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

Hagen, M. van

Chapter 3

Inhibition of Hypoxia-Inducible Factor-1α by conjugation of Small Ubiquitin-like Modifier to a lysine in a non-consensus site

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Inhibition of Hypoxia-Inducible Factor-1α by conjugation of Small Ubiquitin-like Modifier-1 and -2 to a Lysine in a Non-Consensus Modification Site

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ABSTRACT

HIF-1α is a master regulator of the cellular response to hypoxia. Under normoxic conditions, HIF-1α is degraded by the ubiquitin-proteasome system. During hypoxia, HIF-1α is stabilized and conjugated to the ubiquitin-like proteins SUMO-1 and SUMO-2. Previously, it has been shown that lysines 391 and 477 of HIF-1α are SUMO acceptor sites. Here we show that lysine 377 of HIF-1α, a lysine that is situated in the non-consensus sumoylation site TKVE, is also used as a SUMO acceptor site. Sumoylation of this lysine was detected by mass spectrometry. Mutating all three sumoylation sites in HIF-1α caused a significant increase in transcriptional activity compared to mutating only lysines 391 and 477. Overexpressing the SUMO protease SENP1 also caused an increase in HIF-1α transcriptional activity. We conclude that sumoylation inhibits the activity of HIF-1α. Stimulating HIF-1α sumoylation could be a novel strategy to limit the activity of this key drug target.

INTRODUCTION

Hypoxia Inducible Factors (HIFs) are dimeric transcription factors that play key roles in the cellular response to hypoxia (Lee *et al.*, 2004;Pouyssegur *et al.*, 2006;Ke and Costa, 2006). Hypoxia is linked to cancer, cardiovascular disease and stroke and also plays an important role during embryonic development. HIF-1 acts as a dimer that is composed of HIF-1α and HIF-1β. Alternative HIF dimers consist of HIF-1β and either HIF-2α or HIF-3α (Lee *et al.*, 2004;Ke and Costa, 2006). HIF controls an extensive set of target genes, including erythropoietin (EPO), vascular endothelial growth factor (VEGF) and glucose transporters, that regulate oxygen supply, cellular metabolism, cell growth and apoptosis (Wenger *et al.*, 2005). HIF-1α deficient mice die during embryonic development due to a lack of vascularization and major cardiac malformation including defective ventricle formation (Ryan *et al.*, 1998;Iyer *et al.*, 1998;Compernolle *et al.*, 2003). This is due to a reduced expression of the HIF-1α target gene myocyte enhancer factor 2C (Compernolle *et al.*, 2003).

HIF-1β is constitutively present in cells. In contrast, the activity of HIF-1α is primarily regulated via protein stability (Lee *et al.*, 2004;Pouyssegur *et al.*, 2006;Ke and Costa, 2006;Berra *et al.*, 2006). Under normoxic conditions, HIF-1α is continuously synthesized, but rapidly targeted for degradation via post-translational modifications (Salceda and Caro, 1997;Huang *et al.*, 1998). This is initiated by prolyl-hydroxylation of HIF-1α carried out by the prolyl hydroxylases PHD1, PHD2 and PHD3 (Bruick and McKnight, 2001;Epstein *et al.*, 2001;Yu *et al.*, 2001;Berra *et al.*, 2006). These PHDs are the major oxygen sensors in cells and are inactivated during hypoxia to allow HIF stabilization and activity (Epstein *et al.*, 2001;Ivan *et al.*, 2001;Jaakkola *et al.*, 2001;Gerald *et al.*, 2004;Nakayama *et al.*, 2004). Hydroxylation of proline 564 of HIF-1α enables the binding of the von Hippel-Lindau-containing ubiquitin E3 ligase complex responsible for the ubiquitination of HIF-1α (Maxwell *et al.*, 1999;Bruick and McKnight, 2001;Ivan *et al.*, 2001;Jaakkola *et al.*, 2001). Ubiquitination of HIF-1α on K532, K538 and K547 targets the protein to the proteasome for degradation (Salceda and Caro, 1997;Tanimoto *et al.*, 2000;Paltoglou and Roberts, 2007).

In addition to these modifications, other post-translation modifications tightly control the activity of HIF-1 (Brahimi-Horn *et al.*, 2005). Recently, it has been found that ubiquitin-like proteins control the activity of HIF (Bae *et al.*, 2004;Shao *et al.*, 2004;Matic *et al.*, 2007;Carbia-Nagashima *et al.*, 2007;Berta *et al.*, 2007;Cheng *et al.*, 2007;Ulrich, 2007). We

have studied the conjugation of Small Ubiquitin-like Modifiers (SUMOs) to HIF-1α in detail and identified three acceptor lysines, K377, K391 and K477. Conjugation of HIF-1α to SUMO significantly decreased its transcriptional activity but did not alter its subcellular localization. Thus, the activity of HIF-1α during hypoxia can be regulated by sumoylation.

MATERIALS AND METHODS

Plasmids

A plasmid containing the human HIF-1α cDNA (IMAGE clone 3842146) was obtained from the Mammalian Gene Collection and this cDNA was sub-cloned into pDONR207 (Invitrogen). HIF-1α was transferred to eGFP-, T7-His_6 - and Gal4DBD Destination vectors using standard Gateway cloning technology (Invitrogen). The N-terminal truncation mutant HIF-$1\alpha_{1\text{-}420}$ was generated by PCR using oligonucleotides 5'-*aaaaagcaggctcc*atggagggcgcc-3' and 5'- *agaaagctgggtc***tta**agttctgtgtcgttgctg-3' (HIF-1α- specific sequence underlined, stop codon added to primer in bold). A secondary PCR was performed using a primer set complementary to part of the first primer set (sequence in italics) to introduce Gateway-compatible sequences: 5'-ggggacaagtttgtacaaaaaagcaggct-3' and 5'-ggggaccactttgtacaagaaagctgggt-3'. The secondary PCR product was also cloned into pDONR207 (Invitrogen).

SENP1 cDNA was amplified by PCR from IMAGE clone 5298667 obtained from the Mammalian Gene Collection and inserted into the PCS2 vector (Roukens *et al.*, 2008) digested with *Stu*I. The FLAG-tag sequence was included in the forward oligonucleotide (sequence underlined). SENP1 cDNA was amplified using oligonucleotides 5'-atggactacaaggatgacgacgataaggatgatattgctgataggatgag-3' and 5'-tcacaagagttttcggtggaggatc-3'.

