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## THE HUMAN HYPOXIA-INDUCIBLE FACTOR (HIF)-3 $\alpha$ GENE IS A HIF-1 TARGET GENE AND MAY MODULATE HYPOXIC GENE INDUCTION

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**Running title:** The human HIF-3 $\alpha$  gene is a target of HIF-1

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**Abbreviations used:** ARNT, arylhydrocarbon receptor nuclear translocator; bHLH, basic helix-loop-helix (domain); ChIP, chromatin immunoprecipitation; CTAD, C-terminal transactivation domain; DMEM, Dulbecco's modified eagle medium; DP, 2,2'dipyridyl; DMOG, dimethylxalylglycine; GFP, green fluorescence protein; HIF, hypoxia-inducible factor; HRE, hypoxia-responsive element; IPAS, inhibitory PAS domain protein; NTAD, N-terminal transactivation domain; ODD, oxygen-dependent degradation domain; PAS, per arnt sim (domain); PHD, prolyl hydroxylase (domain) protein; RT-PCR, reverse transcriptase polymerase chain reaction; siRNA, small interfering RNA; pVHL, von Hippel-Lindau tumor suppressor protein

## SYNOPSIS

Hypoxia-inducible factor (HIF)-3 $\alpha$  is the third member of the HIF transcription factor family. Whereas HIF-1 $\alpha$  and -2 $\alpha$  play critical roles in the cellular and systemic adaptation to hypoxia, little is known about the regulation and function of HIF-3 $\alpha$ . At least five different splice variants may be expressed from the human HIF-3 $\alpha$  locus, which are suggested to exert primarily negative regulatory effects on hypoxic gene induction. Here we report that hypoxia induces the human HIF-3 $\alpha$  gene at the transcriptional level in a HIF-1-dependent manner. HIF-3 $\alpha$ 2 and HIF-3 $\alpha$ 4 transcripts, the HIF-3 $\alpha$  splice variants expressed in Caki-1 renal carcinoma cells, rapidly increased after exposure to hypoxia or chemical hypoxia mimetics. SiRNA-mediated HIF- $\alpha$  knock-down demonstrated that HIF-3 $\alpha$  is a specific target gene of HIF-1 $\alpha$ , but not affected by HIF-2 $\alpha$  knock-down. In contrast to HIF-1 $\alpha$  and HIF-2 $\alpha$ , HIF-3 $\alpha$  is not regulated at the level of protein stability. HIF-3 $\alpha$  protein could be detected under normoxia in cytoplasm and nuclei, but increased under hypoxic conditions. Promoter analyses and chromatin immunoprecipitation experiments localized a functional hypoxia-responsive element 5' to the transcriptional start of HIF-3 $\alpha$ 2. SiRNA-mediated knock-down of HIF-3 $\alpha$  increased transactivation of a HIF-driven reporter construct and mRNA expression of lysyl oxidase. Immunohistochemistry revealed an overlap of HIF-1 $\alpha$ -positive and HIF-3 $\alpha$ -positive areas in human renal cell carcinomas. These findings shed light on a novel aspect of HIF-3 $\alpha$  as a HIF-1 target gene and point to a possible role as a modulator of hypoxic gene induction.

**Key words:** transcriptional regulation, hypoxic response, promoter, RNA interference, renal carcinoma

## INTRODUCTION

Of the three hypoxia-inducible transcription factor (HIF) family members identified in mammals so far, HIF-3 is the least characterized and understood. HIF-1 and -2 are heterodimeric transcription factors which mediate the systemic and cellular response to hypoxia and consist of two basic helix-loop-helix (bHLH) PAS (Per Arnt Sim) domain proteins, of which the  $\alpha$ -subunit is oxygen-regulated and the  $\beta$ -subunit constitutively expressed (for review see [1]). HIF- $\beta$  is identical to the arylhydrocarbon receptor nuclear translocator (ARNT) and thus also part of transcription factor complexes involved in the xenobiotic response [2]. Two of the alternative  $\alpha$ -subunits, HIF-1 $\alpha$  and HIF-2 $\alpha$ , transactivate many functionally heterogeneous genes involved e.g. in anaerobic glucose metabolism, erythropoiesis and angiogenesis.

HIF-1 $\alpha$  and HIF-2 $\alpha$  show significant structural homology and are primarily regulated at the protein level by oxygen-dependent destabilization, which is preceded by enzymatic hydroxylation of two conserved prolyl residues in their oxygen-dependent degradation domains. At least three prolyl hydroxylase domain proteins (PHD1-3) have been identified which modify the HIF- $\alpha$  subunits using dioxygen and 2-oxoglutarate as co-substrates. This modification enables binding of the von Hippel-Lindau tumor suppressor protein (pVHL), which targets the HIF- $\alpha$  subunits for proteasomal degradation. Under hypoxia, the HIF- $\alpha$  subunits are thus stabilized, dimerize with HIF- $\beta$ , translocate to the nucleus and transactivate their target genes. In addition to regulation of protein stability the activity of HIF-1 $\alpha$  is repressed in an oxygen-dependent manner by the asparagyl hydroxylase factor inhibiting HIF-1 (FIH-1) (for review see [1]). The vast majority of HIF target genes is regulated by HIF-1 $\alpha$ , whereas exclusively HIF-2 $\alpha$ -dependent genes are scarce and cell type-dependent [3-6].

In 1998 a third HIF- $\alpha$  isoform was identified which exhibits about 55% amino acid sequence identity with HIF-1 $\alpha$  and HIF-2 $\alpha$  in its bHLH PAS domain [7]. It also contained an oxygen-dependent degradation domain (ODD) and a N-terminal transactivation domain (NTAD), similar to HIF-1 $\alpha$  and HIF-2 $\alpha$ , but no CTAD. In 2001 the murine inhibitory PAS domain protein (IPAS) was shown to be a repressor of HIF-dependent gene induction by virtue of its ability to bind to HIF-1 $\alpha$  [8]. Subsequently, IPAS was demonstrated to be a splice variant originating from the murine HIF-3 $\alpha$  locus. Highly expressed in the mouse brain and eye hypoxic IPAS induction impairs upregulation of vascular endothelial growth factor (VEGF) activity in the avascular cornea during sleep and thereby prevents angiogenesis [8]. Interestingly IPAS, but not HIF-3 $\alpha$ , is upregulated under hypoxia by differential splicing and transcriptional induction [9, 10].

The human HIF-3 $\alpha$  locus may give rise to at least five alternatively spliced transcripts, of which three (HIF-3 $\alpha$ 1-3) contain the ODD and NTAD but differ in their C-terminal and N-terminal ends, whereas the other splice variants lack the complete C-terminal half of the protein including ODD and NTAD [11]. Moreover, HIF-3 $\alpha$ 1-3 differ by alternative first exons resulting in different translational start sites. HIF-3 $\alpha$ 2 thereby lacks most of the DNA binding bHLH domain. Functional discrimination of the HIF-3 $\alpha$  splice variants are impaired by the lack of cell lines expressing HIF-3 $\alpha$  at high levels and of isoform-specific antibodies.

Interestingly, the human IPAS orthologue HIF-3 $\alpha$ 4 was detected in the kidney and shown to be reduced in renal cell carcinomas [12]. Since its downregulation may enhance hypoxic gene induction in kidney tumors, this effect may contribute to tumorigenesis [12].

To gain insight into the function and regulation of HIF-3 $\alpha$ , we screened a number of cell types and identified renal carcinoma Caki-1 cells as a cell line with high HIF-3 $\alpha$  expression. We found that hypoxia induces different HIF-3 $\alpha$  splice variants at the transcriptional level,

mediated by HIF-1 $\alpha$  and that HIF-3 $\alpha$  may exert a modulating effect on specific HIF target genes.

## MATERIALS AND METHODS

**Cell culture:** Caki-1 renal carcinoma cells, Hep3B and HepG2 hepatoma cells, HeLa cervical cancer cells, Kelly neuroblastoma, HT1080 fibrosarcoma, and HEK293 human embryonic kidney cells were purchased from the ATCC or the DSMZ (Braunschweig, Germany). The VHL-deficient and -reconstituted (+VHL) renal carcinoma cell lines 786-0 and RCC4 were provided by P. Ratcliffe (Oxford, U.K.) and P. Maxwell (London, U.K.), and HKC-8 renal tubular cells by L. Racusen (Baltimore, MD, USA). Carcinoma cells were grown in DMEM, 1.0 g glucose/L, 10% fetal bovine serum and antibiotics. Human primary renal tubular epithelial (hPT) cells were prepared from tumor nephrectomies and cultured as described before [13]. Hypoxic stimulation was accomplished by exposing cells to 1% oxygen in a Jouan IG750 incubator (Thermo Electron). For chemical HIF induction the iron chelator 2,2'-dipyridyl (DP; 100  $\mu$ M) and the iron-independent hydroxylase inhibitor dimethylxalylglycine (DMOG; 1 mM) were used.

