Hypoxia-Induced SUMOylation of E3 Ligase HAF Determines Specific Activation of HIF2 in Clear-Cell Renal Cell Carcinoma

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Abstract

Clear-cell renal cell cancer (CRCC) is initiated typically by loss of the tumor-suppressor VHL, driving constitutive activation of hypoxia-inducible factor-1 (HIF1) and HIF2. However, whereas HIF1 has a tumor-suppressor role, HIF2 plays a distinct role in driving CRCC. In this study, we show that the HIF1α or HIF3α ligase hypoxia-associated factor (HAF) complexes with HIF2α at DNA to promote HIF2-dependent transcription through a mechanism relying upon HAF SUMOylation.

Introduction

The hypoxia response promotes the adaptation to low oxygen by shifting cells towards anaerobic metabolism, neovascularization, and resistance to apoptosis. Hypoxia occurs in all solid tumors and their metastases, and drives responses that contribute to tumor aggressiveness, such as increased invasion, metastasis, and a poorly differentiated phenotype. This occurs largely through activation of the hypoxia-inducible factors (HIF), HIF1 and HIF2, which are central players in the regulation of the physiologic and pathophysiologic responses to hypoxia (1, 2). HIF1 and HIF2 are nonredundant and play unique and complementary roles during bone and vascular development, and also in regulating cellular transcriptional responses to acute and chronic hypoxia (3, 4). In this regard, HIF1 and HIF2 can drive distinct downstream target genes and also exhibit antagonism in regulating the hypoxia response (5). The highly divergent outcomes of HIF1 and HIF2 signaling on tumor growth and progression occur despite their high degree of similarity in structure and mechanisms of regulation, and appear to be dependent on hypoxic intensity and duration, and other isoform specific HIF regulators that are beginning to be identified (2). We have established that the hypoxia-associated factor (HAF) can promote the switch from HIF1α to HIF2α-dependent signaling, that it achieves by selectively degrading HIF1α independently of oxygen or pVHL, and promoting HIF2α transcription without affecting HIF2α levels (4, 6).

Clear-cell renal cell carcinoma (CRCC) is the most common type of kidney cancer, and is highly refractory to standard chemotherapy and radiation. The etiology of CRCC is uniquely linked to loss of the von Hippel–Lindau (pVHL) tumor-suppressor protein, whereby more than 90% of cases of both sporadic and hereditary CRCC show pVHL deficiencies (7–9). pVHL is the substrate recognition component of the E3 ligase complex that targets the oxygen labile HIF1α and HIF2α subunits for proteasomal degradation under aerobic conditions. Under hypoxic conditions, or in the presence of pVHL deficiency, HIF1α and HIF2α are stabilized, and enter the nucleus where they heterodimerize with HIF1β, forming the HIF1 or HIF2 transcriptional complexes respectively, and activate the transcription of hundreds of genes critical for the adaptation to hypoxia, and for tumor progression (1, 10). pVHL loss of function is a critical event for CRCC initiation, promoting the constitutive activation of HIF1α and HIF2α, which play a dominant role in the progression of CRCC (11). Indeed, CRCC is one of the best-perfused of solid tumors due to the overproduction of HIF-dependent proangiogenic factors, VEGFA and PDGFB. Nevertheless, CRCC tumors still experience relatively low oxygen tensions due to the already low physiologic oxygen tensions within the kidney (CRCC is believed to originate from proximal tubular epithelial cells within the renal cortex), and to the inherent abnormality of the tumor vasculature (12–14).

Converging lines of evidence support a driving role for HIF2α and not of HIF1α in CRCC. First, although elevated HIF1α is apparent in the earliest preneoplastic lesions in VHL patients, the
appearance of HIF2α is associated with increased dysplasia and cellular atypia (15, 16). Hence, CRCC cells and tumors can be subdivided into two subtypes: those that express both HIF1α and HIF2α (pVHL mutant or pVHL wild-type), or those that express HIF2α exclusively (pVHL mutant only; refs. 17, 18). Second, overexpression of HIF2α promotes, whereas overexpression of HIF1α inhibits, CRCC growth (19–21). Third, inhibition of HIF2α by shRNA is sufficient to suppress the growth of pVHL-null CRCC cells (19, 22). Fourth, type IIB pVHL mutants, associated with high risk of CRCC, retain some ability to downregulate HIF1α, but have reduced ability to downregulate HIF2α, compared with type IIA pVHL mutants, associated with low risk of CRCC (23, 24). Taken together, the data suggest that there may be a temporal nature to HIF activation in CRCC, whereby VHL loss is associated with early elevation of HIF1α, which then shifts to HIF2α, which promotes dysplasia and CRCC progression. There is also increasing evidence implicating HIF1A as a kidney cancer-suppressor gene, particularly in advanced CRCC. Loss of heterozygosity in chromosome 14q in the locus spanning HIF1A has been reported in approximately 40% of human CRCC, whereas homozygous deletion of HIF1A was detected in approximately 50% of CRCC cell lines (25, 26). Although the mechanism of the shift to HIF2α is unclear, the specific protumorigenic effect of HIF2α in CRCC may be due its increased potency compared with HIF1α in driving protumorigenic factors such as cyclin D1, TGFβ, and VEGFα, and in potentiating c-Myc activity, which in contrast is inhibited by HIF1α (18, 21).

