

Role of Ubiquitin and SUMO in Intracellular Trafficking

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Abstract

Precise location of proteins at a given time within a cell is essential to convey specific signals and result in a relevant functional outcome. Small ubiquitin-like modifications, such as ubiquitin and SUMO, represent a delicate and diverse way to transiently regulate intracellular trafficking events of existing proteins in cells. Trafficking of multiple proteins is controlled reversibly by ubiquitin and/or SUMO directly or indirectly via regulation of transport machinery components. Regulation is dynamic and multilayered, involving active crosstalk and interdependence between post-translational modifications. However, in most cases regulation appears very complex, and the mechanistic details regarding how ubiquitin and SUMO control protein location in cells are not yet fully understood. Moreover, most of the findings still lack *in vivo* evidence in multicellular organisms.

behaviour. In addition to e.g. chemical groups, such as phosphate groups, proteins can be modified by small polypeptides, such as ubiquitin and SUMO. Ubiquitin and SUMO share a similar three-dimensional structure and are principally covalently linked at lysine (K) residues of substrate proteins by a similar conjugation pathway (Hershko *et al.*, 1998; Hay, 2013). Unlike ubiquitin, SUMO is preferentially attached at SUMO consensus sites ΨKxE in substrates Ψ = hydrophobic residue with high preference for I or V, x = any amino acid) under steady state conditions, but under stress conditions in particular more non-consensus sites are SUMOylated (Hendriks *et al.*, 2016). Mammals express ubiquitously SUMO1, SUMO2 and SUMO3, whereas SUMO4 and SUMO5 are only expressed in some tissues and their functional role is unclear (Pichler *et al.*, 2017). SUMO2 and SUMO3 are nearly identical and resemble approximately 50% to SUMO1 (Geiss-Friedlander *et al.*, 2007).

Precursors of ubiquitin and SUMO are processed to mature forms prior to conjugation and activated by an E1 enzyme in a reaction depending on ATP. Subsequently ubiquitin and SUMO are transferred to an E2 enzyme, and E3 ligase alone or with E2 ligates them to substrates by an isopeptide bond (Hershko *et al.*, 1998; Hay, 2013). Modification is reversible and specific proteases can cleave ubiquitin and SUMO from substrates (Williamson *et al.*, 2013; Kunz *et al.*,

Posttranslational modifications in regulation of cellular processes

General principles of ubiquitination and SUMOylation

Posttranslational modification (PTM) of proteins is a powerful, fast and often transient way to control the fate of existing proteins and cell

2018). Proteins can be tagged by a single ubiquitin or SUMO either at one or at multiple lysines. In addition, modifications can exist as chains. Ubiquitin contains seven internal lysine residues (K6, K11, K27, K29, K33, K48, K63) that are involved in the formation of ubiquitin-ubiquitin polymers, known as polyubiquitin chains (Pickart *et al.*, 2004; Heride *et al.*, 2014). In addition, the linkage between the amino-terminal amino group of methionine on a ubiquitin can be conjugated with a target protein and the carboxy-terminal carboxy group of the incoming ubiquitin for linear chains (Walczak *et al.*, 2012). SUMOs can also form polymeric chains through internal lysine residues (Geiss-Friedlander *et al.*, 2007). Monoubiquitination and different chain types determine the fate of the modified protein (Piper *et al.*, 2014). For example, K48 ubiquitin chains are considered classical signals for proteasomal degradation and K63 ubiquitin chains are linked to trafficking and DNA damage response (Pickart *et al.*, 2004). Recently basic principles of the field have been challenged by e.g. discoveries of mixed polyubiquitin chains and ubiquitination of non-lysine residues (Piper *et al.*, 2014). Less is known regarding the consequences of attachment of either single or many SUMO moieties. SUMO chains are at least implicated in recruitment of SUMO-targeted ubiquitin ligases (Lallemand-Breitenbach *et al.*, 2008).

