Regulation of hypoxia-inducible factors by small ubiquitin-like modifiers

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Chapter 7

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Complex multicellular organisms require oxygen for their normal metabolism but must also keep the toxic potential of oxygen in check. Therefore, oxygen levels in cells are carefully balanced by complex and intricate networks. At the center of the network of proteins regulated by oxygen levels in cells are the hypoxia-inducible factors, themselves stringently regulated proteins. Among the many post-translational modifications that govern the stability and activity of the HIFs is sumoylation. The research presented in this thesis investigates sumoylation in general and its role in regulating the HIFs in particular.

In chapter 3, we present data supporting an inhibitory role for sumoylation on HIF-1α transcriptional activity. Furthermore, we show that SUMO conjugation can take place on Lys-377 of HIF-1α, in addition to Lys-391 and Lys-477. While the latter two SUMO conjugation sites lie in sequence stretches corresponding to the consensus for sumoylation sites, the former does not. Disrupting any single one of the three identified SUMO acceptor sites in HIF-1α increased its transcriptional activity to a similar degree. Mutating two or all three SUMO acceptor sites had a cumulative effect with each additional mutation increasing HIF's transcriptional activity further.

Sumoylation of HIF-1α was first reported in 2004 (1). Since then, several independent groups have studied the functional consequences of SUMO conjugation to HIF-1α. However, different results obtained by these groups have lead to conflicting reports on SUMO's role in HIF-1α regulation. Our results indicating that HIF-1α sumoylation leads to inhibition of transcriptional activity are consistent with those reported by Berta *et al*. Using a similar approach with transiently over-expressed wild-type and mutant HIF-1α they mapped sumoylation to Lys-391 and Lys-477. Their sumoylation-impaired HIF-1α mutants also exhibited an increase in transcriptional activity and displayed no difference in protein stability compared to the wild-type. However, while they considered Lys-377 as a potential non-conventional sumoylation site, it did not appear to be used as such in their experiments (2). Carbia-Nagashima *et al* characterized RSUME, a general stimulator of sumoylation. HIF-1α was shown to be among the proteins affected by RSUME and increased HIF-1α sumoylation resulted in increased stability and transcriptional activity (3). Finally, Cheng *et*

al proposed a model where sumoylation of HIF-1α provided an oxygen-independent mechanism to recruit VHL, allowing HIF-1α to be degraded during hypoxia (4).

It is important to note that SAE2, SENP1 and RSUME have much broader roles in regulating sumoylation (3, 5-7). Depleting cells of these proteins would therefore affect many other proteins besides HIF-1α, making it difficult to compare these studies directly. This is only exacerbated when taking into account the complex network that regulates HIF-1α stability and activity. A specific way to study the regulation of HIF-1α by sumoylation is the analysis of HIF-1α mutants that lack SUMO acceptor lysines, as described in this thesis.

The results described in chapter 4 demonstrate cooperation between sumoylation and ubiquitination in the regulation of a subset of SUMO-targeted proteins. We identified ubiquitin as one of the proteins to accumulate in SUMO-2-enriched samples from HeLa cells. Treating the cells with proteasome-inhibiting drugs prior to SUMO-2 purification greatly increased the amount of ubiquitin present in the enriched samples. These data imply cooperation between SUMO-2 and ubiquitin in regulating proteasomal degradation of at least a subset of SUMO-2 targets.

Using quantitative proteomics, we identified seventy-three SUMO target proteins that undergo subsequent ubiquitination and proteasomal degradation. Another forty SUMO target proteins were identified that showed a reduction in sumoylation upon inhibition of the proteasomal degradation machinery. These may represent transiently sumoylated proteins with the most short-lived modified forms. With the free pool of SUMO-2/3 diminished after inhibition of the proteasome, insufficient amounts of free SUMO-2/3 were available for conjugation to this second set of target proteins.

