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Title: Hypoxia-induced changes in SUMO conjugation affect transcriptional regulation under low oxygen.

Running title: Hypoxia-induced changes in SUMO conjugation.

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Abstract

Hypoxia occurs in pathological conditions, such as cancer, as a result of the imbalance between oxygen supply and consumption by proliferating cells. HIFs are critical molecular mediators of the physiological response to hypoxia but also regulate multiple steps of carcinogenesis including tumor progression and metastasis. Recent data support that sumoylation, the covalent attachment of the Small Ubiquitin-related MOdifier (SUMO) to proteins, is involved in the activation of the hypoxic response and the ensuing signaling cascade. To gain insights into differences of the SUMO1 and SUMO2/3 proteome of HeLa cells under normoxia and cells grown for 48 h under hypoxic conditions, we employed endogenous SUMO-immunoprecipitation in combination with quantitative mass spectrometry (SILAC). The group of proteins whose abundance was increased both in the total proteome and in the SUMO IPs from hypoxic conditions was enriched in enzymes linked to the hypoxic response. In contrast, proteins whose SUMOylation status changed without concommitant change in abundance were predominantly transcriptions factors or transcription regulators. Particularly interesting was transcription factor TFAP2a (Activating enhancer binding Protein 2 alpha), whose sumoylation decreased upon hypoxia. TFAP2a is known to interact with HIF-1 and we provide evidence that deSUMOylation of TFAP2a enhances the transcriptional activity of HIF-1 under hypoxic conditions. Overall, these results support the notion that SUMO-regulated signaling pathways contribute at many distinct levels to the cellular response to low oxygen.

Introduction

Human tissues or cells are frequently exposed to reduced oxygen concentration, a state known as hypoxia. Hypoxia occurs both during physiological and pathological processes like intense exercise, high altitude, ischemia and cancer (1). Hypoxia promotes a dramatic reprogramming in gene expression followed by a cascade of events which facilitate adaptation and survival of cells in the hypoxic environment (2). These include increased transport and delivery of oxygen to tissues, switching to anaerobic production of energy in cells and invasion and metastasis of cancer cells.

Essential to these responses are the hypoxia-inducible transcription factors HIF-1 and HIF-2. Under normal oxygen conditions the regulatory alpha subunit (HIF-α) of HIFs is continuously produced and destroyed, in a process involving its hydroxylation by specific prolyl hydroxylases (PHDs). HIF-α is subsequently ubiquitinated in a VHL-dependent manner and is recognized by the proteasome for degradation. When oxygen concentration is low, hydroxylation is impaired and HIF-α is stabilized, translocates to the nucleus, dimerizes with HIF-1β (Aryl hydrocarbon receptor nuclear translocator-ARNT) to form HIF and binds to hypoxia-responsive elements (HREs) in the promoters/enhancers of its target genes (3).

Another type of post-translational modification that regulates HIF-1α, HIF-2α (Endothelial PAS Domain Protein 1-EPAS1) and other critical components of the hypoxic response pathway is sumoylation (reviewed in (4)). Sumoylation is the covalent and reversible attachment of small (10–11 kDa) Ubiquitin-related proteins called SUMOs (Small Ubiquitin-related MOdifiers) (5-7). Sumoylation mechanistically resembles ubiquitylation and requires E1 (a heterodimer comprised by Aos1-Uba2) and E2 (Ubc9) enzymes as well as suitable E3 ligases for the formation of an isopeptide bond between the C-terminus of SUMO proteins (SUMO-1, 2, and 3 in mammals) and the ε-amino group of an acceptor lysine within the target protein (8, 9).

Sumoylation regulates many normal cellular processes including transcription, recombination, chromosome segregation, nuclear transport and DNA damage (8, 10-12) but requirements for sumoylation are often cell and tissue specific (13).

Several reports have shown that sumoylation fine-tunes the activity of critical mediators of the hypoxia-signaling cascade including HIF-1α, HIF-2α, HIF-1β and VHL (4), via multiple pathways including SUMO de-conjugation by the Sumo-Specific Isopeptidases (SENPS). For example, SENP1, which can be induced in hypoxia (14), was found to contribute to HIF-1α stability during hypoxia (15). On the other hand, enhanced SUMOylation of a subset of cellular proteins observed 24 hours into hypoxic exposure (16), was linked to inactivation of SENP1 and SENP3. Interestingly, in addition to regulatory sumoylation of individual target proteins, hypoxia or ischemia also cause global changes in the SUMO proteome or "SUMO-ome" (17-19). The effects of these changes on the adaptation to low oxygen concentration are poorly characterized even though they are essential for cell survival (18, 19).

To obtain further insights into the interplay between SUMOyaltion and hypoxia, we decided to compare the total, SUMO1 and SUMO2/3 proteomes of normoxic Hela cells with those incubated under hypoxia for 48 hours, a time point at which cells have normally adapted transcriptionally to low oxygen conditions. For quantification, we used a SILAC-based approach. Although we did not observe a massive change in the SUMO-ome upon hypoxia, we could identify a significant number of proteins with altered sumoylation levels, both for SUMO-1 and for SUMO-2/3, upon hypoxia. For a subset of these proteins modulation of their sumoylation level occurred with concomitant changes in their expression level. More interesting were proteins whose SUMOylation changed without concomitant changes in abundance. Those proteins may be targets and/or effectors in the hypoxic signal transduction cascade. One of these proteins is the transcription factor TFAP2a (Activating enhancer binding Protein 2 alpha), a

general transcription factor involved in apoptosis, growth and differentiation (20). We demonstrate that sumoylation of TFAP2a is reduced by hypoxia and that under-modified TFAP2a contributes to the transcriptional activation of HIF-1 suggesting direct involvement of TFAP2a and its sumoylation in the cellular response to the hypoxic environment.

Experimental Procedures

Plasmid Constructions

CMV-AP2α wt was kindly provided by Dr. Bhattacharya (University of Oxford) and contains the cDNA for the full length TFAP2α (Transcription Factor Activator Protein 2-alpha, AP2α, isoform a) (21). CMV-AP2α mut (K10R) was modified from CMV-AP2α wt using site directed mutagenesis. The primers used are available upon request. Point mutation was confirmed by sequencing. AP2α wt and AP2α mut were further subcloned as EcoRI-XhoI fragments into the pcDNA3.1-HA vector (22). pcDNA-flag-HIF1a and pcDNA-flag-HIF2a that were used for co-tranfection experiments were described previously (23, 24). pcDNA-His-SUMO-2 was previously described. p3xAP2-Bluc was kindly provided by Dr. Bhattacharya (University of Oxford) (21)and contains three copies of the AP-2 binding site in the human metallothionein IIa promoter. pGL3–5HRE-VEGF and the *Renilla* luciferase expressing plasmid pCI-*Renilla*, were generously provided by Dr. A. J. Giacia (Stanford University) and Dr. M. U. Muckenthaler (University of Heidelberg, Germany) and were previously reported (25).

Cell lines, transfection and luciferase assays

Human HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Biosera) containing 10% fetal bovine serum (FBS) and 100 U/ml penicillin–streptomycin (Biosera). Transient transfections were carried out in 10-cm, 6 or 12-well plates by using a PEI (polyethylenimine) transfection reagent and were incubated for 24h. Briefly 15 μg of DNA was

added in a 10cm plate in 1.5ml DMEM without FBS and PS. After mixing 30 μg PEI (1μg/μl), was added and the mixture was incubated for 10 minutes. 4.5ml of DMEM containing 5% FBS were added and the mixture was added on top of the cells for 4hours. Cells were incubated in fresh DMEM with 10% FBS with PS for 24-48 hours. When required, cells were treated for the indicated times under hypoxia, (1% O_2, 94% N_2 and 5% CO_2 in an IN VIVO$_2$ 200 hypoxia workstation (Ruskinn Life Sciences)). Reporter gene assays were performed as described previously (25).

