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Regulation of Protein-Protein Interactions by the SUMO and Ubiquitin Pathways

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1. Introduction

Post-transcriptional modifications of proteins by ubiquitin and ubiquitin-like proteins (UBLs) such as SUMO (Small Ubiquitin-related Modifier) regulate the function of protein-networks, enable cells to respond to signaling cues during development and to cope with the changing environment during adult life. The ubiquitin and SUMO pathways have profound impacts on protein stability, localization, protein-protein interactions and function.

In this chapter we will review mechanistic and biological aspects of protein-protein interactions that are regulated by ubiquitin and SUMO. We will describe the covalent tagging of proteins by ubiquitin and SUMO, and the enzymatic machineries that regulate these modifications. Subsequently, we will discuss how ubiquitylated or SUMOylated proteins are recognized by ubiquitin and SUMO recognition motifs present on interacting proteins. We will also illuminate how these non-covalent interactions regulate diverse cellular processes such as DNA repair, transcription, signaling, and autophagy in health and disease. Finally, we will address the crosstalk between the ubiquitin and SUMO pathways by SUMO-Targeted Ubiquitin Ligases (STUBLs).

2. Covalent modification of proteins by ubiquitin and SUMO

Ubiquitylation is a post-transcriptional modification where ubiquitin, a 76 amino acids long polypeptide, is covalently attached to proteins. Originally, ubiquitylation was considered as a “death-tag” targeting proteins for degradation by the 26S proteasome. However, over the last two decades non-proteolytic roles of ubiquitylation have also been found to impact protein function, cellular localization and protein-protein interactions. Furthermore, in addition to ubiquitin, ubiquitin-like proteins were identified and collectively termed UBLs. These proteins include among others SUMO (Small Ubiquitin Like Modifier), Nedd8 (Neural precursor cell expressed developmentally down-regulated 8), ISG15 (interferon stimulated gene 15) and FAT10, and all share at least one of the ubiquitin canonical folds (Hershko, 1983; Hochstrasser, 2009).

Among UBLs, the most studied modifier is SUMO. Vertebrates possess four different SUMO isoforms, termed SUMO1-4. While SUMO1-3 are efficiently conjugated to target proteins by

specific SUMO enzymes, it is less clear if SUMO4 is conjugated to proteins *in vivo*. SUMO conjugation of target proteins results in a change in their activity, affecting protein localization, and modulates composition of protein complexes. In some cases SUMOylation may act as a priming modification that promotes ubiquitylation via a specific specialized sub-type of E3 ubiquitin ligases termed SUMO-Targeted Ubiquitin Ligases (Abed et al., 2011b; Praefcke et al., 2011). Within the scope of this chapter we will focus solely on the interactions mediated by ubiquitin and SUMO.

2.1 SUMO and Ubiquitin pathways

Ubiquitylation or SUMOylation are both mediated by a tripartite enzymatic cascade comprised of specific sets of enzymes. Ubiquitylation is mediated by the E1 ubiquitin-activating enzyme, E2-ubiquitin-conjugating enzyme (Ubc), and an E3 ubiquitin ligase (Hershko 1983; Pickart 2001). SUMOylation is similarly carried out by a different set of specialized SUMO specific E1, E2 and E3 enzymes. Both Ubiquitin and SUMO are covalently conjugated via their C-terminus Gly residue to free NH₂ amine group of the target protein that may reside on the ϵ -amino group of a Lys residue along the protein sequence, or on the amino terminus of the protein. Post-transcriptional regulation by ubiquitin and UBLs are tightly regulated, as covalent modification by ubiquitin or SUMO requires ATP. In the human genome only two genes coding for E1, ubiquitin-activating enzymes have been found. It is estimated that about one hundred E2s (ubiquitin-activating enzymes), and hundreds or more E3 ubiquitin-protein ligases exist. Interestingly, in plants the ubiquitin and SUMO pathways are greatly expanded. Collectively, these observations probably reflect the high degree of specificity and regulatory role of these pathways (Hershko & Ciechanover, 1998; Weissman et al., 2011).

E3 ligases are the “match makers” that directly recognize the targeted protein substrate. Ubiquitin E3 ligases can be classified into two main functional classes. The first is the HECT (Homologous to the E6-AP Carboxyl Terminus) domain ligases that accept ubiquitin from an E2 enzyme in the form of a thio-ester via a Cys residue in their catalytic domain, thus forming a thio-ester bond directly with the ubiquitin molecule. The second and most abundant class is the RING (Really Interesting New Gene) finger E3 ligases which utilizes a metal binding domain harbouring Zn²⁺ ions to facilitate ubiquitylation (Deshaies & Joazeiro, 2009). RING ligases are a diverse subclass, encompassing several hundreds of proteins in the human genome. This large family of ligases is further divided into modular sub-classes: 1. Single subunit ligases such as c-Cbl and parkin, that directly bind to both the E2 enzymes and the targeted ubiquitylation protein substrate, and 2. RING E3 ligases that function as a multi-protein-complex that recruits substrates via separate subunits. Examples for this subclass are the well-characterized APC/C (Anaphase promoting complex; McLean et al., 2011) and, the Cullin-based RING E3 ligases (SCF). A subclass of RING-like ligases is the U-box ligases, that contain a modified RING motif lacking canonical cysteine residues for Zn²⁺ coordination (Hindley et al., 2001, Patterson, 2002).