**RNase protection assay (RPA):** Templates for RNA probes were generated by reverse transcriptase PCR using the primers listed in **Supplemental table 1**. Amplified fragments were cloned into the pSP72 vector (Promega). <sup>32</sup>P-labeled RNA probes were generated by *in vitro* transcription and RPAs were carried out as described [3, 14]. Quantification of signals was performed using a Phosphoimager (FujiBAS 2000, Japan) and the AIDA<sup>®</sup> image analysis software (Raytest, Germany).

**Reverse transcriptase-polymerase chain reaction (RT-PCR):** RNA was transcribed using random hexamer primers and M-MuLV Reverse Transcriptase (MBI Fermentas). For the differentiation of HIF-3 $\alpha$  splice variants, PCR reactions were carried out with isoform-specific primers (**Suppl. table 1**) and analyzed on 2% agarose gels. For quantification Real-time PCR was performed on an ABI-Prism7000 thermocycler with SYBR Green Master Mix (Applied Biosystems). Data were analyzed using the 2<sup>(- $\Delta\Delta$ Ct)</sup> method. Relative expression levels were calculated using  $\beta$ -actin mRNA as reference. Primers are listed in **Suppl. table 1**.

**SiRNA transfections:** Cells were transfected with HIF-1 $\alpha$  and HIF-2 $\alpha$  siRNAs at 50 nM as described before [3, 14]. SiRNAs directed against luciferase (luc) and green fluorescence protein (GFP) were used as controls. For HIF-3 $\alpha$  knock-down normal siRNAs (HIF-3 $\alpha'$  and HIF-3 $\alpha''$ ; Qiagen and Eurogentec) and stabilized Stealth<sup>®</sup> siRNAs (3 $\alpha$ 69 and 3 $\alpha$ 70; Invitrogen) and a GC content-matched Stealth<sup>®</sup> control siRNA (H) were used (see **Suppl. table 1**). All HIF-3 $\alpha$  siRNAs were targeting HIF-3 $\alpha$ 1-4.

**Promoter constructs and luciferase reporter assays:** Candidate HREs in the HIF-3 $\alpha$  genomic locus were localized using the MatInspector<sup>®</sup> software [15]. DNA fragments comprising the HREs were PCR-amplified and cloned into the pGL2basic vector (Promega). The functional HRE was inactivated by site-directed mutagenesis. Luciferase reporter assays were carried out as described [3]. The functional role of HIF-3 $\alpha$  was investigated by co-transfection of HIF-3 $\alpha$  siRNAs with a 6xHRE luciferase reporter (6xHRE-luc). Because of

the long half-life of HIF-3 $\alpha$  protein, cells were stimulated 32 h after transfection for further 16 h.

**Chromatin immunoprecipitation (ChIP):** Binding of HIF-1 to the candidate HREs of the HIF-3 $\alpha$  gene was demonstrated by ChIP analyses as described previously [16]. Cells were fixed with 1% formaldehyde, lysed by 1% SDS and sonicated. Samples were then diluted 10-fold, divided into two parts, and precipitated with anti-HIF-1 $\alpha$  rabbit polyclonal antibody (NB 100-449, Novus Biologicals) or normal rabbit serum and protein A agarose (Roche). Precipitated DNA was used for PCR with the primers listed in **Suppl. table 1**.

**Immunoblotting:** Protein expression of HIF- $\alpha$  subunits was analyzed as described [3, 14] with polyclonal anti-HIF-3 $\alpha$  (ab10134, Abcam), anti-HIF-1 $\alpha$  (NB100-449) or anti-HIF-2 $\alpha$  (NB100-122, Novus Biologicals) antibodies;  $\beta$ -actin (monoclonal antibody AC-74, Sigma) was used as loading control. X-ray films were scanned and signals quantified densitometrically using the AIDA<sup>®</sup> software.

**Tumor collection:** Tissue specimens were collected from total nephrectomy in patients with renal clear cell carcinoma [17]. All patients gave their informed consent. The study was approved by the local ethics committees. Tissue samples were immediately snap frozen after surgical removal or fixed overnight in 4% paraformaldehyde.

**Immunohistochemistry:** HIF-3 $\alpha$  and HIF-1 $\alpha$  protein was immunostained in human kidney tumor sections as described [17]. For immunofluorescence Alexa fluor<sup>®</sup> 488 labelled secondary antibodies were used.

**Statistical analysis:** Data are expressed as mean $\pm$ SD or mean $\pm$ SEM, as appropriate. Statistical analyses were carried out using a Stat-View software (Version 5.0 SAS Institute, USA). Comparisons among groups were made using ANOVA, with the correction of Bonferroni/Dunn's method, where applicable. P values of <0.05 were considered significant.

## RESULTS

**Expression and hypoxic induction of HIF-3 $\alpha$  splice variants in Caki-1 cells.** We first screened human cell lines for HIF-3 $\alpha$  expression by RNase protection assay (RPA) using a probe for the PAS-B domain of HIF-3 $\alpha$ , which is contained in all splice variants (**Fig. 1 A**). Among ten cell lines tested, Caki-1 renal carcinoma cells expressed HIF-3 $\alpha$  mRNA by far at the highest level, showing a moderate increase after stimulation with the hypoxia mimetic 2,2'-dipyridyl (DP; **Fig. 1 B**). Hep3B and HeLa cells expressed about 10% of HIF-3 $\alpha$  transcript levels found in Caki-1 cells. In VHL-deficient and VHL-reconstituted 786-0 and RCC4 renal carcinoma cells HIF-3 $\alpha$  was not detectable by RPA or RT-PCR (**Fig. 1 B** and data not shown). Therefore, we primarily used Caki-1 cells for subsequent experiments. Hypoxia (1% O<sub>2</sub>) and the hypoxia mimetics DP and DMOG stimulated HIF-3 $\alpha$  mRNA expression approximately 3-fold as determined by RPA (hypoxia 3.0 $\pm$ 0.9-fold, DP 3.9 $\pm$ 0.7-fold and DMOG 3.6 $\pm$ 0.3-fold; each p<0.05 vs. control, n=3-4; **Fig. 1 C**). Five splice variants have been reported and confirmed for the human HIF-3 $\alpha$  locus (**Fig. 1 A**). The ODD

containing transcripts for HIF-3 $\alpha$ 1, 2 and 3 differ structurally in terms of their transcriptional start sites thus allowing further differentiation. RT-PCR analyses identified a specific band for HIF-3 $\alpha$ 2, while those corresponding to HIF-3 $\alpha$ 1 and 3 were absent within the amplification range of up to 40 cycles (**Fig. 1 D**). HIF-3 $\alpha$ 6 was also absent (not shown).

Next we quantified mRNA levels of the HIF-3 $\alpha$  splice variants by RPA using two different probes directed against the oxygen-dependent degradation domain (ODD: for HIF-3 $\alpha$ 1, 2 and 3) and HIF-3 $\alpha$ 4, respectively (**Fig. 1 A and E**). After 4 and 16 h hypoxia, HIF-3 $\alpha$ ODD containing transcripts (here: HIF-3 $\alpha$ 2) and HIF-3 $\alpha$ 4 were clearly increased, reaching  $2.2\pm 0.2$ - and  $2.8\pm 0.4$ -fold, respectively (both  $p < 0.05$  vs. unstimulated controls,  $n=3$ ; **Fig. 1 F**). Although total HIF-3 $\alpha$  expression was markedly lower, a similar induction was observed in Hep3B cells (not shown). In addition, qRT-PCR analysis identified HIF-3 $\alpha$  transcripts in human primary renal tubular epithelial cells (hPT, passage 1-3) and in RNA samples from human normal kidney tissue obtained from tumor nephrectomies. In hPT cells total HIF-3 $\alpha$  mRNA expression was induced significantly by hypoxia and DP suggesting that HIF-3 $\alpha$  is expressed in tubular cells under physiological conditions and that its hypoxic induction may also be preserved in the kidney (**Fig. 1 G**).

