

RESEARCH ARTICLE

PHD3–SUMO conjugation represses HIF1 transcriptional activity independently of PHD3 catalytic activity

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ABSTRACT

By controlling HIF α hydroxylation and stability, the prolyl hydroxylase domain (PHD)-containing proteins are essential to the maintenance of oxygen homeostasis; therefore these enzymes are tightly regulated. Small ubiquitin-like modifier (SUMO) is a 10-kDa protein readily conjugated to lysine residues of the targeted proteins in a process termed SUMOylation. In this study, we introduce SUMO conjugation as a novel regulator of PHD3 (also known as EGLN3). PHD3 SUMOylation occurs at a cluster of four lysines at the C-terminal end of the protein. Furthermore, PHD3 SUMOylation by SUMO2 or SUMO3 contributes to PHD3-mediated repression of HIF1-dependent transcriptional activity. Interestingly, PHD3–SUMO conjugation does not affect PHD3 hydroxylase activity or HIF1 α stability, providing new evidence for a dual role of PHD3 in HIF1 regulation. Moreover, we show that hypoxia modulates PHD3–SUMO conjugation and that this modification inversely correlates with HIF1 activation. PHD3 SUMOylation highlights a new and additional layer of regulation that is likely required to fine-tune HIF function.

KEY WORDS: HIF α , Hypoxia, PHD, EGLN, SUMO, Transcriptional repression

INTRODUCTION

Maintaining oxygen homeostasis is essential for most organisms as hypoxia, even when transient, could trigger irreversible damage. Adaptation to reduced oxygen availability is indeed a major physiological challenge during embryonic development and in adulthood, but it is also associated with many human diseases (Benizri et al., 2008). This adaptation is primarily led by activation of hypoxia inducible factor (HIF), which controls the expression of hundreds of direct target genes, secondary transcription factors and non-coding RNAs (Semenza, 2012). HIF is a heterodimer formed by the interaction of the constitutive HIF β subunit with a tightly regulated HIF α subunit (Huang et al., 1996; Salceda and Caro, 1997; Wang et al., 1995). Oxygen-dependent hydroxylation by the prolyl hydroxylase domain (PHD)-containing proteins drives HIF α ubiquitylation and proteasomal degradation (Bruick and McKnight, 2001; Epstein et al., 2001; Ivan et al., 2001; Jaakkola et al., 2001). Hydroxylation is indeed limiting for HIF α degradation and hence HIF activation.

Caenorhabditis elegans and *Drosophila melanogaster* have a single PHD family member (EGL-9), whereas the genomes of higher metazoans encode at least three paralogous PHD proteins (PHD1, PHD2 and PHD3; also known as EGLN2, EGLN1 and EGLN3, respectively) (Bruick and McKnight, 2001; Epstein et al., 2001). All three PHD proteins catalyze site-specific hydroxylation of HIF1 α polypeptides at P564 *in vitro*. However, PHD2 appears to be primarily responsible for basal HIF α levels, although PHD1 and PHD3 contribute to HIF α regulation in certain settings (Berra et al., 2003; Ginouvès et al., 2008; Takeda et al., 2006). Thereby, we found previously that PHD3, through isoform-specific regulatory mechanisms, participates in a negative-feedback loop in response to prolonged hypoxia (Ginouvès et al., 2008). Moreover, recent reports highlight specific HIF-independent targets for PHDs. In this regard, PHD1 controls cyclin D1 levels and thus mammary gland proliferation (Zhang et al., 2009). PHD2 has been found to modulate protein translation through regulation of eEF2 phosphorylation (Romero-Ruiz et al., 2012). PHD3 mediates oxygen-dependent stability of activating transcription factor 4 (ATF4), PKM2 and β_2 -adrenergic receptor, and prevents degradation of myogenin (Fu et al., 2007; Köditz et al., 2007; Luo et al., 2011; Xie et al., 2009). PHD3 also negatively regulates NF- κ B and Pax2 (Fu and Taubman, 2010; Xue et al., 2010; Yan et al., 2011). Furthermore, PHD3 appears to be necessary and sufficient for apoptosis of neuronal cells after nerve growth factor withdrawal (Lee et al., 2005). The absence of PHD3 is indeed related to the failure of developmental apoptosis, which might play a role in pheochromocytoma pathogenesis. Clearly if the PHD proteins are to be targeted, it will be important to understand the basis of their specificities.

Post-translational modifications (PTMs) act to fine-tune the functions of encoded proteins, and they play a crucial role in conferring plasticity. Among several PTMs, SUMOylation, the conjugation of the small ubiquitin-related modifier (SUMO) to target proteins, has emerged as a fundamental strategy to modulate protein function (Geiss-Friedlander and Melchior, 2007). SUMOylation requires a sequential enzymatic cascade implicating SUMO-specific E1 and E2 enzymes and, for some substrates, a SUMO-E3 ligase. As is the case for many other PTMs, SUMOylation is a reversible process, with SUMO proteases acting to remove the modification (Hay, 2007). SUMOylation occurs often, but not exclusively, at the lysine residue within the consensus SUMO-acceptor site sequence Ψ KxE/D, in which Ψ is an aliphatic branched amino acid (Rodríguez et al., 2001). Altered patterns of protein SUMOylation are associated with stress, suggesting that SUMO modification is essential in the cellular adaptation to environmental changes (Tempé et al., 2008). Accordingly, previous reports have provided insight into the direct implications of SUMO conjugation on the regulation of

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essential factors required for hypoxia adaptation, such as HIF α , HIF β , VHL and p300 (Núñez-O'Mara and Berra, 2013).

To gain further insight into PHD3 regulation, we evaluated whether SUMO conjugates to PHD3. In the present study, we show for the first time that PHD3 can be a SUMO target. Surprisingly, our results demonstrate that PHD3 SUMOylation represses HIF1 transactivation activity without affecting HIF1 α stability. Moreover, we present evidence that hypoxia modulates PHD3 SUMOylation and that this modification correlates with the inhibition of HIF1. PHD3 SUMOylation appears to be a newly identified additional step in the control of HIF.

RESULTS

PHD3 can be SUMOylated

To study new mechanisms of PHD3 regulation, we evaluated whether PHD3 can be modified by SUMO. We incubated [35 S]-methionine-labeled HA-PHD3 that had been *in vitro* transcribed and translated (IVTT) with a SUMOylation mixture, including the E1 (SAE1 and SAE2) and E2 (UBC9) enzymes, in the absence or

in the presence of SUMO or GST-SUMO. As shown in Fig. 1A, we detected the non-modified PHD3 as well as higher-molecular-mass bands in the presence of SUMO1, 2 and 3 or GST-SUMO1, 2 and 3, suggesting that PHD3 is a target for SUMO. The identity of the high-molecular-mass band was revealed by double immunoblotting with anti-HA and anti-SUMO (Fig. 1B), which confirmed the presence of PHD3-SUMO conjugates.

Among SUMO proteins, SUMO1 shares only 50% identity with SUMO2 and SUMO3, which are almost identical (Su and Li, 2002). Hereafter, we use the nomenclature SUMO2/3 to refer to both proteins, as the two paralogs cannot be functionally distinguished. In view of the *in vitro* results, we next transfected (His) $_6$ -tagged PHD3 together with HA-SUMO1 or HA-SUMO2/3 into COS-7 cells. PHD3 was purified under denaturing conditions on Ni-NTA beads, and the purified material was analyzed by western blotting (Fig. 1C). In the presence of SUMO2/3, we were able to detect high-molecular-mass PHD3 bands with the anti-(His) $_6$ antibody. The anti-HA antibody also recognized these bands, confirming that they corresponded to