The SUMO-1, SAE1/2 and Ubc9 bacterial expression vector (pE1E2S1) (Uchimura *et al.*, 2004a) was a kind gift from Dr. H. Saitoh (Kumamoto University, Japan). The 5xGal4-E1B-Luciferase reporter (Roche *et al.*, 2007) was a kind gift from Dr. N.D. Perkins (Wellcome Trust Biocentre, Dundee, U.K.) and the 5xHREpGL3-Luciferase reporter (Duyndam *et al.*, 2003) was a kind gift from Dr. M.C.A. Duyndam (VUMC, Amsterdam, the Netherlands).

RNAi

Plasmids were generated encoding puromycin resistance and shRNAs directed against the indicated components using the following oligonucleotides: SAE2: 5'-gatcccatagaccagtgcagaacaattcaagagattgttctgcactggtctatttttttggaaa-3' and 5'-agcttttccaaaaaatagaccagtgcagaacaatctcttgaattgttctgcactggtctatgg-3', Lamin A/C: 5'-gatcccatgatcccttgctgacttattcaagagataagtcagcaagggatcatttttttggaaa-3' and 5'-agcttttccaaaaaatgatcccttgctgacttatctcttgaataagtcagcaagggatcatgg-3' Luciferase: 5'-gatccccttacgctgagtacttcgattcaagagatcgaagtactcagcgtaagtttttggaaa-3' and 5'-agcttttccaaaaacttacgctgagtacttcgatctcttgaatcgaagtactcagcgtaaggg-3'. Inserts were verified by sequence analysis. Transfected cells were selected for 48 hours using 5 μM puromycin.

Mutagenesis

The K377R, E379A, K391R and K477R mutations in HIF-1α were generated by site-directed mutagenesis using the Quickchange II kit according to the instructions of the manufacturer (Stratagene). The following oligonucleotides were used to generate these mutations: K377R, 5'-cagctattcaccagagttgaatcagaag-3' and 5'-cttctgattcaactctggtgaatagctg-3'; E379A, 5'-caccaaagttgcatcagaagatac-3', 5'-gtatcttctgatgcaactttggtg-3'; K391R, 5'-gacaaacttaggaaggaacctgatgc-3' and 5'-gcatcaggttccttcctaagtttgtc-3'; K477R, 5'-gaagttgcattaagattagaaccaaatcc-3' and 5'-ggatttggttctaatcttaatgcaacttc-3'; K532R, 5'-gtcaatgaattcaggttggaattggtag-3' and 5'-ctaccaattccaacctgaattcattgac-3'. All mutants were sequence verified.

Cell culture and transfection

HeLa cells stably expressing His_6-SUMO-1 or His_6-SUMO-2 were previously described (Vertegaal *et al.*, 2006). HeLa cells were grown in Dulbecco's modified Eagle medium (DMEM), supplemented with 10% FCS and 100 U/ml penicillin and streptomycin (Invitrogen). Hypoxic stabilization of HIF-1α was performed at 1% O_2 for the time periods indicated. Transient transfections were performed at 70–80% confluency using 3.3 μl ExGen500 (Fermentas) per μg DNA.

Antibodies

Peptide antibody AV-SM23-0100 (Eurogentec) against SUMO-2/3 was described previously (Vertegaal *et al.*, 2004). Monoclonal antibody 610958 against HIF-1α and monoclonal antibody 611602 against SAE2 (UBA2) were obtained from BD Biosciences, monoclonal

antibody M2 against the FLAG tag and monoclonal antibody T6199 against α-tubulin were obtained from Sigma Aldrich. Secondary antibodies used were anti-rabbit HRP and anti-mouse HRP (Pierce Chemical Co.). The secondary used for immunofluorescence was goat anti mouse Alex488 (Invitrogen).

Proteins

SUMO-1 and SUMO-2 proteins were produced in *E. coli* and purified as described previously (Tatham *et al.*, 2001). GST-SAE2-SAE1 an GST-Ubc9 were produced in *E. coli* and purified as described previously (Tatham *et al.*, 2001;Mohrmann *et al.*, 2002). The GST-tag was removed from Ubc9 by thrombin cleavage to increase the enzymatic activity.

***In vitro* sumoylation assays**

In vitro transcription / translation reactions were carried out using the TnT Quick Coupled Transcription/Translation kit according to the manufacturer's instructions (Promega). *In vitro* sumoylation reactions were carried out in 10 µl volumes containing 120 ng SAE1/2, 200 ng Ubc9, 500 ng SUMO-1 or -2, 2 mM ATP, 0.6 U/ml Inorganic Pyrophosphatase, 10 mM Creatine Phosphate, 3.5 U/ml Creatine kinase (Sigma), 5 mM $MgCl_2$, 50 mM Tris-HCl pH 7.5 and protease inhibitor cocktail. Assays were incubated for 3 hours at 37°C before addition of LDS sample buffer. As a substrate, either 1 µl of the *in vitro* translated HIF-1α mix or SART1 mix or 0.3 nmol HIF-1α peptide was used.

Bacterial sumoylation

Sumoylated His_6-HIF-1α and sumoylated His_6-HIF-$1\alpha_{1-420}$ were produced in the *E. coli* strain BL21(DE3) (Stratagene) . 50 µl of competent bacteria were transformed with 100 ng pT7- His_6-HIF-1α expression plasmids with or without 50 ng pE1E2S1 or pE1E2S2 and plated on a LB plate containing the appropriate antibiotics. Single colonies were selected from the plates and transferred to 50 ml of LB medium containing the appropriate antibiotics. Bacterial cultures were grown overnight at 37°C with shaking. They were then diluted to 500 ml with LB containing the appropriate antibiotics and grown until an OD_{600} of 0.6 was reached. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a concentration of 1 mM and cultures were incubated overnight at 25°C.

