofactors Ufd1-Npl4. This segregase is well-known for its roles in extracting proteins from complexes. It interacts preferentially with sumoylated Rad52 via SIMs in both Ufd1 and Cdc48, therefore suggesting that it could compete with Rad51 for binding to Rad52. Epistasis between a hypomorphic allele of *cdc48* and a *rad51* SIM mutant with respect to DNA damage sensitivity favors this model. Also,

Cdc48/p97 can displace Rad51/Rad52 from DNA, and it does so more effectively when Rad52 is fused to SUMO (Bergink et al. 2013).

Identification of a robust function for Rad52 sumoylation has probably been hampered by the fact that it represents only one of many sumoylation events that coordinately target and therefore likely jointly regulate the HR pathway (see Sect. 4.3). Mechanistically, this phenomenon could involve SUMO acting as a "molecular glue" to control the interactions amongst the relevant proteins (Matunis et al. 2006). In fact, Rad52 preferentially interacts with the sumoylated forms of Rfa1, as part of RPA, and Rad59 (Psakhye and Jentsch 2012; Silva et al. 2016). Likewise, Rfa1 preferentially interacts with the sumoylated forms of Rad52 (Psakhye and Jentsch 2012). Fusing SUMO to either Rad52 or Rad59, to mimic constitutively modified versions of these proteins, also enhances their respective interactions, but, surprisingly, occluding their sumoylation does not appreciably inhibit it (Psakhye and Jentsch 2012; Silva et al. 2016). Phenotypically, mutations of the known sumoylation sites of RPA (in Rfa1, Rfa2 and Rfa3), Rad52, and Rad59 impair growth upon chronic exposure to MMS and, unlike the *rad52*^{K10,11,220R} single mutant, significantly reduce the rates of both spontaneous and damage-induced interchromosomal recombination (Psakhye and Jentsch 2012). As expected from the function of Siz2 in mediating bulk sumoylation of HR factors, *siz2Δ* is epistatic with the "SUMO-less" RPA/Rad52/Rad59 mutant (Psakhye and Jentsch 2012). Overall, these results suggest that the DNA damage-induced and -coordinated sumoylation of recombination factors, including Rad52, stabilizes the interactions amongst such proteins, promoting repair.

Like in yeast, human RAD52 is also sumoylated in vivo and in vitro, at K411 and K412, which are close to the C-terminus of this protein. In vitro, this modification does not affect Rad52's binding to ssDNA or dsDNA, its ssDNA annealing activity or its interaction with Rad51. In vivo, mutating K411 and K412 to arginine restricts RAD52, which is normally a nuclear protein, to the cytoplasm. It remains to be determined whether this phenotype actually results from loss of sumoylation, or from a disruption of RAD52's nuclear localization signal, which overlaps with K411 and K412 (Saito et al. 2010).

4.5.4 Sumoylation of PCNA

In budding yeast, the homotrimeric DNA polymerase processivity factor PCNA is sumoylated mainly at K164 by Siz1, and to a lesser extent at K127 (Hoege et al. 2002; Stelter and Ulrich 2003). PCNA sumoylation normally takes place during S phase (Hoege et al. 2002), which is consistent with the observation that the protein is efficiently modified only when loaded onto DNA (Parker et al. 2008). In fact, the relative abundance of loaded and sumoylated PCNA suggests that a large proportion, if not all, of the loaded trimer could be modified during unperturbed replication (Parker et al. 2008). Yet, abolishing PCNA sumoylation does not compromise the replication process per se. PCNA is not only targeted by SUMO: in response to DNA damage it is also modified by mono- and poly-ubiquitin by a set of ubiquitin E2s and E3s known as the *RAD6* pathway (Hoege et al. 2002). PCNA mono-ubiquitylation at K164 by the E3 Rad18 promotes the bypass of DNA lesions by recruiting damage-tolerant polymerases to stalled replication forks, while poly-ubiquitylation activates a poorly understood error-free damage avoidance pathway that likely involves template switching (Ulrich 2005). Although they target the same site on PCNA, sumoylation does not seem to compete or act antagonistically with ubiquitylation. Instead, SUMO appears to channel damage processing away from HR into the *RAD6* pathway via two cooperating mechanisms (Fig. 4.2).

The first clue to the roles of PCNA sumoylation counteracting HR came from the observation that an unsumoylatable PCNA mutant (*pol30*^{K127,164R}) or a deletion of *SIZ1* strongly suppresses the DNA damage sensitivity of *rad18Δ* cells, where PCNA cannot be ubiquitylated (Papouli et al. 2005; Pfander et al. 2005). Interestingly, deleting *SRS2* suppresses the *rad18*Δ phenotype to a similar extent (Lawrence and Christensen 1979), suggest-

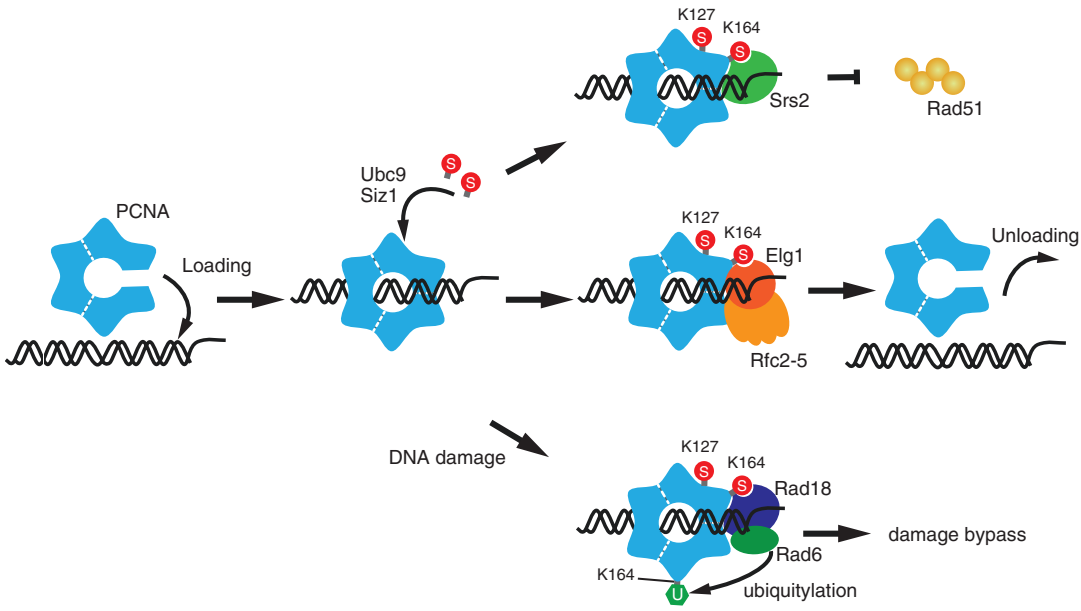

Fig. 4.2 *SUMO modification of PCNA in budding yeast.* During S phase, PCNA is loaded onto DNA, thus becoming a favorable substrate for sumoylation by Ubc9 and Siz1. The resulting PCNA-SUMO conjugate recruits the Srs2 helicase to replication forks, where this helicase counteracts the accumulation of recombinogenic Rad51 filaments. Sumoylation of PCNA also contributes to the recruitment of Elg1, which facilitates the unloading of PCNA from the chromatin. After replication fork stalling due to DNA damage, the E2-E3 complex Rad6-Rad18 associates preferentially with sumoylated PCNA through a SIM within Rad18. Rad6-Rad18-dependent ubiquitylation of PCNA then allows DNA damage bypass via translesion synthesis or template switching. (S) = SUMO, (U) = ubiquitin

ing that Srs2 may act in the same pathway as PCNA sumoylation (Papouli et al. 2005; Pfander et al. 2005). In fact, deleting *SRS2* in *rad18*Δ *siz1*Δ or *rad18*Δ *pol30*^{K127,164R} cells does not suppress their damage sensitivity any further, and the effects of both *SRS2* and *SIZ1* were found to depend on the presence of an intact HR pathway. This genetic relationship between PCNA^{SUMO} and *SRS2* was elaborated mechanistically by showing that sumoylation enhances the affinity of Srs2 for PCNA both in vivo and in vitro, due to a tandem receptor motif consisting of a PCNA-interacting protein box (PIP-box) and a SUMO interaction motif (SIM) at the C-terminus of Srs2 (Papouli et al. 2005; Pfander et al. 2005; Armstrong et al. 2012). These findings have given rise to a model whereby sumoylated PCNA recruits Srs2 to replication forks, where the helicase counteracts the assembly of Rad51 filaments onto DNA. Conversely, when PCNA cannot be sumoylated, Srs2 fails to associate with replication forks efficiently, resulting in an increased rate of sister chromatid recombination due to the elevated levels of Rad51 on DNA (Robert et al. 2006). In addition, Srs2 recruitment by PCNA^{SUMO} has been shown to induce the dissociation of Polδ and Polη from a recombination intermediate (Burkovics et al. 2013).

At the same time, the attachment of SUMO to PCNA greatly enhances the activity of the ubiquitin ligase Rad18 towards the DNA-bound clamp (Parker and Ulrich 2012, Fig. 4.2). This effect is attributable to a SIM in Rad18 and suggests that PCNA^{SUMO} is Rad18's physiological substrate. Altogether these observations indicate that sumoylation of PCNA, by means of recruiting the anti-recombinogenic Srs2 and the ubiquitin E3 Rad18, may allow stalled replication forks to use PCNA ubiquitylation for damage bypass rather than the possibly deleterious recombination pathway (Papouli et al. 2005; Pfander et al. 2005; Parker and Ulrich 2012).

There is also good evidence for a more direct contribution of SUMO to the regulation of Srs2. Sumoylation of Srs2, mediated through the C-terminal SIM of this protein, has been described to inhibit the interaction with $PCNA^{SUMO}$, thus likely ensuring an appropriate balance between PCNA-bound and free Srs2 (Kolesar et al. 2012). Furthermore, the SUMO-like domain protein Esc2 can counteract the Srs2-mediated inhibition of HR by promoting Srs2 turnover on chromatin and thereby facilitating Rad51 recruitment (Urulangodi et al. 2015).

Sumoylation of PCNA is not limited to budding yeast, but has also been observed in *Xenopus* egg extracts, chicken DT40 B lymphocytes and more recently human cells (Leach and Michael 2005; Arakawa et al. 2006; Gali et al. 2012; Moldovan et al. 2012). Although SUMO-dependent Rad18 recruitment and stimulation of PCNA ubiquitylation do not appear to be conserved in vertebrates (Parker and Ulrich 2012), a potential homologue of Srs2 has been identified: similar to Srs2 in yeast, the protein PARI harbors a UvrD-like helicase domain, interacts with Rad51 and preferentially binds to a PCNA-SUMO1 fusion construct via a C-terminal PIP-box and a SIM. Depletion of PARI in U2OS or DT40 cells significantly stimulates HR rates, suggesting that vertebrate PARI acts like Srs2 in the suppression of inappropriate HR events (Moldovan et al. 2012). In accordance with these findings, overexpression of a PCNA-SUMO1 fusion inhibits recombination at stalled replication forks, and overexpression of sumoylation-deficient PCNA mutants induces DNA DSBs (Gali et al. 2012). The relevance of this mechanism in a physiological setting needs to be determined, however, as sumoylation of human PCNA has been detected only after overexpression of tagged SUMO1 in 293T or HeLa cells (Gali et al. 2012; Moldovan et al. 2012).

In addition to enhancing mono-ubiquitylation via Rad18 and controlling HR via Srs2, SUMO may affect other aspects of PCNA biology. The yeast protein Elg1 bears homology to the largest subunit of the replication factor C (RFC) complex, and it has been proposed to act as an unloader of PCNA in complex with the Rfc1–4 subunits (Kubota et al. 2013, Fig. 4.2). As a consequence, both sumoylated PCNA and Srs2 strongly accumulate on the chromatin of *elg1* mutants. Similar to Srs2, Elg1 preferentially binds to sumoylated PCNA via two SIMs (Parnas et al. 2010); however, the Elg1 complex likewise acts on unmodified PCNA.

Finally, SUMO modification of PCNA can also interfere with the binding of interaction partners, such as Rfc1 and Eco1 (Moldovan et al. 2006). It has been noted that K127 is located within a region of PCNA that serves as its major partner-interaction site. Sumoylation of such residue might therefore compromise the association of PCNA with its partners, essentially acting as an "off-switch" to clear the clamp (Moldovan et al. 2006).

4.5.5 Sumoylation of SGS1/BLM

The budding yeast RecQ-like helicase Sgs1 is an important player in the repair of DSBs via HR. A *sgs1* knockout mutant accumulates Rad51 foci at damaged replication forks, a phenotype that has similarly been described for a *ubc9* mutant (Liberi et al. 2005; Branzei et al. 2006). This indicates that both sumoylation and Sgs1 functions are needed for the regulation of HR at damaged replication forks. Sgs1 has been identified as a SUMO target; however, this sumoylation event *per se* does not affect HR efficiency (Lu et al. 2010). Recently, the STUbL Slx5/8 has been reported to interact with Sgs1 and to negatively affect the formation of Sgs1 foci after replication fork stalling, indicating that Sgs1 might be removed from damaged replication forks in a STUbL-dependent manner (Bohm et al. 2015). However, overall protein levels of Sgs1 after damage induction remain unaltered, suggesting that removal of Sgs1 does not involve proteasomal degradation. Interestingly, expression of the human STUbL RNF4 in a *slx8Δ* mutant background also reduces the formation of Sgs1 foci.

A triple *slx5Δ slx8Δ sgs1Δ* mutant is synthetically lethal, but overexpression of the protease Wss1 in this background rescues this phenotype (Tong et al. 2001; Pan et al. 2006; Mullen et al. 2010). A *wss1Δ sgs1Δ* double mutant also exhibits synthetic lethality, suggesting that Slx8 and Wss1 act synergistically in this pathway.

Accumulation of the human homologue of Sgs1, BLM, in repair foci is also diminished after depletion of RNF4 in human cells, clearly indicating that the phenomenon of STUbL-mediated modulation of RecQ-like helicases is conserved in evolution (Bohm et al. 2015). Accordingly, BLM is indeed sumoylated, and cells producing an unsumoylatable BLM mutant accumulate higher levels of DNA damage after replication fork stalling than those expressing the WT protein, indicating that the modification is important to resolve replication problems (Eladad et al. 2005). Abolishing sumoylation of BLM prevents the recruitment of RAD51 and subsequent HR at stalled replication forks. Indeed, RAD51 preferentially binds to sumoylated BLM in vitro, providing a first mechanistic explanation for how sumoylation of BLM may stimulate HR at stalled replication forks (Ouyang et al. 2009).

4.5.6 Sumoylation of Thymidine DNA Glycosylase in Base Excision Repair

Base excision repair processes a variety of chemical lesions inflicted on the nitrogenous bases of the DNA. It relies on several highly specialized glycosylases that recognize and cleave a narrow spectrum of damaged or modified bases. The resulting abasic sites, regardless of the enzyme that generated them, feed into a common core pathway that involves the initial displacement of the glycosylase from DNA and the nicking of the damaged duplex by the APE1 endonuclease. DNA polymerase β then removes the baseless sugar residue and polymerizes across the gap. Finally, the XRCC1-ligase III complex seals the nick in the DNA (reviewed by Memisoglu and Samson 2000; Barnes and Lindahl 2004).

Thymidine DNA glycosylase (TDG) is best known for its ability to protect DNA against C → T transitions by recognizing thymine or uracil within G•T and G•U mismatches arising from spontaneous deamination of 5-methyl-cytosine or cytosine, respectively (Barnes and Lindahl 2004). More recently, TDG has also been implicated in regulating DNA methylation. TDG actively demethylates DNA by excising 5-carboxylcytosine and 5-formylcytosine, the products of iterative oxidation of 5-methylcytosine by TET dioxygenases (Cortazar et al. 2011; Cortellino et al. 2011; He et al. 2011; Maiti and Drohat 2011; Kohli and Zhang 2013). This process is critical for the development of higher eukaryotes and could explain why, unlike other DNA glycosylases, TDG is essential for viability in mice (Hu et al. 2010).

Human TDG is sumoylated at K330 in vivo and in vitro. In vitro, TDG is preferentially modified by SUMO1, and to a lesser extent by SUMO2, under equivalent reaction conditions. This could be partly explained by the observation that TDG also non-covalently interacts with SUMO1 through a SIM at the C-terminus of the central catalytic core, and possibly a second one within the N-terminal regulatory domain (Hardeland et al. 2002; Baba et al. 2005; Steinacher and Schar 2005; Takahashi et al. 2005; Mohan et al. 2007; Smet-Nocca et al. 2011; Coey et al. 2014). Both covalent and non-covalent interactions influence the functions of TDG.

Initially, sumoylation was proposed to reduce TDG's affinity for DNA, thereby relieving the strong product inhibition exhibited by this enzyme and promoting catalytic turnover. The crystal structure of a central region of TDG conjugated to SUMO1 appears to support this model because it shows that the covalent and non-covalent interactions between SUMO and TDG may result in the protrusion of a helix from the surface of the glycosylase. When DNA is modeled into this structure, the protruding helix sterically clashes with the duplex, suggesting that a SUMO-induced conformational change may force the enzyme to dissociate from DNA (Baba et al. 2006, 2005). This conformational change

does not strictly depend on the covalent modification of TDG, but can apparently also be triggered by the non-covalent binding of SUMO to the glycosylase (Smet-Nocca et al. 2011). An interaction between SUMO and the N-terminus of TDG, which is required for tight binding to G•T, may also be involved in this process because deleting this domain enhances TDG turnover in a way that is "epistatic" with SUMO modification. Consistently, early studies show that the N-terminus of TDG undergoes a conformational change in response to sumoylation of the enzyme (Steinacher and Schar 2005). More recently, however, NMR analysis reported no change in the structure of TDG's N-terminal regulatory domain upon sumoylation. It rather seems that the interaction between SUMO and the C-terminal SIM of TDG competes with its regulatory domain for binding to the catalytic domain. Therefore, SUMO could dislodge the regulatory domain from the catalytic interface of TDG, leading to an extended conformation that is poised for catalysis (Smet-Nocca et al. 2011). Observations showing that sumoylation reduces the affinity of TDG for DNA and thereby stimulates its catalytic turnover also corroborated the above-described model (Hardeland et al. 2002). However, subsequent studies show that sumoylated TDG can still bind to DNA fairly tightly, albeit less so than the unmodified enzyme (Coey et al. 2014). In addition, in contrast to what the model described above would predict, DNA-bound TDG is not sumoylated more efficiently than the free enzyme, at least in vitro and in the absence of an E3 (Coey et al. 2014). Free SUMO can also boost the catalytic turnover of TDG in vitro in a SIM-independent manner, which possibly suggests a more indirect influence of SUMO on TDG activity (Smet-Nocca et al. 2011). In vivo, sumoylation does not appear to be important for TDG activity either, as neither preventing sumoylation nor disrupting non-covalent interactions with SUMO compromise TDG's ability to excise 5-carboxylcytosine. Likewise, overexpressing SUMO or altering the cellular sumoylation/desumoylation balance does not affect TDG's in vivo activity (McLaughlin et al. 2016).

Given that APE1 can also relieve product inhibition of TDG (Waters et al. 1999; Fitzgerald and Drohat 2008; McLaughlin et al. 2016), it is conceivable that TDG sumoylation may actually regulate some other process, e.g. binding to other proteins. In fact, in exponentially growing cells TDG is found exclusively in the nucleus and is enriched within PML nuclear bodies. This localization relies on the interaction of TDG's two SIMs with sumoylated PML (Takahashi et al. 2005; Mohan et al. 2007; McLaughlin et al. 2016). Consistently, TDG preferentially binds to sumoylated PML in vitro (Takahashi et al. 2005). This association appears to be incompatible with DNA binding (Mohan et al. 2007). As a consequence, deleting the DNA-binding N-terminus of TDG enhances co-localization with PML, probably by exposing TDG's SIMs (Mohan et al. 2007). At the same time, TDG sumoylation prevents the non-covalent, intermolecular interaction with a SUMO moiety on PML (Mohan et al. 2007). Taken together, these observations suggest that when TDG is released from DNA, it exposes its SIMs that would mediate its translocation to PML bodies unless the intermolecular interaction with sumoylated PML is prevented by sumoylation of TDG itself (Mohan et al. 2007). Why unsumoylated TDG localizes to PML bodies is unclear, but it may involve CBP/p300, an acetyl-transferase responsible for the transcriptional activation of several genes in mammalian cells (Goodman and Smolik 2000). CBP/p300 can interact with and acetylate TDG (Tini et al. 2002), but only when the glycosylase is unmodified, suggesting that TDG localization to PML bodies may promote its acetylation (Mohan et al. 2007).

The STUbL RNF4 may also affect the functions of sumoylated TDG, as determined by work on DNA methylation. Overexpressing RNF4 reduces the methylation levels of a methylation-sensitive reporter promoter, leading to its activation. This effect requires both the SUMO-binding and ubiquitin ligase activities of RNF4, as well as TDG and APE1. Vice versa, RNF4$^{-/-}$ mouse embryonic fibroblasts (MEFs) show increased levels of global and locus-specific DNA methyla-

tion compared to WT cells. Via its SIM-containing N-terminal region, RNF4 physically interacts with TDG and APE1, synergizing with them in the activation of DNA demethylation (Hu et al. 2010). Therefore, RNF4 controls DNA demethylation via TDG/APE1 as a STUbL. It remains to be determined whether these functions actually depend on the sumoylation and subsequent ubiquitylation of TDG or on its SUMO-binding activity: although the interaction between RNF4 and TDG can be recapitulated in vitro, it does not apparently require, or is enhanced by, prior sumoylation of the glycosylase (Moriyama et al. 2014).

4.6 SUMO in the Maintenance of Telomere Function

Telomeres are structural elements at the ends of chromosomes that protect these from being recognized as DSBs and provide a solution to the end replication problem, which would otherwise cause a shortening of linear DNA molecules after every round of replication (Watson 1972; Verdun and Karlseder 2007; Arnoult and Karlseder 2015). Telomeric sequences consist of tandemly repeated dsDNA that terminates in a G-rich single-stranded 3′-overhang (Blackburn et al. 2015). They are covered by a group of proteins collectively called shelterin complex that, together with a range of accessory factors, controls telomere length and function (Fig. 4.3). Telomere length is maintained by an RNA-dependent DNA polymerase named telomerase, an enzyme that uses an RNA cofactor as a template to elongate telomeres (Autexier and Lue 2006). In the absence of telomerase, chromosome ends progressively shorten, leading to senescence and/or cell death. Hence, all immortal cell lines appear to have acquired some mechanism to maintain telomeres. Frequently, this involves re-expression of telomerase (Granger et al. 2002), but it is also possible by means of a mechanism called alternative lengthening of telomeres (ALT), as observed in a few cancers. Although the exact molecular aspects of ALT remain unclear, increasing evidence suggests that this process involves some type of HR-mediated DNA replication that uses telomeric DNA, in cis or in trans, as a template (Pickett and Reddel 2015).

SUMO plays an important role in telomere biology. Not only do telomeres become longer than usual in budding and fission yeast sumoylation mutants (Tanaka et al. 1999; Xhemalce et al. 2004; Zhao and Blobel 2005; Hang et al. 2011), but in *S. cerevisiae* compromising sumoylation causes cells to senesce more quickly than normal (Chavez et al. 2010). Conversely, senescent cells also show increased levels of total sumoylation (Chavez et al. 2010).

In both budding and fission yeast, sumoylation controls telomerase activity. Early studies hinted at this possibility by showing that the unusually long telomeres observed in *S. pombe* SUMO mutants probably originate from extension of the telomeric 3′-overhangs, a hallmark of uncontrolled telomerase activity (Xhemalce et al. 2007). The main player in this process appears to be the shelterin factor Tpz1. Tpz1 sumoylation peaks with telomere replication in late S phase and is catalyzed by the SUMO E3 Pli1 (Garg et al. 2014; Miyagawa et al. 2014). An unsumoylatable allele, $tpz1^{K242R}$ does not affect shelterin stability but leads to longer-than-usual telomeres. This phenotype is suppressed by loss of telomerase activity but is not further enhanced by loss of *pli1* or *pmt3*, indicating that Tpz1 likely is the main sumoylation target in telomere homeostasis in fission yeast, and that this process is mediated via telomerase. In fact, $tpz1^{K242R}$ cells show both a loss of the telomerase-inhibitory complex Stn1/Ten1 from telomeres and an increased association of telomerase. Stn1 binds non-covalently and independently to both SUMO and Tpz1, resulting in a synergistic enhancement of binding to a covalent SUMO-Tpz1 fusion. These observations suggest a model where Tpz1 sumoylation prevents telomere elongation by recruiting Stn1/Ten1 to telomeres and thereby restraining telomerase activity (Garg et al. 2014; Miyagawa et al. 2014). In support of this model, fusing Stn1 to SUMO or even directly to Tpz1, to mimic a constitute interaction between these two proteins, suppresses the telomeric phenotypes of *pmt3Δ* cells (Miyagawa et al. 2014, Fig. 4.3a).

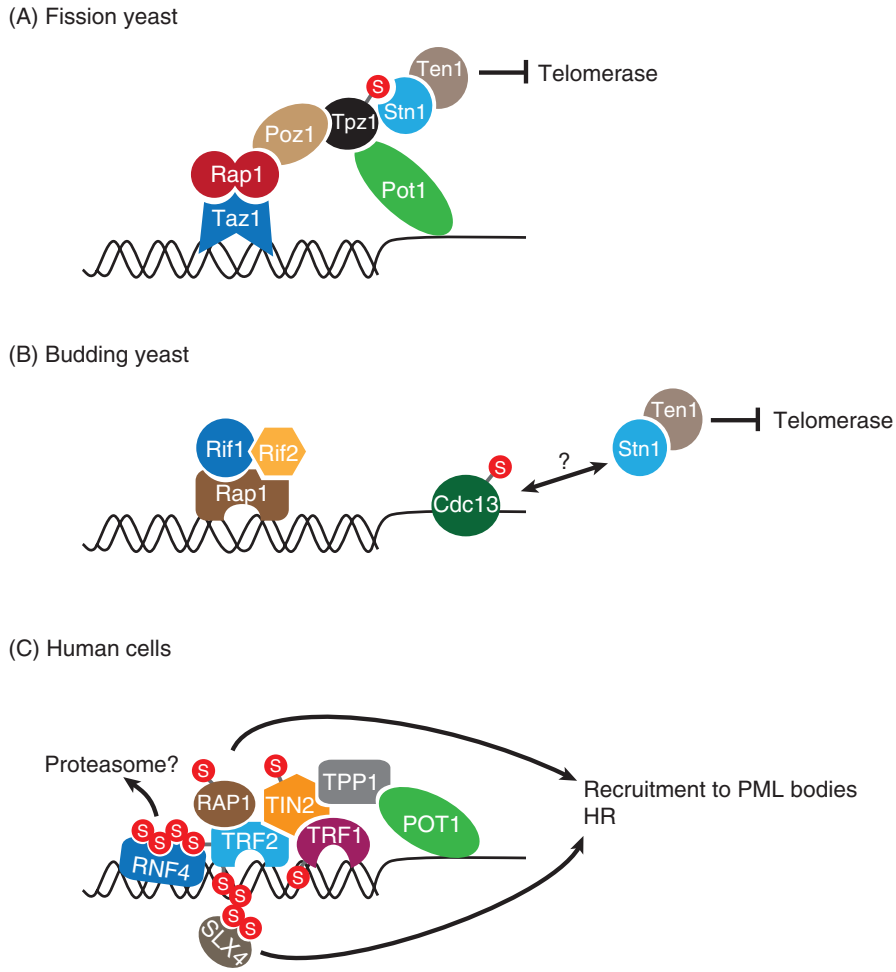

Fig. 4.3 *Telomere composition and contributions of the SUMO system in yeast and human cells.* (**a**) In fission yeast, the dsDNA-binding protein Taz1 coats double-stranded telomeric repeats (Spink et al. 2000) and interacts with Rap1 and Poz1. Via Tpz1, Poz1 interacts with Pot1, which directly recognizes the 3′ telomeric overhang (Baumann and Cech 2001). (**b**) In budding yeast, dsDNA telomeric repeats are bound by Rap1, which interacts with Rif1/2, while the CST complex recognizes the 3′-overhangs via Cdc13. (**c**) Human telomeres harbor a set of proteins similar to those found in fission yeast, which are collectively called shelterin complex: TRF1 and TRF2 (the orthologues of Taz1) bind to RAP1 and TIN2. In turn, TIN2 recognizes TPP1, which interacts with POT1 (de Lange 2005). In addition to shelterin, another complex is important for telomere regulation. It is called CST, after its constituent human proteins CTC1 (Cdc13 in budding yeast), STN1 and TEN1, and it is reminiscent of RPA. (S) = SUMO

A surprisingly similar contribution of SUMO to telomere homeostasis is observed in budding yeast (Hang et al. 2011), although both the molecular mechanism and the key modification target are probably different. In *S. cerevisiae*, several telomeric proteins are sumoylated: yKu70, Rap1, the helicase Pif1 and the 3′ overhang binding protein Cdc13 (Zhao and Blobel 2005; Hang et al. 2011; Hang et al. 2014; Chymkowitch et al. 2015). Similar to sumoylation of Tpz1 in *S. pombe*, budding yeast Cdc13 sumoylation peaks in late S phase. The modification, catalyzed by Siz1 and Siz2, occurs at K909 within Cdc13's Stn1-binding domain. Cells bearing an unsumoylatable *cdc13*^{K909R} allele have longer-than-usual telomeres but show normal

levels of telomeric 3′-overhangs, indicating that chromosome end protection is unaffected. The *cdc13*^{K909R} allele is epistatic with *siz1*/*siz2* and *stn1*, strongly suggesting that Cdc13 is the main target of Siz1/Siz2-dependent sumoylation in telomere control in budding yeast, and that the modification acts via Stn1. In fact, inhibiting Cdc13 sumoylation reduces the binding of Cdc13 to Stn1 in the yeast two-hybrid system, while a permanent fusion of SUMO to Cdc13 increases the interaction in vitro and leads to shorter telomeres in vivo. Given that the telomeric phenotype of *cdc13*^{K909R} cells depends on active telomerase and is suppressed by overexpressing *STN1*, Cdc13 sumoylation has been proposed to facilitate Stn1/Ten1 recruitment to telomeres (Hang et al. 2011). However, unlike fission yeast Stn1, budding yeast Stn1 does not appear to bind appreciably to SUMO; therefore, it is unlikely that it directly recognizes sumoylated Cdc13 (Hang et al. 2011). Rather, sumoylation may change other properties of Cdc13, such as its structure or DNA binding, which could ultimately facilitate association of Stn1 with telomeres (Fig. 4.3b).

In budding yeast, Siz2-dependent sumoylation appears to regulate telomerase activity by a second mechanism, involving telomere clustering (Ferreira et al. 2011; Ferreira et al. 2013). As in a number of other organisms (Ferreira et al. 2013), chromosome ends cluster at the nuclear envelope in *S. cerevisiae*. This effect is mediated by two pathways: one dependent on the partially redundant Sir2/3/4 proteins and the other on Yku70/80 (yeast Ku70/80; Hediger et al. 2002). Both Sir4 and Yku80, and to some extent Yku70, are sumoylated in a Siz2-dependent manner (Ferreira et al. 2011), and deletion of *SIZ2* leads to a loss of telomere clustering. Both pathways appear to be affected, because *siz2Δ* cells are unable to tether a reporter locus to the nuclear envelope via either Yku70/80 or Sir4. Clustering can be rescued by permanently fusing SUMO to Yku70 or Yku80 to mimic a constitutively sumoylated Yku70/80 complex, indicating that sumoylated Yku70/80 directly controls telomere clustering. Sumoylation of Sir4 does not seem to be as critical. Telomere lengthening upon loss of *siz2* is epistatic with deletion of *PIF1*, encoding a helicase with an inhibitory effect on telomerase. It has therefore been proposed that SUMO-dependent clustering of telomeres at the nuclear envelope may antagonize telomerase activity (Ferreira et al. 2011). Although interesting, definitive proof for this model will require a demonstration that the increase in telomere length observed in *siz2Δ* cells actually depends on, for example, Yku70 sumoylation, rather than another functionally analogous sumoylation event, such as the modification of Cdc13.

The roles of SUMO in telomere regulation go beyond controlling telomerase activity. For instance, sumoylation of the anti-recombinogenic helicase Sgs1 by Siz1/Siz2 at K621 stimulates recombination between telomeres (Lu et al. 2010). In addition, sumoylation of Yku70 by Mms21, which appears to be functionally distinct from that catalyzed by Siz2, may also play a role in this process. This modification event, which occurs at a cluster of lysines within the DNA-binding domain of Yku70, does not in fact act via telomerase (Hang et al. 2014). In addition, a *yku70* mutant that shows reduced levels of Yku70 sumoylation has shortened, rather than elongated, telomeres and an increased length of telomeric 3′-overhangs. These results are consistent with the Yku70/80 complex's well-established ability to inhibit end resection by DNA binding, thereby preventing HR and promoting NHEJ. Inhibiting sumoylation of Yku70 reduces its binding to DNA ends at both telomeres and DSBs and, consequently, has been reported to speed up resection of the latter. It follows that Yku70 sumoylation could facilitate the association of the Yku70/80 complex with DNA ends in general and thereby contribute to telomere maintenance (Hang et al. 2014). The exact mechanism of this process is unclear but could involve HR, because inhibiting Mms21 E3 activity boosts the levels of recombination intermediates at telomeres in senescing telomerase-deficient mutant yeast (Chavez et al. 2010).

SUMO's roles in regulating NHEJ at budding yeast telomeres also involves STUbLs. Cells

devoid of Uls1 show increased levels of chromosomal end-to-end fusions (Lescasse et al. 2013). This phenotype requires a functional NHEJ pathway and the ability to form poly-SUMO chains and is recapitulated by inhibiting Uls1's ubiquitin E3 activity (Lescasse et al. 2013). This implies that Uls1's poly-sumoylation-mediated ubiquitylation activity protects telomeres by inhibiting NHEJ at these loci. Consistently, Uls1 binds to telomeres. The relevant SUMO target in this pathway might be Rap1, as it was not only shown to be sumoylated, but its modification increases upon loss of *uls1*. Also, a *rap1*^{K240,246R} allele, which largely but not completely abolishes sumoylation, can partially suppress the end-to-end fusions observed in *uls1* cells (Lescasse et al. 2013). Although it seems clear that Uls1-dependent degradation of Rap1 is important for inhibiting NHEJ at telomere fusions, the molecular details of this mechanism remain to be determined.

Another STUbL contributing to telomere function is the Slx5/8 complex. Like human cells, budding yeast cells devoid of telomerase accumulate critically short telomeres and eventually undergo senescence. Under these conditions, eroded telomeres relocalize from their typical position around the nuclear envelope to the nuclear pore complex (Khadaroo et al. 2009). This accrual of dysfunctional telomeres is thought to facilitate HR amongst them, thereby allowing some cells to maintain telomeres without telomerase by a mechanism similar to ALT in human cells. These cells can escape senescence and are called "survivors". Mutants devoid of STUbL activity (*slx5Δ*) poorly concentrate telomeres to the nuclear pore complex and also show reduced levels of survivors (Churikov et al. 2016). A similar, although not as penetrant, phenotype is observed in the *siz1 siz2* mutant (Churikov et al. 2016), suggesting that Slx5/8 controls recombination at dysfunctional telomeres by mechanisms that are dependent on its ability to ubiquitylate poly-sumoylated proteins. Accordingly, Slx5/8 has been suggested to recruit telomeres to the nuclear pore complex, since it also associates with the latter (Nagai et al. 2008). This model appears, however, unlikely because artificially tethering a telomere to the nuclear pore complex cannot restore recombination at this locus in the *slx5Δ* mutant (Churikov et al. 2016). On the other hand, both SUMO and Slx8 are recruited to eroding telomeres with similar kinetics (Churikov et al. 2016). The relevant sumoylated proteins on which Slx5/8 acts in this process are unclear. RPA has been suggested as a candidate, because Rfa1 is sumoylated in response to telomere attrition, and deleting *SLX5* also boosts this modification (Churikov et al. 2016). However, this scenario seems improbable because RPA is sumoylated not exclusively upon telomere erosion, but also in response to other kinds of DNA damage (see Sects. 4.5.2 and 4.5.3), all of which produce large amounts of ssDNA as repair intermediates. Therefore, ssDNA is the likely trigger for RPA sumoylation, rather than eroded telomeres *per se*.

Sumoylation plays an important role at human telomeres as well (Fig. 4.3c): several components of human shelterin are sumoylated. The main SUMO E3 involved in this process is the SMC5/6-associated ligase MMS21, which appears to be of particular importance for the ALT pathway (Potts and Yu 2007). In ALT cells only, telomeres co-localize with specialized PML bodies containing many HR factors, suggesting that they could act as sites of telomere elongation (Yeager et al. 1999). This co-localization depends on both an intact SMC5/6 complex and MMS21-dependent sumoylation. Consistently, MMS21 can sumoylate four (TRF1, TRF2, TIN2 and RAP1) out of the six components of the shelterin complex. In ALT cells, over-producing unsumoylatable TRF1 or TRF2 mutants reduces the recruitment of telomeres to PML bodies. Blocking shelterin sumoylation by silencing MMS21 also inhibits recombination between sister telomeres in these cells, possibly explaining why they develop progressively shorter telomeres and eventually senesce (Potts and Yu 2007). The functions of shelterin sumoylation could be mediated, at least partially, via SLX4, an interaction partner of TRF2. SLX4 acts as a scaffold protein by interacting with several complexes involved in different DNA repair pathways, possibly orchestrating their activities. SLX4 contains

three SIMs that allow it to interact preferentially with SUMO2/3 chains and have been shown to contribute to the SLX4-TRF2 interaction in ALT cells (Ouyang et al. 2015). Overall, these results suggest that sumoylation of telomeric factors, including but not limited to TRF2, helps telomeres to associate with specialized PML bodies and SLX4 in ALT-positive cells.

TRF2 (and TRF1) is also modified by PIAS1 with SUMO1 and SUMO2/3, but the relevant target lysines are different from those sumoylated by MMS21 (Her et al. 2015). The molecular consequences are also distinct. Poly-sumoylation of TRF2, together with its ability to physically interact with the STUbL RNF4, leads to its polyubiquitylation and subsequent proteasome-mediated degradation (Her et al. 2015). The exact downstream functions of this process are unknown, but they could be related to TRF2's roles in telomere protection because depleting RNF4 leads to telomere end-to-end fusions (Groocock et al. 2014).

4.7 Sumoylation in Chromosome Topology

Not only DNA repair pathways, but also the mechanisms that control chromosome segregation, contribute to genome maintenance, as errors in the distribution of the genetic material during cell division result in aneuploidy and thus genomic instability. Not surprisingly, SUMO plays a prominent role in this area as well. Accordingly, SUMO has been detected in association with essentially the entire chromosome segregation apparatus, i.e. centromeres and kinetochores, the chromosomal passenger complex and the mitotic spindle, and numerous components within these structures have been identified as SUMO targets by mass spectrometry in yeast and mammalian cells. The functions of SUMO in controlling chromosome segregation, especially in the context of centromere and kinetochore function, have been the subject of several excellent reviews (Dasso 2008; Wan et al. 2012) and are also covered in more detail elsewhere in this volume [see Chap. 9]. Here we will only discuss those aspects that directly impinge on the genetic material itself, namely DNA damage-induced cohesion and the processing of topological stress by means of topoisomerase II.

4.7.1 Sumoylation of Cohesin

In *S. cerevisiae*, both core components of the cohesin complex (Smc1 and Smc3) and its ancillary factors (Mdc1/Scc1, Scc3 and Pds5) are sumoylated (Almedawar et al. 2012). Although all three mitotic SUMO E3s (Siz1, Siz2 and Mms21) contribute to modification of Scc1, Mms21 appears to be responsible for the bulk of it. During unchallenged growth, modification peaks at the beginning of S phase (Almedawar et al. 2012). As determined by genetically arresting cohesin at different stages of its cohesion cycle, modification occurs at a point between loading of cohesin onto chromatin and DNA entrapment (Almedawar et al. 2012). Functionally, SUMO regulates the core activities of cohesin. Overproducing Scc1 fused to the catalytic domain of Ulp1 (Scc1-Ulp1^{CD}), as a means to reduce the sumoylation of adjoining proteins, is toxic to cells, but only when such an appendage is catalytically active. Although it is not possible to unambiguously ascribe this effect to the sumoylation of cohesion itself, as Ulp1^{CD} could also promote the desumoylation of other proteins, it strongly suggests that SUMO is required to establish cohesion during unchallenged growth. In fact, cells carrying an unsumoylatable mutant of Scc1 experience defects in sister chromatid cohesion (McAleenan et al. 2012). Cohesin sumoylation is also induced by exposure to DNA damaging agents, such as MMS (McAleenan et al. 2012). Since nucleotide depletion by HU treatment does not induce this modification, it may be triggered by genuine DNA damage. In fact, a single DSB is sufficient to boost the reaction (McAleenan et al. 2012). Hence, SUMO appears to affect cohesin's ability to facilitate DSB repair by promoting cohesion around them. Inhibiting the SUMO E3 activity of Mms21, compromising the integrity of the Smc5/6 complex (by using the *smc6–9* allele), or rendering

Scc1 refractory to sumoylation reduces the recruitment of cohesin to a DSB and impairs the establishment of damage-induced cohesion at such lesions (McAleenan et al. 2012).

Human Scc1 is also sumoylated at several lysines by MMS21 but, unlike in yeast, this modification is not induced by DSBs (Wu et al. 2012). Although an unsumoylatable SCC1 mutant ($SCC1^{K15R}$) can fully rescue the chromatid separation phenotype exhibited by SCC1-depleted cells, it cannot suppress the increased rate of sister chromatid exchanges that such cells experience, and it also sensitizes them to ionizing radiation (Wu et al. 2012). Also, in contrast to the yeast system, sumoylation is dispensable for recruiting human cohesin to DNA damage because SCC1 proficiently localizes to DNA lesions upon depletion of MMS21 or SMC5 (Wu et al. 2012). Instead, sumoylation of human SCC1 appears to exert its functions through the negative regulator of cohesion, WAPL. Knocking down this protein suppresses both the increased sensitivity to ionizing radiation and the defects in sister chromatid recombination caused by depleting MMS21 or rendering SCC1 unsumoylatable (Wu et al. 2012).

4.7.2 Sumoylation of Topoisomerase II

During DNA replication, the topological stress associated with opening the template DNA generates catenanes between homologous chromosomes (Lucas et al. 2001). Catenanes are inter-locked structures of two topologically constrained DNA molecules. They pose a serious problem to cells because they have to be resolved prior to chromosome segregation in order to avoid damage to or partial loss of the genome. Topoisomerase II (Topo II) resolves catenanes by covalently binding to both strands of a DNA helix and creating a DSB that is used as a gate to allow passage of a second duplex. It then reseals the initial break to avoid lasting damage (reviewed by Champoux 2001). Inhibiting Topo II results in metaphase arrest and extensive chromosome bridging, which likely arises from a defect in chromosome disjunction due to impaired decatenation (DiNardo et al. 1984; Downes et al. 1991; Shamu and Murray 1992).

Analysis of Topo II function, especially in higher eukaryotes, has relied on the use of pharmacologically relevant inhibitors such as ICRF-193, which traps the enzyme on its target DNA and prevents strand cleavage (Roca et al. 1994), and poisons such as etoposide, which stabilizes the covalent DNA-Topo II complex (Froelich-Ammon and Osheroff 1995). Topo II sumoylation is conserved from budding yeast to metazoans. Although the way in which this modification is regulated is analogous in the two systems, its functions appear to be distinct. A pivotal role for the centromere may in fact be the only similarity. In *Xenopus* egg, mouse and human cell extracts Topo II is preferentially sumoylated by SUMO2/3 in metaphase (Azuma et al. 2003; Agostinho et al. 2008; Dawlaty et al. 2008). PIAS4 facilitates this reaction in *Xenopus* egg extracts and possibly in some human cell lines (Azuma et al. 2005; Diaz-Martinez et al. 2006). In *Xenopus* egg extracts, depletion of PIAS4 eliminates nearly the entire pool of SUMO conjugates on mitotic chromosomes, demonstrating a general role for this SUMO ligase during mitotic progression (Azuma et al. 2005). Depletion of PIAS4 in HeLa cells blocks the localization of Topo II at centromeres and thereby faithful chromosomal segregation (Diaz-Martinez et al. 2006). Conversely, in MEFs, RanBP2, but not PIAS4, promotes Topo II sumoylation (Dawlaty et al. 2008). Agostinho et al. (2008) also showed, by using chemicals that arrest Topo II at different catalytic stages, that the modification occurs at the covalent DNA-Topo II complex state, implying that sumoylation may depend on Topo II activity.

Regardless of the mechanism of regulation, PIAS4-depleted HeLa cells or *Xenopus* egg extracts and RanBP2-deficient MEFs show a similar phenotype: chromosomes correctly condense, attach to the spindle and align onto the metaphase plate but then fail to disjoin properly (Azuma et al. 2005; Diaz-Martinez et al. 2006; Dawlaty et al. 2008). When anaphase naturally occurs or is induced, chromosomes show exten-

sive bridging, and lagging chromosomes and mis-segregation frequently cause aneuploidy (Diaz-Martinez et al. 2006; Dawlaty et al. 2008). This phenotype is independent of cohesin because in cells co-depleted of PIAS4 and the cohesin guardian hSGO1 chromosomes are still unable to separate (Diaz-Martinez et al. 2006). Such studies demonstrate that in the absence of PIAS4/RanBP2-mediated sumoylation, chromosome segregation is impaired most likely because of an unusually high level of catenation. This phenotype in turn suggests that Topo II function is compromised in the absence of sumoylation. In support of this view, Topo II cannot localize to the axial core of chromosomes and centromeres in both PIAS4-depleted *Xenopus* egg extracts and HeLa cells and in RanBP2-deficient MEFs (Azuma et al. 2005; Diaz-Martinez et al. 2006; Agostinho et al. 2008; Dawlaty et al. 2008). In the latter system, an over-produced SUMO-Topo II fusion protein localizes to centromeres and prevents chromosome bridging, suggesting that Topo II is indeed the relevant substrate whose sumoylation is required for efficient chromosome decatenation (Dawlaty et al. 2008). Interestingly, sumoylation of Topo II strongly inhibits decatenation activity in vitro (Ryu et al. 2010). Using a mass spectrometric approach, *X. laevis* Topo II has been found to be sumoylated within its DNA-binding domain at K660. Modification is enhanced by DNA binding. Mutation of K660 abolishes the inhibitory effect on decatenation, suggesting that SUMO specifically modifies active Topo II on centromeric DNA and thereby regulates decatenation activity (Ryu et al. 2010). Additional SUMO sites at the C-terminus of Topo II have no influence on the decatenation activity, but facilitate its interaction with other proteins, such as Claspin, an essential regulator of checkpoint arrest (Ryu et al. 2015). Sumoylation of Topo II and SIMs within Claspin are needed for the efficient recruitment of Claspin to centromeric DNA. Thus, sumoylation has different effects on the functionality and interactome of Topo II.

In budding yeast, the roles of Topo II sumoylation are somewhat different. Siz1 and Siz2 mediate the modification of the enzyme at K1220, K1246 and K1277 during metaphase (Bachant et al. 2002; Takahashi et al. 2006). A yeast strain carrying an unsumoylatable Topo II mutant ($top2^{K1220,1246,1277R}$) grows normally and shows only a mild chromosomal phenotype. This includes low levels of bridged chromosomes (Bachant et al. 2002), which possibly explains why $top2^{K1220,1246,1277R}$ cells lose artificial mini-chromosomes at a slightly higher rate than the WT (Takahashi et al. 2006). The most notable phenotype of the $top2^{K1220,1246,1277R}$ mutant is an increased stretching of centromeres during precocious separation of chromatids (Warsi et al. 2008). In budding yeast, as homologues pair during metaphase, they separate and recoil over a ~10 kbp region surrounding the centromere (Goshima and Yanagida 2000). It has been proposed that cells may use this mechanism to sense and control for correct tension at kinetochores and bi-orientation of homologous chromosomes prior to anaphase (Yeh et al. 2008). The $top2^{K1220,1246,1277R}$ allele is also able to stabilize spindle attachment in a genetic background where kinetochores are weakened. Both of these phenotypes occur in the absence of catenation (Warsi et al. 2008). Thus, these observations indicate that in budding yeast sumoylation impinges on a new role of Topo II in the maintenance of centromere compaction and separation that is independent of its function in decatenation.

4.8 Conclusion

In this chapter we have reviewed the contributions of the SUMO system to several aspects of genome stability, including homologous recombination, base excision repair, telomere maintenance and chromosome segregation. On one hand, these examples illustrate the diversity of the effects that the modifier can exert on cellular metabolism. Here, the pervasive influence of a few key factors, such as the SUMO-targeted ubiquitin ligases, throughout the various pathways of genome maintenance is particularly noteworthy. On the other hand, the cases discussed here exemplify two distinct mechanistic principles in the action of sumoylation: by

becoming attached to a substrate, the modifier can either create a binding surface for a particular downstream effector that recognizes SUMO through a dedicated interaction motif (SIM), as observed in the recruitment of Srs2 through sumoylated PCNA or the intricate modulation of inter- and intramolecular interactions involving human TDG. Alternatively, there are many examples of SUMO targets for which the consequences of the modification are much less well understood because of the difficulties in identifying the modification sites and/or relevant SUMO-interacting proteins. Here, a clear-cut one-to-one relationship between a SUMO target and its downstream effector may not even apply. This appears to be particularly relevant for those SUMO substrates that are part of multi-protein complexes or macromolecular assemblies where more than one subunit is modified, such as the nuclear PML bodies or the chromatin-associated HR machinery. In these cases, SUMO is believed to either maintain the structural integrity of a subcellular compartment via multiple covalent and non-covalent interactions among the different components or mediate effective recruitment and complex formation of an ensemble of factors involved in a common process, thus acting as a "molecular glue" in a relatively substrate-independent fashion. Therefore, in the future it will be necessary to differentiate between the target-specific and the bulk interaction effects of sumoylation on a case-by-case basis. Last, but not least, a better understanding of how SUMO protects DNA will require assigning the phenotypes exhibited by global sumoylation defects to the relevant target proteins.

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Regulation of Cellular Processes by SUMO: Understudied Topics

5

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Abstract

SUMO plays a multiple role in maintenance of cellular homeostasis, both under normal conditions and under cell stress. Considerable effort has been devoted to unraveling the functions of SUMO in regulation of transcription and preservation of genome stability. However, it is clear from high-throughput SUMO proteome studies that SUMO likely regulates many more cellular processes. The function of SUMO in these processes has hardly been explored. This review will focus on the emerging function of SUMO in regulation of several of these processes.

Keywords

SUMO • Transcription • Chromosome stability • Cell cycle • Lipogenesis • mRNA modification • Autophagy • Protein folding

5.1 Introduction

Protein modification by the Small Ubiquitin-like Modifier SUMO is conserved from yeast to plants and vertebrates. Protein sumoylation has been shown to regulate many cellular processes, including nuclear/cytoplasmic transport, signaling, DNA repair, cell cycle progression, the cellular stress response, and transcription (Chymkowitch et al. 2015a). SUMO is covalently linked to a plethora of proteins to regulate their enzymatic activity, subcellular localization, or their interactions with other proteins. The physiological significance of many of these sumoylation events remains unknown, which is in part due to the fact that Sumo can be attached to multiple components of an entire complex, and preventing the attachment of Sumo to a single component of the complex often has little or no clear effect (Enserink 2015). The vast majority of SUMO modifications appear to occur on DNA repair proteins and proteins involved in chromatin modification and transcription. However, large-scale SUMO proteome studies indicate that SUMO likely regulates many other cellular processes. Here, I will briefly discuss the involve-

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ment of SUMO in some well-studied processes, and then provide several examples of the emerging function of SUMO in a number of additional cellular processes.

5.2 Examples of Well-Studied SUMO-Regulated Processes

5.2.1 Genome Stability

The function of SUMO in regulation of genome stability has been studied extensively and will only be mentioned here briefly. Instead, for reviews see the following publications (Bergink and Jentsch 2009; Jackson and Durocher 2013).

Genome integrity is continuously challenged by internal and external factors that damage DNA and that interfere with DNA replication, such as DNA-damaging chemicals, ionizing radiation, UV light, and spontaneous errors that occur during DNA replication. In response to DNA damage, the cell activates the DNA damage response, which is an integrated and well-coordinated cellular response aimed at maintaining homeostasis. The DNA damage response includes, but is not limited to, cell cycle arrest, activation of DNA repair mechanisms, rewiring of transcriptional programs, and modulation of cell morphogenesis (Santocanale and Diffley 1998; Lopes et al. 2001; Lisby et al. 2004; Enserink et al. 2006; Dotiwala et al. 2007).

One of the best-studied SUMO targets in maintenance of genome stability is proliferating cell nuclear antigen (PCNA). PCNA is modified by SUMO during unperturbed DNA replication, primarily at K164 and to a lesser extent at K127 (Garcia-Rodriguez et al. 2016). Sumoylation of PCNA results in recruitment of the helicase Srs2, which prevents unscheduled homologous recombination. Sumoylation of PCNA also stimulates recruitment of the ubiquitin ligase Rad18, which, when a DNA replication-stalling lesion is encountered, promotes processing of the DNA damage through a lesion bypass pathway (Garcia-Rodriguez et al. 2016). Furthermore, when DNA damage is detected, such as a DNA double strand break, one of the cell's responses is to simultaneously sumoylate a large group of DNA repair proteins, including the three replication protein A (RPA) complex components Rfa1, Rfa2 and Rfa3, the helicase Sae2, the homologous recombination proteins Mre11, Rad50, Rad52 and Rad59, and the DNA damage checkpoint proteins Rad9 and Mrc1 (Psakhye and Jentsch 2012). Sumoylation of multiple proteins in the same pathway provides multiple low-affinity DNA interaction sites, which is believed to promote stability of the complexes to facilitate efficient DNA repair (Psakhye and Jentsch 2012).

5.2.2 Cell Cycle Regulation

Another process well known to be regulated by SUMO is the cell cycle [for a recent review see (Eifler and Vertegaal 2015)]. Numerous proteins required for efficient cell cycle progression are SUMO targets (Eifler and Vertegaal 2015). For instance, cyclin dependent kinases (CDKs) have been found to be targets of SUMO, including Cdk1, Cdk2 and Cdk6. While the exact function of sumoylation of these proteins has not been fully elucidated, sumoylation of Cdk6 in M phase appears to interfere with ubiquitination and degradation of this kinase during G1 phase, thereby promoting a subsequent round of the cell cycle by stimulating the G1/S transition (Bellail et al. 2014).

Furthermore, efficient formation of the mitotic spindle and progression through mitosis requires the E2 SUMO conjugase Ubc9, several E3 SUMO ligases including PIASy and RanBP2, as well as the SUMO proteases SENP1 and SENP2 (Eifler and Vertegaal 2015). These proteins control sumoylation of a large group of proteins required for mitotic spindle dynamics, including the kinetochore component CENP-I and the mitotic passenger proteins INCENP, Aurora B, and Borealin, as well as the mitotic checkpoint protein BubR1 (Eifler and Vertegaal 2015). Interfering with protein sumoylation during M phase, for instance by depleting the E3 ligase RanBP2, results in severe chromosomal instability, underscoring the importance of protein sumoylation in mitotic progression (Dawlaty et al. 2008).

5.2.3 Transcription

One of the best-studied processes regulated by SUMO is transcription. Numerous transcription factors and chromatin modifying enzymes have been shown to be sumoylated [for recent reviews see (Chymkowitch et al. 2015b; Enserink 2015)]. Sumoylation of the overwhelming majority of these SUMO targets appears to result in down-regulation of transcription, leading to the commonly held belief that SUMO inhibits transcription (Chymkowitch et al. 2015b). However, this view may - at least in part - be the result of a bias in the selection of research subjects and/or reporting of results, and it has recently become clear that SUMO can have a profound stimulatory effect on transcription (Chymkowitch et al. 2015b). For instance, sumoylation of the yeast transcription factor Rap1 promotes its association with TFIID, resulting in recruitment of the basal transcription machinery to Rap1-regulated promoters, which increases expression of these genes (Chymkowitch et al. 2015a). Similar findings were reported in mammalian cells (Liu et al. 2015). Nonetheless, the physiological relevance of sumoylation of the vast majority of transcription factors and chromatin-modifying enzymes remains to be established.

5.3 Examples of Less-Studied SUMO-Regulated Processes

5.3.1 RNA Editing

ADAR1 (adenosine deaminase that acts on RNA) is a member of the family of enzymes that convert adenosine to inosine in double-stranded RNA. This conversion in coding sequences can lead to amino acid changes that affect protein function. A-to-I RNA editing also occurs in 5′ and 3′ UTR, introns, and splicing branch sites (Nishikura 2016). ADAR1 enzymes also modify miRNAs, and they interact with Dicer to promote miRNA processing (Nishikura 2016). Interestingly, ADAR1 is sumoylated on I$\underline{\mathbf{K}}_{418}$LE, which reduces the editing activity of the enzyme. Although the physiological consequences of ADAR1 sumoylation were not explored, given the large number of RNAs that are edited in mammalian cells (Athanasiadis et al. 2004), this could have widespread consequences for cell homeostasis. The exact conditions that govern ADAR1 sumoylation also remain to be explored.

5.3.2 snoRNA

Small nucleolar RNAs (snoRNAs) are a large class of small noncoding RNAs that play a central role in ribosome biogenesis, guiding the sequence-specific chemical modification of pre-rRNA (ribosomal RNA) by 2′-O-methylation or pseudouridylation (Stepanov et al. 2015). Some snoRNAs also regulate alternative splicing and posttranscriptional modification of mRNA (Stepanov et al. 2015).

Interestingly, multiple proteins involved in snoRNA maturation have been found to be sumoylated. For instance, Nhp2 is mainly sumoylated on I$\underline{\mathbf{K}}_{5}$AD, and Nop58 on V$\underline{\mathbf{K}}_{467}$VE and on I$\underline{\mathbf{K}}_{497}$EE (Westman et al. 2010). Importantly, sumoylation of Nop58 is essential for its interaction with snoRNAs, and expression of a non-sumoylatable Nop58 mutants results in mislocalization of U3 snoRNA, suggesting that sumoylation of Nop58 is important for snoRNP biogenesis. The E3 ligase that mediates sumoylation of Nhp2 and Nop58 has not yet been identified, and the upstream pathway that regulates sumoylation of these proteins remains unknown.

5.3.3 mRNA Translation

Sumo regulates the efficiency of translation at multiple levels. For instance, it promotes the synthesis of tRNA, as well as the transcription and maturation of ribosome components and ribosome biogenesis factors (Finkbeiner et al. 2011; Liu et al. 2012, 2015; Chymkowitch et al. 2015a).

However, SUMO likely has more direct effects on mRNA translation. For instance, in mammalian cells a key SUMO target in regulation of

translation is eukaryotic translation initiation factor 4E (eIF4E), which is sumoylated on K36, 49, 162, 206 and 212 (Xu et al. 2010). eIF4E binds to the mRNA 5′ cap and brings the mRNA into a complex with other protein synthesis initiation factors and ribosomes, which is important for the translation of capped mRNAs. Sumoylation of eIF4E is important for dissociation of eIF4E from 4E–BP1 and for the formation of the eIF4F complex (which consists of eIF4E, eIF4G and eIF4A) (Xu et al. 2010), and expression of unsumoylatable eIF4E impairs translational efficiency (Xu et al. 2010). Interestingly, sumoylation of eIF4E is dependent upon phosphorylation of residue Ser209, which is mediated by Mnk kinases in response to mitogens, and which has been shown to increase the affinity of eIF4E for capped mRNA and for the associated scaffolding protein eIF4G (Waskiewicz et al. 1999). However, the molecular mechanism by which Mnk kinases activate eIF4E has remained mysterious. The fact that eIF4E phosphorylation promotes eIF4E sumoylation and subsequent stabilization of the eIF4F complex raises the interesting possibility that the SUMO moieties are recognized by SIMs in other components of the complex, resulting in complex stabilization. In support of this hypothesis, data from several studies indicate that other components of eIF4F are also sumoylated, i.e. eIF4A and eIF4G, although the exact physiological relevance of these sumoylation events remains unclear (Matafora et al. 2009; Bruderer et al. 2011; Jongjitwimol et al. 2014).

In addition to translation initiation factors, the ribosome itself may also be targeted by SUMO, since multiple ribosomal proteins have been found to be sumoylated in whole-proteome studies (Panse et al. 2004; Wohlschlegel et al. 2004; Zhou et al. 2004; Denison et al. 2005; Hannich et al. 2005; Albuquerque et al. 2013). Furthermore, many ribosomal proteins have been reported to physically interact with SUMO (Sung et al. 2013). The function of ribosome sumoylation, as well as the nature of the physical interactions between ribosomal components and SUMO, remains unknown. However, given that SUMO most likely stimulates translation by increasing the synthesis of ribosomal proteins and by promoting ribosome biogenesis, the effect of ribosome sumoylation is probably to enhance the translational capacity of the cell. In this model, sumoylation of ribosomal proteins may create multiple low-affinity binding sites for other ribosomal components to stabilize the ribosomal complex.

5.3.4 Protein Folding

An unexpected role for SUMO in cellular homeostasis was recently reported in yeast, i.e. regulation of protein folding (Mollapour et al. 2014). The critical SUMO target in this process is HSP90, which is sumoylated on L$\underline{K}_{178}$DD, an optimal SUMO consensus motif. Interestingly, sumoylation of HSP90 is specifically important for the interaction of HSP90 with the co-chaperone Aha1, but not of other HSP90 cochaperones, such as Cdc37^{p50}, Sti1^{HOP} and Sba1^{p23}. HSP90 molecules form dimers *in vivo*, and, surprisingly, only HSP90 dimers in which just one protomer is sumoylated are able to recruit Aha1. Unexpectedly, sumoylation of HSP90 facilitated binding of HSP90 inhibitors and sensitized cells to these drugs. These findings may provide an explanation for the previous observations that tumor cells retain HSP90 inhibitors for a much longer period of time than wild-type cells (Trepel et al. 2010), and that tumor cells are often killed more efficiently by HSP90 inhibitors than healthy cells (Bisht et al. 2003).

Proteomic studies in yeast indicate that other HSPs may also be sumoylated, such as HSP42, and the HSP70 family members Ssa1, Sse1, Ssb1 and Ssb2. The physiological function of sumoylation of these proteins remains unclear. HSP42 is a small heat shock protein that suppresses unfolded protein aggregation; Ssa1 is involved in many cellular processes including protein folding and NLS-directed nuclear transport; Sse1 is the ATPase component of the HSP90 holocomplex; and Ssb1 and Ssb2 are ribosome-associated proteins that stabilize long, slowly translated, and aggregation-prone nascent polypeptides (Willmund et al. 2013). It will be

interesting to see how SUMO regulates the activity of all these proteins.

5.3.5 Lipogenesis

SUMO is well known to regulate multiple aspects of cell metabolism (Wilson 2009). However, the effect of SUMO on one particular aspect of metabolism, i.e. lipogenesis, is not well studied. SUMO is likely to play a key role in this process, because SUMO-1 knock-out mice fed on a high-fat diet gain less weight and have smaller and fewer adipocytes than wild-type mice (Mikkonen et al. 2013).

One important SUMO target in lipogenesis is the nuclear receptor peroxisome proliferator-activated receptor γ (PPARγ) (Ohshima et al. 2004). PPARγ is sumoylated on I**K**$_{107}$VEP, an optimal SUMO motif. Sumoylation of PPARγ inhibits its ability to transactivate transcription, possibly by stabilizing a repressive complex, resulting in downregulation of a multitude of genes involved in lipid synthesis (Ohshima et al. 2004).

Another target of SUMO are the transcription factor sterol regulatory element-binding proteins 1 and 2 (SREBP1/2), which regulate a wide variety of genes involved in cholesterol and fatty acid synthesis and low density lipoprotein (LDL) uptake (Goldstein et al. 2006). Both SRBP1a and SRBP2 are sumoylated, on I**K**$_{123}$EE and on V**K**$_{418}$TE, and on V**K**$_{464}$DE, respectively (Hirano et al. 2003). Sumoylation of these transcription factors results in recruitment of the repressive HDAC3 complex, leading to decreased transactivation capacity of these transcription factors (Arito et al. 2008).

Together, these findings indicate that SUMO inhibits the transcriptional programs involved in cholesterol and fatty acid homeostasis, which may be important to turn off lipogenesis during nutritional deprivation (Lee et al. 2014). While the examples above are relatively well-studied SUMO targets, SUMO likely also affects lipogenesis at other levels. For instance, in yeast SUMO is important for inositol synthesis, although its critical substrate remains elusive (Felberbaum et al. 2012). One potential target of SUMO in this process is Scs2, which is an endoplasmic reticulum (ER) membrane protein that regulates phosphatidylinositol synthesis and lipid trafficking. Scs2 is sumoylated on V**K**$_{180}$KE, however preventing sumoylation of this residue did not appear to have any effect on inositol synthesis. Therefore, the SUMO substrates that control inositol synthesis remain to be identified.

5.3.6 Cell Morphology

Maintenance of correct cell morphology is essential to well-being of any organism (Rodriguez-Boulan and Macara 2014). Cell morphogenesis is controlled by numerous proteins and regulatory pathways, including septins (Howell and Lew 2012). Septins are eukaryotic GTP-binding proteins that form filaments at the cell cortex and which associate with actin and microtubule cytoskeletal networks. The septin cytoskeleton has multiple functions, including coordination of cell division, cell polarity, remodeling of the membrane and establishment of diffusion barriers (Bridges and Gladfelter 2015). Septins were among the first SUMO targets to be identified (Johnson and Blobel 1999). The yeast septin Cdc3 is sumoylated on L**K**$_{4}$EE, I**K**$_{11}$QD, I**K**$_{30}$QE, V**K**$_{63}$VE and possibly A**K**$_{287}$SD. The septins, Cdc11 and Shs1, are primarily sumoylated on IK$_{412}$QE and on I**K**$_{426}$QE and I**K**$_{437}$TE of Shs1, respectively. Preventing sumoylation of septins resulted in a septin disassembly defect, although the underlying molecular mechanism remains unclear (Johnson and Blobel 1999).

In mammalian cells, SUMO has a more pronounced effect on cell morphogenesis. For instance, sumoylation of the GTPase Rac1 by PIAS3 is required for optimal cell migration (Castillo-Lluva et al. 2010). Rac1 is sumoylated on a stretch of non-consensus sites: V**K**$_{183}$**K**$_{184}$R**K**$_{186}$R**K**$_{188}$CL, and substitution of these lysines with arginine residues resulted in decreased Rac1 GTP loading, although the exact mechanism remains unclear. Importantly, sumoylation of Rac1 promotes formation of

lamellipodia-ruffle, cell migration and invasion (Castillo-Lluva et al. 2010).

Another important target of SUMO in cell morphogenesis is Rho GDP dissociation inhibitor (RhoGDI) (Yu et al. 2012). RhoGDI binds small Rho-family GTPases and keeps them in a biologically inactive state in cytoplasm, thereby affecting actin polymerization and cell motility. RhoGDI is sumoylated on V**K**$_{138}$ID. RhoGDI sumoylation increases the affinity of RhoGDI for the small GTPase Rho, thereby inhibiting actin polymerization, cytoskeleton formation and cell motility (Yu et al. 2012).

These two studies find seemingly opposing roles of SUMO in cell migration; sumoylation of Rac1 promotes cell migration, whereas sumoylation of RhoGDI inhibits this process. How the cell sumoylation of these targets to regulate cell migration remains to be established.

5.3.7 Autophagy

Autophagy is a key element of the response to nutrient starvation is activation of macroautophagy (Efeyan et al. 2015) (here referred to simply as autophagy). Autophagy is an evolutionarily conserved lysosomal degradation pathway that mediates the recycling of cytoplasmic components to supply nutrients (Nakatogawa et al. 2009; Kaur and Debnath 2015). Yeast mutants that fail to undergo autophagy rapidly lose viability under nitrogen-limiting conditions (Nakatogawa et al. 2009), and deregulation of autophagy in mice leads to early developmental defects, neurodegeneration, or cancer (Kuma et al. 2004; Mizushima et al. 2008; Tsukamoto et al. 2008; Nakatogawa et al. 2009; Galluzzi et al. 2015; Menzies et al. 2015), emphasizing the physiological importance of this catabolic process. Although autophagy occurs constitutively at a low basal level, starvation, growth factor deprivation, protein aggregation, as well as other cellular stresses rapidly increase its activity (Mizushima et al. 2008). Under these conditions, autophagy is crucial for generating nutrients or removing damaged cytoplasmic components, serving mainly as a protective cellular response (Mizushima et al. 2008).

Given the importance functions of autophagy, it is tightly controlled by several cellular signaling pathways (Feng et al. 2015; Kaur and Debnath 2015). There are indications that SUMO also regulates autophagy. For instance, activation of autophagy is enhanced by overexpression of SUMO1 and decreased by SUMO1 depletion (Cho et al. 2015).

Several targets of SUMO implicated in regulation of autophagy have been identified. One potential target of SUMO is the acetyltransferase Tip60/KAT5 (Naidu et al. 2012). Tip60 acetylates p53 on K120 within the DNA binding domain, which promotes the transcriptional activity of p53 (Sykes et al. 2006; Tang et al. 2006). Tip60 is sumoylated by PIASy on L**K**$_{430}$SE and on I**K**$_{451}$KE, and expression of a non-sumoylatable Tip60 mutant reduces p53 acetylation on K120, indicating that sumoylation of Tip60 stimulates its catalytic activity (Naidu et al. 2012). Because acetylation of K120 of p53 promotes autophagy (Naidu et al. 2012), sumoylation of Tip60 may therefore help activate autophagy.

Another potential target of SUMO in regulation of autophagy may be Vps34 (Yang et al. 2013). Vps34 is a class III phosphatidylinositol-3-OH-kinase (PI3K) important for activation of the autophagic machinery (Simonsen and Tooze 2009). Vps34 is the catalytic subunit of the Vps34 complex, which also includes Vps15, ATG14L and Beclin. Under autophagy-inducing conditions, HSP70 binds the Beclin-Vps34 complex. Subsequently, HSP70 recruits the E3 ligase KAP1, which in turn sumoylates Vps34 on V**K**$_{840}$KV. Sumoylated Vps34 increases the activity of Vps34 to stimulate autophagy (Yang et al. 2013).

These examples show that SUMO has a stimulatory effect on autophagy. However, given the complexity of the autophagy process, it is likely that additional SUMO targets remain to be identified.

5.4 Conclusion

SUMO is well known to be important for processes like transcriptional regulation and DNA repair, and numerous proteins involved in these processes are direct SUMO targets. However, the examples described above indicate that SUMO has many more functions in maintenance of homeostasis. Most of these processes remain poorly understood, and the upstream signals and pathways that control sumoylation, as well as the critical SUMO targets, remain almost completely unknown.

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The Molecular Interface Between the SUMO and Ubiquitin Systems

6

Jeff L. Staudinger

Abstract

The SUMO conjugation system regulates key cellular processes including cell growth, division, mitochondrial dynamics, and the maintenance of genome stability in eukaryotic cells. The ubiquitin conjugation system regulates the stability of a myriad of vital cellular proteins in a signal-dependent manner by targeting them for destruction via the proteasome-mediated degradation pathway. Recent research efforts have unveiled an evolutionarily conserved and fundamental molecular interface between the SUMO and ubiquitin systems. A coordinated and integrated interaction between these two pathways plays a key role in adapting the SUMO-related stress response to alterations in sub-cellular protein localization, specific protein recruitment strategies, and the regulation of stress-inducible protein stability. This chapter will describe the interconnected and interdependent role of the SUMO and ubiquitin systems in mediating DNA damage repair and the genesis and the resolution of inflammatory-related diseases such as cancer. New insights regarding the interdependence of these two important post-translational modifications with nuclear receptor superfamily members will also be highlighted.

Keywords

DNA damage repair • Inflammatory-related disease • STUbls • RNF4

6.1 DNA Checkpoints, DNA-Repair

The genomes of all living organisms on earth endure genotoxic stressors such as exposure to ultraviolet light, as well as foreign chemicals that produce oxidative DNA damage. If left unrepaired, the resulting damage to the genome often

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leads to cell death. In mammals, the loss of genome stability is connected with a plethora of human diseases including cancer, as well as both neurological and developmental disorders. Hence, all living organisms have evolved mechanistic safeguards which promote the accurate passage of their replicated genomes during mitosis to daughter cells. Such safeguards in eukaryotic systems include both cell cycle checkpoints that halt genome replication, as well as several programs of sophisticated and faithful DNA-repair. The initiation of these molecular events comprises an evolutionarily conserved pathway that functions as the guardian of genomic stability and DNA integrity. The DNA integrity checkpoints prevent mitosis from proceeding in the presence of damaged DNA or other problematic replication errors. These checkpoints also provide the replicating cell with the opportunity to coordinate the appropriate DNA-repair response.

6.2 Key Historical Discoveries in the Field

When first discovered in 1996, post-translational modification of proteins by SUMO was initially thought to be un-related to the ubiquitin system (Johnson 2004). Subsequent investigations revealed that the SUMO and ubiquitin systems shared certain target lysine residues in key proteins involved in regulating the inflammatory response and DNA repair (Desterro et al. 1998; Hoege et al. 2002; Huang et al. 2003). It was therefore speculated that the SUMO and ubiquitin systems were thought to be, in part, competitive events that target identical lysine residues. However, it soon became clear that their actions could only rarely be explained by simple competition (Ulrich 2005). Nonetheless, it would be another 2 years before researchers discovered that the SUMO and ubiquitin systems were linked in a very specific manner that eventually led to the current understanding of the process of SUMO-dependent ubiquitination.

The key discovery that led to the unveiling of the interdependence of these two processes was the identification of an evolutionarily conserved family of enzymes that serve as the scaffold upon which recently sumoylated proteins become the subsequent target of the ubiquitin-mediated protein degradation system. This family of enzymes have been termed SUMO-targeted ubiquitin ligases (STUbLs), also known as ubiquitin ligases for sumoylated proteins (ULS) (Hunter and Sun 2008; Perry et al. 2008; Prudden et al. 2007; Sun et al. 2007; Uzunova et al. 2007). Two key protein domains/motifs contained in all STUbL/ULS proteins include (1) the RING-finger domain and (2) the SUMO-interacting or SUMO-binding motif (SIM/SBM). For simplicity, I will refer to the SUMO-targeted ubiquitin ligase enzymes as 'STUbLs' and the SUMO-interacting motifs as 'SIMs', respectively, throughout the remainder of this chapter.

A RING- (Really Interesting New Gene) finger is a protein structural motif that comprises a zinc-finger domain containing the Cys_3-His-Cys_4 amino acid sequence. RING finger domains function to coordinately bind to two zinc cations forming two finger-like structures (Borden and Freemont 1996; Lovering et al. 1993). Each RING finger domain contains from 40 to 60 amino acids that bind target proteins, such as certain ubiquitin ligase enzymes, with high levels of specificity. While many proteins contain RING finger domains, several key RING-finger domain-containing proteins play a pivotal role in the crosstalk between the SUMO and ubiquitin systems. They are all collectively referred to as STUbLs. All of the presently characterized STUbL proteins also contain a tandem array of SIMs.

The SIM, apart from the consensus sumoylation site motif, was originally defined as a unique concept using yeast two-hybrid methods (Hecker et al. 2006; Minty et al. 2000). Additional studies using a nuclear magnetic resonance approach revealed the presence of a SIM in RanBP2, the best characterized SUMO-E3 ligase enzyme for RanGap (Song et al. 2004). The authors further identified the presence of SIMs in nearly all proteins known to be involved in SUMO-dependent processes. This study clearly identified the general role of SIMs in sumoylation-dependent cellular functions. Further analysis

using genetic approaches in yeast and mass spectrometry identified several functional categories of SUMO-modified proteins and potential SIMs in SUMO conjugation system enzymes including E3 SUMO-ligase enzymes, chromatin- and gene silencing-related factors, DNA repair and genome stability proteins, stress-related proteins, transcription factors, proteins involved in translation and RNA metabolism, and a variety of metabolic enzymes (Hannich et al. 2005). Thus, the SIM was discovered to play a central role in mediating the effects of the sumoylation signal transduction pathway. Since then it has become apparent that all SUMO substrates, E3 SUMO-ligase and STUbL enzymes likely contain a canonical, atypical, or undefined SIM or series of SIMs by necessity. The SIM, as a unique concept, has therefore only recently been revealed as the central mediator of the physical interface between the SUMO and ubiquitin conjugation systems.

6.3 More Recent Key Discoveries in the Field

The STUbL family of enzymes includes the founding members from *Schizosaccharomyces pombe* called Slx8-Rfp1, and from budding yeast *Saccharomyces cerevisiae* Slx5 and Slx8, respectively. Their involvement in the promotion of genome stability and their ability to interact with each other and to bind directly to DNA was characterized in 2006 (Wang et al. 2006; Yang et al. 2006; Zhang et al. 2006).

In a remarkable series of studies using yeast model systems several groups identified the fact that STUbL enzymes are recruited to SUMO-modified proteins to mediate their subsequent ubiquitination and degradation (Burgess et al. 2007; Ii et al. 2007a, b; Mullen and Brill 2008; Xie et al. 2007). In perhaps the most elegant of the studies mentioned here, Sun et al., revealed that mammalian RNF4, complements the growth and genomic stability defects of mutant yeast cells lacking Rfp1, Rfp2, and Slx8 (Sun et al. 2007). These authors further showed that both the Rfp-Slx8 complex and RNF4 specifically ubiquitinate artificial SUMO-containing substrates in vitro in a SUMO-binding-dependent manner. Moreover, they showed that a large number of sumoylated proteins accumulate in rfp1-rfp2 double-null yeast cells, suggesting that Rfp/Slx8 proteins may promote ubiquitin-dependent degradation of a myriad of sumoylated targets in vivo. In mammalian cell lines, the overexpression of $(His)_6$-tagged SUMO2 enabled the co-purification of poly-SUMO2-modified proteins that were also found to be highly ubiquitinated, but only in the presence of proteasome inhibitors (Schimmel et al. 2008). Thus, the concept that sumoylation of a protein can help trigger its subsequent ubiquitination and likely degradation through the recruitment of a ubiquitin ligase through a SIM, or possibly multiple tandem SIMs, was born.

This new understanding of the molecular interface between the SUMO and ubiquitin systems has become particularly important in the present day because of its newly identified and prominent role in maintaining the integrity of the human genome (Nie and Boddy 2016; Sriramachandran and Dohmen 2014). Moreover, the discovery of its mechanistic role in the arsenic-mediated cure of promyelocytic leukemia has shed new light on the importance of this concept in cancer research and treatment (Geoffroy et al. 2010; Lallemand-Breitenbach et al. 2008; Percherancier et al. 2009; Tatham et al. 2008). I will now focus on the recent discoveries regarding the human RNF4 protein, alternatively referred to as '**s**mall **nu**clear **R**ING **f**inger protein' or SNURF (Moilanen et al. 1998). For simplicity I will use the RNF4 nomenclature to indicate the RNF4/SNURF protein.

6.4 The Identification of RNF4

The gene encoding the really interesting new gene (RING)-domain-containing 4 (RNF4) protein was originally identified in 1998 (Chiariotti et al. 1998). The authors found that RNF4 messenger RNA is expressed at low levels in all human tissues examined, however, very high expression of RNF4 mRNA is detected in human testis. The mouse homolog of RNF4 is abundantly

expressed very early in embryonic tissues and exhibits a ubiquitous pattern of expression. Interestingly, the gene encoding RNF4 maps between the huntingtin (HD) and the fibroblast growth factor receptor 3 (FGFR3) genes in both the human and mouse genomes.

Also in 1998, the RNF4 protein was identified in a yeast two-hybrid screen using the androgen receptor DNA-binding domain as bait (Moilanen et al. 1998). The biochemical function of RNF4 was hypothesized to be that of a novel coactivator protein. This was due to the fact that androgen receptor transactivation capacity was increased by co-expression of RNF4. These authors further identified a more pleotropic role on nuclear receptor mediated transactivation capacity based on the observation that overexpression of RNF4 in cultured mammalian cells enhanced transcription from several endogenous steroid-regulated promoters. They further observed that mutations in the RING finger which abolished zinc-binding also abrogated RNF4-mediated enhanced basal transcription. In contrast, RNF4 containing these same RING-finger mutations retained its ability to co-activate steroid receptor-dependent transcription. The authors therefore suggested that there are separate domains in RNF4 that interact with different regulatory factors to mediate these two separate biochemical effects. These early studies suggested that RNF4 functions as a bridging factor to regulate steroid receptor-dependent transcription by a mechanism different from those of canonical histone deacetylase/histone acetyl transferase nuclear receptor co-regulatory proteins. This study revealed a fundamental biochemical interface between the RNF4 protein and nuclear receptor proteins. It is now evident that RNF4 functions to modulate the transactivation capacity of key members of the nuclear receptor superfamily of ligand-activated transcription factors. This is a significant and important concept that I will return to later in this chapter.

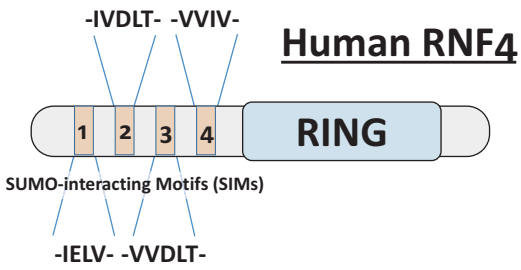

Fig. 6.1 Schematic Representation of Human RNF4. The RNF4 protein has four tandem SUMO-Interacting Motifs (SIMs) labelled as (*1–4*) at its N-terminal region. The amino acid sequences that comprise each respective SIM are shown. The SIMs are followed by a RING-finger domain that binds to ubiquitin ligase enzymes through protein-protein interactions

The RNF4 protein is 190 amino acids in length and represents the best characterized STUbL to date (Fig. 6.1) (Lallemand-Breitenbach et al. 2008; Lallemand-Breitenbach et al. 2012; Prudden et al. 2007; Sun et al. 2007; Tatham et al. 2008; Weisshaar et al. 2008). The human RNF4 protein has four characterized SIMs arranged in tandem that mediate high-affinity binding to poly-SUMO2/3 chains of at least three SUMO moieties in length (Tatham et al. 2008). Remarkably, a recent study using mass spectrometry exploited this fact to isolate and characterize over 300 proteins as potential RNF4-target substrate proteins using a non-denaturing affinity pull-down approach (Bruderer et al. 2011). Structural studies indicate that the RNF4 protein functions as a heterodimer, and the stability of the E2~ubiquitin thioester bond is regulated by RING domain dimerization. Additional structural studies indicate that the RNF4 dimer facilitates ubiquitin transfer by preferentially binding E2-charged ubiquitin-thioester across the dimer and activating this bond for catalysis (Liew et al. 2010; Plechanovova et al. 2011, 2012). Taken together, these studies collectively revealed the existence of a family of SIM-containing RING-finger proteins that regulate eukaryotic poly-sumoylated protein stability by linking SIM-containing STUbL proteins with ubiquitin conjugation and protein degradation in an evolutionarily conserved manner (Fig. 6.2).

The question arises as to why cells adopt the strategy of ubiquitinating and subsequently degrading highly poly-sumoylated proteins. The answer appears to be that this approach defends the cell against massive accumulation of poly-sumoylated proteins which are necessarily generated under

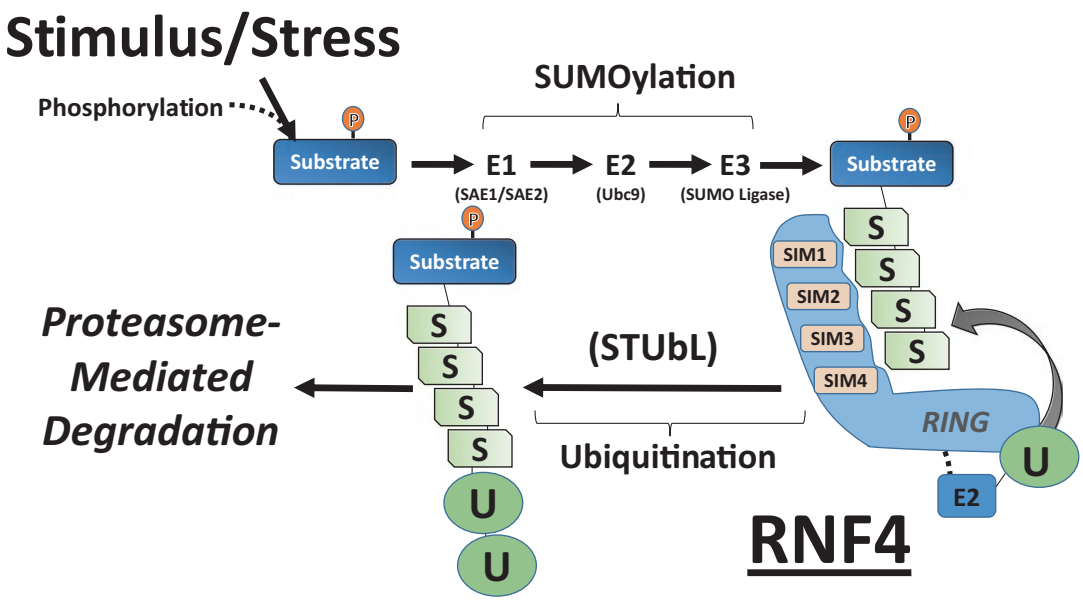

Fig. 6.2 Schematic of a General Model of the Molecular Basis of STUbL-mediated Interaction Between the SUMO and Ubiquitin Systems. An extracellular stress stimulates a phosphorylation event on a potential poly-SUMO-substrate protein. The canonical sumoylation cascade consisting of E1, E2, and E3 enzymes promotes modification of the given substrate. The formation of poly-SUMO chains allows recruitment of the RNF4 STUbL enzyme through its tandem array of four SIMs in close association with an E2-Ubiquitin ligase. This complex promotes the formation mixed SUMO-ubiquitin chains that are subsequently recognized by the proteasome, thereby leading to the SUMO-dependent ubiquitin mediated degradation of the target protein

conditions of high cellular stress. The stress conditions can include stimuli such as DNA damage, oxidative, hypoxic, and xenobiotic/chemical insults. This tactic allows the cell to eventually down-regulate SUMO-related stress responses by preventing their over-accumulation via signal-dependent and targeted protein degradation.

More recent proteomic and biochemical research indicates that a significant pool of poly-SUMO2/3 chains exist as hybrid molecules with ubiquitin to form interacting surfaces with binding proteins that contain both tandem SIMs and ubiquitin-binding motifs (Hay 2013). For example, the DNA repair function of the breast cancer susceptibility protein, BRCA1, depends upon its interaction with RAP80. The RAP80 protein targets BRCA1 to DNA double-strand breaks through recognition of poly-ubiquitin chains and also requires sumoylation (Guzzo et al. 2012). These authors discovered that, in addition to high affinity ubiquitin-interaction motifs, RAP80 also contains a SIM that functions cooperatively with its ubiquitin-binding motif. In other words, in combination with the ubiquitin-binding activity, the SIM in RAP80 enables this protein to bind with nanomolar affinity to hybrid SUMO-ubiquitin chains. Moreover, it is now clear that RNF4 synthesizes hybrid SUMO-ubiquitin chains, and this activity is necessary for the recruitment of both RAP80 and BRCA1 to sites of DNA damage. These findings connect RNF4-synthesized hybrid SUMO-ubiquitin chains to directly BRCA1-recruitment and DNA repair of double-stranded DNA breaks.

6.5 Cancer, DNA Damage, and the Best Known Substrate of RNF4

Acute promyelocytic leukemia is characterized by a very specific chromosomal translocation involving the retinoic acid receptor alpha (RARα) on chromosome 17 with the promyelocytic

leukemia (PML) gene on chromosome 15. Patients with this form of leukemia aberrantly express large amounts of the PML-RARα oncoprotein. Remarkably, these patients have achieved outstanding cure rates following a treatment regimen comprised of retinoic acid (RA), arsenic trioxide, or a combination of both compounds. Treatment with arsenic trioxide by itself cures nearly 70% of these patients, whereas those treated with the combination of RA and arsenic trioxide reach an astounding 90% cure rate.

It is therefore important to note that the first substrate identified for the human RNF4 STUbL was the PML protein, as well as the oncogenic fusion PML-RARα (Lallemand-Breitenbach et al. 2008). Treatment with retinoic acid and arsenic trioxide has been shown, after the fact, to target the PML-RARα fusion protein at several levels. When exposed to arsenic trioxide and retinoic acid, both the sumoylated form of PML and the oncoprotein PML-RARα recruit RNF4, ubiquitin and proteasomes onto PML nuclear bodies (Geoffroy et al. 2010; Lallemand-Breitenbach et al. 2012; Percherancier et al. 2009; Tatham et al. 2008; Weisshaar et al. 2008). Collectively, these studies have revealed that the sumoylated form of PML and the PML-RARα oncoprotein utilizes the STUbL function of RNF4 to integrate its subsequent ubiquitination to the proteasome-mediated protein degradation pathway. It is noteworthy that PML sumoylation recruits not only RNF4, ubiquitin and proteasomes, but also recruits many other sumoylated proteins onto PML nuclear bodies. It will therefore likely be important to determine the identity of additional substrates for RNF4.

6.6 Additional Substrates and Molecular Functions of RNF4

Specific knowledge regarding additional substrates for RNF4 and other STUbLs has shed new light on the regulation of DNA damage checkpoints, DNA repair pathways and additional important and highly regulated metabolic pathways. For example, more recent research indicates that RNF4 participates in the repair of double-stranded breaks at telomeres and that RNF4 likely binds directly to nucleosomes through its RING domain (Groocock et al. 2014; Hakli et al. 2001). Recruitment of RNF4 to double-stranded breaks in DNA is likely a cooperative process involving both the tandem array of SIMs together with the RING domain-interaction with telomere-specific proteins and nucleosomes, respectively (Galanty et al. 2012; Lescasse et al. 2013; Mullen and Brill 2008; Yin et al. 2012). Additional protein substrates of RNF4 include the kinetochore protein CENP-I (Mukhopadhyay et al. 2010). RNF4 interaction with the CENP-I protein regulates the kinetochore CENPH-CENPI-CENPK multi-protein complex by targeting poly-sumoylated CENPI for proteasomal degradation. Martin et al., found that RNF4 mediates the heat-shock-inducible ubiquitination of PARP-1 (Martin et al. 2009). RNF4-mediated ubiquitination of poly-sumoylated PARP-1 regulates its stability and is a positive regulator of gene expression in response to heat shock. The RNF4 protein also binds to and promotes the ubiquitination of poly-sumoylated hypoxia-inducible factors HIF1α/HIF2α to regulate the cellular responses to hypoxia (Keith et al. 2012; van Hagen et al. 2010). RNF4 binds and co-activates the PEA3 transcription factor, which plays a key role in mammary oncogenesis (Guo and Sharrocks 2009). In the case of PEA3, in contrast to most transcription factor sumoylation events which typically impart a repressive function, sumoylation of PEA3 is required for maximal activation of its target genes including the inflammatory modulators MMP-1 and COX-2. Importantly, the sumoylation of PEA3 is required for its subsequent ubiquitination to promote its degradation. This is in keeping with the 'promoter clearance' model of the role of ubiquitin in imparting maximal transcriptional regulation (Dennis and O'Malley 2005; Lonard and O'Malley 2009). Specific knowledge of the identity of additional RNF4 substrates is expected to contribute to a deeper understanding of the DNA damage response, metabolic pathways, and the inflammatory response. In particular, recent research efforts have revealed an integrated inter-

action of the SUMO and ubiquitin systems with several important nuclear receptor superfamily members.

6.7 Sumoylation, Ubiquitination, Nuclear Receptors, Bile Acid Homeostasis, Cholesterol Metabolism, and the Inflammatory Response

It is now well-recognized that the interaction between SUMO and ubiquitin at the level of key nuclear receptor proteins forms an essential biological interface that underlies a sophisticated post-translational regulatory scheme among specific members of this superfamily of ligand activated transcription factors. The targeting of SUMO and ubiquitin to nuclear receptor superfamily members and their and co-regulatory proteins generally modulates their transactivation capacity by altering their stability and protein partner interaction profile. Future research efforts should focus on the potential role of RNF4 and other mammalian STUbLs in these processes.

The molecular differences in the biochemistry and mode of SUMO or ubiquitin attachment have very distinct consequences on the biology of nuclear receptor proteins. For example, mono-ubiquitination –versus- poly-ubiquitination, as well as the site and type of poly-ubiquitin linkage on a given nuclear receptor family member can lead to very distinct biological outcomes. The same is also true for sumoylation attachment modes, that is, whether a nuclear receptor is the target of mono-sumoylation by SUMO1 –versus- poly-sumoylation by SUMO2/3 gives rise to various nuclear receptor-mediated biological outcomes.

Indeed, it is worth noting here that from the outset, the nuclear receptor superfamily emerged as an important target of both the sumoylation and ubiquitin proteasome systems. This fact is aptly demonstrated by the original identification and biochemical analysis of the RNF4 STUbL STUbL enzyme as an androgen receptor-interacting protein in a yeast two-hybrid screen (Moilanen et al. 1998). Hence, nuclear receptor-mediated transcription is inextricably linked to both the SUMO-signaling pathway and the ubiquitin proteasome system. Indeed, both the ubiquitin and SUMO signaling pathways have integrated regulatory functions in vital nuclear receptor-mediated gene activation programs. Specifically, gene activation programs including those mediating the initiation and resolution of the acute inflammatory response, regulation of cholesterol and bile acid homeostasis are profoundly impacted by the SUMO and ubiquitin systems at the level of nuclear receptor proteins.

A recent thrust of research has uncovered key insights into how post-translational modification of nuclear receptor proteins in eukaryotic organisms regulate the expression and activity of cholesterol metabolism, bile acid metabolism, and inflammatory mediators in a coordinated manner to inversely affect these important biological outcomes (Ghisletti et al. 2007; Kim et al. 2015; Pascual et al. 2005). The integration of sumoylation and ubiquitination plays a key role in regulating the xenobiotic and inflammatory responses in liver (Cui et al. 2015; Hu et al. 2010; Sun et al. 2015). The involvement of counter-regulatory systems mediated by an integration of the SUMO- and ubiquitin-signaling pathways at the level of Nuclear Factor Kappa B (NFkB) in combination with several important nuclear receptor proteins has been previously described (Ghisletti et al. 2007; Kim et al. 2015; Pascual et al. 2005). Nuclear receptor super family members PPARγ, FXR, LXRα and LXRβ are all targeted by the SUMO-and ubiquitin-signaling pathways to diminish the acute inflammatory response. The acetylation of nuclear receptor proteins is also being implicated in the integrated response to aberrant extracellular and pathophysiologic signals (Kim et al. 2015).

6.8 SUMO, Ubiquitin, Acetyl, Pregnane X Receptor, and Drug Metabolism

Much like the regulation of DNA repair processes, the removal of xenobiotic insult and the initiation and eventual resolution of the appropriate inflammatory response share the central characteristic of being tightly regulated at the transcriptional level. Similar to the DNA damage response, both of these biochemical functions are absolutely necessary for the organism's survival. In higher mammalian systems, the enzymes that function to regulate drug and xenobiotic metabolism also metabolize steroid hormones and bile acids, and are thus key in avoiding a toxicological episode. In mammals, the liver is the major organ responsible for mediating these processes. A coordinated program of hepatic gene expression ensues upon exposure to specific xenobiotics, drugs, bile acids, and environmental toxicants.

The coordinated program of gene expression is highly inducible and is comprised of multiple cytochrome P450 family members, several key hepatic uptake and efflux drug transporter proteins, and other detoxification systems to include pivotal glutathione-s-transferase enzymes, several UDP-glucuronosyl transferase enzymes, and numerous carboxylesterase enzymes (Staudinger 2013; Staudinger et al. 2003). In this way, the organism is protected from xenobiotic insult by upregulating the activity of these protective systems in liver to aid in their eventual excretion (Staudinger et al. 2006). All of these protective responses are under the control of the 'master regulator' of drug metabolism, excretion, and efflux- pregnane x receptor (PXR, NR1I2), a member of the nuclear receptor superfamily of ligand-activated transcription factors (Kliewer et al. 2002). A very important clinical phenomenon has been recognized for years in which patients that present with chronic or acute inflammatory conditions are compromised with respect to these hepatic detoxification systems, and are thus at increased risk for toxicity due to their propensity to retain drugs in their body. This is especially clinically relevant in cancer patients as the cytochrome P450 3A4 enzyme, the prototypical hepatic PXR-target gene, is the major metabolizer of anti-cancer agents that is dramatically suppressed during acute inflammation (Kacevska et al. 2008). Moreover, the SUMO and ubiquitin systems have recently been found to play crucial and interdependent roles in the regulation of drug and xenobiotic metabolizing genes in mammals (Cui et al. 2015, 2016; Hu et al. 2010; Staudinger et al. 2006, 2011; Sun et al. 2015).

It is important to note here that the initiation of the acute inflammatory response pathway is centrally regulated in liver in mammals by the 'acute phase response' system (Baumann and Gauldie 1994). The liver is also centrally involved in the appropriate resolution of the inflammatory response by virtue of its expression of compensatory anti-inflammatory mediators (Serhan et al. 2008). What is even more remarkable is the fact that drug metabolism and inflammation are inversely regulated in hepatocytes. That is, when inflammation is high, drug metabolism and transport activities are severely compromised (Aitken et al. 2006; Morgan et al. 2008). In a reciprocal manner, when drug metabolism pathways are elevated, the ability to mount an effective immune response is compromised in mammalian systems. The central involvement of PXR in this response is now currently well-accepted (Cheng et al. 2012; Dou et al. 2012). Our recent investigations reveal that PXR is highly modified by multiple PTMs to include phosphorylation, sumoylation, and ubiquitination. Using both primary cultures of hepatocytes and cell-based assays, research from our laboratory has revealed that, in addition to modification with SUMO and ubiquitin, PXR is modified through acetylation on lysine residues (Fig. 6.3). We have also shown that increased acetylation of PXR stimulates its increased SUMO-modification to support active transcriptional suppression. Importantly, both the acetylation and sumoylation status of the PXR protein is affected by its ability to associate with the lysine de-acetylating enzyme histone de-acetylase (HDAC3) in a complex with silencing mediator of retinoic acid and thyroid hormone receptor (SMRT). Taken together, our data support a model in which a SUMO-acetyl 'switch' occurs

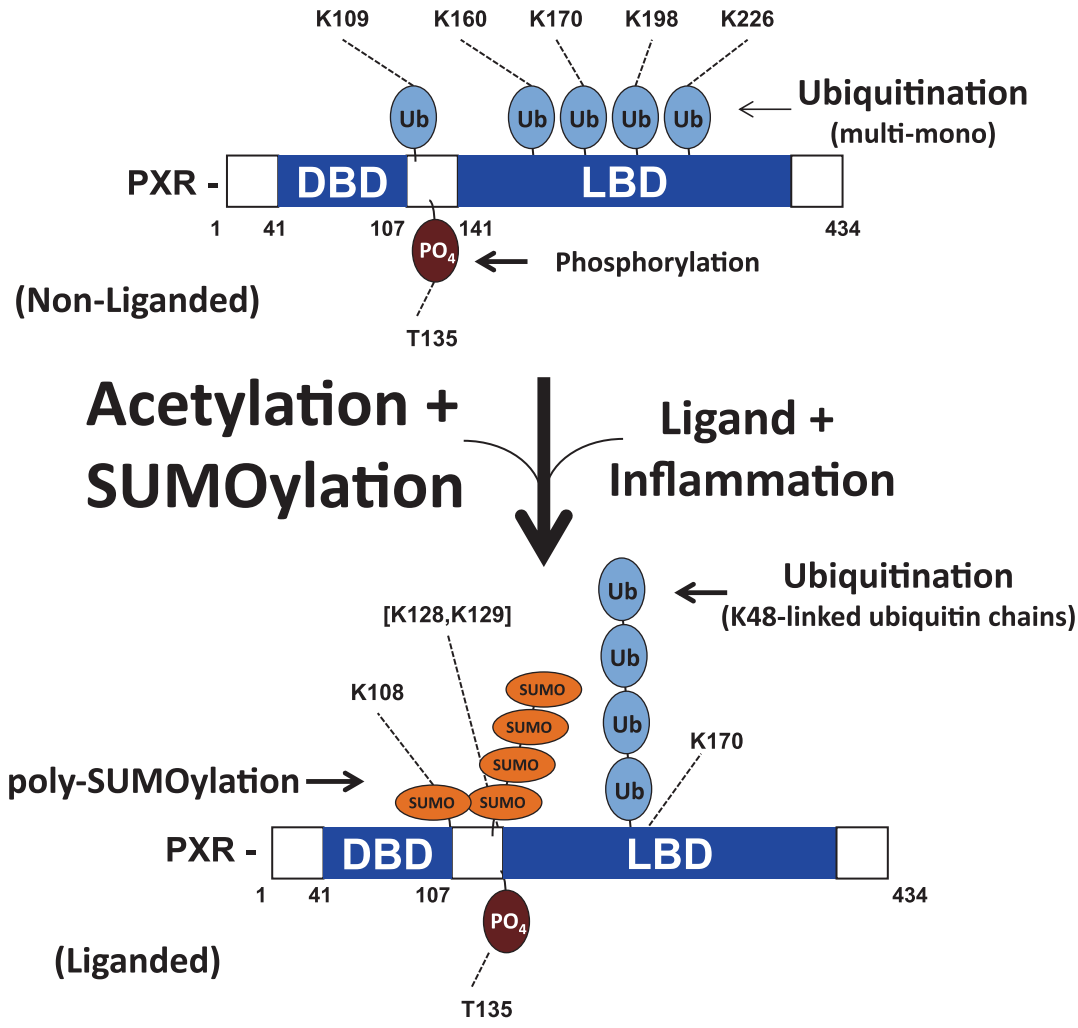

Fig. 6.3 Schematic of Nuclear Receptor Superfamily Member Pregnane X Receptor (NR1I2) Post-Translational Modification and Interaction Between the Acetyl, SUMO, and Ubiquitin Systems. Mass spectrometry of human PXR protein isolated from primary hepatocytes reveals multi-mono ubiquitination on several lysine residues and a phosphorylation event at threonine 135. Following treatment with ligand and an inflammatory stimulus (TNFα, 10 ng/ml), acetylation marks the PXR protein as competent for sumoylation. The poly-sumoylation of PXR ensues at lysine 128 and/or lysine 129 in the consensus site (−KKSE-). The PXR protein isolated at this stage reveals extensive poly-ubiquitin chains primarily at lysine 170 in the PXR protein

such that acetylation of PXR likely stimulates SUMO-modification of PXR to promote the active repression of PXR-target gene expression through SUMO-dependent processes. However, while the precise molecular mechanisms remain to be elucidated, fact that PXR is involved in the regulation of drug metabolism and resolution of the inflammatory response it is likely to play an integral and important role in mediating the reciprocal connections between these two hepatic processes. The likelihood that integrated signaling between acetylation, sumoylation and ubiquitination pathways in mediating these central hepatic processes is also very high.

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SUMO and Nucleocytoplasmic Transport

7

Christopher Ptak and Richard W. Wozniak

Abstract

The transport of proteins between the nucleus and cytoplasm occurs through nuclear pore complexes and is facilitated by numerous transport factors. These transport processes are often regulated by post-translational modification or, reciprocally, transport can function to control post-translational modifications through regulated transport of key modifying enzymes. This interplay extends to relationships between nucleocytoplasmic transport and SUMO-dependent pathways. Examples of protein sumoylation inhibiting or stimulating nucleocytoplasmic transport have been documented, both through its effects on the physical properties of cargo molecules and by directly regulating the functions of components of the nuclear transport machinery. Conversely, the nuclear transport machinery regulates the localization of target proteins and enzymes controlling dynamics of sumoylation and desumoylation thereby affecting the sumoylation state of target proteins. These inter-relationships between SUMO and the nucleocytoplasmic transport machinery, and the varied ways in which they occur, are discussed.

Keywords

SUMO • Nuclear pore complex • Nucleocytoplasmic transport

7.1 Introduction

In eukaryotes, the cytoplasm and nucleoplasm are separated by the nuclear envelope (NE) that consists of an impermeable double membrane. The nucleocytoplasmic transport of macromolecules across this barrier is facilitated by nuclear pore complexes (NPCs), which extend across the NE at numerous positions where the inner and

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outer nuclear membranes are fused. A typical mammalian NE contains ~5000 NPCs. Each of these large macromolecular assemblages is composed of ~30 different proteins termed nucleoporins or nups. Nups form specific subcomplexes that assemble into ultrastructurally defined components, including the cytoplasmic filaments, the nuclear basket, and an elaborate core structure that provides the framework for the central transport channel (reviewed in Tran and Wente 2006).

Transport through the central channel can occur by diffusion if molecules, including metabolites and proteins, are less than ~40 kDa in size. However, efficient translocation of most proteins and macromolecular complexes, such as ribosomes and RNPs, requires active transport through NPCs. In general, these active transport pathways are characterized by: 1) the interaction of cargoes with a nuclear transport factor (NTF), 2) the NTF-facilitated translocation of the cargo complex through the NPC, 3) the release of cargo upon arrival within the target compartment and, 4) the recycling of the NTF back to its compartment of origin (reviewed in Cook et al. 2007; Terry et al. 2007; Stewart 2007).

Many of the NTFs belong to the karyopherin or kap family of proteins. Kaps are subdivided into importins that direct cargo transport into the nucleus, and exportins that direct cargo transport out of the nucleus. Kaps recognize and interact with a specific amino acid sequence within their cognate cargoes. These include nuclear localization signals (NLS), recognized by importins, and nuclear export signals (NES), recognized by exportins (reviewed in Wozniak et al. 1998; Marelli et al. 2001; Pemberton and Paschal 2005).

Most importins directly bind their cargo. However, importin-β also known as Kap-β1 or, in yeast, Kap95p) requires the adaptor protein importin-α (also known as Kap-α or, in yeast, Kap60p) (Enenkel et al. 1995; Görlich et al. 1995; Moroianu et al. 1995a, b). Importin-α interacts with cargoes containing a classical NLS (cNLS) that is rich in basic amino acid residues (Kalderon et al. 1984; Robbins et al. 1991), and importin-β through distinct binding domains, forming a trimeric complex that is competent to be transported through the NPC (Rexach and Blobel 1995; Görlich et al. 1996a; Moroianu et al. 1996; Weis et al. 1996; Conti et al. 1998; Cingolani et al. 1999). Upon entering the nucleus, transport is completed by disruption of the trimeric complex through the binding of the small GTPase Ran, in its GTP-bound state, to importin-β (Rexach and Bloebel 1995; Vetter et al. 1999; Lee et al. 2005). This, and other factors, aids in the release of importin-β and ultimately the cargo from importin-α (Fig. 7.1, Import) (reviewed in Cook et al. 2007; Stewart 2007).

Following dissociation of the import complex, importin-α and importin-β are recycled back to the cytoplasm (Fig. 7.1, export). The importin-β•RanGTP heterodimer is, itself, exported back to the cytoplasm (Görlich et al. 1996b). Export of importin-α requires its interaction with the exportin CAS (Cse1 in yeast) together in complex with RanGTP (Kutay et al. 1997; Hood and Silver 1998; Solsbacher et al. 1998). The cooperative formation of a trimeric complex between the exportin, RanGTP, and the NES containing cargo is a prerequisite for exportin-mediated transport (reviewed in Pemberton and Paschal 2005). After export, the recycled importin or the exportin is released into the cytoplasm. The final step in this process is the release of the kap from Ran. This is driven by the conversion of RanGTP to RanGDP, in a reaction stimulated by RanGAP1 (Bischoff et al. 1994) and other factors (Floer et al. 1997). Importins are then free to associate with other cargoes, while exportins translocate through the NPC back to the nucleoplasm for another round of export. RanGDP is also transported back to the nucleus by the transport factor NTF2 (Ribbeck et al. 1998; Smith et al. 1998) where the Ran guanine nucleotide exchange factor RCC1 facilitates the exchange of GDP for GTP on Ran and the formation of RanGTP (Bischoff and Ponstingl 1991).

