Review

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Deciphering the emerging role of SUMO conjugation in the hypoxia-signaling cascade

Abstract: By driving the primary transcriptional response, the hypoxia inducible factor (HIF) is a master player of the hypoxia-signaling cascade, activation of which is essential to maintain oxygen homeostasis. HIF is formed by the interaction of a constitutive HIF-1β subunit with a HIF-α subunit tightly regulated through the concerted action of the prolyl hydroxylase domain containing proteins (PHDs) and factor inhibiting HIF. In well-oxygenated cells, HIF-α prolyl-hydroxylation by PHDs is the recognition signal for the binding of the ubiquitin E3 ligase pVHL, allowing protein poly-ubiquitination and degradation by the proteasome. Factor inhibiting HIF-mediated asparaginyl hydroxylation prevents interaction with the CBP/p300 coactivator and hence reduces HIF-dependent transcriptional activity. Upon low oxygen availability, HIF-α hydroxylation is blocked, resulting in protein stabilization and HIF complex activation. Post-translational modifications other than hydroxylation appear to be important in the cellular response to hypoxia. Small ubiquitin-like modifier (SUMO) is a 10 kDa protein readily conjugated to the lysine (K) residues of numerous cellular substrates in a sequential process termed SUMOylation. Recent data support the idea that a fine balance in SUMOylation/deSUMOylation is required for the adequate activation of the hypoxia-signaling cascade. In the present review, we will concentrate on the mechanisms of SUMOylation and its consequences in the cellular response to hypoxia.

Keywords: cancer; hypoxia inducible factor (HIF); hypoxic pathway; oxygen homeostasis; prolyl hydroxylase domain containing proteins (PHDs); SUMOylation.

Post-translational modification by SUMO

Post-translational modifications (PTMs) of encoded proteins play a critical role in conferring signaling pathway plasticity. The effectiveness of these modifications relies on their fast and reversible nature. Among other PTMs, SUMOylation, the conjugation of the small ubiquitin-related modifier (SUMO) to target proteins has emerged as a fundamental strategy for many proteins (Matunis et al., 1996; Mahajan et al., 1997). The small fraction of a target protein that is normally modified at a given moment explains why the SUMOylation was not detected until a few years ago as a PTM. One exception is RanGAP1, which was the first SUMO targeted protein detected (Mahajan et al., 1997). Subsequently, hundreds of functionally diverse substrates have been found to be related to the SUMO pathway. Such a modification still has a substantial physiological impact. The functional consequences of SUMOylation are diverse and depend on the modified protein. For example, SUMOylation of the androgen receptor reduces its hormone-induced transcriptional activity (Poukka et al., 2000). In the case of the heat-shock factor HSF2, SUMOylation impedes the DNA binding (Anckar et al., 2006), whereas SUMOylation of DNMT1 and PTP1B enhances methyltransferase activity and reduces phosphatase activity, respectively (Dadke et al., 2007; Lee and Muller, 2009).

SUMO homologues have been detected from Saccharomyces cerevisiae, Caenorhabditis elegans, Drosophila melanogaster and Arabidopsis thaliana to Homo sapiens, supporting the belief that SUMOylation is an evolutionarily conserved cellular process. Whereas lower eukaryotes express a single SUMO protein called Smt3 (suppressor of the mitotic fidelity gene 3), plants and vertebrates have several SUMO paralogues (Choudhury and Li, 1997; Johnson et al., 1997; Lapenta et al., 1997; Mahajan et al., 1997; Chen et al., 1998; Hanania et al., 1999; Bhaskar et al., 2000; Kurepa et al., 2003; Bohren et al., 2004). The human genome encodes four different SUMO genes (SUMO 1 to SUMO 4), although only SUMO1, SUMO2 and SUMO3 are

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functional (Lapenta et al., 1997; Mahajan et al., 1997; Chen et al., 1998; Bohren et al., 2004). SUMO proteins belong to the family of the ubiquitin-like proteins (UBLs), and SUMO1 exhibits about 18% amino acid sequence homology to ubiquitin, although their overall three-dimensional structure is extremely well conserved (Bayer et al., 1998). SUMO1 shares only 50% identity with SUMO2 and SUMO3, which are almost identical. SUMO proteins modify a distinct yet overlapping group of proteins, although they appear to be redundant, as underscored by the observation that SUMO1 knockout mice are viable (Zhang et al., 2008).