Luciferase assays

Cells were cultured in 24 well plates and transfected with 0.2 µg luciferase reporter plasmid and 0.5 µg expression plasmid as indicated. Experiments were carried out in triplicate and additional wells were prepared for control immunoblotting experiments. Cells were lysed in Reporter Lysis Buffer (Promega) for luciferase activity measurements or in NuPage LDS protein sample buffer (Invitrogen) for immunoblotting.

Microscopy

Microscopy experiments were carried out using a confocal microscope system (model TCS/SP2; Leica). Images were acquired with a 100x NA 1.4 plan Apo objective and were analyzed with Leica confocal software.

Purification of His_6-SUMO conjugates

Cells were cultured in 14 cm diameter culture dishes. Purification of His_6-SUMO-conjugated proteins was essentially carried out as previously described (Vertegaal *et al.*, 2006).

Sample preparation and mass spectrometry

The Coomassie stained protein bands containing SUMOylated HIF1-alpha were excised from an SDS-PAGE gel and digested with trypsin in-gel as previously described (Shevchenko *et al*., 2006). Briefly, the gel-slices were destained by consecutive washed with a 50% acetonitrile in a 50 mM ammonium bicarbonate buffer, pH 8.5. Protein disulfides were reduced by treatment with 10 mM dithiothreitol for 30 minutes followed by alkylation with 55 mM iodoacetamide for 45 minutes. SUMOylated proteins were digested in-situ overnight using trypsin (Promega, modified sequencing grade) and the resulting peptides were extracted, desalted and concentrated on a reversed-phase C18 STAGE tip (Rappsilber *et al*., 2007). The peptide mixtures were analyzed by online nanoflow liquid chromatography tandem mass spectrometry (LC-MS/MS) as described previously (Olsen *et al*., 2005) with a few modifications: All LC-MS/MS analyses were performed on an EASY-nLC™ system (Proxeon Biosystems, Odense, Denmark), coupled to a hybrid LTQ-Orbitrap XL (Thermo Scientific, Bremen, Germany) using a modified nanoelectrospray ion source (Proxeon Biosystems) interface. Binding and chromatographic separation of peptides were achieved in a 20-cm fused silica emitter (75-µm inner diameter) packed in-house with ReproSil-Pur reversed-phase C18-AQ 3-µm resin (Dr. Maisch GmbH). The tryptic peptide mixtures were

auto-sampled at a flow rate of 500 nl/min onto the column and then eluted with a linear gradient of acetonitrile in 0.5% acetic acid at a flow rate of 250 nl/min for one hour. Raw Orbitrap full-scan MS and MS/MS spectra were processed in the MaxQuant software suite (Cox and Mann, 2008), and SUMOylated HIF1-alpha peptides were identified by Mascot (Matrix Science, London, UK) via automated database matching of all tandem mass spectra against an in-house curated target/decoy database of human International Protein Index (IPI) sequence database supplemented with a hSUMO1- and a hSUMO2-modified version of the recombinant his-tagged HIF1-alpha protein as well as common contaminants such as human keratins, bovine serum proteins and porcine trypsin. Tandem mass spectra were initially matched with a mass tolerance of 7 ppm on precursor masses and 0.5 Da for fragment ions, and strict trypsin specificity. The resulting Mascot output files were processed by MaxQuant, where we fixed the estimated false discovery rate (FDR) of all peptide and protein identifications at 1%, by automatically filtering on peptide length, mass error and Mascot score of all forward and reversed peptide identifications.

In-gel tryptic digestions of SUMO-conjugated His_6-HIF-$1\alpha_{1-420}$ were performed as described previously (Steen *et al.*, 2002). After digestion, peptides were resolved using two rounds of extraction with 20 µl of 0.1% TFA and stored at –20°C prior to analysis by mass spectrometry. For LC-MS analysis, samples were injected onto a capillary HPLC system (Ultimate, Dionex) equipped with a peptide trap column (Pepmap 100, 0.3 i.d. x 1 mm, LC Packings) and an analytical column (Pepmap 100, 0.075 i.d. x150 mm, LC Packings). The mobile phases consisted of (A) 0.04% formic acid/0.4% acetonitrile and (B) 0.04% formic acid/90% acetonitrile. A 45 min linear gradient from 0 to 60% mobile phase B was used at a flow rate of 0.2 µl/min. The outlet of the HPLC system was coupled to an HCT IonTrap (Bruker Daltonics, Bremen) using a nanoelectrospray ionisation source. The spray voltage was set at 1.2 kV and the temperature of the heated capillary was set to 165°C. Eluting peptides were analyzed using the data dependent MS/MS mode over a 400-1600 *m/z* range. The five most abundant fragments in an MS spectrum were selected for MS/MS analysis by collision-induced dissociation using helium as the collision gas.

Immunoblotting

Protein samples were size fractionated on Novex 4-12% Bis-TRIS gradient gels using 4-morpholinepropanesulfonic acid buffer (Invitrogen). Size fractionated proteins were

subsequently transferred onto Hybond-C extra membranes (Amersham Biosciences) using a submarine system (Invitrogen). The membranes were incubated with specific antibodies as indicated. Bound antibodies were detected via chemiluminescence with ECL Plus (Amersham Biosciences).

