

Roles of the human hypoxia-inducible factor (HIF)-3 α variants in the hypoxia response

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Abstract The hypoxia-inducible transcription factor (HIF) controls (in an oxygen-dependent manner) the expression of a large number of genes whose products are involved in the response of cells to hypoxia. HIF is an $\alpha\beta$ dimer that binds to hypoxia response elements (HREs) in its target genes. Human HIF- α has three isoforms, HIF-1 α , HIF-2 α and HIF-3 α , of which the roles of HIF-3 α are largely unknown, although it is usually regarded as a negative regulator of HIF-1 α and HIF-2 α . The human HIF-3 α locus is subject to extensive alternative splicing, leading to at least seven variants. We analyzed here the effects of the long variants and the short variant HIF-3 $\alpha 4$ on the hypoxia response. All these variants were found to interact with HIF- β , HIF-1 α and HIF-2 α . The long HIF-3 α variants were localized in the nucleus in hypoxia, while HIF-3 $\alpha 4$ was cytoplasmic. Interaction of the HIF-3 α variants with HIF-1 α inhibited the nuclear translocation of both. None of the long HIF-3 α variants was capable of efficient induction of an HRE reporter in overexpression experiments, but instead inhibited the transcriptional activation of the reporter by HIF-1 and HIF-2. Unexpectedly, siRNA knock-down of the endogenous HIF-3 α variants led to downregulation of certain HIF target genes, while overexpression of individual long HIF-3 α variants upregulated certain HIF target genes in a variant and target gene-specific manner under conditions in which HIF- β was not a limiting factor. These data indicate that the HIF-3 α variants may have more versatile and specific roles in the regulation of the hypoxia response than previously anticipated.

Keywords Hypoxia response · Hypoxia-inducible factor · Hypoxia-inducible factor 3 isoform · Prolyl 4-hydroxylase · Hypoxia response element

Abbreviations

HIF	Hypoxia-inducible factor
ODDD	Oxygen-dependent degradation domain
P4H	Prolyl 4-hydroxylase
C-TAD	C-terminal transactivation domain
bHLH	Basic helix-loop-helix
PAS	Per-ARNT-Sim
HRE	Hypoxia response element
IPAS	Inhibitory PAS domain protein
LZIP	Leucine zipper
qPCR	Quantitative real-time RT-PCR

Introduction

The hypoxia-inducible transcription factor (HIF) is a master regulator of adaptive cellular responses to decreased oxygen levels, controlling in an oxygen-dependent manner the expression of a large number of genes whose products are involved in functions such as hematopoiesis, angiogenesis, iron transport, glucose utilization, extracellular matrix synthesis, cell proliferation, survival and apoptosis, and tumor progression [1–4]. HIF is a dimer consisting of an unstable α subunit (HIF- α) and a stable β subunit (HIF- β) and binds to specific sequences termed hypoxia response elements (HREs) in its target genes. Hydroxylation of at least one of two conserved prolines in HIF- α mediates its interaction with the von Hippel-Lindau E3 ubiquitin ligase complex, which results in rapid proteasomal degradation of HIF- α in normoxia [5–7]. This

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hydroxylation is catalyzed in humans by three oxygen-dependent HIF prolyl 4-hydroxylases (HIF-P4Hs 1–3, also known as PHDs 1–3, and EglN2, 1 and 3, respectively) [8–12]. Another hydroxylase, factor inhibiting HIF (FIH), acts on an asparagine in the C-terminal transactivation domain (CTAD) of HIF- α , thereby preventing binding of the coactivator p300 and thus full activity of HIF [13–15]. Hypoxia inhibits both these hydroxylations and leads to stabilization and full transcriptional activity of HIF.

Human HIF- α has three isoforms, HIF-1 α , HIF-2 α and HIF-3 α , of which HIF-1 α and HIF-2 α have been studied most extensively [1–4]. These two are closely related and have similar domain structures, with an N-terminal basic helix-loop-helix (bHLH) domain followed by two Per-ARNT-Sim (PAS) domains, the oxygen-dependent degradation domain (ODDD) which harbors the two conserved prolines Pro402 and Pro564 in HIF-1 α that are hydroxylated by HIF-P4Hs and regulates the stability of the subunit, and N-terminal and C-terminal transactivation domains (TADs), of which the NTAD partially overlaps with the ODDD [16] (see Fig. 1). Analyses of HIF-DNA interactions at various hypoxia-responsive gene loci have identified a core HRE motif RCGTG that is bound by both HIF-1 α and HIF-2 α . This binding results in efficient activation of HRE-driven reporter genes [17–19], although HIF-1 α and HIF-2 α show some specificity in their endogenous target genes [16, 20]. The first HIF-3 α variants to be identified, mouse HIF-3 α and human HIF-3 α 1, differ from HIF-1 α and HIF-2 α in that they lack the CTAD [21, 22] and thus cannot bind p300. They also differ from HIF-1 α and HIF-2 α in that their ODDD contains only one of the

two conserved prolines, that corresponding to Pro564 in HIF-1 α . The mouse HIF-3 α and human HIF-3 α 1 have been shown to dimerize with HIF- β and to induce expression of an HRE reporter when overexpressed together with HIF- β , but much more weakly than do HIF-1 α and HIF-2 α , due to the lack of the CTAD [21, 22]. They suppress the activation potential of HIF-1 α and HIF-2 α under conditions where HIF- β is a limiting factor, however, probably by competing for binding with the latter [21, 22].

Three alternatively spliced forms are generated from the mouse HIF-3 α locus. The 307-residue mouse inhibitory PAS domain protein (IPAS) differs from the 662-amino-acid mouse HIF-3 α in that the first eight amino acids are coded by the IPAS-specific exon 1a instead of exon 1b as used in HIF-3, and in addition to lacking the NTAD and CTAD, it also lacks the ODDD [23, 24]. IPAS does not dimerize with HIF- β , but instead forms an inactive complex with HIF-1 α , thus acting as a dominant negative regulator of HIF-1 [23]. A third mouse HIF-3 α variant, NEPAS, is a 664-residue polypeptide that differs from mouse HIF-3 α only in that exon 1b is replaced by the IPAS exon 1a [25]. Like HIF-3 α , NEPAS dimerizes with HIF- β and suppresses HIF-1 and HIF-2 activity [25]. It is expressed mainly during the embryonic and neonatal stages, and its inactivation in mice leads to abnormal heart development and lung remodeling [25].

The human HIF-3 α locus is subject to even more extensive alternative splicing. Database analyses first suggested the existence of six splice variants, HIF-3 α variants 1–6, utilizing three alternative transcription start sites [26]. We have recently shown that full-length human HIF-3 α 1, HIF-3 α 2 and HIF-3 α 4 are expressed in several human tissues and cell lines (Fig. 1), but we obtained no evidence for the generation of the predicted HIF-3 α 3 or HIF-3 α 5 variants [27]. The existence of the predicted HIF-3 α 6 is also unlikely, as its DNA sequence does not correspond to the predicted domain structure [26] but contains partial duplications of exons 3 and 4 after exon 6 [28]. In addition, we identified four novel human HIF-3 α variants, HIF-3 α 7 to HIF-3 α 10, that are expressed in many tissues and cell types (Fig. 1), [27]. The short human HIF-3 α 4 variant is similar to, but not identical with, the mouse IPAS and it has been shown to form a complex with both HIF-1 α and HIF- β , and like the mouse IPAS, to inhibit the transcriptional activity of HIF-1 in a dominant negative manner [28, 29].

We set out to study the effects of the long human HIF-3 α variants 1, 2, 7, 8 and 9, as well as those of HIF-3 α 4, on the hypoxia response. We excluded analysis of HIF-3 α 10 as it consists of only seven amino acids (Fig. 1), [27]. Our data show that all the human HIF-3 α variants are able to interact with HIF- β , HIF-1 α and HIF-2 α . The long HIF-3 α variants were localized in the nucleus in hypoxia in

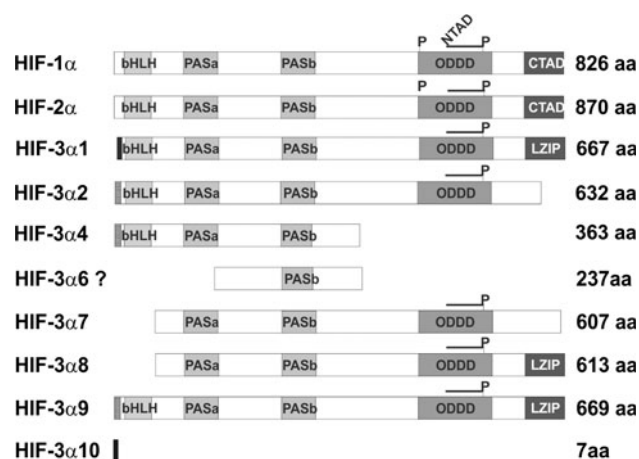


Fig. 1 Schematic representation of the HIF-1 α , HIF-2 α , and the HIF-3 α variant polypeptides. The structural motifs basic helix-loop-helix (bHLH), Per/Arnt/Sim (PAS), the oxygen-dependent degradation domain (ODDD), the N and C-terminal transactivation domains (NTAD and CTAD), the leucine zipper (LZIP), and the lengths of the polypeptides are indicated. The positions of the prolines hydroxylated by HIF-P4Hs in the ODDD are indicated by P

overexpression experiments, while the short variant HIF-3 α 4 was cytoplasmic. Interaction of the HIF-3 α variants with HIF-1 α inhibited nuclear translocation of both HIF-3 α and HIF-1 α . None of the long variants was capable of efficient induction of an HRE-reporter in overexpression experiments, but instead inhibited the transcriptional activation of the reporter by HIF-1 and HIF-2 under conditions where HIF- β is likely to be a limiting factor. Unexpectedly, siRNA knock-down of the endogenous HIF-3 α variants led to downregulation of certain HIF target genes, while overexpression of individual long HIF-3 α variants upregulated HIF target genes in a variant and target gene-specific manner under conditions in which HIF- β was not a limiting factor. These data indicate that the HIF-3 α variants may have more versatile and specific roles in regulation of the hypoxia response than previously anticipated.