The SUMOylation machinery

The mechanism underlying the reversible and covalent conjugation of SUMO requires a sequential enzymatic cascade (Figure 1).

Maturation

The initial step for SUMOylation needs the cleavage of the C-terminal end of the immature SUMO proteins by the hydrolyzing activity of the sentrin-specific proteases (SENPs; Matunis et al., 1996; Li and Hochstrasser, 1999). This cleavage reveals a glycine-glycine motif that is required for further SUMO conjugation. The human genome codifies for six different SENPs (SENP1, SENP2, SENP3, SENP5, SENP6 and SENP7; Gong et al., 2000; Kim et al., 2000; Nishida et al., 2000; Zhang et al., 2002; Gong and Yeh, 2006; Lima and Reverter, 2008). Among them, SENP1 and SENP2 hydrolyze all three SUMO isoforms, SENP3 and SENP5 exhibit preferences for SUMO2 and SUMO3, and SENP6 and SENP7 show low endopeptidase activity (Nishida et al., 2001; Zhang et al., 2002; Xu and Au, 2005; Di Bacco et al., 2006; Gong and Yeh, 2006; Mukhopadhyay et al., 2006; Shen et al., 2006, 2009).

Activation and conjugation

The mature SUMO is activated (in an ATP-dependent reaction) by the formation of a thioester bond between the C-ter glycine and the catalytic cysteine residue of the E1 enzyme, the heterodimer SAE1/2 (or AOS1/UBA2; Johnson and Blobel, 1997; Desterro et al., 1999; Okuma et al., 1999;
Lois and Lima, 2005). Next, SUMO is transferred from the E1 to the E2 enzyme (UBC9), forming a similar thioester linkage with the catalytic cysteine residue of UBC9 (Desterrho et al., 1997; Johnson and Blobel, 1997).

**Modification**

Finally, an isopeptide bond is formed between the C-ter glycine of SUMO and the ε-NH$_2$ group of a lysine residue on the substrate. This process can be facilitated by specific SUMO E3 ligases. The largest family of SUMO E3 ligases is characterized by the presence of a SP-RING motif, which is essential for its function (Kahyo et al., 2001; Takahashi et al., 2001). These ligases comprise two groups according to their functional domains: the PIAS family (PIAS1, 2, 3 and 4; Kahyo et al., 2001; Sachdev et al., 2001; Kotaja et al., 2002; Schmidt and Muller, 2002) and MMS21 (Potts and Yu, 2005). Proteins such as the nuclear pore protein RanBP2 (Pichler et al., 2002), the human polycomb group member Pc2 (Kagey et al., 2003), the topoisomerase I-binding RING finger protein (TOPORS; Weger et al., 2005) and the histone deacetylase 4 (HDAC4; Zhao and Blobel, 2005) have also been identified as SUMO E3 ligases.