RESULTS

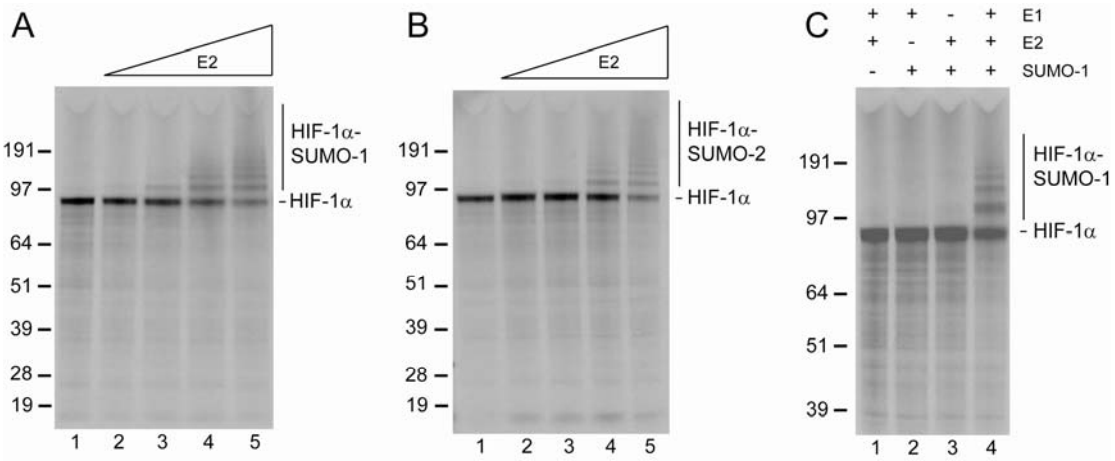

Figure 1. *In vitro* sumoylation of HIF-1α. *In vitro* translated, full-length HIF-1α was incubated for 3 hours with recombinant SAE1/2 (E1), Ubc9 (E2) and either SUMO-1 or SUMO-2. (A) a titration experiment was carried out using HIF-1α SUMO-1 and increasing amounts of the E2 enzyme Ubc9 (0-800ng). (B) a similar experiment was performed, using SUMO-2 instead of SUMO-1. (C) Control experiments were carried out lacking different sumoylation machinery components. In the absence of either SUMO, E1 or E2 enzyme, modified forms of HIF-1α could not be observed, showing the specificity of the assay.

Sumoylation of HIF-1α *in vitro* and in cells

Previously, we have identified endogenous HIF-1α as a target protein for SUMO-2 (Matic *et al.*, 2007). *In vitro*, HIF-1α is efficiently sumoylated by both SUMO-1 and SUMO-2 (Figure 1). To test whether endogenous HIF-1α is conjugated to SUMO-1 and SUMO-2 in cells under hypoxic conditions, we made use of our published stable cell lines expressing either His_6-SUMO-1 or His_6-SUMO-2 (Vertegaal *et al.*, 2006). In a time course experiment, endogenous HIF-1α was found to be conjugated to one or more molecules of His_6-SUMO-2 after culturing cells for 6 or 24 hours at 1% O_2 (Figure 2A). We found that HIF-1α was conjugated to His_6-SUMO-1 and His_6-SUMO-2 (Figure 2B).

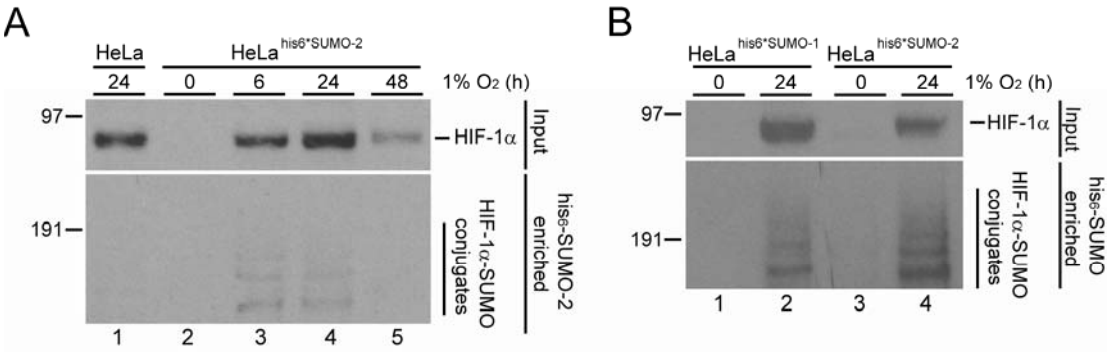

Figure 2. Endogenous HIF-1α is conjugated to SUMO-1 and SUMO-2. (A) HeLa$^{his6\text{-}SUMO\text{-}2}$ cells were cultured for 6, 24 or 48 hours at 1% O_2 to stabilize HIF-1α, or were cultured at 20% O_2 as a control. Control HeLa cells were cultured for 24 hours at 1% O_2. His_6-SUMO-2 conjugates were purified from nuclei of these cells using metal affinity chromatography. Eluted samples were analyzed by immunoblotting using a monoclonal antibody against HIF-1α. Total cell lysates were included as input controls. Sumoylated forms of HIF-1α were specifically detected in the His_6-SUMO-2 purified fractions from hypoxic cells (lanes 3 and 4). (B) SUMO-modified forms of HIF-1α could be detected both in His_6-SUMO-1- and in His_6-SUMO-2-enriched samples purified from stable cells that were cultured at 1% O_2 for 24 hours.

SAE2 knockdown increases the activity of endogenous HIF-1α

In order to study the effect of the endogenous sumoylation machinery on the transcriptional activity of endogenous HIF-1α, an shRNA encoding plasmid was generated directed against the SUMO E1 component SAE2 (Figure 3). Reducing sumoylation resulted in an increase in HIF-1α-dependent transcriptional activity (Figure 3A).

Sumoylation has been reported to increase the stability of HIF-1α during hypoxia, but has also been reported to decrease stability (Bae *et al.*, 2004;Carbia-Nagashima *et al.*, 2007;Cheng *et al.*, 2007). These conflicting data raise the question what happens to the stability of endogenous HIF-1α upon interfering with the

