

The Hypoxia-Inducible Factor 2 α N-Terminal and C-Terminal Transactivation Domains Cooperate To Promote Renal Tumorigenesis In Vivo^{∇†}

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Hypoxia-inducible factor (HIF) is a heterodimeric transcription factor, consisting of an alpha subunit and a beta subunit, that controls cellular responses to hypoxia. HIF α contains two transcriptional activation domains called the N-terminal transactivation domain (NTAD) and the C-terminal transactivation domain (CTAD). HIF α is destabilized by prolyl hydroxylation catalyzed by EglN family members. In addition, CTAD function is inhibited by asparagine hydroxylation catalyzed by FIH1. Both hydroxylation reactions are linked to oxygen availability. The von Hippel-Lindau tumor suppressor protein (pVHL) is frequently mutated in kidney cancer and is part of the ubiquitin ligase complex that targets prolyl hydroxylated HIF α for destruction. Recent studies suggest that HIF2 α plays an especially important role in promoting tumor formation by pVHL-defective renal carcinoma cells among the three HIF α paralogs. Here we dissected the relative contribution of the two HIF2 α transactivation domains to hypoxic gene activation and renal carcinogenesis and investigated the regulation of the HIF2 α CTAD by FIH1. We found that the HIF2 α NTAD is capable of activating both artificial and naturally occurring HIF-responsive promoters in the absence of the CTAD. Moreover, we found that the HIF2 α CTAD, in contrast to the HIF1 α CTAD, is relatively resistant to the inhibitory effects of FIH1 under normoxic conditions and that, perhaps as a result, both the NTAD and CTAD cooperate to promote renal carcinogenesis in vivo.

HIF (hypoxia-inducible factor) is a master transcriptional regulator of hypoxia-inducible genes and is composed of the α subunit HIF1 α , or one of its paralogs HIF2 α or HIF3 α , and a HIF β subunit, such as HIF1 β (also called the aryl hydrocarbon receptor nuclear translocator [ARNT]) (12, 14, 17, 20, 50, 53). Whereas HIF1 β is constitutively present, the HIF α members are highly unstable except under low-oxygen conditions (hypoxia). Under hypoxic conditions, the HIF α subunits accumulate, bind to a HIF β subunit, and transcriptionally activate hypoxia-inducible genes bearing canonical HIF DNA-binding sites called hypoxia-responsive elements (HRE). These genes include genes that control angiogenesis (such as vascular endothelial growth factor [VEGF]), energy metabolism (for example, the glucose transporter 1 [GLUT1] and carbonic anhydrase IX [CAIX]), erythropoiesis (such as erythropoietin [EPO]), and mitogenesis (for example, transforming growth factor α and platelet-derived growth factor B) (18, 49). In the presence of oxygen, HIF α proteins are hydroxylated on conserved prolyl residues by members of the egg-laying-defective nine (EglN) prolyl hydroxylases (also called PHD or HPH hydroxylases) (2, 13, 26–28, 55). Prolyl hydroxylation of HIF α creates a binding site for the von Hippel-Lindau (VHL) ubiquitin ligase complex, which then targets HIF α for polyubiq-

uitination and proteasomal degradation (30). In cells lacking functional pVHL, HIF α is not degraded properly, leading to activation of HIF target genes. More than half of clear cell renal cell carcinomas (RCC) are pVHL-defective as a result of VHL mutations or hypermethylation of the VHL locus (30). Accordingly, overproduction of HIF, and its downstream targets, are hallmarks of this disease.

HIF2 α , also known as EPAS1, HLF, HRF, or MOP2, shares 48% overall identity with HIF1 α (12, 14, 20, 53). Both HIF1 α and HIF2 α are capable of regulating a variety of broadly expressed hypoxia-inducible genes (21). Recent studies suggest that HIF2 α is more important for renal carcinogenesis than HIF1 α (4, 33, 40, 41). For example, tumor suppression by pVHL in RCC can be overridden by an HIF2 α variant that escapes control by pVHL but not by a similar HIF1 α variant (33, 41). Conversely, elimination of HIF2 α in *VHL*^{-/-} RCC cells is sufficient to suppress their ability to form tumors in vivo (32, 56). Therefore, inhibition of HIF2 α is both necessary and sufficient for pVHL to suppress tumor formation by RCC. The importance of HIF2 α is also suggested by careful analysis of preneoplastic lesions arising from the kidneys of patients who carry germ line *VHL* mutations (VHL disease) (40). In this setting, there is an apparent switch from HIF1 α to HIF2 α in association with increased cellular atypia.

HIF α contains two transcriptional activation domains: the N-terminal transactivation domain (NTAD) and the C-terminal transactivation domain (CTAD) (11, 29, 42, 43, 46, 48). In addition to inducing HIF α stability, hypoxia promotes the interaction of the HIF α CTAD with coactivators such as p300/CBP and allows HIF to recruit a larger transcriptional appa-

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ratus to hypoxia responsive genes (8, 11). During normoxic conditions, this interaction between p300/CBP and HIF α CTAD is blocked when a conserved asparaginyl residue within the CTAD is hydroxylated by factor inhibiting HIF1 (FIH1) (19, 37, 39). This modification inhibits HIF α CTAD activity by sterically blocking the interaction between HIF α and p300/CBP (5, 6, 9, 15, 19, 36, 38). Like the EglN prolyl hydroxylases, FIH1 is an Fe(II) and 2-oxoglutarate-dependent dioxygenase, and its activity can be blocked with competitive inhibitors of 2-oxoglutarate and with iron chelators (19, 36). Downregulation of FIH1 expression with small interfering RNA leads to activation of HIF target genes, while FIH1 overexpression leads to suppression of these genes (7, 51). In summary, HIF is regulated at the level of protein turnover by prolyl hydroxylation and at the level of coactivator recruitment by asparaginyl hydroxylation.

The role of HIF in tumor development is not restricted to *VHL*^{-/-} cells. Most solid tumors contain hypoxic regions in which HIF α is stabilized and active, as determined by the induction of HIF target genes that promote survival in a hypoxia environment (18). In most but not all models, genetic disruption of HIF1 α or its partner, ARNT1, leads to decreased tumor formation in vivo (44, 47, 52), as does blocking the interaction of HIF α with the coactivator proteins p300/CBP (34, 35).

The fact that HIF target genes are activated upon pVHL loss under normoxic conditions (24) creates a paradox if, however, the HIF α CTAD is inactivated by FIH1 in the presence of oxygen. Clearly, one explanation would be that activation of HIF target genes in this setting is solely due to the HIF α NTAD. In this regard, a naturally occurring HIF1 α splice variant that encodes the NTAD, but not the CTAD, is capable of activating at least some HIF target genes (16). To address the relative contribution of HIF2 α NTAD and CTAD to hypoxic gene activation and tumor growth, we created a panel of HIF2 α mutants in which either the NTAD, the CTAD, or both were altered. Comparison of these mutants showed that the NTAD is indeed sufficient to transcriptionally activate a variety of HIF-responsive promoters. We also discovered that the HIF2 α CTAD, in contrast to the HIF1 α CTAD, is relatively resistant to FIH1 and cooperates with the HIF2 α NTAD to promote tumor growth in vivo.