However, this SUMO-dependent degradation pathway only regulates the stability of the SUMO-modified fraction of a protein, which is typically very small, and does not seem to affect the total pool. Despite only affecting a small fraction of the total pool of any given protein, this degradation pathway is an important regulator of protein stability. In a study using SENP1 -/- mice, Cheng *et al* found that SUMO-1-modified HIF-1α can undergo subsequent ubiquitination and proteasomal degradation under hypoxic conditions. Normal cells can balance this degradation by removing the SUMO moieties from HIF-1α through desumoylation. In mouse embryos lacking SENP1, however, the rate of HIF-1α degradation was high enough to cause the embryos to die from severe anemia (4).

Among the proteins that we identified in our screen was PML (Ch. 4, supplemental table S1), a known target for SUMO-directed ubiquitination (8). Other proteins known to be

regulated by cross-talk between sumoylation and the ubiquitin-proteasome system are HIF-1α (4) and HIF-2α (Ch. 5, this thesis), yet neither protein was detected in our experiment. However, the cell samples used in the experiment described in chapter 4 had been cultured at high levels of oxygen (21% O_2) with a relatively short treatment to inhibit proteasomal degradation. These conditions may have been insufficient to accumulate HIF-α to detectable levels.

Several ubiquitin E3 ligases mediate SUMO-directed ubiquitination. Cheng *et al* demonstrated the involvement of VHL in the ubiquitination of SUMO-modified HIF-1α during hypoxia (4) while RNF4 was found to be involved in mediating ubiquitination of SUMO-modified PML (8). RNF4 had previously been shown to possess ubiquitin E3 ligase activity (9) and to be able to interact with SUMO through several SIM motifs (10).

In chapter 5 we present data that HIF-2α undergoes regulation through sumoylation, similar to HIF-1α. Similar to HIF-1α, sumoylation of HIF-2α impaired its transcriptional activity without affecting the stability of the total pool of the protein (Ch. 5, fig. 3). We also demonstrated that SUMO-modified HIF-2α undergoes ubiquitination and proteasomal degradation under hypoxic conditions, facilitated by VHL and RNF4.

While the functional consequences of HIF-α sumoylation are starting to be unraveled, the context in which this mechanism occurs is still unclear. With the complex regulatory network already in place to govern the stability, expression and activity of the HIF-α proteins, it seems counter-intuitive to have one that is active specifically when the activity of the HIF-α proteins is required. Perhaps this degradation pathway functions as an overflow mechanism, keeping HIF-α levels in check even during hypoxia. Another possibility is that HIF-α sumoylation functions as a release switch. Sumoylation of HIF-α could stimulate it to release from the DNA and/or transcriptional complex, promoting a quick turn-over of the protein and a rapid decrease in its activity that would be particularly useful upon reestablishing a normoxic state.

Chapter 6 presents the data obtained on SUMO chain formation as well as the optimization of a method to detect specific peptides using mass spectrometry in complex samples. From low-complexity *in vitro* sumoylated samples information was obtained that enabled us to interpret the data gathered from complex cell-derived samples. Using this approach, we studied the formation of SUMO polymer formation *in vitro* and *in vivo*.

Ubiquitin chain formation is a well-studied phenomenon. In contrast, little is known about SUMO polymerization. SUMO chain formation was first described taking place on

PML and HDAC4 (11). SUMO proteins can be conjugated to a specific lysine, situated in a consensus sumoylation site, in another SUMO-2 or -3 moiety. Like SUMO-2 and -3, SUMO-1 can also be added to a SUMO chain but, lacking such an internal sumoylation site, limits further growth. SUMO chain formation has been demonstrated *in vitro* and in yeast and mammalian cells. Mechanistically, *in vitro* assays have shown that Ubc9's ability to bind a second SUMO protein non-covalently is required for proper SUMO chain formation. Several E3 enzymes are also capable of catalyzing SUMO chain formation (12, 13) whereas SUMO proteases SENP6 and SENP7 were found to preferentially process SUMO chains rather than monosumoylated targets (14, 15). SUMO chain formation is balanced by specific SUMO proteases (SENP6 and SENP7 in mammals and Ulp2 in yeast), however, our *in vitro* data implied a potential role for SUMO-1 in limiting the length of SUMO chains as well.

