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Genetic and Functional Studies Implicate HIF1α as a 14q Kidney Cancer Suppressor Gene

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Abstract

Kidney cancers often delete chromosome 3p, spanning the *VHL* tumor suppressor gene, and chromosome 14q, which presumably harbors one or more tumor suppressor genes. pVHL inhibits the HIF transcription factor and HIF2α is a kidney cancer oncoprotein. Here we identify focal, homozygous, deletions of the *HIF1α* locus on 14q in clear cell renal carcinoma cell lines. Wild-type HIF1α, but not the products of these altered loci, suppress renal carcinoma growth. Conversely, downregulation of HIF1α in HIF1α-proficient lines promote tumor growth. HIF1α activity is diminished in 14q deleted kidney cancers and all of the somatic *HIF1α* mutations identified in kidney cancers tested to date are loss of function. Therefore *HIF1α* has the credentials of a kidney cancer suppressor gene.

Keywords

14q deletion; HIF1α; kidney cancer; tumor suppression; von Hippel-Lindau; hypoxia

INTRODUCTION

Kidney cancer causes over 10,000 deaths each year in the United States (1). Although surgery is potentially curative for kidney cancers that are detected at an early stage, recurrences after surgery remain common and late stage, inoperable, kidney cancer is usually fatal.

Clear cell renal carcinoma is the most common form of kidney cancer and is usually linked to biallelic inactivation of the von Hippel-Lindau *VHL* tumor suppressor gene, which is located on chromosome 3p25. Individuals who carry a mutant *VHL* allele in the germline (von Hippel-Lindau disease) are at increased risk of clear cell renal carcinoma, in addition to

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pheochromocytomas and central nervous system hemangioblastomas. Somatic mutation, or hypermethylation, of the *VHL* locus is also common in sporadic clear cell renal carcinomas (2).

The *VHL* gene product, pVHL, has multiple functions including serving as the substrate recognition subunit of an ubiquitin ligase complex that targets the alpha subunits of the heterodimeric transcription factor HIF (hypoxia-inducible factor) for polyubiquitination and proteasomal degradation when oxygen is present (3). Accordingly, deregulation of HIF target genes, such as *VEGF*, is a signature abnormality in pVHL-defective neoplasms and the degree of HIF deregulation correlates well with renal carcinoma risk linked to different *VHL* alleles (4–6). Notably, a number of drugs that inhibit VEGF, or its receptor KDR, have demonstrated significant activity in the treatment of metastatic kidney cancer (7).

Multiple lines of evidence suggest that HIF2α, and not its more intensively studied paralog HIF1α, acts as a driver in pVHL-defective renal carcinomas. For example, pVHL-defective renal carcinoma cell lines and tumors produce both HIF1α and HIF2α or HIF2α alone (6, 8) and the appearance of HIF2α in preneoplastic lesions in the kidneys of VHL patients correlates with increased histological evidence of impending malignancy(9). Moreover HIF2α, but not HIF1α, can override pVHL's tumor suppressor activity (10–12) whereas eliminating HIF2α is sufficient to suppress tumor formation by pVHL-defective renal carcinoma cells in preclinical models (13, 14). A recent genome-wide association study linked the risk of renal carcinoma to *HIF2α* polymorphisms (15). Finally HIF2α, rather than HIF1α, appears to be responsible for much of the pathology that develops following pVHL inactivation in the mouse (16, 17).

Although HIF1α and HIF2α are similar to one another they can clearly antagonize one another in certain settings. For example, in some models HIF1α antagonizes, while HIF2α potentates, c-Myc activity (8, 18, 19). In addition, HIF1α and HIF2α reciprocally regulate each other's protein levels in some contexts such that, for example, loss of HIF1α leads to induction of HIF2α and vice-versa (10). In keeping with these observations, overproduction of wild-type HIF1α in pVHL-defective renal carcinoma cells suppresses tumor formation (10), whereas overproduction of HIF2α promotes tumor growth (10, 11). On the other hand, HIF1α is believed to promote, rather than inhibit, many other tumor types of non-renal origin (20).

A number of chromosomal abnormalities, in addition to chromosome 3p loss, have been described in clear cell renal carcinoma including, most commonly, amplification of 5q and loss of chromosome 14q. Loss of 14q has been associated with poorer outcomes in renal carcinoma in numerous studies (21–24). The knowledge that *HIF1α* is located at chromosome 14q, together with the considerations outlined above, led us to explore further whether HIF1α might be a clear cell carcinoma tumor suppressor gene.

RESULTS

Loss of Chromosome 14q Spanning the *HIF1α* Locus is a Common Feature of Human Kidney Cancer

Kidney cancers frequently undergo deletions affecting chromosome 14q. To ask if this abnormality occurs more often in kidney cancers than in other forms of cancer, we examined a recently published collection of copy number data generated with high density SNP arrays from 3131 cancers representing 26 different tumor types (25). The frequency of large deletions affecting most of chromosome 14q was highest in kidney cancer, followed by melanoma, gastrointestinal stromal tumor (GIST), and esophageal cancer (Figure 1A). As expected, loss of chromosome 3p, which harbors the *VHL* tumor suppressor gene and other

tumor suppressor genes such as *PBRM1* (26), as well as amplification of 5q, were also extremely common in kidney cancer relative to other tumor types (Figure 1B and 1C). These data do not, however, reflect a general proclivity for copy number alterations in kidney cancer because other copy number changes, such as loss of chromosomes 17p and 13q, which harbor *p53* and *RB1*, respectively, did not occur more commonly in kidney cancer than in other cancers (data not shown).

HIF1α expression is lost in many *VHL*−/− renal carcinoma lines, can suppress tumor formation by *VHL*−/− renal carcinoma cells when overexpressed (10), and maps to 14q23. On the other hand, previous studies, including our own, pinpointed 14q31-ter as the most likely region to harbor a kidney cancer tumor suppressor gene (23, 27, 28). Nonetheless, the 14q deletions in kidney cancer are typically very large, with the localization to 14q31-ter based on relatively rare kidney cancers with smaller deletions. For example, in our recent analysis of 90 clear cell renal carcinomas, 39 tumors (43%) had sustained 14q deletions (27). Of these, 36 (93%) were large deletions that also encompassed the *HIF1α* locus (27). This suggests the existence of multiple tumor suppressor genes on 14q, including perhaps *HIF1α*. Consistent with this hypothesis, deletions affecting *HIF1α* are more common in kidney cancer than in the other 16 tumor types for which we have 40 or more samples (Figure 1D). This strong bias toward kidney cancer is not apparent, however, if one includes deletions elsewhere on 14q (Figure S1).

Frequent Homozygous Deletions of the HIF1α Locus in Kidney Cancer Cell Lines

We next surveyed a panel of 16 clear cell renal carcinoma lines, most of which had undergone biallelic *VHL* inactivation, for HIF1α protein and mRNA production, along with HK-2 immortalized, diploid, human renal epithelial cells. In keeping with earlier reports (6, 8, 29), we found that many *VHL*−/− renal carcinoma lines produce no detectable wild-type HIF1α mRNA or protein whereas all the *VHL*−/− lines produce HIF2α (Figure 2, Figure S2, and data not shown). Interestingly, some lines, such as RCC4, SKRC-20, A498, and 786-O cells, produce mRNAs with increased electrophoretic mobility (Figure 2B). The truncated HIF1α mRNA in 786-O has also been noted by others (30). SKRC-20, A498, and 786-O also produce aberrantly migrating HIF1α proteins (Figure 2A). These findings raised the possibility that the HIF1α locus, in addition to undergoing copy number loss, is rearranged in a subset of renal tumors.