While only one ubiquitin isoform exists, three functional SUMO isoforms have been characterized: SUMO1 and SUMO2/3 that share 97% sequence identity. SUMO pathway components are much less diverse, comprising of only one known E2 conjugating enzyme termed Ubc9 and a few known E3 ligases. A key difference between SUMO1 and SUMO2/3

is the lack of a SUMOylation motif in SUMO1. Therefore, SUMO2/3 can form high molecular weight SUMO polymers with greater affinity than SUMO1. Similarly to ubiquitin ligation, SUMOylation is mostly facilitated via one of two main mechanisms: by recruiting an E2-SUMO to the substrate or by enhancing the conjugation of SUMO to a substrate already bound to the E2 (Ulrich, 2009). Like E3 ubiquitin ligases, SUMO E3 ligases are diverse and have been categorized into three families. The first class is PIAS like (Protein Inhibitor of Activated STAT-signal transducer and activator of transcription) proteins which possess an SP-RING domain functioning similarly to the ubiquitin RING domain and interacting with Ubc9. The second class is the RanBP2/Nup358 (Nuclear pore proteins Ran binding protein 2 and nucleoporin 358) like proteins, which harbour tandem elements that are capable of binding both Ubc9 and SUMO. A third group includes proteins like polycomb group protein Pc2, TOPORS (Topoisomerase I-binding RING finger protein) and likely HDAC4 (histone deacetylase 4) whose molecular mechanism of SUMO ligation is not fully understood (Gareau & Lima, 2010; Hannoun et al., 2010).

2.1.1 Mono ubiquitylation and SUMOylation

Mono-ubiquitylation is formed in most cases by a covalent attachment of the carboxy terminal glycine of the ubiquitin polypeptide to the ϵ -amino group within the side chain of Lys residues on the target protein. In some cases the attachment site could be the free NH₂-group of the protein's first amino acid. Mono-ubiquitylation or mono-SUMOylation are mediated by ubiquitin-conjugating enzymes (Ubcs, E2s) or in the case of SUMO by the single SUMO conjugating enzyme, Ubc9. A single protein can be modified by several mono-ubiquitin monomers, resulting in multi-mono-ubiquitylation.

Mono-ubiquitylation most commonly has a regulatory function as a cellular signal, marking transmembrane receptors for recycling in the lysosome, or alternatively marking specific histone tails, thereby impacting chromatin structure. Yet, it can also serve as a degradation signal. As for SUMO, the three SUMO isoforms differ in their conjugation ability, but all three forms can be conjugated to form mono-SUMOylated substrates. Like ubiquitin, SUMO is bound to the target protein via a covalent attachment of its C-terminal carboxyl group to the ϵ -amino group on the Lys residue of the modified protein. Subsequently, SUMOylated and ubiquitylated proteins are recognized by a specific interaction motif that functions as a 'receptor' for these proteins on their interaction partners. In both cases multiple Lys residues within the target substrate can undergo ubiquitin/UBL modifications, generating multi-ubiquitylated or SUMOylated proteins (Hurley, 2006; Gareau & Lima, 2010).

2.1.2 Poly ubiquitylation and SUMOylation

Successive rounds of ubiquitylation or SUMOylation of a single covalently attached mono-ubiquitin or mono-SUMO molecules will generate poly-ubiquitin or poly-SUMO chains. Ubiquitin- or SUMO-protein ligase enzymes (E3), together with distinct E2s are essential for catalyzing poly-ubiquitylation, SUMOylation, and determine substrate specificity as well as govern chain structure. Following mono-ubiquitylation, E2 and E3 ligases conjugate subsequent ubiquitin units, forming additional iso-peptide bonds between the carboxyl group of the newly added ubiquitin molecule and the ϵ -amino group of the Lys residue of the already covalently attached ubiquitin molecule. Recent work suggests that these chains serve

as a versatile “code” that regulates protein fate, and that the internal structure and length of the chain directly impacts its recognition by “reader” proteins (Weismann et al., 2011).

Poly-ubiquitylation may be linked through any of the seven Lys residues present in the ubiquitin molecule - Lys⁶, Lys¹¹, Lys²⁷, Lys²⁹, Lys³³, Lys⁴⁸ and Lys⁶³ or the ubiquitin N-terminal Met (Met¹). Additional ubiquitin chains can be linked through the same Lys residue within ubiquitin, forming homotypic chains. One unique form are linear poly-ubiquitin chains where the C-terminal Gly of the ubiquitin molecule is sequentially linked to the N-terminal Met of the next ubiquitin molecule. In addition, chains linked through different Lys side chains can form mixed poly-ubiquitin chains (i.e. harbouring alternating Lys linkage types) or even branched trees of ubiquitin molecules in which more than one Lys residue is extended. Thus, while only one isoform of ubiquitin exists, diverse arrays of ubiquitin chains are formed, dictating different globular structures. Aside from the role in proteasomal degradation mediated by chains with a Lys⁴⁸ linkage, other types of poly-ubiquitylation chains regulate protein-protein interactions in processes such as DNA repair or immune signalling. For example, linear poly-ubiquitin chains are important for the regulation of NFκB signalling (as shortly described below, and Harper & Schulman, 2006; Ikeda & Dikic, 2008; Iwai & Tokunaga, 2009; Weismann et al., 2011; Kim et al., 2011; Behrends & Harper, 2011).

K63-linked ubiquitin chains have been reported to function as scaffolds for the recruitment of other signaling proteins upon cytokine stimulation, and recently an emerging unique role for ubiquitin conjugation in TNF-R signaling was characterized. TNF receptor-1 (TNF-R) activation results in K⁶³-linked ubiquitin chains. These chains are specifically generated by two ubiquitin RING E3 ligases named Haeme-Oxidized-IRP2-ubiquitin-Ligase-1 (HOIL-1) and Haeme-Oxidized-IRP2-ubiquitin-Ligase-1-Interacting-Protein (HOIP). HOIL-1 and HOIP along with a third protein, SHARPIN, form the linear ubiquitin chain assembly complex (LUBAC) that catalyzes linear head-to-tail ubiquitylation by ligating the N-terminal Met¹ residue of the ubiquitin molecule to the C-terminal Gly residue of another ubiquitin molecule. TNF- induced LUBAC complex binds to an activator of the NFκB pathway named NFκB Essential Modifier (NEMO). LUBAC conjugates linear poly-ubiquitin chains to NEMO and enhances the interaction between NEMO and the TNF-R signaling complex. Since NEMO is required for efficient activation of the TNF-R signaling complex, LUBAC activity influences activation of the NFκB pathway. Taken together, linear ubiquitylation in this case serves as a survival machinery required for the proper activation of the TNF-R signaling complex, NFκB gene induction, and protection from TNF-induced cell death (Haas et al., 2009; Gerlech et al., 2011; Iwai, 2011; Niu et al., 2011).