The HAF (located at 11q13.1) is a mediator of the switch from HIF1α to HIF2α, by selectively degrading HIF1α, and promoting HIF2α transcription (4). HAF overexpression enhances the ability of glioblastoma cells (which express both HIF1α and HIF2α) to initiate tumors as intracranial xenografts in mice. However, HAF overexpression decreases xenograft tumor growth of HT29 colon carcinoma cells that only express HIF1α (6). Hence, HAF may either inhibit or promote tumor progression, depending on the dominant HIF isoform expressed in a given cellular context. However, the mechanism by which HAF elicits its selective effects on HIF1α and HIF2α in the context where both HIF isoforms are present remains unknown.

Here, we elucidate the mechanism and regulation of HAF-mediated HIF2α transactivation. We show that HAF promotes the transcription of a subset of HIF2 target genes by binding to a DNA consensus site located within close proximity to the hypoxia-responsive element (HRE), thus forming a transcriptional complex and driving transcription. Significantly, we demonstrate that the ability of HAF to bind and transactivate HIF2α is dependent upon HAF SUMOylation, which is induced by exposure to hypoxia. In contrast, the ability of HAF to bind and degrade HIF1α is independent of HAF SUMOylation. Thus, in the context of pVHL loss that results in the constitutive stabilization of HIF1α and HIF2α, HAF preferentially promotes HIF2-specific activation, and patients with CRCC with high HAF show significantly decreased progression-free survival (PFS) than those with low HAF. This suggests that HAF may be the determinant of HIF2-specific activation in CRCC, thus providing a novel avenue for therapy.

Materials and Methods

Tissue culture

A498, 786-0, PANC-1, MIAPaCa-2, and ACHN cells were from ATCC and were maintained in RPMI (A498) and DMEM (ACHN, 786-0, PANC-1, MIAPaCa-2; Invitrogen Corp., Life Technologies), supplemented with 10% fetal bovine serum. The identities of all cell lines were confirmed by the Molecular Cytogenetics Facility at MD Anderson Cancer Center (MDACC; Houston, TX) using short tandem repeat (STR) DNA fingerprinting upon receipt from ATCC. Cells were frozen, thawed, and restested after 3 months in culture, after which cells were discarded and a fresh vial thawed. Hypoxic incubations (1% O2) were performed using the InVivo2 Hypoxia Workstation (Biotrace International Inc.).

Plasmid construction and transfections

Stable cell lines overexpressing HAF and HAF double mutant (DM; HAF K94R K141R), or shHIF2 were generated by retroviral infection using pMX-IRES-GFP or pSUPER, respectively (27). The pCMVF/LAG14 construct was used for transient transfections with HAF or HAF DM (6). The P117 luc reporter was generated by cloning the P117 sequence (28) upstream of luciferase and a minimal CMV promoter (pCL4-17; Promega Corp.). P117 HRE containing the HRE and HIF ancillary sequence (29) was generated by cloning the HRE downstream of P117 (Supplementary Methods). HAF–GAL4 fusion protein was generated by ligating full-length FLAG–HAF downstream of the GAL4 DNA-binding domain (DBD) using the pBIND plasmid from the Checkmate Mammalian Two-Hybrid System (Promega). Transactivation activity was confirmed using the pG5 luc vector containing five GAL4 binding sites upstream of luciferase, normalized to intronic Renilla luciferase encoded within the pBIND vector. GAL4–VP16 (pBIND-ïd and pACT-MycD) was used as a positive control according to the manufacturer’s protocol. Transient transfections were performed using Lipofectamine 2000 (Invitrogen) and cells were assayed 48 hours after transfection. HIF2α siRNA duplexes were from Dharmacon (GE Healthcare): siGENOME D-004814-02 (DNA oligos were ligated into pSUPER to generate shH2), D-004814-03 (siH2_2). HAF (SART1) siRNA was ON-TargetPlus SMARTpool (Dharmacon L-017283, siHAF_1), and Silencer #15787 (siHAF_2; Ambion, Life Technologies). Hypoxic exposure was at 1% O2, performed 24 hours after transfection, and harvested after 16 hours unless otherwise indicated.

TaqMan quantitative RT-PCR

Total RNA was isolated using the RNeasy Kit with DNase I step. TaqMan qRT-PCR was performed using the ABI 7300 system with One-Step RT-PCR Master Mix Kit and predesigned primer/probes, and normalized to β-2 microglobulin as previously described (6). Statistical significance was determined using MS Excel (t test).

Western blotting, immunoprecipitation, and immunohistochemistry

Western blotting was performed using HIF2α (NB100-122; Novus Biologicals), actin (Santa Cruz Biotechnology Inc.), HIF1α (Cell Signaling Technology), SUMO-1 (Cell Signaling Technology; #4930S), SUMO-2/3 (ab61371; Abcam), and HAF antibodies (6, 30). Immunoprecipitation to show SUMOylation of HAF was performed using RIPA buffer (with 20 mmol/L NEM) with sonication. Denaturing immunoprecipitation was performed by boiling immunoprecipitated proteins conjugated to Protein A beads in 50 μL 1% SDS solution for 5 minutes, resuspending the supernatant in 1 mL of RIPA buffer, and repeating immunoprecipitation with fresh protein A beads overnight. Immunocytochemistry was performed using purified mouse monoclonal anti-HAF antibody raised against GST-
HAF (Biogenes GmbH) and rabbit polyclonal anti-HIF2α (NB 100-122), with Cy3 goat anti-mouse, and Alexa Fluor 488 chicken anti-rabbit secondary antibodies, respectively (Invitrogen). Images were taken using the Nikon A1Rsi confocal microscope at a single plane and Z-stack (0.8 μm/level). Immunohistochemistry (IHC) was performed using HIF2α or HAF mAb antibody, using citrate buffer antigen retrieval (Leica microsystems), and the Bond Polymer Intense Detection Kit (Leica Microsystems).