Materials and methods

Cell culture

Hep3B hepatoma cells were cultured in Eagle's minimum essential medium (Sigma) and ChoK1 cells in Dulbecco's minimum essential medium (Biochrom AG) with 0.375% sodium bicarbonate (Sigma). Both culture media were supplemented with 0.1 mM non-essential amino acids (Sigma), 1 mM sodium pyruvate (Sigma), 10% fetal bovine serum (HyClone), 2 mM L-glutamine (Sigma) and 100 U/ml penicillin and 0.1 mg/ml streptomycin (Gibco). In the hypoxia experiments, the cells were incubated in 1% O₂, 5% CO₂, and 94% N₂ in an Invivo₂ 400 hypoxic workstation (Ruskin Technologies). As the ChoK1 cell line is easily transfectable and allows good expression levels of the transfected constructs, it was selected as a host for the interaction, reporter, and localization studies of the recombinant human HIF-3 α polypeptides. Hep3B cells were used in experiments where the effects of the knock-down of endogenous human HIF-3 α variants or overexpression of recombinant human HIF-3 α variants on the expression of endogenous human hypoxia responsive genes was studied.

Expression plasmids

The HIF-1 α -C-ODDD-luciferase reporter plasmid (a gift from FibroGen Inc.) contains the C-terminal region of the HIF-1 α ODDD between an NF- κ B activation domain and a Gal4 DNA-binding domain (Stratagene) followed by a Gal4-dependent luciferase reporter. The HIF-3 α -ODDD-luciferase reporter plasmid was generated in a similar way by cloning cDNA coding for the full-length ODDD (amino acids 411–552) of HIF-3 α into the reporter plasmid.

Mammalian expression vectors for full-length human untagged HIF-1 α and HIF-2 α , and for HIF-2 α with a C-terminal V5 tag were gifts from FibroGen Inc, and the expression plasmid for HIF- β with an N-terminal V5 tag was a gift from Dr. Katchinski, University of Göttingen. Cloning of the full-length HIF-3 α variants into the pcDNA3.1/Zeo(–) vector has been described previously [27]. To generate expression plasmids for HIF-1 α and the HIF-3 α variants [27] with C-terminal V5 tags, the full-length cDNAs were amplified by PCR and cloned into the pcDNA3.1–V5–His-A vector (Invitrogen). The HIF-3 α 2Pro_Ala mutant plasmid in which the codon for the single hydroxylatable Pro was changed to Ala was generated using the QuickChangeXL site-directed mutagenesis kit (Stratagene).

An expression plasmid for untagged HIF- β was generated by amplifying the full-length HIF- β cDNA and cloning it into the pcDNA3.1(+) vector (Invitrogen). For generation of an expression plasmid for HIF-2 α with an N-terminal hemagglutinin (HA) tag, the HIF-2 α cDNA was amplified by PCR so that the 5' oligo contained the sequence for the HA-tag followed by the codon for the second HIF-2 α amino acid, and the amplified product was then cloned into the pcDNA3.1/Zeo(–) vector (Invitrogen).

The glutathione S-transferase (GST) fusion constructs were generated by amplifying full-length HIF-1 α and HIF-2 α and HIF- β cDNAs using primers with an artificial BamHI restriction site at the 5' end and an XbaI or SalI restriction site at the 3' end to facilitate cloning into a BamHI-XbaI/SalI-digested pGEX-4T1 vector (GE Healthcare Life Sciences) using the Rapid DNA Ligation Kit (Roche).

EGFP-tagged constructs of the full-length human HIF-3 α variants 2, 4, 7, and 9 were generated by cloning into an XhoI-HindIII-digested pEGFP-N1 expression vector (BD Biosciences Clontech). The DsRed-monomer-C1-mHIF-1 α expression plasmid was a gift from Dr. Teresa Pereira (Karolinska Institutet, Stockholm, Sweden).

All sequences were verified using an automated DNA sequencer (ABI Prism 377, Applied Biosystems).

Analysis of degradation of HIF-3 α ODDD and selected full-length long HIF-3 α variants by HIF-P4Hs

On 24-well plates, 7×10^4 ChoK1 cells were seeded and cotransfected after 24 h with 300 ng of the HIF-1 α -C-ODDD-Luc or HIF-3 α -ODDD-Luc plasmids together with 300 and 600 ng of the plasmids coding for HIF-P4H-1, HIF-P4H-2 or HIF-P4H-3 or an empty pcDNA3.1/Zeo(–) vector, and 60 ng of a Renilla luciferase plasmid (Promega) with LipofectamineTM 2000 (Invitrogen) according to the supplier's protocol. The cells were cultured for 24 h in normoxia and assayed for luciferase activity using the

Dual-luciferase reporter assay system (Promega) according to the manufacturer's protocol and Luminoskan RS (Lab-systems) for measuring the luciferase activity.

In 6-well plates, 3×10^5 ChoK1 cells were seeded and cotransfected after 24 h with 750 ng of the expression plasmids coding for full-length HIF-1 α or V5-tagged HIF-3 α 1, HIF-3 α 2 or HIF-3 α 2_ProAla, in which the single hydroxylatable proline in the ODDD had been mutated to alanine, together with increasing amounts (750, 1,000, 1,250, or 1,500 ng) of HIF-P4H-2 with LipofectamineTM 2000 (Invitrogen) according to the supplier's protocol. The cells were cultured for 48 h in normoxia and lysed in a buffer containing 150 mM NaCl, 1 mM EDTA, 10% glycerol, 0.1% Tween-20, 1% NP-40, 2.5 mM EGTA, and 50 mM Hepes, pH 7.5 [30], supplemented with Complete protease inhibitor cocktail + EDTA (Roche), 1 mM NaVO₄, 50 mM NaF and 0.5 mM dithiothreitol upon incubation on ice for 20 min, and centrifuged at $12,000 \times g$ for 20 min. The cell lysates were analyzed by 8% SDS-PAGE under reducing conditions followed by ECL-Western blotting (Amersham Biosciences) with primary antibodies against HIF-1 α (BD Biosciences Pharmingen), V5 (Invitrogen) and α -tubulin (Sigma) at 1:1,000, 1:2,000 and 1:10,000 dilutions, respectively.

Protein interaction assays

For the immunoprecipitation analyses, 1.8×10^6 ChoK1 cells were seeded on 10-cm plates and transfected with 5 μ g of V5-tagged HIF-3 α 1, HIF-3 α 2, HIF-3 α 4, HIF-3 α 7, HIF-3 α 8 or HIF-3 α 9 together with 2 μ g of untagged HIF-1 α , HA-tagged HIF-2 α or untagged HIF- β using LipofectamineTM 2000 (Invitrogen) according to the supplier's protocol. Negative control samples were obtained by transfecting 5 μ g of the V5-tagged HIF-3 α variants together with 2 μ g of an empty pcDNA3.1/Zeo(-) vector (Invitrogen), and positive control samples by transfecting 5 μ g of V5-tagged HIF-1 α with 2 μ g of untagged HIF- β and 5 μ g of V5-tagged HIF- β with 2 μ g of untagged HIF-1 α or HA-tagged HIF-2 α . The transfected cells were cultured for 24 h in normoxia followed by a 24 h exposure to hypoxia. The cells were lysed as above. Immunoprecipitation of the lysates was performed with the V5 antibody and a Protein A/G PLUS-Agarose Immunoprecipitation reagent (Santa Cruz) according to the manufacturer's protocol. The immunoprecipitated samples were analyzed by 8% SDS-PAGE under reducing conditions followed by ECL-Western blotting (Amersham Biosciences) with primary antibodies against V5, HIF-1 α and HA (Sigma) at dilutions of 1:2,000, 1:1,000 and 1:1,000, respectively.

For the in vitro binding assays the GST-tagged HIF-1 α and HIF-2 α expression constructs were transformed into

the *E. coli* BL21 strain. Cells were grown from a single colony at 37°C overnight in 100 ml of Luria-Bertoni medium containing ampicillin (50 μ g/ml). The overnight culture was diluted 1:100 and isopropyl- β -D-thiogalactoside was added to a final concentration of 0.1 mM, at which an A₆₀₀ of 0.6–2 was reached. The cells were harvested by centrifugation 3 h after the induction, resuspended in 10 ml of ice-cold PBS and disrupted by sonication. Triton X-100 was added to a final concentration of 1% and the lysate was centrifuged at $10,000 \times g$ for 5 min at +4°C. The GST-fusion proteins expressed were bound to Protino Glutathione Agarose 4B beads (Macherey-Nagel) according to the manufacturer's protocol. The ChoK1 cells were transfected with the V5-tagged HIF-3 α variants or HIF- β , which served as a positive control. Cells transfected with the empty vector pcDNA3.1/Zeo(-) served as negative controls. The transfected cells were cultured for 24 h in normoxia followed by a 24 h hypoxia treatment. The cells were lysed in a buffer containing 150 mM NaCl, 1% Nonidet P-40, 10% glycerol, 10 mM MgCl₂, 1 mM EDTA and 0.25% sodium deoxycholate, 25 mM Hepes, pH 7.5, supplemented with Complete protease inhibitor cocktail + EDTA (Roche), 1 mM NaVO₄ and 50 mM NaF. The cell lysates were centrifuged at $12,000 \times g$ for 20 min at +4°C, precleared with mouse IgG together with Protino Glutathione Agarose 4B beads, and incubated with 50 μ l of Protino Glutathione Agarose 4B with or without the bound GST-HIF-1 α and GST-HIF-2 α fusion proteins for 2 h at 4°C. The beads were washed four times with the above buffer, boiled for 5 min in SDS-PAGE loading buffer, centrifuged at $12,000 \times g$ for 2 min at room temperature and analyzed by 8% SDS-PAGE under reducing conditions followed by ECL-Western blotting (Amersham Biosciences) with the primary antibody against V5 at a dilution of 1:2,000.