A link between nucleocytoplasmic transport, NPCs, and sumoylation was first uncovered in vertebrate cells when sumoylated RanGAP1 was shown to co-localize with NPCs (Matunis et al. 1996; Mahajan et al. 1997, 1998). The sumoylation of RanGAP1 was shown to be required for its interaction within a trimeric complex that includes the nucleoporin RanBP2/RanBP2/

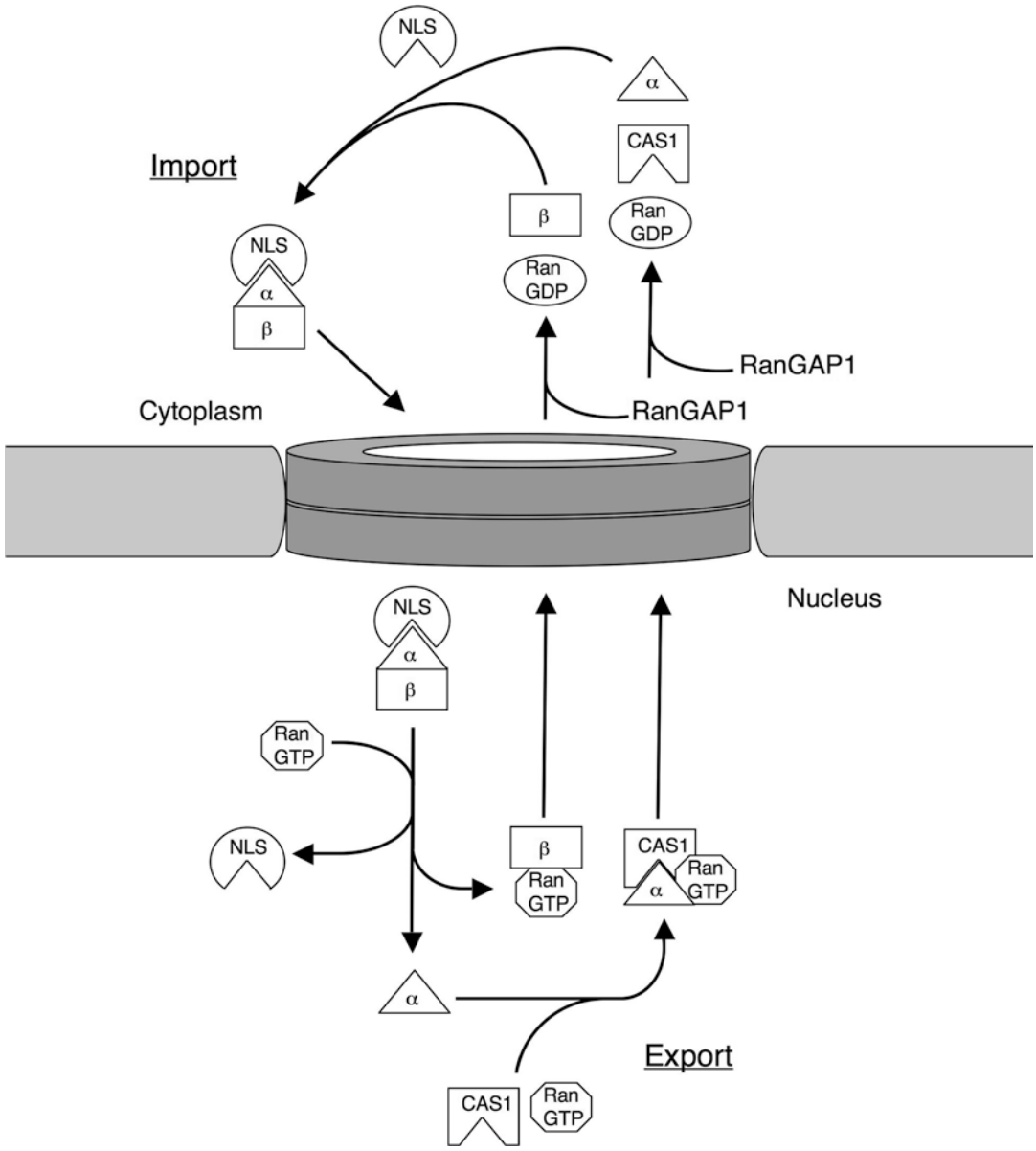


Fig. 7.1 Importin-α/β mediated nucleocytoplasmic transport. Shown are some of the key steps involved in the import of cargoes recognized by importin-α/βand the recycling of the kaps. A trimeric complex in which importin α interacts through distinct domains with the cNLS of the cargo protein and importin-β, is translocated from the cytoplasm to the nucleus through the NPC. Once in the nucleus, RanGTP interacts with importin-β inducing release of importin-α and the cargo. The cargo is released from importin α with the aid of factors not shown here. Importin-α is then exported from the nucleus in a trimeric complex with the export factor CAS and RanGTP. Importin-β in complex with Ran GTP is exported back to the cytoplasm and released concomitant with the conversion of RanGTP to RanGDP, a reaction stimulated by RanGAP1

Nup358 (Mahajan et al. 1997; Matunis et al. 1998), which contains an E3 SUMO ligase domain (Pilcher et al. 2002), and the SUMO conjugating enzyme Ubc9 (Saitoh et al. 1997, 1998; Lee et al. 1998). This complex is positioned on the cytoplasmic face of NPCs (Zhang et al. 2002).

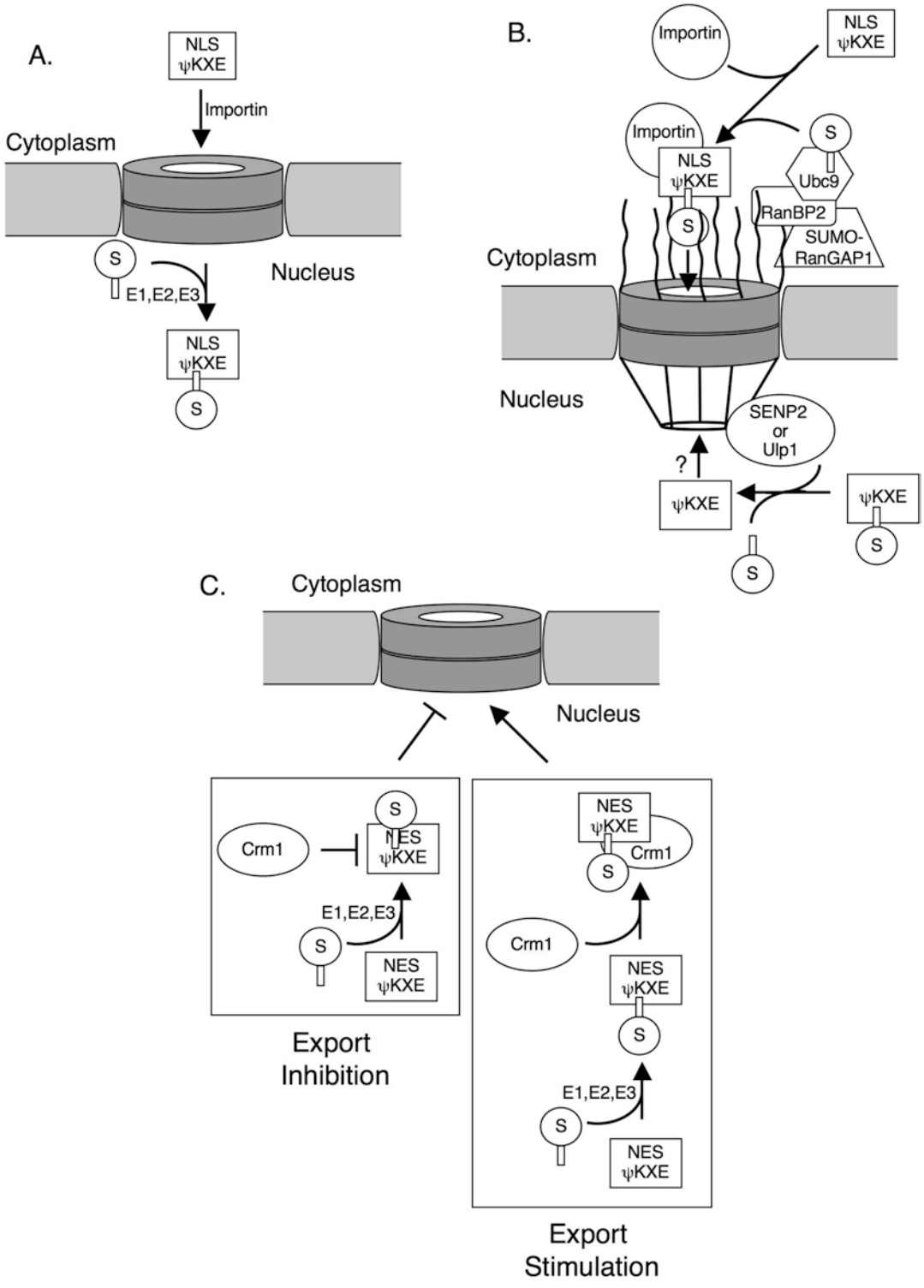
The positioning of a key transport regulator, RanGAP1, in association with a sumoylating enzyme complex, Ubc9•RanBP2/Nup358, at NPCs has long suggested a link between sumoylation and nucleocytoplasmic transport (Pilcher and Melchior 2001). Studies carried out over the past decade support this conclusion, providing examples that highlight this inter-relationship. We review these processes providing salient examples for the interplay between SUMO-dependent reactions and the nucleocytoplasmic transport machinery.

7.2 Compartment-Specific Sumoylation

As with any biological reaction, SUMO modification requires that the position of a protein substrate be coordinated in space and time with the position of the enzymes required for its sumoylation. A classical representation of this compartmentalization comes from experiments showing that a fusion protein consisting of a sumoylation site fused to the cytoplasmic protein pyruvate kinase is not sumoylated, while the same fusion protein containing an NLS is sumoylated (Rodriguez et al. 2001). Importantly, this study suggested that nuclear import is a key step in the sumoylation of many proteins (Fig. 7.2a). Consistent with SUMO-dependent processes being principally nuclear, most SUMO-specific enzymes from vertebrates to yeast, including the E2 conjugating enzyme Ubc9, SUMO E3 ligases of the PIAS family, and most desumoylating enzymes, are found primarily within the nucleus (reviewed in Melchior et al. 2003). In agreement with this, the majority of sumoylated proteins appear to be nuclear as evidenced by proteomic studies carried out using yeast (Panse et al. 2004; Wohlschlegel et al. 2004; Denison et al. 2005; Hannich et al. 2005; Wycoff and O'Shea 2005), and human cells (Li et al. 2004; Vertegaal et al. 2004; Zhao et al. 2004).

While most SUMO targets and enzymes are nuclear, this is not exclusively the case. Targets have been identified within the cytoplasm, including in association with mitochondria, the endoplasmic reticulum, and the plasma membrane. Consistent with this, SUMO-specific enzymes may localize to sites within a cell other then the nucleoplasm (Melchior et al. 2003). Germane to this review is the localization of SUMO-specific enzymes at NPCs. For example, the SUMO conjugating enzyme, Ubc9, is found on both the cytoplasmic and nucleoplasmic faces of NPCs (Zhang et al. 2002). On the cytoplasmic side, Ubc9 is found in a trimeric complex that includes the SUMO E3 ligase RanBP2/Nup358 and RanGAP1 (Saitoh et al. 1997; Zhang et al. 2002). In principle, the NPC localization of these enzymes permits nucleocytoplasmic transport and sumoylation reactions to be coupled (Fig. 7.2b). A representative example would appear to be the sumoylation of the deacetylase HDAC4. Its sumoylation requires both Ubc9•RanBP2/Nup358 and its NLS, suggesting that HDAC4 is

Fig. 7.2 Inter-relationships between SUMO-dependent reactions, and nucleocytoplasmic transport. (**a**). Compartment localized sumoylation. A cargo protein, containing a consensus sumoylation site (ψKXE) and an NLS, is transported through the nuclear pore to the nucleus by an importin. Once in the nucleus the target is sumoylated (S identifies SUMO) by a sumoylating enzyme (Ubc9, or Ubc9•E3 complex) present in the nucleoplasm. (**b**). Proposed models for SUMO-dependent reactions at NPCs. Sumoylation on the cytoplasmic face of NPCs may occur as the importin•cargo complex contacts the cytoplasmic filaments through RanBP2/Nup358. Ubc9, found in complex with RanBP2/Nup358 and SUMO-RanGAP1, subsequently sumoylates the cargo. This is followed by cargo translocation through the central channel. Desumoylation may occur at the nuclear basket where the vertebrate desumoylating enzyme SENP2 or its yeast counterpart, Ulp1, are located. It remains to be determined if export (question mark) or, for that matter, import of sumoylated cargoes is coupled with desumoylation at the nuclear basket. (**c**). Proposed models for SUMO-dependent export inhibition and stimulation. *Export inhibition:* Sumoylation, at a consensus site, occurs in close proximity to an NES. In these cases, the NES is typically leucine rich and recognized by the exportin Crm1. The SUMO moiety occludes the NES, preventing Crm1 from binding and inhibiting export. *Export stimulation:* SUMO modification of a protein at a consensus sumoylation site facilitates the interaction between Crm1 and an NES leading to efficient cargo export


A.
NLS
ψKXE
Importin
Cytoplasm
S
E1,E2,E3
Nucleus
NLS
ψKXE
S
B.
Importin
NLS
ψKXE
Importin
S
NLS
ψKXE
Ubc9
RanBP2
S
SUMO-
RanGAP1
Cytoplasm
Nucleus
SENP2
or
Ulp1
?
ψKXE
ψKXE
S
S
C.
Cytoplasm
Nucleus
S
Crm1
NES
ψKXE
E1,E2,E3
S
NES
ψKXE
Export
Inhibition
NES
ψKXE
Crm1
S
Crm1
NES
ψKXE
S
E1,E2,E3
S
NES
ψKXE
Export
Stimulation

sumoylated as it traverses the NPC during nuclear import (Kirsch et al. 2002).

Desumoylation reactions may also occur at NPCs. The vertebrate desumoylating enzyme, SENP2, associates with the nucleoplasmic filaments of the NPC through interactions with the nucleoporin Nup153 (Zhang et al. 2002). In yeast, the SENP2 homolog, Ulp1p, has also been shown to localize to the nucleoplasmic face of NPCs. This localization is dependent upon the interactions of Ulp1p with the kaps Kap121p and the Kap60p/Kap95p complex (Panse et al. 2003), as well as a number of NPC associated components included Nup60p, Mlp1p, and Mlp2p (Zhao et al. 2004), the Nup84 subcomplex (Palancade et al. 2007), and the nuclear envelope associated protein Esc1 (Lewis et al. 2007). Ulp1p localization to the nuclear basket functions, in part, to stabilize the protein (Zhao et al. 2004; Palancade et al. 2007) and, possibly, to prevent the unregulated desumoylation of proteins within the nucleoplasm (Li and Hochstrasser 2003; Zhao et al. 2004; Palancade et al. 2007; Lewis et al. 2007), both of which can result in multiple cellular defects including increased DNA damage. In light of this, it is thought that the regulated desumoylation of target proteins by Ulp1p requires that they be recruited to the nucleoplasmic face of NPCs (reviewed in Palancade and Doye 2008). In some of these cases, Ulp1 mediated desumoylation at NPCs might also be coupled with nuclear export of the target protein(s) (Fig. 7.2b). For example, Ulp1 appears to function in parallel with the export factor Mtr2 to promote the export of 60S preribosomal complexes, suggesting that the desumoylation of ribosomal proteins may be a prerequisite for 60S export (Panse et al. 2006). Cumulatively, these studies imply that the NPC can function as a platform onto which SUMO-specific enzymes bind, thereby providing a focal point within the cell to which proteins can be targeted for the regulated modulation of their sumoylation state.

7.3 Regulation of Nuclear Transport by Sumoylation

Among the many effects that sumoylation has on the function of modified proteins are changes in their interaction with the nuclear transport machinery. As a consequence, the addition or removal of SUMO from a target protein can lead to alterations in its localization. Coupled with the dynamic nature of sumoylation, this modification provides a mechanism for regulating transport that is conceptually similar to another well-established regulator of transport, phosphorylation. Phosphorylation of a cargo molecule can alter import or export by changing its ability to interact with a NTF, either by blocking the access of the NTF to an NLS or NES, or, by enhancing the binding of the NTF to an NLS or NES. Such modification can orchestrate rapid changes in the distribution of a protein between the nucleus and the cytoplasm causing upstream affects on transcription, DNA replication, and chromosome segregation pathways (reviewed in Poon and Jans 2005). Growing evidence suggests sumoylation can similarly regulate the nuclear transport of proteins, and examples of these are discussed below.

7.3.1 SUMO-Dependent Inhibition of Nuclear Export

As much of the sumoylation machinery lies within the nucleus, it is strategically positioned to regulate nuclear export pathways. A number of observations support this idea, including data identifying proteins that fail to concentrate in the nucleus when their sumoylation is inhibited (Table 7.1). This observation could be explained by a mechanism in which SUMO modification functions to inhibit protein export, resulting in the retention of the protein within the nucleus.

The nuclear localization of the adenovirus 5 E1B-55 kDa protein, referred to here as E1B, in

Table 7.1 Proteins exhibiting SUMO-dependent transport

SUMO target	References	SUMO target	References
I. Export inhibition		III. Export stimulation	
Adenovirus E1B E1B 55 K[a]	Kindsmüller et al. (2007)	BPV E1	Rosas-Acosta and Wilson (2008)
CtBP1[a]	Lin et al. (2003)	DdMEK1	Sobko et al. (2002)
CREB[a]	Comerford et al. (2003)	p53	Carter et al. (2007)
Dorsal	Bhaskar et al. (2000)	SMAD3	Imoto et al. (2008)
ELK1[a]	Salinas et al. (2004)	TEL	Wood et al. (2003)
HIPK1	Li et al. (2008)	IV. Import stimulation	
KLF5[a]	Du et al. (2008)	PAP	Venthantham et al. (2008)
NEMO[a]	Huang et al. (2003)	V. Undefined	
		Bicoid	Epps and Tanda (1998)
NFAT1[a]	Terui et al. (2004)	Caspase-8	Besnault-Mascard et al. (2005)
MDM2[a]	Miyachui et al. (2002)		
TAX	Lamsoul et al. (2005)	PDX1	Kishi et al. (2003)
II. Import inhibition			
ATF7	Hamard et al. (2007)	SMAD1	Shimada et al. (2008)
c-MYB	Morita et al. (2005)	SMAD4	Shimada et al. (2008)

[a]Proteins with a confirmed or putative Crm1 NES and a sumoylation site that are in close proximity within their primary amino acid sequence (Du et al. 2008)

virus-infected cells provides an example of this phenomenon. E1B participates in multiple aspects of virus production requiring its shuttling between cytoplasmic and nuclear compartments within a host (Flint and Gonzales 2003). In the nucleus, E1B shows both a diffuse localization as well as subnuclear localization to viral replication centers. Nuclear export of E1B is mediated by the exportin Crm1 and it can be inhibited using leptomycin B (LMB), a drug that specifically targets Crm1 (Nishi et al. 1994), or by introducing amino acid substitutions within the E1B NES. Export inhibition increases E1B localization to viral replication centers with a concomitant increase in the level of sumoylated E1B. By contrast, sumoylation deficient E1B derivatives, generated by amino acid substitutions, do not localize to viral replication centers and show increased cytoplasmic localization. These results suggest that upon entry into the nucleus, Crm1 and sumoylating enzymes compete for E1B binding. However, once sumoylated, export via Crm1 is inhibited and E1B is directed to subnuclear sites, likely through interactions with SUMO binding proteins. Within these sites it has been proposed that E1B is sequestered from Crm1 and also undergoes desumoylation, thus accounting for sustained nuclear localization coupled with low sumoylation levels (Kindsmüller et al. 2007).

While SUMO-dependent subnuclear localization of a protein provides a basis for export inhibition, inspection of the primary amino acid sequence of E1B and similarly regulated proteins indicate that an alternate mechanism may also be at play (Du et al. 2008). These proteins are characterized by the presence of a Crm1 specific NES that is found in close proximity to their sumoylation site(s), raising the possibility that sumoylation sterically hinders Crm1 binding to the NES, thus preventing nuclear export (Table 7.1). However, such steric inhibition has proven difficult to confirm experimentally. This stems from the common occurrence wherein the sumoylated form of a protein is found in far less abundance than its unmodified form (discussed in Johnson 2004; Hay 2005). For example, a study on the transcription factor KLF5 showed that fusion proteins containing the KLF5 NES and sumoylation sites always exhibited an apparent interaction with Crm1, regardless of whether the fusion could be sumoylated or not. However, this resulted from the low levels of sumoylated

protein in these experiments, such that any effect that sumoylation might have had on its interaction with Crm1 was masked by interactions between Crm1 and the unmodified fusion (Du et al. 2008).

Considering these observations, any model defining the SUMO-dependent inhibition of nuclear export must account for both the profound effect that sumoylation has on the nuclear localization of these proteins and the low cellular abundance of their sumoylated forms (Johnson 2004; Hay 2005). In one proposed mechanism protein sumoylation functions to both sterically inhibit Crm1 binding and direct sequestration of the protein to subnuclear sites, resulting in the efficient inhibition of their nuclear export (Kindsmüller et al. 2007; Du et al. 2008). Desumoylation would rapidly follow these export inhibitory and sequestration steps, thereby limiting the abundance of the sumoylated protein (Kindsmüller et al. 2007). Support for desumoylation at these sites derives, at least in principle, from the observation that an active site mutant of the desumoylating enzyme SENP1 can localize to subnuclear foci containing sumoylated components (Bailey and O'Hare 2004).

The execution of SUMO-dependent export inhibition mechanisms may also be coupled to cellular signaling. For example, the stimulation of T-cells, through engagement of the T-cell antigen receptor or through the artificial influx of Ca^{2+} using inonomycin, alters the localization of the transcription factor NFAT1 from the cytoplasm to the nucleus. Nuclear localization initially requires the stimulation of nuclear import upon NFAT1 dephosphorylation by calcineurin (Liu et al. 1991). Once imported, NFAT1 retention within the nucleus requires the inhibition of Crm1-mediated nuclear export through the stimulation of its sumoylation; first at a site in close proximity to a putative NES (Du et al. 2008), and then at a second site that directs NFAT1 localization to subnuclear foci (Terui et al. 2004). These observations conform to the model proposed above, except that NFAT1 sumoylation is stimulated as a consequence of cell signaling events. The mechanism by which signaling stimulates sumoylation, however, remains in question. In particular, does dephosphorylation expose sumoylation sites to permit E3 binding, does signaling function to activate an E3, or is some other mechanism at play?

In some proteins, the NES and sumoylation site(s) are not found adjacent to one another suggesting that sumoylation does not necessarily sterically inhibit exportin binding. An example is the HTLV-1 encoded transcriptional activator Tax, which possesses two sumoylation sites that are some eighty amino acid residues away from its NES. Sumoylation at both of these sites is required to direct the localization of Tax to nuclear bodies, indicating that Tax export is inhibited by its sequestration at these sites. However, while the loss of one Tax sumoylation site inhibits its localization to nuclear bodies, this Tax derivative mislocalizes to the nucleoplasm, rather than to the cytoplasm, consistent with sumoylation inhibiting export in some manner other than by sequestration (Alefantis et al. 2003; Lamsoul et al. 2005). Sumoylation may sterically inhibit exportin binding to Tax if the tertiary structure of Tax places its NES and sumoylation sites in close proximity. Alternatively, the NES may be blocked by SUMO in some other way, such as SUMO-dependent conformational changes in Tax or through the interaction between Tax and a SUMO interacting protein (Alefantis et al. 2003). Further study, particularly into the structure of Tax, will be required to address this issue.

7.3.2 SUMO-Dependent Stimulation of Nuclear Export

Sumoylation is not always inhibitory with respect to nuclear export. A case in point is the transport of the bovine papilloma virus E1 enzyme (BPV E1). Within infected cells, BPV E1 shuttles between both cytoplasm and nucleus. Nuclear export of BPV E1 is Crm1 dependent, but also SUMO-dependent, as a derivative that cannot be sumoylated appears absent from the cytoplasm and concentrated within the nucleus and at the nuclear envelope. *In vitro* binding studies suggest that BPV E1 sumoylation stabilizes its interac-

tion with Crm1, implicating sumoylation in the formation of an export complex. How these interactions would impact on the translocation of the Crm1•BPV E1 complex through the NPC or the terminal dissociation of the export complex is not clear. Perhaps SUMO functions to stabilize the Crm1•BPV E1 complex until entry into the NPC where desumoylation by an NPC associated desumoylase contributes to the final disassembly of the complex in the cytosol (Rosas-Acosta and Wilson 2008).

The role of sumoylation in enhancing nuclear export has also been linked to inducible export events. In *Dictostylium*, activation of the MAP kinase MEK1, in response to external stimuli, leads to its SUMO-dependent export. The chemoattractant cAMP initiates signaling from the plasma membrane, activating a MAP kinase cascade and leading to the phosphorylation of MEK1 within the nucleus (Ma et al. 1997). Apart from activating the kinase activity of MEK1, phosphorylation also directs its release from sequestration by the E3 ubiquitin ligase MIP1 and promotes its sumoylation. Sumoylation then stimulates MEK1 nuclear export leading to its final localization at the cell cortex where it activates ERK1. How sumoylation stimulates this process and which NTF(s) mediates MEK1 export remains to be determined (Sobko et al. 2002).

Sumoylation may also contribute to the stimulation of nuclear export events that are dependent upon other post-translational modifications, such as ubiquitination. An example is the nuclear export of mammalian p53, which requires its monoubiquitination (Li et al. 2003). Monoubiquitination has multiple effects including the unmasking of the p53 NES and stimulation of its sumoylation. These post-translational modifications are thought to inhibit the binding of the ubiquitin E3 ligase Mdm2 to p53, thus preventing p53 polyubiquitination and degradation. In the monoubiquitinated and sumoylated form, the p53 NES remains exposed, allowing for the formation of an export complex between Crm1 and p53 and the translocation of p53 to the cytoplasm (Carter et al. 2007). This example highlights the capacity of multiple post-translational modifications, including sumoylation, to act in concert to direct the nuclear export of a protein.

7.3.3 Sumoylation and Nuclear Import

Direct links between SUMO-dependent reactions and the regulation of nuclear import have also been documented, but currently remain few in number (Table 7.1). In one case, import of the mammalian poly(A) polymerase (PAP) is dependent upon two NLS elements (Raabe et al. 1994), one of which contains two sumoylatable lysine residues. Sumoylation at these sites is thought to stimulate the nuclear import of PAP. This conclusion was based on the use of PAP derivatives possessing conservative lysine to arginine substitutions at these sites. These substitutions, while not expected to significantly affect NLS function (Blackwell et al. 2007 provides an example), caused both the loss of PAP sumoylation at these positions and the loss of PAP nuclear import. Moreover, over expression of the desumoylase SENP1, which induces the desumoylation of PAP, resulted in the cytoplasmic accumulation of PAP. The observed loss of nuclear localization also does not appear to stem from SUMO-dependent export inhibition as PAP contains no consensus NES sequence in the vicinity of these sumoylation sites, nor is it exported in a Crm1 dependent manner (Venthantham et al. 2008). Together, these observations implicate PAP sumoylation as a prerequisite for its nuclear import, however, further experimentation will be required to categorically eliminate SUMO-dependent inhibition of nuclear export, possibly invoking a Crm1 independent pathway, as a potential mechanism. Important issues to address include where in the cell is PAP sumoylated, how does sumoylation affect importin binding, and does PAP desumoylation by SENP1 occur in the nucleus or in the cytoplasm as this enzyme may localize to either compartment (Bailey and O'Hare 2004; Kim et al. 2005; Li et al. 2008). Further support for SUMO-dependent import would also come from experiments testing the proposed idea that sumoylation

prevents access to a nearby lysine residue whose acetylation functions to inhibit importin-α/β binding to PAP (Venthantham et al. 2008).

Cytoplasmic sumoylation of a target may also function to inhibit nuclear import, as exemplified by c-Myb, a proto-oncogene product. Using vertebrate cells that constitutively express c-Myb, immunoprecipitation from subcellular fractions showed that cytosolic c-Myb is exclusively in the sumoylated form, while nuclear localized c-Myb occurs in both unmodified and SUMO modified forms. These populations of c-Myb appear to arise from compartment specific modification of the protein. In the cytoplasm, c-Myb sumoylation is dependent on the E3 TRAF7, resulting in the inhibition of c-Myb nuclear import and its retention within the cytoplasm. This suggests that unsumoylated c-Myb within the cytosol is imported into the nucleus where it may be sumoylated by the E3 PIASy and, possibly, by TRAF7 as well. Thus, while c-Myb can be sumoylated in multiple compartments by distinct E3s, its modification within the cytoplasm functions to inhibit its nuclear import (Morita et al. 2005).

Unlike c-Myb, where cytoplasmic sumoylation prevents its nuclear import, in other cases sumoylation appears to have more subtle affects on nuclear transport. For example, sumoylation of the transcription factor ATF7 appears to reduce the rate at which it is imported into the nucleus. To observe this effect, the normally low cellular abundance of sumoylated ATF7 was artificially increased. This was accomplished by fusing SUMO to the amino-terminus of an ATF7 derivative lacking specific sumoylation sites, generating a constitutively sumoylated derivative, SUMO-ATF7. To assess the effect of SUMO on import, the carboxy-terminal ATF7 NLS was replaced with a hormone responsive element that stimulated nuclear import of the fusion upon addition of hormone (Love et al. 1998). After hormone addition, it was observed that import of SUMO-ATF7 was significantly delayed as compared to unsumoylated controls, leading to the conclusion that sumoylation of ATF7 functions to reduce its rate of import. A model for how this might occur was further proposed primarily on the basis of two results: the identification of the nucleoporin RanBP2/Nup358 as the putative ATF7 E3 ligase and the SUMO-dependent co-localization of ATF7 with NPCs. The model implies that sumoylated ATF7 interacts with NPC components, leading to its increased residence time at NPCs and, ultimately, a slower rate of import (Hamard et al. 2007). The applicability of this and related models to the regulated import of other sumoylated proteins, specifically those modified at the NPC (Pichler and Melchior 2001), will be of interest to follow as more examples are uncovered and analyzed.

7.4 Control of the Nuclear Transport Machinery by Sumoylation

The role of SUMO in nuclear transport pathways extends to its effects on the function and localization of components of the nuclear transport machinery. The first, and defining example of this was the observation that SUMO modification of RanGAP1 is essential for its association with the NPC. Sumoylation of RanGAP1 occurs through the action of Ubc9 (Lee et al. 1998; Saitoh et al. 1997). Both the SUMO modification as well as Ubc9 itself contribute to the binding of RanGAP1 to the nucleoporin RanBP2/Nup358, leading to the formation of a trimeric RanGAP1•Ubc9•RanBP2/Nup358 complex on the cytoplasmic face of the NPC (Matunis et al. 1996, 1998; Mahajan et al. 1997; Saitoh et al. 1997; Lee et al. 1998; Zhang et al. 2002). Through these SUMO directed events, RanGAP1 is positioned adjacent to RanBP2/Nup358 where these proteins are proposed to work synergistically to bind RanGTP•importin-β complexes and facilitate the hydrolysis of GTP by Ran, the release of Ran from importin-β and the assembly of importin-α/β•cargo complexes (Yokoyama et al. 1995; Yassen and Blobel 1999; Hutten et al. 2008).

Beyond its role in the assembly of the RanGAP1•Ubc9•RanBP2/Nup358 complex, it is unclear what role NPC-associated Ubc9 plays in nuclear transport. Similarly, the function of the E3 ligase activity of RanBP2/Nup358 in the

transport process has not been defined. It is likely that as more sumoylation targets of Ubc9•RanBP2/Nup358 complex are identified a clearer understanding of the significance of their function will become apparent. Among the possible targets of this complex and other sumoylating enzymes are the nucleoporins and karyopherins. Such modifications could globally affect transport or the transport of cargos controlled by specific karyopherins. In yeast, the SUMO modification of one or more nucleoporins or transport components could explain the observation that sumoylation appears to play a role in Kap60p/Kap95p (yeast importin-α/β) mediated import by promoting the recycling (i.e. export) of Kap60p. Sumoylation was implicated in this pathway as a thermolabile derivative of Uba2p, a component of the yeast SUMO E1 enzyme, exhibits both an import defect specific to cNLS containing cargoes and the nuclear retention of Kap60p. In the absence of sumoylation, Kap60p is not recycled back to the cytoplasm for a subsequent round of import, leading to the accumulation of cNLS cargoes within the cytoplasm (Stade et al. 2002). Sumoylation of Kap60p itself, however, does not appear to occur, suggesting that some other component(s) of the pathway may be modified to direct its export, such as its export factor Cse1p. Another candidate is Nup2p (Nup50 in vertebrates), which large-scale proteomic studies have shown is sumoylated (Wohlschlegel et al. 2004; Hannich et al. 2005). Nup2p interacts with Kap60 and assists in cargo release and promotion of Kap60 export (Booth et al. 1999; Hood et al. 2000; Dilworth et al. 2001; Gilchrist et al. 2002; Solsbacher et al. 2000). Mutations in Nup2p also lead to an accumulation of Kap60p in the nucleus (Dilworth et al. 2001). While suggestive of a link between Nup2 sumoylation and Kap60p export, this remains to be established, especially since the import defect associated with the *uba2* mutant strain is not accompanied by changes in the cellular distribution pattern of Nup2. While Nup2p sumoylation may have subtler affects on the pore association of Kap60p or its function, it is also possible that other sumoylation events account for the inhibition of cNLS-mediated import (Stade et al. 2002).

To appreciate the potential significance of the interplay between the importin-α/β mediated import pathway and the sumoylation machinery, it is important to grasp the scope of proteins whose nuclear transport is controlled by the importin-α/β complex. For instance, bioinformatic analyses of the yeast proteome have identified ~2600 proteins with a potential cNLS. Furthermore, of the ~1500 proteins that are observed to localize to the nucleus, more than half possess a putative cNLS. In addition, ~100 proteins containing a cNLS have so far been detected in association with Kap60p (reviewed in Lange et al. 2007). These cNLS containing proteins direct a myriad of biological processes, underscoring the critical role of importin α/β--mediated import in cell physiology and thus the potential wide spread affects of sumoylation and desumoylation in its regulation.

7.5 Nucleocytoplasmic Transport of SUMO-Specific Enzymes

As stated in Sect. 7.2, the sumoylation state of a protein is dependent upon the localization of the SUMO-specific enzyme(s) that act upon it. This often entails transport of the target protein to the location in which the enzyme is found, but may also result from the regulated transport of components of the sumoylation machinery between different compartments. An example is the varied nuclear and cytoplasmic localization of the vertebrate desumoylating enzyme SENP1 (Bailey and O'Hare 2004; Kim et al. 2005; Li et al. 2008). In bovine aortic embryonic cells, SENP1 is retained within the cytoplasm as a consequence of its interaction with the thioredoxin Trx1, a redox protein that neutralizes oxidizing agents such as reactive oxygen species (ROS). In response to ROS-mediated stress, cells can commit to apoptosis. Apoptotic signaling relies, in part, upon the sensing of ROS by Trx1, which induces the release of proteins from Trx1, including SENP1. Once released, SENP1 is translocated to the nucleus where it desumoylates proteins required for apoptotic signaling, including the kinase

HIPK1. The sumoylated form of HIPK1 functions to inhibit its nuclear export. As a result, SENP1 desumoylation releases HIPK1, allowing it to be translocated to the cytoplasm where it phosphorylates a number of targets that function to further stimulate apoptotic signaling (Li et al. 2008).

Nucleocytoplasmic shutting of other SUMO-specific enzymes, such as SENP2 in vertebrates, has also been reported (Itahana et al. 2006). In yeast, shuttling of the SENP2 homolog Ulp1p is coordinated with shuttling of the PIAS E3 ligase Siz1 to modulate the sumoylation state of cytoplasmically localized septins in a cell cycle dependent manner (Mahknevych et al. 2007). Septin sumoylation is found to peak during mitosis and decrease prior to cytokinesis (Johnson and Blobel 1999). The requisite sumoylation and desumoylation cycles accompanying these septin modifications correlate with differential localization of Siz1p and Ulp1p. During mitosis Siz1p becomes phosphorylated (Johnson and Gupta 2001) and is exported from the nucleus by Msn5p/Kap142p (Mahknevych et al. 2007), a kap previously shown to export phosphorylated cargoes (Kaffman et al. 1998; Boustany and Cyert 2002; Jaquenoud et al. 2002). Once in the cytoplasm, Siz1p accumulates at the budneck through an association with septins, ultimately leading to septin sumoylation (Johnson and Gupta 2001). Cytoplasmic localization of Siz1p during mitosis also likely alters the sumoylation state of other targets as evidenced by the recent identification of sumoylated Kar9p (Leisner et al. 2008). Upon exit from mitosis, Siz1p is dephosphorylated and is reimported into the nucleus through a mechanism that is Kap95p dependent (Johnson and Gupta 2001; Mahknevych et al. 2007).

As cells exit mitosis, septins are desumoylated. Surprisingly, this cytoplasmic event requires Ulp1p, which is localized to the nucleoplasmic face of the NPCs. As mentioned above (see Sect. 7.2), this interaction is thought to be mediated by Kap121p and the Kap60p/Kap95p complex, which bind directly to Ulp1p and are required for its association with NPCs (Panse et al. 2003). The ability of Ulp1p to desumoylate septins requires that it be released from the NPC and, in a process mediated by Kap121p, targeted to the septin ring (Mahknevych et al. 2007). This transient redistribution of Ulp1p during mitotic exit may be triggered by molecular rearrangements in the NPC that alter the interaction of Ulp1p and Kap121p with NPC binding sites (Mahknevych et al. 2003). Thus, the cycle of septin sumoylation and desumoylation provides an example of regulated nucleocytoplasmic transport of SUMO-specific enzymes and the key function of these transport events in the timing of specific target modifications.

7.6 Conclusion

The intersection of the nucleocytoplasmic transport machinery with SUMO-dependent processes has been shown to occur in a variety of different ways, exposing the interdependence of these two processes. Examples highlight the requirement of transport to position targets in proximity to the SUMO-specific enzymes that act upon them. Protein sumoylation or desumoylation itself may regulate nucleocytoplasmic transport by either inhibiting or directing the translocation of a cargo or by regulating the activity of transport factors. Beyond these examples are a growing list of SUMO-dependent events controlled by the regulated transport and facilitated targeting of the sumoylation and desumoylation machinery to structures such as the septin ring. While some general principals have been uncovered, future studies will be required to elucidate specific molecular details. Among these interesting questions are how cargo sumoylation may either inhibit or stimulate Crm1 mediated export and what components of the nucleocytoplasmic transport machinery are sumoylated in order to mediate the export of Kap60p. As answers to these and other questions present themselves, it will be interesting to see what themes arise and how they coalesce into the broader picture of how SUMO-dependent processes and nucleocytoplasmic transport function in concert to regulate specific biological pathways.

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Sumo Modification of Ion Channels

8

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Abstract

Recently, a role for SUMO modification outside of the nucleus has emerged. Although the number of extranuclear proteins known to be sumoylated is comparatively small, ion channels represent one important new class of these proteins. Ion channels are responsible for the control of membrane excitability and therefore are critical for fundamental physiological processes such as muscle contraction, neuronal firing, and cellular homeostasis. As such, these ion-conducting proteins are subject to precise regulation. Recently, several studies have identified sumoylation as a novel mechanism of modulating ion channel function. These studies expand the list of known functions of sumoylation and reveal that, in addition to its more established role in the regulation of nuclear proteins, this modification plays important roles at the cytoplasmic face of membranes.

Keywords

Potassium channels • Voltage-gated channels • Ligand-gated channels

8.1 Introduction

Ion channels are transmembrane proteins that form pores to allow the rapid passage of ions across a lipid bilayer down their electrochemical gradient. They constitute an ancient class, present in the earliest known organisms and are critical for cell survival. Although not limited to a particular cell type, they are perhaps best know for their role in excitable cells that have the ability to generate and propagate electrical activity, such as neurons and muscle cells. With hundreds of related members, ion channels comprise one of the largest families of signal transduction proteins in the human genome. Ion channels can be broadly categorized based on the gating mechanism that controls their opening and closing. The two most prominent classifications include voltage-gated and ligand-gated channels. The superfamily of voltage-gated channels represents

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the largest and most diverse subclass of ion channels (Gutman et al. 2005). These channels open and close in response to fluctuations in the transmembrane charge separation (or *potential*) near the protein. The ensemble average of hundreds, or even thousands of voltage-gated channels opening and closing in orchestrated concert, shapes the complex electrical responses of excitable cells.

Gating criterion, sequence similarity and ion selectivity further subdivide ion channels into several subtypes. The voltage-gated family includes channels that are selective for cations (primarily sodium, calcium, or potassium) as well as anions (primarily chloride). These ion-selective subclasses of channels can be categorized based on membrane topology. Of particular relevance to our discussion in this chapter are the groups of potassium-selective channels composed of subunits with either four or six transmembrane domains. Mammalian K2P channels define a family of channels whose conducting subunits have four transmembrane domains and two pore-forming domains. In humans, the K2P family includes 15 members that are discriminated based on their modulation by physiological stimuli such as temperature, mechanical stretch, pH, or cellular lipids (Honore 2007; Lotshaw 2007). These channels are constitutively open at rest and are believed to control the resting membrane potential of cells by providing a background "leak" of K^+ that reduces excitability. In the second group, mammalian voltage-dependent potassium (Kv) channels are composed of tetrameric α subunits with six transmembrane domains. The central conducting pore of the channel tetramer is formed by transmembrane domains 5 and 6, which include voltage-dependent activation and inactivation gates as well as the selectivity filter of the channel. The first four transmembrane domains arrange in the periphery of this central axis and serve as a voltage-sensing domain. The voltage-dependent gating of these channels allows them to play critical roles in establishing the resting membrane potential and dictating the duration and frequency of action potentials throughout the cardiovascular and nervous systems.

The second canonical family of channels is composed of ligand-gated ion channels that open in response to binding of specific ligands to extracellular sites. These channels operate at chemical synapses and transduce electrical signals in response to neurotransmitters such as acetylcholine, glutamate, γ-aminobutyric acid, or glycine. Depending on their ion selectivity, ligand-gated channels act to excite or inhibit postsynaptic cells. Constituent members of this class include nicotinic acetylcholine (nACh) receptors, GABA receptors, and ionotropic glutamate receptors (GluR) (Collingridge et al. 2008).

Given the fundamental importance of ion channels to cell homeostasis and membrane excitability, it is no surpise that these proteins are tightly regulated. Recent studies have identified regulatory mechanisms throughout the lifecycle of channels spanning from their transcription, to their assembly and trafficking, to their insertion and removal from the plasma membrane. One important regulatory process is the post-translational modification of channel proteins. Although we understand much of the structure and function of most ion channels, the post-translational mechanisms that regulate their assembly and function are not yet as fully understood and likely constitute important therapeutic intervention points. Given the diversity of ion channels and the number of potential post-translational modifications, it is difficult to review this topic in its entirety. However, it is noteworthy that numerous modifications have been reported including: glycosylation, phosphorylation, fatty acylation, nitrosylation, sulfation, and ubiquitination (Schulz et al. 2008; Schmidt and Catterall 1987; Zhang et al. 2007; Swope et al. 1999; Nunez et al. 2006).

Very recently, post-translational modification by Small Ubiquitin-like Modifier (SUMO) proteins has emerged as a novel mechanism of channel regulation. SUMO proteins are approximately 11 kDa proteins that share a similar structural fold and enzymological pathway of conjugation to that of ubiquitin (Johnson 2004; Saitoh and Hinchey 2000; Seeler and Dejean 2003; Su and Li 2002). Post-translational modification of target proteins by SUMO was only recently

discovered in 1996 (Matunis et al. 1996; Mahajan et al. 1997). Sumoylation has rapidly been found to be an essential process in *S. cerevisiae* (Johnson et al. 1997), *C. elegans* (Fraser et al. 2000), *Arabidopsis thaliana* (Saracco et al. 2007) and mice (Nacerddine et al. 2005). This modification has served as the founding class of a growing group of ubiquitin-like (Ubl) proteins including Nedd8, Apg12, Hub1, FAT10, and ISG15 (Geiss-Friedlander and Melchior 2007). The amino acid sequence, surface charge distribution, and functions of SUMO are distinct from those of ubiquitin and other Ubl proteins, however, and represent a unique and widespread form of post-translational regulation.

As with other post-translational modifications, and illustrated by the examples described throughout this book, the functional consequence of sumoylation varies greatly depending on the specific target protein. Since the discovery of SUMO just over ten years ago, well over 100 targets have now been identified, and this modification has been found to alter properties as diverse as the stability, subcellular localization, trafficking, and activity of its substrates. This chapter will focus on the extranuclear actions of SUMO and will discuss ion channels as new substrates for SUMO modification.

8.2 Extranuclear Targets of Sumoylation

The overwhelming majority of targets of sumoylation described to date are located in, or near the nucleus. These proteins regulate transcription, DNA repair, RNA, RNA processing, genome integrity, chromatin modification, and nucleocytoplasmic transport, among many other functions (see other chapters in this book). It is important to note however, that members of a group of cytoplasmic proteins that form the septin ring at the bud neck of budding yeast were some of the first proteins shown to be sumoylated (Johnson and Blobel 1999). Recently, a wealth of data that supports a clear role for SUMO modification outside of the nucleus has begun to emerge. Numerous global proteomics analyses have repeatedly identified non-nuclear targets for sumoylation. Furthermore, the sumoylation machinery has long been known not to be limited to the nuclear compartment. Recent reports have identified important roles for sumoylation in the cytoplasm, mitochondria, endoplasmic reticulum, and plasma membrane ((Martin et al. 2007b, Geiss-Friedlander and Melchior 2007). Although long anticipated, it is only recently that the function and role of the sumoylation machinery has been demonstrated to play an important regulatory role for integral membrane proteins at the plasma membrane.

The first evidence for a role of sumoylation at the plasma membrane derived from studies of the glucose transporters GLUT1 and GLUT4 (Giorgino et al. 2000). In multiple cell lines expressing these transporters, endogenous Ubc9 was found (although to differing extents) in subcellular fractions from the plasma membrane, high- and low-density microsomal membranes, nuclei/mitochondria, and (to a low extent) the cytosol. Interestingly, stable overexpression of Ubc9 in skeletal muscle cells, resulted in a 65% reduction in the expression of GLUT1 and an almost eightfold increase in the expression of GLUT4. This led to the important functional consequence of decreased basal glucose transport, which is mediated by the GLUT1 transporter, and significantly increased insulin--stimulated glucose transport mediated by the GLUT4 transporter. Although the sumoylation targets responsible for these effects were not identified, the GLUT1 and GLUT4 transporters were found to interact with Ubc9 through an 11-amino acid sequence on their C-termini. Moreover, a SUMO immunoreactive protein of higher molecular weight was recovered in GLUT1 and GLUT4 immunoprecipitates. Although this was interpreted as evidence of sumoylation of the transporters, the identity of this modified species was not confirmed. Nevertheless, this report established that Ubc9 is present at the plasma membrane, and that SUMO modification can have important functional consequences for plasma membrane proteins.

Following this initial report, the C terminal domains of multiple metabotropic glutamate

receptor subunits (Wilkinson et al. 2008; Tang et al. 2005), and the ion channels K2P1 (Rajan et al. 2005), Kv1.5 (Benson et al. 2007), GluR6 (Martin et al. 2007a)., and GluR7a/b (Wilkinson et al. 2008) as well as the excitatory amino acid transporter 2 (EAAT2) (Gibb et al. 2007) have been identified as targets for SUMO modification.

Metabotropic (mGluR) glutamate receptors are a family of G-protein coupled receptors (GPCRs) that bind glutamate and by modulating the activity of nearby ion channels, affect pre- or postsynaptic activity in neurons. The Group III presynaptic mGluR8a and b were the first of these receptor subunits found to be sumoylated (Tang et al. 2005). Using a yeast-two-hybrid assay, screening of an adult rat brain cDNA library identified that the cytoplasmic C-termini of mGluR8a and b interact with SUMO1, Ubc9, PIAS1, PIASγ, and PIASxβ. While verification of these interactions using recombinant protein pull-down assays proved difficult, recombinant PIAS1 was found to bind mGluR8a and b robustly, and this interaction was mapped to a region just upstream of the consensus sumoylation motif on the C-termini of both receptor subunits. Interestingly, PIAS1 was also found to bind to all six members of the Group III glutamate receptor subunits (mGluR4, −6, −7a/b, and −8a/b). Analysis of the isolated cytoplasmic C-terminal domains of mGluR8a and b in a heterologous mammalian system indicated that they can be SUMO modified, and that this modification could be disrupted by mutation of the target lysine in the identified consensus sumoylation motifs. Although not as extensively characterized, the C terminal domains of mGluR2, 4, 7a, and 7b have also been shown to serve as substrates of SUMO modification using a bacterial sumoylation assay (Wilkinson et al. 2008). Whether the intact receptors are modified in their native membrane environment however, has not been demonstrated. Moreover, the functional consequences of the SUMO modification of these metabotropic glutamate receptor subunits are still unknown and await further study.

Recently, a C-terminal fragment of the EAAT2 transporter has been shown to be SUMO modified in a mouse model of amyotrophic lateral sclerosis (ALS) (Gibb et al. 2007). EAAT2 is a Na^+-dependent glutamate transporter in glial cells. In patients with ALS or in a mutant superoxide dismutase 1 (SOD1) mouse model of ALS, the activity of this transporter is reduced significantly. Interestingly, a source of this inhibition appears to derive from the caspase-3 cleavage of EAAT2 at a defined locus on the C-terminus of the transporter in response to oxidative stress. Western blot analysis of this C-Terminal fragment of EAAT2 (called the CTE) in spinal cord homogenates of mutant SOD1 mice revealed that the fragment migrated at a higher molecular weight than expected. Based on the observation that the CTE contained a consensus sumoylation motif and that this high migration seemed not to be due to oligomeric aggregation, the CTE was tested for SUMO modification. The CTE could be immunoprecipitated from mutant SOD1 mouse spinal cord homogenates using a monoclonal anti-SUMO1 antibody. Additionally, the CTE was found to interact with Ubc9 and SUMO1 in a yeast-two-hybrid assay, and to be sumoylated *in vitro*. Furthermore, sumoylated CTE, but not unmodified CTE, was targeted to promyelocytic leukemia (PML) nuclear bodies. Based on this observation and given that PML bodies are known to regulate transcription, the authors propose the idea that EAAT2, through SUMO-mediated targeting of the CTE to PML bodies, may contribute to the pathology of ALS. While this example of the SUMO modification of a glutamate transporter is unique, it is clear that sumoylation of glutamate signaling components is emerging as a widespread and likely important regulatory mechanism in pathological and pathophysiological states.

8.3 Sumoylation of Ion Channels

Four ion channels, including the K^+ leak channel K2P1 (Rajan et al. 2005), the voltage-gated potassium channel Kv1.5 (Benson et al. 2007), the ionotropic kainate receptor subunit GluR6 (Martin et al. 2007a), and the GluR7a/b subunits (Wilkinson et al. 2008) have been shown to be

SUMO modified. While GluR7a/b has simply been shown to serve as a substrate for SUMO modification using a bacterial sumoylation assay, sumoylation of K2P1, Kv1.5, and GluR6 has been characterized to various extents and shown to have important regulatory consequences on the currents mediated by these channels.

8.3.1 K2P1

The potassium leak channel K2P1 was the first ion channel found to be SUMO modified (Rajan et al. 2005). K2P channels are a family of potassium-selective pores that influence the resting membrane potential and activity of excitable cells by remaining open across a physiological range of transmembrane potentials. Since the original discovery of K2P0 in *Drosophila melanogaster,* over fifteen K2P channels have been identified and characterized. These channels have the common feature of being composed of four transmembrane segments with two pore-forming "P" loops. While many members of the K2P family do not encode measurable currents when expressed in heterologous systems, many others encode outward K^+ currents dynamically regulated by a number of mechanisms including phosphorylation, pharmacological outer pore blockers, lipid interactions, G-protein interactions, and mechanical stretch. A major unanswered question in the field involved the observation that while mRNA encoding the founding mammalian member of these channels, K2P1, could be detected in the heart, brain, and kidney, currents from this channel could not be measured. In 2005, Rajan et al. reported that exogenous K2P1 could be abundantly expressed on the plasma membrane of *Xenopus* oocytes and Cos7 cells and proposed that failure to detect current through the channel was due to constitutive sumoylation of lysine 274 on the C-terminus of the channel. Mutation of the proposed receptor lysine to glutamic acid, or treatment with SENP, unmasked a readily detected K^+-selective, pH-sensitive, openly rectifying, macroscopic current.

Confocal microscopy of oocytes over expressing GFP-tagged Ubc9 revealed that GFP-Ubc9 could be detected in a uniform, non-polarized distribution on the plasma membrane. In contrast, K2P1 was found to be restricted to the animal pole of these cells. When co-expressed, the localization of GFP-Ubc9 reorganized and was enriched at the animal pole, suggesting an interaction between the channel and Ubc9. Concordant results were observed for endogenous Ubc9. An interaction between the two proteins was also detected in a yeast-two-hybrid assay. These observations provided evidence that components of the SUMO conjugation machinery are present at the plasma membrane of oocytes, and suggested that targets of SUMO modification may be able to actively recruit this machinery while at the membrane.

Co-expression of HA-tagged SUMO1 and K2P1 in oocytes revealed that K2P1 appears to be a target for SUMO modification. Immunoprecipitation of K2P1 and subsequent separation by SDS-PAGE and western blotting revealed the presence of ~45 kDa protein species that was immunoreactive to antibodies directed against K2P1, HA-SUMO1, and endogenous SUMO1. Furthermore, replacement of K2P1 lysine 274 by glutamic acid shifted the migration of this species to an apparent lower molecular weight and disrupted the immunoreactivity to either anti-HA or anti-SUMO1 antibodies. This suggests that K2P1 can be SUMO modified, and that this modification takes place at acceptor lysine 274. Similarly, treatment of immunoprecipitated K2P1 with SENP1 caused the same shift in molecular weight, and the channel was no longer found to be immunoreactive to anti-HA-SUMO1 antibodies. This effect appears to be dependent on the catalytic activity of SENP since a catalytically inactive C603S mutant had no effect on K2P1.

Functional data indicated that mutation of the proposed acceptor lysine 274 to glutamic acid or co-expression of WT channel with SENP1 activated K2P1 and revealed a K^+-selective, open rectifying, macroscopic current. This was observed both in oocytes and Cos7 cells, and found to be dependent on the catalytic activity of

SENP as the C603S SENP1 mutant had no effect on K2P1 current. A striking set of experiments support the view that the effect of the protease is acutely reversible. Single-channel currents could be recorded from membrane patches excised from oocytes co-expressing WT K2P1 and SENP1. Remarkably, these single-channel currents were suppressed within 13 s upon insertion of the patch into naïve oocytes expressing endogenous sumoylation machinery. This implies that acute exposure of the channel to SUMO could serve to block channel conductance. When this same patch was withdrawn from the cell and re-inserted into an oocyte over-expressing SENP1, single-channel currents could be again detected within 18 s, arguing that the effects of sumoylation are also acutely reversible. Furthermore, K274E K2P1 current was not inhibited by insertion into naïve cells, suggesting that the suppressive effect of these cells on K2P1 current was dependent on an intact lysine residue. Similarly, insertion of WT channel in oocytes expressing C603S SENP1 did not induce detectable currents, suggesting that the activating effects of SENP were dependent on its catalytic activity. These sets of experiments were remarkable as they argued that sumoylation could acutely control the function of ion channels expressed on the plasma membrane.

Despite the attractive nature of the model proposed by Rajan et al., many of the observations in this initial study have since been challenged. Specifically, Feliciangeli et al. (2007), failed to detect any sumoylation of K2P1 using similar western blotting techniques, instead detecting only the lower migrating K2P1 species that Rajan et al. interpreted as unmodified channel. Additionally, while Feliciangeli et al. confirmed that the K274E mutation unmasked K2P1 current as described by Rajan et al., the effect depends on the nature of the substituting residue since the K274R mutation, which should also inhibit channel sumoylation while preserving the basic charge of this residue, was unable to unmask K2P1 macroscopic currents. Although these studies were undertaken with a slightly different construct of K2P1 that had a *Heteractis crispa* red (HcRed) fluorescent protein fused to the N-terminus of the channel, the authors argue that there is no evidence that this should affect the results of the study or the ability of the channel to be SUMO modified. In their original report, Rajan et al. described, but did not present data arguing that mutations of lysine 274 to arginine, glutamine, alanine, and cysteine all led to macroscopic currents in oocytes. Their data from Cos7 cells did indicate that the K274R mutation leads to the induction of K2P1 macroscopic current, albeit only to about one-fourth the extent of that induced by the K274E mutation. This is an important point. If the effects of the K274E mutation are due to loss of sumoylation of the channel, the K274R mutation should also recapitulate this effect. On the other hand, if the effect of the K274E mutation on the channel were due instead to a charge effect by replacing the positively-charged lysine with a negatively-charged glutamic acid, the K274R mutation would be predicted to have a much less severe effect on K2P1 current density. Future experiments will clearly have to resolve this issue.

Several other aspects of the sumoylation pattern of K2P1 are highly intriguing. The large majority of known sumoylation targets are modified at very low stoichiometries. A common feature of sumoylation appears to be that this modification can have significant functional effects on target proteins even though only 1–5% of the total cellular target protein normally appears to SUMO modified at any given time. It has been speculated that sumoylation of target proteins may be a very transient process, thus allowing for only a very small percentage of target protein to be captured in the modified state using biochemical techniques. How this low stoichiometry can exert significant effects however, remains a major, unanswered issue in the sumoylation field. The apparent stoichiometric sumoylation of K2P1 under basal conditions would thus be exceptional. The observation that Ubc9, even when over-expressed, can be completely redistributed to membrane K2P1 would imply that the high rate of steady-state K2P1 sumoylation may be a result of a biased balance favoring Ubc-9-mediated sumoylation over low SENP-mediated deconjugation. What is

surprising about this, however, is that the same quantitative sumoylation of K2P1 was observed in Cos7 cells, in which numerous targets of sumoylation, including the plasma membrane-expressed Kv1.5 that we have previously described, are found to have low stoichiometries of sumoylation. This would imply that the complete sumoylation of K2P1 is not merely caused by a generalized high-level sumoylation or low-level protease activity at the plasma membrane. As the conjugation of SUMO to target proteins is an ATP-dependent process, if K2P1 sumoylation is dynamic as proposed, the high stoichiometry raises the interesting point that to maintain it would require a significant expenditure of energy on the part of the cell. Feliciangeli et al. have noted this unusual finding and have reported that, in their hands, again using the slightly different HcRed-K2P1 fusion construct, they are unable to detect any sumoylation of the channel, l*et alone* stoichiometric amounts of it.

One last unique finding of K2P1 sumoylation involves the context of the sumoylation site. The motif was identified as L-K-K-F, which does not conform to the canonical ψ-K-X-E/D description of sumoylation motifs (where ψ is a hydrophobic residue, and x is any residue). While there are numerous examples of proteins targeted by sumoylation at non-canonical sumoylation motifs, this observation begs the question if there is any significance to the fact that this is an altered motif. The properties of the sequence surrounding the acceptor lysine in sumoylation motifs are thought to affect Ubc9 binding dynamics to the target protein. Is it thus possible that this non-canonical motif may increase the affinity of Ubc9 for K2P1 and provide a possible mechanism for the observed quantitative sumoylation of the channel? The authors make the point that numerous proteins in the nucleus are sumoylated at non-canonical motifs, thus suggesting that this is not a distinctive feature of extra-nuclear or plasma membrane-expressed target of sumoylation. However the significance of this newly-identified motif remains to be more fully understood.

While there are a number of distinct features of the reported K2P1 sumoylation, and the sumoylation patterns and role of the K274E mutation clearly remain controversial, this report was the first to identify a potential role for the sumoylation of ion channels. The observations also laid the groundwork for further exploration of ion channel sumoylation by confirming the presence of components of the sumoylation machinery at the plasma membrane. This original report was published, as our own efforts to investigate the role of sumoylation in the regulation of Kv1.5 were under way. We reported our findings in 2006, which represented the first description of the sumoylation of a voltage-gated ion channel (Benson et al. 2007), and soon thereafter, the GluR6 kainate receptor was reported to be regulated by SUMO modification (Martin et al. 2007a), as described below.

8.3.2 Kv1.5

Kv1.5 is an important voltage-gated K^+ channel in the cardiovascular system underlying the I_{Kur} ultra-rapid rectifying potassium current. I_{Kur} is a major repolarizing current in atrial myocytes that regulates the resting membrane potential and excitability of smooth muscle cells. In the human heart, Kv1.5 is selectively expressed in the atria (Lagrutta et al. 2006; Wang et al. 1993), but not the ventricle (Amos et al. 1996). Therefore, Kv1.5 has emerged as an extremely promising target for a ventricular-sparing antiarrhythmic therapy, and significant effort has been made to identify novel blockers of the channel to treat atrial fibrillation. Very recent work has provided a handful of candidate drugs that are able to inhibit Kv1.5 activity with varying degrees of specificity (Lagrutta et al. 2006; Regan et al. 2006; Stump et al. 2005; Brendel and Peukert 2003; Camm and Savelieva 2004; Pecini et al. 2005). While very promising, none of these drugs has progressed to clinical use, presumably due to unanticipated side effects related to their incomplete specificity for Kv1.5. Based on these developments, it is clear that a better understanding of the basic mechanisms that cells utilize to regulate the functional properties of Kv1.5 are likely to provide novel insights and therapeutic opportunities for the treatment of AF.

Our laboratory has identified multiple mechanisms of Kv1.5 regulation including thioacylation (Zhang et al. 2007), microdomain localization (Mcewen et al. 2008; Martens et al. 2000), and the constitutive internalization and recycling of the channel (Mcewen et al. 2007). Building on our experience in the analysis of transcription factor sumoylation, and using a bioinformatic approach, we identified that a number of Kv channels contain sequences that conform well to sumoylation motifs. Kv1.5 contains two consensus sumoylation sequences, centered on K221 and K536, that are highly conserved across species and that are located on cytoplasmic regions of the channel. Structural modeling of Kv1.5, using the crystal structure of Kv1.2 (Long et al. 2005), placed the motifs of each α-subunit in close proximity to each other exposed to the side portals that provide cytoplasmic access to the pore of the channel. This suggested that SUMO attachment to either K221 or K536 can be accommodated without undue alterations of the overall structure. Concurrent with this observation, the Kv associated protein KChAP, which is known to modulate the surface expression and whole-cell current densities of several Kv channels, was identified as a member of the PIAS family of SUMO E3 ligases. This raised the intriguing possibility that the consensus sumoylation sites that we identified in Kv1.5 may be functional and may contribute to a novel form of Kv channel regulation.

Using multiple approaches, we have provided evidence that Kv1.5 interacts specifically with the SUMO conjugating enzyme Ubc9 and serves as a target for post-translational modification by SUMO1, 2, and 3 in a heterologous Cos7 cell system. Isolation of epitope-tagged full-length hKv1.5 from cells co-expressing HA-SUMO-3 and Ubc9 by Ni^{+}-chelate chromatography under denaturing conditions revealed that two distinct major HA-immunoreactive bands can be detected only in samples derived from cells co-expressing SUMO and Kv1.5. These bands correspond to SUMO-modified Kv1.5 since (1) they are also visible as minor species when probed using a channel directed antibody, (2) their detection is dependent on inhibition of endogenous SUMO proteases and (3) they are sensitive to *in vitro* treatment with- or co-expression of- SUMO proteases. As in the case of most sumoylated proteins, the extent of Kv1.5 modification appears to be relatively low (~1%). In addition, purified recombinant Kv1.5 serves as a substrate in a minimal *in vitro* reconstituted sumoylation reaction. Consistent with the bioinformatic analysis, replacement of the predicted acceptor lysines by arginine in the first or second motifs led to the selective loss of the higher and lower migrating SUMO conjugated species respectively and the double mutant led to loss of both species. Consistent with the sumoylation consensus, replacement of other key amino acids such as the downstream acidic residue by an arginine or the first hydrophobic position of both motifs to asparagine also severely compromised Kv1.5 sumoylation. In contrast, disruption of the motifs with the K221/536R mutations did not alter the recovery of ubiquitinated species in the presence of a proteasomal inhibitor. Thus, the data support the view that the proposed motifs in Kv1.5 serve as the major sites of SUMO conjugation. These findings are significant as they represent the first report of the SUMO modification of a voltage-gated ion channel and only one of a handful of reports demonstrating the sumoylation of a protein that localizes to the plasma membrane. One significant difference in our findings and that of the original reports for K2P1 channels is in the stoichiometry of modification. Whereas in the case of the K2P1 channel the biochemistry suggested an all-or-nothing modification, we detected sumoylation of a relatively low (~ 1%) percentage of Kv1.5 channel. This does not appear to be due to preferential modification of the Kv1.5 fraction found at the plasma membrane since subsequent unpublished data using a surface biotinylation approach indicate that Kv1.5 is SUMO modified at the plasma membrane with approximately the same stoichiometry as that for total cellular channel. While the low stoichiometry is consistent with data for transcription factors, the precise explanation remains elusive.

An important aspect of our work identifying Kv1.5 as a substrate for SUMO modification was the discovery that although sumoylation of the

channel is not a prerequisite for assembly and delivery of functional channels to the plasma membrane, this modification specifically modulates its biophysical properties. Analysis of channel activity using whole-cell patch clamp electrophysiology revealed that disruption of the conjugation sites leads to a selective ~15 mV hyperpolarizing shift in the voltage-dependence of steady-state inactivation with no associated effects on the voltage-dependence of activation or total current density. Similarly, co-expression of a cytoplasmically localized form of the SUMO protease SENP2 also leads to a comparable shift in the voltage dependence of inactivation. Importantly, this effect of SENP2 is likely due to a direct effect on the channel since it requires the presence of functional sumoylation motifs in the channel. These results again contrast with the initial description of K2P1 channels. Whereas in the case of K2P1, sumoylation is proposed to function as an absolute gatekeeper preventing any access to conducting states of the channel (Rajan et al. 2005), this modification appears to play a more modulatory role in the case of Kv1.5. Importantly, however, our results clearly suggest that alterations in the sumoylation of Kv1.5 have the potential to alter the excitability of both atrial myocytes and vascular smooth muscle cells by modulating either the action potential duration or resting membrane potential.

The suggestion of an endogenous regulatory role for sumoylation of Kv1.5 highlights important unanswered questions. For example, our original report did not address whether this regulatory mechanism is operational in the native cellular context of cardiomyocytes where Kv1.5 serves its physiological functions or whether sumoylation has additional roles in the regulation of Kv1.5-mediated currents. In addition, it is important to note that the studies in heterologous systems involved complete and persistent loss of sumoylation through mutations or co-expression of SENP2, which could reflect stable alterations in the biosynthesis and assembly of Kv1.5 complexes. Thus, these experiments did not address whether sumoylation could serve as an acute and rapid regulatory mechanism acting on pre-existing channels at the plasma membrane. Recent experiments in our laboratory have begun to address these issues and suggest that SUMO modification acutely regulates the voltage-dependence of inactivation of endogenous I_{Kur} current in cardiomyocytes, and that modulation of the sumoylation pathway can lead to significant functional consequences on the shape and duration of the cardiac action potential. These initial data raise the intriguing possibility that manipulation of the sumoylation of Kv1.5 may provide a novel target for the highly specific pharmacological manipulation of I_{Kur} current in patients with atrial fibrillation.

8.3.3 GluR6

The kainate receptor GluR6 subunit is widely expressed throughout the brain, being most enriched in the hippocampus. By regulating neuronal excitability at both pre- and post-synaptic sites, GluR6 is thought to play important roles in synaptic plasticity, learning and memory, and epilepsy. As is the case with other kainate receptor (KAR) subunits, GluR6 subunits are composed of an extracellular N-terminus, three transmembrane segments, a P loop, and an intracellular C-terminus. These subunits can homo- or heterotetramerize to form a functional channel that is able to conduct Na^+ or K^+ ions in response to extracellular binding of the excitatory neurotransmitter glutamate.

GluR6 was initially identified as a potential substrate of sumoylation when it was found to interact with Ubc9 and PIAS3 in a yeast-two-hybrid screen (Martin et al. 2007a). These interactions were verified using co-immunoprecipitation studies and mapped to a 13-amino acid sequence that contains a consensus sumoylation motif on the C-terminus of the channel. Interestingly, this consensus sumoylation motif was found to be absent in all other KARs subunits. In rat brain extracts, a slow-migrating, GluR6-immunoreactive band was detected by western blot analysis and found to be dependent on the SUMO protease inhibitor N-ethylmaleimide (NEM). Furthermore, GluR6 was shown to co-immunoprecipitate with SUMO1 in cultured

hippocampal rat neurons, and WT, but not K886R GluR6 was able to serve as a substrate for SUMO modification in a bacterial sumoylation assay.

GluR6 is known to internalize from the cell surface in response to stimulation by kainate or N-methyl-D-aspartate (NMDA) (Martin and Henley 2004). This agonist-mediated endocytosis was found to be dependent on SUMO modification of the channel (Martin et al. 2007a). Application of kainate or glutamate led to the internalization of GluR6, as expected, but also led to a rapid increase in the amount of channel that could be recovered in the sumoylated state. This agonist-induced stimulation of GluR6 sumoylation occurred rapidly, as it could be detected in less than one minute and reached maximal conjugation (at three times basal levels) within ten minutes. The effect was specific to GluR6 as kainate did not induce global increases in cellular sumoylation. Similarly, the effect was also agonist-specific as NMDA was able to induce strong GluR6 internalization, but was found to have no effect on channel sumoylation.

In immunocytochemical studies of cultured hippocampal neurons, co-localization of internalized GluR6 with SUMO-1 dramatically increased with time when treated with kainate, but showed no effect when treated with NMDA. Moreover, transduction of these cells with SENP1 dramatically inhibited this kainate-induced GluR6 internalization, but had no effect on NMDA-induced internalization. Importantly, a catalytically-inactive mutant (C603S) of SENP1 was found to have no effect on either kainate- or NMDA-induced endocytosis. Consistent with the idea that agonist-induced sumoylation of GluR6 leads to the rapid internalization of the channel, sumoylation of surface-expressed GluR6 could not be detected unless endocytosis was inhibited by treatment with sucrose. Furthermore, disruption of the consensus sumoylation motif with the K886R mutation led to a dramatic loss of kainate-induced internalization in Cos7 cells.

The SUMO modification of GluR6 subunits was also found to have important functional consequences on the channel. KAR-mediated excitatory postsynaptic currents (KAR-EPSCs) were recorded from CA3 neurons at the mossy fiber synapse in hippocampal slices. When recombinant SUMO-1 was loaded in the recording pipette and allowed to diffuse into the patched neurons, the amplitude of KAR-EPSCs was found to decrease rapidly, reaching maximal inhibition within approximately five minutes of break-in. Conversely, when SENP1 was allowed to diffuse into these CA3 neurons, KAR-EPSC amplitude rapidly increased within a similar period. Importantly, no changes were detected in KAR-EPSC amplitudes when either C603S SENP1 or conjugation-deficient SUMO-1-ΔGG were delivered into these cells. Since AMPA receptor-mediated EPSC amplitudes recorded from the same cells were found to be unaffected by SUMO-1 or SENP1 treatment, the effects are specific to kainate induced responses. Taken together, these data provide strong evidence that upon kainate or glutamate-induced stimulation, GluR6 is sumoylated and subsequently internalized. This SUMO-mediated removal of channel from the plasma membrane surface has important functional consequences on the post-synaptic amplitude of KAR-mediated EPSCs, and thus may have a potential role in the processes of learning and memory.

8.4 Ion Chanels as Probes of Sumo Modification at the Plasma Membrane

Together, these reports clearly identify an emerging role for the SUMO modification of ion channels at the plasma membrane. The extent to which this regulatory mechanism contributes to membrane protein function however, remains to be established. Our own unpublished data indicate that the complement of SUMO-modified proteins present at the plasma membrane is readily detected and the pattern is distinct from that observed for soluble proteins. This indicates that analysis of sumoylation in this compartment is likely to be quite revealing. Although the identity

Table 8.1 Identification of membrane ion channels that contain synergy control motif sumoylation sequences[a]

Acc # | Symbol | (Size aa)	Description	SUMOylation Motif
|Q09470|KCNA1| (495)	Voltage-gated potassium channel subunit Kv1.1	135 - 145: Gf...IKeEerpIP
		190 - 200: Pe...LKdDkdftG
|P16389|KCNA2| (499)	Voltage-gated potassium channel subunit Kv1.2	131 - 141: Gy...IKeEerpIP
|P22460|KCNA5| (613)	Voltage-gated potassium channel subunit Kv1.5	218 - 228: Gf...IKeEekpIP
		532 - 540: Pav..LKeEq...G
|Q03721|KCNC4| (635)	Voltage-gated potassium channel subunit Kv3.4	20 - 32: PsktcLKeEmak.G
|Q12809|KCNH2| (1159)	Voltage-gated potassium channel subunit Kv11.1	114 - 120: P....VKnEd...G
|Q9ULD8|KCNH3| (1083)	Voltage-gated potassium channel subunit Kv12.2	115 - 121: P....IKnEk...G
		786 - 797: GragaLKaEag..P
|Q9UQ05|KCNH4| (1017)	Voltage-gated potassium channel subunit Kv12.3	115 - 121: P....IKnEm...G
|Q9H252|KCNH6| (994)	Voltage-gated potassium channel subunit Kv11.2	114 - 120: P....VKnEd...G
|Q9NS40|KCNH7| (1196)	Voltage-gated potassium channel subunit Kv11.3	1093-1105: Peas.IKtDrsfsP
|Q96L42|KCNH8| (1107)	Voltage-gated potassium channel subunit Kv12.1	115 - 121: P....IKnEk...G
|Q9UNX9|IRK14| (436)	ATP-sensitive Inward rectifier K(+) channel Kir2.4	50 - 57: Grf..VKkD....G
|Q9NZI2|KCIP1| (227)	Kv channel-interacting protein 1 (KChIP1)	175 - 182: Pv...LKeDt...P
|Q6PIL6|KCIP4| (250)	Kv channel-interacting protein 4 (KChIP4)	198 - 205: Pv...LKeDa...P
|Q9Y244|POMP| (141)	Voltage-gated K channel beta subunit 4.1	34 - 44: Gfsc.VKnEll..P
|Q8IZS8|CA2D3| (1091)	Voltage-gated calcium channel subunit alpha-2/delta-3	225 - 235: Pg...IKwEpdenG
|Q15878|CAC1E| (2312)	Voltage-gated calcium channel subunit alpha Cav2.3	852 - 860: Ggs..LKgDg...G
|O43497|CAC1G| (2377)	Voltage-gated calcium channel subunit alpha Cav3.1	653 - 663: Pc...LKaDsgacG
|O95180|CAC1H| (2353)	Voltage-gated calcium channel subunit alpha Cav3.2	938 - 948: GckfsLKtDt...G
|Q9P0X4|CAC1I| (2223)	Voltage-gated calcium channel subunit alpha Cav3.3	1932-1945: PvrswLKhDssqaP
|Q13698|CAC1S| (1873)	Voltage-gated calcium channel subunit alpha Cav1.1	1472 - 1481: GtalkIKtE....G
|Q92736|RYR2| (4967)	Cardiac muscle ryanodine receptor-calcium channel (RyR2)	4381 - 4389: Gld..LKrEg...G
|A8K7I4|CLCA1| (914)	Calcium-activated chloride channel family member 1) (hCLCA1)	754 - 766: GqitdLKaEihg.G
|Q9UQC9|CLCA2| (943)	Calcium-activated chloride channel family member 2) (hCLCA2)	662 - 670: Gadv.IKnD....G
|Q9Y696|CLIC4| (253)	Intracellular chloride ion channel protein p64H1	6 - 17: Plng.LKeEdke.P
|P35498|SCN1A| (2009)	Voltage-gated sodium channel subunit alpha Nav1.1	1748-1759: Pgss.VKgDcgn.P
|Q99250|SCN2A| (2005)	Voltage-gated sodium channel subunit alpha Nav1.2	1738-1749: Pgss.VKgDcgn.P
|Q9NY46|SCN3A| (2000)	Voltage-gated sodium channel subunit alpha Nav1.3	1733-1744: Pgss.VKgDcgn.P
|P35499|SCN4A| (1836)	Voltage-gated sodium channel subunit alpha Nav1.4	1560-1571: Pgts.VKgDcgn.P
|Q9UQD0|SCN8A| (1980)	Voltage-gated sodium channel subunit alpha Nav1.6	1134-1147: GstidIKpEveevP
|P51172|SCNND| (638)	Nonvoltage-gated sodium channel 1 subunit delta) (SCNED)	580-592: Gass.IKpEasqmP
|Q8IWT1|SCN4B| (228)	Sodium channel subunit beta-4 precursor	82 - 91: Gt...VKnEksd.P
|Q9BX84|TRPM6| (2022)	Melastatin-related TRP cation channel 6	1995-2006: Gle..IKiEsaeeP
|Q9Y5S1|TRPV2| (764)	osm-9-like TRP channel 2) (TrpV2)(OTRPC2)	421 - 428: Ph...LKaEv...G
|Q9BQR3|PRS27| (290)	Channel-activating protease 2 (CAPH2)(Marapsin) (Pancreasin)	205 - 216: Pkt..IKnDmlcaG
|Q8TDI7|TMC2| (906)	Transmembrane channel-like protein 2 (cochlear-expressed)	7 - 15: G....LKeEarg.G

[a]Using the ExPASy: ScanProsite Tool, the UniProtKB/Swiss-Prot protein database was scanned using the sequence [PG]-X(0,4)-[ILV]-K-X-[ED]-X(0,4)-[PG] with the species filter "human" and the description filter "channel" and the match mode "greedy/overlaps/no includes." All 38 hits on 35 sequences are shown

of the target proteins remains to be determined, scanning of the human ProSite database using a search profile based on functionally characterized synergy control motifs in transcription factors (Iniguez-Lluhi and Pearce 2000) and a description filter of "channel", identifies that numerous ion channels contain high-scoring motifs (Table 8.1). Included in these are multiple members of the voltage-gated K^+, Ca^{2+}, and Na^+ families. Interestingly, while members of the Kv4 family known to mediate I_{to} current in cardiomyocytes are absent from this list, the HERG channel (Kv11.1), which is thought to mediate the I_{ss} current in cardiomyocytes (Snyders 1999), contains a high-scoring motif on its cytoplasmic N-terminus (Table 8.1). Notably, recent data from our laboratory suggest that infusion of sumoylation machinery components does not appear to affect the current density of HERG-encoded I_{ss}. This clearly raises the possibility that

if present, SUMO modification may have other effects on the biophysical properties of this important channel.

Intriguingly, as well, a recent genotyping screen has revealed the occurrence of a naturally-occurring missense mutation in a patient with long-QT syndrome that disrupts a flanking proline residue in a putative sumoylation motif in HERG (P114S) (Lupoglazoff et al. 2001). This finding is very interesting in light of a recent report of a similar nonsynonymous polymorphism in Kv1.5 (P532L) that maps to the well-conserved upstream proline of the second Kv1.5 sumoylation motif. Remarkably, this polymorphism, which is carried by 1.1% of African Americans, confers a substantial reduction in the ability of quinidine to block Kv1.5 (Simard et al. 2005; Drolet et al. 2005). The conservation of Pro/Gly residues flanking the core sumoylation motifs reflects their role as terminators of secondary structure elements and suggests that they facilitate exposure of the sumoylation site to the conjugation machinery. Consistent with this idea, analysis of transcription factor sumoylation by our group indicates that substitution of the proline residues flanking the core sumoylation sites with amino acids other than glycine leads to a reduced interaction with the SUMO E2 conjugating enzyme Ubc9 and a concomitant reduction in sumoylation (Mukherjee et al. in preparation). An examination of whether the putative sumoylation site in HERG is indeed functional and the potential relation with the P114S mutation associated with long-QT syndrome is likely to be revealing.

It is also interesting to note that the Kv accessory proteins KChIP1, KChIP4, and Kvβ 4.1 all contain high-scoring sumoylation motifs. While little is known about the interactions or functional effects of Kvβ4.1, the other two proteins, KChIP1 and KChIP4 are well-known to interact with members of the Kv4 family and to alter the current densities and biophysical properties of these channels (Pourrier et al. 2003). As Kv4 family members mediate the I_{to} current in cardiomyocytes, this raises the possibility that while Kv4 family members do not appear to contain sumoylation motifs, critical regulators of these pore-forming subunits may be subject to this form of regulation.

A less stringent scan of the human ProSite database using a profile containing only the core sumoylation motif ([ILV]-K-X-[ED]) and a description filter of "channel" yielded many additional hits not identified in the above search (205 hits in 123 sequences). Included in these hits were multiple isoforms of serotonin receptors, ATP-binding cassette (ABC) transporters, cyclic nucleotide-gated (CNG) channels, inward rectifier potassium (Kir) channels, ryanodine receptors (RyR), and two-pore potassium channels (among many other families of membrane-associated proteins). Many of these identified motifs appeared to be located in regions of the proteins accessible to the cytoplasmic SUMO conjugation machinery.

There are some important observations that can be made from these first reports of SUMO modified ion channels. The first observation involves the finding that the SUMO conjugation machinery appears accessible to integral membrane proteins on the plasma membrane. As already mentioned, studies of GLUT1 and GLUT4 transporters, as well as of the K2P1 channel identified that Ubc9 is associated with the plasma membrane. Additionally, immunocytochemical studies of cultured hippocampal neurons in the GluR6 study, demonstrated that endogenous SUMO, Ubc9, and PIAS3 are all present not only in the nucleus, but also throughout dendrites and at synapses. Future studies that characterize the subcellular localization of the E3 ligases and SENP proteases may offer insights into additional layers of regulation of SUMO modification at the membrane. For example, SENP1 is found to be primarily localized in the nucleus whereas SENP2 is enriched at the nuclear pore complex but can shuttle in and out of the nucleus. In a further example, SENP5 is enriched in the nucleolus but is also found in association with mitochondria. It will thus be interesting to determine which SENP isoforms are enriched or have access to the cytoplasmic domains of integral membrane proteins in the cell. Such enzymes might acutely modulate GluR6, Kv1.5 and K2P1 in different ways.

The finding that both K2P1 and GluR6 can be acutely regulated by SUMO and SENP may also provide some information as to where in the cell ion channel sumoylation occurs. One could imagine that sumoylation of K2P1 or GluR6 could take place at the plasma membrane or during the synthesis, trafficking, or recycling of the channel. Transient sumoylation of GluR6 during its synthesis, for example, could induce conformational changes or promote assembly with other binding partners that could persist throughout the lifecycle of the channel and affect its function at the plasma membrane. This does not seem to be the case, however, since SUMO and SENP were found to be able to regulate both GluR6 and K2P1 channel activity within seconds to minutes of application. Given these kinetics, it seems more likely that sumoylation affects channels in or near the plasma membrane where this modification can have immediate effects on function.

It has been suggested that sumoylation may serve to inhibit ion channel activity (Geiss-Friedlander and Melchior 2007) since sumoylation reduced current in both K2P1 and GluR6, while SENP served to increase these currents. This generalization seems perhaps premature, however, since the mechanisms by which SUMO inhibits K2P1, GluR6 and Kv1.5 are very different. Thus, while GluR6 current was inhibited by sumoylation-promoted endocytosis, internalization does not appear to be involved in sumoylation-induced inhibition of K2P1-mediated currents. This suggests that SUMO may be acting by different mechanisms in each channel. Indeed, as described above, SUMO does not seem to inhibit the activity of Kv1.5 directly, but rather to protect the channel from inactivation. Thus, while further work may reveal a common mechanism for channel sumoylation (by promoting interactions with other binding partners, or inducing conformational changes, or modulating the local electrostatic environment, for example) the functional consequences of sumoylation will most likely be dependent on the individual target channel.

Finally, these studies provide some insight into the regulation of SUMO modification of ion channels. GluR6 sumoylation was clearly found to be induced by treatment with certain known agonists of the channel. The mechanism by which agonist binding regulated sumoylation of the channel is not clear, however. It seems reasonable that conformational changes induced by ligand binding might serve to make the sumoylation motif of the channel more accessible to SUMO conjugation machinery. Alternatively, such a conformational change could serve to stabilize transient sumoylation of the channel by inhibiting access by SUMO proteases. Interestingly, treatment of oocytes with a broad range of volatile anesthetics, long-chain free fatty acids, lysophospholipids, and classical regulators of kinases and phosphatases was shown to have no effect on the constitutive sumoylation status of K2P1. This suggests that regulation of channel sumoylation is specific and potentially dependent on distinct conformational changes that modulate the accessibility of the sumoylation motif by the SUMO machinery. Clearly, further work is needed to fully understand this regulatory mechanism.

8.5 Conclusions

Although the number of ion channels currently shown to be post-translationally modified by sumoylation is small, it represents an important class of targets that extend the reach of the SUMO conjugation machinery to the plasma membrane. Future work identifying additional channel targets, as well as the factors regulating the modification of target proteins by SUMO, will be critical for the advancement of this new field. In addition, important questions regarding stoichiometry and control of the balance between the forward conjugation of SUMO to the channel and the reverse de-conjugation of the SUMO moieties remain. Nevertheless, these intriguing observations suggest that ion channels may be a unique class of proteins that are useful to probe both the mechanisms and functional significance of SUMO modification for protein substrates at the plasma membrane. Together they support the view that SUMO modification is likely to be an important and widespread regulatory mechanism

at the plasma membrane and that it can have a significant impact on the control of the electrical properties of excitable cells.

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The Roles of SUMO in Metabolic Regulation

9

Elena Kamynina and Patrick J. Stover

Abstract

Protein modification with the small ubiquitin-related modifier (SUMO) can affect protein function, enzyme activity, protein-protein interactions, protein stability, protein targeting and cellular localization. SUMO influences the function and regulation of metabolic enzymes within pathways, and in some cases targets entire metabolic pathways by affecting the activity of transcription factors or by facilitating the translocation of entire metabolic pathways to subcellular compartments. SUMO modification is also a key component of nutrient- and metabolic-sensing mechanisms that regulate cellular metabolism. In addition to its established roles in maintaining metabolic homeostasis, there is increasing evidence that SUMO is a key factor in facilitating cellular stress responses through the regulation and/or adaptation of the most fundamental metabolic processes, including energy and nucleotide metabolism. This review focuses on the role of SUMO in cellular metabolism and metabolic disease.

Keywords

SUMO • Metabolism • Metabolic disease • One-carbon metabolism • Lipids

Abbreviations

SUMO	Small Ubiquitin-related Modifier
SIM	SUMO interacting motif
SREBPs	sterol regulatory element binding proteins
bHLH-Zip	basic helix-loop-helix leucine zipper
SRE	sterol response element
SCAP	SREBP cleavage activating protein
INSIG	insulin inducing gene
MAPKs	mitogen-activated protein kinases
HDAC3	histone deacetylase 3
ROS	reactive oxygen species
SENPs	sentrin specific proteases

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PPARs	peroxisome proliferators-activated receptors
KLF5	Krüppel like transcription factor 5
Cpt1b	carnitine palmitoyl transferase
Ucp2	uncoupling proteins
ICA512	islet cell autoantigen 512
STAT5	signal transducer and activator of transcription
GLUTs	glucose transporters
DRP1	dynamin related protein 1
FIS1	fission protein 1
AICARTfase	aminoimidazole-4-carboxamide ribonucleotide transferase
DHFR	dihydrofolate reductase
GARTfase	10-formyltetrahydrofolate:5′-phosphoribosylglycinamide N-formyltransferase
MTHFD1	methylenetetrahydrofolate dehydrogenase 1
NADPH	nicotinamide adenine dinucleotide phosphate
SHMT1	cytoplasmic serine hydroxymethyltransferase 1
SHMT2	mitochondrial serine hydroxymethyltransferase 2
TYMS	thymidylate synthase
THF	tetrahydrofolate
FPLD	familial partial lipodystrophy
LMNA	lamin A/C
PIAS3	protein inhibitor of activated STAT 3
UBC9	SUMO-conjugating enzyme UBC9, ubiquitin carrier protein 9
PKM2	pyruvate kinase, muscle, isozyme 2

9.1 Introduction: Functions of SUMO in Metabolism

Protein modification with the small ubiquitin-related modifier SUMO can affect protein function, enzyme activity, protein-protein interactions, protein stability, protein targeting and cellular localization. The number of identified Sumoylated proteins, and proteins that interact with SUMO through SUMO interacting motifs (SIM), continues to grow (Becker et al. 2013; Bruderer et al. 2011; Eifler and Vertegaal 2015; Hendriks et al. 2014; Impens et al. 2014; Jardin et al. 2015; Jentsch and Psakhye 2013; Kaminsky et al. 2009; Kroetz and Hochstrasser 2009; Lamoliatte et al. 2014; Makhnevych et al. 2009; Subramonian et al. 2014; Tammsalu et al. 2014, 2015; Yang and Paschen 2015; Yang et al. 2012). Like other forms of post-translational modification, sumoylation is now known to be involved in most, if not all cellular processes (Flotho and Melchior 2013; Gareau and Lima 2010; Hecker et al. 2006; Makhnevych et al. 2009; Stehmeier and Muller 2009; Wilkinson and Henley 2010). Sumoylation is a reversible modification: SUMO/sentrin-specific proteases (SENPs) are capable of removing SUMOs from target proteins, contributing to a dynamic control of sumoylation (Flotho and Melchior 2013; Hay 2007; Hickey et al. 2012; Mukhopadhyay and Dasso 2007; Yeh 2009).

Not surprisingly, SUMO is now known to modify and affect the function and/or regulation of specific metabolic enzymes within pathways, and in some cases regulates entire metabolic pathways by affecting the activity of master control proteins or facilitating the translocation of entire metabolic pathways to subcellular compartments (Gareau and Lima 2010). In addition to its established roles in maintaining metabolic homeostasis, there is increasing evidence that SUMO is a key factor in facilitating cellular stress responses. This occurs through the regulation of some of the most fundamental metabolic processes, including energy and nucleotide metabolism, and permits physiological adaptation in response to cellular and environmental queues (Enserink 2015; Makhnevych et al. 2009). SUMO has been implicated in complex human diseases and developmental anomalies that are also associated with nutritional and/or metabolic perturbations including Alzheimer's disease (Dorval and Fraser 2007; Hoppe et al. 2015; Lee et al. 2013, 2014b; Martins et al. 2016; McMillan et al. 2011; Sarge and Park-Sarge 2009), Parkinson's disease (Guerra de Souza et al. 2016) (Eckermann 2013; Krumova et al. 2011), type

I diabetes (Li et al. 2005; Wang and She 2008), familial partial lipodystrophy (Simon et al. 2013), diabetes-mediated cardiovascular disease (Chang and Abe 2016), congenital heart disease (Wang et al. 2011), cardiomyopathy (Kim et al. 2015c; Zhang and Sarge 2008), arthritis (Yan et al. 2010), amyotrophic lateral sclerosis (Dangoumau et al. 2016; Foran et al. 2013; Niikura et al. 2014), and cleft lip and/or palate (Alkuraya et al. 2006; Song et al. 2008; Tang et al. 2014).

9.2 SUMO and Transcriptional Regulation of Metabolic Pathways

9.2.1 SUMO and Master Regulation of Lipid Biosynthesis

Cholesterol and fatty acids, which are essential for cellular membrane and lipoprotein particle biosynthesis, can be acquired through diet and/or through cellular biosynthesis (Brown and Goldstein 2009; Goldstein and Brown 2015; Goldstein et al. 2006; Soyal et al. 2015; Weber et al. 2004). Changes in their availability at the cellular level, both deficiencies and excesses, can affect membrane fluidity, the function of membrane-associated proteins, and the maintenance of cellular homeostasis and risk of chronic disease. Many of the enzymes that comprise the cholesterol and fatty acid biosynthesis pathway, as well as proteins required for low density lipoprotein uptake, are regulated at the level of transcription through common feedback loops that involve the sterol regulatory element binding proteins (SREBPs) (Brown and Goldstein 2009; Goldstein and Brown 2015; Goldstein et al. 2006; Jeon and Osborne 2012; Soyal et al. 2015; Weber et al. 2004). SREBPs are members of the family of basic helix-loop-helix leucine zipper (bHLH-Zip) transcription factors and are master regulators that govern the expression of over 30 genes that encode enzymes required for lipid biosynthesis and accumulation. There are three mammalian SREBP isoforms (SREBP-1a, SREBP-1c and SREBP-2) encoded by two genes (SREBP-1 and SREBP-2). SREBP-1a is a master regulator of genes necessary for fatty acid, triacylglyceride and phospholipid biosynthesis, as well as the generation of NADPH and acetyl-CoA cofactors that are required for fatty acid synthesis. SREBP-1a is expressed in proliferating tissues, whereas SREBP-1c is expressed in liver and adipose tissue (Brown and Goldstein 2009; Goldstein et al. 2006; Weber et al. 2004; Yokoyama et al. 1993). SREBP-2 exhibits more ubiquitous expression, but targets cholesterol biosynthesis almost exclusively (Hua et al. 1993; Weber et al. 2004). SREBPs are present in the endoplasmic reticulum as precursor resident transmembrane proteins in a complex with the lipid-sensing SREBP cleavage-activating protein (SCAP) and the insulin-inducing gene (INSIG), which sequester SREBPs and confine them to the endoplasmic reticulum when membrane cholesterol levels are elevated. SCAP contains a sterol sensing domain, and escorts SREBPs to the Golgi when membrane sterol concentrations are depleted. In the Golgi, SREBPs are processed by two proteolytic cleavage events that liberate the mature protein from the membrane. SREBPs translocate to the nucleus, bind to sterol response elements (SREs) and function to upregulate the expression of genes required for fatty acid and cholesterol biosynthesis (Hirano et al. 2003). Whereas the genes involved in cholesterol biosynthesis are almost exclusively regulated by SREBPs, transcriptional regulation of the genes encoding enzymes required for lipid biosynthesis is complex and responds to many intra- and extracellular signaling pathways (Goldstein and Brown 2015; Weber et al. 2004).

SREBPs that have been liberated from the Golgi by proteolysis are subject to an additional level of regulation in the nucleus by an antagonistic interplay between two posttranslational modifications, sumoylation and phosphorylation (Arito et al. 2008). The SREBPs are targets for the growth-hormone-induced mitogen-activated protein kinases (MAPKs) ERK1 and ERK2. ERK-dependent phosphorylation occurs in close proximity to the site of SUMO modification but elicits very different functional effects on SREBP activity (Arito et al. 2008). Phosphorylation of SREBP-2 at S455 increases its transcriptional

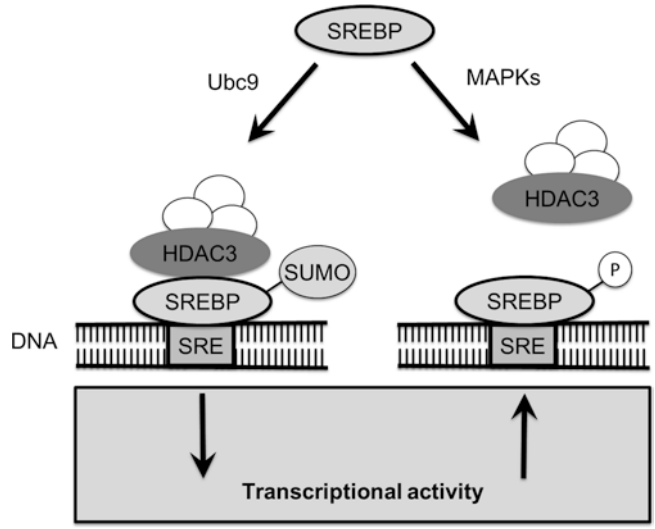

Fig. 9.1 The sumoylation of SREBPs recruits a co-repressor complex which includes HDAC3. The recruitment of HDAC3 containing complex reduces the transcriptional activity of SREBPs. SUMO is only required for the formation of the co-repressor complex, as the complex continues to repress once SUMO is removed. Alternatively, SREBPs can be phosphorylated by MAPKs which inhibit sumoylation by competing for sites nearby the sumoylation motif. SREBP phosphorylation allows for transcriptional activation of lipid biosynthesis by expressing genes that contain sterol-response elements

activity, whereas SUMO modification at K464 inhibits its activity (Arito et al. 2008). SREBP-2 phosphorylation and sumoylation were shown to be competitive modifications; mutation of S455 to an alanine residue enhances sumoylation whereas the S455G mutant SREBP-2, a phosphorylated SREBP-2 mimic, exhibited impaired sumoylation (Arito et al. 2008). As no direct effects of S455 phosphorylation on SREBP structure or function have been reported, the physiological function of S455 phosphorylation may be to prevent SREBP sumoylation (Fig. 9.1).

SREBP modification with SUMO elicits functional consequences. SREBP sumoylation inhibits its SREBP transcriptional activity indirectly through the recruitment of a co-repressor complex that includes histone deacetylase 3 (HDAC3). Following recruitment and establishment of the repressor complex, the SREBP-2 SUMO moiety is likely not essential as most SREBP present in the repressor complex lacks the SUMO modification. HDAC3 activity directly affects lipid homeostasis, as both LDL uptake and expression of the LDL receptor were elevated in HepG2 cells when HDAC3 expression was repressed (Arito et al. 2008). Growth-hormone induced ERK-dependent phosphorylation of SREBPs ensures that lipid synthesis occurs for membrane biosynthesis during periods of growth, whereas sumoylation can repress lipid biosynthesis and attenuate lipid-sensing signals that originate in the endoplasmic reticulum and generate SREBP protein.

There is also evidence that sumoylation of SREBP-1plays a role in glucogon secretion by the alpha-cells of pancreas. Glucagon reduces hepatic lipid synthesis, and it's been known that SREBP-1c is downregulated by fasting. The metabolic effects of glucagon are mediated through the cAMP-dependent protein kinase A (PKA). It has been recently demonstrated that SREBP-1 is one of the substrates for PKA phosphorylation (Dong et al., 2014). During nutritional deprivation, PKA is activated, resulting in induction of SREBP-1c sumoylation by PIASy. This cascade results in increased sumoylation of SREBP-1, repression its transcriptional activity, and turning off of hepatic lipogenesis (Lee et al. 2014a). This pathway represents a dynamic fine-tuning of SREBP-1 transcriptional activity in response to

various nutritional and hormonal changes to turn off lipid synthesis during fasting or nutritional deprivation. Thus, sumoylation of SREBPs plays an important role in the suppression of the hepatic lipogenic program upon fasting-induced signals.

9.2.2 SUMO and Metabolic Nuclear Receptors

Many metabolic pathways are regulated by the orchestrated action of nuclear receptors (NRs) that exert crucial functions by regulating gene expression, both positively and negatively, in response to changes in metabolite levels (Treuter and Venteclef 2011). Nuclear receptors are a class of cellular proteins that have the ability to sense and respond to the changes in concentration of various small molecules that specifically bind to the receptor and serve as receptor ligands. NRs are generally divided into 3 classes based on the nature of their ligands: **(1)** The first group consists of nuclear receptors that bind steroid hormones, such as the glucocorticoid receptor (GR), receptor (GR), the mineralocorticoid receptor (MR) and the estrogen receptor (ER); receptor (ER); **(2)** The second group consists of orphan nuclear receptors for which ligands has not been yet identified, such as the estrogen-related receptors alpha (ERRα) and gamma (ERRβ); upon identification of the ligand, the orphan NRs usually are reclassified and leave this subgroup; **(3)** The third group is comprised of ligand-dependent NRs that are regulated by a diverse group of endogenous and exogenous small molecules, such as PPARs, which bind fatty acids and fatty acid metabolites, liver X receptors (LXRs), which bind oxysterols, Farnesoid X receptor (FXR), which binds bile acid, and retinoic acid receptors (RXR), which bind retinoic acid but can also respond to other ligands by forming heterodimers with other NRs. Several of the NRs are targeted by synthetic ligands in therapy: RXR and its fusion product RXR-PML are targeted by retinoic acid administration in acute promyelocytic leukemia, ERα (NR3A1) is targeted in breast cancer by the synthetic antagonist tamoxifen, GR is targeted in inflammatory diseases by dexamethasone, peroxisome proliferator-activated receptor gamma (PPARγ) is targeted in type II diabetes by administration of thiazolidinediones (Germain et al. 2006). Sumoylation modulates the transcriptional activities of many NRs, thus affecting metabolism (summarized in Table 8.1). More than 20 nuclear receptors have been reported to be reversibly sumoylated (Treuter and Venteclef 2011). Other modifications of nuclear receptors can inhibit their sumoylation. Acetylated FRX cannot be sumoylated, leading to activation of inflammatory genes (Kim et al. 2015b). In fact, acetylation of SUMO protein itself prevents SUMO binding to proteins containing SIMs.

9.3 SUMO in Familial Partial Lipodystrophy

Familial partial lipodystrophy (FPLD) is a metabolic disorder characterized by abnormal regional and progressive adipose tissue loss after puberty due to adipocyte degeneration. FPLD is often associated with insulin-resistant diabetes with acanthosis nigricans and hypertriglyceridemia. A subset of FPLD cases, also called Dunnigan-type familial partial lipodystrophy, or familial partial lipodystrophy type 2 (FPLD2), is caused by mutations in LMNA gene encoding structural nuclear proteins Lamin A and C (Cao and Hegele 2000; Speckman et al. 2000). Lamin A/C is sumoylated (Boudreau et al. 2012; Zhang and Sarge 2008) and also binds SUMO through a SUMO-interacting motif (SIM) (Moriuchi et al. 2016). Lamin A mutations linked to familial partial lipodystrophy alter lamin A sumoylation (Simon et al. 2013). The FPLD-causing mutations decrease lamin A binding to SREBP1 and upregulate a large number of SREBP1 target genes (Lloyd et al. 2002; Vadrot et al. 2015). Thus, a model has been proposed where lamin A K486 modification by SUMO blocks binding of interacting proteins, including SREBP1 (Simon et al. 2013). These studies implicate altered lamin A sumoylation in familial partial lipodystrophy;

however, the molecular mechanisms of FPLD and the metabolic consequences of altered lamin A sumoylation remain to be elucidated.

9.4 Metabolic Adaptation to Cellular and Oxidative Stress

Metabolic response to cellular stress is essential to maintain cell viability and reestablish homeostasis, and several observations indicate a key role for SUMO as a component of the metabolic stress response (Enserink 2015; Guo and Henley 2014; Nunez-O'Mara and Berra 2013; Yang et al. 2014; Zhou et al. 2004). Hibernation torpor has been used as a model system for studying reduction in blood flow to various organs and thus oxygen and glucose starvation in those tissues. During torpor of ground squirrels, a substantial increase in global sumoylation was observed within brain, liver, and kidney tissues which occurred coincident with increased Ubc9 expression in these tissues (Lee et al. 2007). Likewise, Ubc9 over-expression was shown to protect SH-SY5Y neuroblastoma cells in response to oxygen and glucose starvation, and mice subjected to transient ischemia also showed an increase in SUMO2/3 conjugates in the hippocampus and cerebral cortex (Cimarosti et al. 2008; Yang et al. 2008a, b, c). Hippocampal HT22 cells exposed to arsenite, an inducer of oxidative stress and inhibitor of pyruvate dehydrogenase also increased global protein sumoylation (Yang et al. 2008c). In primary cortical neuronal cultures from rodents and is SH-SY5Y human neuroblastoma cells, sumoylation participates in induction of ischemic tolerance (Lee et al. 2009b). Transgenic mouse lines that overexpress Ubc9 up to 5-fold higher than the wild type mice exhibit resistance to brain ischemia (Lee et al. 2011). These data support a role of sumoylation in cytoprotection responses elicited by cellular stresses (Lee et al. 2014c, 2016b; Tong et al. 2015; Lee and Hallenbeck 2013; Yang et al. 2012).

Although not thoroughly characterized, SUMO-2/3 may play a role in response to cellular stresses such as protein damage (Seifert et al. 2015). In adult differentiated cells, a very small pool of unconjugated SUMO-1 is present in the cell whereas a much larger pool of free SUMO-2/3 is available (Saitoh and Hinchey 2000). Upon exposure to protein-damaging insults such as heat shock, oxidative stress, ethanol, osmotic stress, or replicative stress, an increase in the amounts of SUMO-2/3 conjugated proteins occurs (Bursomanno et al. 2015; Manza et al. 2004; Saitoh and Hinchey 2000). It has been suggested that the activation of SUMO-2/3 conjugation under these conditions stabilizes and thereby increases protein half-lives to enable cell survival. Some debate surrounds the role of reactive oxygen species (ROS) in the SUMO-mediated cell survival. Studies demonstrating an increase in SUMO-2/3 conjugates in response to stress is in contrast to reports that ROS negatively regulates Ubc9 catalyzed sumoylation (Bossis and Melchior 2006). Under physiologically relevant concentrations of H_20_2, it has been observed that nearly all SUMO conjugation is inhibited as a result of the formation of a disulfide linkage between the catalytic cysteines of Uba2 and Ubc9 (Bossis and Melchior 2006). Sumoylation is a reversible post-translational modification and sentrin specific proteases (SENPs) can efficiently and rapidly desumoylate proteins (Flotho and Melchior 2013). Because the activity of SENPs is unaffected by H_20_2, ROS exposure is anticipated to result in the rapid deconjugation of sumoylated proteins. Indeed, the regulation of sumoylation by ROS may function to control the redox state of the cell during oxidative stress. In support of this concept, sumoylation represses the transcriptional activity of c-Fos and c-Jun which are potent regulators of oxidative stress responsive genes (Bossis et al. 2005; Tempe et al. 2014).

The promyelocytic leukemia (PML) protein is the main constituent and a scaffold of PML nuclear bodies (NBs), which assemble in response to stress. During oxidative stress, PML

protein is oxidized and forms disulfide-mediated spherical meshes that recruit UBC9, which enhances PML sumoylation (Sahin et al. 2014). Sumoylated PML recruits SUMO-binding proteins that become sequestrated within the NB inner core. In response to oxidative stress, NBs promote sumoylation of these so-called partner proteins (Sahin et al. 2014).

9.5 SUMO and Energy Metabolism

9.5.1 SUMO in Muscle Metabolism

Perturbations of energy metabolism in skeletal muscle are associated with metabolic syndromes, and contribute to the mechanisms leading to obesity and obesity related disorders. Peroxisome proliferator-activated receptors (PPARs) are a family of nuclear receptor proteins that function as ligand-activated transcription factors which play essential roles in regulating cellular lipid, protein and carbohydrate metabolism (Desvergne and Wahli 1999; Grygiel-Gorniak 2014). The cellular ligands that activate PPARs include fatty acids, and eicosanoids. The nuclear receptor PPARβ/δ plays a major role in cardiac lipid metabolism, and mice with cardiac-specific deletion of this receptor develop myocardial lipid accumulation and cardiomyopathy (Cheng et al. 2004; Vazquez-Carrera 2016). PPARβ/δ is expressed highly in skeletal muscle, and is a determinant of the oxidative capacity of the cell. PPARβ/δ forms an obligate heterodimer with retinoic acid receptor (RXR or NR2B) (DiRenzo et al. 1997). It initiates transcription of target genes by binding to peroxisome proliferator response elements (PPREs). Selective disruption of PPARβ/δ expression in mouse skeletal muscle myocytes has been shown to lead to the onset of obesity and diabetes (Schuler et al. 2006). Similarly, in other mouse models, selective overexpression of PPARβ/δ in skeletal muscle has been shown to rescue mice from diet-induced obesity by enhancing the expression of genes that encode enzymes in the fatty acid oxidation pathway. Thus, PPARβ/δ is a key regulator of fat accumulation (Wang et al. 2004). Another key regulator of lipid metabolism, Krüppel-like transcription factor 5 (KLF5) is present in nuclear protein complexes with PPARβ/δ and these two proteins interact in mammalian two-hybrid and co-immunoprecipitation experiments (Oishi et al. 2008). KLF5$^{+/-}$ mice are resistant to diet-induced obesity, hypercholesterolemia, and glucose intolerant phenotypes despite consuming more food than control mice (Oishi et al. 2008).

Sumoylated KLF5 is found in transcriptionally-repressive regulatory complexes with unliganded PPARβ/δ and co-repressorsrepressors (Table 9.1). This complex represses carnitine-palmitoyl transferase-1b (Cpt1b), and uncoupling proteins 2 and 3 (Ucp2 and Ucp3) expression, which are regulators of lipid oxidation and energy uncoupling respectively. Cpt1b catalyzes the rate-limiting step in mitochondrial fatty acid import into mitochondria for β-oxidation. Ucp2 and Ucp3 uncouple oxidative phosphorylation from ATP generation and thereby generate heat, but also function to regulate mitochondria-derived ROS. Upon PPARβ/δ ligand binding, KLF5 is desumoylated and becomes associated with transcriptionally active complexes, leading to the enhancement of Cpt1b, Ucp2, and Ucp3 expression (Fig. 9.2). In this regard, KLF5 desumoylation is a component of nutrient signaling within PPARβ/δ containing transcription factor complexes, and acts as a regulatory switch mediating the transcriptional activation of energy consumption (Oishi et al. 2008).