SDS page, western blot and antibodies

SDS page and immunoblotting was performed as previously described (25). The following antibodies were used: rabbit monoclonal antibody against TFAP2a (1:1000 dilution) from Thermo- Fisher Scientific (Rockford IL, USA) , mouse monoclonal antibody against HA epitope (1:2500 dilution) from Covance (Princeton N.J. USA) , mouse monoclonal antibody against His epitope (1:2000 dilution) from Qiagen (Hilden, Germany), affinity purified rabbit polyclonal antibody against HIF-1α (Lyberopoulou et al., 2007), mouse monoclonal antibodies against ARNT (611079, 1:500 dilution) from BD Biosciences (San Jose, CA, USA), rabbit polyclonal antibody against HIF-2α (NB100-122, 1:1000 dilution) from Novus Europe (Cambridge, UK) or against Flag (F4042, 1:10000 dilution) from Sigma- Aldrich (St Louis, MO, USA) and mouse monoclonal antibody against actin (3700,1:5000 dilution) from Cell Signaling (Danvers, MA, USA). Antibodies for Exosc10, KCTD1, KCTD15 were purchased from Abcam (Cambridge, UK), ZFP106, NFRKB, ZBTB38, ZNF687 were from Bethyl-Biomol (Hamburg, Germany) and IRF2BP2 antibody was from Proteintech (Manchester, UK). Affinity purified polyclonal antibodies against SUMO-2/3 and SUMO-1 were also used. These antibodies were raised in rabbits using as antigen recombinant SUMO3-GST and SUMO1-GST respectively, and were

affinity-purified against untagged SUMO. Western blot images were taken using an Uvitec Cambridge Chemiluminescence Imaging System equipped with Alliance Software (ver. 16.06) and quantified (values from three 4 independent experiments) by Uviband Software (ver. 15.03) provided with the instrument (Uvitec Cambridge, Cambridge, UK).

SILAC (Stable Isotopic Labelling in Cell cultures)

For performing quantitative proteomic experiments cells were differentially labelled using SILAC as described (26). Briefly, cells were grown in Dulbecco's modified Eagle's medium lacking L-lysine and L-arginine, which were replaced with stable isotope (SILAC) forms (Cambridge Isotope Laboratories). Medium was supplemented with 10% dialyzed fetal calf serum (FCS). Two independent SILAC experiments were performed. The 1st SILAC experiment compared unlabelled cells growing in normoxia (Lys0 and Arg0) with labelled cells growing for 48hours under 1% hypoxia (4,4,5,5-D4-lysine, Lys4, and 13C6-arginine, Arg6). The 2nd SILAC experiment compared labelled cells growing in normoxia (4,4,5,5-D4-lysine, Lys4, and 13C6-arginine, Arg6) with unlabelled cells growing for 48hours under 1% hypoxia (Lys0 and Arg0). Cells were grown in 150-mm-diameter dishes and 30 dishes were used per SILAC condition.

Immunoprecipitation

200 mg of total mixed HeLa protein lysates from cells growing in normoxia and hypoxia (see above) were used for the SUMO-1 and SUMO-2/3 IPs. Endogenous SUMO-1 and SUMO-2/3 conjugates immunoprecipitation was described previously (27, 28). In brief, HeLa adherent cells were lysed using a denaturing lysis buffer with 1%SDS. To immunoprecipitate SUMOylated proteins, the lysate was diluted 10-fold to achieve Ripa buffer conditions and was incubated with monoclonal anti-SUMO1 and anti-SUMO2/3 antibody (SUMO1 21C7 and SUMO2 8A2)

coupled beads at 4°C over night. Upon extensive washing and mock-elution for 30 min at 37 C without peptide, SUMO conjugates were eluted twice with an excess of epitope spanning peptides. Eluted proteins were precipitated with trichloroacetic acid and resuspended to 50µl before analysis by Coomassie-stained SDS-PAGE (Invitrogen, NuPAGE 10% Bis-tris). 30 µg of the input protein lysate was also analysed by Coomassie-stained SDS-PAGE. The lanes containing SUMO-1 and SUMO-2/3 –purified proteins and the input sample lanes were sliced into twenty-three sections before undergoing in-gel tryptic digestion.

For the anti-HA non denaturing Immunoprecipitation the following procedure was followed: Briefly, HeLa cells were washed with cold PBS 24 h after transfection and lysed (20 min, 4 °C) in buffer containing 25 mM Hepes, pH 7.6, 150 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, EDTA-free protease inhibitor mixture (Roche, Basel, Switzerland), 50 mM glycerolphosphate, and 10 mM Na_3VO_4. After centrifugation, the sample volume was adjusted to 800 µl with lysis buffer and samples were incubated for 3 h at 4 °C with 1 µl of anti-HA antibody. 20 µl of Protein A-Sepharose (Amersham Biosciences) bead slurry was added and incubation continued for 16 h at 4 °C under gentle shaking. Beads were collected by centrifugation, washed 3 times with lysis buffer, and bound proteins were eluted by SDS sample buffer.

Mass spectrometry

Proteins were separated by one-dimensional SDS–PAGE (4–12% NuPAGE Bis-Tris Gel, Invitrogen) and the entire lane of the Coomassie blue-stained gel was cut into 23 slices. All slices were reduced with 10 mM DTT for 55 min at 56°C, alkylated with 55 mM IAA for 20 min at 26°C and digested with modified trypsin (Serva) overnight at 37°C. Tryptic peptides were injected into a C18 precolumn (2.5 cm, 360 µm o.d., 150 µm i.d., Reprosil-Pur 120 Å, 5 µm,

C18-AQ, Dr Maisch GmbH) at a flow rate of 10 µl/min. Bound peptides were eluted and separated on a C18 capillary column (15 cm, 360 µm o.d., 75 µm i.d., Reprosil-Pur 120 Å, 3 µm, C18-AQ, Dr Maisch GmbH) at a flow rate of 300 nl/min, with a gradient from 7.5 to 37.5% ACN in 0.1% formic acid for 50 min using an Agilent 1100 nano-flow LC system (Agilent Technologies) coupled to an LTQ-Orbitrap XL hybrid mass spectrometer (Thermo Electron). The mass spectrometer was operated in the data-dependent mode to automatically switch between MS and MS/MS acquisition. Survey MS spectra were acquired in the Orbitrap (*m*/*z* 350–1600) with the resolution set to 30 000 at *m*/*z* 400 and automatic gain control target at 5×10^5. The eight most intense ions were sequentially isolated for CID MS/MS fragmentation and detection in the linear ion trap. Ions with single and unrecognized charge states were excluded. The Raw Data was analysed with Maxquant 1.3.0.5 and searched against Uniprot_human_270812 database (2012_06 86725 sequences). Precursor and fragment ion tolerance was set to 20 ppm. Trypsin was allowed to cleave after Lysine and arginine with one missed cleavage. Fixed modification was Carbamidomethyl (C) and variable modification was Oxidation (M) and Acetyl (Protein N-Term). False discovery rate was 0.01 for precursor and fragments. Raw data and Maxquant tables have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository (29) with the dataset identifier PXD010946.

The following statistics have been applied: Log 2 ratios (median) obtained from MaxQuant analyses (Supplementary Table S1) of Hypoxia treated vs normoxic cells were tested to be different from zero (no changes) using limma package version 3.36.5 (30) within R statistical environment (R version 3.5.0). Specifically, a one-sample t-test was approximated using a model of the form $y = a$ with a being a mean log2 ratio from the two biological experiments for a given protein in a particular experiment (Input, Sumo1 and Sumo2/3 IP). Only proteins having log2-

ratios reported in both experiments were included in the analysis. Moderated *t*-statistics and *p*-values resulting from empirical Bayes approach were used for the statistical interpretation of the intercept parameter *a* being non-zero. For multiple testing correction, an approach proposed by Storey et al (31, 32) was applied as implemented within an R package 'qvalue' (version 2.12.0).