Luciferase assays
These were performed using the Luciferase Dual-Glo Assay System (Promega). Activities of reporters were normalized to constitutive Renilla luciferase (p-actin).

Chromatin immunoprecipitation
Chromatin immunoprecipitation (ChiP) assays were performed using 786-0 or PANC-1 cells stably expressing HAF, HAF DM, or shHIF2 and the respective empty vector controls pMX-IRES-GFP (Vec) or pSUPER, using the EZ ChIP Kit (EMD Millipore). ChiP antibodies were HIF2α (NB100-122), HAF (6), FLAG (Sigma-Aldrich), and control IgG (Bethyl Labols). Semiquantitative PCR was performed using standard protocols with indicated primer pairs (Supplementary Methods). Quantitation was performed using the AlphaView Q Imaging software (ProteinSimple).

In vivo tumor growth studies
Nu, nude mice (10 per group) were injected subcutaneously with 10⁷ 786-0 cells. Tumor diameters were measured twice weekly at right angles (dshort and dlong) using electronic calipers and tumor volumes were calculated by the formula volume = (dshort ² × dlong ²) / 2 (31). Orthotopic renal models were generated by injection of 5 x 10⁶ 786-0 cells (in 50 μl saline) in the right renal subcapsule of nude mice (6 per group) as described (32). Tumors were harvested after 2 months. Tumor weight was calculated by subtracting the weight of the normal left kidney from the weight of the implanted right kidney.

CRCC tumor microarray preparation and analysis
The tumor microarray (TMA) comprised pretreatment nephrectomy specimens of patients with metastatic grade 3/4 CRCC treated with sorafenib or sorafenib plus interferon (33). All patients showed haploinsufficiency at 3p, indicating pVHL deficiency, although formal sequencing of pVHL was not undertaken. Three cores per patient in replicate arrays were stained for HIF1α (1:100; 2015-1; Epitomics), HIF2α, or HAF. Image capture and analysis for HIF1α and HIF2α were performed using the Ariol system (Applied Imaging). Cores were scanned using TMA Navigator software (Applied Imaging). Regions of viable tumor were identified by subtracting the weight of the normal left kidney from the weight of the implanted right kidney.

Statistical analysis
These were performed using IBM SPSS (Vs.19) comparing nuclear HAF staining and PFS with patients stratified to groups expressing low and high HAF, respectively. The log-rank test and Cox regression were used to compute the value for Kaplan–Meier curves and hazard ratios, respectively.

Results
HAF promotes the transcription of HIF2 target genes
CRCC cells offer a unique opportunity to study the effects of HAF on HIF2α, independently of HIF1α, as many CRCC cells do not express HIF1α (34). CRCC also provides a setting in which HIF2α has a clear driving role in tumor progression. To identify HIF2 target genes in 786-0 CRCC cells (pVHL deficient, constitutive HIF2α, no HIF1α), we stably expressed HIF2α shRNA (shHIF2) or empty vector (pSUPER) in these cells. Knockdown of HIF2α in 786-0 shHIF2 cells decreased the transcription of a panel of HIF2 genes compared with the empty vector control, confirming that these genes were HIF2 dependent (Fig. 1A). Similar results were obtained by transient transfection of parental 786-0 cells with a different siRNA duplex targeting HIF2α and normalized to a nontargeting control siRNA (siH2_2; Fig. 1A). To investigate the role of HAF specifically on HIF2-dependent transcription, we overexpressed HAF in the 786-0 cells and found that it induced the transcription of the aforementioned HIF2 target genes without affecting HIF2α expression (Fig. 1B). Among these genes, we observed that the greatest induction due to HAF overexpression was in the levels of the pluripotency genes OCT-3/4, SOX2, and NANOG. The induction of these genes was also observed in 786-0 cells stably overexpressing HAF, and this occurred in both normoxia and hypoxia (Fig. 1C). Similar results were obtained using A498 CRCC cells (pVHL-deficient, constitutive HIF2α; Supplementary Fig. S1A and S1B). Consistent with constitutive HIF2α expression due to loss of pVHL, the 786-0 cells did not show any general induction of HIF2 target genes upon exposure to 16 hours of hypoxia. However, we did observe significant temporal inductions of OCT-3/4, SOX2, and NANOG after 8 hours of exposure to hypoxia (Fig. 1D), suggesting an alternate, pVHL-independent hypoxia-sensing mechanism for these genes. To investigate the impact of HAF overexpression in cells that have functional pVHL, and therefore hypoxia-inducible HIF1α and HIF2α, we overexpressed HAF in the ACHN CRCC cells. Here, HAF overexpression decreased the levels of HIF1α protein without affecting HIF2α levels, and attenuated the hypoxic induction of HIF1-specific target genes, BNIP3, BNIP4L, NIX), and PKI (Fig. 1E and F). HAF overexpression also significantly induced transcription of the HIF2 target genes OCT-3/4, SOX2, NANOG, and VEGFA to a level above that induced by hypoxia (Fig. 1G). Hence, HAF promotes the shift toward HIF2-dependent transcription in both pVHL wild-type and pVHL-deficient CRCC cells.