The nuclear receptor peroxisome proliferator-activated receptor α (PPARα) is highly expressed in tissues with high fatty acid catabolic activity such as skeletal muscle, liver and brown adipose tissue, small intestine, heart, and kidney. It regulates the anti-inflammatory response and energy homeostasis in response to endogenous ligands such as arachidonic acid as well as other polyunsaturated fatty acids (Kersten 2014). It is activated under conditions of energy deprivation and fasting and promotes expression of genes involved in adipose tissue lipolysis. In addition to directly binding DNA and regulating the expression of metabolic genes, PPARs can also regulate expression of inflammation related genes by

Table 9.1 Role of Sumoylation in metabolism through the regulation of the activities of transcription factors

Metabolic transcription factor	Affected metabolic pathways	SUMO connections/effect of sumoylation on activity and metabolism	References
Pregnane and Xenobiotic Receptor (PXR)	Metabolism and elimination of noxious endobiotics and xenobiotics; role in inflammatory bowel disease and cancer; glucose and lipid metabolism in liver	Sumoylation by SUMO-1/ SUMO-acetylation switch/ enhanced activity, increased expression of CYP3A4, CYP2C9, MDR1 and UGT1A1 genes	Cui et al. (2016), Hu et al. (2010) and Priyanka et al. (2016)
Steroidogenic Factor 1 (SF-1) nuclear receptor	Energy balance; development of the ventral medial nucleus of the hypothalamus (VMH) and the maintenance of normal body weight; sexual development and reproduction; gonadal and adrenal steroidogenesis	Sumoylation by SUMO via PIASy and PIASxalpha/ SUMO-mediated repression of activity	Lee et al. (2005), Komatsu et al. (2004) and Lee et al. (2016a)
Liver Receptor Homolog 1 (LRH-1, or Nr5a2, PHR-1, hB1F)	Binds phospholipids;steroid synthesis and ovulation; production and secretion of the pancreatic digestive juice; lipid metabolism in inflammation	Sumoylation by SUMO-1/ repression of activity through association with PML nuclear bodies; anti-inflammatory hepatic transrepression (acute phase response)	Stein and Schoonjans (2015), Treuter and Venteclef (2011) and Venteclef et al. (2010)
Peroxisome Proliferator-Activated Receptor alpha (PPARα)	Female-specific repression of hepatic genes involved in steroid metabolism and immunity	Sumoylation/protection against estrogen-induced intrahepatic cholestasis	Leuenberger et al. (2009) and Pourcet et al. (2010)
Peroxisome Proliferator-Activated Receptor beta/delta (PPARβ/δ)	Fatty acid metabolism in skeletal muscle	Desumoylation via SENP2/ desumoylation of PPARβ/δ, recruitment to the promoters of genes involved in fatty acid oxidation; fatty acid metabolism in C2C12 myotubes	Koo et al. (2015)
Peroxisome Proliferator-Activated Receptor gamma (PPARγ)	Adipogenesis, glucose homeostasis and inflammation in adipocytes; antagonism of inflammatory responses by transrepression of nuclear factor kappa B (NF-kappaB) target genes	Sumoylation by SUMO-1, SUMO-2 via PIAS1 and PIASxβ/ desumoylation via SENP2/Ligand binding-mediated decrease in sumoylation; SUMO-mediated repression of transcriptional activity; transrepression ofNF-kB signaling in inflammation; sumoylation-mediated prevention of clearance of the corepressor complex	Chung et al. (2011), Diezko and Suske (2013), Ghisletti et al. (2007), Jennewein et al. (2008), Ohshima et al. (2004), Pascual et al. (2005), Shimizu et al. (2006), Wadosky and Willis (2012) and Yamashita et al. (2004)

(continued)

Table 9.1 (continued)

Metabolic transcription factor	Affected metabolic pathways	SUMO connections/effect of sumoylation on activity and metabolism	References
Sterol Regulatory Element Binding Protein 1 and 2 (SREBP-1, SREBP-2)	Lipid biosynthesis and accumulation	Sumoylation via PIASy/ Inhibition of SREBP transcriptional activity indirectly through the recruitment of a co-repressor complex that includes histone deacetylase 3 (HDAC3); turning off of hepatic lipogenesis	Arito et al. (2008) and Lee et al. (2014a)
Krüppel-like transcription factors 4 and 5 (KLF4, KLF5)	Cell proliferation; cell cycle; apoptosis; migration, differentiation, and stemness; pluripotency; energy consumption	Sumoylation of KLF5/ sumoylation leads to nuclear localization and transcriptionally-repressive regulatory complexes with unliganded PPARβ/δ and co-repressors/desumoylation is a regulatory switch mediating the transcriptional activation of energy consumption	Du et al. (2008), Oishi et al. (2008) and Tahmasebi et al. (2013)
Promyelocytic Leukemia (PML)	Transcription; mRNA transport; DNA damage response; telomere maintenance; cellular stress response; apoptosis; stem cell maintenance and senescence	Sumoylation by SUMO-1, SUMO-2, SUMO-3/assembly of PML nuclear bodies (PML_NB); stabilization of p53	Bernardi and Pandolfi (2007), Borden (2002), de The et al. (2012), Fu et al. (2005), Ishov et al. (1999), Ivanschitz et al. (2015), Kamitani et al. (1998), Zhong et al. (2000)
Hypoxia-inducible factor 1 alpha (HIF-1α)	Adaptive response to hypoxia; enhanced glycolysis; overexpressed in solid tumors	Protein inhibitor of activated STAT3 (PIAS3)-mediated sumoylation/SENP1-mediated desumoylation/	Agbor et al. (2011), Ao et al. (2015), Bae et al. (2004), Bhattacharjee et al. (2016) and Chan et al. (2011), Gu et al. (2014), Kang et al. (2010), Nakagawa et al. (2016), Shao et al. (2004) and Tojo et al. (2002)
Retinoid X receptor alpha (RXRα)	Heterodimeric partner necessary for PPAR action	Sumoylation by SUMO-1/ desumoylation by SUSP1 (SENP6)/SUMO-mediated repression of activity	Choi et al. (2006)
Nuclear Bile Acid Receptor, Farnesoid X receptor (FXR)	Bile acid homeostasis; control of lipid and glucose levels; expression of target genes in response to bile acid signaling in enterohepatic tissues	Sumoylated by SUMO-1/ repression of activity; dysregulated acetyl/SUMO switch of FXR in obesity- related metabolic disorders	Balasubramaniyan et al. (2013), Kemper (2011) and Kim et al. (2015b)

(continued)

Table 9.1 (continued)

Metabolic transcription factor	Affected metabolic pathways	SUMO connections/effect of sumoylation on activity and metabolism	References
PPARγ coactivator 1 alpha (PGC1α)	Co-activator of PPARs, ERRα, FoxO1, HNF4α, NRF1; regulation of mitochondrial biogenesis and energy expenditure; adaptive thermogenesis; hepatic gluconeogenesis; mitochondrial biogenesis and respiration in muscle	Sumoylation by SUMO-1/ SUMO-mediated repression of activity; decreased mitochondrial biogenesis	Fernandez-Marcos and Auwerx (2011), Guerra de Souza et al. (2016) and Rytinki and Palvimo (2009)
Nuclear Factor kappa B (NFκB)	Inhibitor protein IkBα; multiple myeloma; innate immunity and energy metabolism; chronic inflammation in metabolic diseases	Desumoylation via SENP-1/	Chen et al. (2014) Huang et al. (2013), Kauppinen et al. (2013), Kim et al. (2011), Lee and Miyamoto (2011), Leidner et al. (2014) and Xu et al. (2015)
Oxysterol or Liver X Receptors alpha and beta (LXRα and LXRβ)	Cholesterol homeostasis; inhibition of inflammation;	Sumoylation by PIAS1 or HDAC4/anti-inflammatory transrepression in macrophages;suppression of pro-inflammatory TLR4-induced transcription by preventing the NCoR clearance step	Ghisletti et al. (2007), Huang et al. (2011), Lee et al. (2009a), Venteclef et al. (2010) and Zhang et al. (2012)
Glucocorticoid (GR)	Anti-inflammatory effects through tethered transrepression	Sumoylation/formation of (SUMOs)-NCoR1/SMRT-HDAC3 repressing complex	Druker et al. (2013), Holmstrom et al. (2008), Hua et al. (2016a, b) and Paakinaho et al. (2014)
Mineralocorticoid Receptor (MR)	Regulation the ions and water reabsorption in epithelial tissues (nephron, trachea colon,salivary glands); tissue-specific response in non-epithelial tissues	Sumoylation/negative regulation of synergy	Faresse (2014), Tallec et al. (2003), Tirard et al. (2007) and Yokota et al. (2007)
Estrogen-Related Receptors alpha and gamma (ERRα, ERRγ)	Metabolic reprogramming during TLR-induced inflammation; key metabolic regulator; mitochondrial biogenesis; glycolysis; lactate oxidation in cancer metabolism	Sumoylation/association with coregulators; repression of activity	Park et al. (2016), Tremblay et al. (2008) and Vu et al. (2007)

inhibiting the activity of other transcription factors via direct protein–protein interactions. This action of PPARs is referred to as transrepression. PPARα is sumoylated by SUMO-1 on lysine 185 in the hinge region by the activity of the E2-conjugating enzyme Ubc9 and the SUMO E3-ligase PIASy (Table 9.1). SUMO-1 modification of PPARα leads to the specific recruitment of corepressor NCoR, which results in a decrease of PPARα trans-activity and down-regulation of a subset of PPARα target genes (Kim et al. 2015c; Pourcet et al. 2010).

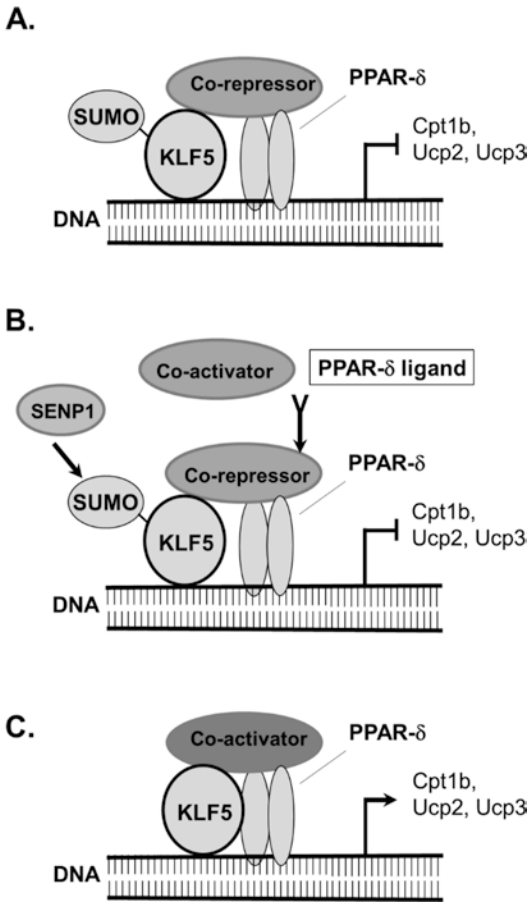

Fig. 9.2 (**a**) Under basal conditions, SUMO modified KLF5 is part of a co-repressor complex that contains unliganded PPAR-δ. The KLF5 co-repressor complex inhibits the transcription of the lipid oxidation gene Cpt1b and uncoupling protein genes Ucp2 and Ucp3. (**b**) Upon PPAR-δ ligand stimulation, KLF5 is desumoylated by SENP1 allowing for the exchange of co-repressors for co-activators. (**c**) Desumoylation of KLF induces the interaction of KLF5 and PPAR-δ allowing for the induction of Cpt1b, Ucp2, and Ucp3 transcription

9.5.2 SUMO in Insulin Synthesis and Secretion

The insulin signaling pathway plays an essential role in whole-body glucose homeostasis and in downstream metabolic pathways of energy metabolism. Nutrients and growth hormones promote the production of insulin and lead to the propagation of β-cells in the pancreas. Insulin is secreted from secretory granules within β-cells in response to hyperglycemia. However, the mechanisms whereby β-cells maintain near constant secretory granule stores remain to be fully understood (Mziaut et al. 2006; Trajkovski et al. 2004). Islet cell autoantigen 512 (ICA512) is a key enhancer of insulin gene expression and is an intrinsic granule membrane protein. ICA512 knockout mice have elevated blood glucose levels and impaired insulin secretion, whereas over expression of ICA512 enhances insulin secretion and levels of secretory granules within β-cells. ICA512 is a member of the receptor protein tyrosine phosphatase family but is catalytically inactive. Following granule exocytosis, the cytosolic domain of ICA512 is cleaved generating a soluble cytoplasmic fragment ICA512-CCF, which translocates to the nucleus and, in conjunction with phosphorylated signal transducer and activator of transcription 5 (STAT5), enhances the transcription of genes required for granule synthesis including insulin and ICA512 (Trajkovski et al. 2004). Upon sumoylation of ICA512, the interaction between ICA512 and STAT5 is weakened, suggesting that ICA512 sumoylation represses the activity of STAT5 and thus regulates insulin production and secretory granule formation (Mziaut et al. 2006).

In pancreatic β-cells, glucose stimulates insulin secretion by a process that involves cell depolarization followed by opening of voltage-dependent Ca^{2+} channels, which leads to insulin granule exocytosis. SUMO1 impairs glucose-stimulated insulin secretion by inhibiting the β-cell exocytotic response to Ca^{2+}. Sumoylation negatively regulates insulin secretion by β-cells by directly and reversibly inhibiting exocytosis in response to an increase in intracellular to Ca^{2+} (Manning Fox et al. 2012). The process involves SUMO1 attachment to synaptotagmin VII. The sentrin/SUMO-specific protease-1 (SENP1) is required to reverse SUMO-mediated inhibition of exocytosis and thus rescues glucose-dependent insulin secretion (Dai et al. 2011). NADPH reducing equivalents and reduced glutathione activate SENP1 function, thus increasing insulin secretion (Ferdaoussi et al. 2015). Conversely, overexpression of SENP1 reduces insulin secretion and impairs intracellular Ca^{2+} handling by inducing cell death

(Hajmrle et al. 2014). SUMO-1 has an opposite effect on exocytosis in pancreatic α-cells: it enhances exocytosis and glucagon secretion in cAMP-dependent manner (Dai et al. 2014). Recently, a metabolomics profiling of the insulinoma cell line 832/13 demonstrated a significant glucose-induced changes in purine pathway intermediates: a decrease in inosine monophosphate (IMP) and an increase in adenylosuccinate (S-AMP). SENP1 expression modified the effect of S-AMP on exocytosis in patch-clamp experiments in isolated human islet beta cells, suggesting a functional metabolic link between sumoylation, purine biosynthesis, and glucose-stimulated insulin secretion (GSIS) from human islet beta cells (Gooding et al. 2015). Taken together, these findings implicate SUMO pathway and the redox state of the cell in the regulation of glucose homeostasis. Whether SUMO plays a broader role in NADPH-dependent regulation of exocytosis in other cell types and whether NADPH levels generally play a role in the dynamic regulation of sumoylation and desumoylation in other cellular compartments remains to be determined.

9.5.3 SUMO in Glucose Transport and Metabolism

Glucose transporters (GLUTs) are a family of transmembrane glycoproteins that transport glucose across the plasma membrane (Chen et al. 2015; Giorgino et al. 2000; Thorens and Mueckler 2010). In insulin-responsive tissues including skeletal muscle and adipose, GLUT1 and GLUT4 are expressed. GLUT1 is responsible for basal glucose uptake whereas GLUT4 is responsible for insulin-responsive glucose uptake. The glucose transporters GLUT1 and GLUT4 were the first identified integral membrane proteins that are targets of sumoylation. Although the effects of sumoylation on these transporters are still not well understood, the interaction of Ubc9 with GLUT1 and GLUT4 may play a role in regulating glucose transport. Over expression of Ubc9 in L6 skeletal muscle cells decreased GLUT1 abundance by 65% while increasing GLUT4 abundance 8-fold. This effect is not explained by changes in mRNA levels, but was associated with increased protein stability (Giorgino et al. 2000; Liu et al. 2007). Similarly, Ubc9 over expression in adipocytes led to a decrease in GLUT4 degradation whereas inhibited Ubc9 expression by siRNA increased GLUT4 degradation. Over expression of the catalytically inactive Ubc9-C93A elicited the same effect on protein stability, suggesting that GLUT4 stabilization did not result from SUMO modification, but rather was the result of Ubc9 binding (Liu et al. 2007). GLUT4 localizes to storage vesicles in insulin responsive cell types, and over expression of Ubc9 also mediated the increased targeting of GLUT4 to these storage compartments, leading to an increase in insulin-responsive glucose transport capacity (Liu et al. 2007). Although no direct role of SUMO in glucose transport has been demonstrated, the SUMO conjugation pathway clearly affects glucose-stimulated insulin secretion, and perturbations of this pathway may contribute to metabolic disease.

Cancer cells shift their metabolism toward aerobic glycolysis at the expense of oxidative mitochondrial respiration, a phenomenon known as Warburg effect (Warburg 1925; Warburg et al. 1927). Desumoylation has been recently linked to these metabolic changes. Over-expression of SUMO-specific protease 2 (SENP2) reduced glucose uptake and lactate production while increasing the cellular levels of ATP in MCF7 cells. On the other hand, SENP2 knockout in MEF cells showed increased glucose uptake and lactate production along with the decreased ATP levels. Thus, desumoylation by SENP2 enzyme is implicated as a negative regulator of aerobic glycolysis in MCF7 and MEF cells, although mediator proteins that undergo SENP2-dependent desumoylation remain largely unknown (Tang et al. 2013). One of the glycolytic enzymes emerging as being regulated by sumoylation is pyruvate kinase PKM2. PKM2 is a master regulator of metabolism and has been shown to promote tumorigenesis by regulating the Warburg effect (Filipp 2013; Luo and Semenza 2012; Wong et al. 2015. PKM2 is sumoylated, probably by PIAS3, in human osteosarcoma U-2 OS cells and

the mouse NIH3T3 cell line (Spoden, 2009 #14710). PKM2 is differentially sumoylated in breast cancer metastasis (Subramonian et al. 2014). Taken together, these findings implicate sumoylation of glycolytic enzymes as an important contribution to SUMO-mediated metabolic regulation. Future studies will clarify the mechanistic details of the SUMO-mediated regulatory mechanisms of glucose metabolism and will unravel the impact of enzyme sumoylation on reprogramming metabolite fluxes through the glycolytic pathway in normal and transformed cells.

9.5.4 SUMO in Mitochondrial Biogenesis and Metabolic Disease

Mitochondria function, including oxidative capacity and rates of oxidative phosphorylation, are reduced in skeletal muscle during metabolic disease (Aon et al. 2014; Civitarese and Ravussin 2008). Type II diabetes, obesity and insulin resistance are associated with abnormal mitochondrial morphology, reduced function and an overall reduced number of mitochondria (Goodpaster 2013; Kelley et al. 2002; Koves et al. 2008). Declining cellular oxidative capacity leads to hepatic steatosis, which in turn increases hepatic glucose production and hyperglycemia (Anderson et al. 2009; Sonoda et al. 2007). Mitochondria are dynamic organelles that continuously undergo fission and fusion events in response to changes in cellular metabolism. Fusion of mitochondria has been suggested to be a mechanism by which intact mitochondria can recover the activities of damaged, depolarized mitochondria in order to maintain metabolic efficiency (Mishra and Chan 2014; Twig et al. 2008). On the other hand, mitochondrial biogenesis is stimulated in response to mitochondria damage, but also when ATP requirements are increased (Frazier et al. 2006; Toyama et al. 2016). Excessive mitochondrial fission may cause apoptosis (Bueler 2010). Therefore, regulation of fission and fusion events is essential to ensure an appropriate balance is achieved between the number and quality of mitochondria in response to cellular metabolic needs and/or to maintain metabolic and oxidative capacity.

SUMO is required for mitochondria fission events, and may regulate rates of mitochondrial biogenesis, as elevations in SUMO-1 expression were shown to increase mitochondrial fission events (Harder et al. 2004). Fission is controlled by the GTPase dynamin-related protein 1 (DRP1) and fission protein I (Fig. 9.1). These proteins are key components of the fission machinery in mitochondria and peroxisomes that function both during cellular homeostasis and apoptosis (Reddy et al. 2011; Wasiak et al. 2007). Fission is initiated by the recruitment of DRP1 from the cytoplasm to the mitochondrial outer membrane by FIS 1, an outer mitochondrial membrane associated protein, and a number of other recruitment factors (MFF, MiD49, and MiD51). The recruitment of DRP1 to the mitochondria is regulated by phosphorylation events in response to energy stress. One of the targets for phosphorylation is the mitochondrial fission factor (MFF), which is phosphorylated by the energy-sensing adenosine monophosphate (AMP)–activated protein kinase (AMPK) is response to electron transport chain inhibitors such as rotenone- and antimycin A. Phosphorylated MFF is better able to recruit DRP1 to mitochondria for fission initiation (Toyama et al. 2016). DRP1 mediates membrane scission, and its activity is also regulated by two posttranslational modifications: Sumoylation and phosphorylation. DRP1 is activated by CDK1/cyclinB-mediated phosphorylation at Ser 618 resulting in mitochondrial fragmentation (Taguchi et al. 2007), whereas its GTPase activity is inhibited by phosphorylation at Ser637 by cAMP-dependent kinase A, which induces mitochondrial fusion (Chang and Blackstone 2007; Suen et al. 2008). DRP1 is also covalently modified by sumoylation, and also has been shown to directly interact with SUMO-1. Fluorescence microscopy studies revealed that SUMO-1 was present at sites of fission between fragmented mitochondria, and co-localized with DRP1 (Harder et al. 2004).

DRP1 is recruited to the mitochondrial outer membrane from the cytosol by several receptors/

recruitment factors (FIS1, MFF, MiD49, and MiD51). DRP1 carries out fission of the outer membrane, and thus contributes to mitochondrial and peroxisomal division (Wai and Langer 2016). It self-assembles into a ring-like oligomeric structures wrapping around the future constriction points during fission, similarly to dynamin collars at the necks of budding vesicles during endocytosis (Smirnova et al. 2001). The mitochondrial division allows the cell to isolate defective mitochondrial segments, to degrade the damaged pieces, and to prevent damage accumulation through a process referred to as mitophagy (Yamano et al. 2016). Mutations in DRP1 cause encephalopathy, a lethal infantile neurodegenerative disease due to defective mitochondrial and peroxisomal fission (Chang et al. 2010; Waterham et al. 2007). DRP1 is a key regulator of mitochondrial fission and a direct target of sumoylation by all three SUMO isoforms. DRP1 is sumoylated by the mitochondrial-anchored SUMO E3 ligase MAPL (Braschi et al. 2009). DRP1 does not contain consensus sumoylation sequences, but instead harbors noncanonical conjugation sites present as clusters of lysine residues within the B domain (Figueroa-Romero et al. 2009). Increased SUMO-1 expression leads to increased sumoylated DRP1 protein and mitochondria' fragmentation; SUMO-1 may have a direct and protective effect on stabilizing DRP1 for mitochondrial fission (Harder et al. 2004). The activation of apoptosis triggers sumoylation of DRP1 by MAPL/ by MAPL/MUL1, which leads to stabilized ER/mitochondrial contact sites that serve as a platform for cytochrome c release (Prudent et al. 2015). The sumoylation of DRP1 can be reversed by at least three different desumoylating enzymes: SENP2, SENP3, and SENP5. Silencing of SENP5 results in accumulation of sumoylated DRP1 leading to increased mitochondrial fission. COS-7 cells with SENP5 RNAi knockdown exhibit increased generation of ROS, presumably due to the inhibition of fusion events that eliminate dysfunctional mitochondria. On the other hand, overexpression of SENP5 in COS-7 cell line rescues SUMO-1-induced mitochondrial fragmentation (Zunino et al. 2007). DRP1 is also desumoylated by SUMO isopeptidase SENP2. Regulated sumoylation/desumoylation of DRP1 protein is critical for mitochondrial morphogenesis and is particularly important for the survival of neural cell types. SENP2 regulates DRP1 desumoylation and stability, and mice with neural-specific disruption of SENP2 develop movement difficulties, which progress to paralysis with associated dysregulation of mitochondrial dynamics (Fu et al. 2014). Therefore, appropriate levels of SUMO-1 expression and DRP1 sumoylation are essential to achieve a balance between mitochondrial fission and fusion for maintenance of mitochondria quantity and quality. SENP3 is a SUMO-2/3-specific protease that is degraded during oxygen/glucose deprivation (OGD), an in vitro model of ischemia. DRP1 is one of the key targets for SENP3-mediated desumoylation. Depletion of SENP3 by RNAi knockdown lengthens the duration of DRP1 sumoylation, leading to the suppression of DRP1-mediated cytochrome c release and caspase-mediated cell death. Thus, knockdown of SENP3 protects cells from reoxygenation-induced cell death, in support of the notion that global increases in SUMO-2/3 conjugation are a neuroprotective response to severe stress (Guo et al. 2013). These studies highlight the importance of sumoylation and desumoylation in the regulation of mitochondrial metabolism.

9.6 SUMO and Folate-Mediated One-Carbon Metabolism

Folate is a B-vitamin that functions in the cell as a metabolic cofactor that carries and chemically activates single carbons as three different oxidation states for folate-mediated one-carbon metabolism (Fox and Stover 2009). One-carbon metabolism is a metabolic network comprised of three interconnected biosynthetic pathways: the *de novo* synthesis of purines, *de novo* thymidylate biosynthesis, and the remethylation of homocysteine to form methionine (Fox and Stover 2009). Methionine can be subsequently adenosylated to form S-adenosylmethionine which serves as a cofactor for numerous cellular methylation reactions in the cell, including chro-

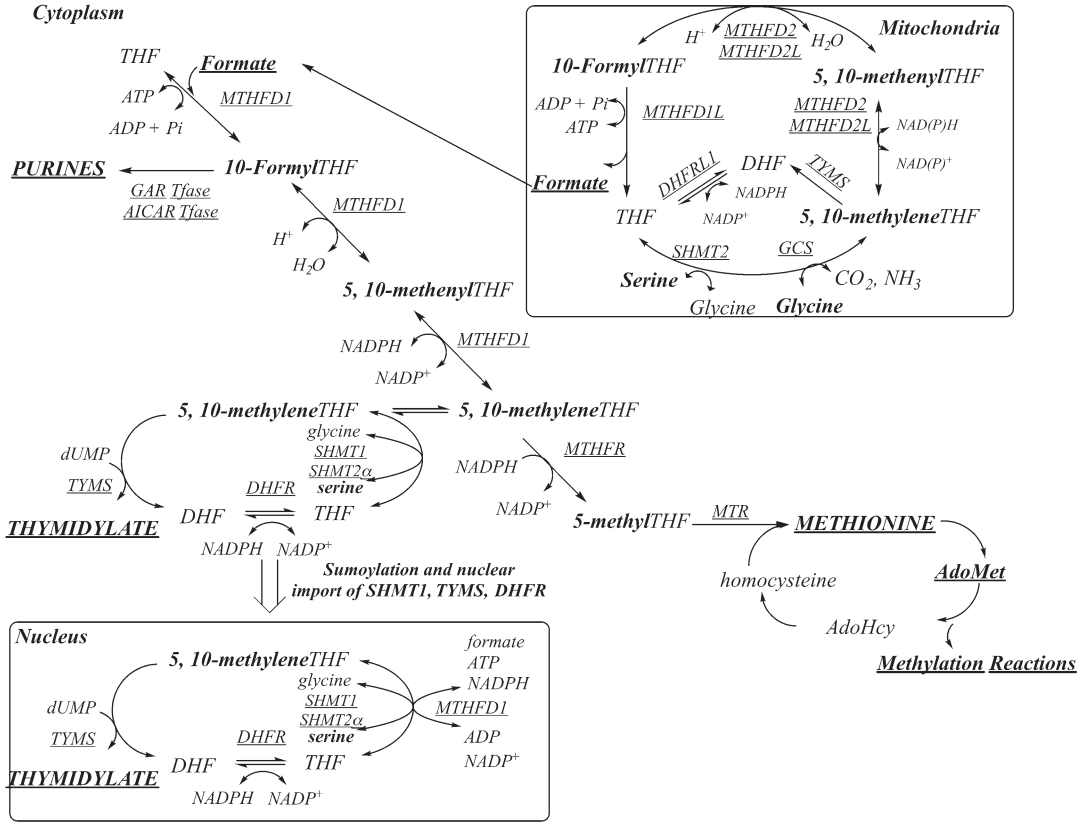

Fig. 9.3 Compartmentation of folate-mediated one-carbon metabolism in the cytoplasm, mitochondria, and nucleus. One-carbon metabolism in the cytoplasm is required for the de novo synthesis of purines and thymidylate, and for remethylation of homocysteine to methionine. One-carbon metabolism in the nucleus synthesizes dTMP from dUMP and serine. *AICARTfase*, aminoimidazole-4-carboxamide ribonucleotide transferase; *DHFR*, dihydrofolate reductase; *GARTfase*, 10-formyltetrahydrofolate:5′-phosphoribosylglycinamide N-formyltransferase; *MTHFD1*, Methylenetetrahydrofolate Dehydrogenase; *NADPH*, nicotinamide adenine dinucleotide phosphate; *SHMT1*, Cytoplasmic Serine Hydroxymethyltrasferase; *TYMS*, Thymidylate Synthase; *THF*, tetrahydrofolate

matin methylation (Fig. 9.3) (Fox and Stover 2009). Disruptions in folate metabolism are associated with numerous pathologies and developmental anomalies including cancer, cardiovascular disease, neural tube defects and cleft palate, although mechanisms have yet to be established (Stover 2004).

Folate mediated one-carbon metabolism is known to be compartmentalized in the cell; cellular folates are found in the cytoplasm, mitochondria and in the nucleus (Shin et al. 1976). Mitochondria generate one-carbons in the form of formate from the catabolism of the amino acids serine and glycine. Formate derived in mitochondria traverses to the cytoplasm to support one-carbon transfer reactions in that compartment (Fox and Stover 2009).

In the cell, the concentration of folate-activated one-carbons is limiting relative to the concentration of folate-dependent enzymes, indicating that folate-dependent biosynthetic pathways compete for a limiting pool of folate cofactors (Stover and Field 2011). This competition is most pronounced for the thymidylate and methionine biosynthesis pathways which compete for a limiting pool of methylenetetrahydrofolate (Herbig et al. 2002), which is generated by the reduction of 10-formyl-

tetrahydrofolate catalyzed by methylene-tetrahydrofolate dehydrogenase 1 (MTHFD1) (Fig 9.3) (Field et al. 2014, 2015, 2016).

Tissues including kidney, liver, brain, and colon express serine hydroxymethyltransferase 1 (SHMT1), which functions in the cytoplasm as an alternative source of one-carbon units for the synthesis of methylene-tetrahydrofolate. Isotope tracer studies demonstrate that SHMT1-derived methylenetetrahydrofolate is preferentially partitioned towards the thymidylate synthesis pathway at the expense of methionine biosynthesis (Herbig et al. 2002). The mechanism by which SHMT1 preferentially partitions one-carbons to thymidylate synthesis was shown to involve the SUMO-dependent translocation of the entire thymidylate biosynthesis pathway into the nucleus (Anderson et al. 2007; Woeller et al. 2007) and the assembly of the metabolic complex at the sites of DNA replication (Anderson et al. 2012a, b). The three enzymes in the de novo thymidylate pathway, cytoplasmic serine hydroxymethyltransferase (SHMT1), thymidylate synthase (TYMS), and dihydrofolate reductase (DHFR) are substrates for Ubc9 catalyzed sumoylation and subsequent translocation to the nucleus during the S and G2/M phases of the cell cycle (Anderson et al. 2007; Woeller et al. 2007). In the nucleus, SHMT1, SHMT1 is responsible for the generation of methylene-tetrahydrofolate by catalyzing the transfer of one-carbon units from serine to tetrahydrofolate. TYMS uses methylenetetrahydrofolate as the methyl donor for the conversion of deoxyutidylate to deoxythymidylate and dihydrofolate. DHFR catalyzes the reduction of dihydrofolate to tetrahydrofolate, thereby allowing the de novo thymidylate synthesis cycle to continue. The thymidylate biosynthesis is compartmentalized in the nucleus during DNA biosynthesis and repair, S and G2/M phases respectively. During G1, the thymidylate biosynthesis pathway enzymes localize exclusively to the cytoplasm. During the S phase, SHMT1 anchors the complex to nuclear lamina and DNA replication sites (Anderson et al. 2012b). TYMS was identified as SUMO-2-conjugated protein in four large-scale proteomics studies (Golebiowski et al. 2009; Hendriks et al. 2014, 2015; Tammsalu et al. 2014).

SHMT1 interacts with several nuclear and nuclear pore associated proteins including Ubc9, RanBP9 and PCNA. SHMT1. SHMT1 sumoylation is essential for its compartmentalization to the nucleus; K38R/K39R SHMT1 mutants are not substrates for Ubc9-mediated sumoylation, and these SHMT1 mutant proteins do not localize to the nucleus during S-phase (Woeller et al. 2007). Furthermore, a common SHMT1 variant, L474F, alters the SHMTI-Ubc9 binding interface. SHMTI L474F protein is not an effective substrate for Ubc9 sumoylation and is impaired in its nuclear localization. SHMT1 localization to the nucleus is dependent on the G-protein Ran; expression of a dominant negative RanT24N also impairs SHMT1 accumulation in the nucleus.

The SUMO-dependent compartmentation of thymidylate biosynthesis pathway in the nucleus accounts for the preferential partitioning of methylene-tetrahydrofolate towards *de novo* thymidylate biosynthesis. When DNA is damaged, several DNA repair mechanisms require dNTP synthesis (Mathews 2015). Exposure of cultured cells to ultraviolet radiation induces SHMT1 sumoylation and nuclear localization of the thymidylate biosynthesis pathway, and impairment in nuclear thymidylate synthesis sensitizes cells to UV-induced cell death (Fox et al. 2009; Fox and Stover 2009). *De novo* thymidylate synthesis is unique from other nucleotide biosynthetic pathways which are believed to occur in the cytoplasm, with the exception of synthesis during DNA damage (Mathews 2015). Nuclear thymidylate biosynthesis serves to limit the misincorporation of uracil into DNA and thereby promotes genome stability (Field et al. 2014, 2015, 2016; MacFarlane et al. 2008, 2011a, b).

While SHMT1 is involved in partitioning of folate cofactors towards nuclear thymidylate biosynthesis (Herbig et al. 2002; MacFarlane et al. 2008), SHMT2 is a mitochondrial isozyme of SHMTs that converts serine to glycine and serves as a major source of glycine in proliferating cells (Jain et al. 2012; Kim et al. 2015a). *Shmt2* gene encodes several alternatively spliced transcripts, one of which lacks exon 1 and expresses SHMT2α protein lacking mitochondrial targeting sequence

(Stover et al. 1997). Both SHMT1 and SHMT2α enzymes are cytosolic and translocate into the nucleus in S phase, and are functionally redundant in the nuclear thymidylate biosynthesis (Anderson and Stover 2009). A mouse model for folate-responsive neural tube defects has been recently developed through disruption of the *Shmt1* gene in the mouse genome (Beaudin et al. 2012; MacFarlane et al. 2008). Homozygous deletion of the *Shmt1* gene in mice results in viable and fertile animals that exhibit depressed rates of *de novo* thymidylate synthesis and elevated levels of uracil in nuclear DNA (MacFarlane et al. 2008). The viability of these mice is attributed to the functional redundancy with SHMT2α (Anderson and Stover 2009). When the dams are placed on a folate-deficient diet, $Shmt1^{+/-}$ and $Shmt1^{-/-}$ embryos exhibit sporadic exencephaly, a type of neural tube defects, independently of maternal genotype (Beaudin et al. 2011, 2012;). Interestingly, the rate of neural tube defects associated with embryonic *Shmt1* disruption can be increased by maternal uridine supplementation but rescued with maternal deoxyuridine supplementation, through stimulation of thymidylate synthesis (Martiniova et al. 2015). Moreover, the $Shmt1^{+/-}$ mice crossed to the $Apc^{Min/+}$ mouse model show increased susceptibility to intestinal tumors compared to wild-type mice (Macfarlane et al. 2011b). These studies connect impairments in folate- and SUMO-dependent nuclear thymidylate biosynthesis not only to the pathogenesis of folate-responsive neural tube defects but also to cancer pathophysiology. Additional roles of SUMO in folate-dependent nuclear thymidylate biosynthesis, outside its role in nuclear translocation, have yet to be established.

9.7 Conclusions

A growing body of publications over the past 10 years indicate that modification of proteins with SUMO plays an important role in maintaining metabolic homeostasis as well as in metabolic adaptation to stress. Several metabolic pathways, including but not limited to energy balance, lipid metabolism in inflammation, hepatic gluconeogenesis, glycolysis, mitochondrial biogenesis and respiration in muscle, chronic inflammation in metabolic diseases, and ions and water reabsorption in epithelial tissues have been reported to be regulated through controlled sumoylation of transcription factors. Changes in cellular sumoylation patterns have been observed under various stress conditions, such as torpor, hypoxia, glucose starvation, and oxidative stress. Sumoylation has emerged as important regulator of mitochondrial fission and fusion events, thus controlling energy homeostasis. SUMO pathway has been implicated in insulin production and secretion. Furthermore, SUMO plays an important role in mediating compartmentation of folate-metabolizing enzymes to the nucleus during DNA biosynthesis for thymidylate production at the sites of DNA replication. The scope of metabolic processes under the control of SUMO modification, from energy to glycolysis to nucleotide synthesis and beyond, suggests that SUMO plays an important role as a regulator and integrator of metabolic responses. Therefore, understanding the commonality in these diverse processes through future studies will allow for a unified model for SUMO control of metabolism.

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Part II

Cell Growth Regulation

The SUMO Pathway in Mitosis

10

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Abstract

Mitosis is the stage of the cell cycle during which replicated chromosomes must be precisely divided to allow the formation of two daughter cells possessing equal genetic material. Much of the careful spatial and temporal organization of mitosis is maintained through post-translational modifications, such as phosphorylation and ubiquitination, of key cellular proteins. Here, we will review evidence that sumoylation, conjugation to the SUMO family of small ubiquitin-like modifiers, also serves essential regulatory roles during mitosis. We will discuss the basic biology of sumoylation, how the SUMO pathway has been implicated in particular mitotic functions, including chromosome condensation, centromere/kinetochore organization and cytokinesis, and what cellular proteins may be the targets underlying these phenomena.

Keywords

Mitosis • SUMO • Condensin • Topoisomerase • Kinetochore

10.1 Introduction

Mitosis is the most visually dramatic stage of the cell cycle, wherein the replicated genetic material is segregated into two daughter cells. Although there are morphological variations between different cells types and between species, mitosis invariably involves chromatin condensation, bipolar spindle assembly, separation of sister chromatids and ultimately cytokinesis (Matsumoto and Yanagida 2005). All these steps are stringently regulated because any mistake will lead to aneuploidy and its consequences. Post-translational modifications like phosphorylation and ubiquitination have been well-documented as mechanisms of temporal and spatial control of mitosis (Devoy et al. 2005). Many lines of evidence suggest that sumoylation

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is an additional post-translational modification that is essential to proper progression of mitosis (Dasso 2008). Here, we will discuss how the sumoylation pathway has been implicated in particular mitotic functions, including chromosome condensation, centromere/kinetochore organization and cytokinesis.

10.2 The SUMO Pathway

SUMO proteins are a family of small ubiquitin-related modifiers that become covalently conjugated to cellular proteins in a reversible process called sumoylation. There is only one SUMO in fungi (called Smt3p in *S. cerevisiae*), while there are three widely expressed SUMOs in vertebrates (SUMO1–3). Mature SUMO2 and 3 are around 97% identical, while each is roughly 45% identical to SUMO1. It is clear that SUMO1 has different dynamics and a distinct profile of target proteins from SUMO2 and 3 (Ayaydin and Dasso 2004), and that some proteins with SUMO interacting motifs (SIMs) distinguish SUMO1 from the other paralogues (Hecker et al. 2006). No functional differences have yet been found between SUMO2 and SUMO3, and they will collectively be called SUMO2/3 in circumstances where they are indistinguishable.

The enzymes responsible for sumoylation and desumoylation have been the objects of intensive study during the past decade (Johnson 2004; Mukhopadhyay and Dasso 2007). All SUMOs are synthesized as pro-peptides that must undergo proteolytic maturation by SUMO-specific proteases, called SENPs (Sentrin-specific protease) in vertebrates and Ulps (Ubiquitin-like protein protease) in yeast; this cleavage exposes a C-terminal diglycine motif. The carboxyl terminus of mature SUMOs is activated by ATP-dependent thioester linkage with the Aos1/Uba2 hetero-dimer (E1 enzyme), then transferred to a conserved cysteine of Ubc9 (E2 enzyme) and ultimately to substrates with the help of SUMO ligases (E3 enzymes). SENP/Ulp proteases mediate deconjugation of SUMOs from their target proteins (Mukhopadhyay and Dasso 2007). There are two Ulp proteases in yeast, Ulp1 and Ulp2, and there are six SUMO-specific SENPs in humans, SENP1, 2, 3, 5, 6, 7. These enzymes display a considerable degree of specialization with respect to their enzymatic specificity and their localization (Mukhopadhyay and Dasso 2007).

Proteins that possess a variant RING-finger motif (SP-RING domain) are a conserved family of SUMO E3 enzymes found in all eukaryotes. Budding yeast SP-RING family members are called Siz (SAP and miz-finger domain) proteins, while the major vertebrate family members are called PIAS (protein inhibitor of activated STAT) proteins (reviewed in Palvimo 2007). Siz1p and Siz2p are responsible for the bulk of Smt3p conjugation in budding yeast, although other E3 enzymes of this class (Zip3p and Mms21p) are important for meiotic synaptonemal complex assembly (Cheng et al. 2006) and DNA repair (Potts and Yu 2005), respectively. The five vertebrate PIAS proteins (PIAS1, PIAS3, PIASxα, PIASxβ and PIASy) are important for a broad variety processes, including gene expression, genome maintenance and signal transduction. Mammals also express an Mms21p homologue, as well as two other SP-RING proteins (hZIMP7 and hZIMP10) that have been implicated in androgen receptor-dependent gene expression (Beliakoff and Sun 2006). Other vertebrate E3 enzymes, including the polycomb group protein Pc2 (Kagey et al. 2003) and the nucleoporin RanBP2 (Pichler et al. 2002), lack obvious homologues in yeast.

10.3 Outcomes of SUMO Modification

Sumoylation has been demonstrated to cause a variety of different outcomes, dependent upon the target protein, including changes in the target's sub-cellular localization, activity, protein-protein interactions, and stability (Fig. 10.1). Mechanistically, these changes can reflect the loss or acquisition of interaction surfaces upon conjugation of the SUMO group. An exciting recent development in this field has been the description of SUMO-interacting motifs (SIMs) in many proteins (Minty et al. 2000; Song et al.

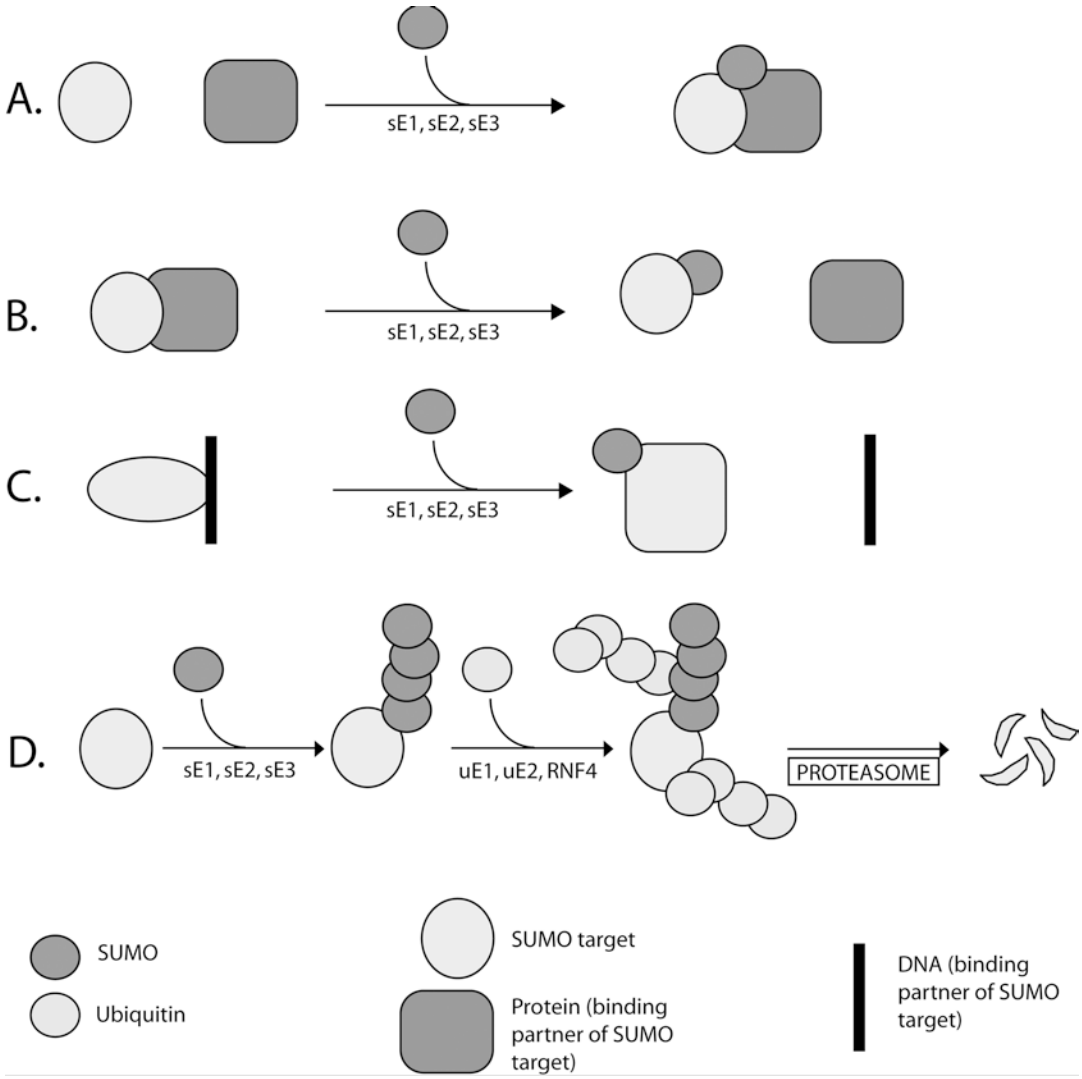

Fig. 10.1 Alternative outcomes of sumoylation. (**a**) SUMO modification can lead to higher order complex formation. (**b**) Sumoylation can disrupt protein-protein interaction. (**c**) SUMO modification can induce conformational alteration, leading to decreased DNA binding affinity. (**d**) PolySUMO chains can act as a signal for RNF4-mediated ubiquitination and subsequent degradation by the proteasome. SUMO pathway enzymes and ubiquitin pathway enzymes are indicated with "s" and "u" prefix, respectively

2005; Hannich et al. 2005; Hecker et al. 2006) SIMs are characterized by consensus sequence, V/I-V/I-X-V/I, with hydrophobic residues frequently punctuated at the third position (X) by an acidic amino acid. This core is frequently flanked by a stretch of acidic residues near its N- or C-terminus. SIMs allow low-affinity, non-covalent interactions between SIM-containing proteins and free or conjugated SUMOs. They may discriminate between different SUMOs, facilitating paralogue recognition and establishing functional distinctions between different SUMOs (Zhu et al. 2008; Hecker et al. 2006). SIMs are found in enzymes of the SUMO pathway as well as many SUMO target proteins. In some cases, SIM-based interactions allow targets to form higher-order protein complexes after sumoylation (Shen et al. 2006; Lin et al. 2006).

For other SUMO target proteins, SIM motifs help to recruit SUMO-linked Ubc9, thereby promoting conjugation in an E3-independent manner (Meulmeester et al. 2008; Zhu et al. 2008). Finally, if a sumoylated protein possesses a SIM, intramolecular binding of the SIM to the conjugated SUMO can cause a change in the conformation of the target, thus influencing its activity or behavior (Geiss-Friedlander and Melchior 2007; Baba et al. 2005).

Protein-protein interactions mediated by SIMs have recently been shown to contribute to target protein instability: A class of RING finger ubiquitin E3 ligases (RNF4 in vertebrates, Slx5p-Slx8p in budding yeast) possess multiple SIMs that they utilize to specifically recognize highly sumoylated proteins (Xie et al. 2007; Tatham et al. 2008; Lallemand-Breitenbach et al. 2008). These enzymes ubiquitinate the sumoylated proteins and target them for proteasomal degradation. Notably, specialized SUMO chain editing enzymes like Senp6 (Mukhopadhyay et al. 2006) and Ulp2p in yeast (Bylebyl et al. 2003) appear to antagonize this degradation pathway.

10.4 The Role of SUMO in Mitotic Chromosome Structure

At the onset of mitosis, decondensed interphase chromatin undergoes condensation. In vertebrates, this results in well-defined structures wherein the replicated sister chromatids can be clearly observed microscopically. Condensation facilitates chromatid separation and segregation during anaphase, preventing damage to the chromosomes. The molecular events involved in mitotic chromosome condensation are poorly understood (Belmont 2006). However, it is well established that condensins, cohesins and topoisomerase II are major structural components of condensed chromosomes, which are required for their assembly and maintenance (Fig. 10.2).

Condensins and Cohesin are large multiprotein complexes that play central roles in mitotic chromosome structure and in accurate chromosome segregation (Haering and Nasmyth 2003; Hirano 2005). Each of these complexes possesses two subunits that belong to the family of SMC (structural maintenance of chromosome) proteins. Common motifs within all SMC family members include an ATP binding domain and a DNA-binding globular domain, separated by a long anti-parallel coiled- coiled region. All SMCs exist as dimers: the Smc1p/Smc3p heterodimer is present in Cohesin, which maintains sister chromatid cohesion until the onset of anaphase. The Smc2p/Smc4p heterodimer are constituents of the Condensin I and II complexes, which are required for chromosome condensation and segregation. All SMC proteins have been demonstrated to be sumoylated (Takahashi et al. 2008).

In addition to Smc1p and Smc3p, yeast Cohesin complexes contain Scc3p and a "kleisin" subunit, called Scc1p (also known as Rad21p or Mcd1p) (Haering and Nasmyth 2003). Vertebrates

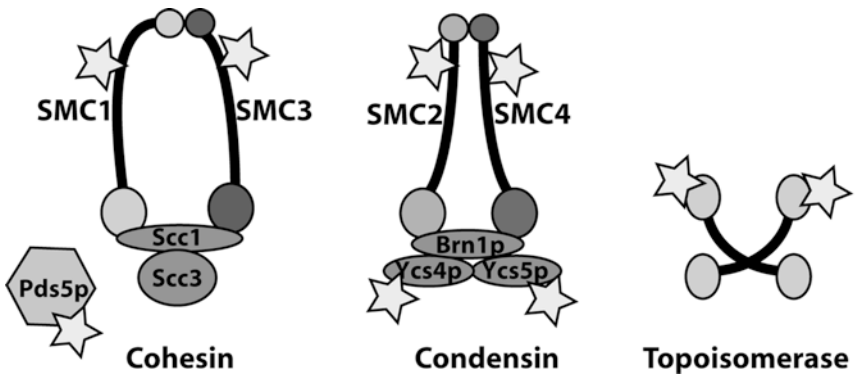

Fig. 10.2 Sumoylation targets involved in chromosome condensation and sister chromatid cohesion. *Stars* indicate subunits of each complex that have been experimentally confirmed as sumoylation targets

possess two mitotic orthologs of Scc3p, called SA1 and SA2, which are incorporated into Cohesin complexes in a mutually exclusive manner. Cohesins may act as clip, holding the sister chromatids until anaphase and thereby preventing their premature separation (Michaelis et al. 1997). Cohesin release from chromatin during mitosis is bi-phasic: during prophase, Cohesin is released from the chromosome arms in a manner that is dependent on the Polo-like kinase 1 (Plk1) and Aurora B kinases (Hauf et al. 2005). During this initial phase of Cohesin release from the arms, the centromeric cohesion is protected by Shugoshins (Wang and Dai 2005). Shugoshins are released in metaphase when sister chromosomes bi-orient and tension is established, (Lee et al. 2008). This allows the second phase of Cohesin release from centromeric regions at anaphase by cleavage of the Scc1p subunit by separase. The sumoylation of Smc1p and Smc3p in nocodazole-arrested cells depends on Mms21p, and correlates with proper distribution of cohesin within the ribosomal DNA (rDNA) (Takahashi et al. 2008). The functional significance of this distribution is not entirely clear.

Another way that sumoylation may regulate Cohesin is through Pds5p, a Cohesin-associated protein important for cohesion maintenance (Denison et al. 1993). Budding yeast Pds5p is sumoylated in a cell cycle dependent manner, peaking just before anaphase onset (Stead et al. 2003). Ulp2p over expression causes desumoylation of Pds5p and ameliorates the temperature sensitivity and cohesion defects of *pds5-ts* alleles. Conversely, Siz1p over expression causes Pds5p hypersumoylation and exacerbates temperature sensitivity in these strains. These results are consistent with a model wherein the strength of sister chromatid cohesion is enhanced by Pds5p-Cohesin interaction, while sumoylation disrupts this interaction to facilitate cohesion release.

The yeast Condensin complex contains Smc2p and Smc4p, as well as the kleisin Brn1p and non-SMC subunits Ycs4p and Ycs5p (Hirano 2006). Smc2p, Smc4p, Ycs4p and Ycs5p are all subject to mitotic sumoylation (Takahashi et al. 2008), and Brn1p has been identified as a potential sumoylation target in yeast proteomic screens (Denison et al. 2005; Wohlschlegel et al. 2004). Mms21p has been implicated as the E3 enzyme responsible for Smc2p modification, as well as a contributor to the sumoylation of Smc4p and Ycs4p (Takahashi et al. 2008). Regulation of Condensin by sumoylation was initially indicated by the findings that Ulp2p over expression suppresses conditional lethality of yeast with a temperature sensitive *smt2-6* allele (Strunnikov et al. 2001). Although yeast Condensin is constitutively associated with rDNA, GFP-tagged Condensin localizes to the inner kinetochore region during S phase, immediately after spindle pole body duplication (Bachellier-Bassi et al. 2008). This recruitment to kinetochores is disrupted in *ulp2* mutants, indicating that it is controlled by sumoylation. Accurate segregation of the rDNA and telomeres requires the protein phosphatase Cdc14, which is also required for the efficient sumoylation of Ycs4p during anaphase (D'Amours et al. 2004). These findings might suggest that Cdc14p promotes sumoylation of Condensin at anaphase, which in turn promotes its recruitment to rDNA. This model would predict that inactivation of Ulp2p and increased Condensin sumoylation should enhance Condensin concentration at the rDNA. Although one study has validated this prediction (Bachellier-Bassi et al. 2008) others have found the opposite effect of Ulp2p inactivation (D'Amours et al. 2004; Strunnikov et al. 2001). The precise role of sumoylation in Condensin targeting to rDNA thus remains unclear, and the process might be more complex than it has been proposed.

Topoisomerase II is an important sumoylation substrate in both mitotic yeast and vertebrate cells. Siz1p and Siz2p appear to be the primary ligases for yeast Topoisomerase II (Top2p) (Takahashi et al. 2006). The closely similar chromosome segregation defects observed in *siz1 siz2* double mutants and non-sumoylatable *top2* mutants suggests that Top2p is among the most important substrates for these enzymes. In ulp2 strains, centromeric cohesion is disrupted, but this defect can be suppressed by over expression of Top2p or by physiological levels of a *top2*

mutant lacking sumoylation sites (Bachant et al. 2002). These observations particularly indicate that elevated sumoylation of Top2p promotes precocious centromeric separation. Notably, a tagged Top2p-Smt3p fusion protein is preferentially concentrated in pericentromeric chromatin, as measured through chromatin immunoprecipitation assays (Takahashi et al. 2006), suggesting that sumoylation may contribute toward the loss of cohesion through increased Top2p recruitment to centromeres. Sumoylation is also involved in nucleolar localization of Top2p: insertion of multiple Smt3p repeats within the Top2p polypeptide near the natural site of sumoylation targets a GFP-Top2p fusion protein to a sub-nucleolar compartment (Takahashi and Strunnikov 2008). Optimal nucleolar targeting was observed in fusion proteins possessing four or five tandem Smt3p polypeptides. It is attractive to speculate that such targeting may contribute significantly toward the requirement for sumoylation in rDNA segregation (Strunnikov et al. 2001; Takahashi et al. 2008).

Vertebrate Topoisomerase II (Topo II) is a major sumoylated species associated with mitotic chromosomes (Azuma et al. 2003). This modification has been most intensely studied in Xenopus egg extracts (XEEs), where Topo II is conjugated preferentially to SUMO-2/3. Inhibition of sumoylation also causes a striking failure of sister chromatid separation during anaphase that closely resembles defects observed after treatment of XEEs with Topo II inhibitors (Azuma et al. 2003; Shamu and Murray 1992). Sumoylation does not appear to affect Topo II decatenation activity (Azuma et al. 2003). One interesting alternative possibility is that different levels of sumoylation might direct Topo II to different chromosomal loci, analogous to observations in yeast. Perhaps consistent with this idea, a small proportion of Topo II is tightly associated with mitotic chromatin in XEEs; inhibition of sumoylation causes dramatic increase of this population (Azuma et al. 2003), indicating that sumoylation promotes dynamic remodeling of Topo II on mitotic chromosomes.

Analogous to the conjugation of Top2p by Siz1p and Siz2p in yeast, PIASy is responsible for SUMO modification of Topo II in XEEs (Azuma et al. 2005). PIASy modifies Topo II in a chromatin-dependent manner (Azuma et al. 2005). Consistent with the findings in Xenopus, PIASy-depleted HeLa cells fail to properly localize Topo II to mitotic chromosome axes and to centromeres (Díaz-Martínez et al. 2006). PIASy-depleted cells also show delayed anaphase, caused by activation of an Aurora B- and Mad2-dependent checkpoint. Anaphase can be induced in PIASy-depleted HeLa cells through chemical inhibition of Aurora B, but they show a strong defect in sister chromatid disjunction, indicating a defect in cohesion release (Díaz-Martínez et al. 2006). Interestingly, these defects do not appear to result from abnormal retention of Cohesin. It has been reported that unconventional SUMO ligase RanBP2 is responsible for mitotic sumoylation of Topo II in mice (Dawlaty et al. 2008). While this observation potentially explains why PIASy is not required during mouse development (Wong et al. 2004), it is surprising that a pathway conserved between yeast, frogs and humans is not apparently utilized in mice.

10.5 SUMO and Centromere/Kinetochore Organization

Centromeres are specialized chromatin domains on each sister chromatid. In budding yeast, the cen DNA constitutes the cis element where centromeres form. In higher eukaryotes, centromeres are maintained epigenetically by a set of centromeric proteins that associates with highly repetitive centromeric satellite DNA and are typically present at the primary constriction of mitotic chromosomes. Centromeric nucleosomes contain a histone variant, CenpA (Cse4p in *S. cerevisiae*), which plays a central role in the recruitment and maintenance of other centromeric proteins (Cenps) (Cleveland et al. 2003). During every mitosis, centromeres serve as sites for assembly of kinetochores, proteinaceous structures that provide the sites of attachment for microtubules (MTs)) of the kinetochore fibers (k-fibers) that connect sister chromatids to spindle poles (Musacchio and Salmon 2007; Cheeseman and

Desai 2008). Correct attachment of k-fibers to opposite spindle poles is central to the accurate segregation of sister chromatids. Kinetochores also have a key role in the spindle assembly checkpoint (SAC), which prevents loss of sister chromatid cohesion and mitotic exit until all chromosomes are attached and aligned correctly on the metaphase plate. Proteins responsible for MT attachment and stability as well as for the SAC largely reside in the outer kinetochore and fibrous corona (FC), vertebrate kinetochore domains defined by electron microscopic imaging. The inner centromeric region (ICR) is the chromatin domain between the sister kinetochores that contains factors responsible for centromeric cohesion and chromosomal passenger proteins, which play essential roles in detecting and correcting mis-attachments of k-fibers (Andrews et al. 2004; Lan et al. 2004). Multiple functions of sumoylation have been proposed within mitotic centromeres and kinetochores, and recent reports have suggested that many proteins within these domains are sumoylation targets (Fig. 10.3).

There are over 60 proteins associated with the yeast centromere (McAinsh et al. 2003), many of which were found as sumoylation targets in proteomic screens (Denison et al. 2005; Wohlschlegel et al. 2004). Yeast centromeric components can be grouped into six major complexes (McAinsh et al. 2003), three of which contain confirmed sumoylation substrates:

1. The Cbf3 complex contains four subunits: Ndc10p/Cbf2p, Ctf13p, Cep3p and Skp1p. The sumoylation of Ndc10p and Cep3p have been demonstrated (Montpetit et al. 2006). This complex plays a critical role in kinetochore assembly because it is required for association of all other kinetochore components to the yeast centromere (McAinsh et al. 2003). Mutants that eliminate Ndc10p sumoylation cause mislocalization of Ndc10p from the mitotic spindle, abnormal anaphase spindles, and chromosome instability (Montpetit et al. 2006). In these mutants, Cep3p was mislocalized, indicating improper targeting of the Cbf3 complex in absence of Ndc10p sumoylation.

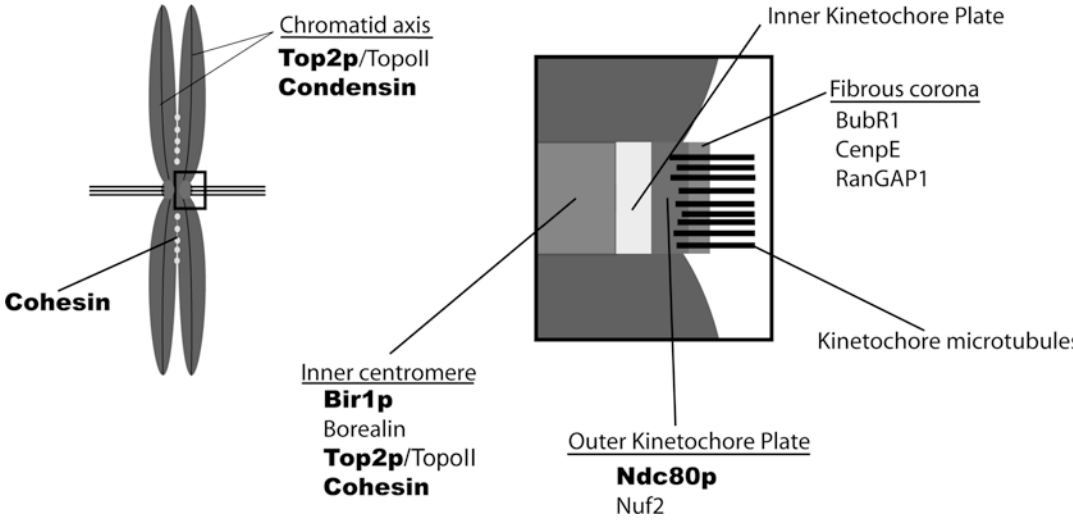

Fig. 10.3 Localization of chromosomal sumoylation targets. The distribution of confirmed targets is represented schematically, based upon previously reported localization of the vertebrate homologues. These reported localizations generally reflect the bulk of each vertebrate protein on mitotic chromosomes, not specifically the sumoylated forms. The names of yeast proteins are indicated in boldface type, while vertebrate proteins are in standard type.

2. The Bir1 complex contains Bir1p, Sli15p and Ipl1. The sumoylation of Bir1p has been confirmed *in vivo* (Montpetit et al. 2006). This complex can link CBF3 to MTs *in vitro*, and may sense tension to activate the Ipl1p kinase in the vicinity of syntelic k-fiber attachments (Sandall et al. 2006). The vertebrate counterpart of Bir1 complex is the chromosomal passenger complex (CPC): Survivin (Bir1), INCENP (Sli15) and Aurora B (Ipl1) in combination with Borealin constitute the CPC (Ruchaud et al. 2007). The CPC is an important mitotic regulator which has key roles in controlling sister cohesion, kMT attachment, and cytokinesis. Interestingly, *ndc10* sumoylation mutants altered Bir1p sumoylation, but did not affect its localization on the spindle (Montpetit et al. 2006). The absence of Cbf3p did not produce a comparable effect, suggesting that Ndc10p sumoylation has a Cbf3p-independent role in modulation of Bir1p sumoylation (Montpetit et al. 2006).
3. The Ndc80 complex contains Ndc80p, Nuf2p, Spc24p, and Spc25p. This complex plays a central role in kinetochore-MT attachment. It is also essential for SAC function and for the recruitment of many kinetochore proteins (McAinsh et al. 2003). The modification of Ndc80p has been confirmed *in vivo* (Montpetit et al. 2006). While the majority of Ndc80p sumoylation can be attributed to modification of a single lysine residue (K231), phenotypic consequences of mutations at this site have not been reported. Ndc80p sumoylation is distinguished from modification of Ndc10p, Bir1p, and Cep3p by the fact that it remains sumoylated after exposure of yeast cells to the microtubule poison nocodazole and activation of the SAC. Ndc10p, Bir1p, and Cep3p become desumoylated under these circumstances, suggesting that that they are regulated differently than Ndc80p.

In vertebrates, sumoylation has been demonstrated for ICR, outer kinetochore and fibrous corona proteins (Zhang et al. 2008; Klein et al. 2009). Chromatin assembled in XEEs shows pronounced accumulation of SUMO-2/3-conjugated species at the ICR in a PIASy-dependent manner (Azuma et al. 2005). Much of this signal may arise from Topo II sumoylation, but other ICR proteins are likely to be modified as well. Recently, Borealin was been demonstrated to be SUMO-2/3 conjugated in HeLa cells during early metaphase (Klein et al. 2009). Borealin interacts with RanBP2, which promotes its mitotic sumoylation. It can also interact with SENP3, and co-localizes with SENP3 in interphase nucleoli, leading to its desumoylation. The biological role of Borealin sumoylation is not clear as non-conjugatable Borealin mutants localize correctly to the ICR and causes no obvious mitotic defect.

CENP-C is an inner kinetochore protein that is the vertebrate homologue of budding yeast Mif2p (Meluh and Koshland 1995). It is important for outer kinetochore assembly, checkpoint signaling and proper chromosome segregation (Kwon et al. 2007). CENP-C is a substrate for sumoylation *in vitro* (Chung et al. 2004), and a number of genetic observations suggest that it may be regulated through this modification. DT40 chicken lymphoma cell lines were engineered to express mutant CENP-C cDNA constructs with changes in conserved amino acids (Fukagawa et al. 2001). One of these cell lines (ts4-11 cells) was temperature sensitive, displaying metaphase delay and chromosome missegregation under restrictive conditions, eventually arresting in the G(1) phase of the cell cycle. A HeLa cDNA library was screened for the capacity to rescue these defects, and SUMO-1 was identified as a suppressor. This relationship is reminiscent of the discovery of budding yeast SMT3 as a suppressor of the *mif2* phenotype (Meluh and Koshland 1995). There is currently no evidence that CENP-C becomes sumoylated *in vivo*, so it remains possible that suppression of the ts4-11 phenotype results from of sumoylation of a CENP-C interacting (Zhang et al. 2008).

CENP-E is a plus end-directed microtubule motor of the kinesin superfamily that localizes to the outer plate of the kinetochore and FC (Yen et al. 1991; Cooke et al. 1997). It is important for congression of chromosomes with single unattached kinetochores to the metaphase plate (Kapoor et al. 2006), for the maintenance of bipolar attachment of microtubules to kinetochores (McEwen et al. 2001), for generation of

tension across sister kinetochores (Kim et al. 2008), and for the SAC (Putkey et al. 2002). Suppression of mitotic sumoylation in HeLa cells by over expression of SENP2 leads to a chromosome segregation defect through disruption of CENP-E targeting to kinetochores (Zhang et al. 2008). Moreover, CENP-E itself is both a SUMO-2/3 target and poly-SUMO-2/3 binding protein. The latter activity is particularly critical, since mutation of SUMO-2/3 interacting motifs (SIM-2/3) blocked kinetochore recruitment of CENP-E. To identify conjugated species that may be recognized by the SIM-2/3 motifs of CENP-E, Zhang et al. (2008) examined the sumoylation of proteins that were previously implicated in targeting of CENP-E to kinetochores. Two of these proteins, Nuf2 and BubR1 displayed sumoylation when expressed as FLAG-tagged fusion proteins in HeLa cells. Nuf2 is the vertebrate homologue of Nuf2p in budding yeast (McAinsh et al. 2003), and it resides at the outer kinetochore in a conserved complex that also contains Hec1, the vertebrate homologue of Ndc80, as well as Spc24p and Spc25p homologues (Bharadwaj et al. 2004; Wei et al. 2005; Cheeseman and Desai 2008). As in yeast, this complex plays a pivotal role in the kinetochore-MT interface (Cheeseman and Desai 2008). BubR1 is an outer kinetochore kinase that has an essential role in the SAC (Musacchio and Salmon 2007). Notably, Nuf2 and BubR1 are both modified in a SUMO-2/3-specific fashion that is antagonized by SENP2 over expression. Together, these findings suggest that CENP-E binds kinetochore-associated species containing multiple SUMO-2/3 conjugates that may include BubR1 and Nuf2, and that this binding is essential for CENP-E localization and function.

RanGAP1 is the GTPase activating protein for the small GTPase Ran, which controls interphase nuclear transport and mitotic spindle assembly (Dasso 2006). RanGAP1 is an extremely efficient target for SUMO-1 conjugation (Mahajan et al. 1998; Matunis et al. 1998). Sumoylation promotes RanGAP1 assembly into a complex containing RanBP2 and Ubc9 (Saitoh et al. 1997, 1998), which is stable throughout the cell cycle (Joseph et al. 2004). During interphase, this complex is incorporated into nuclear pores, the primary conduits of nuclear-cytoplasmic trafficking (Mahajan et al. 1998; Matunis et al. 1998). During mitosis, it is targeted to the outer kinetochore or FC in a MT-dependent fashion (Joseph et al. 2002), where it performs an important role in k-fiber assembly (Arnaoutov et al. 2005). The binding of SUMO-1-conjugated RanGAP1 and Ubc9 occurs at the same domain of RanBP2 that has been shown to possess SUMO ligase activity, the IR domain (Pichler et al. 2002). Formation of this complex blocks IR activity *in vitro* (Reverter and Lima 2005), and it will be interesting to determine how its assembly may modulate RanBP2 ligase activity *in vivo*. While some species have developed sumoylation-independent mechanisms for targeting of RanGAP1 to spindles and kinetochores (Jeong et al. 2005), IR domain-containing proteins are only found in vertebrates (Dasso 2002), indicating that this mechanism of RanGAP1 localization is vertebrate specific.

10.6 SUMO and Cytokinesis

At the end of mitosis, daughter cells are physically separated by formation of a contractile ring composed of actin, myosin and septins (Glotzer 2005). Septins are the most prominent nonchromosomal mitotic sumoylation targets in budding yeast (Johnson and Blobel 1999). Septins exist as GTP binding hetero-oligomers, which forms polymeric filaments and are crucial for bridging microtubules to the contractile ring (Versele and Thorner 2005). There are five different types of yeast Septins: Cdc3, Cdc10, Cdc11, Cdc12 and Ssh1/Sep7. Among these, Cdc3, Cdc11 and Ssh1 become highly sumoylated during mitosis (Johnson and Blobel 1999). Septin modification is tightly controlled both temporally and spatially by the action of Siz1p and Ulp1p (Makhnevych et al. 2007). Septins form a collar at the bud neck of dividing yeast cells. At the onset of anaphase, sumoylation of Septins occurs abruptly and exclusively on the mother cell side septin collar (Johnson and Blobel 1999). At the beginning of cytokinesis, the septin collar splits laterally (Versele and Thorner 2005). Desumoylation of the Septin ring correlates with this splitting and the onset of cytokinesis (Johnson

and Blobel 1999). The asymmetry of Septin sumoylation may be related to Septin ring separation or to the polarized distribution of kinases and other cell cycle regulators involved in the budding yeast morphogenesis checkpoint (Keaton and Lew 2006). While sumoylation of Septins has been demonstrated in other fungi (Martin and Konopka 2004), it has not been reported in metazoans. On the other hand, *Drosophila* Septins can interact with components of the SUMO conjugation machinery *in vitro*, and *Drosophila* SUMO localizes to the midbody, suggesting that sumoylation of septins or other midzone proteins may occur during cytokinesis in Drosophila (Shih et al. 2002).

Eliminating sumoylation sites of Cdc3p, Cdc11p and Shs1p drastically decreased sumoylation at the bud neck in *S. cerevisiae*, and markedly lowered the overall level of sumoylation within G_2/M phase cells (Johnson and Blobel 1999). These triple mutants were defective in dismantling the septin ring of the mother cell bud neck, suggesting that sumoylation is important for Septin ring dynamics during the cell cycle. This conclusion is supported by the improper separation of Septin collars of cells arrested in mitosis in *ts-ubc9* strains (Johnson and Blobel 1999). Notably, the triple mutants show synthetic lethality with the *cdc12-1* temperature sensitive allele at normally permissive temperatures. Cdc10p and Cdc12p have been subsequently identified as sumoylation targets in proteomic screens (Panse et al. 2004; Denison et al. 2005), raising the possibility that low-level sumoylation of Cdc10p and Cdc12p may compensate for the absence of Cdc3p, Cdc11p and Shs1p sumoylation, and thus explain the absence of any overt cell cycle defects in the triple mutants cells.

10.7 Conclusions and Perspectives

It has been slightly more than a decade since the discovery of post-translational modification through the SUMO pathway (Mahajan et al. 1998; Matunis et al. 1998). During this time, there has been rapid progress in understanding both the enzymology of this pathway and its role in different cellular processes, including progression through mitosis. It is now clear that sumoylation is involved with many mitotic events, including remodeling of chromosome structure, kinetochore function, and cytokinesis. Much remains to be understood, however, regarding the mechanisms through which sumoylation facilitates these events.

Important aspects for future study will include:

1. Identification of new sumoylation substrates: while a growing number of substrates have been documented in both yeast and metazoans, it seems highly likely that many more remain to be discovered. This notion is supported by the large number of proteins identified through proteomic screens of sumoylated proteins in budding yeast (Panse et al. 2004; Wohlschlegel et al. 2004; Zhou et al. 2004; Denison et al. 2005; Hannich et al. 2005; Wykoff and O'Shea 2005) The identification and verification of individual sumoylation targets will be a major task for the foreseeable future, as will be the study of the physiological circumstances under which they are modified.
2. Determination of molecular consequences of sumoylation: the capacity of SIM-containing proteins to distinguish sumoylated species should allow discrimination based upon paralogues, as well as upon the extent of multi-sumoylation or SUMO chain assembly. We are only beginning to investigate how this capacity may be used. This topic will be particularly fascinating under circumstances where SIM-containing proteins may compete with each other to determine the fate of the conjugated target. For instance, the fact that CENP-E is targeted by multiple SUMO-2/3 conjugated proteins (Zhang et al. 2008), while these same proteins are substrates for both SENP6-mediated deconjugation (Mukhopadhyay et al. 2006) and for ubiquitin-mediate proteolysis (Tatham et al. 2008), may hit at extremely dynamic and sensitive mechanisms whereby sumoylation can control of kinetochore composition.
3. Coordination of different events through sumoylation: sumoylation is important for

many aspects of mitotic function. In this sense, it may be well-suited to coordinate different cellular events with each other, in a manner similar to previously described regulatory pathways that feature mitotic kinases of regulated proteasomal protein degradation. It will be important both to establish how sumoylation is coordinated between targets, and to understand the interplay between sumoylation and previously described regulatory pathways.

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Wrestling with Chromosomes: The Roles of SUMO During Meiosis

11

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Abstract

Meiosis is a specialized form of cell division required for the formation of haploid gametes and therefore is essential for successful sexual reproduction. Various steps are exquisitely coordinated to ensure accurate chromosome segregation during meiosis, thereby promoting the formation of haploid gametes from diploid cells. Recent studies are demonstrating that an important form of regulation during meiosis is exerted by the post-translational protein modification known as sumoylation. Here, we review and discuss the various critical steps of meiosis in which SUMO-mediated regulation has been implicated thus far. These include the maintenance of meiotic centromeric heterochromatin, meiotic DNA double-strand break repair and homologous recombination, centromeric coupling, and the assembly of a proteinaceous scaffold between homologous chromosomes known as the synaptonemal complex.

Keywords

Double-strand break repair • Homology sorting • Meiosis • SUMO • Synaptonemal complex

11.1 Introduction

Sexually reproducing organisms depend on the formation of haploid gametes (eggs and sperm) for successful propagation of their species. This requires a specialized cell division process known as meiosis through which chromosome number is reduced by half, generating haploid gametes that upon fertilization will reconstitute a diploid state. The precise reduction in chromosome number is accomplished by following a

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single round of DNA replication with two consecutive rounds of chromosome segregation (meiosis I and II). Homologous chromosomes segregate away from each other in the first (reductional) division, whereas sister chromatids segregate from each other in the second (equational) division. To accurately accomplish a reductional division, chromosomes undergo a series of well-orchestrated steps which are unique to meiosis I. These include homologous chromosome pairing, the formation of a "zipper-like" structure (the synaptonemal complex or SC) between aligned homologs, and the completion of meiotic recombination leading to physical attachments (chiasmata) between homologs. All of these events play a critical role in ensuring the proper alignment of homologous chromosomes at the metaphase I plate, and their subsequent orderly segregation to opposite ends of the spindle upon onset of meiosis I. Significantly, errors in any of these steps lead to chromosome nondisjunction and the formation of aneuploid gametes with tremendously deleterious consequences. Aneuploidy accounts for 30% of miscarriages in humans and is a contributing factor to infertility and birth defects such as Down syndrome (Hassold and Hunt 2001).

Given the importance of achieving accurate chromosome segregation during meiosis, it is not surprising that this is a tightly regulated process. This chapter highlights new findings implicating sumoylation as a key post-translational modification underlying the specificity of several important meiotic events ranging from the sorting of homology, to meiotic double-strand break (DSB) repair and SC morphogenesis.

11.2 Sumoylation

Sumoylation is a post-translational protein modification analogous to ubiquitination, where the SUMO (small ubiquitin-related modifier) protein is covalently linked to lysine residues present in a sumoylation consensus sequence on its target proteins. These target proteins include histones, transcription factors, DNA repair factors and proteins involved in multiple other cellular functions (reviewed in Gill 2004; Hay 2005; Johnson 2004). Similarly to ubiquitination, sumoylation proceeds via a stepwise transfer of SUMO to its substrate by an E1 activating enzyme, an E2 conjugating enzyme and sometimes an E3 ligase enzyme. In yeast, mature SUMO (cleaved from a precursor form by SUMO-specific proteases or SENPs), forms a thioester bond with the heterodimeric E1 enzyme, Aos1/Uba2, and is then transferred to the E2 conjugating enzyme Ubc9 (Table 11.1). Unlike the ubiquitination pathway, which requires an E3 ligase to proceed, SUMO-conjugated Ubc9 is competent to sumoylate targets *in vitro* (Bencsath et al. 2002). However, several SUMO E3 ligases have been identified that promote sumoylation *in vivo*, suggesting that the E3 ligases may be important for regulating sumoylation in the cellular context (Gill 2004). Unlike ubiquitination, which is frequently associated with proteasomal degradation of its targets, the biological function of sumoylation is less clear. Sumoylation has been linked to transcriptional repression and protein localization, and not surprisingly, appears to affect protein:protein interactions (Gill 2004; Hay 2005). Some insight into the biological roles of sumoylation can be gained from both the phenotypes of sumoylation-deficient model organisms and the recent influx of large-scale proteomic screens that have identified many SUMO-modified substrates. Taken together, both these types of studies indicate a conserved and important role for sumoylation during meiosis, as we will explore below.

11.2.1 Sumoylation in Meiosis: A Phenotypic Survey

Meiosis involves numerous and tightly coordinated chromosomal processes that must be temporally regulated. Therefore, it is not surprising that a link between a dynamic post-translation modification such as SUMO and meiotic processes has been observed from budding yeast to humans (Tables 11.1 and 11.2). In *Saccharomyces cerevisiae*, sumoylation intersects with at least two proteins required for SC formation. Zip1 is a

Table 11.1 SUMO pathway enzymes and meiotic phenotypes

Species	Sumo Pathway Component	Gene Name	Meiotic Expression and/or *Relevant Phenotype(s)*
S. cerevisiae	SUMO	*SMT3*	
	E1	*AOS1*	
		UBA2	
	E2	*UBC9*	localizes to SC; *perturbed replication fork repair*
	E3	*SIZ1*	*siz1siz2 mutant has mild sporulation defect*
		SIZ2	
		MMS21	*perturbed replication fork repair*
		ZIP3	SC component; *inefficient SC formation; reduced and delayed crossovers*
	De-conjugating	*ULP1*	*synthetic lethal with recombination protein Srs2*
		ULP2/SMT4	increased upon sporulation; *cell cycle arrest at meiotic prophase*
S. pombe	SUMO	*pmt3*	
	E1	*fub2*	
	E2	*hus5*	*aberrant asci; reduced spore viability*
	E3	*pli1*	*aberrant asci; reduced spore viability; reduced crossovers*
		nse1	
	De-conjugating	*ulp1*	
D. melanogaster	SUMO	*smt3*	embryonic germline
	E1	*Aos1*	embryonic germline
		Uba2	embryonic germline
	E2	*lesswright*	embryonic germline; *suppresses mild chromosomal non-disjunction*
	E3	*tonally*	down-regulated in female germline post-mating
		Su(var)2–10	oogenesis
	De-conjugating	*Ulp1*	
C. elegans	SUMO	*smo-1*	high expression in somatic gonad; *sterile, abnormal germline; genetic interaction with zhp-3*
	E1	*aos-1*	
		uba-2	
	E2	*ubc-9*	
	E3	*zhp-3*	SC localization; marker of crossover events; *sterile, high incidence of male progeny (suggesting chromosomal non-disjunction)*
		gei-17	*genetic interaction with mus-101 (required for DNA replication and DNA damage response)*
	De-conjugating	*ulp-1*	*sterile progeny*
		ulp-2	*reduced brood size, sterile, sterile progeny*
Mouse/Human	SUMO	*SUMO-1*	SC and constitutive heterochromatin during spermatogenesis
		SUMO-2/3	constitutive heterochromatin during spermatogenesis
		SUMO-4	
	E1	*SAE1*	
		SAE2	
	E2	*UBE2I*	
	E3	*PIAS* family	PIASx/PIAS1 upregulated during spermatogenesis
		RanBP2	
		Pc2	
		HDAC4	
	De-conjugating	*SENP* family	

Information on the sumoylation pathway members and their expression (normal text) and reported meiotic phenotypes (*italicized*) is gathered from the following online resources, reviews and primary sources. For *S. cerevisiae*: the Saccharomyces Genome Database (SGD), (Cheng et al. 2007; de Carvalho and Colaiácovo 2006; Soustelle et al. 2004; Agarwal and Roeder 2000); for *S. pombe*: (Watts et al. 2007); for *D. melanogaster*: (Talamillo et al. 2008); for *C. elegans*: Wormbase, (Bhalla et al. 2008; Holway et al. 2005; Jones et al. 2002); and for mammalian: (Brown et al. 2008; Yan et al. 2003)

Table 11.2 Budding yeast sumoylated proteins and their roles in meiosis

Name	Known or predicted role in meiosis
DSBR	
Ecm11	Crossover recombination
Mlh3	DNA mismatch repair and meiotic crossover recombination
Rad52	DSB repair during vegetative growth and meiosis
Sgs1	Prevents aberrant crossing over during meiosis
Srs2	Required for proper timing of commitment to meiotic recombination and the transition from Meiosis I to Meiosis II
Top2	Localizes to axial cores in meiosis; meiotic crossover recombination
Structural and chromosome segregation	
Ndc1	Required for nuclear pore complex assembly and spindle pole body duplication; required for chromosome segregation in Meiosis II
Red1	SC axial element component; involved in chromosome segregation during Meiosis I
Slk19	Kinetochore-associated protein required for normal segregation of chromosomes in meiosis and mitosis
Smc4	Structural Maintenance of Chromosomes (SMC) condensin protein
Smc5	SMC condensin protein
Transcriptional	
Sth1	Required for expression of early meiotic genes
Ume1	Negative regulator of meiosis; represses meiotic gene expression during mitotic growth

The proteins are subdivided into functional categories of DSBR (Double-strand break repair), Structural and Chromosome Segregation, and Transcriptional based on published literature. Identification of sumoylation and description of meiotic roles are consolidated from the Saccharomyces Genome Database (SGD) and the following primary sources: (Branzei et al. 2006; Cheng et al. 2006; Denison et al. 2005; Hannich et al. 2005; Panse et al. 2004; Sacher et al. 2006; Zavec et al. 2008)

structural component of the SC which may recognize SUMO-conjugated proteins on the chromosomal axes, and Zip3 is a SUMO E3 ligase which appears to regulate Zip1 polymerization (Cheng et al. 2006). Conversely, mutations in the *S. cerevisiae* SUMO deconjugating enzyme *ulp2/smt4* lead to arrest in meiotic prophase (Li and Hochstrasser 2000), further linking control of sumoylation to meiosis. In *Schizosaccharomyces pombe*, mutation of the SUMO E3 ligase *pli1* leads to reduced spore viability and aberrant asci, a phenotype resulting from defective meiotic recombination (Watts et al. 2007). Mutations in *lesswright*, the *Drosophila* homolog of the E2 enzyme Ubc9, were found to suppress a mild meiotic nondisjunction phenotype, implicating sumoylation in the regulation of accurate meiotic chromosome segregation (Apionishev et al. 2001). Meanwhile, the *C. elegans* genome contains a single SUMO homolog, *smo-1*, and *smo-1* mutants display a pleiotropic phenotype including highly aberrant germlines (Broday et al. 2004). A partially rescued *zhp-3* (the Zip3 ortholog) mutation appears to phenocopy *smo-1* mutations (Bhalla et al. 2008), suggesting a potential conservation of the SUMO and SC connection first reported in budding yeast. In rodents and humans, SUMO shows a stage-specific and chromosomal-specific localization during spermatogenesis (Brown et al. 2008; Metzler-Guillemain et al. 2008; Rogers et al. 2004; Vigodner et al. 2006; Vigodner and Morris 2005), as we describe further below. Moreover, infertile men show a decrease in SUMO in the Sertoli cells, implicating sumoylation in human infertility (Vigodner et al. 2006).

11.2.2 Targets of Sumoylation in Meiosis

Large-scale proteomic studies to identify sumoylated targets have been predominantly done in *S. cerevisiae* thus far. These studies have identified several sumoylated proteins with roles in meiosis, underscoring the breadth of regulatory control exerted by this mode of post-translational modification during this cell division program (Table 11.2). These sumoylated targets can be separated into several groups, including DNA repair proteins and proteins involved in the structural organization of chromosomes during meiosis.

The function of sumoylation has been studied further for at least two proteins with roles in meiotic DSB repair in *S. cerevisiae*. First, the homologous recombination protein Rad52 has been reported to be sumoylated upon an accumulation of meiotic DSBs, in a manner that interferes with its proteasomal degradation and results in its stabilization (Sacher et al. 2006). Second, the budding yeast protein Ecm11, with important functions in DNA replication and meiotic crossover recombination, is sumoylated during meiosis and not mitosis. Mutation of the Ecm11 sumoylation site phenocopies the sporulation defect of the *ecm11* mutant, suggesting sumoylation is required for the meiotic function of this protein (Zavec et al. 2008). Taken together, these studies thus far implicate sumoylation as essential for promoting the stability and function of at least two proteins with known roles in meiotic DSB repair.

Another set of important processes during meiosis involve the assembly and disassembly of the SC, as well as the subsequent chromosomal segregation events that depend on proper SC formation earlier in prophase. Several key players in these processes are sumoylated: one notable example being the axial element component Red1 (Cheng et al. 2006). This sumoylation appears to serve as a recognition site for the SC component Zip1, and has therefore been suggested to play a role in SC assembly (Cheng et al. 2006). Several proteins involved later in chromosome segregation, such as the integral membrane protein Ndc1 and the Separase-binding protein Slk19, are sumoylated (Table 11.2). Although it is currently unknown how sumoylation affects their function, these proteins are involved in the regulation of proper chromosome redistribution and therefore suggest at least a potential role for sumoylation in this process.

Taken together, the analysis of mutant phenotypes and sumoylated substrates hints at interesting functions for sumoylation in meiosis and further highlights the importance of studies in various organisms to determine its degree of conservation.

11.3 Centromeric Heterochromatin and Sumoylation

Centromeric function is important for both mitosis and meiosis, and sumoylation seems to play a particularly important role in the establishment and/or maintenance of heterochromatin at the centromere from yeast to mammals. Both Smt3 (the *S. cerevisiae* SUMO-1 homolog) and Smt4 (a SUMO de-conjugating enzyme) were originally identified as suppressors of mutations in the centromere binding protein Mif2/Cenp-C (Meluh and Koshland 1995), supporting an important functional connection between sumoylation and centromeres. In *S. pombe*, deletion of *pli1*, which encodes for a SUMO E3 ligase, results in a mild dysfunction of the kinetochore and/or centromere (Xhemalce et al. 2004). Moreover, de-silencing of a reporter gene located in the centromeric region in *pli1* mutants suggests a defect in heterochromatin maintenance in this region (Xhemalce et al. 2004). In *Drosophila*, SUMO is seen localizing to heterochromatic sites (Lehembre et al. 2000), and in *S. pombe*, sumoylation has more recently been shown to play a role in heterochromatin maintenance at the centromere and other heterochromatic regions of the genome (Shin et al. 2005).

Heterochromatin can either be transiently induced ("facultative") or be permanent ("constitutive"), and sumoylation has been implicated in both types of heterochromatin. One classic example of meiotic facultative heterochromatin that has been linked with sumoylation is the sex body or XY body, formed by the mammalian sex chromosomes during pachytene spermatogenesis (Rogers et al. 2004; Vigodner et al. 2006; Vigodner and Morris 2005). However, a recent study suggests that at least in humans, the observation of XY body sumoylation (which would be considered facultative heterochromatin) may actually be the result of a large region of constitutive heterochromatin on the Y chromosome (Metzler-Guillemain et al. 2008) as opposed to an XY body-specific process. This is in agree-

ment with the observation of other large SUMO-1 signals on chromosomes 1, 9 and 16 (Brown et al. 2008; Metzler-Guillemain et al. 2008), which also contain large regions of constitutive heterochromatin. These data, along with the frequent observation of SUMO-1 at mammalian centromeres (known sites of constitutive heterochromatin) during meiosis (Brown et al. 2008; La Salle et al. 2008; Metzler-Guillemain et al. 2008; Vigodner et al. 2006), suggests that sumoylation may in fact be more specific to constitutive heterochromatin. However, numerous studies link sumoylation to transcriptional repression (reviewed in Gill 2004), suggesting that sumoylation may contribute to facultative heterochromatin in non-meiotic situations. Further studies are therefore required to determine the extent to which sumoylation plays a role, if any, in facultative heterochromatin during meiosis.

11.4 Centromeric Coupling

The establishment of stable pairing between homologous chromosomes is a critical step for successful meiosis I progression. Before homologous chromosomes can synapse and progress through meiosis, they must first search for homology and pair, and recent studies in yeast have uncovered an important link between centromeric sumoylation and these early pairing events. Pairing and synapsis of homologous chromosomes during meiosis in *S. cerevisiae* relies on both recombination-dependent and -independent mechanisms. Once homologous chromosomes are paired, several proteins, including Zip1, Zip2 and Zip3, form the Synapsis Initiation Complex (SIC) at sites called Axial Associations (AA) where the chromosomes are in close contact (Rockmill et al. 1995). Synapsis (polymerization of the SC between paired and aligned homologous chromosomes) is believed to then proceed from these sites. In many organisms, synapsis is dependent upon DSB formation and subsequent recombination (reviewed in Page and Hawley 2004). This and other lines of evidence have suggested that the SICs form at the sites of crossover recombination (reviewed in Henderson and Keeney 2005).

However, several recent studies have implicated sumoylation in a recombination-independent form of early chromosomal pairing termed "centromeric coupling" (Cheng et al. 2006; Hooker and Roeder 2006; Tsubouchi et al. 2008; Tsubouchi and Roeder 2005) (Fig. 11.1). In a *spo11* mutant that lacks DSB formation and fails to synapse, the SC component Zip1 and the SUMO E3 ligase Zip3 do not polymerize along chromosomes, but instead, form foci at (or near) the centromeres (Tsubouchi et al. 2008; Tsubouchi and Roeder 2005). Moreover, the number of observable centromere-associated foci is approximately half that of the number of chromosomes, suggesting that even in the absence of recombination and synapsis, the chromosomes are pairing at or near the centromeres (Tsubouchi and Roeder 2005). This "centromeric coupling" occurs even in the absence of bouquet formation, a process of telomere clustering that is important for efficient homolog pairing (Trelles-Sticken et al. 2000). Interestingly, the earliest centromeric coupling is not between homologous chromosomes, although over time the proportion of paired homologs increases (Tsubouchi and Roeder 2005). In a *spo11 zip1* double mutant the number of centromere foci double, indicating that Zip1 is required for centromeric coupling (Tsubouchi and Roeder 2005). In a wild-type background, AAs can be found at the centromeres, and Zip1 and Zip3 linear staining appears to initiate from the centromeres, further supporting a model where SC formation during early stages of meiosis initiates from the sites of centromeric coupling (Tsubouchi et al. 2008; Tsubouchi and Roeder 2005).

Taken together, the authors propose a model in which homologous pairing is modulated not just by the previously-studied bouquet formation and recombination, but also by Zip1-dependent centromeric coupling. Thus, centromeric coupling and/or bouquet formation may serve to sequentially match together different chromosomes until homology is determined (Tsubouchi and Roeder 2005). They propose that Spo11 then initiates recombination via the production of DSBs,

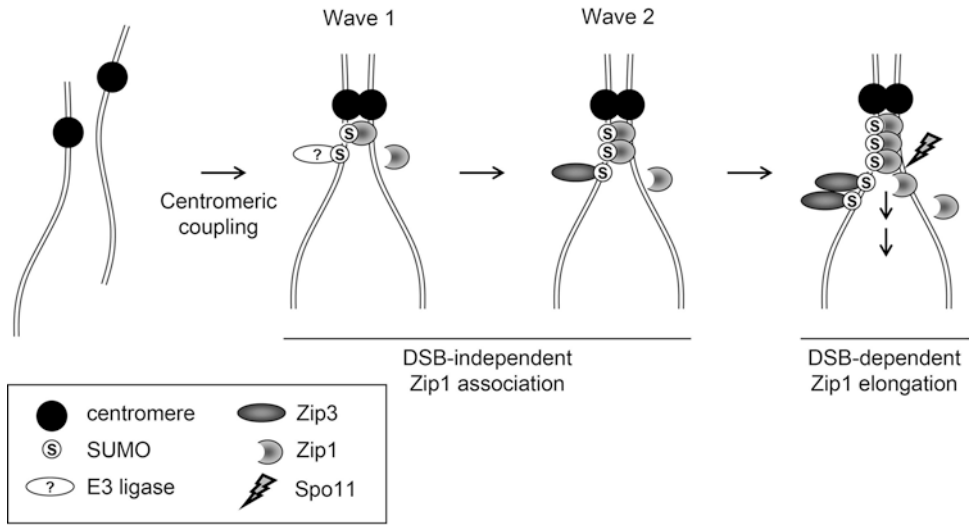

Fig. 11.1 *A model for the roles of sumoylation in meiotic chromosome dynamics: Centromeric coupling and SC assembly.* In budding yeast, Zip1, a structural component of the synaptonemal complex, is required for centromeric coupling early in meiotic prophase I. Once homologous chromosomes are coupled, synapsis ensues. Two distinct waves of sumoylation are believed to participate in these processes. **Wave 1** involves centromeric (or pericentromeric) sumoylation and the recognition of SUMO-conjugated products at the centromeres by Zip1 in a Zip3-independent fashion. Thus, centromeric sumoylation may be the result of the activity of an as yet unidentified E3 ligase. **Wave 2** involves the Zip3 SUMO E3 ligase and results in the formation of short Zip1 stretches. Initiation of synapsis is not DSB–dependent, however, DSB formation via Spo11 function is required for the Zip3-dependent Zip1 elongation resulting in a fully-formed SC. Therefore, it appears that sumoylation is important both in the early stages of chromosome pairing/homology sorting and later on in the assembly of the mature SC

further linking the homologs together and promoting SC formation (Tsubouchi and Roeder 2005). Therefore, the SUMO-mediated centromeric coupling observed in yeast, along with the observations by immunofluorescence studies that centromeric regions are sumoylated during meiosis in mouse, rat and human (Brown et al. 2008; Metzler-Guillemain et al. 2008; Vigodner et al. 2006; Vigodner and Morris 2005), suggest that sumoylation may play a conserved role in centromere function as it relates to early chromosome pairing in meiosis.

11.5 SUMO-Mediated Regulation of SC Dynamics

After homologous chromosomes find and pair with one another, they undergo synapsis via assembly of the SC. The establishment of this proteinaceous scaffold is crucial for the stabilization of homologous pairing interactions and the completion of crossover recombination (Page and Hawley 2004). Interestingly, despite the ubiquitous presence of the SC from yeast to humans, and its fundamental importance for reproductive biology, the regulation of the assembly and disassembly of this macromolecular structure remains poorly understood. However, recent studies in several model systems are linking sumoylation with the regulation of SC morphogenesis.

11.5.1 ZIP1 and ZIP3: A SUMO Connection

Analysis of human testes samples has shown that SCP1 and SCP2, structural components of the SC, are sumoylated, and that SUMO-1 localizes to the SC (Brown et al. 2008). Although the co-localization observed in mammals is still controversial (Metzler-Guillemain et al. 2008), recent studies observed a co-localization of the yeast

SUMO homolog Smt3 to the SC in budding yeast (Cheng et al. 2006; Hooker and Roeder 2006). The SIC components Zip1 and Zip3, and the topoisomerase-like enzyme Spo11, involved in generating programmed meiotic DSB breaks, are required for this localization (Cheng et al. 2006; Hooker and Roeder 2006), suggesting that these proteins are involved in SC sumoylation in yeast. In synapsis-defective mutants, both Smt3 and Zip1 co-localize to non-SC aggregates termed polycomplexes (reviewed in de Carvalho and Colaiácovo 2006; Zickler and Kleckner 1999), further supporting their SC-related interaction.

Earlier in prophase, Smt3 is present at the Zip1 foci implicated in centromeric coupling (Cheng et al. 2006; Hooker and Roeder 2006; Tsubouchi and Roeder 2005). In both wild type and *zip3* mutants, these early Zip1 foci disappear by mid-prophase, but in a *zip3* mutant background, an additional mutation of the Smt3 deconjugating enzyme, *ulp2*, leads to prolonged maintenance of these foci on chromosomes (Cheng et al. 2006), suggesting sumoylation may support their stability. In addition, Hooker and Roeder 2006 find that mutations in the yeast SUMO E2 conjugating enzyme, Ubc9, lead to delays in synapsis, further supporting the importance of sumoylation for proper synapsis (Hooker and Roeder 2006).

Zip3 acts as a Smt3 E3 ligase *in vitro* leading Cheng et al. (2006) to conclude that Zip3-dependent sumoylation is necessary for proper SC formation (Cheng et al. 2006). They also describe Zip1 as a Smt3-conjugate binding protein, with both Zip3-independent (to Smt3-Top2 during early prophase) and Zip3-dependent (to Smt3-Red1 during mid-to-late prophase) interactions, implicating sumoylation in both centromeric coupling and subsequent SC formation (Cheng et al. 2006) (Fig. 11.1).

These findings suggest that at least two "waves" of sumoylation may be involved in the association of Zip1 onto chromosomes during meiosis in budding yeast (Fig. 11.1). The first wave results in Zip1 localization to centromeric and pericentromeric regions in early meiotic prophase, thereby promoting centromeric coupling and early synapsis, and involves sumoylation mediated by an as of yet unidentified SUMO E3 ligase. The second wave results in the extensive polymerization of Zip1 along the full length of chromosomes, thereby promoting completion of SC assembly, and is Zip3-dependent (Cheng et al. 2006; Tsubouchi et al. 2008; reviewed in de Carvalho and Colaiácovo 2006). Furthermore, taken together these studies suggest that the Zip1 foci implicated in centromeric coupling could also be sites of synapsis initiation (Fig. 11.1).

Interestingly, studies of ZHP-3 (the Zip3 homolog) function during meiosis in the nematode *C. elegans* reveal it is required for crossover recombination in a SC-dependent manner (Jantsch et al. 2004). However, in contrast to yeast, SC assembly is not impaired in either *zhp-3* or *smo-1* (the SUMO homolog) mutants (Bhalla et al. 2008; Jantsch et al. 2004). Instead, comparisons between a *zhp-3::gfp* integrated transgene which partially complements a *zhp-3* null mutant, *smo-1* and *smo-1; zhp-3::gfp* double mutants revealed that ZHP-3 coordinates recombination with SC disassembly and bivalent differentiation (Bhalla et al. 2008). Therefore, both Zip3 and ZHP-3 may function to coordinate crossover recombination with SC morphogenesis. However, in *S. cerevisiae*, where DSB-formation is critical to promote synapsis, Zip3 coordinates crossover formation with SC assembly (Agarwal and Roeder 2000). Meanwhile, in *C. elegans*, where synapsis is DSB-independent (Dernburg et al. 1998), ZHP-3 coordinates crossover formation with SC disassembly and bivalent formation. The role of SUMO in these processes during *C. elegans* meiosis remains to be further examined and its potential role in the formation of functional bivalents (stably attached through chiasmata) needs to be investigated across species. Taken together, these studies further highlight the importance of identifying additional meiotic SUMO targets and pursuing the analysis of their roles in SC assembly and disassembly to understand the crucial regulation of SC dynamics.

11.6 Meiotic DSB Repair/ Recombination

Proper control of DNA double-strand break repair (DSBR) is essential for promoting interhomolog recombination resulting in crossovers and subsequent accurate chromosome segregation. Several proteins with roles in meiotic DSBR are known to be sumoylated, therefore implicating this post-translational modification in the critical regulation of this meiotic process (Table 11.2). Many of the proteins involved in DSBR are highly conserved across species (reviewed in Villeneuve and Hillers 2001), however, their roles in meiotic DSBR have been more extensively investigated in yeast, and therefore, we will primarily focus on the roles of the yeast proteins with links to sumoylation.

In yeast and metazoans, the endonuclease Spo11 creates the DSBs during the early stages of prophase I (Villeneuve and Hillers 2001). The Mre11/Rad50/Xrs2 complex then resects the 5′ ends of the DSBs thereby creating 3′ overhangs, where the ssDNA binding factor RPA binds, allowing Rad51 and Rad52 to participate in the homology search and strand invasion that allows homologous recombination to proceed. The Srs2 helicase opposes this activity by disrupting Rad51 binding and serves an important function in preventing inappropriate recombination events from proceeding (Veaute et al. 2003). The RecQ helicase homolog Sgs1 also acts to prevent inappropriate crossovers, although its mechanism is less well understood (Rockmill et al. 2003). The MutL homologs, Mlh1 and Mlh3, act downstream to promote crossover formation (Hoffmann and Borts 2004). Finally, topoisomerases such as Top2 are proposed to "untangle" recombined chromosomes upon completion of DSBR, thereby allowing for efficient segregation (Hartsuiker et al. 1998).

Several of these proteins are known to be sumoylated: specifically, Rad52, Sgs1, Srs2, Mlh3 and Top2 (Table 11.2). In *S. cerevisiae*, Rad52 is sumoylated on at least two sites upon induction of DSBs (Sacher et al. 2006). While Rad52 mutants that lack the sumoylation sites are still able to complete meiotic DSBR, the sumoylation does appear to stabilize Rad52 and promote its activity (Sacher et al. 2006). More recent studies uncovered a remarkable link between Rad52 sumoylation and relocalization of damage sites to "damage foci" for repair, where repair of ribosomal DNA sites requires Rad52 sumoylation for formation of Mre11 and Rad52-containing extranucleolar foci (Torres-Rosell et al. 2007). Additional studies have implicated the SUMO E3 ligase Slx5/8 in the relocalization of damaged DNA to nuclear pore complexes (Nagai et al. 2008), suggesting that sumoylation plays a role in relocalizing damaged DNA to sites of repair after experimentally-induced damage and perhaps during endogenous meiotic DSBR as well.

The anti-recombinogenic helicases Sgs1 and Srs2 are both known to be sumoylated (Table 11.2), and Srs2 is also known to interact specifically with sumoylated PCNA earlier in pre-meiotic S phase in order to prevent inappropriate recombination at stalled replication forks (Pfander et al. 2005). The *in vivo* functions of Sgs1 sumoylation are not yet known, however sumoylation of the mammalian Sgs1 homolog BLM is required for DNA damage-induced foci (Eladad et al. 2005). Formation of these foci involves relocalization of sumoylated BLM (Eladad et al. 2005), further supporting a general role for sumoylation in subnuclear relocalization during DSBR. However, these studies have yet to be repeated in the context of meiosis, so future studies are critical to see whether SUMO does in fact play a role in meiotic DSBR-induced relocalization.

Another sumoylated protein that plays an important role in meiosis is the topoisomerase Top2. During mitosis, Top2 is known to be sumoylated, and mutation of the Top2 sumoylation sites contributes to mitotic chromosomal missegregation (Bachant et al. 2002; Takahashi et al. 2006). During meiosis, immunofluorescence analysis shows colocalization of Top2 and the yeast SUMO homolog Smt3 (Cheng et al. 2006), suggesting Top2 is sumoylated during meiosis as well. Furthermore, sumoylated Top2 (localized near the centromeres) is believed to interact with the sumo-binding SC component

Zip1 (Cheng et al. 2006), suggesting that sumoylated Top2 may act both early and late in meiosis with functions in SC assembly and chromosome segregation.

Finally, *C. elegans* ZTF-8, a functional analog of mammalian RHINO, which plays roles in both DSBR and DNA damage-induced apoptosis, is a direct target for sumoylation at its consensus CKxE sites *in vivo* (Cotta-Ramusino et al. 2011; Kim and Colaiácovo 2014; Kim and Colaiácovo 2015). Non-sumoylatable transgenic worms mimic the phenotypes observed in the null mutants such as reduced fertility, impaired DNA damage repair, and mislocalization of the 9–1-1 complex component HUS-1, suggesting that sumoylation is indispensable for DSBR and DNA damage-mediated checkpoint activation in the germline. However, while mutants for components acting in the sumoylation pathway fail to properly localize ZTF-8, its localization is not altered in the ZTF-8 non-sumoylatable mutants. These observations suggest that while direct sumoylation of ZTF-8 is required for its roles in DSBR and DNA damage response, it is not required for its localization. Instead, another factor may be a target for sumoylation, and it in turn may be required for proper localization of ZTF-8.

11.7 Conclusions

Sumoylation has been implicated in various ways for several essential events of meiosis, including homologous pairing, synapsis, and DSBR leading to crossover events. The importance of sumoylation in meiosis is highlighted by the meiotic phenotypes of sumoylation pathway mutants across species (Table 11.1). Furthermore, many proteins with known important roles in meiotic processes are known to be sumoylated (Table 11.2), although further studies are needed to determine the precise role or function for the sumoylation undergone by some of these proteins. In yeast, sumoylation is involved in both centromeric coupling and the subsequent polymerization of the SC (Fig. 11.1), and at least one report of SC sumoylation in human spermatocytes suggests a general conservation of this role (Brown et al. 2008). In contrast, in the nematode *C. elegans*, sumoylation is apparently not required for SC assembly and instead is important for proper SC disassembly (Bhalla et al. 2008). Further work is therefore needed in mammalian and other model systems to determine whether the role of SUMO in centromeric coupling and SC morphogenesis is in fact conserved across species.

The role of sumoylation in meiotic DSBR is supported by mutant phenotypes and the identification of sumoylated DSBR proteins (Tables 11.1 and 11.2), but even more intriguing is the potential connection of sumoylation with DNA damage-induced re-localization and repair (Eladad et al. 2005; Nagai et al. 2008; Torres-Rosell et al. 2007; Kim and Colaiácovo 2014, 2015). Sumoylation has long been implicated in intracellular re-localization (reviewed in Gill 2004), and future studies may specifically implicate this re-localization in meiotic DSBR, potentially uncovering entirely novel mechanisms of DSBR regulation in meiosis. Taken together, the studies reviewed here hint at many possible avenues for research, and future studies will undoubtedly strengthen the connections between sumoylation and meiotic processes.