MATERIALS AND METHODS

Plasmids. pcDNA3.0-HA-HIF2 α P405A/P531A/N847A was generated from pcDNA3.0-HA-HIF2 α P405A/P531A plasmid (32) by site-directed mutagenesis using sense primer A (GACTGTGAGGTGCCCGTGGCTGG) and the QuikChange site-directed mutagenesis kit (Stratagene). The HIF2 α P405A/P531A/N847A cDNA was excised by digestion with BamHI and MfeI and ligated into the pBABE-puro-HA vector (33) cut with BamHI and EcoRI to make pBABE-puro-HA-HIF2 α P405A/P531A/N847A.

pBABE-puro-HA-HIF2 α P405A/ Δ 450-572 and pBABE-puro-HA-HIF2 α P405A/ Δ 450-572/N847A were generated by two-step PCR. pcDNA3.0-HA-HIF2 α P405A/P531A and pcDNA3.0-HA-HIF2 α P405A/P531A/N847A were first amplified with primer B (ATTGACGCAAATGGCGGTAG) and primer C (CTACAGGGGCCAGTGGGCTCTGGGTGCTGTGG) or primer D (CCA CAGCACCCAGAGCCCACTGGCCCTGTAG) and primer E (GGCAAAC AACAGATGGCTGG). Aliquots of these two PCR mixtures were then mixed and amplified with primer B and primer E. The resulting PCR product was digested with BamHI and MfeI and ligated into pBABE-puro-HA vector cut with BamHI and EcoRI. Similarly, pBABE-puro-HA-HIF2 α P405A/ Δ 450-572/ Δ 820-870 was generated by two-step PCR. pcDNA3.0-HA-HIF2 α P405A/P531A was first amplified with primer B and primer C or primer D and primer F

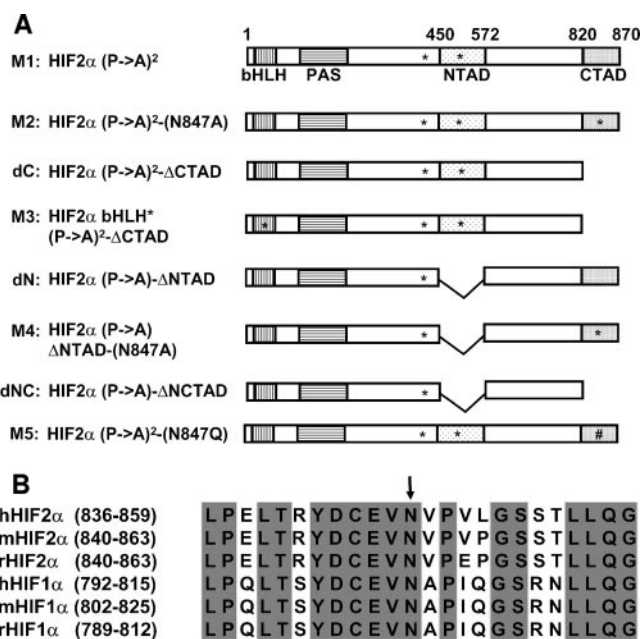


FIG. 1. HIF2 α mutants used in the study. (A) Schematic of the HA-HIF2 α mutants used in this study. The bHLH, PAS, NTAD, and CTAD domains are indicated by boxes. The bHLH mutation, Pro405-to-Ala, Pro531-to-Ala, and Asp847-to-Ala substitutions are indicated by asterisks in the corresponding regions. The Asp847-to-Gln substitution is indicated (#) in the corresponding region. (B) Alignment of the C-terminal regions of HIF1 α and HIF2 α from human (h), mouse (m), and rat (r). The fully conserved amino acids are indicated in gray, and the Asn hydroxylation site is indicated by the arrow.

(CCGGCAATTGTACACCTGTGGGCTGACGAC). Aliquots of these two PCR mixtures were then mixed and amplified with primer B and primer F. The resulting PCR product was digested with BamHI and MfeI and ligated into pBABE-puro-HA vector cut with BamHI and EcoRI.

pcDNA3.0-HA-HIF2 α P405A/P531A was PCR amplified with primers B and F, digested with BamHI and MfeI, and ligated into pBABE-puro-HA vector cut with BamHI and EcoRI to make pBABE-puro-HA-HIF2 α P405A/P531A/ Δ 820-870. pBABE-puro-HA-HIF2 α bHLH*/P405A/P531A/ Δ 820-870 was generated by two-step PCR. pcDNA3.0-HA-HIF2 α P405A/P531A was first amplified with primer B and primer G (CATAGAACACCTCCGTCTCAGCGCTAGCAGCG CAAGCCGACGATCCCGGGAC) or primer H (GTCCCGGATGCTGCG GCTTGCCTGCTAGCGCTGAGACGGAGGTGTCTATG) and primer F. Aliquots of these two PCRs were then mixed and amplified with primer B and primer F. The resulting PCR product was digested with BamHI and MfeI and ligated into pBABE-puro-HA vector cut with BamHI and EcoRI.

pcDNA3.0-HA-HIF2 α P405A/ Δ 450-572, pcDNA3.0-HA-HIF2 α P405A/ Δ 450-572/N847A, pcDNA3.0-HA-HIF2 α P405A/ Δ 450-572/ Δ 820-870, pcDNA3.0-HA-HIF2 α P405A/P531A/ Δ 820-870, and pcDNA3.0-HA-HIF2 α bHLH*/P405A/P531A/ Δ 820-870 were made by excising the cDNAs from the corresponding pBABE vectors with BamHI and BstXI and ligating them into pcDNA3-HA (33) cut with these two enzymes.

pcDNA3.0-HA-HIF2 α P405A/P531A/N847Q was made by site-directed mutagenesis of pcDNA3.0-HA-HIF2 α P405A/P531A (32) with sense primer I (G ACTGTGAGGTGCAAGTGCCCGTGG) using the QuikChange site-directed mutagenesis kit (Stratagene). pcDNA3.0-HA-HIF1 α P402A/P564A/N803A and pcDNA3.0-HA-HIF1 α P402A/P564A/N803Q were made from pcDNA3.0-HA-HIF1 α P402A/P564A using the QuikChange site-directed mutagenesis kit (Stratagene) with the primers described previously (19). All plasmids were verified by DNA sequencing.

The pGL2-VEGF promoter (VEGF-Luc) plasmid was a kind gift from Deb Mukhopadhyay (Harvard Medical School). 3 \times HRE luciferase (3 \times HRE-Luc) plasmid (a kind gift from Andrew Kung, Dana-Farber Cancer Institute) was created by inserting 3 copies of the canonical hypoxia-response element from the *EPO* gene and a minimal thymidine kinase promoter into pGL3 (Promega).