**De-SUMOylation or de-modification**

The SENPs that exhibit isopeptidase or deconjugase activity catalyze de-SUMOylation. SENP1 and SENP2 can cleave the isopeptide bond between the glycine residue of all the three SUMO isoforms and the lysine residue of the target, while the other four SENPs are more efficient at deconjugating SUMO2 and SUMO3 than SUMO1 (Gong et al., 2000; Nishida et al., 2001; Di Bacco et al., 2006; Gong and Yeh, 2006; Mukhopadhuy et al., 2006; Shen et al., 2006, 2009; Mikolajczyk et al., 2007). In addition, differences in the subcellular localization of the SENPs contribute to the selectivity: SENP1, SENP6 and SENP7 mostly localize in the nucleoplasm, SENP2 in the nuclear pore complex, and SENP3 and SENP5 in the nucleolus (Gong et al., 2000; Nishida et al., 2000; Hang and Dasso, 2002; Bailey and O’Hare, 2004; Di Bacco et al., 2006; Gong and Yeh, 2006; Mukhopadhuy et al., 2006; Shen et al., 2009). More recently, three new de-SUMOylases (DeS11, USPL1 and Wss1) have been identified (Mullen et al., 2010; Schulz et al., 2012; Shin et al., 2012).

SUMOylation occurs generally, but not exclusively, on the lysine residue at the consensus SUMO-acceptor site sequence ($\psi$KxE), in which $\psi$ is an aliphatic branched amino acid (Rodriguez et al., 2001). This motif is found in proteins such as RanGAP1, PML, p53 and 1x8 (Rodriguez et al., 2001). E2,55K (Pichler et al., 2005), UBC9 (Knipscheer et al., 2008) or DRP1 (Figueroa-Romero et al., 2009), however, are well-known substrates for SUMO that lack this consensus sequence.

SUMOylation may result in the conjugation of a single SUMO molecule (monomeric modification); however, a substrate can be mono-SUMOylated at multiple sites (multiple monomeric modification) or poly-SUMOylated as a chain (polymeric modification). Smt3, SUMO2 and SUMO3, but not SUMO1, contain an internal consensus SUMOylation site (K^x$\psi$) that allows SUMO chain formation (Tatham et al., 2001). Nevertheless, SUMO1 can also form mixed chains with SUMO2 and SUMO3 by its association at the end of the chain, forming a ‘cap’ and functioning as an inhibitor of the polymerization (Matic et al., 2008). These different modifications represent a unique signal that can result in different biological outcomes. Conjugated SUMO(s) represent additional surfaces for protein–protein interactions, which can recruit a range of effectors that alter the fate of the modified proteins. These effectors usually contain a short motif that interacts non-covalently with SUMO, called SUMO Interacting motif/SUMO binding motif or SIM/SBM. The consensus sequence for a SIM is hhxh/hxhh, where h is valine, isoleucine or leucine and x is any amino acid (Chupreta et al., 2005; Song et al., 2005; Hecker et al., 2006). This sequence forms a β-strand (Song et al., 2004) and is usually flanked, N- or C- terminally, by acidic and/or serine residues (targets for phosphorylation). In this context, it has been proposed that the presence of negative charge(s) increases the affinity for SUMO1 (Hecker et al., 2006). Furthermore, the SIM motif can be localized in the SUMO-target protein promoting the SUMOylation process (Lin et al., 2006).

The interplay between SUMOylation and other PTMs has been extensively reported. SUMO modification of PARP-1, for instance, was found to completely abrogate p300-mediated acetylation of the protein (Messner et al., 2009). Modification of a target protein by ubiquitin and SUMO may have opposite biological consequences, but both pathways can also converge toward a common goal. Recent data have reported that SUMO acts as a recognition signal to recruit the ubiquitin E3 ligase RNF4, resulting in PML SUMO-dependent ubiquitination and proteasome-mediated degradation (Lallemand-Breitenbach et al., 2008). In contrast, 1x8 ubiquitination triggers protein degradation, whereas SUMO1 modification stabilizes the protein, and thus inhibits the NF-xB pathway (Desterro et al., 1998; Culver et al., 2010).
Relevance of SUMO conjugation