As for SUMO, three SUMO1-3 genes encode for proteins that differ from one another in their ability to form SUMO chains. Only SUMO2/3 possess a Lys residue within a consensus motif ΨKXE that facilitates the formation of SUMO chains. SUMO-1 lacks this consensus site and therefore formation of poly-SUMO chains is less favorable. Yet, *in vitro* it can form polymeric chains and can serve as the chain terminator of SUMO chains. Of the eight Lys residues encoded in the SUMO molecule, SUMO chains are predominantly formed via Lys¹¹. SUMOylation of proteins was thought to enhance transcriptional repression; however, new findings suggest a more diverse function for SUMOylation. SUMOylation was found to affect sub-cellular localization and is also involved in intra-nuclear localization, transport and apoptosis, as well as in targeting proteins for ubiquitin-mediated degradation (Ulrich 2008; Matic et al., 2008; Ulrich, 2009).

2.1.3 Ubiquitin and SUMO chain editing

Covalent ubiquitylation or SUMOylation is a reversible process in which de-ubiquitylating enzymes (DUBs) or sentrins/SUMO specific proteases (SENPs), promote the cleavage of the iso-peptide bond and release ubiquitin or SUMO molecules, respectively. About 80 known DUBs, and less than 10 SENPs, are devoted to removing covalent ubiquitin or SUMO modifications. DUBs serve to perform three distinct roles in the cell; First, DUBs can cleave some of the ubiquitin molecules that are transcribed as a linear fusion chain for future conjugation processes. Second, DUBs mediate the removal of ubiquitin from tagged proteins prior to their degradation, allowing for recycling of ubiquitin molecules for future conjugation processes. Third, DUBs can trim ubiquitin chains, subsequently changing their length and structure (Komander, et al., 2009a). Editing of ubiquitin chains by specific ubiquitin peptidases may impact their recognition by the proteasome, or affect protein-protein interaction. Interaction between DUBs and ubiquitylated proteins is mediated through ubiquitin interacting motifs within the DUBs (UIMs and UBD, ubiquitin binding domains, see below and chapter 3.1.1). DUB-mediated chain editing is essential for regulation of chromatin structure, and is involved in DNA damage repair pathways as well as endosomal targeting of membrane bound receptors (Katz, 2010).

One of the well-studied DUBs is the tumor suppressor CYLD (cylindromatosis associated DUB). CYLD is a negative regulator of Wnt, NF κ B and JNK signaling pathways in immunity and inflammation. Among CYLD's targets are substrates with Lys⁶³-linkage ubiquitylation chains like TRAF6, BCL3, PLK1 that regulate cell cycle proliferation and apoptosis (Massoumi, 2010). CYLD also forms a protein complex with the ubiquitin ligase Itch. Together this editing complex inhibits TAK1, which is required for termination of the immune response (Wertz, 2011). Importantly, mutations in CYLD are associated with cylindromatosis, a predisposition to benign tumors of the hair follicle in the skin and other secretory glands. Another work established that CYLD functions as a tumor suppressor and its loss is associated with cancer (Bignell et al., 2000). In this regard, DUBs are emerging as excellent targets for small molecule inhibitors. For example, recent work from the Dixit group revealed that genetically or chemically targeting USP1 induces muscle stem-cell differentiation, and can serve as a molecular target for therapy of osteosarcoma that is highly resistant to conventional chemotherapy (Williams, 2011).

Like ubiquitylation, SUMOylation is also a reversible process, and a family of seven sentrin-specific proteases (SENPs) catalyzes de-SUMOylation. Family members differ from one another based on SUMO chain specificity and cellular localization that is determined by distinct N-terminal domains. Biochemical and genetic experiments revealed that SENPs show high degrees of specificity. Towards example, the SENP6 and SENP7 have greater affinity de-conjugation of di- and poly-SUMO2/3 chains than SUMO1 (Lima & Reverter, 2008). SENPs harbor a Ulp domain at their C-terminus which facilitates the cleavage of the isopeptidic bond between the SUMO molecule and the Lys group on the modified protein. SENP-mediated de-conjugation plays an important role in the regulation of developmental and signaling processes. It is also important for tightly regulating the levels of free SUMO in the cell. The regulatory function of SENPs is biologically relevant in development. SENP3/5 are required for ribosomal biogenesis, and targeting SENP2 during cardiac development impairs the expression of key cardiac factors Gata2 and Gata6 (Yun et al, 2008; Kang et al., 2010). Specifically, in SENP2 null embryos, SUMOylated polycomb group Pc2/CBX4

complex accumulates on the promoters of PcG target genes, leading to repression of Gata4 and Gata6 transcription. SENPs also play a key role in tumorigenesis; expression of SENP1 transforms prostate cancer cells and activates Androgen Receptor (AR) signaling. In addition, SENP3 regulates angiogenesis via its impact on HIF1 α -associated coactivator p300, and elevated mRNA levels of SENP6, 7 are linked to breast cancer (Cheng, 2010; Bawa-Khalfe & Yeh, 2010).