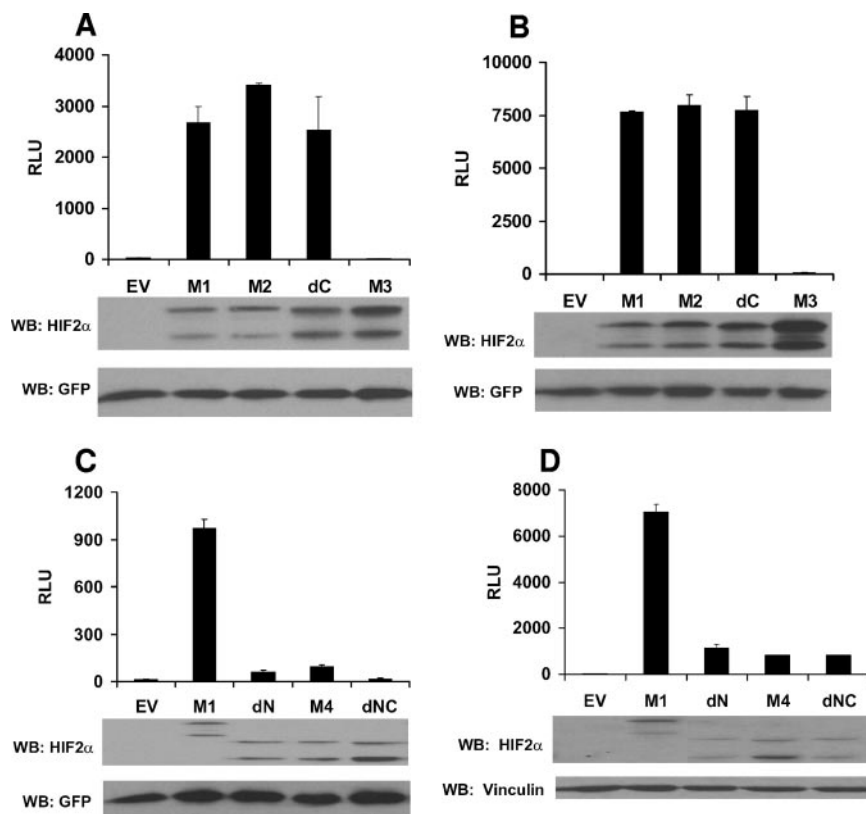


FIG. 2. Transcriptional activation by the HIF2 α mutants. Normalized firefly luciferase values of U2OS cells transiently transfected to produce the indicated HIF2 α variants along with a reporter plasmid containing firefly luciferase under the control of the VEGF promoter (VEGF-Luc) (A and C) or 3 tandem HIF-response elements and a minimal promoter (3 \times HRE-Luc) (B and D). Transfection mixes also contained plasmids encoding *Renilla* luciferase and, in some experiments, GFP for normalization purposes. Data are the averages of results from duplicate experiments \pm standard errors. Production of the HIF2 α mutants and GFP was assessed by Western blot (WB) analysis (lower panel). EV, empty vector control; RLU, relative luciferase unit.

Cell culture. 786-O and RCC4 renal carcinoma cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal clone I (HyClone) in a 37°C, 10% CO₂ incubator. 786-O renal carcinoma cell subclones stably transfected with pRc-CMV-HA-VHL (WT7) (27) were grown in DMEM containing 10% fetal clone I (HyClone) containing 1 mg/ml G418 in a 37°C, 10% CO₂ incubator. Retrovirally infected cells were selected and maintained in the presence of 1.5 μ g/ml puromycin.

Phoenix cells (a generous gift of Gary Nolan, Department of Molecular Pharmacology, Stanford University) and U2OS cells were grown in DMEM containing 10% fetal bovine serum (HyClone) in a 37°C, 10% CO₂ incubator.

Retroviruses. Retroviral plasmids were transfected into the Phoenix packaging cell line using FuGene 6 reagent (Roche Molecular Biochemicals) according to the manufacturer's instructions. Tissue culture supernatant was harvested 48 h later, passed through a 0.45- μ m filter, and added to cells in the presence of 4 μ g/ml Polybrene.

Immunoblot analysis. Cells were lysed in EBC lysis buffer (50 mM Tris [pH 8.0], 120 mM NaCl, 0.5% NP-40) supplemented with Complete protease inhibitor cocktail (Roche Molecular Biochemicals). Approximately 30 μ g of cell extract per lane, as determined by the Bradford method, was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Bio-Rad). After blocking in Tris-buffered saline with 4% nonfat milk, the membranes were probed with antihemagglutinin (anti-HA) mouse monoclonal antibody (HA.11; Covance), anti-HIF2 α mouse monoclonal antibody (NB100-132; Novus Biologicals), anti-GLUT1 rabbit polyclonal antibody (GT11-A; Alpha Diagnostic), anti-green fluorescent protein (anti-GFP) monoclonal antibody (BD Biosciences), antivinculin mouse monoclonal antibody (clone hVIN-1; Sigma-Aldrich), anti- α -tubulin mouse monoclonal antibody (clone B-5-1-2; Sigma-Aldrich), or anti-HIF α rabbit polyclonal antibody (a kind gift from Jacques Pouyssegur) diluted in Tris-buffered saline with 4% nonfat milk. Bound antibody was detected with the appropriate horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G or goat anti-mouse immunoglobulin

G (Pierce) and SuperSignal West Pico or Dura chemiluminescent substrate (Pierce) according to the manufacturer's instructions.

Luciferase assays. For transcriptional transactivation experiments, U2OS or WT7 cells were transfected in six-well plates using FuGene 6 reagent (Roche Molecular Biochemicals). At 24 h after transfection, cells were lysed and luciferase activity was measured using the dual-luciferase reporter assay system (Promega). Relative luciferase units were determined by dividing the luciferase activity of the VEGF or 3 \times HRE reporters by that of the pRL-CMV *Renilla* luciferase control reporter.

Microarray experiments. Total RNA was isolated using the RNeasy mini kit with on-column DNase digestion (QIAGEN) and processed as described previously (22) for hybridization to microarrays containing approximately 25,000 oligonucleotides. Ratio hybridizations were performed with fluorescent label reversal to eliminate dye bias. Microarrays were purchased from Agilent Technologies. The error models for data processing have been previously described (22). Data were analyzed using Rosetta Resolver version 6.0 software.

In vitro proliferation assays. Cell proliferation was measured colorimetrically using cell proliferation kit II (Roche Diagnostics) according to the manufacturer's protocol. Briefly, 2,000 cells per well were cultured in 96-well cell culture plates. At the indicated time points, sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) was added to the cells. Four hours later, the spectrophotometric absorbance at 450-nm wavelength was measured using a microtiter plate reader.

Nude mouse xenograft assays. Nude mouse xenograft assays were performed as described previously (33). In brief, cells were released by trypsinization and resuspended in phosphate-buffered saline. Viable cells (10^7), as determined by trypan blue staining, were injected subcutaneously into the flanks of Swiss nude mice. Both flanks were used for each mouse. The animals were sacrificed 5 to 6 weeks after injection. Tumors were weighed and fixed in formalin for immunohistochemical analysis.

Real-time reverse transcription-PCR. Total RNA was isolated using the RNeasy mini kit with on-column DNase digestion (QIAGEN). First-strand cDNA was generated using the StrataScript First-Strand synthesis system (Stratagene). Real-time PCR was performed in triplicate using QuantiTect SYBR green PCR master mix (QIAGEN) and the Mx3000P QPCR system (Stratagene). All values were normalized to the level of β -actin mRNA abundance. Primers specific for *actin* were actinF (CTCTTCCAGCCTTCCTCCT) and actinR (AGCACTGTGTTGGCGTACAG). Primers specific for *EglN3* were EglN3F (TCTCCCGAGAGTTGCGAGAAA) and EglN3R (AGAGGGAACG ATCTACACGAG) (54). Primers specific for *VEGF* were VEGFF (AAGGAG GAGGCAGAAATCAT) and VEGFR (CACACAGATGGCTTGAAGA). Primers specific for *Glut1* were as described previously (3). Primers specific for *CITED* were CITEDF (GGGCGAGCACATACACTACG) and CITEDR (AC CCATGAACTGGGAGTTGTTA) (54). Primers specific for *BNIP3* were BNIP3F (CAGGGCTCTGGGTAGAACT) and BNIP3R (CTCCGTCCAGA CTCATGCTG) (54). Primers specific for *ENO1* were ENO1F (CTGGTGCCG TTGAGAAGGG) and ENO1R (GGTGTGGTAAACCTCTGCTC) (54). Primers specific for *PGK1* were PGK1F (TTAAAGGGAAGCGGGTCTGTTA) and PGK1R (TCCATTGTCCAAGCAGAATTTGA) (54).