To explore this further, we isolated genomic DNA from the 16 renal carcinoma lines and performed multiplex ligation-dependent probe amplification (MLPA) analysis to look for copy number changes affecting specific *HIF1α* exons (31). As controls, we also interrogated randomly chosen exons on chromosome 1, 10, and 17. Two cell lines (SLR20 and SLR21) appeared to be diploid across the HIF1α locus (Figure 2C and Figure S3). Both of these cells lines, which are phenotypically *VHL*+/+, were tested by us previously using high density SNP arrays and did not exhibit 14q loss (27). Three cell lines (RCC4, Caki-2, and A704) appeared to have lost one *HIF1α* allele in its entirety and to have retained the other (Figure 2C and Figure S3). Interestingly, 7 out of 16 cell lines (SKRC20, A498, 769P, 786-O, UOK101, SLR24, and SLR26) had clearly sustained homozygous deletions, which in some cases were very focal and involved only a subset of contiguous HIF1α exons (Figure 2C and Figure S3). The remaining 4 cell lines (UMRC2, UMRC6, SLR23, SLR25) displayed more complex MLPA patterns that were intermediate between haploid and diploid across the HIF1α locus, with preferential loss of particular exons (Figure S3).

Several other putative tumor suppressor genes reside on chromosome 14 including the Hippo pathway genes *SAV1* (14q22) and *FRMD6* (14q22) (32). In contrast to HIF1α, we did not detect altered transcripts for these genes (Figure S4B and S4C). We also did not detect homozygous deletions of *SAV1* by MLPA, with the exception of the previously reported

deletion in 786-O cells (33) (Figure S4A). The discovery of focal, homozygous, *HIF1α* deletions provides genetic evidence for a tumor suppressor role for HIF1α in clear cell renal carcinoma.

HIF1α Suppresses Kidney Cancer Proliferation *In Vitro* and *In Vivo*

To address this further, we made retroviral HIF1α expression vectors in which the amount of HIF1α produced can be regulated by the addition of doxycycline. *VHL* −/− renal carcinoma cells were then infected with these viruses and maintained in pools. Immunoblot analysis of these cells grown in the presence or absence of 1 μg/ml doxycycline confirmed that HIF1α expression was induced by doxycyline and that the HIF1α levels achieved were similar to the levels observed after treating HK2 immortalized renal epithelial cells with the prolyl hydroxylase inhibitor DMOG or the proteasomal inhibitor MG132 (Figure 3A and Figure S5). Reexpression of HIF1α in the *VHL* −/− renal carcinoma cell lines A498, 769-P, UOK101, and SLR24, all of which produce HIF2α but not wild-type HIF1α, impaired proliferation *in vitro* (Figure 3B). This effect was specific because induction of HIF1α did not impair the proliferation of RCC4 and UMRC-2 *VHL* −/− renal carcinoma cells, which express both HIF1α and HIF2α (Figure 3B and Figure S5). Therefore HIF1α can suppress the proliferation of *VHL* −/− renal carcinoma cells when expressed at levels approximating those achieved after *VHL* inactivation.

In a reciprocal set of experiments, we downregulated HIF1α or HIF2α in three *VHL* −/− renal carcinoma cell lines that express both HIF1α and HIF2α (Caki2, RCC4, and SLR25) (Figure 4A). In contrast to a recent report, we did not observe an increase in HIF2α in the cells treated with HIF1α shRNA (10) (Figure 4A). The significance of this discrepancy is unclear. In all three cases downregulation of HIF1α with 2 independent shRNAs enhanced proliferation *in vitro* compared to cells expressing a scrambled control shRNA or HIF2α shRNA (Figure 4B and Figure S6), in keeping with a recent study using RCC4 cells (8). The very modest inhibition of cell proliferation observed with the HIF2α shRNA is consistent with earlier studies using cells grown under standard serum conditions.

In addition to affecting proliferation *in vitro*, downregulation of HIF1α promoted the growth of RCC4 renal carcinoma cells that had been implanted in the kidneys of nude mice (Figure 5A–5C). Similar results were obtained with Caki-2 cells grown subcutaneously in nude mice (Figure S6F and S6G), whereas SLR25 cells did not form tumors in nude mice irrespective of HIF1α status (data not shown). Downregulation of HIF1α in UMRC2 renal carcinoma cells also dramatically enhanced tumor growth (Figure 5D–5G) despite having inconsistent effects *in vitro* (Figure S6D). Therefore HIF1α suppresses tumor formation by *VHL* −/− renal carcinoma cells.

HIF1α Variants Resulting from HIF1α Genomic Deletions are Defective as Tumor Suppressors

Next we attempted to recover the aberrant mRNAs that we had detected by Northern Blot analysis in a subset of renal carcinoma lines. mRNA was harvested from these cells lines, converted to cDNA, and amplified by PCR. As predicted by the MLPA analysis, the HIF1α transcript in SKRC-20 cells lacked specifically exons 3 and 4 (Δ3–4), the HIF1α transcript in A498 cells lacked exons 2–6 (Δ2–6), and the HIF1α transcript in SLR26 cells lacked exons 5–10 (Δ5–10) (Figure 6A). These transcripts presumably reflect the homozygous deletion detected by MLPA together with alternative splicing. In addition, PCR primers based on the 5’ and 3’ HIF1α untranslated regions detected a transcript lacking exon 2–12 in RCC4 and A498 cells, perhaps responsible for the faster migrating Northern Blot band detected in these cells (Figure 2B)(data not shown). This variant (Δ2–12), in contrast to the other mRNA variants, was also detected in some normal kidney mRNA samples (Table S2).

In 786-O cells we recovered a transcript lacking exons 13–15 using 3' RACE, in keeping with the MLPA data (Figure S3 and data not shown) and with western blot data indicating that these cells produce a HIF1α variant that reacts with a polyclonal antibody but not a C-terminal monoclonal antibody (Figure 2A). This transcript is predicted to encode a fusion protein as it contains several in-frame exons from a neighboring gene (data not shown). For unclear reasons, however, this transcript greatly exceeds the apparent MW of the mRNA detected in these cells by Northern Blot analysis (Figure 2B).

The Δ3–4, Δ2–6, and Δ5–10 HIF1α variants, as well as wild-type HIF1α, were introduced into 769-P *VHL*−/− renal carcinoma cells using the doxycycline-inducible retroviral expression vector described above. Notably, each variant preserves the proper reading frame, with the exception of the Δ5–10 variant. An N-terminal hemagglutin (HA) epitope tag was introduced to facilitate the detection of the wild-type and mutant HIF1α proteins after induction with doxycycline. Each variant migrated in accordance with its predicted molecular weight and, with the exception of the Δ2–6 variant, was produced at levels that were similar to the levels of wild-type HIF1α (Figure 6B).

Notably, wild-type HIF1α suppressed the proliferation of 769P cells to a greater extent than did any of the 3 variants tested (Figure 6C). In the next set of experiments the 769P cells that had been engineered to inducibly express wild-type or mutant HIF1α were pooled and propagated *in vitro* in the presence or absence of doxycycline (Figure 6D). Growth in the presence of doxycyline led to the progressive loss of cells expressing wild-type HIF1α relative to the cells expressing the mutants, indicating that cells expressing wild-type HIF1α are at a growth disadvantage in such competition assays (Figure 6E). Similar results were obtained when the cells were injected into the kidneys of NOD/SCID mice and propagated *in vivo* (Figure 6F), indicating that the HIF1α variants tested here are enfeebled as tumor suppressors relative to wild-type HIF1α.