HIF-3 α overexpression experiments

To study the ability of overexpressed HIF-3 α variants to activate an HRE reporter, 3×10^5 ChoK1 cells were seeded per well on 6-well plates and transfected 24 h later with a 3xHRE-secreted alkaline phosphates (HRE-SEAP) reporter plasmid (a gift from FibroGen Inc.) together with increasing amounts (500, 1,000, 2,000 ng) of pcDNA.3.1/Zeo(-) (negative control) or expression plasmids for full-length V5-tagged HIF-1 α , HIF-2 α , HIF-3 α 1, HIF-3 α 2, HIF-3 α 4, HIF-3 α 7, HIF-3 α 8 or HIF-3 α 9 using LipofectamineTM 2000 (Invitrogen) according to the supplier's protocol. The cells were cultured for 48 h in normoxia and medium samples were collected and assayed for SEAP activity using the SEAP Reporter Assay Kit (InvivoGen). Absorbance at 405 nm was measured with a VictorTM 1420 Multilabel Counter (Wallac). The cells were lysed in a buffer containing 150 mM NaCl, 1 mM EDTA, 10%

glycerol, 0.1% Tween-20, 1% NP-40, 2.5 mM EGTA, and 50 mM Hepes, pH 7.5 [30], supplemented with Complete protease inhibitor cocktail + EDTA (Roche), 1 mM NaVO₄, 50 mM NaF and 0.5 mM dithiothreitol upon incubation on ice for 20 min, and centrifuged at 12,000 \times g for 20 min. Expression of the transfected HIF- α polypeptides was analyzed by 8% SDS-PAGE under reducing conditions followed by ECL-Western blotting (Amersham Biosciences) with the primary antibody against V5 at a dilution of 1:2,000.

To study the capacity of overexpressed HIF-1 α and HIF-2 α to activate an HRE reporter in the presence of overexpressed HIF-3 α variants, 3×10^5 ChoK1 cells were seeded on 6-well plates and transfected after 24 h with 500 ng of the expression plasmids for full-length HIF-1 α or HIF-2 α together with 500 ng of pcDNA3.1/Zeo(-) or the expression plasmids for the HIF-3 α variants together with 750 ng of a 3xHRE-luciferase (HRE-LUC) reporter plasmid (a gift from Dr. Pasi Tavi) and 100 ng of a Renilla luciferase plasmid (Promega) using LipofectamineTM 2000 (Invitrogen) as above. The cells were cultured for 48 h in normoxia and assayed for luciferase activity as above.

To study the effect of overexpressed human HIF-3 α variants on the transactivation capacity of an HRE reporter with respect to endogenous human HIF-1 α and HIF-2 α , Hep3B cells were transfected as above but without the expression plasmids for human HIF-1 α and HIF-2 α , cultured for 24 h in normoxia followed by 24 h in hypoxia and assayed for luciferase activity.

To study the effect of overexpressed HIF-3 α variants on selected endogenous HIF target genes 3.5×10^6 Hep3B cells were seeded on 6-well plates and transfected 24 h later with 250–1,000 ng of the expression plasmids for the HIF-3 α variants together with 1,000 ng of the HIF- β or pcDNA3.1/Zeo(-) plasmid using LipofectamineTM 2000 (Invitrogen) as above. In the experiments where HIF- β was not limited, i.e., in the presence of the HIF- β plasmid, the cells were cultured in normoxia for 48 h followed by 90 min in hypoxia, while in those where HIF- β should become limiting, i.e., in the presence of the pcDNA3.1/Zeo(-) plasmid, the cells were cultured for 24 h in normoxia followed by 24 h in hypoxia. Total RNA was isolated from the cells using the E.Z.N.A.TM total RNA kit (Omega) and mRNA expression levels of the HIF target genes for erythropoietin (Epo), angiopoietin-related protein 4 (AngPTL4), phosphofructokinase L (Pfk1) and glucose transporter 1 (Glut1) were analyzed by qPCR with normalization to the expression of the TATA-binding protein gene using gene-specific primers (Table 1), iTaqTM SYBR[®] Green Supermix with ROX (Bio-Rad) or SsoFast EvaGreen[®] Supermix (Bio-Rad) and the MX3005 (Stratagene) equipment.

RNA interference

siRNA oligos against HIF-3 α (siGENOME Set of 4, MQ-010068-03-0005, Thermo Fisher Scientific) were obtained from Dharmacon, and three siRNA oligos from this set were used either individually (3 α _a, b or c) or in combination (3 α _a+b+c) in the siRNA experiments. The sequences of the siRNA oligos are given in Table 1. Firefly pGL2 luciferase control duplex siRNA (Eurogentec) was used as a negative siRNA control. All siRNAs were reconstituted under RNase-free conditions according to the manufacturer's protocol, using the buffers supplied. Hep3B cells were transfected with the siRNAs at a confluence of 80–90% with LipofectamineTM 2000 (Invitrogen) in OPTI-MEM[®]-1 medium (GIBCO[®]) according to the supplier's protocol. The final concentration of each individual siRNA oligo in the cell culture medium was 0.08 pmol/ μ l. The transfection was repeated after 24 h and the cells were cultured for an additional 24 h in hypoxia. Forty-eight hours after the first transfection, the culture medium was removed from the cells and stored in -70°C and total RNA and protein were isolated from the cells with the ParisTM kit (Ambion). No distinct differences in the viability of the HIF-3 α and luciferase siRNA treated cells and the mock-transfected cells were detected as the cell cultures were confluent at the time of harvest and the amounts of total RNA and protein isolated from the cells were highly similar. The RNA samples were treated with DNase using the Turbo-DNA-freeTM kit (Ambion) and converted to cDNA with the iScriptTM cDNA synthesis kit. The protein samples were analyzed by 8% SDS-PAGE under reducing conditions followed by ECL-Western blotting (Amersham Biosciences) with primary antibodies against HIF-1 α (BD Biosciences Pharmingen), HIF-2 α (Novus Biologicals) and α -tubulin (Sigma) at dilutions of 1:1,000, 1:200, and 1:10,000, respectively. The amount of Epo protein in the medium was analyzed with a Quantikine[®] IVD[®] Erythropoietin ELISA kit (R&D Systems Inc.) according to the manufacturer's protocol. The amount of Epo was measured with a VictorTM 1420 Multilabel Counter (Wallac) using 450 nm as the primary wavelength and 540 nm as the reference wavelength. mRNA expression levels of EPO, ANGPTL4, PFKL, GLUT1, lysyl oxidase (LOX), hexokinase 2 (HK2), transferrin (TRF), urokinase receptor (uPAR), BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3), heme oxygenase 1 (HO1) and vascular endothelial growth factor (VEGF) were analyzed by qPCR as above.

Confocal microscopy

To study the localization of the EGFP-tagged HIF-3 α variants in cells, 2.2×10^5 ChoK1 cells were seeded on

Table 1 Sequences of the oligos used in RNA interference and qPCR analyses of the HIF-3 α variants

Primer	Sequences (5'-3') and locations of the oligos	Use
3 α _a (D-010068-02)	GCAAGAGCAUCCACACCUU, exon 7 and 8	RNA interference
3 α _b (D-010068-05)	CGACAGGAUUGCAGAAGUG, exon 6 and 7	RNA interference
3 α _c (D-010068-21)	UACAGGGCAGUAUCGCUU, exon 8	RNA interference
TBP_F	GAARARAARCCCAAGCGGTTTG	qPCR
TBP_R	ACTTCACATCACAGCTCCCC	qPCR
HIF1a_Q_F	CTAGCTTTGCAGAATGCTCAG	qPCR
HIF1a_Q_R	GTAGTAGCTGCATGATCGTCTG	qPCR
HIF2a_Q_F	CCCAGATCCACCATTACAT	qPCR
HIF2a_Q_R	ACTCCAGCTGTCGCTTCA	qPCR
Epo_F	CTCCGAACAATCACTGCT	qPCR
Epo_R	GGTCATCTGTCCCCTGTCCT	qPCR
LOX_QF	ACGGCACTGGCTACTTCCAG	qPCR
LOX_QR	GGCCAGACAGTTTTCTCCCG	qPCR
Q_GLUT_F	GATTGGCTCCTTCTCTGTGG	qPCR
Q_GLUT_R	TCAAAGGACTTGCCCAGTTT	qPCR
Q_HK2_F	CAAAGTGACAGTGGGTGTGG	qPCR
Q_HK2_R	GCCAGGTCCTTCACTGTCTC	qPCR
Q_PFKL_F	CCCGAGGACGGCTGGGAGAA	qPCR
Q_PFKL_R	GGGCTTCCCCTTGCAGTCAA	qPCR
Q_TRF_F	GTCGCTGGTCAGTTCGTGATT	qPCR
Q_TRF_R	AGCAGTTGGCTGTTGTAACCTCTC	qPCR
Q_uPAR_F	GGTGACGCCTTCAGCATGA	qPCR
Q_uPAR_R	CCCACTGCGGTACTGGACAT	qPCR
Q_BNIP_F	GGACAGAGTAGTCCAGAGGCAGTTC	qPCR
Q_BNIP_R	GGTGTGCATTTCCACATCAAACAT	qPCR
Q_AngPT4_F	TCTCCGTACCCTTCTCCACT	qPCR
Q_AngPT4_R	AGTACTGGCCGTTGAGGTTG	qPCR
Q_HO1_F	CTATGTGAAGCGGCTCCACG	qPCR
Q_HO1_R	GCTCTGGTCCTTGGTGTCTAT	qPCR
Q_VEGF_F	AGCCTTGCCCTTGCTGCTCTA	qPCR
Q_VEGF_R	TCAGGGTACTCCTGGAAGATG	qPCR

35 mm glass-bottomed culture dishes (MatTek Corporation) and transfected 24 h later with 800 ng of the expression plasmids for EGFP-tagged HIF-3 α 2, HIF-3 α 4, HIF-3 α 7 or HIF-3 α 9 using LipofectamineTM 2000 (Invitrogen) as above. The cells were incubated for 12 h in normoxia \pm 1 h in 1% oxygen, fixed with 3% paraformaldehyde for 3 min, washed with PBS and analyzed using an Olympus FluoView FV 1000 confocal laser scanning microscope system. To study the possible colocalization of HIF-3 α variants with HIF-1 α , cells were seeded as above and transfected with 800 ng of the expression plasmids for each of the EGFP-tagged HIF-3 α variants together with DsRed- monomer-C1-mHIF-1 α . The cells were cultured for 24 h in normoxia followed by 8 h in 1% oxygen. Cells were fixed and washed as above and stained with DAPI (Sigma) diluted 1:10,000 in PBS for 15 min at room temperature. After staining, the cells were washed with PBS three times and examined as above.