RESULTS

We showed before that an HIF2 α variant in which both prolyl hydroxylation sites (proline 405 and 531) were replaced by alanine (Fig. 1A, M1) is no longer recognized by pVHL and is therefore stable, yet it can still bind to DNA and activate transcription (32). Moreover, this variant can override pVHL's ability to inhibit tumor growth by *VHL*^{-/-} RCC in vivo (32). We mutagenized the NTAD and CTAD within this HIF2 α variant to explore the relevant contributions of these two domains to transcriptional activation and tumor promotion by HIF2 α (Fig. 1A).

The HIF1 α CTAD can be hydroxylated on asparagine 803 by FIH1. The region surrounding asparagine 803 is highly conserved across species and between HIF1 α and HIF2 α (Fig. 1B). Substitution of the corresponding asparagine of HIF2 α in the context of a Gal4-HIF2 α CTAD fusion protein leads to enhanced transcriptional activity that is no longer sensitive to changes in oxygen availability (36, 37). We created two HIF2 α mutants in which the HIF2 α CTAD was altered. One mutant lacked the CTAD because of a premature stop codon (Fig. 1A, dC). In the other mutant, Asn847 was converted to Ala (Fig. 1A, M2), which we predicted would lead to enhanced transcriptional activation capability. As an additional control, we introduced a 4-amino-acid loss-of-function mutation in the basic helix-loop-helix (bHLH) DNA binding domain of the HIF2 α dC mutant (Fig. 1A, M3).

The HIF2 α CTAD mutants were next introduced into *VHL*^{+/+} U2OS osteosarcoma cells by transient transfection in the presence of a luciferase reporter plasmid containing either the VEGF promoter (Fig. 2A) or a minimal artificial promoter containing 3 copies of the HIF-response element from the *EPO* gene (Fig. 2B). Surprisingly, neither deletion of the CTAD nor replacement of Asn847 with alanine had a marked effect on HIF2 α -dependent transcriptional activation function. The similarities between the M1, M2, and dC mutants were seen over a wide range of plasmid input concentrations (data not shown; see Fig. 7B). Transcriptional activation by the dC mutant was dependent upon DNA-binding capability because it was abolished by the HIF2 α bHLH mutation (Fig. 2A and B, M3). All of the mutants were produced at comparable levels, as determined by Western blot analysis. This suggested that the

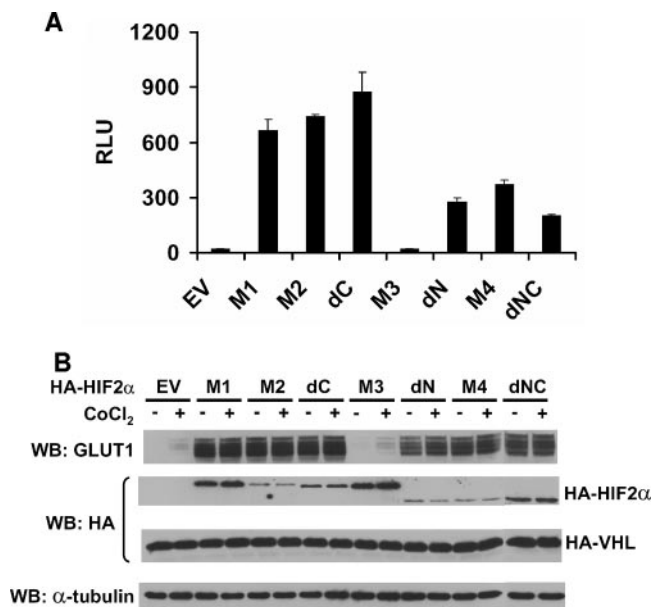


FIG. 3. Behavior of HIF2 α variants when introduced into *VHL*^{-/-} renal carcinoma cells stably transfected to produce wild-type pVHL. (A) Normalized firefly luciferase values of WT7 cells transiently transfected to produce the indicated HIF2 α variants along with a reporter plasmid containing firefly luciferase under the control of 3 tandem HIF-response elements upstream of a minimal promoter. Transfection mixes also contained plasmids encoding *Renilla* luciferase for normalization purposes. Data are the averages of results from duplicate experiments \pm standard errors. RLU, relative luciferase unit. (B) Western blot (WB) analysis of WT7 cells after stable infection with retroviruses encoding the indicated HIF2 α mutants. Where indicated, media contained 100 μ M CoCl₂ for 16 h prior to harvest. EV, empty vector control.

HIF2 α NTAD is sufficient, and the HIF2 α CTAD unnecessary, for transcriptional activation by HIF2 α .

To pursue this further, we generated an HIF2 α variant in which the residues that are essential for the NTAD function were eliminated by an in-frame deletion (Fig. 1A, dN) (11, 42). The HIF2 α dN mutant was impaired with respect to transcription activation of the VEGF (Fig. 2C) or 3 \times HRE reporters (Fig. 2D) relative to the M1 variant, suggesting that NTAD is necessary for transcriptional activation of HRE-regulated genes. A caveat in these experiments is that the NTAD deletion might affect the folding of the CTAD or its proper orientation once bound to DNA.

These results left open the possibility that the CTAD might substitute for the NTAD were it not for the repressive effects of asparagine hydroxylation. To address this, we introduced the Asn847Ala substitution into the dN mutant (Fig. 1A, M4). However, transcriptional activation by this mutant was not significantly enhanced relative to dN itself (Fig. 2C and D). Nonetheless, the HIF2 α dN variant did activate reporters weakly. To determine whether CTAD contributes to that residual activity, we combined the NTAD deletion mutation with the CTAD truncation mutation (Fig. 1A, dNC). This mutant retained weak transcriptional activation capability on the 3 \times HRE reporter but not the VEGF promoter (Fig. 2C and D). Although the significance of this finding is not clear, it suggests the presence of a cryptic transcriptional activation