SUMOylation allows the regulation of a range of protein parameters, including stability, structure, function, activity, intracellular location and interaction with other proteins. SUMOylation is a dynamic and fundamentally important PTM implicated in a multitude of processes. Consistent with the pleiotropic role of SUMOylation, a correct balance between SUMOylation and de-SUMOylation is necessary for normal development and physiology. Accordingly, genetic inactivation of Ubc9 or Senp1 and Senp2 is lethal in mice due to the overall depletion of or increase in SUMO conjugation (Nacerddine et al., 2005; Cheng et al., 2007; Chiu et al., 2008). SUMO has also been shown to play an important role in keratinocyte differentiation (Deyriefux et al., 2007), the total number of SUMO-modified proteins being highest in non-differentiated cells, decreasing after the induction of differentiation and slowly rebounding as differentiation progressed. However, within this overall trend the pattern of change for individual SUMOylated proteins is highly variable (Heaton et al., 2012). In addition, SUMO modification has been shown to be critical for the maintenance of genomic integrity. Mice lacking Ubc9 show chromosome condensation and segregation defects and also severe deficiency in nuclear organization (Nacerddine et al., 2005). Moreover, proteins involved in the DNA damage response, such as SGS1, YKU70 and RAD52 in yeast, or BLM, XRCC4 and RPA in mammals, are SUMO targets (Eladad et al., 2005; Zhao and Blobel, 2005; Branzei et al., 2006; Sacher et al., 2006; Yurchenko et al., 2006; Dou et al., 2010). SUMOylation is essential to stabilize these proteins or locate them in the adequate DNA breakage points optimizing their activity. Consistent with these reports, the activity of the SUMO E3 ligase MMS21 in yeast is essential for DNA reparation (Zhao and Blobel, 2005). Furthermore, DNA damage-induced SUMOylation contributes to genome integrity independently of the Mec1 checkpoint (Cremona et al., 2012).

Changes in the abundance and activity of SUMO conjugation–deconjugation machinery have been reported in different types of cancer; in particular, the altered expression of SENPs has been observed in several carcinomas. Increased expression of SENP1 and SENP3 has been assessed in human prostate cancer patients (Wang and Banerjee, 2004; Cheng et al., 2006). Unlike prostate cancer, increased SUMOylation associated with reduced SENP6 and increased UBC9 expression has been found in several breast cancer tissue arrays (Mo et al., 2005; Mooney et al., 2010). So far, however, it has been difficult to establish whether changes in the levels of SUMOylation or de-SUMOylation persist throughout the different stages and/or facilitate the progression of a given cancer.

Altered patterns of protein SUMOylation are associated with stresses, such as heat shock, chemotherapy, ultraviolet exposure or ionizing radiations, osmotic and oxidative stress, suggesting that SUMO modification is essential in the cellular adaptation to environmental changes (Saitoh and Hinchey, 2000; Comerford et al., 2003; Manza et al., 2004; Golebiowski et al., 2009). Although a global increase in SUMOylation has been proposed under these conditions, the situation is not that sample at the levels of individual targets (Bossis et al., 2005; Golebiowski et al., 2009; Huang et al., 2009).

SUMOylation and the adaptation to hypoxia

In addition to environmental stresses, metabolic stress including hypoxia is associated with changes in the levels of SUMO conjugation. Increased in vitro SUMO global protein conjugation upon hypoxia has been reported and further supported by in vivo studies (Comerford et al., 2003; Shao et al., 2004). Hypoxia-induced transcription of SUMO1 or RWD-containing SUMOylation enhancer (RSUME) might explain the global effects on SUMOylation by directly targeting the conjugation machinery (Comerford et al., 2003; Shao et al., 2004; Carbia-Nagashima et al., 2007), while regulation of specific SUMO E3 ligases, such as PIAS4, might account for more restricted effects (Kang et al., 2010). Furthermore, SUMO1 overexpression has been related with an increase in glycolysis and glucose uptake (Agbor et al., 2011). It has been proposed that SUMO could play a role in the metabolic switch to glycolysis, contributing to the cellular adaptation to hypoxia, which is consistent with the fact that GLUT-1 and GLUT-4 are substrates for SUMO (Giorgino et al., 2000).