Statistical analysis

The statistical analyses were performed using Student's two-tailed *t* test. Data are shown as means \pm SD. Values of $p < 0.05$ were considered statistically significant (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Results

Regulation of the HIF-3 α ODDD by HIF-P4Hs

Synthetic 20–35-residue peptides representing the single hydroxylation site of the human HIF-3 α ODDD have been shown to be efficiently hydroxylated in vitro by all three recombinant human HIF-P4Hs [11, 31]. The human HIF-3 α ODDD and the full-length human HIF-3 α 1 have been shown to be targeted for ubiquitylation and

proteasomal degradation in a VHL and oxygen-dependent manner, but the full-length HIF-3 α 1 is ubiquitinated less robustly than the ODDD, suggesting that the full-length protein may have structural elements that inhibit ubiquitylation [26]. It has recently been reported that full-length human HIF-3 α 2 does not accumulate in cells upon chemical inhibition of HIF-P4Hs, indicating that it is not subject to oxygen-dependent destabilization [32].

To study this aspect further, we first analyzed the effect of overexpressed HIF-P4Hs on degradation of the HIF-3 α ODDD by cotransfecting ChoK1 cells with (a) a constant amount of a luciferase reporter plasmid for the HIF-3 α ODDD or the C-terminal region of the HIF-1 α ODDD, and (b) increasing amounts of a plasmid encoding HIF-P4H-1, 2 or 3. The cells were cultured for 24 h in normoxia and assayed for luciferase activity. Overexpression of all three HIF-P4Hs led to a 44–60% reduction in the level of the C-terminal region of the HIF-1 α ODDD (Fig. 2b). The level of the HIF-3 α ODDD was reduced by 30–44% by overexpression of HIF-P4H-1 and by 69–77% with HIF-P4H-2 or 3 (Fig. 2a), indicating that the HIF-3 α ODDD is efficiently hydroxylated by all three HIF-P4Hs in cellulo, leading to subsequent VHL-dependent ubiquitylation and proteasomal degradation.

To study the HIF-P4H-dependent degradation of selected full-length long HIF-3 α variants, we cotransfected ChoK1 cells with (a) a constant amount of a plasmid encoding full-length V5-tagged HIF-1 α , HIF-3 α 1, HIF-3 α 2

or HIF-3 α 2Pro_Ala mutant polypeptides, and (b) increasing amounts of a plasmid encoding HIF-P4H-2, the main hydroxylase regulating the hypoxia response [33–35]. The cells were cultured for 48 h in normoxia, lysed, and analyzed by Western blotting. The amounts of the HIF-1 α , HIF-3 α 1 and HIF-3 α 2 polypeptides all decreased in a HIF-P4H-2 dose-dependent manner, while that of the HIF-3 α 2Pro_Ala mutant polypeptide remained constant (Fig. 2c). These data show that the HIF-3 α ODDD also mediates HIF-P4H-dependent degradation in the context of full-length HIF-3 α polypeptides, including HIF-3 α 2.

All human HIF-3 α variants interact with HIF- β , HIF-1 α , and HIF-2 α

ChoK1 cells were cotransfected with vectors coding for V5-tagged HIF-3 α variants together with non-tagged HIF- β , non-tagged HIF-1 α or HA-tagged HIF-2 α (Fig. 3a–c). Immunoprecipitation with an anti-V5 antibody showed that HIF- β was coprecipitated with all the V5-HIF-3 α variants and with the V5-HIF-1 α used as a control for positive interaction (Fig. 3a). The interaction of the HIF-3 α variants with HIF- β seemed to be just as efficient as that of HIF-1 α when the input amounts of the HIF-3 α variants and HIF-1 α were taken into consideration (Fig. 3a). Likewise, HIF-1 α was coimmunoprecipitated with all the V5-HIF-3 α variants in the experiments where interaction of HIF-1 α with V5-HIF- β served as a positive control

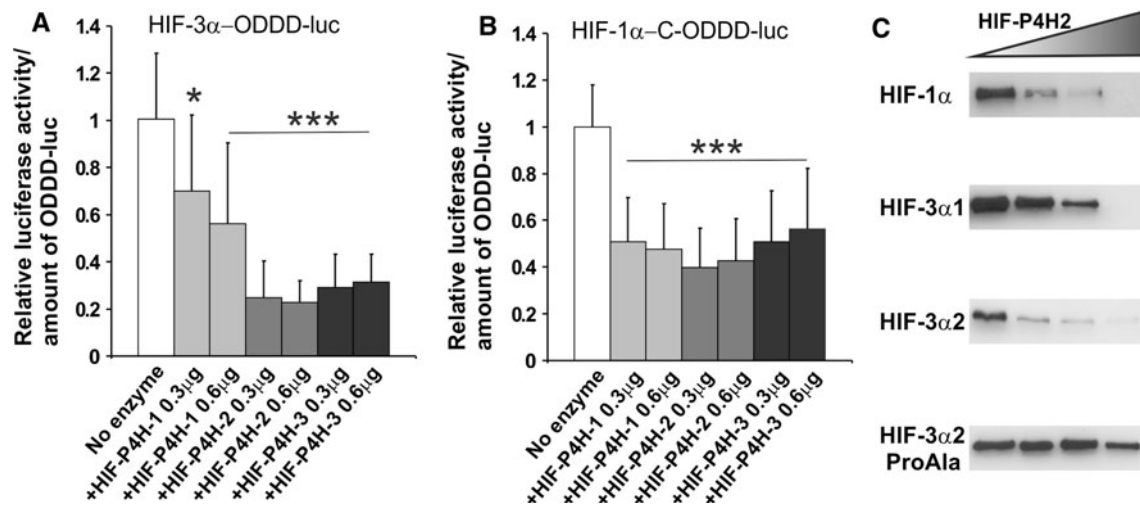


Fig. 2 Overexpression of HIF-P4H enzymes leads to degradation of a HIF-3 α -ODDD reporter and full-length HIF-3 variants. ChoK1 cells were transfected with a HIF-3 α -ODDD-luc (a) or HIF-1 α -C-ODDD-luc (b) reporter plasmid together with 0.3 or 0.6 μ g of HIF-P4H-1, HIF-P4H-2 or HIF-P4H-3 expression plasmids. The transfected cells were cultured for 24 h in normoxia and assayed for luciferase activity. The luciferase activity of the control sample without HIF-P4H overexpression was set as 1. The data represent means \pm SD from at least four independent experiments. * p < 0.05, *** p < 0.001. To

study the degradation of full-length HIF- α polypeptides, ChoK1 cells were transfected with plasmids encoding full-length HIF-1 α , V5-tagged HIF-3 α 1, HIF-3 α 2 or HIF-3 α 2ProAla together with increasing amounts of HIF-P4H-2 plasmid (c). The cells were cultured for 48 h in normoxia, lysed, and analyzed by 8% SDS-PAGE under reducing conditions followed by Western blotting with HIF-1 α , V5 and α -tubulin antibodies. The results of a typical experiment are shown

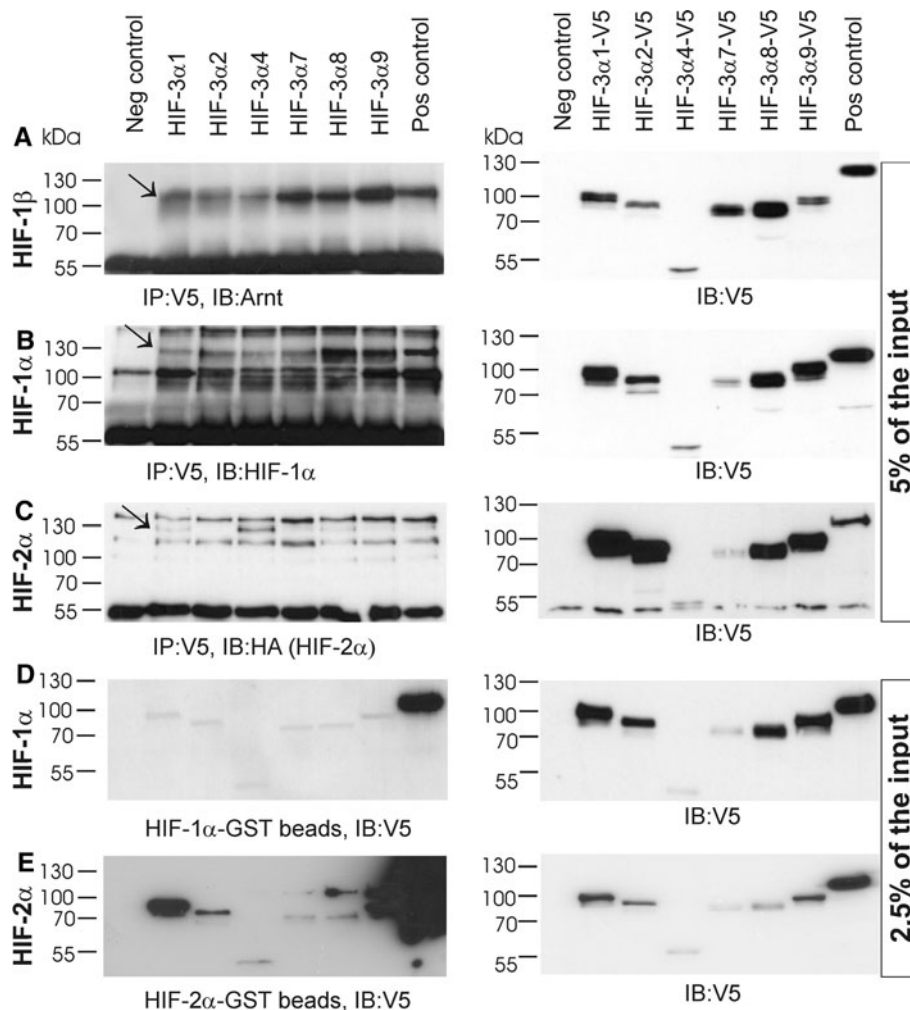


Fig. 3 All HIF-3 α variants can bind HIF-1 α , HIF-2 α and HIF-1 β . ChoK1 cells were transfected for immunoprecipitation studies with plasmids for V5-tagged HIF-3 α 1, HIF-3 α 2, HIF-3 α 4, HIF-3 α 7, HIF-3 α 8 and HIF-3 α 9 with or without untagged HIF-1 β (a), HIF-1 α (b), or HA-tagged HIF-2 α (c). In the positive control samples the cells were transfected with plasmids for HIF-1 β and V5-tagged HIF-1 α (a), HIF-1 α and V5-tagged HIF-1 β (b), and HA-tagged HIF-2 α and V5-tagged HIF-1 β (c). The cells were cultured for 24 h in normoxia followed by 24 h in 1% oxygen. The *left-hand panels* in a–c show the amounts of the HIF-1 β , HIF-1 α and HIF-2 α polypeptides immunoprecipitated with the V5-tagged HIF-3 α variants or the positive controls, and the *right-hand panels* show the amounts of the V5-tagged polypeptides present in the whole cell lysates (5% of the input amount) used in the

(Fig. 3b). HA-HIF-2 α was coimmunoprecipitated with all the V5-HIF-3 α variants except HIF-3 α 2 (Fig. 3c). The amount of HA-HIF-2 α immunoprecipitated with the V5-tagged HIF-3 α variants or HIF-1 β was in general low, except for V5-HIF-3 α 4, which pulled down the largest amount of HA-HIF-2 α despite the relatively low input amount of V5-HIF-3 α 4 (Fig. 3c).