**Hypoxic induction of HIF-3 $\alpha$  requires HIF-1 $\alpha$ .** A time-course analysis of HIF-3 $\alpha$  mRNA induction following DP-stimulation revealed kinetics similar to those of known HIF-1 target genes such as angiopoietin-like 4 (ANGPTL4 [18]; **Fig. 2 A**). We therefore hypothesized that HIF-3 $\alpha$  might be a direct transcriptional target of HIF-1 and examined the effects of siRNAs directed against HIF-1 $\alpha$  and HIF-2 $\alpha$  on HIF-3 $\alpha$  mRNA expression. Knock-down of HIF-1 $\alpha$ , but not of HIF-2 $\alpha$ , significantly reduced the induction of ODD containing transcripts and HIF-3 $\alpha$ 4 indicating that HIF-3 $\alpha$  requires HIF-1 $\alpha$  for transcriptional upregulation (**Fig. 2 B**). HIF- $\alpha$  immunoblotting confirmed the efficiency of the HIF- $\alpha$  knock-down (**Fig. 2 C**). To address the possibility that HIF-1 might indirectly increase HIF-3 $\alpha$  mRNA levels through the induction of a mediator, we investigated the effect of HIF- $\alpha$  knock-down after short-term DP-stimulation. After 4 h DP-stimulation, HIF-1 $\alpha$  knock-down already reduced the stimulated mRNA levels of both splice variants by about 30%, and after 8 h by 40% ( $n=3$ ), suggesting that HIF-3 $\alpha$  may be a direct target of HIF-1 (**Fig. 2 D**). In unstimulated normoxic Caki-1 cells, i.e. in the absence of HIF-1 $\alpha$  protein, HIF-1 $\alpha$  siRNA transfection did not reduce HIF-3 $\alpha$  mRNA expression, as determined by the use of qRT-PCR for reasons of sensitivity (**Fig. 2 E**).

**HIF-1 $\alpha$  binds to regulatory elements in the human HIF-3 $\alpha$  gene.** Next we searched the HIF-3 $\alpha$  genomic locus for consensus (R)CGTG/C HIF binding motifs by the use of the MatInspector<sup>®</sup> software (Genomatix, [15]). Screening 20 kbp of the 5' flanking region up to exon 2, we identified six candidate HIF binding sites (**Fig. 3 A** and **Supplemental table 2**). We then determined whether HIF-1 binds to these sequences using chromatin immunoprecipitation (ChIP) assays. Of three sets of PCR primers used, primer pair 2 yielded an amplified fragment when hypoxic cell lysates were immunoprecipitated with a HIF-1 $\alpha$  antibody, which indicated that HIF-1 binds to the HRE at nt 42496-42508 (termed 'c' in **Fig. 3 A**) in the HIF-3 $\alpha$  gene (**Fig. 3 B**).

**Hypoxic regulation of HIF-3 $\alpha$  promoter constructs.** The functionality of this HIF binding motif was corroborated in luciferase reporter assays. We cloned three different HIF-3 $\alpha$  promoter fragments into the pGL2basic vector to cover all candidate HREs and examined their hypoxic induction in different cell types (**Fig. 3 A and C**). Following DP-stimulation in Hep3B cells, luciferase activity of the reporter driven by the HIF-3 $\alpha$  promoter fragment nt

42365-43656 (seq.2-luc) increased 2.9-fold, whereas all other reporter constructs retained activation levels similar to the pGL2 control vector (1.8- to 1.9-fold). Identical results were obtained by co-transfection of a normoxically stable HIF-1 $\alpha$  triple mutant (mHIF-1 $\alpha$ TM) in HEK293 cells. Again, the seq.2-luc reporter responded more markedly than the other constructs including pGL2 control (16.9-fold vs. 5.0- to 5.6-fold), suggesting that the seq.2 contains a functional HIF binding motif. Thus, the motif 'c' was considered to play a critical role for the hypoxic induction of HIF-3 $\alpha$  mRNA.

The region spanning nt 42365-43656 (seq.2) was therefore further characterized. 3' terminal deletion of this promoter fragment from nt 42741 (seq.22) to nt 42484 (seq.23) blunted the hypoxic induction markedly (2.6 $\pm$ 0.5 vs. 1.5 $\pm$ 0.1-fold,  $p$ <0.01; **Fig. 3 D**). In contrast, deleting the 5' end from nt 42365 (seq.22) to 42484 (seq.25) did not affect the hypoxic response significantly (2.6 $\pm$ 0.5 vs. 2.3 $\pm$ 0.3-fold,  $p$ =n.s.), which suggested that a functional hypoxia-responsive sequence was located between nt 42484 and 42741. Mutagenesis of the HRE candidate motif 'c' at nt 42496-42508 from ACGTC to AGATC (seq.2mut) markedly reduced the hypoxic activation (3.0 $\pm$ 0.5 vs. 1.8 $\pm$ 0.3-fold,  $p$ <0.01; **Fig. 3 D**). These results further supported that the HRE at nt 42496-42508 upstream of exon 1b is critical for the hypoxic induction of the HIF-3 $\alpha$  promoter.

**Expression and subcellular localization of HIF-3 $\alpha$  protein.** The HIF-3 $\alpha$  antibody used in the present study was raised against a peptide within the region AA 300-400 of HIF-3 $\alpha$  and therefore predicted not to detect HIF-3 $\alpha$ 4. Thus, the band of approximately 60 kDa which was marked by the antibody in Caki-1 cells was considered to represent HIF-3 $\alpha$ 2 according to the results of the RNA analyses. In some experiments, at longer exposures a second, slower migrating band appeared, which may represent a different splice variant or modified HIF-3 $\alpha$ 2. In keeping with this notion pre-incubation of the antibody solution with the HIF-3 $\alpha$  peptide used for immunization eliminated both bands in immunoblot assays (data not shown).

Hypoxic induction of HIF-3 $\alpha$  was also observed at the protein level, albeit less strikingly than at the transcript level. In Caki-1 cells, hypoxia induced HIF-3 $\alpha$  protein 1.5 $\pm$ 0.2-fold and DP 1.2 $\pm$ 0.09-fold ( $n$ =4;  $p$ <0.05, **Fig. 4 A**). The differences between normoxic and stimulated cells may have been underestimated due to the method of quantification (scanning and densitometric analysis of x-ray films). In concordance with mRNA data, Caki-1 cells expressed HIF-3 $\alpha$  protein more abundantly than HeLa cells. HIF-3 $\alpha$  protein was also detectable in hPT cells (not shown). When 200  $\mu$ g protein were loaded, three hypoxia-inducible bands were marked by the antiserum.

In contrast to HIF-1 $\alpha$ , HIF-3 $\alpha$  did not accumulate during 2 h DP-stimulation, which indicates that HIF-1 $\alpha$ , but not HIF-3 $\alpha$ , is subject to oxygen-dependent protein destabilization, which is rapidly inhibited by DP (**Fig. 4 B**). Translational inhibition by cycloheximide revealed that HIF-3 $\alpha$  has a distinctly longer protein half-life (approximately 18 h) in Caki-1 cells than HIF-1 $\alpha$ , indicating that these two HIF- $\alpha$  family members are regulated by different molecular mechanisms (**Fig. 4 C**).

As observed at the mRNA level, siRNA-mediated knock-down of HIF-1 $\alpha$ , but not HIF-2 $\alpha$ , again notably offset the DP-stimulated increase in HIF-3 $\alpha$  protein (**Fig. 4 D**, for HIF-2 $\alpha$  protein knock-down see Fig. 2 C), which confirmed that HIF-3 $\alpha$  induction requires HIF-1 $\alpha$  activation. HIF-1 $\alpha$  knock-down reduced hypoxic HIF-3 $\alpha$  protein expression by 32 $\pm$ 17% ( $p$ <0.05,  $n$ =4), whereas HIF-2 $\alpha$  knock-down had no significant effect (102 $\pm$ 13% of luc siRNA-transfected DP-stimulated cells).

Immunofluorescence analysis was performed to determine the subcellular localization of HIF-3 $\alpha$  in Caki-1 cells (**Fig. 4 E**). HIF-3 $\alpha$  was found in both cytoplasm and nuclei under normoxia. Overnight exposure to hypoxia or DP enhanced in particular the nuclear signals as determined by co-staining of the nuclei with Hoechst dye.