Immunofluoresence

HeLa cells were grown on coverslips incubated at normoxia or hypoxia for 8-48hours and analysed by immunofluoresence as previously described (23). Coverslips were incubated with a rabbit monoclonal anti-TFAP2a antibody (1:100 dilution), and with an Alexa 488-conjugated anti-rabbit secondary antibody (1:1000, Jackson ImmunoResearch). Images were taken on a Zeiss Axioplan fluorescence microscope using an AxioCam MRm CCD sensor and 100× objective with suitable filters.

Experimental Design and Statistical Rationale

Two biological experiments were performed in order to compare protein expression profile and the SUMO proteome in cells growing in normoxia versus cells growing for 48hours under 1% hypoxia. The Stable Isotope Labeling of Amino acids in Cell culture (SILAC) technique was used for quantitation of the proteomic results (26). The 2-state SILAC labelling was reversed between the two experiments (see above). Cell lysates from cells growing in normoxia and hypoxia in each experiment were pooled and subjected to SUMO-1 and SUMO2 immunoprecipitation. Input and SUMO-1 and SUMO-2/3 immunoprecipitated proteins were subjected to trypsin in-gel digestion, analysis by high-resolution LC-MS/MS and changes were estimated on precursor peptide intensities in MAxQuant (see above). Intensity ratios (log2) of Hypoxia treated cells/ untreated (normoxic) cells for the proteins that detected in the two

biological experiments was calculated and plotted in heat map and x/y scatter plots. (Supplementary file S1 and Fig 1).

Values shown for luciferase activity assays are derived from a minimum of three independent experiments performed in triplicates. For Statistical analysis the Graph Pad Instat Statistical package for Mac was used. Data are expressed as mean±s.e.m. Differences were examined by Student's t-test (two-tailed) between two groups. $P<0.05$ was considered statistically significant.

Nomenclature

SUMO-1 (Smt3C; P63165), SUMO2 (Smt3A; also known as SUMO3, P55854) and SUMO3 (Smt3B; also known as SUMO2, P61956).

Results

Identification of endogenous SUMO-1 and SUMO-2/3 conjugates and their response to hypoxia.

Although a few studies report global changes to the SUMO proteome in ischemic models (oxygen and glucose deprivation) (33), specific targets and their physiological relevance are yet unclear. To identify specific cellular targets whose sumoylation is altered 48 hours after initiation of hypoxia, we undertook a quantitative proteomic approach combined with a recently established protocol for denaturing immunoprecipitation of endogenous Sumo species (27, 28). The Stable Isotope Labeling of Amino acids in Cell culture (SILAC) technique was used for quantitation of the proteomic results (26). Specifically, HeLa cells were grown either under normoxia in SILAC medium containing non-labelled amino-acids (Lys^0Arg^0-Light) or incubated under 1% O_2 for 48 hours in SILAC medium containing isotopically labelled amino-acids (Lys^4Arg^6-Heavy) (Fig 1A). The experiment was repeated with reverse labelling (Fig 1A) in order to identify and reject nonspecific contaminants of the immunoprecipitation.

Analysis of the cell lysates demonstrated the expected induction of HIF-1α expression under hypoxia (Fig S1A, upper panel), while global SUMO-1 and SUMO-2/3 patterns showed little differences at this time point compared to normoxia (Fig S1A, lower panel). Analysis of cell lysates, flow-through and the eluates of the anti-SUMO-1 or anti-SUMO-2/3 immunoprecipitates (IPs) revealed efficient enrichment of SUMO-1 and SUMO-2/3 conjugates in the IP eluates (Fig 1A-coomassie and Fig S1B). As expected, SUMOylated RanGAP1 (Fig S1B upper panel, marked with asterisk, 90kDa) was enriched in the SUMO-1 IP eluates and free SUMO-2 (Fig S1B lower panel marked with arrow, 20kDa) was highly enriched in SUMO-2/3 IP eluates.

Analysis of the SUMO-1 and SUMO-2/3 IP eluates by coomasie stain, followed by trypsin digestion (Fig S2) and liquid chromatography–tandem mass spectrometry (LC-MS/MS) yielded quantitative data for several hundred sumoylated proteins in each IP (592 proteins were identified in the 1st SUMO1-IP and 430 in the 2nd IP, 554 were identified in the 1st SUMO2/3-IP and 332 in the 2nd IP, Supplementary files S1, S2 and S3). From our total proteins identified, 350 were recovered from both of the SUMO-1 IPs and 272 proteins from both of the SUMO-2/3 IPs (Supplementary file S1). The SUMO conjugates identified by our analysis showed significant overlap with the ones initially identified by Becker et al (28) from cells grown under normoxic conditions (309 common targets were found for SUMO-1 and 255 for SUMO-2/3). Moreover the identified sumo conjugates show almost 70-80% overlap with targets from SUMO proteomics data identified from 22 previous studies as referred to the study of Hendriks et al 2016 (34) and others (28, 35-39) (Fig S1c and Supplementary file S5), verifying the specificity of our SUMO immunoprecipitations. Finally, our SUMO1 conjugates show good overlap with SUMOylated proteins identified by Kunz et al (16), who employed a similar procedure to investigate the SUMO1 proteome of HeLa cells 24 hours after initiation of hypoxia.

As shown in Fig. 1B&C, the number of proteins modified by SUMO-1 or SUMO-2/3 was not massively affected by exposure to hypoxia (marked as unchanged in Fig 1C). Interestingly, close to 10% of our total proteins identified as SUMO conjugates were found to modulate their sumoylation after hypoxia (34 proteins for SUMO-1 and 28 for SUMO-2/3, Fig 1C, marked in the light red squares, see also Table 1). There were several proteins (17 modified by SUMO-1 and 11 by SUMO-2/3) that were enriched in the SUMO IP eluates from hypoxic cells but were also expressed at higher levels in the same cell lysates (Table 2, see also Supplementary file S4), suggesting that the increased sumoylation level of the corresponding polypeptides correlated to the increased abundance of the proteins upon hypoxia. These proteins included glycolytic enzymes, such as ALDOA, ENO1 and PGK1, and LDHA, all of which are known targets of hypoxia and HIF-1 (40), and upregulation of their gene expression by hypoxia could explain their enrichment in our SUMO IPs. There has been substantial evidence for sumoylation of several glycolytic enzymes in the literature. AldoA, ENO1, LDH and PGK1 have been found in a number of SUMO-IP experiements in S. cerevisiae (41) and human cells (16, 37, 42, 43) both under normoxia and hypoxia (16, 41).

On the other hand, our analysis also identified proteins with altered sumoylation levels (Table 1 and Table 3, see also Supplementary file S4), whose expression levels did not seem to significantly differ between normoxia and hypoxia. This group was enriched for factors linked to transcription and RNA stability. To validate their hypoxia-dependent changes in the sumoylation status, we turned to immunoprecipitation followed by immunoblotting, using specific antibodies for each target. As shown in Figures 2A, Fig S3 and Fig. S4, we could validate both, stable expression and changes in SUMOylation for several proteins involved in transcriptional regulation such as TFAP2a (Fig. 2A), KCTD15, NFRKB, ZBTB38 (Fig. S3), ATRX, IRFBP2 (Fig. S4), two proteins with unknown function, ZNF687 (Fig. S3) and ZFP106

(Fig. S4), and for Exosc10 (Exosome subunit 10, known as Rrp6), a subunit of the RNA exosome (Fig. S4). Most of the sumoylated proteins migrated approximately 20 kDa higher than the unmodified proteins as shown in the immunoblot analysis and by the apparent MW at which they were identified in the MS analysis (Fig S2), indicative of mono-SUMOylation. Some proteins such as KCTD15, ZNF687 and NFRKB gave rise to multiple higher migrating bands in the SUMO-2/3 IPs, indicating poly-sumoylation (Fig. S3, marked with asterisks).

TFAP2a is a target of sumoylation and its modification is inhibited under hypoxia

Amongst the nine SUMO targets for which we could validate modification changes in response to hypoxia that are independent of expression levels, we were most interested in TFAP2a, as it had previously been linked to hypoxia. TFAP2a was reported to interact with HIF-1α, HIF-2α and CITED2 (21, 44, 45), and although it was known that TFAP2a could be sumoylated (46), nothing was known about the involvement of its sumoylation in these interactions or the hypoxic response.