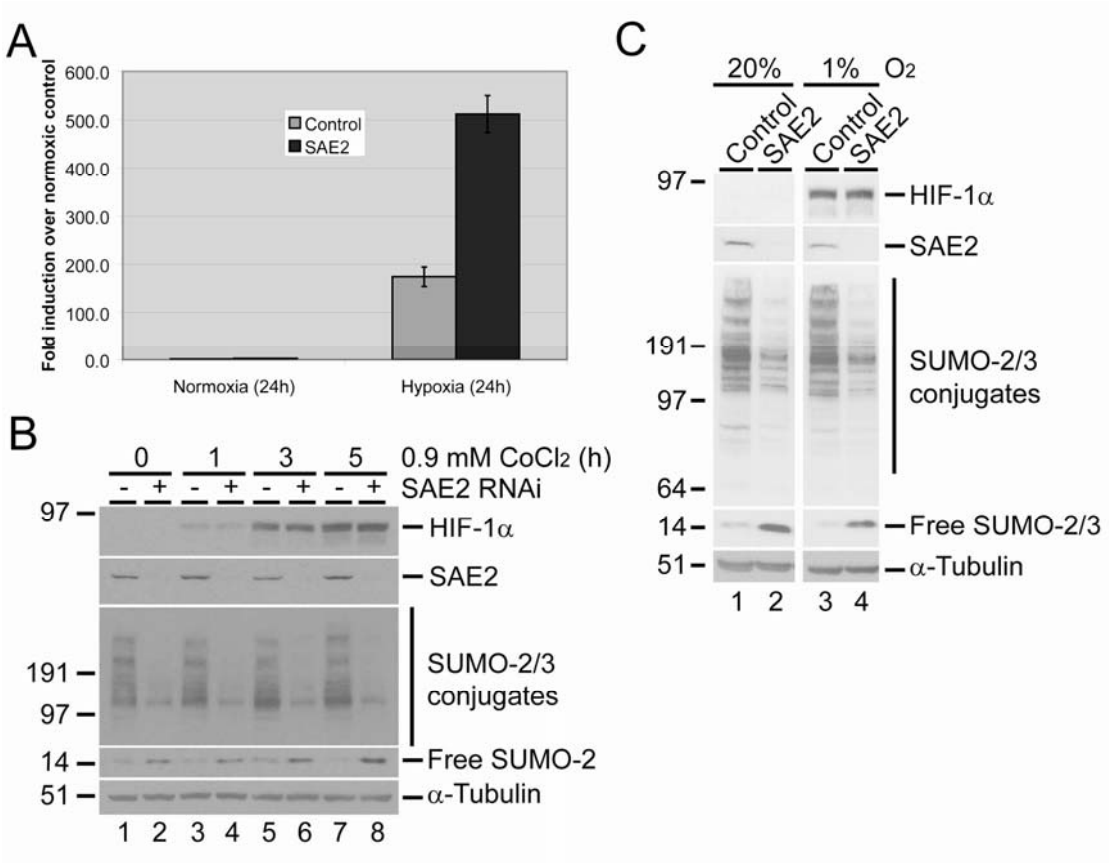

Figure 3. Stabilization of HIF-1α during hypoxia is not dependent on the endogenous sumoylation machinery. (A) HeLa cells were transiently co-transfected with the 5xHRE-luciferase reporter vector and the shRNA vectors against Lamin A/C (control) or SAE2. Cells were incubated at 1% O_2 for 24 hours to stabilize endogenous HIF-1α or were kept normoxic. HRE mediated transcriptional activity was increased upon knock down of SAE2. (B and C) HeLa cells were transiently transfected with shRNA vectors targeting SAE2 or luciferase. Transfected cells were selected by puromycin treatment starting 24 hours post-transfection and cells were lysed 72 hours post-transfection. HIF-1α was stabilized by (B) $CoCl_2$ treatment of cells for the indicated periods of time or by (C) incubating the cells at 1% O_2 for 24 hours. Immunoblotting results showed that the expression levels of SAE2 were significantly reduced by RNAi, causing a reduction in sumoylation. No significant effect on HIF-1α protein levels could be observed upon knock down of SAE2.

endogenous sumoylation machinery in cells with unaltered levels of endogenous SUMO proteases. Knockdown of endogenous sumoylation using the shRNA encoding plasmid against SAE2 did not alter the levels or stabilization of total endogenous HIF-1α (Figure 3B and C).

Sumoylation of HIF-1α on lysine 377

SUMOs are frequently, but not exclusively conjugated to lysines in target proteins situated in the sumoylation consensus site (V/I/L/M/F)KX(E/D) (Gill, 2004;Johnson, 2004;Hay, 2005). Three sumoylation consensus sites are present in HIF-1α, K391, K477 and K532 (Figure 3A). Previously, we have shown by mass spectrometry that K391 of HIF-1α is an acceptor lysine for SUMO-2 (Matic *et al.*, 2007) and Bae *et al.* (2004) have shown by mutational analysis that K391 and K477 are *in vitro* SUMO acceptor sites located in the ODD domain of HIF-1α.

Using a published bacterial sumoylation system (Uchimura *et al.*, 2004a;Uchimura *et al.*, 2004b) combined with protein purification, tryptic digestion and mass spectrometry, we identified K377 as an alternative acceptor site for SUMO-1 in a truncation mutant of HIF-1α (Figure 4B) and in full length HIF-1α (Figure 4C and D). The amino acid preceding the sumoylated lysine 377 is a threonine, which is unusual.

Sumoylation decreases the transcriptional activity of HIF-1α

To investigate the functional consequences of HIF-1α sumoylation, the transcriptional activity of wild-type HIF-1α and sumoylation site mutants were studied (Figure 5). To avoid interference of endogenous HIF-1α in our experiments, we made use of chimeric HIF-1α proteins fused to the GAL4 DNA binding domain (Ross *et al.*, 2002). HIF-1α mutants that lacked single SUMO acceptor lysines displayed a modest increase in transcriptional activity compared to the wild-type protein (data not