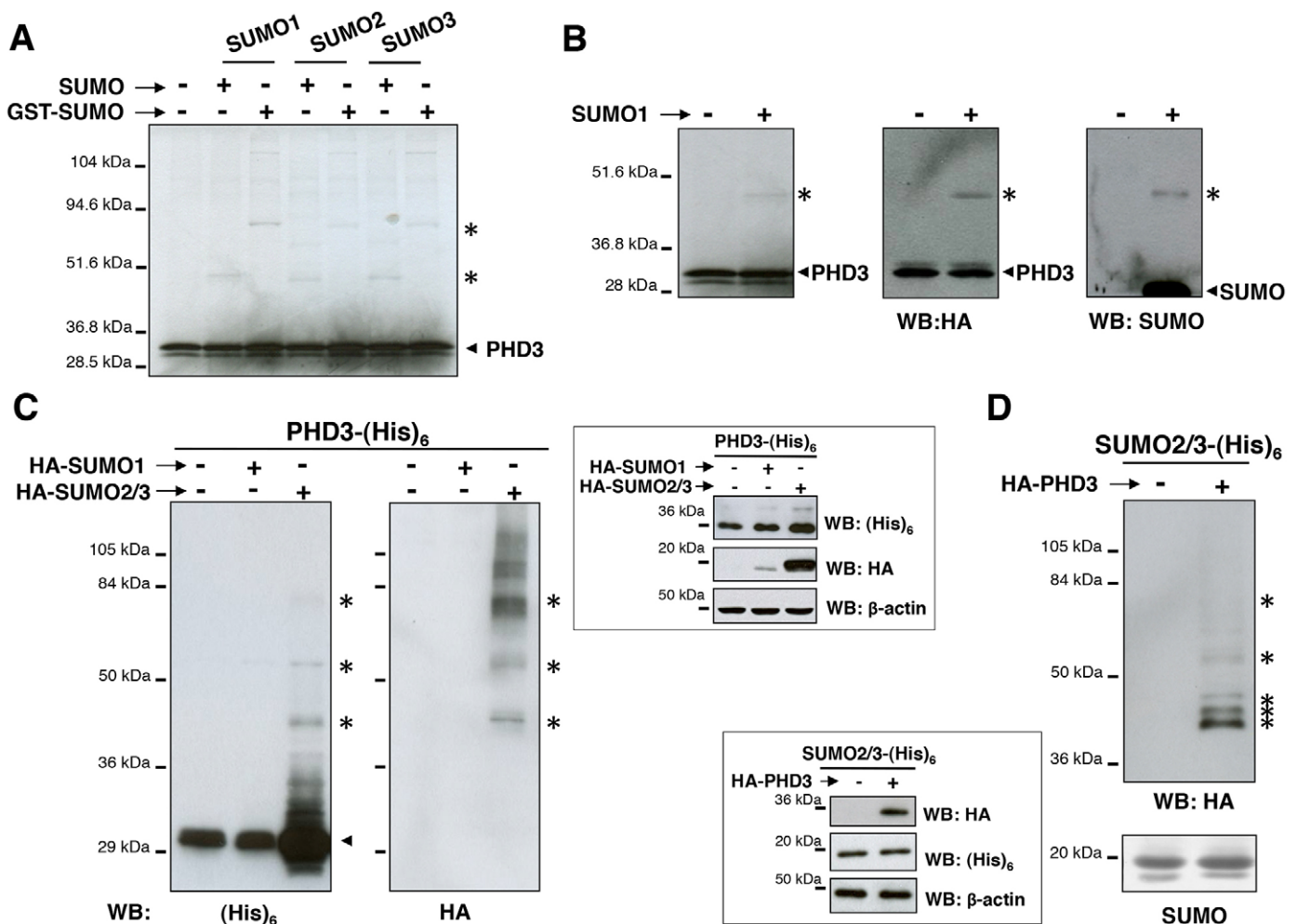


Fig. 1. PHD3 is a SUMO target. (A) [35 S]-labeled IVTT PHD3 was incubated in the presence of the SUMOylation reaction buffer and the indicated SUMO proteins, resolved by SDS-PAGE and detected by autoradiography. The arrowhead and asterisks correspond to the non-modified and the conjugated forms of PHD3, respectively. (B) The PHD3 forms detected by autoradiography (left panel) were identified by western blotting (WB; central and right panel). Note that the left panel shows the first two lanes of the autoradiogram presented in A, for comparison with the western blotting data. COS7 (C) and HEK293T (D) cells were transfected as indicated. Ni-NTA affinity purification was performed after 48 h, and the purified proteins were resolved by SDS-PAGE and finally visualized by using western blotting. Insets show the protein expression levels from total cell extracts transfected in parallel. β -actin was used as the protein loading control.

PHD3–SUMO conjugates. Note that SUMO2/3 was expressed at higher levels compared with SUMO1 (inset Fig. 1C), which might explain the absence of PHD3–SUMO1 conjugates (Fig. 1C). To validate the identification of PHD3 as a new SUMO target protein, we performed the reciprocal purification (Fig. 1D). We could show that PHD3 does co-purify with (His)₆-tagged SUMO2/3 when both proteins were ectopically expressed. Taken together, these data confirm that PHD3 can be a SUMO target protein not only *in vitro* but also in cultured cell lines.

Looking for the PHD3 SUMOylation motif(s)

Although PHD3 lacks any consensus SUMO acceptor site, an analysis of the sequence showed 12 evolutionary conserved lysines that might be potential SUMO-modified residues. To facilitate the identification of the target residue(s), we generated two overlapping truncated forms of PHD3 (PHD3 Nter and PHD3 Cter) that were subjected to *in vitro* SUMOylation assays as described previously. Interestingly, PHD3 Nter was no longer SUMOylated, whereas PHD3 Cter was still modified like the wild-type protein (Fig. 2A), suggesting that PHD3 is SUMOylated at the C-terminal region of the protein. We replaced each of the putative SUMO target lysines located within PHD3 Cter (K119, K154, K159, K172, K222, K223, K224 and K231) with arginines (R). However, none of the individual mutations prevented PHD3

SUMOylation (supplementary material Fig. S1). We next adopted a three-dimensional homology approach using an online server (<http://www.sbg.bio.ic.ac.uk/phyre/>) to better characterize the SUMO-modified residues (Kelley and Sternberg, 2009). This approach allowed us to identify two clusters of lysines at the C-terminal region of PHD3. These clusters correspond to lysines 154, 159 and 172 and lysines 222, 223, 224 and 231, respectively. Mutation of the two different clusters independently allowed us to identify lysines 222, 223, 224 and 231 as the PHD3 SUMOylation motif, at least *in vitro* (Fig. 2B).

To confirm these results, we transfected HEK293T cells with (His)₆-tagged SUMO2/3 together with wild-type PHD3 or PHD3 K222/223/224/231R. Consistent with the *in vitro* assays, PHD3 SUMOylation was almost abolished in the K222/223/224/231R mutant compared with that of the wild-type protein (Fig. 2C). Thus, we confirmed the PHD3 SUMOylation motif and accordingly identified the SUMOylation dead mutant (K222/223/224/231R), which we will refer to hereafter as PHD3 Δ SUMO.

PHD3 SUMOylation affects HIF activity but does not affect the NF- κ B pathway

Although it was originally identified as a repressor of the HIF complex, additional roles have been reported for PHD3. We wondered whether SUMOylation might affect specific PHD3

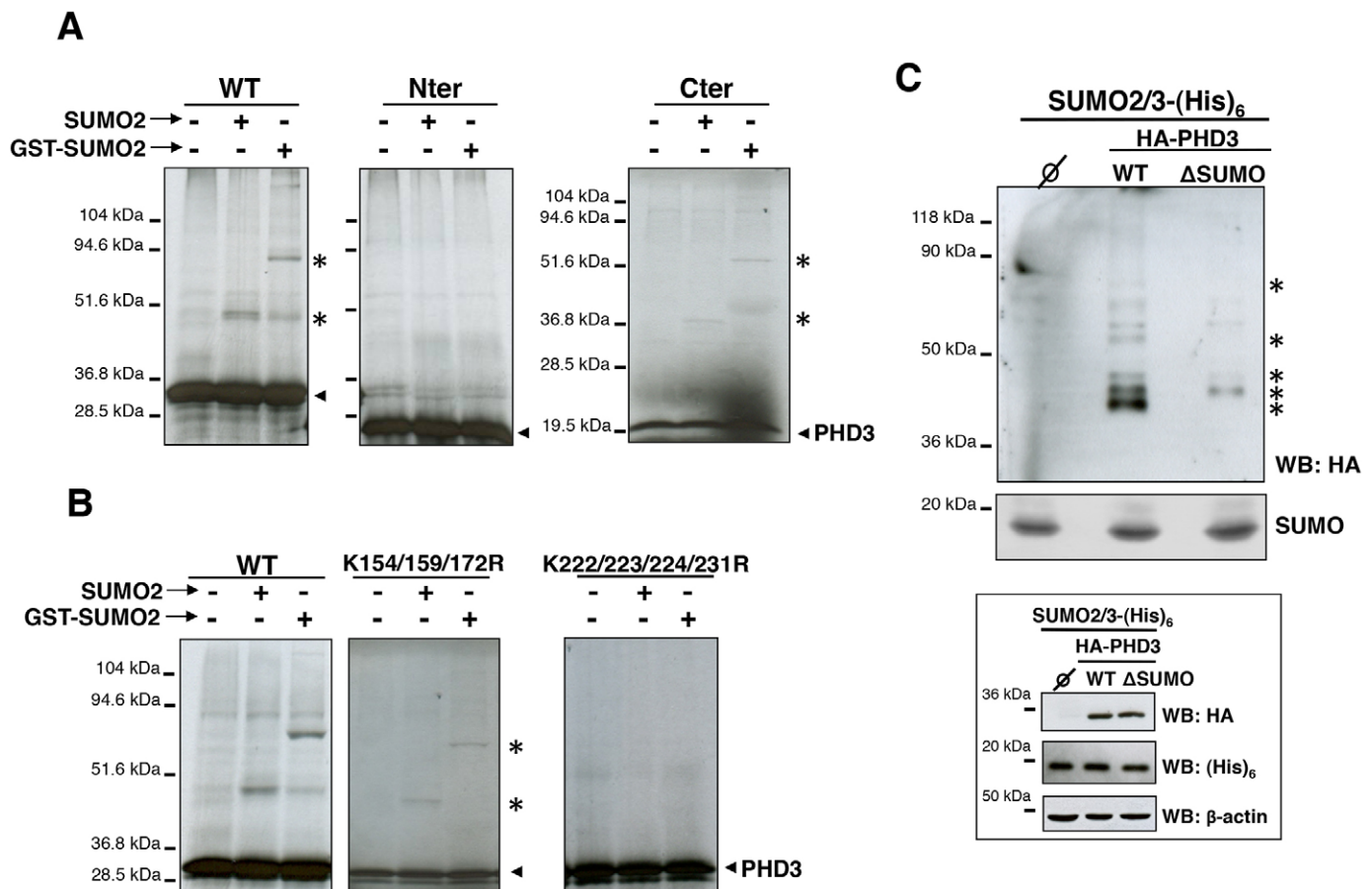


Fig. 2. Identification of the PHD3 SUMOylation site(s). (A) The wild type (WT) and truncated (Nter and Cter) [³⁵S]-labeled IVTT PHD3 constructs were incubated as described for Fig. 1A. The non-modified (arrowhead) and the modified (asterisk) forms of PHD3 were detected by autoradiography. (B) The indicated PHD3 constructs were subjected to an *in vitro* SUMOylation as described for Fig. 1A. (C) HEK293T cells were transfected as indicated, the SUMO-conjugated proteins were purified on Ni-NTA beads, resolved by SDS-PAGE and finally visualized by using western blotting (WB). The inset shows the protein expression levels from total cell extracts transfected in parallel. β -actin was used as the protein loading control. Ø, empty vector.

functions. We initially focused on two signaling pathways, the HIF and NF- κ B pathways, as they implicate PHD3 hydroxylase activity-dependent and -independent mechanisms, respectively. To address this issue, we co-transfected HEK293T cells with the different PHD3 mutants and luciferase reporter plasmids under the control of HIF- or NF- κ B-responsive elements (HRE-Luc or NF- κ B-Luc, respectively). In addition to PHD3 Δ SUMO, we generated chimeric PHD3 mutants (PHD3-SUMO1 and PHD3-SUMO2/3) by fusing SUMO1 or SUMO2/3 to the C-terminal end of PHD3 (Fig. 3; supplementary material Fig. S2). This strategy has been successfully used to mimic constitutive SUMOylation and study the impact of SUMOylation on target proteins (Ross et al., 2002). Note that the two glycine residues, which are crucial for generating the covalent link between SUMO and the target proteins were removed in the chimera to exclude the possibility of the fusion protein acting as a SUMO-like protein.