Consistent with the altered patterns of SUMO conjugation highlighted in prostate cancer, SENP3 promotes the expression of vascular endothelial growth factor (VEGF) that facilitates the development of new blood vessels, and thus contributes to cancer cell survival under hypoxic conditions (Huang et al., 2009). SUMO therefore plays an additional role in the cellular adaptation to hypoxia by regulating angiogenesis.

The relevance of SUMOylation has been clearly shown in brain and heart upon hypoxia. Mice exposed to focal or global ischemia show a massive increase in SUMO conjugation (Cimarosti et al., 2008; Yang et al., 2008a,b). Moreover, the silencing of SUMO1 or SUMO2/3 in primary neurons induces cellular death under oxygen and glucose deprivation, suggesting that SUMOylation...
is an essential mechanism in tolerance against hypoxia (Lee et al., 2009; Datwyler et al., 2011). The SUMO protection hypothesis is further supported by the fact that SUMOylation is increased during hibernation torpor (Lee et al., 2007), which is characterized by a reduction of blood flow, body temperature, energy consumption and protein synthesis (Frerichs et al., 1994, 1998; Frerichs and Hallenbeck, 1998). In this context, by using a stable isotope labeling by with amino acids in cell culture-based proteomic approach, Yang and co-workers have recently identified SUMO3 targets and quantified their modulation in an experimental model of transient ischemia (Yang et al., 2012).

**SUMOylation of hypoxia-signaling cascade mediators**

Previous reports have provided insight into the direct implication of SUMO conjugation on the regulation of the hypoxia signaling cascade mediators required for cellular adaptation to reduced oxygen availability. Figure 2 is a schematic summary of the data described below.

**HIF-1α**

The impact of SUMOylation on this subunit is highly documented. HIF-1α conjugation of to up to seven SUMO molecules *in cellulo* has been even confirmed by high accuracy mass spectrometry, although very few peptides modified by SUMO have been identified by this technique, contrary to its successful application for the identification of many other PTMs (Matic et al., 2008). It remains technically challenging to map SUMOylation sites due to the long tryptic peptides attached to the modified lysines, which are generated as a consequence of the lack of an arginine or a lysine residue in the proximity of the C-ter of SUMO, and results in complex fragmentation patterns that are not readily interpretable. The consequences of SUMO conjugation on HIF-1α expression and/or activity are, however, a matter of debate and additional studies will be required to resolve this rather contentious area. There are three apparently conflicting findings about how SUMO affects HIF1α:

- SUMOylation decreases HIF-1α stability;
- SUMOylation stabilizes HIF-1α; and
- SUMOylation does not affect HIF-1α stability. Instead, SUMOylation decreases HIF-1α transactivation activity.

Cheng and co-workers have convincingly shown the simultaneous accumulation of SUMOylated HIF-1α and the decrease in HIF-1α stability in SENP1−/− cells during hypoxia (Cheng et al., 2007). These authors demonstrate that deletion of the Senp1 gene in mice results in early embryonic lethality as a consequence of a severe anemia due to a marked reduction of HIF-1α stabilization and
deficient erythropoietin production during hypoxia. They propose that HIF-1α SUMOylation promotes its interaction with pVHL through a proline-independent mechanism, leading to its ubiquitination and degradation in the absence of SENP1. Furthermore, SENP1 is induced under hypoxia, contributing to a positive feedback loop in HIF-1α stabilization (Xu et al., 2010). However, the requirement for consecutive SUMOylation and de-SUMOylation events remains to be explained. PIAS4 has been proposed as the specific SUMO E3 ligase for hypoxia-induced HIF-1α modification by SUMO1 (Kang et al., 2010). Moreover, PIAS4 expression inversely correlates to angiogenesis in colon cancer (Kang et al., 2010).