As shown in Figure 2A, analysis of input samples and SUMO-2/3 immunoprecipitates with anti-TFAP2a antibodies confirmed a) that levels of endogenous TFAP2 do not change and b) that TFAP2a SUMOylation is lost upon hypoxia. Moreover, the apparent MW of SUMOylated TFAP2a in the IP, which is 20 kDa larger than the calculated MW of unmodified TFAP2a, is indicative of mono-SUMOylated TFAP2 species. Independent experiments confirmed that the protein expression levels of TFAP2a did not significantly change after incubation of cells for 24 or 48h under hypoxia (Fig 2B). Furthermore, hypoxic treatment did not affect the subcellular localization of TFAP2a, which remained nuclear under all conditions (Fig 2C). To begin to address the molecular mechanisms that may account for the loss of TFAP2 SUMOylation in hypoxia, we tested for transcriptional induction of SUMO isopeptidases by Q-PCR. As shown in

Figure S5, we did not detect significant changes in SENP mRNA levels. Taken together, our data confirmed that endogenous TFAP2a is a target for post-translational modification by SUMO-2/3 and revealed that its sumoylation is specifically inhibited under hypoxic conditions. The underlying molecular mechanism will have to await further investigations (see discussion).

SUMOylation of TFAP2a on lysine 10 is lost under hypoxia,

TFAP2a is a member of the developmentally regulated family of AP-2 transcription factors. Individual members have both overlapping and distinct roles in development and in tumor biology. For example, although TFAP2a and TFAP2c sit on the same luminal gene cluster in breast cancer epithelial cells, only TFAP2c is able to induce the expression of luminal breast cancer genes. As shown by Bogachek et al (46), this difference can be attributed to SUMOylation of TFAP2a on lysine 10, which has a repressive function. Our finding that TFAP2a is desumoylated in response to hypoxia suggests that this may revert TFAP2a from a repressor to an activator, at least on a subset of genes.

To follow up on this idea, we mutated the only consensus sumoylation site at Lys10 (46) (highlighted in Fig 3A) into Arg. Wild-type HA-tagged TFAP2a and its SUMO-deficient K10R mutant were then overexpressed in HeLa cells and analysed by immunoblotting. Both wild-type HA-TFAP2a and the K10R mutant were detected in HeLa cell lysates as 55kDa protein bands by an anti-HA antibody (Fig 3B). Furthermore, an additional band with higher MW was recognized by the anti-HA antibody only in the case of wild-type HA-TFAP2a but not in the K10R TFAP2a mutant (marked with asterisk, Fig 3B). To confirm the identity of this band as the sumoylated form of TFAP2a, we co-expressed a His-tagged form of SUMO2 and performed immunoprecipitation of the HA-TFAP2a forms with an anti-HA antibody. Upon analysis of the immunoprecipitates with an anti-HA antibody (Fig 3B right upper panel, full blot can be found

in Fig S6a) we detected the same higher MW band (marked with asterisk), which was also recognized in another blot by an anti-His antibody (Fig. 3B, right bottom panel, full blot can be found in Fig S6a) confirming that it indeed corresponded to the sumoylated form of TFAP2a. The absence of this form from the K10R mutant TFAP2a expressing cells verified that Lys 10 is the dominant SUMO2 conjugation site in TFAP2a. Even though we cannot exclude that residual sumoylation may exist in the TFAP2a K10R mutant due to other putative previously suggested non-consensus sumoylation sites (36, 47), sumoylation at the K10 residue appears, according to our data, to be the predominant one in our system.

We next compared the expression of wild-type TFAP2a and its K10R mutant form under normoxia or after 24-hour exposure to hypoxia (Fig 3D). No apparent changes could be observed in the expression levels of the wild-type HA-TFAP2a under normoxia or hypoxia (Fig 3C left panels, full blot can be found in Fig S6b) but following immunoprecipitation, the sumoylated form of HA-TFAP2a wt, as detected by both anti-HA and anti-SUMO-2 antibodies, was clearly reduced under hypoxia (Fig 3C right panels, marked with asterisk, full blot can be found in Fig S6b), showing that sumoylation of the tagged over-expressed form of TFAP2a is regulated by hypoxia in a similar manner as endogenous TFAP2a. Of note, consistent with the observation that endogenous TFAP2a was only identified in SUMO2/3 - but not in SUMO1 IPs, analysis of immunoprecipitated tagged TFAP2a did not yield specific signals with SUMO1 antibodies (Fig S6c).

SUMOylation deficient TFAP2a is as transcriptionally active as wt TFAP2a To test whether sumoylation of TFAP2a plays a role in its transcriptional activity, we used a luciferase reporter assay with plasmid p3xAP2-Bluc (see Materials & Method). We could show that both wild-type and K10R mutant forms of TFAP2a were transcriptionally active, under both normoxia and

hypoxia (Fig 3D). In both cases the K10R mutant form of TFAP2a exhibited slightly higher activity than the wild-type form but the difference did not reach statistical significance, suggesting that neither the K10R mutation per se nor the lack of sumoylation at Lys10 affected significantly the transcriptional activity of TFAP2a. Although we can conclude from these experiments that SUMOylation is not required for TFAP2a activity, it remains possible that SUMOylation inhibits TFAP2a since the small fraction of sumoylated TFAP2a may not be sufficient to cause a significant decrease in the activity of the reporter constructs (Fig. 3B).

TFAP2a enhances the transcriptional activity of HIF-1 in a sumoylation-dependent manner.

As described above, TFAP2a is known to interact with other transcription factors including HIF-1. It is thus conceivable that SUMOylation of TFAP2a does not (only) serve to regulate all TFAP2a - dependent genes, but genes whose expression depends on both HIF-1 and TFAP2a. To test this hypothesis, wild-type HA-TFAP2a and its K10R mutant sumo-deficient form were expressed in HeLa cells carrying an HRE-dependent luciferase reporter plasmid (pGL3–5HRE-VEGF). Overexpression of HA-TFAP2a stimulated (close to 3-fold) the HRE-dependent transcriptional activity observed under hypoxia (Fig. 4A) and this stimulation was similar for both wild-type and mutant forms of TFAP2a, as anticipated by the fact that sumoylation of TFAP2a is lost under hypoxia (Fig 3D). To test whether the effect of TFAP2a on HRE-dependent transcription involves HIF-1, wild-type HA-TFAP2a and its K10R mutant sumo-deficient form were co-expressed with Flag-HIF-1α in HeLa cells carrying the HRE- reporter and grown under normoxia. The transcriptional activity of HIF-1 in these cells was stimulated by both the wild-type and K10R mutant sumo-deficient forms of HA-TFAP2a but the effect of the K10R mutant form was significantly stronger (Fig 4B), suggesting that lack of sumoylation

facilitates a positive effect of TFAP2a on the activity of HIF-1. We could further show that the sumoylated form of TFAP2a was not affected in cells overexpressing HIF-1α under normoxia (Fig 4C), suggesting that desumoylation of TFAP2a under hypoxia is not HIF-1 dependent.

TFAP2a interacts physically with HIF-1 and HIF-2 in a sumoylation-independent manner

Consistent with previous studies (45), the effect of TFAP2a on HIF-1α activity indicated that the two proteins may be interacting. To test whether SUMO contributes to this interaction, wild-type and K10R mutant sumo-deficient forms of HA-TFAP2a were immunoprecipitated from normoxic cells expressing also either Flag-HIF-1α or Flag-HIF-2α. Analysis of the IPs showed association of TFAP2a with both HIF-α isoforms (Fig 5A and B, right panels) and this association was similar for both wild-type and K10R mutant sumo-deficient forms of HA-TFAP2a. ARNT (HIF-1β) was also equally recovered in the IPs of both TFAP2a forms. Given that the HIF-1α/ARNT (HIF-1) or HIF-2α/ARNT (HIF-2) heterodimers are exclusively nuclear (48), these data suggest that TFAP2a can bind to both HIF-1 and HIF-2 inside the nucleus and may facilitate their transcriptional activity. Sumoylation of TFAP2a appears not to be required for the TFAP2a-HIF interaction although it enhances HIF-1 activity, suggesting that de-sumoylation of TFAP2a triggered by hypoxia may be required for the transcriptional activation of the TFAP2a-HIF complexes. However, we can not exclude that SUMO to some extend could also inhibit the TFAP2a-HIF-1α interaction, but is not observed in our experiment due to low level of sumoylated TFAP2a in our IPs.