HIF1α Activity is Diminished in Human Kidney Cancers Harboring 14q Deletions

To determine if our cell line data were relevant to human kidney cancers, we next asked if HIF1α activity is diminished in human kidney cancers that have sustained 14q deletions encompassing the HIF1α locus. Toward this end we performed gene expression profiling on the cell lines described above that either expressed both HIF1α and HIF2α (either naturally or by virtue of induced expression of HIF1α) or expressed HIF2α alone (either naturally or by virtue of a HIF1α shRNA) followed by supervised clustering to arrive at a "HIF1α transcriptional signature", which included known HIF1α specific targets such as *BNIP3*, *PGK1*, *HK1*, and *TPI1* (Figure 7A) (10, 34). Gene set enrichment analysis using this HIF1α signature and gene expression data from 52 *VHL*−/−clear cell renal carcinomas, including 32 without a *HIF1α* deletion and 20 with a *HIF1α* deletion, confirmed that HIF1α activity is diminished in tumors that have sustained a 14q deletion spanning the *HIF1α* locus ($p < 0.01$) (Figure 7B).

Somatic HIF1α Mutations in Human Clear Cell Carcinomas are Loss of Function

We have not yet been able to unambiguously identify focal, homozygous, deletions in renal tumor DNA by MLPA (data not shown). This might be technical because MLPA is very sensitive to contamination by host DNA. We did, however, detect presumptively pathogenic *HIF1α* mRNA splice variants (absent in normal kidney RNA samples and absent in publicly available databases), analogous to those present renal carcinoma cell lines, in 3/23 primary clear cell renal carcinomas (Table S2).

Somatic *HIF1α* mutations have been described at low frequency in human kidney cancer (29, 35), (H. Greulich, M. Meyerson, and W.G.K.-unpublished data). We therefore made

doxycycline-inducible retroviral vectors corresponding to 3 of these mutations (c.2120delA, c.2180 C→A, and V116E) as well to a presumed benign single nucleotide polymorphism (A475S)(Figure 7C and 7D). All 3 of the mutations suspected to be pathogenic compromised HIF1α's ability touppress renal carcinoma growth whereas the presumptive SNP did not (Figure 7E). A fourth mutant that was recently reported (35), Exon 4 -5 del (tacagTTTGAACTAACTGGA), was not tested because it would be predicted to induce a frameshift after exon 3. Collectively, these results support that HIF1α activity is diminished in a subset of *VHL*−/− kidney cancers because of a reduction in gene dosage and, in some cases, as a result of intragenic, loss of function, mutations.

DISCUSSION

Loss of the region of chromosome 3p spanning the *VHL* gene is the most frequent genomic change in clear cell renal carcinoma, which is the most common form of kidney cancer. The next two most common genomic abnormalities in kidney cancer are chromosome 5q amplification and chromosome 14q loss. Moreover, we show here that kidney cancer has the highest rate of chromosome 3p loss, chromosome 5q amplification, and 14q loss amongst a wide variety of tumor types.

VHL loss leads to increased abundance of HIF1α and HIF2α and deregulation of HIF-dependent transcription is a signature abnormality in kidney cancer. Mounting evidence suggest that HIF2α, rather than HIF1α, promotes pVHL-defective renal carcinogenesis. Indeed, many pVHL-defective renal carcinomas produce low, or undetectable, levels of HIF1α (6, 8, 36) and restoring HIF1α expression in a *VHL*−/− renal carcinoma line was shown before to suppress tumorigenesis (10). We confirmed this later finding and showed that HIF1α, at levels comparable to those seen in when pVHL function is impaired, suppresses renal carcinoma proliferation and tumor growth.

Prompted by this knowledge we asked whether HIF1α, which resides on chromosome 14q23.2, might be one of the genes targeted by 14q deletions in kidney cancer. Indeed, we found that the vast majority of 14q deletions detected in renal carcinoma encompass HIF1α. Moreover, we documented that HIF1α, but not neighboring genes on chromosome 14q, is often subject to focal deletions in kidney cancer cell lines. Some of these deletions led to the production of aberrant mRNAs and proteins that compromised HIF1α's ability to suppress *VHL*−/− renal carcinoma proliferation and tumorigenesis. We also discovered that downregulation of wild-type HIF1α promotes renal carcinoma growth *in vivo*. Finally, we showed that somatic, presumably pathogenic, *HIF1α* mutations in human clear cell carcinomas enfeeble HIF1α as a tumor suppressor in cell proliferation assays. Collectively, these genetic and functional data credential HIF1α as a clear cell renal carcinoma suppressor gene. Hence loss of pVHL simultaneously leads to activation of an oncoprotein (HIF2α) and a tumor suppressor protein (HIF1α). This would explain the frequent loss of chromosome 14q in kidney cancer and is consistent with the observation that loss of HIF1α protein in preneoplastic lesions in the kidneys of VHL patients heralds further malignant transformation (9).

In a recent study the percentage of clear cell renal carcinomas with low HIF1α expression approximated the frequency of 14q loss for this tumor type (8) and 14q loss was enriched amongst the HIF1α negative tumors (Kate Nathanson-personal communication). Moreover, we confirmed that HIF1α transcriptional activity is indeed decreased in *VHL*−/− kidney tumors that have sustained 14q deletions encompassing *HIF1α* compared to those that have not. Although homozygous *HIF1α* deletions appear to be common in clear cell renal carcinoma cell lines we have not, however, documented a similarly high frequency in primary renal tumors. Although this discrepancy might be due to technical factors it raises

the possibility that HIF1α haploinsufficiency is sufficient to promote primary tumor growth *in vivo*, and that reduction to nullizgyosity is selected for during tumor progression *in vivo* or the propagation of clear cell carcinoma lines *in vitro*.

HIF1α is usually thought to promote tumor growth but there is precedence for it functioning as a tumor suppressor. For example, loss of HIF1α enhances tumor formation by embryonic stem cell-derived teratoma cells and by murine astrocytes (37–39). In the context of renal carcinoma, HIF1α might act as a tumor suppressor specifically by antagonizing HIF2α. For example, transactivation by one of the two HIFα transactivation domains (the C-terminal transactivation domain or CTAD) is inhibited by the asparaginyl hydroxylase FIH1. HIF2α is less sensitive than HIF1α to FIH1-mediated inhibition (40, 41). Competitive displacement of HIF2α by HIF1α from HIF-responsive promoters that depend upon the CTAD for full activation would therefore potentially decrease promoter activity. Moreover, HIF1α can suppress HIF2α levels via as yet unclear mechanisms in some contexts (10). In short, loss of HIF1α might, paradoxically, increase the activity of certain HIF-responsive promoters in pVHL-defective tumor cells. Experimental evidence exists to support this contention (10) and (C.S. and W.G.K.-data not shown).