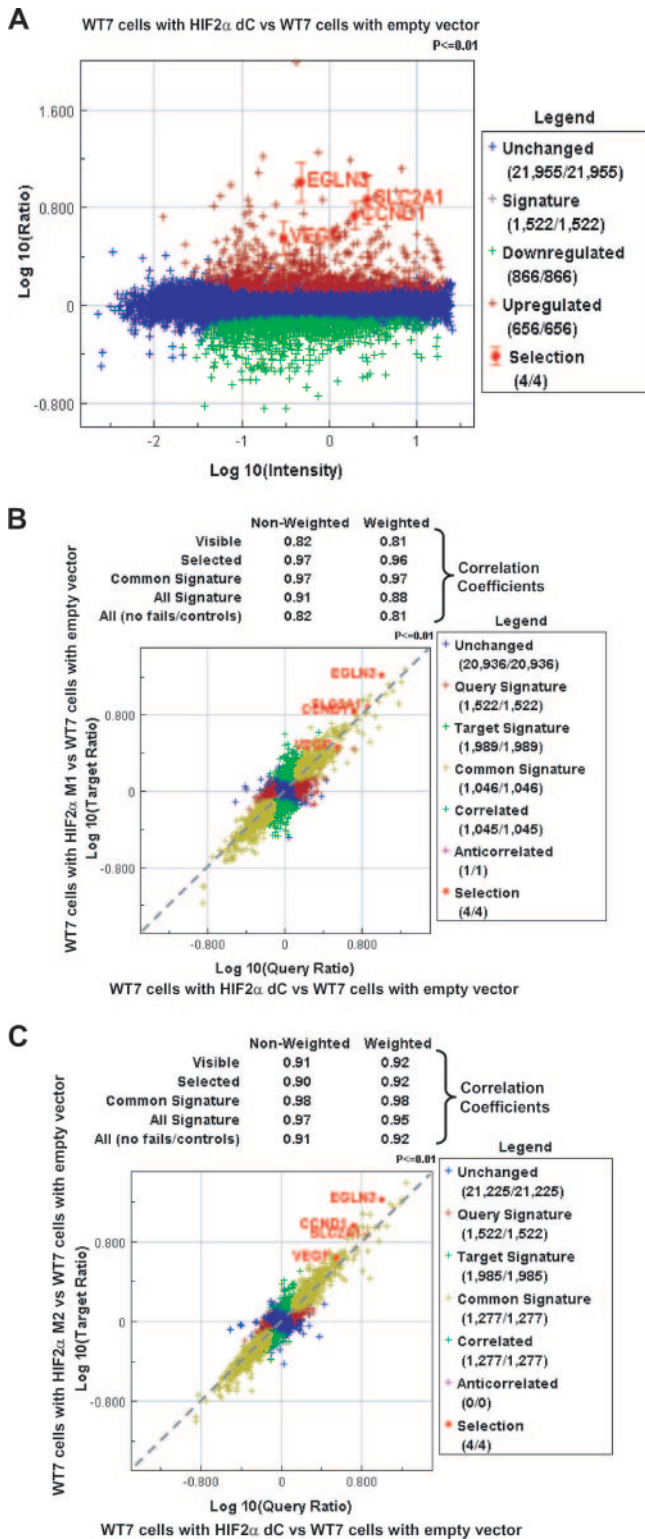


FIG. 4. The NTAD is sufficient for HIF2 α regulation of target gene expression. (A) The HIF2 α dC variant up-regulates expression of canonical HIF target genes. RNA isolated from WT7 cells expressing HIF2 α dC was compared to WT7 cells with the empty vector. Significantly up-regulated (red) or down-regulated (green) genes were determined as described previously (22). Canonical HIF target genes such as *cyclin D1* (*CCND1*), *GLUT1* (*SLC2A1*), *VEGF*, and *EglN3* were highlighted. (B) The HIF2 α dC and M1 variants up-regulate ex-

pression of canonical HIF target genes to similar levels. RNA isolated from WT7 cells expressing HIF2 α dC or M1 variants were compared to WT7 cells with the empty vector, and these two data sets were compared to each other. Genes regulated in both the HIF2 α dC and M1 experiments are yellow, those in the dC but not the M1 experiment are red, those in the M1 but not the dC experiment are green, and those in neither experiment are blue. Canonical HIF target genes such as *cyclin D1* (*CCND1*), *GLUT1* (*SLC2A1*), *VEGF*, and *EglN3* were similarly up-regulated in HIF2 α dC-expressing cells and M1-expressing cells. (C) HIF2 α dC and M2 variants up-regulate expression of canonical HIF target genes to similar levels. RNA isolated from WT7 cells expressing HIF2 α dC or M2 variants were compared to WT7 cells with the empty vector, and these two data sets were compared to each other. Genes regulated in both the dC experiment and the M2 experiment are yellow, those in the dC but not the M2 experiment are red, those in the M2 but not the dC experiment are green, and those in neither experiment are blue. Canonical HIF target genes such as *cyclin D1* (*CCND1*), *GLUT1* (*SLC2A1*), *VEGF*, and *EglN3* were similarly up-regulated in HIF2 α dC-expressing cells and M2-expressing cells.

domain within HIF2 α , or perhaps ARNT, that can contribute to the activation of certain genes. Reintroduction of wild-type pVHL into 786-O *VHL*^{-/-} cells blocks their ability to form tumors in nude mice (23). In such cells (wild-type [WT] pVHL restored), introduction of an HIF2 α M1 variant promotes tumor formation (32). Transcriptional activation by the HIF2 α variants described above after introduction into WT7 cells by transient transfection was qualitatively similar to the results obtained with U2OS cells (Fig. 3A). In WT7 cells, the amount of HIF2 α produced was below the limit of detection due to a lower transfection efficiency (data not shown).

Next, WT7 cells were infected with retroviruses encoding the HIF2 α variants or with the empty vector. Successfully infected cells were selected with puromycin and maintained as pools. In contrast to the U2OS transient-transfection assays, the levels of M2, dC, dN, and M4 were somewhat lower than that of M1 after stable infection of WT7 cells (Fig. 3B). This might, among several possibilities, reflect selection against cells producing higher levels of these variants. As expected, because of the proline-to-alanine substitutions, none of the variants was further induced by the hypoxia mimetic cobalt chloride, which inhibits EglN function (Fig. 3B). All of the variants, with the exception of the bHLH mutant M3, induced the expression of the endogenous HIF-responsive gene *GLUT1*.

To look more globally at the behavior of endogenous HIF-responsive genes, we performed microarray experiments. In keeping with the experiments described above, the HIF2 α dC variant, which lacks the CTAD, retained the ability to activate a wide variety of canonical HIF targets, including *EglN3*, *VEGF*, *cyclin D1*, and *GLUT1* (*SLC2A1*), relative to cells infected with an empty retrovirus (Fig. 4A; also see Table S1 in the supplemental material). Remarkably, the induction of HIF targets by dC was highly similar to the induction observed with the M1 variant, which contains both the NTAD and CTAD (Fig. 4B; also see Table S1 in the supplemental material). This similarity was not merely due to neutralization of the M1 CTAD by FIH1 because the dC profile was also highly similar to the profile observed with M2, which lacks the FIH1 hydroxylation site (Fig. 4C; also see Table S1 in the supplemental material).

pression of canonical HIF target genes to similar levels. RNA isolated from WT7 cells expressing HIF2 α dC or M1 variants were compared to WT7 cells with the empty vector, and these two data sets were compared to each other. Genes regulated in both the HIF2 α dC and M1 experiments are yellow, those in the dC but not the M1 experiment are red, those in the M1 but not the dC experiment are green, and those in neither experiment are blue. Canonical HIF target genes such as *cyclin D1* (*CCND1*), *GLUT1* (*SLC2A1*), *VEGF*, and *EglN3* were similarly up-regulated in HIF2 α dC-expressing cells and M1-expressing cells. (C) HIF2 α dC and M2 variants up-regulate expression of canonical HIF target genes to similar levels. RNA isolated from WT7 cells expressing HIF2 α dC or M2 variants were compared to WT7 cells with the empty vector, and these two data sets were compared to each other. Genes regulated in both the dC experiment and the M2 experiment are yellow, those in the dC but not the M2 experiment are red, those in the M2 but not the dC experiment are green, and those in neither experiment are blue. Canonical HIF target genes such as *cyclin D1* (*CCND1*), *GLUT1* (*SLC2A1*), *VEGF*, and *EglN3* were similarly up-regulated in HIF2 α dC-expressing cells and M2-expressing cells.