HAF promotes the binding of HIF2α to the HRE
To determine the mechanism by which HAF promotes HIF2 transcription, we investigated the impact of HAF overexpression on HIF2α DNA binding activity within the promoter of VEGFA. HAF has been shown to bind the VEGFA promoter at a site located approximately 600 bp downstream of the HRE (Fig. 2A, i; ref. 35). Using specific primers flanking the VEGFA HRE in 786-0 cells (Fig. 2A), we show that HAF overexpression significantly increased the binding of HIF2α to the HRE (Fig. 2A, ii, left, 2C). In addition, HAF itself bound the HRE but binding was reduced when HIF2α was knocked down, suggesting that HAF binding to the HRE is HIF2α-dependent.
Figure 1.
A, the effect of HIF2α knockdown using stable expression of HIF2α shRNA or transient transfection with an alternative HIF2α siRNA duplex on levels of HIF2α transcript and a panel of HIF2α target genes as determined by qRT-PCR normalized to stably or transiently expressed nontargeting controls, respectively. B, the effect of transient HAF overexpression in 786-0 cells on HIF2α protein levels determined by Western blotting (i) and transcription of HIF2α and a panel of HIF2α target genes as determined by qRT-PCR normalized to vector control (ii). C, the effect of stable HAF overexpression in 786-0 cells on transcription of a subset of HIF2α target genes in both normoxia and hypoxia as determined by qRT-PCR normalized to vector cells in normoxia. D, qRT-PCR showing effect of duration of hypoxic exposure on the transcription of the panel of HIF2 target genes. Data shown are the mean of at least two independent experiments ± SE; *, P < 0.05. E and F, the effect of HAF overexpression in ACHN cells on HIF1/2α protein levels by Western blotting (E) and HIF1 (F), or HIF2-dependent target genes (G) in normoxia and hypoxia. Data are representative of two independent experiments ± SE; *, P < 0.05.
Using specific primers flanking the HAF site in the VEGFA promoter, we confirmed that both endogenous and overexpressed HAF bound to the HAF site (Fig. 2A, iii, left; 2D and F). HAF binding to this site was unchanged when HIF2α was knocked down, suggesting that HAF binding to the HAF site is HIF2α independent (Fig. 2A, iii, right; 2G). In
A schematic showing the P117 reporter construct containing the HAF site inserted upstream of a CMV minimal promoter and luciferase. B, effects of HAF overexpression on luciferase activity of cotransfected P117 Luc or P117 containing a deletion of the HAF site (P117D) in 786-0 cells in normoxia. Results are given as relative light units (RLU) of luciferase normalized to a cotransfected luciferase construct. C, effects of HAF or HIF2α (siH2) knockdown using two different siRNA duplexes on activity of P117 in 786-0 cells in normoxia. D, transcriptional activity of the p65 luciferase reporter when cotransfected with the GAL4 DBD alone, GAL4 DBD-HAF fusion, or positive control GAL4-VP16. Results given as RLU of luciferase normalized to a cotransfected Renilla vector. E, schematic showing the P117 HRE reporter construct containing the P117 sequence in tandem with the HRE fused to luciferase. F and G, the effect of HAF overexpression in ACHN CRCC cells when cotransfected with P117 HRE Luc (F) or P117del HRE (G) in normoxia and hypoxia. Results are given as RLU of luciferase normalized to a cotransfected constitutive Renilla luciferase construct. H, effect of deletion of the HRE reporter construct containing the P117 sequence in tandem with the HRE fused to luciferase. I, effect of deletion of the HRE reporter construct containing the P117 sequence in tandem with the HRE fused to luciferase. J, cartoon depicting the formation of HAF/HIF2 transcriptional complex that promotes HIF2α-dependent transcription.

To investigate another HIF2-dependent target gene, we examined the promoter sequence of the stem cell factor, OCT-3/4. Four conserved regions (CR) between the promoters of the human, mouse, and bovine orthologs of OCT-3/4 have been reported (36). Although six putative HREs have been identified within these CRs, only two (CR3 and CR4) have been described to bind HIF2α (Fig. 2B, i; ref. 37). We found that endogenous HIF2α bound the OCT-3/4 promoter within the HRE on CR3, but not within CR4, and the binding of HIF2α to CR3 was significantly increased when HAF was overexpressed (Fig. 2B, ii, iii; 2C). Similar to the VEGFA promoter, HAF overexpression increased its recruitment to the CR3 HRE, but its binding was decreased when HIF2α was knocked down, suggesting, again, that the binding of HAF to the HRE is HIF2α dependent (Fig. 2B, ii, right; 2F). HIF2α binding to the HRE in CR4 was undetectable, and hence the impact of HAF overexpression on HIF2α binding to this site could not be assessed (Fig. 2B, iii, left). Hereafter, the HRE within CR3 will be referred to as the OCT-3/4 HRE. Because HAF binds to similar sites (CCCCCCTCCCCC and CCCCCAGCCCCC) within the EPO and VEGFA promoters, respectively (35, 38), we searched the CRs within the OCT-3/4 promoter for putative HAF binding sites based on the CCCCCRCCCC motif deduced from the two HAF binding sites. We identified a putative HAF site (CCCCCTCCCCC) within a highly CR of CR4, known as the OCT-3/4 distal enhancer (39). Using ChIP primers flanking this region, we observe binding of HAF to CR4, which was not affected by HIF2α knockdown, suggesting that HAF binds to CR4 in a HIF2α-independent manner (Fig. 2B, iii, right, 2G).

To evaluate the robustness of the data, we quantitated and combined ChIP results from three independent experiments. Hence, we confirm that HAF overexpression promotes a significant increase in the binding of HIF2α to the HREs of both VEGFA and OCT-3/4 (CR3), and that HAF itself is also recruited to the HRE in a HIF2α-dependent manner (Fig. 2C–E). In addition, we
confirm the binding of both endogenous and overexpressed HAF to the HAF site within VEGFA, and to its putative binding site within OCT-3/4 (CR4), both of which occur independently of HIF2α (Fig. 2F and G).