Components, regulation and function of ubiquitin and SUMO machinery

Multiple enzymes involved in ubiquitin conjugation have been recognized. The human ubiquitin machinery comprises a network including two ubiquitin E1 enzymes, approximately 40 ubiquitin E2s, and more than 600 E3 ubiquitin ligases in the human genome (Heride *et al.*, 2014). Three major types of E3 ligases are really interesting new gene (RING) type E3s comprising most of human E3s, homologous to E6-AP carboxyl terminus (HECT) and RING-between-RING (RBR) E3s ligases (Dessaies *et al.*, 2009; Rotin *et al.*, 2009; Wenzel *et al.*, 2012). Ubiquitin is cleaved by approximately 100 deubiquitinating enzymes (DUBs) (Williamson *et al.*, 2013; Heride *et al.*, 2014). Intriguingly, only one heterodimeric

E1 (Sae1/Aos1–Sae2/Uba2) and E2 (Ubc9) are known to be involved in SUMO conjugation (Hay, 2013). Three classes of SUMO E3s have been widely accepted and characterized including SP-RING Siz/PIAS ligases, RanBP2 and ZNF451 ligases (Geiss-Friedlander *et al.*, 2007; Rytinki *et al.*, 2009; Cappadocia *et al.*, 2015). Both E2 and E3 can select substrates for SUMOylation, and spatial and temporal regulation of co-localization appears integral for substrate selection (Pichler *et al.*, 2017). Cysteine proteases of the sentrin-specific protease (SENP) family members reverse SUMO conjugation in mammals (Kunz *et al.*, 2018). Moreover, desumoylating isopeptidases 1 and 2 and ubiquitin-specific protease-like 1 can deSUMOylate proteins (Shin *et al.*, 2012; Schulz *et al.*, 2012). Whereas ubiquitin machinery is widely expressed within a cell, components of SUMO conjugation pathway mainly localize at the nuclear pores and nucleus. Thus, most of SUMOylated substrates are nuclear proteins, although SUMO modified proteins outside of nucleus exist (Geiss-Friedlander *et al.*, 2007).

Ubiquitination is regulated by extracellular stimuli including growth factors and cytokines, stress and cell cycle changes (Pickart *et al.*, 2004; Heride *et al.*, 2014). Different types of stress stimuli such as heat shock, hypoxia, reactive oxygen species, DNA damage and proteotoxic stress regulate the activity of SUMOylation machinery (Hietaakangas *et al.*, 2003; Shao *et al.*, 2004; Bossis *et al.*, 2006; Galanty *et al.*, 2009; Morris *et al.*, 2009; Seifert *et al.*, 2015). Both ubiquitin and SUMO modifications can alter the function, activity, location and stability of their targets. Ubiquitin and SUMO are recognized by either ubiquitin-binding domains (UBD) or sumo-interacting domains (SIM), respectively, and serve as platforms for non-covalent protein–protein interactions (Seet *et al.*, 2006). These domains have been identified in hundreds of proteins. PTMs are also involved in regulation of conjugation specificity and activation (Pichler *et al.*, 2017). For example, SUMO-targeted ubiquitin ligase (STUbL) RNF4 contains multiple SIMs and a RING-domain to bind SUMOylated proteins and an E2 ubiquitin-conjugation enzyme (Sun *et al.*, 2007; Tatham *et al.*, 2008).

Ubiquitin and SUMO as signals regulating membrane trafficking and endocytosis

General principles of membrane protein trafficking and endocytosis in cells

Internalization and endocytosis of cell surface proteins including different receptors often occurs via the clathrin-dependent endocytic pathway. Cell surface receptors are clustered to pits coated with clathrin that pinch off of the membrane forming vesicles and early endosomes. From early endosomes cargo can be directed back to plasma membrane via recycling endosomes or destined to lysosomal degradation via late endosomes and multivesicular bodies (Mellman and Yarden, 2013). Also, other types of endocytic routes exist, including cholesterol-rich membrane structures, such as lipid rafts and caveolae (Barbieri *et al.*, 2016). Several adaptor proteins and PTMs control these processes (Piper *et al.*, 2014). Membrane protein trafficking and endocytosis system are tightly connected to protein homeostasis.

