The Oxygen Sensor Factor-Inhibiting Hypoxia-Inducible Factor-1 Controls Expression of Distinct Genes through the Bifunctional Transcriptional Character of Hypoxia-Inducible Factor-1α

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Abstract

The function of the hypoxia-inducible factor-1 (HIF-1), the key transcription factor involved in cellular adaptation to hypoxia, is restricted to low oxygen tension (pO_2). As such, this transcription factor is central in modulating the tumor microenvironment, sensing nutrient availability, and controlling anaerobic glycolysis, intracellular pH, and cell survival. Degradation and inhibition of the limiting HIF-1α subunit are intimately connected in normoxia. Hydroxylation of two proline residues by prolyl hydroxylase domain (PHD) 2 protein earmarks the protein for degradation, whereas hydroxylation of an asparagine residue by factor-inhibiting HIF-1 (FIH-1 or FIH) reduces its transcriptional activity. Indeed, silencing of either PHD2 or FIH in normoxia partially induced hypoxic genes, whereas combined PHD2/FIH silencing generated a full hypoxic gene response. Given the fact that HIF-1α possesses two transcriptional activation domains [TAD; NH_2-terminal (N-TAD) and COOH-terminal (C-TAD)], we hypothesized on a possible bifunctional activity of HIF-1α that could be discriminated by FIH, an inhibitor of the C-TAD. In human cell lines engineered to overexpress or silence FIH in response to tetracycline, we show by quantitative reverse transcription-PCR that a set of hypoxic genes (*ca9, phd3, pgk1*, and *bnip3*) respond differently toward FIH expression. This finding, extended to 26 hypoxia-induced genes, indicates differential gene expression by the N-TAD and C-TAD in response to the hypoxic gradient. We propose that the oxygen-sensitive attenuator FIH, together with two distinct TADs, is central in setting the gene expression repertoire dictated by the cell pO_2. (Cancer Res 2006; 66(7): 3688-98)

Introduction

Tumor progression is highly regulated by hypoxia, a low level of oxygen, which occurs after excessive tumor cell proliferation that distances cells from oxygen-rich blood vessels. The hypoxia-inducible factor-1 (HIF-1) is the most important factor involved in the cellular response to hypoxia and consequently has been studied intensely this last decade (see ref. 1 for review). HIF-1 is a heterodimer of two basic helix-loop-helix (bHLH) and PER-aryl hydrocarbon receptor nuclear translocator (ARNT)-SIM (PAS) domains: HIF-1α, HIF-2α, and HIF-3α, the highly regulated subunits, and HIF-1β, the constitutive subunit also called ARNT (2). The NH_2-terminal half of HIF-1α contains bHLH and PAS domains that are required for dimerization and DNA binding. The COOH-terminal half contains domains required for degradation and transactivation: the oxygen-dependent degradation domain (ODDD) that confers oxygen-dependent instability and two independent transcriptional activation domains [TAD; NH_2-terminal (N-TAD; amino acids 531-575) and COOH-terminal (C-TAD; amino acids 786-826; refs. 3, 4)] in its COOH-terminal half and in between, an inhibitory domain that negatively regulates the TADs.

HIF-1α is tightly regulated by post-translational modifications by at least two separate oxygen-dependent mechanisms: direct hydroxylation of proline residues by prolyl hydroxylase domain (PHD) protein triggers the degradation of HIF-1α (5, 6), whereas asparagine hydroxylation by factor-inhibiting HIF-1 (FIH-1 or FIH) inactivates the C-TAD of HIF-1α (7–9). The activity of these enzymes is governed by the O_2 concentration within the cell, which defines these proteins as oxygen sensors. Three human PHD isoforms have been described in the presence of dioxygen, the PHD hydroxylate prolyl residues (402 and/or 564) located in the ODDD of HIF-1α (5). Proline hydroxylation on HIF-1α constitutes the signal for binding of the tumor suppressor protein von Hippel-Lindau, a component of an E3 ubiquitin ligase multiprotein complex involved in protein ubiquitination. Thus, hydroxylated HIF-1α is covalently modified by ubiquitin that earmarks the protein for destruction by the proteasomal system. FIH has also been characterized more recently as an oxygen sensor involved in the regulation of the activity of HIF-1α. FIH possesses an asparaginyl hydroxylase activity that by targeting Asn^{803} represses HIF-1α transcriptional activity by preventing binding of the transcriptional coactivator p300/CBP to the HIF-1α C-TAD. FIH does not influence HIF-1α stability but allows for modulation of HIF-1α transactivation. Thus, cells have evolved a dual mechanism to control a low HIF-1 activity in "normoxic conditions" by inducing destruction and inhibition of HIF-1α. In contrast, a progressive decrease in the O_2 tension will inhibit both PHDs and FIH, resulting in HIF-1α stabilization and activation.

Several studies have pointed out that the two α subunits, HIF-1α and HIF-2α, have contrasting roles in cell physiology because they are capable of eliciting the transcription of different sets of genes (10–13). A good example of gene selectivity is shown for carbonic anhydrase 9 (*ca9*; ref. 14) and HIF PHD3 (*phd3*; ref. 15), where the former is predominantly HIF-1 and the latter is both HIF-1 and HIF-2 regulated.

In this context of selectivity, we were intrigued by the existence of two separate TADs, N-TAD and C-TAD, present in both HIF-1α and HIF-2α. We decided to investigate whether some genes will be

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differentially sensitive to inactivation of the C-TAD, a domain exclusively modulated by FIH. We believe that this question is extremely relevant to gene expression *in vivo* where cells are exposed to graded O_2 concentrations, particularly if PHDs and FIH have distinct affinities for O_2, as *in vitro* studies have recently highlighted (16).

In this study, we used three different means to stabilize HIF-1α: by either silencing the key HIF-1α destabilizing enzyme PHD2 (17), exposing cells to various low oxygen concentrations, or using cell confluency. Together with HIF-1α stabilization, FIH was either silenced or overexpressed to evaluate the respective transactivation potential of the N-TAD and C-TAD. We focused our attention on four HIF-1-dependent genes: *ca9*, a major regulator of pH (18); *phd3* (19); the glycolytic enzyme phosphoglycerate kinase 1 (*pgk1*; ref. 20); and *bnip3*, a proapoptotic member of the BH3-only subfamily of Bcl-2 family (21). Our results highlight the differential effect of the N-TAD and C-TAD on regulation of HIF-1-dependent genes and allow us to propose two groups of hypoxia-regulated genes based on their sensitivity to FIH expression: the FIH-inhibited and the non-FIH-inhibited groups. We finally propose a model for the expression of genes along the oxygen gradient within the pathophysiologic environment of a solid tumor based on the bifunctional transcriptional character of HIF-1α.

Materials and Methods

Cell Culture

HeLa and LS174Tr cells were grown in DMEM supplemented with 5% and 10% fetal bovine serum, respectively, and antibiotics (50 units/mL penicillin G and 50 μg/mL streptomycin + 10 μg/mL blasticidin specifically for the LS174Tr cells; Life Technologies, Inc., Gaithersburg, MD). LS174Tr cells expressing the tetracycline (Tet) repressor were kindly provided by Dr. van de Wetering (Netherlands Cancer Institute, Amsterdam, the Netherlands; ref. 22).

Hypoxic conditions were produced by incubation of cells in a sealed Bug-Box anaerobic workstation (Ruskinn Technology Biotrace International Plc, Bridgend, Wales, United Kingdom). The oxygen level was maintained at 9%, 3%, or 0.2% with the residual gas mixture of 86% to 95% nitrogen and 5% carbon dioxide.

siRNA Preparation

The 21-nucleotide RNAs were chemically synthesized (Eurogentec, Seraing, Belgium). The set of siRNAs targeting FIH (Genbank accession no. AF395830) correspond to the coding region from 992 to 1,011 relative to the start codon and were as follows: forward 5′-GAUGCUUGGAGAGGCCUUGTT-3′ and reverse 5′-CAAGGCCUCUCCAAGCAUCTT-3′. Small interfering RNA (siRNA) sequences targeting PHD2 and SIMA have been described previously (17). The latter was used as a control and targets the *Drosophila* HIF-1 called Similar. siRNA targeting human HIF-1α (17) and HIF-2α (12) have also been described. Annealing of siRNAs was done as described previously by Elbashir et al. (23, 24).