In addition to quantitative differences on shared HIF-responsive promoters, there are a number of qualitative differences between HIF1α and HIF2α that might relate to HIF1α scoring as a tumor suppressor protein. For example, some genes that are regulated by HIF1α are not regulated by HIF2α and vice versa. Conceivably, some of the genes that are preferentially activated by HIF1α decrease renal carcinoma cell fitness. In this regard, 3 of the genes found in our HIF1α signature, *TXNIP*, *KCTD11* and *PLAGL1*, have been implicated as tumor suppressors in other contexts (42–45). Moreover, HIF1α and HIF2α differ in terms of their ability to engage collateral signaling pathways such as those involving c-Myc and Notch. For example, HIF1α, via a variety of mechanisms, can inhibit c-Myc activity in certain settings whereas HIF2α does not (8, 18, 19).

The 14q deletions in kidney cancer are typically very large and usually span HIF1α, as noted above. Nonetheless, rare tumors with small deletions had pinpointed 14q31-ter as the likely location for a kidney cancer tumors suppressor gene (23, 27, 28). The simplest reconciliation of these findings would be the existence of multiple kidney cancer tumor suppressor genes on 14q, in addition to *HIF1α*, with perhaps some acting through haploinsufficiency. Alternatively, these small deletions might have been passenger, rather than driver, mutations.

It will be of interest to determine whether pVHL-defective clear cell renal carcinomas that retain wild-type HIF1α expression utilize alternative mechanisms to circumvent HIF1α's tumor suppressor activity. We note, for example, that *PLAGL1* maps to a region of 6q that is frequently deleted in VHL-associated neoplasms and sporadic clear cell renal carcinomas (27, 46, 47). Moreover, it will be important to determine whether retention of HIF1α expression alters the response of pVHL-defective tumors to targeted agents that directly or indirectly target HIF. In this regard, it is possible that the salutary effects of rapamycin-like mTOR inhibitors (rapalogs) in kidney cancer are partially mitigated by their ability to downregulate HIF1α (48), especially in light of a recent report predicting that HIF2α would be relatively resistant to such agents (49).

METHODS

Cell Lines

HK-2, 786-O, A704, 769P, Caki-2, RCC4, and A498 cells were purchased from the ATCC. UMRC-2 (50), UMRC-6 (50) and UOK101 (51) cells were provided by Drs. Bert Zbar and

Martson Linehan (National Cancer Institute). SLR20, SLR21, SLR23, SLR24, SLR25, and SLR26 cells were provided by Drs. Mark A. Rubin and Kirsten Mertz (Weill Cornell Medical College) (52). SKRC20 cells (53) were provided by Drs. Gerd Ritter and Beatrice Yin (Memorial Sloan-Kettering Cancer Center). DNA from these cell lines was subjected to SNP analysis by us previously (27) and the cell lines were monitored regularly for mycoplasma contamination. No other validation was performed. Whenever possible freshly thawed vials of cells were used at early passage. HK2 immortalized human renal epithelial cells were maintained in Keratinocyte Serum Free Medium (K-SFM) supplemented with 0.05 mg/ml bovine pituitary extract (BPE) and 5 ng/ml human recombinant epidermal growth factor (EGF). Renal carcinoma cells 786-O, A498, RCC4, UMRC-2, UMRC-6, and UOK101 were maintained in DMEM containing 10% fetal bovine serum (FBS); 769P, A704, SKRC20, SLR20, SLR21, SLR23, SLR24, SLR25, and SLR26 were maintained in RPMI-1640 containing 10% FBS; and Caki2 were maintained in McCoy's 5A containing 10% FBS. Following retroviral or lentiviral infection, cells were maintained in presence of puromycin (2 μg/ml) or G418 (500 μg/ml) depending on the vector. All cells were maintained at 37°C in 10% CO_2.

Plasmids

The wild-type and variant HIF1α cDNAs (HIF1αΔ3–4, HIF1αΔ2–6, & HIF1αΔ5–10) were PCR amplified from HK-2, SKRC-20, A498 & SLR26 cells, respectively, with a 5′ primer that introduced a BamH1 site and an HA epitope and a 3′ primer that introduced a MluI site. The products were digested with BamHI and MluI and cloned into pRetroX-Tight-Pur vector (Clontech) cut with these two enzymes.

The tumor-derived HIF1α mutations in Figure 7 were generated by the site-directed mutagenesis of the pRetroX-Tight-HIF1α-WT by using QuikChang II XL Site-Directed Mutagenesis Kit (Agilent Technologies) and confirmed by DNA sequencing. The forward primers for mutagenesis of HIF1α mu1-mu4 are

5'-CAGTTCCTGAGGAAGAACTAATCCAAAGATACTAGCTTTGCAG-3';

5'-GGAACATGATGGTTAACTTTTTCAAGCAGTAGG-3';

5'-CATGATTTACATTTCTGATAATGAGAACAAATACATGGGATTAAC-3'; and 5'-CTGCACTCAATCAAGAAGTTTCATTAAAATTAGAACCAAATCC-3', respectively.

Lentiviral shRNA vector pLKO.1, lentiviral HIF1α shRNA vector (TRCN0000003810, target sequence: 5'-GTGATGAAAGAATTACCGAAT-3'; TRCN0000003811, target sequence: 5'-CGGCGAAGTAAAGAATCTGAA-3'; TRCN0000003809, target sequence: 5'-CCAGTTATGATTGTGAAGTTA-3') and lentiviral HIF2α (EPAS1) shRNA vector (TRCN0000003806, target sequence: 5'-CAGTACCCAGACGGATTTCAA-3') were obtained from the Broad Institute TRC shRNA library.

Immunoblot Analysis

Cells were lysed with 1× EBC buffer (50 mM Tris [pH8.0], 120 mM NaCl, 0.5% NP-40) supplemented with a proteaseinhibitor cocktail (Complete; Roche Applied Science, Indianapolis,IN), resolved by SDS-PAGE (30 μg/lane) and transferred to nitrocellulose membranes (Bio-Rad). Primary antibodies were rabbit polyclonalanti-HIF1α (NB100-479, Novus, Littleton, CO), mouse monoclonal anti-HIF1α (BD Transduction Laboratories), mouse monoclonal anti-HA (HA-11, Covance Research Product), and mouse monoclonal anti-tubulin(B-512, Sigma-Aldrich). Bound antibody was detected with horseradishperoxidase-conjugated secondary antibodies (Pierce, Rockford, IL) and Immobilon Western chemiluminescent horseradishperoxidase substrate (Millipore).

Northern Blot Analysis

Total RNA was isolated using TRIzol reagent (Invitrogen), resolved by agarose-formaldehyde gel electrophoresis (10 μg RNA/lane), transferred to nitrocellulose membranes, and probed with a HIF1α AgeI-PstI fragment labeled with [α-^{32}P]dCTP using a Prime-It II Random Primer Labeling Kit (Stratagene). Hybridizations were performed in QuikHyb (Stratagene) according to the manufacturer's instructions. Signals were detected by X-ray film.

Multiplex Ligation-dependent Probe Analysis (MLPA)

_____Multiplex ligation-dependent probe amplification was performed as described in (31) using the probe sets listed in Supplemental Table 1 and reagents provided by MRC-Holland (54).

The capillary electrophoresis and peak height determination of amplification products were performed by Mei Lin at DNA Sequencing Facility, Brigham and Women's Hospital. Briefly, amplification products were 10× diluted in HiDi formamide (ABI, Foster city, CA, USA) containing 1/16 volume of ROX500 size standard (ABI) and then were separated by size on an ABI 3100 Genetic Analyzer (ABI). Electropherograms were analyzed by GeneMapper v3.5 (ABI), and peak height data were exported to an Excel table.