**Effect of siRNA-mediated HIF-3 $\alpha$  knock-down on HIF-1 $\alpha$  activity.** HIF-3 $\alpha$  has been proposed to serve as a competitive inhibitor of other HIFs, notably HIF-1 $\alpha$ . Therefore we examined the effect of HIF-3 $\alpha$  knock-down on HIF-1-driven reporter gene induction in Caki-1 and Hep3B cells. Probably due to the long half-life of HIF-3 $\alpha$  protein, knock-down proved to be difficult. The first siRNA (HIF-3 $\alpha$ '') reduced HIF-3 $\alpha$  protein levels by 12-16% after 48 hours and the second (HIF-3 $\alpha$ '') by about 50% in both Caki-1 and Hep3B cells, whereas the expression of HIF-1 $\alpha$  protein was not affected (**Fig. 5 A and B**). In Hep3B cells cotransfection of siRNA HIF-3 $\alpha$ '' with a 6xHRE-luc reporter significantly increased DP-stimulated reporter activity in comparison with GFP control siRNA-transfected, DP-stimulated cells (**Fig. 5 C**). SiRNA HIF-3 $\alpha$ ' had no significant effect. We tried to improve knock-down efficiency with chemically stabilized Stealth<sup>®</sup> siRNAs. SiRNAs 3 $\alpha$ 69 and 3 $\alpha$ 70 did not significantly alter HIF-1 $\alpha$  or HIF-2 $\alpha$  protein induction (**Fig. 5 D**). SiRNA 3 $\alpha$ 69 reduced DP-stimulated HIF-3 $\alpha$ ODD transcript levels in Caki-1 cells to 66% $\pm$ 13 and HIF-3 $\alpha$ 4 transcript levels to 71 $\pm$ 3%, siRNA 3 $\alpha$ 70 HIF-3 $\alpha$ ODD mRNA to 32 $\pm$ 5% and HIF-3 $\alpha$ 4 mRNA to 50 $\pm$ 11% compared with control siRNA H as determined by RPA (n=3, **Fig. 5 E**). Using the more sensitive qRT-PCR analysis, total HIF-3 $\alpha$  knock-down amounted to 18% of DP-stimulated control values by the 3 $\alpha$ 69 and 7% by the 3 $\alpha$ 70 siRNA, respectively (**Fig. 5 E**, bar graph). HIF-2 $\alpha$  mRNA expression was reduced to 72 $\pm$ 5% (p<0.05) by the 3 $\alpha$ 69 siRNA and HIF-1 $\alpha$  mRNA to 76 $\pm$ 17% (n.s.) of controls by the 3 $\alpha$ 70 siRNA. In reporter assays in Caki-1 cells, DP-stimulated 6xHRE-driven luciferase activity significantly increased by 70 $\pm$ 16% in the presence of the siRNA 3 $\alpha$ 69 and by 110 $\pm$ 55% in the presence of the siRNA 3 $\alpha$ 70 (both p<0.05, n=6; **Fig. 5 F**). These data indicate that HIF-3 $\alpha$  splice variants in Hep3B and Caki-1 cells may exert inhibitory effects on HIF-1 $\alpha$ -dependent gene activation. The modest reduction of HIF-1 $\alpha$  and HIF-2 $\alpha$  mRNA may have been due to non-specific effects of siRNA transfection.

Searching for endogenous HIF target genes that were regulated by HIF-3 $\alpha$  in Caki-1 cells, we analyzed RNA samples of DP-stimulated and siRNA 3 $\alpha$ 69- or 3 $\alpha$ 70-transfected cells on Affymetrix microarrays and compared them with DP-stimulated control siRNA-transfected cells (data not shown). However, despite good RNA quality the reverse transcription and labelling process was obviously impaired by the Stealth<sup>®</sup> siRNAs, resulting in low detection calls. A high proportion of genes not related to the hypoxia response were altered by the two HIF-3 $\alpha$  siRNAs, which may suggest a high incidence of off-target effects. However, it cannot be excluded that the expression of genes other than HIF-1 $\alpha$  and HIF-2 $\alpha$  targets may be regulated by HIF-3 $\alpha$ , since HIF- $\beta$ /ARNT, with which HIF-3 $\alpha$  is considered to interact, is also part of other transcription factor complexes. In the microarray the HIF target genes *Lox*, *ETS1*, *IRS1*, *TOP2B* and *IGFBP3* appeared in the group of transcripts slightly upregulated by both HIF-3 $\alpha$  siRNAs, whereas *ANGPTL4*, *HIG2* and *EGLN2* (=PHD1) were among the downregulated genes. We investigated mRNA expression of (amongst others) *Lox*, *ANGPTL4*, *HIG2* as well as *VEGF-A* and *PDGF-B*, because these two genes were previously associated with IPAS/HIF-3 $\alpha$  function, in Caki-1 cells by the use of qRT-PCR. *Lox* was significantly induced by HIF-3 $\alpha$  knock-down and *ANGPTL4* reduced (**Fig. 5 G**). For the other genes consistent effects of the two HIF-3 $\alpha$  siRNA could not be confirmed.

**Immunohistochemical analyses of human renal cell carcinomas reveal co-localization of HIF-3 $\alpha$  and HIF-1 $\alpha$ .** To investigate whether HIF-3 $\alpha$  expression correlates with HIF-1 $\alpha$  protein accumulation *in vivo*, we analysed human renal cell carcinomas (RCCs), the majority of which are associated with inactivation of the *VHL* gene and thus characterized by constitutive HIF- $\alpha$  protein expression [17, 19]. Staining of semi-serially prepared RCC

sections with HIF-1 $\alpha$  and HIF-3 $\alpha$  antibodies confirmed HIF-3 $\alpha$  expression in 14 out of 15 kidney tumors, and comparison of the distribution patterns revealed co-localization of HIF-1 $\alpha$  and HIF-3 $\alpha$  (**Fig. 6** and **Suppl. fig. 1**). Finally, we analysed RNA from HIF-positive and HIF-negative tumors by RPA. In HIF-positive tumors the expression of HIF-3 $\alpha$ , both of ODD-containing splice variants and HIF-3 $\alpha$ 4, tended to be higher than in normal kidneys, whereas the expression level in HIF-negative tumors was similar to, or even lower than that in normal kidneys (**Fig. 7**). Altogether these observations indicate that HIF-3 $\alpha$  may also be induced by HIF-1 $\alpha$  *in vivo*. However, the lack of HIF-3 $\alpha$  expression observed in some HIF-1 $\alpha$  positive tumors and in the RCC cell lines *in vitro* hints at the contribution of other factors to basal HIF-3 $\alpha$  expression.

## DISCUSSION

In the present study we analysed the molecular mechanisms governing the hypoxic regulation of the human HIF-3 $\alpha$  gene in Caki-1 renal carcinoma cells, a cell line with high endogenous HIF-3 $\alpha$  expression. The HIF-3 $\alpha$  splice variants identified in Caki-1 cells were simultaneously and rapidly upregulated by hypoxia and DP. Induction kinetics and the effects of HIF-1 $\alpha$  knock-down indicated that, for both splice variants, transcriptional activation by HIF-1 $\alpha$  was the molecular mechanism underlying mRNA induction. In contrast, the alternatively spliced transcripts of the murine HIF-3 $\alpha$ /IPAS gene were shown to be regulated through two complementary mechanisms: transcriptional induction and enhanced alternative splicing [10, 20]. Notably, however, total HIF-3 $\alpha$  mRNA has been reported to be induced by systemic hypoxia in rat organs [21] as well as in A549 human lung cancer cells [22].

Three of the human HIF-3 $\alpha$  splice variants (HIF-3 $\alpha$ 1-3) encompass an ODD with a single prolyl hydroxylation motif predicting destabilization by molecular oxygen. However, in the context of the full length protein modulation of protein stability by the HIF-3 $\alpha$  ODD was greatly diminished [11]. Our results are in keeping with these data and show that protein stabilization is of limited, if any, significance for the hypoxic regulation of HIF-3 $\alpha$ .