Plasmids

pSuper-FIH. To construct pSUPER-FIH, gene-specific oligonucleotides were chosen as described previously (25). The oligonucleotides used for FIH were as follows: forward 5′-GATCCCCGATGCTTGGAGAGGCCTTGTTCAAGAGACAAGGCCTCTCCAAGCTACTTTTTGGAAA-3′ and reverse 5′-AGCTTTTCCAAAAAGATGCTTGGAGAGGCCTTGTCTCTTGAACAAGGCCTCTCCAAGCATCGGG-3′. The mixture (1 μL) was ligated into the pSUPER vector that had been digested with *Bgl*II and *Hind*III (kind gifts from Dr. Agami; ref. 25).

Short hairpin RNA-FIH. The plasmid pTer-FIH was constructed as described above for pSUPER-FIH. The same oligonucleotides were inserted into the pTer vector (22) digested with *Bgl*II and *Hin*dIII.

pFIH. Conventional cloning procedures were used to insert the FIH cDNA obtained first by PCR using the primers (forward) 5′-CTATCGGATCCATGGCGGCGACAGCGGCGG-3′ and (reverse) 5′-CCACTAAGCTTCTAGTTGTATCGGCCCTT-3′ into *Bam*HI and *Hin*dIII sites of the pCRII-TOPO (Invitrogen, Carlsbad, CA). The pFIH plasmid was obtained by subcloning the *Eco*RI-*Eco*RI fragment from the pCRII-TOPO FIH into the *Eco*RI site of pTREX-C vector (also named pcDNA4/TO/myc-His C; Invitrogen).

Transient Transfection and Luciferase Assay

HeLa and LS174Tr cells were transfected using the calcium phosphate method (26).

Transient transfection. Cells were transfected with siRNAs (siRNA to FIH, HIF-1α, and HIF-2α) twice at a 24-hour interval or once with the pFIH vector. Combination of siRNA to PHD2 (20 nmol/L) and FIH (10 μg) overexpression exposed the N-TAD activity by increasing the endogenous pool of HIF-1α while inhibiting the C-TAD activity. In a similar manner, a combination of siRNA to PHD2 (100 nmol/L) and FIH (20 nmol/L) mimicked hypoxic conditions with full activity. Cells were lysed 48 hours after transfection.

Luciferase assay. Cells were transfected with siRNAs [or short hairpin RNA (shRNA)-FIH vector] twice at a 24-hour interval or once with the pFIH vector (1 μg/six-well plate), transfected with the reporter vector 24 hours after the last siRNA transfection, and analyzed on the day following the last transfection. HeLa and LS174Tr cells were transfected with or without siRNAs (0.2, 2, and 20 nmol/L), 300 ng reporter vector, p3HRE-Δptk-LUC, described previously by Berra et al. (17), containing three copies of the hypoxia-responsive element (HRE) from the erythropoietin gene and β-galactosidase-expressing plasmid (50 ng). As a control for HIF-1 activation, cells were incubated under hypoxic conditions (1-2% O_2) for 24 hours. Cells were lysed 48 hours after transfection, and the luciferase and β-galactosidase activities were measured as described previously (27).

Stable Clones

HeLa cells were cotransfected with pSUPER-FIH and pBabepuro. Puromycin-resistant clones were tested for their ability to down-regulate FIH by immunoblotting. LS174Tr cells were transfected with shRNA-FIH or pFIH vectors. Zeocin-resistant clones incubated in the absence or presence of 10 μg/mL Tet, to down-regulate or up-regulate FIH, respectively, were screened by immunoblotting.

RNA Extraction

Total RNA was extracted from HeLa or LS174Tr cells using Trizol reagent (Life Technologies) according to the manufacturer's instructions. Briefly, cultured cells were homogenized in 1 mL Trizol, centrifuged after adding chloroform and precipitating RNA with isopropanol, and washed with 75% ethanol. The RNA purity was evaluated by spectrophotometry. Total RNA (2 μg) from HeLa and LS174Tr cells was added to a 20-μL reverse transcription-PCR (RT-PCR) reaction using the Omniscript kit (Qiagen, Inc., Valencia, CA).

Real-time Quantitative PCR

Real-time quantitative amplification of RNA of 36B4, BNIP3, CA9, FIH, PGK1, PHD2, and PHD3 was done according to the manufacturer's instructions (Roche Diagnostics Corp., Indianapolis, IN). Extracted cDNA (2 μL; 10-fold dilutions) was used as template for PCR amplification in a LightCycler PCR instrument (Roche Diagnostics/Idaho Technology). The amplification mixture (20 μL final volume) contained the following volumes and concentrations of reactants: 1 μL of both sense and antisense primers (10 μmol/L), 2 μL LC FastStart DNA Master SYBR Green I Mix (Roche Diagnostics/Idaho Technology), and $MgCl_2$ (3 mmol/L final concentration). Master Mix and extracted DNA were added to a capillary tube, which was sealed, centrifuged, and placed in the LightCycler carousel.

The relative expression level of 26 transcripts was quantified by real-time RT-PCR using the Taqman PCR Master Mix (Eurogentec) on an ABI Prism 7300 Sequence Detection System (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The relative amount of each gene to the 36B4 internal control and fold stimulation was calculated by using the equation $2^{-\Delta\Delta CT}$, where $\Delta C_T = C_{T(gene)} - C_{T(36B4)}$ and $\Delta\Delta C_T =$

$\Delta C_{T(\text{stimulated condition})} - \Delta C_{T(\text{unstimulated condition})}$. Each gene was amplified using the appropriate specific primers. Each individual induction is the mean of two amplifications. Each difference in gene expression was estimated within the limits of a 95% confidence interval (95% CI). The results are representative of at least three separate experiments.

Immunoblotting

HeLa and LS174Tr cells were lysed in 1.5× Laemmli buffer. The protein concentration was determined using the bicinchoninic acid assay. Whole-cell extracts (10 μg for FIH or 40 μg for PHD2 and HIF-1α) were resolved by SDS-PAGE (7.5% for HIF-1α detection and 10% for FIH and PHD2) and transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA). Immunoreactive bands were visualized with the enhanced chemiluminescence system (Amersham Biosciences, Buckinghamshire, United Kingdom).

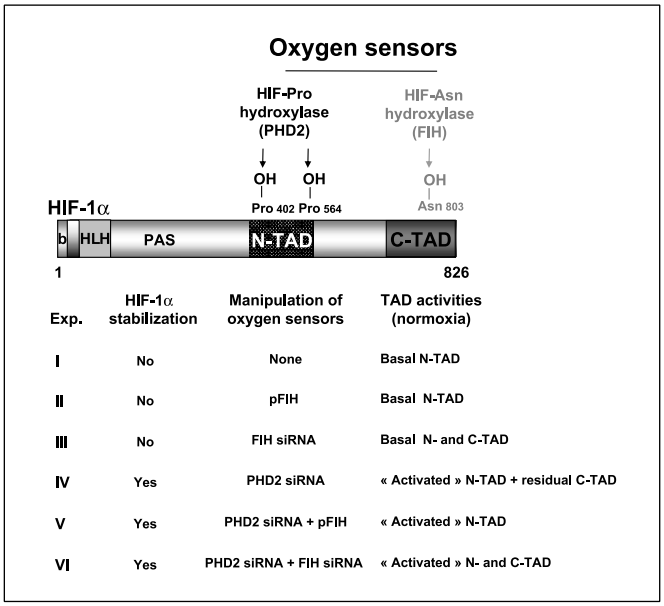
Antibodies

Anti-HIF-1α (antiserum 2087), p42MAPK (antiserum E1B4), and anti-PHD2 (antiserum 804) were produced and characterized in our laboratory (17, 27, 28). Anti-FIH (antiserum 810) was raised in rabbits immunized against the last 21 amino acids of the COOH-terminal end of human FIH. The mouse anti-c-Raf-1 antibody (clone 53) was from BD Transduction Laboratories (Franklin Lakes, NJ).

Results

Cosilencing of PHD2 and FIH shows a synergistic effect on the activity of HIF-1 in normoxia in HeLa cells. To study the effect of FIH (overexpression or ablation) on the HIF-1α transcriptional activity and a possible role for both TADs of HIF-1α, we did the experiments under well-oxygenated conditions (21% O_2) as described in Fig. 1.