Cell Proliferation Assays

Renal carcinoma cells that had been infected to inducible express HIF1α were plated in 96 well plates (~500 cells/well; 6 wells per condition and timepoint) in RPMI-1640 media supplemented with 5% FBS in the presence or absence of the indicated amount of doxycycline, with a media change every two days. The number of viable cells per well at each time point was measured using an XTT assay (Cell Proliferation Kit II, Cat. No. 11465015001, Roche) according to the manufacturer's instructions. Spectrophotometricalabsorbance at 450 nm was measured 6 hours after the addition of the XTT labeling reagent/electron coupling reagent using a microtiter plate reader(Perkin Elmer Life and Analytical Science).

Renal carcinoma cells stably infected with lentiviral shRNAs were plated, in triplicate, in 6 well plates (10^4 cells/well) in RPMI (RCC4, SLR25 and UMRC-2) or McCoy's 5A (Caki2) supplemented with 5% FBS. Cells were trypsinized and collected at the indicated time points. The number of viable cells, as determined by Trypan blue staining, was determined with a hemocytometer.

Renal carcinoma 769P cells that had been infected to inducible express tumor-derived HIF1α mutants were plated, in triplicate, in 6 well plates (5,000 cells/well) in RPMI with 5% FBS. Cells were trypsinized and collected at the indicated time points. The number of viable cells was determined by using an automated cell counter (Countess, Invitrogen).

Xenograft assays and bioluminescence

Orthotopic and subcutaneous growth of tumor cells was as described in (5). For RCC4 cells, 1×10^6 viable RCC4 HIF1α shRNA cells (right kidney) and 1×10^6 viable RCC4 scrambled shRNA cells (left kidney) were injected orthotopically into Swiss nude mice (Taconic, Hudson, NY). Bioluminescent detection and quantification of tumor burden were performed as described in (55). For each mouse the total photons from the right kidney were divided by the total photons from the left kidney and normalized to the ratio for that mouse the first week after tumor cell injection. For UMRC-2 cells, 2×10^7 viable UMRC-2 HIF1α shRNA cells (right side) and 2×10^7 viable UMRC-2 scrambled shRNA cells (left side) were injected subcutaneously into Swiss nude mice. The mice were sacrificed 4 weeks post

injection and tumors were excised and weighed. For Caki-2 cells, 1×10^7 viable Caki-2 HIF1α shRNA cells (right side) and 1×10^7 viable Caki-2 scrambled shRNA cells (left side) were injected subcutaneously into Swiss nude mice. The mice were sacrificed 16–20 weeks post injection and tumors were excised and weighed.

5-week old female NOD scid gamma (NSG) mice (The Jackson Laboratories, Bar Harbor, ME) were orthotopically injected with a 1×10^6 viable 769P cells (1:1:1:1 mixture of 769P cells stably infected with retroviruses encoding wild-type HIF1α or three different HIF1α variants). The mice were randomized to chow that did or did not contain 6 g doxycycline/kg chow (Bioserv) 1 week after tumor cell implantation and sacrificed 6 to 8 weeks later. 300 ng of genomic DNA, extracted from the injected kidney by using DNeasy Blood & Tissue Kit (Qiagen), was PCR amplified using a forward primer (5'-ATGTACCCATACGATGTTCCAGATTACGC-3') based on the sequence encoding the shared HA epitope tag and a reverse primer (5'-TGTGCTTTGAGGACTTGCGCTTTC-3') based on the HIF1α C-terminus.

Microarray & GSEA analysis

Gene expression profiling was performed at the Microarray Core at Dana-Farber Cancer Institute using Gene 1.0ST Arrays (Affymetrix) and the data deposited in the Gene Expression Omnibus database (GSE27415). In brief, total RNA was extracted by using a RNeasy mini-kit with on-column DNase digestion (Qiagen). Biotin labeled cRNA was prepared from 1 μg of total RNA, fragmented, and hybridized to a Human Gene 1.0ST array. The arrays were scanned and the raw expression data was normalized using DNA-Chip Analyzer (dChip) custom software (56). Genes were assessed for significantly altered expression between H1H2 and H2 subgroups by T-test analysis using software GENE-E (57). The 71 genes with P values ≤ 0.05 and an average signal ratio ≥ 1.3 between two subgroups were selected as positively regulated by HIF1α. This gene set was evaluated using Gene Set Enrichment Analysis (GSEA) (58) across a panel of 52 ccRCC tumors with biallelic inactivation of *VHL* (with 32 non-*HIF1α* deletion tumors and 20 *HIF1α* deletion tumors) for which expression data were previously obtained (GSE14994) (27). We used the GSEA parameters: weighted scoring, signal-to-noise metric, and gene-set permutations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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SIGNIFICANCE

Deletion of 14q is a frequent event in clear cell renal carcinoma and portends a poor prognosis. In this study, we provide genetic and functional evidence that HIF1α is one of the targets of 14q loss in kidney cancer.