Luciferase activity following the expression of wild-type PHD3 and H196A (the hydroxylase dead mutant) was consistent with the predicted impact of these proteins on hypoxia-induced HIF activation (Fig. 3A). PHD3-SUMO2/3 was a significantly stronger HIF repressor compared with wild-type PHD3, whereas PHD3 Δ SUMO showed a clear tendency to be a less active repressor (Fig. 3A). This tendency is overcome by fusing SUMO2/3 to PHD3 Δ SUMO, supporting the role of SUMO conjugation in PHD3-mediated HIF repression (Fig. 3A). Moreover, the effect of the SUMO2/3 chimera was specific, as PHD3-SUMO1 behaved similarly to the wild-type protein (supplementary material Fig. S2). In contrast to the effects on

the HIF pathway, all of the PHD3 mutants inhibited TNF α -triggered activation of NF- κ B in a similar manner. Therefore, our data imply that PHD3 SUMOylation optimizes its HIF repressor activity without affecting the NF- κ B pathway (Fig. 3B).

To test whether endogenous SUMOylation at physiological levels contributes to HIF repression, HEK293T cells were transfected with short hairpin (sh)RNAs targeting HIF1 α (used as an internal control), UBC9 or SUMO3 and the luciferase reporter plasmids. According to our data, UBC9 or SUMO3 silencing, and therefore the inhibition of the SUMOylation machinery, increased hypoxia-triggered HIF activation (supplementary material Fig. S2).

Impact of PHD3 SUMOylation on protein stability and localization

Although the functional consequences of SUMOylation depend on the modified protein, SUMO conjugation is very often associated with temporal and spatial regulation of its targets. Therefore, to evaluate the impact of SUMO conjugation on PHD3 behavior, we analyzed the stability of PHD3 and PHD3-SUMO conjugates. We co-transfected PHD3 and (His)₆-tagged SUMO2/3 plasmids. After transfection, we treated cells with the protein synthesis inhibitor cycloheximide and harvested cells at the indicated times (Fig. 4A). SUMOylated proteins were recovered on Ni-NTA beads, and PHD3-SUMO conjugates were identified by immunoblotting (Fig. 4A, left panel). In parallel, the expression of the non-modified PHD3 was resolved using total

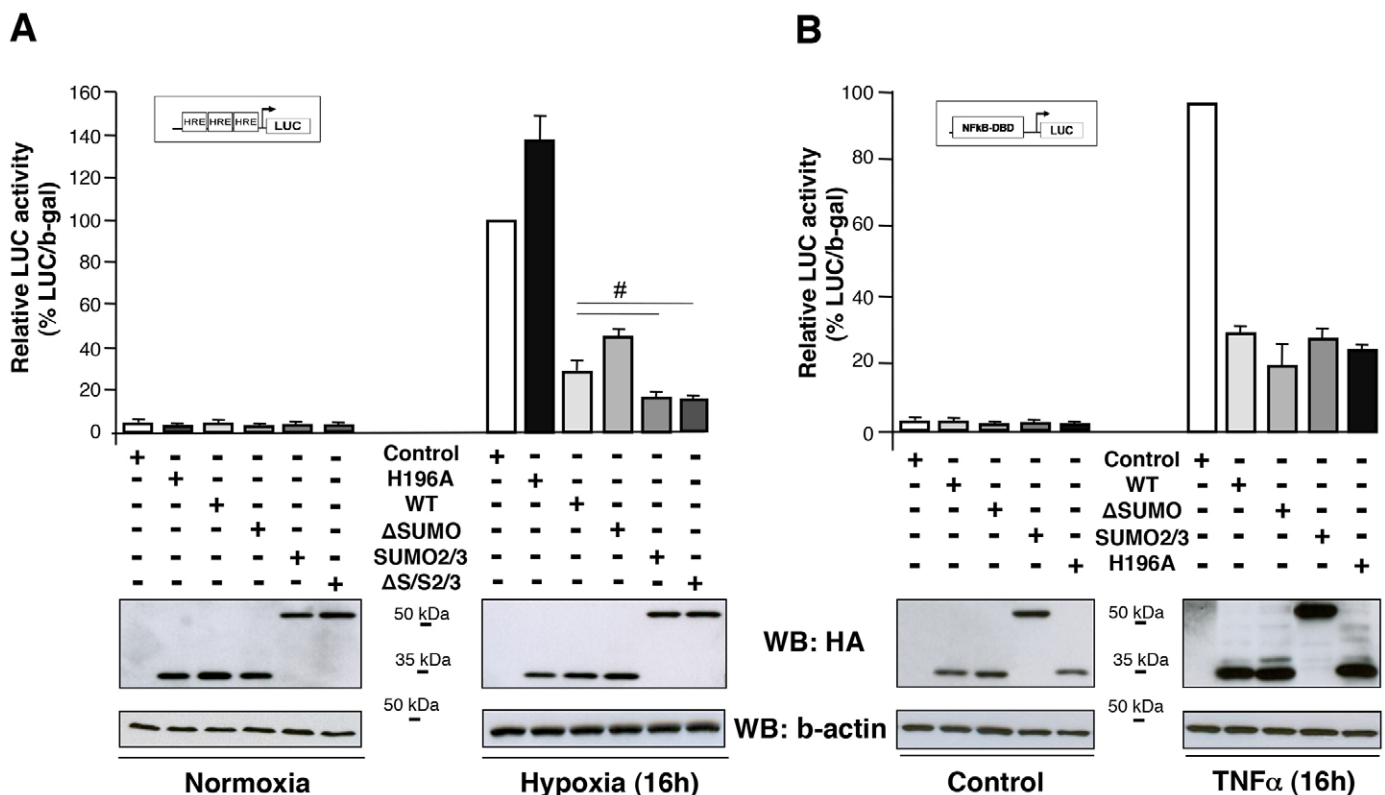


Fig. 3. The impact of SUMOylation on PHD3-dependent regulation of the HIF and NF- κ B pathways. (A) The indicated PHD3 constructs were transfected into HEK293T cells together with a reporter vector (pRE- Δ tk-Luc) containing three copies of the HRE from the erythropoietin gene and CMV- β gal to normalize for transfection efficiency. Cells were incubated in normoxia (20% O₂) or hypoxia (1% O₂) for 16 h and luciferase (LUC) and β -galactosidase (b-gal) activity were measured. WT, wild-type PHD3. (B) HEK293T cells were transfected with the PHD3 constructs, an NF- κ B luc-reporter and SV40- β gal. Luciferase and β -galactosidase activity was measured after 16 h of TNF α (20 ng/ml) treatment. The values represent the percentage of the LUC: β -gal activity relative to that of the control cells (treated). In parallel, cells were transfected to analyze protein expression levels by western blotting (WB). Results show the mean \pm s.e.m. (at least three independent experiments performed in triplicate); $^{\#}P < 0.05$ versus wild-type PHD3.

cellular extracts (Fig. 4A, right panel). No major differences in the stability of PHD3–SUMO conjugates compared with that of the non-modified PHD3 were observed. Furthermore, we evaluated the half-life of the different PHD3 mutants. As shown in Fig. 4B, the three constructs showed similar behavior, suggesting that SUMOylation does not affect PHD3 stability. To consolidate these data, we also evaluated the impact of Siah2, the ubiquitin E3 ligase that is reported to trigger PHD3 proteasomal degradation (Nakayama et al., 2004). We transfected cells with the different PHD3 mutants with and without Myc–Siah2 and analyzed their expression levels (Fig. 4C). Siah2 overexpression induced degradation of wild-type PHD3, PHD3–SUMO2/3 and PHD3 Δ SUMO constructs to a similar extent. These results suggest again that SUMOylation is unlikely to affect PHD3 stability. The data shown are supported by the fact that PHD3 Δ SUMO is similarly ubiquitinated to wild-type PHD3 (data not shown). Therefore, unlike other targets such as I κ B α , SUMO would not compete with ubiquitin to stabilize PHD3 (Desterro et al., 1998).

We next analyzed the effect of SUMO on PHD3 intracellular localization. We transfected the different HA-tagged constructs

into HeLa cells and performed immunofluorescence assays (supplementary material Fig. S3). In agreement with previous reports (Metzen et al., 2003), wild-type PHD3 showed nuclear and cytoplasmic localization. PHD3 SUMO mutants exhibited similar localization compared with that of the wild-type protein, indicating that SUMO does not affect PHD3 localization.

Consequences of PHD3 SUMOylation on HIF1 α protein levels

Among the complex mechanisms underlying HIF activation, PHD-mediated regulation of HIF α stability through hydroxylation plays an essential role. Thus, one scenario could be that SUMOylation optimizes PHD3 hydroxylase activity. We tested this possibility using a commercially available antibody that specifically recognizes hydroxylated P564 (P564OH), as we have confirmed (supplementary material Fig. S4A,B). In contrast to our initial hypothesis, wild-type PHD3, PHD3–SUMO2/3 and PHD3 Δ SUMO proteins were equally competent to hydroxylate HIF1 α P564, either in the presence or in the absence of the proteasome inhibitor, MG132 (Fig. 5A,B). Hence, we found no evidence that SUMOylation directly affects PHD3 hydroxylation activity, at least on HIF1 α P564, and therefore, HIF1 α protein levels.