We first evaluated the endogenous pool of active HIF-1α in normoxia using a very sensitive HIF-1-inducible reporter gene vector (p3HRE-Δptk-LUC) and a clone of HeLa cells, in which siRNA transfection is very efficient. Specific silencing of HIF-1α markedly reduced the activity of this reporter in both normoxic and hypoxic conditions, 90% and 80% inhibition of the luciferase activity, respectively (data not shown). This inhibition in normoxia indicates that there is a basal level of active



Exp.	HIF-1α stabilization	Manipulation of oxygen sensors	TAD activities (normoxia)
I	No	None	Basal N-TAD
II	No	pFIH	Basal N-TAD
III	No	FIH siRNA	Basal N- and C-TAD
IV	Yes	PHD2 siRNA	« Activated » N-TAD + residual C-TAD
V	Yes	PHD2 siRNA + pFIH	« Activated » N-TAD
VI	Yes	PHD2 siRNA + FIH siRNA	« Activated » N- and C-TAD

Figure 1. Domain structure of HIF-1α showing the proline and asparagine residues involved in its regulation and indicating the different conditions used to differentiate between the two TADs. HIF-1α possesses bHLH and PAS domains that are involved in dimerization with HIF-1β and DNA binding. HIF-1α contains N-TAD and C-TAD where the N-TAD lies within the ODDD. The stability of HIF-1α is regulated through the ODDD via recognition of the hydroxylation state of the Pro^{402} and/or Pro^{564} residues. Enzyme termed PHD2 catalyzes this hydroxylation. Hydroxylation of Asn^{803} by the enzyme FIH inhibits activation by preventing binding of p300/CBP, a HIF-1α coactivator. Description of the six different conditions used to differentiate between the activities of the N-TAD and/or C-TAD in normoxia (21% O_2). Normoxic condition, with no detectable HIF-1α protein expression, was used as a control (*I*). pFIH (*II*) and siRNA to FIH (FIH siRNA; *III*) were used to abolish and release the C-TAD activity, respectively. siRNA to PHD2 (PHD2 siRNA; *IV*) was used to increase the pool of HIF-1α. Combination of siRNA to PHD2 and pFIH (PHD2/pFIH siRNA; *V*) permitted examination of the N-TAD activity, whereas the combination of siRNA to PHD2 and siRNA to FIH (PHD2 siRNA/FIH siRNA; *VI*) was used to mimic hypoxic conditions. Detection of stable HIF-1α was determined by immunoblotting in all conditions and detectable only in conditions IV, V, and VI. *Exp.,* experiment.

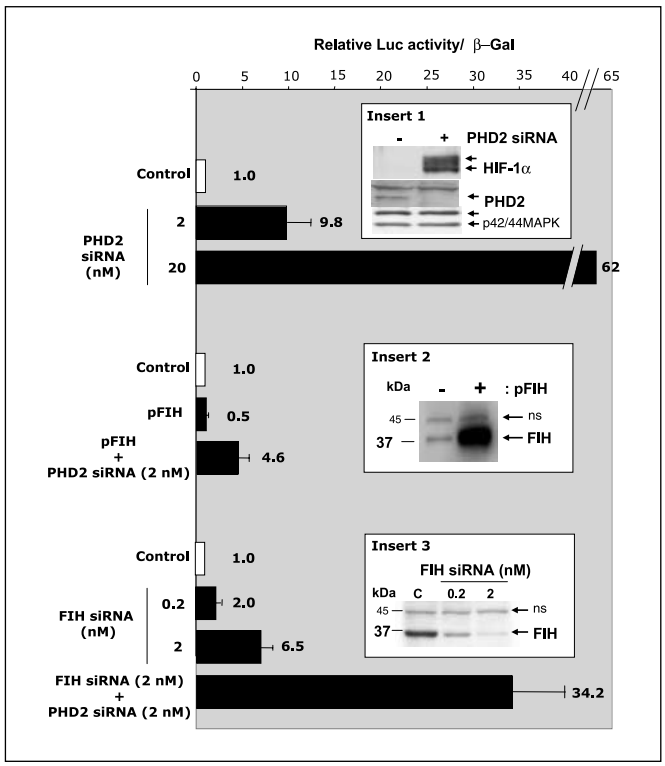


Figure 2. Comparison of the endogenous N-TAD and N/C-TAD activities in HeLa cells in normoxia. *Inset 1,* effect of siRNA silencing of PHD2 on HIF-1 transcriptional activity. Immunoblot: Silencing of PHD2 up-regulates HIF-1α expression in normoxia. HeLa cells were transfected with siRNAs (20 nmol/L) and were analyzed by immunoblotting using the anti-HIF-1α antibody 48 hours later. Luciferase assay: HeLa cells were transfected with siRNA to PHD2 (2 and 20 nmol/L) twice at a 24-hour interval together with 300 ng reporter vector (p3HRE-Δptk-LUC). siRNA to SIMA (2 nmol/L) was used as a control. Cells were then incubated in normoxia (21% O_2) for 24 hours. *Inset 2,* N-TAD endogenous activity in normoxia. Immunoblot: Overexpression of the FIH protein. HeLa cells were transfected with pFIH (3 μg/six-well plate), and FIH expression was analyzed by immunoblotting 72 hours after transfection. Clone strongly expressing FIH protein levels (6.5-fold above the endogenous) under normoxia. *Left,* molecular mass standards. *ns,* nonspecific. Luciferase assay: HeLa cells were transfected twice at a 24-hour interval with siRNA to PHD2 (2 nmol/L) together with 300 ng reporter vector (p3HRE-Δptk-LUC). Plasmid (1 μg) containing the human FIH coding sequence (pFIH) was added during the second transfection with siRNA. siRNA to SIMA (0.2 nmol/L) was used as a control. *Inset 3,* N-TAD and C-TAD endogenous activity in normoxia. Immunoblot: Specific silencing of FIH in a dose-dependent manner using siRNA. Cell extracts were prepared from HeLa cells incubated in normoxia (21% O_2) 48 hours after transfection with a control siRNA (*C*) and siRNA to FIH (0.2 and 2 nmol/L). *Left,* molecular mass standards. Luciferase assay: HeLa cells were transfected twice with siRNA to FIH (0.2 and 2 nmol/L) and PHD2 (0.2 nmol/L), single or in combination, at a 24-hour interval together with 300 ng reporter vector (p3HRE-Δptk-LUC). siRNA to SIMA (2.2 nmol/L) was used as a control. Luciferase assay: Plasmid expressing β-galactosidase (100 ng; 12-well plate) was cotransfected to normalize for the transfection efficiency. Luciferase activity was measured 48 hours after the last transfection. *Columns,* mean of at least three independent experiments done in triplicate; *bars,* SD.

HIF-1 even when the HIF-1α protein is not detectable by immunoblotting.

HeLa cells were then transfected with siRNA targeting PHD2 and a control siRNA (SIMA) together with the LUC reporter gene. HIF-1α stabilization in normoxia with siRNA to PHD2 has been described previously by our team (17). Cell lysates were then assayed for luciferase activity. Figure 2 (*inset 1*) shows an increase in the luciferase activity and HIF-1α pool when cells were incubated in the presence of siRNA to PHD2 and maintained in normoxia.

To investigate whether HIF-1α possesses a bifunctional activity implicating both TADs, N-TAD and C-TAD, we first overexpressed FIH (pFIH) in HeLa cells. Immunoblot analysis showed increased levels of FIH protein in samples of cells transiently transfected with pFIH (Fig. 2, *inset 2*). As expected, overexpression of FIH diminished the luciferase activity (Fig. 2) in a dose-dependent manner (data not shown) resulting in ~50% decrease in induction. In combination with controlled HIF-1α expression obtained with 2 nmol/L siRNA to PHD2, the transcriptional activity was lower (4.6-fold induction versus 9.8-fold induction after HIF-1 stabilization) as anticipated if HIF-1α N-TAD activity alone was present.

We next used a RNA interference strategy to silence FIH expression. We used two independent sets of FIH siRNA duplexes, one targeting the 5′ end and the other targeting the 3′ end of the coding mRNA region, to specifically target human FIH. The 3′ end at 2 nmol/L suppressed the expression of FIH by 70%. The level of inhibition was concentration dependent in human HeLa cells (Fig. 2, *inset 3*), whereas the other siRNA gave a 50% inhibition at the same concentration (data not shown). Based on these results, we selected the FIH siRNA (3′ end) shown in Fig. 2 for further studies. When siRNA to FIH (2 nmol/L) was used in combination with the same amount of siRNA to PHD2 (2 nmol/L), a higher luciferase activity than that obtained without siRNA to FIH (2 nmol/L) was obtained as anticipated if the activities of both N-TAD and C-TAD were maximal (Fig. 2). Cosilencing of PHD2 and FIH (2 nmol/L siRNA each) showed a synergistic effect on the luciferase activity comparable with that obtained in hypoxia when HIF-1α is fully active (data not shown).