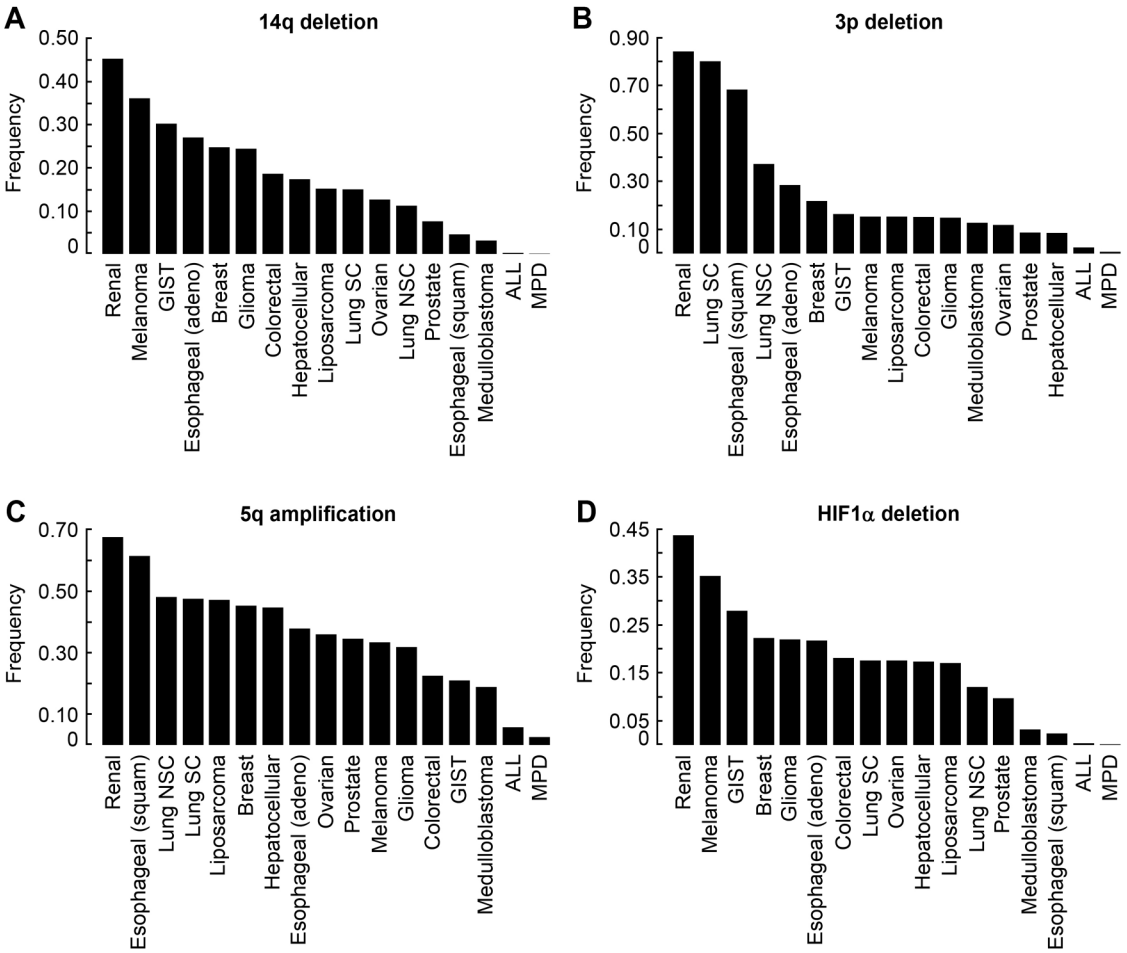

Figure 1. Frequencies of Chromosomal Abnormalities Across Different Cancers

(A) Large deletions affecting most of 14q arm.

(B) Large deletions affecting most of 3p arm.

(C) Amplification of any region of 5q.

(D) Deletions affecting *HIF1α* locus. See also Figure S1.

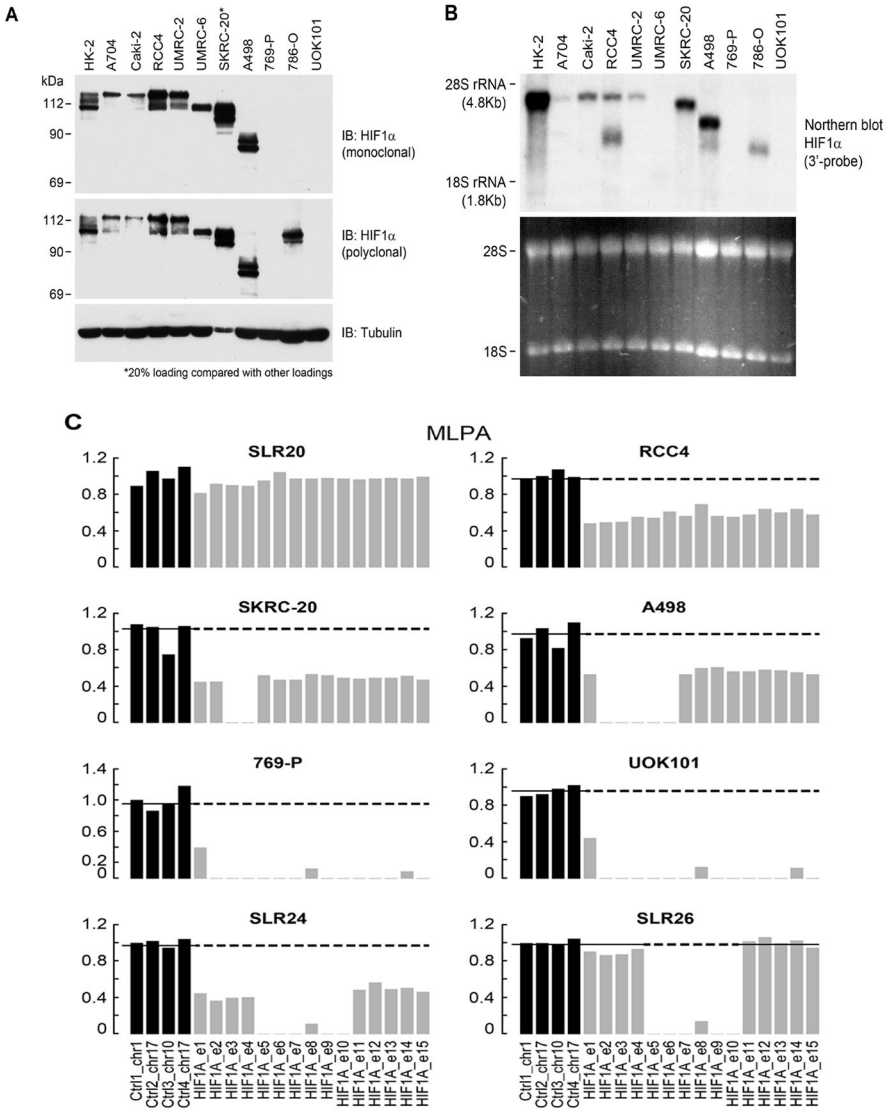

Figure 2. *HIF1α* Deletions and Altered *HIF1α* Gene Products in Renal Carcinoma Cells

Immunoblot (A) and Northern blot (B) analysis of the indicated cell lines. The difference in HIF1α electrophoretic mobility between HK-2 immortalized renal epithelial cells and the HIF1α-positive lines such as A704, Caki-2, RCC4, and UMRC-2 might reflect differential phosphorylation. See also Figure S2.

(C) Shown are MLPA data for the indicated cell lines, normalized to HK-2 immortalized renal epithelial cells (diploid = 1). Black bars = selected control exons on chromosomes 1, 10, and 17. Grey bars = HIF1α exons. See also Figure S3, Figure S4 and Table S1.