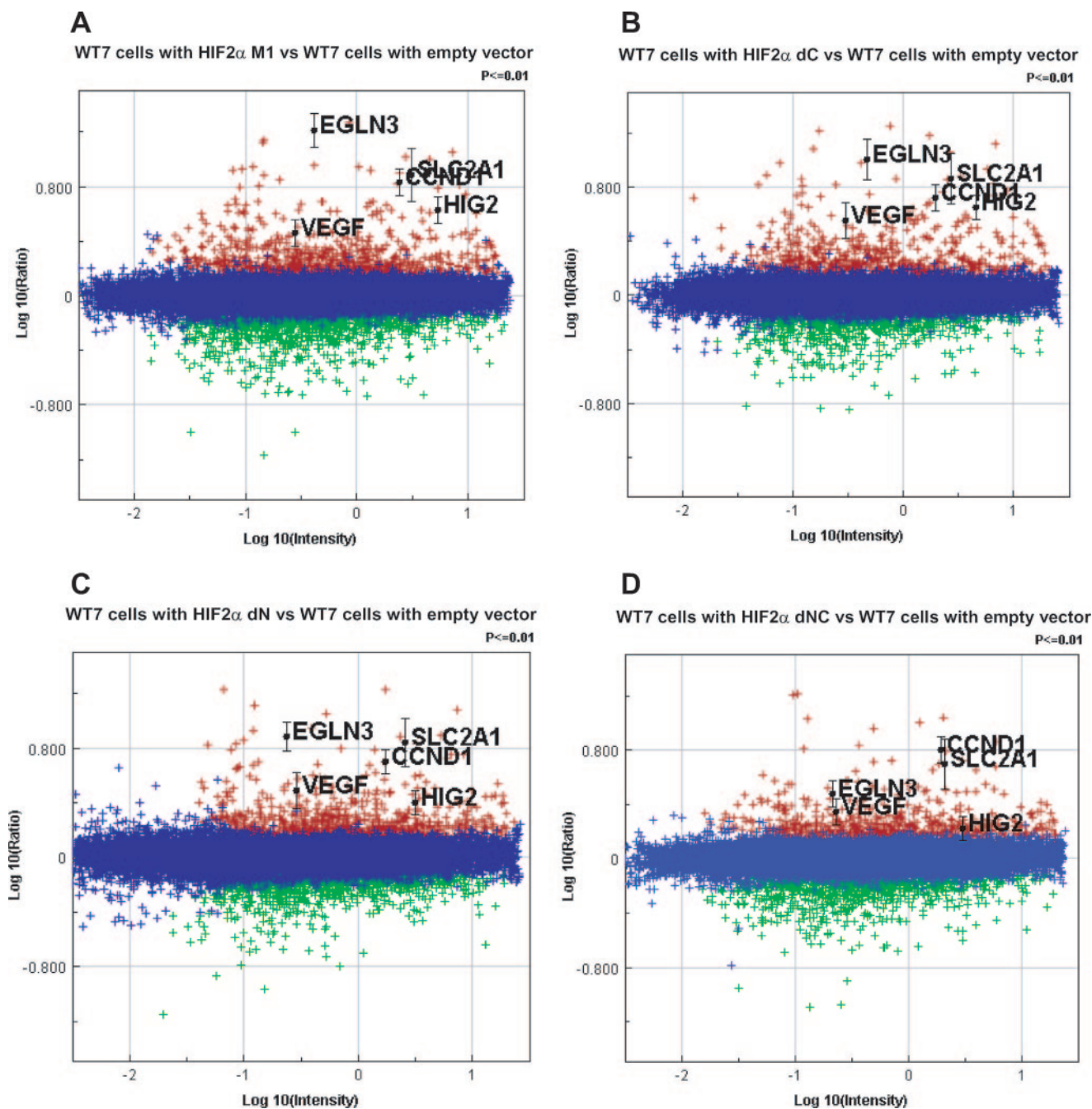


FIG. 5. Comparison of gene expression profiles of WT7 cells expressing various HIF2 α mutants. RNA isolated from WT7 cells expressing HIF2 α M1 (A), dC (B), dN (C), and dNC (D) variants were compared to WT7 cells with the empty vector. Significantly up-regulated (red) or down-regulated (green) genes were determined as described previously (22). Canonical HIF target genes, such as *cyclin D1* (*CCND1*), *GLUT1* (*SLC2A1*), *VEGF*, *EglN3*, and *HIG2*, are highlighted.

In keeping with the data shown in Fig. 3, the HIF2 α dN and dNC variants, which lack the NTAD, also retained the ability to activate many HIF target genes (Fig. 5C and D; also see Fig. S1 and Table S2 in the supplemental material), although the dNC variant was clearly impaired relative to variants containing the NTAD (Fig. 5A and B, note that Fig. 4A is reproduced as Fig. 5B for comparative purposes) or the CTAD (Fig. 5C). Upon careful inspection, loss of either the NTAD or CTAD

resulted in subtle quantitative differences in gene expression relative to M1, as exemplified by genes such as *EglN3*, *cyclin D1*, and *HIG2* (also see Fig. S1 and Table S2 in the supplemental material).

None of the HIF2 α variants substantially altered the proliferation of WT7 cells in tissue culture in the presence of serum (Fig. 6A and B), in keeping with earlier results after introduction of M1 into WT8 cells (32). Also as expected, WT7 cells