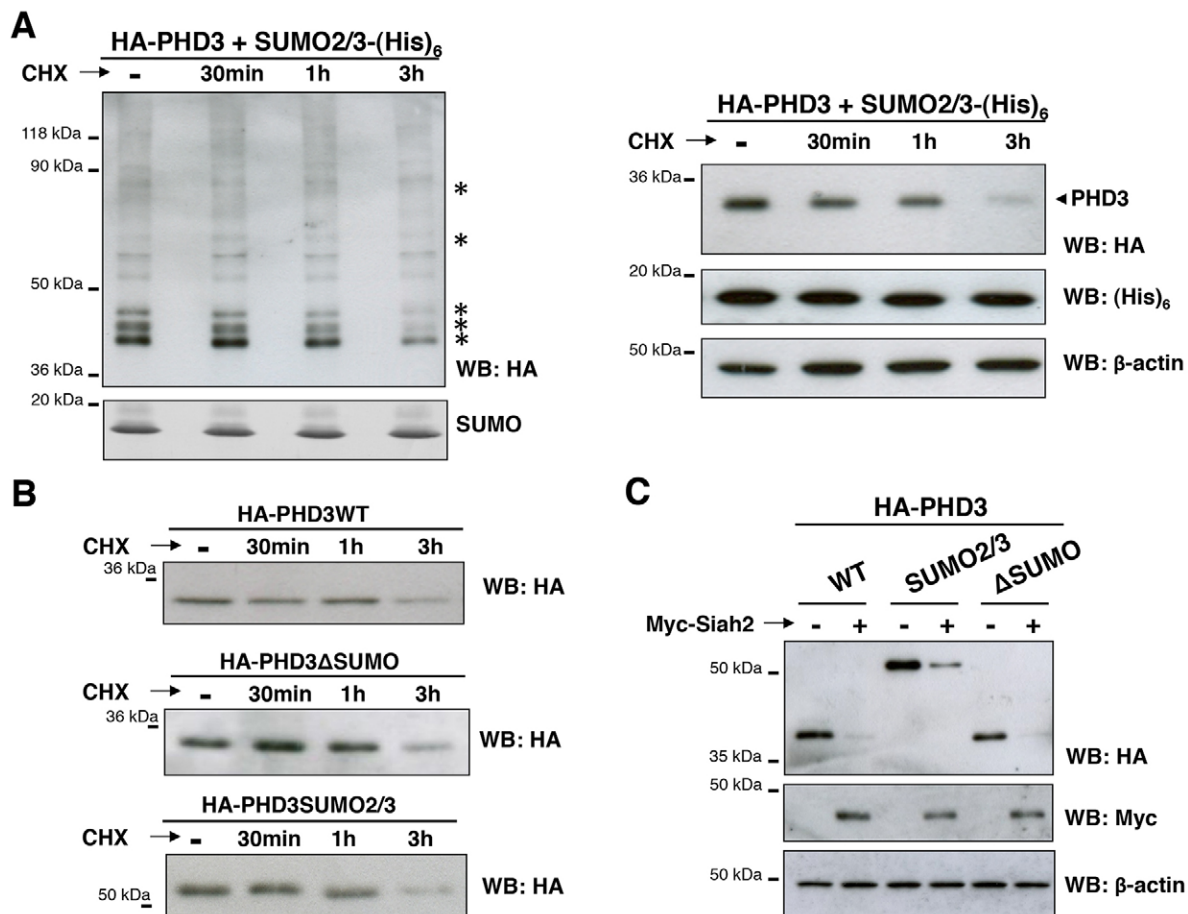


Fig. 4. PHD3–SUMO conjugation does not affect protein stability. (A) HEK293T cells transfected with PHD3 and SUMO2/3-(His)₆ were incubated in the presence of the protein synthesis inhibitor cycloheximide (CHX, 20 μ g/ml) for the indicated periods of time. SUMO-conjugated proteins (asterisks) were purified on Ni-NTA beads, resolved by SDS-PAGE and visualized by western blotting (WB, left panel). In parallel, the expression levels of the non-modified PHD3 (arrowhead) were analyzed by western blotting from the total cell extracts (right panel). (B) HEK293T cells were transfected with the different PHD3 constructs and incubated in the presence of the protein synthesis inhibitor cycloheximide (20 μ g/ml) for the indicated periods of time. Finally, the expression levels of the PHD3 constructs were analyzed by western blotting from the total cell extracts. (C) HEK293T cells were transfected with the different PHD3 constructs with or without Myc–Siah2. Total protein extracts were resolved by SDS-PAGE and expression levels were visualized by using western blotting. β -actin was used as the protein loading control. WT, wild-type PHD3.

PHD3 SUMOylation optimizes repression of HIF1 transactivation activity

An alternative scenario could be that SUMOylation affects HIF1 transactivation activity per se. To investigate this issue, we analyzed the impact of PHD3 mutants on the transcriptional activity triggered by a constitutively expressed HIF1 α (HIF1-DM). As shown in Fig. 5C, the expression of wild-type PHD3 significantly repressed HIF-dependent activation of the HRE-Luc reporter gene. Surprisingly, PHD3 Δ SUMO was a weaker inhibitor, whereas PHD3-SUMO2/3 and PHD3 Δ SUMO-SUMO2/3 behaved as stronger inhibitors compared with the activity of the wild-type protein (Fig. 5C). Note that mutation of the two proline residues released HIF1 α from PHD3-dependent degradation, but it did not prevent inhibition of HIF-dependent transactivation. By contrast, silencing of endogenous UBC9 or SUMO3 expression by shRNAs significantly reduced PHD3-mediated HIF repression (supplementary

material Fig. S4C). To determine whether PHD3 hydroxylase activity was implicated in the repression of HIF1 transactivation activity, we transfected cells as described previously but including the PHD3 H196A mutant (supplementary material Fig. S4D). This experiment clearly showed that PHD3 catalytic activity is not required to repress HIF transactivation.

Finally, we analyzed the impact of PHD3 mutants on the expression levels of two endogenous HIF1 target genes – *CA9* and *GLUT1* (also known as *SLC2A1*). Supporting our data on reporter gene expression, wild-type PHD3 repressed *CA9* and *GLUT1* mRNA levels. Furthermore, PHD3 Δ SUMO and PHD3-SUMO2/3 were a weaker and a stronger repressor, respectively, compared with the activity of the wild-type protein (Fig. 5D). Taken together, these data demonstrate that PHD3 represses HIF1 transcriptional activity in a hydroxylase-independent manner and also that PHD3 SUMOylation optimizes such repression.