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Sumoylation in Development and Differentiation

12

Adeline F. Deyrieux and Van G. Wilson

Abstract

Tissue morphogenesis is a fascinating aspect of both developmental biology and regeneration of certain adult organs, and timely control of cellular differentiation is a key to these processes. During development, events interrupting cellular differentiation and leading to organ failure are embryonic lethal; likewise, perturbation of differentiation in regenerating tissues leads to dysfunction and disease. At the molecular level, cellular differentiation is orchestrated by a well-coordinated cascade of transcription factors (TFs) and chromatin remodeling complexes that drive gene expression. Altering the localization, stability, or activity of these regulatory elements can affect the sequential organization of the gene expression program and result in failed or abnormal tissue development. An accumulating body of evidence shows that the sumoylation system is a critical modulator of these regulatory cascades. For example, inhibition of the sumoylation system during embryogenesis causes lethality and/or severe abnormalities from invertebrates to mammals. Mechanistically, it is now known that many of the TFs and components of chromatin remodeling complexes that are critical for development and differentiation are targets for SUMO modification, though the specific functional consequences of the modifications remain uncharacterized in many cases. This chapter will address several of the models systems that have been examined for the role of sumoylation in differentiation and development. Understanding the profound regulatory role of SUMO in different tissues should lead not only to a better understanding of developmental biology, stem cell linage control,

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and the mechanisms of cellular differentiation, but may also lead to the identification of new targets for drug therapy and/or therapeutic manipulation of damaged organs and tissues.

Keywords
Ubc9 • SENP • Keratinocytes • Gonads • Germ cells • Hematopoietic cells • Neural cells • Stem cells

12.1 Introduction

Embryonic development and post-embryonic differentiation are complex processes that depend on exquisitely coordinated networks of gene expression. Orchestrating this network relies on diverse regulatory mechanisms that control the expression, localization, and activity of the pertinent transcription factors (TFs), co-regulators, and chromatin modifying complexes that collectively determine global patterns of transcription. Among these mechanisms is post-translational modification of the TFs and their co-factors. Over the last 20 years, sumoylation has emerged as a significant functional modifier of TFs, their co-activators and co-repressors, and components of the chromatin remodeling machinery (see Chaps. 2, 3, and 5). This broad target range is consistent with reports that sumoylation has a global impact protein networks, at least under certain conditions (Heaton et al. 2012; Xiao et al. 2015; Hendriks et al. 2014, 2015). There is also growing evidence that sumoylation can provide fine tuning to these networks by altering and coordinating activities of network components, and several example pertaining to development and differentiation are presented in the following sections, as well as in more detail in Chaps. 14, 15, and 19.

Initially, the importance of sumoylation in development and differentiation was underscored by studies using knockouts or knockdowns of the sumoylation system in various model organisms. Many studies have focused on Ubc9 as loss of this sole SUMO conjugating enzyme totally abrogates sumoylation. In *C. elegans*, RNAi knockdown of Ubc9 results in severe developmental defects and embryonic arrest after gastrulation (Jones et al. 2001). Likewise, deletion of smo-1, the sole SUMO encoding gene in the *elegans* genome, results in sterile adults with severe defects in the reproductive system (Broday et al. 2004). Interestingly, overexpression of SUMO also perturbed the reproductive system, suggesting that precise levels of sumoylation are critical for normal development (Rytinki et al. 2011). Developmental defects are also observed for Ubc9 mutation in zebrafish (Nowak and Hammerschmidt 2006). In *Drosophila*, dysregulation of sumoylation by targeting Ubc9 (Huang et al. 2005), SUMO (Nie et al. 2009; Kanakousaki and Gibson 2012), the E1 activating enzyme (Kanakousaki and Gibson 2012), or an E3 SUMO ligase (Betz et al. 2001) all yielded developmental defects [reviewed in (Smith et al. 2012)]. Both SUMO (Yukita et al. 2007) and SUMO proteases (Wang et al. 2009) have been shown to be essential for normal *Xenopus* development. Many of these sumoylation effects are even more pronounced during mouse development where loss of Ubc9 function leads to apoptosis and early embryonic lethality (Nacerddine et al. 2005). Embryonic lethality in mice was also seen for PIAS1 knockouts (Constanzo et al. 2016), SENP1 mutants (Sharma et al. 2013), and SENP2 knockouts (Kang et al. 2010). This critical role for sumoylation in development is not confined to animals, and is likewise observed for plants (see Chap. 14). Thus, the combined literature supports a critical role for sumoylation in development, though the individual targets and mechanisms appear to vary highly between species. The following sections will discuss the role of sumoylation in several diverse systems.

12.2 The Reproductive System

As cited in the previous section, reproductive tract defects were one of the first developmental abnormalities associated with defective sumoylation. Subsequent publications have reported abundant expression of SUMO and SUMO proteins in male and female germ cells of several species. High concentrations of sumoylation components were detected in testis and sperm cells in worms, mice, rats, and humans, emphasizing a conserved mechanism across species in the development of the male reproductive organs (Broday et al. 2004; Kim et al. 2000; Vigodner et al. 2006; Vigodner and Morris 2005; Brown et al. 2008; Santti et al. 2003; La Salle et al. 2008; Metzler-Guillemain et al. 2008; Yan et al. 2003). Likewise, SUMO proteins has also been reported in female germ cell oocytes (Li et al. 2006), and sumoylation appears to be critical for oocyte maturation (Wang et al. 2010; Yuan et al. 2014), though the contribution of sumoylation to the male reproductive system remains better characterized (Vigodner 2011). Together these observations underscore the importance of sumoylation for sex organ development and gamete maturation is a wide variety of species as described in more detail below.

12.2.1 Vuval Morphogenesis

To investigate the role of SUMO (SMO-1) in *C. elegans* development, a deletion mutant was constructed and analyzed (Broday et al. 2004). While earlier *smo-1* RNAi studies showed 100% embryonic lethality (Fraser et al. 2000; Jones et al. 2002), the *smo-1* −/− mutants survived, but were sterile. This mutation is associated with physiological disturbance of vulval uterine connection in *C. elegans* and also with somatic gonad and germ line abnormal differentiation. These results resembled the phenotype seen with mutations in LIN-11, a transcription factor which demonstrates important regulatory properties for vulval precursor cell division as well as uterine morphogenesis (Newman et al. 1999). Broday et al. showed that LIN-11 can be SUMO modified at lysines 17 and 18, and that the double mutant form of LIN-11 could partially rescue vulva formation in a LIN mutant background, but was still significantly impaired for uterine seam cell (utse) formation. They additionally showed that expression of a SUMO-LIN11 fusion in the *smo-1* mutant *C. elegans* background rescues π-cell differentiation, but accentuates impairment of late stages in vulval development. These combined results support the conclusion that sumoylation is critical for normal vulval development and that LIN-11 is an important sumoylation target in this system. Subsequent work showed that sumoylation of LIN-1 promoted transcriptional repression and interaction with MEP-1, a component of the NuRD transcriptional repressive complex which may be an important pathway in controlling vulval development (Leight et al. 2005). Nevertheless, it is likely that LIN-11 is not the only factor whose sumoylation state plays an essential role for gonadal uterine-vulval morphogenesis in worms. For example, recent work showed that the nuclear hormone receptor, NHR-25, is sumoylated and that this modification is critical for normal vulval formation (Ward et al. 2013). While these results underscore the impact of sumoylation on the formation of the *elegans* reproductive system, similar observations in vertebrates are lacking. In contrast, a large literature supports a role for sumoylation in vertebrate gametogenesis.

12.2.2 Sperm Differentiation

Spermatogenesis is a process of the reproductive system by which male germ cells enter into meiosis, divide, and differentiate into mature spermatozoa. During meiosis homologous chromosome pairs, including the heterochromosome XY, are distributed equally to the daughter cells. The timing, sub-cellular recruitment, and assembly of chromatin remodeling proteins are crucial for proper synapsis and chromosomal recombination during spermatogenesis. Initial reports indicated the presence of SUMO1 in mouse, rat, and human

spermatids (Rogers et al. 2004; Vigodner and Morris 2005; Vigodner et al. 2006), suggesting an active role for the sumoylation process.

Two groups described the dynamic expression pattern of SUMO during SC formation in the mouse and human models, respectively. La Salle et al. (La Salle et al. 2008) examined the sumoylation genes and proteins during prophase meiosis I in male mouse germ cells. They compared localization and expression levels of SUMO1 versus SUMO2/3 by immunolabeling on surface spread chromatin and RT-PCR, respectively. SUMO1 clearly localized to the XY body and the chromocenter of pachytene spermatocytes, as did SUMO2/3. However, as prophase progressed, SUMO1 and SUMO2/3 presence decreased from both the XY body and the chromocenter. Interestingly, while the presence of SUMO1 was completely absent in metaphase I, SUMO2/3 could still be detected in centromeres, suggesting a functional distinction for these 2 SUMO types. To further characterize the implication of the sumoylation in the division and development of male sperms, they determined that the only known SUMO conjugation enzyme (UBE2I) showed overlapping localization with SUMO1 and SUMO2/3 in prophase and with SUMO2/3 in metaphase. This strongly suggests that conjugation of proteins by SUMO is taking place at these locations, and thus infers that sumoylation plays a functional role in sperm maturation.

To further characterize the dynamics of this system, La Salle et al. looked at the relative transcription levels of 19 sumoylation system genes using quantitative RT-PCR. *Sumo1* transcript levels peaked in early prophase (Zygotene) and then quickly decreased as prophase progressed. *Sumo2/3*, *Sae1/2*, and *Ube2i* showed a similar pattern though with a peak expression at the adult pachytene spermatocyte stage and decreased expression in mature spermatids. The SUMO proteases showed a more varied pattern of expression with *Senp1*, *2*, and *6* having patterns similar to *Sae1/2* and *Ube2i*, while *Senp5* was similar to *Sumo1*. *Senp3* and *7* were distinct in that they had highest expression at the leptotene/zygotene stage followed by declining expression with subsequent sperm maturation, a pattern shared by the *Pias3* SUMO ligase. In contrast to *Pias3*, the other ligases (*Pias1*, *2*, and *4*) had low expression at the leptotene/zygotene stage with a dramatic increase in expression in spermatids. Unlike the changing expression patterns of the sumoylation system genes, expression of *Senp8*, a NEDD8 protease, was relatively constant throughout spermatogenesis, suggesting that changes in the sumoylation gene expressions is biologically significant. Overall, two main patterns emerged from RNA and protein expression studies: high expression during meiosis follow by low expression post-meiosis for SUMO1/2/3, SAE1/2, UBE2i, and SENP1/2/5/6; low expression during meiosis and high expression post-meiosis for PIAS 1, 2, and 4. These differential expression patterns clearly indicate a dynamic process during spermatogenesis and are consistent with a requirement for the sumoylation during this process. It is not yet clear if specific proteins modified by SUMO and it enzymes regulate chromosome dynamics during meiosis in male germ cells, although the co-localization and common expression patterns of SUMO and its enzymes does suggest that protein modification takes place on the chromatin. Consistent with this possibility, studies in yeast indicate that sumoylation of TOP2 is most important for proper chromosome segregation in mitosis, suggesting that sumoylation is important in maintaining proper cell division (Bachant et al. 2002) (Azuma et al. 2003, 2005).

In contrast to mouse, human spermatocytes show a much different distribution of SUMO1 (Metzler-Guillemain et al. 2008). Human pachytene spermatocytes showed the presence of SUMO1 in constitutive heterochromatin, but lacked SUMO1 on the XY body, underscoring a different regulatory regime associated with human compared to mouse spermatogenesis; why these XY results differ from those of Vigodner et al. is not yet clear (Vigodner et al. 2006). Additionally and consistent with mouse studies, there was no SUMO1 detected on synaptonemal complex (SC) structures, so unlike yeast there may be no role for SUMOs in mammalian SC assembly. In addition to SUMO1

localization to the constitutive heterochromatin, Guillemain et al. demonstrated that increased SUMO1 staining correlates with decreased histone H4-K20me3 staining. This result suggests a competition between sumoylation and methylation at lysine 20 and raises the possibility that sumoylation of lysine 20 is an important epigenetic mark for constitutive heterochromatin in human spermatocytes.

In summary, numerous bioimaging studies have localized SUMO1 versus SUMO2/3 throughout spermatogenesis and have shown differences in their distributions that suggests different functional roles [reviewed in (Vigodner 2011; Rodriguez and Pangas 2016)]. While some discrepancies exist in the literature, in general SUMO1 is found in association with the sex chromosomes of meiotic spermatocytes and with centrosome in spermatids (Brown et al. 2008; Vigodner and Morris 2005). All three SUMO are also found co-localized with XY bodies in spermatocytes (La Salle et al. 2008; Vigodner and Morris 2005) Interestingly, SUMO has been found at double-strand DNA break sites, indicating a possible role in meiotic recombination (Shrivastava et al. 2010; Vigodner 2009). While the numerous localization studies are supportive of a biological role for sumoylation in spermatogenesis, these studies are mostly observational, and functional evidence has been limited by the constraints of this cell system, including the paucity of identified SUMO targets in sperm cells. Three more recent reports are beginning to provide evidence for a functional role. First, it was reported that defective spermatozoa have excessive sumoylation in the tail and neck regions compared to normal sperm, a result that hints strongly towards a requirement for finely balanced sumoylation in normal sperm development (Vigodner et al. 2013). Second, cigarette smoke extract exposure which is known to cause oxidative stress in sperm results in desumoylation of many sperm proteins which may be at least partially responsible for the reduce sperm function (Shrivastava et al. 2014). Third, a large scale isolation and identification of sumoylated proteins from spermatocytes and spermatids revealed 120 substrates, including many with unique roles in spermatogenesis (Xiao et al. 2016). Having specific substrates proteins to evaluate for sumoylation effects should greatly accelerate the understanding of mechanisms and pathways through which SUMO modification affect the spermatogenesis process.

12.2.3 Oocyte Maturation

The role of sumoylation during oocyte formation is less studied than for spermatogenesis, but a few studies have examined the SUMO pathway and its components (Ihara et al. 2008; Wang et al. 2010; Yuan et al. 2014). SUMOs 1–3 can be detected throughout oocyte maturation and there is agreement that SUMO 2/3 localizes to the nucleoplasm (Ihara et al. 2008; Yuan et al. 2014). Likewise, Ubc9 is mostly found in the nucleoplasm (Ihara et al. 2008). In contrast, the location of SUMO1 has been reported to be either nuclear membrane associated (Ihara et al. 2008) or in germinal vesicle in meiotically competent oocytes (Yuan et al. 2014). During oocyte maturation there are differences in the localization of SUMO1 versus SUMO2/3 suggestive of different substrates and functional differences in their roles.

To begin to address the biologic function of sumoylation in oocytes, individual components of the sumoylation system have been overexpressed or inhibited. Overexpression of the SUMO protease, SENP2, led to defects in spindle organization, consistent with an important role of sumoylation (Wang et al. 2010). Consistent with the SENP2 result, blocking SUMO1 with antibodies or reducing Ubc9 levels with siRNA also led to spindle disruption and altered subcellular localization of gamma-tubulin, a known spindle organization protein (Yuan et al. 2014). Surprisingly, SUMO1 overexpression had no discernible effect. Similar studies with SUMO2/3 are not available so little is known about its mechanistic role in oocytes. Identification of specific SUMO substrates in these cells, such as was recently done for sperm cells, would greatly facilitate further functional characterization.

12.3 Embryonic Development

As discussed in Sect. 12.1, a general requirement for sumoylation during embryonic development is common to many organisms as loss of Ubc9, the sole SUMO conjugating enzyme, is typically lethal. In zebrafish, deficiency in any of the three SUMO paralogs is well tolerated during development, but loss of all three is led to severe defects, consistent with a requirement for sumoylation though with considerable redundancy between the paralogs (Yuan et al. 2010). Interestingly, even though all three SUMO paralogs are widely expressed throughout embryonic development in the mouse model, the essential SUMO paralog appears to be SUMO2 (Wang et al. 2014). It was initially reported that knocking out SUMO1 was embryonic (Alkuraya et al. 2006), but subsequent studies failed to find a phenotype for SUMO1 null mice suggesting that SUMO2/3 compensated for the loss of SUMO1 (Zhang et al. 2008). By constructing separate SUMO2(−/−) and SUMO3(−/−) mice, Wang et al. demonstrated that loss of SUMO2 was embryonic lethal while SUMO3(−/−) mice were viable (Wang et al. 2014). Somewhat surprisingly based on the SUMO2 result, SENP1 which specifically desumoylates SUMO1 conjugates was also found to be essential for mouse embryogenesis (Sharma et al. 2013). One possible role that might explain this requirement is removal of SUMO1 from poly-SUMO2/3 chains. Inability to degrade these chains could account for the accumulation of SUMO2/3 product observed. Embryonic lethality was also observed for SENP2 null mice (Kang et al. 2010). In these embryos there was a significant cardiac defect due to accumulation of the sumoylated form of the Pc2/CBX4 subunit of the polycomb repressive complex which led to reduced transcription of two genes essential for cardiac development, Gata4 and Gata6. Similar defects in cardiac development were also seen using a condition SENP2 knockout mouse model (Maruyama et al. 2016). Consistent with this result, the SUMO E3 ligase, PIAS1, is also critical for cardiac development during embryogenesis (Constanzo et al. 2016). Gata4 is sumoylated (Collavin et al. 2004), and PIAS1 co-localizes with Gata4 (Constanzo et al. 2016) suggesting that it may be the SUMO ligase that promotes modification of Gata4 by SUMO. A critical role for sumoylation is also seen in neural crest and muscle development where SUMO modification of the Pax7 transcription factor is essential proper morphogenesis (Luan et al. 2013). Thus, while many questions remain unaddressed, the evidence to date clearly indicates a critical role for sumoylation in the embryonic development of a wide range of organ systems.

12.4 Stem Cells

Stem cells, whether embryonic or adult, are relatively pluripotent cells with the capacity to differentiate into one or more cell types. These cells are critical for normal development and for certain maintenance needs in adult tissues. Additionally, stem cells have enormous potential for therapeutic applications in human disease and injury, so understanding their biology and function is a major focus in the medical sciences today (Sayed et al. 2016; Wang and Zhou 2016). It is becoming clear that sumoylation has significant roles in stem cell propagation and differentiation, and specific examples of SUMO modification in stem cell populations are beginning to be identified. As in the reproductive system, sumoylation in stem cells appears important for regulation of critical TFs that contribute to the differentiation decision switches that control cell fate. Better understanding of the role sumoylation plays in stem cell biology may provide new means for controlling and directing stem cell growth and differentiation.

12.4.1 Embryonic Stem Cells

One of the initial observations connecting sumoylation with embryonic stem cells was the phenotype of the Ubc9 null mice (Nacerddine et al. 2005). While the Ubc9 knockout is embryonic lethal, normal appearing blastocysts can be isolated at E3.5. However, the endogenous pluripotent stems cells in the blastocyst do not

expand and exhibit apoptosis, consistent with a requirement for sumoylation to develop beyond this stage. Further evidence for a role of sumoylation in development has come from studies examining the distribution and expression of sumoylation components during murine brain development. Loriol et al. demonstrated high levels of SUMO1 modified proteins in neuronal nuclei early in development (Loriol et al. 2012). As development progressed there was an overall reduction in sumoylation but an increase at synapses. They also noted developmental-dependent changes in SENP1 and SENP2 levels that may in part account for changing sumoylation levels in various regions of the brain. A subsequent study also observed developmental regulation of SUMO1, SUMO2/3, and Ubc9 in developing mice brains, again with a decrease in total sumoylated proteins as development proceeded (Hasegawa et al. 2014). Strong signal was observed for SUMO1 and SUMO2/3 in neural stem cells with persistence of the SUMO2/3 signal, suggestive of different functional roles for SUMO1 versus SUMO2/3 modification in neural stem cell differentiation. Analysis of SENP2 function in mouse embryos revealed a similar important role for sumoylation in trophoblast development (Chiu et al. 2008). In this system, SENP2 is required for desumoylation of Mdm2, a key regulator of p53 (Jiang et al. 2011). In the absence of SENP2, Mdm2 remains sumoylated and interferes with p53 degradation. The increased levels of p53 cause cellular stress and disrupt the G-S phase transition.

The sumoylation system is also critical for hematopoiesis in zebrafish via hematopoietic stem/progenitor cells (HSPCs). The tango(hkz5) zebrafish mutant is defective for hematopoiesis and this mutation maps to the gene encoding the SAE1 subunit of the SUMO E1 activating enzyme (Li et al. 2012). Embryos with this mutation show drastically reduced numbers of HSPCs, and this phenotype could be reproduced with an Ubc9 knockdown, strongly linking the HSPC decrease to defective sumoylation. Similar effects on hematopoiesis were seen with mopholino-mediated knockdown of either SUMOs or Ubc9 (Yuan et al. 2015). In this study, the CCAAT/enhancer-binding protein α (C/EBPα) was shown to be a critical sumoylation target as a SUMO- C/EBPα fusion could rescue the hematopoietic defect in embryos deficient for SUMO.

While the accumulating literature reveals a potent role for sumoylation during embryonic development, the specific mechanistic pathways in different organ systems are still mostly undefined. One general effect of sumoylation is likely to involve direct modification of the small pool of transcription factors that are critical for regulating embryonic stem (ES) cells. Octamer4 (Oct4) is known to be required for maintenance of stem cell pluripotency and their undifferentiated state; even slight variation in expression levels significantly impacts embryonic cellular differentiation (Niwa et al. 2000). Oct4 is a POU transcription factor which can act as a repressor or activator controlling over 600 genes in the genome. High expression of Oct4 in ES cells leads to differentiation commitment to endoderm or ectoderm. Persistence of high Oct4 expression leads to embryonic carcinoma while down regulation of Oct4 transactivation leads to mesoderm differentiation (Kuijk et al. 2008; Looijenga et al. 2003). Therefore, transient regulation of Oct4 is crucial for cell fate commitment and proper embryonic cell differentiation. Mouse Oct4 has 3 lysines that have a SUMO consensus sequence and can be modified *in vivo* and *in vitro* (Wei et al. 2007). Two of these lysines are conserved in human Oct4, including lysine 118. Lys118 is located near the N-terminal DNA binding domain and is poly-sumo modified *in vitro* and *in vivo*. Surprisingly, Oct4 sumoylation with SUMO1 doesn't decrease its transcriptional activity, but instead increases its stability, its DNA binding, and its transactivation. Although these combined studies support a role for sumoylation in the regulation of Oct4 activity, only suggestive data have been generated on the actual contribution of SUMO-Oct4 towards cell differentiation commitment. Similar to Oct4, other factors critical for stem cell regulation such a KLF4 (Du et al. 2010), Nanog (Wu et al. 2012), and Sox2 (Tsuruzoe et al. 2006) are sumoylated, suggesting that sumoylation may provide a complex coordination of the activity of these factors dur-

ing development. Much further work will be needed to define the precise functional roles of sumoylation in control of ES cell differentiation.

In addition to being SUMO modified, Oct4 can bind to other sumoylated proteins without being itself SUMO modified, and this may also account for changes in its transactivation as well. For instance, Sox2, another stem cell marker, forms a tight complex with Oct4 and regulates its transactivation as well (Rodda et al. 2005). Sox2 is also conserved from mice to humans. Sox2 is sumoylated (Tsuruzoe et al. 2006), but how sumoylation of either Sox2 or Oct4 affects complex formation or function of the complex in ES cell differentiation is unknown. Also of interest is the observation that Oct4 is sumoylated only by SUMO1 and not by SUMO2 (Wei et al. 2007). This observation may imply specific functional regulation through SUMO1 that could be tied to distinct patterns of SUMO1 versus SUMO2/3 expression in developing ES cells.

12.4.2 Post-natal Stem Cells

In addition to its role in embryonic development and ES cell regulation, numerous examples are accumulating that demonstrate an important role for sumoylation in adult stem cells such as bone marrow hematopoietic stem cells. These adult stem cells can split into two lineages: the myeloid and lymphoid lineages. Myeloid progenitor cells can be further divided into sub-classes of blood cells including monocytes, macrophages, neutrophils, basophils, eosinophils, erythrocytes, megakaryocytes/platelets, and dendritic cells. Their differentiation is regulated by lineage specific TFs leading multipotent cells to become specialized blood cells. For instance, expression of the MafB transcription factor in myeloid progenitor cells forces differentiation into macrophages and prevents myeloid lineage differentiation toward erythroid or dendritic cell types (Tillmanns et al. 2007). Conversely, high level of myb expression preserves immature myeloid cell proliferation, controlling timing of the differentiation process (Emambokus et al. 2003). Thus MafB and c-myb, two transcription factors, act as antagonists in the balance of the hematopoietic system. Moreover, induction of myb in macrophages leads to rapid de-differentiation (Beug et al. 1987).

To determine the mechanism driving the antagonist effect between MafB and v-myb, Tillmanns et al. investigated the sumoylation control of those two transcription factors and found that MafB is SUMO modified at two lysine residues *in vivo* and *in vitro* (Tillmanns et al. 2007). Interestingly, preventing MafB sumoylation led to macrophage differentiation and inhibition of myeloid progenitor expansion. Furthermore, the MafB SUMO site mutant could not be repressed by v-myb and committed to macrophage differentiation even with expression of v-myb, suggesting that repression of MafB by v-myb is dependent on the MafB sumoylation state. Likewise, c-myb is itself sumoylated via TRAF7, a SUMO ligase. Sumoylated c-myb is sequestered by TRAF in the cytoplasm, and therefore, negatively regulated by SUMO (Morita et al. 2005). The dual negative effect on both TFs suggests a finely tuned regulation of these competing activities by the sumoylation system. Consequently, the presence or absence of effector proteins such as SUMO ligases may be a key determinant in the balance between cellular differentiation versus proliferation, and the degree of sumoylation may coordinate the antagonist transcription factors to control hematopoietic cell differentiation. Evidence in support of a role for SUMO ligases in hematopoiesis was recently reported (Liu et al. 2014). PIAS1 was shown to control the switch for HSPCs between self-renewal and differentiation through another member of the Gata family, Gata1.

To more generally examine the role of sumoylation in adult mice, Demarque et al. developed an inducible knockout mouse line (Demarque et al. 2011). The major phenotypic effect was observed in the small intestine where the stem cell population was rapidly depleted leading to death within 6 days. At the subcellular level, defects were observed in nuclear positioning and in polarization of actin, with keratin 8 identified as a major SUMO target. The combined molecular defects resulted in diminished proliferative capacity and detachment of enterocytes

from the basal lamina. A critical role for Ubc9, and hence sumoylation, was likewise observed for reprogramming of mouse embryonic fibroblasts into induced pluripotent stem (iPS) cells and for survival of embryonic stem (ES) cells (Tahmasebi et al. 2014). In the absence of Ubc9, iPS induction was greatly impaired and ES cells underwent apoptosis. Interestingly, reduced Ubc9 was correlated with decreases in the protein levels for Nanog, Oct4, KLF4, and Sox2, all critical transcription factors for stem cell differentiation and themselves targets for sumoylation. While these two studies clearly demonstrate the requirement for sumoylation, adult stem cells, like embryonic stem cells, appear to require a delicate balance between sumoylation and desumoylation (Nayak et al. 2014). In human dental follicle stem cells, siRNA knockdown of the SENP3 desumoylating enzyme also prevented differentiation in the osteogenic pathway. A number of critical regulatory factors accumulated in the sumoylated form in the absence of SENP3, suggesting that inability to turn over the SUMO moieties on these substrates impaired the differentiation program in these cells. Thus, the reoccurring theme is that a delicate balance between sumoylation and desumoylation is essential for proper maintenance and/or differentiation of stem cells.

12.5 Tissue and Cellular Differentiation

In addition to its roles in embryonic development, SUMO also plays critical roles in differentiation in adult tissues. Several examples have been clearly identified and characterized in recent years, and while much remains unknown, the contribution of sumoylation to the differentiation process in distinct tissue types is now well established. In some cases, specific TFs serve as the critical sumoylation targets for differentiation, while in other cases the targets are unidentified and the mechanism by which sumoylation contributes to the differentiation process is undefined. The following subsections discuss the currently evaluated systems where sumoylation has a known effect on initiation or completion of differentiation.

12.5.1 Epithelial Tissue

The epidermis has been intensely studied both as a convenient model of tissue differentiation (Gandarillas 2000; Werner and Smola 2001) and for its medical importance in wounds, oncogenesis, congenital and acquired skin dysfunctions, and infections (Angel et al. 2001; Ghoreishi 2000). Human keratinocytes are easily induced to differentiate in culture so that state-specific differences can be explored at the biochemical and molecular level (Poumay and Leclercq-Smekens 1998). Furthermore, the development of organotypic cultures has allowed the recapitulation of nearly authentic epidermal histology and morphology *in vitro* (Benbrook et al. 1995). Nonetheless, regulation of epidermal differentiation is still poorly understood (Koster et al. 2002). Many studies have focused on changes in transcriptional programs that result from differentiation induction signals and have identified a number of TFs relevant to the differentiation process. More recently, Deyrieux et al. studied the role of sumoylation in skin biology using the human HaCaT line as a model system (Deyrieux et al. 2007). In both undifferentiated and differentiated HaCaT cells the sumoylation system was expressed and active with numerous substrates modified. Interestingly, at both the RNA and protein levels, expression of the sumoylation system components was transiently upregulated during the active differentiation process with a peak expression observed as late differentiation markers appeared. Immunohistochemical analysis of HaCaT cells stratified in organotypic cultures revealed that Ubc9 expression increased in the suprabasal cells, just beneath where keratin K1 expression commenced, and then waned in the upper layers, consistent with the transient expression increase seen in differentiating monolayer cultures. When sumoylation was prevented during differentiation the monolayer

HaCaT cells showed delayed and reduced expression of the late differentiation markers and grossly abnormal morphology, suggesting that sumoylation is needed for successful completion of the differentiation program. Global 2-dimensional gel analysis of the SUMO3 substrates during HaCaT differentiation revealed a complex profile (Heaton et al. 2012). The number of SUMO3-modified proteins was highest in basal cells with an abrupt decrease immediately following induction of differentiation followed by a gradual increase at 2–3 days post-induction. However, within this overall trend there was great variability in the level of sumoylation of individual proteins; some increased, some decreased, and some were unchanged. While the specific critical target(s) has not yet been identified, these results strongly support a role for sumoylation in the differentiation of skin, likely through modulatory effects on pertinent TFs.

A role for sumoylation in keratinocyte biology has also been observed through studies of the Cbx4 protein, a component of the polycomb repressive complex 1 (PCR1) (Luis et al. 2011; Mardaryev et al. 2016). Cbx4 has SUMO ligase activity that is important for its regulatory activity (Kagey et al. 2003; Wotton and Merrill 2007). In epidermal stem cells a ligase-minus mutant of Cbx4 stimulated proliferation and increased differentiation, suggesting that sumoylation of one or more targets contributes to restricting growth and keeping these cells in the undifferentiated state (Luis et al. 2011). Consistent with these observations in cultured cells, deletion of Cbx4 in mice results in altered epidermis with enhanced expression of differentiation markers and premature expression of these markers in the suprabasal layers (Mardaryev et al. 2016). Transfection studies with domain-deletion versions of Cbx4 confirmed that these effects on keratinocyte growth and differentiation were dependent on the SUMO-ligase activity. To further understand this pathway it will be critical to identify SUMO substrates for Cbx4.

Like keratinocytes, differentiation of ocular lens epithelial cells also requires sumoylation, with SUMO1 and SUMO2/3 exhibiting distinct functions (Gong et al. 2014). Differentiation in this system can be triggered by treatment with basic fibroblast growth factor (bFGF), and overexpression of SUMO2/3 inhibits this bFGF-induced differentiation while overexpression of SUMO1 has no effect. Conversely, knockdown of SUMO2/3 did not affect differentiation while knockdown of SUMO1 again inhibited the bFGF-induced differentiation. These results suggest that SUMO1 expression is required for differentiation and the SUMO2/3 is inhibiting this process. Mechanistically, the transcription factor Sp1 is known to be a major regulator of lens-specific gene transcription, and Sp1 was shown to be differentially regulated by the SUMO paralogs. Sp1 was activated by SUMO1 while it was repressed by SUMO2 conjugation at K683. Addition of the SUMO2 moiety at K683 reduced both DNA binding capacity of Sp1 and its ability to interact with the coactivator, p300. This antagonistic activity of SUMO1 versus SUMO2 on Sp1 function is consistent with the effects of these two paralogs on lens cell differentiation and suggests that varying levels of the different SUMOS may be a major pathway for regulating differentiation in this cell system.

12.5.2 Myocytes

Like basal keratinocytes, the muscle precursor cells known as myoblasts are proliferative cells that can stop replicating and enter terminal differentiation (Pownall et al. 2002). Upon differentiation the myoblasts start to fuse and form multinucleated myotubes, a process driven by the activity of the MyoD family of TFs in cooperation with the myocyte enhancer factor (MEF2) family (Tapscott 2005). Using the well-established C2C12 myoblast differentiation model, Riquelme et al. examined sumoylation during the differentiation process (Riquelme et al. 2006a). In contrast to keratinocytes, they showed that overall sumoylation of cellular targets declined for both SUMO1 and SUMO2/3 after induction of differentiation. Additionally, Ubc9, which is expressed in both myocytes and myotubes, changes its distribution during differentiation and became more homogenously

distributed throughout the nuclei of myotubes. Ubc9 knockdown with siRNA reduced global sumoylation, but had no effect on MyoD or myogenin expression, localization, or activity, suggesting that the effect of Ubc9 in not mediated directly through the MyoD family. Somewhat surprisingly since overall sumoylation decreases during myocyte differentiation, Ubc9 knockdown inhibited differentiation and resulted in decreased formation of myotubes. Neither apoptosis nor G2/M arrested cells increased under the knockdown conditions, so the mechanism of the Ubc9 effect is unclear, but must reflect subtle differences in target modification during knockdown compared to the sumoylation decrease seen during normal differentiation.

While the sumoylation of the MyoD family is uncertain, sumoylation of other myogenesis regulatory factors is now well documented. SnoN is an oncoprotein that also plays a role in muscle differentiation and was recently shown to be sumoylated at a single lysine residue in C2C12 cells (Wrighton et al. 2007). Mutation of the sumoylation site to arginine imbued SnoN with enhanced myogenic activity and enhanced transcriptional synergy with MyoD. During C2C12 cell differentiation, sumoylation of SnoN decreased slightly, consistent with decreased sumoylation promoting myocyte differentiation and myotube formation. Similarly, several members of the MEF2 family have been shown to be sumoylated, including MEF2A (Riquelme et al. 2006b), MEF2C (Gocke et al. 2005), and MEF2D (Gregoire et al. 2006), and at least for MEF2A (Riquelme et al. 2006b) and MEF2C (Kang et al. 2006) sumoylation is a negative regulator of transcriptional activity. While the role of MEF2 sumoylation in myocyte differentiation remains to be explored, the modification of these important regulatory factors by SUMO is clearly consistent with a functional role for sumoylation in growth and differentiation of this cell type. Furthermore, cross-talk between MEF2 sumoylation and other post-translational modifications such as phosphorylation (Gregoire et al. 2006) and acetylation (Gregoire et al. 2007) suggests exciting and complex regulatory feedback that may be critical for proper response to external stimuli and subsequent control of differentiation.

Several additional studies have begun to identify and characterize other sumoylation targets that are critical for muscle cell development. One of the members of the Pax family of transcriptional regulators, Pax7 is sumoylated on K85, and this modification is necessary to prevent myogenic differentiation of murine skeletal muscle cells (Luan et al. 2013). A lysine to arginine mutant of Pax7 at residue 85, which cannot be sumoylated, fails to transactivate known Pax7 target genes, which suggests that one or more of these gene products is critical for maintaining the cells in the undifferentiated state. Sharp-1 is another inhibitor of skeletal muscle differentiation that is also sumoylated, in this case at lysines 240 and 255 (Wang et al. 2013). Mutation of the SUMO addition sites or overexpression of SENP1 reduces the ability of Sharp-1 to repress differentiation, strongly linking this ability to SUMO modification. Mechanistically, the sumoylation of Sharp-1 promotes interaction with G9a, a histone methyltransferase with co-repressor activity. In the absence of Sharp-1 sumoylation G9a occupancy of muscle promoters is reduced, likely leading to transcription of genes promoting differentiation. A third inhibitor of muscle cell differentiation, BS69, is also a substrate for sumoylation at lysine 367, and in this case PIAS1 appears to be an important SUMO ligase to enhance BS69 sumoylation (Yu et al. 2009). However, sumoylation deficient mutants of BS69 showed no obvious phenotype so the role of sumoylation in the differentiation function of BS69 remains uncertain.

In contrast to Pax7, Sharp-1, and BS69 which act as inhibitors of muscle differentiation, skNAC appears to be a positive regulator of differentiation through a sumoylation-dependent process (Berkholz et al. 2014). skNAC bind to both the Mms21/Nse2 complex, which is known to function as a SUMO ligase (Potts and Yu 2005) and to a myogenic regulator known as Smyd1 (Li et al. 2009). Knockdown of Mms21/Nse2 partially inhibits myogenesis and decreases Smyd1 sumoylation muscle cells, suggesting that sumoylation regulates the activity of the skNAC/

Smyd1 complex to control muscle differentiation. The results with these different factors, Pax7, Sharp-1, BS69, and skNAC/Smyd1 all highlight a role for sumoylation in coordinating events that regulate the transition from undifferentiated to differentiated state in muscle cells. Pax7 and BS69 play a similar role in both muscle and neural cells (Luan et al. 2013), and contribution of sumoylation to neural cell differentiation is discussed in the next section.

12.5.3 Neuronal Cells

In addition to their role in myocyte differentiation, MEF2 proteins are also critical factors for neuronal biology (Heidenreich and Linseman 2004). The MEF2 family members are widely expressed in developing brains and have been implicated in control of proliferation, differentiation, and apoptosis [reviewed in (McKinsey et al. 2002)]. One important function of the MEF2 proteins is to function as integrators of calcium signals mediated through calmodulin and the calcium/calmodulin-dependent protein kinase (CaMK). CaMK stimulates MEF2 transcriptional activity and appears to act through disruption of MEF2 interactions with the HDAC transcriptional repressors (Lu et al. 2000). Functional regulation of the MEF2 family is known to involve phosphorylation, and several studies have now shown that at least MEF2A (Riquelme et al. 2006b), MEF2C (Gocke et al. 2005), and MEF2D (Gregoire et al. 2006) are sumoylated, implying that post-translational modifications will be an important mechanism for controlling MEF2 activity.

Among the MEF2 family members, the role of MEF2A in neuronal differentiation is the best characterized. MEF2A is required for postsynaptic differentiation of cerebellar dendrites into dendritic claws, and this activity is regulated by sumoylation (Shalizi et al. 2006). MEF2 is sumoylated on lysine 403 in a process that is promoted by phosphorylation at lysine 408. When lysine 408 is dephosphorylated by calcium-dependent activation of calcineurin, sumoylation at lysine 403 is reduced and acetylation of K403 is promoted. Sumoylation of MEF2A reduces its transcriptional activating function and represses Nur77, a factor that normally prevents dendritic claw formation. In a subsequent publication, Shalizi et al. demonstrated that PIASx (αor β) were the SUMO E3 ligases responsible for sumoylation of MEF2A, while the other 3 PIAS family members were inactive on MEF2 (Shalizi et al. 2007). PIASx knockdown reduced dendritic claw formation, but this reduction could be overcome by expression of a MEF2A-SUMO fusion protein, indicating that PIASx is normally acting through stimulation of MEF2A sumoylation. Thus, sumoylation is a key component of the regulatory switch that controls morphogenesis of the claw. Interestingly, a previous report indicated that PIAS1 could enhance sumoylation of MEF2A (Riquelme et al. 2006b), and while the reason for this discrepancy is unknown it does support a role PIAS proteins in MEF2 sumoylation. As very little is known about the expression patterns of PIAS proteins in neural tissues, it will be important to determine how PIAS expression is regulated and what effect that has on sumoylation of different MEF2 members and their isoforms.

The calcium/calmodulin-dependent serine protein kinase (CASK), a member of the membrane-associated guanylate kinase (MAGUK) family, is also important for dendritic spine stabilization or maintenance in hippocampal neurons (Chao et al. 2008). CASK appears to function by linking plasma membrane proteins with the actin skeleton through its interaction with the 4.1 protein. CASK is sumoylated at a single site, lysine 679, and sumoylated CASK shows reduced 4.1 binding and a more cytosolic location, suggesting that sumoylation may promote dissociation of CASK from the membrane. Expression of a CASK-SUMO fusion protein impaired spine formation which implies that sumoylation is a negative regulator of this event, in contrast to the positive regulation of cerebellar dendritic claw formation. Unfortunately, regulation of these neuronal processes is likely to be complex and difficult to resolve due to the numerous potential SUMO targets involved.

Consistent with several other of the systems described above, desumoylation is also critical for neuronal differentiation (Juarez-Vicente et al. 2016). Using a mouse teratocarcinoma cell line, Juarez-Vicente et al. demonstrated a generalized increase in free SUMO following neuronal induction with retinoic acid. Examination of the expression levels for the components of the sumoylation system found no changes except for upregulation of SENP5 and SENP7, whose desumoylation activity could account for the increase in free SUMO. Consistent with this deconjugation of SUMO during induction being functionally significant, overexpression of SUMO1 or SUMO2 impaired differentiation. Likewise, SENP7 knockdown impaired differentiation and reduced free SUMO levels, specifically of SUMO2/3, implicating these paralogs as the important regulators. Identification of specific SUMO2/3 targets and exploration of their functional roles will be highly informative for elucidating the pathways and molecular mechanisms that contribute to differentiation control in neuron cells.

12.5.4 Hematopoietic Cells

In the adult, hematopoietic stem cells (HSCs) give rise to multiple lineages, including B cells, T cells, neutrophils, and monocyte/macrophages (Wang and Ema 2016). Diverse studies have now shown that sumoylation plays a regulatory role in many of these pathways. For example, MafB is a transcription factor that promotes macrophage differentiation from myeloid precursors, and MafB is sumoylated at K32 and K297 (Tillmanns et al. 2007). Sumoylation is required for repression of MafB by v-Myb, so sumoylation-defective mutants of MafB exhibit increased differentiation and suggest that the level of sumoylation can control the switch between maintenance of the precursor and differentiation into macrophages. Sumoylation-dependent regulation was also observed for another critical factor controlling hematopoietic development, GATA-1 (Lee et al. 2009). Like MafB, GATA-1 is sumoylated, and in this case sumoylation is required for binding to FOG-1 and transcriptional activation of FOG-1-dependent genes. While not directly tested, loss of GATA-1 sumoylation would like cause significant disruption in the differentiation program. Similarly, GFI1 is another multipotent regulatory factor that plays important roles in mammalian neutrophil differentiation (van der Meer et al. 2010). GFI1 is sumoylated at K239, and SUMO conjugation at this residue is required for GFI1 to support granulocytic differentiation (Andrade et al. 2016). Interaction between GFI1 and its partner, the LSD1/CoREST lysine demethylase complex, is disrupted by mutation of lysine 239 to arginine, implicating this pathway as the critical step that is regulated by sumoylation.

Like macrophages and neutrophils, sumoylation has also been implicated in development of B and T cells (Van Nguyen et al. 2012). STAT5 is a key regulator that is critical for both B and T cells (Yao et al. 2006), and it is modified by SUMO (Van Nguyen et al. 2012). Sumoylation of STAT5 on K696 blocks acetylation at this same residue; lack of acetylation prevents STAT5 dimerization and results in transcriptionally inactive STAT5. Unless the SUMO moiety can be removed by SENP1, the inactive form of STAT5 accumulates resulting in impairment of B and T cell development. Thus, a cycle of sumoylation-desumoylation is essential for the normal function and regulation of B and T cell lineage development by STAT5.

In addition to a regulatory role in normal hematopoietic cell development, it has been noted that sumoylation is disrupted in several type of hematopoietic malignancies. Driscoll et al. observed that patients with multiple myeloma had enhanced overall levels of sumoylation (Driscoll et al. 2010). Levels of Ubc9 and PIAS1 were also elevated in many patients, and this elevation of the conjugation and ligase could explain the increase in sumoylation. High expression levels of these two components correlated with lower patient survival suggesting that increased sumoylation was advantageous for the tumors. Consistent with positive role for sumoylation in tumor cells, acute myeloid

leukemia (AML) cells have reduced levels of SENP5 compared to normal cells (Federzoni et al. 2015). While not addressed in the study, reduction in this desumoylating enzyme would be expected to elevate overall sumoylation. Knocking down SENP5 in an AML neutrophil model prevented differentiation, so this pathway may be contributing to the occurrence of the undifferentiated blast cells that are characteristic of this disease. While a great deal more work is needed to fully understand how sumoylation relates to hematopoietic cancers, there is great potential here for possible diagnostic and/or therapeutic approaches.

12.6 Conclusions

Numerous publications over the last 10 years have shown that the sumoylation system is an important regulator of cellular fate and differentiation, and several systems have been described in this chapter. Examples of both positive and negative effects on differentiation by sumoylation have been reported, so the possibility exists of opposing pathways that are co-regulated by sumoylation to provide fine control of the commitment to differentiate. At the molecular level, SUMO conjugation modulates transcriptional activity both for specific TFs and more globally via changes in chromatin structure. Through its covalent attachment to transcription factors and other chromatin regulatory proteins, such as the histones and chromatin remodeling enzymes, SUMO can influence the recruitment and formation of multi-protein complexes that are critical mediators of the cellular transcriptional program. Additionally, sumoylation of non-TFs, such as kinases or structural proteins, also appears to have important contributions to regulation of cell fate. Consequently, understanding the precise functions of sumoylation in different developmental and differentiation systems may provide new targets for specific or global modulation of these processes. Being able to exert subtle control on developmental and differentiation systems should have important therapeutic benefits for treatment of diseases and repair of injuries.

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The Role of Sumoylation in Senescence

13

Lyndee L. Scurr, Sebastian Haferkamp, and Helen Rizos

Abstract

Cellular senescence is a program initiated by many stress signals including aberrant activation of oncogenes, DNA damage, oxidative lesions and telomere attrition. Once engaged senescence irreversibly limits cellular proliferation and potently prevents tumor formation *in vivo*. The precise mechanisms driving the onset of senescence are still not completely defined, although the pRb and p53 tumor suppressor pathways converge with the SUMO cascade to regulate cellular senescence. Sumoylation translocates p53 to PML nuclear bodies where it can co-operate with many sumoylated co-factors in a program that activates pRb and favors senescence. Once activated pRb integrates various proteins, many of them sumoylated, into a repressor complex that inhibits the transcription of proliferation-promoting genes and initiates chromatin condensation. Sumoylation is required for heterochromatin formation during senescence and may act as a scaffold to stabilize the pRb repressor complex. Thus, SUMO is a critical component of a tumor-suppressor network that limits aberrant cell proliferation and tumorigenesis.

Keywords

Heterochromatin formation • p53 • PML nuclear bodies • pRb • Senescence • Telomeres

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13.1 Introduction

Cellular senescence was first recognized when Hayflick and Moorehead observed that primary human fibroblasts ceased proliferating after serial cultivation *in vitro* (Hayflick and Moorhead 1961). The arrested cells remained metabolically active for many weeks, but did not initiate DNA

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replication despite adequate culture conditions. In contrast, cancer cells failed to undergo proliferative arrest but continued to divide indefinitely. Today, cellular senescence is regarded as a stress response and is applied to the irreversible proliferative arrest of cells induced by various stress signals, including telomere attrition (a response often referred to as replicative senescence), activated oncogenes (a process known as oncogene-induced senescence), DNA damage, oxidative lesions and suboptimal culture conditions (reviewed in Collado and Serrano 2006). Irrespective of the initiating trigger, the hallmark of cellular senescence is permanent proliferative arrest, and whereas quiescent cells can be stimulated to resume proliferation, senescence cells cease to respond to mitogenic stimuli.

Senescent cells have been identified both *in vitro* and *in vivo* using a series of markers that are not exclusive to the senescent state but act as powerful predictors of senescence when used in combination (reviewed in Collado and Serrano 2006; Campisi and d'Adda di Fagagna 2007). Increased activity of acidic ß-galactosidase, termed senescence-associated ß-galactosidase (SA-ß-gal) is the most widely accepted marker of senescent cells (Dimri et al. 1995). The expression of this enzyme correlates strongly with the senescence state (Fig. 13.1), although it can also be induced by stresses such as serum withdrawal and prolonged cell culture (Severino et al. 2000). SA-ß-gal activity derives from residual lysosomal ß-galactosidase activity at the suboptimal pH 6.0 (pH 4.5 is optimal) and reflects the increased lysosomal content of senescent cells (Kurz et al. 2000; Lee et al. 2006). More recently, the appearance of DAPI-stained heterochromatic regions, known as senescence-associated heterochromatic foci (SAHF) (Fig. 13.1), which result in the stable repression of some E2F target genes is involved in the irreversible growth arrest associated with senescence (Narita et al. 2003). These foci are enriched for markers of heterochromatin, including histone H3 methylated at lysine 9 (H3K9Me) (Fig. 13.2), its binding partner heterochromatin protein-1 γ (HP-1 γ) and the non-histone chromatin protein, HMGA2 (Narita et al. 2003; reviewed in Adams 2007). Several other

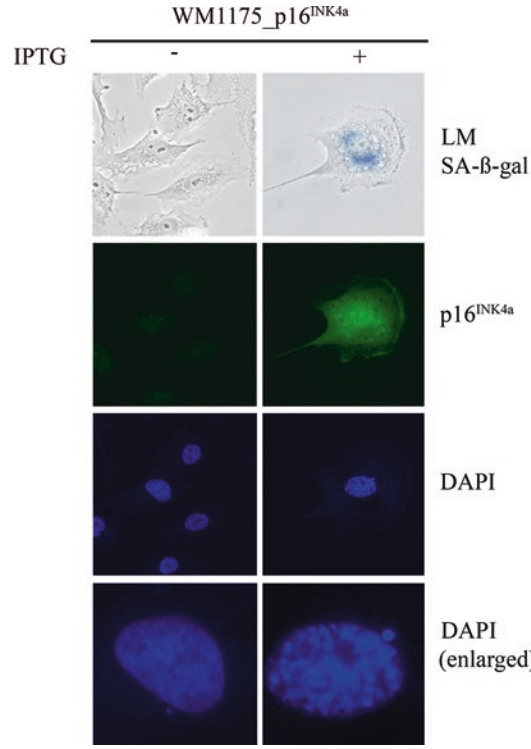

Fig. 13.1 Senescence is associated with positive SA-ß-gal activity and appearance of condensed chromatin. WMM1175_p16^{INK4a} melanoma cells were either left untreated (−) or induced to express p16^{INK4a} using 4 mM IPTG for five days (+) (Haferkamp et al. 2008). The accumulation of p16^{INK4a} induced senescence that was associated with positive SA-ß–gal activity, chromatin condensation (DAPI), and enlarged cells

markers of senescence have also been described and validated, including the cyclin dependent kinase (CDK) inhibitors p16^{INK4a}, p15^{INK4b}, an anti-apoptotic bcl-2 member, Mcl-1 and the transcription factor, Dec1 (Collado and Serrano 2005). Morphological changes such as cell enlargement, vacuolisation and cell flattening are also typical of senescent cells (Fig. 13.1). In addition, senescence renders many cell types resistant to apoptotic cell death and promotes changes in gene expression that appear unrelated to proliferative arrest. For example, senescent cells can secrete proteins that degrade the extracellular matrix and stimulate the growth of neighbouring pre-malignant cells (reviewed in Campisi and d'Adda di Fagagna 2007).

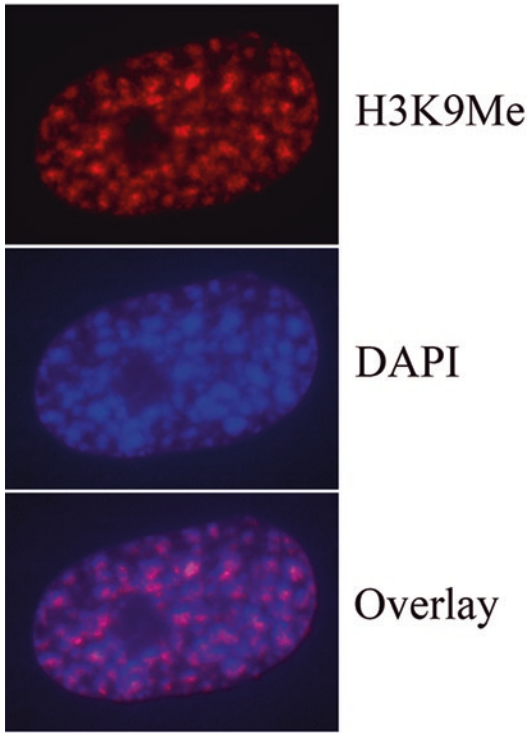

Fig. 13.2 Features of senescence-associated heterochromatin foci. Melanocytes induced to undergo senescence with oncogenic N-RAS^{Q61K} were stained with DAPI and an antibody to H3K9Me to highlight senescence-associated heterochromatin foci, 15-days post infection

Once established, senescence permanently limits cellular proliferation and protects against the development of malignant cancer. Accordingly, senescent cells are abundant in pre-malignant lesions of the skin, the lung and the pancreas whereas they are almost completely absent in malignant tumors (Collado and Serrano 2005; Michaloglou et al. 2005). For instance, pre-malignant lesions in the lung (which developed in a conditional knock in mouse model expressing oncogenic K-RAS^{V12}) contained abundant senescence cells, whereas lung adenocarcinomas were almost completely devoid of cells positive for markers of oncogene-induced senescence, including p16^{INK4a}, p15^{INK4b}, SAHF and SA-ß-gal (Collado and Serrano 2005). Likewise, human naevi (moles) are benign tumours of melanocytes that frequently harbour oncogenic mutations in B-RAF (Pollock et al. 2003), a protein kinase and downstream effector of Ras. Nonetheless naevi typically remain growth arrested for decades and rarely become melanomas (Kuwata et al. 1993; Maldonado et al. 2004), presumably because B-RAF signalling induces a growth inhibitory response that displays many features of senescence, including increased p16^{INK4a} expression and positive SA-ß-gal activity (Michaloglou et al. 2005; Mooi and Peeper 2006). It should be noted that the presence SA-ß-gal positive senescent naevus cells *in vivo*, remains controversial (Cotter et al. 2007, 2008; Michaloglou et al. 2008).

Evidence is also accumulating that replicative (telomere-associated) senescence limits the regenerative capacity of tissues and might contribute to age-related decrements in tissue structure and function. Certainly cells with a senescent phenotype accumulate in the skin of elderly people (Dimri et al. 1995) and have been found at sites of age-related diseases, such as osteoarthritis and atherosclerosis (Chang and Harley 1995; Price et al. 2002). Moreover, there is strong correlation of *in vitro* cell lifespan with the age of the donor (Schneider and Mitsui 1976; Bruce et al. 1986) and a reduced *in vitro* lifespan of cells derived from patients with the premature aging disorder, Werner syndrome (reviewed in Davis et al. 2007). Recent evidence in mice that express low amounts of the mitotic checkpoint protein BubR1 and develop age-related phenotypes at an early age indicated that inactivation of p16^{INK4a} attenuated the development of age-related pathologies and decreased the expression of senescence-associated proteins (Baker et al. 2008). Taken together, these findings suggest that irreversibly growth- arrested senescent cells act as a barrier against tumor formation in young organisms, but their net accumulation may reach a point that compromises tissue function and may promote the development of deleterious phenotypes with age (Campisi and d'Adda di Fagagna 2007).

Although diverse stimuli can induce a senescence response, they appear to converge on two pathways that initiate and maintain the senescence program. These pathways are regulated by the tumor suppressor proteins p53 and pRb, both of which are frequently lost in human cancer cells (Sherr 1996). Importantly, although cancer

cells have partially lost the capacity to signal senescence, the senescence response can be re-engaged, and tumor regression through senescence may be achieved by restoring the p53 and pRb pathways. It has been shown, for instance, that re-instating $p16^{INK4a}$ in $p16^{INK4a}$-null human melanoma cells established and maintained a senescence response (Haferkamp et al. 2008), and restoration of p53 in p53-deficient human tumor cells promoted senescence (Sugrue et al. 1997). Importantly, DNA damaging strategies are more effective in tumors that can engage the senescence program, compared to those that do not (Schmitt et al. 2002; te Poele et al. 2002; Roninson 2003)

13.2 Sumoylation and Senescence

Senescence initiation and maintenance requires the activity of the p53 and pRb tumor suppressor proteins (Ben-Porath and Weinberg 2005) both of which are subject to sumoylation. Not surprisingly, sumoylation has also been shown to play a role in promoting cellular senescence (Bischof et al. 2006). The levels of endogenous E3 SUMO ligase, PIASy increase during replicative senescence, as do the levels of hypersumoylated proteins (Bischof et al. 2006). Over expression of PIASy in normal human fibroblasts induces premature senescence program that is accompanied by increased sumoylation of exogenous p53 and the appearance of PIASy in SAHF (Bischof et al. 2006). Similarly, over expression of SUMO-2/3 in HEK293 cells induces sumoylation of exogenous pRb and p53 and premature senescence that is dependent on both these tumour suppressor proteins (Li et al. 2006). The formation of promyelocytic leukemia (PML) bodies, where p53 is targeted and activated, is also dependent on sumoylation of PML (Kang et al. 2006; Shen et al. 2006) and the size and number of nuclear bodies containing SUMO-1/2 and PML is significantly increased in senescent cells (Yates et al. 2008) (Fig. 13.3). Finally, the increased sumoylation of target proteins, such as RBP1 and APA-1 has also been implicated in senescence (Benanti et al. 2002; Binda et al. 2006).

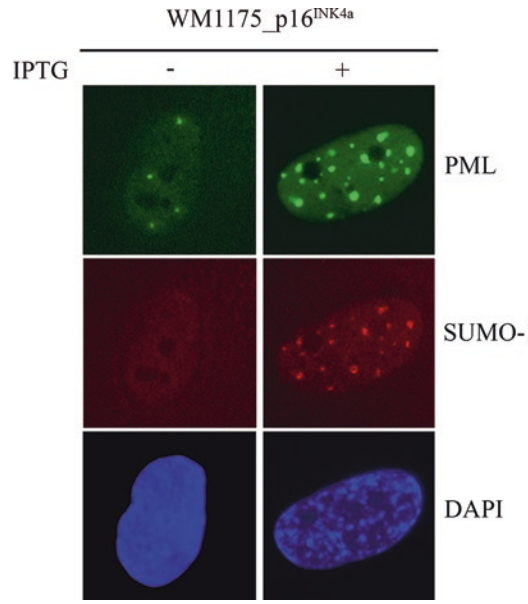

Fig. 13.3 Increase in the size and number of PML/SUMO bodies in senescent cells. Senescent WMM1175_$p16^{INK4a}$ melanoma cells accumulate DAPI-stained heterochromatin and display an increase in the number and size of PML/SUMO-1 nuclear bodies

Senescent cells have decreased SUMO-protease activity and repression of endogenous SUMO proteases SENP1, SENP2 and SENP7 acutely induces senescence in low passage human fibroblasts (Yates et al. 2008). Cells induced to senesce following SENP1 repression displayed many of the phenotypic changes typical of replicative senescence, including cell enlargement, cell flattening, elevated SA-ß-gal activity, cell cycle arrest and accumulation of SUMO/PML bodies (Yates et al. 2008). These data suggest that persistent protein sumoylation is increased in senescence cells and sumoylation is involved in the execution and maintenance of p53- and pRb-dependent cellular senescence (Bischof and Dejean 2007).

13.3 Cellular Senescence and P53 Sumoylation

The p53 transcription factor is a critical regulator of cell survival in response to cellular stress signals including DNA damage, oncogene activation, hypoxia and viral infection (reviewed

in Rubbi and Milner 2003). Many of these stresses induce the covalent modification and subsequent stabilization of p53, usually by disrupting the interaction between p53 and Mdm2. Mdm2 is one of several E3 ligases (others include Pirh2, COP1, TOPORS and ARF-BP1) described for p53, and targets p53 for proteasomal degradation (Haupt et al. 1997). Once stabilized, p53 accumulates in the nucleus and activates a program of gene expression that induces either cell cycle arrest or apoptosis (reviewed in Sherr and McCormick 2002). Stress induced post-translational modifications of p53 include phosphorylation, ubiquitination, acetylation, neddylation and sumoylation. Whereas monoubiquitination of p53 is strongly associated with nuclear export (Brooks et al. 2004), the functional consequences of p53 sumoylation are less clear, possibly because only a small fraction (probably less than 5%) of total cellular p53 is sumoylated (Chen and Chen 2003). Nevertheless, there is persuasive evidence that p53 sumoylation enhances its transcriptional activity to contribute to the initiation of senescence.

SUMO-1, -2 and -3 can be covalently conjugated to lysine-386 in the highly conserved C-terminal region of p53, which contains three nuclear localization motifs (Gostissa et al. 1999; Rodriguez et al. 1999; Li et al. 2006). SUMO modification is not affected by p53 ubiquitination, which does not compete for the lysine-386 acceptor site, but is abrogated by p53 hyperphosphorylation (Rodriguez et al. 1999; Muller et al. 2000). Initial reports demonstrated that sumoylation targeted p53 to PML bodies (Fogal et al. 2000) and enhanced p53 transcriptional activity (Gostissa et al. 1999; Rodriguez et al. 1999; Fogal et al. 2000). These results have been validated by additional studies including work with Drosophila p53 (Mauri et al. 2008) and a study of the human p53-Mdm2 network in yeast models (Muller et al. 2000; Bischof et al. 2006; Li et al. 2006; Di Ventura et al. 2008). Moreover, p14ARF, a key activator of p53 acts co-operatively with Mdm2 to stimulate the sumoylation of p53 *in vivo* (Chen and Chen 2003) and p53 modification with SUMO-2/3 was stimulated in response to oxidative stress (Li et al. 2006). However, in a few studies sumoylation induced no change in p53 activity or was associated with p53 transcriptional repression or nuclear export of p53 (Kwek et al. 2001; Schmidt and Müller 2002; Carter and Vousden 2008). This discrepancy may reflect differences in experimental cell models and reporter constructs. In most reports, p53 was over expressed in cancer cells, which may carry alterations affecting the integrity of the SUMO-p53 pathway. Certainly, the level of sumoylated p53 in response to DNA damage and its localization within PML bodies varied with the type of cancer cell line used (Kwek et al. 2001).

Perhaps the most informative data on the functional impact of SUMO-conjugation on p53 function come from studies utilizing silencing, rather than protein over expression strategies. The repression of SUMO-specific proteases SENP1, SENP2 and SENP7 in primary human fibroblasts enhanced p53 transcriptional activity, with significant induction of the p53-responsive CDK gene, *p21*. p53 activation led to the potent induction of cellular senescence and the accumulation of PML bodies that contained SUMO-1, SUMO-2 and p53 (Yates et al. 2008). The (microrchidia3)-ATPase (MORC3) was recently shown to promote p53 translocation into PML nuclear bodies and induce senescence. Importantly, in MORC3-deficient cells, p53 was stabilized but was not relocated to PML bodies and was not efficiently activated in response to the genotoxic drug adriamycin (Takahashi et al. 2007). These data indicate that p53-dependent senescence requires the augmented assembly of PML bodies and translocation of p53 into these bodies (Lin et al. 2006; Shen et al. 2006). PML bodies recruit many sumoylated proteins and/or proteins containing SUMO-binding motifs, such as p53, Sp100 and the p53-regulators Mdm2, Daxx and CBP. The assembly of co-factors may allow p53 to engage a transcriptional program that can induce senescence, apoptosis or differentiation depending on the initiating cellular stress (Fig. 13.4) (Pearson et al. 2000; Bischof et al. 2002; Tang et al. 2006; Di Ventura et al. 2008).

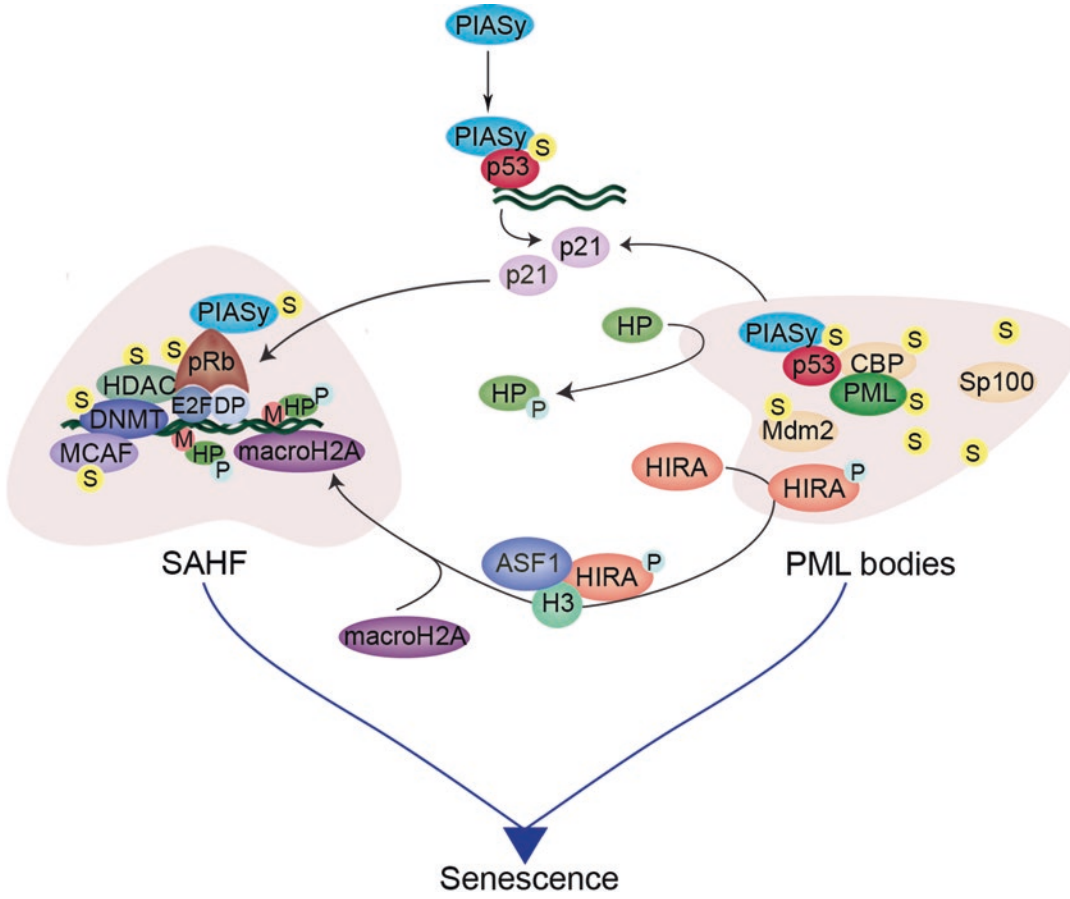

Fig. 13.4 A model for the role of sumoylation in regulating the senescence program. The SUMO cascade influences the senescence program at multiple stages. First, SUMOylation of PML initiates the formation of PML nuclear bodies. These foci are rich in SUMO modified proteins and are required for the modification of the chromatin regulator HIRA and HP1 and the subsequent formation of transcriptionally silenced chromatin domains, called senescence-associated heterochromatin foci (SAHF). Second, SUMOylation of p53 by the PIASy SUMO ligase stimulates p53 activity and re-localizes p53 to PML bodies. Current data suggest that sumoylated, PML-bound p53 activates an expanded transcriptional program to promote cell senescence. An important transcription target of p53 is the p21 CDK inhibitor. p21 maintains pRb in an active hypophosphorylated state, which is prone to SUMOylation. Third, PIASy interacts with pRb creating a transcriptional repressor complex that inhibits the expression of E2F-regulated genes involved in cell cycle progression. Many proteins within the repressor complex are sumoylated, and this may stabilize protein interactions and intensify pRb-mediated transcriptional repression. The activation HIRA/ASF1 pathway cooperates with the active pRb-repressor pathway to drive chromatin condensation, leading to the incorporation of HP1 and macroH2A proteins and formation of SAHF. S, SUMO; P, phosphorylation; M, methylation. Figure adapted from (Fogal et al. 2000; Bischof et al. 2006; Adams 2007; Bischof and Dejean 2007)

13.4 Sumoylation, Senescence and the Retinoblastoma Protein

The retinoblastoma protein (pRb) is a negative regulator of cell proliferation that must be transiently inactivated to allow entry into the mammalian cell cycle. pRb interacts with more than 100 cellular proteins, many of which are transcription factors and chromatin-associated proteins (Morris and Dyson 2001). The E2F transcription factors are critical partners of pRb and the highly regulated progression of the cell cycle relies on the association between pRb and E2F.

This family of transcription factors associates with a DP protein to repress or activate the expression of genes involved in cell cycle progression. Active pRb associates with a subset of E2F transcription factors to constrain the proliferation-promoting activity of E2F and this control mechanism is disrupted in most tumor cells (reviewed in Trimarchi and Lees 2002).

Distinct post-translational modifications of pRb, including phosphorylation, acetylation and sumoylation regulate its interaction with specific cellular partners. For instance, phosphorylation of pRb induces the release of E2F, resulting in its activation and transcription of E2F-responsive genes in late G1 (Trimarchi and Lees 2002). SUMO also conjugates to pRb and preferentially targets lysine-720 of active, hypophosphorylated pRb (Ledl et al. 2005; Li et al. 2006). This residue is part of the lysine cluster that binds proteins with LxCxE-motifs and binding of viral and cellular LxCxE-motif proteins, including papillomavirus E7 and adenovirus E1A proteins, have been shown to repress sumoylation of pRb *in vitro* (Ledl et al. 2005). In contrast, E2F-1, which lacks an LxCxE-motif does not affect sumoylation of pRb, and when SUMO was co-expressed with an E2F-driven luciferase reporter, there was only a modest decrease in the repressive activity of pRb towards E2F (Ledl et al. 2005). It seems likely, however, that over expression of SUMO alone may not have been sufficient to reveal the impact of the SUMO cascade on pRb activity. In particular, co-expression of the E3 ligase PIASy and SUMO potently repressed E2F-regulated promoters in a pRb-dependent manner. The co-repressive effect of PIASy and pRb on E2F-transcriptional activity does not appear to require the sumoylation of pRb, but may involve the interaction between PIASy and pRb (Bischof et al. 2006). It has been hypothesized that the PIASy-pRb complex recruits SUMO machinery and chromatin modifying proteins, such as histone deacetylases (HDACs), DNA methyltransferases (DNMTs) and HP1 to initiate heterochromatin formation at the promoters of E2FE2F target genes (Gonzalo and Blasco 2005; Adams 2007). Sumoylation may facilitate anchorage between repressor proteins and enhance the activity of the repressor complex. In fact many proteins involved in transcriptional repression, including DNMT3A, HDACs 1 and -4, MCAF1 and MBD1, are susceptible to SUMO modification (David et al. 2002; Ling et al. 2004l; Yang and Sharrocks 2004; Li et al. 2007; Sekiyama et al. 2008). Recruitment of this SUMO-repressor complex onto affected genes would eventually lead to an irreversible silencing of these genes via SAHF formation (Bischof and Dejean 2007) (Fig. 13.4). Recent evidence supports the notion that SUMO functions as an epigenetic modulator for heterochromatin formation as the depletion of SUMO disrupted the assembly of MCAF1, H3K9Me, HP1ß, HP1γ at MBD1-contaning heterochromatin (Uchimura et al. 2006).

13.5 Role of PML and Sumoylation in the Regulation of Senescence

PML is a tumour suppressor that localises to distinct subnuclear structures known as PML nuclear bodies. These doughnut shaped nuclear foci (0.2–1.0 μM) are dynamic structures that are present in most mammalian cell nuclei and typically number 1–30 bodies per nucleus. They contain more than 40 constitutive or transient proteins and they have been implicated in essential cellular processes, including DNA damage response and repair, apoptosis, antiviral responses and senescence (reviewed in Bernardi and Pandolfi 2007). The PML protein is the essential component of PML nuclear bodies and PML sumoylation is required for nuclear body formation (Ishov et al. 1999; Zhong et al. 2000). Many of the proteins found in PML bodies are also sumoylated and components of the sumoylation machinery, including SUMO-1, -2/3 and the E3 SUMO ligase PIASy, are localized within these foci (Sachdev et al. 2001; Ayaydin and Dasso 2004). Indeed PML nuclear bodies, together with the nuclear rim and nucleolus are the main sites for sumoylation in the cell (see Fig. 13.3) (Saitoh et al. 2006).

PML is covalently modified by SUMO-1, -2 and -3 at three lysine residues (lysine-65, -160 and -490) and PML contains a SUMO-binding domain in its carboxy terminus (Duprez et al. 1999). Interaction between sumoylated PML proteins via the SUMO-binding domain is thought to initiate the formation of PML bodies that also contain other proteins recruited to the PML foci through sumoylation, protein-protein interactions or SUMO binding domains (reviewed in Bernardi and Pandolfi 2007).

PML impacts on the senescence program in a number of ways. First, expression of PML is induced by oncogenic RAS and in cells undergoing replicative senescence (Ferbeyre et al. 2000). Second, the number and size of PML nuclear bodies increases in senescent cells, and these recruit and activate p53 via sumoylation and acetylation (Pearson et al. 2000; Yates et al. 2008). Third, PML nuclear bodies are involved in the formation of SAHF. The development of SAHF is driven by two histone chaperones, HIRA and anti-silencing factor-1 (ASF1), which promote the deposition of the macroH2A and heterochromatin protein 1 (HP1) into chromatin. In cells approaching senescence, regardless of the initiating trigger, HIRA is translocated into PML bodies, prior to the formation of SAHF. The translocation of HIRA is essential for the formation of SAHF, which do not develop if the function of PML bodies is inhibited (reviewed in Adams 2007). Like HIRA, HP1 proteins also translocate transiently into PML bodies and become phosphorylated at some point prior to accumulating in SAHF (Fig. 13.4) (Zhang et al. 2005, 2007; Ye et al. 2007). Although, it is tempting to suggest that heterochromatic proteins become sumoylated as they move through PML bodies and on export they initiate chromatin condensation, this has yet to be explored. Nevertheless, there is mounting evidence that sumoylation is involved in chromatin condensation. The SUMO-1 and SUMO-2/3 proteins are enriched in areas of heterochromatin, and knockdown of SUMO proteins dissociated the heterochromatin proteins, MCAF, H3K9Me and HP1 from heterochromatic foci (Uchimura et al. 2006) (Fig. 13.4). Further, sumoylation of the Sp3 transcription factor was associated with the establishment of compacted chromatin and transcriptional repression (Stielow et al. 2008), and SUMO deletion in yeast impaired silencing at heterochromatic regions (Shin et al. 2005).

13.6 Telomere Maintenance and SUMO

Telomeres are protein bound, repetitive DNA sequences that comprise 5–15 kilobases of the ends of each chromosome (Blackburn 2001). These sequences progressively shorten with each cell division owing to the end-replication problem of the lagging DNA strand (Harley et al. 1990). As telomeres become critically shortened, normal human cells respond by entering cellular senescence (Bodnar et al. 1998). Cancer cells overcome telomere shortening by either activating telomerase, the enzyme that synthesises new telomeric repeats or by activating homologous recombination mechanisms for lengthening telomeres, known as ALT (alternative lengthening of telomeres) (Bryan et al. 1997; Shay 1997). A hallmark of ALT cells is the localization of telomeres in PML-bodies, and because these may be unique to ALT cells they are called ALT-associated PML bodies (APBs) (Yeager et al. 1999). APBs contains many DNA repair proteins (many of which are also prone to sumoylation) including RAD51, RAD52, BLM and WRN and the ‘structural maintenance of chromosomes’ (SMC) 5/6 complex that promotes repair of double stranded breaks (Yeager et al. 1999; Potts and Yu 2007). SMC5/6 consists of the SMC5-SMC6 heterodimer and at least six non-SMC proteins including the MMS21 SUMO ligase. This ligase sumoylates multiple components of the shelterin telomere-binding complex, including TRF1 and TRF2. Shelterin protects telomeres from being recognized as damaged DNA (telomere ends are similar to double stranded breaks) and prevents telomere lengthening by homologous recombination. It has been proposed that sumoylation of shelterin induces its dissociation from telomere ends, which are then recognized as double stranded breaks and lengthened within ABPs via

homologous recombination. Importantly, long term depletion of SMC5 or MMS21 caused progressive telomere shortening and senescence in ALT cells, but not in a telomerase-positive cell line (Potts and Yu 2007). Perhaps it is not surprising that the function of MMS21 in regulating telomeres in ALT cancer cells is not conserved in all cancer cells, and possibly not in normal cells. In fact, in contrast to the role of SUMO in ALT cells, deletion of SUMO and PIAS homologues in yeast resulted in hyperactivation of telomerase (Xhemalce et al. 2007) and increased telomere length (Tanaka et al. 1999; Xhemalce et al. 2007). The inhibition of telomere lengthening by SUMO would seem more appropriate with its described roles in activating PML, pRb and p53 pathways, but confirming this SUMO function requires further work.

13.7 Conclusions

Sumoylation has emerged as a critical post-translational modification that impacts many cellular responses, including the onset of cellular senescence. This permanent form of proliferative arrest is induced when a cell is exposed to strong and persistent stress signals and many of these stresses also intensify sumoylation. In senescence cells, there is a dramatic increase in the level of protein sumoylation and a clear accumulation of SUMO-modified proteins in subnuclear domains that are critical in initiating and maintaining cellular senescence. The presence of so many SUMO-conjugated proteins complicates defining the precise mechanisms by which SUMO contributes to senescence, and this contribution will no doubt vary according to cell type and stress signal. It is also difficult to investigate the contribution of any particular sumoylated protein, as it may only constitute a small fraction of the total cellular protein pool. Nevertheless, current models are beginning to highlight the central role of sumoylation in senescence; the enhanced p53 activity, the formation of PML-bodies as factories of protein sumoylation and the assembly of SAHF to irreversibly repress gene transcription. Many questions remain. The precise impact of SUMO on the ASF1/HIRA pathway and on pRb remains unresolved. The function of PML nuclear bodies in activating HP1 and the impact of sumoylation on this process is not understood. The role of sumoylation in maintaining telomeres in normal human cells and the contribution of the SMC5/6 complex needs clarification. Also, cellular senescence is a tumour suppressive mechanism and studies are required to clarify the biological role of protein sumoylation in human tumorigenesis.

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Regulation of Plant Cellular and Organismal Development by SUMO

14

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Abstract

This chapter clearly demonstrates the breadth and spectrum of the processes that SUMO regulates during plant development. The gross phenotypes observed in mutants of the SUMO conjugation and deconjugation enzymes reflect these essential roles, and detailed analyses of these mutants under different growth conditions revealed roles in biotic and abiotic stress responses, phosphate starvation, nitrate and sulphur metabolism, freezing and drought tolerance and response to excess copper. SUMO functions also intersect with those regulated by several hormones such as salicylic acid, abscisic acid, gibberellins and auxin, and detailed studies provide mechanistic clues of how sumoylation may regulate these processes. The regulation of COP1 and PhyB functions by sumoylation provides very strong evidence that SUMO is heavily involved in the regulation of light signaling in plants. At the cellular and subcellular levels, SUMO regulates meristem architecture, the switch from the mitotic cycle into the endocycle, meiosis, centromere decondensation and exit from mitosis, transcriptional control, and release from transcriptional silencing. Most of these advances in our understanding of SUMO functions during plant development emerged over the past 6–7 years, and they may only predict a prominent rise of SUMO as a major regulator of eukaryotic cellular and organismal growth and development.

Keywords

SUMO • SIZ1 • MMS21/HPY2 • OTS1/OTS2 • ESD4 • COP1 • PhyB • DELLA • Arabidopsis • Plant development • Stress response • Hormone • Light • Chromatin

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14.1 Introduction

Plants, like other eukaryotes, evolved elaborate mechanisms to tightly regulate cellular and developmental processes so that they may grow and interact with their environment successfully. An added degree of complexity, though, is that plants are sessile organisms. They cannot migrate seasonally, walk towards sources of food, or run away from danger. So, whereas animals may react behaviorally to a changing environment, plants can only react chemically in response to environmental distress. Consequently, plants may need to exceptionally invest in their regulatory mechanisms to adapt with changing environments. Post-translational modification of proteins by the small ubiquitin-like modifier (SUMO) is one of these regulatory mechanisms that impact the function of a great number of proteins, pathways and cellular and developmental processes throughout eukaryotes, including plants. The mechanistic features of SUMO conjugation and deconjugation of proteins are conserved in eukaryotes. However, it seems that plants have diversified the SUMO system by, for instance, amplifying the number of genes encoding SUMO and some of the SUMO pathways components (Augustine et al. 2016; Karan and Subudhi 2012; Novatchkova et al. 2012). Additionally, assessment of the several hundreds of SUMO targets identified in the plant model organism *Arabidopsis thaliana* and the thousands of targets identified in mammalian cells suggests that whereas many core protein families and basic cellular pathways are similarly regulated by SUMO in plants and mammals, some of the biological processes these proteins are involved in are unique to plants (Elrouby 2015). Efforts to identify plant SUMO protein targets have not been as prolific as those in other eukaryotic models (mammalian cells and yeast), but with the advent of new technologies this is likely to change soon. The identification of more plant SUMO targets will undoubtedly permit the delineation of common processes and those that plants evolved to adapt with their specific environment.

Plants also provide the opportunity to study SUMO functions at an organismal level. Except for core SUMO conjugation pathway mutants, mutants of SUMO ligases and proteases are viable. Together with the study of SUMO roles in the functional regulation of individual proteins, this permits detailed assessment of SUMO functions at the whole-organism level, during organ development, in tissue and cell differentiation, and in the coordination of plant growth with hormone action, light signaling, and environmental cues. So, unlike yeast and mammalian cell cultures, plants afford functional analyses of SUMO during the development of multicellular eukaryotes. In this chapter I discuss recent studies that address some of the roles of SUMO during plant growth, development and interaction with the environment. Despite the diversity of the cellular, physiological and developmental aspects these studies address, it is likely that they have just barely scratched the surface of a regulatory mechanism the magnitude of which will be uncovered in the decades to come.

Arabidopsis thaliana (Arabidopsis) is a member of the Brassicaceae, an economically important family of flowering plants that include mustard species as well as many important vegetable species and cultivars such as cabbage, broccoli, cauliflower, kale and Brussels sprouts. Arabidopsis itself is not grown for food but its small size (can be grown in small greenhouse or growth chamber space) and its amenability to genetic, biochemical, physiological, and molecular biology analyses makes it an excellent model for plant biology. The genetic, molecular and genomic resources that were subsequently generated transformed Arabidopsis into a model system for plant development the way the yeast *Saccharomyces cerevisiae* is for cell biology. Naturally, the concepts discussed in this chapter are derived from research performed in Arabidopsis. However, emerging studies of the SUMO system in other plant species suggest that it is much more complex than that of the comparatively "streamlined" Arabidopsis SUMO system, as reflected by the amplification of genes involved in SUMO conjugation and deconjugation into large gene families.

14.2 Phenotypes Associated with SUMO Pathway Mutants

Arabidopsis contains nine SUMO genes, potentially encoding eight SUMO isoforms and a pseudogene (the yeast *Saccharomyces cerevisiae* contains one gene and human encodes three conjugatable isoforms) (Novatchkova et al. 2012). Arabidopsis SUMO1 and SUMO2 are essential for normal growth since their double knockout leads to early embryonic lethality (Saracco et al. 2007). Loss of SUMO3 function delays the transition from vegetative to reproductive development (floral transition, or flowering) suggesting a role in this important developmental switch (van den Burg et al. 2010). Like SUMO1/2 double knockouts, mutations that inactivate the SUMO activating enzyme (E1 or SAE) or the SUMO conjugating enzyme (E2 or SCE) lead to embryonic arrest (Saracco et al. 2007). These enzymes are encoded by single genes in Arabidopsis (SAE contains two subunits: the small SAE1 subunit is encoded by two genes but the large SAE2 catalytic subunit is encoded by a single gene). These studies indicate that sumoylation is essential for plant survival, growth and development, but they do not reveal specific phenotypes that may promote our understanding of the molecular and developmental functions of SUMO. Mutants of SUMO ligases (E3) and SUMO proteases, on the other hand, are viable and implicate SUMO in a diverse set of physiological responses and adaptations as well as functions during development. Assessing the role of sumoylation in the function of individual proteins also promotes our understanding of how SUMO regulates specific biological processes and provides detailed mechanistic understanding of this regulation. In the next sections I will discuss a few of these roles taking into consideration that the goal is not to present all available data but rather to highlight some of the work for which we have a better understanding.

Mutants of the major SUMO ligase (SIZ1) and SUMO protease (ESD4) are stunted, early flowering and exhibit complex pleiotropic phenotypes including defects in plant phyllotaxis (the organization of leaves and branches with respect to the main stem), in leaf size and shape, and in numerous physiological responses. Except for HPY2/MMS21, mutants of other ligase or protease genes exhibit less dramatic phenotypes. *siz1* mutant plants contain elevated levels of salicylic acid (SA) (Lee et al. 2006). SA is a signaling molecule that regulates plant stress responses including response to pathogen attack. Congruent with increased SA levels, the *siz1* mutant exhibits increased expression of pathogenesis-related genes, increased resistance to the bacterial pathogen *Pseudomonas syringae* pv *tomato* DC3000, and constitutive systemic acquired resistance (Lee et al. 2006). Genetic and epistatic analyses suggested that SIZ1 regulates SA signaling through the disease resistance genes EDS1 and PAD4 and that it may negatively regulate innate immunity (Lee et al. 2006). Increased SA levels in the *siz1* mutants is also associated with the dwarf and reduced leaf size phenotypes of these mutants. Expression of the bacterial *nahG* gene (which encodes salicylate hydroxylase and converts SA to inactive catechol) suppressed these phenotypes (Lee et al. 2006; Miura et al. 2010). The *siz1* mutants are also hypersensitive to heat shock as exhibited by reduced germination rates at elevated temperatures (Yoo et al. 2006) (see below for more details). This phenotype is independent of SA since reducing SA levels in *siz1* does not have an effect on its thermosensitivity (Yoo et al. 2006). Domain and functional complementation experiments suggested that the SP-RING domain of SIZ1 plays important roles in the regulation of SA accumulation and SA-dependent phenotypes such as enhanced disease resistance and SA-associated dwarf phenotypes, as well as in the SA-independent thermosensitivity of seed germination (Cheong et al. 2009).

SIZ1 is also involved in drought tolerance since drought stress induces SIZ1-dependent accumulation of SUMO-protein conjugates and *siz1* mutants exhibit enhanced drought tolerance (Catala et al. 2007). The high SA levels characteristic of the *siz1* mutants appear to regulate this process by inducing the production of reactive oxygen species, which in turn mediate stomatal closure (Miura et al. 2013). These results suggest

that SIZ1 negatively affects stomatal closure and drought tolerance. The *siz1* mutants are also hypersensitive to the plant phytohormone abscisic acid (ABA) which regulates plant responses to water deficit (Miura et al. 2009), suggesting that *siz1* mutants respond more readily to water deficit. ABA treatment leads to inhibition of growth and seed germination in wild type plants, and this inhibition is enhanced in *siz1* mutants (Miura et al. 2009). Domain analyses revealed that the SXS domain of SIZ1 modulates the inhibitory effects of ABA (Cheong et al. 2009). Additional roles of SIZ1 in the regulation of phosphate starvation, nitrate and sugar metabolism, copper tolerance, cold acclimation, regulation of basal thermotolerance, and modulation of ABA responses are discussed later.

Arabidopsis encodes three other SUMO ligases. Two proteins that are related to animal Protein Inhibitor Of Activated STAT (PIAS)-type SUMO E3 ligases (called PIAL1 and PIAL2) specifically mediate SUMO chain formation (Tomanov et al. 2014). Mutants of PIAL1 and PIAL2 are indistinguishable from wild type plants but they grow better under salt stress and *pial12* double mutants contain elevated levels of sulfate (Tomanov et al. 2014). The third protein, MMS21 (also known as HPY2), is another SUMO ligase that appears to be involved in the regulation of drought tolerance (Zhang et al. 2013). Mutants of MMS21/HPY2 are also dwarf and display defects in meristem patterning due to altered transition from the mitotic cycle into the endocycle (see below), which results in much shorter roots and highly reduced seedling survival rate (Huang et al. 2009; Ishida et al. 2009). *Mms21/hpy2* mutants also exhibit enhanced drought tolerance and hypersensitivity to ABA. Constitutive overexpression of MMS21/HPY2, on the other hand, reduces tolerance to water deficiency, directly demonstrating that like SIZ1, MMS21/HPY2 negatively regulate drought tolerance (Zhang et al. 2013).

MMS21/HPY2 and SIZ1 are the major SUMO ligases in Arabidopsis and they likely perform non-redundant functions and sumoylate different proteins. This is suggested by several lines of evidence. First, double knockouts of MMS21/HPY2 and SIZ1 are embryo lethal (Ishida et al. 2012). Second, reciprocal complementation experiments revealed that expression of SIZ1 in *mms21/hpy2* does not rescue this mutant phenotype, and similarly, expression of MMS21/HPY2 in *siz1* failed to complement *siz1* mutant phenotypes (Ishida et al. 2012). Third, consistent with these results, expression analyses suggested that SIZ1 is expressed in all cells including proliferating cells of the root tip and distal cells that were fully differentiated whereas MMS21/HPY2 is strongly expressed in proliferating cells of the meristem (Ishida et al. 2012). The expression profiles of the two genes is consistent with their mutant phenotypes, which are caused specifically by meristem defects in *mms21/hpy2* but are generally associated with elevated SA levels in *siz1* (many of the *siz1* mutant phenotypes are reversed by reducing SA levels back to wild-type levels whereas *mms21/hpy2* mutant phenotypes are not affected by reduction of SA levels) (Ishida et al. 2009; Ishida et al. 2012). Collectively, these findings suggest that SIZ1 and MMS21/HPY2 have distinct functions during plant growth and development.

Four SUMO proteases have been characterized in Arabidopsis. Like *siz1* mutants, mutants of the major SUMO protease ESD4 are stunted, display a pleiotropic phenotype highlighted by extreme early flowering (most notably when grown in short days), phyllotaxis defects, early termination of the shoot apical meristem, and several leaf size and shape phenotypes (Reeves et al. 2002; Villajuana-Bonequi et al. 2014). Interestingly, like the *siz1* mutant, *esd4* mutants contain elevated levels of SA, and a second-site mutation in a gene encoding Isochorismate Synthase I (ICSI) reduced SA levels and partially rescued some *esd4* mutant phenotypes (Villajuana-Bonequi et al. 2014). Although we do not have a solid understanding of how both SUMO ligase (SIZ1) and SUMO protease (ESD4) mutants contain elevated levels of SA, it is clear that there is an important relationship between SUMO homeostasis and SA levels, and that elevated SA may contribute to numerous phenotypes associated with these mutants. Mutants of an ESD4-like SUMO protease, ELS1,

do not exhibit strong phenotypes and are barely different from wild type plants (Hermkes et al. 2011). Although the two genes display distinct expression patterns, the functions of ELS1 during plant growth are yet to be determined.

Double mutants of two other SUMO protease genes (OTS1 and OTS2) are hypersensitive to salt whereas overexpression of OTS1 enhanced salt tolerance, indicating that OTS1/2 positively regulate salt tolerance in plants (Conti et al. 2008). Salinity leads to accumulation of SUMO-protein conjugates in wild type plants, and this is enhanced even further in the *ots1 ots2* double mutant, suggesting that OTS SUMO proteases enhance salt tolerance by deconjugating SUMO from target proteins (Conti et al. 2008). This implicates protein sumoylation as a negative regulator of salt tolerance responses. Consistent with this, the abundance of the OTS proteins themselves is downregulated in response to salt stress, suggesting that salt sensitivity occurs, in part, after the likely degradation of OTS proteins (Conti et al. 2008). Interestingly, like *siz1* and *esd4* mutants, *ots1 ots2* double mutants contain elevated levels of SA (and consequently enhanced disease resistance and increased cell death), and SA was found to induce the proteasomal degradation of OTS1/2 (Bailey et al. 2016), suggesting that salt stress may result in increased SA levels, which in turn may induce OTS1/2 degradation and consequently salt sensitivity. Curiously, *ots1 ots2* plants exhibit much higher expression levels of the SA biosynthesis gene ICS1 whereas overexpression of OTS1 resulted in significant reduction in ICS1 expression, consistent with previous identification of an ICS1 mutation as a suppressor of *esd4* (Bailey et al. 2016; Villajuana-Bonequi et al. 2014). These data suggest that OTS1/2 negatively regulate SA signaling (to prevent inappropriate activation of defense) and propose a regulatory feedback loop where OTS1/2 repress ICS1 expression while SA induces OTS1/2 degradation (Bailey et al. 2016).

SUMO-targeted ubiquitin ligases (STUbLs) are another set of proteins that regulate the SUMO modification pathway (Elrouby 2014; Elrouby et al. 2013; Uzunova et al. 2007; Xie et al. 2007). STUbLs target polysumoylated proteins for degradation by the proteasome, and hence provide an alternate route to deconjugation by SUMO proteases. Arabidopsis encodes at least six STUbLs, some of which may have maintained a conserved molecular function in DNA damage repair, but we still do not know much about their functions during plant development (Elrouby et al. 2013). Nevertheless, Arabidopsis mutants of AT-STUbL4 are late flowering and AT-STUbL4 may regulate the floral transition by regulating the abundance of the flowering time protein CDF2 (Elrouby et al. 2013).

14.3 SUMO Functions in Physiological Adaptation and Stress Responses

Growth under stress conditions such as elevated temperatures, drought, salinity, and elevated levels of reactive oxygen species lead to enhanced protein sumoylation (Kurepa et al. 2003). SIZ1 mediates most of this stress-induced sumoylation (Saracco et al. 2007; Yoo et al. 2006), and consequently it is involved in the regulation of drought and salt tolerance and in innate immunity (Lee et al. 2006). For example, *siz1* mutants are sensitive to elevated growth temperatures (thermosensitive). This phenotype is manifested by substantial and rapid growth inhibition as well as severe chlorosis upon direct heat shock (45 °C) (Yoo et al. 2006), suggesting that SIZ1 is required for basal (innate) thermotolerance. On the other hand, sumoylation of HsfA2 (which is required for acquired thermotolerance) represses its transcriptional activity and reduces expression of several downstream targets, hinting that sumoylation of this transcription factor negatively regulates acquired thermotolerance (Cohen-Peer et al. 2010). The mechanism that triggers and maintains cellular responses to stress conditions is not fully understood. Interestingly, both SUMO protease and SUMO ligase mutants contain elevated levels of SA which acts as a signaling molecule in several biotic and abiotic stress responses. However, unlike the *esd4* and *ots1 ots2* mutants which additionally accumulate SUMO-protein conjugates, the *siz1* mutants

contain reduced levels of sumoylated proteins. Although the accumulation of SUMO conjugates in the *esd4* mutants is primarily caused by the ESD4 mutation itself, which abolishes its protease activity and prevents the recycling of SUMO protein targets into their unsumoylated forms, it seems that the stability of these SUMO conjugates is also enhanced by elevated SA levels (Villajuana-Bonequi et al. 2014). Similarly, SA induces the degradation of OTS1/2 (Bailey et al. 2016) and would consequently enhance the stability of SUMO conjugates as well. The relationship between SA and SUMO homeostasis in wild type conditions is in need for further examination. A possible model is that stress conditions instantly cause SA accumulation, which would lead to inactivation of SUMO proteases as observed for the SA-induced degradation of OTS1/2 (Bailey et al. 2016), and consequently to enhanced stability of SUMO-protein conjugates. SUMO-modified proteins are hence likely to play important roles in plant physiological responses to stress.

SIZ1 regulates tolerance to freezing and low temperatures. *siz1* mutant seedlings are hypersensitive to freezing temperatures where they sustain elevated electrolyte leakage and where prior exposure to non-lethal low temperatures (cold acclimation) did not enhance their freezing tolerance (Miura et al. 2007). *siz1* plants are also sensitive to chilling (cold, above freezing temperatures) since they display chlorosis and necrosis upon prolonged exposure to 4 °C (Miura et al. 2007). Consistent with a role for SIZ1 in cold and freezing tolerance, massive SIZ1-mediated sumoylation of plant proteins occurs in a time-dependent fashion when seedlings are exposed to 0 °C (Miura et al. 2007). Miura and colleagues found that ICE1, a MYC-like basic helix-loop-helix transcription factor that positively regulates cold responsive genes to promote low temperature tolerance, was sumoylated *in vitro* and *in vivo* in a transient expression assay. SIZ1 is required for ICE1 sumoylation, and lysine 393 was found to be the SUMO acceptor site (Miura et al. 2007). The physiological function of ICE1 sumoylation was evident when ICE1 or ICE1^{Lys393Arg} was overexpressed in wild type plants. Whereas overexpression of ICE1 enhanced freezing tolerance, overexpression of ICE1^{Lys393Arg} rendered plants sensitive to freezing temperatures. In agreement with this, cold-induced upregulation of CBF3/DREB1A, COR15A and COR47 (ICE1 transcriptional targets) was greater in lines overexpressing ICE1 and reduced in lines overexpressing ICE1^{Lys393Arg} (Miura et al. 2007). Experiments that assessed the regulatory function of ICE1 sumoylation revealed that sumoylation does not alter its transactivation properties but enhances its protein stability by reducing its polyubiquitination by the ubiquitin E3 ligase HOS1 and its subsequent degradation by the proteasome (Dong et al. 2006; Miura et al. 2007). SUMO, thus, may enhance ICE1 stability and potentially its positive regulation of freezing tolerance.

Preliminary findings suggest that SUMO may regulate sugar metabolism in plants. The *siz1* and *pial12* mutants contain altered levels of glucose and fructose, and several starch and sucrose degradation genes are upregulated in *siz1*, and consequently *siz1* plants contain much reduced levels of starch (Castro et al. 2015; Tomanov et al. 2014). *Siz1* mutant seeds also displayed reduced germination rate when germinated on elevated sucrose concentrations, and while glucose suppresses germination of both wild type and *siz1* seeds, higher concentrations were needed to suppress wild type seeds. These results suggest that the *siz1* mutant is hypersensitive to sucrose and glucose (Castro et al. 2015).

Protein sumoylation and the SUMO E3 ligase SIZ1 play important roles in the regulation of plant responses to phosphate deficiency (Miura et al. 2005). *Siz1* mutant plants display inhibition of primary root growth, extensive lateral root and root hair development, elevated root/shoot mass ratio, and enhanced anthocyanin accumulation. These phenotypes are consistent with constitutive activation of phosphate starvation signaling. Expression of phosphate-starvation responsive genes was enhanced in *siz1* plants even when phosphates were present in sufficient concentrations. However, these phenotypes were suppressed by the addition of inorganic phosphates to the growth media. A transcriptional factor,

PHR1, which is involved the regulation of some aspects of phosphate starvation signaling was found to be sumoylated by SIZ1 (Miura et al. 2005).

The Arabidopsis SUMO E3 ligase SIZ1 also facilitates plant tolerance to excess levels of copper (Chen et al. 2011a). Copper is required for normal plant growth, however, it has a high redox activity, and excess copper can induce the production of reactive oxygen species (ROS), potentially leading to toxicity (Burkhead et al. 2009; Yu et al. 2008). Chen and colleagues demonstrated that the *siz1* mutant is hypersensitive to excess copper (Chen et al. 2011a). Total fresh weight of *siz1* seedlings was lower than that of wild type seedlings when grown on elevated levels of $CuSO_4$ (Chen et al. 2011a). Estimates of shoot-to-root ratio of copper content indicated that *siz1* plants possessed twice the levels of copper compared to wild type plants when grown on high levels of $CuSO_4$, and that this was due to hyper accumulation of copper in the shoot system of *siz1* plants, suggesting that copper sensitivity of the *siz1* mutant is possibly due to aberrant copper translocation from the root to the shoot. Consistent with this, the expression pattern of several copper-related transporter genes is altered in *siz1*. In particular, *YSL1* and *YSL3* are upregulated in the shoot of *siz1* compared to wild type. Genetic analyses suggested that *YSL3*, specifically, may act downstream of *SIZ1* to control copper levels in the shoot (Chen et al. 2011a). Interestingly, the *siz1* mutation leads to increased activity of several superoxide dismutases (SOD), and copper and the *siz1* mutation lead to increased Zn/Cu SOD activity and increased ROS concentrations (Chen et al. 2011b; Yu et al. 2008). As has been previously demonstrated, ROS may lead to accumulation of SUMO-protein conjugates as observed in Arabidopsis seedlings treated with H_2O_2 (Kurepa et al. 2003). Consistent with copper-induced production of ROS and with copper toxicity observed in the *siz1* mutant, Chen and colleagues demonstrated that copper induces accumulation of sumoylated proteins in wild-type roots treated with $CuSO_4$, and that this process requires SIZ1 since the *siz1* mutant seedlings failed to accumulate these conjugates completely (Chen et al. 2011a), directly demonstrating that SUMO plays an active role in the regulation of copper tolerance in Arabidopsis. These results, together with the upregulation of *YSL1* and *YSL3* in the *siz1* mutant, suggest that SIZ1-mediated sumoylation may downregulate the expression of genes encoding copper transporters to control copper levels in the shoot.

Soil nitrate is the main source of nitrogen for plants and also acts as a signal that regulates plant growth and development (Stitt et al. 2002). Nitrate is transported into plant cells through nitrate transporters and subsequently reduced into nitrite and then ammonium by nitrate reductases. Park and colleagues (Park et al. 2011) found that supplementing soil with ammonium, but not nitrate or a variety of other inorganic nutrients, restored wild type phenotypes to the *siz1* mutant, both at the vegetative and the reproductive phases. Ammonium also reduced the high SA levels associated with the *siz1* mutation and restored disease-resistance gene expression to wild type levels. Compared with wild type plants, *siz1* plants were found to contain much less nitrogen and elevated levels of nitrate, suggesting that the *siz1* mutant is not defective in nitrate transport into plant cells but rather in nitrate reduction into ammonium. In support of this, nitrate reductase activity is much reduced in *siz1* (Park et al. 2011). Park and colleagues (Park et al. 2011) demonstrated that SIZ1 strongly interacts with and sumoylates both of the Arabidopsis nitrate reductase proteins NIA1 and NIA2 at lysines 356 and 353, respectively. NIA1 and NIA2 form dimers, but sumoylation did not affect their dimerization. Rather, sumoylation enhanced both of their nitrate reductase activity and proteins stability (Park et al. 2011). Interestingly, NIA1 and NIA2 steady state transcript levels were higher in *siz1* than in wild type plants indicating that while NIA1/NIA2 mRNA levels are higher in *siz1*, their proteins are unstable in this mutant, proposing an active degradation process that is suppressed by SUMO.

14.4 Flowering Time

Both the *esd4* and *siz1* mutants are early flowering (Jin et al. 2008; Miura et al. 2005; Reeves et al. 2002). These mutants also display complex pleiotropic characteristics and may exhibit early flowering as a consequence of overall altered growth. In support of this idea, both mutants exhibit extremely early flowering phenotypes when grown under short day conditions (8-h light) and are only marginally early flowering when grown in long days (16-h light) (Jin et al. 2008; Reeves et al. 2002). However, emerging evidence also points to a possible active role for sumoylation in flowering time control. Several flowering time proteins were identified as putative SUMO targets (Elrouby and Coupland 2010; Miller et al. 2010), and a genetic screen for suppressors of *esd4* (*sed*) identified 120 mutants that suppressed several aspects of the *esd4* phenotype including delaying flowering (Villajuana-Bonequi et al. 2014). Additionally, mutations of AT-STUbL4 are late flowering, and this SUMO-targeted ubiquitin E3 ligase likely mediates proteasomal degradation of a floral and transcriptional repressor protein called CDF2 (Elrouby et al. 2013).

It is intriguing that both the SUMO ligase (*siz1*) and SUMO protease (*esd4*) mutants exhibit very similar flowering time attributes. Both mutants are much earlier than wild type in short day but flowering is only slightly accelerated in long days. Both mutations also reduce the mRNA abundance of the floral repressor gene FLC, and consequently enhance the expression of the floral promoter gene SOC1 (Jin et al. 2008; Reeves et al. 2002). Both mutants contain elevated levels of SA, and a second-site mutation in ICS1 (which reduces SA levels) suppressed the early flowering phenotype of *esd4*, and similarly expression of *nahG* (which also reduces SA levels) also suppressed the early flowering phenotype of *siz1* (Jin et al. 2008; Villajuana-Bonequi et al. 2014). The early flowering phenotype of *siz1* and *esd4* suggests that these genes negatively regulate the floral transition, possibly by enhancing the expression of floral repressor genes such as FLC and MAF4 (Jin et al. 2008; Reeves et al. 2002). How can a SUMO ligase and a SUMO protease affect the floral transition (and many other aspects of their phenotypes) in the same manner? SA is at the center of this dilemma, where it seems that perturbation of SA cellular levels may regulate the transcript abundance of members of the MAF transcriptional repressor genes, including FLC and MAF4, such that high SA levels are associated with lower mRNA levels and accelerated flowering, implicating SA in the promotion of flowering (Jin et al. 2008). In addition, genetic analyses suggested that SIZ1 may also activate FLC expression through an SA-independent pathway that requires the flowering time gene FLD (Jin et al. 2008). On the other hand, the FLC protein itself interacts with SIZ1 and is sumoylated on its lysine 154 as indicated by *in vitro* sumoylation assays (Son et al. 2014) (Elrouby and Coupland, unpublished). However, rather than mediating its sumoylation, SIZ1 appears to inhibit FLC sumoylation *in vitro* (Son et al. 2014). Despite these findings, we are lacking overwhelming evidence for a direct mechanistic explanation of the enormous difference in flowering time between wild-type and *siz1* or *esd4* plants.

14.5 Hormone Signaling

ABA regulates various aspects of plant development including embryo maturation, seed dormancy, germination, cell division and elongation, and coordinates growth and adaptation to environmental stresses such as drought, salinity, cold and pathogen attack (Finkelstein 2013). Water deficiency induces ABA biosynthesis to arrest germination until conditions are favorable. Slightly elevated ABA concentrations promote root growth but inhibit shoot proliferation (a response characteristic of mild water stress), whereas highly elevated ABA levels associated with severe water stress inhibit both root and shoot growth. Transgenic plants that overexpressed SUMO1 or SUMO2 and that displayed increased sumoylation of endogenous targets exhibited a reduction in ABA-mediated root growth inhibition (Lois et al. 2003), suggesting

that sumoylation may suppress ABA-mediated growth inhibition. *siz1* mutants are hypersensitive to ABA and exhibited delayed seed germination and increased inhibition of primary root expansion in response to ABA treatment (Miura et al. 2009). Additionally, ABA treatment induced higher expression levels of ABA-responsive genes in *siz1* compared with wild type. These results directly implicate SIZ1 (and sumoylation) as a negative regulator of ABA signaling. ABI5, which acts upstream of ABA-responsive genes and physically interacts with their promoters (through ABA-responsive elements) in response to ABA treatment, was found to be sumoylated at lysine 391 in a SIZ1-dependent manner (Miura et al. 2009). The ABI5 (ABA insensitive) mutant *abi5-4* is less responsive to ABA-mediated inhibition of germination and root elongation. This phenotype was rescued by a transgene expressing wild type ABI5 but a transgene expressing ABI5^{Lys391Arg} in the *abi5-4* background led to ABA hypersensitivity (plants exhibited enhanced inhibition of seed germination and root elongation), suggesting that SUMO modification of ABI5 may suppress ABA-mediated growth inhibition (Miura et al. 2009). Mutants of the transcription factor MYB30, unlike those of ABI5, are hypersensitive to ABA, exhibiting increased inhibition of seed germination (Zheng et al. 2012). ABI5 and MYB30 regulate expression of different sets of genes and MYB30 expression is reduced by ABA treatment. MYB30 was found to be sumoylated at lysine 283, and SIZ1 is required for this modification (Zheng et al. 2012). Expression of wild type MYB30 in the *myb30-2* mutant complemented the ABA hypersensitivity phenotype (that is to say, it reduced the sensitivity of the plant to ABA to wild type levels) whereas expression of MYB30^{Lys283Arg} only partially rescued the mutant phenotype, substantiating the evidence that SUMO is required for MYB30 function in ABA signaling and suggesting that it may do so by reducing sensitivity to ABA. Interestingly, ABA reduces the abundance of MYB30 in the *siz1* mutant but not in wild type plants, suggesting that sumoylation may stabilize MYB30 (Zheng et al. 2012). This is an interesting case where sumoylation of two proteins involved in ABA-mediated signaling acts in different ways to remove the inhibitory block imposed by ABA on germination and growth.