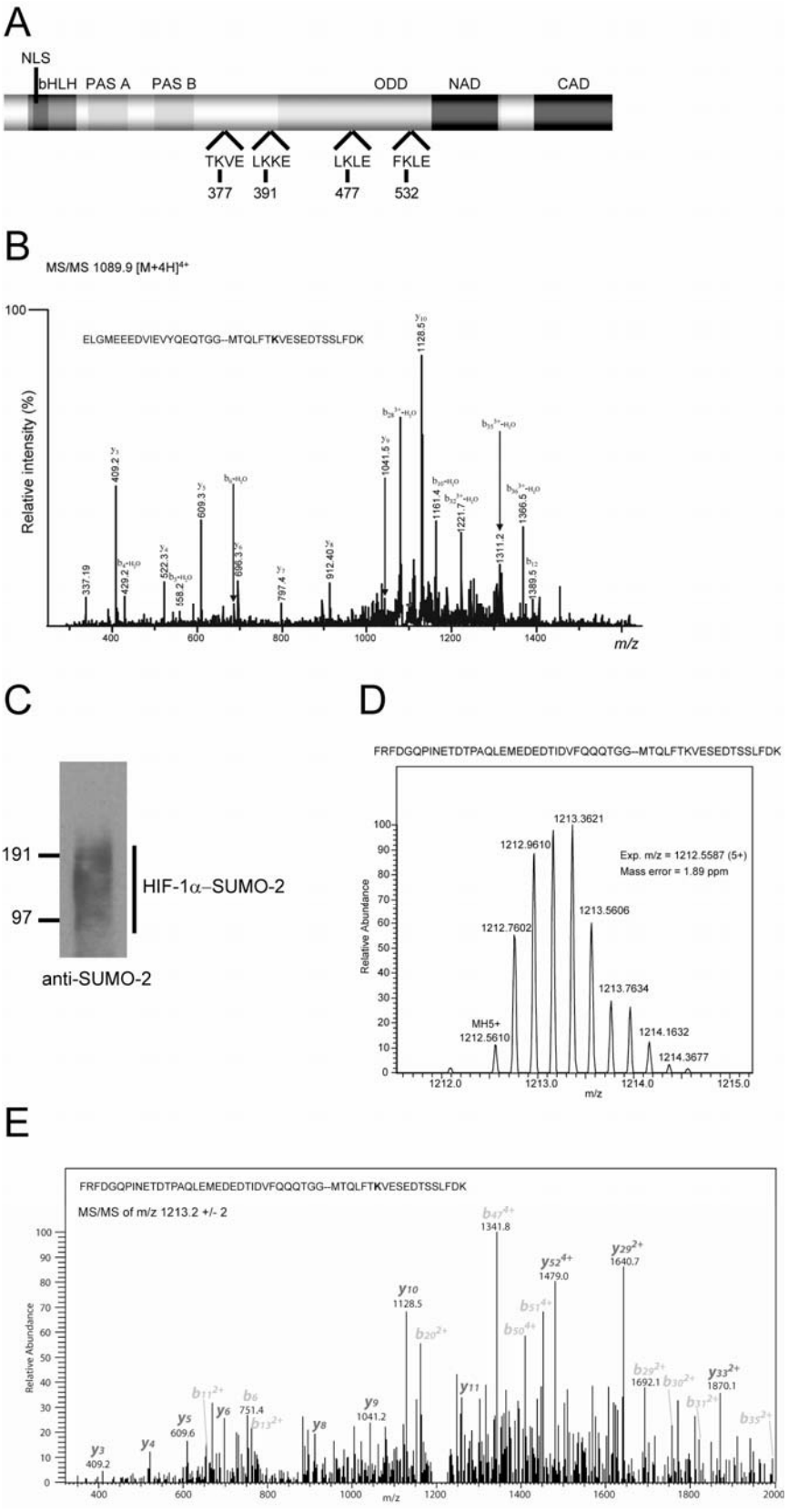

Figure 4. Lysine 377 of HIF-1α is a novel SUMO-acceptor site. (A) structural domains of HIF-1α and location of lysines 377, 391, 477 and 532. bHLH, basic helix-loop-helix; PAS, Per/Arnt/Sim domain; ODD, oxygen dependent degradation domain; NAD, N-terminal transactivation domain; CAD, C-terminal transactivation

domain. (B) MS/MS spectrum of a tryptic SUMO-1-HIF-1α branched peptide showing the conjugation of SUMO-1 to lysine 377 of HIF-1α. A truncation mutant of HIF-1α (His_6-HIF-$1\alpha_{1-420}$) was sumoylated in bacteria, purified by metal affinity chromatography, size separated by SDS-PAGE and stained using Colloidal Blue. Sumoylated His_6-HIF-$1\alpha_{1-420}$ was in-gel digested with trypsin and analyzed by mass spectrometry. (C-D) SUMO conjugation of lysine 377 was confirmed using full-length HIF-1α. MS spectrum (C) and MS/MS spectrum (D) of HIF-1α lysine 377 conjugated to SUMO-2.

shown). Mutating K391 and K477 significantly increased the transcriptional activity and the triple mutants K377,391,477R and K391,477R+E379A were the most active mutants indicating that the HIF-1α SUMO acceptor lysine 377 is functionally relevant. The expression levels of wild-type and mutant proteins were verified by immunoblotting (Figure 5B). We conclude that sumoylation reduces the transcriptional activity of HIF-1α.

Wild-type and sumoylation impaired HIF-1α localize to the nucleoplasm

Sumoylation can cause a redistribution of target proteins in cells (Geiss-Friedlander and Melchior, 2007). To test whether sumoylation alters the subcellular localization of HIF-1α, a set of plasmids was generated encoding wild-type or mutant HIF-1α proteins fused to GFP. Cells were transfected with these plasmids, incubated for 24 hours at 1% O_2, fixed and the localization of the fusion proteins was determined using confocal microscopy (Figure 6). As a control, the subcellular localization of endogenous HIF-1α is shown. All fusion proteins localized to the nucleoplasm, thus sumoylation appears not to alter the subcellular localization of HIF-1α.