HIF-1α-dependent gene expression in HeLa cells. To further evaluate the putative bifunctional activity of HIF-1α, endogenous expression of four HIF-1-dependent genes was measured by quantitative PCR (q-PCR) in HeLa cells: bnip3, *pgk1, phd3*, and *ca9*. First, it was necessary to identify the relative expression and contribution of HIF-1α and HIF-2α on induction of these four genes.

We therefore silenced HIF-1α and HIF-2α by transfecting HeLa cells with the corresponding siRNAs and incubated the cells in hypoxia (24 hours, 1.5% O_2). To exclude the possibility of non-specific effects due to a particular siRNA, we selected two different

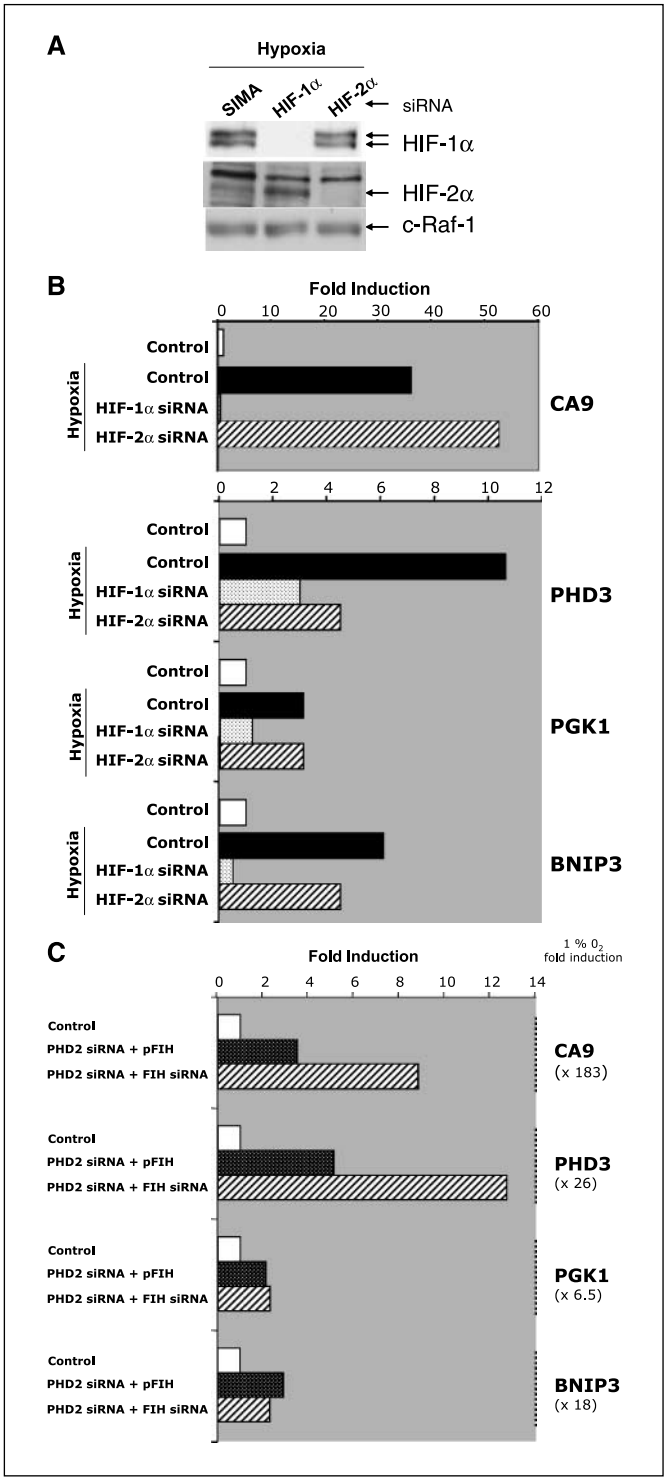
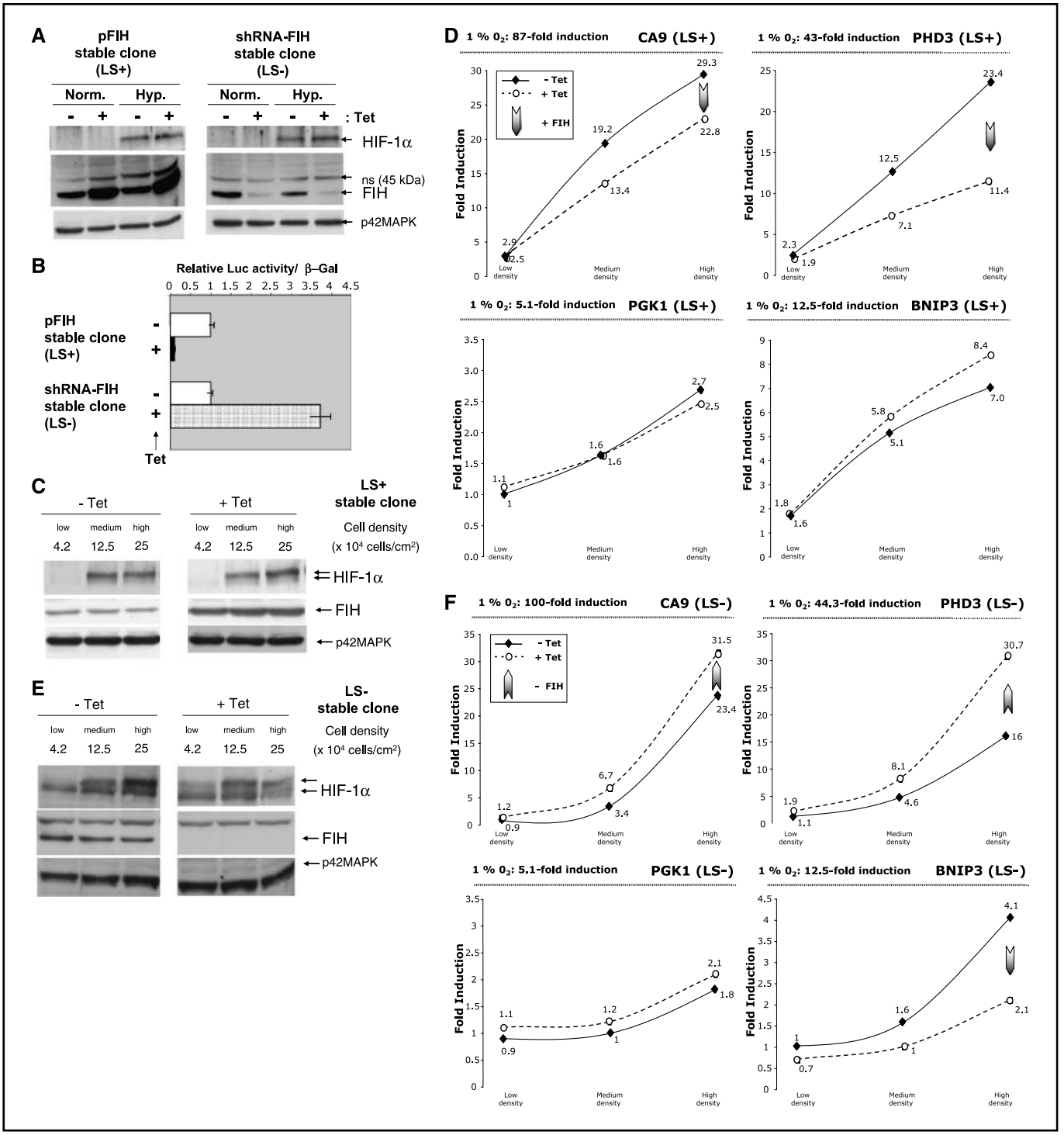