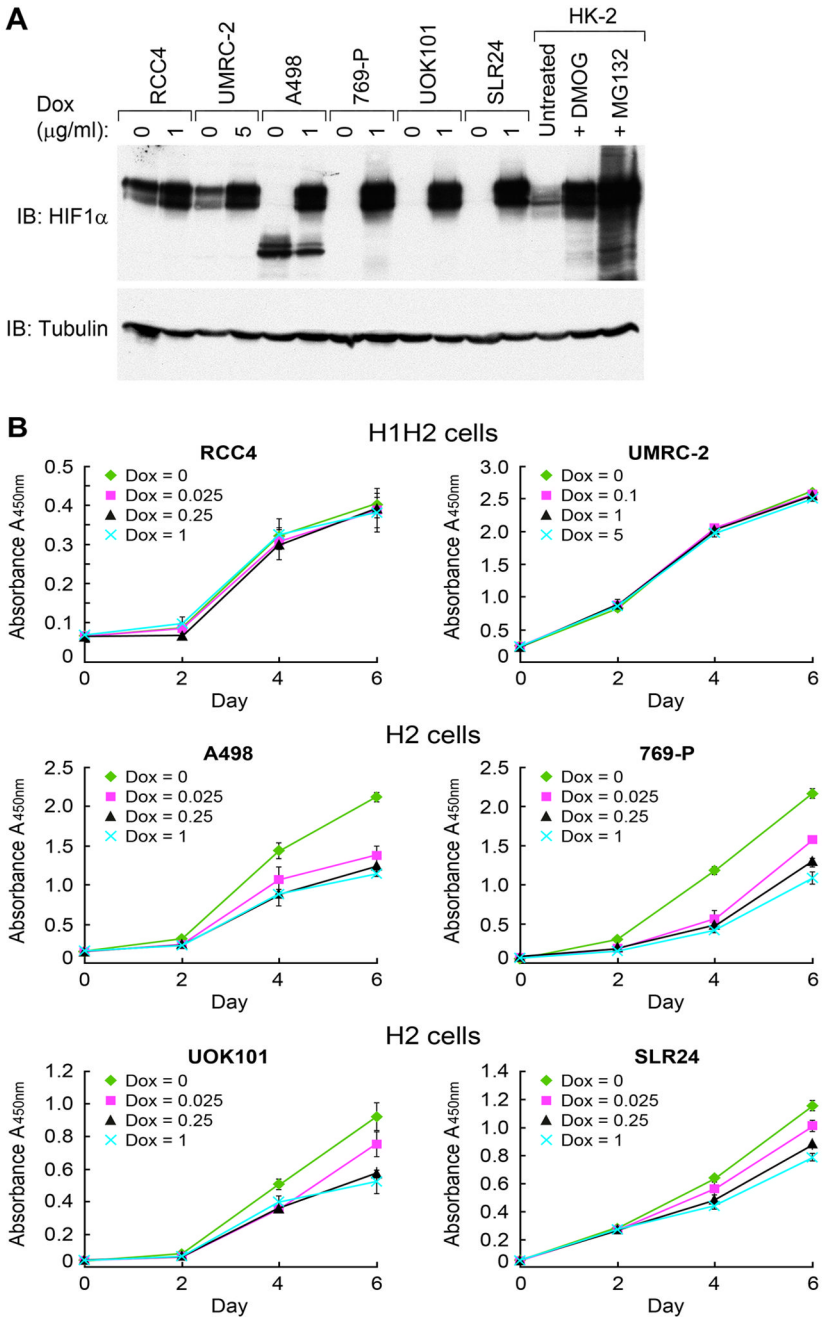

Figure 3. Suppression of *VHL*−/− renal carcinoma proliferation by HIF1α

Immunoblot (A) and proliferation (B) assays of indicated cell lines infected with a doxycycline-inducible retrovirus encoding HIF1α and propagated in 5% serum in the presence or absence of doxycycline. HK-2 cells treated with vehicle, DMOG (1 mM), or MG132 (10 μM) were included as a control in (A). See also Figure S5.

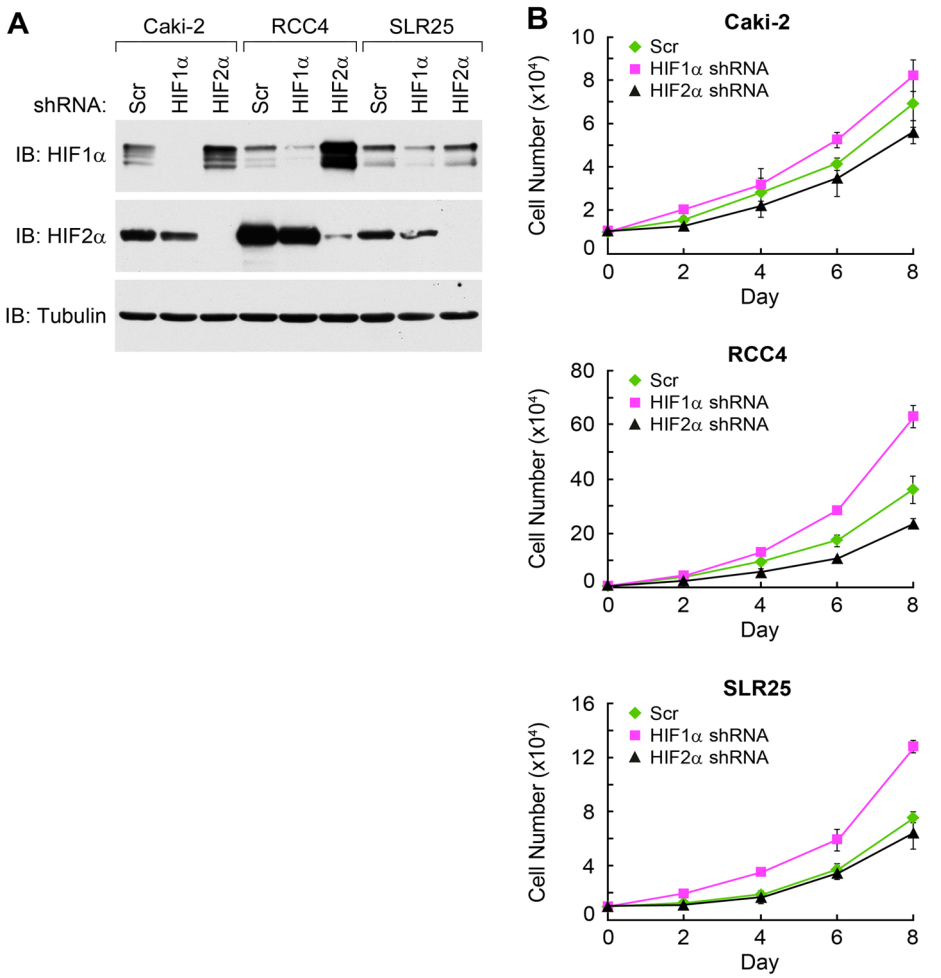

Figure 4. Downregulation of HIF1α in *VHL*−/− renal carcinoma cells enhances cell proliferation

Immunoblot (A) and proliferation (B) assays of the indicated cell lines after infection with lentiviruses encoding HIF1α shRNA, HIF2α shRNA, or scrambled control shRNA and grown in the presence of 5% serum. See also Figure S6.

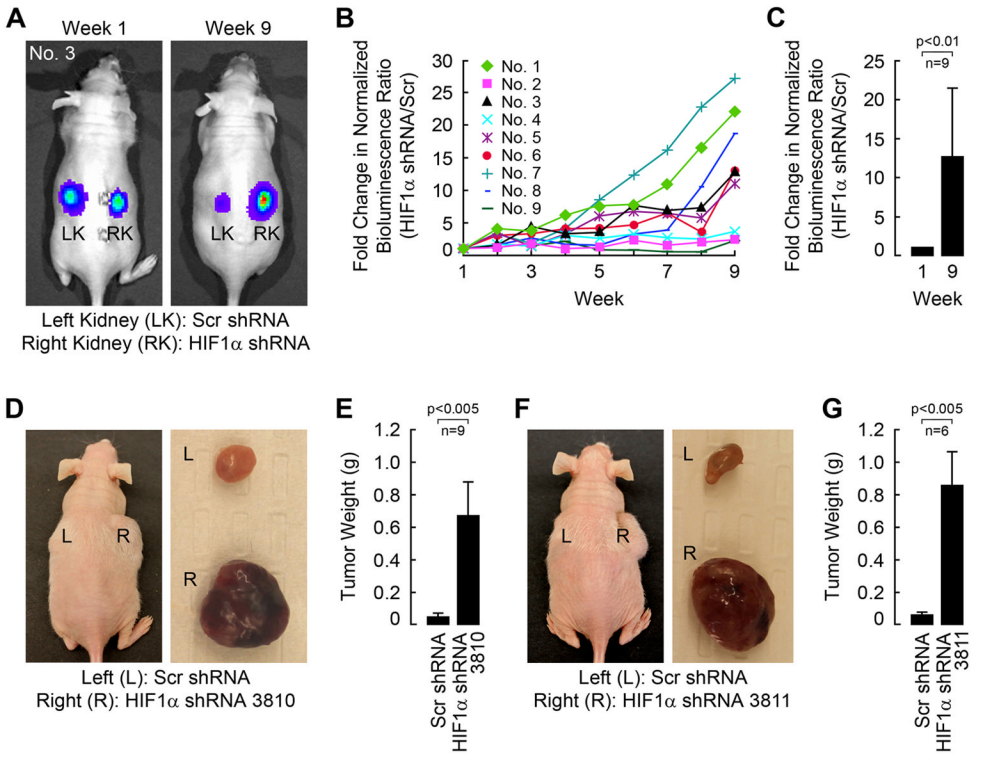

Figure 5. Downregulation of HIF1α Promotes Tumor Cell Growth *In Vivo*

(A) Representative bioluminescent images of nude mice orthotopically injected with RCC4 renal carcinoma cells that stably express firefly luciferase and an shRNA directed against HIF1α (right kidney) or a scrambled shRNA control (left kidney). Shown are images 1 week and 9 week after tumor cell implantation.