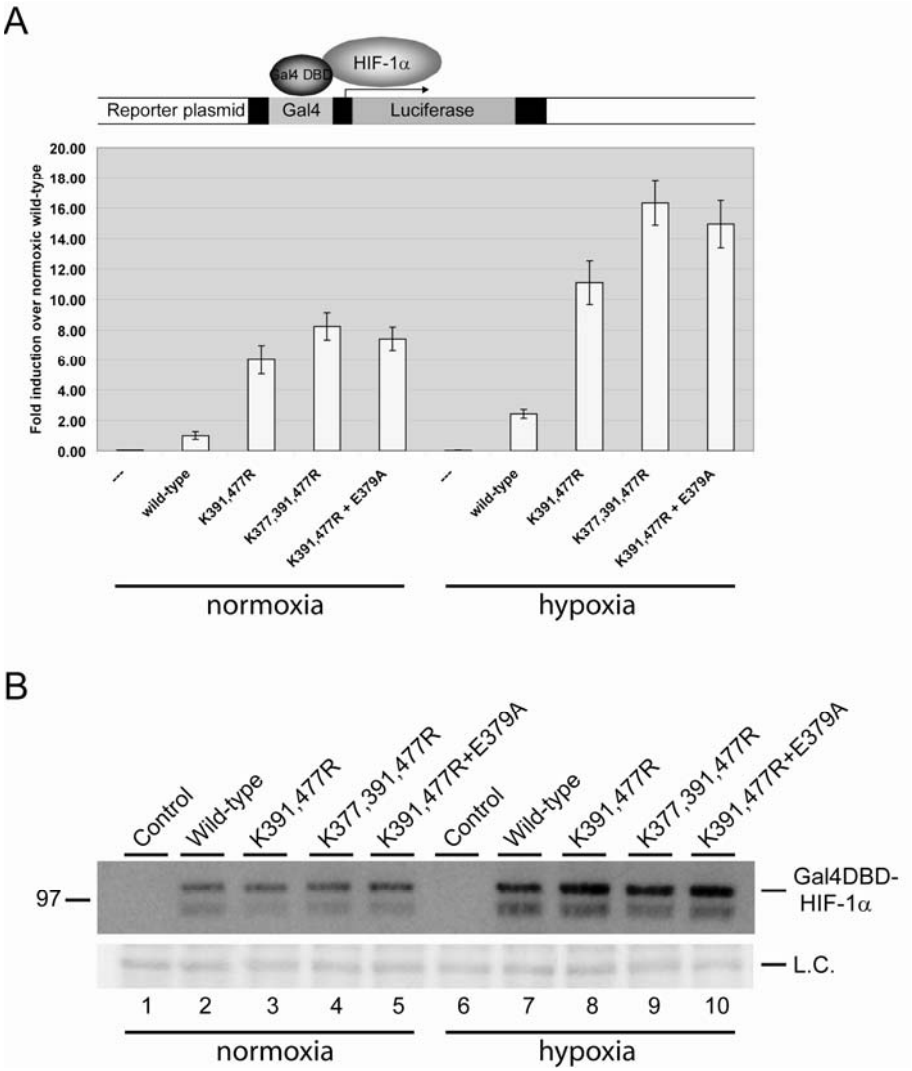

Figure 5. Sumoylation decreases the transcriptional activity of HIF-1α. HeLa cells were transiently transfected with luciferase reporter plasmids and wild-type or mutant Gal4DBD-HIF-1α expression plasmids. 24 hours post-transfection, cells were incubated at 1% O_2 for 24 hours or were kept normoxic. Cells were lysed 48 hours post-transfection and luciferase activity was measured. Control cell lysates were prepared in LDS sample buffer and analyzed by immunoblotting to determine the Gal4DBD-HIF-1α expression levels. (A) the transcriptional activity of Gal4DBD-HIF-1α wild-type was compared to sumoylation-impaired mutants using a Gal4BD-luciferase reporter plasmid. Mutating SUMO-acceptor lysines K391 and K477 resulted in an increase in transcriptional activity compared to the wild-type protein. The transcriptional activity of the K377,391,477R mutant and the K391,477R+E379A mutant was increased compared to the K391,477R mutant. (B) the expression levels of wild-type and mutant Gal4DBD-HIF-1α proteins were determined by immunoblotting using a monoclonal anti-HIF-1α antibody.

SENP1 regulates HIF-1α activity

The SUMO protease SENP1 is a physiological regulator of HIF-1α (Cheng *et al.*, 2007). We used a SENP1 expression vector to study the effect of SENP1 overexpression on HIF-1α activity (Figure 7). As expected, SENP1 overexpression caused a significant increase in the activity of HIF-1α.

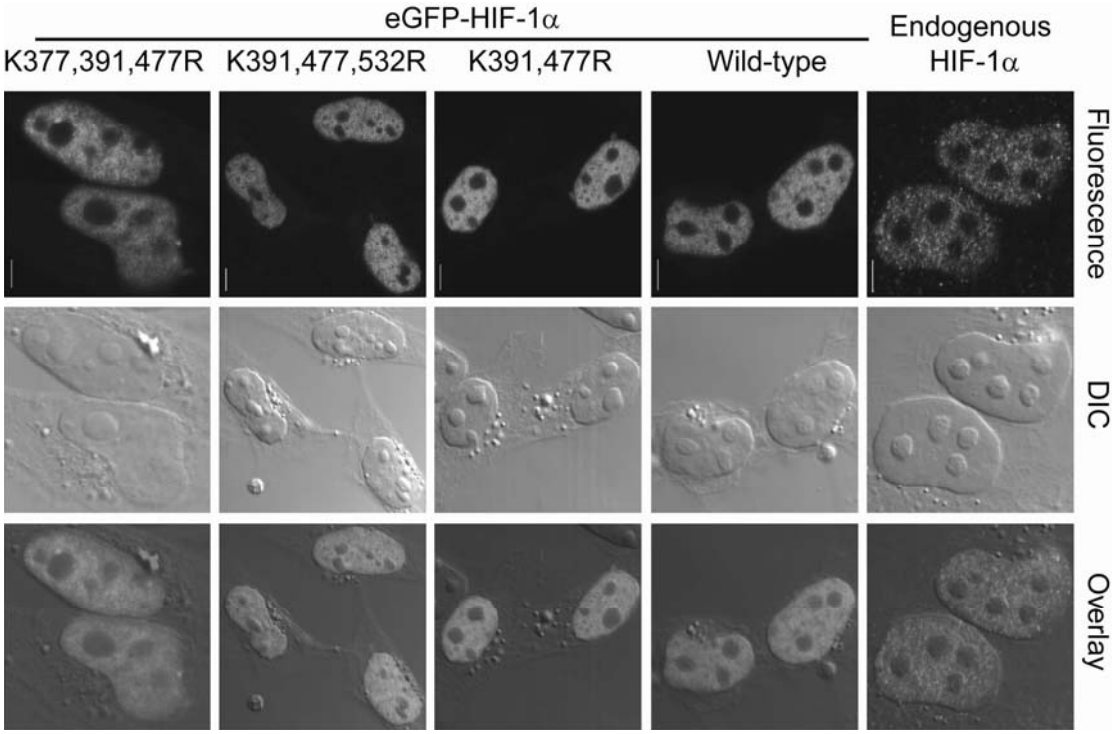

Figure 6. Sumoylation does not alter the subcellular localization of HIF-1α. HeLa cells were transfected with the indicated plasmids encoding wild-type or mutant eGFP-HIF-1α and 24 hours post-transfection cells were incubated at 1% O_2 for 24 hours prior to fixation. Images were captured using a confocal laser-scanning microscope. eGFP-HIF-1α was present diffusely throughout the nucleoplasm but excluded from nucleoli. No difference in localization could be observed between wild-type and mutant proteins. As a control, the localization of endogenous HIF-1α is shown. Scale bar 5μm.