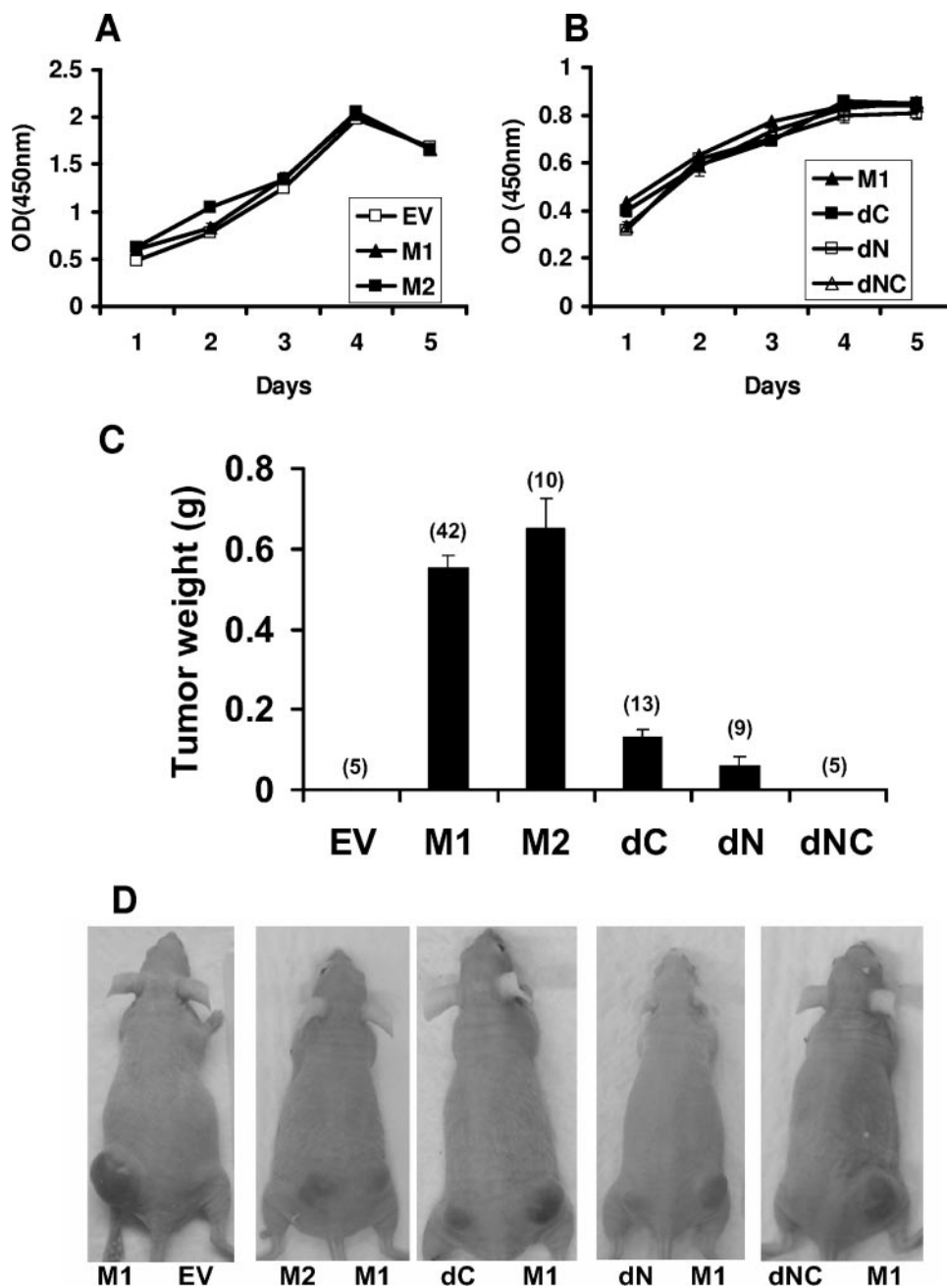


FIG. 6. Proliferation of WT7 cells expressing various HIF2 α mutants. (A and B) XTT assay of WT7 cells stably expressing the indicated HIF2 α mutants after seeding in 96 well plates. Optical density (OD) at 450 nm values reflect the number of viable cells. Data are the averages of results from triplicate experiments \pm standard errors (SE). (C) Tumor weights approximately 5 to 6 weeks after subcutaneous injection of WT7 cells stably expressing the indicated HIF2 α mutants indicated in flanks of the nude mice. The number of tumors analyzed is indicated in parentheses. Error bars indicate \pm SE. (D) Representative photographs of nude mice analyzed in panel C. EV, empty vector control.

producing HIF2 α M1 formed large tumors 5 to 6 weeks after subcutaneous injections in the flanks of nude mice, whereas WT7 cells infected with an empty retrovirus did not (Fig. 6C and D). The behavior of M2, lacking the Asn847 site, in these assays was similar to that of M1, in keeping with the results of our transcriptional assays. Surprisingly, deletion of either the NTAD or the CTAD dramatically reduced tumor forming capability and simultaneous deletion of both essentially eliminated tumor formation (Fig. 6C and D). The tumors formed

by the cells expressing the different HIF2 α variants were all very similar histologically and expressed similar numbers of vessels, as determined by CD34 staining (data not shown).

The decreased oncogenicity of the dN and dC HIF2 α variants presumably reflects the many small, quantitative, differences with respect to the induction of HIF target genes we documented by gene expression profiling (Fig. 5; also see Fig. S1 and Table S2 in the supplemental material). It is also possible that these differences in gene expression would be mag-

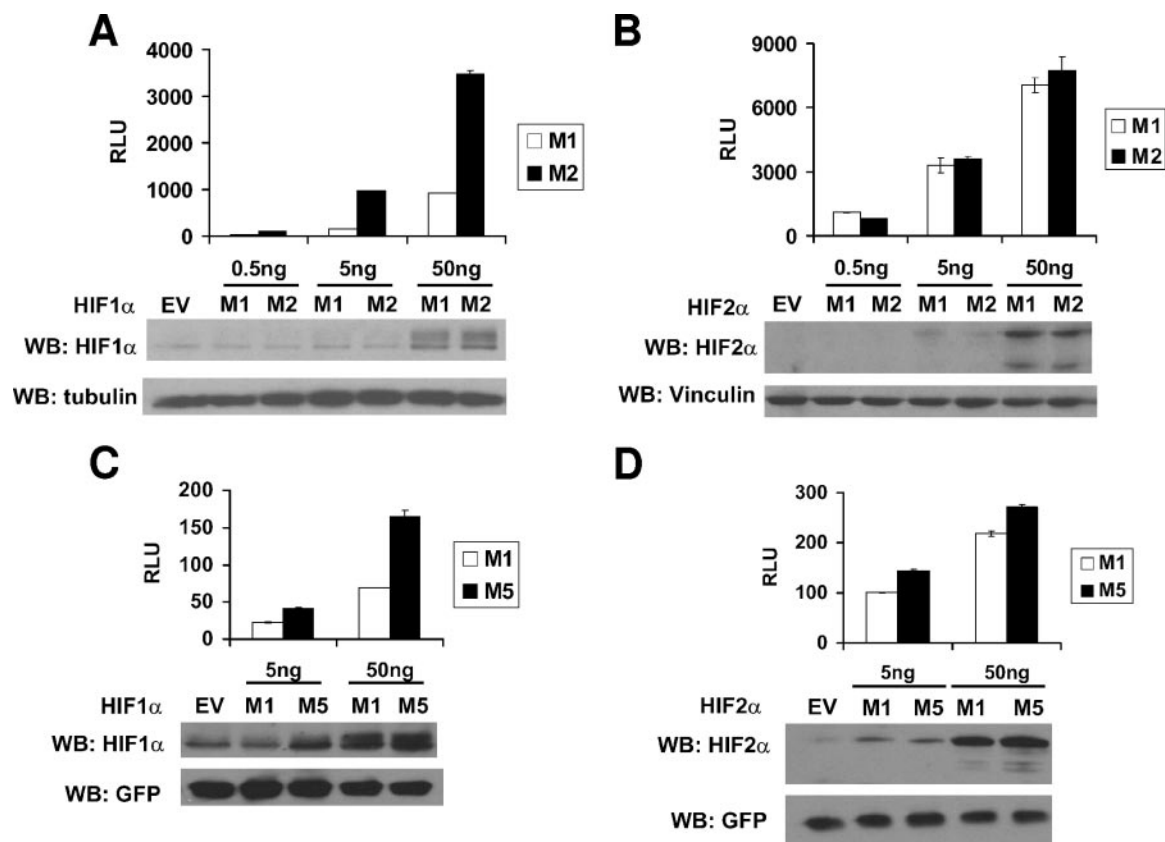


FIG. 7. Transcriptional activation by the HIF1 α and HIF2 α mutants. Normalized firefly luciferase values of U2OS cells transiently transfected to produce the indicated HIF1 α (A and C) or HIF2 α (B and D) variants along with a reporter plasmid containing firefly luciferase under the control of 3 tandem HIF-response elements upstream of a minimal promoter. Transfection mixes also contained plasmids encoding *Renilla* luciferase and, in some experiments, GFP for normalization purposes. Data are the averages of results from duplicate experiments \pm standard errors. Production of the HIF α mutants, GFP, vinculin, and tubulin was assessed by Western blot (WB) analysis (lower panel). RLU, relative luciferase unit; EV, empty vector control.

nified under in vivo conditions, although this remains to be tested.

Several groups have shown that mutation of Asn803 in the HIF1 α CTAD and the corresponding Asn in the HIF2 α CTAD to Ala or Gln renders these transactivation domains, as GAL4 DNA-binding domain fusions, constitutively active and insensitive to oxygen (19, 36, 37). Furthermore, FIH1 inhibits some HIF transcriptional target genes, presumably through inactivation of HIF α CTAD by asparaginyl hydroxylation (7, 51). In our studies, however, replacement of Asn847 with Ala in the context of full-length HIF2 α had no effect on HIF2 α 's ability to activate transcription (Fig. 2A and B) and promote tumor growth (Fig. 6C and D). To address this paradox, we created HIF1 α variants analogous to HIF2 α M1 and M2 and conducted HIF-responsive reporter gene assays in U2OS cells. Replacement of Asn803 with Ala in HIF1 α , in contrast to the results observed for HIF2 α , markedly enhanced transcriptional activation by HIF1 α (Fig. 7A and B). Similar results were obtained when HIF1 α Asn803 and HIF2 α Asn847 were replaced with Gln, which more closely resembles Asn but cannot be hydroxylated by FIH1 (Fig. 1A, M5, and Fig. 7C and 7D). Therefore, HIF2 α is relatively inured to the effect of FIH1 relative to HIF1 α .