While SUMO chain formation has been demonstrated to occur in yeast and mammalian cells, their role in cells is not yet clear. Our own experiments in mammalian cells suggested that SUMO chains were not required for the role of sumoylation in mediating proteasomal degradation (Ch. 4, this thesis). Data obtained from experiments in yeast suggest roles for SUMO chains during meiosis and replication arrest responses. Replacing wild-type Pmt3 with a polymerization-deficient mutant in *Saccaromyces pombe* lead to abnormal cell and nuclear morphologies. In addition, these cells displayed sensitivity to hydroxyurea, an inhibitor of DNA synthesis, but not other DNA damaging agents (16). In *Saccharomyces cerevisiae* strains a similar Smt3 mutant could functionally replace wild-type Smt3 under normal growth conditions (17). Sporulation in these strains was however strongly reduced and Smt3 polymerization was shown to be required for the formation of the synaptonemal complex during meiosis (18).

In addition, deletion of one of the two yeast SUMO proteases, Ulp2, *in S. cerevisiae* resulted in accumulation of Smt3 polymers and gave rise to various phenotypes that included slow growth and sensitivity to several stresses. Replacing wild-type Smt3 with polymerization-impaired Smt3 mutants in the Δ*ulp2* strains suppressed these sensitivities (17). It is not clear whether the presence of large Smt3 polymers by itself is problematic or that they simply become Smt3 sinks, reducing the pool of free Smt3 available for conjugation to other proteins below critical levels.

In his_6-SUMO-2 enriched fractions isolated from HeLa nuclear extracts we found that HIF-1α could be modified by at least seven SUMO proteins (Ch. 6, this thesis). So far, only three SUMO acceptor sites (two consensus and one non-consensus) have been identified.

While closer examination of HIF-1α may yet reveal the existence of additional acceptor sites, this implies that one or more sites may be modified by multiple SUMO moieties. Our *in vitro* data demonstrated that SUMO chain formation can indeed occur on HIF-1α. Typically, sumoylation regulates proteins by blocking existing interaction sites or providing new ones. Proteins that bind preferentially to SUMO chains rather than to SUMO monomers have been reported, such as ZIP1 in *S. cerevisiae* (18) and the microtubule motor protein centromere-associated protein E (CENP-E) in mammalian cells (19). Proteins able to distinguish between mono- and polysumoylated proteins based on preferential interactions would greatly expand the regulating capabilities of the sumoylation system. One or more HIF-1α interactors may require the presence of multiple SUMO moieties to provide the necessary binding interface. Alternatively, SUMO chains may simply provide a more potent blocking mechanism than single SUMOs can provide.

Taken together, we have presented data that demonstrate a role for sumoylation in the regulation of the activity of HIF-1α and -2α. Modification by SUMO proteins reduces the transcriptional activity of both proteins. For HIF-2α, we have shown that sumoylated forms of the protein are degraded by the proteasome even during hypoxia, mediated by VHL and RNF4. Others have shown that sumoylated HIF-1α is targeted for ubiquitination and proteasomal degradation by VHL (4).

The HIF-α proteins are critical survival factors of solid tumors. A better understanding of HIF-α regulation by sumoylation might enable the modulation of HIF-α activity. Increasing HIF-α sumoylation could be achieved by either increasing the activity of the sumoylation machinery or decreasing the activity of the SENPs. The SUMO E3 ligases provide the target specificity of the sumoylation machinery. Therefore, activating the E3 enzyme(s) responsible for HIF-α sumoylation may increase its sumoylation relatively specifically. Unfortunately, it is currently not clear which E3 ligase is involved in sumoylation of either HIF-1α or -2α. Alternatively, HIF-α sumoylation could be increased by inhibiting the activity of the SENPs. The potential for a reduction in SENP1 activity to affect HIF-1α *in vivo* has already been shown in SENP1 -/- mice (18), albeit not in a cancer-related setting. SENP1 has also been implicated in the development of prostate cancer where it was demonstrated to enhance the transcriptional activity of the androgen receptor and c-Jun and the expression of cyclin D1 (20). Taken together, the SENPs are an attractive target for designing novel anti-cancer drugs.

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