The interplay between SUMO and Gibberellins (GA) in the coordination of plant growth in response to stress conditions has been elegantly demonstrated (Conti et al. 2014). The phytohormone GA binds its receptor GID1 which consequently associates with a group of repressor proteins called DELLA (Griffiths et al. 2006; Murase et al. 2008; Sun 2010; Ueguchi-Tanaka et al. 2005). In addition to regulating plant growth in response to GA, DELLA proteins integrate GA and light signals to coordinate plant development, such as in flowering (de Lucas et al. 2008; Feng et al. 2008; Galvao et al. 2012). GA binding, which results in the association of GID1 with DELLA, targets the latter for degradation by the proteasome, releasing their repressor effects and promoting growth (Fu et al. 2002; McGinnis et al. 2003). Conti and colleagues demonstrated that DELLA proteins are sumoylated at a highly conserved lysine residue, that their levels increase in the SUMO protease double mutant *ots1 ots2*, and that treatment of sumoylated DELLA with OTS1 resulted in dramatic reduction of the sumoylated forms of the protein, indicating that OTS1/OTS2 likely deconjugate SUMO from DELLA proteins and that sumoylation may enhance their stability. Salinity enhances the accumulation of DELLA proteins whereas GA treatment induces their rapid disappearance (Conti et al. 2014). Genetic, transcriptional and physiological analyses suggested that OTS function in the regulation of DELLA protein levels is independent of GA (Conti et al. 2014). When OTS1 or OTS2 was overexpressed in the *ga1-5* mutant (which is deficient in bioactive GA and hence permits DELLA protein accumulation), DELLA proteins were destabilized due to increased SUMO deconjugation and this mimicked the effect of GA treatment to attenuate growth inhibition brought about by DELLA. Additionally, Conti and colleagues tested the effect of abolishing sumoylation of the DELLA protein GAI by converting its SUMO attachment lysine (Lys49) into arginine and ectopically expressing this mutant version in wild

type plants. $GAI^{Lys49Arg}$ levels were much lower compared with its control wild type form (GAI), and $GAI^{Lys49Arg}$ plants exhibited increased root growth, consistent with a lift of the growth repressor functions of DELLA proteins. These results provide additional evidence that sumoylation regulates DELLA function through a GA-independent mechanism. SUMO appears to co-opt the GA signaling pathways to coordinate growth in response to environmental changes (such as increased salinity). The GA receptor GID1 contains a SUMO-interaction motif (SIM). Under stress conditions such as salinity, DELLA proteins are sumoylated and interact with GID1 through its SIM, reducing its binding to DELLA and their subsequent degradation (Conti et al. 2014; Nelis et al. 2015; Willige et al. 2007). This allows for the levels of these growth repressors to build up, inhibiting growth till favorable conditions are resumed.

A further involvement of SUMO in the regulation of GA signaling was revealed by the demonstration that SLEEPY1 (SLY1) is sumoylated by SIZ1 (Kim et al. 2015). SLY1 is an F-box protein component of an SCF complex (SCF^{SLY1}) that mediates interaction of GID1 with DELLA proteins in response to GA, thereby facilitating DELLA degradation (McGinnis et al. 2003). SIZ1 sumoylates SLY1 at lysine 122, and this increases SLY1 stability and interaction with DELLA proteins, consequently promoting plant growth due to enhanced degradation of the growth repressors DELLA proteins (Kim et al. 2015). Interestingly, GA induces SIZ1 expression and also stimulates sumoylation of SLY1 (Kim et al. 2015). These data propose a model in which GA stimulates growth by inducing SIZ1 expression, which in turn leads to SLY1 sumoylation, stabilization, and interaction with DELLA proteins. SLY1 interaction with DELLA leads to subsequent DELLA degradation. This model implicates SUMO as a positive regulator of GA-mediated plant development (Kim et al. 2015). Consistent with this model, the *siz1* mutant exhibits short hypocotyl phenotype and overall stunted growth.

The results presented above suggest that SUMO fine-tunes plant growth in response to GA and environmental conditions. Whereas the sumoylation of the growth repressor DELLA proteins in response to salt stress stabilizes them and consequently inhibits growth in adverse conditions, sumoylation of the F-box protein SLY1 in response to GA perception facilitates its interaction with DELLA proteins and the latter's subsequent degradation by the proteasome, allowing a lift of growth inhibition. These results put SUMO at the center of this regulatory module.

An interesting study by Miura and colleagues (Miura et al. 2011) revealed a relationship between the phosphate starvation phenotype of the *siz1* mutant (Miura et al. 2005) and auxin signaling. *siz1* root mutant phenotype (inhibition of primary root elongation and promotion of lateral root and root hair growth) phenocopies effects of phosphate starvation, and several phosphate-starvation responsive genes are upregulated in this mutant, suggesting that SIZ1 negatively regulate the remodeling of root architecture in response to phosphate starvation (Miura et al. 2005). Interestingly, low external phosphate levels mimic the effects of auxins on lateral root development (Nacry et al. 2005), suggesting that phosphate starvation may lead to auxin redistribution. Additionally, auxin receptor (TIR1)-dependent signaling is implicated in lateral root development caused by phosphate starvation (Perez-Torres et al. 2008). Miura and colleagues showed that under low phosphate levels, auxin enhanced the inhibition of primary root elongation and substantially increased lateral root density in *siz1* compared with wild type. On the other hand, inhibitor of auxin efflux carriers suppressed lateral root proliferation in *siz1* to wild-type levels (Miura et al. 2011). These results suggest that regulation of phosphate starvation responses by SIZ1 involves changes in auxin accumulation. Although we are still lacking a clear mechanism of how sumoylation and SIZ1 activity may regulate phosphate starvation responses, findings linking this process with changes in root architecture and alterations of auxin accumulation patterns in the root may provide the path towards that goal. In addition to SIZ1, auxin also plays a regulatory role in the function of MMS21/HPY2 in the root meristem. Exogenous auxin

application (using the synthetic auxin α-naphthaleneacetic acid) strongly induces *HPY2* expression in lateral root primordia, while the use of auxin antagonists such as PEO-IAA narrows *HPY2* expression domain in primary roots (Ishida et al. 2009). Additionally, expression of the auxin-inducible Plethora genes (*PLT1* and *PLT2*) dramatically enhances HPY2 expression in the root meristematic region suggesting that *HPY2* functions downstream of auxin and the auxin-inducible transcriptional factors PLT1 and PLT2 (Ishida et al. 2009).

In addition to the roles of SUMO in ABA, GA, and auxin signaling, its intimate regulation of SA-mediated plant growth, development and response to biotic and abiotic stress is evident from the discussion of the SUMO ligase and protease mutants presented above. SUMO also controls cell division and elongation through SA-dependent mechanisms. The *siz1* mutant is dwarf, and this phenotype is associated with reduced cell volume and number (Miura et al. 2010), which is suppressed by the expression of genes that restore the normal SA levels, suggesting that SIZ1 regulates cell and plant growth by regulating the levels of SA.

Action of hormones such as ABA, GA, SA and auxin (and likely others) must be regulated in the context of growth conditions such as water and nutrient availability, salinity, elevated temperatures, pathogen attack, and other forms of biotic and abiotic stress conditions. Recent work strongly indicates that protein sumoylation is a powerful regulatory mechanism of both stress and hormonal responses, and that it coordinates between hormone signaling and plant growth and responses to the environment.

14.6 Sumo and Light Signaling

Plants perceive light through a battery of photoreceptors and convey changes in light quality and quantity through signaling pathways that program cellular and molecular events to ultimately regulate various aspects of plant growth and development (Franklin and Quail 2010). For example, in the dark (skotomorphogenesis), seedlings have elongated hypocotyls, closed cotyledons and an apical hook. Light-grown seedlings undergo "photomorphogenesis", leading to short hypocotyls and fully open and expanded cotyledons. At the center of this is an Ubiquitin E3 ligase called COP1 (for CONSTITUTIVE PHOTOMORPHOGENIC 1) that acts as a repressor of photomorphogenesis, and consequently, mutants of which exhibit light-grown phenotypes (short hypocotyl) even when grown in dark [reviewed in (Lau and Deng 2012)]. Similar to *cop1* mutants, Lin and colleagues (Lin et al. 2016) found that *siz1* seedlings exhibit a weak constitutive photomorphogenic phenotype characterized by short hypocotyls when grown in darkness or in white, red, blue, or far-red light, and expression of light-regulated genes was altered in this mutant. This phenotype was complemented by a transgene expressing SIZ1 from its native promoter but not by a transgene expressing a bacterial salicylate hydroxylase (*nahG*), suggesting that it is not caused by the elevated SA levels in this mutant. A mutant in which SUMO1 is null and SUMO2 is much downregulated by a microRNA approach [*sum1–1 amiR-SUM2*; (van den Burg et al. 2010)] also exhibits short hypocotyl, which together with the *siz1* phenotype, suggests that this phenotype is due to impaired SUMO1/2 modification and that SIZ1 acts as a negative regulator of photomorphogenesis (Lin et al. 2016).

The phenotypic similarity of *siz1* and *cop1* mutants and the common role of these proteins as negative regulators of photomorphogenesis suggested that they may act together to suppress photomorphogenesis. Lin and colleagues (Lin et al. 2016) found that the two proteins physically interact, the two genes genetically interact, and that COP1 is sumoylated by SIZ1 at lysine 193. Interestingly, sumoylation of COP1 is increased in darkness, and whereas overexpression of COP1 in wild type plants led to increased hypocotyl length, its expression in *siz1* or expression of $COP1^{Lys193Arg}$ in wild type plants failed to produce the same effect. These results indicate that SIZ1 and sumoylation are required for COP1 activity. In agreement with this, protein extracts from wild type plants or from plants

overexpressing COP1 supported HY5 ubiquitination (HY5 is a known COP1 target) more efficiently than extracts from COP1^{Lys193Arg} or *siz1* plants, and like in the *cop1* mutant, HY5 protein levels are elevated in *siz1* and are comparable in *siz1*, *cop1*, and *siz1 cop1*, suggesting that SIZ1 regulates HY5 levels by enhancing COP1 activity. In fact, experiments attempting to elucidate the mechanism by which sumoylation of COP1 may regulate its activity ruled out roles in its stability, dimerization, or target (such as HY5) interaction. Rather, sumoylation enhances COP1 transubiquitination activity since sumoylated COP1 is more efficient in HY5 ubiquitination than non-sumoylated COP1 (Lin et al. 2016). Tight regulation of COP1 activity is important for proper photomorphogenesis. While sumoylation of COP1 enhances its transubiquitination activity, a feedback loop exists to downregulate COP1 activity. Lin and colleagues also found that COP1 may ubiquitinate and degrade SIZ1 in a proteasome-dependent manner, thereby controlling its negative impact on photomorphogenesis (Lin et al. 2016). It is not known, however, whether sumoylation of COP1 is also important for its function in SIZ1 degradation.

Plants encode a variety of photoreceptors that regulate their responses to ambient light conditions by interacting with a suite of downstream proteins to relay changes in light quality and quantity. The photoreceptors include the cryptochromes and phototropins (absorb light in the blue/UVA spectrum), UVR8 (UVB-specific), and the phytochromes (phys) which absorb red/far red light (Franklin and Quail 2010). In Arabidopsis, five phys have been identified (phyA, phyB, phyC phyD and phyE). PhyB is especially important during seedling development. Sadanandom and colleagues elegantly demonstrated that SUMO negatively regulates phytochrome B-mediated light signaling (Sadanandom et al. 2015). PhyB is sumoylated at lysine 996 and the SUMO proteases OTS1 and OTS2 catalyze its desumoylation (Sadanandom et al. 2015). PhyB-mediated photomorphogenesis regulates seedling development by inhibiting hypocotyl elongation and promoting cotyledon opening and expansion. *phyB* mutants exhibit long hypocotyls whereas phyB overexpression inhibits hypocotyl elongation [reviewed in (Franklin and Quail 2010)]. When a phyB-GFP transgene was constitutively expressed in a phyB mutant (*phyB-9*), the phyB-GFP fusion protein accumulated in red light, far red light, and in dark but sumoylated phyB-GFP accumulated only during the day under red light conditions (Sadanandom et al. 2015). phyB overexpression led to hypersensitivity to red light and resulted in higher levels of inhibition of hypocotyl elongation in response to red light (hypocotyls were shorter than those of wild type plants). Expression of phyB^{Lys996SArg}, which is no longer sumoylated, led to even higher levels of inhibition of hypocotyl elongation in red light. Similarly, expression of phyB^{Lys996Arg} led to enhanced cotyledon opening and expansion, at levels higher than those obtained by expression of wild type phyB (Sadanandom et al. 2015). These results indicate that SUMO modification negatively represses phyB function and negatively regulates phyB-mediated light signaling.

The mechanism by which SUMO regulates phyB function was also examined (Sadanandom et al. 2015). PhyB exists in two forms in plant cells. It is synthesized in its biologically inactive form (Pr), which upon absorbing red light is converted into the biologically active form (Pfr). Pfr can readily convert back to its inactive form by absorbing far red light. Experiments performed by Sadanandom and his colleagues ruled out a direct effect of the Lys^{996}Arg mutation on the photochemical properties of phyB. Instead, they found that red light specifically enhances the abundance of sumoylated phyB, and that the levels of this modified form of phyB increase with increasing length and intensity of red light. In addition, they observed that the constitutively active phyB^{Tyr276His} mutant is hypersumoylated in a light-independent fashion, and that the nuclear pool of phyB is more prone to sumoylation. These data suggest a model in which red light may lead to the accumulation of sumoylated phyB in its Pfr form in the nucleus, possibly to dampen Pfr activity. Desumoylation (or the Lys^{996}Arg mutation) may free Pfr from SUMO's inhibitory effects, allowing hypersensitivity of

phyB responses (Sadanandom et al. 2015). An added complexity to this model is the interplay with phyB-interacting proteins such as PIF5. PIF5 negatively regulates light signaling by binding to phyB Pfr to initiates PIF5 phosphorylation and subsequent degradation of both proteins (Al-Sady et al. 2006). Sumoylation of phyB Pfr reduces its binding to PIF5 and moderately enhances phyB stability (Sadanandom et al. 2015). Given its complex signaling network and large number of interactors and transcriptional targets, fine-tuning of phyB-mediated light signaling is achieved at multiple levels, including sumoylation.

14.7 Roles in Cell Division, Meristem Proliferation, Meiosis, and Gametophyte Development

Studies with the SUMO E3 ligase MMS21/HPY2 revealed that it functions as a repressor of the transition from the mitotic cycle into the endocycle in Arabidopsis (Ishida et al. 2009). Endoreplication permits multiple rounds of DNA replication without subsequent cell division (cytokinesis), leading to successive doubling of the nuclear DNA content of the cell (De Veylder et al. 2007). The transition from a mitotic cell cycle into an endocycle is often associated with the switch of meristematic cells from division to expansion and differentiation, and acts to maximize cellular activity to support a specialized differentiated state.

Human and yeast MMS21 is a component of the SMC5-SMC6 complex required for sister chromatid cohesion (Zhao and Blobel 2005). The Arabidopsis ortholog of MMS21 (HPY2) was identified as a mutant with a severe dwarf phenotype and severely compromised meristems (Ishida et al. 2009; Xu et al. 2013) and by homology with human MMS21 (Huang et al. 2009). The *hpy2-1* mutant seedlings survive for only a few weeks under normal growth conditions, but a few seedlings eventually form shoot systems that exhibit fasciation and defects in phyllotaxis (Ishida et al. 2009; Ishida et al. 2012). Their root meristems contain abnormally enlarged cells and a higher proportion of cells in the endocycle (Ishida et al. 2009). These endocyclic cells also contain higher DNA content (reaching 64C and 128C) and much larger nuclei compared to wild type (Ishida et al. 2009). These results indicated that *hpy2-1* cells enter the endocycle prematurely and that the *hpy2* mutation also allows additional DNA replication cycles. In order to exit the mitotic cycle and enter the endocycle, a reduction in mitotic cyclins and cyclin-dependent kinase activity occurs (De Veylder et al. 2007). Interestingly, transcript and protein levels of some cyclin-dependent kinases (CDKB1 and CDKB2) and cyclins (CYCB1;1 and CYCB1;2) are much reduced in *hpy2-1* compared with wild type (Huang et al. 2009; Ishida et al. 2009), suggesting that HPY2 is involved in the repression of entry into the endocycle, probably by the persistent promotion of the mitotic cycle.

MMS21/HPY2 is also required for meiosis and gametophyte development. Mutants of MMS21/HPY2 that survive through the reproductive stage display severe fertility defects exemplified by a much reduced seed set and increased rate of seed abortion (Liu et al. 2014). Reciprocal pollination experiments suggested that most of the reduced fertility of *hpy2* mutants are likely due to pollen defects, although some defects in female gametophyte may also contribute to sterility but to a lesser extent. These and other experiments also suggested that *hpy2* pollen tube growth was defective even in wild type pistils (Liu et al. 2014). These results are consistent with expression analyses which indicated that MMS21/HPY2 is expressed in anther and pollen (Ishida et al. 2012; Ling et al. 2012; Liu et al. 2014). Further characterization revealed that *mms21/hpy2* anthers show morphological defects, are generally variable in size and shape, and produce fewer and nonviable pollen grains compared with wild type (Liu et al. 2014). Collectively, these results indicate that MMS21/HPY2 is required for the normal development of the male gametophyte.

Pollen defects prompted Liu and colleagues to assess whether meiosis and gametogenesis are impaired in *mms21/hpy2* mutants. In Arabidopsis,

anther development is divided into 14 morphologically distinct stages (Sanders et al. 1999). The earliest defects in *mms21/hpy2* anthers are seen at stage 7 where instead of the expected tetrads that appear in wild type anthers, *mms21/hpy2* mutant anthers contain a mixture of dyads, triads, and tetrads (Liu et al. 2014). Defects continue during subsequent stages where *mms21/hpy2* microspores were aberrant in shape and size in stage 8, and eventually degenerate by stage 11. Defects in female gametogenesis were also observed in *mms21/hpy2* where some megaspore mother cells aborted either before or after meiosis (Liu et al. 2014). These findings indicate that MMS21/HPY2 is required for normal gametogenesis (meiosis) in Arabidopsis. Consistent with this, the earliest chromosomal defects in *mms21/hpy2* were seen at the end of prophase I where chromosomes did not condense to the same level as in wild type anthers, and subsequently a distinct metaphase I was not clear in this mutant. Defects in sister chromatid segregation at anaphase II were also observed, leading to irregular meiotic products with variable DNA content (Liu et al. 2014). These defects in meiotic chromosome behavior are consistent with the molecular function proposed for MMS21 in yeast and mammals as a component of the smc5-smc6 complex that ensures proper chromosome behavior during meiosis (Pebernard et al. 2004; Potts and Yu 2005).

Independent studies revealed that SIZ1 and MMS21/HPY2 are expressed in reproductive organs, but while MMS21/HPY2 is expressed in anther and pollen SIZ1 is not expressed in these male reproductive cells but is expressed in flowers of different stages, with strongest expression in petals, sepals and pistils (Ishida et al. 2009; Ling et al. 2012). In pistils, expression was particularly high in the style and the whole ovule. Consistent with a role in ovule development, ~23% of *siz1* ovules were found to be desiccated (compared to 1% in wild type), and consequently seed and silique development is impaired in this mutant, suggesting that SIZ1 is implicated in female gametophyte development (Ling et al. 2012). As discussed earlier, several *siz1* mutant phenotypes are associated with the elevated SA levels in this mutant. Interestingly, the ovule defects in this mutant are not caused by high SA levels because expressing *nahG* in *siz1* did not restore ovule viability (Ling et al. 2012), suggesting that SIZ1 plays a direct role in ovule development. Reciprocal pollination and pollen viability analyses revealed that *siz1* pollen is indistinguishable from wild type pollen and when used to pollinate wild type pistils, siliques with normal seed set and ovule development formed (Ling et al. 2012). On the other hand, *siz1* female gametophyte could not support full fertilization of wild type pollen and scanning electron microscopy revealed that pollen grains germinate and pollen tubes migrate through the style of *siz1* plants but fail in the final stage of pollen tube guidance to reach the micropylar opening and hence fail to enter the embryo sac (Ling et al. 2012). Collectively, these findings suggest that SIZ1 is required for normal female gametophyte development and that its mutations do not alter normal male gametogenesis or embryogenesis.

14.8 SUMO and Chromosome Functions

Substantial evidence, mostly from yeast and mammalian studies, revealed an intimate relationship between SUMO and chromosome, chromatin, and nucleic acids structure and function (Bachant et al. 2002; Rosonina et al. 2010). In Arabidopsis, assessment of SUMO targets identified a large number of transcription factors and proteins involved in nucleic acids and chromatin metabolism and modification such as helicases, polymerases, methyltransferases and demethylases, heavily implicating SUMO in chromosome biology (Elrouby et al. 2013; Elrouby and Coupland 2010; Miller et al. 2010). Arabidopsis STUbLs could complement the DNA repair defects of the yeast *stubl* mutants suggesting that at least some of these STUbLs are involved in similar functions in plants (Elrouby et al. 2013). Meiotic defects (including defects in sister chromatid segregation) associated with mutations of the SUMO E3 ligase HPY2/MMS21 (see above) also strongly suggest that SUMO is intimately

involved in chromosome functions. Recent studies, however, directly provide mechanistic understanding of how plant SUMO regulates centromere disassembly, heterochromatin status, activation of ribosomal RNA genes, and transcriptional silencing.

Centromeres attach chromosomes to spindle fibers, ensuring proper segregation during mitosis and meiosis. Centromeric nucleosomes contain a centromere-specific histone variant called CenH3 (or CENP-A), which is required for subsequent kinetochore assembly (Henikoff and Furuyama 2012). Centromeres are associated with heterochromatin that is rich in repetitive satellite DNA and transposons (Folco et al. 2008; Henikoff and Furuyama 2012). In actively dividing cells, centromeric heterochromatin remains condensed and is associated with repressive chromatin marks (such as DNA cytosine methylation and histone H3 Lys9 methylation) and reduced transcription (Jenuwein and Allis 2001). On the other hand, terminally differentiated cells (cells that exited the cell cycle), contain chromosomes with very little CenH3, presumably because centromere functions during cell division (chromosome segregation) are not needed anymore (Steiner and Henikoff 2014). Failure to remove CenH3 and disassemble kinetochores in differentiated cells may lead to oncogenesis in mammals. In plants, haploid pollen microspores produced by meiosis undergo two additional mitotic divisions to produce a vegetative cell engulfing two sperm cells. Pollen vegetative cells become terminally differentiated to devote their activity towards the formation and growth of pollen tubes necessary to bring sperm cells to the female gametophyte. Centromeres of pollen vegetative cells, hence, decondense, and lose their CenH3 and heterochromatin marks (Schoft et al. 2009). In a forward genetics screen to identify genes mediating terminal differentiation of pollen vegetative cells, a mutation (called *izanagi*, or *igi*) in CDC48/p97 caused pollen vegetative cells to retain condensed CenH3 centromeres and consequently to lose their ability to form pollen tubes (Merai et al. 2014). Due to these defects in pollen tube formation, *igi/cdc48* mutants are infertile.

CDC48 (in yeast) and its mammalian homolog p97 is an AAA-ATPase that was initially described as a molecular chaperone and then found to be involved in the regulation of many cellular processes including the ER-associated protein degradation (ERAD) pathway that targets misfolded proteins for degradation by the ubiquitin-proteasome system (UPS) (Braun et al. 2002; Rape et al. 2001; Wolf and Stolz 2012). CDC48 forms a complex with its co-factors Ufd1 and Npl4, which can bind ubiquitin, and hence allow the interaction of the complex with ubiquitinated proteins. Through ATP hydrolysis, CDC48 derives the required energy to pull proteins out of cellular structures such as membranes. For examples, during ERAD, misfolded proteins are polyubiquitinated, recognized and untethered from the ER by the $CDC48^{NPL4\text{-}Ufd1}$ complex, and subsequently brought for degradation by the UPS [reviewed in (Wolf and Stolz 2012)]. Accordingly, $CDC48^{NPL4\text{-}Ufd1}$ was described as a "segregase" (Braun et al. 2002) and exemplified with a "Swiss army knife" (Baek et al. 2012). In addition to its role in ERAD, CDC48 has been implicated in the extraction and degradation of proteins from other cellular components such as chromatin (Wilcox and Laney 2009) and in the nonproteolytic extraction of other proteins allowing chromatin decondensation and nuclear envelope formation (Ramadan et al. 2007) and transcriptional activation (Ndoja et al. 2014). $CDC48^{NPL4\text{-}Ufd1}$ also acts as a SUMO-targeted segregase (Bergink et al. 2013; Nie et al. 2012). In fission yeast, Ufd1 interacts with SUMO and is important for maintaining genome stability by providing an alternate mechanism in which $CDC48^{NPL4\text{-}Ufd1}$ may remove poly-sumoylated repair proteins from chromatin (Nie et al. 2012). Additionally, budding yeast $CDC48^{NPL4\text{-}Ufd1}$ binds sumoylated Rad52 through the Ufd1 SIM, which curbs Rad52's interaction with (and loading of) Rad51 at DNA double strand breaks, dislodging the recombination complex (Rad52-Rad51) from DNA and preventing unwarranted recombination (Bergink et al. 2013).

The persistence of CenH3 and condensation of pollen vegetative cell centromeres in the *igi/*

cdc48 mutant suggested that CDC48 acts by removing CenH3 from centromeres as a prerequisite for heterochromatin decondensation and vegetative cell differentiation. To assess the mechanism by which Arabidopsis CDC48 may mediate this process, Mérai and colleagues identified proteins that co-immunoprecipitated with CDC48-Myc in extracts from early-stage pollen by mass spectrometry. A prominent di-sumo-modified CenH3 and to a much lesser extent unmodified CenH3 were identified (Merai et al. 2014), suggesting that CDC48 may have higher affinity to sumoylated CenH3. This was confirmed in transgenic plants that express CDC48-Myc and CenH3-GFP in pollen where equal amounts of di-sumoylated and unmodified CenH3 were pulled down using anti-GFP antibodies, suggesting that the higher levels of di-sumoylated CenH3 that co-immunoprecipitated with CDC48A-Myc resulted from higher affinity of CDC48 to sumoylated CenH3. In addition, FISH and immunolocalization experiments suggested that whereas SUMO1 is uniformly distributed in the nucleoplasm of wild type pollen vegetative cell nuclei, it heavily accumulates with condensed centromeres in the *igi/cdc48* mutant pollen vegetative cell nuclei, confirming the role of CDC48 in binding and removing sumoylated CenH3 at the centromeres. Intriguingly, in addition to sumoylated and unsumoylated CenH3, immunoprecipitates of CDC48-Myc also contained the SUMO E3 ligase HPY2/MMS21, consistent with its roles in meiosis and expression domain in anthers and pollen, and probably implicating it as the SUMO E3 for CenH3. In addition, NPL4, UFD1, and three proteasomal subunits, but notably no ubiquitin, were co-precipitated with CDC48-Myc (Merai et al. 2014). Arabidopsis contains three genes encoding Ufd1 and two genes encoding Npl4. Double Npl4 mutants (*npl4a npl4b*) phenocopy *igi/cdc48* mutants as, similar to *igi/cdc48*, the *npl4a npl4b* mutant is infertile due to the persistent condensation of its centromeric repeat loci and continued association with sumoylated CenH3 (Merai et al. 2014). These results suggest that CDC48 forms a complex with Npl4 and Ufd1 to remove CenH3 from centromeres of pollen vegetative cell nuclei, and that sumoylation of CenH3 is required for its interaction with the CDC48 complex, directly implicating SUMO in the regulation of pollen vegetative cell differentiation. This is highly consistent with recently reported functions of $CDC48^{NPL4\text{-}Ufd1}$ as a SUMO-targeted segregase in fission and budding yeast, and provides an additional function for this complex in centromere disassembly and cell differentiation. Subsequent to CenH3 removal, H3K9 dimethylation is lost and centromeric heterochromatin is decondensed, and this coincides with transcriptional activation of ribosomal RNA genes (Merai et al. 2014). This is particularly important in cells with higher demands for ribosomes and protein synthesis (Grummt and Pikaard 2003). Consistent with this, all major rDNA variants were found to be transcribed in pollen vegetative cells but totally silenced in leaves (Merai et al. 2014). These results suggest the $CDC48A^{NPL4\text{-}Ufd1}$ complex is recruited to sumoylated CenH3 which leads to their removal from the centromeres, centromeric repeat decondensation, and activation of rRNA gene transcription, permitting higher levels of protein synthesis and the differentiation of pollen vegetative cells to facilitate pollen tube growth and fertilization.

A recent study also suggested that the Arabidopsis SUMO E3 ligases PIAL1 and PIAL2 interact with MOM1 (MORPHEUS MOLECULE 1) to form a novel complex required for transcriptional silencing (Han et al. 2016). MOM1-mediated whole genome transcriptional silencing appears to be independent of changes in DNA methylation and H3K9 dimethylation (Probst et al. 2003; Vaillant et al. 2006), and to only require MOM1's CMM2 domain that facilitates its dimerization, suggesting that MOM1 may act as an adaptor molecule in a protein complex involved in transcriptional silencing (Caikovski et al. 2008; Nishimura et al. 2012). The study by Han and colleagues revealed that PIAL1 and PIAL2 are part of this complex. A forward genetics screen identified a mutation in PIAL2 that released silencing of a reporter construct, and insertional mutants of PIAL1 and PIAL2 (and double mutants) as well as rescue experiments confirmed the redundant roles of these proteins in

transcriptional silencing (Han et al. 2016). Furthermore, analysis of target loci whose transcription is silenced by MOM1 indicated that ~90% of these loci were also regulated by PIAL1/PIAL2 since they were similarly up-regulated in *mom1* and *pial1 pial2*. Co-immunoprecipitation, gel filtration, and yeast two-hybrid experiments indicated that MOM1, PIAL1, PIAL2, and likely a Bromo-adjacent homology domain-containing transcriptional regulator (BDT1), and a PHD domain-containing protein (PHD1) interact to form a high molecular weight complex, and that MOM1 is required for complex assembly (Han et al. 2016). The CMM2 domain in MOM1 is necessary and sufficient for complex formation and a domain in PIAL1 and PIAL2 (called the IND domain) mediates dimerization and interaction with MOM1. Interestingly, PIAL1/PIAL2 SUMO ligase activities are not required for transcriptional silencing, and their interaction with MOM1 does not lead to MOM1 sumoylation. On the other hand, MOM1 was identified in a screen for Arabidopsis SUMO-interacting proteins and found to interact with SUMO noncovalently (Elrouby et al. 2013). It is possible that sumoylation of other members of the complex may regulate complex dynamics, for example, interaction with MOM1. It is also conceivable that initial sumoylation of these proteins may be mediated by other SUMO ligases whereas PIAL1/PIAL2 may catalyze subsequent SUMO chain formation, which might be important for downstream purposes such as removal of the complex from chromatin. Alternatively, PIAL1/PIAL2 may play a novel role in transcriptional silencing completely independent of their sumoylation activity.

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SUMO in Drosophila Development

15

Joseph Cao and Albert J. Courey

Abstract

The ubiquitin-like protein SUMO is conjugated covalently to hundreds of target proteins in organisms throughout the eukaryotic domain. Genetic and biochemical studies using the model organism *Drosophila melanogaster* are beginning to reveal many essential functions for SUMO in cell biology and development. For example, SUMO regulates multiple signaling pathways such as the Ras/MAPK, Dpp, and JNK pathways. In addition, SUMO regulates transcription through conjugation to many transcriptional regulatory proteins, including Bicoid, Spalt, Scm, and Groucho. In some cases, conjugation of SUMO to a target protein inhibits its normal activity, while in other cases SUMO conjugation stimulates target protein activity. SUMO often modulates a biological process by altering the subcellular localization of a target protein. The ability of SUMO and other ubiquitin-like proteins to diversify protein function may be critical to the evolution of developmental complexity.

Keywords

SUMO • Ubc9 • Ubiquitin-like proteins • Ras/MAPK signaling • Dpp • Medea • Drosophila development • Scm • Groucho • Bicoid • Spalt

15.1 The SUMO Pathway

Small Ubiquitin-related Modifier (SUMO) is one of many ubiquitin-like proteins with diverse functions in cell biology and development. SUMO has a structure very similar to that of ubiquitin, and like ubiquitin, is covalently conjugated to a large variety of target proteins. Sumoylation and ubiquitylation are homologous processes catalyzed by homologous enzymes

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(Bayer et al. 1998; Smith et al. 2012). Sumoylation is reversible and like many post-translational modifications (e.g., phosphorylation, acetylation, methylation, etc.) functions as a switch to modulate target protein activity. Depending on the target, sumoylation can alter protein function in different ways, often by regulating target protein subcellular localization, interactions with other proteins, and protein stability (Seeler and Dejean 2003; Smith et al. 2012).

SUMO is conserved throughout the eukaryotic domain. The single SUMO family protein in Drosophila is encoded by the *smt3* gene, while the human genome encodes four SUMO family proteins, SUMO1, SUMO2, SUMO3, and SUMO4 (Huang et al. 1998; Smith et al. 2004, 2012). Drosophila SUMO is more closely related to human SUMO2 and SUMO3 than to the other human SUMO family members (Smith et al. 2012). *smt3* is an essential gene that is required both maternally and zygotically (Nie et al. 2009).

Sumoylation of a target protein requires three steps, which are catalyzed by enzymes generally termed E1 (the activating enzyme), E2 (the conjugating enzyme), and E3 (the ligase) (Fig. 15.1). Drosophila SUMO is first expressed in an immature form, containing a two-amino acid C-terminal extension, which is removed by one of the ubiquitin-like proteases, Ulp1 or Ulp2, to expose a required C-terminal Gly-Gly motif (Smith et al. 2004). Coupled to the hydrolysis of ATP to AMP and pyrophosphate, SUMO becomes covalently attached to the E1 enzyme, a heterodimer consisting of SUMO Activating Enzyme 1 (SAE1) and SUMO Activating Enzyme 2 (SAE2) subunits, via a thioester linkage between a cysteine residue in SAE1 and the C-terminal carboxyl group of SUMO. SUMO is then transferred to a cysteine residue in the E2 enzyme Ubc9 (Long and Griffith 2000). While ubiquitylation employs multiple alternative conjugating enzymes, Ubc9 is the only known

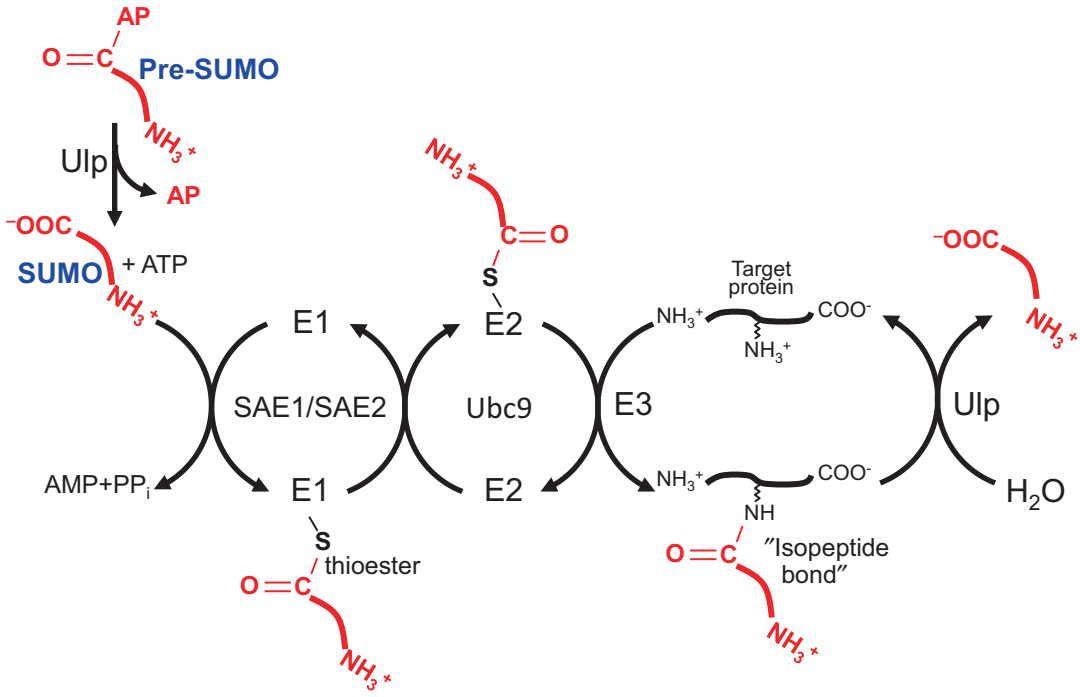

Fig. 15.1 *SUMO conjugation and deconjugation.* SUMO is initially synthesized as a pre-protein with a two amino acid C-terminal extension (AP). The AP is cleaved off by a Ulp family protease to generate mature SUMO. SUMO is then attached to a target protein via a three-step pathway involving the E1 activating enzyme SAE1/SAE2, the E2 conjugating enzyme Ubc9, and a ligation step, which may or may not require an E3 enzyme. The resulting isopeptide bond between the target protein and the C-terminus of SUMO can be hydrolyzed by a Ulp family protease

conjugating enzyme in the SUMO pathway. SUMO is then ligated to an acceptor lysine residue in the target protein. This residue frequently falls within a sequence with similarity to a ΨKXE consensus motif (Ψ is any hydrophobic amino acid and X is any amino acid) (Rodriguez et al. 2001). Unlike ubiquitin conjugation, which has an obligate requirement for an E3 ligase to catalyze the transfer of ubiquitin from the E2 to the target, there is no absolute requirement for a ligase in the catalysis of SUMO conjugation. However, E3 ligases often help Ubc9 select its target and a number of proteins have been found that have SUMO ligase activity, including the PIAS family proteins, RanBP2, and Pc2 (Agrawal and Banerjee 2008; Pichler et al. 2002; Schmidt and Muller 2002; Smith et al. 2012). SUMO modification can be reversed by either Ulp1 or Ulp2, both of which catalyze the hydrolysis of the isopeptide (amide) linkage between SUMO and the lysine side chain in the target protein (Smith et al. 2004).

SUMO-modified proteins are able to interact non-covalently with other proteins through SUMO interaction motifs (SIMs). These motifs possess a hydrophobic core with the consensus sequence V/I-V/I-X-V/I (X is any amino acid) (Hecker et al. 2006; Song et al. 2004). The SIM forms a β strand that interacts with the β2 strand of SUMO in either a parallel or an anti-parallel orientation (Baba et al. 2005; Kerscher 2007). Serine and threonine residues adjacent to the SIM hydrophobic core can be phosphorylated, and the phosphate group forms a salt bridge to a conserved lysine residue within SUMO (Hecker et al. 2006).

15.2 SUMO and Drosophila Development

The remainder of this review will focus on a few of the many roles of SUMO in regulating embryogenesis and imaginal development in *Drosophila melanogaster*. Several of the signaling pathways required for oocyte and embryonic patterning as well as imaginal disc patterning, such as the Ras/MAPK pathway, the Decapentaplegic (Dpp) pathway, and the Jun N-terminal Kinase (JNK) pathway, are regulated by SUMO. In addition, multiple spatially regulated sequence-specific transcription factors that control Drosophila development, such as the maternal morphogen Bicoid and the wing determinant Spalt, are regulated by SUMO. Finally, important ubiquitously localized transcriptional corepressors, including the Polycomb group (PcG) protein Scm and Groucho (Gro), are also regulated by SUMO.

It may be that SUMO most often acts as a negative regulator of target protein activity, e.g., it negatively regulates JNK signaling, Dpp signaling, Gro function, and Scm function. However, SUMO is also sometimes used to enhance pathway activity, e.g., in the case of the Ras/MAPK signaling, Bicoid function, and Spalt function.

15.2.1 Regulation of Signal Transduction by SUMO

15.2.1.1 Ras/MAPK Signaling

The Ras/MAPK signal transduction pathway is required to pattern the follicle cell epithelium during egg chamber development (Reeves and Stathopoulos 2009). This requires the secretion of the TGF-α-like protein Gurken from the presumptive dorsal side of the oocyte, and the binding of Gurken to the Torpedo receptor tyrosine kinase (RTK) in the membranes of the overlying follicle cells. Subsequent dimerization and cytoplasmic autophosphorylation of Torpedo leads to the formation of a docking site for the adaptor protein DRK (Pawson and Gish 1992; Simon et al. 1991, 1993). DRK, in turn, recruits the GTP exchange factor Son of Sevenless (SoS) for Ras activation through the exchange of GDP for GTP in the membrane tethered Ras protein (Bonfini et al. 1992). Ras then stimulates a phosphorylation cascade involving the sequential activation of three Ser/Thr kinases, Raf, MEK, and MAPK (Leevers et al. 1994; McCubrey et al. 2007; Wellbrock et al. 2004), thus triggering the adoption of a dorsal follicle cell fate. These follicle cells then secrete dorsal eggshell structures such

as the pair of dorsal appendages that act as respiratory filaments (Brand and Perrimon 1994; Hsu and Perrimon 1994; Schnorr and Berg 1996).

Early evidence that SUMO has a role in Ras signaling came from a study demonstrating that reduction of *smt3* gene dosage enhanced the eggshell pattering defect resulting from a hypomorphic *ras* mutation. Specifically, mothers homozygous for a weak *ras* allele and heterozygous for an *smt3* P-allele exhibited fused dorsal appendages, which is indicative of a ventralized egg chamber (i.e., the partial loss of the dorsal follicle cell fate) (Schnorr et al. 2001). This is consistent with a requirement for SUMO in Ras signaling. Subsequently, a number of proteins known to influence Ras/MAPK signaling were found to be SUMO-conjugation targets (Nie et al. 2009). Furthermore, RNAi knock down of SUMO in S2 cells revealed that SUMO is required for robust Ras/MAPK signaling in response to the RTK ligands insulin and Spitz. SUMO knockdown led to reduced levels of activated MEK and MAPK in the stimulated cells indicating that SUMO likely acts upstream of MEK and downstream of the RTK in the pathway. Several of the Ras pathway SUMO conjugation targets in the early embryo, including protein phosphatase 2A, and 14-3-3 family proteins are known to function via the Raf protein (Abraham et al. 2000; Light et al. 2002; Rommel et al. 1996, 1997; Wassarman et al. 1996), suggesting that SUMO may influence Ras signaling at the level of Raf.

15.2.1.2 Dpp Signaling

Dpp signaling, which is required for many developmental pathways, including embryonic dorsoventral patterning and imaginal disc patterning, is also regulated by SUMO. In this case, SUMO appears to interfere with signaling in contrast to its role in enhancing Ras/MAPK signaling. Dpp is a member of the BMP subfamily of TGFβ family ligands and signals through a heteromeric receptor consisting of a type I subunit (Saxophone or Thickveins) and a type II subunit (Punt) (Shimmi et al. 2005). Both type I and type II subunits possess Ser/Thr kinase activity. After activation of the receptor by Dpp, the type II subunit phosphorylates the type I subunit, and then the type I subunit phosphorylates the Smad family transcription factor Mothers against Dpp (Mad). Phospho-Mad then interacts with the co-Smad Medea (Med) and activates downstream targets at the level of transcription (Affolter et al. 2001).

A yeast two-hybrid screen uncovered an interaction between Med and Ubc9, and tissue culture experiments using S2 cells demonstrated Med sumoylation (Miles et al. 2008). Furthermore, overexpression of SUMO in the embryo inhibited the transcription of the Medea target genes *Ance* and *ush*, while expression of the two targets increased upon expression of a Med mutant containing a defective SUMO acceptor site. Fluorescent Recovery After Photobleaching (FRAP) studies showed that sumoylation of Med occurs in the nucleus and allows for the shuttling of Med out of the nucleus, thus explaining how Med sumoylation interferes with Dpp signaling.

15.2.1.3 Jun N-Terminal Kinase Signaling

The Jun-N-terminal Kinase (JNK) pathway, another highly conserved MAPK signaling pathway, regulates multiple processes during Drosophila development, including dorsal closure in embryos, thorax closure in pupae, and stress induced apoptosis (Etter et al. 2005; Igaki 2009; Luo et al. 2007). Intrinsic and external

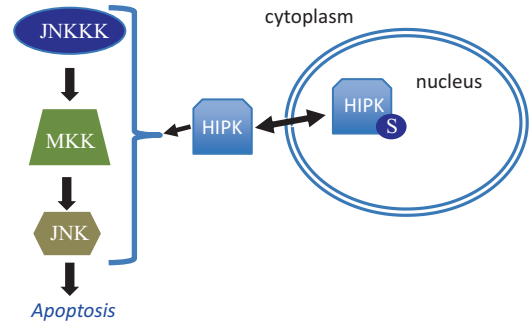

Fig. 15.2 *Regulation of apoptosis by SUMO.* Apoptosis can be triggered by activation of the JNK pathway, which involves the sequential action of JNKKK, MKK, and JNK. This pathway is activated at an unknown step by HIPK. Sumoylation (S) of HIPK prevents excess apoptosis by sequestering HIPK in the nucleus

stimuli triggers the pathway by activating JNK Kinase Kinase (JNKKK), which then phosphorylates MAPK Kinase (MKK) for the subsequent phosphorylation and activation of JNK (Biteau et al. 2011).

As mentioned above, the JNK pathway upregulates apoptosis and SUMO antagonizes this process since SUMO knockdown by RNAi led to increased apoptosis in the wing disc (Huang et al. 2011). However, when SUMO and JNK were knocked down at the same time, increased apoptosis was not observed. In addition, SUMO knockdown in the wing disc led to increased expression of the JNK target genes *puckered* and *matrix metalloproteinase I*.

Further genetic analysis suggests that SUMO may regulate JNK activity via homeodomain-interacting protein kinase (HIPK) (Huang et al. 2011) (Fig. 15.2). HIPK knockdown attenuates SUMO knockdown-induced apoptosis. Furthermore, HIPK is a SUMO conjugation target and SUMO is required for retention of HIPK in the nucleus. Apparently, when cells are depleted of SUMO, HIPK enters the cytoplasm where it encounters and activates the JNK pathway leading to increased apoptosis.

15.2.2 Regulation by SUMO of Spatially Restricted Sequence-Specific Transcription Factors

15.2.2.1 Bicoid

Lesswright (*lwr*), the gene encoding Ubc9, was independently discovered for its role in anterior patterning. Hence, an alternative name for *lwr* is *semushi*, which means "hunchback" in Japanese, reflecting the similarity between the *semushi* phenotype and that of the gap gene *hunchback* (*hb*). Loss-of-function mutations in either gene perturb segmentation of the anterior portion of the early embryo (Epps and Tanda 1998). Further examination of the *semushi* mutants revealed reduced expression of *hb*.

SUMO may mediate anterior patterning by controlling the function of the transcription factor Bicoid, a classical morphogen that is distributed in an anteroposterior gradient in the early embryo and that functions as an activator of *hb*. In particular, Ubc9 function may be required for the nuclear translocation of Bicoid (Epps and Tanda 1998). Paradoxically, however, a cell culture assay using an *hb* enhancer element to drive reporter gene expression revealed that sumoylation of Bicoid inhibits its ability to activate transcription (Liu and Ma 2012).

15.2.2.2 Spalt

Spalt (Sal) and Spalt-related (Salr) are highly conserved zinc-finger transcription factors that regulate wing vein formation and the expression of *knirps* during wing development (Barrio and de Celis 2004; de Celis and Barrio 2000; de Celis et al. 1996). Both proteins contain two SUMO-acceptor lysine residues and mutations in the genes encoding SUMO and Ubc9 enhance the ectopic wing vein phenotype observed in flies heterozygous for a small deficiency that removes both *sal* and *salr* (Sanchez et al. 2010). Wild-type Sal overexpression results in ectopic vein formation, while expression of Sal containing mutations in the SUMO acceptor sites does not, thus suggesting that SUMO conjugation is required for Sal activity. In contrast, mutagenesis of the SUMO acceptor lysine residues in Salr enhanced the wing venation defect due to overexpression suggesting that sumoylation of Salr interferes with its activity. These contrasting effects of the mutations in Sal and Salr on wing venation were paralleled by contrasting effects on *knirps* expression. Mutagenesis of the SUMO acceptor sites in Sal interfered with its ability to up-regulate *knirps*, while mutagenesis of the acceptor sites in Salr enhanced its ability to up-regulates *knirps*.

The mechanism by which SUMO influences Sal and Salr function may be related to the ability of SUMO to control the subnuclear localization of these two proteins. For example, while Sal exhibits diffuse nuclear localization in wild-type wing discs, reduced levels of Ubc9 (presumably leading to reduced levels of sumoylation) result in the appearance of large punctate Sal-containing nuclear bodies.

15.2.3 Regulation by SUMO of Co-repressors

15.2.3.1 The Polycomb Group Protein Scm

While the spatially regulated transcription factors (i.e., the products of the gap and pair rule genes) that initiate homeotic gene expression are only present in the early embryo, the spatially restricted patterns of homeotic gene expression are somehow maintained throughout embryonic and imaginal development. This cellular memory is thought to be provided by two groups of genes termed the Polycomb Group (PcG) and the Trithorax Group (TrxG), with the former being required for epigenetic stability of the repressed state, while the latter is required for epigenetic stability of the active state.

Many of the PcG proteins are members of one of three different complexes, the Pleiohomeotic Repressive Complex (PhoRC), Polycomb Repressive Complex 1 (PRC1), and Polycomb Repressive Complex 2 (PRC2) (Schwartz and Pirrotta 2013). PhoRC, which contains Pleiohomeotic (Pho) and Scm-related gene containing four MBT domains (Sfmbt), binds to cis-regulatory elements in the homeotic gene complex termed polycomb response elements (PREs), where they are thought to recruit PRC2. This complex contains Enhancer of zeste (E(z)), a SET family histone methyltransferase, which catalyzes the trimethylation of lysine 27 on histone H3 (H3K27me3). H3K27me3 then serves as a docking site for PRC1. This complex ubiquitinates histone H2A and directs the compaction of chromatin, with this latter function serving to reduce the accessibility of associated genes to a TrxG-encoded chromatin remodeling complex that opens up the chromatin allowing the transcriptional machinery to gain access to the DNA template. An additional PcG gene product that is essential for PcG function is Sex combs on midleg (Scm), which may be a peripheral component of PRC1 (Fig. 15.3).

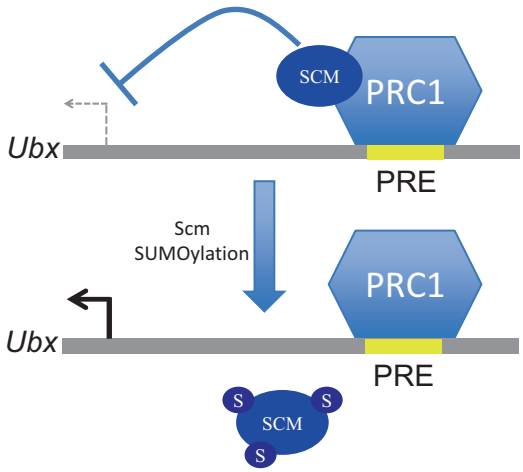

Fig. 15.3 *Regulation of Scm-mediated repression by SUMO.* Scm, a peripheral component of PRC1, is an essential of Polycomb group protein. Polycomb group-mediated repression of genes such as *Ubx* requires the recruitment of PRC1 along with Scm to the PRE. Sumoylation (S) of Scm results in the release of Scm from the PRE and the loss of repression

Like its *C. elegans* homolog SOP-2 (Zhang et al. 2004), Drosophila Scm is regulated by SUMO. Knockdown of SUMO in S2 cells was found to increase association of Scm with a PRE in the homeotic gene complex and to result in the derepression of the homeotic gene *Ultrabithorax* (*Ubx*). Conversely, knockdown of the SUMO deconjugating enzyme Ulp1 was found to decrease Scm association with the PRE. These findings are consistent with the idea that SUMO acts to negatively regulate Scm activity and, through Scm, to alleviate PcG-mediated repression. In support of this idea, mutagenesis of three consensus SUMO acceptor sites in Scm significantly reduced Scm sumoylation and led to increased association of Scm with the PRE (Smith et al. 2011). Consistent with the idea that SUMO negatively regulates Scm activity and therefore positively regulates *Ubx* expression, knockdown of SUMO in developing haltere discs results in an *Ubx*-like phenotype, i.e., a partial haltere-to-wing transformation.

The mechanism by which SUMO controls Scm and therefore polycomb group activity is unclear. Both Scm and the PRC1 component Polyhomeotic contain sterile alpha motif (SAM) domains, which are capable of mediating the formation of long protein filaments, and that may be required for chromatin compaction (Boettiger et al. 2016; Peterson et al. 2004). The functions

of the Scm SAM domain are complex: it is required for recruitment of Ubc9 and thus sumoylation, but it also appears to have an independent requirement in the recruitment of Scm to the PRE (Smith et al. 2011). We speculate that Scm sumoylation could modulate PcG function by modulating the role of the SAM domain in such processes as Scm recruitment, filament formation, and chromatin compaction.

15.2.3.2 Groucho

Groucho is a transcriptional co-repressor required for function of many of the transcriptional repressors that act throughout Drosophila development, including, the Hairy-Enhancer of split family factors, the Runt family factors, Engrailed, Dorsal, Capicua, and Brinker (Dubnicoff et al. 1997; Hasson et al. 2001; Jimenez et al. 1997; Paroush et al. 1994). Groucho functions, in part, by mediating the recruitment of Histone Deacetylase 1 (HDAC1) to its target genes (Turki-Judeh and Courey 2012).

Groucho is a sumoylation target (Nie et al. 2009). In mammalian cells, SUMO appears to positively regulate Groucho function by helping to mediate the recruitment of HDAC1 through a SIM in HDAC1 (Ahn et al. 2009). On the other hand, work in Drosophila suggests that SUMO antagonizes Groucho-mediated repression. Degringolade (Dgrn) a SUMO Targeted Ubiquitin Ligase (STUbL) appears to bind Groucho in a SUMO dependent manner leading to the sequestration and therefore inactivation of Groucho. Thus in the absence of SUMO, sequestration does not occur allowing for Groucho-mediated repression (Abed et al. 2011).

15.3 Conclusion

SUMO acts as a genetic switch that targets hundreds or thousands of proteins to regulate a wide variety of essential cellular and developmental processes. Illuminating its biological roles is as challenging as trying to arrive at a comprehensive understanding of the roles of other common protein modifications, such as phosphorylation, acetylation, and glycosylation (Lomeli and Vazquez 2011). Due to the pleiotropic functions of SUMO in development, global disruption of sumoylation is not usually instructive. Therefore, approaches such as mapping and mutating individual SUMO acceptor sites, SUMO-substrate fusions, and tissue-specific overexpression or knockdown of SUMO pathway components must be utilized to dissect specific SUMO functions from one another.

Another challenge to understanding the many biological roles of SUMO is the so-called "SUMO enigma" (Hay 2005). In most cases, it appears that only a small fraction of any given sumoylation target is conjugated to SUMO at any one time. Paradoxically, however, sumoylation of proteins such Scm, Sal, Groucho, HIPK, and Med often leads to near quantitative effects on the activity or subcellular localization of these proteins. While this enigma remains unresolved, two speculative non-mutually exclusive explanations are as follows. First, it is possible that cyclic rounds of conjugation and deconjugation are required for progress through a pathway. Second, perhaps deconjugation leaves behind a protein that still retains the memory of being sumoylated. For example, sumoylation could be required to overcome a kinetic barrier to the formation of a protein complex that remains stable after deconjugation has occurred.

The ease with which the Drosophila genome can be manipulated has allowed us to overcome the challenges described above. Since pathways regulated by SUMO are highly conserved across the eukaryotic domain, studies of sumoylation in Drosophila may provide insight into how SUMO leads to increased developmental complexity by diversifying protein function.

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Part III

Diseases

Sumoylation: Implications for Neurodegenerative Diseases

16

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Abstract

The covalent posttranslational modifications of proteins are critical events in signaling cascades that enable cells to efficiently, rapidly and reversibly respond to extracellular stimuli. This is especially important in the CNS where the processes affecting synaptic communication between neurons are highly complex and very tightly regulated. Sumoylation regulates the function and fate of a diverse array of proteins and participates in the complex cell signaling pathways required for cell survival. One of the most complex signaling pathways is synaptic transmission.

Correct synaptic function is critical to the working of the brain and its alteration through synaptic plasticity mediates learning, mental disorders and stroke. The investigation of neuronal sumoylation is a new and exciting field and the functional and pathophysiological implications are far-reaching. Sumoylation has already been implicated in a diverse array of neurological disorders. Here we provide an overview of current literature highlighting recent insights into the role of sumoylation in neurodegeneration. In addition we present a brief assessment of drug discovery in the analogous ubiquitin system and extrapolate on the potential for

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development of novel therapies that might target SUMO-associated mechanisms of neurodegenerative disease.

Keywords
Alzheimer's disease • Amyotrophic lateral sclerosis • Ischemia • Neuronal intranuclear inclusion disease • Parkinson's disease • Polyglutamine diseases • Drug targets

16.1 Introduction

Abnormalities in neuronal pathways can give rise to a diverse range of disease states. Prominent among these are neurodegenerative disorders that result in the progressive loss of neuronal populations. Clinical symptoms include impairments in speech and motor function, cognitive defects, and dementia. At the cellular level, pathologies relating to protein trafficking and degradation have been strongly implicated in neurodegeneration by the presence of ubiquitin and proteasome-related proteins in inclusion bodies in diseased neuronal tissues [reviewed in (Ciechanover and Brundin 2003)]. SUMO proteins have also been detected in inclusion bodies of patients suffering from various polyglutamine (polyQ) neurodegenerative disorders. Given its close relationship to the ubiquitin pathway and its ability to modify cellular processes, several groups have hypothesized that defects in protein sumoylation may be a key factor in these pathophysiological processes (Table 16.1).

Thus, the mechanisms, target proteins and consequences of sumoylation in the central and peripheral nervous systems have become the increasing focus of interest in recent years and several molecular mechanisms are emerging by which SUMO might contribute to neuropathologies. In particular, attention has been directed towards the potential roles of neuronal sumoylation on aberrant protein aggregation and cellular inclusion bodies or on polyQ-related pathologies since these are common to many neurological disease states (Ross and Poirier 2004). In addition, several reports suggest altered patterns of SUMO conjugation in situations of acute cell stress such as ischemia. Here we provide a summary of the current state of understanding of the field [for related reviews see also (Dorval and Fraser 2007; Henley et al. 2014; Krumova and Weishaupt 2013; Martin et al. 2007b)].

16.2 Parkinson's Disease

Parkinson's disease (PD) is a degenerative disorder of the CNS that impairs motor function and speech. It is the most common movement disorder and motor deficits are caused by progressive loss of dopaminergic neurons in the substantia nigra that project to the striatum. A defining cellular feature of PD is the accumulation of Lewy bodies, which are cytosolic protein aggregates often staining positive for ubiquitin and α-synuclein, in the affected neurons (Baba et al. 1998; Spillantini et al. 1997; Wakabayashi et al. 2007). Sumoylation is implicated in the neuropathology of PD with respect to a number of proteins [for reviews see Eckermann 2013; Guerra de Souza et al. 2016; Vijayakumaran et al. 2015)]; one such example is α-synuclein. While there has been extensive study of the role of α-synuclein in the pathogenesis of PD, the normal function of α-synuclein is as yet undefined.

Evidence suggests that α-synuclein is involved in presynaptic membrane trafficking where it might function as a molecular chaperone, assisting in the folding and refolding of synaptic

Table 16.1 Neurological conditions and putative SUMO substrates

Neurological disorder	SUMO substrate	References
Parkinson's disease	α-synuclein Parkin (non-covalent) DJ-1	Abeywardana and Pratt (2015), Dorval and Fraser (2006), Kim et al. (2011), Krumova et al. (2011), Kunadt et al. (2015), Oh et al. (2011), Shahpasandzadeh et al. (2014), Um and Chung (2006), and Shinbo et al. (2006)
Alzheimer's disease	Amyloid-β Tau BACE1	Li et al. (2003), Dorval and Fraser (2006), Luo et al. (2014), Takahashi et al. (2008) and Yun et al. (2013)
Ischemia	Drp1 HIF-1α	Guo et al. (2013) and Chan et al. (2011)
Huntington's disease	Rhes - HTT	Subramaniam et al. (2009, 2010)
DRPLA	Atrophin-1	Terashima et al. (2002)
SBMA	Androgen Receptor	Chan et al. (2002), Chua et al. (2015) and Mukherjee et al. (2009)
SCA-1	Ataxin-1 Ataxin-3 Ataxin-7	Guo et al. (2014), Ryu et al. (2010), Ueda et al. (2002), Zhou et al. (2013) and Janer et al. (2010)
ALS	SOD1 EAAT2	Fei et al. (2006), Niikura et al. (2014) and Foran et al. (2011, 2014)
NIID	unknown	Pountney et al. (2003)
Cell Stress	PML XBP1 HIF-1α	Han et al. (2010), Jiang et al. (2012) and Huang et al. (2009)

DRPLA denaturubro-pallidoluysian, *SBMA* spinobulbar muscular atrophy *SCA*-1 spinocerebellar ataxia 1, *ALS* amyotrophic lateral sclerosis, *NIID* neuronal intranuclear inclusion disease, *ALS* amyotrophic lateral sclerosis, *DJ-1* protein deglycase 1, *BACE1* beta secretase 1, *Drp1* dynamin-related protein 1, *HIF-1α* hypoxia-inducible factor 1 alpha, *Rhes* small guanine nucleotide-binding protein, *HTT* huntingtin, *SOD1* superoxide dismutase 1, *EAAT2* excitatory amino acid transporter2, *PML* promyelocytic leukemia protein, *XBP1* X-box binding protein 1

SNARE proteins, which facilitate synaptic transmission by mediating the fusion of synaptic vesicles with the cell membrane at the synapse (Chandra et al. 2005). Sumoylation has recently been shown to regulate the sorting of α-synuclein into extracellular vesicles in the CNS via the endosomal-sorting complex required for transport (ESCRT) (Kunadt et al. 2015). α-synuclein was identified as a SUMO1 substrate (Dorval and Fraser 2006). Coupled with the finding that SUMO1 marks subdomains within glial cytoplasmic inclusions of the synucleinopathy Multiple System Atrophy (MSA) (Pountney et al. 2005), these data suggest a role for sumoylation in the formation or maintenance of Lewy bodies in PD, via either α-synuclein or other, as yet unidentified, substrates. SUMO1 was also found in the halo of Lewy bodies colocalizing with α-synuclein in the brains of patients with PD and dementia with Lewy bodies. Proteasome inhibition induced the formation of aggregates and inclusions containing sumoylated α-synuclein. However, sumoylation did not affect the ubiquitination of α-synuclein, suggesting that proteasomal dysfunction results in the accumulation, and subsequently aggregation, of sumoylated α-synuclein (Kim et al. 2011). In a mouse model of PD, the levels of α-synuclein and SUMO1 were increased in the rotenone-lesioned hemisphere of both aged and adult mice. In comparison to adult mice, aged mice showed a smaller increase in SUMO1 and greater increase in α-synuclein, suggesting some of the changes observed were age-related (Weetman et al. 2013).

Sumoylation of α-synuclein is facilitated by the human polycomb protein 2 (hPc2), which acts as a SUMO E3 ligase, promoting its aggregation in fibroblasts treated with the proteasome

inhibitor MG-132. Surprisingly, this SUMO-mediated α-synuclein aggregation decreased staurosporine-induced cellular death, probably by directing the aggregates to aggresomes that are responsible for sequestering damaged proteins (Oh et al. 2011). Contradictorily, in another study, SUMO-modified α-synuclein remained soluble, whereas unmodified α-synuclein formed fibrils. An α-synuclein double mutant, where lysines 96 and 102 were mutated to arginines, showed impaired sumoylation and increased propensity for aggregation and cytotoxicity, supporting a role for sumoylation in promoting the solubility of aggregation-prone proteins (Krumova et al. 2011). In order to elucidate the effects of sumoylation on α-synuclein aggregation, a recent study used homogeneously sumoylated α-synuclein obtained from protein semi-synthesis. SUMO1 was found to be better aggregation inhibitor than SUMO3 and sumoylation at lysine 102 was better at preventing aggregation than modification at lysine 96 (Abeywardana and Pratt 2015). In agreement with SUMO increasing α-synuclein solubility, impaired sumoylation increased the number of cells with α-synuclein inclusions and reduced yeast growth, further suggesting that sumoylation may have a protective role. Moreover, and considering that one of the main pathophysiological mechanisms of neurodegenerative diseases is autophagy, sumoylation inhibition has been shown to prevent autophagy-mediated aggregate clearance. Interestingly, a defect in α-synuclein sumoylation was compensated by its phosphorylation, not only by partially rescuing autophagic degradation, but also by promoting proteasomal degradation (Shahpasandzadeh et al. 2014).

Several proteins involved in the pathogenesis of PD, including α-synuclein and ubiquitin carboxy-terminal-hydrolase-L1 (UCH-L1), are linked to the ubiquitin proteasome system [reviewed in (Ciechanover and Brundin 2003)]. Indeed, interplay between SUMO and ubiquitin of the same target protein has been demonstrated in a number of cases. One protein whose interactions with both the SUMO and ubiquitin systems have been studied in relation to PD is parkin, which non-covalently interacts with SUMO1 *in vitro* and *in vivo* (Um and Chung 2006). Parkin is an E3 ligase in the ubiquitin pathway. Importantly, mutations in the parkin gene have been reported to account for many of the familial cases of PD [reviewed in (Tan and Skipper 2007)]. Parkin ubiquitinates the SUMO E3 ligase RanBP2, leading to its degradation. Further, a non-covalent interaction between SUMO1 and parkin up-regulates the ubiquitin-ligase activity of parkin in a negative feedback loop (Um et al. 2006). This example also draws attention to the growing importance of conjugation-independent SUMO functions (Kerscher 2007) and underscores the significance of non-covalent SUMO interactions in PD pathogenesis.

Modulation of transcriptional regulation by SUMO has also been shown to play a role in the neuropathology of PD [reviewed in (Eckermann 2013; Guerra de Souza et al. 2016; Vijayakumaran et al. 2015)]. The multifunctional protein DJ-1 plays a role in regulation of transcription of a number of genes, many of which are involved with the cellular response to oxidative stress (Taira et al. 2004; Takahashi et al. 2001). The onset of PD has been linked to the loss of DJ-1 function, which has been shown to interact with SUMO E3 ligases (Takahashi et al. 2001), and was identified as a direct SUMO substrate (Shinbo et al. 2006).

The significance of the SUMO pathway in DJ-1 function is supported by the identification of two DJ-1 mutants. Firstly, mutation of the SUMO acceptor lysine, K130, abolishes all known functions of DJ-1 in cultured cells, including ras-dependent transformation, cell growth promotion, and anti-UV-induced apoptosis (Shinbo et al. 2006). A second mutation, L166P, has been found in PD patients. This mutant form of DJ-1 has been reported to be unstable and to be degraded, in part, by the ubiquitin-proteasome system (Olzmann et al. 2004; Moore et al. 2003). The L166P mutant also exhibits increased levels of sumoylation compared to wild-type DJ-1 (Moore et al. 2003). One interpretation of these results is that the mutants are either poly-sumoylated on target lysine residues or aberrantly sumoylated on residues that are not sumoylated under normal conditions. Thus,

incorrect sumoylation of the L166P mutant may promote aggregation of DJ-1, which could be the cause of the increase in protein insolubility. In turn, decreased DJ-1 solubility might lead to the observed increase in protein degradation by the proteasome (Shinbo et al. 2006).

16.3 Alzheimer's Disease

Alzheimer's disease (AD) is recognized as the most common cause of chronic dementia among the aging population. The onset of the disease is characterized by a progressive decline in cognitive function whereby mild impairments in memory are surpassed by increasingly significant higher cognitive deficits in language, recognition and skilled movements. Pathological studies have indicated that certain areas of the brain are predisposed to exhibiting neuritic plaques and neurofibrillary tangles, which are hallmarks of AD (Tiraboschi et al. 2004).

It is widely believed that amyloid-beta (Aβ) peptide, produced by β-secretase (BACE) processing of the amyloid precursor protein (APP) via the amyloidogenic proteolytic pathway, is a primary causative factor in AD (Wolfe 2006). Accumulation of extracellular Aβ results in the formation of neuritic plaques. Using an *in vitro* translation expression cloning strategy, APP was identified as a potential SUMO1 substrate (Gocke et al. 2005). Subsequently, it has been shown that SUMO proteins covalently modify two lysines of APP *in vivo* and that sumoylation of these lysine residues is associated with decreased levels of Aβ aggregates (Zhang and Sarge 2008). Furthermore, over-expression of the SUMO E2 enzyme Ubc9 and SUMO1 results in decreased levels of Aβ aggregates in cells transfected with the familial AD-associated mutant APP, suggesting a potential therapeutic effect of up-regulating the activity of the cellular sumoylation machinery as an approach against AD (Zhang and Sarge 2008). In contrast, however, SUMO1 has been reported to increase Aβ levels (Yun et al. 2013). The same authors have further shown that over-expression of SUMO1 increased autophagic activation in neuroglioma H4 cells and autophagy inhibitors reduced SUMO1-mediated increase in Aβ. These findings suggest that SUMO1 might accelerate the accumulation of autophagic vacuoles and promote Aβ production (Cho et al. 2015a).

Apparently contradictory results have been reported regarding the effects of SUMO2/3 on Aβ production. One study found that over-expression of SUMO3 dramatically reduces Aβ production and that dominant-negative SUMO3 mutants significantly increase Aβ production (Li et al. 2003). The same study also reported that a mutant poly-sumoylation-deficient SUMO3 had an opposite effect on Aβ generation to that by wild-type SUMO3, which can form polymeric chains on target proteins. These data suggest that poly-sumoylation reduces whereas mono-sumoylation enhances Aβ generation (Li et al. 2003). In contrast, however, a study has found that over-expression of SUMO3 significantly increased Aβ secretion and that these effects were independent of its covalent attachment or chain formation, since mutants lacking the motifs responsible for SUMO chain formation or SUMO conjugation caused similar changes in Aβ (Dorval et al. 2007). These data further suggest that SUMO3 may be acting non-covalently in regulating Aβ production; clearly, additional work will be required to determine the exact role of SUMO3 in this pathway. Adding further complexity to the role of sumoylation in APP processing and Aβ generation, a study has shown that over-expression of SUMOs inhibits the activity of a gene promoter for BACE (Fang et al. 2011), whereas another study has shown that over-expression of SUMOs increased BACE1 levels (Yun et al. 2013). This later study has also shown that SUMO1 protein levels were increased in the Tg2576 transgenic mice, as well as in primary neurons exposed to Aβ. Contrastingly, in primary astrocytes exposed to Aβ, SUMO1 and Ubc9 levels decreased whereas glial fibrillary acidic protein (GFAP) levels increased. SUMO1 over-expression prevented GFAP increase suggesting a role for SUMO-1 conjugation in keeping astrocytes in a non-reactive state (Hoppe et al. 2013).

Neurofibrillary tangles are another pathological hallmark of AD. The main component of

tangles is a hyper-phosphorylated form of the microtubule-associated protein Tau. Pathological aggregation of Tau is a prominent feature of many neurodegenerative diseases collectively called tauopathies, and the phosphorylation of Tau has been proposed to be a key factor in the neurodegenerative process (Ballatore et al. 2007). Tau has been identified as a target for sumoylation preferentially by SUMO1, compared with SUMO2/3 (Dorval and Fraser 2006). Inhibition of the proteasome pathway increases Tau ubiquitination and decreases Tau sumoylation, suggesting that a competitive mechanism between ubiquitin and SUMO may regulate Tau stability (Dorval and Fraser 2006). A study utilized a transgenic mouse expressing a mutant form Tau (P301L) which has been identified as a causative element for fronto-temporal dementia and parkinsonism linked to chromosome 17 (FTDP-17) to examine the role of Tau sumoylation in the pathogenesis of PD (Takahashi et al. 2008). Analysis of the localization of SUMO1 protein in APP transgenic mice and mutant Tau transgenic mice found that SUMO1 immunoreactivity co-localized with phosphorylated Tau aggregates in amyloid plaques of APP transgenic mice. By contrast, no SUMO1 immunoreactivity was observed in phosphorylated Tau aggregates of mutant Tau transgenic mice, implying that SUMO might preferentially or exclusively associate with normally phosphorylated Tau, and not hyper-phosphorylated Tau. Recently, SUMO1 conjugation at lysine 340 was shown to cause Tau hyper-phosphorylation, which could be blocked by mutation of Tau at this specific site, as well as by ginkgolic acid-mediated SUMO inhibition. Likewise, Tau hyper-phosphorylation promoted its sumoylation, which decreased Tau solubility and inhibited its ubiquitination and subsequently degradation (Luo et al. 2014). These findings potentially indicate a novel pathway for sumoylation in the regulation of Tau, which could underlie disease-related processes.

Global SUMO1 and SUMO2/3 conjugation, as well as Ubc9 and SENP1 levels, were unchanged in the hippocampus, cortex and cerebellum of 9-month old Tg2576 transgenic AD mouse model, whereas some individual high molecular SUMO1 and SUMO2/3 bands were decreased in the cortex (McMillan et al. 2011). Similarly, global SUMO2/3 conjugation was decreased in 17-month old mice, whereas global SUMO1 conjugation, as well as Ubc9 and SENP-1 levels, were increased in the cortex and hippocampus of 3- and 6-month old Tg2576 mice (Nisticò et al. 2014). Sumoylation was also impaired in both Tg2576 mice and human post-mortem AD hippocampal tissue (Lee et al. 2014a). The same study also showed that enhanced sumoylation via Ubc9 over-expression rescued Aβ-induced deficits in long-term potentiation (LTP) and hippocampal-dependent learning and memory. Interestingly, the reduced learning and memory abilities found in 25-month old mice, when compared to 7-month old mice, were accompanied by a matching decrease in SUMO3 conjugation levels (Yang et al. 2012b).

Genetic variations of the Ubc9 gene (UBE2I), the sole SUMO conjugating enzyme, were analyzed in blood samples from AD and mild cognitive impairment patients, as well as healthy controls, where some single nucleotide polymorphisms were associated with late onset AD and mild cognitive impairment (Ahn et al. 2009). More recently, elevated SUMO1 protein levels were found in the plasma of patients with AD, compared to cognitively normal controls (Cho et al. 2015b). This increased SUMO1 correlated well to decreased Mini-Mental State Examination, suggesting that SUMO may be useful as a biomarker for those at risk for AD. For further detailed reviews on the role of protein sumoylation in AD see (Hoppe et al. 2015; Martins et al. 2016).

16.4 Polyglutamine Diseases

Polyglutamine (polyQ) disorders are a family of neurodegenerative disorders characterized by expression of a disease-specific protein containing a toxic stretch of glutamine repeats. The number of polyQ repeats varies in number from 35 to over 300 and the presence of the polyQ stretches in specific genes has been shown to be causative in a number of neurodegenerative

disorders that include Huntington's disease (HD), denatorubro-pallidoluysian atrophy (DRPLA), spinocerebellar ataxias (SCAs), and spinobulbar muscular atrophy (SBMA) [reviewed in (Craig and Henley 2015; Gatchel and Zoghbi 2005)]. Although the proteins involved in each of these disorders have unique neurological functions and localizations, the common phenotype of abnormal aggregation of polyQ-containing proteins and formation of cytoplasmic and/or intranuclear inclusions in neurons is a common feature. An increasing number of SUMO targets have been implicated in the neurodegeneration associated with polyQ disorders. Coupled with a body of evidence suggesting that ubiquitin has been found in the inclusion bodies of most, if not all, polyQ disorders, sumoylation, as well as ubiquitination, is emerging as a critical player in the formation of protein aggregates and the associated neurodegeneration in the family of polyQ diseases.

16.4.1 Huntington's Disease

Huntington's disease (HD) is a hereditary, progressive neurodegenerative disease characterized by the gradual atrophy of neurons of the striatum (Gil and Rego 2008). HD is the result of an abnormal polyQ stretch located in the N-terminal domain of the Huntingtin (HTT) protein, which accumulates in affected neurons. The same lysine residues in the N-terminal domain of HTT have been shown to be a target for both SUMO and ubiquitin (Steffan et al. 2004). Although the relationship between sumoylation and ubiquitination of HTT has not yet been clarified, a study in a *Drosophila* model demonstrated that sumoylation of polyQ HTT resulted in an increase in protein solubility and a reduction in aggregation. Furthermore, its abundance in neurons was increased upon sumoylation (Ehrnhoefer et al. 2011; Steffan et al. 2004). One theory, given that SUMO and ubiquitin share the target lysines in HTT, is that a balance between HTT sumoylation and ubiquitination is responsible for the stability, abundance, and function of HTT in normal neurons and polyQ HTT in diseased brain. It is important to note that disruption of the target lysines, which destroys both sumoylation and ubiquitination, reduces neurodegeneration in the *Drosophila* HD model, suggesting that SUMO plays a role in HTT function and Huntington's neuropathology beyond merely competing with ubiquitination. Adding further complexity to the role of sumoylation in HD, Rhes (small guanine nucleotide-binding protein) was demonstrated to bind to polyQ HTT and elicit its sumoylation, which is associated with polyQ HTT disaggregation and cell death (Subramaniam et al. 2009, 2010).

16.4.2 Denatorubro-Pallidoluysian Atrophy

DRPLA is a familial, progressive neurodegenerative disorder, characterized by symptoms including ataxia and other motor dysfunctions, dementia, and seizure. A polyQ expansion in the atrophin-1 protein has been identified as the causative mutation in DRPLA. Atrophin-1 can be found in both the nuclear and cytoplasmic compartments of neurons. Although its function has not yet been fully elucidated (Schilling et al. 1999; Yazawa et al. 1995), atrophin-1 belongs to a family of nuclear co-repressors (Wang et al. 2008). Atrophin-1 has been identified as a SUMO1 substrate (Terashima et al. 2002). Acceleration in the formation of nuclear aggregates and increased apoptosis has been demonstrated in PC12 cells when polyQ atrophin-1 was co-expressed with wild-type SUMO1. Conversely, polyQ atrophin-1 aggregates were reduced and cell survival was enhanced in the same system upon co-expression of non-conjugatable SUMO1 with polyQ atrophin-1 (Terashima et al. 2002). Several mechanisms might account for these results. It is plausible that sumoylated polyQ atrophin-1 is aberrantly trafficked and/or that sequestration of the sumoylated protein promotes nuclear aggregation. Another possible explanation is that SUMO disrupts or competes with proteasome-mediated degradation, again promoting aggregation of sumoylated polyQ atrophin-1.

16.4.3 Spinobulbar Muscular Atrophy

SBMA is an X-linked neuromuscular and endocrine disease, resulting from a pathogenic polyQ expansion in the androgen receptor (AR) that provides another example of the interplay between sumoylation and ubiquitination. AR is a nuclear hormone receptor, which functions as both a transcription factor and a signaling protein, activating a number of cellular processes [reviewed in (Michels and Hoppe 2008)]. Wild-type AR has been shown to be sumoylated, and the sumoylated form has reduced transcriptional activity (Poukka et al. 2000). In a *Drosophila* model, nuclear and cytoplasmic aggregates of polyQ AR have been detected (Chan et al. 2002), resulting in progressive neurodegeneration. In the same model, a catalytically inactive form (C175S) of the SUMO activating enzyme subunit Uba2, the *Drosophila* homolog of human SAE2, was shown to significantly enhance degeneration caused by polyQ AR. Sumoylation of AR significantly reduced polyQ-mediated aggregation (Mukherjee et al. 2009). In addition, impaired polyQ AR sumoylation enhanced its transcriptional activity, rescuing exercise endurance and muscular atrophy, as well as extending survival (Chua et al. 2015).

With respect to the role of ubiquitination in the pathogenesis of SBMA, compromise of the proteasome pathway by over-expression of an inactive proteasome beta-subunit leads to a degenerative phenotype (Chan et al. 2002). Hsp70, a molecular chaperone involved in protein folding, appears to act in an additive manner to regulate aggregation and the resulting neurodegeneration. Molecular chaperones have been reported in the inclusions formed by polyQ AR and a dominant-negative mutant form of Hsp70 produces the degenerative phenotype observed by blockade of the ubiquitin-proteasome system, similar to the degenerative phenotype of the SUMO E1 C175S mutant. However, functional Hsp70 is unable to rescue degeneration induced by the mutant SUMO E1 enzyme, consistent with sumoylation playing a role down-stream of protein aggregation and/or degradation. Taken together, these data suggest that both sumoylation and ubiquitination pathways are functional and intertwined in diminishing the pathogenic nature of the polyQ AR in SBMA.AR in SBMA.

16.4.4 Spinocerebellar Ataxias

The SCAs are a family of dominantly inherited progressive neurodegenerative disorders characterized by slowly progressive defects in coordination of gait and are often associated with poor coordination of hands, speech, and eye movements. Atrophy of the cerebellar Purkinje layer is a hallmark of the SCAs, of which over 20 specific disorders have been identified. PolyQ expansion in ataxins, a family of phospho-proteins, has been identified as the disease-causing mutation in a number of SCA disorders. SCA1 is the result of expanded polyQ in ataxin-1 (Zoghbi and Orr 2000). Wild-type ataxin-1 has been shown to be sumoylated on at least five lysine residues (Riley et al. 2005), and polyQ ataxin-1 displays reduced levels of sumoylation. Furthermore, an increase in the length of the ataxin-1 polyQ expansion negatively regulates its sumoylation level. Sumoylation of ataxin-1 appears to be phosphorylation-dependent, as sumoylation levels of phosphorylation-deficient mutant (S776A) polyQ ataxin-1 are similar to that of wild-type ataxin-1. The implications of sumoylation of ataxin-1 in SCA are not yet fully understood, though these provide intriguing examples of inhibitory interplay between phosphorylation and the SUMO pathway. Sumoylation of ataxin-1 promotes its aggregation, with oxidative stress further increasing sumoylation/aggregation, which can be prevented by c-Jun N-terminal kinase (JNK) inhibition (Ryu et al. 2010). In a mouse model of SCA1, promyelocytic leukemia (PML), which is a protein involved in polyQ aggregates that also has SUMO ligase activity, was shown to sumoylate misfolded proteins, which were then ubiquitinated by the SUMO-dependent ubiquitin ligase RNF4 and targeted for proteasomal degradation (Guo et al. 2014).

Sumoylation of ataxin-1 also appears to be dependent on nuclear localization (Riley et al.

2005). Ataxin-1 with a mutant nuclear localization signal exhibits significantly reduced sumoylation although levels of ataxin-1 nuclear localization and the presence of nuclear inclusions are identical in wild-type and sumoylation deficient ataxin-1. These findings suggest a role for SUMO modification in modulating the efficiency of nuclear import/export of ataxin-1, or in regulation of ataxin-1 trafficking within the nucleus, both in keeping with the key role SUMO plays in regulating subcellular localization and transport of many proteins between the nucleus and cytoplasm.

In addition to ataxin-1, ataxin-7 was shown to be sumoylated *in vitro* and *in vivo*, the modified lysine identified as K257. The expanded polyQ motif did not disrupt ataxin-7 sumoylation, whereas impaired sumoylation of site-directed mutated poyQ ataxin-7 increased aggregates and caspase-3 positive inclusions, which are both cytotoxic (Janer et al. 2010). More recently, SUMO1 conjugation of ataxin-3 on lysine 166 was shown to increase protein stability and apoptotic cell death, despite not affecting its subcellular localization, ubiquitination or aggregation (Zhou et al. 2013).

16.4.5 Neuronal Intranuclear Inclusion Disease

Neuronal intranuclear inclusion disease (NIID) is a rare neurodegenerative disorder, either sporadic or familial, characterized by ataxia. NIID presents as ataxia in younger patients and progressive ataxia and dementia in adults. Although no polyQ expansions have been reported, NIID shares some of the histopathology of the polyQ disorders. A defining feature of NIID pathology is the presence of insoluble intranuclear inclusions in nearly all central, peripheral and autonomic neurons. Inclusions from NIID show weak immunoreactivity to polyQ antibodies (Lieberman et al. 1998; Pountney et al. 2003) and ataxin-1 and -3, both implicated in the SCAs, have been detected in aggregates from NIID patients (Lieberman et al. 1999).

Immunohistochemical data from human samples has implicated SUMO in the neuropathology of NIID. SUMO1 immunostaining is extensive in inclusions from unrelated cases of familial (Lieberman et al. 1998) and juvenile NIID (McFadden et al. 2005), as well as in sporadic cases of NIID (Takahashi-Fujigasaki et al. 2006). Ubiquitin has also been detected in NIID inclusions, in immunostaining patterns that overlap completely with SUMO1 (Pountney et al. 2003; Wiltshire et al. 2010). Intranuclear inclusions, identical to those found in neurons, are also present in adipocytes, fibroblasts, and sweat gland cells. All these inclusions display positive staining for anti-ubiquitin and anti-SUMO1 antibodies, suggesting that this similar pathological background between dermal and neuronal cells could be useful for diagnosis (Sone et al. 2011).

While specific SUMO substrates have yet to be identified in the pathology of NIID, studies of major proteins components of NIID aggregates (Takahashi-Fujigasaki et al. 2006) might provide clues to the identity of the SUMO targets. For example, the transcriptional co-repressor histone deacetylase HDAC4 is a known SUMO substrate (David et al. 2002; Zhao et al. 2005). Interestingly, HDAC4 has been proposed to function as a SUMO E3 ligase (Zhao et al. 2005) and therefore might be responsible for the presence of SUMO in NIID aggregates. A separate study utilized a SUMO immuno-capture method coupled with mass spectrometry to identify SUMO-associated components in inclusions from NIID patients (Pountney et al. 2008). The proteins NSF, unc-18-1 and dynamin, all involved in membrane trafficking of proteins, and the molecular chaperone Hsp90 were identified and confirmed by immunohistochemistry. The potential significance of these findings in NIID neuropathology, however, remains to be determined.

Another potential target through which SUMO might contribute to aggregates found in NIID inclusions is PML nuclear bodies (NB), nuclear structures implicated in proteasome-mediated protein degradation. Inclusions from both sporadic and familial NIID patients have been shown to be immunoreactive for the protein PML, as well as for ubiquitin and SUMO

(Takahashi-Fujigasaki et al. 2006). These findings support the theory that proteins targeted to the proteasome are retained in NBs due to dysfunction of either the SUMO or ubiquitin pathways.

Collectively the polyQ disorders and NIID comprise a set of rare but relatively well-studied neurodegenerative diseases. Although a very small subset of the population is afflicted with these disorders, the roles of sumoylation in regulating protein aggregation and accumulation that are implicated in more common disorders like PD are conserved, and the insights gained from studying the effects of sumoylation in this family of disorders will certainly aid in the characterization of sumoylation in more prevalent neurodegenerative diseases.

16.5 Cellular Stress

When cells are exposed to metabolic, thermal, physical or toxic stress, various cellular responses are activated, which help cells to withstand the stressful conditions and to restore cell functions. A growing body of evidence suggests rapid changes in SUMO conjugation in response to various cellular stresses such as oxidative stress, osmotic stress, or heat shock (Navascues et al. 2008; Sramko et al. 2006; Tempe et al. 2008; Wuerzberger-Davis et al. 2007; Zhang et al. 2008). While the role of sumoylation in cellular stress with regard to neuronal function is less well studied than in the periphery, it is likely that the recurring themes of transcriptional regulation and protein degradation/recycling observed in the periphery will also be relevant in the brain.

The initial observation suggesting modulation of SUMO conjugation in response to cellular stress was a study reporting that various environmental stresses (osmotic and oxidative stress, heat shock) increase global sumoylation by SUMO2/3 isoforms, but have little effect on SUMO1 conjugation (Saitoh and Hinchey 2000). This report suggested that the difference between the isoforms was due to the lower amounts of free SUMO1 compared to SUMO2/3 and that free SUMO2/3 might act as a pool for immediate response to cellular stresses.

Cellular stresses affect the sumoylation status of various transcription factors involved in the response to stress through a variety of mechanisms, which include regulation of expression of specific substrates, modulation of the sumoylation machinery abundance or activity, or modulation of sumoylation through induction of stress-induced substrate phosphorylation [reviewed in (Tempe et al. 2008)]. For example, changes in global sumoylation levels were observed in HeLa cells exposed to hydrogen peroxide (H_2O_2), accompanied by SENP3 increase and redistribution from the nucleolus to the nucleoplasm. Nucleoplasmic SENP3 enhanced hypoxia-inducible factor-1 (HIF-1) transcriptional activity by de-sumoylating HIF-1 co-activator p300 (Huang et al. 2009). Similarly, mild oxidative stress induced by low doses of H_2O_2 increased SENP3 levels and promoted SENP3 co-localization with PML bodies (Han et al. 2010). As another example, XBP1 (transcription factor X box-binding protein 1), which is a key component of the endoplasmic reticulum (ER) stress response, can be sumoylated (Chen and Qi 2010). De-sumoylated XBP1 shows increased transcriptional activity, whereas SENP1 knockdown caused accumulation of sumoylated XBP1 and down-regulation of XBP1 target genes in response to ER stress (Jiang et al. 2012).

Oxidative stress conditions increase activation of JNK and promote cell death in SH-SY5Y cells. SUMO1 over-expression further increased phosphorylation of JNK and exacerbated cell death, whereas increased SENP1 de-sumoylation reduced H_2O_2-induced cell death (Feligioni et al. 2012). The SUMO E3-ligase PIAS1 (protein inhibitor of activated STAT1) regulates reactive oxygen species (ROS)-mediated JNK activation. Under oxidative stress conditions, PIAS1 knockdown prevented ROS-induced hyper-sumoylation and increased JNK activity in human endometrial stromal cells, suggesting that sumoylation inhibition is needed for induction of several cellular protective genes in response to oxidative stress (Leitao et al. 2011).

16.6 Amyotrophic Lateral Sclerosis

A potential link between oxidative stress, sumoylation, and disease exists in amyotrophic lateral sclerosis (ALS), also known as motor neuron disease. ALS is a degenerative disorder that affects motor neurons in the brain and spinal cord. ALS is characterized by generalized weakness, and is accompanied by muscle atrophy and progressive paralysis. ALS is usually sporadic but can also be inherited. Mutations in superoxide dismutase 1 (SOD1), an abundant metalloenzyme, have been identified in a number of familial cases of ALS (Johnson and Giulivi 2005). SOD1 acts as a cellular antioxidant, and plays a key role in detoxification of cells during oxidative stress. Wild-type human SOD1 has been reported to be sumoylated (Fei et al. 2006), as has an ALS-related mutant SOD1, which resulted in enhanced aggregation in cell culture models. Inclusion bodies can be detected in these models that co-localize with SUMO1, consistent with accumulation of sumoylated SOD1. Both SUMO1 and SUMO2/3 were able to modify lysine 75 of mutant SOD1 in a cell line model. However, only SUMO3 conjugation increased SOD1 protein stability and accelerated intracellular aggregate formation, suggesting that sumoylation by SUMO3 contributes to the formation of the intracellular inclusions underlying the pathogenesis of ALS (Niikura et al. 2014).

Another SUMO target that might be involved in ALS is the astroglial glutamate transporter EAAT2 (excitatory amino acid transporter 2). In the SOD1-G93A mouse model of ALS, the cytosolic carboxy-terminal domain of EAAT2 is cleaved and conjugated to SUMO1. This fragment was shown to accumulate in the nucleus of spinal cord astrocytes where it triggers astrocyte-mediated neurotoxic effects (Foran et al. 2011). A fraction of full-length EAAT2 was shown to be constitutively sumoylated in primary astrocytes *in vitro* and in the CNS *in vivo*. The extent of EAAT2 sumoylation did not change during the course of ALS in the SOD1-G93A mouse model and was not affected by mutant SOD1 expression in cultured astrocytes. Sumoylated EAAT2 displayed intracellular location, whereas non-sumoylated EAAT2 was mostly found in the plasma membrane. Consequently, EAAT2 desumoylation increased EAAT2-mediated glutamate uptake in primary astrocytes (Foran et al. 2013, 2014). These findings suggest that in addition to the role sumoylation plays in the cellular adaptation to oxidative stress, it can also play a role in counterbalancing excitotoxicity, both of which are intimately associated with ALS.

16.7 Ischemia

Cerebral ischemia is a severe form of hypoxic stress characterized by the excitotoxic death of neurons in the infarct region. Interestingly, levels of SUMO1 expression are enhanced following hypoxic stress (Shao et al. 2004), as are levels of SUMO2/3 following various cellular stresses (Saitoh and Hinchey 2000). This increase in global sumoylation as a result of hypoxic stress has been confirmed in multiple models of cerebral ischemia [reviewed in (Cimarosti and Henley 2008; Yang et al. 2008a)]. The first studies to indicate that sumoylation contributes to the protection of neurons under hypoxic conditions examined hibernation of the ground squirrel (Lee and Hallenbeck 2006; Lee et al. 2007). Hibernating squirrels lower their energy consumption, blood flow and body temperature to otherwise lethal levels. However, due to specialized adaptive changes, the animal suffers no discernable CNS damage or cellular loss. Intriguingly, massive sumoylation by both SUMO1 and SUMO2/3 occurs during hibernation of the ground squirrel. Although the cellular mechanisms and target proteins underlying this effect remain to be determined, these studies suggest that SUMO may play a similar role in response to pathological human conditions.

The hypothesis that sumoylation serves as a neuroprotective response to hypoxia is supported by the observation that both transient and permanent global or focal cerebral ischemia induce a rapid, dramatic and long lasting increase in SUMO conjugation (Cimarosti et al. 2008; Yang et al. 2008b, c). After transient focal cerebral

ischemia, increased SUMO conjugation was particularly prominent in neurons located at the border of the ischemic territory where SUMO proteins were found to accumulate in the nucleus (Yang et al. 2008b). The authors attributed this observation to the nuclear translocation of sumoylated proteins. In addition, decreases in the levels of excitatory AMPA- and kainate-type glutamate receptors have been reported following transient occlusion of the middle cerebral artery in rats (Cimarosti et al. 2008). Coupled to the report that sumoylation regulates endocytosis of the kainate receptor subunit GluR6 (Martin et al. 2007a), these findings suggest the possibility that sumoylation may protect against injury during ischemia via down-regulation of glutamate-mediated synaptic transmission and the resulting excitotoxic damage.

This SUMO-mediated neuroprotection hypothesis was further investigated in cultured cell lines and neurons exposed to oxygen and glucose deprivation (OGD), a model of ischemia. Preconditioning neurons with OGD increased SUMO1 conjugation levels (and, to a lesser extent those of SUMO2/3) and decreased vulnerability to OGD. Similarly, SUMO1 or SUMO2 over-expression in neurons and SH-SY5Y cells increased survival after OGD. In contrast, RNAi knockdown of SUMO1 reduced cellular survival after OGD and attenuated preconditioning-mediated protection (Lee et al. 2009). Paradoxically, an increase in SUMO2/3, but not SUMO1, conjugation of proteins was found in neuronal cultures exposed to OGD, which was reduced when cultures were preconditioned with OGD or hypothermia (Loftus et al. 2009). Moreover, silencing SUMO2/3 in primary cultured neurons did not show any detrimental effects under basal conditions. However, when these neurons lacking SUMO2/3 were exposed to OGD a significant increase in cellular death was observed in comparison to neurons expressing SUMO2/3 (Datwyler et al. 2011). In agreement with these findings, SUMO1 and SUMO2/3, as well as SENP1 were increased, whereas Ubc9 remained unchanged in hippocampal neurons exposed to OGD. SENP1 over-expression decreased neuronal survival; further supporting that sumoylation might be part of an endogenous neuroprotective response to stress (Cimarosti et al. 2012). These *in vitro* findings were corroborated by an *in vivo* study showing that genetically modified mice, which overexpress Ubc9 (and consequently increased SUMO1 conjugation), presented a smaller infarction volume following focal ischemia than wild-type mice (Lee et al. 2011).

Further studies have shown that OGD treatment under hypothermic conditions increased global sumoylation and prevented cell death in SH-SY5Y cells and rat cortical neurons. Hypothermia in wild-type mice subjected to permanent focal ischemia also increased global sumoylation and protected from ischemic damage (Lee et al. 2014b). Rats exposed to moderate hypothermic cardiopulmonary bypass showed increased levels and nuclear accumulation of SUMO2/3-conjugated proteins, whereas deep hypothermia in primary neurons caused only a moderate rise in SUMO2/3-conjugated proteins (Wang et al. 2012). In differentiated SH-SY5Y cells, harmful OGD decreased Ubc9 levels and promoted apoptosis, which was prevented by isoflurane preconditioning. Ubc9 knockdown in rats increased cerebral infarct volumes and attenuated the neuroprotective effect mediated by isoflurane preconditioning (Tong et al. 2015). Taken together, these findings suggest that increased global sumoylation may be part of the molecular mechanisms involved in preconditioning and hypothermia-induced ischemic tolerance.

Various non-neuronal studies have begun to uncover the mechanisms underlying enhanced sumoylation and potential SUMO targets in the cellular response to hypoxic stress. For example, RSUME protein (RWD-containing sumoylation Enhancer) that is exclusively expressed under hypoxic conditions has been reported to globally enhance sumoylation (Carbia-Nagashima et al. 2007), suggesting a possible mechanism for the up-regulation of sumoylation during hypoxia. OGD-induced changes in levels of specific sumoylated proteins were quantified in neuroblastoma B35 cells expressing HA-SUMO3 using stable isotope labeling with amino acids in cell culture (SILAC). Hundreds of putative

SUMO3-conjugated proteins were identified, including several transcription factors (Yang et al. 2012a).

As mentioned above, and widely accepted, many SUMO targets are transcription factors (Heun 2007; Seeler and Dejean 2003) and one such protein likely to play a role in the neuronal response to hypoxic stress induced by ischemia is the transcription factor HIF1α. Sumoylation of HIF1α and the resulting increase in HIF1α stability and transcriptional activity in response to hypoxia has been well documented (Bae et al. 2004; Shao et al. 2004), but the mechanisms and consequences of this increase remain unclear. HIF1α is responsible for the transcription of various proteins expressed during the cellular response to hypoxic stress, including RSUME, which is required for the hypoxia-mediated increase in HIF1α sumoylation (Carbia-Nagashima et al. 2007). However, a study proposes that rather than stabilizing HIF1α, sumoylation actually targets HIF1α to the proteasome during hypoxia (Cheng et al. 2007). The authors provide evidence for an essential role of SENP1 in the rescue of sumoylated HIF1α from degradation by reversal of sumoylation of HIF1α despite global up-regulation of sumoylation during hypoxia. Future studies will be required to elucidate the specific roles of the SUMO pathway on hypoxia-induced HIF1α transcription and stability. Nevertheless, SUMO1 conjugation to HIF-1α ameliorated brain stem cardiovascular regulatory failure in an experimental model of brain death (Chan et al. 2011).

Another protein likely to be involved in the neuronal response to hypoxic stress induced by ischemia is the GTPase dynamin-related protein 1 (Drp1), which plays a major role in regulating mitochondrial fission. It has recently been shown that OGD-induced decrease in SENP3 prevented Drp1 de-sumoylation (Guo et al. 2013). Sumoylated Drp1 localization at the cytosol suppressed Drp1-mediated cytochrome c release and caspase-mediated cell death. SENP3 recovery during the reoxygenation period after OGD allows de-sumoylation of Drp1, which facilitates Drp1 localization at the mitochondria and promotes fragmentation and cytochrome c release. This study identified another example of a specific insult-modified SUMO substrate, which is important during cell stress, and revealed a potential therapeutic target for promoting neuroprotection after ischemia or other neurodegenerative conditions.

In summary, the above outlined potential roles of sumoylation in response to cellular stress, both at the neuronal membrane and the nucleus, suggest that the increased SUMO conjugation induced by cerebral ischemia is likely to have a major effect on the fate of cells exposed to a transient reduction or interruption of blood supply, and also imply that the sumoylation process could provide an exciting new target for therapeutic intervention [reviewed in (Lee and Hallenbeck 2013; Silveirinha et al. 2013; Yang et al. 2008a)].

16.8 Sumoylation as a Potential Drug Target

Greater understanding of the roles of sumoylation in neurological disorders leads to the exploration of the potential of modulating SUMO for therapeutic effect. For example, several lines of evidence suggest that sumoylation is massively increased under ischemic conditions. If general or specific protein sumoylation should prove to be neuroprotective, therapies might be designed to temporarily enhance sumoylation in the region surrounding the infarct core. Conversely, several of the disease states discussed in this chapter implicate increased levels of sumoylation in the neuropathology associated with neurodegenerative disease states. In this case, down-regulation of target-specific sumoylation may prove therapeutically beneficial.

The SUMO pathway plays a central role in a number of cellular pathways. Therefore, care must be taken in the design of SUMO modulators. There is, however, precedence for the successful development of modulators of posttranslational modification. In the ubiquitin system, analogous in many ways to the SUMO pathway, a number of drugs have shown promise in clinical trials (Nalepa et al. 2006). Potential

therapeutics could be designed in a number of ways. For example, drugs might be designed to modulate any of the four enzymatic activities central to the sumoylation pathway. Alternatively a target-based approach might prove effective in which drugs could be developed to enhance or antagonize binding and/or conjugation of specific SUMO substrates to proteins involved in the sumoylation/de-sumoylation machinery.

Looking first at the sumoylation enzymes, it is important to note that SAE1/SAE2 and Ubc9 are, respectively, the only E1 and E2 enzymes in the SUMO pathway. Thus pharmacological agents targeted to either of these enzymes will likely result in global modulation of sumoylation. Given that sumoylation has a central role in cell survival, pharmacological intervention at this point would likely be harmful. However, in certain instances brief intervention (immediately following stroke, for example) might be of therapeutic benefit. Structural data suggest that structure-based drug design of small molecular modulators of these activities might be feasible. Studies on SAE1/SAE2 (Lois and Lima 2005) and Ubc9 (Reverter and Lima 2005; Yunus and Lima 2006; Capili and Lima 2007), for example, have begun to elucidate the active sites of the E1 complex and the interaction interface between Ubc9 and the SUMO target protein. It might be possible in the future to use this information to design specific modulators of E1 or E2 function. Very recently, thiazole and pyrazole urea containing compounds were identified as moderate SUMO E1 protein inhibitors, serving as a starting point for the development of therapeutic strategies for neurodegenerative diseases (Kumar et al. 2016).

Indeed, analogous work in the ubiquitin system has proved fruitful, as E1-specific inhibitors of ubiquitination have been identified (Guedat and Colland 2007). In the case of the SUMO pathway, inhibitors of E1 enzymatic activity could target any of a number of functions including occlusion of ATP binding to SAE1, binding of SUMO to the E1 complex, or binding of the E1 complex to Ubc9. A cause for some optimism for this approach comes from the compound imatinib that occludes the ATP binding sight in the ubiquitin system. Imatinib has been successfully used in the clinical setting for the treatment of chronic myelogenous leukemia (Ren 2005).

From a drug development perspective, it is preferable to target a pathway for which there are multiple enzymes, leading to greater specificity and limiting the negative effects of global modulation of sumoylation. Therefore the E3-mediated ligation of SUMO to its substrate protein would appear a more tractable target since multiple SUMO E3 ligases have been identified. Clinical results in the ubiquitin system support this approach, as the most successful ubiquitin pathway drugs have targeted the E3 ligase complexes and de-ubiquitinating (DUB) enzymes [reviewed in (Nalepa et al. 2006)] of which there are multiple enzymes in each case. Additionally, structure-based drug design is now possible for these classes of enzymes since structural data for the SUMO E3 enzyme RanBP2, in complex with the other components of the SUMO pathway, is now available (Reverter and Lima 2005; Yunus and Lima 2006).

Beyond the E3 ligases, the de-sumoylating SENP enzymes represent another potential focus for therapeutic intervention. In this respect, a SENP1 protease inhibitor, 1-[4-(N-benzylamino) phenyl]-3-phenylurea derivative 4 (GN6958), was recently identified (Uno et al. 2012), as well as compounds with 1,2,5-oxadiazole in the central region were shown as a novel class of SENP2 inhibitors (Kumar et al. 2014). However, development of modulators of the SENPs must be undertaken carefully. Because SENP enzymes are responsible not only for the de-sumoylation of target proteins but also the maturation of immature SUMO after translation (Mukhopadhyay and Dasso 2007), SENP-specific enhancers or inhibitors would therefore not necessarily lead to the desired increase or decrease in sumoylation of a given target protein.

Notwithstanding these possible caveats, the SENP family represents a more favorable group of targets for therapeutic intervention than the E1 complexes or Ubc9. Six SENP family members specific to SUMO have been identified, each with specific sub-cellular locations and preferences

between SUMO isoforms (Mukhopadhyay and Dasso 2007), although there is a high level of conservation among the catalytic subunits of the different SENPs. Therefore, it might be possible to design small molecular inhibitors specific to SUMO1 versus SUMO2/3. Indeed, structural data on the SENP enzymes has illuminated potential differences in substrate preference and catalytic activity of the SENPs (Reverter and Lima 2006; Shen et al. 2006). As is the case for other enzymes of the SUMO pathway, this information will be instructive in the design of small molecule modulators of SENP activity.

Potentially, the most appealing approach for modulation of sumoylation levels is the targeting of individual substrate proteins. Although there are multiple SUMO E3 and SENP enzymes, the number of ubiquitin E3 and DUB enzymes is significantly higher, increasing the likelihood of successful modulation of a single ubiquitin related pathway or limited pathways with lower off-target activity. Again, there is precedent in the ubiquitin system for successful inhibition of the interaction between the ubiquitination machinery and specific substrate proteins. A particular example is the multifunctional protein p53 and its ubiquitin E3 ligase Mdm2. A small molecule inhibitor of this interaction has been shown to induce apoptosis *in vitro* and inhibit xenograft tumor growth *in vivo* (Issaeva et al. 2004; Vassilev et al. 2004).

Development of a similar reagent for modification of the interaction between the SUMO E2 or E3 enzyme and its substrate is more complex. In many cases, the SUMO E2 enzyme Ubc9 is capable of direct transfer of SUMO to its substrate in the absence of an E3 enzyme, and the residues on the substrate protein that are modified by SUMO overlap with the residues which interact with Ubc9 (Sampson et al. 2001). Therefore, any inhibitor that interferes with the interaction of Ubc9 with its substrate will likely affect a large number of targets. Consequently, the feasibility of designing specific substrate blockers is currently limited. While it is theoretically possible to modulate the interaction of Ubc9 with its substrate by targeting regions close to the substrate sumoylation site, detailed structural information will be required for each potential target.

It is also plausible to consider the development of compounds that interfere with the interaction of a substrate with its E3 ligase, though the specific E3 ligase responsible for SUMO conjugation to most substrates is not yet known. This problem is compounded by the observation that, at least for some substrates, Ubc9 is capable of SUMO transfer in the absence of E3 involvement. A review speculated on the potential use of small molecular inhibitors of the E3 ligase PIAS1 in the treatment of inflammatory diseases (Liu and Shuai 2008). The proinflammatory molecules TNFα and lipopolysaccharide have been shown to elevate levels of PIAS1, which is involved in the TNFα-mediated signaling cascade as well as being an E3 ligase. As numerous reports have linked elevated levels of TNFα to a host of neurological diseases for which an inflammatory element has been described including ALS, AD, PD, and Multiple Sclerosis [reviewed in (McCoy and Tansey 2008)], such therapeutics could also have applications in neurodegeneration.

It is clear that there are a number of potential points at which the sumoylation cascade might be modulated to therapeutic effect for the treatment of the neurological conditions in which sumoylation is implicated. Accomplishing this, however, is dependent on significant advances in the basic understanding of the sumoylation enzymes and the desired targets. Furthermore, a greater understanding of the neuropathology associated with the SUMO target of interest might also influence which of the above approaches might be most beneficial.