ChIP analyses in conjunction with luciferase reporter assays served to pinpoint a functional hypoxia-responsive element in the human HIF-3 $\alpha$  gene. The sequence of this DNA element upstream of exon 1b was ACGTC and thus slightly different from the canonical HIF binding site RCGTG. However, the transcriptional induction of the murine IPAS gene was also shown to be mediated by a non-canonical novel hypoxia-responsive DNA element with no similarity to previously described HIF binding sites [10]. Inactivation of the hypoxia-responsive element by site-directed mutagenesis led to a reduction of promoter activation confirming its functional significance. However, we cannot exclude the possibility that other *cis*-active elements may contribute to the hypoxic regulation of HIF-3 $\alpha$ .

A previous study reported a decrease of HIF-3 $\alpha$ 4 mRNA in human renal carcinomas irrespective of the presence of functional pVHL [12]. We rather found a tendency for a co-localization of HIF-3 $\alpha$  and HIF-1 $\alpha$  protein in human renal carcinomas, in accordance with the notion of a transcriptional induction of HIF-3 $\alpha$  by HIF-1 $\alpha$ . However, there were HIF-1 $\alpha$  positive tumors lacking immunodetectable HIF-3 $\alpha$ . In addition, HIF-3 $\alpha$  mRNA was not detectable in the RCC cell lines RCC4 and 786-0. Instead, early passage primary human tubular epithelial cells (hPT) expressed considerable amounts of HIF-3 $\alpha$  (comparable to Caki-1 cells), which was hypoxia-inducible in a HIF-1-dependent manner (**Fig. 1 G** and data not shown). It should be noted that HIF-3 $\alpha$  mRNA was downregulated after passage 3 and also

reduced in Caki-1 cells after extensive passaging, which suggests that dedifferentiation processes may affect basal HIF-3 $\alpha$  expression.

To date the current knowledge on human HIF-3 $\alpha$  function is largely confined to the variant HIF-3 $\alpha$ 4, the human ortholog of murine IPAS. In contrast to IPAS, which inhibits HIF-1 $\alpha$  function by binding to HIF-1 $\alpha$ , human HIF-3 $\alpha$ 4 was demonstrated to bind to both HIF-1 $\alpha$  and ARNT [12, 23] and may thus also compete with other ARNT-dimerization partners, e.g. transcription factors involved in the xenobiotic response [2]. Therefore, the modulation of a large number of genes not associated with the hypoxic response, as they were detected in a microarray experiment (data not shown), do not necessarily reflect siRNA off-target effects, but may be related to functions of HIF-3 $\alpha$  outside of the HIF system.

In our attempt to identify HIF target genes whose induction was further enhanced by HIF-3 $\alpha$  knock-down, only Lox (lysyl oxidase) could be confirmed, indicating that the functional significance of HIF-3 $\alpha$  as a modulator of the hypoxic response is limited and may be target gene- and cell type-specific. Whether the unexpected reduction of hypoxic ANGPTL4 expression by HIF-3 $\alpha$  knock-down was indeed HIF-3 $\alpha$ -dependent remains to be clarified. As one of the HIF-1 $\alpha$  target genes with the highest induction amplitudes [14], ANGPTL4 is supposed to be highly sensitive towards even subtle alterations in HIF-1 $\alpha$  transcription and translation, as they may occur during long-term exposure of cells to siRNAs.

Reports on stimulatory effects of other HIF-3 $\alpha$  splice variants on hypoxic gene expression are rare [24, 25]. The majority of studies on HIF-3 $\alpha$  function, which were predominantly based on overexpression experiments, point towards inhibitory effects on HIF-dependent gene regulation. Thus, along with the PHDs and the hypoxia-inducible transcriptional repressor of HIF-dependent gene induction CITED2 [26], HIF-3 $\alpha$  may represent a further piece of a multipart system of feedback mechanisms which attenuate the hypoxic response and thereby may prevent detrimental effects of an unopposed, prolonged HIF activation. Interestingly, HIF-3 $\alpha$  is most highly expressed in the human placenta and the myocardium [11], tissues in which the knock-out of HIF-1 $\alpha$  or its modulators such as PHD2 and CITED2 has severe developmental consequences. This implies that for proper development and function of these organs a precisely tuned control of HIF activity may be critical. In keeping with this notion genetic deletion recently revealed the functional significance of HIF-3 $\alpha$  and its splice variant NEPAS for the regulation of post-natal remodelling in the heart and the lung [27]. It also became evident in that study that HIF-3 $\alpha$  splice variants do not act as global repressors of hypoxic gene induction, but may modulate specific target genes in individual cell types.

In conclusion, we identified a cell culture system suitable for the analysis of endogenous HIF-3 $\alpha$  and demonstrated that the human HIF-3 $\alpha$  gene is a transcriptional target of HIF-1 $\alpha$ . The contribution of HIF-3 $\alpha$  to gene regulation *in vivo* remains an important issue for further studies.

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limited and modulated by cell type-specifi

## FIGURE LEGENDS

### Fig. 1. mRNA expression and hypoxic regulation of HIF-3 $\alpha$ splice variants.

**A**, design of RPA probes generated to differentiate HIF-3 $\alpha$  splice variants (schematic representation, based on acc. no.: HIF-3 $\alpha$ 1 NM\_152794; HIF-3 $\alpha$ 2 NM\_022462; HIF-3 $\alpha$ 3 NM\_152795; HIF-3 $\alpha$ 4 AB118749; HIF-3 $\alpha$ 6 AK024095). **B**, expression of HIF-3 $\alpha$  mRNA in various cell lines as determined by RPA. U6 small nuclear RNA (U6sn) served as loading control; where indicated cells were stimulated with DP (100  $\mu$ M) for 16 h. **C**, induction of total HIF-3 $\alpha$  mRNA after 16 h exposure to 1% O<sub>2</sub> (hyp), DP or DMOG (DG, 1 mM). **D**, RT-PCR analysis for the three PAS-B domain-containing HIF-3 $\alpha$  splice variants (HIF-3 $\alpha$ 1, 2 and 3) revealed that HIF-3 $\alpha$ 2 is the predominant HIF-3 $\alpha$  transcript in Caki-1 cells. Genomic DNA served as positive control for HIF-3 $\alpha$ 1 and the cDNA clone acc. no. BC026308 as control for HIF-3 $\alpha$ 3. **E**, mRNA induction of ODD containing HIF-3 $\alpha$  splice variants (HIF-3 $\alpha$ 2) and HIF-3 $\alpha$ 4 in Caki-1 cells. **F**, quantification of RPA results; data given are means $\pm$ SD, #*p*<0.05 vs. normoxia, *n*=4. **G**, in hPT cells total HIF-3 $\alpha$  mRNA induction after 16 h hypoxia or DP was quantified by qRT-PCR. Means of three independent experiments with three different hPT preparations.

### Fig. 2. Hypoxic induction of HIF-3 $\alpha$ mRNA in Caki-1 cells is HIF-1 $\alpha$ -dependent.

**A**, HIF-3 $\alpha$ ODD and HIF-3 $\alpha$ 4 mRNA induction after DP-stimulation paralleled the induction of the HIF-1 target gene ANGPTL4. Quantitative data are means of *n*=2. **B**, effect of HIF-1 $\alpha$  (1 $\alpha$ ) siRNA, but not HIF-2 $\alpha$  (2 $\alpha$ ) or luciferase (luc) control siRNA, on HIF-3 $\alpha$  mRNA induction after 16 h DP stimulation; for quantification of knock-down effects, mRNA expression of luc siRNA-transfected, DP stimulated cells were set to 1. Data in bar graphs are means $\pm$ SD of *n*=3. **C**, HIF-1 $\alpha$  and HIF-2 $\alpha$  immunoblots demonstrating the efficiency of the HIF- $\alpha$  knock-down in Caki-1 cells. **D**, effect of HIF-1 $\alpha$  knock-down at earlier time points (4 h and 8 h DP); line graph: quantification of HIF-3 $\alpha$  transcript levels in HIF-1 $\alpha$  siRNA-transfected cells in comparison with luc siRNA-transfected cells (=100%), representative of *n*=3. **E**, in unstimulated cells, HIF-1 $\alpha$  knock-down did not reduce HIF-3 $\alpha$  transcripts. Upper three panels: immunoblots for HIF-1 $\alpha$ , HIF-2 $\alpha$  and  $\beta$ -actin; lower four panels: RPA of different HIF-3 $\alpha$  transcripts; bar graph: qRT-PCR analysis of total HIF-3 $\alpha$  mRNA expression (means of *n*=3).