HAF binding to DNA cooperates with the HRE for maximal hypoxic induction

To determine whether HAF binding to its DNA binding site confers transcriptional activation, we used the minimal promoter of EPO, P117 (40), which contained a HAF binding site (Supplementary Materials) fused to a CMV minimal promoter upstream of luciferase (P117 Luc). HAF overexpression significantly increased P117 Luc activity in 786-0, but did not affect the activity of P117del, which contained the flanking sequences of P117, and a deletion of the HAF binding sequence (CCCCCCCCCCCC, Fig. 3B). Similar results were obtained using PANC-1 cells (Supplementary Fig. S2A). Furthermore, P117 Luc activity was significantly decreased in the presence of HAF siRNA, but was not affected by transfection with HIF2α siRNA, suggesting that HAF binding to its site confers transcriptional activity independently of HIF2α (Fig. 3C). Efficiency of siRNA knockdown was verified using Western blotting (Supplementary Fig. S2B). To determine whether HAF on its own is sufficient to confer transcriptional activation, we used the yeast GAL4/UAS system. The GAL4 DBD binds a consensus UAS site on DNA, but is unable to activate transcription unless fused to a transactivator, such as the herpes simplex virus protein, VP16. When we generated a GAL4DBD–HAF fusion, we observed a significant induction of luciferase from a cotransfected 5xUAS domain containing luciferase reporter construct, compared with transfection with the GAL4DBD alone, suggesting that HAF is sufficient to confer transcriptional activation when tethered to DNA (Fig. 3D). It should be noted that the HAF-induced increase in luciferase was substantially lower than that with GAL4-VP16, which has been described as an unusually potent transcriptional activator, possibly due to its viral origin (41).

To assess the contribution of HAF and HIF binding to hypoxia-induced gene transcription, we inserted the HRE contained within the EPO enhancer in tandem with P117 Luc, to generate P117 HRE Luc (Fig. 3E). Using ACHN CRCC cells expressing wild-type pHL for hypoxia-inducibility, we found that cotransfection of P117 HRE with HAF in normoxia, or transfection with P117 HRE alone with exposure to hypoxia, resulted in significant inductions of luciferase activity (1.5-fold each), compared with P117 HRE in normoxia (Fig. 3F). When we cotransfected P117 HRE with HAF and also exposed cells to hypoxia, we obtained a 3-fold increase in luciferase when compared with P117 HRE in normoxia, suggesting that the HAF and the HRE both contribute to hypoxia-induced gene transcription (Fig. 3G). In contrast, cotransfection of HAF with P117del HRE (P117 HRE containing a deletion of the HAF binding sequence), did not significantly affect luciferase activity, suggesting that the binding of HAF to the HAF site is required for its transcriptional activity (Fig. 3G). To address the effects of potential nonspecific activity conferred by flanking sequences, we also evaluated the transcriptional activities of constructs containing various combinations of the deletion of the HAF site (P117del), or of the HRE (HREdel). We found that deletions of the HRE but not of the HAF site abrogated the hypoxia-inducibility of P117 HRE, suggesting that the HAF site does not contribute to the hypoxia-inducibility of transcription, the latter of which is entirely dependent on the presence of the HRE (Fig. 3H).

Taken together, the results suggest that HAF, by binding to a conserved HAF DNA binding sequence within close proximity to the HRE, cooperates specifically with HIF2α to promote HIF2-dependent transcription (Fig. 3I).

HAF SUMOylation is required for HIF2 activation

HAF has been reported to be a target of conjugation with small ubiquitin-related modifiers (SUMO), and two SUMOylation sites (K94 and K141) have been confirmed using mass spectrometric approaches (42, 43). Protein SUMOylation and deSUMOylation can regulate a variety of diverse outcomes, including changes in protein stability, localization, transcriptional activation, and protein–protein interactions (44). To determine whether the reported sites are indeed the sites of HAF SUMOylation, we overexpressed FLAG-tagged wild-type HAF (referred to as HAF), or FLAG–HAF containing lysine to arginine mutations of the residues known to be SUMOylated (K94R and K141R), referred to as HAF DM. We observed constitutive SUMO-1 conjugation of overexpressed wild-type