16.9 Conclusions and Perspectives

There have been rapid and significant advances in the basic understanding of the mechanisms and consequences of sumoylation on cellular processes in both normal and disease states. Observations of SUMO immunoreactivity within neuronal inclusions have been reported in a

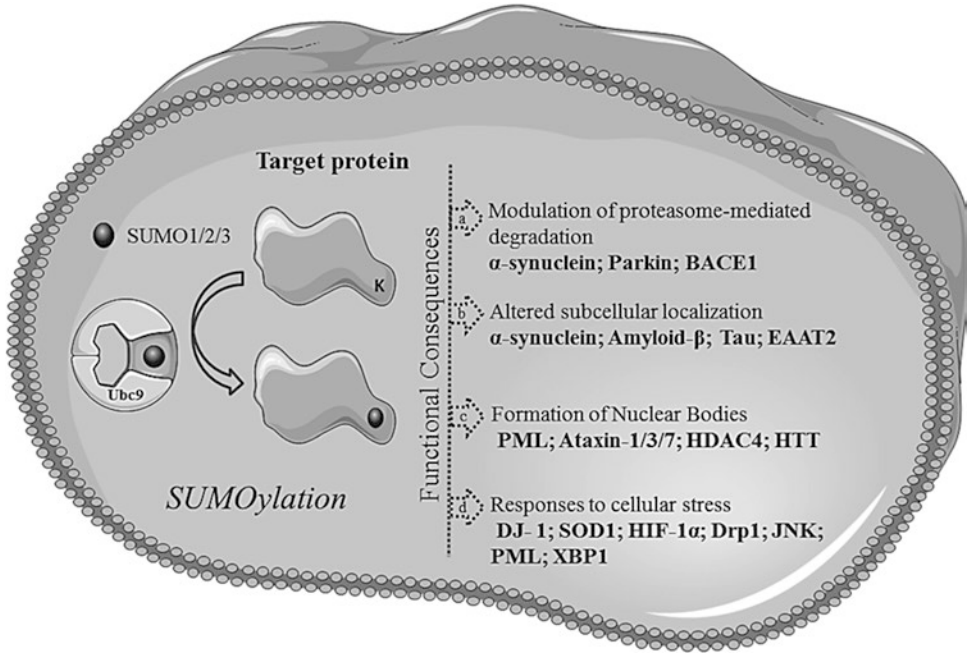

Fig. 16.1 Potential mechanisms of sumoylation in neurological diseases. Modulation of neuronal function in disease by protein sumoylation can be categorized in multiple ways. (*a*) Competition between SUMO and ubiquitin can modulate protein levels and affect aggregation; (*b*) Sumoylation of target proteins can cause sequestering of proteins to abolish critical cellular function or promote aggregation; (*c*) Formation of nuclear bodies leads to proteasome--mediated degradation; (*d*) Sumoylation is involved in the response to a number of cellular stresses; while the response is robust (ischemia), much targets have been described but much others remain unidentified

number of neurodegenerative diseases characterized by aberrant protein aggregation in the nucleus and or cytoplasm, and these findings have led to the identification of a growing list of sumoylated targets found within these protein lesions. Additionally, a number of non-covalent SUMO interactions have also been implicated in these processes.

A review of the current literature surrounding neurological disorders and SUMO reveals a number of themes regarding the roles of SUMO in neuropathology of degenerative disease states. Broadly, these include regulation of cellular transport of proteins, altering subcellular localization to enhance proteasome-mediated degradation (either positive or negative modulation), participating in the formation of nuclear aggregation, or in response to stress (Fig. 16.1). The effects of sumoylation have been reported to be both beneficial (for example in cellular responses to stress in ischemia) and detrimental (for example, the SUMO-induced increased in toxic HTT in HD).

The development of novel sumoylation-targeted therapeutics, for which currently none are available, will require a deeper understanding of the mechanisms, regulation, and targets for sumoylation. Though recent advances have been made in the generation of pharmacological agents that target the ubiquitin-proteasome system, care must certainly be taken in the design of small molecule modulators of the sumoylation pathway. However, targeted screens for chemical modulators of the sumoylation pathway will undoubtedly enhance our understanding of this modification and potentially lead to promising compounds for the treatment of the various neurological disorders in which sumoylation is involved.

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Sumoylation and Its Contribution to Cancer

17

Jason S. Lee, Hee June Choi, and Sung Hee Baek

Abstract

Post-translational modifications play an important role in regulating protein activity by altering their functions. Sumoylation is a highly dynamic process which is tightly regulated by a fine balance between conjugating and deconjugating enzyme activities. It affects intracellular localization and their interaction with their binding partners, thereby changing gene expression. Consequently, these changes in turn affect signaling mechanisms that regulate many cellular functions, such as cell growth, proliferation, apoptosis, DNA repair, and cell survival. It is becoming apparent that deregulation in the SUMO pathway contributes to oncogenic transformation by affecting sumoylation/desumoylation of many oncoproteins and tumor suppressors. Loss of balance between sumoylation and desumoylation has been reported in a number of studies in a variety of disease types including cancer. This chapter summarizes the mechanisms and functions of the deregulated SUMO pathway affecting oncogenes and tumor suppressor genes.

Keywords

SUMO • Sumoylation • Cancer • Metastasis • Ubc9 • PIAS1 • SENP • PML • TEL • p53 • Reptin • Pontin • IκBα • NEMO

17.1 Introduction

Cells are continually exposed to extra- and intracellular stimuli and appropriate responses to these signals that regulate proliferation, differentiation and apoptosis are tightly orchestrated to maintain homeostasis as a whole organism. When this process is deregulated, cells grow and divide in an uncontrolled manner, invade normal

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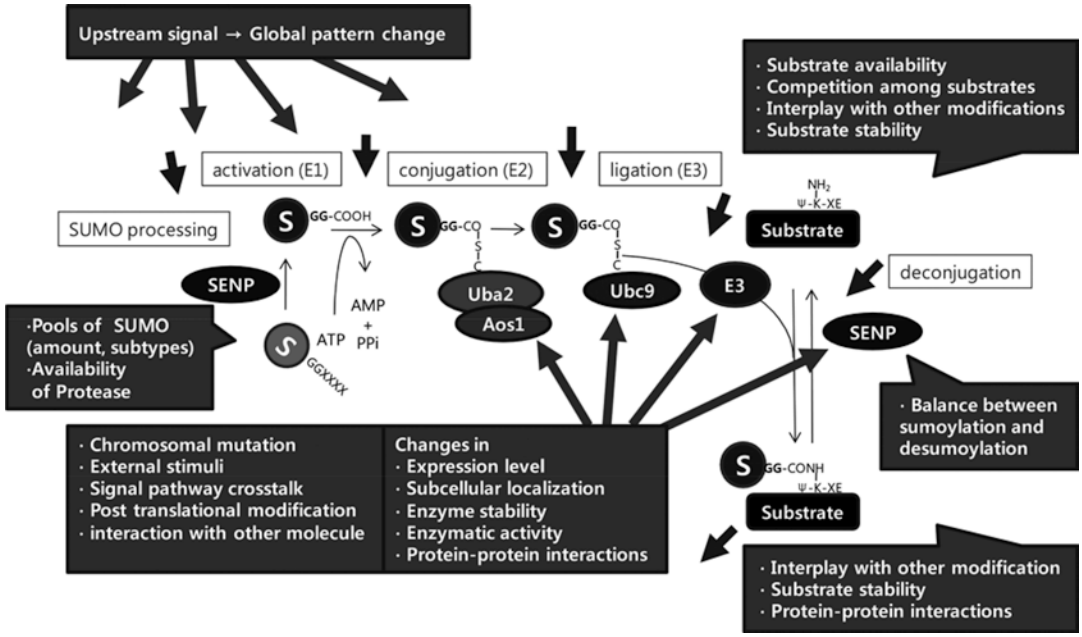

Fig. 17.1 The possible mechanisms to regulate SUMO-conjugation system

tissues and organs, and this loss of control leads to cancer (Lee and Thorgeirsson 2004; McDoniels-Silvers et al. 2002; Mo et al. 2005; Wang and Banerjee 2004). Sumoylation is a three step enzymatic process which requires E1 activating enzyme, E2 conjugating enzyme, and E3 ligase (Johnson 2004). SUMO is first translated as an inactive precursor form and needs to be processed by SUMO protease to be conjugated (Johnson 2004). SUMO protease also cleaves SUMO from modified substrates. Each step is a highly dynamic process that can be regulated in response to cellular stimuli or pathogenic challenges. Altered activity of SUMO conjugation can be achieved through regulation of the expression of various components of the sumoylation pathway, the activity of enzymes, component localization, and through crosstalk with other post-translational modifications including phosphorylation, ubiquitination, and acetylation (Fig. 17.1).

Sumoylation is implicated in cellular growth control such as cell cycle regulation, senescence, and apoptosis (Meinecke et al. 2007; Bischof and Dejean 2007; Gutierrez and Ronai 2006). Targets of sumoylation include molecules involved in DNA damage repair and maintenance of genome integrity. The sumoylation of transcription factors, cofactors or chromatin--remodelling factors, which comprise almost half of targets of the modification, modulate transcriptional activity and regulate many signaling pathways, such as the Wnt (Yamamoto et al. 2003; Ihara et al. 2005), NF-κB (Mabb and Miyamoto 2007) and steroid hormone receptor pathways that are known to be related to cancer progression (Faus and Haendler 2006). Furthermore, sumoylation is a highly dynamic process that can be rapidly reversed by desumoylating enzymes, and many proteins are modified by SUMO following extra- and intra-cellular stimuli. All these features of sumoylation imply deregulation of this enzymatic system can lead to cancer progression.

17.2 Upstream Signals Regulating SUMO-Conjugation System in Cancer

Examination of the involvement of SUMO pathway in cancer in clinical samples has been largely limited to correlating the expression level of com-

Table 17.1 Deregulations in gene expression and locations of SUMO conjugating system in cancer

Deregulated protein	Type of deregulation	Tumor type/disease	References
SUMO1	Upregulation	Anaplastic large-cell lymphoma	Villalva et al. (2002)
SUMO2, UBA2	Upregulation	Hepatocellular carcinoma	Lee and Thorgeirsson (2004)
UBC9	Upregulation	Ovarian tumor	Mo et al. (2005)
		PA-1, OVCAR-8	Mo et al. (2005)
		Human lung adenocarcinomas	McDoniels-Silvers et al. (2002)
		AML with mutations in Cebpa gene	Geletu et al. (2007)
		Metastatic cancer cell line, LNCaP	Kim et al. (2006)
PIAS3	Upregulation	Lung, breast, prostate, colon, rectum and brain tumour	Wang and Banerjee (2004)
PIASy	Downregulation	Myelodysplastic syndrome	Ueda et al. (2003)
SENP1	Upregulation	Thyroid oncocytic adenoma	Jacques et al. (2005)
	Upregulation	Human prostate cancer specimens	Cheng et al. (2006)
	Downregulation	Metastatic cancer cell line, LNCaP	Kim et al. (2006)
	Transgenic expression	Early neoplastic lesions in the prostate	Tagawa et al. (2002)
SENP1-MESDC2	Chromosomal translocation	Infantile teratoma	Veltman et al. (2005)
SENP6-TCBA1	Chromosomal translocation	Human T cell lymphoblastic lymphoma cell line HT-1	Tagawa et al. (2002), Takahashi et al. (2003)
PML-RARα	Chromosomal translocation	APL	Takahashi et al. (2003), Wood et al. (2003)
TEL	Chromosomal translocation	Pediatric B cell acute lymphoblastic leukemia	Wood et al. (2003)
RB	Mutation	Retinoblastoma tumor	Ledl et al. (2005)
$p14^{ARF}$	Mutation	Melanoma	Rizos et al. (2005)

ponents of the SUMO pathway with cancer state or prognostic indicators (Table 17.1). One such example is in hepatocellular carcinoma patients, where over expression of SUMO-2 and the E1 subunit, Uba2, has been correlated with poor survival (Lee and Thorgeirsson 2004). Although not much is known about upstream signals which govern the SUMO pathway leading to pathological states, stress signals have been suggested to affect this pathway. A global increase in sumoylation has been observed upon various stress stimuli such as heat shock, osmotic stress, and hibernation (Kurepa et al. 2003; Lee et al. 2007b; Saitoh and Hinchey 2000), while loss of global conjugation occurs under conditions of oxidative stress (Boggio et al. 2004). Genotoxic stress comprises stress generating DNA double- or single-strand break and it is one of the main causes of cancer. Such breaks are induced by chemotherapeutic agentsagents (etoposide, doxorubicin, cisplatin, camptothecin, etc.), ionizing radiation, and UV exposure. Many targets are reported to be sumoylated or desumoylated under genotoxic stress (Table 17.2). Mostly, these are achieved by targeting E1, E2 conjugating machinery since they are both unique and required for sumoylation.

Table 17.2 Effects of genotoxic stress on SUMO conjugation system

Genotoxic stress	Target protein	SUMO-ylation	Effect	References
etoposide	NEMO	Increase	IKK activation and subsequent NF-κB signaling	Huang et al. (2003)
UV	DJ1	Increase	transcription of XPC, which is involved in DNA repair	Shinbo et al. (2006)
UV	Tip60	Increase	relocation of TIP60 from nucleoplasm to the promyelocytic leukemia body p53 damage response	Cheng et al. (2008)
camptothecin	Topo1	Increase	nucleolar delocalization of topo I	Mo et al. (2002), Mao et al. (2000)
UV	hRIPβ	Decrease	a protein involved in DNA repair	Park et al. (2005)
Doxorubicin	KAP-1	Decrease	transcriptional repression	Lee et al. (2007c)
Doxorubicin	p53	Decrease		Lin et al. (2004)

17.3 Regulation of SUMO E2 Conjugating Enzyme, UBC9, in Cancer

Ubc9, the single SUMO E2 enzyme catalyzing the conjugation of SUMO to target proteins, is upregulated in certain tumors (Moschos and Mo 2006) and over expression of a dominant-negative mutant form of Ubc9 is associated with increased sensitivity to anticancer drugs (Mo et al. 2004). Ubc9 over expression was found in several tumor types including lung and ovarian carcinoma as well as ovarian and prostate cancer cell lines including PA-1, OVCAR-8, and LNCaP (McDoniels-Silvers et al. 2002; Mo et al. 2005; Moschos and Mo 2006). Much more is known about the role of Ubc9 in cancer in that the inhibition of Ubc9 function in MCF-7 breast cancer cells injected in nude mice resulted in attenuation of tumor growth and increased Bcl-2-dependent apoptosis (Mo et al. 2005), suggesting that Ubc9 may be a potential target of therapeutic regime. In acute myeloid leukemia (AML),), transcriptional upregulation of Ubc9 by a 30-kDa dominant-negative isoform of CCAAT/enhancer-binding protein α (C/EBPα) is found, and this enhances the sumoylation of C/EBPαp42 to inhibit granulocytic differentiation (Geletu et al. 2007).

Activity of Ubc9 can be modulated by protein-protein interaction. P14ARF is known to be a tumor suppressor and commonly altered in human cancer. The interaction between p14ARF and Ubc9 enhances sumoylation of p14ARF binding partners, and this enhancement is abrogated in a subset of melanoma which harbor mutation in p14ARF (Rizos et al. 2005). RWD-containing sumoylation enhancer (RSUME) is another protein which interacts with Ubc9 and promotes the conjugation activity by increasing non-covalent binding of SUMO to Ubc9. Under hypoxic condition, RSUME is induced and enhances the sumoylation of HIF-1alpha, promoting its stabilization and transcriptional activity (Carbia-Nagashima et al. 2007). Stress can also directly regulate enzymatic activity by modifying catalytic regions. ROS (reactive oxygen species) directly and reversibly inhibits Uba2 and Ubc9 by forming disulfide bonds involving the catalytic cysteines (Bossis and Melchior 2006). Recently, it has been reported that autosumoylation of Ubc9 at Lys14 regulates target discrimination, thus adding another layer of regulation (Knipscheer et al. 2008).

17.4 Involvement of SUMO E3 Ligases in Cancer

While there are a single E1 and E2 required for sumoylation of all substrates, a number of E3 ligases are localized to specific subcellular complexes and shows target specificity. Therefore, effects observed with regulating SUMO-E3s appear to be more restricted and specific compared to the effect observed with that of SUMO-E1 or E2. Overexpression of PIAS E3 has been reported in a variety of human tumor types including those of the brain, colorectal, breast and prostate (Wang and Banerjee 2004), suggesting the importance of sumoylation in cancer development of a variety of tissue types. Interconnection between ubiquitination and sumoylation pathways has been reported through the modulation of PIAS at the protein level. hSiah proteins possess ubiquitin-E3-ligase activity that triggers their partners to proteasomal-dependent degradation (Depaux et al. 2007). PIAS1 has been found as a novel hSiah2-interacting protein. Degradation of PIAS1 regulated by hSiah2 thereby relieves the effect of sumoylation on PIAS1 targets, for example, c-Jun (Depaux et al. 2007).

The regulation of E3 can also be carried out by substrates as the substrate itself can modulate the activity of a SUMO E3 ligase. Polycomb 2 (Pc2) binds to homeodomain-interacting protein kinase 2 (HIPK2) and acts as a SUMO E3 ligase for HIPK2 (Swaminathan et al. 2004). Upon DNA damage, HIPK2 is activated and then phosphorylates Pc2. The E3 ligase activity of phosphorylated Pc2 toward HIPK2 is enhanced, and sumoylation promotes the ability of HIPK2 to act as a transcriptional repressor (Roscic et al. 2006). Additional examples of post-translational modification of SUMO E3 ligase which regulates the activity of the enzyme can be seen as in nitrosation of PIAS3 (Qu et al. 2007) and phosphorylation of PIASxα (Yang and Sharrocks 2006). PIAS1 E3 ligase is localized in the nucleus and can regulate the activity of a number of transcription factors such as NF-κB, STAT1, and PPARγ upon ligand stimulation (Desterro et al. 1998; Hoege et al. 2002). These factors are rapidly phosphorylated in response to pro-inflammatory stimuli such as TNFα and LPS. The involvement of E3 ligase activity on signal-dependent phosphorylation can be illustrated in the case of IKKα which has been identified as the kinase that mediates PIAS Ser 90 phosphorylation. It is intriguing that the ability of IKKα to phosphorylate PIAS1 on Ser 90 requires the SUMO ligase activity of PIAS1. Consistently, elevated sumoylation can enhance the IKKα-mediated PIAS1 phosphorylation (Liu et al. 2007).

17.5 Involvement of SUMO-Specific Proteases in Cancer

By the action of specific proteases, sumoylation is a reversible process. In mammalian cells, there are at least seven desumolating enzymes known as SENP (SUMO/sentrin-specific protease). Regulation of SENP1 expression level has been reported to be highly correlated with progression of prostate cancer. SENP1 is overexpressed in prostate cancer tissues, and this increase of SENP1 is induced by androgen and IL-6 (Cheng et al. 2006). Induction of SENP1 enhances AR-dependent transcription, c-Jun dependent transcription, and expression of cyclin D1, eventually leading to increased cellular proliferation, which promotes development of prostate cancer (Cheng et al. 2006). SENP1 expression has been also reported to be elevated in thyroid adenomas (Cheng et al. 2006; Jacques et al. 2005). In a mouse model of SENP1, overexpression leads to early prostate intraepithelial neoplasia suggesting that excessive desumoylation in a specific tissue may lead to increased propensity to tumor development. By contrast, a patient developed an infantile sacrococcygeal teratoma caused by the disruption and fusion of the SENP1 gene and the embryonic polarity-related mesoderm development gene (MESDC2) as a result of chromosomal translocation at t(12;15)(q13;q25) (Veltman et al. 2005). This disruption causing teratoma suggests that perhaps spatio-temporal disturbances in desumoylating activities during embryonic development might be important. Similar to SENP1, a chimeric gene

SENP6-TCBA1 has been identified in a T-cell lymphoblastic lymphoma cell line, HT-1 (Tagawa et al. 2002). Similarly, expression of SENP1 was downregulated in LNCaP invasive prostate cancer cell line compared to that in RWPEI normal prostate epithelial cells (Kim et al. 2006). Thus, the correlation between SENP1 expression and cancer appears to be somewhat tissue- and context-specific.

17.6 Regulation of Sumoylation at the Substrate Level and Implications in Cancer

Regulation of sumoylation can also occur at the level of target itself, which involves the interplay with other post-translational modifications. A number of reactions are regulated by phosphorylation of substrate prior to sumoylation. The phosphorylation-dependent sumoylation motif (PDSM) is the extension of classical SUMO consensus site ψKxE (in which ψ is an aliphatic branched amino acid and x is any amino acid) (Rodriguez et al. 2001) followed by a Ser and a Pro residue, of which the phosphorylation is induced by stress-activated kinases such as p38 and JNK, or ATM in the case of genotoxic stress (Hietakangas et al. 2006). When phosphorylation occurs within a PDSM, it leads to increased sumoylation, probably through increased binding affinity to Ubc9. This is exemplified in HSF1 and MEF2 (Hilgarth et al. 2003; Gregoire et al. 2006). However, phosphorylation can also be a negative regulator for sumoylation as has been reported for IκBα, p53, c-Fos and c-Jun (Desterro et al. 1998; Muller et al. 2000; Bossis et al. 2005; Lin et al. 2004). Phosphorylation of Elk-1 induced by the MAP kinase pathway results in a concomitant desumoylation on Elk-1 and activation of transcription (Yang et al. 2003). In addition to phosphorylation, competition on the same lysine residue with acetylation or ubiquitination also negatively regulates substrate sumoylation. The direct antagonism between sumoylation and ubiquitination can be seen in IκBα (Desterro et al. 1998) and PCNA (proliferating-cell nuclear antigen) (Hoege et al. 2002). Acetylation has been shown to antagonize sumoylation of the same lysine residues in substrates such as MEF2 (myocyte enhancer factor 2) (Zhao et al. 2005; Shalizi et al. 2006) and HIC-1 (hypermethylated in cancer 1) (Stankovic-Valentin et al. 2007).

Recently, another layer of regulation at the substrate level has been found by modulating the stability of sumoylated substrates. SUMO-targeted ubiquitin ligases (STUbLs) are a novel family of RING-finger ubiquitin ligases that recognize and selectively ubiquitinate sumoylated substrates via their tandem SUMO interaction motifs (Anckar and Sistonen 2007; Prudden et al. 2007; Uzunova et al. 2007). They down-regulate global levels of protein sumoylation by promoting target protein desumoylation and/or degradation. Cells lacking STUbLs display genomic instability and hypersensitivity to genotoxic stress (Mullen et al. 2001; Zhang et al. 2006). The human STUbL, RNF4, has been implicated in several human diseases including cancer (Hirvonen-Santti et al. 2003; Pero et al. 2001). Chromosomal mutation of RNF4 is frequent (Pero et al. 2001) and expression of RNF4 is strongly attenuated in testicular germ-cell cancers (Hirvonen-Santti et al. 2003; Pero et al. 2001). In addition, RNF4 potentiates the transcriptional activation by several members of the nuclear steroid-receptor family that are deregulated in a variety of disease processes (Moilanen et al. 1998). Examples of regulation on SUMO conjugation system mentioned so far are summarized in Fig. 17.2.

17.7 SUMO Modification of Oncogenes and Tumor Suppressors

Sumoylation induces alteration of inter- or intramolecular interactions of the modified targets. This change leads to the regulation of subcellular localization, interference or promotion of protein-protein interaction, modulation of protein stability, interplay with other modifications, and regulation of the activities of substrates (Geiss-Friedlander and Melchior 2007). Both the amount and variety of substrates are daunting, making it

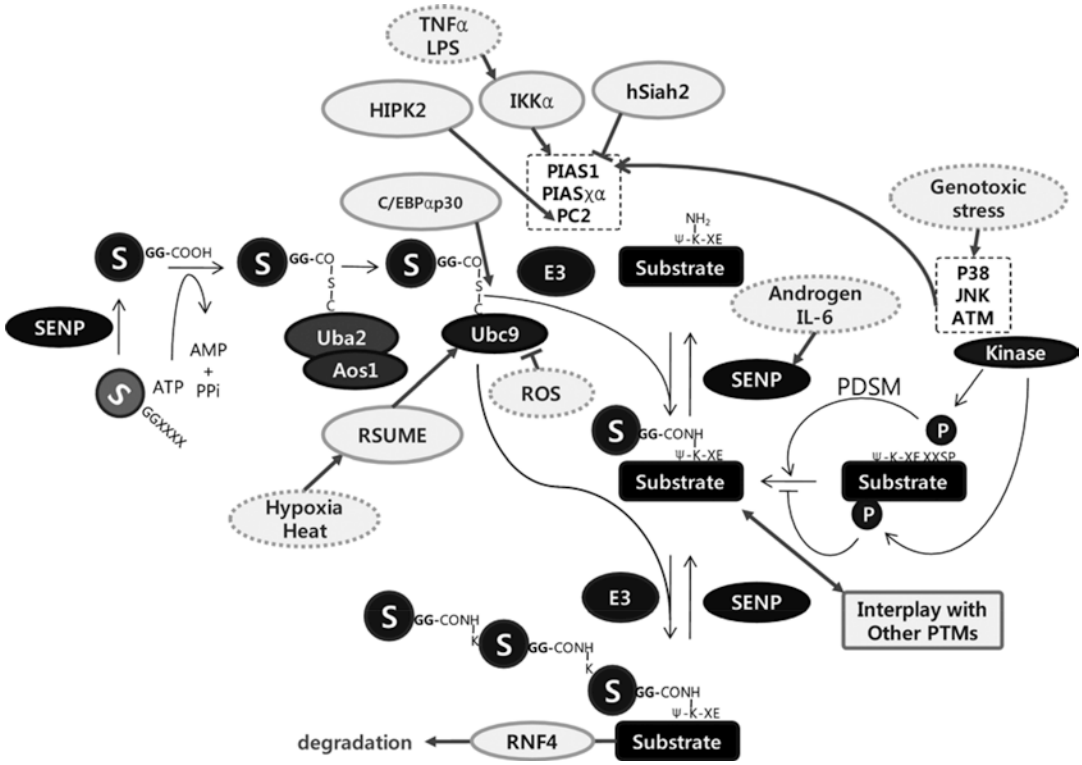

Fig. 17.2 Summary of factors which regulates SUMO-conjugation system

impossible to define the role of sumoylation in cancer progression succinctly. Proteins known to play oncogenic or tumor suppressive role are listed in Table 17.3. Perhaps the best example illustrating the success in targeting SUMO pathway in the development of therapeutic intervention would be the case of the acute promyelocytic leukemia (APL) where PML (promyelocytic leukemia) gene and retinoic acid receptor (RAR) gene are fused to yield an oncogenic fusion protein, PML-RAR. PML is a tumor suppressor required for PML nuclear body formation and is necessary for the recruitment of other components into the nuclear bodies (Ishov et al. 1999; Zhong et al. 2000). Sumoylation plays a key role in this process, since sumoylation on three lysine residues (Duprez et al. 1999; Kamitani et al. 1998) and a SUMO interaction motif (SIM) of PML (Minty et al. 2000) is a prerequisite to allow nuclear body formation (Shen et al. 2006). PML nuclear bodies appear as dense spherical particles tightly associated with the nuclear matrix. In APL, PML nuclear bodies are disrupted indicating pathological correlation of this peculiar structure with disease. A tumor suppressive role of PML nuclear bodies has been reported in several different types of cancer (Koken et al. 1995; Gambacorta et al. 1996; Paul et al. 2007; Lee et al. 2007a; Zhang et al. 2000). The sumoylation on PML was shown to be induced by arsenic on lysine 160 (Lallemand-Breitenbach et al. 2001; Zhu et al. 1997) and recruits 11S proteasome activator complex and releases transcriptional repression (Zhu et al. 2002; Lallemand-Breitenbach et al. 2005). Arsenic has been found to be effective in APL patients by triggering degradation of PML-RAR and resulted in differentiation and apoptotic death of APL cancer cells (Lallemand-Breitenbach et al. 2005; Shen et al. 2004). These patients showed about 32-fold reduction in APL cells and 16 patients out of 18 were disease-free even 12 months after treatment (Shen et al. 2004).

Table 17.3 Substrates of sumoylation

Substrate	Function	Role of sumoylation	References
DNA replication/repair			
Topo I	DNA replication, DNA repair	Nucleolar delocalization	Mao et al. (2000), Mo et al. (2002)
Topo II	DNA replication, DNA repair	Facilitates TopoII enrichment to CEN regions	Mao et al. (2000), Dawlaty et al. (2008)
PCNA	DNA replication factor	Recruitment of Srs2	Ulrich (2007)
TDG	Base-excision repair	Conformational change	Hardeland et al. (2002)
Nuclear bodies			
PML	Tumor suppressor	Allows formation of NBs and recruitment of Daxx/p53/p53 to NBs	Muller et al. (1998)
Sp100	Transcriptional repression	Allows formation of heterochromatin through increased affinity to HP1	Sternsdorf et al. (1997)
HIPK2	Transcriptional repression	Mediates localization of HIPK2 to nuclear dots	Kim et al. (1999)
Daxx	Transcriptionl repression	Recruitment of Daxx to NBs	Ishov et al. (1999), Li et al. (2000)
TEL	Transcriptional repression	Mediates the localization of TEL to nuclear dots	(Chakrabarti et al. (2000), Chakrabarti et al. (1999)
Transcriptional regulation			
AR	Transcriptional activation	Reduces transcriptional activity of androgen receptor	Poukka et al. (2000)
p53	Tumor suppressor	Activates p53 transactivation and apoptosis	Gostissa et al. (1999), Rodriguez et al. (1999)
p73α	p53 homologue		Minty et al. (2000)
c-Jun	Transcriptional activation	Reduces transcriptional activity of c-Jun	Muller et al. (2000)
IκBα	Signal transduction, NF-κB inhibition	Inhibits ubiquitylation of IκBαBlocks NF-κB activity	Desterro et al. (1998)
MDM2	E3 ubiquitin ligase for p53	Inhibits ubiquitylation of Mdm2Activates the E3 function of Mdm2	Buschmann et al. (2000)

Numerous proteins that transiently associate with nuclear bodies such as p53, Daxx, Daxx, Sp100, Sp100, HIPK2 are also sumoylated (Kwek et al. 2001; Jang et al. 2002; Sternsdorf et al. 1999; Gresko et al. 2005), suggesting that sumoylation modulates their interaction in the nuclear body. The SIM enables PML to interact non-covalently with sumoylated proteins including PML itself (Shen et al. 2006). Thus multiple sumoylated PML molecules interact with each other to form a PML network, a macromolecular structure on which other proteins can be dynamically attached depending on their sumoylated status or through their SIM (Shen et al. 2006). PML nuclear bodies serve as a molecular platform for diverse cellular process, such as post-translational modification of proteins, regulation of transcriptional activities by sequestration of transcription factors or by formation of repressive complex, and heterochromatin formation (Bernardi and Pandolfi 2007). All these processes regulate transcriptional regulation of gene expression, DNA repair, genome stability and eventually lead to suppression of tumor (Negorev and Maul 2001) (Fig. 17.3).

Upon sumoylation of PML, the transcriptional corepressor Daxx relocalizes to nuclear bodies, followed by a decrease in repression at Daxx target genes (Ishov et al. 1999; Li et al. 2000; Lehembre et al. 2001). The Daxx SIM mediates

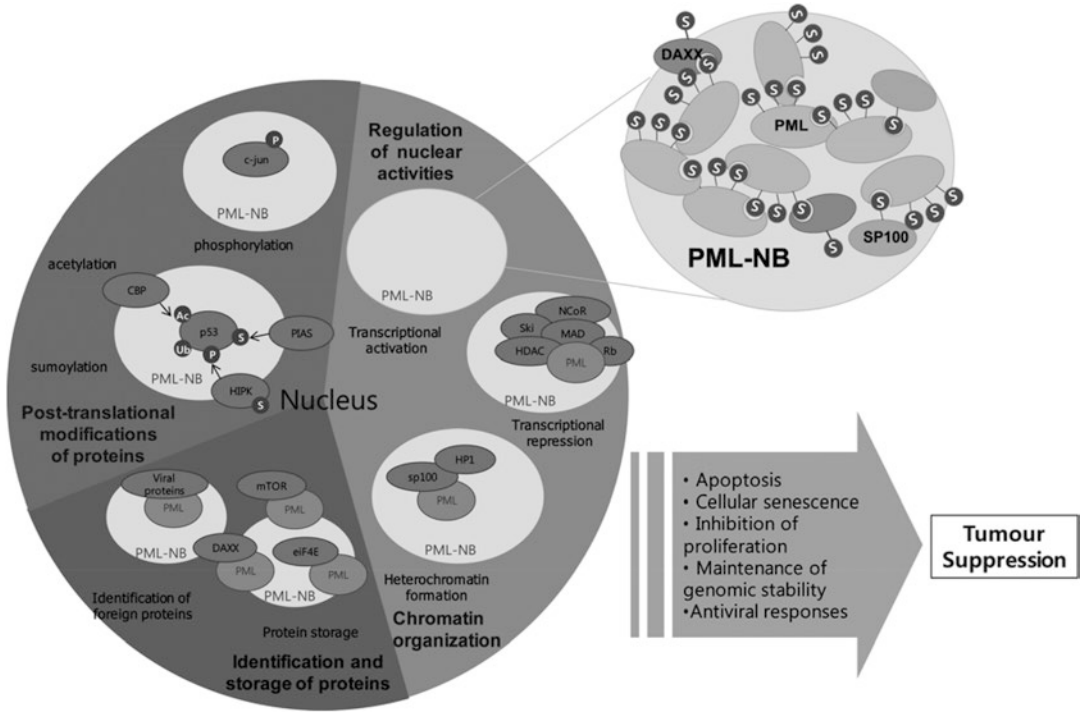

Fig. 17.3 Roles of PML in tumor suppression

its binding to sumoylated PML and is required for targeting to PML-nuclear bodies (Lin et al. 2006). PML nuclear bodies compete with sumoylated transcription factors at promoters for a limited amount of Daxx, thus regulating Daxx-mediated transcriptional repression. Sp100 is another core component of PML nuclear bodies, which is also found to be sumoylated (Sternsdorf et al. 1999). *In vitro* experiments have indicated that SUMO might be important for the interaction of Sp100 with HP1, thus implicating a role in heterochromatin formation (Sternsdorf et al. 1999). Moreover, PML has been shown to bind p53 directly and trigger its recruitment to nuclear bodies (Fogal et al. 2000). Sequestration of p53 into nuclear bodies allows the assembly of a CBP/p53 complex promoting acetylation and activation of p53 (Fogal et al. 2000; Pearson et al. 2000). TEL is a frequent target of chromosomal translocations in both myeloid and lymphoid leukemias, and the second allele of TEL is deleted suggesting that TEL is a tumor suppressor (Golub et al. 1997; Rowley 1999). TEL acts as a transcriptional repressor (Lee and Thorgeirsson 2004; McDoniels-Silvers et al. 2002; Mo et al. 2005; Wang and Banerjee 2004), and can inhibit Ras-dependent transformation (Fenrick et al. 2000; Van Rompaey et al. 1999; Rompaey et al. 2000). Recently, it was demonstrated that TEL is actively exported from the nucleus in a leptomycin B-sensitive manner and this export depends on sumoylation at lysine 99 (Wood et al. 2003) suggesting that tumor suppressor function of TEL is negatively regulated by sumoylation (Wood et al. 2003). Recently in prostate cancer cells, 5α-dihydroxytestosterone significantly increased sumoylation of pontin chromatin-remodelling factor and led to further activation of androgen receptor-dependent transcription which consequently resulted in a more aggressive cancer phenotype (Kim et al. 2007). Ubc9 is among many proteins that were identified as binding partners of pontin from purification of pontin chromatin-remodelling complex, suggesting that symoylation can act as a signal integration code (Kim et al. 2007). In addition to the above

mentioned factors, there are several oncogenic proteins which participate in the processes of cell proliferation and survival that are targeted by sumoylation. These include signalling intermediates or transcription factors downstream of growth factor signalling pathways such as c-fos (Bossis et al. 2005) and c-jun (Muller et al. 2000), both of which result in reduced transcriptional activity following sumoylation.

Sumoylation also targets tumor suppressors thereby regulate cellular growth, differentiation and apoptotic responses. The tumor suppressor, p53 is a master regulatory protein which controls these cellular processes and is a target of various types of post-translational modifications (Bode and Dong 2004). Cellular p53 in the normal state is maintained at low levels and under stress, p53 is stabilized and accumulates in the nucelus and leads to the induction of cell cycle arrest, DNA repair, and apoptosis (Dai et al. 2006; Brooks and Gu 2006). Sumoylation of p53 on lysine 386 has been known for a long time (Rodriguez et al. 1999; Gostissa et al. 1999). However, the functional consequence of SUMO-modification is inconsistent in that both activation and repression was reported (Schmidt and Muller 2002; Nelson et al. 2001; Bode and Dong 2004; Bischof et al. 2006; Gostissa et al. 1999; Rodriguez et al. 1999). This inconsistency might be due to the difference in the system or assays that were used in each study and suggests that p53 may in fact function both in activation and repression. Crosstalk with other post-translational modification of p53 has attracted much attention in studying the functional role of p53. One example of this crosstalk is between sumoylation and ubiquitination of p53 in that *in vivo* sumoylation requires direct interaction with MDM2 (Chen and Chen 2003). Considering that both sumoylation and ubiquitination can occur on lysine residues, sumoylation targeting lysine 386 and several other lysines being targeted by ubiquitination make it unlikely that a simple competitive model exists.

Recent studies have highlighted the contribution of inflammation to cancer development and the NF-κB transcription factors play a central role in regulating innate and adaptive immune responses as well as in cancer development and progression (Karin 2006). SUMO modification has been reported for IκBα and competes with ubiquitination on the same lysine residue directly affecting IκBα's inhibitory role on NF-κB (Desterro et al. 1998). Sumoylation results in stabilization of IκBα by preventing it from ubiquitin-dependent degradation, consequently inhibiting NF-κB-dependent transcriptional activation (Desterro et al. 1998). Thus, sumoylation of IκBα might be beneficial for blocking excessive signalling by NF-κB. Responses to apoptotic stimuli are also regulated by NF-κB pathway. Genotoxic stress induces activation of cytoplasmic IκB kinase (IKK) complex which is mediated by NEMO, the regulatory subunit of the cytoplasmic IKK complex (Ghosh and Karin 2002). IKK-unbound cytoplasmic NEMO is sumoylated upon genotoxic stress and this modification causes nuclear localization of NEMO. At the same time, ATM in the nucleus is activated by genotoxic stress and translocated NEMO undergoes subsequent ATM-dependent ubiquitination, which shuttles NEMO back to the cytoplasm to ultimately activate cytoplasmic IKK. In this way, nuclear DNA damage can regulate the activity of cytoplasmic IKK complex. In contrast to the case of IκBα, ubiquitin and SUMO do not counteract each other but act cooperatively in coordinated sequential events (Huang et al. 2003).

Sumoylation is not only involved in regulating transcription of genes that affect tumorigenesis, but also metastasis. *KAI1* is a NF-κB responsive gene in which transcription activation is brought about by Interleukin-1β in normal and tumor prostate epithelial cells (Baek et al. 2002; Li et al. 2001). Although there is evidence supporting *KAI1* as a metastasis suppressor, it was only recently that the transcriptional control mechanism of KAI1 has been elucidated (Kim et al. 2005). This gene promoter activity is tightly controlled by the balance between Tip60 transcriptional coactivators and β-catenin/reptin chromatin-remodelling complex proteins. For activation, KAI1 requires Tip60 while β-catenin/reptin appears to function as a transcriptional repressor. SUMO-modification of reptin plays an important role in keeping *KAI1* gene expression

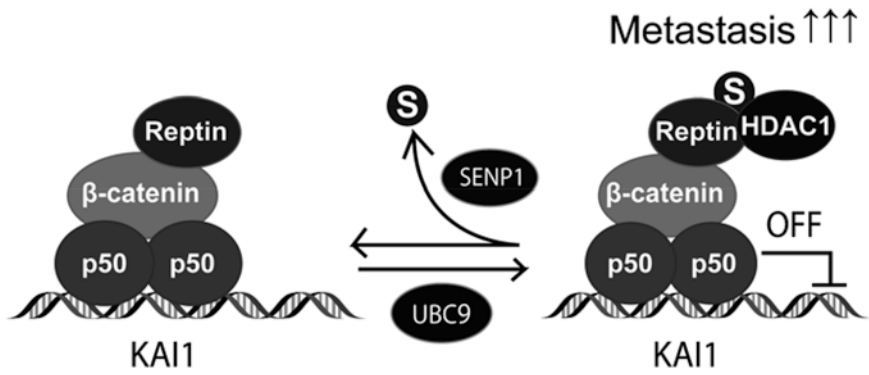

Fig. 17.4 Regulation of metastasis suppressor gene expression by reptin sumoylation

in its OFF state by recruiting histone deacetylase (HDAC1) and thereby increasing invasive potential of cancer cells (Kim et al. 2006). Purification of reptin chromatin-remodelling complex revealed the presence of specific desumoylating enzymes that can remove SUMO from reptin, which provided the novel link between SUMO-modification and cancer metastasis (Kim et al. 2006) (Fig. 17.4). With the advent of many proteins that can regulate cancer progression and metastasis being identified to be SUMO-modified, the balance of the levels of sumoylating and desumoylating enzymes would dictate whether a specific tumor type would progress to an aggressive state.

17.8 Conclusions

Together these data suggest that both aberrant expression of sumoylating and desumoylating enzymes contribute to tumor development in a tissue and context-dependent manner. It is no doubt that sumoylation plays an important role by modulating specific substrates affecting many biological processes. As many of the substrates that are targeted by sumoylation are known to play key roles in cellular proliferation, growth, DNA repair, apoptosis and survival, perturbation of sumoylating and desumoylating enzymes are expected to contribute to the development and progression of cancer. Not surprisingly, cancers of many types show deregulated levels of sumoylating and desumoylating enzymes affecting a range of oncogenes and tumor suppressor genes. The understanding of the mechanism of how the sumoylation pathway contributes to cancer is unclear in a clinical setting at present. However, considering how much wealth of knowledge we have gained in a decade of SUMO research, it will only be a matter of time until we decipher the code of sumoylation of each substrates as well as each specific disease.

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18 Sumoylation Modulates the Susceptibility to Type 1 Diabetes

Jing Zhang, Zhishui Chen, Zhiguang Zhou, Ping Yang, and Cong-Yi Wang

Abstract

Susceptibility to type 1 diabetes (T1D) is determined by interactions of multiple genes with environmental triggers. Thus far, more than 50 T1D susceptibility regions have been suggested from genetic studies by employing either genome-wide or candidate gene approaches. Because the lack of a linear correlation between the presence of risk genes and the onset of disease, the exact susceptible genes encoded in these regions remain largely elusive. In 2004, we first reported the cloning of a novel small ubiquitin-like modifier (*SUMO*) gene, *SUMO4*, in the *IDDM5* region on chromosome 6q25, and presented strong genetic and functional evidence suggesting that *SUMO4* could be a novel T1D susceptibility gene. Follow up studies have consistently confirmed this association in multiple Asian populations despite controversial observations in Caucasians, which could be caused by genetic heterogeneity. In this chapter, we will summarize and validate genetic data for *SUMO4* association studies in type 1 diabetes. The functional properties and possible molecular mechanisms by which altered sumoylation function modulates the development of type 1 diabetes will be also discussed based on published data.

Keywords

SUMO • Type 1 diabetes • Autoimmunity • Sumoylation • Beta-cell

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Abbreviations

SUMO	Small ubiquitin-like modifier
T1D	Type 1 diabetes
IDDM	insulin dependent diabetes mellitus
SNP	single-nucleotide polymorphism
NOD	mouse: nonobese diabetic mouse
STAT	signal transducer and activator of transcription
PIAS1	protein inhibitor of activated STAT1
SIM	SUMO-interacting motif
SAP	scaffold attachment factor-A/B/acinus/PIAS
AP-1	activator protein-1
ROS	reactive oxygen species
NO	nitrite oxide
DCs	dendritic cells
Tregs	regulatory T cells

18.1 Introduction

Type 1 diabetes (T1D) once known as juvenile diabetes or insulin-dependent diabetes mellitus (IDDM), is a multifactorial and highly heritable disease resulted from chronic autoimmune destruction of the insulin-secreting beta cells within the pancreatic islets of Langerhans (Hu et al. 2015; Chiu and Palmer 2004; Kay et al. 2000; Kurrer et al. 1997; Yoon and Jun 2005). It occurs worldwide and is common in childhood and adolescence. As autoimmune destruction of the pancreatic beta cells may proceed subclinically over a long time, overt diabetes develops when beta cells drop to a very low level that the patients are no longer able to secret adequate levels of insulin to control their blood glucose levels. Therefore, at the time of clinical diagnosis, the patients had already lost a major proportion of their beta cells. The consequence of beta cell loss would result in a complete dependence on exogenous insulin for survival. Unfortunately, due to the inability of the exogenous insulin to regulate glucose as accurately as the endogenous insulin released by the functioning pancreatic islets, blood glucose cannot be regulated at a perfect level and eventually would lead to devastating complications. It was found that even short-term hyperglycemic spikes can cause persistent epigenetic changes contributing to dysregulated gene expressions, which then serve as risk factors for the development of diabetic complications (El-Osta et al. 2008). Diabetic ketoacidosis, nonketotic hyperosmolar coma and hypoglycemia are examples of short-term complications resulted from improperly manipulated blood glucose levels (Maahs et al. 2015; Lamb 1994; Couch et al. 2008). On the other hand, diverse complications such as cardiovascular disease (Nadeau et al. 2010; Anselmino et al. 2008; Inoguchi and Takayanagi 2008; Marwick 2008), nerve damage (Son et al. 2015; Lotosh et al. 2012), chronic renal failure (Monhart 2008; Navarro-Gonzalez and Mora-Fernandez 2008), retinal damage (Bandurska-Stankiewicz and Wiatr 2007; Cheung and Wong 2008; Studholme 2008), and poor wound healing (Amin and Doupis 2016; Uckay et al. 2014), can be resulted from long-term impaired control of blood glucose levels. All of these complications are associated with considerable morbidity, mortality, and high costs to patients, families, and the health care systems (Maahs et al. 2015).

The pathogenesis of T1D is complex and multifactorial and involves a genetic susceptibility that predisposes to abnormal immune responses in the presence of ill-defined environmental insults to the pancreatic islets (Kahaly and Hansen 2016; Brilot and Geenen 2005; Gillespie 2006; Knip and Akerblom 1999; Scherbaum 1992; von Herrath 2004; Ziegler and Standl 1994). The inherited genetic factors are a major component implicated in T1D pathogenesis (Wei et al. 2008; Wang et al. 2006; She and Marron 1998; She 1996; Polychronakos and Li 2011). There is compelling evidence that diabetes susceptibility is likely linked to a major locus and that several other minor loci may contribute to diabetes risk in an epistatic way (Kumar et al. 2009; She and Marron 1998; She 1996; Pugliese et al. 1995; Pugliese and Eisenbarth 2004). Around 10–20% of newly diagnosed childhood T1D cases are characterized by an affected first-degree relative. The subjects with an affected sibling or parent manifest a cumulative risk of 3–7%

up to about 20 years of age, as compared with those subjects in the general populations (<1%) (Tuomilehto 2013). This increased risk in relatives compared to the general population prevalence suggests a familial aggregation. The degree of familial aggregation for T1D (λs) can be estimated by a ratio of the risk for siblings of patients and the general population prevalence (i.e., λs = 6/0.4 = 15). Therefore, relatives have much higher risk to develop T1D as they share genes to a greater extent with patients than that of unrelated individuals. However, T1D only shows ~40% concordance rate in monozygotic twins (MZ), suggesting a role for environmental or epigenetic factors in disease etiologies (Stefan et al. 2014). There is an ever-increasing body of evidence demonstrating that T1D development and progression are associated with diverse environmental triggers such as viral infections. The most popular hypothesis circulating within and beyond the scientific community is that viral infections accelerate or delay autoimmune disorders such as T1D (van der Werf et al. 2007; Diana et al. 2011; Gallagher et al. 2015; Moore and Adler 2016; Crevecoeur et al. 2015). Indeed, the environmental triggers often used for the explanation of differences of disease frequency across many populations and the rapid rise in disease frequency in the last few decades (Atkinson 2005). The lack of a perfect correlation between genotype and phenotype renders the identification of susceptibility genes for this disorder a formidable challenge.

Despite the above discussed difficulties, the genetic factors for T1D are probably the best known among complex diseases, and significant progress has been made during the last two decades. To date, Genetic studies of type 1 diabetes (T1D) have identified more than 50 susceptibility regions (Onengut-Gumuscu et al. 2015; Bradfield et al. 2011; Barrett et al. 2009) revealing major pathways contributing to risk (Virgin and Todd 2011), with some loci shared across immune disorders (Cotsapas et al. 2011; Smyth et al. 2008; Wellcome Trust Case Control et al. 2012). However, the susceptibility genes encoded in these regions remain largely elusive. In 2004, we first reported *small ubiquitin-like modifier 4* (*SUMO4*) within the *IDDM5* region in T1D susceptibility (Guo et al. 2004). We demonstrated that the *M55V* substitution within the *SUMO4* gene is associated with increased risk for T1D development in multiple ethnic groups (Wang et al. 2005; 2006; Wang and She 2008). In this chapter, we will summarize and validate genetic data for *SUMO4* association studies in type 1 diabetes. We will also discuss the functional properties and possible molecular mechanisms by which altered sumoylation function is implicated in the pathogenesis of type 1 diabetes.

18.2 Characterization of *SUMO4* in T1D Susceptibility

IDDM5, one of the T1D susceptibility loci located on chromosome 6q25, was initially introduced by Davis and coworkers (Davies et al. 1994). A significant linkage evidence was actually obtained by follow up studies from our research group through analysis of a large Caucasian data set (MLS = 4.5) (Luo et al. 1995, 1996). Our results were then confirmed by studies from multiple groups with observations of genetic heterogeneity in the UK population (Davies et al. 1996; Delepine et al. 1997; Zhoucun et al. 2001). By combined analysis of these published data we further narrowed the *IDDM5* locus between markers *D6S476* and *D6S473*, a region with 5-cM of genetic distance (Luo et al. 1996). In order to characterize the susceptible gene, we performed fine-mapping using high density of single nucleotide polymorphisms (SNPs) flanking the entire region. Our initial study employed a case-control study design which included 703 patients and 916 ethnically and geographically matched controls. SNPs with suggestive association were then expanded to intra-family based association study by analysis of 944 families originated from multi-ethnic populations. Suggestive association was detected in several SNPs in the initial case-control phase study. However, subsequent intra-family based study only detected strong association for a particular SNP, *001Msp*, in the US Caucasian data set ($P = 9.7 \times 10^{-5}$) and French/Spanish data set

(P = 0.03). More importantly, similar trend of transmission was also observed in the Mexican American, Italian, Chinese/Korean data sets although statistical significance in each data set was not reached due to small sample size. Overall, the association between *001Msp* and T1D was highly significant in the combined data set ($P = 3 \times 10^{-7}$).

Next, 13 SNPs flanking *001Msp* which encompass a genomic region of 320 kb of DNA were used to further determine the disease interval (Fig. 18.1). Two SNPs (*932Taq* and *107Hind*) without association for T1D susceptibility were used to define telomeric and centromeric boundaries. SNPs adjacent to *001Msp* were found to be significantly associated with T1D, while the association dropped dramatically from those markers to both telomeric and centromeric sides. Based on these results, we have finally localized the disease interval to a 197 kb of genomic DNA between SNPs *493Ras* and *454Msp* (Guo et al. 2004). Only one known candidate gene, *TAB2*, was found within this newly defined region. Part of the *ZC3H12D* gene was also found within the region. However, sequence analysis of DNA pools originated from both T1D patients and controls failed to identify any polymorphism with functional relevance in these two genes. We next sought to examine the existence of novel transcript(s) within the disease interval. By sequence analysis we have characterized and molecularly cloned a novel transcript within the intron of *TAB2* which was later named as *SUMO4*. *SUMO4* belongs to the *SUMO* gene family which is evolutionarily conserved between diverse species and is required for viability of most eukaryotic cells, including yeast, nematodes, fruit flies, and vertebrate cells in culture (Podolsky et al. 2009; Isogai and Shirakawa 2007; Johnson 2004). Interestingly, a 163A>G mutation was identified in the *SUMO4* gene which changes amino acid methionine (Met) to valine (Val) at position 55 (M55V). Met55 is located within the CUE domain of *SUMO4* and is evolutionarily conserved among diverse species (Fig. 18.2, in italic and indicated by an arrow). M55V was then subjected to genetic analysis of our entire collection of diabetic samples at that time. It was noted that the percentage for transmission of G allele which encodes Val55 is significant higher in all families originated from multi-ethnic populations except for the UK families. Taken together, *M55V* is significantly associated with increased risk for type 1 diabetes ($P = 1.9 \times 10^{-7}$), suggesting that *SUMO4* could be a novel susceptibility gene for type 1 diabetes (Guo et al. 2004).

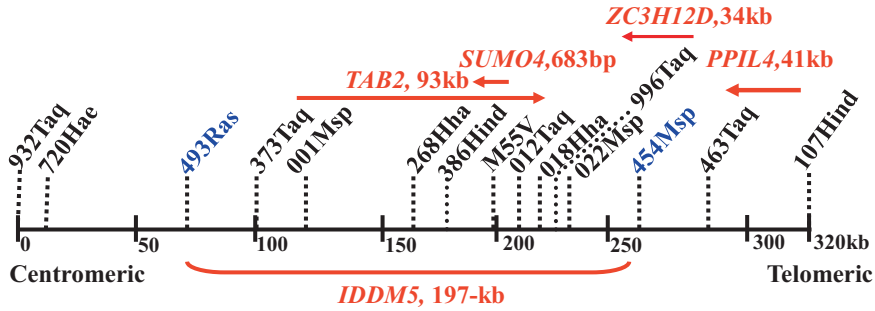

Fig. 18.1 A physical and transcriptional map for the newly defined *IDDM5* interval. The map covers a region of 320 kb genomic DNA on chromosome 6q25. Two SNPs (*932Taq* and *107Hind*) without association to T1D susceptibility were used to determine the centromeric and telomeric boundaries. The disease interval was defined by *493Ras* and *454Msp* to a region within 197 kb genomic DNA. Candidate genes are shown in the map, and the transcriptional direction for each candidate gene is indicated by an *arrow*

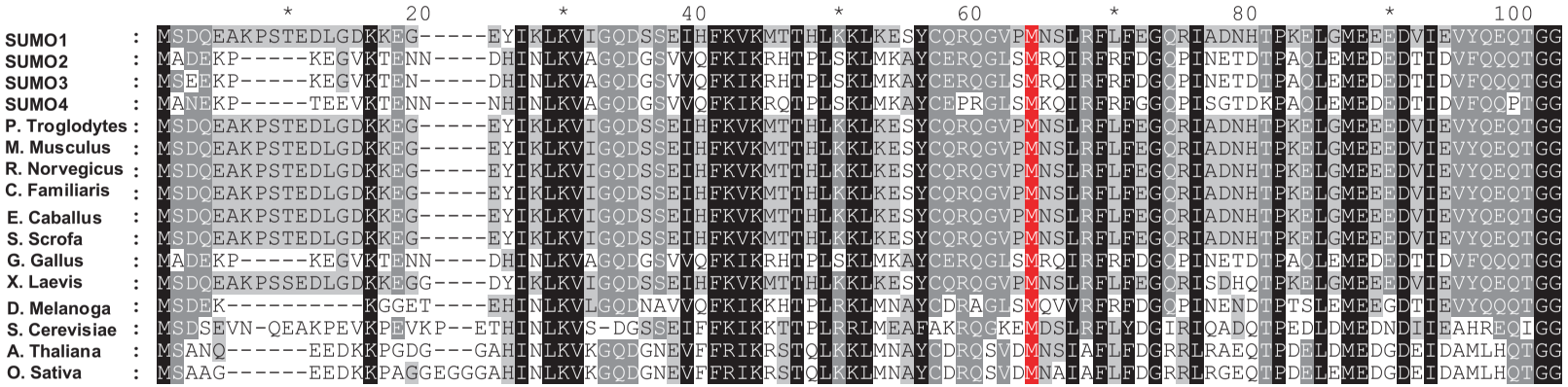

Fig. 18.2 Multiple sequence alignment showing the conservation for Met55 of SUMO4 in diverse species. SUMO amino acid sequences originated from *O. Sativa* (P_001045207), *A. Thaliana* (NP_194414), *S. Cerevisiae* (NP_010798), *D. Melanoga* (NP_477411), *X. Laevis* (NP_001090274), *G. Gallus* (NP_989466), *M. Musculus* (NP_033486), *R. Norvegicus* (NP_001009672), *S. Scrofa* (NP_001106146), *C. Familiaris* (XP_536034), *E. Caballus* (XP_001497276), and *P. Troglodytes* (XP_516035) were aligned with human SUMO1 to 4. Met55 of SUMO4 (in *italic* and indicated by an *arrow*) was found to be evolutionarily conserved ranging from plants to mammals

18.3 Genetic Heterogeneity for *SUMO4* in the European Caucasians

Interestingly, while we were publishing our data, Owerbach and co-workers also reported their study for *SUMO4* in T1D susceptibility almost at the same time (Owerbach et al. 2004). They have analyzed 478 families, of which 222 were originated from UK. To our surprise, in contrast to our finding in the non-UK data set but consistent with our finding in the UK data set, the A allele (Met55) of *SUMO4* is transmitted more frequently from parents to the affected children (57.1%, $P < 0.0004$) (Owerbach et al. 2004). Furthermore, several follow up studies using families with European Caucasian origins failed to replicate the association between *M55V* of *SUMO4* and T1D (Podolsky et al. 2009; Qu et al. 2005; Smyth et al. 2005). These results raised some doubt about the validity of the reported association.

The possibilities responsible for the discrepant association for *SUMO4* could include genotyping mistakes, random variation due to small sample size, spurious association, and genetic heterogeneity or population differences in gene-gene and gene-environment interactions. We ruled out genotyping mistakes by re-genotyping a subset of samples. Spurious association is unlikely responsible for the observed association because of the intra-family based study design. Our sample size was also sufficiently large, and the possibility for association caused by random variation was very unlikely.

18.4 Validation of *SUMO4* as a Novel T1D Susceptibility Gene

In contrast to the association observed in the European Caucasians, *SUMO4* was found to be consistently associated with T1D in the Asian populations. In our initial report, we observed significant association between *SUMO4* and T1D both in Chinese and Korean populations (Guo et al. 2004). The association was then independently confirmed in a Korean case-control cohort consisting of 386 T1D patients and 553 normal controls. Park and colleagues demonstrated that the G allele (Val55) had a significant higher frequency in diabetic patients (62.0%) than that in matched controls (52.1%), with a relative risk (RR) of 1.5 ($P < 0.003$) (Park et al. 2005). The association was further confirmed by a follow up study from Noso and coworkers (Noso et al. 2006, 2007). They have analyzed a large cohort of 1113 Japanese (472 cases and 641 controls) and 171 Korean subjects (69 cases and 102 controls). Consistent with our observation, the G allele was significantly associated with T1D in Japanese patients (OR = 1.43, $P < 0.005$). A similar trend was also observed in Korean subjects (OR = 1.75). In combined data from Japanese and Korean subjects, the G allele was significantly associated with T1D (OR = 1.46, $P = 0.00083$). Subsequently, Tsurumaru and coworkers performed another case-control study using a total of 1480 samples of Japanese origin (Tsurumaru et al. 2006). They demonstrated a significant association between the *M55V* of *SUMO4* and T1D (OR = 1.42, $P = 0.0072$). They also demonstrated the association between *M55V* of *SUMO4* and autoimmune thyroid disease (AITD) (OR = 1.52, $P = 0.0041$), as well as rheumatoid arthritis without amyloidosis (OR = 1.53, $P = 0.027$), but not with primary Sjögren's syndrome. Interestingly, the association was stronger in T1D patients with AITD (OR = 1.62, $P = 0.023$), and in patients without either *DRB1*0405* or *DRB1*0901*, the two HLA class II alleles with the highest risk to T1D in the Japanese population (OR = 2.28, $P = 0.0018$). A meta-analysis of all above non-overlapped case/control data sets suggested a significant association between *SUMO4* and T1D (OR = 1.45, $P = 3.9 \times 10^{-8}$), and no genetic heterogeneity was detected (Wang and She 2008). More recent studies mainly conducted in Asian populations provided additional evidence suggesting that *SUMO4* could be a common genetic factor predisposing to higher risk for other autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus, Vogt–Koyanagi–Harada syndrome and Behcet's disease (Fakhfakh Karray

et al. 2011; Hou et al. 2008a, b; Orozco et al. 2006; Park et al. 2012; Alzolibani et al. 2015).

Given the results from Asian populations, there is little doubt that *SUMO4* is a T1D susceptibility gene. However, the issue is less settled in the Caucasian populations. Similar trend of association between *SUMO4* and T1D was observed in several populations studied in our initial report, including Florida, French, Spanish and Mexican Americans (Guo et al. 2004). The strongest evidence was from the North-Central Florida data set which includes both families and case-control cohorts (Guo et al. 2004; Wang et al. 2006). To confirm our initial observations, we further analyzed a new Florida cohort consisting of 196 T1D patients and 1060 matched controls (Wang et al. 2005). A significant association for the G allele (Val55) of *SUMO4* was obtained (OR = 1.5, P = 0.01). When we combined this new cohort with the initial Florida case/control data set (244 cases and 274 controls), the evidence is further enhanced (OR = 1.6, P = 0.0001, Table 18.1) (Wang et al. 2005, 2006; Wang and She 2008). It is noteworthy that in the large UK case-control dataset studied by Smyth and co-workers (Smyth et al. 2005), there is also marginally significant association consistent with our initial finding (Table 18.1). In addition, recent studies further demonstrated that *M55V* of *SUMO4* is associated with T1D in association with high risk *HLA-DR3* and *DR4* genotypes in Caucasians with Swedish origin (Sedimbi et al. 2007).

The above results provided strong evidence suggesting that *SUMO4* is also associated with T1D in Caucasians. The question is, then, why association is not observed on some other populations. The discrepancies may be explained by two distinct possibilities. The first one is genetic heterogeneity, e.g., *SUMO4* is only implicated in T1D in some populations but not others. The human population is not homogenous in terms of the risk of disease. Different patients or ethnic groups may have a different set of genes that in combination are responsible for their disease onset. Also, depending on the genetic background (gene-gene interactions or gene-environment interactions), an etiological mutation may or may not exert its effect. Furthermore, a plethora of environmental insults have been characterized to be able to induce alterations in the epigenome such as DNA methylome (Al-Haddad et al. 2016; Arroyo-Jousse et al. 2015; Elboudwarej et al. 2016; Ke et al. 2013). More importantly, these epigenetic alterations resulted from environmental exposures can be accumulated during one's lifetime (Gomes and Waterland 2008), which renders an individual with increased susceptibility to disease development and progression (Fraga et al. 2005; Strickland and Richardson 2008; Wilson 2008). In line with this assumption, an increase in the incidence of T1D in children has been reported in recent years along with living style and dietary changes (Gao et al. 2016; Hummel et al. 2012; Teeaar et al. 2010). Genetic heterogeneity resulted from environmental exposures has often been blamed for the inconsistent observations on disease association across different populations or ethnic groups. Few studies have truly distinguished genetic heterogeneity from false association. It remains to be determined whether genetic heterogeneity is truly responsible for the inconsistent association evidence for *SUMO4* and T1D. The second explanation for the inconsistent association is that the *IDDM5* interval may contain multiple T1D susceptibility genes which may contain both susceptible and protective alleles that are in different linkage disequilibrium patterns in different populations. This phenomenon has already been observed for the *DRB1* and *DQA1/DQB1* genes in the HLA region (She 1996). Further studies should be performed to determine whether similar phenomenon also occurs in the *IDDM5* region.

18.5 The Effect of M55V Supports SUMO4 in T1D Susceptibility

Analysis of the functional differences between SUMO4*M55 and *V55 variants provided additional evidence supporting its susceptibility in T1D pathogenesis. In humans, four SUMO genes, named SUMO1 to 4, have been identified. Unlike other SUMO members which have a wide tissue distribution, SUMO4 expression, however,

Table 18.1 Summary of published case-control association results for M55V of *SUMO4*

Datasets		GG (%)	AG (%)	AA (%)	GG + AG (%)
Guo et al.					
Florida dataset:	T1D: 244	83 (34.0)	114 (46.7)	47 (19.3)	197 (80.7)
	Control: 274	58 (21.2)	134 (48.9)	82 (29.9)	192 (70.1)
	P value	0.001	NS	0.005	0.005
Spanish dataset	T1D: 170	47 (27.6)	93 (54.7)	30 (17.7)	140 (82.3)
	Control: 151	39 (25.8)	81 (53.6)	31 (20.6)	120 (79.4)
	P value	NS	NS	NS	NS
Taiwan, China	T1D: 96	5 (5.2)	51 (53.2)	40 (41.6)	56 (58.4)
	Control: 191	18 (9.4)	78 (40.8)	95 (49.8)	96 (50.2)
	P value	NS	0.048	NS	NS
Mainland China	T1D: 96	18 (18.8)	38 (40.0)	40 (41.2)	63 (58.8)
	Control: 188	15 (7.9)	86 (45.7)	87 (46.4)	101 (53.6)
	P value	0.007	NS	NS	NS
Korean dataset	T1D: 97	19 (19.6)	47 (48.5)	31 (31.9)	66 (68.1)
	Control: 112	12 (10.7)	48 (42.9)	52 (46.4)	60 (53.6)
	P value	NS	NS	0.03	0.03
Wang et al.					
Florida dataset:	T1D: 197	66 (33.5)	90 (45.7)	41 (20.8)	156 (79.2)
	Control: 1060	268 (25.3)	534 (50.4)	258 (24.3)	802 (75.7)
	P value	0.01	NS	NS	NS
Park et al.					
Korean data set	T1D: 386	52 (13.5)	187 (48.5)	147 (38.0)	239 (62.0)
	Control: 553	58 (10.5)	230 (41.6)	265 (47.9)	288 (52.1)
	P value	NS	<0.04	<0.003	<0.003
Noso et al .					
Japanese dataset	T1D: 472	43 (9.1)	234 (49.6)	195 (41.3)	277 (58.7)
	Control: 641	64 (9.9)	256 (40.0)	321 (50.1)	320 (50.1)
	P value	NS	0.001	<0.004	<0.004
Korean dataset	T1D: 69	9 (13)	31 (45.0)	29 (42)	40 (58)
	Control: 102	11 (10.8)	34 (33.3)	57 (44.1)	57 (55.9)
	P value	NS	NS	NS	NS
Tsurumaru et al.					
Japanese dataset	T1D: 411	40 (9.7)	201 (48.9)	170 (41.4)	241 (58.6)
	Control: 551	55 (10.0)	220 (39.9)	276 (50.1)	275 (49.9)
	P value	NS	0.005	0.007	0.007
Smyth et al.					
UK dataset	T1D: 3442	898 (26.2)	1769 (51.7)	775 (22.1)	2667 (77.9)
	Control: 3788	1007 (26.6)	1856 (49.0)	925 (24.4)	2863 (75.6)
	P value	NS	0.04	0.056	0.056
Combine Florida dataset					
	T1D: 441	149 (33.8)	204 (46.3)	88 (19.9)	353 (80.1)
	Control: 1334	326 (24.4)	668 (50.1)	340 (25.5)	994 (74.5)
	P value	0.0001	NS	0.02	0.02

is more restricted to immune cells, kidney and pancreatic islets and its distribution is manifested by a tissue- or organ-dependent manner (Wang and She 2008; Guo et al. 2004). By RT-PCR analysis, high levels of SUMO4 expression were detected in immune cells such as dendritic cells

(DCs) and macrophages. Moderate levels of SUMO4 expression were also detected in the kidney and pancreatic islets (Wang and She 2008). This tissue distribution pattern supports its role in T1D susceptibility which is characterized by autoimmune destruction of the pancreatic islets.

Initial functional study revealed that SUMO4 could act as a negative regulator for the NFκB transcriptional activity (Guo et al. 2004), a pivotal molecule implicated in the initiation and progression of autoimmunity in type 1 diabetes. The M55 residue, located in the CUE domain of SUMO4, has been found to be evolutionarily conserved among diverse species (Fig. 18.2, indicated by an arrow) (Li et al. 2005). Functional study revealed that M55V is associated with a significant reduction for the sumoylation function of SUMO4. When HEK293 cells transfected with the *V55 variant, a 5.5-fold higher NFκB-dependent transcriptional activity was observed as compared with that of cells transfected with the *M55 variant upon IL-1β stimulation. Consistent with this observation, a threefold higher *IL-12p40* was observed in peripheral blood mononuclear cells (PBMCs) originated from subjects with G/G genotype (Val55) as compared to that of individuals with A/A genotype (Met55) upon stimulation (Guo et al. 2004). The effect of M55V has been also demonstrated by another independent study. Bohren and coworkers reported that M55V is associated with a significant reduced capability for SUMO4 to enhance HSF transcriptional activity (Bohren et al. 2004). Taken together, the biological effect of M55V provided additional support for a role of SUMO4 in T1D pathogenesis.

Extensive studies in the past few years have demonstrated intriguing evidence for sumoylation in the regulation of immune responses (Decque et al. 2016; Maarifi et al. 2015; Wang et al. 2015), insulin secretion and pancreatic beta cell destruction (Ferdaoussi et al. 2015; Attie 2015; Hajmrle et al. 2014; Manning Fox et al. 2012), and glucose metabolism (Simpson-Lavy and Johnston 2013; Castro et al. 2015; Tang et al. 2013; Liu et al. 2007). Particularly, it was interestingly noted that mice deficient in sumoylation function develop severe diarrhea and die shortly after depletion of the only conjugating enzyme Ubc9 (Demarque et al. 2011), while mice with DC specific diminished sumoylation function confer susceptibility to endotoxin shock and resistance to viral infection (Decque et al. 2016). In the next several sections, we will discuss the functional properties and the possible molecular mechanisms of SUMO4 in the regulation of T1D development based on the experimental evidence.

18.6 Stress-Dependent SUMO4 Functionality

To be functionally active, SUMO proteins need to be hydrolyzed to expose their C-terminal di-glycine (GG) motif. This di-glycine motif is prerequisite for SUMO proteins to covalently conjugate to their substrate proteins (Li et al. 2005). Unlike SUMO1 precursor that has four extended amino acids after the di-glycine motif (GG-HSTV), but similar to the SUMO2 precursor, the precursor SUMO4 only has two extended amino acids after the GG motif (GG-VY). In an effort to determine SUMO4 maturation by the endogenous hydrolase, we prepared cell lysates from overnight cultures of 1×10^7 HEK293 cells, and 20 μl of which was then used to hydrolyze recombinant SUMO4 (rSUMO4) as reported (Wei et al. 2008). To our surprise, after 2 h of incubation, even in the presence of protease inhibitors, the amount of detectable rSUMO4 remained in the reactions was significantly decreased. In contrast, the same cell lysates failed to degrade rSUMO1 even in the absence of protease inhibitors. We then performed a time course study and demonstrated that under physiological condition, SUMO4 undergoes a rapid and progressive degradation as manifested by a time-dependent manner. We next examined SUMO4 stability in the stressed condition. For this purpose, we first stressed HEK293 cells by serum starvation or cytokine stimulation for 36 h and then performed similar study as above. Interestingly, lysates derived from stressed cells failed to degrade rSUMO4. Together, these data suggest that cells under physiological condition probably mediate a rapid and progressive degra-

dation for SUMO4. However, SUMO4 becomes stable once cells under stressed condition, suggesting that SUMO4 could only exert its functionality in cells after stressful insults.

Based on the above observations, appropriate experiments were designed to examine the capacity of stressed cells to mediate SUMO4 maturation. As SUMO4 expression is more restricted to immune cells and pancreatic islets (Guo et al. 2004; Wang and She 2008), DC2.4 cells (a dendritic cell line), Jurkat cells, RAW264.7 cells and CM cells (a pancreatic islet-derived cell line) were selected for the study. It was found that all of these cells showed the capability to mediate SUMO4 maturation, and the highest capacity was observed in RAW264.7 cells (Wei et al. 2008). A time course study further revealed that stimulation of cells with 16 h serum starvation induced the capability to mediate SUMO4 maturation, while the capacity was much higher in cells after longer serum starvation, and the highest capacity was observed in 48 h starved-cells. Co-immunoprecipitation was then carried out in cells transfected with a full-length SUMO4 plasmid to further address the capability for SUMO4 sumoylation of its substrate proteins *in vivo*. Upon stressful insults such as inflammatory cytokine stimulation or serum starvation, SUMO4 showed high capacity to sumoylate its substrate proteins as characterized by the existence of SUMO4-substrate complexes on Western blots (Wei et al. 2008). These observations further support that SUMO4 could only be functionally active after cells insulted by stressful stimulations.

18.7 SUMO4 Acts as a Negative Regulator for the NFκB Signaling Pathway

The nuclear factor κB (NFκB) is a family of transcription factors implicated in the regulation of inflammatory immune response, cell viability and programmed cell death. Thus far, five subunits named p50, p52, p65 (RelA), c-Rel, and RelB, respectively, have been identified in vertebrates (Jimi and Fukushima 2016; Siggers et al. 2015). These subunits usually form various homo- and heterodimers, while the most common active form in mammals is the p50/RelA or p52/RelA heterodimer (Wang and She 2008). Under physiological conditions, the NFκB complexes are sequestered in the cytoplasm in an inactive form *via* a non-covalent interaction with inhibitory proteins known as IκBs such as IκBα. In response to multiple stimuli such as inflammatory cytokines, viral and bacterial infections, and stressful insults, the NFκB/IκBα complex becomes dissociated *via* phosphorylation of the conserved serine residues in the N-terminal portion of IκBα. The dissociated IκBα is then rapidly degraded by the ubiquitylation/proteasome pathway, which leads to NFκB translocation into the nucleus where it binds to the target sequences and activates the transcription of immune responsive genes (Li et al. 2005) (Fig. 18.3).

SUMO1 has been previously suggested to negatively regulate NFκB transcriptional activity by conjugating to IκBα (Desterro et al. 1998). The link between SUMO4 and NFκB was initially established by a yeast two-hybrid screening of a pretransformed spleen cDNA library. SUMO4 was found to interact with IκBα in the yeast two-hybrid system which was then confirmed by the co-immunoprecipitation assays (Guo et al. 2004). More recently, we have demonstrated that upon stressful insults, SUMO4 becomes matured and is able to conjugate to its substrate proteins such as IκBα (Wei et al. 2008). Similar as SUMO1, SUMO4 sumoylation can prevent IκBα from signal-induced degradation and, as a result, it negatively regulates NFκB transcriptional activity (Fig. 18.3). More interestingly, an NFκB binding site has been characterized within the SUMO4 promoter. By luciferase and chromatin immunoprecipitation (ChIP) assays we found that NFκB binds to the SUMO4 promoter and regulates its transcription (Wang et al. 2009). Based on these observations, we postulate that SUMO4 could be a negative feedback regulator for the NFκB signaling pathway. Upon signal-induced activation, NFκB could initiate transcriptions for three groups of genes: 1) autoregulatory genes (i.e., p50 & p65); 2) immune responsive genes (e.g., IL-1α & β, IL-2, -6, -12,

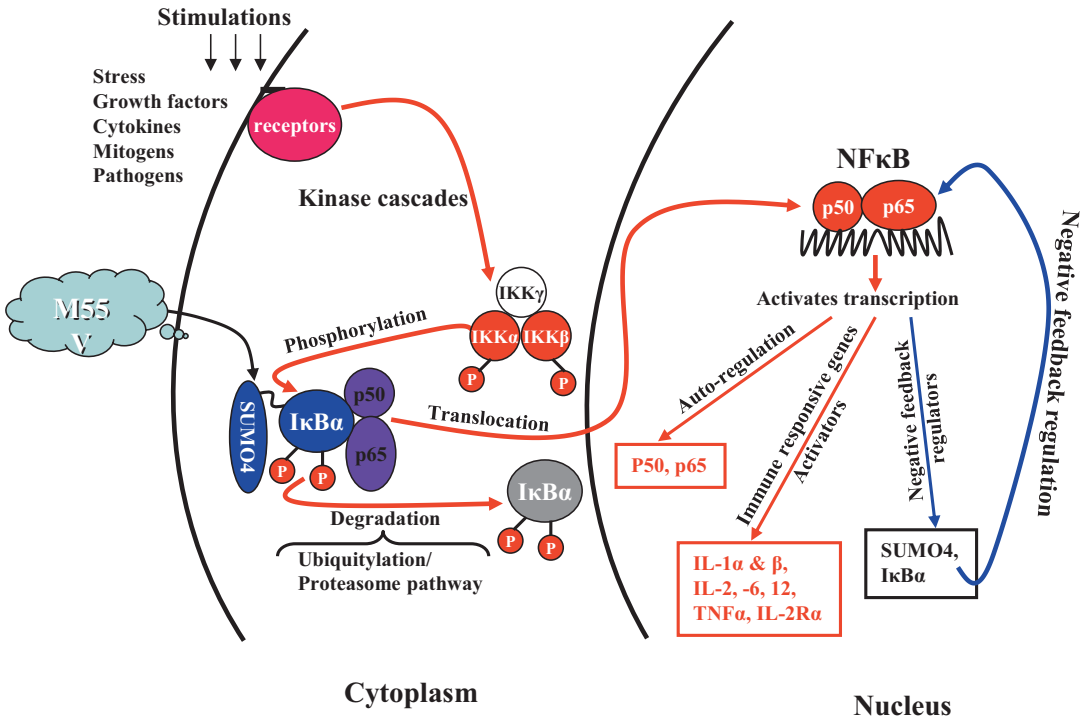

Fig. 18.3 A negative feedback model for SUMO4 regulation of NFκB signaling pathway. Upon activation, NFκB not only activates transcription of genes responsible for immune response, but also transcribes SUMO4 expression to tightly control its activity. In diabetic patients, the M55V substitution is associated with a reduced sumoylation function for SUMO4, leading to a higher NFκB transcriptional activity upon stimulation, which in turn would activate genes necessary for the initiation and progression of autoimmunity during T1D development

TNFα, IFNγ and IL-2Rα); and 3) negative feedback regulators (e.g., SUMO4 and IκBα) that tightly control the immune response (Fig. 18.3). In diabetic patients, the M55V substitution in SUMO4 could be associated with a reduced sumoylation function, which would result in a higher cellular immune response capacity to stimulation, thereby leading to higher levels of activated NFκB, which in turn activates transcription for genes implicated in the development of type 1 diabetes.

Consistent with the above assumption, altered NFκB activities have long been shown to be involved in the pathogenesis of a number of human autoimmune diseases such as rheumatoid arthritis, Crohn's disease and so on (Gregersen et al. 2009; Jiang et al. 2014; Matmati et al. 2011; Sisto et al. 2016; Zheng and Abraham 2013). Aberrant activation of the NFκB signaling pathway has also been linked to the pathogenesis of type 1 diabetes (Fukaya et al. 2016; Salem et al. 2014; Zhao et al. 2011; Katarina et al. 2007). Both T1D patients and non-obese diabetic (NOD) mice, a model for human type 1 diabetes, show altered NFκB activity in DCs and macrophages (Mollah et al. 2008; Poligone et al. 2002; Sen et al. 2003; Weaver et al. 2001), and as such, NFκB activation seems to be essential for the initiation and progression of autoimmunity during T1D development. NFκB has also been found to be involved in the formation of free radicals which may play a pivotal role in beta cell death (Ho and Bray 1999; Quan et al. 2001). In line with these observations, both *c-Rel* and *NFκB1* (*p50/p105*) were found to be essential for multiple low-dose streptozotocin-induced diabetes in mice (Lamhamedi-Cherradi et al. 2003; Mabley et al. 2002), and adoptive transfer of DCs defi-

cient for NFκB can prevent the development of diabetes in NOD mice (Ma et al. 2003).

18.8 SUMO4 Regulates Cytokine-Initiated JAK/STAT Signalings

It has been well demonstrated that inflammatory cytokines are implicated in the pathogenesis of type 1 diabetes (Bobbala et al. 2016; Alnek et al. 2015; He et al. 2014). Studies have shown that cytokines are not only involved in the initiation and progression of autoimmunity, but also play a critical role in mediating islet inflammation (i.e., insulitis) and beta cell destruction during T1D development (Barthson et al. 2011; Bazzaz et al. 2014). Cytokine receptors both functionally and physically associate with numbers of the Janus kinase (JAK) family. JAK are cytosolic tyrosine kinases (JAK1, JAK2, JAK3, and TYK2) and are involved in the signal transduction of both type I receptors (e.g., IL2, IL3, IL4, IL5, IL6, IL7, IL13, GM-CSF, GH, PRL, EPO and TPO) and type II receptors (e.g., IFN-α, −β, −γ) (Marrero et al. 2006; Li and Watowich 2014). Cytokine signaling is initiated when liganded receptors dimerize to bring the associated JAK kinases into apposition. The JAK kinases are then activated by a transphosphorylation event and proceed to activate tyrosine residues in the receptor endodomain, which then recruit signal transducers and activators of transcription (STAT) to the activated receptor complex (Schindler 1999; Schindler et al. 2007; O'Shea et al. 2015) . Subsequent JAK kinase phosphorylation of tyrosine residues in the STAT would lead to their dimerization and nuclear translocation which then transduce the biological response to many inflammatory cytokines. Thus far, a total of seven mammalian STAT (Stat1, 2, 3, 4, 5a, 5b and 6), ranging in size from ~ 75 to 95 kDa, have been identified (O'Shea et al. 2015). Upon activation, STAT have been shown to play a central role in cytokine-dependent gene expression implicated in the regulation of a variety of cellular processes, including immune response, differentiation, cell survival and cell proliferation.

The JAK/STAT signaling pathway is tightly controlled by multiple negative regulatory mechanisms including the SHP family (dephosphorylation of JAK and cytokine receptors), the SOCS family (inhibition of JAK enzymatic activities), and the PIAS family (repression of STAT DNA binding activity). Recent studies have identified sumoylation as another negative regulatory mechanism. More interestingly, PIAS, originally characterized as a family of inhibitors for activated STAT, were found to be SUMO E3 ligases that stimulate the attachment of SUMO to its target proteins (Naidu et al. 2012; Liu et al. 2013; Yan et al. 2015). PIAS consists of at least five members, PIAS1, PIAS3, the α and β splice variants of PIASx, and PIASy (Shuai and Liu 2005). PIAS proteins share important similarities with the RING-type ubiquitin ligases (Jackson 2001), and as a result, they can promote Sumoylation in a manner that resembles the action of RING-type ubiquitin E3 ligases (Palvimo 2007). For example, PIAS1 and PIASx stimulate SUMO modification of STAT1 at a distinct lysine residue (i.e., K703) that is conserved in other members of the STAT family (Rogers et al. 2003; Ungureanu et al. 2003; Yamashina et al. 2006). In addition, PIAS-mediated inhibition of STAT activity, which is independent of sumoylation, may also require its SUMO E3 ligase activity (Yamashina et al. 2006). Moreover, PIAS proteins do not operate merely as SUMO E3s, since their co-regulator effects are often independent of their RING finger but dependent on their capability to noncovalently interact with SUMOs or DNA through their SIM (SUMO-interacting motif) or SAP (scaffold attachment factor-A/B/acinus/PIAS) domain, respectively (Palvimo 2007; Rytinki et al. 2009).

Interestingly, co-immunoprecipitation and Western blot analysis revealed that SUMO4 sumoylates STAT1 and 3 when HEK293 cells undergone serum starvation (Guo et al. 2005). Recent study further demonstrated that SUMO4 sumoylation suppresses STAT1 DNA binding activity (Gronholm et al. 2012). Based on the genetic and functional evidence for M55V of SUMO4 in T1D susceptibility, it is reasonable to postulate that the M55V substitution of SUMO4

could result in a higher STAT transcriptional activity which would predispose to the increased risk for T1D development. In line with this assumption, dysregulation of the JAK/STAT signaling pathway has been associated with T1D development by actions at both the immune system and β-cell level (Davoodi-Semiromi et al. 2012; Rondas et al. 2015; Russell and Morgan 2014; Villarino et al. 2015). NOD mice harboring beta-cells expressing SOCS-1, an inhibitor for the JAK/STAT signaling, have a markedly reduced incidence of diabetes (Flodstrom-Tullberg et al. 2003). Consistent with this observation, beta cells deficient in *STAT1* are protected against IL-1β/IFNγ–induced apoptosis (Cnop et al. 2005). In addition, STAT3 has been associated with IL-1β signaling in beta cells which could be implicated in beta cell destruction predisposing to T1D development (Morton et al. 1999).

18.9 SUMO4 Modulates AP-1 Tanscriptional Activity

Activator protein-1 (AP-1) regulates a wide range of cellular processes including proliferation, differentiation, and apoptosis. As a transcription factor, AP-1 is commonly found as a heterodimer comprised of c-Jun and c-Fos proteins (Lee et al. 2012). AP-1 is often portrayed as a general, nuclear decision-maker that determines life or death cell fates in response to a plethora of physiological stimuli and environmental insults including pro-inflammatory cytokines, growth factors, oxidative stress, and tumor promoters (Ferraris et al. 2012; Guinea-Viniegra et al. 2009; Thomsen et al. 2013; Xia et al. 2013; Chanda et al. 2003; Shaulian and Karin 2002). AP-1 signaling is mediated by mitogen-activated protein kinase (MAPK) cascades including the JNK, p38 and ERK cascades (Chang and Karin 2001; Westwick et al. 1994), while the induction of AP-1 by pro-inflammatory cytokines and genotoxic stress is mostly mediated by the JNK and p38 MAPK cascades (Chang and Karin 2001). As autoimmunity involves multiple immune cells, cell receptors and cytokines, the potential scope for AP-1 to T1D pathogenesis is extensive.

Upon activation, AP-1 not only transcribes numerous cytokines (e.g. IL-2, -6, -8 and MCP-1) and induces adhesion molecules relevant to autoimmune responses (Funakoshi-Tago et al. 2003), but also activates genes necessary for autoimmune-mediated beta cell destruction (Eizirik and Mandrup-Poulsen 2001). Studies in animal models revealed that embryos lacking c-Jun die at midgestation and exhibit defects in heptogenesis, while fetal livers undergo massive apoptosis in the absence of c-Jun (Eferl et al. 1999). In line with these observations, AP-1 has been found to be implicated in autoimmune-mediated beta cell destruction (Ammendrup et al. 2000; Lgssiar et al. 2004; Pavlovic et al. 2000). Nevertheless, whether AP-1 is a foe or friend to beta cells has been somehow controversial. Studies in multiple low-dose-streptozotocin-induced diabetes in C57BL/6 mice indicated that activation of AP-1 is associated with enhanced beta cell destruction (Ohly et al. 2000; Schott-Ohly et al. 2004). In contrast, studies in NOD mice revealed that AP-1 activation in the pancreatic islets protects beta cell from autoimmune-mediated destruction (Schott-Ohly et al. 2004). An emerging scenario is that the effects of AP-1 activities on beta cell death are heavily dependent on the specific Fos and Jun subunits contributing to AP-1 dimers and the cellular context. Consistent with this assumption, in cellular models of type 1 diabetes (cytokine-treated ***β***-cells), JunB was shown to promote ***β*** **-**cell survival by inhibiting NF-***κ***B activity (Gurzov et al. 2008) and regulating ATF3 expression (Gurzov et al. 2012; Cunha et al. 2014).

T cells are the most important effector cells in mediating T1D associated beta cell destruction. Studies have shown that TCR/CD28 mediated c-Jun activation is critical for T-cell development, differentiation, and activation (Chen et al. 1998; Rincon et al. 1998; Su et al. 1994). c-Jun forms either homodimers or heterodimers with other members of Fos-Jun family of transcription factors. It has been suggested that reduced c-Jun transcriptional activity is associated with T cell

anergy. For example, stimulation of anergic T cells failed to activate the binding of IL-2 promoter by Jun-Jun homodimer and the formation of Jun-Jun/Oct complex (Powell et al. 1999; Wotton et al. 1995). Studies have shown that the promoter DNA-binding activity of AP-1 transcription factors is selectively inhibited in the naturally occurring CD4+ CD25+ regulatory T cells (Tregs) from mice (Lee et al. 2008). In line with this observation, blockade of AP-1 in donor splenocytes exhibited reduced Th17/Th1 population and enhanced Treg population in acute graft-versus-host disease. Beneficial Treg expanding property of AP-1 blocker was associated with the induction of Foxp3 and STAT5 transcription factor, where the inhibiting property of Th17 was achieved by suppressing the phosphorylated form of STAT3 and enhancing SOCS3 activity (Park et al. 2014). Aberrant Th2 response has been observed both in T1D patients and NOD mice, and therefore, altered AP-1 activity may also contribute to T1D pathogenesis by limiting the protective Th2 responses (Cameron et al. 1997; Fox and Danska 1997; Rapoport et al. 1998).

Despite extensive studies, the mechanisms underlying the control of AP-1 transcriptional activity remain poorly understood. In 2005, Bossis and coworkers demonstrated that c-Fos can be sumoylated at lysine 265, while c-Jun can be sumoylated at both lysine 229 and 257, and their sumoylation is associated with a decreased DNA binding activity (Bossis et al. 2005; Tempe et al. 2014). Consistent with their report, AP-1 devoid of sumoylation sites had much higher transcriptional activity than its wild-type counterpart (Muller et al. 2000, 2004; Schmidt and Mueller 2002), and PIAS stimulation enhances sumoylation of the AP-1 complex (Kotaja et al. 2002; Muller et al. 2000). Studies suggest that sumoylation of JunB at lysine 237 in activated CD4+ T cells induces activation of the endogenous IL-2 promoter. Blockade of JunB sumoylation by mutation or dominant-negative form of Ubc-9 attenuates its ability to transactivate IL-2 and IL-4 reporter genes (Garaude et al. 2008). It is noteworthy that SUMO represses AP-1 activity not only by direct sumoylation, but also by indirect inhibition of the AP-1 upstream activators. For example, sumoylation inhibits the activity of both SRF (serum response factor) and Elk-1, the upstream activators for c-Fos transcription (Matsuzaki et al. 2003; Salinas et al. 2004). Of importantly note, similar as other SUMO members, SUMO4 has been found to possess the capacity to regulate AP-1 activity (Guo et al. 2005). *In vitro* studies revealed that SUMO4 possesses the capability to sumoylate c-Fos by conjugation assay. By luciferase reporter assay SUMO4 has been found to repress AP-1 DNA binding activity (Guo et al. 2005). It would be interesting to examine the effect of SUMO4 sumoylation on each of the AP-1 subunit, which would shed pivotal information to delineate the molecular mechanisms underlying SUMO4 regulation to T1D development.

18.10 SUMO4 Wrestles with Intracellular Stress

Although the precise mechanisms for autoimmune-mediated beta cell destruction leading to diabetes remain elusive, it has been well accepted that oxidative stress resulted from autoimmunity plays a pivotal role in T1D pathogenesis (Malaguti et al. 2014; Padgett et al. 2013). T cell-mediated beta cell destruction is initiated by the release of cytotoxic cytokines such as IL-1, IFN-γ and TNF-α, which contribute to beta cell death by up-regulation of Fas and Fas ligand and stimulation of nitrite oxide (NO) and free radical production (Suarez-Pinzon and Rabinovitch 2001). Autoreactive T cells can also directly deliver beta cell death signals *via* the Fas signaling characterized by the activation of caspase pathway, which is also associated with actions of NO and oxygen-derived free radicals (Kawasaki et al. 2004). *β*-cells are more prone to oxidative damage than most other tissues because the *β*-cell mitochondria have exceptionally low levels of glutathione peroxidase, superoxide dismutase, and catalase activity (Delmastro and Piganelli 2011). In line with this observation, beta cells or pancreatic islets with exogenous antioxidant enzyme expression are protected from cytokine-induced destruction

(Hohmeier et al. 1998; Lortz et al. 2000; Lortz and Tiedge 2003; Tran et al. 2004). Of note, ROS can be also used by cells of both adaptive and innate immune system as regulators of signal transduction (Griffiths 2005; Larbi et al. 2007; Lu et al. 2007; Williams and Kwon 2004; Kasahara et al. 2011; Park et al. 2015; West et al. 2011). There is compelling evidence that ROS act as requisite second messengers essential for ligand-mediated regulation of protein kinase activation, gene expression, and/or proliferative responses. Therefore, ROS are important in the regulation of T cell signal transduction, gene expression and function. A typical example demonstrating the role of ROS in T1D associated autoimmune response and beta cell destruction is manifested in ALR/Lt mice. ALR mice are selectively bred for resistance to alloxan free-radical-induced diabetes from the same outbred Swiss progenitors as the NOD strain (Graser et al. 1999), and therefore, they share 70% of the NOD genome . However, their unique 30% genome encodes protective determinants at both immune cell and islet cell levels, which renders this strain of mice with unusual resistance to oxidative stress (Yang et al. 2008). As a result, these mice are protected not only from spontaneous autoimmune diabetes, but also from autoimmunity and subsequent beta cell destruction after adoptive transfer of diabetogenic immune cells (Chen et al. 2008; Mathews et al. 2005).

Oxidative stress is a result of imbalanced ROS production and scavenging. Pathways for ROS production have been well recognized during T1D development, while the regulation of the enzymes involving ROS scavenging in the pancreatic islets remains poorly understood. Zhou and co-workers performed a global analysis of sumoylated proteins in *Saccharomyces cerevisiae* by tandem mass spectrometry (Zhou et al. 2004), the observation of increased protein sumoylation in cells under oxidative and ethanol stresses suggested a role for sumoylation in the regulation of cellular stress. A subsequent study in mammalian cells demonstrated a global shift of protein sumoylation in response to oxidative stress (Manza et al. 2004). When cells under oxidative stress, SUMO targets distinct proteins for sumoylation including antioxidant enzymes, chaperones and DNA damage signaling proteins, which could function as a cytoprotective mechanism against oxidative stress. More recently, a number of studies consistently suggested a role for SUMO in prevention of apoptosis by specific targeted sumoylation (Yang et al. 2015; Han et al. 2015; Hajmrle et al. 2014). Interestingly, unlike other SUMO members, SUMO4 undergoes a rapid and progressive degradation in cells under physiological condition. However, SUMO4 becomes matured and is able to sumoylate its substrate proteins in cells after stressful insults (Wei et al. 2008), which supports a role for SUMO4 in the regulation of cellular stress. By 2-D PAGE coupled with MALDI/TOF/TOF analysis, we specifically analyzed the substrates of SUMO4 during cellular stress of serum starvation (Guo et al. 2005). This study identified ninety SUMO4 substrates, which included molecular chaperones (e.g., HSPs), DNA repair regulators (e.g., DNA topoisomerase II), RNA processing proteins (e.g., hnRNP), some anti-stress transcription factors (e.g., glucocorticoid receptor, GR), and antioxidant enzymes (e.g., catalase and peroxiredoxins). It was found that SUMO4 enhances glucocorticoid receptor (GR) DNA binding activity, an endogenous antagonist for NFκB and AP-1 (Guo et al. 2005), but represses AP-2 activity, an endogenous repressor for MnSOD transcription. As a result, cells with exogenous SUMO4 expression were prevented from intracellular H_2O_2 accumulation. In line with our results, recent genetic studies suggested that *M55V* of *SUMO4* could be associated with increased risk for type 2 diabetes and diabetic nephropathy (Sinha et al. 2016; Sozen et al. 2014), both of which are strongly associated with altered cellular oxidative stress. Taken together, all of these results suggest that SUMO4 may play a pivotal role in the regulation of cellular oxidative stress relevant to autoimmune initiation, progression and final beta cell destruction during T1D development.

18.11 Conclusions

Despite the discrepant associations between *SUMO4* and T1D susceptibility in the Caucasian populations, *SUMO4* has been found to be consistently associated with T1D in the Asian populations. The discrepant associations observed in certain Caucasians could be caused by genetic heterogeneity as manifested by the differences of gene-gene interactions or gene-environment interactions, or by the differences in linkage disequilibrium (LD) patterns within the *IDDM5* locus. For example, *IDDM5* may contain multiple T1D susceptibility genes with both susceptible and protective alleles and, as such, the overall association for the markers in the *IDDM5* region would depend on the LD patterns in a given population. In the past two decades, extensive studies have demonstrated that sumoylation is a remarkably versatile regulatory mechanism of protein functions involved in the regulation of immune responses, pancreatic beta cell destruction, and glucose metabolism. In this chapter, however, we limited our discussions to several important pathways with experimental evidence regarding to the possible role of SUMO4 in T1D pathogenesis. As the research evolves, additional molecular pathways could be discovered and animal models such as dendritic cell-specific, T cell-specific and pancreatic islet-specific transgenic models would be important tools to dissect those intriguing questions. Elucidation of the regulatory role for SUMO4 in these signaling pathways should shed novel insights for better understanding of the pathogenesis of type 1 diabetes, and possibly developing novel prevention/intervention strategies against this devastating disorder.

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Sumoylation in Craniofacial Disorders

19

Erwin Pauws and Philip Stanier

Abstract

Craniofacial development requires a complex series of coordinated and finely tuned events to take place, during a relatively short time frame. These events are set in motion by switching on and off transcriptional cascades that involve the use of numerous signalling pathways and a multitude of factors that act at the site of gene transcription. It is now well known that amidst the subtlety of this process lies the intricate world of protein modification, and the posttranslational addition of the small ubiquitin-like modifier, SUMO, is an example that has been implicated in this process. Many proteins that are required for formation of various structures in the embryonic head and face adapt specific functions with SUMO modification. Interestingly, the main clinical phenotype reported for a disruption of the *SUMO1* locus is the common birth defect cleft lip and palate. In this chapter therefore, we discuss the role of SUMO1 in craniofacial development, with emphasis on orofacial clefts. We suggest that these defects can be a sensitive indication of down regulated SUMO modification at a critical stage during embryogenesis. As well as specific mutations affecting the ability of particular proteins to be sumoylated, non-genetic events may have the effect of down-regulating the SUMO pathway to give the same result. Enzymes regulating the SUMO pathway may become important therapeutic targets in the preventative and treatment therapies for craniofacial defects in the future.

Keywords

SUMO • Craniofacial development • Cleft lip and palate • Transcription • Stress

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19.1 Key Role for Sumo in Development

Post-translational protein modifications can have many and variable consequences, but in general, they play a key role in regulating and expanding the diversity of function in the proteome. As documented in this book, the reversible conjugation of SUMO to protein substrates (sumoylation) has emerged as a major post-translational regulatory process. In the last two decades, numerous proteins have been identified that undergo SUMO modification and this list has been greatly expanded with the advent of mass spectroscopy approaches to study the SUMO proteasome (Seeler and Dejean 2003; Geiss-Friedlander and Melchior 2007; Eifler and Vertegaal 2015). The precise action of SUMO modification can vary considerably depending upon the substrate, but in many cases the specific functional effect still remains to be elucidated. For those proteins involved in regulation of gene transcription, SUMO modification usually plays an important role either with (sub)nuclear localisation or the functional activity of the transcription factor in the nucleus. It is therefore not surprising that sumoylation is now being increasingly recognised as a crucial regulator of embryonic morphogenesis. Overall the biological significance of the SUMO pathway in mammalian development can be judged as essential, based on observations of mice deficient for the key E2 conjugating enzyme Ubc9 (Nacerddine et al. 2005). Although heterozygous animals are essentially normal, null embryos die during the period between the early postimplantation stage and prior to embryonal day (E)7.5. In *C. elegans*, knock down of ubc-9 causes severe pharyngeal defects, partly resulting from an altered sub-nuclear distribution of the sumoylated transcription factor tbx-2 (Roy Chowdhuri et al. 2006; Crum and Okkema 2007). The ability to successfully sumoylate individual target proteins and precisely regulate this process is likely to be more subtle but will nevertheless be an essential part of embryonic development. As suggested by the *C. elegans* data and the over-representation of sumoylated proteins involved in craniofacial development, the most sensitive readout of this process in developmental terms may occur during formation of the embryonic head (Pauws and Stanier 2007).

19.2 Sumo1 Haploinsufficiency Causes Cleft Lip and/or Palate

The most direct evidence implicating a role for SUMO in craniofacial development came originally from the analysis of a female patient with a cleft lip and palate who was found to be carrying a balanced reciprocal translocation between human chromosomes 2q and 8q (Alkuraya et al. 2006). Mapping the breakpoint on chromosome 2 revealed an interruption within the gene encoding *SUMO1*, and was predicted to result in haploinsufficiency. The functional significance was then investigated in mice. In wild-type animals, strong *Sumo1* expression in the upper lip, primary palate and medial edge epithelia of the secondary palate was demonstrated by whole mount *in situ* hybridisation (Alkuraya et al. 2006). Next, a mouse with a GeneTrap mutation (RRQ016) in *Sumo1* that generated a null allele was investigated. A low penetrance (8.7%) of cleft palate (CP) was observed in heterozygote animals, while homozygote embryos were embryonic lethal prior to palate closure, indicating that SUMO1 is required for other important developmental functions. *EYA1* is a homolog of the *Drosophila* absent eyes gene, which is mutated in human patients with brachio-oto-renal syndrome (Abdelhak et al. 1997). *Eya1* is important for palate development as evidenced by the fact that it is expressed in the developing mouse palate and mice completely lacking Eya1 have a cleft palate (amongst other defects) (Xu et al. 1999b). This is in contrast to heterozygous animals that show normal palate development. The expression of *Eya1* was noted to overlap with that of *Sumo1* and it has been shown to be a SUMO1 substrate (Alkuraya et al. 2006). Moreover, a significant increase (36%) in the penetrance of CP was observed in compound heterozygous mutants of *Eya1* and *Sumo1*, suggesting a genetic interaction between the two.

This data is not without controversy though, since two independent reports describe how SUMO1 is dispensable throughout development and question the validity of the original findings in the gene trap model. In the first of these, Zhang et al. (2008), describe a mouse in which *Sumo1* was targeted by homologous recombination to make either heterozygous (haploinsufficient) or homozygous null animals. These null animals do not produce any SUMO1 protein, yet they do not have an overt palate defect, nor do they have any disruption to adipogenesis, postnatal growth rate, reproductive function or any other noticeable phenotype. Interestingly, RanGAP1, usually modified by SUMO1, was demonstrated to show increased modification by the SUMO2 paralog instead. Many proteins are specifically modified with one paralog or another, and mechanisms regulating this specificity are only just coming to light (Meulmeester et al. 2008). In the Zhang et al. study, it seems that SUMO2 is able to rescue the SUMO1 deficient mice. However, as the authors point out, their *Sumo1* knockout mice are on a different genetic background to the animals described by Alkuraya et al. (2006) and a different set of genetic modifiers might be involved. This is not unusual when comparing inbred laboratory strains as evidenced by the differences to palate defects seen in C57BL/6 J $Eya^{-/-}$ mice compared to those seen for 129/Sv and Balb/C $Eya1^{-/-}$ mice (Xu et al. 1999b). Perhaps most significantly, the type of gene disruption is also different in the two reports. Unlike the targeted homologous gene targeting strategy employed by Zhang et al. (2008), Alkuraya et al. (2006) used mice generated using a gene trapping strategy, which can be leaky through processes such as mis-splicing (Galy et al. 2004). Whilst it is possible that a gain of function mutant may have been generated, it is also possible that the level of available SUMO1 protein may impact on the ability of other SUMO paralogs to compensate. Alternatively, environmental variables such as diet or stress factors may differ between laboratories and are not taken into account.

These ideas were further brought into question by a third study, where Evdokimov et al. (2008) investigated an independent *Sumo1* GeneTrap (XA024). It was found that resulting homozygous mice were phenotypically normal. This could partially be explained by alternate splicing leading to leaky translation, albeit of a protein lacking 25 amino acids which was predicted to be a loss-of-function allele. Interestingly, like Zhang et al. (2008), these authors also found that RanGAP1 sumoylation could be compensated for by SUMO2/3 in the absence or down regulation of SUMO1 in the XA024 GeneTrap. In order to try to resolve the developmental inconsistencies, Evdokimov et al., went on to reinvestigate the original GeneTrap mice derived from the same RRQ016 ES cells used by Alkuraya et al. (2006). Surprisingly, they found that these mice were normal and fertile. However, a possible explanation to the lack of phenotype was a complex rearrangement at this locus, potentially disrupting the GeneTrap. This was supported by the detection of normal SUMO1-RanGAP1 conjugation in these animals. They surmise that an independent mutation of another gene may have been present and the fundamental cause in the mice analysed by Alkuraya et al. (2006). It now appears that SUMO2 is the most important isoform during development, where embryonic deficiency in mice resulted in severe developmental delay and death at around E10.5 (Wang et al. 2014). As previously suggested by Zhang et al. (2008) and Evdokimov et al. (2008), SUMO2 appears to have some ability to compensate for loss of other SUMO isoforms, all though the reciprocal arrangement is less obvious. Moreover, the precise role of the SUMO pathway in embryonic development still remains to be fully elucidated since embryos deficient for other components of the SUMO regulatory machinery are observed to result in lethality at different stages of embryonic development, presumably acting through different mechanisms (Nacerddine et al. 2005; Cheng et al. 2007; Kang et al. 2010; Sharma et al. 2013).

Despite the controversies over the effect of SUMO1 in mice, independent evidence for a role in cleft lip and palate has come from genetic studies in human CL/P cohorts. It was noted that 2q32-q33 where the *SUMO1* gene resides was previously reported as a region where copy

number variants or translocations were implicated in craniofacial dysmorphology (Brewer et al. 1998, 1999; Van Buggenhout et al. 2005; Shi et al. 2009). The 2q32-q35 locus was also was identified by a meta-analysis of GWAS studies for NSCL/P (Marazita et al. 2004). Therefore, along with the Alkuraya et al. (2006) report, these collective findings prompted a closer look at the *SUMO1* locus, primarily by association studies. The first of these was from Song et al. (2008), who reported a positive association with NSCLP especially between a common haplotype of 4 SNPs within the *SUMO1* gene. This was followed by several further reports finding either association (Carter et al. 2010; Jia et al. 2010; Guo et al. 2012), borderline association (Mostowska et al. 2010) or no association (de Assis et al. 2011; Carta et al. 2012). In addition, de Assis et al. Sanger-sequenced *SUMO1* in a cohort of NSCL/P patients as did Carta et al. who also included *SUMO2*, *SUMO3*, *PIAS1* and *PIAS2* but both failed to identify sequence variants that could be implicated as disease causing. To analyse these apparently conflicting results further, a meta-analysis including 1381 NSCL/P patients and 2054 controls reports empirical evidence implicating a role for *SUMO1* in the etiology of NSCL/P in both Caucasian and Asian populations (Tang et al. 2014).

19.3 Sumoylation Regulates Craniofacial Developmental Genes

The underlying cause of cleft lip and/or cleft palate (CL/P) has been the subject of a great deal of attention (Murray and Schutte 2004: Stanier and Moore 2004; Lidral and Moreno 2005; Setó–Salvia and Stanier 2014). In general, oral clefts can be classified as non-syndromic (NS) when they occur as isolated defects or syndromic, when they occur together with one or more other anomaly. The underlying cause of NSCL/P still remain elusive, partly because they appear to be a sensitive developmental effect accruing from many different genetic and environmental factors. Consequently, any large collection of patients is likely to be extremely heterogeneous and refractory to the standard techniques of genome wide association studies frequently employed to investigate their aetiology. The study of syndromic cases has been much more successful since it has been possible to categorise patients more accurately according to the presence of a second phenotypic feature, such as hypodontia, lip pits, ectodermal dysplasia or ankyloglossia (Stanier and Moore 2004). This has allowed specific genes and etiologic mutations to be identified, but has also had the bonus of identifying the molecular basis of some forms of NSCL/P too, most notably for IRF6 (Kondo et al. 2002) and TBX22 (Braybrook et al. 2001). In addition to the direct role of SUMO1 in lip and palate development described above, it is now becoming apparent that many of the proteins associated with clefts are targets of SUMO modification (Table 19.1).

The sumoylated protein SATB2 is a homeobox transcription factor that was first implicated in NS cleft palate (NSCP) in a patient with a translocation in 2q32-q33 interrupting the gene (FitzPatrick et al. 2003). More recently mutations in *SATB2* were found in syndromic patients with CP, osteoporosis and mental retardation (Leoyklang et al. 2007) as well as NSCP (Vieira et al. 2005). *Satb2* knockout mice also show a distinct CP phenotype combined with skeletal defects (Dobreva et al. 2006). SATB2 has been shown to require SUMO conjugation to mediate its sub-nuclear localisation, protein stability and its transcriptional activity as a repressor (Dobreva et al. 2006).