### Fig. 3. The human HIF-3 $\alpha$ gene is a transcriptional target of HIF-1.

**A**, sequence analysis identified six putative HIF binding sites (a-e) in the 5' flanking region and the first intron of the HIF-3 $\alpha$  gene (upper). PCR primer design for ChIP analysis (middle) and design of promoter luciferase reporter constructs (lower) are shown in parallel; numbering of nucleotide positions according to acc. no. AC007193. **B**, ChIP analysis revealed binding of HIF-1 $\alpha$  protein to the DNA region comprising HRE 'c' amplified by primer pair 2 (nt 42369-42613). Representative of four independent experiments. **C**, activation of HIF-3 $\alpha$  promoter constructs (as depicted in Fig. 3A) in Hep3B cells after DP-stimulation (upper panel), and in HEK293 cells after co-transfection of a normoxically stable mHIF-1 $\alpha$  triple mutant (HIF-1 $\alpha$ TM, lower panel). Luciferase activities were normalized to the respective  $\beta$ -galactosidase activities. Data are representative of at least four independent experiments with three replicates per sample (means $\pm$ SD). **D**, characterization of the HIF-3 $\alpha$  promoter fragment nt 42365-43656 (acc. no. AC007193) in Hep3B cells. Inactivation of the candidate HRE significantly reduced hypoxic activation. Data are means $\pm$ SEM, # *p*<0.01, *n* $\geq$ 3.

**Fig. 4. Expression, hypoxic induction and subcellular localization of HIF-3 $\alpha$  protein.**

**A**, immunoblot analysis of Caki-1 and HeLa extracts demonstrating the hypoxic induction of HIF-3 $\alpha$  protein;  $\beta$ -actin served as loading control. **B**, in comparison with HIF-1 $\alpha$ , HIF-3 $\alpha$  protein induction by DP was distinctly delayed. **C**, translation inhibition by cycloheximide (CHX, 450  $\mu$ M) after DP pre-stimulation revealed that HIF-3 $\alpha$  has a much longer protein half-life than HIF-1 $\alpha$ . **D**, siRNA-mediated knock-down of HIF-1 $\alpha$ , but not HIF-2 $\alpha$ , abrogates the induction of HIF-3 $\alpha$  protein in Caki-1 cells (see **Fig. 2 C** for HIF-2 $\alpha$  knock-down), for HIF-3 $\alpha$  two independent experiments are depicted (panel HIF-3 $\alpha$  (exp. 2)), representative of n=4. **E**, immunofluorescence staining of HIF-3 $\alpha$  in Caki-1 cells after 16 h hypoxia (hyp) or DP. Hoechst staining was used to visualize the nuclei. Overlay (merge) of HIF-3 $\alpha$  signals with Hoechst-staining demonstrated that HIF-3 $\alpha$  is present in cytoplasm and nuclei already under normoxia (no). Hypoxia and DP enhanced HIF-3 $\alpha$  immunoreactivity,

**Fig. 5. Effect of HIF-3 $\alpha$  knock-down on HIF-1-dependent reporter gene induction**

**A**, immunoblot analysis demonstrating different knock-down efficiencies of two HIF-3 $\alpha$  siRNAs in Caki-1 cells: HIF-3 $\alpha$  protein levels were reduced by siRNA HIF-3 $\alpha''$  by 50%; the HIF-3 $\alpha'$  siRNA was virtually inactive. HIF-1 $\alpha$  protein expression was not affected. **B**, immunoblot analysis of Hep3B cells showed that HIF-3 $\alpha$  splice variants are more effectively reduced by siRNA HIF-3 $\alpha''$  than by siRNA HIF-3 $\alpha'$ . **C**, DP-stimulated induction of a 6xHRE-driven luciferase reporter was increased by the HIF-3 $\alpha''$  siRNA (#p<0.05 vs. GFP-siRNA transfected, DP-stimulated cells; mean $\pm$ SD of n=4). HIF-1 $\alpha$  siRNA served as positive control. **D-F**, two Stealth<sup>®</sup> siRNAs (3 $\alpha$ 69 and 3 $\alpha$ 70) directed against all HIF-3 $\alpha$  variants also reduced HIF-3 $\alpha$  protein, but not HIF-1 $\alpha$  or HIF-2 $\alpha$  (**D**). HIF-3 $\alpha$  transcripts after 3 $\alpha$ 69 and 3 $\alpha$ 70 siRNA transfection (**E**, bar graph=quantification by qRT-PCR, n=5). HIF-3 $\alpha$  knock-down increased 6xHRE-driven luciferase activity in Caki-1 cells (**F**) in comparison with the control siRNA H (#p<0.05, means $\pm$ SD of n=6). **G**, HIF-3 $\alpha$  knock-down increased DP-induced Lox mRNA expression and decreased ANGPTL4 induction as determined by qRT-PCR. Mean of n=3-4 (#p<0.05, ##p<0.01 vs. DP-stimulated controls.)

**Fig. 6. HIF-3 $\alpha$  and HIF-1 $\alpha$  expression patterns in human renal cell carcinomas support the notion of a HIF-1 $\alpha$ -inducible HIF-3 $\alpha$  expression.**

Immunostaining of semi-serially prepared human renal carcinoma sections revealed co-localization for HIF-1 $\alpha$  (**A** and **C**) and HIF-3 $\alpha$  (**B** and **D**); magnification 200x.

**Fig. 7. HIF-3 $\alpha$  mRNA expression in renal cell carcinomas.**

RPA showing mRNA expression of ODD containing HIF-3 $\alpha$  transcripts and HIF-3 $\alpha$ 4 in a HIF-1 $\alpha$ /-2 $\alpha$  positive and a HIF-1 $\alpha$ /-2 $\alpha$  negative tumor in comparison with normal kidney tissue. Positions of specific bands are indicated by arrows.