3. Recognition motifs for ubiquitin and SUMO ligases

Are there preferred (consensus) sites for ubiquitylation? While recognition motifs for recruitment of ubiquitin ligases (“Degrons”) exist, it appears that site-specific ubiquitylation is more promiscuous. This variability may stem from the different structural requirements for the diverse interactions of heterogeneous substrates with a variety of E2-E3 complexes. In contrast, the acceptor Lys residues in many SUMOylated proteins reside within a consensus motif Ψ KXE (Ψ - hydrophobic residue; X, any amino acids; E, glutamic or aspartic), forming a unique conformation, which interacts directly with the specific hydrophobic groove on the Ubc9 enzyme. In addition to the canonical SUMOylation consensus sequence, longer consensus sequences with specific characteristics have been identified. Among them are the inverted form of the canonical sequence, a hydrophobic cluster motif enriched with a consecutive sequence of large hydrophobic residues, a phosphorylation dependent motif - PSDM (Ψ KXEXXS^P) and a negatively charged amino-acid motif - NDSM (Ψ KXEXXEEEE). The existence of this highly characterized consensus motif correlates with to the existence of a single unique E2-SUMO conjugating enzyme. Yet, some proteins can be SUMOylated without the presence of the characterized consensus sequence (Ulrich, 2009).

3.1 Recognition of ubiquitylated and SUMOylated proteins

Regardless of the final fate of the modified proteins, covalent tagging by ubiquitin or SUMO is “sensed” by protein motifs that subsequently mediate protein-protein interactions involved in numerous cellular processes. In this section we will discuss the currently known domains that recognize mono and poly-ubiquitin/UBL chains. While our understanding of these interactions is in its infancy, it is the focus of intensive research. For simplicity we will focus on “sensing” ubiquitin and SUMO monomers and polymers.

Proteins modified by ubiquitylation or SUMOylation interact non-covalently with other proteins via ubiquitin binding domains (UBD), or SUMO interacting motifs (SIM), respectively. These motifs are present in many proteins and thereby mediate multiple interactions, and have the potential to induce conformational changes and to impact the avidity of existing protein complexes or to form new protein complexes (see Fig 1).

3.2 Ubiquitin binding domains

UBDs are motifs that enable the association of proteins with either mono ubiquitin or ubiquitin polymers. More than 20 domains have already been identified, and more than 150 different human proteins harbor a versatile combinations of UBDs. Most UBDs interact with ubiquitylated proteins via a hydrophobic patch that include Leu8, Ile44 and Val44 within the ubiquitin molecule on one hand, and a α -helix motif of the UBD on the other hand.

Intensive structure analysis identified that UBD includes, among others, zinc finger interacting domains (ZnF/PAZ/UBZ), plekstrin homology fold (PH fold), and ubiquitin association domain (UBA) as well as ubiquitin-conjugating-like domains (Dikic et al., 2009).