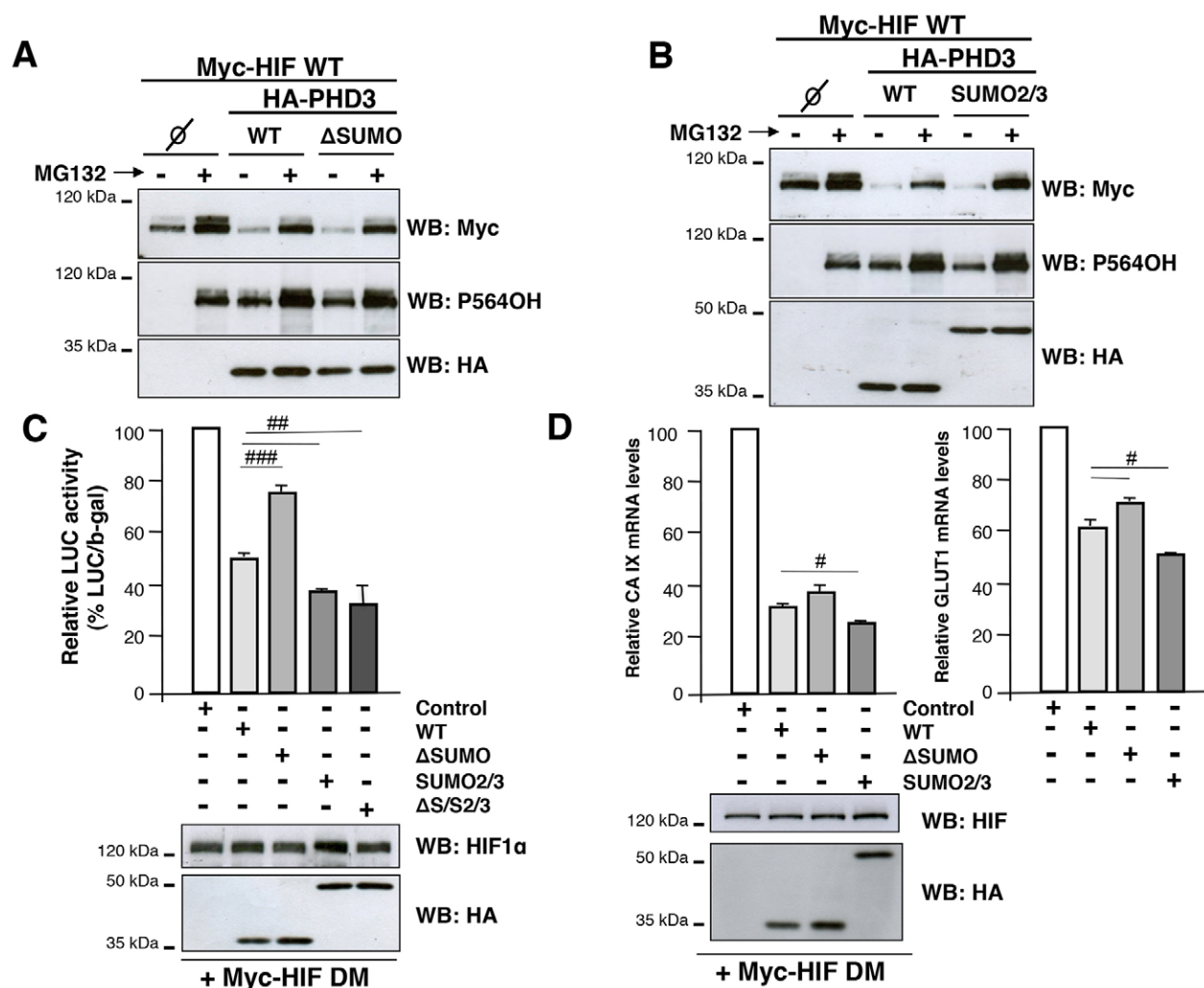


Fig. 5. PHD3 SUMOylation represses HIF1-dependent transactivation without affecting HIF1 α protein levels. (A,B) HEK293T cells were transfected with wild-type (WT) Myc-HIF1 and wild-type PHD3 or the different PHD3-SUMO mutants PHD3 Δ SUMO (A) and PHD3-SUMO2/3 (B). Cells were lysed after 4 h of MG132 treatment (10 μ M) and proteins were detected by using western blotting (WB). \emptyset , empty vector. (C) HEK293T cells were transfected with Myc-HIF1-DM (P402/564A), the indicated PHD3 constructs, the HRE-Luc reporter vector and CMV- β gal to normalize for transfection efficiency. Luciferase (LUC) and β -galactosidase (b-gal) activity were measured in cells incubated in normoxia (20% O₂). (D) HEK293T were transfected with Myc-HIF1-DM (P402/564A) and the indicated PHD3 constructs. The endogenous levels of CA9 (CA IX) and GLUT1 were analyzed by qRT-PCR. The values represent the percentage of the mRNA relative to that of the control cells. In parallel, cells were transfected to analyze protein expression levels by western blotting. Results are the mean \pm s.e.m. (at least three independent experiments performed in triplicate); # P < 0.05; ## P < 0.01; ### P < 0.001 versus wild-type PHD3.

Hypoxia modulates PHD3 SUMOylation

We have previously shown that PHD3 participates in a feedback loop to suppress HIF1 activation in response to prolonged hypoxia (Ginouves et al., 2008). The role of PHD3 SUMOylation in the repression of HIF-dependent transactivation prompted us to investigate whether hypoxia might regulate the conjugation of SUMO to PHD3. In line with our previous work, we conducted a timecourse study to evaluate the levels of PHD3–SUMO conjugates at different time-points of hypoxia (Fig. 6A). Interestingly, we observed that PHD3 SUMOylation was reduced after 4 h of hypoxia, whereas the levels of SUMOylated PHD3 recovered at prolonged hypoxia. These results demonstrate that hypoxia modulates PHD3 SUMOylation and show that the levels of PHD3–SUMO inversely correlate with an active HIF response.

The hypoxic response is primarily triggered by the inactivation of enzymes that belong to the family of 2-oxoglutarate and iron-dependent dioxygenases. Consequently, we wondered whether the activity of these enzymes might also affect PHD3 SUMOylation. Thus, we expressed PHD3 together with (His)₆-tagged SUMO2/3 and treated cells with DMOG, a pan-hydroxylase inhibitor. We found no evidence that hydroxylase activity affects PHD3 SUMOylation (Fig. 6B). Therefore, hypoxia-mediated modulation of PHD3 SUMO conjugation is independent of dioxygenase activity.

DISCUSSION

PHDs drive oxygen-dependent HIF α hydroxylation, which triggers protein ubiquitylation and further degradation by the proteasome. Therefore, oxygen is a master regulator of PHDs, although additional players have been shown to modulate these enzymes. The present study focused on SUMOylation as a potential PHD3 regulator. So far, hypoxia-dependent ubiquitin modification, catalyzed by the ubiquitin E3 ligase Siah2, is the only PTM described for PHD3 (Nakayama et al., 2004). Such ubiquitylation leads to PHD3 targeting for proteasomal degradation, and therefore modulates the response to hypoxia. In this report, we have demonstrated for the first time that PHD3 can be a SUMO target *in vitro* and in cultured cells. However, as reported for most of the SUMO targets, only a small fraction of PHD3 appears to be modified (Da Silva-Ferrada et al., 2012).

Although the *in vitro* SUMOylation assay showed one major PHD3–SUMO band, our mutational analysis indicates that mutation of four lysines (corresponding to the minimal PHD3 Δ SUMO mutant) was required to abolish PHD3 SUMOylation. Based on the close proximity of the four lysines within the cluster, it is tempting to speculate that although they are all likely to be modified, SUMOylation only occurs on one of the residues each time. PHD3 SUMOylation takes place at a non-consensus site in the C-terminal end of the protein. However, the SUMO acceptor site in PHD3 is

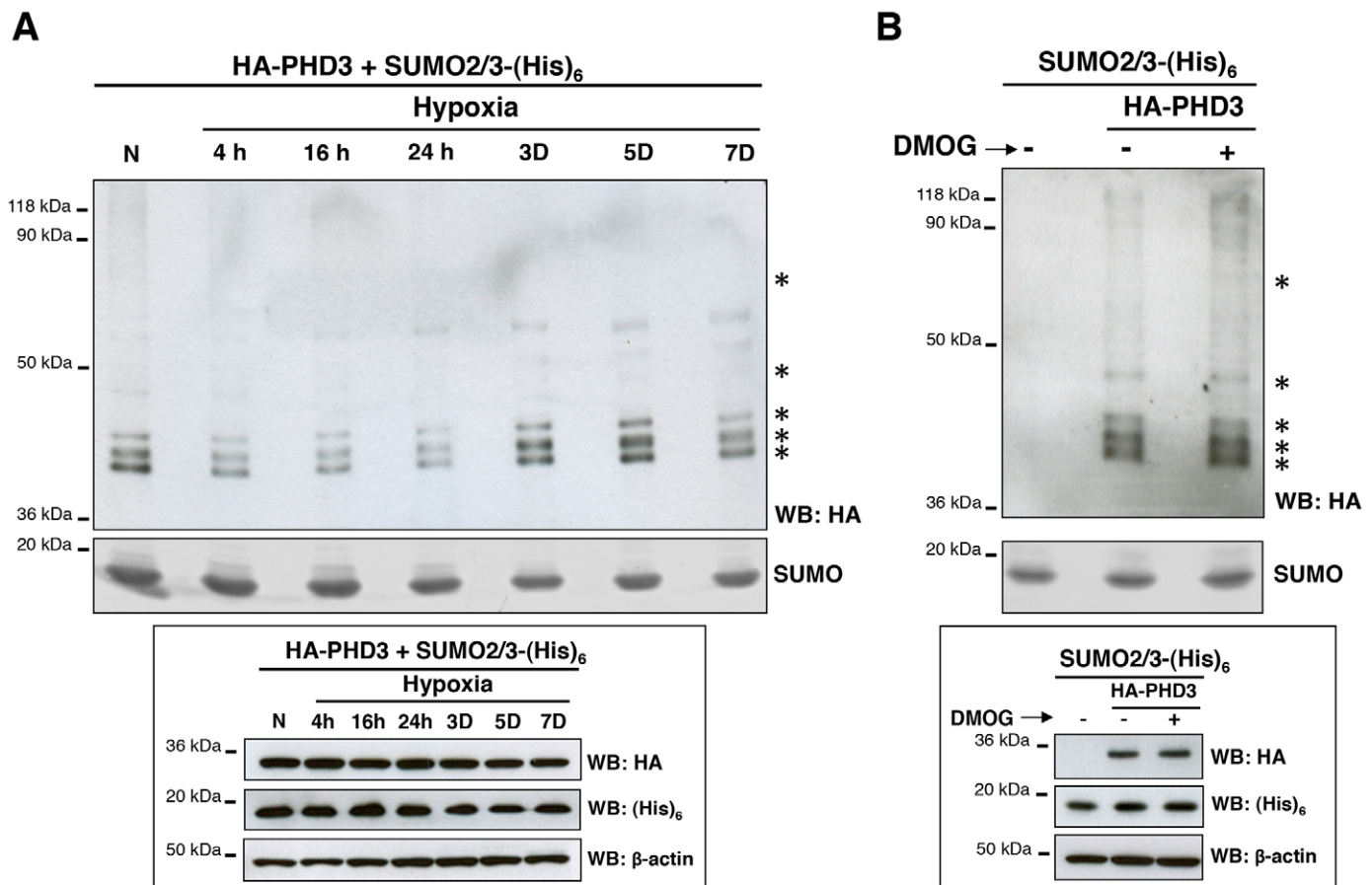


Fig. 6. Hypoxia but not DMOG regulates PHD3 SUMOylation. (A) HEK293T cells incubated in normoxia (N; 20% O₂) or hypoxia (1% O₂) for different times were transfected with PHD3 and SUMO2/3–(His)₆ 48 h before cell lysis. (B) HEK293T cells were transfected as described for A and incubated in the absence or the presence of the hydroxylase inhibitor DMOG (1 mM) for 4 h prior to cell lysis. SUMO-conjugated proteins were purified on Ni-NTA beads, resolved by SDS-PAGE and visualized by using western blotting (WB). SUMO-conjugated proteins are indicated by asterisks. Insets show the protein expression levels from total cell extracts transfected in parallel. β -actin was used as the protein loading control.