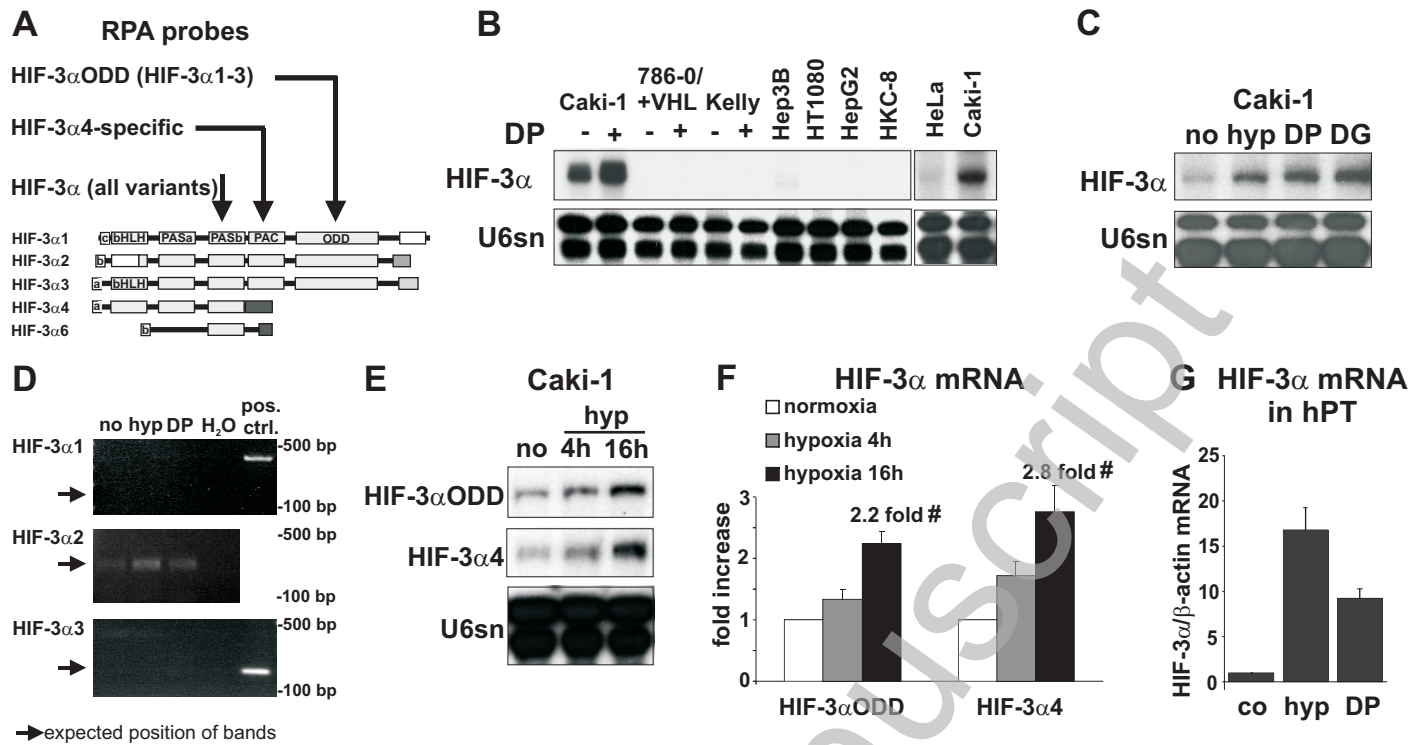


Fig.1

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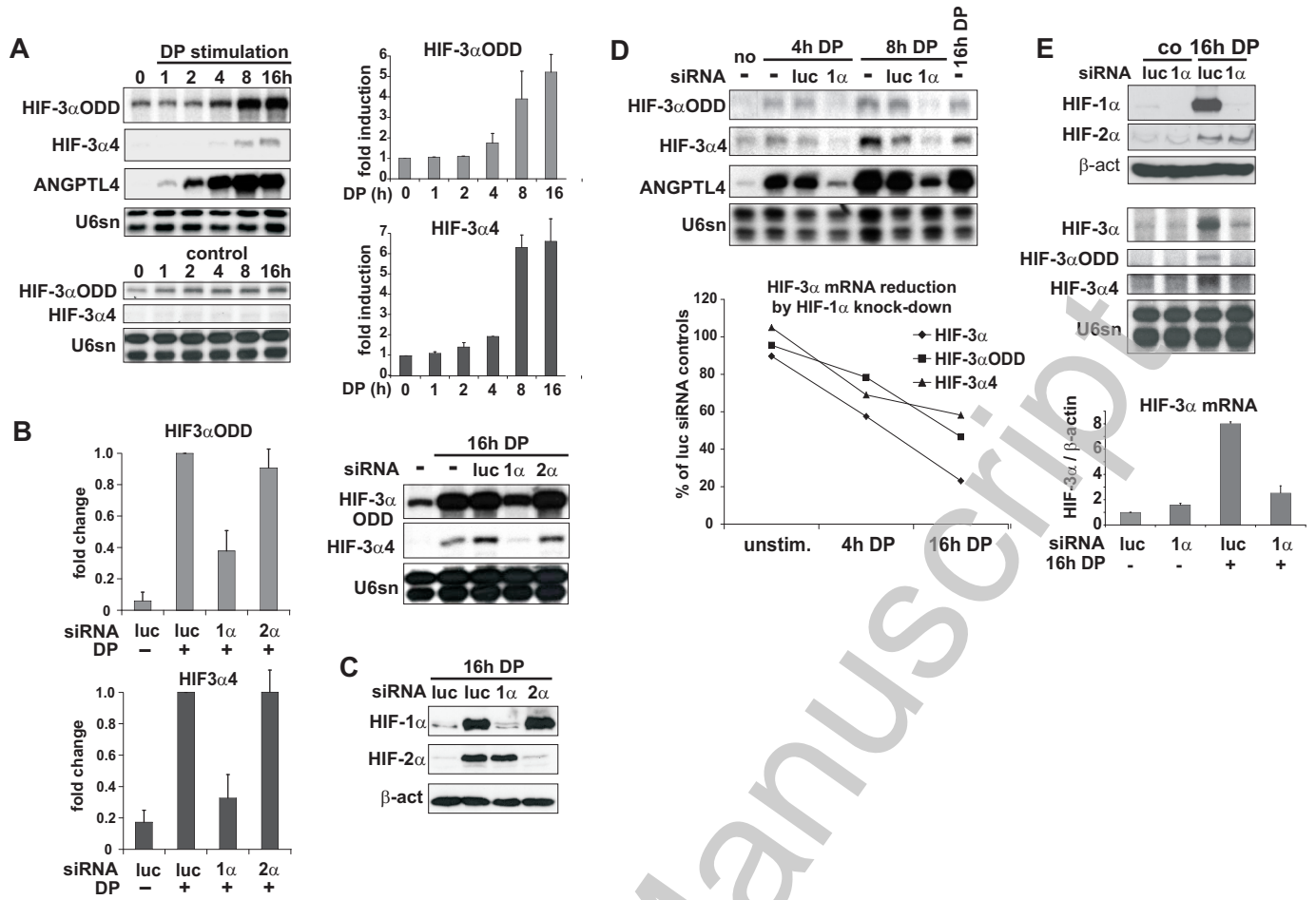


Fig.2

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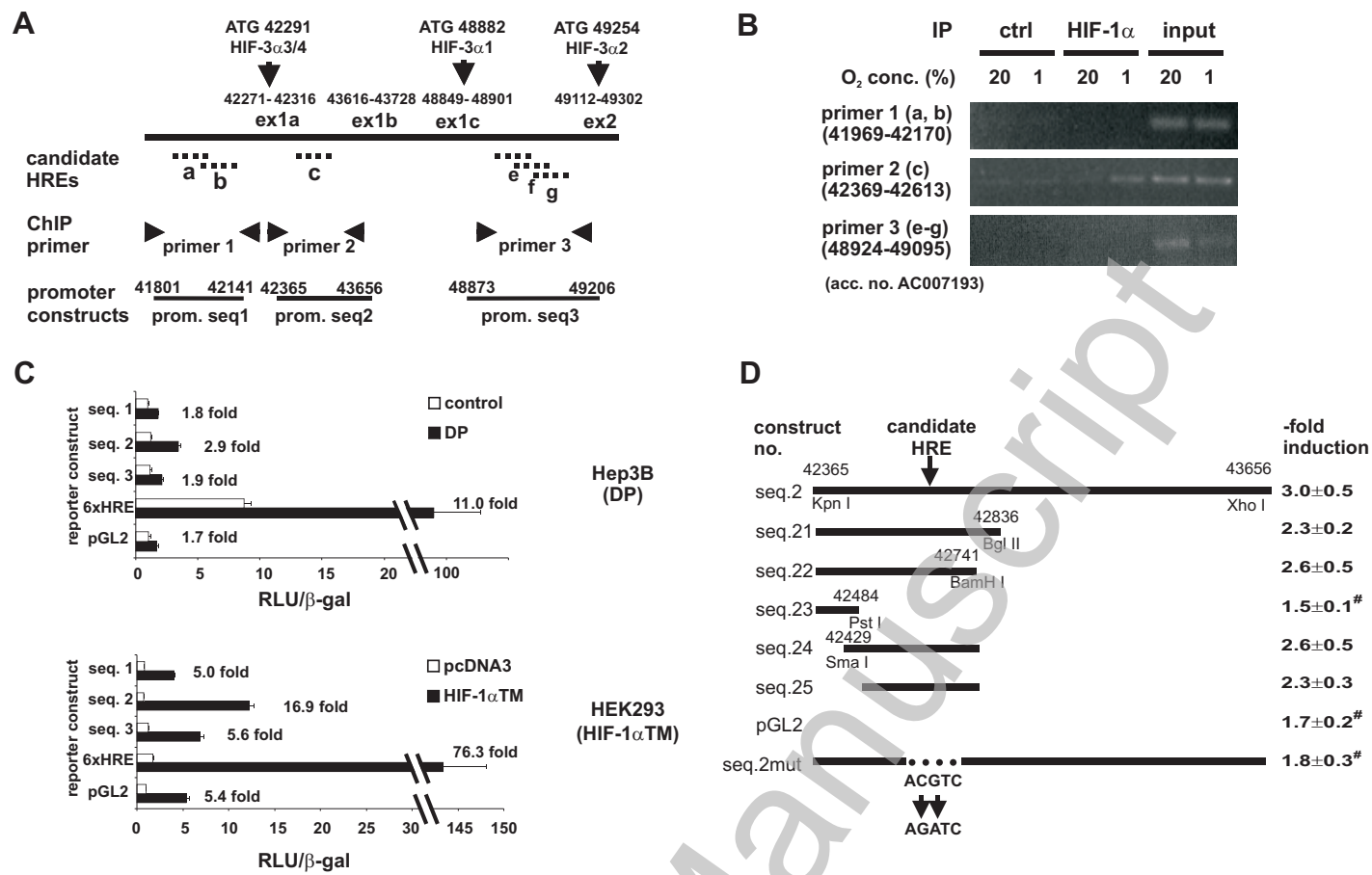