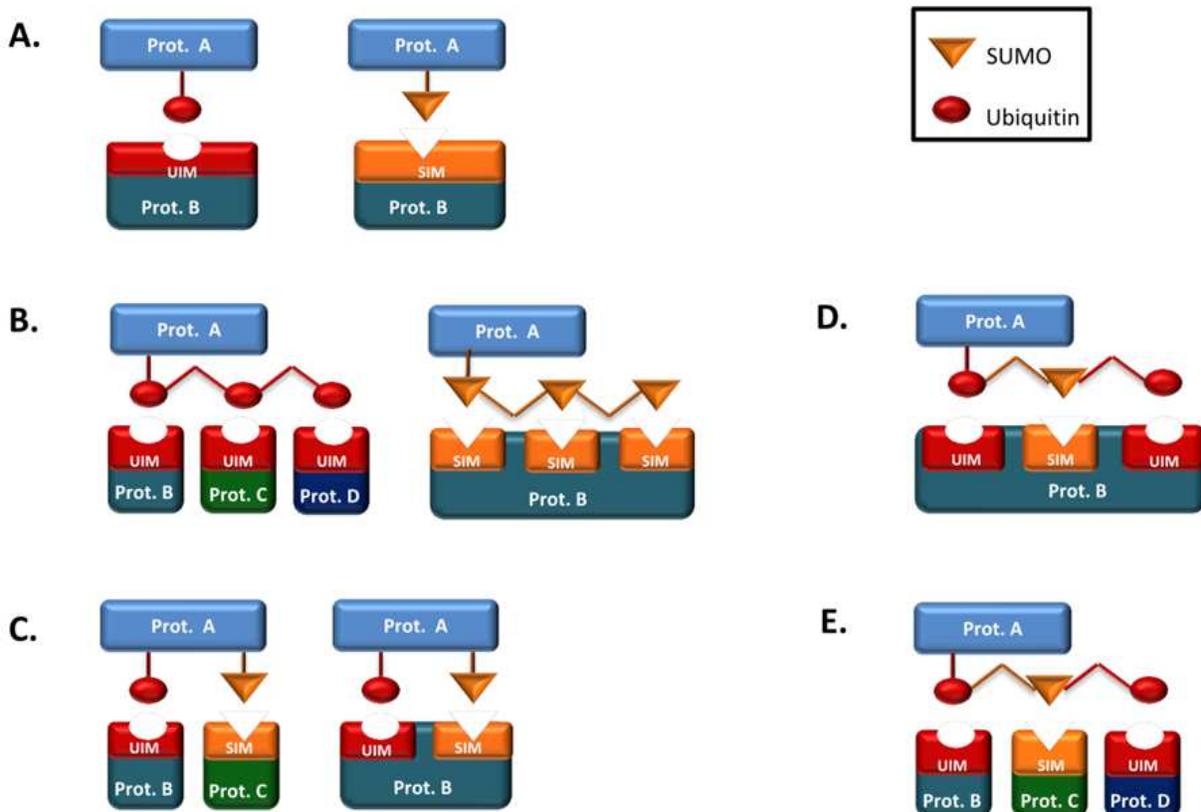


Fig. 1. Regulation of protein-protein interactions by Ub/SUMO signalling

(A). Mono-ubiquitin or mono-SUMO modified substrates are recognized by Ubiquitin Interacting Motif (UIM/UBDs), or by SUMO interacting motifs (SIMs). (B). Poly-ubiquitin or poly-SUMO chains can mediate interaction with several proteins in tandem, or with a single protein harboring several UIM/SIM domains. (C). Combinatorial modifications by mono ubiquitin or SUMO can be sensed by multiple proteins each harboring a discrete interaction motif, or via multiple domains within a single protein. (D-E). Similar to mono ubiquitylation and SUMOylation, modification by UB/SUMO mixed-chains can be recognized by multiple domains either within a single protein (D), or in the context of several proteins within a protein complex (E).

Different UBDs recognize and interact with different ubiquitin polymers. Some UBDs bind specifically to mono-ubiquitylated proteins. For example the mammalian Eap45 subunit of the endosomal sorting complex harbors a UBD domain termed GLUE domain that interacts with the hydrophobic patch of the ubiquitin molecule (Hirano et al., 2006).

Interestingly, the classical α -helix ubiquitin-interacting motif UIM, such as the one that is present in the proteasome S5a subunit and yeast Rpn10 as well as RAP80, can be found in some cases in an “inverted” orientation to form “inverted UIM” (IUIM/MIU). In most cases, each UBD interacts with a single ubiquitin molecule. Yet, ZnF binding domains, such as the

one found in the guanine nucleotide exchange factor RABEX; interact with multiple surfaces on ubiquitin. Hence, in this case a single ubiquitin molecule can interact with three ZnF motifs simultaneously (Penengo et al., 2006). Tandem repeats of UBDs may dictate chain specific interaction. For example, RAP80, which is recruited together with BRCA1 to damage sites, harbors two adjacent UIMs that binds Lys⁶³ but not of Lys⁴⁸ Linked-poly-ubiquitin chains (Hicke et al., 2005; Harper & Schulman, 2006; Komander 2009b; Dikic et al. 2009). In contrast, the ubiquitin receptor RAD23A, that is required for targeting proteins to the proteasome, has a C-terminal UBA domain. RAD23 UBA domain has a 6.3 fold higher affinity to Lys⁴⁸ than to Lys⁶³ chains and a 70 fold higher affinity to Lys⁴⁸ chains than to free ubiquitin (Raasi et al., 2005).

The specificity of different UBDs toward chain linkage is greatly dependent on UBDs present in DUBs. For example, while isoT is dedicated for the de-conjugation of Lys⁴⁸ chains, CYLD is specific for Lys⁶³. Recently a novel UBD that recognizes linear poly-ubiquitin chains (UBAN) was characterized. Importantly, the ability of a cell to respond to TNF α and to activate the IKK kinase complex is compromised in cells that have a mutated UBAN domain within the NEMO protein that is required for activation of the IKK complex (Rahighi et al., 2009; Lo et al., 2009). Likewise, recent work has shown that the ESCRT sorting complex that is involved in targeting the EGF receptor for lysosomal degradation is based on a combination of various UBDs on different ubiquitin receptors. Together these ubiquitin receptors form a large protein complex harboring a high avidity interaction surface with an ubiquitylated cargo (Raiborg & Stenmark, 2009).

An interesting case where binding to ubiquitin chains via ubiquitin receptors plays a key role is autophagy. Autophagy is used by macrophages as a defense mechanism against infection by invading intracellular bacteria. A molecular link between autophagy and ubiquitylation was established following the identification of autophagy receptors, which simultaneously bind both ubiquitin and autophagy-specific ubiquitin-like modifiers (Atg8). Several ubiquitin-related autophagy pathways have already been characterized such as the ubiquitin-NDP52-LC3 pathway, which targets group-A-Streptococcus, *Salmonella typhimurium*, or the ubiquitin-p62-LC3 pathway, which targets *Mycobacterium tuberculosis* and *Listeria monocytogenes*. *Listeria* is a gram-positive pathogen that expresses several virulence proteins including a hemolysin (listeriolysin O, LLO). LLO bacterial proteins in macrophages infected with *Listeria* were found to form small aggregates associated with poly-ubiquitin chains and to undergo selective autophagy by the p62-LC3 pathway (Ogawa et al., 2011). A recent report determined that targeting the SUMO conjugating E2, Ubc9, for degradation and subsequently inhibiting SUMOylation mediate part of the virulence of *Listeria*. Furthermore the Dikic lab recently showed that invading *Salmonella* are coated with poly-ubiquitin chains ligated by a yet to be identified ligase. Subsequently, the ubiquitin chain binding proteins p62 and NDP52 bind to the poly-ubiquitin chains and recruit the protein Optineurin (OPTN) that upon its phosphorylation by Tank Binding Kinase (TBK) connects the coated pathogen to LC3 autophagy receptors allowing the engulfment and autophagy of the pathogen. Thus, an emerging network of interactions between the SUMO pathway, ubiquitin chains, ubiquitin binding proteins and ubiquitin like proteins (Atg8) plays a key role in elimination of bacteria and innate immunity (Wild et al., 2011; Weidberg et al., 2011).