Interaction of the HIF-3 α variants with HIF-1 α and HIF-2 α was studied further by means of an in vitro binding assay. GST-tagged HIF-1 α and HIF-2 α were produced in

immunoprecipitation analysis (d–e). For in vitro binding studies HIF-1 α and HIF-2 α were expressed as GST-fusion proteins in *E. coli*, purified and bound to glutathione agarose beads. ChoK1 cells were transfected with plasmids for the V5-tagged HIF-3 α variants or HIF-1 β (positive control) or the empty vector pcDNA3.1/Zeo(–) (negative control) and cultured for 24 h in normoxia followed by 24 h in 1% oxygen. The cell lysates were incubated with the GST beads \pm GST-HIF-1 α or GST-HIF-2 α . The *left-hand panels* show the amounts of the captured V5-tagged HIF-3 α variants or positive controls, and the *right-hand panels* show the amounts of the V5-tagged polypeptides present in the whole cell lysates (2.5% of the input amount) used in the in vitro binding assay

E. coli, bound to glutathione beads, and lysates from ChoK1 cells expressing V5-tagged HIF-3 α variants or V5-tagged HIF-1 β were incubated with the beads (Fig. 3d, e). All the HIF-3 α variants were bound by the GST-tagged HIF-1 α and HIF-2 α , but in both cases the interaction of HIF-1 α and HIF-2 α with HIF-1 β seemed to be much stronger than with any of the HIF-3 α variants (Fig. 3d, e). In the case of GST-HIF-2 α the amount of bound HIF-1 β was so high that it partially masked the neighboring lane representing binding of HIF-3 α 9, and leakage of HIF-1 β into

the next two lanes representing binding of HIF-3 α 7 and HIF-3 α 8 also occurred (Fig. 3e). Bands of the correct size representing bound HIF-3 α 7, HIF-3 α 8 and HIF-3 α 9 were nevertheless seen in these lanes in addition to HIF- β (Fig. 3e).

Overexpression of the human HIF-3 α variants does not lead to efficient induction of an HRE-reporter

To study the transactivation potential of the HIF-3 α variants with respect to a reporter gene driven by an HRE, we transiently transfected ChoK1 cells with a HRE-SEAP reporter plasmid together with increasing amounts of expression plasmids coding for HIF-1 α , HIF-2 α or the HIF-3 α variants. To exclude the effect of endogenous HIF- α subunits, the cells were cultured for 48 h under normoxic conditions after the transfection and assayed for recombinant HIF- α subunit expression and SEAP activity. The amount of overexpressed HIF- α subunits exceeded the hydroxylation capacity of the endogenous HIF-P4Hs, resulting in the accumulation of stable recombinant HIF- α subunits in a dose-dependent manner under normoxic conditions (Fig. 4a), as also reported by others [21–23, 25]. Overexpression of HIF-1 α and HIF-2 α led to a dose-dependent 14- to 18-fold induction in the HRE-driven

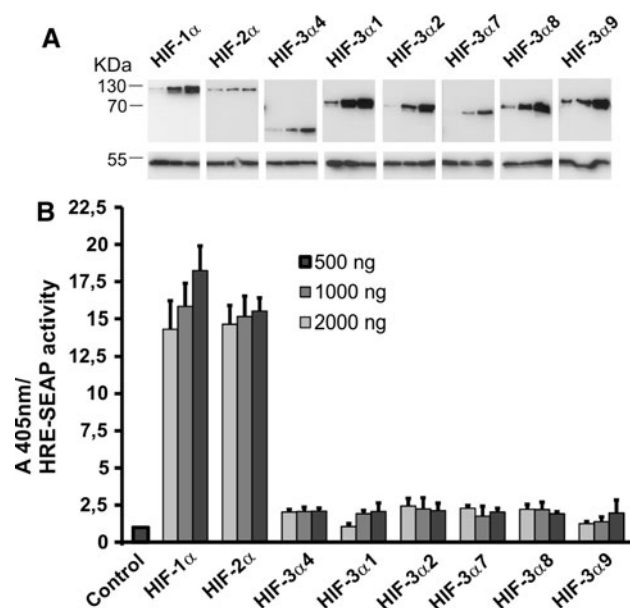


Fig. 4 Effects of the HIF-3 α variants on the activation of a HRE reporter. ChoK1 cells were transfected with an HRE-SEAP reporter plasmid together with increasing amounts (500, 1,000, 2,000 ng) of expression plasmids for HIF-1 α , HIF-2 α or the HIF-3 α variants. The cells were cultured for 48 h in normoxia and analyzed for expression of the HIF- α polypeptides by Western blotting (a) and assayed for SEAP activity (b), which was set to 1 in the control sample without any overexpressed HIF- α polypeptide. The results of a typical experiment are shown in a, and the data in b represent means \pm S.D. from at least 4 independent experiments

SEAP activity, while overexpression of the HIF-3 α variants led only to a 2.5-fold induction at most, and this was in most cases independent of the dose (Fig. 4b). It should be noted, however, that overexpression of even the short HIF-3 α 4 variant, which does not contain any known transactivation domain (Fig. 1), led to about a twofold induction in the SEAP activity (Fig. 4b). This finding may indicate that the 2- to 2.5-fold induction seen with the HIF-3 α variants is not a true effect of these variants on HRE but may result from some indirect phenomenon in the experimental setup. This suggestion is supported by the differences in the cellular localization of the short and long HIF-3 α variants (see Fig. 8a).

Overexpression of the human HIF-3 α variants inhibits the transcriptional activation of an HRE-reporter by HIF-1 α and HIF-2 α

ChoK1 cells were cotransfected with a HRE-LUC reporter plasmid and the HIF-1 α or HIF-2 α expression plasmids in the presence or absence of an expression plasmid for each of the HIF-3 α variants. The cells were cultured for 48 h under normoxic conditions after the transfection and assayed for luciferase activity. Coexpression of all the HIF-3 α variants markedly reduced the HRE-LUC induction potential of overexpressed HIF-1 α and HIF-2 α , the reduction being 52–83% for HIF-1 α and 30–50% for HIF-2 α (Fig. 5a, b). This suggests that the HIF-3 α variants act as dominant negative regulators of HIF-1 α and HIF-2 α -mediated gene expression, at least under conditions where the amount of HIF- β becomes limiting.

To study the effect of overexpressed HIF-3 α variants on the HRE induction potential of endogenous HIF-1 α and HIF-2 α in hypoxia, Hep3B cells were cotransfected with the HRE-LUC reporter in the presence or absence of an expression plasmid for each of the HIF-3 α variants. The cells were cultured for 24 h under normoxic conditions after transfection, followed by a 24 h culture in 1% O₂. All the long HIF-3 α variants efficiently reduced the hypoxia-induced HRE-LUC activity by 45–66% (Fig. 5c). The inhibitory efficiency of HIF-3 α 4 was lower, 28% (Fig. 5c), but this may be due to the lower expression level of this variant relative to the long ones (Fig. 4a).

siRNA knock-down of endogenous HIF-3 α variants unexpectedly leads to downregulation of many HIF target genes

We next studied the effect of knock-down of the endogenous HIF-3 α variants on the expression of a known HIF target gene, Epo, in Hep3B cells. Three individual siRNA oligos targeting all the HIF-3 α variants were used either singly or in combination and their efficiency was analyzed

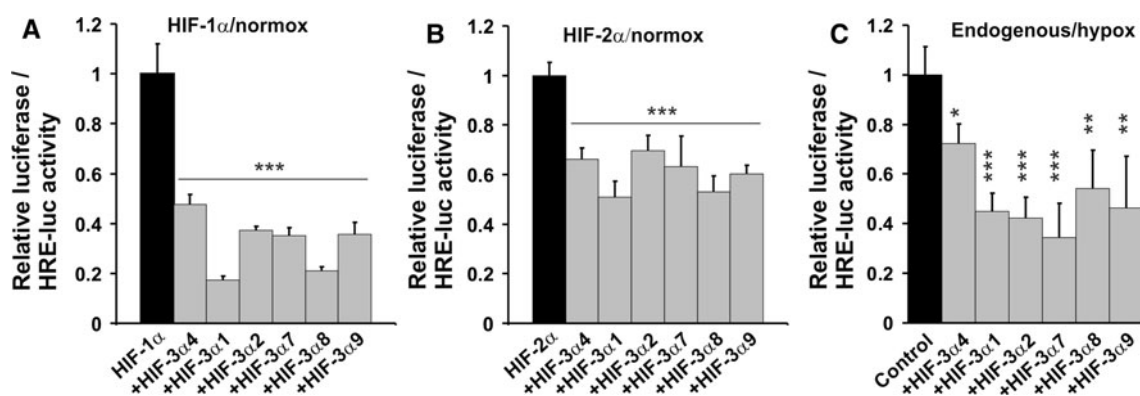


Fig. 5 HIF-3 α variants inhibit the transcriptional activation of an HRE-reporter by HIF-1 and HIF-2. ChoK1 cells were transfected with a 3xHRE-LUC reporter plasmid together with plasmids for HIF-1 α (a) or HIF-2 α (b) and with or without plasmids for the HIF-3 α variants. The cells were cultured for 48 h in normoxia and assayed for luciferase activity. The luciferase activity of cells transfected only with HIF-1 α or HIF-2 α was set to 1. The data represent means \pm SD

from at least six independent experiments. *** $p < 0.001$. Hep3B cells were transfected with a 3xHRE-Luc reporter plasmid together with a empty vector or certain HIF-3 α variants (c). Transfected cells were cultured for 24 h in normoxia followed by 24 h in hypoxia and then assayed for luciferase activity. The luciferase activity of the control samples was set to 1

by qPCR using oligos that amplify a region from exons 5 and 6 that is common to all the HIF-3 α variants. All three HIF-3 α siRNAs, either alone or in combination, efficiently downregulated expression of the HIF-3 α variants at the mRNA level (80–87% reduction) (Fig. 6a). We also attempted to analyze the siRNA efficiency by Western blotting using a monoclonal HIF-3 α antibody or a polyclonal hHIF-3 α 2 antibody. All the recombinant HIF-3 α variants could be identified with these antibodies when overexpressed in Hep3B cells, but no bands of the corresponding sizes representing endogenous HIF-3 α variants at the protein level in Hep3B cells were detectable (data not shown). This is most likely due to the generally low expression levels of the HIF-3 α variants in various cell lines and tissues [27]. One of the siRNA oligos, 3 α _a, led to a non-specific reduction in HIF-1 α and HIF-2 α mRNA levels by about 13 and 23%, respectively, and the combination of the HIF-3 α siRNA oligos also reduced the level of HIF-2 α mRNA by about 11.5% (Fig. 6a). These non-specific reductions did not lead to any noticeable reduction in the amounts of stabilized HIF-1 α and HIF-2 α protein in the hypoxic cells, however (Fig. 6b).