This model is inconsistent with two independent reports supporting SUMOylation contribution to HIF-1α activation as a functional consequence of HIF-1α stabilization (Bae et al., 2004; Carbia-Nagashima et al., 2007). According to these authors, HIF-1α is upregulated through SUMO1 modification at K391 and K477 residues located within the HIF-1α oxygen dependent degradation domain (Bae et al., 2004). This reaction seems to be catalyzed by RSUME, which increases the non-covalent binding of SUMO to UBC9, and hence enhances overall SUMO conjugation (Carbia-Nagashima et al., 2007). One possibility for the difference observed by these authors may be due to an indirect effect on HIF-1α through the SUMOylation of other proteins caused by over-expression of SUMO1 or RSUME in their systems in the presence of SENP1. In contrast, Berta and coworkers propose that HIF-1α SUMOylation by SUMO1 and SUMO2/3 at K391 and K477 residues is stimulated by the SUMO E3 ligase RanBP2 in an in vitro system (Berta et al., 2007). In this model, SUMOylation does not affect HIF-1α stability but decreases its transscriptional activity. The contrasting results from the previous studies may be attributed to the use of a HIF construct mutated in 4Ks, which might be a target for PTMs other than SUMOylation.

**HIF-1β**

This protein has been shown to be SUMOylated at K345 within the dimerization PAS domain (Tojo et al., 2002). SUMOylation represses HIF-1β transactivation, although this SUMOylation does not seem to have an impact on the transactivation activity of the HIF complex.

**CBP/p300**

CBP and p300 contain three (K999, K1034, and K1057) and two (K1020 and K1024) consensus SUMOylation sites, respectively (Girdwood et al., 2003; Kuo et al., 2005). SUMOylation of these proteins decreases their transactivation capacity. Such repression is mediated through similar mechanisms involving the interaction between HDAC2 and CBP and HDAC6 and p300 (Girdwood et al., 2003; Kuo et al., 2005). Consistent with SUMOylation-mediated p300 repression, de-SUMOylation of the protein by SENP3 enhances its binding to HIF-1α. Furthermore, the de-SUMOylating activity of SENP3 is required for reactive oxygen species-enhanced HIF-1-dependent transactivation (Huang et al., 2009).

**pVHL**

The pVHL SUMOylation that occurs at K771 represses ubiquitin E3 ligase activity, and subsequently HIF-1α degradation. Hypoxia increases pVHL SUMOylation. Mechanistically, it has been reported that SUMO modification blocks the interaction between pVHL and elongin C, and thus impairs HIF-1α poly-ubiquitination (Cai et al., 2010). SUMOylation of pVHL also enhances its nuclear localization, while ubiquitination of the protein favors its cytoplasmic accumulation (Cai and Robertson, 2010). PIAS4 is the specific pVHL SUMO E3 ligase, which is increased under hypoxia. Intriguingly, as mentioned previously, PIAS4 has been proposed to contribute to HIF-1α SUMOylation and to promote its interaction with pVHL (Kang et al., 2010). These are again apparently conflicting findings.

Taken together, all these data suggest that SUMO affects each one of these mediators in a different manner. It also remains to be determined whether additional hypoxia-signaling cascade mediators, such as PHDs and factor inhibiting HIF, may be modified by SUMO. Further studies will be required to address these issues, their mechanisms and physiological relevance. In addition to the above-mentioned mediators, IκB SUMOylation is increased under hypoxic conditions (Carbia-Nagashima...
et al., 2007; Culver et al., 2010). Similarly, CREB SUMOylation under hypoxia is implicated in a negative feedback mechanism that controls tumor necrosis factor α expression (Comerford et al., 2003). Many important questions regarding the function of SUMOylation and de-SUMOylation in the context of hypoxia, therefore, remain unanswered.

Conclusion

Given the importance of the hypoxia signaling cascade in different pathologies it is tempting to speculate that a better understanding of the emerging role of SUMO conjugation in this cascade would help in the preclinical development of new selective inhibitors/activators to combat these pathologies. In this context, promoting a general change in the overall level of SUMOylation may not be the most effective method of managing pathologies. The specific targeting of hypoxic SUMO mediators provides a more attractive alternative.

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