Ubiquitin and SUMO in receptor internalization at the cell surface and in endocytic compartments

Initial studies in yeast suggested that ubiquitin can function as a sorting signal regulating the internalization and endosomal targeting of cell surface receptors (Kölling *et al.*, 1994; Hicke *et al.* 1996, Terrell *et al.*, 1998). After that several studies in different systems and organisms have confirmed that ubiquitin is important regulator of endocytosis and its most critical functional role is likely at the sorting endosomes (Mellman and Yarden, 2013). Endocytosis of human receptor tyrosine kinases (RTKs) is suggested to be regulated by multimono-ubiquitination and K63-polyubiquitination, and there is some controversy regarding the significance of specific ubiquitination type due to methodological challenges to address this complex regulatory system (Haglund *et al.*, 2003; McCullough *et al.*, 2004; Huang *et al.*, 2006; Sundvall *et al.*, 2008; Huang *et al.*, 2013). During endocytosis the ubiquitin moieties of cargo are recognized by different endocytic adaptors and regulators via UBDs, such as Eps15,

epsin and endosomal sortin complex required for transport (ESCRT) (Piper *et al.*, 2014). Ubiquitin is also indirectly involved in control of endocytosis as components of endocytic machinery are actively regulated by ubiquitination (Piper *et al.*, 2014).

SUMOylation has been implicated in the regulation of endocytic processes, although when compared to ubiquitin the evidence is less extensive, and more work is needed to make general conclusions. Nevertheless, endocytosis of kainate receptor GluR6 is regulated by SUMOylation and non-SUMOylated mutant of GluR6, GluR6-K886R, is endocytosis-impaired due to unknown mechanisms (Martin *et al.*, 2007). SUMOylation of Smoothed (Smo) promotes its localization at the cell surface (Ma *et al.*, 2016). Intriguingly, mechanistically SUMO interferes with efficient Smo ubiquitination by recruiting deubiquitinase UBPY/USP8 in a SIM-dependent manner (Ma *et al.*, 2016). SUMOylation regulates also cell surface expression and activity of VEGFR2 receptor tyrosine kinase (Zhou *et al.*, 2018). VEGFR2-SUMO1 fusion protein but not SUMOylation defective mutant VEGFR2 accumulated at the Golgi suggesting that mechanistically SUMO regulates exocytosis of VEGFR (Zhou *et al.*, 2018). SUMOylation can also indirectly regulate endocytosis. Components of endocytic machinery are modified and regulated by SUMO, such as CIN85 (Tossidou *et al.*, 2012) and arrestin (Wyatt *et al.*, 2011). Interestingly, also dynamin interacts with several members of the SUMOylation machinery (Mishra *et al.*, 2004). Moreover, SUMO can be important for membrane binding of proteins. SUMOylation of PTEN at lysine 266 within CBR3 loop fosters binding of PTEN to plasma membrane via electrostatic interactions (Huang *et al.*, 2012).

Ubiquitin and SUMO as signals regulating nucleocytoplasmic shuttling and subnuclear targeting

General principles of nuclear import, subnuclear targeting and export

Passage of proteins in and out of the nucleus through nuclear pore complexes is tightly regulated.

In principle, many of the nuclear proteins contain nuclear localization signal (NLS) and nuclear export signal (NES) that facilitate trafficking via associations with karyopherins including importins and exportins (CRM1) together with Ran-GTP, respectively (Stewart M, 2007).

Ubiquitin and SUMO in nuclear targeting and trapping

Molecular mechanism by which ubiquitin machinery controls protein functions are very complex but, nevertheless, some evidence exists to suggest the role for ubiquitin as a signal controlling nucleocytoplasmic trafficking. Ubiquitination of p53 contributes to nuclear export, and although the regulation of p53 ubiquitination and trafficking has turned out to be very complicated and likely dependent of conditions, attachment of mon-ubiquitin in particular is suggested to play a role in trafficking (Lohrum *et al.*, 2001; Li *et al.*, 2003). Moreover, similar type of regulation has been suggested for another tumour suppressor protein, PTEN and NF- κ B essential modulator NEMO (Huang *et al.*, 2003; Trotman *et al.*, 2007).

SUMO was initially discovered as a modifier of RanGap1 targeting it to nuclear pore complex (Matunis *et al.*, 1996, Mahajan *et al.*, 1997). Later SUMO has been implicated in both increasing and decreasing the nuclear accumulation of some proteins. Mechanistically, covalent linkage of SUMO may directly block protein interactions relevant for transport or generate SIM-mediated interaction platform facilitating or interfering with transport. SUMOylation is suggested to regulate nuclear import of full-length IGF receptor (Sehat *et al.*, 2010). The levels of SUMOylation defective mutant IGFR are similar at the cell surface compared with wild type receptor, but the mutant receptor cannot translocate to nucleus unlike wild type (Sehat *et al.*, 2010). Specifically IGFR is suggested to interact with RanBP2 at nuclear pores and that RanBP2 acts as an SUMO E3 ligase for IGFR (Pancham *et al.*, 2015). SUMOylation may also increase the stability of IGFR (Pancham *et al.*, 2015). Trafficking of SUMO machinery components is also under the control of SUMO. SUMOylation of Sae2 in the c-terminus within functional NLS efficiently increases nuclear accumulation (Truong *et al.*, 2012).