The effect of HIF-3 α knock-down on Epo expression was analyzed by qPCR and ELISA. Surprisingly, instead of being upregulated, the Epo mRNA level was reduced by 39–60% and the Epo protein level by 28–73% in the HIF-3 α siRNA-treated cells (Fig. 6c). This finding cannot be due to non-specific effects on the HIF-1 α and HIF-2 α levels, as the reduction was obtained with similar efficiency with the 3 α _b and 3 α _c siRNA oligos, which had no unspecific effects on HIF-1 α or HIF-2 α (Fig. 6a). To study this finding further, we analyzed the effect of HIF-3 α siRNA on the expression of several other known HIF target

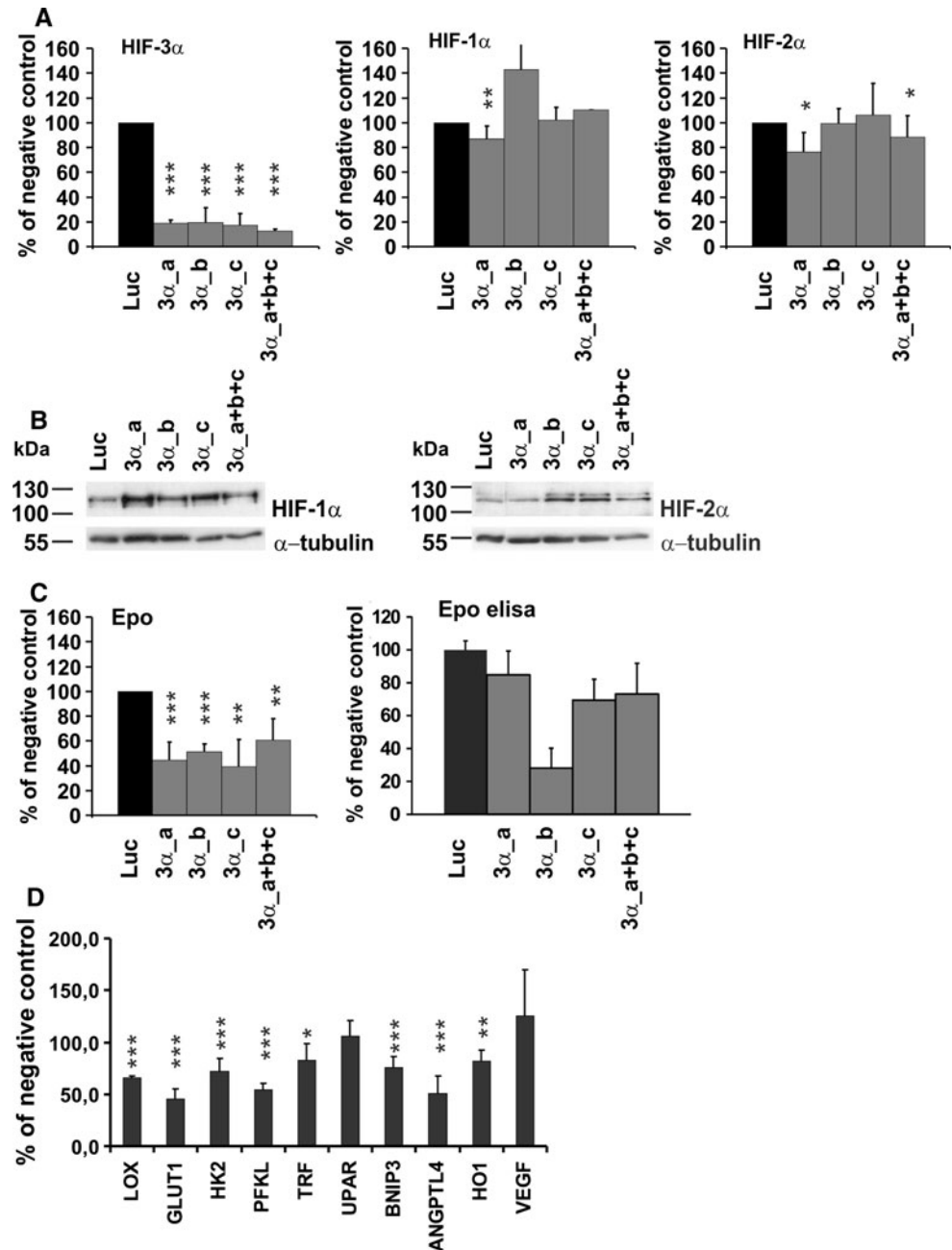
genes. The mRNA expression levels of Lox, Glut1, Hk2, Pfk1, Trf, Bnip3, AngPTL4 and HO1 were likewise significantly downregulated by about 18–54%, while those of uPAR and VEGF were not affected (Fig. 6d).

Certain HIF-3 α variants upregulate selected HIF target genes when HIF- β is not a limiting factor

The above data suggest that the long HIF-3 α variants may be required for maximal activation of certain HIF target genes in hypoxia. We analyzed this possibility by first cotransfecting Hep3B cells with the expression plasmids for each of the long HIF-3 α variants together with the HIF- β plasmid, i.e., under conditions where HIF- β should not be a limiting factor. The cells were cultured for 48 h in normoxia followed by 90 min in hypoxia and the mRNA levels of the HIF target genes Epo, AngPTL4, Pfk1, Glut1, HO-1 and VEGF were determined by qPCR (Fig. 7a). These genes were selected for analysis as knock-down of HIF-3 α had the strongest downregulating effect on the expression levels of Epo, AngPTL4, Pfk1 and Glut1, an intermediate effect on HO-1 and no effect on VEGF (Fig. 6d). The HIF-3 α 1, HIF-3 α 2 and HIF-3 α 9 variants gave about a four to six fold increase in the hypoxic induction of the Epo mRNA level, while the HIF-3 α 7 and HIF-3 α 8 variants gave about a two to three fold increase (Fig. 7a). The AngPTL4 mRNA level was increased by about 1.5- to 2-fold with four of the variants, and the Glut1 mRNA level by up to about 1.4-fold with three variants, while none of the variants had any inductive effect on the Pfk1, HO-1 or VEGF mRNA levels (Fig. 7a).

We next studied the effect of the overexpressed HIF-3 α variants on these same HIF target genes in the absence of

Fig. 6 siRNA knock-down of endogenous HIF-3 α leads to downregulation of many HIF target genes. Hep3B cells were transfected with a negative control siRNA (Luc, pGL2 luciferase control duplex siRNA) or with HIF-3 α siRNAs (three independent siRNAs a, b, c alone or in combination) twice at an interval of 24 h and cultured in 1% oxygen for 24 h after the second transfection. The expression of HIF-3 α (all variants), HIF-1 α , HIF-2 α (a) and Epo (c) at the mRNA level was analyzed by qPCR and the expression of HIF-1 α and HIF-2 α (b) and Epo (c) at the protein level by Western blotting and ELISA, respectively. The effect of the HIF-3 α siRNA on selected HIF target genes was analyzed by qPCR (d). The data represent means \pm SD from at least four independent experiments. * p < 0.05, ** p < 0.01, *** p < 0.001. In the case of the Epo ELISA analysis, the data represent the results of a typical experiment



overexpressed HIF- β , i.e., under conditions where the amount of endogenous HIF- β should become limiting. Hep3B cells were transfected with the HIF-3 α long variant plasmids, the cells were cultured for 24 h in normoxia followed by 24 h in hypoxia, and the mRNA levels of the above genes were determined by qPCR (Fig. 7b). The expression level of the Epo mRNA was increased by about 1.3-fold with the HIF-3 α 8 and HIF-3 α 9 variants and unaffected with the other variants (Fig. 7b). The level of AngPTL4 mRNA was downregulated by about twofold with overexpression of the HIF-3 α 1, HIF-3 α 8 and HIF-3 α 9

variants, and HIF-3 α 7 also showed a trend of downregulation, while overexpression of HIF-3 α 2 seemed to have a slight inductive effect (Fig. 7b). The Glut1 mRNA level was in general not affected by the HIF-3 α variants, except for HIF-3 α 9, which caused a small, but statistically significant reduction (Fig. 7b). In the case of the Pfk1 mRNA, all the long HIF-3 α variants showed a trend for downregulation, about a twofold reduction being obtained with the HIF-3 α 2, HIF-3 α 8 and HIF-3 α 9 variants (Fig. 7b). None of the HIF-3 α variants had a statistically significant effect on the HO-1 and VEGF mRNA levels (Fig. 7b).