Fig.3

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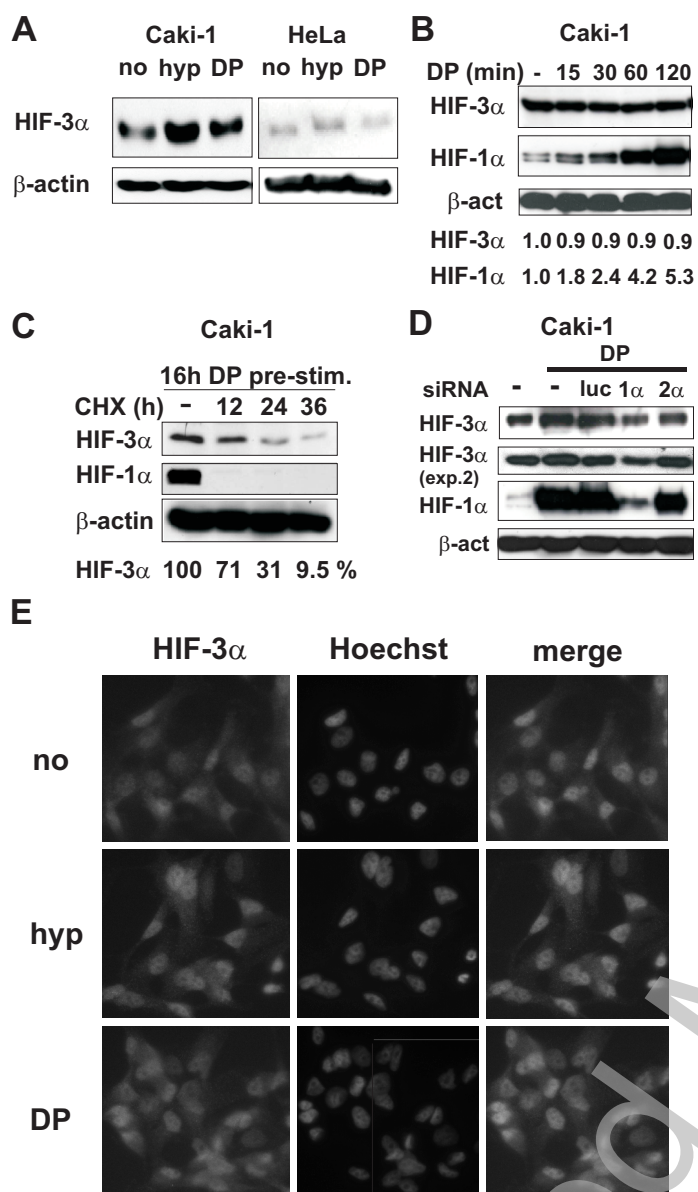


Fig. 4

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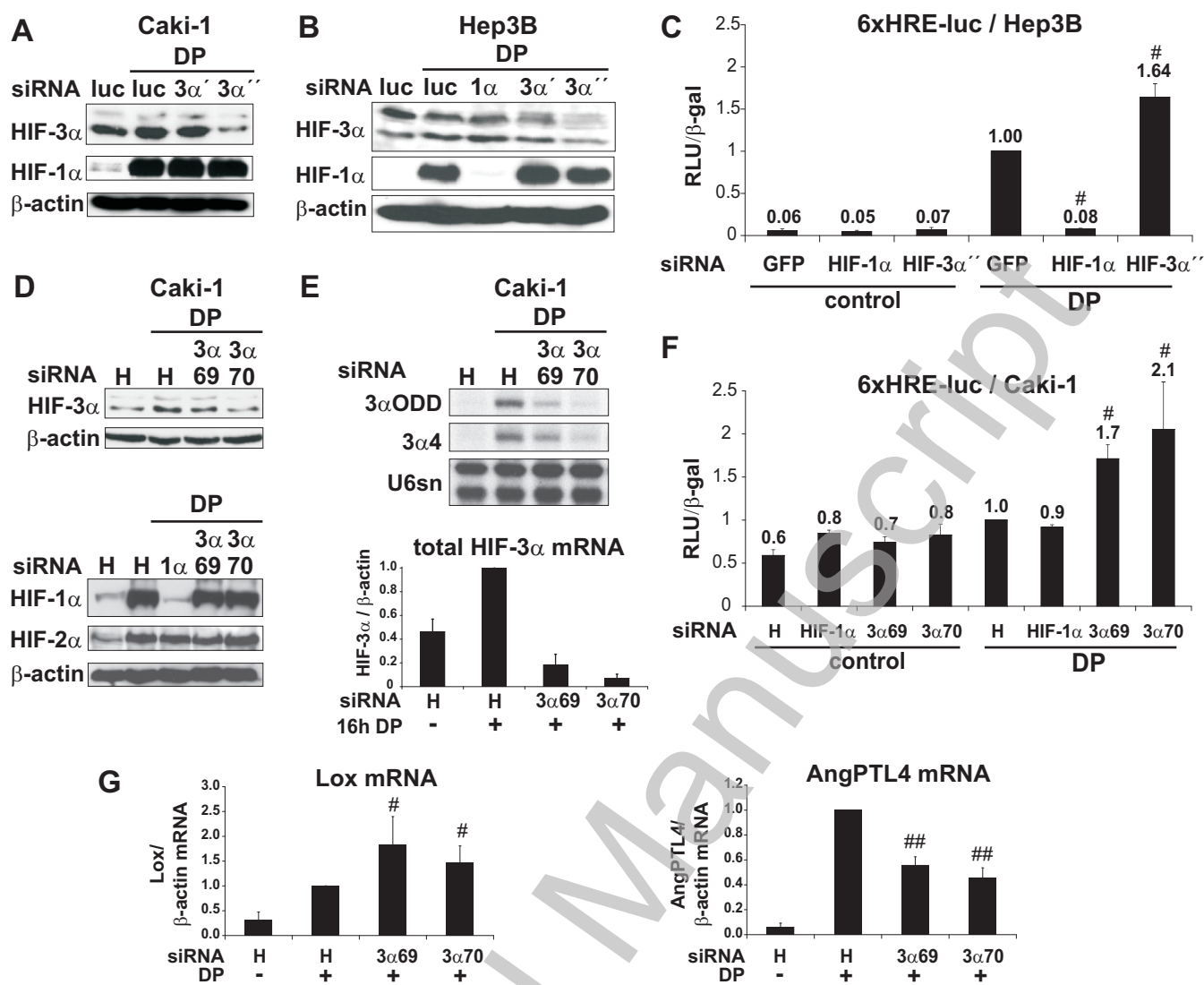


Fig. 5

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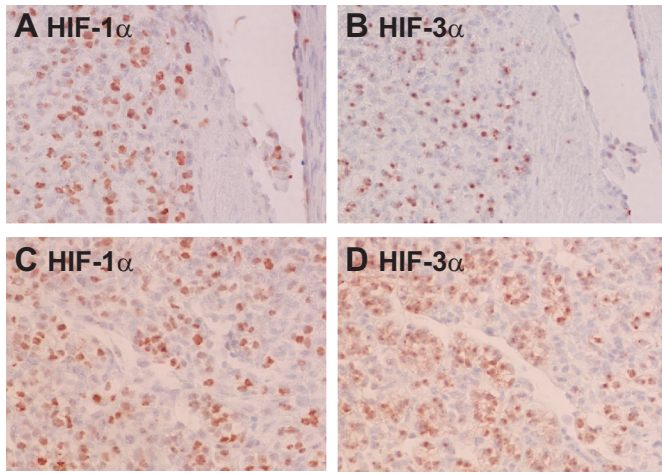


Fig. 6

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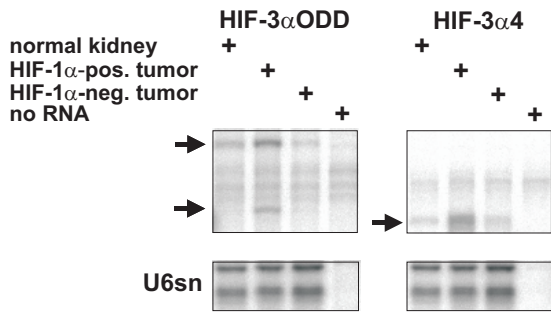


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