Figure 4.
A, i, Western blotting showing immunoprecipitation (IP) of FLAG from 786-0 cell lysates overexpressing FLAG–HAF or a SUMOylation-deficient mutant of HAF (FLAG–HAF DM), then probed for SUMO-1. A, ii, Western blotting showing effect of denaturing immunoprecipitation on HAF SUMOylation. Lyssates were immunoprecipitated with anti-FLAG, boiled in 1% SDS, and supernatant was subjected to a second IP with anti-FLAG, then probed for SUMO-1. Molecular weights consistent with unconjugated HAF or HAF with SUMO-1 are shown. B, Western blotting showing SUMO-1 modification of immunoprecipitated FLAG–HAF in 786-0 cells after 4 hours of exposure to indicated oxygen tensions. C, time course of HAF SUMOylation in response to hypoxia. Stable FLAG–HAF expressing 786-0 cells were exposed to the indicated durations of 1% O2 and analyzed as in B. D, Western blotting showing levels of FLAG–HAF, FLAG–HAF DM, and HIF2α in normoxia, and after exposure to indicated durations of hypoxia in 786-0 cells. E, Western blotting showing SUMO-1 modification of endogenous HAF. Endogenous HAF was immunoprecipitated from lysates of ACHN cells after 8 hours of hypoxia and probed with SUMO-1. F and G, impact of transient HAF or HAF DM overexpression on P117 HRE luciferase activity (F) or transcription of HIF2 target genes OCT-3/4 and SOX2 in 786-0 cells (G) in normoxia determined by qRT-PCR normalized to vector control. H, i, top, Western blotting showing immunoprecipitation of HIF2α from lysates of 786-0 cells (exposed to 4 hours of hypoxia) stably expressing FLAG–HAF or FLAG–HAF DM, then probed with FLAG. Bottom, Western blotting showing immunoprecipitation using 786-0 cell lysates, as in top, of cells grown in normoxia or exposed to 4 hours of hypoxia. ii, Western blotting showing immunoprecipitation of HIF2α from parental 786-0 cells in normoxia or exposed to 4 hours of hypoxia, then probed for HAF (to detect endogenous HAF). I, Western blotting showing levels of HIF1α and HIF2α in normoxia and hypoxia ± proteasome inhibitor MG-132 (5 μmol/L) in PANC-1 cells stably overexpressing FLAG–HAF or FLAG–HAF DM. J, luciferase activity in FLAG–HAF or FLAG–HAF DM overexpressing in PANC-1 cells transiently transfected with P117 HRE and normalized to Renilla luciferase. K and L, quantitation of ChIP assays for CR4 (HAF site; K) and CR3 (HRE site; L) on the OCT-3/4 promoter. Antibodies used for ChIP are indicated below arrows. *P < 0.05. M, transcriptional activity of the pGL4 luciferase reporter when cotransfected with the GAL4 DBD alone, GAL4–DBD–HAF fusion, or GAL4–DBD–HAF DM mutant. Results given as relative light units (RLU) of firefly luciferase normalized to Renilla encoded in the GAL4 DBD (pBIND) vector. *P < 0.05. N, model for the regulation of HAF–mediated HIF2 activation. HAF, by binding to its consensus site, regulates baseline transcription of target genes. SUMOylation of HAF (which can be induced by hypoxia) promotes the binding of HAF to HIF2α, hence driving maximal activation of HIF2 target genes.
HAF but not of cells overexpressing HAF DM, confirming that K94R and K141R are indeed sites of HAF SUMOylation (Fig. 4A, i). To confirm that the SUMOylation observed was specific for HAF, we immunoprecipitated FLAG–HAF, then boiled the samples in 1% SDS to dissociate all associated proteins, and performed a second immunoprecipitation using anti-FLAG. Indeed we found that the SUMOylated bands were retained even after denaturation, confirming that it was indeed HAF that was SUMOylated (Fig. 4A, ii). Two bands were observed above the band for unmodified FLAG–HAF (120 kD) with molecular weight shifts consistent with conjugation to one or two SUMO-1 molecules (each SUMO ~10 kD). To determine whether HAF SUMOylation is dependent on oxygen tension, we immunoprecipitated FLAG–HAF (wt and DM) from 786-0 CRCC cells grown in normoxia (20% O2) or exposed to 3% O2 or 1% O2 for 4 hours. Oxygen tensions of 3% to 6% O2 or 1% to 3% (30–50 mmHg; 8–15 mmHg) are physiologic in the kidney cortex and medulla, respectively, whereas tensions of <1% O2 are typical of solid tumors (45). Intriguingly, we observed that exposure to 1% O2 resulted in a marked increase in SUMOylation (by SUMO-1) compared with exposure to 3% or 20% O2 (Fig. 4B). The increase in HAF SUMOylation was sustained, although slightly decreased after 24 hours at 1% O2, whereas levels of HAF and HIF2α were unchanged (Fig. 4C and D). In contrast, modification by SUMO-2/3 was weak and barely detectable (Supplementary Fig. S3A), and further studies hence focused exclusively on SUMO-1 modification. To determine whether SUMOylation of endogenous HAF was also hypoxia dependent, we immunoprecipitated endogenous HAF from cells grown in normoxia, or from cells exposed to 4 hours of hypoxia (1% O2). Indeed, we found that SUMOylation of endogenous HAF was also increased by hypoxia, thus supporting our findings with overexpressed HAF (Fig. 4E). To assess the impact of HAF SUMOylation on its regulation of HIF2 activity, wild-type HAF or HAF DM was transiently transfected into 786-0 cells, together with the P117 HRE reporter. Indeed, we found that HAF DM, unlike HAF wt, was unable to induce the activity of cotransfected P117 HRE (Fig. 4F). Using qRT-PCR, we confirmed that HAF overexpression did not promote the transcription of the HIF2 target genes OCT-3/4 and SOX2, supporting our observations with P117 HRE (Fig. 4G). Because we have previously shown that HAF-mediated HIF2 transactivation requires HAF binding to HIF2α (4), we examined the ability of HAF DM to bind HIF2α. Here, we found a clear reduction in the amount of FLAG–HAF DM that could be immunoprecipitated with HIF2α compared with that with FLAG-tagged wild-type HAF (Fig. 4H, i, top). In addition, we observed a marked increase in the coimmunoprecipitation of FLAG–HAF (but not of HAF DM) with HIF2α after exposure of 786-0 cells to 4 hours of hypoxia, although equal amounts of HIF2α were immunoprecipitated, suggesting that the binding of HAF to HIF2α is enhanced by hypoxia-dependent SUMOylation (Fig. 4H, i, bottom). We also observed increased coimmunoprecipitation of endogenous HAF with HIF2α in hypoxia, thus supporting our findings with overexpressed HAF (Fig. 4H, ii). To investigate the impact of HAF SUMOylation in the context of functional pVHL in cells expressing both HIF1α and HIF2α, we also overexpressed HAF and HAF DM in PANC-1 cells. Similar to VHL-deficient cells, we observed that HAF SUMOylation in pVHL–competent PANC-1 cells was also increased in hypoxia (Supplementary Fig. S3B).