Discussion

Sumoylation facilitates responses to physiological and pathophysiological events by either promoting global changes to the SUMO conjugation of proteins or by targeting specific and critical substrates of the ensuing signalling cascades. Increased global protein SUMO-

conjugation under hypoxia was initially reported as a result of increased SUMO-1 expression (49), which was also confirmed by an *in vivo* study in hypoxic mouse brains and hearts (50). Global changes in the "SUMO proteome" were also reported in ischemic and OGD (oxygen glucose deprivation) models (17-19, 51). A significant increase in the SUMO-2/3 (but not SUMO-1) conjugates was observed in the recovery period following harmful ischemia but the functional consequences of these global changes were poorly understood although they were required for survival (18, 19).

In contrast to previous studies that focused on acute stress response, we investigated the SUMO proteome of cells that have already been kept for 48 hours under hypoxic condition, and which have had significant transcriptional response to adapt to the stress (adaptive response included upregulation of several SUMO targets; see below). At this late time point, we did not observe any significant increase in the amount of high molecular weight SUMO conjugates (Fig S1), consistent with our quantitative proteomic results obtained from the 48 hour timepoint, which did not show a massive increase in the number of SUMO-1 or SUMO-2/3 modified proteins. Previous studies have shown an increase in SUMO-1 conjugates in different cells of under early hours of hypoxia (16, 49). We did however observe significant sumoylation-status changes in a small group (20-30) of proteins. In line with this, down-regulation of Ubc9 acetylation via SIRT1 under hypoxia was previously shown to affect sumoylation/ desumoylation of a specific group of protein targets (52).

Our proteomic approach identified two categories of sumoylation targets, proteins whose expression and SUMOylation increased under hypoxia, and proteins whose expression remained constant while they lost or gained SUMO. The first group consisted mostly of glycolytic enzymes and known targets of HIF-1, the major transcription factor induced under hypoxia. This finding suggests that both SUMO-1 and SUMO-2/3 modification may be important for the

function of enzymes like ALDOA, ENO1, PGK1 and LDHA that mediate metabolic adaptation to hypoxia. Sumoylation of several glycolytic enzymes like AldoA, ENO1, LDH and PGK1 have been found in a number of SUMO-IP experiements in S. cerevisiae (41) and human cells (16, 37, 42, 43) confirming once more the specificity of our method.

Intriguingly, the second group of proteins consisted mostly of transcription factors or regulators involved in activation or repression of transcription such as TFAP2a, ATRX, IRF2BP2, KCTD15, NFRKB and ZBTB38. Some of these targets were also identified as hypoxia-affected SUMO1 targets in a recent study 24 hours after hypoxia (16).

Focusing on the last group of proteins, we singled out transcription factor TFAP2a and could show that hypoxia reduced its sumoylation (by SUMO-2) without affecting its protein expression levels. TFAP2a coordinates a variety of cell processes, including cell differentiation during embryogenesis, growth, apoptosis and survival. The TFAP2 family of transcription factors is known to mediate both activation or repression of target genes (20). Changes in TFAP2A expression have been frequently detected in different types of cancer including melanoma, neuroglioma and prostate or breast cancers (44, 53-55), indicating that TFAP2A plays a role in tumorigenesis, tumor invasion and metastasis. Sumoylation of TFAP2a has been previously shown to be involved in maintaining the basal cancer phenotype in breast cancer cells (46). Inhibiting sumoylation of TFAP2a by mutating the SUMO-acceptor site enhanced the positive effect of TFAP2a on the transcriptional activity of HIF-1. In line with previous reports (44, 45) , we could also show that TFAP2a can physically interact with both HIF-1α and HIF-2α. However, sumoylation of TFAP2a was not critical for this interaction suggesting that the stimulatory role of TFAP2a de-sumoylation involves the interaction of the TFAP2a/HIF complex with chromatin or other transcriptional co-activators. TFAP2a as well as HIF-1α are known to bind to co-activators such as CITED-2 and p300/CBP. It has been suggested that TFAP2a may

actually compete with HIF-1α for binding to CITED2 (56). However, our results suggest that these two factors rather cooperate than compete as part of the cellular response to hypoxia and such a cooperation has been documented in melanoma cells (44). Our data are also in line with a previous report showing that knockdown of TFAP2a inhibited expression of VEGF, a known HIF target, and impaired cell viability under hypoxia (45).

Another question raised by our findings is what triggers de-sumoylation of TFAP2a under hypoxia. One possibility is activation of a specific SUMO isopeptidase (SENP) responsible for the de-sumoylation of TFAP2a. This seems unlikely, considering that very few targets are deSUMOylated and that isopeptidases are not transcriptionally upregulated in our experiments. Moreover, Kunz et al (16) have reported that SENP1 is inhibited in hypoxic conditions. Alternatively, a specific E3 ligase for TFAP2a sumoylation is impaired under hypoxia, or TFAP2a aquires a modification that prevents or allows recruitment of unregulated E3 ligases or isopeptidases. Further investigation of the links between hypoxia and the sumoylation machinery is required to address this question. Our demonstration that lack of oxygen can either stimulate or inhibit the sumoylation of a specific set of proteins that, as shown for TFAP2a, can in turn modulate the transcriptional response to hypoxia underlines the significance of the connections between hypoxia and sumoylation and their implications for hypoxia related diseases.

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Data availability: The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository (29) with the dataset identifier PXD010946.

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Tables

Table 1. Table summarizing the number of proteins identified as SUMO-1 and SUMO-2/3 conjugates from two SUMO IPs and their modulation upon hypoxia.

	SUMO1	SUMO2/3
Putative SUMO substrates identified from both Ips	350	272
altered SUMO conjugation in hypoxia	34	28
with altered protein expression levels	22	16
with no change in protein expression/ or expression nd	12	12
verified in western blot	6	6

Table 2. Selected putative substrates of SUMO-1 and SUMO-2/3 identified in two IPs that modulate both their sumoylation by SUMO1 or SUMO-2/3 and their protein expression levels upon hypoxia (increase in SUMO conjugation and protein expression is marked with + and decrease with -).

Genes names	Protein names	SUMO1 conjugation and protein expression modulation	SUMO2/3 conjugation and protein expression modulation in hypoxia
ALDOA	Fructose-bisphosphate aldolase A;Fructose-bisphosphate aldolase	+	+
ALDOC	Fructose-bisphosphate aldolase C;Fructose-bisphosphate aldolase	+	
ANXA4	Annexin A4;Annexin	+	
DARS;DKFZp781B11202	Aspartate--tRNA ligase, cytoplasmic	+	
ENO1	Alpha-enolase	+	+
GPI	Glucose-6-phosphate isomerase	+	+
LDHA	L-lactate dehydrogenase A chain;L-lactate dehydrogenase	+	+
LGALS3	Galectin-3	+	+
NUMA1	Nuclear mitotic apparatus protein 1	+	+
PFKP	6-phosphofructokinase type C;6-phosphofructokinase	+	
PGK1	Phosphoglycerate kinase 1;Phosphoglycerate kinase	+	+
PKM2	Pyruvate kinase isozymes M1/M2;Pyruvate kinase	+	+
PRDX2	Peroxiredoxin-2	+	
SP100	Nuclear autoantigen Sp-100	+	+
TFRC	Transferrin receptor protein 1;Transferrin receptor protein 1, serum form	+	+
TPI1	Triosephosphate isomerase	+	+
TUBB6	Tubulin beta-6 chain	+	
ZER1	Protein zer-1 homolog	+	
AHCY	Adenosylhomocysteinase		-
DDX21	Nucleolar RNA helicase 2	-	
EEF1G	Elongation factor 1-gamma	-	-
EEF1D	Elongation factor 1-delta		-
IDH1	Isocitrate dehydrogenase [NADP] cytoplasmic		-
IMPDH2	Inosine-5-monophosphate dehydrogenase 2		-
PSMD2	26S proteasome non-ATPase regulatory subunit 2	-	
RPS16	40S ribosomal protein S16	-	
STRAP	Serine-threonine kinase receptor-associated protein	-	

Table 3. Selected putative substrates of SUMO-1 and SUMO-2/3 identified in two IPs that modulate their sumoylation by SUMO1 or SUMO-2/3. Protein expression levels upon hypoxia were not changed or not detected by MS analysis. Further verification of these targets by small scale IPs and WB analysis is marked with an x in a different column.