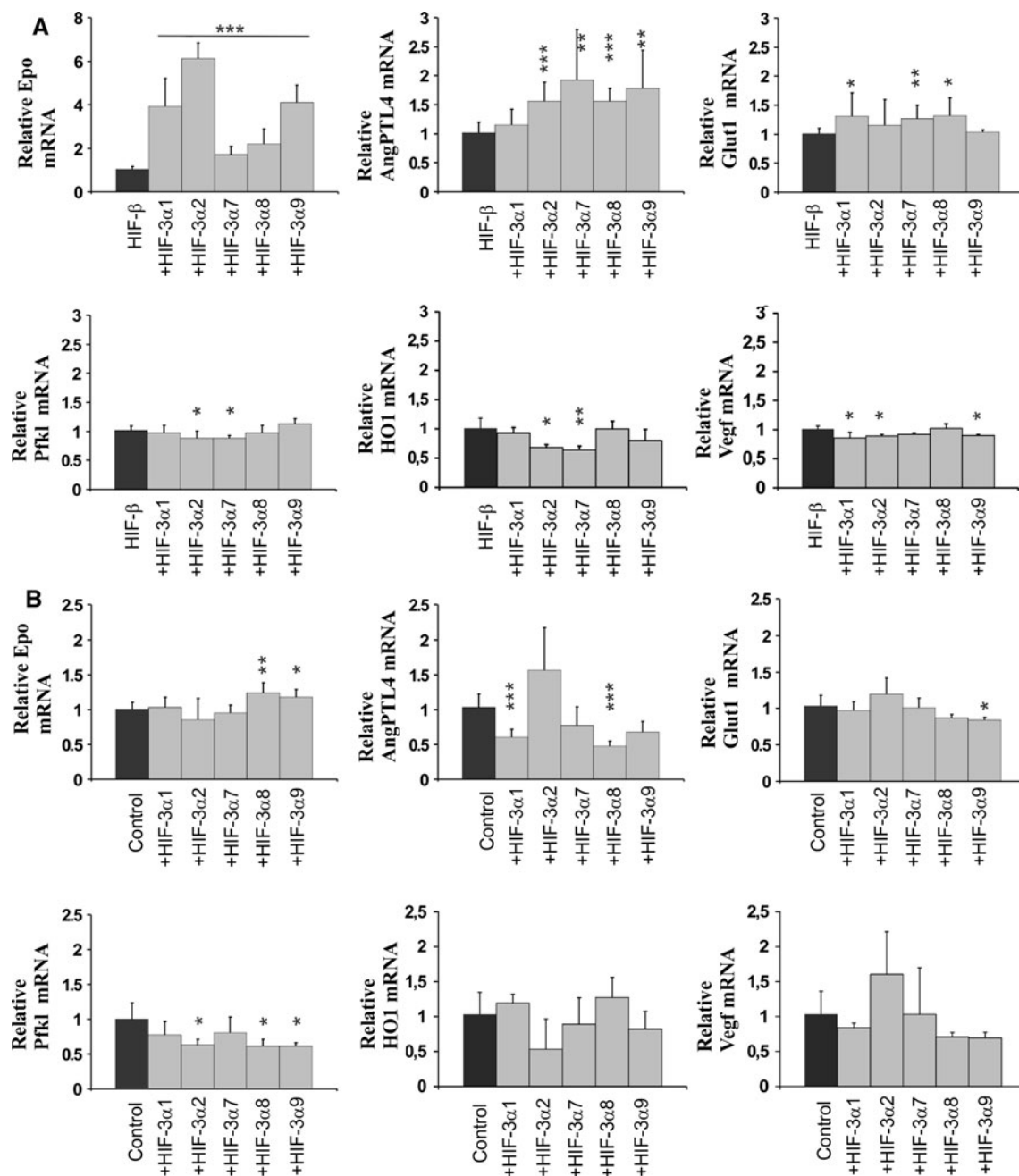


Fig. 7 Certain long HIF-3 α variants upregulate HIF target genes when HIF- β is not limiting. Hep3B cells were transfected with a plasmid encoding the full-length long HIF-3 α variants with (a) or without (b) HIF- β . HIF-3 α 4 was excluded here as the aim was to study the transactivation potential of HIF-3 α variants. The cells were cultured for 48 h in normoxia followed by 90 min in 1% oxygen

(a) or for 24 h in normoxia followed by 24 h in 1% oxygen (b). The expression levels of the EPO, ANGPTL4, GLUT1, PFKL, HO-1 and VEGF mRNAs were analyzed by qPCR. The data represent means \pm SD from at least three independent experiments. * p < 0.05, ** p < 0.01, *** p < 0.001

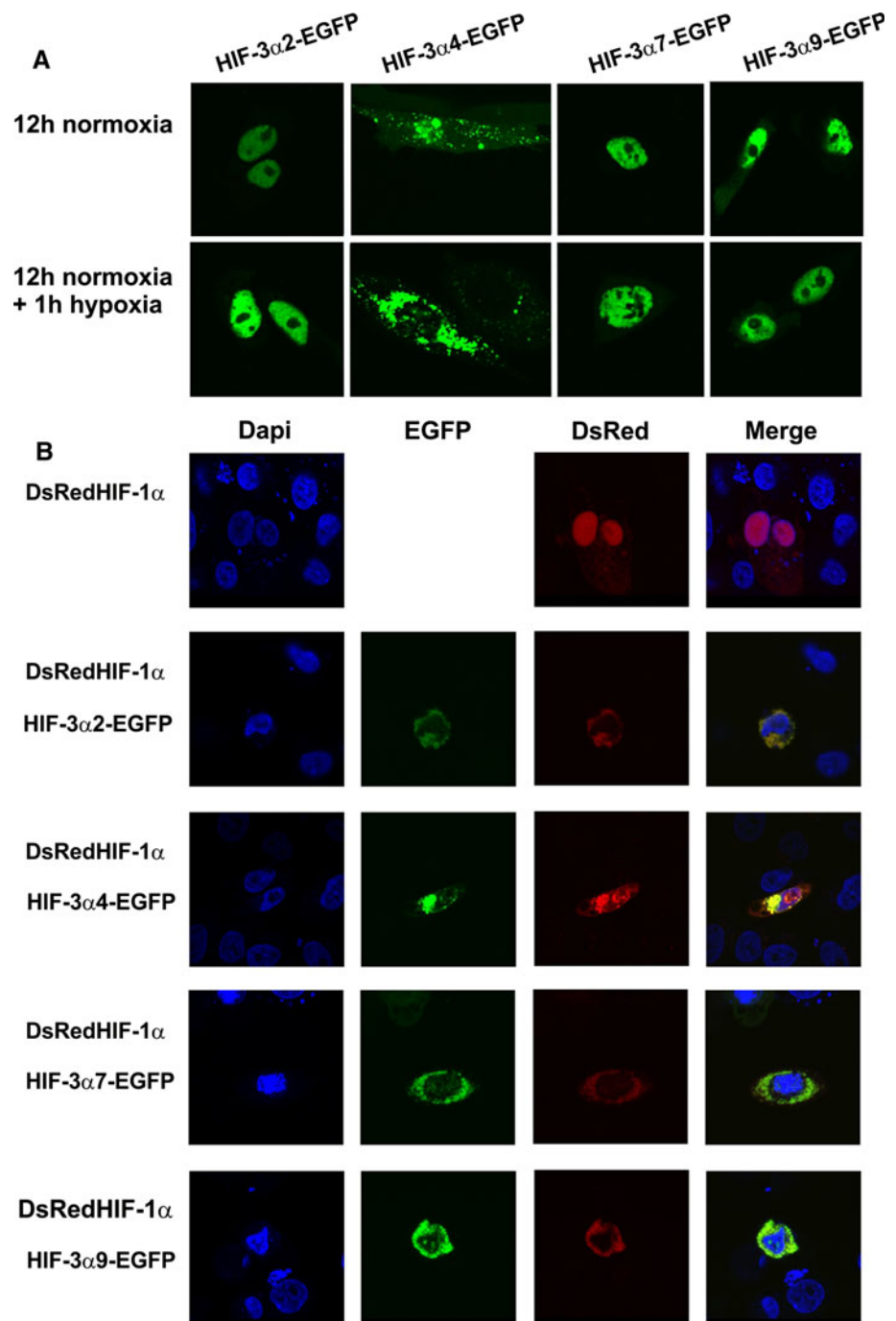
Cellular localization of overexpressed HIF-3 α variants

ChoK1 cells were transfected with EGFP-labeled long HIF-3 α variants 2, 7, and 9, and the short variant HIF-3 α 4 and the cells were cultured for 12 h in normoxia. The long variants accumulated in the nucleus, a similar localization also being observed when culturing of the cells was

extended by means of a 1-h exposure to hypoxia (Fig. 8a). In contrast, HIF-3 α 4 was localized in the cytoplasm and perinuclear regions and did not translocate into the nucleus even in hypoxia (Fig. 8a).

We next analyzed the localization of HIF-1 α and the above HIF-3 α variants when coexpressed in hypoxic cells. ChoK1 cells were transfected with DsRed-labeled HIF-1 α

Fig. 8 Confocal microscopy analysis of the cellular localization of overexpressed HIF-3 α variants. **a** ChoK1 cells were transfected with plasmids encoding EGFP-labeled HIF-3 α variants 2, 4, 7, or 9 and cultured in normoxia for 12 h (upper panel) followed by 1 h in 1% oxygen (lower panel). **b** ChoK1 cells were cotransfected with plasmids encoding EGFP-labeled HIF-3 α variants 2, 4, 7, or 9 and DsRed-labeled HIF-1 α and cultured for 24 h in normoxia followed by 8 h in 1% oxygen and stained with DAPI after fixation



with or without the EGFP-labeled HIF-3 α variants 2, 4, 7, and 9, and the cells were cultured for 24 h in normoxia followed by 8 h in 1% O₂. When the DsRed-labeled HIF-1 α was expressed alone, a nuclear localization was observed after the 8 h hypoxia exposure (Fig. 8b). When coexpressed together with any of the four HIF-3 α variants, the localization of the HIF-1 α and HIF-3 α polypeptides remained cytoplasmic and no accumulation into the nucleus was observed (Fig. 8b). Our interaction studies showed that all the HIF-3 α variants can form a complex

with HIF-1 α (Fig. 3) and the data obtained here indicate that the HIF-1 α /HIF-3 α complexes formed are not capable of nuclear translocation (Fig. 8b).