Figure 3. HIF-1α-dependent genes in HeLa cells. *A,* immunoblot analysis of proteins extracted from HeLa cells after specific siRNA treatment. HeLa cells were transfected twice at a 24-hour interval with either siRNA to the control SIMA (20 nmol/L), HIF-1α (20 nmol/L), or HIF-2α (20 nmol/L), and cells were incubated for 24 hours in hypoxia (1.5% O_2). HIF-1α and HIF-2α expression was analyzed by immunoblotting after transfection for 48 hours. c-Raf-1 immunoreactivity was used as a loading control. *B,* expression of the mRNA levels of CA9, PHD3, PGK1, and BNIP3 determined by real-time q-PCR under the conditions described above. *C,* differential effect of the N-TAD (FIH overexpression) and C-TAD (FIH silencing) on endogenous activities in HeLa cell clones (70% FIH invalidation) in normoxia. Expression levels of CA9, PHD3, PGK1, and BNIP3 mRNA determined by real-time q-PCR of the stable pSUPER-FIH clone transfected with siRNA to PHD2 (20-100 nmol/L) together with transfection with either pFIH (10 μg/100-mm plate; FIH overexpression; N-TAD activity) or siRNA to FIH (20 nmol/L; FIH silencing; N/C-TAD activity) as described above. PHD2 mRNA level was controlled by q-PCR. Similar pools of HIF-1α were present in the N-TAD- and N/C-TAD-activated conditions as checked by immunoblotting of cell lysates with an antibody against HIF-1α. *B* and *C,* experiments were done in duplicate at least thrice. Results from one representative experiment. Each difference in gene expression was estimated within the limits of a 95% CI.

duplexes for both HIF-1α and HIF-2α. Only the more efficient ones are shown. As shown in Fig. 3*A*, HeLa cells transiently transfected with the siRNA duplex corresponding to HIF-1α or HIF-2α displayed a remarkably specific loss of the HIF-1α and HIF-2α protein levels as detected by immunoblotting. The control, siRNA to SIMA, had no effect on either protein, whereas ablation of HIF-1α increased, as expected, the expression of HIF-2α (Fig. 3*A*). We then tested endogenous expression of the four genes under hypoxic conditions in the presence or absence of HIF-1α or HIF-2α. Hypoxia resulted in induction of the mRNA levels of the *ca9, phd3, pgk1*, and *bnip3* genes (Fig. 3*B*). Silencing of HIF-1α fully abolished the induction of CA9, PGK1, and BNIP3 with the exception of PHD3 that remained above basal. As expected, ablation of HIF-2α had no effect on the hypoxic induction of CA9, PGK1, and BNIP3, whereas the expression of PHD3 was reduced by 58% (Fig. 3*B*). Recently, a significant down-regulation of PHD3 by silencing of HIF-1α or



HIF-2α was reported, suggesting that PHD3 is a direct target of both HIF-1α and HIF-2α and that HIF-1 and HIF-2 may activate PHD3 by different mechanisms (15).

Therefore, the HeLa cell system is suitable to analyze the bifunctionality of HIF-1α (N-TAD and C-TAD) on the expression of the HIF-1-sensitive genes (*ca9, pgk1*, and *bnip3*). For the fourth gene, *phd3*, removal of HIF-2α would be required.

Two groups of genes with different sensitivities to FIH in HeLa cells. To determine the effect of FIH on the various genes analyzed, it was important to either abolish FIH by siRNA or overexpress it (4- to 6-fold) above endogenous levels by transfecting cells with the FIH expression plasmid pFIH. To succeed in almost complete abolition of FIH (90% reduction), we transiently transfected siRNA to FIH in a HeLa clone, in which FIH expression was already stably reduced by 70% (expression of a stable shRNA pSUPER-FIH; see Materials and Methods; data not shown).

In this HeLa clone, hypoxia of 1% oxygen produced significant induction of the CA9 (183-fold), PHD3 (26-fold), PGK1 (6.5-fold), and BNIP3 (18-fold) mRNA level (Fig. 3*C*). We then stabilized HIF-1α in normoxia by silencing PHD2 (20 nmol/L siRNA) and ensured full inhibition of the C-TAD by overexpressing FIH (pFIH). This experimental condition that corresponds to "activated" N-TAD (Fig. 1, *V*) led to 3.5-fold (CA9), 5.1-fold (PHD3), 2.1-fold (PGK1), and 2.9-fold (BNIP3) induction in normoxia. Ablation of FIH corresponding to activated N-TAD and C-TAD (Fig. 1, *VI*) resulted in a higher increase in expression of CA9 (8.8-fold versus 3.5-fold) and PHD3 (12.7-fold versus 5.1-fold) for an equivalent pool of stabilized HIF-1α. However, no induction of the mRNA levels of PGK1 or BNIP3 was observed under these conditions. These experiments conducted twice gave the same results. Ablation of FIH had even a slight inhibitory action on the expression of BNIP3. Because PHD3 is induced dually by HIF-1α and HIF-2α, we repeated the above experiments in conditions were HIF-2α was ablated by siRNA. PHD3 expression remained sensitive to the expression of FIH (data not shown). We therefore conclude that HIF-1-dependent genes could have different sensitivities to FIH expression leading to two different groups: *phd3* and ca9, the FIH-inhibited genes, and *bnip3* and *pgk1,* the non-FIH-inhibited genes. These results clearly indicate different actions for N-TAD and C-TAD.

Cell engineering for Tet-induced modulation of expression of FIH (silencing/overexpression) in the adenocarcinoma LS174Tr cell line. We showed in the previous section that the N-TAD and C-TAD of HIF-1α in HeLa cells have a different effect on hypoxia-induced genes. It was important to see whether this finding could be generalized in a different cellular context. We chose the adenocarcinoma LS174Tr cell line for two reasons. First, this cell line was suited for Tet-inducible expression of shRNA expressed with the pTer vector (22). Second, the LS174Tr cells express only the HIF-1α subunit. Indeed, in contrast to HeLa cells, HIF-2α expression measured by either immunoblotting or q-PCR, following exposure of LS174Tr for 24 hours of 1% oxygen, was negative (data not shown). In addition, because this cell line expresses a Tet repressor, we engineered two stable clones expressing either pFIH (clone LS+) or shRNA-FIH (clone LS−) plasmids. Figure 4*A* (*left*) shows that, in response to Tet, 3- to 4-fold overexpression of FIH was observed in normoxia or hypoxia in clone LS+. Figure 4*A* (*right*) shows that, in clone LS−, Tet silenced by 70% the endogenous expression of FIH. We first explored the functionality of this inducible system by monitoring HIF-1α activity using the sensitive hypoxia reporter gene vector (p3HRE-Δptk-LUC). Figure 4*B* shows the effect of overexpression and silencing of FIH expression on the luciferase activity after 2.5 days of Tet treatment in normoxia. As expected, we observed a reproducible almost complete inhibition of the luciferase activity when FIH was overexpressed and a 3.7-fold induction of the luciferase activity after silencing of FIH. These results clearly show (*a*) the efficiency of the inducible system for modulation of FIH expression and (*b*) that endogenous FIH exerts a constant negative control on the hypoxia-induced genes in normoxic conditions.

Two groups of genes with different sensitivities to FIH expression in the LS174Tr cell line. To evaluate the contribution of the HIF-1α N-TAD and C-TAD on gene expression in the LS174 system (LS+ and LS− clones), it was preferable to stabilize HIF-1α in normoxic conditions. Silencing PHD2 by up to 70% (mRNA and protein levels) in LS174Tr with inducible shRNA was not able to trigger HIF-1α stabilization in a satisfactory way (very slight increase compared with HeLa cells). Thus, we used an alternative strategy to increase the endogenous pool of HIF-1α. Cell density has been shown to induce HIF-1α stabilization under so-called normoxic conditions or more precisely pericellular hypoxia (29). Nevertheless, to verify if increasing cell density affects the level of HIF-1α protein in LS+ and LS− stable clones, cells were plated at very low density ($1 \times 10^4/cm^2$), low density ($4.2 \times 10^4/cm^2$), medium density ($12.5 \times 10^4/cm^2$), and high density ($25 \times 10^4/cm^2$) conditions. As shown in Fig. 4*C* and *E*,