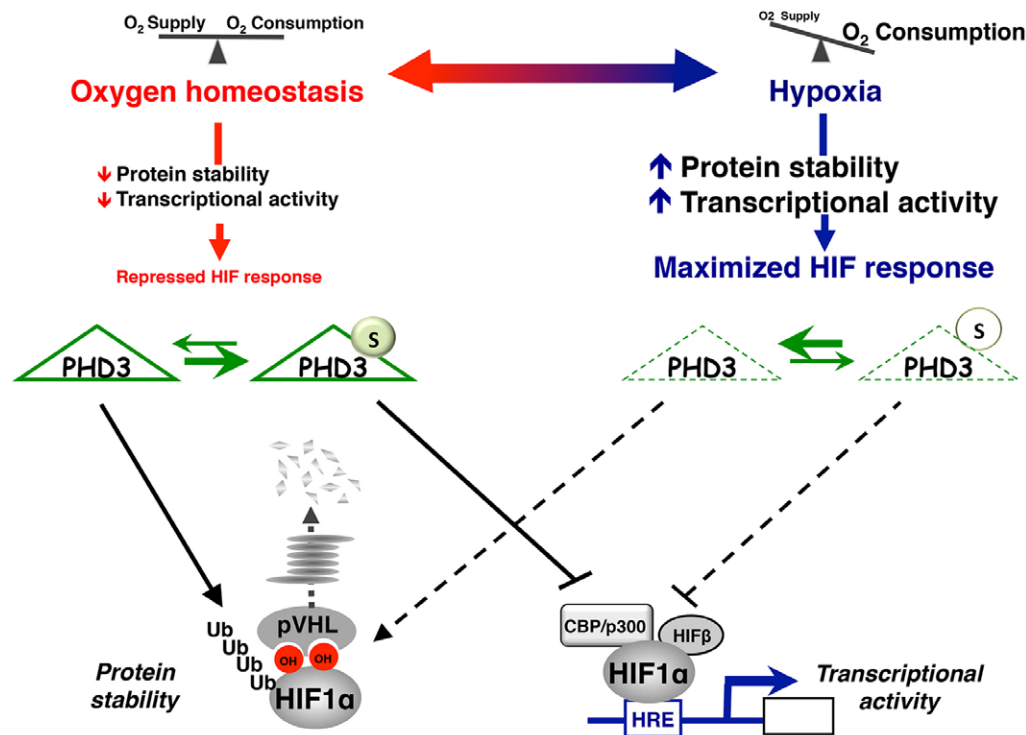


Fig. 7. A dual role for PHD3 in HIF1 repression. Besides the well-characterized role of PHD3 as a HIF1 repressor through destabilizing HIF1 α , PHD3 contributes to the inhibition of HIF1-dependent transcriptional activity per se. Moreover, PHD3 SUMOylation ('S') is required for the optimal repression of HIF1 transactivation. Hypoxia-modulated PHD3 SUMOylation appears to be a newly identified step in fine-tuning the control of HIF1 activation to ensure survival regardless of oxygen availability. Ub, ubiquitylation; OH, hydroxylation.

contained in the context of an alpha helix, as has been reported for substrates that do not conform to the consensus acceptor motif (Knipscheer et al., 2008; Pichler et al., 2005).

The unique PHD3–SUMO band detected *in vitro* also contrasts with the multiple SUMO-conjugates detected in cultured cells, which might result from poly-SUMOylation by SUMO2/3. Indeed, SUMO2 and SUMO3, but not SUMO1, contain an internal canonical SUMOylation site (K¹¹) that allows the formation of SUMO chains (Tatham et al., 2001). Besides the migration pattern of PHD3–SUMO conjugates, our data showing that SUMO2/3-conjugates are more readily detected in cultured cells as well as the functional assays support a central role for SUMO2/3 in the modification of PHD3. Otherwise, the shift of some SUMO-conjugates does not properly fit with the addition of 1, 2, 3 or more SUMO molecules. Thus, we cannot exclude the possibility that this shift might correspond to additional PTMs. Moreover, the interplay between SUMOylation and other PTMs has been extensively reported.

Our data provide new evidence for a dual role of PHD3 in HIF regulation as reported previously for EGL-9 in *Caenorhabditis elegans* (Shao et al., 2009) and PHD2 in mammals (Ozer et al., 2005; To and Huang, 2005). However, these results disagree with a more recent report claiming that PHD3 is a HIF1 coactivator through hydroxylation-mediated recruitment of PKM2 (Luo et al., 2011). In addition to the canonical role as the oxygen sensor that regulates HIF α stability, Powell-Coffman's group showed evidence that EGL-9 (the unique nematode ortholog of the PHD proteins) inhibits HIF1 transcriptional activity through a mechanism that does not implicate the hydroxylase activity. Supporting this prior study, our findings show the first evidence for a role of a mammalian PHD (in this case PHD3) as a HIF repressor independent of the hydroxylase activity. Moreover, we show here that PHD3 SUMOylation by SUMO2/3 mediates the non-canonical role of PHD3 as a HIF1 transcriptional repressor. Direct SUMOylation of transcription factors, which is mostly associated with transcriptional repression, arises as one of the most frequent functions attributed to SUMO in eukaryotic cells

(Ouyang et al., 2009). By contrast, our data support an indirect role of PHD3 SUMOylation in transcriptional repression, as recently shown for I κ B α (Mulero et al., 2013).

Contrary to the global SUMOylation increase induced by hypoxia, de-SUMOylated HIF1 α , HIF2 α , p300 and CBP correspond to the stable and/or active entities responsible for HIF activation (Cai et al., 2010; Cheng et al., 2006; Huang et al., 2009; van Hagen et al., 2010). Our results support this model, because PHD3 de-SUMOylation mirrors maximum HIF activation (Fig. 7). Whether hypoxia affects the machinery for PHD3 SUMOylation and/or de-SUMOylation or whether a second PTM is involved in this regulation remains to be explored.

Here, PHD3 SUMOylation is highlighted as an additional step among the extensive and dynamic mechanisms that are likely to be required to subtly adjust HIF function and to ensure survival regardless of oxygen availability. This complexity reflects not only the crucial role of HIF1 in maintaining oxygen homeostasis but also the need to safeguard against its inappropriate activation. Previous reports have linked the PHD3–HIF and SUMOylation pathways with pathologies such as cancer and ischemia (Cheng et al., 2006; Chen et al., 2011; Flotho and Melchior, 2013; Fox et al., 2011; Loinard et al., 2009; Mo et al., 2005; Silveirinha et al., 2013; Su et al., 2010; Wang and Banerjee, 2004; Xue et al., 2010). In light of these findings, there is a great interest in the development of drugs targeting these pathways. However, targeting global SUMOylation and/or the HIF response might have widespread toxicity and side effects. We anticipate that deciphering the specific SUMO E3 ligase and/or SUMO protease involved in PHD3 SUMOylation would facilitate the identification of more specific and secure targets and, therefore, provide alternative therapies.

MATERIALS AND METHODS

Plasmids

The HA–PHD3 Nter construct was generated by digestion of the pcDNA3–HA–PHD3 plasmid with *Bam*HI and *Eco*RI, filling to give blunt

ends, and re-ligation. HA-PHD3 Cter was generated by PCR amplification using HA-PHD3 as the template and the oligos 5'-GCAA-ATACAGATCTATGGAGAGGTCTAAG-3' and 5'-CTGATGGCGGC-CGCTCAGTCTTCAGTGAG-3'. The PCR product was digested with *Bgl*II and *Nor*I and cloned into the pcDNA3-HA vector that had been digested previously with *Bam*HI and *Nor*I. The pcDNA4-PHD3-(His)₆ construct was generated by digestion of HA-PHD3 with *Hind*III and *Eco*RI and cloning into the pcDNA4-(His)₆ plasmid. The pcDNA4-PHD3 SUMO1-(His)₆ chimera was generated by PCR amplification of SUMO1-(His)₆ with the oligos 5'-CATGGTGCTGCAGTGTCTGAC-3' and 5'-GTGGATCCTAACTCGAGGTTTG-3'. The PCR product was digested with *Pst*I and *Xho*I and cloned into the pcDNA4-PHD3-(His)₆ plasmid that had been digested previously with the same enzymes. pcDNA4-PHD3 SUMO2/3-(His)₆ was generated using the same strategy and the oligos 5'-CATGGTGCTGCAGTGTCCGAG-3' and 5'-GAATTCCTA-CTCGAGGTCTG-3'. The PHD3 mutants were obtained using the QuikChange® II XL Site-Directed Mutagenesis Kit (Stratagene). Primer sequences used for mutagenesis are listed in supplementary material Table S1. pCMV-Myc-HIF wild type and DM (P402/564A), pcDNA3-HA-PHD3, CMV-βgalactosidase, pRE-Δtk-Luc-HRE, pcDNA3-SUMO1-(His)₆, pcDNA3-SUMO2/3-(His)₆, pcDNA3-HA-SUMO1 and pcDNA3-HA-SUMO2/3 were as described previously (Berra et al., 2003; Desterro et al., 1998; Ginouvès et al., 2008; Tatham et al., 2001; Vertegaal et al., 2004). All of the constructs were sequence verified. Validated pools of shRNAs are from Open Biosystems.

In vitro SUMOylation assays

According to the manufacturer's protocol, the indicated PHD3 constructs were synthesized using the TNT T7 Quick Coupled Transcription/Translation Kit (Promega) supplemented with L-[³⁵S]-methionine (PerkinElmer). A volume of 2 μl of the [³⁵S]-labeled IVTT PHDs were used for SUMOylation assays (50 mM Tris-HCl pH 7.5, 5 mM MgCl₂) in the presence of SAE1 and SAE2 (0.109 μM, Enzo/BioMol), ATP (2 mM), creatine phosphate (10 mM), creatine kinase (3.5 U/ml), pyrophosphatase (1 U/ml) and recombinant UBC9 (3.6 μM), SUMO (80 μM) or GST-SUMO (50 μM). After 2 h at 30°C, the reactions were stopped and resolved by SDS-PAGE. Migrated gels were stained with Coomassie Brilliant Blue, dried and exposed. All of the SUMOylation assays were performed at least three times.