SUMO has been suggested to regulate nuclear export of some proteins, including transcriptional repressor TEL, Kruppel-like transcription factor (KLF2), Serine hydroxymethyltransferase 1 (SHMT1), PTEN, p53, ErbB4 and Notch (Wood *et al.*, 2003; Du *et al.*, 2008, Anderson *et al.*, 2009; Bassi *et al.*, 2013, Santiago *et al.*, 2013, Knittle *et al.*, 2017; Antila *et al.*, 2018). SUMOylation adjacent to NES of KLF5 interferes with its interaction with nuclear export receptor CRM1 resulting in the inhibition of efficient export and increased accumulation in the nucleus (Du *et al.*, 2008). ErbB4 RTK undergoes regulated intramembrane proteolysis (RIP) releasing an intracellular domain (ICD) that can translocate to nucleus and regulate transcription. PIAS3 catalysed SUMOylation within NES of ErbB4 increases the nuclear accumulation of a tyrosine phosphorylated ICD by altering the interaction with CRM1 (Sundvall *et al.*, 2012; Knittle *et al.*, 2017). SUMOylation deficient mutant of ErbB4 accumulates less in nucleus and cannot convey efficiently nuclear signalling (Knittle *et al.*, 2017). Another receptor undergoing RIP, Notch, is also SUMOylated in the nucleus and SUMOylation increases nuclear accumulation of the ICD, but mechanisms resulting in accumulation remain to be elucidated (Antila *et al.*, 2018). SUMOylation of SHMT1 increases nuclear accumulation and mutation of the SUMO motif prevents translocation to the nucleus due to unknown mechanisms (Anderson DD *et al.*, 2009). Interestingly, same sites are also ubiquitinated with K63 polyubiquitin chains increasing stability in the nucleus (Anderson *et al.*, 2012). SUMOylation of PTEN (at K254) is suggested to regulate the efficient nuclear accumulation and subsequently DNA damage response. When compared to wild type, SUMOylation deficient mutant of PTEN localizes less into the nucleus and cannot efficiently regulate homologous recombination (Bassi *et al.*, 2013). Nucleocytoplasmic distribution of SUMOylation-deficient transcriptional repressor TEL also changes compared to wild type (Wood *et al.*, 2003). However, the direct mechanism how SUMO regulates subcellular localization of PTEN or TEL is not clear (Wood *et al.*, 2003; Bassi *et al.*, 2013). On the contrary, SUMOylation of p53 stimulates its nuclear export by increasing the

disassembly of p53 from the CRM1 in the cytosol (Santiago *et al.* 2013). Moreover, SUMOylation regulates nuclear export and intranuclear distribution of adenovirus E1B-55K protein (Kindsmüller *et al.*, 2007). Altogether nuclear export and SUMOylation appear to be closely connected due to CRM1-mediated interaction of export complexes with SUMO E3 ligase RanBP2 (Ritterhoff *et al.*, 2015) and SUMOylation also regulates nuclear transport via covalent modification of transport machinery (Rothenbusch *et al.*, 2012).