In addition, similar to wild-type HAF, HAF DM mediated the protaosomal-dependent degradation of HIF1α in both normoxia and hypoxia, while not affecting levels of HIF2α (Fig. 4I). In addition, HAF DM expression did not induce the activity of cotransfected P117 HRE, and showed reduced binding to HIF2α, thus confirming our findings in the 786-0 cells (Fig. 4J and Supplementary Fig. S3C). Furthermore, using ChIP assays, we found that although HAF DM retained the ability to bind the HAF site within the OCT-3/4 promoter in a similar manner to wild-type HAF (Fig. 4K), HAF DM neither bound the OCT-3/4 HRE, nor promoted the binding of HIF2α to the OCT-3/4 HRE, as observed with wild-type HAF (Fig. 4L). However, when tethered to DNA using the GAL4/IUS system, we found that GAL4–HAF DM mutant demonstrated comparable transcriptional activity with GAL4–HAF, suggesting that HAF may also possess SUMOylation-independent transcriptional activity (Fig. 4M).

Hence, we show that HAF SUMOylation is required for the binding of HAF to HIF2α, and the formation of a HAF/HIF2α transcriptional complex that drives maximal induction of HIF2 downstream target genes (Fig. 4N).

HAF promotes tumor metastasis in CRCC cells in vivo

Because HIF2α has been shown to play a unique role in driving CRCC progression, we investigated the role of HAF on tumor growth using the 786-0 CRCC cells. Using Z-stacking multispectral confocal microscopy, we confirmed that endogenous HAF (red) and HIF2α (green) were both localized within close proximity in the nucleus of 786-0 cells (Fig. 5A). Confirmation of the specificities of the antibodies used is shown in Supplementary Fig. S4. When injected subcutaneously into the flanks of nude mice, we found that xenografts from cells overexpressing wild-type HAF showed a trend of increased tumor growth (P < 0.1), whereas cells overexpressing HAF DM grew at a slower rate, similar to the vector control cells, consistent with our findings that SUMOylation of HAF is required for HIF2 transactivation (Fig. 5B). The growth-promoting effect mediated by HAF overexpression was only evident in vivo, as we did not observe any effects of HAF overexpression on cell proliferation in vitro (Supplementary Fig. S5A). Because metastasis is frequently observed in advanced CRCC, we also investigated the impact of HAF overexpression in a 786-0 CRCC mouse renal orthotopic tumor model (46). Here, we found that HAF overexpression increased the incidence and number of metastatic lesions in the gastrointestinal (GI) tract of the mice (5 of 6 in HAF-injected mice) versus 1 of 6 in vector-injected mice (Fig. 5C and D), although there were no significant differences between the sizes of the HAF versus vector-derived primary tumors. The lesions were confirmed by the presence of GFP, encoded by the pMX vector backbone, which was not detected in the GI tract of non-tumor-bearing mice, or in mice bearing vector-derived tumors (Supplementary Fig. S5B). The spleen of mice bearing HAF-overexpressing orthotopic tumors were also enlarged although metastatic lesions were not grossly apparent (Fig. 5E). Hence, HAF overexpression in vivo promotes the growth of subcutaneous tumor xenografts, and in a kidney orthotopic model, results in increased metastasis.

HAF is associated with decreased PFS in CRCC

To investigate the clinical application of our findings, we examined HAF and HIF2α distribution in patient CRCC tissue. When we stained serial sections of tumor tissue obtained from
human stage III CRCC, we observed that regions of the tumor that expressed high levels of HIF2α also expressed high levels of HAF (Fig. 6A). This suggests that the HAF/HIF2α complex may also be present in vivo to promote HIF2-dependent transcription. To investigate the relationship between HAF and CRCC patient prognosis, we stained a TMA from patients with stage III/IV advanced metastatic CRCC (33). Staining intensities for HAF, HIF1α, and HIF2α together with other clinical parameters are shown in Supplementary Fig. S6A. Representative sections of high and low HIF1/2α-expressing cores with nuclear and cytoplasmic HAF expression are depicted in Fig. 6B. Overall, we found that HIF1α staining was almost exclusively nuclear, whereas HIF2α staining was both nuclear and cytoplasmic. HAF staining was also both nuclear and cytoplasmic, and there was a significant correlation between cytoplasmic and nuclear HAF expression (Supplementary Fig. S6B). When we divided the patients into two groups according to the levels of nuclear HAF, we observed a significant correlation between patients with high HAF and decreased PFS, a surrogate endpoint for survival (P = 0.045; Fig. 6C). The hazard ratio for high HAF and time to progression was 2.263 [95% confidence interval (CI), 1.001–5.116]. The median PFS for patients with high versus low HAF was 5.94 ± 0.62 versus 8.97 ± 3.1 months and approached significance (P = 0.08). The only other univariate parameter that also showed significant association with PFS was Motzer criteria (47), with a hazard ratio of 2.34 [95% CI, 0.9985–5.492] for intermediate versus good criteria, and time to progression (Supplementary Fig. S7). Other parameters such as age, sex, and number of disease sites were not significantly associated with PFS (not shown). In addition, we did not detect any significant correlations between levels of HIF1α or HIF2α and PFS (Supplementary Fig. S8). Hence, nuclear HAF may be a novel predictor of poor patient response in high-grade CRCC.
Discussion

We have previously shown that HAF promotes the switch from HIF1α- to HIF2α-dependent transcription in variety of cancer cell lines (4). HAF is an E3 ubiquitin ligase that promotes the ubiquitination and degradation of HIF1α (6). HAF also binds to HIF2α but does not promote degradation, but rather increases HIF2α transactivation. Here, we show that HAF-mediated HIF2α transactivation requires both the binding of HAF to its DNA consensus site, and the hypoxia-dependent SUMOylation of HAF, which promotes its interaction with HIF2α. Hence, SUMOylated HAF contributes to the maximal induction of HIF2 target genes, and this is particularly clear in the pluripotency genes OCT-3/4, SOX2, and NANOG. The fact that this subset of HIF2-dependent genes retains hypoxia inducibility despite loss of VHL (Fig. 1D) supports the existence of an alternative oxygen-sensing mechanism, such as HAF SUMOylation, which may play a role in the progression of VHL-deficient CRCC.