(B) Bioluminescence ratio as a function of time for mice as in (A). For each mouse the ratio was normalized to the week 1 ratio for that mouse.

(C) Mean fold change in normalized bioluminescence ratio for mice analyzed in (B) at week 9. Error bars = 1 standard error of the mean.

(D & F) Representative images of nude mice 4 weeks after subcutaneous injection of UMRC-2 renal carcinoma cells that stably express an shRNA directed against HIF1α (right side) or a scrambled shRNA control (left side).

(E & G) Mean tumor weights at necropsy of mice as in (D & F). Error bars = 1 standard error of the mean. See also Figure S6.

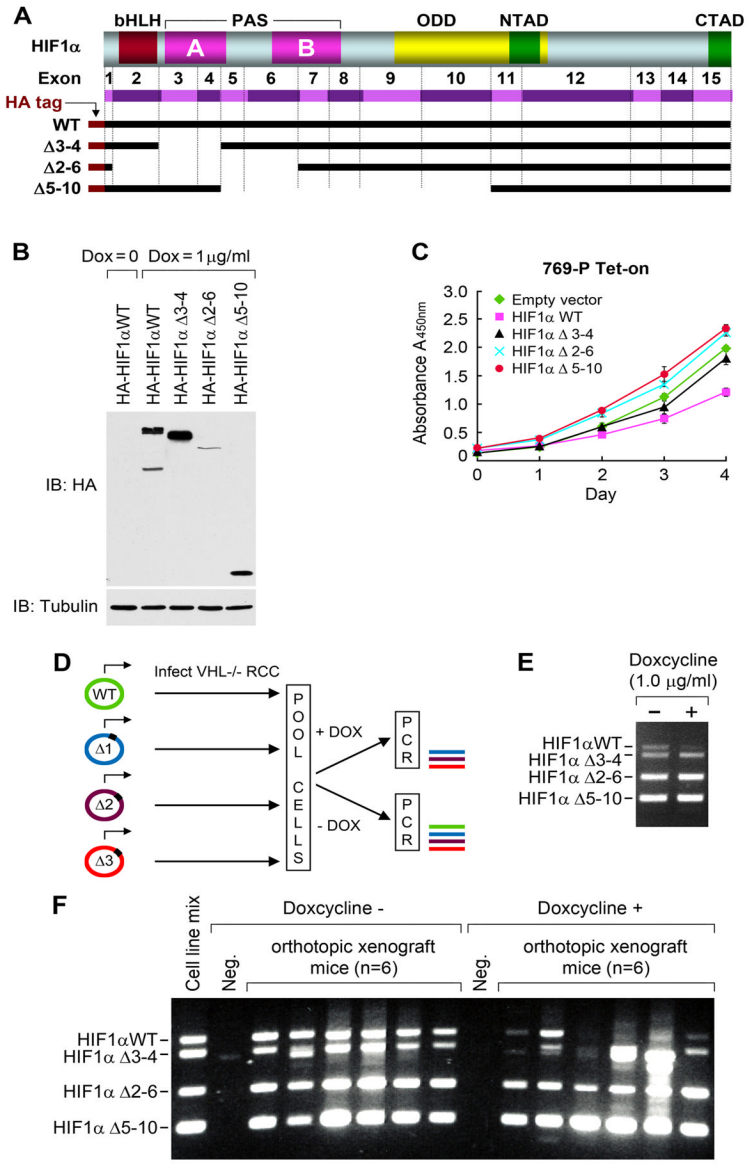

Figure 6. Differential Effects of Cell Line-Derived HIF1α Variants on Proliferation and Tumor Cell Fitness

(A) Schematic of HIF1α variants identified in renal carcinoma lines. (B and C). Immunoblot (B) and proliferation (C) assays of 769P cells infected with a doxycycline-inducible retroviruses encoding the indicated HIF1α variants and propagated in the presence of doxycyline. 769P infected with an inducible virus encoding wild-type HIF1α but grown in the absence of doxycycline were included as a control in (B).

(D) Schematic for *in vitro* and *in vivo* competition assays.

(E) and (F). PCR-based analysis indicating relative abundance of cells as in (B and C) after growth *in vitro* for 9 days (E) or orthotopically *in vivo* for 6 weeks (F) in the presence of absence of doxycycline. Neg. = PCR reaction with no input DNA. See also Table S2.

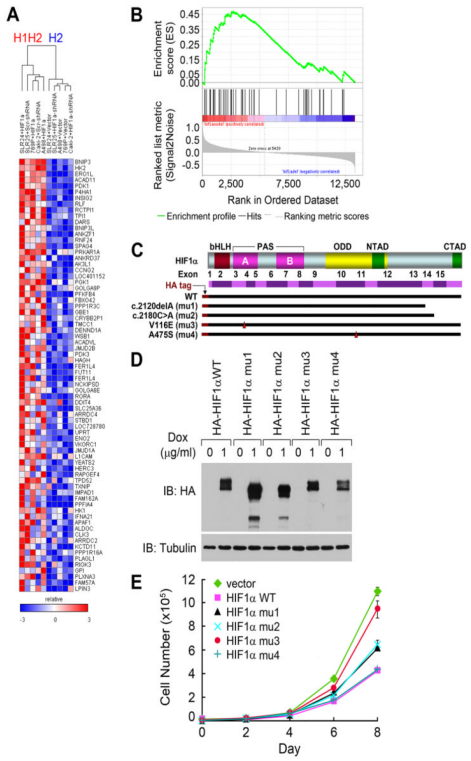

Figure 7. HIF1α Activity is Impaired by Copy Number Changes and Mutations in pVHL-Defective Kidney Cancers

(A) Heat map depicting genes that are differentially expressed between *VHL*−/− renal carcinoma cell lines that do (H1H2) or do not (H2) express high levels of HIF1α. Genes (right) are ordered from top to bottom according to *p* values showing the degree to which they are significantly altered between H1H2 cells compared to H2 cells. T-test and Hierarchical Clustering was performed using software GENE-E at Broad Institute (57).

(B) A Gene Set Enrichment Analysis (GSEA) plot showing the location of the Enrichment Score (ES) of 71 HIF1α up-regulated genes in a set of ccRCC tumors with biallelic *VHL* inactivation. The HIF1α positively regulated genes are significantly enriched ($p \leq 0.01$) in tumors that have not sustained 14q deletions encompassing *HIF1α*.

(C) Schematic of *HIF1α* mutations identified in renal carcinoma patients.

(D and E) Immunoblot (D) and proliferation (E) assays of 769P cells infected with doxycycline-inducible retroviruses encoding wild-type HIF1α or the indicated HIF1α variants and propagated in the presence of doxycyline. Cells grown in the absence of doxycycline were included as controls in (D).