Regulation of subnuclear targeting by ubiquitin and SUMO

Ubiquitin and SUMO system has been indicated in targeting proteins into certain subnuclear structures. Subnuclear targeting can direct proteins into locations essential for their functions, trap protein in locations where they are not available to regulate e.g. transcription or alter their susceptibility to regulators of stability. Both ubiquitin and SUMO are implicated in correct targeting of DNA repair factors to the sites of DNA damage (Ulrich, 2014). For example, BRCA1 targeting to double-strand breaks is regulated by K63-linked ubiquitination and SUMO and the process involves RNF4 (Galanty *et al.*, 2009; Morris *et al.*, 2009; Guzzo *et al.*, 2012). PML bodies are subnuclear structures involved in regulation of transcription and host a lot of transcription factors and their regulators (Zhong *et al.*, 2000a). PML is strongly SUMOylated and SUMOylation regulates the integrity of PML bodies and stability of PML as SUMOylation deficient mutant PML does not form nuclear bodies when expressed in PML null cells (Ishov *et al.*, 1999; Müller *et al.*, 1998; Zhong *et al.*, 2000a,b). Several nuclear proteins are hosted in PML bodies and SIM-mediated interactions are thought to be important for assembly (Shen *et al.*, 2006). SUMO E3 ligases and deSUMOlases have specific nuclear localizations and regulate substrate localization, but regulation is often not SUMO-dependent (Sachdev *et al.*, 2001; Kotaja *et al.*, 2002; Hietakangas *et al.*, 2003). On the other hand, SUMO is suggested to be important in the regulation of subnuclear localization of Nuclear Factor of Activated T-cells (NFAT1) (Terui *et al.*, 2004) or nucleolar localization of Proline-, glutamic acid- and leucine-rich protein 1 (PELP1) involved in ribosome biogenesis

and regulation of transcription (Finkbeiner *et al.*, 2011).

The genetic evidence *in vivo* and the significance of ubiquitin and SUMO mediated regulation of trafficking in human diseases

Genetic studies using targeted gene disruption in mice suggest that ubiquitin and SUMO pathways are essential, but a lot of redundancy is evident with many of the pathway components. For example, SUMO1 and SUMO3 knockout mice are viable, but SUMO2 knockout mice die during embryogenesis as well as Ubc9 knockout is lethal in mice (Nacerddine *et al.*, 2005; Evdokimov *et al.*, 2008; Zhang *et al.*, 2008; Wang *et al.*, 2014). Unfortunately, very little is known regarding genetic models of modification-deficient mutants and their phenotypes *in vivo*.

Deregulation of ubiquitin conjugation machinery and altered protein ubiquitination has been reported in diseases such as neurodegenerative diseases and cancer (Popovic *et al.*, 2014). SUMOylation seems to be a general protective mechanism against the damage caused by stresses such as low oxygen and nutrient deprivation and may also protect neurons after stress and support the growth of cancer cells. Mutations targeting ubiquitin conjugation machinery, such as E3 ligases, and somatic mutations altering ubiquitin ligase binding and subsequent trafficking of targets, including deregulated endocytosis of RTKs, in cancer have been reported (Mellman and Yarden, 2013). SUMOylation seems to be up-regulated in cancer due to e.g. overexpression of the pathway components, such as Ubc9 and some E3 ligases (Seeler *et al.*, 2017). Interestingly, it has been reported that the SUMOylation site is essential for leukaemic transformation mediated by PML-RARalpha in acute promyelocytic leukaemia (Zhu *et al.*, 2005). Moreover, a germline variant of melanoma lineage-specific microphthalmia-associated transcription factor (MITF), MITF-E318K, increases predisposition to sporadic melanoma and renal cell carcinoma. This mutation disrupts SUMOylation site of MITF and results in increased transcriptional activity promoting tumourigenic properties in experimental models, but no changes

in trafficking were observed (Bertolotto *et al.*, 2011; Yokoyama *et al.*, 2011). However, despite extensive sequencing efforts, the evidence is so far lacking for ubiquitin or SUMO binding sites to be systematically targeted by somatic mutations in cancers. Development and research efforts using drugs that specifically inhibit the conjugation pathway activity and components will help to understand the role of SUMO and non-proteolytic functions of Ubiquitin in different contexts.

Conclusions

Emerging evidence strongly implicates both ubiquitination and SUMOylation in the regulation of intracellular trafficking of several proteins at multiple levels. However, these regulation systems are highly dynamic, complex and complicated with several overlapping compensatory mechanisms and the possibility of targeting same residues with different PTMs, making it challenging to design conclusive studies. In most cases the precise convincing mechanistic insights on how this regulation of trafficking happens remain to be elucidated. In addition, the field is still lacking evidence supporting many general findings *in vivo*. Nevertheless, conceptually ubiquitin and SUMO have already emerged as powerful and potent regulators of essential cellular processes, including protein trafficking to control temporal and special protein functions, and these PTMs are definitely an important focus for future research.

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