Another sumoylation target that can result in CL/P when mutated is the MSX1 homeobox transcription factor. Initially, a transgenic mouse devoid of Msx1 was found to have a CP phenotype as well as hypodontia (Satokata and Mass1994). As a result, this gene was considered a good candidate in a 3 generation Dutch family who presented with combinations of tooth agenesis and CP or CLP. This was confirmed by the finding of a nonsense mutation (S105X) which segregated with the affected family members (van den Boogaard et al. 2000). Since then, numerous studies have investigated *MSX1* as a

Table 19.1 Sumoylated proteins involved in mammalian craniofacial development

SUMO target	Protein function	Human disorder and/or phenotype	MIM	Mouse phenotype	SUMO effect
DNMTs3B	*de novo* DNA methylation	ICF – hypertelorism, micrognathia, no CL/P known	242,860	Growth impairment, rostral neural tube defects	Affects nuclear localisation
EYA1	Transcription factor (activator)	BOR - ear defects, branchial clefts, renal dysplasia, no CL/P known	113,650	*Eya1* null – cleft palate	Unknown
				Sumo1/Eya1 double heterozygotes significantly increase CP occurrence	
GSC	Transcription factor (repressor)	None known		Abnormal mandible, tongue, nasal pits	Regulates repressive activity
MSX1	Transcription factor (repressor)	Hypodontia and/or CL/P; NSCL/P	142,983	Homozygous knockout has CP and tooth agenesis	Activates transcriptional repression; sub-nuclear localisation
SATB2	Transcription factor (activator/ repressor)	NSCP	608,148	Knockout has CP and craniofacial abnormalities	Activates transcriptional repression; sub-nuclear localisation
SMAD4	TGFβ signal transduction	None known (associated with colorectal cancer)		Embryonic lethal, mutants lack embryonic germ layers	Interaction with Daxx upregulates Bmp signalling
SnoN	−ve regulator of TGFβ signalling	None known (upregulated in various cancers)		Not known	Activates transcriptional repression
SOX2	Transcription factor (activator)	Micropthalmia	206,900	Inner ear defect, abnormal anterior pituitary development	Down regulates target activation
SOX9	Transcription factor (activator)	CMPD - skeletal defects, CP, micrognathia	114,290	Skeletal defects, CP	Down regulates target activation

(continued)

Table 19.1 (continued)

SUMO target	Protein function	Human disorder and/or phenotype	MIM	Mouse phenotype	SUMO effect
SOX10	Transcription factor (activator)	WS4; Hirschprung – deafness, pigment, megacolon	277,580 609,136	Aganglionic distal bowl	Down regulates target activation
TP63	Transcription factor (activator/ repressor)	EEC - CL/P, ectrodactyly, ectodermal dysplasia; NSCL/P	603,273	Homozygous knockout has bilateral CL and CP	Loss of transcriptional activation; reduced protein stability
TBX22	Transcription factor (repressor)	CPX – CP, ankyloglossia; NSCP	303,400	Not known	Activates transcriptional repression
TRPS1	Transcription factor (repressor)	TRPS – high arched palate	190,350	*Trsp1* loss-of-function mutant mouse model exhibits high-arched palate	Activates transcriptional repression

candidate gene for NSCL/P, both by direct sequencing of patient DNA and in association studies (Lidral and Moreno 2005). It has been suggested that mutations in *MSX1* account for up to 2% of all CL/P (Jezewski et al. 2003). Like SATB2, MSX1 is a transcriptional repressor (Gupta and Bei 2006). Studies suggest that sumoylation is not only required for their repression activity but also plays an important role in sub-nuclear localisation (Lee et al. 2006). Thus, the mode of action might be through appropriate access to its target genes during the period of craniofacial development.

By contrast, TP63, a p53 homolog, is a transcriptional activator, which has several isoforms associated with different disorders affecting ectodermal dysplasia, limb malformations and CL/P (Ghioni et al. 2005). These include split hand/foot malformation (SHFM4), ectodermal dysplasia and CL/P syndrome (EEC3), ankyloblepharon-ectodermal defects-cleft lip/palate syndrome (AEC), Limb mammary syndrome (LMS) and Rapp-Hodgkin syndrome (RHS). Mice deficient for *Tp63* have previously been described with severe craniofacial, limb and skin abnormalities, reflecting loss of the ectodermal cell lineage (Mills et al. 1999; Yang et al. 1999). A recent description of the craniofacial defects in mice deficient for Tp63, showed that they had bilateral cleft lip and cleft palate, which at least in part resulted from downstream effects on *Bmp4*, *Fgf8* and *Shh* expression (Thomason et al. 2008). Numerous mutations have been identified throughout the gene, with some evidence of genotype-phenotype correlations (Rinne et al. 2007). The prevalence of a cleft phenotype varies from 30–80% between these syndromes, whereas mutations in *TP63* are also found in NSCL/P patients (Rinne et al. 2007). SUMO1 conjugation of TP63 regulates its transcriptional activity and protein stability but not its intracellular localization (Ghioni et al. 2005). Several studies have now shown that naturally occurring mutations alter its sumoylation potential thereby strongly upregulating its normal transcriptional activity (Ghioni et al. 2005; Huang et al. 2004).

TBX22 is another SUMO1 target, and this modification has a profound regulatory effect on its transcriptional activity (Andreou et al. 2007). Mutations in *TBX22* were first identified following the study of several large X-linked families (CPX) and then later in collections of isolated CP patients with insufficient family history to predict inheritance (Braybrook et al. 2001; Marçano et al. 2004). Mutations are found in 4–8% of all NSCP patients and, as expected for an X-linked condition, males carrying mutations are most severely affected although 17% of heterozygous females also exhibit CP (Marçano et al. 2004; Suphapeetiporn et al. 2007). TBX22 has been shown to function as a transcriptional repressor with SUMO1 conjugation a necessary requirement for this activity. Functional studies show that most missense mutations in the T-box interfere with DNA-binding, while sumoylation and transcriptional repression are also compromised (Andreou et al. 2007). None of the mutations were located close to the K63 site of SUMO attachment though, which suggests a more general mechanism may be involved. In this case, a more subtle effect on protein conformation might inhibit the process of SUMO conjugation, leading to loss of TBX22 function and the resulting CP phenotype. The recruitment of transcriptional co-factors by SUMO and/or the modified protein seems a likely mechanism, although SUMO interacting motifs (SIMs) haven't been identified in the TBX22 protein yet. This may affect the remodelling of the chromatin structure, resulting in loss of transcriptional repression. These proposed mechanisms might also explain why a low-level sumoylation can be sufficient (Geiss-Friedlander and Melchior 2007).

19.4 Sumo in Developmental Pathways and Syndromes

The importance of SUMO1 for normal craniofacial development in addition to lip and palate formation has also been demonstrated through effects both on specific genes and signalling pathways, For example, the *Xenopus* SUMO1 (XSUMO-1) specific knockdown, using a morpholino antisense oligonucleotide, showed a striking effect, significantly decreasing body axis

formation and causing microcephaly (Yukita et al. 2007). These results appeared to be associated with an inhibitory effect on activin/nodal signalling since injection of XSUMO-1-MO suppressed expression of activin-response genes such as *Xbra, XGoosecoid* and *Chordin*. Meanwhile the observed down regulation was clearly rescued by *myc-XSUMO-1* mRNA. Goosecoid (Gsc) has itself been identified as post-translationally modified by SUMO in mice, (Izzi et al. 2008), while it is known to be essential for the development of mesenchymal-derived craniofacial tissues, with its deletion mainly causing skeletal defects (Rivera-Perez et al. 1999).

The Wnt pathway is essential for correct migration of cranial neural crest cells during development. Wnt signalling molecules Axin, LEF1 and Tcf4 are all modified by Sumo, suggesting that Wnt signal transduction is directly regulated by sumoylation (Rui et al. 2002; Sachdev et al. 2001; Yamamoto et al. 2003). Axin, which acts as a scaffold protein in the canonical Wnt signaling, effectively downregulates β-catenin but fails to activate JNK when mutated at the SUMO attachment site (Rui et al. 2002). The Wnt activated transcription factors LEF1 and Tcf4 are oppositely affected, with sumoylation of LEF1 inhibiting its transcription activity, while sumoylation of Tcf4 promotes it (Sachdev et al. 2001; Yamamoto et al. 2003). More recently, over expression of the SUMO-specific protease XSENP1 was found to cause head defects in *Xenopus* embryos as a consequence of suppressing Wnt signaling (Yukita et al. 2004).

The process of sumoylation also plays an important role in the regulation of Tgfβ signalling and includes both Smad3 and Smad4 as direct targets (Lin et al. 2003). Ubc9 is known to promote the stability of Smad4 and the nuclear accumulation of Smad1 in osteoblast-like Saos-2 cells (Lin et al. 2003; Shimada et al. 2008) with overexpression of E3 ligases upregulating Smad4- or TGFβ -mediated transcriptional activity (Lin et al. 2003; Long et al. 2004; Liang et al. 2004). SUMO1 conjugation of Smad4 also recruits the binding of the transcriptional corepressor, Daxx through its SIM, which downregulates its transcriptional activity (Chang et al. 2005). In *Xenopus*, XPIASy interacts with XSmad2, which enhances its sumoylation, and suppresses its activity required for proper mesoderm induction (Daniels et al. 2004). These findings together suggested that sumoylation of Smads is important for mesoderm formation in *Xenopus* development. The oncoproteins, c-Ski and related SnoN potently repress Tgfβ signaling through interaction with Smads. Their overexpression can result in the induction of skeletal muscle differentiation. SnoN is now also known to be sumoylated (Hsu et al. 2006; Wrighton et al. 2007). However, SUMO modification itself does not alter its ability to repress Tgfβ signaling, instead, it is loss of sumoylation that activates muscle-specific gene expression. Sumoylation of the TGFβ receptor, TGFbRI, meanwhile, controls responsiveness to TGFβ (Kang et al. 2008), with implications for tumor progression, although its role in embryonic development is yet to be investigated.

There are also a number of other human syndromes with a craniofacial involvement that involve sumoylated proteins. *TRPS1*, named after tricho-rhino-phalangeal syndrome (TRPS) is also a transcriptional repressor whose function depends on sumoylation (Kaiser et al. 2007). Mutations in *TRPS1* result in characteristic skeletal and craniofacial malformations including a bulbous nose tip and a long and flat philtrum (Momeni et al. 2000). Mice that are heterozygous for deletions of the *Trps1* GATA-DNA binding domain display facial abnormalities that overlap with those seen in human patients, and consistently have a high-arched palate (Malik et al. 2002).

Several members of the Sox protein family are sumoylated and also function in craniofacial development include Sox2 which is important in eye development and can result in anophthalmia (Tsuruzoe et al. 2006), Sox9 and Sox10, which are both important for neural crest migration and inner ear development (Taylor and Labonne 2005).

The DNA methyltransferase 3B (*DNMT*DNMTs*3B*) gene is mutated in

immunodeficiency, centromere instability, and facial anomalies (ICF) syndrome (Xu et al. 1999a; Hansen et al. 1999). It has been demonstrated that Dnmt3b is post translationally modified by SUMO1 (Kang et al. 2001). Most reported ICF mutations of DNMT3B are missense changes in the C-terminal region, which directly reduce enzymatic activity, however, one exception is the S270P mutation, which has been shown to abrogate SUMO1 attachment (Park et al. 2008). It appears that S270 is important for a non-covalent interaction with SUMO1 and is also the location for interaction with the E3 ligase, PIAS1. Interestingly, the interactions between DNMT3B and either PIAS1 or SUMO1 are inversely affected by increasing concentrations of H_2O_2 treatment, emulating conditions of oxidative stress.

19.5 Sumo, Stress, and CL/P

An environmental component to orofacial clefts has long been recognised with an estimated 50–75% of cases having no recognisable familial history, and monozygotic twins are only concordant for the phenotype approximately 40% of the time (Murray 2002; Wyszynski et al. 1996). It is clear that although the interactions between genes and the environment that are crucial in CL/P development remain elusive, they do converge on the same developmental pathways (Chakravarti and Little 2003). Environmental risk factors thought to play a role in NSCL/P include maternal alcohol use and smoking, whereas exposure to environmental toxins, such as dioxin, folic acid deficiency and increased vitamin A intake during pregnancy have also been suggested to induce syndromic craniofacial abnormalities such as CL/P (Murray 2002). Among these, a study on the effects of maternal smoking in 1244 cleft patients supported a role for genetic-environmental interactions in the pathogenesis of CL/P and suggested that detoxification gene variants were possible risk factors (Shi et al. 2007).

Interestingly, the process of SUMO modification is known to be susceptible to environmental effects that are strikingly similar to some of the risk factors described for orofacial clefts. These include stresses such as heat shock, osmotic and oxidative stress conditions and viral infection, which all trigger changes to the cellular SUMO1 conjugation/deconjugation pathway (Bossis and Melchior 2006; Tempe et al. 2008). Severe oxidative stress is usually associated with an increase in SUMO1 conjugation but lower, more physiologically relevant concentrations of free radicals induce an almost complete loss of SUMO1 modification of target proteins (Bossis and Melchior 2006). A study into the stress response of the transcription factor c-Myb shows that SUMO2/3, rather than SUMO1 conjugation can rapidly inactivate the transcriptional activity of the SUMO target (Sramko et al. 2006). Although SUMO isoforms are similar, it is not clear whether SUMO1 and SUMO2/3 respond similarly to stress within cells. There appears to be a developing link resulting from the interrelationships of environmental stresses with both SUMO and CL/P risk. The finding that several genetic risk factors are regulated by SUMO modification, suggests that further investigation is warranted. This might initially focus on a destabilisation of the normal balance of expression and activity for genes such as *TBX22*, *MSX1*, *SATB2* and *TP63* during early pregnancy that might provide a high-risk environment for CL/P occurrence.

19.6 Conclusions

As described in this chapter and elsewhere in this book, sumoylation is required for many cellular functions. From a developmental perspective, evidence suggests that formation of various craniofacial structures, especially the upper lip and palate are sensitive to varying SUMO1 levels. Moreover, the efficiency of normal SUMO modification in response to local oxidative and osmotic conditions or infection status suggest a potential explanation as to how environmental factors may impact on this birth defect risk. These responses will need to be much more thoroughly investigated, starting with cell based systems and animal models. It is not clear why proteins involved

in craniofacial development are predominantly modified by SUMO1, as opposed to SUMO2/3 has also not yet been addressed, especially since all of these SUMO paralogs are ubiquitously expressed. As demonstrated for the SUMO1 knockout, SUMO2/3 do seem to be able to rescue the phenotype, at least in some circumstances (Zhang et al. 2008; Evdokimov et al. 2008). It is not yet known if these paralogs regularly share targets with SUMO1 or if there is a level of redundancy built in to act as a buffer against catastrophic developmental aberration. Another alternative explanation for discrepancies reported in different animal studies may include local exposure to stress factors such as pathogen load. Global analyses of sumoylated proteins at different stages and sites of development and under different environmental conditions can be used to investigate such effects. Nevertheless, taken together with current evidence from a variety of genes and networks, the process of SUMO protein modification can be seen to play an important role in fine-tuning developmental events required for normal craniofacial morphogenesis. Given the dependency on the SUMO pathway during development, it is likely that we will see future research investigating the regulation of SUMO pathway enzymes as a means of delivering therapeutic and preventative treatments, potentially targeting craniofacial defects specifically.

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Coordination of Cellular Localization-Dependent Effects of Sumoylation in Regulating Cardiovascular and Neurological Diseases

Jun-ichi Abe, Uday G. Sandhu, Nguyet Minh Hoang, Manoj Thangam, Raymundo A. Quintana-Quezada, Keigi Fujiwara, and Nhat Tu Le

Abstract

Sumoylation, a reversible post-transcriptional modification process, of proteins are involved in cellular differentiation, growth, and even motility by regulating various protein functions. Sumoylation is not limited to cytosolic proteins as recent evidence shows that nuclear proteins, those associated with membranes, and mitochondrial proteins are also sumoylated. Moreover, it is now known that sumoylation plays an important role in the process of major human ailments such as malignant, cardiovascular and neurological diseases. In this chapter, we will highlight and discuss how the localization of SUMO protease and SUMO E3 ligase in different compartments within a cell regulates biological processes that depend on sumoylation. First, we will discuss the key role of sumoylation in the nucleus, which leads to the development of endothelial dysfunction and atherosclerosis. We will then discuss how sumoylation of plasma membrane potassium channel proteins are involved in epilepsy and arrhythmia. Mitochondrial proteins are known to be also sumoylated, and the importance of dynamic-related protein 1 (DRP1) sumoylation on mitochondrial function will be discussed. As we will emphasize through-

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out this review, sumoylation plays crucial roles in different cellular compartments, which is coordinately regulated by the translocation of various SUMO proteases and SUMO E3 ligase. Comprehensive approach will be necessary to understand the molecular mechanism for efficiently moving around various enzymes that regulate sumoylation within cells.

Keywords
Shear stress • Atherosclerosis • SENP2 • p90RSK • PKCζ ERK5 • p53 • Potassium channel • DRP1

20.1 Introduction

Sumoylation is an important post-translational modification in which one or more of Small-Ubiquitin like modifier (SUMO) peptides are conjugated to a protein and contributes to the complexity of eukaryotic proteomes. There are four different SUMO family members found in mammals, SUMO 1–4. A comparison of amino acid sequences of SUMO peptides has revealed that SUMO-1 shares 46–48% identity with SUMO-2 and -3, and SUMO-2 and -3 share 95% identity. Therefore, SUMO-2 and -3 together are considered to form a subfamily, which is distinct from SUMO-1. SUMO-4 is mainly expressed in the kidney (Bohren et al. 2004). The SUMO conjugation process is catalyzed by a specific set of enzymes comprising a SUMO activating enzyme called E1, a conjugating enzyme called E2, and a ligase named E3 (Eifler and Vertegaal 2015a, b). Sumoylation is a dynamic and reversible process via conjugation and de-conjugation. First, the mature form of SUMO is activated by dimeric SUMO E1, SAE1/UBA2. Then, SUMO is transferred to Ubc9, an E2 conjugase that binds to SUMO by forming a thioester bond. The last step is regulated by E3 ligase whose function is to transfer SUMO to the free e-amino group of a lysine residue of the target protein. E3 ligases include the family of protein inhibitors such as STAT and Pc2 (Abe and Berk 2014). Protein sumoylation is reversible, and this is achieved by de-sumoylation enzymes called sentrin/SUMO-specific proteases (SENPs; SENP1–7). The SENP family of proteins which consist of 7 enzymes catalyze de-conjugation of sumoylated proteins. Certain SENPs are known to also edit SUMO precursors into matured forms by removing a short peptide from the C-terminus to expose a pair of glycine residues (Li and Hochstrasser 1999; Yeh 2009). In all isoforms of SENPs, the C-terminus is well conserved whereas the N-terminus is poorly conserved (Yeh 2009), suggesting that the N-terminus is important for their enzymatic activity. However, it remains unclear how each specific SENP recognizes its substrate that leads to a variety of biological consequences. In addition, certain SENPs, especially SENP1 and 2 contain both nuclear localization and export signal domains, and shuttling of SENPs from one compartment of the cell to another has an effect on altering sumoylation levels in different cellular regions. In this chapter, we will discuss the role of SENP2 in cardiovascular disease and epilepsy via regulating the sumoylation levels of nuclear and membrane proteins and the regulatory mechanism of SENP2 nuclear import and export. In addition, we will discuss how mitochondrial translocation of SENP5 affects mitochondrial function.

20.2 Sumoylation in the Nucleus Regulates Endothelial Dysfunction and Atherosclerosis

20.2.1 Steady Laminar Flow vs. Disturbed Flow

The luminal surface of blood vessels is made up of a thin monolayer of endothelial cells (ECs). ECsECs in general are atheroprotective as they prevent inappropriate activation of the coagulation system by producing antithrombotic factors (Dawes et al. 1982; Selwyn 2003; Uchiba et al. 2004). This paradigm then suggests that the endothelium plays a central role in the initiation and development of inflammatory atherosclerosis when this thin tissue encounters risk factors for atherosclerosis. A certain form of hemodynamic shear stress is known to induce vascular pathologic conditions such as endothelial dysfunction (van Bussel et al. 2015) and progression of atherosclerosis (Davies et al. 2010; Heo et al. 2013, 2011a, 2015) via regulating local mechanotransduction mechanisms, ultimately activating the shear stress response promoter elements and transcription factors that modulate endothelial gene expression (Davis et al. 2003; Huddleson et al. 2004; Nagel et al. 1999; Urbich et al. 2003). Basically, there are two different types of flow; disturbed flow (d-flow)) and steady laminar flow (s-flow), which exert very different effects on endothelial function. For example, atherosclerotic plaque formation has been reported to be localized in the arterial vasculature where ECs experience d-flow (Libby 2002). D-flow occurs at branch points, bifurcations, and curvatures along the arterial tree and not only down-regulates the atheroprotective mechanisms of ECs and vascular reactivity, but also increases EC inflammation (via upregulated expression of leukocyte adhesion molecules), apoptosis, and proliferation (Heo et al. 2016). In contrast, plaque formation is rare in areas exposed to s-flow (10–20 dyn/cm^2), which can stimulate ECs to release various factors including NO, PGI_2, and tPA to inhibit the inflammatory response of leukocytes, coagulation, and proliferation of smooth muscle cells while simultaneously promoting the survival of ECs (Di Francesco et al. 2009; Diamond et al. 1989; Frangos et al. 1985; Garin et al. 2007; Korenaga et al. 1994; Reinhart-King et al. 2008), all of which have anti-atherogenic effects. Thus, whereas s-flow is anti-atherogenic, d-flow is pro-atherogenic (Gimbrone et al. 2000), and understanding how various signaling pathways in ECs are affected by d-flow and s-flow is of crucial importance when elucidating the molecular mechanism for EC dysfunction and atherosclerosis.

It has been reported that PECAM-1, VE-Cadherin, VEGFR, and integrin receptor are involved in mechanosensory systems of d-flow and s-flow. In particular, PECAM-1 has been established as a first-line mechanosensor (Tzima et al. 2005). PECAM-1 is a type 1 transmembrane glycoprotein with six extracellular Ig-like homology domains and a short cytoplasmiccytoplasmic domain that contains two immune-receptor tyrosine-based inhibitory motifs (ITIMs). When the tyrosine in each ITIM is phosphorylated, ITIMs can recruit Src homology 2 (SH2) domain-containing proteins (Privratsky et al. 2010). Several lines of evidence support that PECAM-1 plays a role as a mechanosensor, but it is also clear that PECAM-1 is unable to distinguish between s-flow and d-flow. For example, PECAM-1 can accelerate the formation of atherosclerotic lesions in the lesser curvature of the aortic arch (d-flow area) (Stevens et al. 2008). In contrast, PECAM-1 can reduce atherosclerotic lesions in the descending thoracic and abdominal aorta (s-flow area) (Goel et al. 2008). These observations suggest that the different responses to s-flow and d-flow are not due to differential PECAM-1 responses to these flow patterns but are due to some downstream modification(s) exerted to PECAM-1 signaling (Osawa et al. 2002; Tzima et al. 2005). Piezo1 (Piezo-type mechanosensitive ion channel component 1 (Li et al. 2014), p130 Crk-associated substrate (Cas) (Sawada et al. 2006), and syndecan 4 (Baeyens et al. 2014) are also proposed as other candidates for mechanosensors. However, it remains unclear if d-flow and s-flow can differentially regulate signaling pathways activated by these mechanosensors.

Further investigation of d-flow mediated EC dysfunction has led to the discovery of post-translational modification (PTM) of proteins via phosphorylation and sumoylation, which play a role in atherogenesis. Our group has reported that sumoylation induced by d-flow affects key nuclear transcriptional molecules such as extracellular signal regulated kinases 5 (ERK5) and p53, resulting in EC inflammation and apoptosis (Heo et al. 2013). Recently, the crucial role of epigenetic factors in regulating flow signaling has become clear. Especially, d-flow--induced DNA methylation by chromatin--based mechanisms (Cheng et al. 2013; Delgado-Olguin et al. 2014; Dunn et al. 2014; Kumar et al. 2013; Lee et al. 1998; Rao et al. 2011; Rexhaj et al. 2013) plays a key role in the regulation of gene expression in a DNA sequence-independent manner (Hamm and Costa 2015; Nazarenko et al. 2015). In this chapter we will discuss the role of sumoylation and DNA methylation on EC dysfunction under d-flow. We believe that the very different and unique physiological consequences are incited by d-flow and s-flow and that delineating signaling pathways activated by these two flow types is critical for understanding the hemodynamic contribution of vascular physiology and pathology.

20.2.2 Nuclear ERK5 Sumoylation and EC Dysfunction

ERK5 is one of the mitogen-activated protein kinases (MAPKs), which along with other MAPKs, has been reported to regulate the downstream transcription factors of genes regulating the growth, proliferation, and differentiation of cells including ECs and cardiomyocytes (Abe et al. 2000a). Kato et al. found that overexpressed ERK5 in (cell type) localizes in the cytoplasm in resting cells but when it is co-expressed with MEK5, which activates ERK5, ERK5 translocates to the nucleus (Kato et al. 1997). However, in ECs, ERK5 is exclusively localized in the nucleus. ERK5 is unique among the MAPK family of kinases because it is not only a kinase but also a transcriptional co-activator with a unique C-terminus transactivation domain (Fig. 20.1a) (Akaike et al. 2004; Kasler et al. 2000). When ERK5 is activated by s-flow in ECs, its transcriptional activity on peroxisome proliferator-activated receptor-γ (PPARs) and Kruppel-like factor 2 and 4 (KLF) is increased, resulting in decreased production of inflammatory chemokines and adhesion molecules while increasing the expression of athero-protective factors such as endothelial nitric oxide synthase (eNOS) (Akaike et al. 2004; Parmar et al. 2006). KLF2 and 4 induction in ECs has been noted to increase thrombomodulin (anti-thrombotic) production, control of vascular permeability, and EC barrier function (Lin et al. 2010). These observations clearly establish the athero-protective effect of ERK5 activation by s-low in ECs (Fig. 20.1b, left).

As explained above, SUMO is covalently attached to certain residues of specific target proteins and alters their functions including the site of protein activity (i.e. subcellular localization), interaction with other molecules including DNA, and transactivation functions of transcription factors (Hilgarth et al. 2004). Our group has reported that H_2O_2 (hydrogen peroxide) and AGE (advance glycation end products) inhibit ERK5 transcriptional activity and promote EC inflammation via up-regulating ERK5 sumoylation (Woo et al. 2008). H_2O_2 triggers ERK5 sumoylation at Lys6 and 22 (K6/22) residues, and this sumoylation inhibits ERK5 transcriptional activity and down-regulates the ERK5/MEF2 (myocyte enhancer factor-2) pathway. Subsequently, the KLF2 promoter activity is reduced due to MEK/ERK5/MEF2/KLF2 inhibition, and this results in the inhibition of KLF2-mediated eNOS expression. Heo et al. reported an increase in ERK5 sumoylation in ECs under d-flow and found that ERK5 sumoylation played a critical role in the mechanism of decreased eNOS expression and increased VCAM-1 expression induced by d-flow (Heo et al. 2013). These studies suggest the importance of ERK5 sumoylation by H_2O_2, AGE, and d-flow for down-regulating ERK5 transcriptional activity and subsequently up-regulating EC inflammation (Fig. 20.1b, right).

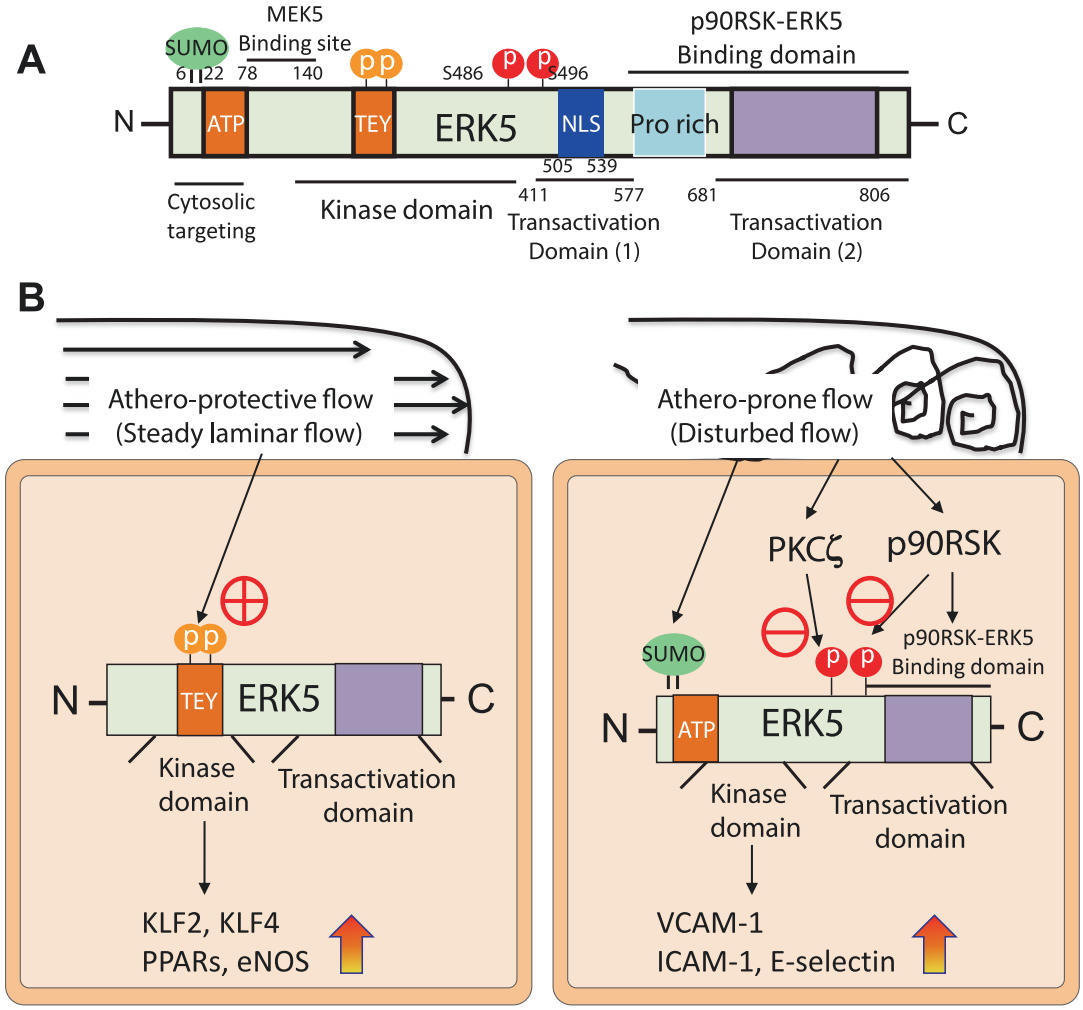

Fig. 20.1 Primary structure of ERK5 and its regulation by shear stress. (**a**) ERK5 is twice the size of other MAPKs and hence the largest kinase within its group. It possesses a catalytic N-terminal domain including the MAPK-conserved threonine/glutamic acid/tyrosine (TEY) motif in the activation loop with 50% homology with ERK1/2, and a unique C-terminal tail including transactivation domains. The activation of ERK5 occurs via interaction with and dual phosphorylation in its TEY motif by MEK. On the other hand, inflammatory stimuli or athero-prone flow (d-flow) leads to ERK5 deactivation via phosphorylation of Ser486 or Ser496, respectively. The N-terminus K6 and K22 sites with small ubiquitin-like modifier (SUMO) modification inhibit its own transactivation. (**b**) After ERK5 kinase activation induced by MEK5 binding or athero-protective flow (s-flow) stimulation and TEY motif phosphorylation with de-sumoylation, ERK5 transcriptional activity at the C-terminus region is fully activated. In contrast, d-flow increases ERK5 sumoylation and ERK5 Ser496 phosphorylation and inhibits ERK5 transcriptional activity. eNOS, endothelial nitric oxide synthase; KLF, Kruppel-like factor; p90RSK, p90 ribosomal S6 kinase; PKCζ, protein kinase C-ζ; and PPAR, peroxisome proliferator-activated receptor (Reprinted and modified from Heo et al. 2016 with permission of the publisher)

20.2.3 Sumoylation Mediated p53 Nuclear Export Leads to EC Apoptosis

In addition to d-flow causing EC inflammation by modulating ERK5 sumoylation as we described above, d-flow is known to induce EC apoptosis. Increased EC apoptosis results in increased EC turnover with accompanying endothelial inflammation and dysfunction (Chiu and Chien 2011; Heo et al. 2011b). Transcription factor p53 has been demonstrated to have a key role in promoting cell death by increasing the production of pro-apoptotic factors and promoting cell arrest (via failed DNA repair) when DNA damage occurs (Garner and Raj 2008). The role of p53 in ECs exposed to s-flow was investigated by Lin et al., who noted that ECs under prolonged s-flow had increased both a JNK-mediated phosphorylation of p53 and p53 expression itself. In addition, s-flow increases p21 and GADD45 (growth arrest and DNA damage inducible protein 45) expression, and consequently inhibits Rb phosphorylation, thus inhibiting cell cycle progression into S-phase. Taken together, p53 inhibits proliferation growth and possibly apoptosis in ECs exposed to s-flow (Lin et al. 2000). Interestingly, it should be noted that these effects of p53 on ECs occur when p53 is localized within the nucleus (Lin et al. 2000).

When cultured ECs are exposed to d-flow, we found that p53 is exported from the nucleus to the cytoplasm. Carter et al. reported that p53 has a NES (nuclear export sequence) on its C-terminus. Initially, p53 NES is masked by its own N-terminal lesion, thus preventing p53 from nuclear export. However, after mono-ubiquitination of p53 by E3 ligase MDM2 (douse double minute 2), sumoylation of p53 by PIAS4 uncovers the masked p53 nuclear export signal (NES),), which then allows p53 nuclear export (Carter et al. 2007). Once exported to the cytoplasm, p53 induces apoptosis by direct association and blocking of the Bax/Bcl anti-apoptotic function (Heo et al. 2011b; Mihara et al. 2003). Our group has reported the crucial role of protein kinase Cζ (PKCζ) activation in p53 sumoylation and consequent nuclear export (Fig. 20.2). D-flow activates PKCζ in ECs which in turn, promotes the association between the PKCζ C-terminus kinase domain (aa401–587) and the RING domain of PIAS4 (protein inhibitor of activated STAT4). Because the PIAS4 RING domain contains a catalytic site, the structure and enzymatic activity of PIAS4 might be altered by this association. The PIAS4-PKCζ association causes an increase in p53 sumoylation and once sumoylated, p53 is exported to the cytoplasm, which then induces EC apoptosis (Heo et al. 2011b) (Fig. 20.2).

20.2.4 Nuclear Export of De-sumoylation Enzyme SENP2 and Its Effects on Nuclear ERK5 and p53

Among the six SENP isoforms that exist in humans (SENP 1–3 and SENP 5–7), we have characterized the functional role of SENP2 in controlling sumoylation of ERK5 and p53 in ECs stimulated by flow (Heo et al. 2013). When SENP2 is deleted, d-flow-induced EC apoptosis and inflammation are up-regulated by increased sumoylation of p53 and ERK5, respectively. Furthermore, aortas from SENP2$^{-/-}$ mice exhibited accelerated atherosclerotic plaque formation in d-flow areas when compared to s-flow areas. As such, one might expect d-flow to decrease SENP2 expression. However, the expression of SENP2 is not regulated by d-flow. Because SENP2 has various nuclear localization/nuclear export signals (NLS/NES), (NLS/NES), one possibility is that SENP2 localization is altered and that the local concentration of SENP2 could be reflected in local de-sumoylation activity (Itahana et al. 2006). Our group found the possible role of p90 kDa ribosomal S6 kinases (p90RSK)) in this possible process (Fig. 20.3).

The family of p90RSK is a unique serine/threonine kinase family that contains two functional kinase domains: the N-terminus and the C-terminus kinase domains (Frodin and Gammeltoft 1999). The N-terminus kinase domain appears to belong to the AGC group of kinases (i.e. PKC and PKA)) and phosphorylates

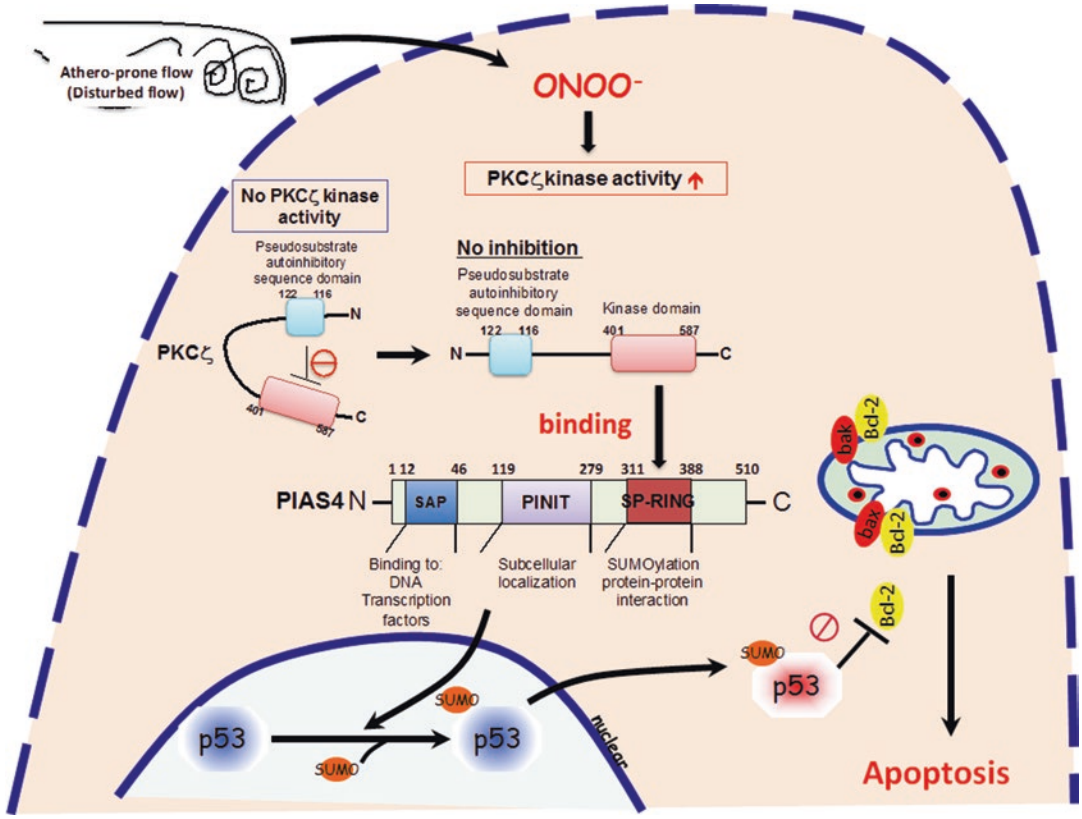

Fig. 20.2 The scheme of PKCζ-mediated p53 SUMOylation and consequent EC apoptosis by atheroprone flow. Atheroprone flow (d-flow) uniquely activates PKCζ via up-regulating ONOO⁻, which increases PKCζ-PIAS4 binding at the SP-RING domain and PIAS4 small ubiquitin-like modifier (SUMO) E3 ligase activity, subsequently increasing p53 sumoylation. Upon binding sumoylated p53, p53 translocates to the cytosol, and the anti-apoptotic effect of Bcl-2 is inhibited, leading to caspase activation and apoptosis. PIAS, protein inhibitor of activated STAT; SAP, scaffold attachment factor-A/B, acinus, and PIAS domain; PINIT, Pro-Ile-Asn-Ile-Thr motif; SP-RING, Siz/PIAS-RING domain Abe and Berk (2014)

p90RSK substrates. The C-terminus kinase domain is a member of the calcium/calmodulin dependent kinase group, which is involved in the p90RSK N-terminal kinase activation. The C-terminus tail contains a short docking motif which is activated by ERK1/2 (Blenis 1993) (Fig. 20.4). It should be noted that p90RSK activation can be achieved by an ERK1/2-independent mechanism (Abe et al. 2000a). Upon activation, p90RSK is able to phosphorylate transcription factors such as CREB, NF-κB, and c-fos. In addition, more recent reports indicate phosphorylation of SENP2 and ERK5 by activated p90RSK (Heo et al. 2016, 2015; Le et al. 2013). Activated p90RSK binds the C-terminal transcriptional domain (amino acids 571–807) of ERK5 and phosphorylates ERK5 S496 with subsequent inhibition of ERK5 transcriptional activity (Le et al. 2013) (Fig. 20.1b). This inhibition of ERK5 transcriptional activity results in decreased KLF2/eNOS expression and at the same time increased adhesion molecules expression, all of which lead to EC dysfunction with accelerated atherosclerotic plaque formation. These effects are similar to what is observed in ECs during ERK5 sumoylation and also in EC-specific ERK5 knockout mice, demonstrating the importance of ERK5 in preventing EC dysfunction.

In addition to the direct role of p90RSK in ERK5 S496 phosphorylation, p90RSK also mediates SENP2 T368 phosphorylation, which also leads to increased p53 and ERK5

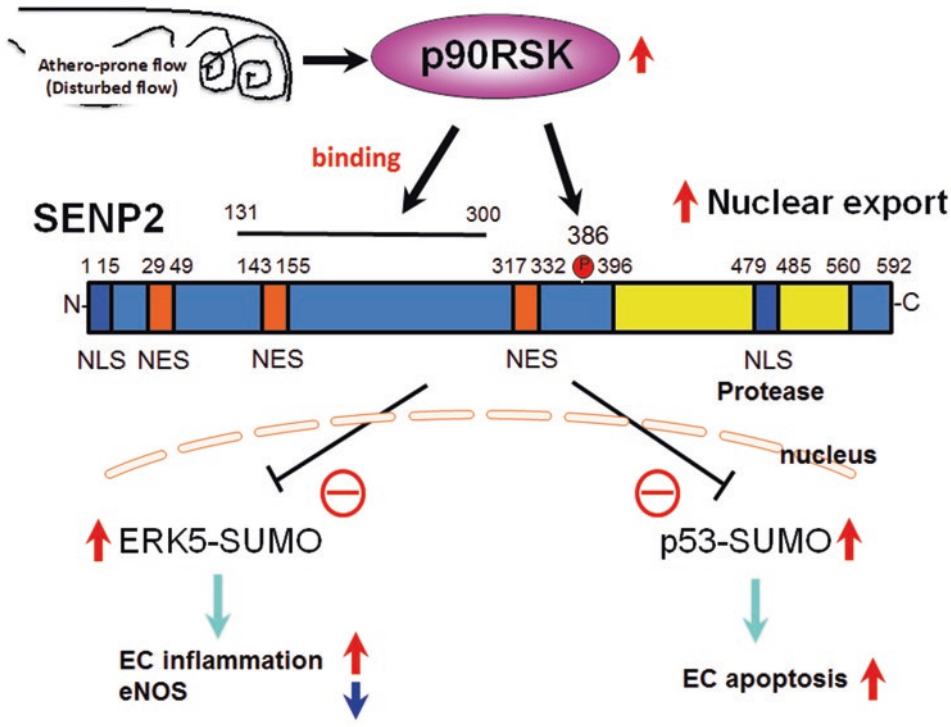

Fig. 20.3 Regulation of p90RSK-SENP2 to increase EC dysfunction by athero-prone flow. p90RSK is uniquely activated by athero-prone (d-flow) flow. SENP2 contains several NLS and NES domains, and we found that p90RSK activation induces SENP2 nuclear export by phosphorylation of SENP2 Thr368 and direct binding to SENP2 aa131–300. This SENP2 nuclear export subsequently up-regulates SUMO modulation of nuclear p53 and ERK5, and increase apoptosis and EC inflammation, respectively. In addition, the increase of ERK5 sumoylation decreases eNOS expression

sumoylation; events exhibited by ECs undergoing d-flow-induced inflammation and apoptosis (Heo et al. 2015) (Fig. 20.3). As already discussed in this chapter, decreased SENP2 expression increases p53 and ERK5 sumoylation with subsequent EC apoptosis and inflammation, respectively (Heo et al. 2013). Although d-flow increases p53 and ERK5 sumoylation, surprisingly, reduced SENP2 expression was not observed. Therefore, it was hypothesized that it is SENP2 post-translational modification (PTM), which plays a critical role in this process. In vitro, SENP2-mediated reduction of p53 sumoylation is inhibited in ECs over-expressing p90RSK. However, the observed inhibitory effect of p90RSK over-expression is lost in ECs expressing the SENP2 T368A phosphorylation mutant, demonstrating that SENP2 phosphorylation at T368 is important for its de-sumoylating function. Furthermore, this study noted that when p90RSK is not able to bind and phosphorylate SENP2 T368 due to over-expression of a decoy fragment (SENP2 aa 131–300 fragment), sumoylation of p53 and ERK5 and subsequent EC apoptosis and inflammation are inhibited. Inhibition of p90RSK activation by FMK-MEA (p90RSK specific inhibitor) or by over-expression of dominant negative p90RSK adenovirus (Ad-DN-p90RSK) both abolish d-flow induced p53 and ERK5 sumoylation via inhibiting SENP2 T368 phosphorylation. Also as previously hypothesized, SENP2 export from the nucleus to the cytoplasm indeed does occur when p90RSK mediated SENP2 T368 phosphorylation occurs. The same phenotype was observed in ECs exposed to d-flow. In contrast, SENP2 is localized in the nucleus in ECs exposed to s-flow. ECs isolated from wild type p90RSK transgenic mice

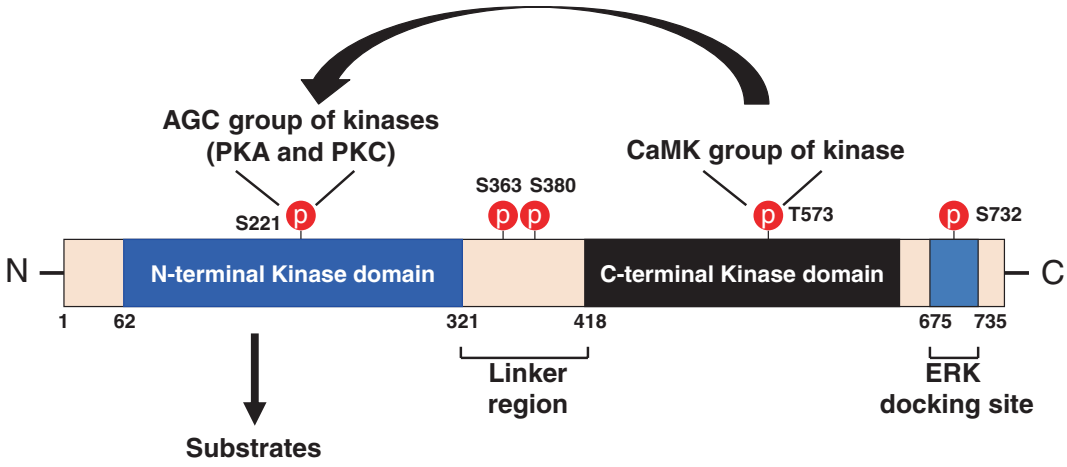

Fig. 20.4 Scheme of p90RSK functional domains. The N-terminus kinase belongs to the AGC group of kinases (i.e., protein kinase A [PKA] and protein kinase C [PKC]), while the C-terminus kinase belongs to the calcium/calmodulin-dependent kinase group. p90RSK is located downstream of the Raf-MEK-ERK1/2 signaling pathway Chung et al. (1991), and ERK1/2 activates the C-terminus kinase of p90RSK, leading to full activation of the N-terminus kinase and subsequent substrate phosphorylation. However, the involvement of an ERK1/2-independent pathway and the role of fyn kinase in regulating ROS-induced p90RSK activation have also been suggested Abe et al. (2000b). Recently, we have reported that p90RSK is activated by d-flow, but not by s-flow Heo et al. (2015) (Reprinted and modified from Heo et al. 2016 with permission of the publisher)

(WT-p90RSK) have increased SENP2 T368 phosphorylation, increased adhesion molecule as well as caspase-3 expression (at both protein and mRNA levels), and decreased eNOS expression. In addition, the p90RSK transgenic mice exhibit increased atherosclerotic lesion size in the aortic arch compared to controls. Overall, these data suggest that p90RSK-mediated SENP2 T386 phosphorylation induces SENP2 nuclear export and plays an important role in atherosclerotic plaque formation in d-flow areas via up-regulating sumoylation of nuclear p53 and ERK5, which leads to EC apoptosis and endothelial inflammation, respectively (Heo et al. 2015) (Fig. 20.3).

20.2.5 D-Flow and DNA Methylation in the Nucleus

DNA methylation at the 5 position of cytosine is a dynamic postsynthetic covalent modification, and more than 98% of DNA methylation occurs in cytosine–phosphate-guanine (CpG) dinucleotides (Guza et al. 2011) in mammals. Methylation of cytosine can cause gene transcriptional silencing via interfering with binding of transcriptional factors or inducing (or forming) a repressive chromatin structure within or near the promoter region (Jaenisch and Bird 2003; Weber et al. 2005). Gene promoters with a cluster of unmethylated CpG dinucleotides are about 50% of genomic DNA, which allow transcription. The dynamic process of DNA methylation is regulated by both methylation and demethylation enzymes (Fig. 20.5).

DNA methyltransferases (DNMTs) which methylate DNA are encoded by different genes on distinct chromosomes: DNMT1, DNMT3A, and DNMT3B (Fig. 20.6). DNMT3A and DNMT3B catalyze de novo methylation during early embryonic development while DNMT1 is crucial to maintaining DNA methylation throughout replication (Okano et al. 1999) DNMT3L has no catalytic activity and belongs to the family of DNMT3A and 3B. It has an important role for stabilizing DNMT3A (Xi et al. 2009). The ten eleven translocation (TET) methylcytosine dioxygenase gene plays a major role in regulating DNA methylation by oxidizing 5-methylcytosine to 5-hydroxymethycytosine (Okano et al. 1999).

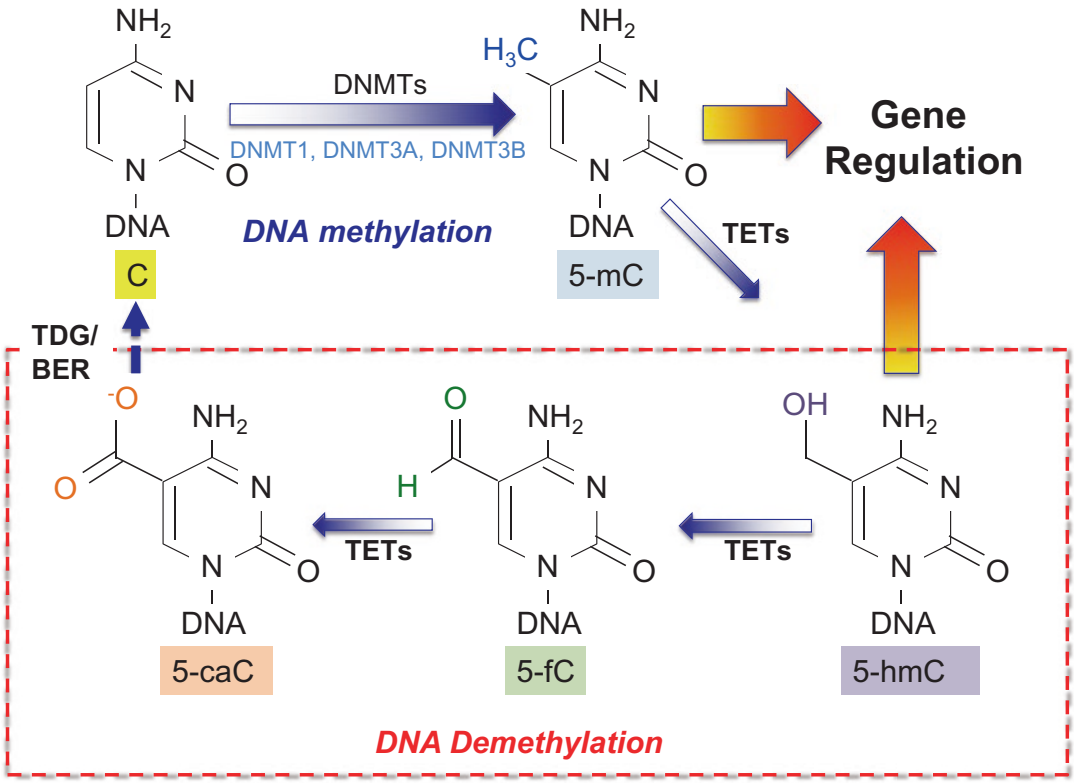

Fig. 20.5 DNA methylation/demethylation enzymes. Methylation of the promoter regions of genes significantly suppresses transcription by direct inhibition of transcription factor binding and recruitment of methyl-CpG-binding proteins within their recognition sites of transcription factors. DNA methylation occurs at carbon 5 of cytosine (5-methylcytosine [5mC]) in cytosine-phosphate-guanine dinucleotides (CpG) dinucleotides. DNA (cytosine-5-)-methyltransferase 1 (DNMTDNMTs1) maintains DNA methylation patterns during cell proliferation via methylation of a hemi-methylated nascent DNA strand. DNMT3A and DNMT3B are required for genome-wide de novo methylation and play crucial roles in the establishment of DNA methylation patterns. Methylation by DNMTs is counterbalanced by DNA demethylation. TET oxidizes 5mC to 5-hydroxymethylcytosine (5hmC) and subsequently to 5-formyl cytosine (5fC) and 5-carboxy cytosine (5caC). The carboxyl group of 5caC is excised by thymine DNA glycolase (TDG) to restore cytosine. An active demethylation pathway through consecutive oxidation of 5-methylcytosine (5mC) mediated by TET (ten eleven translocation) proteins and subsequent base excision repair (BER) in mammalian systems DNA methylation dynamics (Reprinted and modified from Heo et al. 2016 with permission of the publisher)

The involvement of DNA methylation in various cancers (Chik and Szyf 2011; Mizuno et al. 2001; Robert et al. 2003; Roll et al. 2008), immune disorders (Januchowski et al. 2004), neurodegeneration (Chestnut et al. 2011; Martin and Wong 2013), and d-flow-induced EC dysfunction (Jiang et al. 2014) has been reported. Jiang et al. showed different levels of DNA methylation in ECs isolated from swine aortas and human aortas exposed to d-flow and s-flow (Jiang et al. 2014). They found the key role of DNA methylation of CpG islands within the KLF4 promoter in d-flow-mediated inhibition of KLF4 transcription. Using two different DNMT inhibitors, RG-108 and 5-Aza, Jiang et al. found that the reduction of premature KLF4 transcripts was totally recovered but that mature KLF4 was only partially recovered by DNMT inhibition. These data do not only suggest the key role of DNMT activity in regulating d-flow-induced reduction of KLF4 expression, but also the existence of another post-transcriptional inhibition of KLF4 mRNA

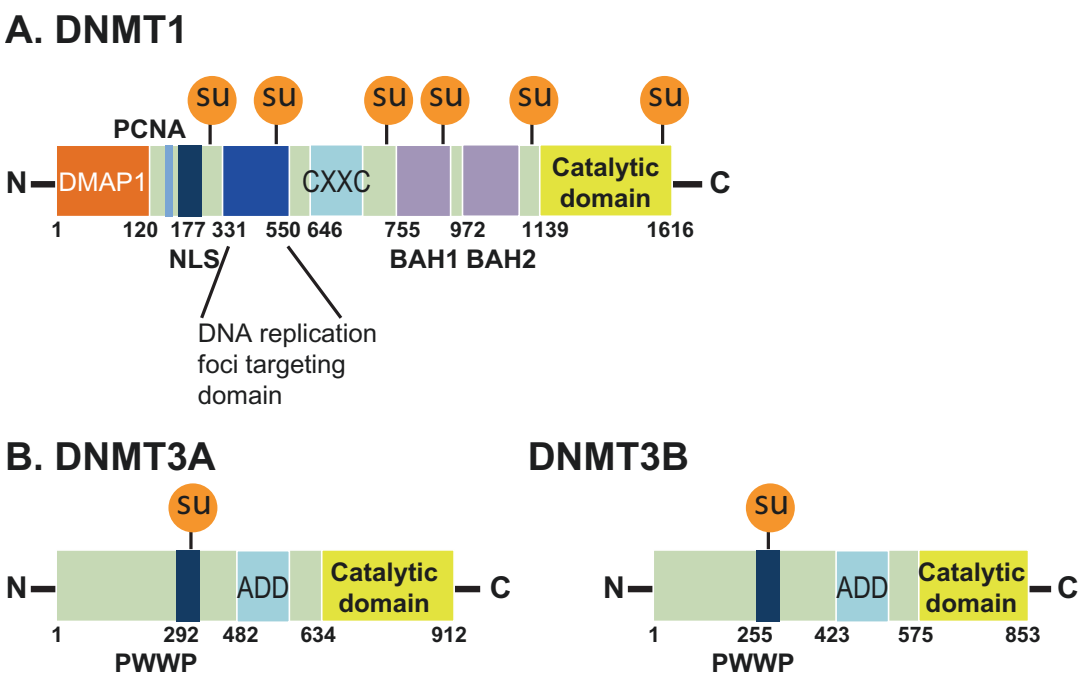

Fig. 20.6 Post-translational regulation of mammalian DNA methytransferases. DNMTs protein domain structure and sumoylation. (**a**) DNMTDNMTs1; DMAP1 domain, PCNA domain, nuclear localization signal domain (NLS), DNA replication foci-targeting domain, CXXC- zinc finger region, bromo-adjacent homology domains (BAH1 and BAH2), and catalytic domain. More than 10 sumoylation sites throughout DNMT1 sequence were suggested. (**b**) DNMT 3A and 3B; a proline-trytophan-proline domain (PWWP), an ATRX-DNMT3-DNMT3L-type zinc finger domain (ADD), and catalytic domain. Sumoylation of the N-terminal regulatory region including the PWWP domain was reported (Reprinted and modified from Heo et al. 2016 with permission of the publisher)

induced by d-flow (Dunn et al. 2014). In addition, d-flow induces DNMT activation and consequent DNA hypermethylation of the KLF4 promoter, which inhibits the expression of eNOS, thrombomodulin, and monocyte chemoattractant protein 1 (Dunn et al. 2014).

The crucial role of DNMTs in d-flow signaling has been reported by Dunn et al. and Zhou et al., but there are some discrepancies in terms of expression of DNMT isoforms (Dunn et al. 2014; Zhou et al. 2014). Dunn et al. reported that d-flow increased both DNMT1 mRNA and protein (Dunn et al. 2014), while Zhou et al. did not observe an increase in DNMTDNMTs1 protein although they did find up-regulation of DNMT1 mRNA expression and nuclear translocation (Zhou et al. 2014). These differences may come from different experimental systems employed. Using ECs isolated from the area exposed to either d-flow or s-flow in swine aortas (Jiang et al. 2014), Jiang et al. found no significant differences in DNMT (DNMT1, 3A, and 3B) expression or cytosine demethylation enzyme mRNA (TET1–3, TDG1, GADD45B, MBD4, and SMUG1) expression in ECs from the d-flow region, but they found a significant increase in DNMT3A protein levels without any change in mRNA levels (Jiang et al. 2014). In contrast, Dunn et al. used the partial carotid ligation model to generate d-flow and compared gene expression and DNA methylation in the carotid artery with (d-flow) or without (s-flow) partial ligation. In in vitro studies, Zhou et al. used a human umbilical vein endothelial cell culture system and Jiang et al. used ECs isolated from swine aorta, which may explain the difference between these studies. Although d-flow-induced induction of nuclear translocation of DNMT1 was reported by Zhou

et al., the regulatory mechanism of this nuclear translocation is not clear. It has been reported that IL-6 causes DNMT1 nuclear translocation by AKT-mediated phosphorylation at the DNMTDNMTs1 nuclear localization signal site (Hodge et al. 2007), suggesting possible involvement of other forms of PTMs including sumoylation as we explain later in d-flow-induced DNMT1 nuclear translocation.

5-Aza is an inhibitor of DNMTs and has been shown to inhibit formation of atherosclerosis in the mouse partial carotid ligation model (Dunn et al. 2014). In this study, DNA methylation in 11 gene promoters was shown to increase by d-flow, and this increase was reversed by 5-Aza. Since a system biology analysis by MetaCore predicted these 11 genes to be regulated by cAMP response element binding protein (CREB1), these authors investigated the CRE site within the promotor region of these genes. Five of the 11 genes contained a CRE site in its differentially methylated regions, and promoters of HoxA5, Klf3, Cmklr1, and Acvrl1 at the CRE CG site were hypermethylated by d-flow, which was also inhibited by 5-Aza. Although a possible role for HoxA5 in vascular remodeling and angiogenesis via EC inflammation has been reported (Dunn et al. 2014), the actual pathological role of d-flow-mediated hypermethylation for each gene promoter remains unclear.

It has been reported that the function of DNMTDNMTss can be regulated by sumoylation (Fig. 20.6). For example, as we discussed above, Jiang et al. have reported that a significant increase in DNMT3A protein levels without changing its mRNA levels, and they have suggested that DNMT3A sumoylation may contribute to this process, because sumoylation can increase the half-life of DNMT3A (Jiang et al. 2014; Ling et al. 2004). Interestingly, DNMT3A sumoylation does not only delay its degradation, but also disrupt the ability of DNMT3A to interact with histone deacetylases (HDACs) and repress transcription of a reporter gene (Ling et al. 2004). These data suggest the possible role of DNMT3A sumoylation in the nucleus, which can up-regulate d-flow-induced transcriptome by hypermethylation of promoters. Not only DNMTDNMTs1 phosphorylation but also the significant role of DNMT1 sumoylation in regulating DNA methylation activity has been reported (Lee and Muller 2009). The relationship between DNMT1 phosphorylation and sumoylation and the way in which this activity stimulates the methylation activity of DNMT1 remain unclear. As we discussed above, DNMTs can shuttle between the cytoplasm and the nucleus, and d-flow may induce DNMTs nuclear translocation (Zhou et al. 2014). Because we have found that d-flow elicits SENP2 nuclear export and increases nuclear ERK5 and p53 sumoylation, it is reasonable to speculate that d-flow also induces DNMT sumoylation, which may increase DNA hypermethylation in the nucleus. Further investigation is needed.

20.2.6 Nuclear Inducible Camp Early Repressor (ICER) Is Regulated by ERK5-Sumoylation in Heart

Reduced expression of cAMP hydrolyzing enzymes including phosphodiesterase 3A (PDE3A) and increased expression of inducible cAMP early repressor (ICER) have been observed in failing hearts. ICER down-regulates Bcl2 via inhibiting the transactivation of cAMP response element binding protein (CREB) and leads to down-regulation of Bcl2 and PDE3A expression. The reduction of PDE3 expression increases cAMP availability and up-regulates PKA signaling, forming an autoregulatory positive feedback loop. Angiotensin II and isoproterenol (β-AR agonist) activate this positive feedback loop, providing a mechanism of how the activation of neurohormonal systems in heart failure affects myocyte apoptosis (Ding et al. 2005, 2000).

Our group has reported that ERK5 plays a critical role in regulating this cardiomyocyte apoptosis pathway (Fig. 20.7). Mice with cardiac-specific ERK5 knockout show accelerated cardiac apoptosis and dysfunction after thoracic aorta constriction (Kimura et al. 2010), while transgenic mice overexpressing cardiac-specific constitutively active MEK5 (CA-MEK5, ERK5's direct upstream kinase) show reduced levels of ICER

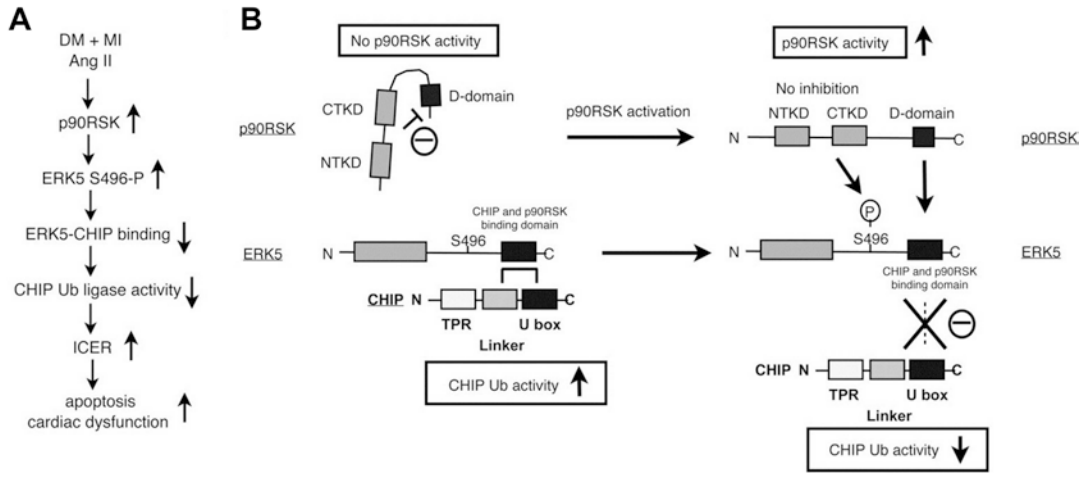

Fig. 20.7 p90RSK regulates ERK5-CHIP module. (**a**) A model of myocardial infarction under diabetes (DM + MI) or angiotensin II (Ang II)-mediated p90RSK-ERK5-CHIP signal transduction pathway that regulates cardiac apoptosis and subsequent cardiac dysfunction. (**b**) A scheme depicting p90RSK-mediated regulation of the ERK5-CHIP module. At the basal level, inactive p90RSK inhibits the D-domain to bind with ERK5 Gao et al. (2010). p90RSK-free ERK5 associates with CHIP at its linker and U-box domain and maintains its CHIP Ub ligase activity to prevent ICER induction and subsequent apoptosis Le et al. (2012). However, once p90RSK gets activated, the inhibition of the kinase domain is released Gao et al. (2010), Woo et al. (2010), and the D-domain of p90RSK associates with the ERK5 COOH-terminal domain, leading to compete with ERK5-CHIP association and ERK5-S496 phosphorylation, which disrupts ERK5-CHIP interaction. The disruption of ERK5-CHIP interaction inhibits CHIP Ub ligase activity Woo et al. (2010), increases ICER induction, and induces apoptosis Esser et al. (2005), Woo et al. (2010) (Reprinted from Le et al. 2012 with permission of the publisher)

induction and myocyte apoptosis upon induction of pressure-overload and myocardial infarction (MI) (Shishido et al. 2008; Yan et al. 2007). Regulation of the PDE3A-ICER mechanism by ERK5 is achieved by an E3 ubiquitin (Ub) ligase called CHIP (carboxyl terminus of HSP70-interacting protein). CHIP has an important cardioprotective role in limiting myocardial damage due to ischemia/reperfusion injury after MI by inhibiting apoptosis. Transgenic CHIP knockout mice exhibit increased infarct sizes and decreased survival compared to wild-type (Zhang et al. 2005). Activation of ERK5 decreased ICER protein stability through CHIP-mediated degradation. ERK5 activation increased ERK5-CHIP binding and subsequently up-regulated CHIP Ub ligase activity and decreased ICER expression after MI (Woo et al. 2010). In diabetic mice with induced MI (DM + MI), CHIP Ub activity and PDE3 expression was decreased, while ERK5-sumoylation and ICER expression was increased, suggesting that ERK5-sumoylation may directly inhibit CHIP Ub activity and increase ICER expression (Shishido et al. 2008). Further investigation is necessary to clarify the role of ERK5-sumoylation on the CHIP-ICER signaling pathway.

20.3 Sumoylation of Potassium Channels at the Plasma Membrane

In the previous section, we have discussed the role of SENP2 in the nucleus, and the nuclear export of SENP2 may enhance sumoylation of nuclear ERK5 and p53. Although SENP1 and 2 can translocate from the nucleus to the cytosol/membrane, the consequence of this translocation on extranuclear proteins remains unclear. In this section, we will review the role of SENP1 and 2 in regulating several potassium channel proteins, which are localized in the plasma membrane.

First, it is known that Kv1.5 (potassium voltage-gated channel subfamily A member 5, KCNA5) is responsible for the I_{Kur} repolarizing

current in atrial myocytes and also regulates vascular tone in peripheral vascular beds. Benson et al. have reported that SENP2 can de-sumoylate Kv1.5 and lead to a substantial hyperpolarizing shift in the voltage dependence of steady-state inactivation (Benson et al. 2007). Of note, they did not see any significant V_{50} shift of (wild type) Kv1.5 in the depolarizing direction by overexpressing SUMO3 and Ubc9, suggesting that neither Ubc9 nor SUMO is a limiting factor for regulating Kv1.5 function. In this study, SENP2 deletion mutant of the first N-terminus residues (SENP2 aa71–590), which shows enhanced desumoylation activity against multiple sumoylated substrates and also localizes to the cytoplasm, was used. Co-expression with the this mutant with wild type Kv1.5 decreased Kv1.5 sumoylation, then caused a significant hyperpolarization shift in the voltage dependence of inactivation without altering the total current density or voltage dependence of Kv1.5 activation. Therefore, it is likely that SENP2 nuclear export can regulate Kv1.5 function via changing its cellular localization. Kv1.5 is widely expressed in the cardiovascular system (Overturf et al. 1994). A loss-of-function mutant of Kv1.5 expressed in the atrium causes a familial form of atrial fibrillation (Olson et al. 2006), and a critical role of Kv1.5 in the pulmonary vasculature for the oxygen-sensitive regulation of arterial tone has also been reported (Hong et al. 2005). Further investigation is necessary to determine the role of Kv1.5 sumoylation and SENP2 in these processes.

Another plasma membrane potassium channel family which can be regulated by sumoylation is potassium voltage-gated channel subfamily Q member (KCNQ). Five KCNQ genes (KCNQ1 to KCNQ5) codify a family of 5 different voltage-gated potassium ion channels (KV7.1 to KV7.5), which are mainly expressed in the nervous and cardiac systems (Brown and Passmore 2009). Using mice homozygous for the floxed SENP2 allele with a neomycin insert ($SENP2^{fxN/fxN}$), Yeh's group has elucidated SENP2's role in sudden death. These $SENP2^{fxN/fxN}$ mice appear healthy at birth, but develop convulsive seizures followed by sudden death at 6–8 weeks of age. The neomycin cassette insertion caused a reduction in SENP2 transcription and protein levels. Reduced expression of SENP2 protein induced an accumulation of SUMO-1 or SUMO-2/3 proteins in the brain and heart, thus leading to the formation of a hyper-sumoylation environment (Qi et al. 2014). SENP2 is abundant in the hippocampal region, an area of the brain of great relevance to seizure, but the exact location with increased SUMO proteins in the hippocampus has not yet been determined. In $SENP2^{fxN/fxN}$ mice, SENP2 levels are markedly reduced and as the result, sumoylation of Kv7.2 is enhanced in hippocampal neurons. Hyper-sumoylation of Kv7.2 potassium channels diminished the M-current (conducted by Kv7 channels), leading to a more positive resting membrane potential and increased excitability of hippocampal neurons. These data suggest the pathophysiological role of SENP2 in epilepsy via regulating plasma membrane Kv7.2 function.

The last potassium channel which can be sumoylated is potassium channel subfamily K member 1 (KCNK1). Both KCNK1 K274E sumoylation site mutation and overexpression of SENP1 increase KCNK1 current (Rajan et al. 2005), suggesting an inhibitory effect of sumoylation (Plant et al. 2010). KCNK1 is widely expressed in the heart and the central nervous system and regulates background leak currents stabilizing neuronal excitability (Silveirinha et al. 2013). Although the possible involvement of SENP1 in regulating KCNK1 has been suggested, it is not clear how the sumoylation status of the plasma membrane KCNK1 current is regulated.

In summary since all the reported sumoylated potassium channels can be regulated by desumoylation enzymes of SENP1 and 2, how SENP1 and 2 change their de-sumoylation activity or localization and regulate the plasma membrane potassium channel function need further investigation.

20.4 Sumoylation of Mitochondrial Proteins

20.4.1 Overview of Mitochondrial Fission and Fusion

Cardiomyocyte mitochondria form a diverse network that is integral to maintaining appropriate cardiomyocyte activity. Mitochondria occupy nearly 33% of cardiac cell volume and produce the energy required to sustain cardiac function (Ong et al. 2015a, b, c). This structure is a dynamic organelle that persistently changes its membrane morphology in response to cellular activity (Jayashankar et al. 2016). These conformational changes are necessary for mitochondrial replication and membrane integration. The mitochondrion undergoes both fission and fusion in order to maintain normal cell function. Mitochondrial fusion is achieved by the process of integrating separate membranes, and fission by the process of separating intact membranes (Twig et al. 2008a, b). The fusion of mitochondrial membranes is mediated by GTPase proteins from the dynamin family including mitofusin 1 (Mfn1), mitofusin 2 (Mfn2), and optic atrophy-1 (Opa1) (Mishra 2016; Mishra and Chan 2016). The overall process of fusion is mainly regulated by ubiquitination via ubiquitin ligases such as membrane-associated RING finger 5 (MARCH5) (Nagashima et al. 2014). Fission of mitochondria occurs in a sequential manner. First, constriction of mitochondrial tubules takes place. Next, the GTPase called dynamic-related protein 1 (Drp1) is mobilized from the cytosol to the outer membrane of mitochondria via several receptor proteins (Ong et al. 2015b). Upon reaching the outer membrane, Drp1 assembles into a scission complex by forming a spiral that surrounds the constricted tubules. Then, functioning in a GTP dependent manner, the Drp1 complex further constricts the tubule to cause scission (Friedman et al. 2011; Ong et al. 2015b). Lastly, the complex is disassembled. (Fig. 20.8)

20.4.2 The Roles of DRP1 Sumoylation in Mitochondrial Fission; SUMO1 vs SUMO2/3

DRP1 sumoylation regulates the process of fission by modifying DRP1 function. In Cos7 and

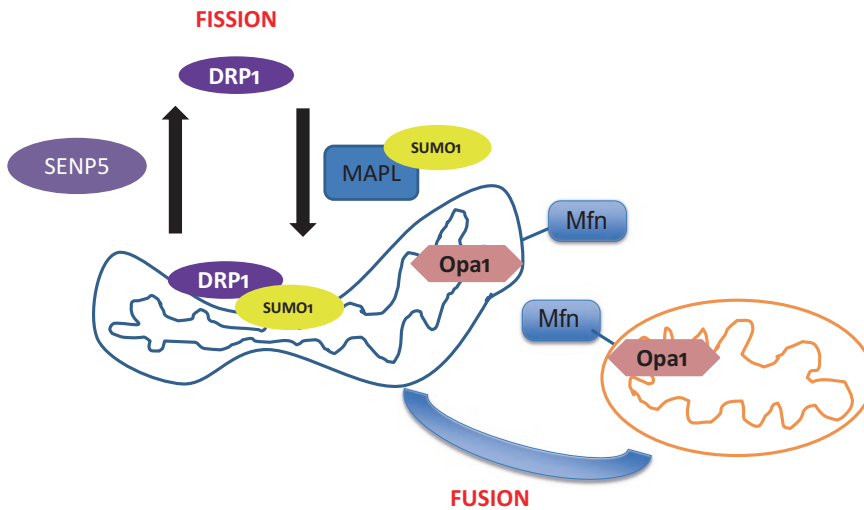

Fig. 20.8 SUMO1 modification of Dynamin Related Protein (DRP1) and mitochondrial fission. The process of mitochondrial fission is regulated by the recruitment and oligomerization of the DRP1. SUMO1 modification of DRP1 increases DRP1 activity, which is regulated by a SUMO E3 ligase MAPL, and deconjugating enzymes include SENP5 (a SUMO protease). The crucial role of GTPase Opa1 and mitofusin has been reported, but the role of sumoylation in this process remains unclear Braschi and McBride (2010)

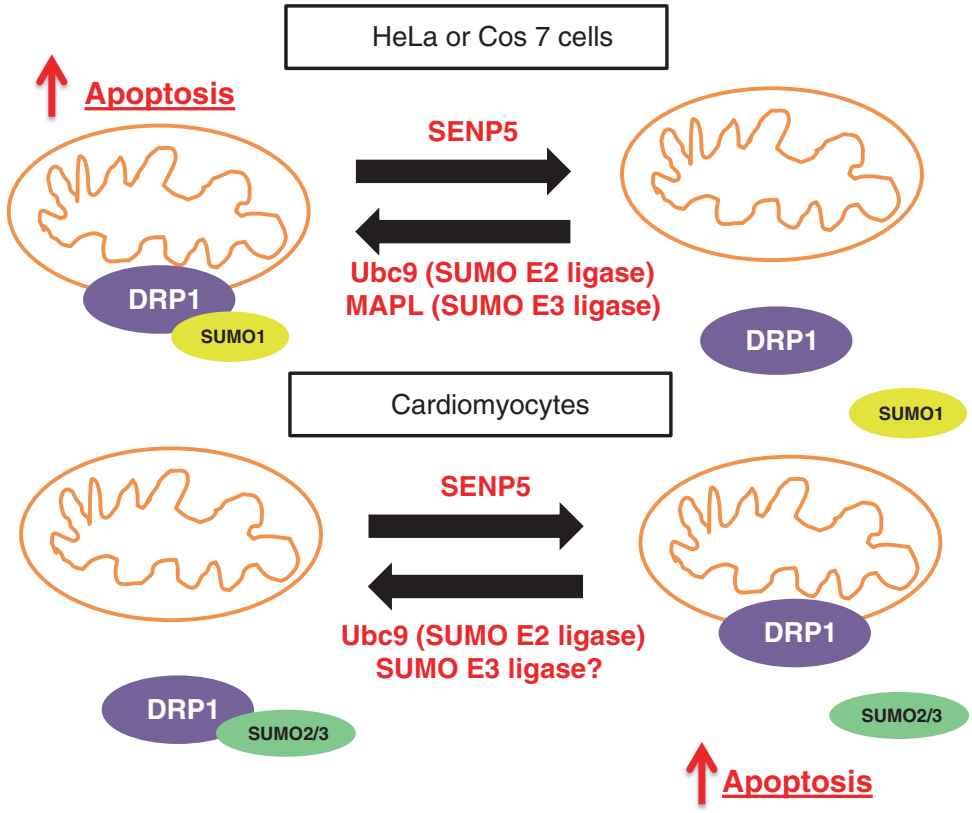

Fig. 20.9 SUMOylation of dynamin related protein (Drp1) in regulating mitochondrial fission. SENP5 was identified as SUMO deconjugases of DRP1 in Hela, Cos7, and cardiomyocytes. Silencing of SENP5 in COS-7 or HeLa cells SUMO1 modification of DRP1 is increased by the depletion of SENP5 and the E3 SUMO ligase mitochondrial anchored protein ligase (MAPL). SUMO1 modification of DRP1 enhances DRP1 binding to mitochondria, leading to mitochondrial fragmentation and cellular apoptosis. In contrast, in cardiomyocytes SENP5 inhibits SUMO2/3 modification of DRP1. De-sumoylated DRP1 can bind to mitochondria, and subsequently induced mitochondrial fragmentation and apoptosis Mendler et al. (2016)

Hela cells, DRP1 SUMO1 modification stimulates mitochondrial fission by enhancing retention of DRP1 on the membrane after its recruitment to mitochondria, followed by disassembly of the Drp1 oligomer via de-sumoylation (SUMO1) once fission is completed (Wasiak et al. 2007; Zunino et al. 2007, 2009). Mitochondrial-anchored protein ligase (MAPL) is a 40 kDa protein located on the outer mitochondrial membrane (Zungu et al. 2011) (Fig. 20.9). Although MAPL can participate into the process of both ubiquitination and sumoylation, under physiologic conditions MAPL preferentially functions as a SUMO E3 ligase for DRP1 sumoylation (SUMO1) (Braschi et al. 2009). MAPL-mediated DRP1 sumoylation (SUMO1) increases mitochondrial fission and hyperfragmentation (Braschi et al. 2009; Neuspiel et al. 2008; Zungu et al. 2011). MAPL-mediated DRP1 sumoylation (SUMO1) has also been identified to play a role in apoptosis. Cytochrome c functions as the terminal trigger for apoptotic cell death and is located in the intermembrane space (Chipuk et al. 2006). Release of cytochrome c has been shown to depend on MAPL-mediated sumoylation (SUMO1) of DRP 1 (Prudent et al. 2015).

SENP5, SENP3, and SENP2 can be a de-sumoylation enzyme for DRP1 (Harder et al. 2004; Mendler et al. 2016). Several experiments have suggested that SENP5 plays a role in regulation of fission via its interaction with DRP1 (Di

Bacco et al. 2006; Zunino et al. 2009). SENP5 participates in de-sumoylation of DRP1 with the ability to remove SUMO1, SUMO2, or SUMO3 (Gong and Yeh 2006; Zunino et al. 2009), but the functional consequence of conjugation of SUMO1 and SUMO2/3 to DRP1 on regulating mitochondrial function is not the same among these isoforms (Mendler et al. 2016). The depletion of SENP5 in COS7 or Hela cells increases DRP1 SUMO1 modification induced by MAPL, leading to mitochondrial binding of DRP1 and increasing mitochondrial fragmentation and cellular apoptosis (Wasiak et al. 2007; Zunino et al. 2007, 2009). In contrast, cardiac-specific overexpression of SENP5 inhibits DRP1 SUMO2/3 modification, which induces apoptosis via promoting the association of DRP1 with mitochondria (Kim et al. 2015) (Fig. 20.9). It has been suggested that DRP1 SUMO2/3 modification prevents DRP1 association with mitochondria, whereas DRP1 SUMO1 modification induces DRP1 binding to mitochondria and induce apoptosis (Mendler et al. 2016). Sumoylation of mitochondrial proteins is an area of ongoing research. Especially, elucidation of detailed mechanisms for different roles of DRP1-SUMO1 and DRP1-SUMO2/3 remains to be critical.

SENP5 is localized primarily to the nucleus, but there is also a substantial amount of this enzyme in the cytosol (Zunino et al. 2007). Zunino et al. have reported that SENP5 translocation from the nucleus to the mitochondria specifically occurs at the G2/M transition (Zunino et al. 2009). Although the regulatory mechanism for SENP5 translocation from the nucleus to the mitochondria remains unclear, this provides another example that translocation of SUMO proteases between different intracellular compartments can regulate various cell responses by modifying sumoylation.

20.5 Conclusions

In this chapter, we have highlighted the role of sumoylation in different cellular locations particularly in cardiovascular disease and epilepsy. Kinases like PKCζ and p90RSK are activated under d-flow or diabetic conditions and play central roles in regulating a complex network of signal transduction that is continuously modified by sumoylation. As discussed in this chapter, sumoylation is an important and dynamic post-translational protein modification occurring at different compartments of cells and this is tightly regulated by the localization of sumoylation and de-sumoylation enzymes. Different roles of SUMO1 and SUMO2/3 in regulating DRP1 function was reviewed, but the contribution of SENP5 mitochondrial translocation and how SENP5 differentially regulate DRP1 modification with SUMO1 and SUMO2/3 remains unclear. Further investigations focused on different roles of sumoylation in different cellular location and how de-sumoylation enzymes including SENP2 and 5 coordinately regulate these processes by changing their localization will be necessary.

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Viral Interplay with the Host Sumoylation System

21

Van G. Wilson

Abstract

Viruses have evolved elaborate means to regulate diverse cellular pathways in order to create a cellular environment that facilitates viral survival and reproduction. This includes enhancing viral macromolecular synthesis and assembly, as well as preventing antiviral responses, including intrinsic, innate, and adaptive immunity. There are numerous mechanisms by which viruses mediate their effects on the host cell, and this includes targeting various cellular post-translational modification systems, including sumoylation. The wide-ranging impact of sumoylation on cellular processes such as transcriptional regulation, apoptosis, stress response, and cell cycle control makes it an attractive target for viral dysregulation. To date, proteins from both RNA and DNA virus families have been shown to be modified by SUMO conjugation, and this modification appears critical for viral protein function. More interestingly, members of the several viral families have been shown to modulate sumoylation, including papillomaviruses, adenoviruses, herpesviruses, orthomyxoviruses, filoviruses, and picornaviruses. This chapter will focus on mechanisms by which sumoylation both impacts human viruses and is used by viruses to promote viral infection and disease.

Keywords

SUMO • Ubc9 • DNA viruses • RNA viruses

21.1 Introduction

Viral proteins were among the first defined substrates for sumoylation, beginning with the demonstration in 1999 that the human cytomegalovirus (HCMV) immediate-early 1 protein (IE1)) was SUMO modified (Muller and Dejean 1999).

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Among the 7 families of DNA viruses that infect humans, five families (Parvoviridae, Papillomaviridae, Adenoviridae, Herpesviridae, and Poxviridae) have one or more sumoylated proteins, illustrating the widespread utilization of the sumoylation system by nuclear viruses. To date sumoylated proteins have not been reported in the Polyomaviridae and the Hepadnaviridae families, though this may be for lack of investigation and not the absence of substrates for these viruses. Additionally, a number of DNA viral proteins can influence sumoylation globally or for specific substrates, and this is likely to be a critical mechanism to alter the host environment to facilitate the viral life cycle and/or overcome host defenses. DNA viral proteins that are sumoylated and/or which regulate sumoylation are listed in Table 21.1.

Among the RNA viruses infecting humans, sumoylation was first observed for retroviruses which also have a nuclear phase to their life cycle. Specifically, both virion antigens (Gurer et al. 2005; Yueh et al. 2006) and the Tax regulatory protein (Lamsoul et al. 2005) of retroviruses were shown to be substrates for sumoylation. Subsequently, however, sumoylation of viral proteins and/or effects on sumoylation by viral proteins has been observed for nine other human RNA virus families. Many of these RNA viral proteins have no nuclear phase, so cytoplasmic sumoylation must be occurring as has now been demonstrated for many cellular proteins (Alonso et al. 2015; Hilgarth et al. 2004; Manning Fox et al. 2012). Whether or not sumoylation is relevant for the other families of human RNA viruses remains to be determined, but seems likely given the growing appreciation of the SUMO system as a key target for viral manipulation. RNA viral proteins that are sumoylated or which influence host sumoylation at listed in Table 21.2.

It is clear from numerous studies over the last nearly two decades that sumoylation is an important post-translational process that contributes to successful infection for a wide range of DNA and RNA viruses. In addition to viral proteins being substrates for and regulated by sumoylation, there is now extensive evidence that some viral proteins can act as surrogate sumoylation enzymes and/or modulate the activity of the authentic host sumoylation system. The ability of viral proteins to usurp the sumoylation system is not unexpected as this system contributes to the regulation of many critical cellular pathways and thus provides viruses with mechanisms to exert control over these pathways. Several recent reviews have covered many aspects of viral sumoylation (Everett et al. 2013b; Hannoun et al. 2016; Mattoscio et al. 2013; Varadaraj et al. 2014; Wilson 2012; Wimmer and Schreiner 2015; Wimmer et al. 2012) and should be consulted for more exhaustive coverage of this subject. In this chapter we will provide an overview of the role of sumoylation in human viral families that have been identified to date.

21.2 DNA Viruses

21.2.1 Parvoviruses

Parvoviruses have small (approximately 5000 bp), single-stranded DNA genomes with only two genes, one which encodes the capsid proteins and the other which encodes the replication proteins (designated NS or Rep proteins). Adeno associated virus type 2 (AAV-2) is a member of this family whose reproductive cycle is dependent upon co-infection with unrelated helper viruses, typically adenovirus or herpesvirus, and which establishes a latent infection in the absence of the helpers (Berns and Linden 1995). The Rep78 protein of AAV-2 binds the SUMO conjugating enzyme, Ubc9, and is sumoylated primarily at lysine 84, though other lysines may also serve as weaker acceptor sites (Weger et al. 2004). Converting lysine 84 to the conserved but nonsumoylatable arginine causes a significant decrease in the half-life of Rep78, implicating sumoylation in the control of Rep78 levels. Functionally the effect of sumoylation was not characterized, but sumoylation could be one of the mechanisms that helps regulate Rep to ensure appropriate intracellular levels to maintain the latent state.

AAV based vectors have become widely used in gene therapy due to their ability to infect a

Table 21.1 DNA virus proteins and sumoylation

DNA Virus family	Virus	Protein	Sumo sites*	SIMs	Effect of sumoylation or effect on sumoylation system
Parvovirus	AAV	Rep78	K84	–	Sumoylation may stabilize Rep78
Papillomavirus	HPV	E1	K559	–	Role of sumoylation unclear
		E2	K292	–	Sumoylation indirectly stabilizes E2
		E6	–	–	Blocks sumoylation of PIASy substrates; Degrades Ubc9
		E7	–	–	Inhibts sumoylation of pRB
		L2	K35	+	Modulate L2 incorporation int capsids
Adenovirus	Ad5	E1A	–	–	Blocks pRB sumoylation; binds Ubc9
		E1B-55K	K104	–	SUMO E3 ligase
		E4orf3	–	–	Increases sumoylation of specific targets
Herpesvirus	HSV	ICP0	–	+	STUbL
	VZV	ORF29p	+	–	Role of sumoylation unknown
		OFR61	–	+	Possible STUbL
	CMV	IE1	K450	–	Sumoylation prevents binding to STAT2
		IE2-p86	K175/K180	+	Sumoylation enhances transactivation activity
		UL44	Multiple	–	Sumoylation enhances DNA binding
		pp71	–	–	Increases sumoylation of Daxx
	HHV6	IE1	K802	–	Role of sumoylation unknown
		IE2	–	–	Binds Ubc9, consequence unknown
	EBV	BZFL1	K12	–	Sumoylation repressions transactivation activity
		BGLF4	–	+	Decreases sumoyation of BZLF1
		EBNA3B	+		Effect of sumoylation unknown
		EBNA3C	+	+	Sumoylation affects nuclear distribution
		LF2	–	–	Enhances sumoylation of Rta
		LMP1	–	–	Binds Ubc9 and increases sumoylation generally and for specific substrates
		Rta (BRLF1)	Multiple	–	Sumoylation may enhance transactivation activity; promotes association with RNF4
	KSHV	K-bZIP	K158/K207	+	SUMO E3 ligase; Sumoylation enhances repressive activity
		K-Rta	–	+	STUbL
		LANA1 (ORF63)	K1140	+	SIM facilitates interaction with host proteins
		LANA2 (vIRF3)	Multiple	+	Enhances or represses sumoylation of specific substrates
Poxvirus	Vaccinia	A40R	K95	–	Sumoyylation solubilizes A40R
		E3	K40/K99	+	Sumoylation represses transactivation activity

*A + symbol indicates that sumoylation has been detected but a specific site has not been mapped. A – symbol indicates that the feature has not been identified or has not been tested for

Table 21.2 RNA virus proteins and sumoylation

RNA virus family	Virus	Protein	Sumo sites*	SIMs	Effect of sumoylation or effect on sumoylation system
Retrovirus	HIV	Integrase	Multiple	–	Unclear
	HTLV	Tax	Multiple	–	Localization
Orthomyxovirus	Influenza A virus	NS1	K70/K219	–	Sumoylation needed to reduce IFN production
		M1	K242	–	Sumoylation required for binding NP
		NP	K4/K7	–	Sumoylation required for intracellular trafickkng of NP
		NS2	+	–	Unknown
Filovirus	Ebola	VP35	–	–	VP35 enhances the sumoylation of host IRF7
Paramyxovirus	Parainfluenza	P	K254	–	Sumoylation required for WT viral titer; no specific defect identified
Rhabdovirus	Rabies/VSV	–	–	–	Increased sumoylation blocks viral mRNA synthesis
Coronavirus	SARS	N	–	–	N binds Ubc9
Flavivirus	Dengue	NS5	+	–	Sumoylation stabilizes NS5
		Envelope Protein	–	–	Binds Ubc9
Picornavirus	Enterovirus 71	3C	K72	–	Sumoylation decreases protease activity and stability of 3C
	EMCV	3C	–	–	3C enhances sumoylation of PML
Reovirus	Rotavirus	VP1	+	+ (tentative)	Unknown
		VP2	+	+ (tentative)	Unknown
		VP6	+	-	Unknown
		NSP2	+	+ (tentative)	Unknown
		NSP5	K19/K82 (tentative)	–	Sumoylation required for viroplasm structure formation
Delta	Hepatitis delta virus	HDAg	Multiple	–	Sumoylation of HDAg increases viral genomic and mRNA production

*A + symbol indicates that sumoylation has been detected but a specific site has not been mapped. A – symbol indicates that the feature has not been identified or has not been tested for

wide variety of dividing and nondividing cells (Hastie and Samulski 2015; Lisowski et al. 2015). However, there are numerous impediments to their practical usage, including limited coding capacity, host immune response, and modest transduction efficiency. Recently, Holshcer et al. demonstrated that host sumoylation is one factor that restricts AAV transduction (Holscher et al. 2015). Using a whole genome siRNA screen they found that genes encoding products in the sumoylation pathway, principally SAE1/SAE2 (the activating enzyme subunits) and Ubc9 (the conjugating enzyme), interfered with AAV transduction. The effect appears to be at the level of capsid entry and may involve sumoylation of capsid protein. Most AAV VP1 capsid proteins contain a putative sumoylation motif, though actual modification of VP1 by SUMO was not experimentally tested. Surprisingly, while this effect was observed for several AAV serotypes

tested, it was not observed for parvovirus H1. It was also not observed when using AAV pseudovirions comprised of human papillomavirus capsid antigens, so this does not appear to be a general antiviral defense. It remains to be determined if sumoylation of AAV virions actually occurs under natural infection conditions and whether or not this impacts the viral infections process in any significant fashion.

21.2.2 Papillomaviruses

The human papillomaviruses (HPVs) are important pathogens with over 200 subtypes that cause benign disease (warts) and promote certain epithelial tumors, including cervical cancer and certain oral cancers (Lee et al. 2016; Madkan et al. 2007). HPVs infecting the mucosa can be classified in two types based on their capacity to cause carcinogenesis: low risk and high risk. Reproduction of papillomaviruses is intimately coordinated with the differentiation process of stratified epithelium (Nguyen et al. 2014). Interruption of the normal virus life cycle and persistent expression of the two oncogene proteins, high risk HPV E6 E6 and E7, underlines the basis for cancer progression (Galloway and Laimins 2015). HPV E7 stimulates the cell cycle by promoting E2F release from pRb, while E6 promotes p53 degradation in an ubiquitin-dependent and independent manner (Doorbar 2006). However, both of these viral proteins are multifunctional and make complex contributions to both oncogenesis and normal viral reproduction. They are also involved in the molecular mechanisms by which HPV hijacks the process of keratinocyte differentiation, leading to the pathology observed with common warts. In addition to E6 and E7, two other viral early proteins, E1 (Bergvall et al. 2013) and E2 (McBride 2013), are essential for normal viral replication.

Currently there are three papillomavirus proteins that have been shown to be sumoylated, two early proteins, E2 (Wu et al. 2007, 2008) and E1 (Rangasamy and Wilson 2000), and the minor capsid protein, L2 (Marusic et al. 2010). The papillomavirus E2 protein is a multifunctional polypeptide with roles in viral DNA replication (Chiang et al. 1992), genome segregation (You et al. 2004), and transcription (Demeret et al. 1997). As a transcription factor, E2 is both an activator and repressor depending on the promoter context (Ledl et al. 2005). E2 has a major sumoylation site at lysine 292, and similar to many transcription factors, E2 modification at this site regulates its transactivation ability (Wu et al. 2008). Furthermore, E2 can interact with a variety of other host cell transcription factors such as Sp1 (Steger et al. 2002), YY1 (Lee et al. 1998), and C/EBP (Hadaschik et al. 2003), all of which are themselves sumoylated (Deng et al. 2007; Kim et al. 2002; Spengler and Brattain 2006). Preliminary results in our lab indicate that E2 reduces the sumoylation of C/EBP *in vivo*. Because sumoylation of C/EBP negatively regulates transcriptional synergy (Subramanian et al. 2003) the E2-mediated reduction of C/EBP sumoylation may account for the observed enhancement of C/EBP activity by E2 protein (Hadaschik et al. 2003). Similar effects of E2 on its other sumoylated binding partners could be a general feature of E2 that contributes to its dysregulation of cellular transcription. In addition to being functionally regulated by direct sumoylation, E2 stability is also sumoylation dependent with increased sumoylation resulting in a dramatically extended half-life (Wu et al. 2009). However, this effect is not due to direct sumoylation of E2 as the lysine 292 mutant that cannot be sumoylated remains stabilized by increasing overall cellular sumoylation levels. This indicates that there is an indirect mechanism connecting sumoylation and turnover of E2 which is likely important during the viral life cycle as sumoylation is known to be a dynamic process during keratinocyte differentiation (Deyrieux et al. 2007; Heaton et al. 2012).

Papillomavirus E1 proteins are replicative helicases that bind to the viral origin of replication, catalyze unwinding of the duplex DNA, and recruits host cell replication factors to direct the synthesis of the viral genome (Bergvall et al. 2013). E1 proteins were the second viral protein

to be identified as a substrate for sumoylation (Rangasamy et al. 2000). Bovine papillomavirus (BPV) E1 is sumoylated at lysine 514 (Rangasamy et al. 2000) and human papillomavirus type 11 is sumoylated at the analogous position which is lysine 559 (Fradet-Turcotte et al. 2009); most other HPV E1 proteins have putative sumoylation sites in a similar location which suggests that E1 sumoylation is a widespread and general feature of this protein. PIAS proteins enhance the sumoylation of several tested HPV types which supports their role as a general SUMO ligase for papillomavirus E1 proteins (Rosas-Acosta et al. 2005). Initially, sumoylation was reported to be required for proper nuclear localization of BPV E1 (Rangasamy et al. 2000), but a subsequent investigation failed to detect a nuclear impairment for either BPV or HPV E1 when sumoylation was inhibited (Fradet-Turcotte et al. 2009; Rosas-Acosta and Wilson 2008), so a functional role of sumoylation has not been defined.

In addition to the two early regulatory proteins, E1 and E2, one of the viral capsid structural proteins, L2, is also sumoylated (Marusic et al. 2010). Like E2 protein sumoylation increases the stability of L2, though is this case it is a direct effect of SUMO addition at lysine 35 of L2. However, while the sumoylated form of L2 is more stable, it cannot bind to L1, the major capsid protein. These results suggest that sumoylation may help regulate virion assembly through modulation of L2 levels and incorporation into the L1 virion. Interestingly, L2 also plays a role in viral infection as it accompanies the incoming viral genome to the nuclear PML bodies after virion disassembly. Bund et al. showed that L2 contains a SUMO interacting motif (SIM) that is critical for this localization, presumably through binding to sumoylated proteins in the PML body (Bund et al. 2014). Mutation of the SIM prevented localization and reduced infectivity in a pseudovirion assay, while sumoylation of L2 itself was not required.

Beyond the modification of some papillomavirus proteins by SUMO, another intriguing feature of papillomaviruses is their ability to dysregulate the sumoylation system through the activity of certain viral proteins. The first HPV protein with known effects on sumoylation is the E7 protein. E7 is an early papillomavirus protein that both helps drive the host cell into a proliferative state and counteracts innate immunity (Hebner and Laimins 2006). A major target of E7 protein is the host pRB protein that regulates the activity of the host E2FE2F transcription factor (Roman and Munger 2013). The papillomavirus E7 protein has an LxCxE motif that binds pRB (Munger et al. 2001), and E7 binding inhibits sumoylation of pRB (Ledl et al. 2005). Since sumoylation appears to reduce the repressive ability of pRB, interaction with E7 is likely to have functional consequences. Additionally, E7 also binds many other cellular targets (Wilson and Rosas-Acosta 2003) and might be able to modulate sumoylation of these proteins to more generally influence cellular protein functions. Interestingly, many of the functions of E7 are inhibited by the cellular tumor suppressor, p14ARF (Pan et al. 2003), which is known to stimulate sumoylation of certain substrates (Rizos et al. 2005; Woods et al. 2004). This suggests that antagonism between the sumoylation inhibition by E7 and the enhancement of sumoylation by p14ARF may be an important determinant of the outcome of infections.

Like E7, the second viral oncoprotein, E6, also influences host sumoylation, though in a broader fashion. In a study published in 2006, Dejean's group showed that high risk HPV-E6, but not low risk E6, was capable of binding to and inhibiting PIASy activity (Bischof et al. 2006). PIASy is a SUMO ligase which enhances sumoylation of specific substrates such as p53 (Schmidt and Muller 2003), and E6 binding blocks sumoylation of PIASy-specific substrates. Functionally, since PIASy act as a promoter of cellular senescence (Bischof et al. 2006), this inhibition of PIASy by E6 may contribute to the ability of E6 to inhibit cellular senescence. Although E6 binds to PIASy and inhibits its ligase activity, it does not target this enzyme for degradation like it does for p53 (Huibregtse et al. 1994). Mechanistically, E6 may be acting simply by sequestering or blocking PIASy in the complex. Whether or not E6 binds to other members of the PIAS family, or to other classes of SUMO

ligases has not been reported. Subsequently, work in our lab demonstrated that high risk E6 proteins can also bind to Ubc9, the SUMO conjugating enzyme (Heaton et al. 2011). Unlike the situation for PIASy, E6 targets Ubc9 for proteasomal degradation via the ubiquitin ligase E6AP which results in a significant perturbation of the overall cellular sumoylation profile. While the consequences of this interaction for viral fitness have not yet been examined, the ability of E6 to dysregulate host sumoylation globally through attacks on PIASy and Ubc9 suggest that this will have important ramifications for the viral life cycle. As will be seen in later sections, this ability of viral proteins to modulate the sumoylation system has now been demonstrated for a number of distinct viruses, and the SUMO system appears to be another important host pathway that is commonly usurped to promote the viral agenda.

21.2.3 Adenoviruses

Adenoviruses are primarily respiratory pathogens that are associated with a number of human diseases (Lynch et al. 2011). Adenoviruses use complex alternative splicing to make multiple proteins from each of their early genes, and these early gene products, particularly from the E1A and E1B genes, are critical for reshaping the cellular environment to facilitate viral gene expression and replication (Imperiale et al. 1995). To date, only one adenoviral protein has been identified as a substrate for sumoylation, the early gene product E1B-55K protein. The E1B-55K protein interacts with the SUMO conjugation enzyme, Ubc9 (Wimmer et al. 2013) resulting in sumoylation of E1B-55K at lysine 104 (Endter et al. 2001). Sumoylation of E1B-55K also requires phosphorylation at a C-terminal motif (Wimmer et al. 2013), a site which is likely modified by cellular protein kinase CK2 (Ching et al. 2012). The phosphorylation site on E1B-55K protein is not part of the SUMO recognition motif so addition of the phosphate moiety is not creating a typical phosphorylation-dependent sumoylation motif (Hietakangas et al. 2006). Instead, phosphorylation appears to be providing a cross-talk mechanism between phosphorylation and sumoylation by an as yet undefined mechanism. Recently, a host cell viral restriction factor, KAP1, was also shown to bind E1B-55K and enhance sumoylation of the viral protein, though again the mechanism is unclear (Burck et al. 2016). Interestingly, adenovirus infection also led to reduced sumoylation of KAP1 itself, resulting in decreased epigenetic silencing, suggesting a complex interplay between E1B-55K and KAP1 that is at least in part involving the sumoylation system.

Functionally, E1B-55K sumoylation is required for nuclear localization of E1B-55K (Kindsmuller et al. 2007). Sumoylation blocks a CRM-dependent nuclear export sequence (NES), thus helping retain E1B-55K in the nucleus. Subnuclear localization of E1B-55K is also regulated by the viral E4orf6 protein such that the intranuclear distribution of E1B-55K changes as E4orf6 levels increase during infection (Leppard and Everett 1999). As E4orf6 levels increase there is a decrease in E1B-55K sumoylation, suggesting that E4orf6 may in part be regulating E1B-55K through controlling sumoylation levels (Lethbridge et al. 2003). Importantly, nuclear retention and subnuclear localization of E1B-55k also relies on its interaction with the type IV and V isoforms of the PML protein, and the interaction with PML IV requires sumoylation of E1B-55K protein while the interaction with PML V is largely independent of sumoylation (Wimmer et al. 2010). Surprisingly, sumoylation of E1B-55K was not required for its interaction with another PML associated protein, Daxx, but is required for the E1B-55K-mediated proteasomal degradation of Daxx (Schreiner et al. 2011; Wimmer et al. 2013). A mutant of E1B-55K lacking the SUMO conjugation site was defective for Daxx degradation and for transformation of primary rodent cells, indicating that sumoylation of E1B-55K is important for its transforming activity (Schreiner et al. 2011). Thus, these combined studies indicate that modification of E1B-55K protein by SUMO is clearly important for various biological activities and functions of this viral regulatory protein.

In addition to being a substrate for sumoylation, the E1B-55K protein itself has SUMO E3 ligase ability. A critical host target for E1B-55K is nuclear p53 (Weitzman and Ornelles 2005). Through direct binding with p53, E1B-55K enhances the sumoylation of p53 (Muller and Dobner 2008). While this study reported that E1B-55K SUMO ligase activity on p53 was not observed *in vitro*, a subsequent study was able to confirm E3 activity by E1B-55K protein in a reconstituted in vitro sumoylation reaction, thus confirming that E1B-55K itself has intrinsic ligase activity (Pennella et al. 2010). The sumoylation of p53 by E1B-55K also requires that E1B-55K itself be sumoylated (Muller and Dobner 2008). Since E1B-55K, in conjunction with E4orf6, mediates ubiquitinylation and proteasomal degradation of p53, this was the first example of a viral protein having dual activity in both the ubiquitin and SUMO pathways. Mutation of the p53 sumoylation site decreased the ability of E1B-55K to repress the transcriptional activity of p53 and to tether p53 in PML bodies (Pennella et al. 2010; Wimmer et al. 2016). These results demonstrate that the E1B-55K protein induced sumoylation is functionally relevant and contributes to the overall abrogation of p53 defenses in the adenoviral infected cell.

Like E1B-55K protein, a second adenoviral protein, the E4orf3 protein, was also shown to affect sumoylation of cellular proteins (Sohn and Hearing 2012). E4orf3 protein induces sumoylation of the host Mre11 and Nbs1 proteins in a process that requires relocalization of these proteins into E4orf3 nuclear tracks and does not require the E1B-55K SUMO ligase activity. The sumoylation of these two proteins is transient and peaks during early infection. Mre11 is modified by SUMO2 while Nbs1 is modified by either SUMO1 or SUMO2, though the functional significance of these paralog differences is not clear. Interestingly, the adenoviral induced sumoylation of these two proteins is only seen with subgroup C adenoviruses. Both the Mre11 and Nbs1 proteins are components of the MRN complex which functions in DNA damage repair and which must be inactivated by many viruses (Hollingworth and Grand 2015), so presumably the sumoylation is helping subgroup C adenoviruses to defeat this defense process. E4orf 3 from type C adenoviruses also induces relocalization and sumoylation of TFII-I, a general transcription factor that can repress the adenoviral intermediate promoter L4P (Bridges et al. 2016). Sumoylation of TFII-I is followed by ubiquitinylation and degradation of TFII-I, leading to derepression of L4P, exemplifying how adenovirus can modulate viral transcription through the host sumoylation system. Furthermore, E4orf3 does not cause an overall increase in host sumoylation (Sohn et al. 2015), so the effect on these three host proteins represents a specific and targeted role for E4orf3 protein. However, these are not the only targets for E4orf3 as a proteomic analysis found that E4orf3 expression influenced the sumoylation of 51 host proteins, most for them showing increased sumoylation (Sohn et al. 2015). E4orf3 protein was subsequently found to specifically sequester the PIAS3 SUMO E3 ligase into its nuclear tracks while not affecting the localization of other PIAS family members, including PIAS1, PIAS2, and PIAS4 (Higginbotham and O'Shea 2015). This result suggests that PIAS3 may be the primary E3 ligase through which E4orf3 protein mediates its effects on sumoylation of host proteins. The ability of E4orf3 protein to target PIAS3 was not restricted to the subclass type C adenoviruses, suggesting that E4orf3-induced effects on sumoylation will have a more general role in the adenoviral life cycle beyond the regulating the MRN and TFII-I proteins.

Another adenovirus protein that influences host sumoylation is the E1A protein (Ledl et al. 2005). E1A is an early protein that regulates viral genome transcription and contributes to cell transformation (Berk 2005). A major target for E1A is the host pRB protein, a regulatory factor that binds to E2FE2F and masks the E2F transcriptional activation of S phase genes (Dyson 2016). In addition, pRB can recruit repressive chromatin remodeling factors to E2F bound promoters to further silence gene expression (Berk 2005). Ledl et al. showed that SUMO is attached to pRB at a single residue, lysine 720, in the B-box motif which interacts with LxCxE-motif proteins such as E1A (Ledl et al. 2005). Mutation

of lysine 720 results in a pRB protein with increased repressive activity on an E2F-responsive promoter, indicating that sumoylation negatively regulates pRB repressive activity. E1A is known to bind pRB leading to the release and activation of E2F. However, binding of E1A to pRB also prevents sumoylation of lysine 720, thereby providing another level of control of pRB by this viral protein (Ledl et al. 2005). Ultimately, E1A seeks to activate cellular S-phase and increase transcription of DNA replication related genes to promote viral genome reproduction, and influencing the sumoylation state of pRB may provide fine regulation of E2F activity. Furthermore, E1A binds to a variety of other cellular proteins (Berk 2005), at least two of which are themselves sumoylated, CtBP (Lin et al. 2003) and p300 (Girdwood et al. 2003). This raises the possibility that E1A might also decrease the sumoylation state of binding partners other than pRB if complex formation also blocks their sumoylation. Additionally, E1A also directly binds the SUMO conjugase, Ubc9 (Hateboer et al. 1996). The binding element in E1A is located within conserved region 2 and is comprised of the sequence EVIDLT (Yousef et al. 2010). This E1A motif interacts with the N-terminal region of Ubc9, a region which also binds SUMO and is involved in polysumoylation (Knipscheer et al. 2007). While E1A binding to Ubc9 did not affect global sumoylation in cultured human cells, this interaction did interfere with polysumoylation in a yeast model, suggesting that subtle alterations in host polysumoylation could be occurring during viral infection (Yousef et al. 2010). For example, Yousef et al. observed that E1A affected PML localization in an Ubc9-dependent manner, and PML is known to be polysumoylated (Tatham et al. 2001), so perhaps E1A is disrupting PML polysumoylation through blocking this activity of Ubc9. Alternatively, since E1A does not block the monosumoylation activity of Ubc9 (Yousef et al. 2010), E1A could be enhancing the sumoylation of specific substrates by redirecting Ubc9 to PML bodies or other E1A targets. Clearly, a great deal of further investigation is required to sort out all the possible ramifications for sumoylation in the adenoviral life cycle.