Discussion

We set out here to investigate the roles of the human HIF-3 α variants in the regulation of the hypoxia response. Our present data demonstrate that HIF-P4H-2 and HIF-P4H-3

hydroxylate the HIF-3 α ODDD and target it for proteasomal degradation with an efficiency at least similar to that of the HIF-1 α ODDD, if not better, while HIF-P4H-1 seemed to act somewhat less efficiently on the HIF-3 α ODDD than on the HIF-1 α ODDD. HIF-P4H-1 and HIF-P4H-3 have been shown to hydroxylate a 20-residue synthetic peptide representing the HIF-3 α hydroxylation site with identical efficiency [11], and thus the less efficient hydroxylation of the HIF-3 α ODDD by HIF-P4H-1 may be attributed to neighboring sequences or conformational effects. Importantly, our results also show that the HIF-3 α ODDD is degraded in a HIF-P4H-2-dependent manner when the ODDD is present in a full-length HIF-3 α polypeptide, in this case HIF-3 α 1 or HIF-3 α 2. In a previous report the full-length human HIF-3 α 1 as produced in an in vitro transcription-translation system was said to be ubiquitinated less robustly than the HIF-3 α ODDD after treatment with a HIF-P4H-enriched extract from rabbit reticulocyte lysate [26], while HIF-3 α 2 was said not to be regulated in an oxygen-dependent manner in Caki-1 or HeLa cells at all [32]. Our data clearly indicate, however, that the full-length HIF-3 α polypeptides, including HIF-3 α 2, are targeted for proteasomal degradation in a HIF-P4H-dependent and thus also oxygen-dependent manner.

In order to understand the potential effects of the human HIF-3 α variants on the hypoxia response, we studied their capacity to form complexes with HIF- β , HIF-1 α and HIF-2 α . The in vitro binding studies showed that all the HIF-3 α variants were able to form a complex with HIF-1 α and HIF-2 α . Furthermore, the coimmunoprecipitation studies showed that the HIF-3 α variants generally associated with HIF-1 α and HIF-2 α as efficiently as did HIF- β , and with HIF- β as efficiently as did HIF-1 α . The only exceptions were the undetectable formation of a HIF-3 α 2/HIF-2 α complex, and the particularly effective formation of a HIF-3 α 4/HIF-2 α complex. The interaction of the HIF-3 α variants 2, 4, 7, and 9 with HIF-1 α was further verified in confocal microscopy studies, where overexpression of these HIF-3 α polypeptides together with HIF-1 α was found to inhibit translocation of HIF-1 α and the long HIF-3 α variants 2, 7, and 9 into the nucleus. The subcellular localization of the short HIF-3 α 4 variant was cytoplasmic irrespective of the oxygen concentration and this variant also effectively inhibited the nuclear transport of HIF-1 α in hypoxia.

Previous analyses of the effects of HIF-3 α on the hypoxia response have mainly been carried out by studying the effects on HRE reporters. It has been shown that dimers formed between the mouse HIF-3 α or human HIF-3 α 1 and HIF- β can induce an HRE reporter, but much more weakly than the HIF-1 α /HIF- β or HIF-2 α /HIF- β dimers [21, 22]. When HIF- β is a limiting factor, however, these HIF-3 α variants have been shown to suppress the activation

potential of HIF-1 and HIF-2 [21, 22]. In the present study we obtained at most a 2.5-fold induction of an HRE reporter with the long HIF-3 α variants under conditions where overexpression of the HIF-1 α and HIF-2 α polypeptides resulted in a 14- to 18-fold induction. Furthermore, about a twofold induction of the HRE reporter was obtained even with the short HIF-3 α 4 variant, which does not contain any known transactivation domain, and therefore the inductive potential of the long HIF-3 α variants with respect to the HRE reporter must be at least very low, if it exists at all. Our results agree with those of previous studies in that all the HIF-3 α variants were found to be effective in inhibiting HIF-1 and HIF-2-mediated HRE-reporter induction in overexpression experiments where HIF- β is likely to be limiting, in addition to which the inhibiting effect was much stronger towards HIF-1 α than towards HIF-2 α , which agrees with the results of the coimmunoprecipitation studies, which generally showed a stronger interaction of the HIF-3 α variants with HIF-1 α than with HIF-2 α . One exception to this was HIF-3 α 4, which seemed to interact particularly strongly with HIF-2 α in the coimmunoprecipitation experiments but still exerted a stronger inhibitory effect on HIF-1 α than on HIF-2 α .

In the light of the data obtained in our HRE reporter experiments, the results of the HIF-3 α siRNA experiments were highly unexpected. The expectation was that knock-down of the endogenous HIF-3 α variants would lead to increased expression of the HIF target genes, but instead it did not have an inductive effect on any of the HIF target genes studied and even significantly reduced the expression levels of certain target genes, especially those of Epo, AngPTL4, Glut1 and Pfk1, where the reduction was 50–60%. Overexpression experiments showed that the HIF-3 α 1, HIF-3 α 2 and HIF-3 α 9 variants induced the Epo mRNA expression level about four to six fold and the HIF-3 α 7 and HIF-3 α 8 variants about two to three fold when HIF- β was not a limiting factor. Under the same conditions the inductive effects of the HIF-3 α variants on AngPTL4 were weaker, up to about twofold, and those on Glut1 less than 1.5-fold, while no inductive effect was detected on Pfk1. As the siRNA experiments were designed so that all the HIF-3 α variants were targeted for knock-down simultaneously, the effects of the individual HIF-3 α variants on selected HIF target genes were studied further in the overexpression experiments. Their results indicate that these target genes may have specific response elements of varying strengths for the individual HIF-3 α variants. In the case of the Epo gene, overexpression of HIF-3 α 1, HIF-3 α 2 and HIF-3 α 9 alone was enough to exert a strong inductive effect and overexpression of HIF-3 α 7 and HIF-3 α 8 alone could exert a somewhat weaker inductive effect, while in the case of the AngPTL4 gene overexpression of HIF-3 α 2, HIF-3 α 7, HIF-3 α 8 and HIF-3 α 9 alone was enough to give a

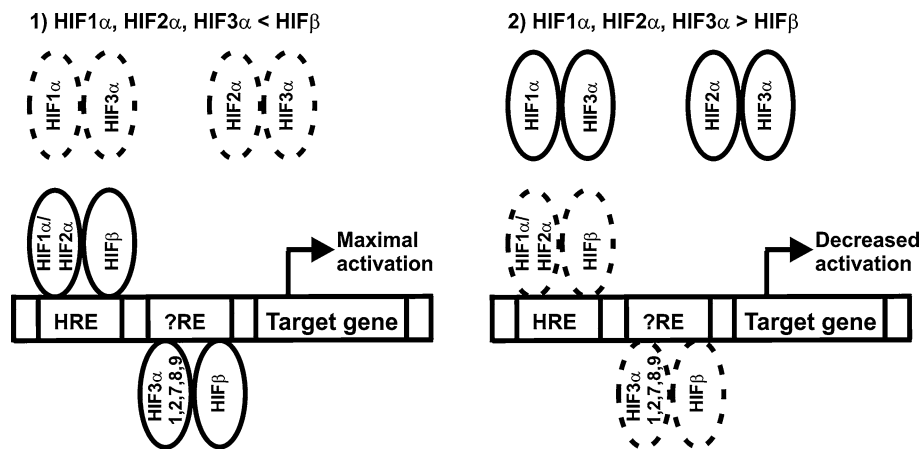


Fig. 9 Schematic representation of the roles of long HIF-3 α variants in the hypoxia response. **a** When HIF- β is not limiting, HIF-1 α , HIF-2 α , and the long HIF-3 α variants mainly associate with HIF- β and activate hypoxia-inducible target genes. Our data suggest that the long HIF-3 α variants are required for maximal activation of certain HIF target genes and are likely to have target elements distinct from the canonical HRE response element. **b** When the amount of HIF- β is

limiting, the long HIF-3 α variants associate with HIF-1 α and HIF-2 α leading to decreased activation of HIF target genes. The short variant HIF-3 α 4, which is not depicted in the scheme, forms inactive complexes with HIF-1 α , HIF-2 α and HIF- β and causes downregulation of HIF target genes independent on whether HIF- β is non-limiting or limiting. Formation of these complexes is also dependent on the relative amounts of HIF-3 α 4, HIF-1 α , HIF-2 α and HIF- β

significant inductive effect and in the case of the *Glut1* gene the same was true of the expression of HIF-3 α 1, HIF-3 α 7, HIF-3 α 8 alone. On the other hand, induction of *Pfk1* and a stronger induction of *AngPTL4* and *Glut1* is likely to require the concerted action of several HIF-3 α variants. Our data also show that the effects of the HIF-3 α variants differ in a variant and target gene-dependent manner under conditions where the amount of HIF- β is likely to be limiting. Certain HIF-3 α variants had a minor effect in increasing the *Epo* mRNA level, a reducing effect on the *AngPTL4* and *Pfk1* mRNA levels and little effect on *Glut1*.

Altogether, our data indicate that the HIF-3 α variants are likely to have more versatile and specific roles in the regulation of the hypoxia-responsive genes than has been anticipated previously (Fig. 9). When HIF- β is not limiting, HIF-1 α , HIF-2 α , and the long HIF-3 α variants mainly associate with HIF- β and activate hypoxia-inducible target genes, the long HIF-3 α variants being required for maximal activation of a subset of HIF target genes. As the inductive potential of the long HIF-3 α variants with respect to an HRE reporter was very low, if it existed at all, the molecular mechanism involved most likely includes binding of the HIF-3 α /HIF- β dimer to a response element that is distinct from the canonical HRE element recognized by HIF-1 and HIF-2 and which is only present in a subset of HIF-1 and HIF-2 regulated genes. When the amount of HIF- β is limiting, the long HIF-3 α variants associate with HIF-1 α and HIF-2 α leading to decreased activation of HIF target genes. The short variant HIF-3 α 4 forms inactive complexes with HIF-1 α , HIF-2 α and HIF- β and causes downregulation of HIF target genes independent on whether HIF- β is non-limiting or limiting. Formation of these

complexes is also dependent on the relative amounts of HIF-3 α 4, HIF-1 α , HIF-2 α and HIF- β . As the HIF-3 α variants themselves are also HIF-1 targets, and as the expression levels of individual HIF-3 α variants vary in different cell types and tissues [27, 32], their contributions to the hypoxia response in cellulo and in vivo will be an important topic for further studies. It is also noteworthy that several other factors can modulate the expression of hypoxia-inducible genes. For example, *Bach1* has been shown to downregulate the hypoxic induction of *HO-1* in certain cell types, whereas in some other cell types *HO-1* is clearly hypoxia-inducible in a HIF-1-dependent manner [37, 38]. Whether HIF-3 α can affect the functions of such modulating factors (or vice versa) remains to be studied.

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