3.3 SUMO interacting motifs

In analogy to ubiquitylation, SUMOylation is also “sensed” by a specific protein motif, termed SUMO Interacting Motif (SIM). SIM motifs are characterized by a sequence motif of hydrophobic amino acids (V/I) X (V/I) (V/I). The SIM domain interacts with the hydrophobic patch on SUMO. This hydrophobic interaction is re-enforced with other weak non-covalent interactions that are formed between basic residues on the SUMO and the acidic residues flanking the SIM domain (Gareau & Lima, 2010). SIM-mediated recruitment plays a key role in transcriptional repression. SUMO/SIM-mediated binding is required for the recruitment of histone de-acetylases and histone de-methylases to co-repressor complexes, as well as impacts the activities of chromatin remodeling factors. Examples for these interactions are the SIM/SUMO dependent recruitment of HDAC2 to SUMOylated Elk2, and the SUMO2/3 and SIM dependent recruitment of the Lys demethylase (LSD) to the CoRest co-repressor complex. In this case, CoREST1 binds directly and non-covalently SUMOylated REST (NRSF) to bridge HDAC2 and LSD (Yang & Sharrocks, 2004; Gill, 2005; Ouyang & Gill, 2009). Furthermore, SUMO/SIM interactions are likely to impact nucleosome remodeling as the recruitment of the ATP-remodeling complex protein Mi-2 to the transcription factor SP3 is SIM/SUMO dependent (Stielow et al., 2006).

SIMs and UBDs are targets for posttranscriptional modification (PTMs). These PTMs impact the structural properties of SIMs, UBDs or their immediate vicinity, resulting in a change in the binding properties of the modified domains. For example a CKII-mediated phosphorylation site near the SIM domain in the co-repressor DAXX shifts specificity between SUMO prologs. The DAXX (Fas death domain associated protein) is a transcriptional co-repressor that binds to a variety of transcription factors at the promoter sites of anti-apoptotic genes. The SIM domain within DAXX facilitates a non-covalent interaction with other SUMOylated proteins. DAXX binding to SUMO-1 but not SUMO2/3 is enhanced by CKII phosphorylation of DAXX Ser^{737, 739} surrounding its SIM domain, enhancing its recruitment and subsequent transcriptional repression of these anti-apoptotic genes (Chang et al., 2011; Mukhopadhyay & Matunis 2011). Thus, the observations that SIMs and likely UBDs are targeted to PTMs by signaling pathways provide evidence for another layer of regulation that establish a direct crosstalk between signaling pathways and ubiquitin/SUMO signals.

4. Cross talk between ubiquitylation, SUMOylation, and the function of SUMO-targeted ubiquitin ligases

While both ubiquitin and SUMO pathways have been well studied individually, the long-suspected nature of the crosstalk between SUMO and ubiquitin pathways has been molecularly enigmatic. Importantly the interplay between SUMOylation and ubiquitylation can be a critical determinant in signaling, transcription, and cancer (Karscher 2006). For example, the equilibrium between SUMOylation and ubiquitylation can influence the balance between p53 nuclear localization and stabilization, cytoplasmic export and degradation, as well as regulating the activity and stability of Hypoxia Induced Factor (HIF; Lee et al., 2006; Carter et al., 2007; Carbia-Nagashima et al., 2007).

The crosstalk between Ubiquitin and SUMO is mediated at several levels. First, SUMOylation or ubiquitylation on the same Lys residue can differentially regulate the

activity and fate of several proteins. For example, ubiquitylation of Lys¹⁶⁴ of the Proliferating Small Nuclear Antigen (PCNA), which is required for replication and DNA damage response, enhances the recruitment of translesion error-prone DNA polymerases. Yet, genetic evidence suggests that SUMOylation at this site by the SUMO ligase Siz-1 at S-phase promotes PCNA association with the Srs2 helicase and restricts the helicase activity (Bergink & Jentch 2009). Second, enzymes within each pathway are targets for modification by the other pathway. For example, the ubiquitin ligase E2-25k undergoes SUMOylation at its core domain that inhibits its activity. Another example is the DUB USP25 that is regulated by both SUMOylation and ubiquitylation. In this case SUMOylation inhibits its function, and ubiquitylation at the same site enhances its enzymatic activity. An interesting case is the ubiquitin/SUMO ligase TOPORS. TOPORS is unique as it can catalyze the formation of either ubiquitin or SUMO chains. Importantly, a phospho-switch induced by the polo like kinase, PLK1 results site-specific phosphorylation of TOPORS, inhibiting its ability to SUMOylate, but enhancing its ubiquitylation activity (Yang et al., 2009).

However, until recently it was not clear how does the cell directly “sense” and integrate the ubiquitin and SUMO signals at the single protein level. A first direct and enzymatic link between the two pathways was established by the identification of SUMO-targeted ubiquitin ligases (STUbLs). STUbLs are a unique group of RING proteins: they bind non-covalently to the SUMO moiety of SUMOylated proteins via several SIM motifs, and subsequently target the SUMOylated protein for ubiquitylation via a RING domain [Sun et al., 2007; Geoffroy & Hay, 2009; Abed et al 2011b]. STUbLs impact protein stability, localization, and are required for the maintenance of genomic integrity, transcription and are involved cancer. Thus, STUbLs integrate the SUMO and ubiquitin pathway and generate a SUMO/ubiquitin dual signal that may serve as an additional level of regulation of protein-protein interactions.