Lastly, while not a human virus, it is germane to mention the GAM1 protein of the avian type 1 adenovirus known as CELO (Chicken Embryo Lethal Orphan virus) (Chiocca et al. 1996). GAM1 has the most dramatic inhibition of global sumoylation of all known viral proteins, and is well-characterized mechanistically. Moreover, a CELO Gam1 negative mutant is replication-defective, which clearly establishes GAM1 as an important protein for the viral life cycle. Interestingly, this 30 kDa viral protein has no homology to other known anti-apoptotic proteins such as E1B from the adenovirus type 5 or to the Bcl2 and Bax family of eukaryotic proteins. GAM1 is predominantly located in the nucleus, and sumoylation is globally reduced when GAM1 is expressed in a dose dependent manner (Boggio et al. 2004; Colombo et al. 2002). GAM1 was also shown to re-distribute SUMO from the nucleus to the cytoplasm, promoting nuclear transcription factor and HDAC (histone deacetylase) de-sumoylation, thus positively influencing cellular transcription. Mechanistically, it was established that GAM1 binds to the SUMO activation enzyme complex, SAE1/SAE2 (Boggio et al. 2004) and reduces the half-life of SAE1/SAE2 by recruiting a cellular ubiquitin E3 ligase to the GAM1-SAE1/SAE2 complex that ubiquitinylates SAE1 leading to proteasomal degradation (Boggio et al. 2007). The loss of SAE1 destabilizes both SAE2 and Ubc9, resulting in reduction in their levels as well. Even though the de-sumoylation process is unaffected by GAM1, the inability to perform new SUMO modification greatly decrease the entire pool of sumoylated subtracts. The net result of this GAM1 effect is an increase in overall cellular transcriptional activity which facilitates viral replication. While GAM1 may be an extreme example, it is likely that other viruses have evolved related strategies to target the sumoylation enzymes in order to enhance the cellular transcriptional environment to favor their replication needs.

21.2.4 Herpesviruses

The family *Herpesviridae* is a diverse group with three subfamilies (α, β, and γ) that encompasses eight human viruses termed human herpesviruses (HHV) 1-8, though most have common names such as cytomegalovirus and Epstein-Barr virus (Grinde 2013). A characteristic feature of this family is that all members can establish latent infections, either with or without an acute disease presentation, leading to very complex interactions with their respective host cells. Studies over the last 15 years have revealed that sumoylation plays important roles in the life cycle of the herpesvirus family members, both through sumoylation of viral proteins and through viral manipulation of the host sumoylation process. Of the eight human herpesviruses, five have been shown to have one or more sumoylated viral proteins: cytomegalovirus (CMV) (Hofmann et al. 2000; Muller and Dejean 1999; Sinigalia et al. 2012), Epstein-Barr virus (EBV) (Adamson and Kenney 2001; Chang et al. 2004; Rosendorff et al. 2004), Kaposi's sarcoma-associated herpesvirus (KSHV) (Izumiya et al. 2005), varicella-zoster virus (VZV) (Stallings and Silverstein 2006), and human herpesvirus 6 (HHV6) (Gravel et al. 2002; Stanton et al. 2002). These five herpesviruses include representatives of the alpha (VZV), beta (HCMV and HHV6), and gamma (EBV and KSHV) subgroups, further demonstrating that sumoylation of herpesvirus proteins is a frequent and common event for members of this family. For the remaining three human herpesviruses (herpes simplex types 1, herpes simplex type 2, and human herpesvirus 7) sumoylation of viral proteins has not been reported, though a recent proteomics study suggested that several HSV 1 proteins may be modified by SUMO2 (Sloan et al. 2015). Based on these observations, it is likely that there will be sumoylated proteins identified for the remaining human herpesviruses types as well.

As discussed in previous sections, papillomaviruses and adenoviruses target the sumoylation system to alter the cellular milieu and/or avoid host defenses, and this strategy is used by several herpesviruses as well. For example, while HSV has not yet been definitely shown to have sumoylated viral proteins, it does modulate host sumoylation through the viral ICP0 protein (Boutell et al. 2011). A number of other herpesviruses also manipulate the host sumoylation system through viral proteins that have SUMO ligase activity, SUMO targeted ubiquitin ligase (STUbL) activity, or undefined mechanisms. In some cases this reflects a global alteration in host sumoylation while in other cases the effects are confined to specific substrates. Several recent reviews examine the role of sumoylation for herpesviruses (Boutell and Everett 2013; Campbell and Izumiya 2012; Chang et al. 2016), and the following sections will provide a brief overview for the individual family members.

21.2.4.1 Herpes Simplex Virus

The initial observations that viruses could impact host sumoylation were made for an alpha herpes virus, herpes simplex (HSV). The HSV ICP0 immediate early gene product (also known as Vmw110) is not itself sumoylated, but causes loss of high molecular weight isoforms of PML (Everett et al. 1998). These forms were eventually determined to be SUMO-modified versions of PML, a major component of nuclear ND10 bodies (Muller and Dejean 1999). Similarly, ICP0 also decreases the amount of sumoylated Sp100, another major constituent of ND10s (Everett et al. 1998). ND10 disruption by HSV is necessary for effective lytic replication, and this disruption requires ICP0. Originally, this loss of sumoylated PML and Sp100 was believed to cause disruption of the ND10s, but subsequent studies showed that the SUMO protease, SENP1, could elicit similar loss of the sumoylated forms of PML and Sp100 without affecting ND10 structures (Bailey and O'Hare 2002). ICP0 does not inhibit PML sumoylation nor does it cause desumoylation of PML *in vitro* (Boutell et al. 2003), though it does recruit SENP1, at least under conditions of transient co-expression, which may contribute to desumoylation of PML components (Bailey and O'Hare 2002). In subsequent studies it became clear that the major effect of ICP0 on ND10 bodies appears to be due to its known ubiquitin ligase activity (Boutell et al. 2002).

ND10 bodies are part of an intrinsic anti-viral defense that blocks HSV infection effectively in the absence of ICP0 (Everett et al. 2006, 2008). Several of the components of ND10 bodies, including PML, Sp100, and Daxx contain SUMO interacting motifs (SIMs) that are critical for recruitment of these proteins to the HSV genome (Cuchet-Lourenco et al. 2011). ICP0 also has SIM motifs that are required for its ability to overcome the anti-viral function of ND10s (Boutell et al. 2011). These SIM motifs help direct ICP0 to sumoylated proteins where it causes their ubiquitinylation and proteasomal degradation. While ICP0 causes a general decrease in cellular sumoylated proteins, sumoylated PML is preferentially degraded (Sloan et al. 2015). One putative SIM in the central region of ICP0 was absolutely required for this general effect on sumoylated host proteins and for PML isoforms other than type 1, while several putative SIMs in the C-terminal region also contributed (Everett et al. 2014). Interestingly, ICP0 can also interact directly with PML type 1 and target it for degradation in a SUMO-independent fashion, suggesting that this isoform may be particularly important for restricting HSV replication (Cuchet-Lourenco et al. 2012). Overall, it appears that ICP0 acts as a SUMO-targeted ubiquitin ligase (STUbL) that is highly active against PML and other components of ND10 bodies, leading to their degradation, and thus overcoming this host defense mechanism sufficiently to allow HSV productive infections (Lanfranca et al. 2014). Since a proteomic analysis of SUMO 2 identified 124 other host proteins whose sumoylation was significantly reduced by ICP0 (Sloan et al. 2015), it will also be of interest to evaluate the biological role of these other proteins in HSV replication.

Further evidence for the importance of sumoylation in the HSV life cycle comes from the recent observations that the PIAS SUMO ligases contribute to the intrinsic anti-viral response to this virus. PIAS1 is localized to ND10 bodies in a SIM-dependent fashion (Brown et al. 2016), likely through interaction with sumoylated PML. PIAS1 causes SUMO1 accumulation at sites of HSV nuclear entry, and helps inhibit HSV replication by a mechanism that is additive to PML. Like PML, the restrictive effect of PIAS1 is overcome by ICP0. Similarly, PIAS4 also associates with HSV nuclear entry sites in a SIM-dependent process and cooperates with PML to restrict HSV (Conn et al. 2016). ICP0 can reduce this accumulation and overcome the restriction imposed by PIAS4. These combined results strongly support an anti-herpes simplex role for PIAS1 and PIAS 4 that is functionally reduced by ICP0.

21.2.4.2 Varicella-Zoster Virus

The second human alpha herpesvirus is varicella-zoster virus, the etiological agent of chickenpox and the reactivation disease known as shingles (Gilden et al. 2015). Sumoylation in this member of the alpha subgroup has not been extensively studied, and there are only two reports concerning sumoylation and this virus. It was reported that one of the viral early proteins, ORF29p, is sumoylated, but the role of sumoylation in the function of this protein was not investigated (Stallings and Silverstein 2006). A second viral protein, ORF61, contains three SIMs that facilitate binding to SUMO1 (Wang et al. 2011). These SIMs are required for ORF61 to interact with and disperse PML in ND10 bodies. During skin infection, the number of ND10 bodies is reduced, but this is not observed in ORF61 SIM mutants. Also, these mutants did not demonstrate typical skin lesions and had reduced viral spread. There results suggest that the SUMO binding function of ORF61 is critical for overcoming the anti-viral effects of the PML bodies, similar to ICP0 of HSV. ORF61 is homologous to ICP0 (Moriuchi et al. 1992) and has ubiquitin ligase (Everett et al. 2010), but whether or not ORF61 has STUbL activity has not been determined.

21.2.4.3 Cytomegalovirus

The family of beta herpesviruses includes three human virus members, cytomegalovirus (CMV), human herpes virus 6 (HHV6), and human herpes virus 7 (HHV7). There is not yet a report on sumoylation in HHV7, but sumoylation plays a role in the life cycle of both HHV6 and CMV. The immediate early IE1 protein is sumoylated for

both CMV (Muller and Dejean 1999) and HHV6 (Gravel et al. 2002). Additionally, IE2-p86 (Hofmann et al. 2000), the UL44 protein (Sinigalia et al. 2012), and the pp71 tegument protein (Hwang and Kalejta 2009) for CMV are sumoylated. Multiple groups identified lysine 450 as the SUMO acceptor site in IE1 (Lee et al. 2004; Nevels et al. 2004; Sadanari et al. 2005; Spengler et al. 2002; Xu et al. 2001), but the role of SUMO modification in IE1 function remains poorly understood. Like HSV ICP0, the CMV IE1 disrupts ND10s and reduces the level of sumoylated forms of PML and Sp100 (Everett et al. 2013a; Muller and Dejean 1999; Tavalai et al. 2011), but unlike ICP0 this disruption is proteasome independent (Lee et al. 2004; Xu et al. 2001). IE1 also does not cause any global decrease in sumoylated proteins so it does not appear to be a STUbL like ICP0 (Scherer et al. 2013). Furthermore, IE1 mutants with the SUMO acceptor site lysine changed are still capable of disrupting PML ND10 bodies and overcoming the anti-viral activity of these elements, indicating that sumoylation of IE1 itself is not involved in this process (Lee et al. 2004; Spengler et al. 2002; Xu et al. 2001). IE1 also does not have an intrinsic SUMO protease activity, and it has been suggested that the desumoylation of PML seen with IE1 expression may be due to disruption of PML aggregation (Kang et al. 2006).

Since sumoylation of IE1 is not related to its function in disrupting ND10 bodies, the actual role of this modification remains vague, though a K450R mutant virus grows more slowly and with reduced yield, suggesting that sumoylation is important for a fully robust virus (Nevels et al. 2004). Conflicting reports exist for a role for sumoylation in IE1 intracellular localization with Nevels et al. finding no sumoylation requirement for proper localization of IE1 (Nevels et al. 2004), while a second report indicated that sumoylated and unsumoylated IE1 exhibited different cellular fraction properties which could reflect different intracellular locations (Sadanari et al. 2005). In other studies, IE1 mutants with the SUMO acceptor lysine altered were unaffected for protein stability and transactivation activity on several promoters, so sumoylation is unlikely to contribute generally to those functions (Spengler et al. 2002). However, while transactivation at several promoters is unaffected by the SUMO site mutation, the mutant exhibited decreased IE2 transcripts and protein expression, so specific regulation of IE1's activity on the IE2 promoter may be a critical function of SUMO modification (Nevels et al. 2004).

To date, the only other reported effect for IE1 sumoylation is on its interaction with the host STAT2 protein (Huh et al. 2008). STAT2 is anti-viral in that it induces interferon-stimulated genes, but this function is antagonized by unmodified IE1 which reduces STAT2 binding to target promoters. Sumoylation of IE1 prevents its binding to STAT2, suggesting that sumoylation is actually a negative regulator of IE1 activity in this pathway. Thus, sumoylation of IE1 appears to have both a positive role in IE2 expression and a negative role in preventing IE1 inhibition of the anti-viral STAT2 pathway.

In contrast to IE1, sumoylation of IE2-p86 protein has more clearly defined functional effects. IE2-p86 is a transactivator of both viral and cellular promoters, and it plays a role in both lytic and latent infections. There are two sumoylation sites in IE2-p86, at lysines 175 and 180, and the protein is effectively modified by either SUMO1 or SUMO2 (Ahn et al. 2001; Hofmann et al. 2000). While targeting of IE2-p86 to ND10 bodies was not dependent upon sumoylation, mutational inactivation strongly decreased the ability of IE2-p86 to activate viral early promoters (Hofmann et al. 2000). Conversely, over-expression of SUMO1 increased the transactivation ability of IE2-p86 (Ahn et al. 2001), so the combined results are consistent with sumoylation of IE2-p86 being a positive regulator of transactivation. In the context of viral infections, this effect of sumoylation on IE2-p86 appears to be biologically important. While it was initially reported that mutation of the IE2-p86 sumoylation sites had no effect on growth of the CMV Towne strain (Lee and Ahn 2004), subsequent studies with other CMV strains, including a clinical isolate, showed a major impact on viral growth for the sumoylation minus IE2-p86 protein (Berndt et al. 2009; Kim

et al. 2010). Viruses with sumoylation defective IE2-p86 had reduced levels of immediate-early gene products and much lower levels of viral DNA replication (Berndt et al. 2009), consistent with IE2-p86 sumoylation being critical for the normal viral replicative cycle, except perhaps in the Towne strain.

In addition to being sumoylated, IE2-p86 non-covalently binds SUMO (Ahn et al. 2001; Hofmann et al. 2000), leading to the identification of a SIM motif at amino acid 200 (Berndt et al. 2009). SIM minus mutants showed reduced sumoylation of IE2-p86 leading to replication impairment similar to SUMO site mutants (Berndt et al. 2009; Kim et al. 2010). Additionally, SIM mutants showed reductions in IE2 association with viral promoters, formation of viral transcription domains, and late protein expression (Kim et al. 2010). The SIM motif in IE2-p86 was also necessary for interaction with the host sumoylated form of the TATA-binding protein (TBP), an interaction which enhanced IE2's transactivation ability (Kim et al. 2010). Given that many other transcription associated factors are known to be sumoylated, the potential for IE2-p86 to recruit numerous other host proteins through SIM-SUMO interaction is intriguing. Lastly, a recent study found that IE2 inhibited IE1 sumoylation (Kim et al. 2014). This negative regulation was found to be mediated through PIAS1 binding by IE2. PIAS1 stimulates sumoylation of IE1, so binding of PIAS1 by IE2 may sequester it and prevent PIAS1-mediated sumoylation of IE1. Consistent with this model, levels of IE1 sumoylation peaked early in infection and decreased in late phase where IE2 levels were highest. Thus, a complex interplay between IE1, IE2, and the SUMO system appears to contribute to the fine regulation of viral protein activities.

The third CMV viral protein known to be sumoylated is the UL44 protein (Sinigalia et al. 2012). UL44 is a subunit of the viral DNA polymerase that confers processivity through binding to the viral DNA template. UL44 both binds Ubc9 and is extensively sumoylated with at least 16 SUMO attachment sites identified by mass spectrometry, leading to modification of approximately 50% of the protein late in infection. Sumoylation with either SUMO1 or SUMO2/3 enhanced DNA binding by UL44, but SUMO over-expression during infection led to a decrease in UL44 association with viral replication centers, suggesting that sumoylation may actually be a negative regulator of UL44 function for viral replication. Paradoxically, over-expression of sumoylation increased levels of viral DNA and resulted in two to three fold higher virus titers, though this effect could be due to sumoylation of the other viral or host proteins, so the biological consequences of UL44 sumoylation remain unclear.

The final CMV protein known to be involved in sumoylation is the pp71 tegument protein (Hwang and Kalejta 2009). Tegument proteins are components of the virion and are delivered to the host cell upon uptake of the virion. Pp71 acts to overcome the repressive effects of the Daxx host protein on viral early promoters by mediating degradation of Daxx. Hwang and Kalejta showed that expression of pp71 causes an increase in the sumoylation of cellular Daxx protein (Hwang and Kalejta 2009). This stimulation of Daxx sumoylation required direct interaction between pp71 and Daxx, but was not required for pp71-induced degradation of Daxx. Furthermore, a functional effect of this pp71-induced sumoylation of Daxx on viral immediate early promoters was not observed, so like UL44 the significance of this pp71 effect is not yet defined.

21.2.4.4 Human Herpesvirus 6

Like CMV, human herpesvirus 6 (HHV6) is a betaherpesviruses and expresses an IE1 protein, though the CMV IE1 and the HHV6 IE1 lack significant identity at the protein level (Gravel et al. 2002). Even without much relatedness to CMV IE1, the HHV6 IE1 still possess a transcriptional activating function and is sumoylated (Gravel et al. 2002; Stanton et al. 2002). There is a single SUMO attachment site in IE1 at lysine 802, and SUMOs 1–3 could each be conjugated (Gravel et al. 2004). Polysumoylation at this site was also observed.

Like CMV IE1, HHV6 IE1localizes to ND10 bodies (Gravel et al. 2002; Stanton et al. 2002).

Also like CMV IE1, sumoylation of HHV6 IE1 is not required for localization to ND10s (Gravel et al. 2004). Surprisingly, HHV6 IE1 does not cause disruption of ND10s when transiently expressed alone. Even during viral infection, ND10s did not disperse and instead condensed into a smaller number of larger bodies (Gravel et al. 2002). Furthermore, it has not yet been reported that HHV6 IE1 affects the sumoylation of PML or any other cellular proteins. These results indicate that HHV6 does impact ND10 structure, though whether or not is functionally inactivates ND10s is unknown. It was shown that over-expression of SUMO increases IE1 levels (Gravel et al. 2004; Stanton et al. 2002), however, this does not require an intact sumoylation site (Gravel et al. 2004) and is apparently an indirect effect whose mechanism in unknown.

There was also no indication that sumoylation affected the transactivation ability of HHV6 IE1, so the functional consequences of IE1 sumoylation, if any, remain to be discovered.

HHV6 also expresses an IE2 immediate early protein that is a transcriptional transactivator. Unlike the CMV IE2 protein, no sumoylation of HHV6 IE2 has been detected (Tomoiu et al. 2006). Interestingly though HHV6 IE2 does bind to Ubc9 and this binding represses transactivation activity. The repression did not require catalytically active Ubc9 so appears not to involve sumoylation, and both the mechanism and functional consequences for viral infection are uncharacterized.

21.2.4.5 Epstein-Barr Virus

The third herpesvirus subgroup, gamma, contains two human virus members, Epstein-Barr virus (EBV) and Kaposi's sarcoma herpes virus (KSHV).). EBV is the etiological agent of infectious mononucleosis and is also associated with several diverse cancers (Hislop 2015). Sumoylation plays an important role in the life cycle of this virus with four viral proteins known to be sumoylated (BZLF1, Rta, EBNA3B, and EBNA3C), three viral proteins that modulate sumoylation (LF2, LMP, and BGLF4), several other viral proteins that have putative SIM motifs (Li et al. 2012a), and one report suggesting that viral microRNAs may modulate the sumoylation system (Callegari et al. 2014). The first reported EBV protein identified as a SUMO substrate is the BZLF1 protein (also known as Z protein or ZTA), one of the EBV immediate early gene products (Adamson and Kenney 2001). Two groups demonstrated that BZLF1 has a single SUMO acceptor lysine at position 12, and that BZLF1 can be modified by SUMOs 1-3 (Hagemeier et al. 2010; Murata et al. 2010). Sumoylation of BZLF1 can be regulated by the viral protein kinase (EBV-PK, also known as BGLF4) whose expression decreases sumoylation of BZLF1 (Hagemeier et al. 2010). This effect on sumoylation of BZLF1 does not require phosphorylation of BZLF1 itself so the actual mechanism is unknown. Functionally, the consensus is that sumoylation represses that transactivation ability of BZLF1 (Adamson 2005; Hagemeier et al. 2010; Murata et al. 2010) as mutation of the SUMO acceptor site increases transactivation activity as does desumoylation of BZLF1 with exogenously expressed SENP. At least part of the repressive effect appears to be due to preferential interaction of cellular histone deacetylase 3 (HDAC3) with the sumoylated form of BZLF1 (Murata et al. 2010). Similarly, the cellular scaffolding protein, RanBPM, was shown to enhance BZLF1 transactivation by binding BZLF1 and inhibiting its sumoylation (Yang et al. 2015b). Consistent with the repressive effect of sumoylation on BZLF1, a nonsumoylatable lysine 12 to alanine mutation exhibited two-fold increased viral production (Hagemeier et al. 2010).

In contrast to the repression of transactivation activity, sumoylation of BZLF1 has no effect on its intracellular localization or stability (Adamson 2005; Hagemeier et al. 2010), though it does contribute to ND10 body disruption by EBV (Adamson and Kenney 2001; Hagemeier et al. 2010). BZLF1 protein expressed alone is sufficient to disrupt ND10 bodies, in part because it reduces the sumoylation of PML in ND10 bodies apparently by competing for limited SUMO1 (Adamson and Kenney 2001). However, a SUMO site mutation in BZLF1 still shows partial ability to disrupt ND10 bodies, so reduction of PML sumoylation by competition cannot be the only

mechanism by which BZLF1 causes ND10 dispersion (Hagemeier et al. 2010). Whether or not BZLF1 affects the sumoylation of other proteins has not been examined, but would be predicted based on the proposed competition mechanism.

A second immediate early protein for EBV is the BRLF1 protein which is also known as Rta. Like BZLF1, Rta is sumoylated, though for this protein there are three sumoylation sites at lysines 19, 213, and 517 (Chang et al. 2004). Members of the PIAS family act as SUMO ligases for Rta as PIAS1 and PIASx (alpha and beta) interact with Rta and stimulate its sumoylation (Liu et al. 2006). In contrast to BZLF1, it was initially reported that the transactivation activity of Rta is enhanced by sumoylation (Chang et al. 2004; Liu et al. 2006). However, a later study demonstrated that sumoylation was not needed for transactivation (Calderwood et al. 2008), so a functional role for Rta sumoylation in transactivation remains questionable. Interestingly, there is additional complex regulation of Rta function, some of which involves sumoylation. Two cellular proteins, RanBPM and RNF4, and one viral protein, LF2, have been shown to influence Rta and affect sumoylation. RanBPM, the nucleocytoplasmic scaffolding protein that inhibits BZLF1 sumoylation has the opposite effect on Rta as RanBPM binds Rta and enhances its sumoylation leading to increased activity on several promoters (Chang et al. 2008). However, sumoylation of Rta promotes its association with a cellular STUbL known as RNF4 through the SIM motifs in RNF4 (Yang et al. 2013). Thus, Rta sumoylation may enhances its activity, but it also leads to its proteasomal degradation, perhaps providing a check and balance possibly to prevent excessive Rta activity. Rta activity is also negatively regulated by the viral LF2 protein (Calderwood et al. 2008). LF2 expression induces Rta modification by SUMO2 and SUMO3 at four additional residues, lysines 426, 446, 517, and 530 (Heilmann et al. 2010). Paradoxically, these SUMO modifications are not required for the negative regulation by LF2 which is due to LF2 binding and relocalization of Rta to the extranuclear cytoskeleton. Consequently, neither endogenous sumoylation nor LF2-induced sumoylation events have a clearly defined effect on Rta and understanding the biological role of SUMO modification for this protein awaits further investigation.

The last known sumoylated EBV proteins are the related EBNA3B and 3C proteins (Rosendorff et al. 2004). EBNA3B and 3C, along with a third related gene product, EBNA3A are transcriptional regulatory factors with both overlapping and distinct functions (Robertson et al. 1996). Both EBNA3B and 3C are sumoylated with sumoylation being much more prominent for EBNA3C; EBNA3A was not seen to be sumoylated (Rosendorff et al. 2004). Mutants of EBNA3C that could not be sumoylated were more diffusely distributed rather than accumulating in nuclear dots. Nonetheless, these mutants were still wild-type in their ability to transactivate the LMP1 promoter in conjunction with EBNA2, so this function does not appear to require sumoylation. Instead of covalent modification by SUMO, one or more SIM-like motifs in EBNA3C are important for LMP1 promoter activation (Lin et al. 2002; Rosendorff et al. 2004). EBNA3C interacts with p300/CBP at the LMP1 promoter, but a p300 mutant that was not sumoylated could still be co-activated by EBNA3C, so p300 is not the target for the EBNA3C SIM motif(s) (Rosendorff et al. 2004). This led Rosendorff et al. to propose that binding of EBNA3C to an unidentified sumoylated repressor counteracts that repressive effect to allow LMP1 promoter activation. The potential role of EBNA3B sumoylation was not investigated.

As mentioned above, the EBV protein kinase (BGLF4) is able to inhibit sumoylation of the BZLF1 protein (Hagemeier et al. 2010). BGLF4 was subsequently shown to have two SIM motifs, one in the N-terminal region and one in the C-terminal region adjacent to a nuclear export sequence (Li et al. 2012a). Both SIM motifs contribute to nuclear accumulation of BGLF4 by blocking nuclear export. SUMO binding by BGLF4 was also required for its ability to inhibit sumoylation of BZLF1, as was the kinase activity of BGLF4. Since it was previously shown that phosphorylation of BZLF1 itself by BGLF4 is

not required for inhibition of sumoylation (Hagemeier et al. 2010), the requisite target for the kinase activity in this process is not known. Interestingly, BGLF4 could also globally reduced cellular sumoylation in a SIM- and kinase-dependent fashion, though what, if any, biological role this has in the viral life cycle was not tested (Li et al. 2012a). Lastly, BGLF4 also contributes to several other viral functions, including ND10 dispersion, DNA damage response induction, and production of extracellular virus, and all these activities required the intact SIMs. Thus, while targets and mechanistic pathways for all of these BGLF4-related functions are relatively undefined, it is likely that the all involve targeting of BGLF4 through interaction with SUMO moieties conjugated to various host and/or viral proteins.

Lastly, a very important effect on sumoylation has been elucidated for latent membrane protein 1 (LMP1). LMP1 is the primary viral oncoprotein and it plays a key role in maintaining the latent state (Li and Chang 2003). While not itself sumoylated, LMP1 interacts with Ubc9 through sequences in its C-terminal activating region 3 (CTAR3) and causes a general increase in host protein sumoylation (Bentz et al. 2011). Subsequently, two specific cellular targets of LMP1-mediated sumoylation were identified, IRF7 (Bentz et al. 2012) and KAP1 (Bentz et al. 2015). IRF7 is an interferon regulatory factor involved in innate immunity. LMP1 promotes sumoylation of IRF7 at lysine 452, resulting in decreased IRF7 function which likely helps abrogate the innate response during EBV latency (Bentz et al. 2012). KAP1 (KRAB-associated protein 1) also is believed to have antiviral activity, likely through its transcriptional repressive activity (Iyengar and Farnham 2011). KAP1 was shown to bind to EBV early promoters and the lytic origin of replication (OriLyt) in a sumoylation-dependent fashion (Bentz et al. 2015). These results suggested that LMP1-mediated sumoylation of KAP1 helps to repress the lytic phase of EBV and promote maintenance of the latent state. It will be of interest to see if there are other targets of LMP1-mediated sumoylation that also contribute to the viral life cycle.

21.2.4.6 Kaposi's Sarcoma-Associated Herpes Virus

Like the other human virus member of the gamma herpesvirus subgroup, sumoylation is also highly important of Kaposi's sarcoma-associated herpes virus (KSHV) (Campbell and Izumiya 2012; Chang and Kung 2014). Four KSHV viral proteins, K-bZIP, K-Rta, LANA1 (ORF73), and LANA2 (vIRF3) modulate sumoylation of host and/or viral proteins. K-bZIP was the first KSHV protein identified as a SUMO substrate with modification occurring at lysine 158 (Izumiya et al. 2005). Sumoylation of K-bZIP is negatively regulated by phosphorylation at threonine 111 catalyzed by the viral ORF36 protein kinase (Izumiya et al. 2007). However, it was later reported that K-bZIP splice variants exist, and that 2 of the variants lack the lysine 158 and are sumoylated on newly generated lysine, residue 207 (Lefort et al. 2010). What, if any, different properties the variants have has not been extensively investigated.

Functionally, K-bZIP is a transcriptional repressor, and sumoylation of K-bZIP enhances its repressive activity (Izumiya et al. 2005). Additionally, K-bZIP binds Ubc9 and recruits it to viral promoters where it likely sumoylates and inactivates other transcription factors to facilitate repression. One pathway that has been characterized for K-bZIP is interferon-stimulated gene expression where K-bZIP represses target genes (Lefort et al. 2010). A SUMO site mutant of K-bZIP is impaired for the ability to repress these target genes, indicating that sumoylation is a positive regulator for this activity of K-bZIP. Subsequently it was shown that K-bZIP is a viral SUMO E3 ligase that can catalyze its own sumoylation (Chang et al. 2010). K-bZIP contains a SIM motif the preferentially binds SUMO2/3 and not SUMO1, and its ligase function also utilizes primarily SUMO2/3. Using this ligase activity K-bZIP can sumoylate known binding partners such as p53 and pRB, and thus regulate their activities. More globally, during KSHV reactivation there is an increase of SUMO2/3 association with promoter regions of both cellular (Chang et al. 2013) and viral genomes (Yang et al. 2015a). For the cellular

genome, the viral factor(s) responsible were not examined, but K-bZIP would be a likely candidate. Consistent with this speculation, K-bZIP was required for the increased SUMO2/3 association with viral DNA as a mutant lacking the SUMO ligase activity showed no increase (Yang et al. 2015a). Importantly, the SUMO ligase defective mutant of K-bZIP showed increased virus production during infection, and the same results were seen with a SUMO2/3 knockdown. These results indicated that K-bZIP induced sumoylation is repressing viral activation and may function to help maintain the latent state. Similarly, enhanced SUMO2/3 modification at cellular promoters is likely to help prevent viral reactivation and/or to repress cellular innate immune responses to the virus.

While K-bZIP has SUMO E3 ligase activity that enhances sumoylation of viral and cellular proteins, the K-Rta protein is a STUbL that targets sumoylated proteins for proteasomal degradation (Izumiya et al. 2013). K-Rta has multiple SIM motifs that provide strong binding to multimerized SUMO, with a preference for SUMO2/3. PML is one of the specific targets for K-Rta leading to degradation of PML and contributing to the dispersal of the ND10 bodies by this virus. K-Rta can also target sumoylated K-bZIP for degradation, suggesting a mechanism for counteracting the repressive function of K-bZIP. Mutations in the SIM motifs of K-Rta prevent PML degradation, decrease transcriptional activation by K-Rta, and reduce viral replication, which is strongly consistent with the STUbL activity being important for all these functions. Further characterization of the opposing functions of K-bZIP and K-Rta will likely provide greater insight into the regulatory balance between viral reproduction and latency.

KSHV has two latency associated nuclear antigens, LANA1 and LANA2, that both contribute to modulation of substrate sumoylation status. LANA1 contains a SIM motif that is specific for SUMO2 and which facilitates the sumoylation of LANA1 at lysine 1140 (Cai et al. 2013). Through this SIM element, LANA1 recruits poly-sumoylated host KAP1 protein which is part of a transcriptional repressive complex that also includes Sin3A. Deletion of the SIM motif prevents association of LANA1 with this complex and results in loss of viral episomal genome maintenance and loss of repression of K-Rta expression, implicating this SIM as a critical part of the mechanism by which LANA1 helps to establish and maintain latency. LANA1 has been shown to enhance sumoylation of Sp100 which promotes its accumulation into nuclear aggregates which are likely ND10 bodies, and it was proposed the ND10-induced restrictions may be necessary for establishment of viral latency (Gunther et al. 2014). Similarly, LANA1 enhances sumoylation of histones in a SIM-dependent which is correlated with repression of viral genes and again may contribute to establishment of latency (Campbell and Izumiya 2012). Interestingly, a broader proteomic study identified 151 host proteins that interacted with the LANA1 SIM (Gan et al. 2015). These results suggest that LANA1 could be modifying and regulating a large and complex protein network to facilitate its latency function for KSHV.

LANA2 is another latency associated protein that modulates the sumoylation state of partner proteins. LANA2 increases sumoylation of PML which ultimately leads to disruption of ND10 bodies due to proteolytic degradation (Marcos-Villar et al. 2009, 2011). Both an intact SIM motif in LANA2 and sumoylation of LANA2 at multiple sites were important for the ND10 dispersion. In contrast to the enhancement of PML sumoylation, LANA2 binding to the pocket proteins (pRB, p107, and p130) (Marcos-Villar et al. 2014) or p53 (Laura et al. 2015) inhibits their sumoylation. For the pocket protein, this effect requires binding via an LxCxE motif in LANA2 that is typical of proteins that interact with pRB and the other pocket proteins (Marcos-Villar et al. 2014). For p53, inhibition of sumoylation requires both the LANA2 SIM motif and sumoylation of LANA2 (Laura et al. 2015). The ability of LANA2 to target and influence the sumoylation of two key cellular regulatory proteins, pRB and p53, strongly implies that modulating these pathways is critical in the viral life cycle.

21.2.5 Poxviruses

Poxviruses are complicated pathogens with complex virions, large genomes, and cytoplasmic genome replication, which is distinct from the typical nuclear location for genome replication other DNA viruses (Lefkowitz et al. 2006). The primary human pathogen was variola, the agent of smallpox, though most studies in recent decades have been conducted on the vaccine strain known as vaccinia virus (Roberts and Smith 2008; Voigt et al. 2016). To date, only two the vaccinia virus proteins, A40R (Palacios et al. 2005) and E3 (Gonzalez-Santamaria et al. 2011) are known to be targets for sumoylation. E3 was originally shown to interact with SUMO1 in a yeast two hybrid assay (Rogan and Heaphy 2000). A subsequent study confirmed the interaction with SUMO and demonstrated that a SUMO interacting motif (SIM) was required for this association (Gonzalez-Santamaria et al. 2011). Interestingly, the SIM motif in E3 was required for protein stability, nuclear localization, and efficient sumoylation of E3. With the SIM motif intact, E3 could be modified by either SUMO1 or SUMO2, with SUMO addition sites mapped to lysines 40 and 99. Sumoylation of E3 occurred both in the context of viral infection and with transfected E3 expression, indicating that no viral components were required for E3 modification. Functionally, the SUMO modification repressed the transcriptional activation activity of E3 on two tested target genes, which suggests that this post-translational modification is likely to be biologically relevant to the viral life cycle.

The other known sumoylated vaccinia protein is the A40R gene product which is modified by SUMO as a single site, lysine 95 (Palacios et al. 2005). Like E3, modification of A40R does not require any viral products and is intrinsic to A40R in the presence of the sumoylation system. A unique feature of A40R is that its sumoylation is nearly quantitative, in contrast to most proteins for which only a small fraction exists in the sumoylated form at any time. The need for extensive sumoylation is likely explained by the observation that A40R is insoluble unless sumoylated. A40R lacking the SUMO acceptor site self-aggregates in the cytoplasm forming rod-shaped bodies rather than localizing to the viral mini-nuclei where viral DNA replication occurs. Nonetheless, because nonsumoylated A40R associates with the cytosolic side of the endoplasmic reticulum (ER), the authors speculated that during viral infection a small fraction of non-sumoylated A40R may function to bring ER membranes together and assist with membrane fusion around the viral replication foci.

21.3 RNA Viruses

Sumoylation was originally thought to be an exclusively nuclear event, so its role in virology was initially examined only for viruses with a nuclear phase (most DNA viruses and a few RNA viruses). As appreciation grew that sumoylation also occurs in the cytoplasm and various membrane compartments, greater attention was placed on searching for contributions of this post-translational modification system to human RNA virus biology. There are now numerous examples of sumoylated proteins from RNA viruses, both positive and negative stranded, as well as examples of RNA virus proteins that influence the sumoylation of other viral and/or host proteins. Two RNA viruses with prominent nuclear aspects, influenza and retroviruses, are the most intensively investigated for sumoylation, while for most other RNA viruses there are currently only limited reports concerning sumoylation. Consequently, detailed mechanistic and functional characterization of the ability of RNA viruses to use and/or manipulate the sumoylation system is still lacking for most RNA viruses. This section will review the current knowledge about the burgeoning list of RNA viruses with a sumoylation connection.

21.3.1 Retrovirus

Sumoylation has a role in the life cycle of two human retroviruses of the lentivirus subgroup, HIV and HTLV. Two HIV proteins, GAG (Jaber et al. 2009) and integrase (Zamborlini et al. 2011),

interact with the sumoylation system. One of the GAG proteolytic products, p6, is sumoylated and this seems to be a negative regulatory event early in infection, perhaps as a host defense (Gurer et al. 2005). Alternatively, later in infection the full-length GAG binds Ubc9, and reduction of Ubc9 by RNAi resulted in decreased incorporation of the ENV glycoprotein into released virions (Jaber et al. 2009). While not affecting total number of virions produced, the resulting virions were eight- to ten-fold less infectious. This effect did not require catalytically active Ubc9 so is unlikely to involve sumoylation of GAG. In contrast, integrase is sumoylated, but the functional consequence is unresolved as two groups have reported different effects. Zamborlini et al. reported that integrase has three SUMO consensus motifs and is multiply sumoylated (Zamborlini et al. 2011). They found that a triple mutant at the three consensus sites had normal stability and intracellular localization, but was replication defective at a step after reverse transcription but before integration, suggesting that sumoylation was necessary for this undefined step. However, Li et al. subsequently reported that overexpression of sumoylation caused integrase to accumulated in nuclear punctate bodies rather than be diffuse (Li et al. 2012b). They also observed that the degree of viral genome integration was inversely correlated with the levels of cellular sumoylation, leading to their suggestion that sumoylation is antiviral for HIV. While their studies did not directly confirm that these effects of sumoylation were mediated through integrase, it does raise questions about what sumoylation actually does for integrase activity. Interestingly, a recent report on a SENP inhibitor supports the antiviral nature of integrase sumoylation (Madu et al. 2015). Treatment of HIV infected cells with this inhibitor reduces the infectivity of progeny virions as the virions had defective integration during subsequent infection. The inhibitor effect could be abrogated by mutation of the SUMO acceptor sites in integrase, implying that sumoylation of integrase is a negative regulator and that the only active integrase molecules are ones that have been desumoylated by SENPs prior to virion packaging. These are clearly intriguing results that will need further study to clarify the precise mechanisms.

A second human lentivirus, human T-lymphotropic virus (HTLV) has one known sumoylated protein, Tax. Tax is a key regulator of the NF-κB pathway and is known to be modified by both ubiquitin and acetyl groups. Tax has also been reported as sumoylated for both HTLV1 (Lamsoul et al. 2005) and HTLV2 (Turci et al. 2009). Several groups have shown that Tax sumoylation is involved with its ability to accumulate in nuclear bodies (Kfoury et al. 2011; Lamsoul et al. 2005; Nasr et al. 2006). Sumoylation of Tax also appears to regulate its targeting of NEMO, a Tax partner, to centrosomes (Kfoury et al. 2011), and to be required for binding of the STUbL, RNF4,, RNF4, which ubiquitinylates Tax to relocalize it from the nucleus to the cytoplasm (Fryrear et al. 2012). Thus, it appears that regulation of Tax intracellular localization is a complex process reflecting the contributions of several post-translation modification events. Unfortunately, the functional consequences of this complex intracellular regulation are still uncertain as initial reports that sumoylation is required for NF-κB activation (Lamsoul et al. 2005; Nasr et al. 2006) have been refuted in later studies (Bonnet et al. 2012; Pene et al. 2014). So as for HIV integrase, it is likely the sumoylation of Tax is important, but our understanding of the relevant mechanism is poor. A similar situation exists for Tax2 from the related HTLV-2B where Tax2 is sumoylated but the functional role of sumoylation is not clear (Journo et al. 2013; Turci et al. 2009, 2012).

21.3.2 Orthomyxovirus

A second RNA virus with significant impact by the sumoylation system is the orthomyxovirus, influenza A virus (IAV). Five IAV proteins (NS1, PB1, NP, M1, and NS2) are modified by sumoylation (Pal et al. 2011), and at least some functional characterization has been performed to evaluate the role of sumoylation on four of the

five targets. The first identified IAV SUMO target was the NS1 protein (Pal et al. 2010). Two groups showed that sumoylation of NS1 was important for viral multiplication and the loss of sumoylation resulted in delayed growth (Santos et al. 2013; Xu et al. 2011). Both groups agreed that lysine 221 is a major SUMO acceptor site, but Santos et al. also found that lysine 70 was a SUMO site (Santos et al. 2013). The groups differed in that Xu et al. found that sumoylation stabilized NS1 (Xu et al. 2011) while Santos et al. found no effect on NS1 stability though they did report an effect on NS1 multimerization (Santos et al. 2013). The difference in the stability results may reflect that Santos et al. examined a double mutant of NS1 with both SUMO sites altered while Xu et al. only eliminated sumoylation at the major SUMO site. Lastly, Santos et al. also reported that sumoylated NS1 was greatly reduced in its ability to prevent interferon production during infection, and this might account for the delay replication (Santos et al. 2013).

For the remaining four sumoylated IAV proteins, there is modest information available about the role of sumoylation in their function, though three of the four are interrelated in their functions so sumoylation may have coordinating effects. The M1 protein is sumoylated at lysine 242, and this modification is required for effective interaction between M1 and the viral ribonucleoprotein (NP) (Wu et al. 2011). Without sumoylation of M1 and formation of this complex, viral maturation and assembly is greatly reduced which produces a strong reduction in viral yield. As cited above, NP is itself sumoylated with acceptor sites mapping to lysines 4 and 7; the lysine residue 7 is highly conserved and is critical for viral reproduction, so it may be the primary SUMO site (Han et al. 2014). The sumoylation deficient mutant of NP exhibits altered intracellular localization which may prevent effective interaction with M1 and contribute to its functional defect in viral replication. Further regulation of this process is mediated by the viral NS2 protein (Gao et al. 2015). NS2 interacts with the cellular AIMP2 protein, and the NS2-AIMP2 complex switches the modification of M1 lysine 242 from ubiquitin to SUMO which would facilitate the M1-NP interaction. Whether or not this requires the reported sumoylation of NS2 itself is unknown.

It is also of interest to note the influenza infection leads to a general dysregulation of cellular sumoylation (Pal et al. 2011). A detailed proteomic study found that influenza infection specifically targeted 63 host proteins for sumoylation while generally promoting global desumoylation (Domingues et al. 2015). The pattern of changes was distinct from other stress responses and involved at least ten potential antiviral factors, so likely reflects a specific influenza-induced process that reflects the battle between host containment of viral infections and viral attempts to interdict these defenses.

21.3.3 Filovirus

Among the human filoviruses, sumoylation has only been examined for the infamous Ebola virus (Chang et al. 2009). The viral VP35 protein binds Ubc9, PIAS1, and IRF7. IRF7 is a transcription factor required for interferon transcription, and VP35 enhances the sumoylation of IRF7 which reduces its transactivation capacity and results in lower levels of interferon production. In this example, the virus appears to be exploiting the host sumoylation system to reduce the innate immune response.

21.3.4 Paramyxovirus

Parainfluenza virus commonly causes respiratory infections and is a frequent cause of childhood illness. The P protein of this virus is sumoylated at lysine 254 (Sun et al. 2011). This protein is a co-factor of viral RNA-dependent RNA polymerase, and mutation of the SUMO acceptor site produced a virus that grew with lower levels of viral RNA and reduced titers. However, the mutant P protein remained wild-type in most

activities that were tested, so no biochemical or molecular mechanism to explain the effect of sumoylation was reported.

21.3.5 Rhabdovirus

This family includes the highly dangerous rabies virus as well as the more benign vesicular stomatitis virus (VSV). While sumoylation of rhabdoviral proteins has not yet been demonstrated, there is a recent report that sumoylation is involved in regulating both rabies and VSV infection (Maarifi et al. 2016). Stable exogenous SUMO expression blocked VSV viral mRNA synthesis, thus inhibiting all the downstream steps needed for viral reproduction. The host MxA protein is a known inhibitor of VSV transcription, and blocking MxA prevents the effect of increased SUMO expression. MxA protein becomes highly stable and accumulates under SUMO overexpression conditions, thus it appears that sumoylation is a host defense that restricts VSV by increasing the levels of this host protein that inhibits early steps in the VSV life cycle. In contrast to VSV, exogenous SUMO expression led to higher titers of rabies virus, an effect that was specific for SUMO3 and not SUMO1. It is known that MxA is not inhibitory for rabies virus, but SUMO3 expression did increase sumoylation of IRF3 which decreases its transcriptional activity and reduces the viral induction of interferon. Thus, for rabies virus increasing sumoylation has a positive effect on viral production, possibly through reduction of the interferon response. The opposing effects of sumoylation on two different viruses in the same family illustrate the complexity of the viral-host interplay and the diversity with which different viruses have evolved to interact with the sumoylation system.

21.3.6 Coronavirus

Human coronavirus are typically associated with mild respiratory diseases, but the SARS coronavirus is a highly lethal agent. There is only one report involving SARS and the sumoylation system, with Fan et al. demonstrating that the SARS nucleocapsid protein (N) bound to and co-localized with Ubc9 (Fan et al. 2006). Unfortunately, there was little functional characterization of this interaction and no follow up studies have been reported.

21.3.7 Flavivirus

Two members of this family, dengue virus and hepatitis C virus (HCV), have known interactions with the sumoylation system. The dengue envelope protein binds Ubc9 and co-localize with Ubc9 on the cytoplasmic side of the nuclear membrane (Chiu et al. 2007). Since overexpression of Ubc9 lead to reduced plaque formation, Ubc9 may be acting as part of the antiviral defense to restrict dengue infection at early stages. Conversely, silencing Ubc9 expression also reduced the replication of dengue, suggesting that the sumoylation system may have both positive and negative effects on dengue (Su et al. 2016). In this case the effect was related to sumoylation of the viral NS5 protein. Sumoylation of NS5 was required for viral RNA replication and suppression of the interferon production. Sumoylation of NS2 required a SIM motif in NS2 and led to increased stability which could account for both the observed effects on replication and interferon. For HCV, it was observed that SUMO1 is upregulated during infection and that knockdown of SUMO reduced viral replication (Akil et al. 2016). No specific viral target was identified, but the results suggest that sumoylation is a positive factor for this virus also.

21.3.8 Picornavirus

The encephalomyocarditis virus (EMCV) can cause disease in many mammals, likely including humans. The EMCV 3C protease enhances sumoylation of PML leading to association of PML with ND10s and subsequent proteasomal degradation of PML (El Mchichi et al. 2010). While the mechanism of 3C action on PML

sumoylation is undetermined, this study does suggest that EMCV has evolved anti-PML activities as have many of the DNA viruses. A second picornavirus, enterovirus 71 is a more definite human pathogen that can cause serious central nervous system infections. EV71 also has a 3C protease, and in this case 3C was shown to bind Ubc9 and be sumoylated at lysine 52 (Chen et al. 2011). Sumoylation decreases protease activity of 3C and promotes 3C degradation. The K52R mutant of 3C that could not be sumoylated had increased viral levels during infection of cultured cells, consistent with sumoylation being a negative cellular regulatory event against EV71.

21.3.9 Reovirus

Rotaviruses are important human pathogens of the reovirus family and are the causative agents of acute gastroenteritis. Campagna et al. found that sumoylation was a positive regulator for rotavirus and that five viral proteins were sumoylated, VP1, VP2, NSP2, VP6, and NSP5 (Campagna et al. 2013). VP1, VP2, and NSP2 were also able to bind SUMO noncovalently and likely have SIM motifs. The NSP5 protein was further investigated and two putative sumoylation sites were identified, lysines 19 and 82. A mutant NSP5 protein that was not sumoylated was mostly wild-type in its activities, but did display hyperphosphorylation and a defect in viroplasm formation when co-expressed with VP2. However, this defect was not sufficient to reduce viral replication so the biological importance of this modification to the overall viral life cycle is unclear.

21.3.10 Deltavirus

The hepatitis delta virus (HDV) is a serious pathogen that is always found in conjunction with hepatitis B virus (HBV) and which exacerbates HBV infections (Sureau and Negro 2016). HDV expresses a nuclear antigen (HDAg) that occurs in a short (S-HDAg) and long (L-HDAg) form. Multiple lysines in the short form are sumoylated, and this modification enhances production of viral genomic RNA and mRNA (Tseng et al. 2010).

21.4 Conclusion

The sumoylation system has been shown to be an important player in many biological processes, such as cellular differentiation, transcriptional regulation, and cell growth (Chymkowitch et al. 2015; Eifler and Vertegaal 2015). Perturbing sumoylation changes cellular response to diverse signaling pathways (Sharrocks 2006), so this system has huge potential as a target for pathogens to use to manipulate the host environment. Sumoylation also helps regulate host defense systems and appears in many cases to contribute to an "antiviral state" (Hannoun et al. 2016), so intracellular pathogens such as viruses may need to alter sumoylation to interfere with these defenses. The large body of evidence presented here indicates that SUMO and its enzymatic pathway play an important role in viral-host interactions for a large and growing number of DNA and RNA viruses. Numerous viral proteins are substrates for sumoylation with example of both positive and negative regulation of their activities. Thus, as with other types of host cell post-translational modification, viruses have clearly adapted to use sumoylation to regulate aspects of their life cycle. Additionally, there is an expanding list of viral proteins that can manipulate sumoylation, either a globally or in a substrate-specific manner. Given the wide range of cellular pathways affected by sumoylation, determining how these changes impact overall viral fitness require extensive experimentation, so in most reported cases the overall effect is still poorly understood. It is likely that many additional viral proteins that impact sumoylation await to be discovered. Identifying and understanding these global and specific viral effects on sumoylation will provide new insight into viral-host interactions and may highlight new targets for therapeutic treatment of viral infections.

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Sumoylation as an Integral Mechanism in Bacterial Infection and Disease Progression

22

Chittur V. Srikanth and Smriti Verma

Abstract

Post translational modification pathways regulate fundamental processes of cells and thus govern vital functions. Among these, particularly the modification with Small Ubiquitin-like Modifiers (SUMO) is being recognized as a pathway crucial for cell homeostasis and health. Understandably, bacterial pathogens intervene with the SUMO pathway of the host for ensuring successful infection. Among the bacterial pathogens known to target host sumoylation varied points of intervention are utilized. Majority of them including *Salmonella* Typhimurium, *Shigella flexneri* and *Listeria monocytogenes* target the E2 conjugating enzyme Ubc9. While others, such as *Xanthomonase compestris*, target the desumoylation machineries mimicking cysteine protease activity. Still others such as *Ehrlichia chaffeensis* and *Anaplasma phagocytophilum* utilize host SUMO-machinery for sumoylating their own effectors. Together such changes lead to modulation of host proteome and transcriptome thereby leading to major alterations in signal transduction that favor invasion and bacterial multiplication. Such interplay between bacterial pathogens and host sumoylation has added a new dimension to host-pathogen biology and its understanding could be vital for developing potential therapeutic intervention strategies.

Keywords

Bacteria • Sumoylation • Post-translational modification • Inflammation • Transcription • Ubc-9 • Host-pathogen interaction • Pathogenesis • Proteome • Transcriptome

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22.1 Introduction

22.1.1 Host-Microbe Interactions

The human body interacts with and is home to a diverse array of microorganisms at all surfaces exposed to the environment particularly the skin and mucosa. Such interactions can either be beneficial, harmful or cause no consequences. Most of the microbes are either beneficial or do not have any effect and are present as a relatively stable population, referred to as the normal microbial flora or microbiota. Microbiota are acquired at the time of birth and develop for each individual according to a dynamic interactions between the microbes, the environmental factors, habits, immune status and genetic background [reviewed in (Grice and Segre 2011, 2012; Wade 2013; Kaiko and Stappenbeck 2014)]. Some microorganisms however cause damage or disease. Of these, microbes that always cause disease when present (like *Yersinia pestis*, the causative agent of plague) and are considered to be pathogens, while that are commonly a part of the normal flora but can cause disease on occasion (like *Escherichia coli*) are referred to as opportunistic pathogens. The damage induced during a pathogenic onslaught can be either mediated by the pathogen or be the result of host-responses. The host-pathogen interactions have evolved to consist of multiple dimensions – the host cell that provides the niche, the immune system and the normal microflora that prevent establishment of the pathogen and the pathogen with its various virulence factors. The interaction of all these factors takes place at a molecular level and has often been referred to as an "*arms race*" between the host and the microorganism where the hosts develop multiple mechanisms to protect themselves from microbial invasion whereas the pathogens generate diverse strategies to invade and exploit the host cell and evade protective mechanisms. The continuous adaptation of each to survive the other has been termed the *Red Queen hypothesis* by Leigh van Valen (1973) for the dialogue "*It takes all the running you can do, to keep in the same place*" by the character in the Lewis Carroll novel '*Through the looking glass*'. In general the hosts protect themselves, either by developing resistance to the virulence factors or growth of the pathogen, or by generating tolerance and diminishing the harmful consequences of the damage induced (Roy and Kirchner 2000; Miller et al. 2005).

Most pathogens have developed strategies to enter a host, proliferate and be transmitted to another host within a time span that is shorter than that required for them to be either cleared off by the host immune system or for the host to be killed by irreparable damage caused by them. The final outcome of a host-pathogen interaction depends both on the virulence of the pathogen and the immune status of a host as exemplified by diseases caused by so called avirulent pathogens in immune compromised individuals and the lack of it in immune-competent individuals. Therefore a virulence factor of a pathogen is a virulence factor only in a susceptible host where it triggers mechanisms that support infection. Mechanisms of adherence to host surfaces are important to prevent being swept away by mucus and other fluids and the ciliary action. Bacteria have specific surface molecules that interact with host cell receptors and mediate adherence. These include pili, lipoteichoic acid, or other specialized ligands such as surface adhesins produced by *Vibrio cholerae*, the causative agent of cholera. These allow the bacteria to adhere to the gut epithelial surface followed by internalization in case of intracellular pathogens. This may occur via active role of the bacteria and a passive role of the host, a process termed "invasion". Many enteropathogenic bacteria such as *Listeria*, *Salmonella*, *Yersinia* and *Shigella* exhibit the ability to induce their uptake by non-phagocytic epithelial cells. They achieve this by triggering massive cytoskeletal rearrangements at the site of contact that result in the bacteria being enveloped by the host membrane. *Yersinia* and *Listeria* induce this by binding to surface bound proteins. Invasins of *Yersinia* bind to integrins of the β1 family (Isberg and Barnes 2001) while the Internalin A of *Listeria monocytogenes* binds to E-cadherin of host {Mengaud, 1996 #61}. *Shigella* and *Salmonella* on the other hand use a triggering mechanism. By using specialised secretory

apparatuses called the type III secretion system (T3SS), these pathogens inject into the host cell, bacterial proteins that go and manipulate the host cytoskeletal to induce membrane ruffling at the point of contact followed by internalization [reviewed in (Sansonetti 2002)]. Several types of toxins are also produced by bacteria, a process referred to as *toxigenicity*, to manipulate host cell function and take control over vital processes to favour microbial proliferation. Endotoxins or Lipopolysaccharide (LPS), constituents of the wall of gram-negative bacteria are released upon bacterial death or during growth; however, their release is not required for their toxic effects. Pore forming toxins are largest class of bacterial toxins (Listeriolysin by *L. monocytogenes*, CyclolysinA by *Salmonella enterica* and *Shigella flexneri*) that insert into membranes resulting in the formation of pores and increased permeability and ion imbalance. These ion imbalances are detected by the host cell and lead to activation of several pathways such as Mitogen activated protein kinase (MAPK), autophagy (Dal Peraro and van der Goot 2016) or those leading to inflammasome and innate immune mechanisms.

Another key virulence strategy involves subversion of existing host machineries wherein pathogens simply modulate the pre-existing molecules in the host for their own benefit thereby minimizing energy costs as well as maintaining the economics of the genome. One of the key modes to achieve this is via perturbation of Post-translational modifications (PTMs). PTMs are the covalent attachment of a group to a target protein that alters its properties and consequently its functions. Such modifications expand the compositional and functional repertoire of an organism and also provide key access points to usurp the molecular machinery. PTMs may involve modifications of already existing protein(s) by either removal of a part from its primary structure or addition of various moieties such as (i) a small chemical group – phosphorylation, hydroxylation, acetylation, methylation; (ii) a complex molecule – AMPylation, ADR-Ribosylation, Glycosylation, Isoprenylation; or (iii) a polypeptide- Ubiquitin and Ubiquitin like proteins – SUMO, Nedd8, IGS15, FAT10 etc. Bacterial pathogens have been recognized to modulate most of these PTMs (Ribet and Cossart 2010). Ubiquitin and SUMO pathways have shown to be crucial pathways of intervention by bacterial pathogens. IpaH9.8 from *S. flexneri* functions as a prokaryotic E3 ubiquitin ligase that catalyse the polyubiquitination of NEMO/IKKγ thus hampering NFκB –mediated inflammatory response during infection (Ashida et al. 2010). *S.* Typhimurium encodes SseL that possesses deubiquitination activity and deubiquitinates p62 bound protein aggregates and reduces recruitment of autophagic machinery, thus favouring bacterial replication (Mesquita et al. 2012; Thomas et al. 2012).

In the recent past, a large number of cellular targets have been identified undergo SUMO-modification and a vast array of cellular processes are understood to be regulated by sumoylation revealing it to be a critical process of the host cell required not only for homeostasis but also to respond to environmental stimuli and pathogenic onslaught (Marx 2005; Wilkinson and Henley 2010; Flotho and Melchior 2013; Guo and Henley 2014). Therefore it is not surprising that pathogens have developed various strategies to manipulate this process for their own benefit, altering it to ensure an environment favourable for their multiplication and persistence. The current chapter covers this unique molecular crosstalk between bacterial pathogens and the host. The different strategies of pathogen mediated sumo-alterations will be covered highlighting the significance in the biology of infection.

22.1.2 Sumoylation Regulates Cellular Processes

SUMO (small ubiquitin-like modifier) is a protein of approximately 101 amino acids that bears limited homology to Ubiquitin while maintaining significant structural conservation. Covalent attachment of this polypeptide to its target protein is referred to as sumoylation. The human genome encodes several SUMO isoforms– SUMO1, SUMO2, SUMO3 and SUMO4. Of these, SUMO1–3 are ubiquitously expressed while the expression of SUMO4 appears to be restricted to

certain organs such as spleen, kidney and lymphatic nodes. Very recently another variant SUMO5 has been identified. SUMO5 also exhibits tissue specificity being detected in testes and peripheral leukocytes and is believed to be important in formation of PML nuclear bodies (Liang et al. 2016). SUMO2 and SUMO3 only differ in three amino acids often being referred to as SUMO2/3 as they are indistinguishable by antibodies. Together they are about 50% similar to SUMO1 and 86% to SUMO4 (Bohren et al. 2004). During sumoylation, an isopeptide bond is formed between a terminal glycine of SUMO and a lysine residue of the target protein within a conserved motif consisting of ΨKxD/E where Ψ represents a hydrophobic amino acid, and x is any amino acid (Rodriguez et al. 2001; Sampson et al. 2001). Not all consensus motifs are sumoylated and not all sumoylations occur at these consensus motifs (Seeler and Dejean 2003; Miller et al. 2010). SUMO2, SUMO3 and SUMO5 also contain the consensus sumoylation motif allowing them to get conjugated to other SUMO moieties thus forming chains. SUMO1 lacks this motif being able to attach single and is often present as a cap at the end of the SUMO2/3 chain. Free SUMO2/3 is more abundant in the mammalian cells as compared to SUMO1 which is present mostly in the conjugated form. SUMO2/3 are believed to respond to environmental stimuli and conjugate target proteins while SUMO1 is believed to play a role in more house-keeping/constitutive functions. SUMO1 and SUMO2/3 have overlapping as well as distinct targets and thus distinct proteomes (Vertegaal et al. 2006).

All eukaryotes have the sumoylation machinery with lower eukaryotes generally having a single SUMO isoform while plants and mammals encoding several. Conjugation of the SUMO isoforms to their target proteins takes place via an enzymatic cascade that is analogous to ubiquitinylation, albeit with differences. The SUMO isoform are processed at the C-terminus to expose the terminal Gly-Gly containing mature isoform by cysteine proteases referred to SENPs/Sentrin proteases. The mature SUMO isoform gets activated by adenylation and is then bound to activating E1 enzyme from where it is transferred to the sole E2 conjugating enzyme Ubc-9. Several E3 ligases have been identified for sumoylation belonging either to PIAS family or the HECT ring ligases. These aid in substrate identification and promote conjugation of SUMO to the target protein {Flotho, 2013 #22}. The deconjugation of sumoylated proteins is carried out by the SENPS (sentrin proteases) or desumoylases. The mammalian genome encodes for 6 such proteins – SENP1, SENP2, SENP3, SENP5, SENP6 and SENP7. Each protease exhibits differential localization and specificity for the SUMO isoform and the target proteins thus their activity can specifically alter the SUMO proteome of a cell (Yeh 2009; Hickey et al. 2012; Flotho and Melchior 2013).

The sumoylated fraction of any protein in the cell usually never exceeds more than five to ten per cent of the cellular pool yet, sumoylation of a protein is known to have detrimental consequences to its functions (Hay 2005). This is referred to as the SUMO enigma. It is believed that a protein has what is referred to as SUMO memory where modification by sumoylation attributes a change in the property of the target protein which is retained even when the SUMO isoform is removed. Thus despite their being only a small fraction of sumoylated pool, the functioning of majority of the pool is altered. It is as yet not known why the cell needs to maintain only a small sumoylated pool for most proteins with some exceptions such as RANGAP1, a protein that is being sumoylated at all times.

SUMO is a large PTM to occur to a protein and every conceivable consequence has been attributed to proteins that get sumoylated. Unlike ubiquitination, the molecular consequences of sumoylation are difficult to predict and underlying principle of sumoylation is that it leads to alteration of inter and intramolecular interactions of the target protein. Crystal structure analyses have revealed that as opposed to ubiquitin, SUMO has a large negative surface charge along with a negative pocket. This can lend itself to modulation of target proteins' interactions as well as structure, both upon direct conjugation as well as by interaction via conserved motif called SUMO interacting motifs or SIM. For example, the human thymine-DNA glycolase a component

of the base excision repair as well transcription, undergoes distinct conformational changes upon direct conjugation to SUMO and interaction via SIM (Smet-Nocca et al. 2011). The poly SUMO2/3 chains act as docking sites for the SUMO-targeted E3 Ubiquitin ligase RNF4 during the processing of misfolded proteins (Gartner and Muller 2014; Guo et al. 2014). In addition, changes in stability, localization and activity are also repercussions of sumoylation or interactions with sumoylated proteins via SIM. The SUMO conjugated proteome of any cell is referred to as its SUMOylome (or SUMOome). A variety of studies have revealed novel proteins that undergo sumoylation with yeast-two-hybrid and mass spectrometry. Over the last decade these studies have enabled our understanding and led to exponential increase in the number of target proteins that are known to undergo sumoylation. These have been shown to be important in varied cellular processes such as genomic stability, transcriptional regulation, cell cycle progression, and apoptosis (Nuro-Gyina and Parvin 2016; Seeler and Dejean 2003; Hilgarth et al. 2004; Hay 2005; Cubenas-Potts and Matunis 2013; Eifler and Vertegaal 2015).

22.2 Bacterial Pathogens and Sumoylation

Infectious diseases continue to cause significant morbidity and mortality. Studying host-pathogen interaction not only aids in our understanding of pathogenesis and therefore helps in the development of intervention strategies, but also helps in providing insight into cellular processes**.** Of the many strategies known for pathogenesis, modulation of PTMs is fast gaining importance because of the all-pervasive effect on almost cellular processes, particularly sumoylation. Several examples of pathogens perturbing host sumoylation pathways are known but the possibility of host-driven SUMO alterations as a response to pathogens or for defence is not known but can't be ruled out.

First observed in infections by viruses (Everett et al. 2013), modulation of host sumoylation has emerged as a widespread phenomenon encompassing bacterial pathogens of both plants and animals. Although themselves lacking the sumoylation machinery, prokaryotes and viruses have devised clever ways of exploiting the host's sumoylation machinery for their benefit.

Some actinobacteria such as *Leptospirillum* sp., *Mycobacterium* sp., *Bifidobacterium longum*, *Corynebacterium glutamicum*, possess a PTM involving tagging proteins for degradation. They possess prokaryotic ubiquitin-like protein (Pup) which gets attached to the target protein via an isopeptide bond with a lysine residue (Pearce et al. 2008; Burns et al. 2009). The ligation of Pup is catalysed by a single enzyme Pup ligase PafA which is counterbalanced by depupylation enzyme, Dop (Burns et al. 2010; Imkamp et al. 2010). The Pup-proteasome system is one of multiple degradation complexes present in these bacteria and is for the most part non-essential (Knipfer and Shrader 1997; Hong et al. 2005; Lamichhane et al. 2006) except for certain conditions such for survival of *Mycobacterium tuberculosis* in host (Darwin et al. 2003; Gandotra et al. 2007). Pup and ubiquitin do not share homology with respect to their amino acid sequence or structure with ubiquitin adopting a characteristic β-grasp fold while Pup remaining largely disordered (Liao et al. 2009; Chen et al. 2009; Sutter et al. 2009). In addition PafA and Dop, which share structural homology with each other, do not share evolutionary relatedness with enzymes of the ubiquitination pathway (Iyer et al. 2008) (Striebel et al. 2009; Ozcelik et al. 2012). Instead, the absence of E3 ligases is slightly similar to the few E3 ligases present in the SUMO pathway.

22.2.1 Strategies Employed by Bacteria to Intercept/ Exploit Host Sumoylation

As sumoylation is a multistep process (Fig. 22.1), there are that many points of intersection for the pathogens. A limited number of pathogen proteins have been demonstrated so far that target

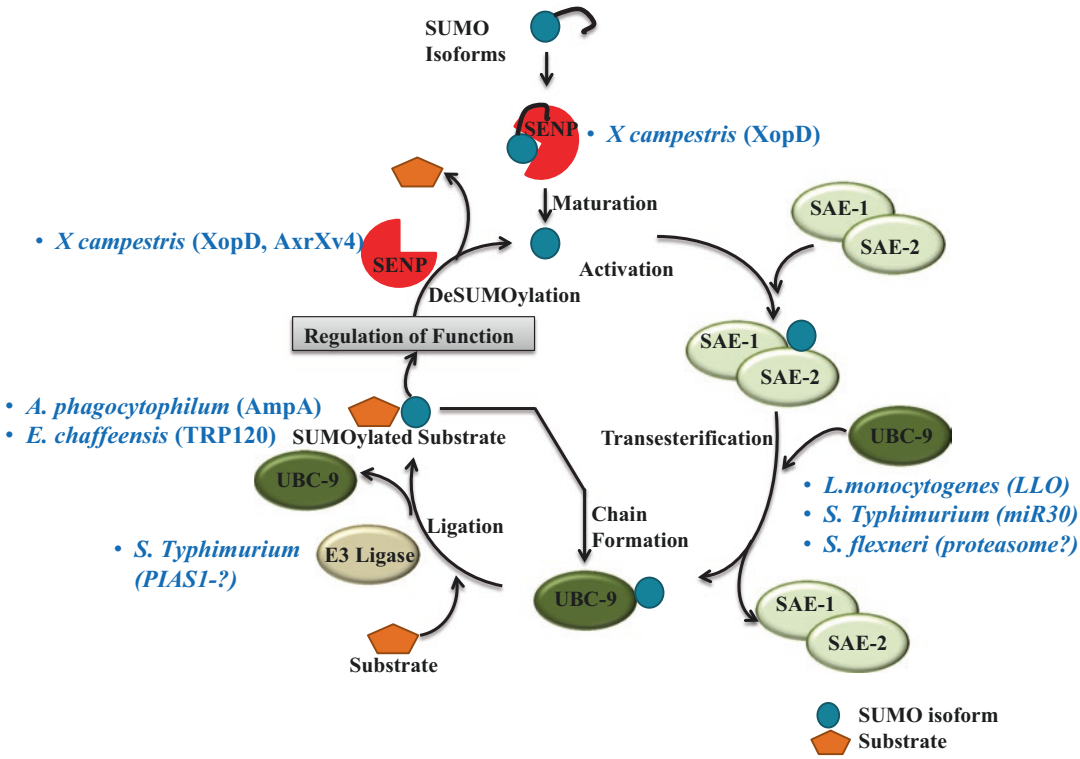

Fig. 22.1 Points of intervention in the sumoylation pathway by bacterial pathogens. Sumoylation cycle: The point of interception by various pathogens is indicated. The immature form of SUMO isoforms are processed at the C-terminal end by cysteine proteases (SENPs) to expose terminal Gly-Gly that take part in the isopeptidase reaction. The Gly-Gly then forms high energy thioester bond with active site cysteine of the E1 activating enzyme, SAE1/SAE2 at the expense of an ATP molecule. This is followed by a transfer of SUMO to the catalytic cysteine of E2 conjugating enzyme, Ubc-9. Ubc-9 can directly interact with the substrate recognizing the SUMO consensus sequence (ΨKxD/E) and with the aid of E3 ligases transfers the SUMO to the lysine (K) residue within the motif. The addition of SUMO isoform(s) leads to alteration/regulation of function of the substrate. The SUMO isoform is deconjugated from the substrate by cleavage of the isopeptidase bond by SENPs

the desumoylases, the E1 conjugating enzymes or the E3 ligase. The E2 conjugation step catalysed by Ubc-9 however has been the target of many pathogens. This corresponds to the critical role of Ubc-9 which is sufficient to bring about sumoylation of most target proteins while E3 ligases enhance the product formation. Ubc-9 also possesses substrate specificity. Apart from the E2 activity, Ubc-9 has also been implicated to have several other functions (Kurihara et al. 2005; Liu et al. 2007; Zhu et al. 2010; Uemura et al. 2013). Several pathogens also harness the host SUMO machinery for sumoylation of their own proteins leading to altered functionality in their favour (Fig. 22.1).

22.2.1.1 Mimicry of Sumoylation Machinery Components

The first report of bacterial pathogen modulating host sumoylation was that of the T3SS effector protein YopJ encoded on a 70 Kb plasmid harboured by *Yersinia* that encodes the *Yersinia* outer membrane proteins (Yops). YopJ was shown to exhibit protease-like features, similar to an adenovirus protease AVP via secondary structure predictions (Orth et al. 2000). In 1999, Li and Hochstrasser had observed that AVP resembles a yeast cysteine protease, Ubiquitin-like protease 1 (Ulp1) which was predicted to process immature SUMO polypeptide as well desumoylate target proteins (Li and Hochstrasser

1999). YopJ shares limited structural similarity with the active site of yeast Ulp1. Thus it was predicted that YopJ may be mimicking the host SENP activity. Indeed, overexpression of YopJ into planta resulted in decreased levels of SUMO conjugated proteins while the mutant for the conserved cysteine was unable to bring about the decrease. However, over expression of YopJ also resulted in decrease in ubiquitinated proteins and later work demonstrated this protein to be a deubiquitinase and/or acetyl transferase (Zhou et al. 2005; Mukherjee et al. 2006) and its effect on sumoylation thought to be an indirect one.

YopJ/HopZ/AvrXv superfamily of type three secretion system (T3SS) effectors is one of the largest and most widely distributed families with effectors found in both pathogens of animals (*Salmonella* AvrA, *Vibrio* VopJ, *Aeromonas* AopP) and plants (*Pseudomonas* HopZ, *Xanthomonas* AvrRxv, AvrXv4, AvrBsT, XopJ, *Rhizobium* Y4LO). AvrXv4 from *Xanthomonas campestris* pv vesicatoria, the causative agent of bacterial leaf spot in pear and tomato, is known to be secreted into the plant cell via T3SS. *In vitro*, the protein is not active, but upon transient overexpression in plants it leads to loss of sumoylated proteins suggesting it to act as a desumoylase towards host proteins (Roden et al. 2004). Another protein family of *Xanthomonas* that shares structural homology with Ulp1 is the XopD family. Its characteristic member, XopD encodes a plant specific cysteine protease that has been demonstrated to cleave tomato and *Arabidopsis thaliana* SUMO isoforms *in vivo* after the invariant C-terminal Gly-Gly residues(Hotson et al. 2003; Chosed et al. 2007). In addition, Kim et al., have demonstrated a role for XopD in desumoylation of transcription factor SlERF4 leading to reduced stability and transcriptional activity (Kim et al. 2013). Precisely, the effector colocalized with sumoylated SlERF4 within the cell nucleus and mediated hydrolysis of SUMO1 from lysine 53 of SlERF4. Physiological outcome of this is a decrease in the synthesis of ethylene, a plant hormone that is responsible for the development of symptoms and generation of host immunity during infection with *Xanthomonas campestis* pv vesicatoria (Kim et al. 2013).

The SUMO system in plants has higher number of genes encoding SUMO proteases (*A. thaliana* has 9 SUMO isoforms and 12 SENPS identified so far) with speculations placing the number of SENPs to be tenfold or greater than the SUMO E3 ligases (Yates et al. 2016). From an evolutionary perspective this implies a more prominent role for desumoylation in the regulation of sumoylation in plants which is reflected in the pathogens acquiring strategies of mimicking SENPs.

22.2.1.2 Alteration of Sumoylation of Specific Proteins

Some bacteria have also been demonstrated to carry out subtle changes to one or a few SUMO conjugated proteins. *Escherichia coli* are typical components of the normal gut microflora. Some strains however, acquire pathogen like features and are called pathobionts. *E. coli* strain containing the pks (polyketide synthase) genomic island is a pathobiont that has been shown to possess tumour inducing properties, such that transient contact with the bacterium increases tumour growth. These bacteria are also over represented in patients with Inflammatory Bowel Disease and colorectal cancer. Cougnoux et al. demonstrated that promotion of tumour growth was due to the development of a senescent phenotype characterized by increased release of growth factors (Cougnoux et al. 2014). This senescence in intestinal epithelial cells was shown to be induced in cells that came in contact with pks+ *E. coli* due to the increased conjugation of p53 to SUMO1. p53 is known to be conjugated by SUMO1, a modification required for its full activity, as well as by SUMO2/3 which correlates with reduction in p53 activation and repression of a subset of its target proteins (Muller et al. 2000; Stindt et al. 2011). The increased p53 sumoylation was a result of cMyc dependent miR20a-5p mediated down modulation of SENP1. Overexpression of SENP1 was shown to reduce senescent phenotype (Dalmasso et al. 2014; Cougnoux et al. 2014). The effects of SENP1-mediated degradation

were not interrogated at the global level. It can be speculated that the effect of SENP1 down modulation would be limited as the enzyme would target only a subset of SUMOylome. However the exact implications of this down regulation of this SUMO machinery enzyme would be clear only upon further examination.

The physiological significance of tumour induction is as yet unclear. It is known that bacteria that associate with tumours are benefitted by the nutrient supply to the tumours and the down modulation of immune response by the tumours. Whether this phenomenon or any other is the driving force behind induction of tumours or the tumours are an unintended result of the sum of interaction of bacterial virulence factors and host immune response, is still to be deciphered.

22.2.1.3 Sumoylation of Virulence Factors and Functional Alterations

The recruitment of the host sumoylation machinery for modification of bacterial proteins has been recently demonstrated by two groups almost simultaneously in members of the family Anaplasmataceae – *Ehrlichia chaffeensis* (Dunphy et al. 2014) and *Anaplasma phagocytophilum* (Beyer et al. 2015). The vacuoles containing both these pathogens were observed to colocalize with host sumoylated proteins.

A high preponderance of proteins containing SUMO interacting motifs (SIMs) was observed in the *E. chaffeensis*-host protein interactome. Type 1 secretion system effector tandem repeat protein TRP120 which during infection is known to be present in the host cell cytosol and nucleus, was found to contain a conventional SUMO consensus motif with lysine 432 (K432) serving as the putative SUMO conjugating lysine. *In vitro* sumoylation assays demonstrated modification of recombinant TRP120 by all three SUMO isoforms which was abrogated upon mutation of K432. Immunoprecipitation of sumoylated TRP120 from HeLa cells overexpressing a GFP-tagged version of TRP120 and HA-tagged SUMO isoforms 1, 2 or 3 however revealed co-immunoprecipitation only with HA-SUMO2 and HA-SUMO3 but not HA-SUMO1. The single protein band was approximately 15 kDa higher in molecular weight suggesting monosumoylation modification. TRP120 has been shown previously by yeast two hybrid analyses to interact with numerous host proteins with a small proportion also migrating to the nucleus and binding directly to DNA. Interaction of TRP120 with the host proteins was shown to be compromised if its sumoylation was prevented. When host sumoylation was inhibited by treatment with anacardic acid, a small molecule inhibitor of the E1 heterodimer, or viomellein, an inhibitor of Ubc-9, the reduced recruitment to the vacuole was observed for, among others, PCGF5 a component of the polycomb repressive complex known to robustly interact with TRP120. The inhibition of host sumoylation also resulted in decreased bacterial burden although bacterial entry remained unaffected. Thus, the bacteria exploit the host sumoylation machinery to enhance the repertoire of protein interactions of TRP120 which benefits its survival and multiplication.

A. phagocytophilum effector AmpA has also been demonstrated to localize to the *A. phagocytophilum* containing vascular membrane (AVM) and secreted into the cytosol. In lysates from infected cells AmpA displays several isoforms whereas the recombinant protein in *E. coli* appears as a single band suggesting it to be post-translationally modified by the host. Analysis of the possible interacting motif revealed the presence of several lysine residues that could be sumoylated. Indeed, a pulldown of sumoylated protein from infected cells reveals the presence of a small percentage of AmpA to be sumoylated. Further analysis showed the protein to be preferentially polysumoylated by SUMO2/3 early during infection while interactions with SUMO1 being evident only 24 hrs post infection. Inhibition of host sumoylation machinery resulted in decreased bacterial burden while ectopic overexpression of AmpA but not K*R isoform, also decreased bacterial burden. This may point towards an inhibitory role of sumoylated isoform of the effector on bacterial replication. AmpA sumoylation could perhaps serve the role

of keeping a check on the rate of bacterial replication in order to prevent overwhelming the host cell.

E. chaffeensis protein TRP120 and *A. phagocytophilum* effector AmpA are both proteins containing repetitive sequences that are secreted into the host cell cytoplasm and localize to the membrane of the pathogen containing vacuole. Both the proteins get preferentially sumoylated with SUMO2/3, although conjugation with SUMO1 has been observed for AmpA later in the infection cycle *in vivo*. TRP120 was found to be monosumoylated while AmpA gets polysumoylated. *In vitro* however, all isoforms are able to sumoylate these bacterial proteins pointing towards a role of host cell factors such as E3 ligases and SENPs in regulating the distinction between the isoform conjugated inside the cell. The fact that both the effectors get sumoylated in the absence of bacterial infection insinuates sumoylation to be a property of the effector protein unaided by any other bacterial effector. Another important characteristic of the modification of these effectors was that infection with the pathogen does not alter global sumoylation levels in the host or levels of the E2 conjugating enzyme Ubc-9. Instead they employ the strategy of recruiting the existing host machinery for the sumoylation of bacterial proteins. Although the mechanism by which the SUMO-switch operates to mediate the function of TRP120 or that of AmpA is still an unsolved puzzle and would be an interesting avenue for further research.

22.2.1.4 Global Modulation of Host Sumoylation Levels

Alteration of global sumoylation has been observed as an adaptive mechanism for many abiotic stresses such as ischemia (Yang et al. 2008a, b; Lee and Hallenbeck 2013), heat-shock (Golebiowski et al. 2009) as well as hibernation torpor (Lee et al. 2007). For examples, increase in global sumoylation was found to protect cells against ischemic challenge (Datwyler et al. 2011; Cimarosti et al. 2012) and mice overexpressing modest levels of Ubc-9 have been shown to possess greater resistance to cerebral ischemia (Lee et al. 2011). In addition, many cancers have been shown to upregulate components of sumoylation machinery such as SAE2 (Kessler et al. 2012), Ubc-9 (Mo et al. 2005) or E3 ligases such as PIAS1 (Hoefer et al. 2012) insinuating a role of sumoylation pathway in cellular proliferation. In contrast, a common theme of global hyposumoylation upon infection with pathogens has emerged from several studies in the recent past suggesting host sumoylation to be generally restrictive or detrimental to bacterial survival and multiplication. These global changes observed in response to the stresses/infections were not merely just increased general sumoylation but rather distinctive alterations to SUMO-conjugated proteomes as shown by quantitative comparisons. Moreover, many of the proteins that underwent changes during abiotic stress were also found to be tuned during bacterial infections. BHLHE40, TOP1, BEND3, FOXP4, HMBOX1, FOXP1, are some examples of proteins that underwent modulation in their sumoylation status during heat shock (Golebiowski et al. 2009) as well as infection with *S. flexneri* (Fritah et al. 2014). It can therefore be hypothesized that the cells have a machinery to respond to stresses and these are regulated differentially by tinkering with their sumoylation status depending on the kind of stress signals received. The true nature of the relationship between the abiotic and biotic response in terms of sumoylation begs further, detailed analyses.

22.2.1.5 *Listeria monocytogenes*

Ribet et al., in 2010 were the first to demonstrate a bacterial pathogen to modulate the global sumoylation of a host epithelial cell (Ribet et al. 2010). They observed that infection with enteric pathogen *Listeria monocytogenes* resulted in decrease in proteins conjugated to SUMO1 as well as SUMO2/3 both *in vitro* and *in vivo* (Fig. 22.2). They identified the *L. monocytogenes* toxin listeriolysin (LLO) to be responsible for the down-regulation of host SUMOylome as treatment of cells with pure toxin was able to bring about the same effect. There were no changes observed in the levels of the SUMO pathway activating enzymes or at the mRNA levels of Ubc-9. Their studies uncovered that LLO acts by

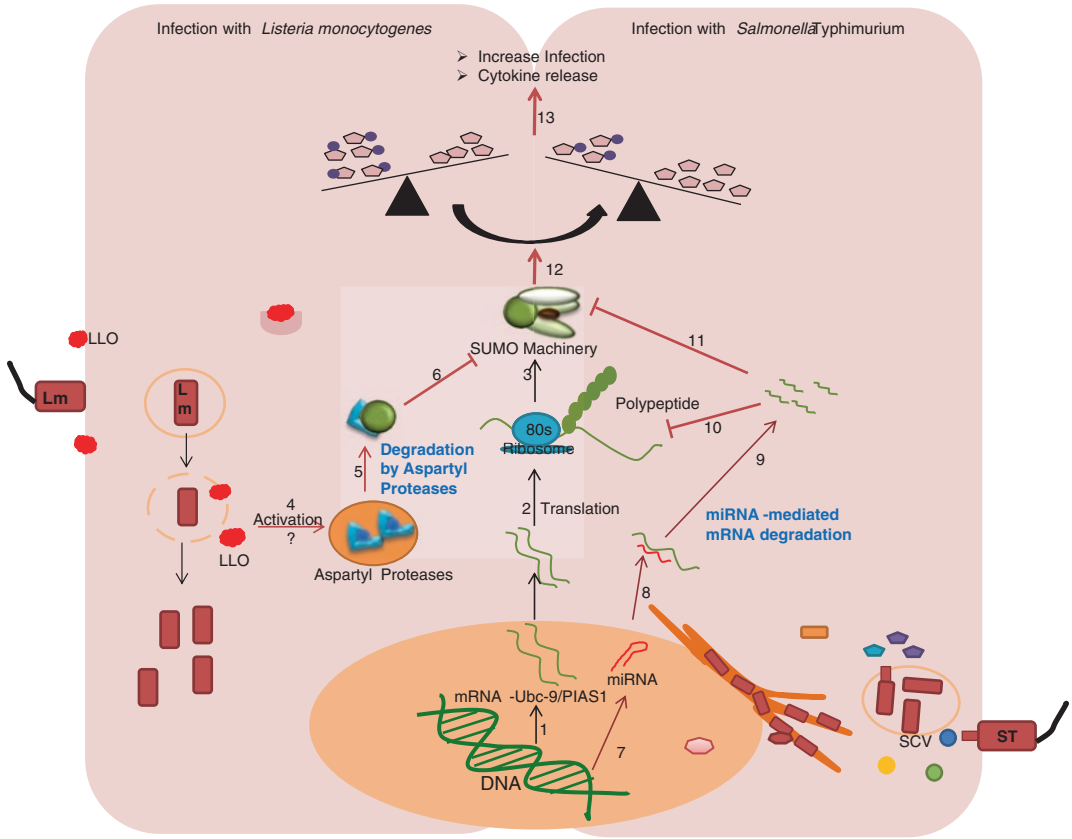

Fig. 22.2 Modulation of sumoylation machinery by enteric pathogens, *L. monocytogenes* and S. Typhimurium. The mRNA for the components of the sumoylation pathway are synthesized in the nucleus (*1*) and then they migrate to the cytoplasm where the protein synthesis machinery of the cell synthesizes each protein of the component which are together termed as the SUMO machinery (*3*). Upon infection with *L. monocytogenes* (*left hand panel*), the pathogen releases the toxin, listeriolysin (LLO) which activates aspartyl proteases within the cell by an as yet unknown mechanism (*4*). These aspartyl proteases degrade the Ubc-9 protein within the cell (*5*) impairing the activity of the sumoylation machinery (*6*). Infection with S. Typhimurium results in the induction of cellular microRNAs (miRNA) of the miR30 family (*7*) which bind to the mRNA of Ubc-9 (*8*) thus leading to miRNA mediated degradation of the Ubc-9 mRNA. This hinders the formation of Ubc-9 protein (*10*) and consequently the activity of the sumoylation machinery (*11*). The impairment of the SUMO machinery results in global desumoylation leading in an alteration in the SUMO proteome balance within the cell (*12*). This culminates in altered cellular functioning of the host cellular processes favouring bacterial survival and multiplication (*13*)

bringing about a degradation of the host Ubc-9 protein both *in vitro* and *in vivo*, in a proteasome independent, aspartyl protease dependent manner. The down modulation of host sumoylation favoured bacterial survival with up-regulation of SUMO1 or SUMO2 inhibiting bacterial growth. The outcome of down modulation of Ubc-9 and consequently the sumoylation as examined on the TGF-β pathway known to be regulated by sumoylation. It was found that a reduction in sumoylation favoured bacterial infection by diminishing host response. Particularly SMAD4, the common mediator Smad (Sma and mother against decapentaplegic) protein responsible for transducing signals from TGF-β family of proteins is known to be stabilized upon sumoylation (Lee et al. 2003). Upon infection with *L. monocytogenes*, a decrease in the level of SMAD4 was observed that could be countered by over-expression of SUMO1. The authors also found other pore forming toxins (Perfringolysin/PFO from *Clostridium perfringens*, Pneumolysin/

PLY from *Streptococcus pneumoniae*) to also bring about similar down modulation of Ubc-9 pointing towards a generic mechanism. Many of the proteins detected to undergo a change in their sumoylation status upon infection (Ribet et al. 2010) with *L. monocytogenes* include those involved in transcription (E.g., aspartyl t-RNA synthase, heterogeneous nuclear ribonucleoprotein K), translation (E.g., 40s ribosomal proteins S3, S14, Elongation factor1A1, Eukaryotic Initiation Factor 4A-I), cytoskeletal rearrangements (keratin, type I cytoskeletal 17, Filamin A, Lamin A/C) and intracellular transport (Protein ERGIC-53 precursor, SEC13,YIF1A), all processes necessarily involved in altering cellular conditions required to generate an optimal niche for the pathogen entry, survival and replication. LLO does not directly interact with Ubc9 and therefore is likely to be triggering a signalling event for Ubc9 degradation. The authors have demonstrated it to be independent of calcium influx and MAPK pathway, the canonical pathways activated upon pore formation by pore forming toxins. What remains to be discovered is the cascade set off by LLO that leads to Ubc-9 degradation and if such signalling mechanisms can be used to disrupt bacterial multiplication and/or survival. .

22.2.1.6 *Shigella flexneri*

Global sumoylation was also demonstrated to be important for *Shigella flexneri* an enteric pathogen responsible for bacillary dysentery (Fritah et al. 2014). Over expression of SUMO2 but not SUMO1 in intestinal epithelial cell lines impaired the invasiveness of the bacterium *in vitro*. When host sumoylation was down modulated *in vitro* using siRNA directed towards the SUMO activating enzyme (SAE2) a significantly higher level of bacterial burden was obtained upon infection. This was also recapitulated *in vivo* using mice haploinsufficient for Ubc-9 that has been demonstrated to have impaired sumoylation capabilities. Further comparative proteomics revealed that proteins were globally less conjugated to SUMO2 upon infection and this resulted in the alteration of a restricted set of transcriptional regulators particularly those known to be involved in gut homeostasis and inflammation. For example, Heat Shock Transcription Factor 2 (HSF2) is a transcription factor that binds to heat shock promoter elements and is associated with the severity of ulcerative colitis (Miao et al. 2014). HSF2 is repressed upon sumoylation and in the study by Fritah et al., was shown to be less conjugated to SUMO2/3 in *S. flexneri* infection (Fritah et al. 2014). Inflammatory regulators such as NFκB, PPARγ and Fos were all hyposumoylated during infection with *S. flexneri*. The mechanism of the down modulation in sumoylation was not elaborated and presupposed to be Ubc-9 mediated. This was however shown to be true by Sidik et al., who demonstrated a down modulation of both SUMO1ylome and SUMO2ylome upon *S. flexneri* infections accompanied by a decrease in Ubc-9 protein levels (Sidik et al. 2015). The degradation of Ubc-9 was shown to be dependent on the proteasome as treatment with MG132 (inhibitor of 26 s proteasome) rescued Ubc-9 from degradation upon infection with *S. flexneri*. The pathogen effector molecule responsible for bringing about the decrease in Ubc-9 is yet to be determined.

22.2.1.7 *Salmonella enterica* serovar Typhimurium

Salmonella Typhimurium the causative agent of gastroenteritis also brings about the down regulation of global SUMOylome by mediating a reduction in the level of Ubc-9 (Verma et al. 2015) both *in vitro* and *in vivo*. The mechanism of the down modulation was distinct from that of *L. monocytogenes* in that reduction was seen at both the mRNA as well as protein level. Interestingly, *S.* Typhimurium was demonstrated to mediate post-transcriptional regulation of Ubc-9 by upregulating the levels of host cellular microRNAs of the miR30 family upon infection. The reduction in SUMOylome was shown to be critical for both bacterial replication and the formation of Salmonella containing filaments (SIFs), filaments that are required for maintenance of *S.* Typhimurium enclosed vacuole. Overexpression of SUMO1 led to reduction of both the number of bacteria within an infected cell and the percentage of cells with SIFs.

Conforming to observations in *L. monocytogenes*, the modulation of global SUMOylome was accompanied by alterations in sumoylation status of protein known to mediate inflammation such as PPARγ and p65 (SUMO2/3 conjugated fraction).

In addition to the E2 enzyme, *S.* Typhimurium targets the sumoylation cycle at the levels of the E3 ligase. Verma et al. demonstrated a time-dependent decrease in the levels of PIAS1, an E3 ligase known to be involved in the regulation of the inflammatory pathways. The precise mechanism and implications of these however remain to be ascertained. Proteomic analysis of infection with related pathogen *S. flexneri* (Fritah et al. 2014) has shown a decrease in SUMO conjugation for several PIAS1 substrates such as SATB2, a chromatin remodelling enzyme, DCLRE1A, a DNA crosslink repair enzyme and PPAR gamma, a nuclear receptor, which was also shown to undergo reduced sumoylation during *S.* Typhimurium infection (Verma et al. 2015). This may thus reflect an attempt by the cell to regulate the specificity of the proteome that undergoes alteration upon infection. The triggers for the upregulation of the microRNAs, as well as the mechanism of PIAS1 down modulation, are as yet unknown.

As evident from the studies from these three bacteria, all enteric pathogens, the strategy of reducing host sumoylation status is crucial for both bacterial multiplication/survival as well as induced inflammation at the intestine. The critical role of regulation by sumoylation in the intestine is further supported by Ubc-9 haploinsufficient mice which die of destruction of colonic tissue, an increase in gut permeability and an increase in proinflammatory cytokines reinforcing the critical requirement of a balance in SUMOylome for intestinal homeostasis and inflammation.

All three pathogens target Ubc-9 to bring about drastic reduction in the levels of proteins conjugated to both SUMO1 and SUMO2/3, albeit by quite diverse mechanisms perhaps reflecting the molecular differences in pathogenicity. *Listeria* enrols the cellular aspartyl proteases and alters only the protein levels of Ubc-9 in a proteasome independent manner. The mechanism by which *S. flexneri* brings about decrease in Ubc-9 levels remains unclear except that it is carried out in a proteasome dependent manner. *S.* Typhimurium on the other hand harnesses the host microRNA machinery and brings about a post transcriptional regulation of Ubc-9 levels thus impairing the conjugation of proteins to SUMO isoforms.

A partial rescue of SUMO1ome was observed to take place by MG132 during infections with *L. monocytogenes* while an almost complete rescue was seen in case of *S.* Typhimurium (Verma et al., unpublished results). It is as yet unclear if the rescue by MG132 reflects the normal turnover of the SUMO conjugated proteins or an active degradation by the proteasome triggered during infection and the differences on the level of rescue is a function of the varying rates of turnover, differences in cell types used or varied experimental designs.

Sumoylation evolved as a post-translational modification in order to assist protein function and regulation for optimal growth of eukaryotic cells. Therefore it is plausible that microorganisms, whose purpose is usurping the host machinery and promoting their benefit to the detriment of host cell, have evolved ways to reduce this modification as an attempt to bring the host cell to a state of impaired regulation that allows the pathogens to use the entire repertoire of host machinery for their own benefit. How the bacteria regulate specificity of the proteome undergoing change, upon modulation of the SUMO machinery in general, is yet to be understood. This is particularly brought to light by the observation that levels of the abundantly sumoylated protein RANGAP1 does not undergo change with alteration of the SUMOylome (Verma et al. 2015). Verma et al. also demonstrate that while the SUMO2/3 conjugated fraction of p65 undergoes a decrease, the SUMO1 conjugated fraction is upregulated upon infection with S. Typhimurium. This is further supported by Zhang et al., who observed an upregulation of sumoylation of Axin-1, a regulator Wnt signalling, upon infection with S. Typhimurium (Zhang et al. 2012) despite the overall decrease in global levels. In addition, the proteomic analysis of SUMOylomes reveal that proteins undergo

both decrease and increase in levels of SUMO conjugation in response to infection (Ribet et al. 2010; Fritah et al. 2014). In each case the down modulation of SUMOylome led to significant reprogramming of various processes most notable being transcription and inflammation, causing better replication/survival of the pathogen as described below (Table 22.1).

22.3 Host Cellular Processes Affected by Perturbation of Sumoylation

22.3.1 Sumoylation and Transcriptional Regulation

One way to alter the state of a cell is to alter the transcriptome. Indeed *Salmonella* is well known to carry out global reprogramming in order to promote its intracellular growth (Hannemann et al. 2013). In consonance a large number of SUMO conjugates identified so far during infection include components of the transcriptional and chromatin modification machinery. For example ETV5 and HSF2 transcription factors which are repressed upon sumoylation were shown to be less conjugated to SUMO2/3 in *S. flexneri* infected cells. Transcriptional repressors BEND3 (Sathyan et al. 2011) and BHLHE40 (Hong et al. 2011), both of which require sumoylation for optimum repression, were also hyposumoylated. Furthermore, studies have uncovered several transcription factors to undergo changes in SUMO status upon infection. Fos (FBJ Murine Osteosarcoma viral Oncogene Homolog) is a transcription factor that plays an important role in inflammation and is regulated by sumoylation. Fos is a leucine zipper protein that dimerizes with members of the JUN family to form the transcription factor AP-1, which activates several inflammatory genes during infection. As a component of the heterodimer, Fos can be sumoylated, an event that leads to loss of transcriptional activity of AP-1 owing to its release from target promoter(s) (Bossis et al. 2005; Tempe et al. 2014). The level of SUMO2/3 conjugated Fos decreased upon *S. flexneri* infection (Fritah et al. 2014) further substantiating a promotion of transcription. All three transcription factors play a key role in the immune response (discussed below) and therefore alterations in their sumoylation status exemplify the effort by the bacteria to subprogram cellular transcriptional machineries favouring infection.

22.3.2 Sumoylation and Cellular Inflammatory Cascade in Bacterial Infection

One of the most prominent pathways of inflammation, the NFκB signalling pathway has been shown to be under tight regulation by the sumoylation pathway. NFκB is a dimeric transcription composed of any of the 5 NFκB/RelA family proteins –p65/RelA, RelB, c-Rel, p105/p50 and p100/p52. In mammalian cells the most common dimer is composed of p65/p50. It is present in the cytosol as an inactive form bound to a repressor protein called IkB. Upon receiving a signal, IkBα is phosphorylated by IkB kinase composed of two kinases IKKα and IKKβ and a regulatory subunit called IKKγ/NEMO. Phosphorylation of IkBα is followed by its conjugation to ubiquitin and proteasome-mediated degradation. This releases p65 and its nuclear localization signal and the transcription factor translocates to the nucleus where it mediates its activity. IkBα has been shown to be modified by SUMO-1 at the same lysine at which it gets ubiquitinylated, thus preventing its conjugation to ubiquitin. This creates a special pool if IkBα that do not dissociate from p65 inhibiting NFκB-dependent transcription (Desterro et al. 1998). In addition, NEMO has also been demonstrated to be conjugated to SUMO-1 by PIASy E3 ligase in response to several cellular stresses including genotoxic stress (Huang et al. 2003). The increase in SUMO-modified NEMO also correlated with augmented NFkB activation. p65 itself has been shown to be SUMO modified with the aid of PIAS3 repressing its transcriptional activity (Liu et al. 2012). The outcome of the NFκB's interaction with sumoylation depends on the cell type

Table 22.1 Enumerating some of the proteins identified in the altered SUMOylomes during infection with enteric pathogens *L. monocytogenes* and *S. flexneri*

Identified protein	Function	SUMOylation status upon infection[a]	Literature analysis
DNA Topoisomerase I (TopI)	Transcription and Replication	Sf- Decreased Lm – Increased S1 conjugation	SUMOylation prevents TopI from binding to nucleoli driving it to nucleoplasm.
Heat Shock Factor 2 (HSF2)	Transcriptional activator	Sf- Decreased	SUMOylation of HSF2 loop impedes HSF2 DNA binding activity thus negatively regulating expression.
Basic helix loop helix family member 40 (BHLHE40)	Transcriptional repressor	Sf- Decreased	SUMOylation is important for nuclear transport and stabilization of repressor activity.
Special AT-rich sequence binding protein 2 (SATB2)	Chromatin remodelling	Sf- Decrease	SUMOylation with aid of PIAS-1 reduces SATB2 mediated gene activation.
Spallt-like transcription factor 4 (SALL4)	Zinc finger transcription factor	Sf- Decrease	SUMOylation is important for stability, sub-cellular localization and transcriptional activity.
Ben domain containing protein 3 (BEND3)	Transcriptional repressor	Sf- Decreases	SUMOylation is essential for transcriptional repression.
FBJ Murine Osteosarcoma Viral Oncogene Homolog (Fos)	Transcription factor (AP-1)	Sf-Decreased	SUMOylation reduces the transcriptional activity by promoting release of AP-1 from promoter
Peroxisome proliferator activated receptor gamma (PPARγ)	Nuclear receptor	Sf- Decreased	SUMOylation promotes its association with NCoR complex mediating transrepression.
(RXR)	Nuclear receptor	Sf- Decreased	SUMOylation promotes its association with NCoR complex mediating transrepression.
Pyruvate kinase	Conversion of Phosphoenol pyruvate to Pyruvate	Lm- Decreased	SUMO-1 conjugation alters localization to nucleus where it serves as a transcriptional co-activator of Aryl hydrocarbon receptor.
Filamin A	Regulation of actin cytoskeleton	Lm- Decreased	Unknown
Nuclear Factor of ACTIVATED T-CELLS C2 (NFATC2)	Transcription	Sf-Increased	SUMOylation converts NFATC2 from activator to a site-specific transcriptional repressor.
Cut-like homeobox (CUX1)	Golgi retrograde transport	Sf- Decreased	Unknown

[a]*Lm* proteomic analysis of *Listeria monocytogenes*, *Sf* Proteomic analysis of Shigella flexneri

and the signals leading to NFκB activation and sumoylation (Mabb and Miyamoto 2007). *S.* Typhimurium causes a differential regulation of SUMO1 and SUMO2/3 conjugated fractions of NFκB with SUMO-1 conjugated fraction increasing upon infection while the SUMO2/3 fraction undergoing a decrease. Understanding of the exact implications of this alteration requires probing into the consequences that result from conjugation to the different isoforms of SUMO and thus wait further in depth studies.

The inflammatory signalling is also controlled by several members of the nuclear receptor family such as the regulation of the NFκB pathway by PPARγ (peroxisome proliferator-activated receptor) and LXR (Liver X receptor). SUMO-conjugation results in targeting of these to the nuclear receptor to the NCoR complex, inhibiting the clearance of NCoR from promoters thus preventing the signal-dependent activation of transcription factors, a process termed as transrepression (Pascual et al. 2005; Venteclef et al. 2010). PPARγ underwent a decrease upon infection in both its SUMO1 (*S.* Typhimurium) as well as SUMO2/3 (*S.* Typhimurium and S. *flexneri*) conjugated fractions. In addition, a decrease was also observed in the levels of PIAS1, the E3 ligase for PPARγ, further implying a reduction in the levels of SUMO conjugation (Fritah et al. 2014; Verma et al. 2015), and suggesting an inhibition of the transrepression mediated by the nuclear receptor and promotion of inflammation. Fritah et al. carried out expression analysis of inflammatory genes, including transcription factor encoding and cytokine encoding genes, by qPCR in Ubc9 wildtype and haploinsufficient mice infected with *S. flexneri*. In intestines, Ubc9 haploinsufficiency lead to enhanced expression of inflammatory genes during *S. flexneri* infection. Specifically they saw dramatic induction of IL-6, cxcl3, IFNβ1 and Tnfrsf1a genes in case of the Ubc9 haploinsufficient samples compared to the wildtype.

Signalling via engagement of TCR and costimulatory CD28 has been demonstrated to sumoylate Protein kinase theta (PKC-θ) aided by the E3 ligase PIASxβ. PKC-θ is a member of calcium independent protein kinase superfamily that mediates TCR and coreceptor CD28 mediated activation of the transcription factor NFkB and AP-1. Sumoylation of PKC-θ is essential for T-cell activation with desumoylation inhibiting the association of CD28 with PKC-θ and filaminA and impairing the formation of a mature immunological synapse (Wang et al. 2015). Filamina A is a protein with actin-binding domain and a protein-protein interacting domain, allowing it to serve as signalling scaffold connecting cellular processes to dynamic regulation of the actin cytoskeleton (Feng and Walsh 2004). Analysis of sumoylated proteome of *L. monocytogenes* revealed filaminA to be one of the proteins which underwent modulation of its sumoylation status, implicating sumoylation in the control of cell migration in response to receptor-mediated signalling that is critical to the immune response.

22.3.3 Sumoylation and Modulation of Cellular Metabolism by Bacteria

The host serves as a source of food for all pathogens and one of the mechanisms of restricting pathogen growth within the host is to limit the nutrient supply, a phenomenon termed as nutrient immunity. Glucose and glucosamine are the major carbon and/or nitrogen sources of mammalian cells. Intracellular pathogens have access to cellular metabolites within the cell but require the cell to produce a continuous supply of energy. In order to obtain suitable energy currency, pathogens manipulate host cell metabolism and carbon &/or energy fluxes (Eisenreich et al. 2010). For example, *A. phagocytophilum* primarily utilizes amino acids as a source of carbon and/or energy. It encodes a type IV secretion system effector, Ats-1 which promotes host autophagy mediated degradation of proteins and thus gains access to amino acids. *L. monocytogenes* uses glycerol as the major carbon source when replicating in the cytosol in macrophages while most of the amino acids are imported from the host (Eylert et al. 2008). Proteomic analyses of SUMOylome carried out by Ribet et al., (Ribet et al. 2010) indi-

cates alteration in the sumoylation status (increase or decrease) during infection for several enzymes critical for glucose metabolism such as Fructose bisphosphate aldolase A, an enzyme which catalyses the interconversion of fructose 1,6 bisphosphate with dihydroxyacetone phosphate & glyceraldehyde-3 phosphate. They also observed alterations in the sumoylation status of Transketolase, an enzyme of the pentose phosphate pathway (PPP) that connects PPP to glycolysis via the intermediates glyceraldehyde-3 phosphate and fructose-6 phosphate and has also been shown to be sumoylated in yeast (Hannich et al. 2005). The tricarboxylic acid (TCA) cycle is the main pathway for generation of ATP in the cell. Several enzymes that are part of this cycle or feed into it were found to be a part of the altered SUMOylomes of *L. monocytogenes* and *S. flexneri* infected cells. These include Pyruvate kinase and Pyruvate dehydrogenase enzymes responsible for generation of pyruvate and its conversion to AcetylCo-enzyme A respectively. Pyruvate kinase has been demonstrated to be sumoylated with the aid of PIAS3 and the SUMO1 conjugated form translocates into the nucleus where it acts as a transcriptional co-activator of ARH receptors (Matsuda et al. 2016). Pyruvate carboxylase directly carboxylates pyruvate to oxaloacetate , an intermediate of the TCA cycle, and glutamate dehydrogenase which converts glutamate into TCA cycle intermediate α-ketoglutarate in *L. monocytogenes* infected cells. In addition to carbohydrate metabolism many enzymes have also been identified in altered SUMOylomes involved in amino acid metabolism (Asparagine synthase, D-3phosphoglycerate dehydrogenase, S methyl 5′ thioadenosine phosphorylase), biosynthesis of nucleotides and DNA replication (C-1 tetrahydrofolate synthase, CTP synthase, DNA topoisomerase, DNA J homolog subfamily A member1), ATP biosynthesis (ATP synthase subunit α) cytoskeleton cellular transport (F-actin capping protein, keratin type I, keratin type II, profiling-1, palladin) and stress responsive proteins (heat shock protein 90 beta, Heat shock protein 70 kDa protein 1, heat shock cognate 71KDa protein). Although these enzymes stated above have been detected in altered SUMOylomes during infection of *L. monocytogenes*, for many of these their sumoylated forms are yet to be experimentally demonstrated. In addition, the impact of sumoylation on the activity (inhibition or activation) is unknown.

22.4 Conclusions

This chapter reflects current knowledge regarding the role of sumoylation in bacterial infection. Sumoylation being involved in several fundamental processes of the cell is emerging to be as one of the preferred pathways of attack by pathogens. The point of intervention in the SUMO-pathway is different for different pathogens. In many cases while global sumoylation of the host cell is affected yet the pathogen ensures a specificity of the altered proteome. Sumoylation dependent alterations in proteomes and transcriptomes of host cells can potentially regulate severity of the infection. Though the details of how this is achieved is not fully understood. These aspects of the pathogen-sumoylation crosstalk will be the future areas of intense investigation in infectious disease biology. Such studies will not only improve our understanding of the process of sumoylation as it functions in the cell but also may help in unravelling potential targets for therapeutic intervention against bacterial disease.

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