Figure 4. Differential effect of the endogenous N-TAD and C-TAD activities in LS+ and LS− Tet-inducible stable cells in normoxia. *A,* characterization of the LS+ and LS− Tet-inducible clones. Cells were incubated in the absence (−) or presence (+) of Tet (10 μg/mL) for 4 days and maintained in either normoxia (*Norm.*; 21% O_2) or hypoxia (*Hyp.*; 1.5% O_2) for the last 24 hours. Total cellular extracts were analyzed by immunoblotting with antibodies against HIF-1α, FIH, and p42MAPK. The latter was used as a loading control. *B,* luciferase activity of LS+ and LS− Tet-inducible clones. Reporter vector (300 ng; p3HRE-Δptk-LUC) was transfected into cells of the LS+ and LS− Tet-inducible clones. Plasmid expressing β-galactosidase (100 ng) was cotransfected to normalize for the transfection efficiency. Tet was added on the day of transfection, and the luciferase activity was measured 72 hours after transfection. *Columns,* mean of three independent experiments done in triplicate; *bars,* SD. *C,* comparisons of different pools of HIF-1α when FIH is overexpressed. LS+ inducible stable cells were grown to different cell densities [4.2×10^4 (low), 12.5×10^4 (medium), and 25×10^4 (high) cells/cm^2] to increase the pool of HIF-1α. Cells were then incubated in the absence (*−Tet*) or presence (*+Tet*) of Tet (10 μg/mL) for 2 days before testing different cell density conditions. Total cellular extracts were examined by immunoblotting with antibodies against HIF-1α, FIH, and p42MAPK. The latter was used as a loading control. *D,* expression of the mRNA levels of CA9, PHD3, PGK1, and BNIP3 in the LS+ clone. The mRNA levels were determined by real-time q-PCR in the stable LS+ inducible pFIH clone, in which FIH was overexpressed in the presence of Tet as described above (*C*). Cells were cultured in the absence (♦) or presence (○) of Tet (10 μg/mL) for 2 days before testing different cell density conditions. *Arrow,* action of FIH overexpression (*+FIH*). Induction of the mRNA of each gene was done first. *E,* comparisons of different pools of HIF-1α when FIH was silenced (LS−). Cells were cultured in the absence or presence of Tet (10 μg/mL) for 11 days before testing different cell densities. Cells from the Tet-inducible clone of LS− cells were grown to different cell densities [4.2×10^4 (low), 12.5×10^4 (medium), and 25×10^4 (high) cells/cm^2] cm^2] for 24 hours to obtain different pools of HIF-1α. Total cellular extracts were examined by immunoblotting with antibodies against HIF-1α, FIH, and p42MAPK. The latter was used as a loading control. *F,* expression of the mRNA levels of CA9, PHD3, PGK1, and BNIP3 in the LS− clone. The mRNA was determined by real-time q-PCR of the Tet-inducible stable clone of LS- cells, in which FIH was silenced as described above (Fig. 4*E*). Cells were cultured in the absence (♦) or presence (○) of Tet (10 μg/mL) for 11 days before testing different cell density conditions. *Arrow,* action of FIH silencing (*−FIH*). *D* and *F,* experiments were done in duplicate at least thrice. Results from one representative experiment. Each difference in gene expression was estimated within the limits of a 95% CI.

the HIF-1α protein level increased in a density-dependent manner in normoxia. Cells ($1 \times 10^4/cm^2$) were used as control, in which the basal expression of HIF-1α was not detectable (data not shown). We were able to increase the pool of endogenous HIF-1α and further investigate the effect of FIH modulation on the HIF-1α transcriptional activity. Thus, the effect in regulating the levels of CA9, PHD3, PGK1, and BNIP3 was evaluated in LS174Tr cells. The stable LS+ clone expressing pFIH was plated at different densities (1×10^4, 4.2×10^4, 12.5×10^4, and 25×10^4 cells/cm^2) for 24 hours in the absence or presence of Tet. As shown in Fig. 4*C*, the amount of HIF-1α protein increased in both conditions (−/+ Tet), showing a slightly higher induction in the presence of Tet at 25×10^4 cells/cm^2. In parallel, a 3-fold overexpression of FIH was observed in the presence of Tet. The effect of FIH overexpression on the mRNA levels of CA9, PHD3, PGK1, and BNIP3 was examined (Fig. 4*D*). Low-density conditions showed no difference in the mRNA levels of the four genes treated with Tet (+Tet) or without Tet (−Tet). However, differences appeared under medium- and high-density conditions. The mRNA levels of CA9 and PHD3 in the presence of Tet were inhibited reproducibly (30% and 50%, respectively) compared with that of cells in the absence of Tet. However, as observed in HeLa cells, no inhibition in mRNA levels of PGK1 was seen. On the contrary, BNIP3 mRNA level was slightly increased (14-20%) when FIH was overexpressed. Taken together, these results clearly confirm the existence of two groups of genes, one group sensitive to FIH overexpression blocking the HIF-1α C-TAD activity (*ca9* and *phd3* genes) and one group insensitive to the blockage of the HIF-1α C-TAD activity (*pgk1* and *bnip3* genes). Interestingly, the mRNA level of BNIP3 is reproducibly "derepressed" by FIH overexpression, suggesting the existence of a particular subgroup in the non-FIH-inhibited group to modulation of FIH expression.

The stable LS− clone expressing the shRNA-FIH was then plated at four densities (1×10^4, 4.2×10^4, 12.5×10^4, and 25×10^4 10^4 cells/cm^2) for 24 hours in the absence or presence of Tet. As shown in Fig. 4*E*, the HIF-1α protein level increased with increasing cell density. A slight decrease in the presence of Tet at 25×10^4 cells/cm^2 was observed. Total silencing of FIH expression was observed in the presence of Tet. Note that to obtain full ablation of FIH, a rather stable protein, LS− cells were pregrown for a week in the presence of Tet before starting the experiment. The effect of FIH silencing on the mRNA levels of four genes was then examined (Fig. 4*F*). Low-density conditions showed no significant differences in the mRNA levels of CA9, PHD3, PGK1, and BNIP3 of treated (+Tet) or untreated (−Tet) cells. However, differences in the mRNA levels of these genes appeared at medium and high density. As observed for HeLa cells (Fig. 3*C*), the mRNA levels of CA9 and PHD3 of treated cells (+Tet) were reproducibly induced (30% and 80%, respectively) compared with untreated cells (Fig. 4*F*). However, we observed no significant variations in the mRNA level of PGK1. As expected from the increase in the mRNA level of BNIP3 observed in FIH overexpression conditions (Fig. 4*D*), the mRNA level of BNIP3 was decreased (up to 50%) when FIH was silenced (Fig. 4*F*). Taken together, these results are in total agreement with results obtained when FIH was overexpressed. They act as a perfect mirror and clearly corroborate the existence of two groups of genes (FIH-inhibited and non-FIH-inhibited genes) with the particular case of BNIP3, which seems to be "FIH stimulated" in both HeLa and LS174Tr cells.

Differential gene expression under partial hypoxia. According to Stolze et al., FIH activity persists at a low oxygen tension (1% O_2) but also in quite severe hypoxia (0.2% O_2) yet only when FIH was overexpressed (30). Based on these results, we decided to increase the endogenous pool of HIF-1α under two different oxygen tensions: 9% and 3%. Under such conditions and according to the differential affinities for O_2 of the two hydroxylases, we could anticipate that the PHD2 activity will gradually be affected leading to HIF-1α stabilization, whereas FIH will retain high activity.

The effect of FIH overexpression (LS+, stable pFIH clone +Tet) and silencing of FIH (LS−, stable shRNA-FIH clone +Tet) on gene expression was then examined. Figure 5 shows the effect of FIH overexpression and silencing of FIH expression on the mRNA levels of CA9, PHD3, PGK1, and BNIP3 following two concentrations of O_2: 9% and 3%. All the studied genes maintained the same behavior (Fig. 5), as observed before, when HIF-1 was stabilized by either silencing of PHD2 in the HeLa cell model (Fig. 3*C*) or increasing the cell density (Fig. 4). The "FIH-dependent" effect observed on the mRNA level of BNIP3 was still observed under these conditions. We were still able to identify the same two groups of genes: FIH-inhibited and non-FIH-inhibited genes.

We broadened our study to a series of 80 HIF-sensitive genes (31). Among these genes, 26 revealed to be expressed and hypoxia induced in the LS174 cell type (Table 1). We analyzed the effect of FIH on our LS clones at 3% oxygen concentration. We then classified these genes in two groups: the FIH-inhibited genes, as expected, induced by the release of the C-TAD, such as *ca9* and *phd3*, and the non-FIH-inhibited genes, which showed no increase with the release of the C-TAD, such as *pgk1* and *bnip3*. This classification has been made in two steps. First, we separated our list in two groups according to the response of the LS− clone. The frontier of FIH-inhibited genes has been arbitrarily fixed to an increase of >25% following the silencing of FIH. We then confronted these two categories with the LS+ clone response. We obtained a coherent classification shown in Table 1. On the one hand, 16 genes, including *glut1, nix, rtp801*, and *vegf*, were indeed sensitive to the FIH-inhibiting effect. On the other hand, 10 genes, including *pgk1, gpi, hgtd-p, cathepsinD, eno1, gapdh*, and *igfbp2*, were not inhibited by the FIH enzyme. Underline the particular behavior of *bnip3* and *pfkl*, which are in the non-FIH-inhibited group: they seem to be even induced by this factor. They belong to a special subgroup of genes acting the opposite way of the expected classic C-TAD inducibility. At last, the classification of transferrin receptor was more challenging because this gene was inhibited by both overexpression and silencing of FIH.