Cell culture and transfections

COS7, HeLa and HEK293T cells were cultured in Dulbecco's modified Eagle medium supplemented with 5% FBS. Cells were incubated at 37°C at 95% humidity and 5% CO₂. Hypoxic conditions were generated by incubation of cells in an anaerobic workstation (*In vivo* 400, Ruskinn). For hypoxic kinetics, HEK293T cells (supplemented with 10% FBS, 20 mM HEPES and ampicillin-streptomycin) were exposed to 1% O₂ for 7, 5 or 3 days or 24, 16 or 4 h prior to lysis. During longer timecourses, cells were split and seeded inside the workstation to obtain 80% confluence at the time of lysis. Cell extraction was performed inside the anaerobic workstation to avoid reoxygenation. Cells were transfected at 60–70% confluence 24 h post-seeding using the indicated DNA with FuGENE6 (Roche), Lipofectamine 2000 (Invitrogen) or ArrestIN (Open Biosystems) as transfection reagents.

Ni-NTA purification and immunoblotting

To purify on Ni-NTA beads, cells were lysed (6 M guanidium, 0.1 M Na₂HPO₄/NaH₂PO₄, 0.01 M Tris-HCl pH 8.0) and the extracts were incubated with the beads (Qiagen) in the presence of 10 mM β-mercaptoethanol for 2.5 h at room temperature. After two sequential washes using urea buffers (8 M urea, 0.1 M Na₂HPO₄/NaH₂PO₄, 0.01 M Tris-HCl, 10 mM β-mercaptoethanol) equilibrated first at pH 8.0 and then at pH 6.3, the purified proteins were eluted with 3× disruption buffer (150 mM Tris-HCl pH 6.8, 6% SDS, 30% glycerol, 10% β-mercaptoethanol, Bromophenol Blue) containing 200 mM imidazole. For total cell extracts, cells were lysed with Laemmli buffer (50 mM Tris-HCl pH 6.8, 1.25% SDS, 15% glycerol). Following quantification using the Lowry assay, the proteins were resolved by SDS-PAGE and electrophoretically transferred onto a PVDF membrane (Millipore). The following antibodies were used for

immunoblotting: mouse anti-HA.11 (BAbCO/Covance), mouse anti-β-actin (Sigma), rabbit anti-SUMO2/3 (Eurogentec), mouse anti-(His)₆ (Novagen), mouse anti-Myc (Cell Signaling Technology), rabbit anti-HIF-P564OH (Cell Signaling Technology), rabbit anti-HIF1α (Richard et al., 1999). Immunoreactive bands were visualized with ECL.

Reporter assays and qRT-PCR

Cells were lysed in 25 mM Tris phosphate pH 7.8, 8 mM MgCl₂, 0.5% Triton X-100, 7.5% glycerol and 1 mM DTT. Luciferase activity measurement was performed using the Steadylite plus™ High Sensitivity Luminescence Reporter Gene Assay System (PerkinElmer). β-galactosidase activity measurement was performed using the Galacto-Light Plus system (Applied Biosystems).

Total RNA was isolated using the RNeasy Mini Kit (Qiagen), reverse transcribed and amplified with the quantitative PCR MasterMix Plus for SYBR Green I (Roche). PCR was carried out in a C1000™ Thermal cycler (Bio-Rad). *RPLP0* was used for normalization. The primer sequences are available upon request.

Immunofluorescence

HeLa cells were fixed in 3% paraformaldehyde for 30 min at 37°C. After several washes with PBS, cells were permeabilized for 5 min with 0.2% Triton X-100 and blocked for 30 min in PBS solution containing 0.2% gelatin and 2% bovine serum albumin (BSA). Then cells were incubated with mouse anti-HA.11, donkey anti-mouse-IgG conjugated to Alexa Fluor 488 (Invitrogen) and DAPI. Cells were analyzed with a fluorescent microscope, the Axio Imager D1 (Zeiss), using structured illumination (Apotome accessory) with a 63× objective. Images were taken with an AxioCam HRm camera (Zeiss) operated by AxioVision Rel 4.8 software.

Statistical analysis

Data are expressed as the mean ± s.e.m. Differences were examined by Student's *t*-test (two-tailed) between two groups or by one-way analysis of variance (ANOVA) within multiple groups. *P* < 0.05 was considered statistically significant.

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Competing interests

The authors declare no competing interests.

Author contributions

A.N., A.G., S.P., O.C., B.U. and F.L. performed the experiments; A.N. and M.R. contributed to the writing of the manuscript and to analyze data; E.B. designed and supervised the project, analyzed data and wrote the manuscript.

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Supplementary material

Supplementary material available online at <http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.151514/-DC1>

References

- Benizri, E., Ginouvès, A. and Berra, E. (2008). The magic of the hypoxia-signaling cascade. *Cell. Mol. Life Sci.* **65**, 1133–1149.
- Berra, E., Benizri, E., Ginouvès, A., Volmat, V., Roux, D. and Pouyssegur, J. (2003). HIF prolyl-hydroxylase 2 is the key oxygen sensor setting low steady-state levels of HIF-1α in normoxia. *EMBO J.* **22**, 4082–4090.
- Bruick, R. K. and McKnight, S. L. (2001). A conserved family of prolyl-4-hydroxylases that modify HIF. *Science* **294**, 1337–1340.