Genes names	Protein names	SUMO1 conjugation in hypoxia	SUMO2/3 conjugation in hypoxia	Verified by WB analysis
ATRX	Transcriptional regulator ATRX	+	+	x
IRF2BP1	Interferon regulatory factor 2-binding protein 1	+		x
IRF2BP2	Interferon regulatory factor 2-binding protein 2	+		x
NFIL3	Nuclear factor interleukin-3-regulated protein		+	
RREB1	Ras-responsive element-binding protein 1	+		
TMPO	Lamina-associated polypeptide 2, isoforms beta/gamma;Thymopoietin;Thymopentin		+	
ZBTB38	Zinc finger and BTB domain-containing protein 38	+	+	x
ZNF687	Zinc finger protein 687		+	x
BEND3	BEN domain-containing protein 3	-	-	
DBF4	Protein DBF4 homolog A	-		
EXOSC10	Exosome component 10	-		x
GPC1	Glypican-1;Secreted glypican-1		-	
KCTD1	BTB/POZ domain-containing protein KCTD1		-	
KCTD15	BTB/POZ domain-containing protein KCTD15		-	x
NFRKB	Nuclear factor related to kappa-B-binding protein		-	x
NOL8	Nucleolar protein 8	-		
PDLIM5	PDZ and LIM domain protein 5	-		
TACC2	Transforming acidic coiled-coil-containing protein 2	-		
TFAP2a	Transcription factor AP-2-alpha;Transcription factor AP-2-beta		-	x
ZFP106	Zinc finger protein 106 homolog	-		x

Figure Legends:

Figure 1: Identification of endogenous SUMO-1 and Sumo 2/3 conjugates under hypoxia.

(A). Overview of the work flow of protein identification and quantification. HeLa cells were either untreated (normoxia) or exposed to 1% O_2 for 48h (hypoxia). Labelling of cells is indicated for both experiments (Light SILAC medium Lys^0Arg^0 and heavy SILAC medium Lys^4Arg^6). After denaturing lysis, the cell lysates were pooled and subjected to SUMO-1 and SUMO2 immunoprecipitation. Input and SUMO-1 and SUMO-2/3 immunoprecipitates were analysed by SDS-PAGE electrophoresis and coomassie staining. Proteins were further subjected to trypsin in-gel digestion, analysis by high-resolution LC-MS/MS and data processing (see Materials and Methods). (**B).** Heat map representing the average of hypoxia treated/ untreated (normoxia) log2 intensity ratios for the proteins that were detected in two biological experiments. First column includes proteins found in the INPUT analysis, the second and third columns includes proteins found in the SUMO-1 and SUMO-2/3 immunoprecipitates, respectively. Increased or decreased abundance of proteins upon hypoxia is represented with the red to blue colour scale. White indicates no change in protein abundance between hypoxia and normoxia or lack of data (protein not detected in any experiment). (**C).** x/y scatter plots, representing comparison of hypoxia treated/ untreated (normoxia) log2 intensity ratios for proteins common to both SILAC-SUMO-2/3 IPs (left) and SILAC-SUMO-1 IPs (right). Each protein is represented by one single point with coordinates coming from both IP experiments (x axis: log2 ratio heavy (hypoxia)/Light (normoxia) intensity ratio, y axis: log2 ratio Light (hypoxia)/heavy (normoxia) intensity ratio). Ratio cut-offs were determined graphically at 0.5 (dotted lines). Proteins that were only found more abundant in the unlabelled (Light) form in both experiments (upper left square), were rejected as external contaminants (in a red circle).

Figure 2: TFAP2a sumoylation is inhibited under hypoxia.

(A). Soluble extracts (Input) and SUMO-2/3 immunoprecipitates, from Hela cells incubated

under normoxia or hypoxia for 24h, were analysed by immunoblotting using a rabbit monoclonal anti-TFAP2a and a rabbit anti-SUMO2 antibody for verification of endogenous SUMO species enrichment. The asterisk shows the sumoylated version of TFAP2a and the arrowhead shows free Sumo2. Beta-actin was used as loading control.

(B). Hela cells were incubated in normoxia or hypoxia for the indicated times and expression levels of endogenous TFAP2a were analysed by immunoblotting. Endogenous TFAP2a expression from 3 independent experiments was normalized against corresponding expression of β-actin and expressed as fold increase in relation to TFAP2a expression under normoxia (p values >0.05).

(C). Hela cells were incubated as in (B) and localization of endogenous TFAP2a was detected by indirect immunofluorescence microscopy. DAPI was used for nucleal visualization.

Figure 3: Desumoylation of TFAP2a under hypoxia does not influence significantly its transcriptional activity.

(A). Schematic representation of the HA-TFAP2a constructs used in this study showing amino acid sequence 7-17. The position of the putative binding motif (IKYE) and the mutation (K10R) producing the sumo deficient mutant are highlighted. (**B).** Hela cells expressing His-SUMO2 and the indicated HA-TFAP2a constructs were subjected to immunoprecipitation with an anti-HA antibody. Inputs and eluates were analysed by immunoblotting using the indicated antibodies. (**C).** Hela cells expressing the indicated HA-TFAP2a constructs were incubated under normoxia or hypoxia for 24h and their lysates were immunoprecipitated with an anti-HA antibody. Inputs and eluates were analysed by immunoblotting using the indicated antibodies. The asterisks show the position of the sumoylated forms of HA-TFAP2a. In all cases β-actin was used as loading control. (**D).** HeLa cells transfected with the p3xAP2-Bluc/pCI-Renilla reporter plasmids and the indicated HA-TFAP2a constructs, were incubated under normoxia or hypoxia for 24h. Values

shown are ratios of firefly luciferase activity over renilla activity expressed as fold increase relative to the corresponding control (cells transfected with HA-empty plasmid under normoxia). Immunoblotting with HA shows the equal expression of wild-type and mutant TFAP2a in all cases.

Figure 4: TFAP2a enhances the transcriptional activity of HIF-1 in a sumoylation-dependent manner.

(A). HeLa cells were transfected with the pGL3–5HRE-VEGF and pCI-Renilla reporter plasmids and the indicated HA-TFAP2a constructs under 24h of hypoxia. (**B).** HeLa cells were transfected as in (A) with the addition of a flag-HIF1α and incubated for 24h in normoxic conditions. Values are ratios of firefly luciferase activity over renilla activity expressed as fold increase relative to the corresponding control (cells transfected with mock HA plasmid in hypoxia in A, or normoxia in B, considered as 1 in both cases). In all cases values represent the mean of three independent experiments performed in triplicate ± s.e.m. (n=9; $*P < 0.05$, $**P < 0.01$) and expression of the transfected proteins was detected with immunoblotting (lower panels).