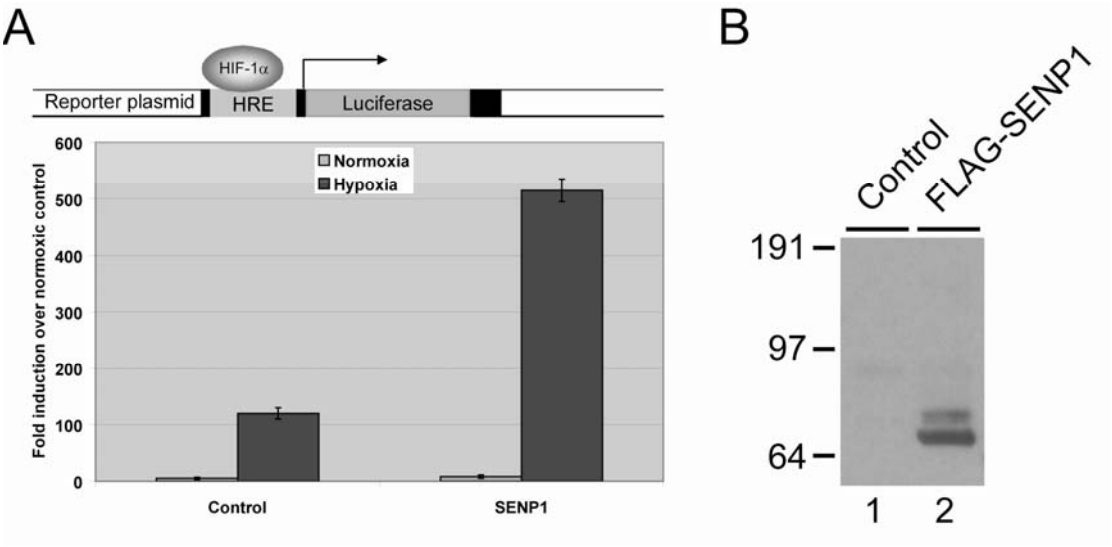

Figure 7. Regulation of HIF-1α activity by SUMO protease 1. (A) HeLa cells were transiently co-transfected with the 5xHRE-luciferase reporter vector and FLAG-SENP1 expression plasmid or control plasmid. 24 hours post-transfection, cells were incubated at 1% O_2 for 24 hours or were kept normoxic. Cells were lysed 48 hours post-transfection and luciferase activity was measured. (B) Control cell lysates were prepared in LDS sample buffer and analyzed by immunoblotting to verify the expression of FLAG-SENP1.

DISCUSSION

Under normoxic conditions, HIF-1α is rapidly targeted for degradation by the ubiquitin-proteasome system (Salceda and Caro, 1997). We have shown here that ubiquitin-like proteins also control the activity of HIF-1α during hypoxia, since the ubiquitin family members SUMO-1 and SUMO-2 play a key role in regulating the activity of HIF-1α during hypoxia. Acceptor lysines for SUMO in HIF-1α are K377, K391 and K477 and conjugation of these lysines to SUMOs results in a significant decrease in transcriptional activity. This is not due to altered subcellular localization since both wild-type and mutant forms of HIF-1α that lack one or more SUMO acceptor lysines all localize to the nucleoplasm.

Lysine 532, the major acceptor lysine of HIF-1α for ubiquitin during normoxia (Tanimoto *et al.*, 2000), is also situated in a consensus sumoylation site, but we have not been able to detect the sumoylation of this lysine. In agreement, mutating K532 in addition to other SUMO acceptor lysines did not alter the transcriptional activity of HIF-1α (data not shown). Thus, there is no evidence that ubiquitin and SUMO are competing for the same acceptor lysine in HIF-1α in a manner analagous to the competition of ubiquitin and SUMO for K21 of IκBα (Desterro *et al.*, 1998).

Two recent papers have proposed contrasting roles for SUMOs covalently attached to HIF-1α during hypoxia. Carbia-Nagashima *et al*. proposed an HIF-1α stabilizing role for SUMO (Carbia-Nagashima *et al.*, 2007), whereas Cheng *et al*. proposed an HIF-1α destabilizing role for SUMO (Cheng *et al.*, 2007). Carbia-Nagashima *et al*. identified RSUME, a small RWD-containing protein that interacts with the SUMO E2 ligase Ubc9 and enhances overall SUMO conjugation. RSUME is induced by hypoxia and enhances the sumoylation of HIF-1α, thereby promoting its stability and transcriptional activity. Reciprocally, HIF-1α controls RSUME levels via an HIF responsive element in the RSUME promoter. Cheng *et al*. (Cheng *et al*., 2007) showed that mouse embryos deficient for SENP1 die early during embryonic development due to a lack of HIF-dependent erythropoietin production, which leads to severe anemia. According to this paper, desumoylation of HIF-1α by SENP1 is required for stabilization of HIF-1α. It is important to note that SENP1 is also responsible for the desumoylation of other SUMO target proteins (Mukhopadhyay and Dasso, 2007) and that RSUME modestly increases overall sumoylation (Carbia-Nagashima *et al*., 2007). Therefore, the effects of RSUME overexpression and SENP1 depletion on target proteins other than HIF-1α might complicate the interpretation of these results. In agreement with another recent study (Berta *et al*., 2007), we have been unable to detect changes in HIF-1α protein levels due to interfering with sumoylation (Figure 4D). Nonetheless, we showed that sumoylation controls HIF-1α transcriptional activity.

Expression of HIF-1α protein levels in tumors often correlates with a poor prognosis (Keith and Simon, 2007). Thus, extensive efforts are made to block the activity of HIF-1α in tumors (Semenza, 2003). The currently available data on the sumoylation of HIF-1α suggest that the sumoylation status of this protein in tumors might influence disease progression. Stimulating the sumoylation of the key drug target HIF-1α could be a novel strategy to block disease progression.

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