We have observed that the HAF mutant that cannot be SUMOylated appears to be more efficient at degrading HIF1α than wildtype HAF (Fig. 4I). This suggests that HAF SUMOylation is a determinant of its HIFα isofrom selectivity. In this regard, HAF binds and ubiquitinates HIF1α through the HAF E3 ligase domain located at the HAF C-terminus, and this occurs independently of...
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HAF SUMOylation. In contrast, HAF SUMOylation, which occurs at the HAF N-terminal region, close to the HIF2α-binding domain (HAF 300–500), facilitates the binding of HAF to HIF2α, thus promoting HIF2-dependent transcription. In this regard, decreased HAF SUMOylation may reduce binding to HIF2α, thus increasing the amount of available HAF to bind and degrade HIF1α, resulting in the increased HIF1α degradation. Alternatively, it is also possible that HAF SUMOylation may interfere with its E3 ubiquitin ligase activity. Whichever the case may be, modulators of HAF SUMOylation may dictate whether it acts primarily as an activator of HIF2α, or as an inhibitor of HIF1α.

On the basis of our findings, we propose that under ambient conditions, when HIF1/2α are largely absent, HAF drives baseline transcription by binding the HAF site within the promoters of target genes. As the HAF site within the OCT-3/4 promoter resides within the distal enhancer, which is required for OCT-3/4 transcription in embryonic stem cells, HAF may also be an important regulator of OCT-3/4 transcription in these cells (39). During VHL loss (CRCC tumor initiation), both HIF1α and HIF2α are stabilized and activate downstream transcription. Under these conditions, HAF functions as an E3 ligase for HIF1α, hence attenuating the increase in HIF1α caused by pVHL loss. As CRCC tumors develop and begin to experience regions of hypoxia, HAF becomes SUMOylated and is able to bind and transactivate HIF2, driving maximal induction of HIF2 target genes (particularly of the HIF2-dependent pluripotency genes OCT-3/4, SOX2, and NANOG), while continuing to degrade HIF1α. Thus, the combination of the non-hypoxic constitutive activation of the HIFs (pseudohypoxia) manifest during pVHL loss, and the presence of SUMOylated HAF in hypoxic CRCC tumors, promote preferential activation of HIF2α, thus mediating the shift to HIF2-dependent transcription frequently observed in pVHL-deficient CRCC (Fig. 7).

In contrast, differences in hypoxia-dependent spatiotemporal stabilization of HIF1α versus HIF2α may limit the extent of HAF-mediated HIF2 specific activation due to fluctuations in HIF2α availability in other solid tumor types not associated with VHL-loss (2).

Hypoxia-dependent SUMOylation has increasingly been shown to regulate the hypoxia response. PIASy, a SUMO E3 ligase upregulated in hypoxia, SUMOylates pVHL, resulting in pVHL oligomerization, abolishing its ability to degrade HIF1α (48). Hypoxia-dependent SUMOylation of HIF1/2α has also been reported, and this promotes HIF1/2α degradation by pVHL, although this is typically reversed by the SUMO-specific protease 1 (SENP1; refs. 49, 50). Conversely, SUMOylation of HIF1α can also promote HIF1α stabilization and transcriptional activity (51). Hence, SUMOylation and deSUMOylation have clear consequences on the HIF pathway, and their physiologic (and pathophysiologic) significance remain to be elucidated. We have not yet identified the SUMO E3 ligase responsible for the hypoxia-dependent SUMOylation of HAF. However, to our knowledge, the hypoxia-dependent SUMOylation of HAF is the first report of SUMO-mediated HIFα isoform-specific regulation.

In human patients, increased HAF levels correlate to decreased PFS in patients with metastatic grade 3/4 CRCC. Indeed, the only other univariate parameter that also showed significant association with PFS was Motzer criteria, a classification system for advanced RCC that has shown excellent predictive value, and that has been used to select and stratify patients for treatment (47, 52). We did not detect any significant correlations between levels of HIF1α or HIF2α and PFS (Supplementary Fig. S8). This may be due to the advanced stage of the tumors used in our study, compared with previously reported relationships between the HIFs and patient survival, which were identified in samples comprising multiple tumor stages (15, 26, 53).

In summary, we have identified a novel mechanism determining the HIFα isoform specificity of HAF. Hypoxia-dependent SUMOylation of HAF enables its binding to HIF2α to promote the transcription of HIF2 target genes without affecting HAFs ability to bind and degrade HIF1α. In the context of the pVHL deficiency frequently observed in CRCC, this may result in preferential HIF2-specific activation that drives tumor progression, resulting in poor patient prognosis. Hence, the targeting of the HAF–HIF2 axis offers a promising therapeutic strategy for the treatment of CRCC.

Disclosure of Potential Conflicts of Interest
G. Powis has ownership interest (including patents) in Oncothyreon. No potential conflicts of interest were disclosed by the other authors.
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