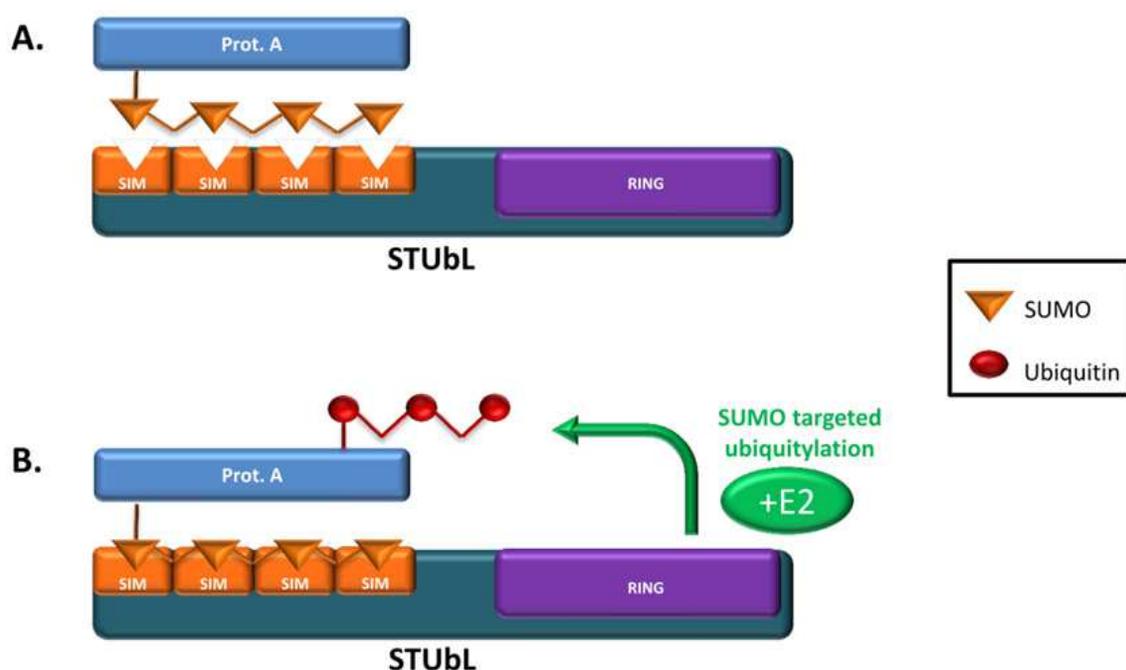


Fig. 2. Classical mode of action of STUbL: STUbL interact with SUMOylated-proteins via its SUMO Interacting Motifs (SIM). Subsequently, dimers of STUbL proteins interact with charged E2 -Ub complex, and catalyze poly-ubiquitylation via the RING domain.

4.1 Characterization of STUbL proteins

STUbLs are highly conserved in eukaryotes. Members of the STUbL family include for example: the yeast *S. pombe* Slx8-Rfp; *S. cerevisiae* Slx5-Slx8; *H. sapiens* RNF4; *D. discoideum* MIP1; and the *D. melanogaster* Degringolade (Dgrn). Yet, no clear STUbL orthologs exist in the worm *C. elegans*. These members are structurally and functionally conserved, as RNF4 protein can substitute for the yeast and fly genes in functional assays (Abed et al., 2011a; Abed et al., 2011b; Barry et al., 2011; Praefcke et al., 2011). Recent structural work from the Hay lab uncovered that RNF4, and likely other STUbLs, function as dimers and that dimer formation is actively required to facilitate SUMO-dependent ubiquitin conjugation (Plechanovova' et al. 2011).

Several observations link STUbLs to protein degradation: 1. STUbLs bind and ubiquitylate SUMO chains, 2. STUbLs enhance the degradation of SUMOylated proteins 3. Genetic ablation of STUbL genes in yeast, flies, and cancer cells results in accumulation of poly-SUMOylated proteins. Among the most studied substrates of RNF4 are the promyelocytic leukaemia protein, PML and its derived oncogene PML-RAR. An elegant set of experiments by several groups established that arsenic-induced phosphorylation enhances poly SUMOylation of PML and recruitment of RNF4. Subsequently, RNF4-dependent ubiquitylation targets SUMOylated and ubiquitylated PML for degradation via the 26S proteasome (Lallemand-Breitenbach et al., 2008; Tathem et al., 2008). This is highly relevant to the treatment of acute promyelocytic leukemia, where a combination of Retinoic Acid (RA) with arsenic treatment can result in 90% cure (Lallemand, 2011). Other bona-fide human substrates of RNF4 are the kinetochore proteins CENP-I and VHL. In addition, proteomic analysis using RNF4 as a bait identified a wide spectrum of proteins that are bound by RNF4 (Makhopadhyay et al., 2010; Tatham et al., 2011). While the exact nature of these interactions requires further characterization, GO analysis already points out that SUMO-dependent ubiquitylation by RNF4 is relevant to a large variety of protein complexes and involves diverse cellular processes.

An interesting issue is the recognition of proteins by STUbLs. While by their definition STUbLs recognize SUMOylated substrates via their SIM motifs, recent reports suggest a more complex picture. For example, the recognition of the Mat2 α repressor by Slx5: Slx8 does not require substrate SUMOylation, but does require intact SIM motifs (Xia et al., 2010). Furthermore, the binding of the *Drosophila* STUbL Dgrn to the Notch-related HES repressor proteins is independent of SUMOylation, and is mediated by Dgrn's RING domain and not the SIM motif. Yet, the SIM motifs are required for ubiquitylation *in vitro* and for Dgrn's impact *in vivo* (Abed et al., 2011a, Barry et al., 2011). Thus, it is highly likely that specific interactions are determined *in vivo* by a dual recognition machinery, where the SIM motif interacts with the poly-SUMO chain and the RING domain interacts with other non-SUMO determinants in the target protein or adjacent proteins. In addition, recent work suggests that RNF4 can interact with proteins such as Nip45 that harbour two SUMO Like Domains (SLDs), but are not SUMOylated, thus expanding the spectrum of RNF4 targets (Sekiyama et al., 2010).