Dayan and coworkers recently reported that some HIF target genes, such as *EglN3*, *VEGF*, *GLUT1*, and *CITED*, are

sensitive to changes in FIH1 activity, whereas others, such as *BNIP3*, *ENO1*, and *PGK1*, are not (7). Our findings predict that hypoxia and hypoxia mimetics should have little effect on FIH1-dependent gene expression in *VHL*^{-/-} cells that exclusively produce HIF2 α , in contrast to *VHL*^{-/-} cells that produce both HIF1 α and HIF2 α . Indeed, the hypoxia mimetic CoCl₂ did not lead to further induction of HIF target genes in 786-O cells, which exclusively produce HIF2 α , but did induce the FIH1 targets *EglN3* and *VEGF* in RCC4 cells, which produce both HIF1 α and HIF2 α (Fig. 8). The two other FIH1 targets examined, *GLUT1* and *CITED*, were also not induced (Fig. 8), which might, among several possibilities, reflect differences between renal carcinoma cells and the colorectal cells employed by Dayan and colleagues. Although 786-O and RCC4 are not isogenic, these results are consistent with the idea that HIF2 α is relatively inured to the effects of FIH1.

DISCUSSION

In this study, we began to dissect the relative contribution of the two HIF2 α transactivation domains to hypoxic gene activation and renal carcinogenesis. We created a panel of HIF2 α mutants in which either the NTAD, CTAD, or both were altered. Comparison of these mutants showed that the HIF2 α NTAD is capable of activating HIF-responsive promot-

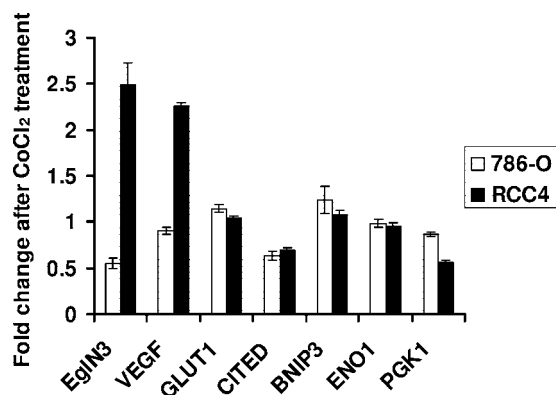


FIG. 8. Induction of HIF target genes in 786-O and RCC4 cells by CoCl_2 treatment. 786-O and RCC4 cells were grown in the presence or absence of $200 \mu\text{M}$ CoCl_2 for 16 h. Expression levels of the indicated HIF target genes were determined by quantitative, real-time reverse transcription-PCR and normalized to β -actin mRNA levels. Shown are relative changes after CoCl_2 treatment. Data are the averages of results from triplicate experiments \pm standard errors.

ers in the absence of the CTAD. We also investigated the regulation of the HIF α CTAD by FIH1. Interestingly, we found that the HIF2 α CTAD, in contrast to the HIF1 α CTAD, is relatively resistant to the inhibitory effects of FIH1 under normoxic conditions and that, perhaps as a result, both the HIF2 α NTAD and CTAD cooperate to promote renal carcinogenesis *in vivo*.

Our finding that the HIF2 α NTAD is sufficient to activate at least some HIF-responsive genes is consistent with earlier work that showed that a naturally occurring HIF1 α splice variant that lacks the HIF1 α CTAD, but retains the HIF1 α NTAD, is likewise able to activate transcription (16). Transcriptional activation by this HIF1 α variant, however, appears to be impaired relative to full-length HIF1 α . The same might be true of the corresponding HIF2 α variant at lower protein concentrations than used here.

Previous studies showed that the HIF1 α and HIF2 α CTADs, as GAL4 DNA-binding domain fusions, can be hydroxylated by FIH1, which impairs their ability to activate transcription. Mutation of the relevant hydroxylation sites renders these GAL4 fusions constitutively active and insensitive to oxygen (19, 36, 37). We found, however, that the HIF2 α CTAD is less sensitive to FIH1 modification than HIF1 α CTAD in the context of their respective full-length proteins. This observation is consistent with a recent study that showed that HIF2 α is a poor FIH1 substrate *in vitro* relative to HIF1 α , in part because of differences in residues immediately following their respective Asn hydroxylation sites (1).

At least some HIF target genes are activated upon pVHL inactivation, even under well-oxygenated conditions. The activation of these targets presumably, based on the considerations outlined above, reflects the fact that the HIF α NTAD is active under normoxic conditions coupled with the relative insensitivity of the HIF2 α CTAD to inhibition by FIH1. The latter finding, coupled with the fact that HIF1 α , but not HIF2 α , is still subject to proteasomal degradation in pVHL-defective cells (25, 31), might explain why HIF2 α plays a more promi-

nent oncogenic role in *VHL*^{-/-} renal carcinoma cells than does HIF1 α .

The EglN family members and FIH1 are iron- and 2-oxo-glutarate-dependent dioxygenases and can be inhibited with drug-like small molecules. Inhibition of these enzymes might be useful for the treatment of ischemic diseases and anemia. Theoretically, drugs that inhibited EglN alone would not induce genes that require the HIF1 α CTAD for activation but would activate genes for which either the HIF1 α or HIF2 α NTAD is sufficient for activation. In addition, such drugs would induce genes activated by the HIF2 α CTAD in cells that produce HIF2 α , which is more restricted in its expression than HIF1 α (12, 14, 20, 53). In summary, such small molecules might differentially regulate HIF targets depending on the degree to which they are regulated by HIF1 α versus HIF2 α and the degree to which they depend upon the NTAD, CTAD, or both for full activation. Support for this general idea comes from gain-of-function and loss-of-function studies involving EglN1 (PHD2), which is the primary HIF prolyl hydroxylase under resting conditions, and FIH1 (7, 51). For example, some HIF-responsive genes, such as *EglN3* and *VEGF*, appear to be inhibited by FIH1, but others do not (7, 51). It is also increasingly clear that some genes are preferentially regulated by different HIF α proteins (3, 10, 21, 45).

Deletion of the HIF2 α NTAD grossly impaired HIF2 α -dependent transcription activation in our reporter gene assays, whereas deletion of the HIF2 α CTAD did not. This raises the possibility that HIF2 α -dependent transcription is largely driven by the NTAD. A caveat, however, is that the in-frame deletion of the NTAD we introduced might alter HIF2 α folding in a way that alters, for example, the folding or presentation of the CTAD. This deletion mutant was not completely unfolded, however, because it retained at least partial transcriptional activation capability, as measured in reporter gene assays and by gene expression profiling. Perhaps more importantly, we found that deletion of either the NTAD or the CTAD impaired tumor promotion by HIF2 α . This strongly argues that both the NTAD and the CTAD contribute to HIF2 α -dependent transcription in the context of the full-length protein *in vivo*. Two, non-mutually exclusive, possibilities can explain this cooperativity. The NTAD and CTAD may act synergistically to activate a common set of genes that promote tumorigenesis *in vivo*. Here the difference between full-length HIF2 α and the two deletion mutants would be largely quantitative. In addition, there may be some tumor-relevant genes that are uniquely activated by the NTAD and some by the CTAD. In this scenario, the difference between full-length HIF2 α and the two deletion mutants would be qualitative. Our gene expression profiling studies are thus far most consistent with the former. A caveat, however, is that our gene expression studies were performed on cells grown under standard culture conditions. It remains possible that some tumor-relevant differences in HIF target gene expression will only manifest themselves under the conditions arising in tumors *in vivo*.

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