Discussion

Few transcription factors possess two TADs. However, this is the case for estrogen receptor-α, Fos, MITF, retinoic acid receptor-γ, HIF-1α, and HIF-2α (32). Moreover, the functional consequences of two TADs are totally unknown, and as such, they have been considered as redundant. The α subunit of the factor HIF exists in three forms: HIF-1, HIF-2, and HIF-3. HIF-1α and HIF-2α contain a C-TAD and a N-TAD, respectively. However, HIF-3α does not have a C-TAD, which suggests that it possesses a simpler form of regulation of its function. Thus,

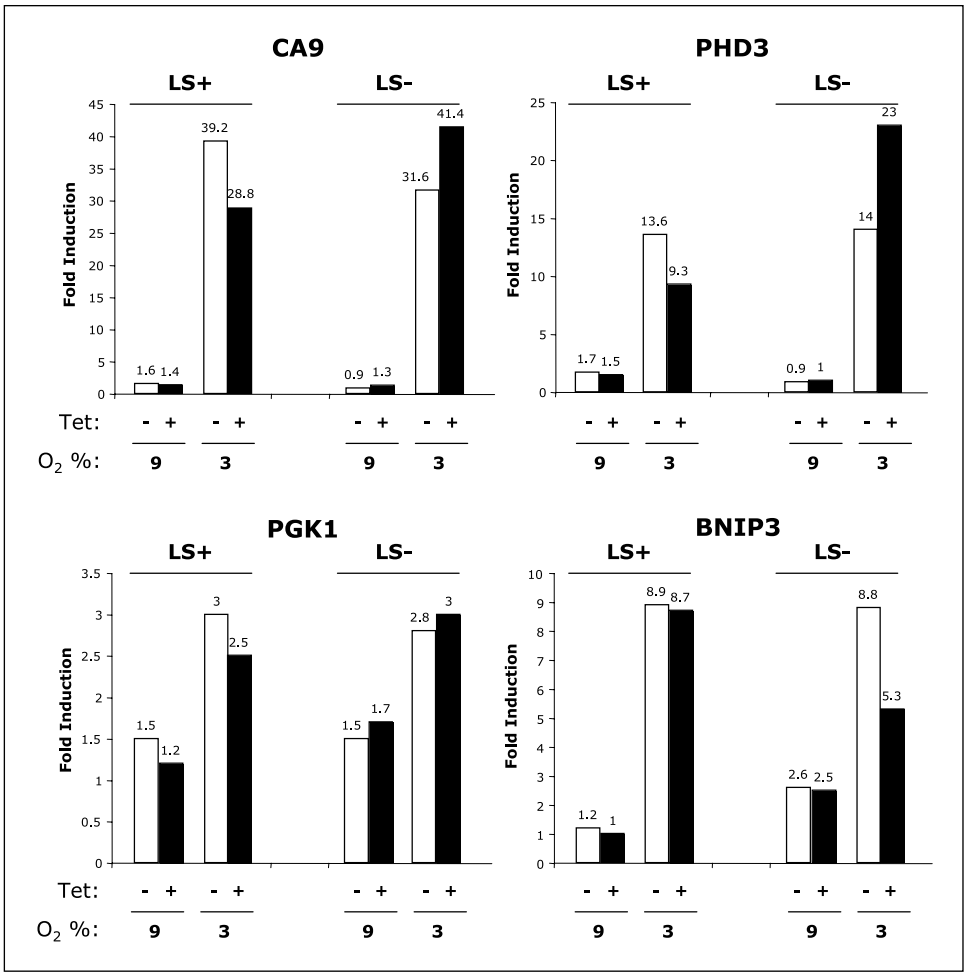


Figure 5. Differential effect of the endogenous N-TAD and C-TAD activities in LS+ and LS− Tet-inducible cells in the presence of 9% and 3% O_2. Expression of the mRNA levels of CA9, PHD3, PGK1, and BNIP3 determined by real-time q-PCR in the Tet-inducible stable LS+ and LS− clones in 9% and 3% O_2. Cells from the Tet-inducible LS+ and LS− clones were grown at 4.2×10^4 cells/cm^2 (low density) in the absence (−) or presence (+) of Tet (10 μg/mL) for 2 and 11 days, respectively, before testing different hypoxic conditions (24 hours, 9% and 3% O_2). Experiments were done in duplicate at least thrice. Results from one representative experiment. Each difference in gene expression was estimated within the limits of a 95% CI.

we questioned the role of these two domains in downstream gene regulation. We first compared the protein sequences of the N-TAD and C-TAD of HIF-1α and found no more than 20% similarity. We then compared the conservation of each TAD of HIF-1α in different species. The N-TAD is highly conserved (>90 %) in *Homo sapiens, Bos taurus, Mus musculus, Rattus norvegicus*, and *Gallus gallus*. The human N-TAD protein sequences of HIF-2α and HIF-3α share 65% and 60% identity with human HIF-1α, respectively. The C-TAD of HIF-1α shows even more interspecies conservation with almost 100% conservation for the above species, suggesting an important role of this domain in its transcriptional function. The human HIF-1α C-TAD is also extremely conserved when compared with human HIF-2α C-TAD (>70 %).

Why does a tightly regulated protein, such HIF-1α, have two TADs? Because FIH exerts only its "repressive" control on the HIF-1α C-TAD and that differential K_m for the two oxygen sensors, PHDs and FIH, have been reported (16), we propose a working model considering the microenvironment of a blood vessel (Fig. 6). According to this model, in areas close to blood vessels where tumor cells are well oxygenated, the fully active PHDs and FIH will lead to maximal degradation and inactivation of HIF-1α (Fig. 6). Down the oxygen gradient, the progressive decrease in the oxygen tension will first inactivate the PHDs resulting in progressive stabilization of the HIF-1α protein, "exposing" N-TAD transcriptional activity, whereas the C-TAD will still remain "repressed" by the active FIH. With a further decrease in the oxygen level, total inhibition of the activity of both sensors will lead to complete stabilization of HIF-1α and a total release of the C-TAD by enabling interaction with cofactors. As reported in Fig. 6, full release of the C-TAD will occur only under drastic hypoxic conditions when FIH is totally blocked as shown by Stolze et al. (30). Our present findings support this model where expression of genes sensitive to the N-TAD activity, such as *bnip3*, will be induced in moderate hypoxia, whereas more severe hypoxia will lead to activation of a family of N-TAD- and C-TAD-sensitive genes.

Using siRNA targeting PHD2 and FIH or a vector expressing FIH in a luciferase-HRE assay, we were able to examine either the full N-TAD or N/C-TAD activities. HIF-1α stabilized with 2 nmol/L siRNA to PHD2 in combination with overexpressed FIH mimics moderate hypoxic conditions and thus reflects only the N-TAD activity. Under these conditions, a 4.6-fold induction of the luciferase activity compared with normoxic conditions was obtained. However, in combination with 2 nmol/L siRNA to FIH, which then mimics total inhibition of both oxygen sensors, 34.2-fold induction was obtained. These results uncovered the potential of the N-TAD to affect gene expression and confirm the predominant role of the C-TAD, which gives six times more activity when both N-TAD and C-TAD are fully released in the context of this sensitive artificial construct.