- Cai, Q., Verma, S. C., Kumar, P., Ma, M. and Robertson, E. S. (2010). Hypoxia inactivates the VHL tumor suppressor through PIASy-mediated SUMO modification. *PLoS ONE* 5, e9720.
- Chen, S., Zhang, J., Li, X., Luo, X., Fang, J. and Chen, H. (2011). The expression of prolyl hydroxylase domain enzymes are up-regulated and negatively correlated with Bcl-2 in non-small cell lung cancer. *Mol. Cell. Biochem.* 358, 257–263.
- Cheng, J., Bawa, T., Lee, P., Gong, L. and Yeh, E. T. (2006). Role of desumoylation in the development of prostate cancer. *Neoplasia* 8, 667–676.
- Da Silva-Ferrada, E., Lopitz-Otsoa, F., Lang, V., Rodríguez, M. S. and Matthiesen, R. (2012). Strategies to identify recognition signals and targets of SUMOylation. *Biochem. Res. Int.* 2012, 875148.
- Desterro, J. M., Rodríguez, M. S. and Hay, R. T. (1998). SUMO-1 modification of I κ B α inhibits NF- κ B activation. *Mol. Cell* 2, 233–239.
- Epstein, A. C., Gleadle, J. M., McNeill, L. A., Hewitson, K. S., O'Rourke, J., Mole, D. R., Mukherji, M., Metzen, E., Wilson, M. I., Dhanda, A. et al. (2001). C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. *Cell* 107, 43–54.
- Flotho, A. and Melchior, F. (2013). Sumoylation: a regulatory protein modification in health and disease. *Annu. Rev. Biochem.* 82, 357–385.
- Fox, S. B., Generali, D., Berruti, A., Brizzi, M. P., Campo, L., Bonardi, S., Bersiga, A., Allevi, G., Milani, M., Aguggini, S. et al. (2011). The prolyl hydroxylase enzymes are positively associated with hypoxia-inducible factor-1 α and vascular endothelial growth factor in human breast cancer and alter in response to primary systemic treatment with epirubicin and tamoxifen. *Breast Cancer Res.* 13, R16.
- Fu, J. and Taubman, M. B. (2010). Prolyl hydroxylase EGLN3 regulates skeletal myoblast differentiation through an NF- κ B-dependent pathway. *J. Biol. Chem.* 285, 8927–8935.
- Fu, J., Menzies, K., Freeman, R. S. and Taubman, M. B. (2007). EGLN3 prolyl hydroxylase regulates skeletal muscle differentiation and myogenin protein stability. *J. Biol. Chem.* 282, 12410–12418.
- Geiss-Friedlander, R. and Melchior, F. (2007). Concepts in sumoylation: a decade on. *Nat. Rev. Mol. Cell Biol.* 8, 947–956.
- Ginouvès, A., Ilc, K., Macias, N., Pouyssegur, J. and Berra, E. (2008). PHDs overactivation during chronic hypoxia “desensitizes” HIF1 α and protects cells from necrosis. *Proc. Natl. Acad. Sci. USA* 105, 4745–4750.
- Hay, R. T. (2007). SUMO-specific proteases: a twist in the tail. *Trends Cell Biol.* 17, 370–376.
- Huang, L. E., Arany, Z., Livingston, D. M. and Bunn, H. F. (1996). Activation of hypoxia-inducible transcription factor depends primarily upon redox-sensitive stabilization of its α subunit. *J. Biol. Chem.* 271, 32253–32259.
- Huang, C., Han, Y., Wang, Y., Sun, X., Yan, S., Yeh, E. T., Chen, Y., Cang, H., Li, H., Shi, G. et al. (2009). SENP3 is responsible for HIF-1 transactivation under mild oxidative stress via p300 de-SUMOylation. *EMBO J.* 28, 2748–2762.
- Ivan, M., Kondo, K., Yang, H., Kim, W., Valiando, J., Ohh, M., Salic, A., Asara, J. M., Lane, W. S. and Kaelin, W. G., Jr (2001). HIF1 α targeted for VHL-mediated destruction by proline hydroxylation: implications for O₂ sensing. *Science* 292, 464–468.
- Jaakkola, P., Mole, D. R., Tian, Y. M., Wilson, M. I., Gielbert, J., Gaskell, S. J., von Kriegsheim, A., Hebestreit, H. F., Mukherji, M., Schofield, C. J. et al. (2001). Targeting of HIF- α to the von Hippel-Lindau ubiquitylation complex by O₂-regulated prolyl hydroxylation. *Science* 292, 468–472.
- Kelley, L. A. and Sternberg, M. J. (2009). Protein structure prediction on the Web: a case study using the Phyre server. *Nat. Protoc.* 4, 363–371.
- Knipscheer, P., Flotho, A., Klug, H., Olsen, J. V., van Dijk, W. J., Fish, A., Johnson, E. S., Mann, M., Sixma, T. K. and Pichler, A. (2008). Ubc9 sumoylation regulates SUMO target discrimination. *Mol. Cell* 31, 371–382.
- Köditz, J., Nesper, J., Wottawa, M., Stiehl, D. P., Camenisch, G., Franke, C., Mjlyharju, J., Wenger, R. H. and Katschinski, D. M. (2007). Oxygen-dependent ATF-4 stability is mediated by the PHD3 oxygen sensor. *Blood* 110, 3610–3617.
- Lee, S., Nakamura, E., Yang, H., Wei, W., Linggi, M. S., Sajjan, M. P., Farese, R. V., Freeman, R. S., Carter, B. D., Kaelin, W. G., Jr et al. (2005). Neuronal apoptosis linked to EglN3 prolyl hydroxylase and familial pheochromocytoma genes: developmental culling and cancer. *Cancer Cell* 8, 155–167.
- Loinard, C., Ginouvès, A., Vilar, J., Cochain, C., Zouggar, Y., Recalde, A., Duriez, M., Lévy, B. I., Pouyssegur, J., Berra, E. et al. (2009). Inhibition of prolyl hydroxylase domain proteins promotes therapeutic revascularization. *Circulation* 120, 50–59.
- Luo, W., Hu, H., Chang, R., Zhong, J., Knabel, M., O'Meally, R., Cole, R. N., Pandey, A. and Semenza, G. L. (2011). Pyruvate kinase M2 is a PHD3-stimulated coactivator for hypoxia-inducible factor 1. *Cell* 145, 732–744.
- Metzen, E., Berchner-Pfannschmidt, U., Stengel, P., Marxsen, J. H., Stolze, I., Klinger, M., Huang, W. Q., Wotzlaw, C., Hellwig-Bürgel, T., Jelkmann, W., Acker, H. and Fandrey, J. (2003). Intracellular localisation of human HIF-1 α hydroxylases: implications for oxygen sensing. *J. Cell. Sci.* 116, 1319–1326.
- Mo, Y. Y., Yu, Y., Theodosiou, E., Ee, P. L. and Beck, W. T. (2005). A role for Ubc9 in tumorigenesis. *Oncogene* 24, 2677–2683.
- Mulero, M. C., Ferrer-Marco, D., Islam, A., Margalef, P., Pecoraro, M., Toll, A., Drechsel, N., Charneco, C., Davis, S., Bellora, N. et al. (2013). Chromatin-bound I κ B α regulates a subset of polycomb target genes in differentiation and cancer. *Cancer Cell* 24, 151–166.
- Nakayama, K., Frew, I. J., Hagensen, M., Skals, M., Habelhah, H., Bhoomik, A., Kadoya, T., Erdjument-Bromage, H., Tempst, P., Frappell, P. B. et al. (2004). Siah2 regulates stability of prolyl-hydroxylases, controls HIF1 α abundance, and modulates physiological responses to hypoxia. *Cell* 117, 941–952.
- Núñez-O'Mara, A. and Berra, E. (2013). Deciphering the emerging role of SUMO conjugation in the hypoxia-signaling cascade. *Biol. Chem.* 394, 459–469.
- Ouyang, J., Valin, A. and Gill, G. (2009). Regulation of transcription factor activity by SUMO modification. *Methods Mol. Biol.* 497, 141–152.
- Ozer, A., Wu, L. C. and Brückner, R. K. (2005). The candidate tumor suppressor ING4 represses activation of the hypoxia inducible factor (HIF). *Proc. Natl. Acad. Sci. USA* 102, 7481–7486.
- Pichler, A., Knipscheer, P., Oberhofer, E., van Dijk, W. J., Körner, R., Olsen, J. V., Jentsch, S., Melchior, F. and Sixma, T. K. (2005). SUMO modification of the ubiquitin-conjugating enzyme E2-25K. *Nat. Struct. Mol. Biol.* 12, 264–269.
- Richard, D. E., Berra, E., Gothi, E., Roux, D. and Pouyssegur, J. (1999). p42/p44 mitogen-activated protein kinases phosphorylate hypoxia-inducible factor 1 α (HIF-1 α) and enhance the transcriptional activity of HIF-1. *J. Biol. Chem.* 274, 32631–32637.
- Rodríguez, M. S., Dargemont, C. and Hay, R. T. (2001). SUMO-1 conjugation in vivo requires both a consensus modification motif and nuclear targeting. *J. Biol. Chem.* 276, 12654–12659.
- Romero-Ruiz, A., Bautista, L., Navarro, V., Heras-Garvín, A., March-Díaz, R., Castellano, A., Gómez-Díaz, R., Castro, M. J., Berra, E., López-Barneo, J. et al. (2012). Prolyl hydroxylase-dependent modulation of eukaryotic elongation factor 2 activity and protein translation under acute hypoxia. *J. Biol. Chem.* 287, 9651–9658.
- Ross, S., Best, J. L., Zon, L. I. and Gill, G. (2002). SUMO-1 modification represses Sp3 transcriptional activation and modulates its subnuclear localization. *Mol. Cell* 10, 831–842.
- Salceda, S. and Caro, J. (1997). Hypoxia-inducible factor 1 α (HIF-1 α) protein is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions. Its stabilization by hypoxia depends on redox-induced changes. *J. Biol. Chem.* 272, 22642–22647.
- Semenza, G. L. (2012). Hypoxia-inducible factors in physiology and medicine. *Cell* 148, 399–408.
- Shao, Z., Zhang, Y. and Powell-Coffman, J. A. (2009). Two distinct roles for EGL-9 in the regulation of HIF-1-mediated gene expression in *Caenorhabditis elegans*. *Genetics* 183, 821–829.
- Silveirinha, V., Stephens, G. J. and Cimarosti, H. (2013). Molecular targets underlying SUMO-mediated neuroprotection in brain ischemia. *J. Neurochem.* 127, 580–591.
- Su, H. L. and Li, S. S. (2002). Molecular features of human ubiquitin-like SUMO genes and their encoded proteins. *Gene* 296, 65–73.
- Su, Y., Loos, M., Giese, N., Hines, O. J., Diebold, I., Görlach, A., Metzen, E., Pastorekova, S., Friess, H. and Büchler, P. (2010). PHD3 regulates differentiation, tumour growth and angiogenesis in pancreatic cancer. *Br. J. Cancer* 103, 1571–1579.
- Takeda, K., Ho, V. C., Takeda, H., Duan, L. J., Nagy, A. and Fong, G. H. (2006). Placental but not heart defects are associated with elevated hypoxia-inducible factor α levels in mice lacking prolyl hydroxylase domain protein 2. *Mol. Cell. Biol.* 26, 8336–8346.
- Tatham, M. H., Jaffray, E., Vaughan, O. A., Desterro, J. M., Botting, C. H., Naismith, J. H. and Hay, R. T. (2001). Polymeric chains of SUMO-2 and SUMO-3 are conjugated to protein substrates by SAE1/SAE2 and Ubc9. *J. Biol. Chem.* 276, 35368–35374.
- Tempé, D., Piechaczyk, M. and Bossis, G. (2008). SUMO under stress. *Biochem. Soc. Trans.* 36, 874–878.
- To, K. K. and Huang, L. E. (2005). Suppression of hypoxia-inducible factor 1 α (HIF-1 α) transcriptional activity by the HIF prolyl hydroxylase EGLN1. *J. Biol. Chem.* 280, 38102–38107.
- van Hagen, M., Overmeer, R. M., Abolvardi, S. S. and Vertegaal, A. C. (2010). RNF4 and VHL regulate the proteasomal degradation of SUMO-conjugated Hypoxia-Inducible Factor-2 α . *Nucleic Acids Res.* 38, 1922–1931.
- Vertegaal, A. C., Ogg, S. C., Jaffray, E., Rodríguez, M. S., Hay, R. T., Andersen, J. S., Mann, M. and Lamond, A. I. (2004). A proteomic study of SUMO-2 target proteins. *J. Biol. Chem.* 279, 33791–33798.
- Wang, L. and Banerjee, S. (2004). Differential PIAS3 expression in human malignancy. *Oncol. Rep.* 11, 1319–1324.
- Wang, G. L., Jiang, B. H., Rue, E. A. and Semenza, G. L. (1995). Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O₂ tension. *Proc. Natl. Acad. Sci. USA* 92, 5510–5514.
- Xie, L., Xiao, K., Whalen, E. J., Forrester, M. T., Freeman, R. S., Fong, G., Gygi, S. P., Lefkowitz, R. J. and Stamler, J. S. (2009). Oxygen-regulated beta(2)-adrenergic receptor hydroxylation by EGLN3 and ubiquitylation by pVHL. *Sci. Signal.* 2, ra33.
- Xue, J., Li, X., Jiao, S., Wei, Y., Wu, G. and Fang, J. (2010). Prolyl hydroxylase-3 is down-regulated in colorectal cancer cells and inhibits IKK β independent of hydroxylase activity. *Gastroenterology* 138, 606–615.
- Yan, B., Jiao, S., Zhang, H. S., Lv, D. D., Xue, J., Fan, L., Wu, G. H. and Fang, J. (2011). Prolyl hydroxylase domain protein 3 targets Pax2 for destruction. *Biochem. Biophys. Res. Commun.* 409, 315–320.
- Zhang, Q., Gu, J., Li, L., Liu, J., Luo, B., Cheung, H. W., Boehm, J. S., Ni, M., Geisen, C., Root, D. E. et al. (2009). Control of cyclin D1 and breast tumorigenesis by the EglN2 prolyl hydroxylase. *Cancer Cell* 16, 413–424.