4.2 Cellular processes regulated by STUbLs

STUbLs are required for normal development, for the cell's ability to cope with genotoxic stress, and to maintain genome stability (Prudden et al., 2007; Nagi et al. 2008; Nagi et al.,

2011 and Barry et al., 2011). During mouse development, RNF4 is highly expressed in the stem cell compartment of the developing gonads and brain (43). RNF4 was also identified as a gene that is specifically expressed in hematopoietic, embryonic, and neural progenitor cells, likely representing its role in “stemness” (Galili et al., 2000; Ramalho-Santos et al., 2002). During early *Drosophila* development Dgrn localizes to centrosomes, and *dgrn* null embryos accumulate SUMOylated proteins. *dgrn* null embryos show genomic instability phenotypes; they fail to incorporate DNA into centrosomes, assemble aberrant mitotic spindles and exhibit chromosomal bridges at anaphase. Cells in *dgrn* null embryos show high SUMO content and fall from the embryo surface into the center of the syncytium (Barry et al., 2011). These findings fit well with those reported for the yeast STUbLs, as yeast lacking *Slx5:Slx8* display genomic instability, and are hypersensitive to replication stress. For example, yeast deficient in *Slx5:Slx8* fail to replicate DNA upon hydroxy-urea treatment (Prudden 2007, Rouse, 2009). While the protein substrates of STUbLs in this context are still unknown, the observations that many of the proteins involved in the DNA damage response are ubiquitylated, SUMOylated, or contain SLD motifs suggest that STUbLs are targeting specific regulatory “nodes” in the DNA response network. Since genomic instability is a hallmark of cancer cells, proteins such as STUbLs may be the “Achilles heel” in specific cancerous settings. Therefore we predict that STUbLs inhibition will result in collapse of the tumorigenic network and cancer elimination.

Interestingly, and prior to the identification of RNF4 as a dedicated STUbL protein, RNF4 was identified by the Palvimo lab as a potent transcriptional regulator that functions both as a co-activator or a co-repressor depending on the cellular context. For example RNF4 was shown to be essential for androgen and steroid receptor-mediated target gene activation (Moilanen et al., 1998; Poukka et al., 2000;). In addition, we found that in transcription the consequences of Dgrn activity are not strictly limited to targeting SUMOylated proteins for degradation (Abed et al., 2011a; Barry et al., 2011). Importantly, Dgrn/RNF4-mediated ubiquitylation impacts the affinity between proteins, inhibiting the interaction of a given protein with one protein but not affecting its ability to bind other protein. Specifically, we found that during fly development Dgrn serves as a molecular selector that determines co-repressor recruitment as described below. Dgrn-mediated ubiquitylation of the HES-related repressor Hairy inhibits its ability to interact with its co-repressor Groucho but not with other Hairy co-repressors such as dCtBP. In addition, we find that Dgrn specifically targets SUMOylated Gro for sequestration. Yet, the exact cellular and molecular details surrounding sequestration require further exploration. Accordingly, Dgrn antagonize Hairy/Groucho-mediated repression in transcription and function in cells and *in vivo*. Genome wide association studies using DamID profiling unveiled that the activity of Dgrn is relevant genome wide. Thus, Dgrn serves as a “molecular selector” that determines protein-protein interactions. We found that this “selector” activity of Dgrn/RNF4 is likely relevant also to HES independent processes and in other types of co-factor switches, and may directly impact chromatin structure (Abed et al., 2011a; Hu et al., 2010; Orian unpublished). We speculate that this activity of STUbLs will be highly relevant not only in transcription, but in the regulation of protein-protein interactions in other cellular process such as the selective recruitment of proteins to DNA repair foci.

5. Conclusion and future challenges

We focused on ubiquitin and SUMO signalling as a mode to regulate protein-protein interactions. We predict that the lessons learned during the last decades regarding ubiquitin and SUMO will be highly relevant for other UBLs and non UBL modifications. An important concept that emerges from these studies is that combinatorial posttranscriptional modifications by ubiquitin/UBLs serves as a molecular tool to regulate and establish diverse and selective, signal-induced protein-protein interactions. Furthermore, proteins that have ubiquitin like or SUMO like domains, and the ability of specific enzymes to catalyse different and distinct chains of ubiquitin/UBL proteins, further add to this diversity. This complexity is also reflected at the level of “reader” proteins that contain several UBD/UBL recognition motifs and that bind only a discrete combinatorial ubiquitin/UBL signal. Thus, we can envision how a relatively small number of signalling pathways and a limited pool of ubiquitin/UBLs can generate discrete protein-protein interactions. We predict that a key objective for future studies will be to understand the enzymatic machinery that dictates selective recruitment in distinct cellular processes. The identification of these enzymes has direct implications beyond basic research. It will pave the way to design highly selective inhibitors that will impact specific pathways with minimal side effects, features that are desired in the clinic such as in the case of cancer treatments.

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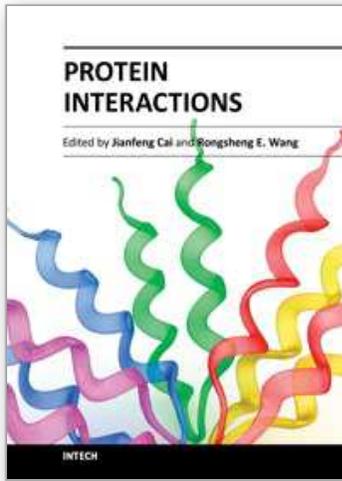
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Protein interactions, which include interactions between proteins and other biomolecules, are essential to all aspects of biological processes, such as cell growth, differentiation, and apoptosis. Therefore, investigation and modulation of protein interactions are of significance as it not only reveals the mechanism governing cellular activity, but also leads to potential agents for the treatment of various diseases. The objective of this book is to highlight some of the latest approaches in the study of protein interactions, including modulation of protein interactions, development of analytical techniques, etc. Collectively they demonstrate the importance and the possibility for the further investigation and modulation of protein interactions as technology is evolving.

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