What will be the behavior of HIF-1-dependent genes using this strategy? Two clear groups of gene family were brought to the light using this model system: one sensitive to the modulation of FIH silencing (*ca9* and *phd3*) and one insensitive to FIH silencing (*pgk1*). However, a special case of sensitive but repressed genes to FIH silencing was identified for the *bnip3* gene. Our results are strengthened by the fact that a similar profile of induction was obtained in different cellular backgrounds. A comparable classification of genes can be made after examination of HeLa cells (data not shown), HeLa cells with a 70% decrease in FIH expression (stable shRNA pSUPER-FIH clone) and LS174Tr cells (the pattern of gene expression of the LS− cells is the inverse image of that of the LS+ cells). Classification of gene expression obtained after stabilization of HIF-1α using various oxygen concentrations (9% and 3% O_2) in the absence or presence of Tet, which modulates FIH expression, totally corroborated with the classification achieved with a gradient of cell density, also used to stabilize HIF-1α. Most genes were positively sensitive to FIH silencing, suggesting that expression of these genes will be increased along the oxygen gradient to very low oxygen conditions. Genes, such as *phd3, nix*, and *rtp801* (also known as *redd1*), responded to the release of the C-TAD activity with a 92%, 66%, and 105% increase in expression, respectively, and to a blockage of the C-TAD activity with a 52%, 51%, and 35% decrease in expression, respectively. However, some genes showed a particular behavior. For example, *plaur*, the urokinase-type plasminogene activator receptor and a key molecule in the regulation of cell-surface plasminogen activation, was very sensitive to the release of the C-TAD with a 129% increase in expression, whereas only a 5% decrease in gene expression was obtained when the C-TAD was repressed. This result suggests that a subgroup of genes exists that are hypersensitive to FIH in comparison with *phd3, ca9*, or even *nix*. Indeed, the endogenous FIH activity seems to be sufficient to maintain the expression of these genes at their basal level, and only FIH silencing reveals the full expression of this subgroup. This group of genes could simply reflect, for expression, a strict requirement for p300/CBP. The classification of insensitive genes to FIH expression was more straightforward. We reproducibly observed no effect on *pgk1, gapdh*, or *igfbp2* expression when FIH expression was modulated either up or down, giving a group of genes that will be more expressed in

Table 1. Differential gene expression as a function of the level of FIH expression

	Gene name	Sequence accession IDs	Fold induction (0.2% O_2)	Fold induction (3% O_2)	Value relative to 100 in LS−*	Value relative to 100 in LS+†
FIH-inhibited genes	*phd3*	NM_022073	× 327	× 13.7	192	48
	ca9	NM_001216	× 102	× 23.2	134	77
	glut1	AY034633	× 27	× 5	125	76
	nix (*bnip3l*)	NM_004331	× 22.5	× 2.5	166	49
	rtp801	AF335324	× 21.5	× 2.3	205	65
	hk2	NM_000189	× 15.8	× 3.4	131	55
	krt19	NM_002276	× 14	× 1.8	181	45
	vegf	NM_003376	× 10.7	× 2.8	147	56
	cyclin G2	NM_004354	× 8.4	× 1.6	149	62
	ldha	NM_005566	× 7.9	× 2	164	65
	adrenomedullin	NM_001124	× 4.7	× 1.4	151	64
	pparγ	NM_138712	× 3	× 0.7	209	83
	trefoil factor 3	NM_003226	× 2	× 1.2	150	73
	cited2	NM_006079	× 7.2	× 1.7	188	90
	plaur	NM_001005377	× 4.8	× 1.2	229	95
	p21	BC001935	× 2.7	× 1.1	210	112
Non-FIH-inhibited genes	*pgk1*	NM_000291	× 6.8	× 2.4	110	93
	gpi	NM_000175	× 4.2	× 2.2	111	104
	hgtd-p	NM_014367	× 3.3	× 1.4	110	83
	cathepsinD	NM_001909	× 3.1	× 1.9	110	97
	eno1	NM_001428	× 2.7	× 1.8	94	93
	gapdh	BC029340	× 1.7	× 2	100	100
	igfbp2	NM_000597	× 1.4	× 1.3	107	114
	transferrin receptor	NM_003234	× 2.8	× 1.5	59	50
	bnip3	NM_004052	× 13.2	× 5.6	51	120
	pfkl	NM_002626	× 2.2	× 2.2	82	107
No induction	*phd1*	NM_080732	× 1	× 1	100	100

NOTE: Expression of the mRNA levels of 26 HIF target genes determined by real-time q-PCR in stable Tet-inducible LS+ and LS− clones. The maximal induction of the mRNA of each gene was first determined by comparing the levels in normoxia and hypoxia (0.2% O_2, 24 hours) using the Tet-inducible LS− clone. The gene expression profile was then examined in the LS+ and LS− clones incubated at 3% O_2 for 24 hours. The value relative to 100 for each gene was obtained from the ratio of the expression in the presence or absence of Tet [10 μg/mL for 2 days (LS+) or 11 days (LS−)].

*Ablation of FIH.

†Overexpression of FIH.

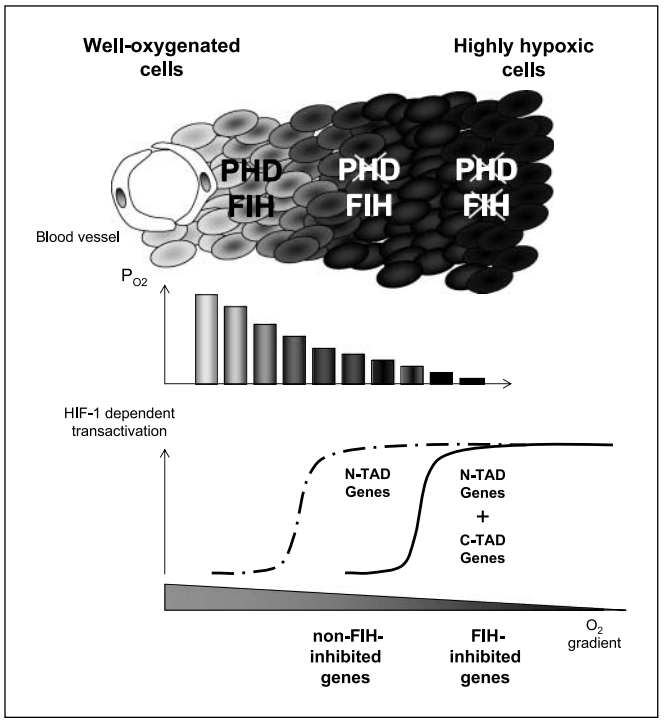


Figure 6. Two TADS of HIF-1α differentially regulate gene expression: a model of gene expression along the oxygen gradient within a solid tumor. Studies into the K_m of the two oxygen sensors, PHD and FIH, indicate that the PHDs require higher oxygen levels than FIH for activity (16). Based on this study, we postulate that, in areas near blood vessels, where cells are maximally oxygenated, both PHD and FIH will be active. Under these conditions, only minimal, basal normoxic levels of HIF-1α will exist but will be inactive. As the cells are distanced from the blood vessels, a progressive drop in the oxygen pressure will occur with stabilization of HIF-1α but maintenance of the lock on the C-TAD by FIH. However, if certain genes are solely driven by the N-TAD (i.e., they do not require the C-TAD), these genes will become activated at the intermediate oxygen level. As the oxygen pressure drops even further, the genes requiring the C-TAD, together with or without the N-TAD, will be induced. Based on this concept, we classified 26 HIF target genes, the expression of which was quantified by real-time PCR, according to their dependence on the C-TAD. Overexpression or silencing of FIH allowed us to identify two groups of genes that are non-FIH and FIH inhibited, respectively, and thus C-TAD independent and C-TAD dependent.

moderate hypoxic conditions where the PHDs are inactivated and FIH is still active. However, transferrin receptor gene expression was at the threshold of insensitive and sensitive to FIH modulation. Surprisingly, a subgroup of genes that were differently sensitive to FIH silencing was observed. Expressions of genes, such as *bnip3* or *pfkl*, were reproducibly repressed when the C-TAD activity was released (49% and 18% decrease in expression, respectively), whereas the individual N-TAD activity revealed their full expression. One hypothesis is that these genes, *bnip3* and *pfkl*, show a bell-shaped induction with an apparent maximum when only the N-TAD is active. We propose that a HIF-dependent gene product, yet to be identify and strongly induced with FIH inactivation (low pO_2), acts as a specific repressor of *bnip3* and *pfkl* genes in a feedback loop. The finding that the proapoptotic gene product BNIP3 is so easily induced at moderate hypoxia, where HIF-1 activation induces cell survival, is rather intriguing. The current hypothesis is that BNIP3, at moderate hypoxia, is cryptic and becomes active under low intracellular pH (21), a condition encountered in severe hypoxic areas in the tumor microenvironment.

In this article, we questioned not only the role of the two TADs in downstream gene regulation of HIF-1α but also the role and the prevalence of FIH in this regulation. We clearly show that both TADs, N-TAD and C-TAD, have their own function. Moreover, FIH does not act in the shadow of the PHDs by only controlling the level of gene expression. FIH definitively controls a spectrum of gene expression that implicates further fine-tuning of HIF-1α regulation, suggesting that FIH has newly acquired an elevated rank in the post-translational modifications of HIF-1α. Our results suggest that specific FIH inhibitors would modify completely the profile of gene expression, which could lead to destabilize tumor growth. Thus, FIH is certainly a potential new target that affects numerous cellular events involved in hypoxic stress adaptation and cancer progression.

Acknowledgments

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