(C). Hela cells were transfected with the indicated HA-TFAP2a constructs, incubated in normoxia or hypoxia for 24h, or in the presence of overexpressed flag-HIF-1α and lysates were immunoprecipitated with anti-HA antibody. Inputs and eluates were analysed by immunoblotting using the indicated antibodies. The asterisks show the position of the sumoylated form of HA-TFAP2a. β-actin was used as loading control.

Figure 5: Binding of TFAP2a to HIF-1 and HIF-2 is sumoylation independent.

(A). Hela cells were transfected with the indicated HA-TFAP2a constructs and Flag-HIF-1α for 24h and lysates were immunoprecipitated with an anti-HA antibody. **(B)** Hela cells were transfected as in (A) with the indicated HA-TFAP2a constructs and Flag-HIF-2α and lysates were immunoprecipitated with an anti-HA antibody. Inputs and eluates were analysed by

immunoblotting using the indicated antibodies. The asterisks show the position of the sumoylated form of HA-TFAP2a. ARNT or β-actin was used as loading control.

Figures

Figure 1:

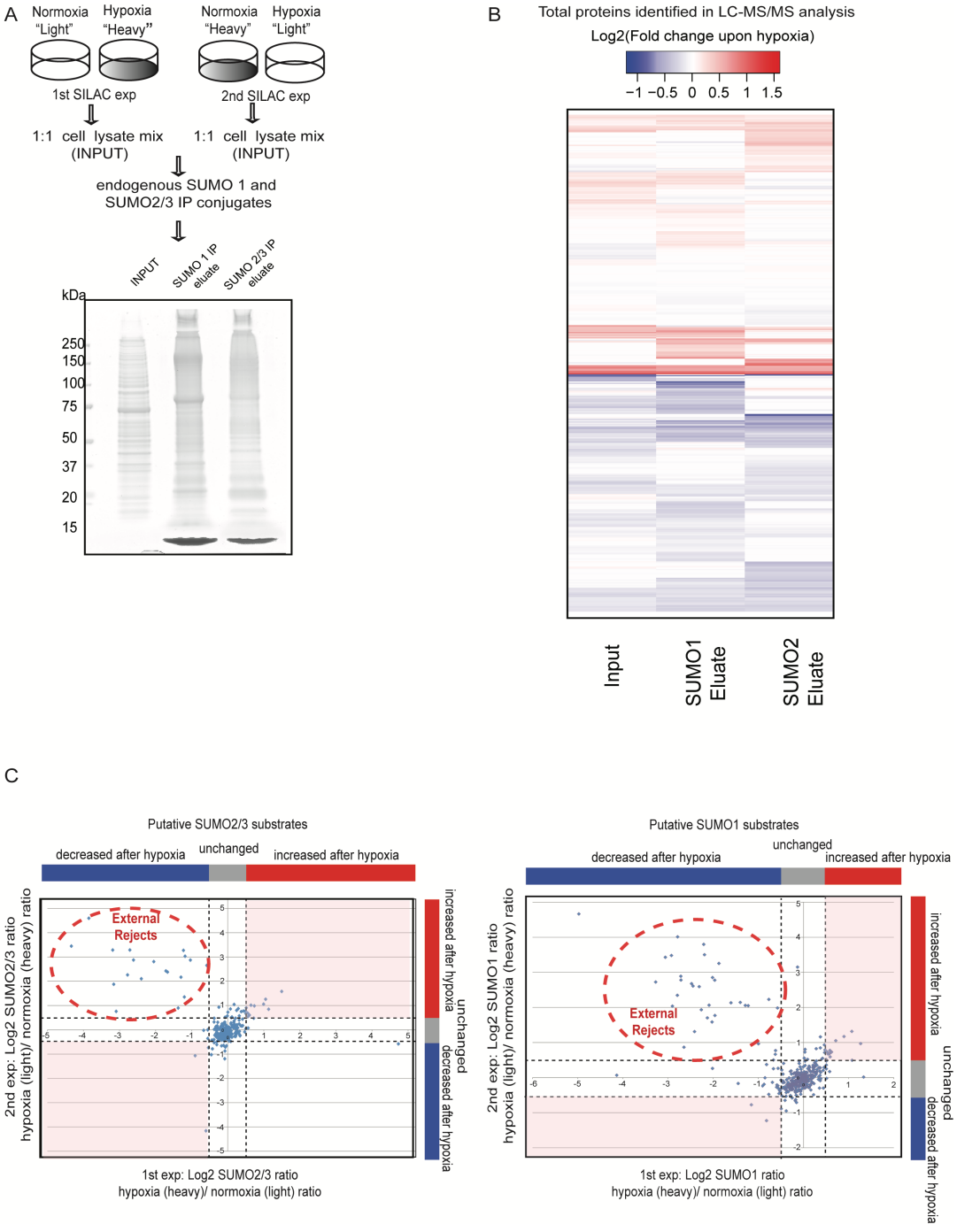

Figure 2:

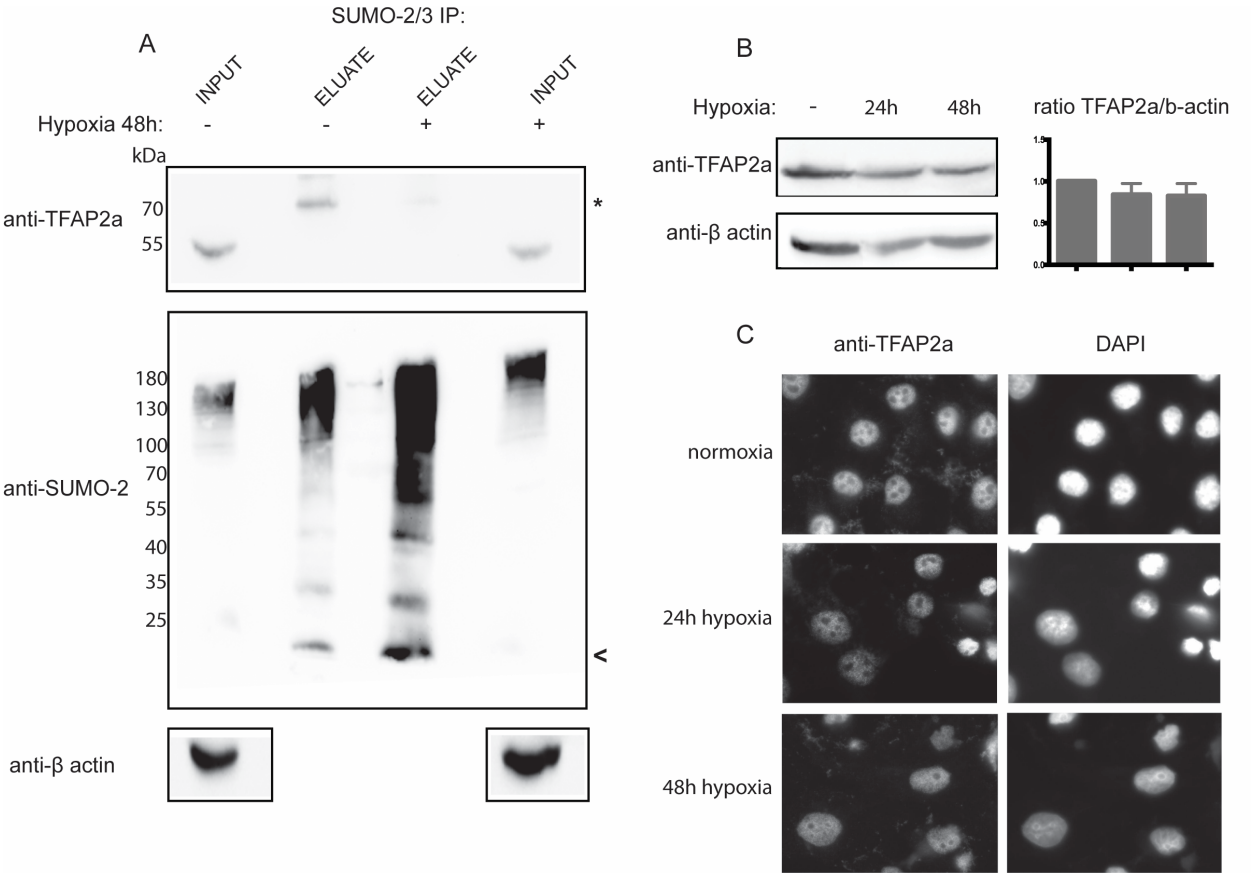

Figure 3:

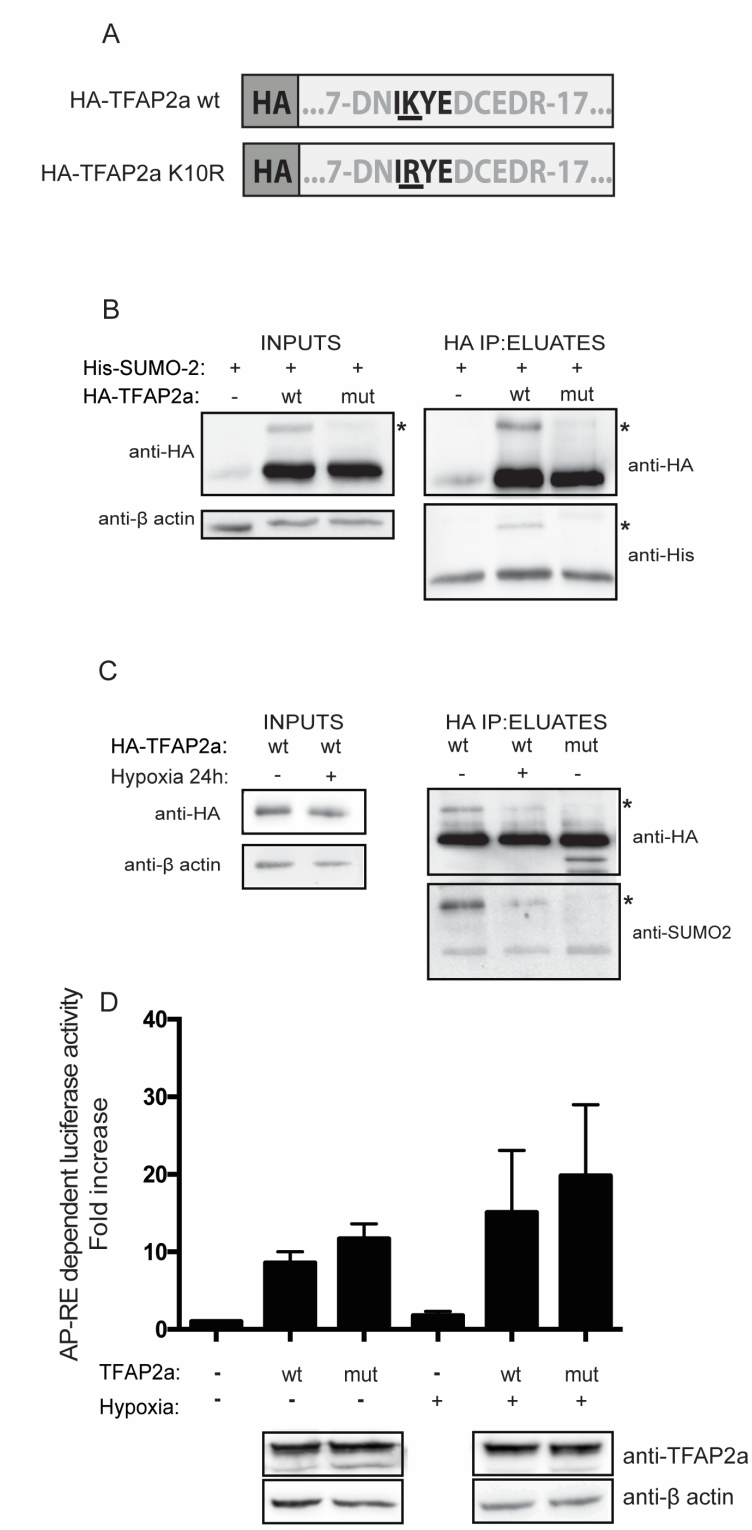

Figure 4:

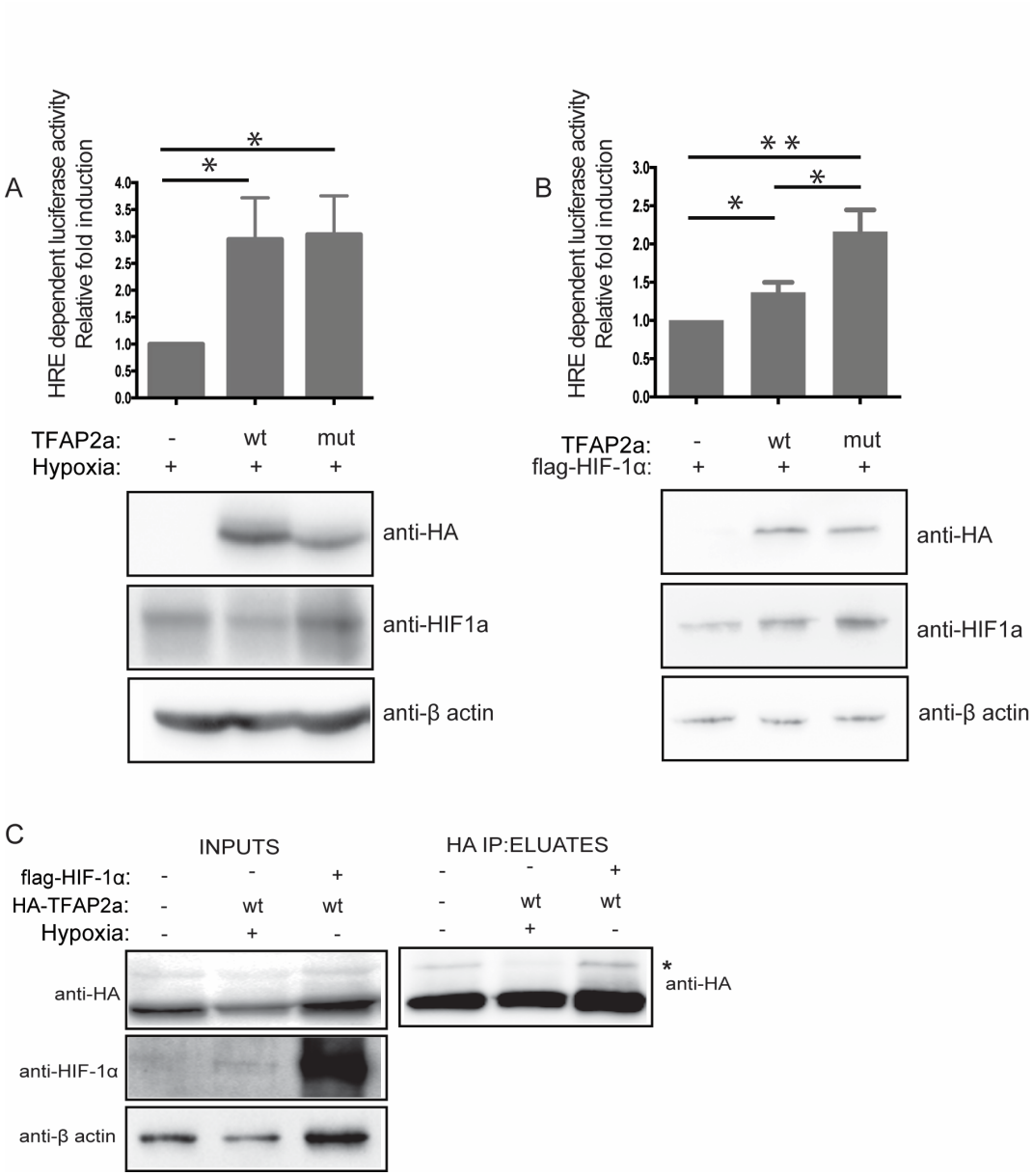

Figure 5: