Hypoxia-Associated Factor, a Novel E3-Ubiquitin Ligase, Binds and Ubiquitinates Hypoxia-Inducible Factor 1α, Leading to Its Oxygen-Independent Degradation

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Hypoxia-Associated Factor, a Novel E3-Ubiquitin Ligase, Binds and Ubiquitinates Hypoxia-Inducible Factor 1α, Leading to Its Oxygen-Independent Degradation▽†

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The hypoxia-inducible factor 1α (HIF-1α) is the master regulator of the cellular response to hypoxia. A key regulator of HIF-1α is von Hippel-Lindau protein (pVHL), which mediates the oxygen-dependent, proteasomal degradation of HIF-1α in normoxia. Here, we describe a new regulator of HIF-1α, the hypoxia-associated factor (HAF), a novel E3-ubiquitin ligase that binds HIF-1α leading to its proteasome-dependent degradation irrespective of cellular oxygen tension. HAF, a protein expressed in proliferating cells, binds and ubiquitinates HIF-1α in vitro, and both binding and E3 ligase activity are mediated by HAF amino acids 654 to 800. Furthermore, HAF overexpression decreases HIF-1α levels in normoxia and hypoxia in both pVHL-competent and -deficient cells, whereas HAF knockdown increases HIF-1α levels in normoxia, hypoxia, and under epidermal growth factor stimulation. In contrast, HIF-2α is not regulated by HAF. In vivo, tumor xenografts from cells overexpressing HAF show decreased levels of HIF-1α accompanied by decreased tumor growth and angiogenesis. Therefore, HAF is the key mediator of a new HIF-1α-specific degradation pathway that degrades HIF-1α through a new, oxygen-independent mechanism.

The hypoxia-inducible factor 1 (HIF-1) regulates the cellular response to oxygen deprivation or hypoxia. HIF-1 is composed of an oxygen-regulated HIF-α subunit and a constitutive HIF-1β subunit (45). To date, three HIF-α isoforms have been described, of which HIF-1α and HIF-2α are the best characterized. HIF-1α is expressed ubiquitously, while HIF-2α displays more tissue-specific expression (51). The HIF-1 heterodimer binds to a conserved HIF binding sequence within the hypoxia-responsive element (HRE) in the promoter or enhancer regions of target genes, causing their transactivation and an adaptive response of the tissue to hypoxia (44). HIF-1 activation is important in development and in normal adult tissues such as in skin during wound healing or in the kidney during hematopoiesis (17, 19). HIF-1 is also upregulated in many solid tumors which contain hypoxic regions because of the inability of the local vasculature to supply sufficient oxygen to the growing tumor (45). HIF-1α is a positive factor in tumor growth, and its increased expression has been correlated with poor patient prognosis (43).

Ubiquitin is a highly conserved eukaryotic protein that when covalently attached as repetitive chains to target proteins via K48 linkages targets them for degradation by the proteasome (8). The ubiquitination process involves a ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzymes (E2), and a substrate-specific ubiquitin-protein ligase (E3) that recognizes and recruits specific target proteins for ubiquitination. Under aerobic conditions, HIF-1α is hydroxylated by specific prolyl hydroxylases (PHDs) 1 to 3 in an oxygen-dependent manner at two conserved proline residues (P402 and P564 in human HIF-1α) that are situated within the oxygen-dependent degradation (ODD) domain of HIF-1α (23). Hydroxylation of these residues allows for recognition of the von Hippel-Lindau protein (pVHL), which together with elongin C, elongin B, cullin-2, and Rbx1 (the pVHL-E3 ligase complex) and the E2 enzyme UbcH5, causes the ubiquitination and subsequent degradation of HIF-1α by the 26S proteasome (34). Under hypoxic conditions where oxygen is limited, the activities of the PHDs are inhibited and HIF-1α is not recognized by pVHL, resulting in HIF-1α stabilization.

The pVHL-dependent degradation of HIF-1α is regulated by other factors such as OS-9, which increases the interaction of HIF-1α with the PHDs, hence increasing its degradation (2), and spermidine/spermine *N*-acetyltransferase 2 (SSAT2), which stabilizes the interaction between HIF-1α, pVHL, and elongin C, thus also promoting HIF-1α degradation (1). Adding to the complexity of HIF-1α regulation, it has recently been shown that hypoxia induces the SUMOylation of HIF-1α, which enables the hydroxylation-independent binding and subsequent degradation of HIF-1α by the pVHL-E3 ligase complex (7). However, under normal conditions, this is prevented by SUMO-specific protease 1 (SENP1), which deconjugates SUMOylated HIF-1α, thus stabilizing HIF-1α (7). Although not directly regulating the ubiquitination of HIF-1α, the Siah1 and Siah2 RING (*r*eally *i*nteresting *n*ew *g*ene) type E3 ligases that bind and degrade PHDs 1 and 3 during hypoxia present another level of control of HIF-1α levels (33). Additionally, Hdm2, the RING E3 ligase that binds and degrades the p53 tumor suppressor protein, can also induce the proteasomal degradation of HIF-1α in a p53-dependent manner (40).

While the pVHL-E3 ligase complex is currently the best known E3-ubiquitin-protein ligase that directly associates with

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and degrades HIF-1α, there is compelling evidence for the existence of other, non-pVHL-mediated mechanisms for HIF-1α degradation (10, 11, 21, 27, 38, 48). HIF-1α degradation mediated by the Hsp90 inhibitor 17-AAG has been attributed to the receptor of activated protein kinase C (RACK1), which binds to HIF-1α when Hsp90 is inhibited and recruits elongin C and other components of the E3 ligase complex to HIF-1α, hence promoting HIF-1α ubiquitination and degradation (30).

Here, we identify hypoxia-associated factor (HAF) as a new E3 ligase for HIF-1α that mediates the degradation of HIF-1α through a novel pVHL- and oxygen-independent pathway. HAF, also known as $SART1_{800}$ (*s*quamous cell carcinoma *a*ntigen *r*ecognized by *T* cells), was originally identified as a nuclear protein expressed in proliferating cells (47). Here, we show that HAF is an important regulator of HIF-1α that, unlike pVHL, is able to ubiquitinate and degrade HIF-1α irrespective of cellular oxygen tension. We also demonstrate the importance of HAF in the regulation of HIF-1α levels under multiple conditions and explore its significance in relation to the pVHL pathway in a panel of cell lines. Hence, our data establish a new mechanism for the regulation of HIF-1α via an oxygen-independent degradation pathway.

MATERIALS AND METHODS

Tissue culture. HT29, PANC-1, DU-145, and PC-3 cells were from ATCC (Manassas, VA). UMRC6, RCC4, and RCC4/VHL cells were gifts from P. Corn (University of Texas M. D. Anderson Cancer Center). Cells were maintained in McCoy's 5A media (HT29), Dulbecco's modified Eagle's medium (PANC-1, DU-145, UMRC6, and RCC4), and Ham's F-12 (PC-3) supplemented with 10% fetal bovine serum and 400 μg/ml G418 where appropriate. Hypoxic incubations (1% O_2) were performed for 16 h using the $InVivo_2$ hypoxia workstation (Biotrace International, Inc., Muncie, IN). Cell lysis was performed under hypoxic conditions to limit the pVHL-dependent polyubiquitination of HIF-1α induced by reoxygenation (23). Human recombinant epidermal growth factor (EGF) was from R&D Systems (Minneapolis, MN), cycloheximide was from Sigma-Adrich (St. Louis, MO), and [^{35}S]methionine/cysteine Easytag express protein labeling mix was from Perkin-Elmer (Waltham, MA).

Plasmid construction. HAF was PCR amplified from pOTB7 (ATCC MGC-2038) and recombined into pcDNA3-DEST-47 using Gateway methods (Invitrogen, Carlsbad, CA). To create FLAG-HAF (F-HAF), HAF was ligated into p3xFLAG-CMV-14 (Sigma-Aldrich), while for recombinant protein production, full-length HAF and truncated HAF were ligated into pGEX-4T1 (Amersham Biosciences, GE Healthcare, Piscataway, NJ). Site-directed mutagenesis for $HAF^{396–800}$ C-A mutations was performed using the QuikChange kit (Stratagene, La Jolla, CA). Insert sequences were verified by sequencing. pcDNA3-HIF-1α was from P. Corn (University of Texas M. D. Anderson Cancer Center), while pcDNA3.1-HIF-1α/P402A/P564A/N803A was from M. Celeste Simon (Abramson Cancer Center, University of Pennsylvania). HIF-1α truncations were generated by PCR and ligated into pcDNA3.2-V5/GW (Invitrogen) using Gateway recombination. For recombinant HIF-1α expression, V5-$HIF^{296–826}$ was excised from pcDNA3.2-V5/GW and religated into pGEX-KG (Amersham).

Antibody production. Rabbit polyclonal antibodies were raised against recombinant glutathione *S*-transferase (GST)-$HAF^{432–800}$. Immunization and antibody purification were performed by Bethyl Laboratories (Montgomery, TX). Antibody validation was by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using FLAG-tagged and endogenous HAF from cell lysates and detected HAF as a single band at ≈120 kDa, as previously reported for HAF (49).

Generation of stable clones. pcDNA3-DEST47-HAF or empty vector was transfected into HT29 cells using Lipofectamine 2000 (Invitrogen). Stable clones were selected using 500 μg/ml G418, and HAF expression was verified by quantitative reverse transcription-PCR (RT-PCR) and Western blotting.

Transient transfections. DNA transfections were performed using Lipofectamine 2000 (HT29 and RCC4) and Fugene 6 (Roche Applied Science, Indianapolis, IN) (UMRC6). Small interfering RNA (siRNA) transfections were performed using siPORT Amine (Ambion, Applied Biosystems, Foster City, CA) for HT29, DharmaFECT 2 (Dharmacon, Lafayette, CO) for PC-3, DharmaFECT 1 for DU-145, X-tremeGENE for UMRC6, and Lipofectamine 2000 for RCC4 and PANC-1 cells, using either Dharmacon SMARTpool siRNAs for HAF (L-017283-00-0005 [siHAF and HAF1]), pVHL (M-003936-00 [siVHL]), or nontargeting pool 1 (Con) or Ambion predesigned siRNA duplexes for HAF (identification no. 15969 [HAF2]). Knockdown efficacy was determined using TaqMan quantitative RT-PCR compared to control siRNA. Cotransfections of DNA and siRNA were performed using Lipofectamine 2000 (PANC-1 and RCC4), siPORT Amine (HT29), and X-tremeGENE (UMRC6).

Quantitative RT-PCR. Total RNA was isolated from cell lysates or tumor sections using the PARIS kit (Ambion, Inc). TaqMan quantitative RT-PCR was performed on the ABI 7300 system using the TaqMan one-step RT-PCR master mix kit and predesigned primers/probes (Applied Biosystems). Normalization and analyses were carried out using β_2-microglobulin (B_2M) as the internal reference by the $2^{-\Delta\Delta CT}$ method (31) and with Applied Biosystems GeneAmp 5700 SDS software. All measurements were performed in triplicate. Significance was determined by analysis of variance or Student's *t* tests where appropriate.

Western blotting and IP. Western blotting was performed with sonicated whole-cell or tumor lysates as previously described (37). Fractionations were performed using NE-PER nuclear and cytoplasmic extraction reagents (Pierce, Rockford, IL). Primary antibodies were obtained from the following sources: HIF-1α RACK1, Elongine, and GST, BD Biosciences, San Jose, CA; HIF-2α (NB100-122), Novus Biologicals, Littleton, CO; hemagglutinin (HA) and FLAG, Sigma-Aldrich; V5, Invitrogen; ubiquitin, lamin A/C, and actin, Santa Cruz Biotechnology, Inc., CA; and control immunoglobulin G1 (IgG1), Bethyl. Immunoprecipitation (IP) was performed with 1 mg lysate and antibody in lysis buffer and protein A-Sepharose beads (Sigma-Aldrich) overnight at 4°C. For sequential IP, 2.5 mg initial lysate was incubated with antibody as for single IP and washed and boiled in 50 μl 1% SDS for 5 min, after which IP was repeated with the supernatant with fresh beads and antibody in 1 ml lysis buffer.

Pulse-chase analysis. RCC4 cells transiently transfected with 50 nM siHAF#1 or control siRNA (one time per six-well plate per time point) were grown for 72 h. Cells were starved for 1 h in methionine/cysteine-free medium (Gibco cell culture; Invitrogen) supplemented with L-glutamine, labeled with 180 μCi/ml [^{35}S]methionine/cysteine in methionine/cysteine-free medium for 2 h, and then washed three times in normal medium and harvested after 0, 1, 2, and 4 h of incubation in normal medium. HIF-1α IP was performed as described above using the entire volume of lysate. Detection of the HIF-1α band was performed by autoradiography and quantitated by densitometry.

Luciferase reporter assay. The HIF luciferase reporter plasmid containing five tandem repeats of the HRE fused to a cytomegalovirus (CMV) minimal promoter (46) in the pGL3 vector backbone (Promega, Madison, WI) was a gift from R. Gillies (Arizona Cancer Center, Tucson, AZ). Assays were performed using the Dual-Glo luciferase assay system (Promega).

VEGF ELISA. The vascular endothelial growth factor (VEGF) concentration in media was measured using the Quantikine human VEGF enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems). Adherent cells were trypsinized and counted using a Neubauer hemocytometer, and VEGF formation was normalized to the cell number in each well.

GST pull-down assays. HIF-1α and truncations were generated using TNT quick coupled transcription/translation systems (Promega). Twenty microliters of in vitro-translated V5-tagged HIF-1α was incubated with 4 μg GST or GST-HAF conjugated to glutathione-agarose beads in binding buffer (20 mM Tris [pH 7.4], 250 mM NaCl, 0.1% NP-40, 1 mM dithiothreitol, 1× protease inhibitor cocktail), rotated at 4°C for 2 h, and washed five times with binding buffer. Proteins were eluted by boiling in sample buffer and analyzed by SDS-PAGE.

Recombinant protein production. GST-$HIF^{296–826}$-V5 and GST-HAF were expressed using BL21 Star (DE3) competent *Escherichia coli* cells (Invitrogen). Briefly, cells growing in log phase were induced with 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 4 h at 25°C at 200 rpm and purified with glutathione-agarose beads (Sigma-Aldrich) as previously described (37).

In vitro ubiquitination assays. Autopolyubiquitination assays were performed using 10 μg glutathione-agarose bound GST-HAF (or mutants) as described previously (4). For polyubiquitination of HIF-1α, glutathione-agarose-bound GST-HAF or GST–HIF-1α was combined in 50-μl reaction mixtures containing 50 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 2 mM ATP, 60 mM phosphocreatine (Fluka, Sigma-Aldrich), 5 U creatine phosphokinase (Sigma-Aldrich), 2 mM dithiothreitol, 50 μM human recombinant ubiquitin (Boston Biochem, Cambridge, MA), 100 nM E1, and 500 nM human recombinant E2 (Boston Biochem) and incubated at 37°C for 3 h with gentle agitation. Beads were washed and boiled, and the supernatant was immunoprecipitated with anti-V5 antibody and analyzed by SDS-PAGE.

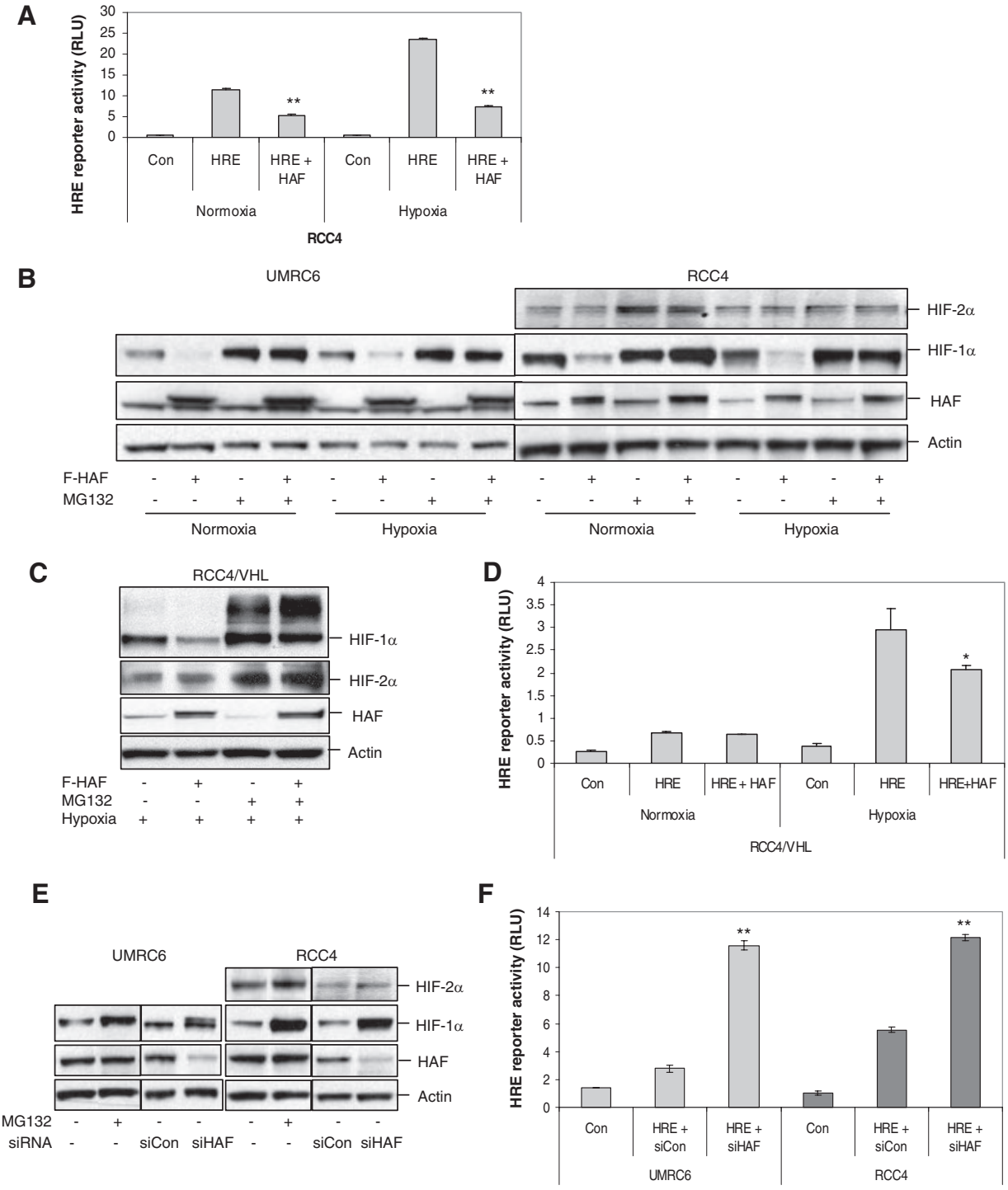


FIG. 1. HAF is the mediator of a novel mechanism of HIF-1α degradation in pVHL-deficient cells. (A) Effect of HAF overexpression on HIF-1 transactivation in RCC4 cells. Cells were transfected with HAF or control DNA together with HRE-firefly luciferase reporter or pGL3 basic vector (HRE and Con, respectively) with pTK-Renilla as a normalization control. Dual-luciferase activity was measured, and the ratio of firefly/*Renilla* luciferase activity was determined in relative light units (RLU). The data shown are from a representative experiment performed in triplicate (± standard error). (B and C) Western blot showing the effect of F-HAF transfection on HIF-α levels in UMRC6 and RCC4 cells (B) and RCC4/VHL cells (C) ± 5 μM MG132 in normoxia (where applicable) and hypoxia. The blot probed with HAF antibody shows the overexpressed F-HAF band as a slightly shifted band above the endogenous HAF. (D) Effect of HAF overexpression on HIF-1 transactivation in RCC4/VHL cells. The experiment was performed as in panel A. (E) Effect of HAF (siHAF) or the nontargeting control (siCon) siRNA transfection on levels of HIF-α in UMRC6 and RCC4 cells in normoxia. Cells were transfected with 50 nM siRNA for 72 h and then subjected to Western blotting. Untransfected cells were treated with MG132 16 h prior to harvest to show HIF-1α levels detected under proteasomal inhibition. Similar results were obtained for cells in hypoxia. (F) Effect of HAF knockdown in panel E on HIF-1 transactivation in UMRC6 and RCC4 cells. Cells were cotransfected with siRNA and DNA as in panel A. In all studies, significance was determined using Student's *t* test comparing data points to the vector control. *, $P < 0.05$; **, $P < 0.01$.

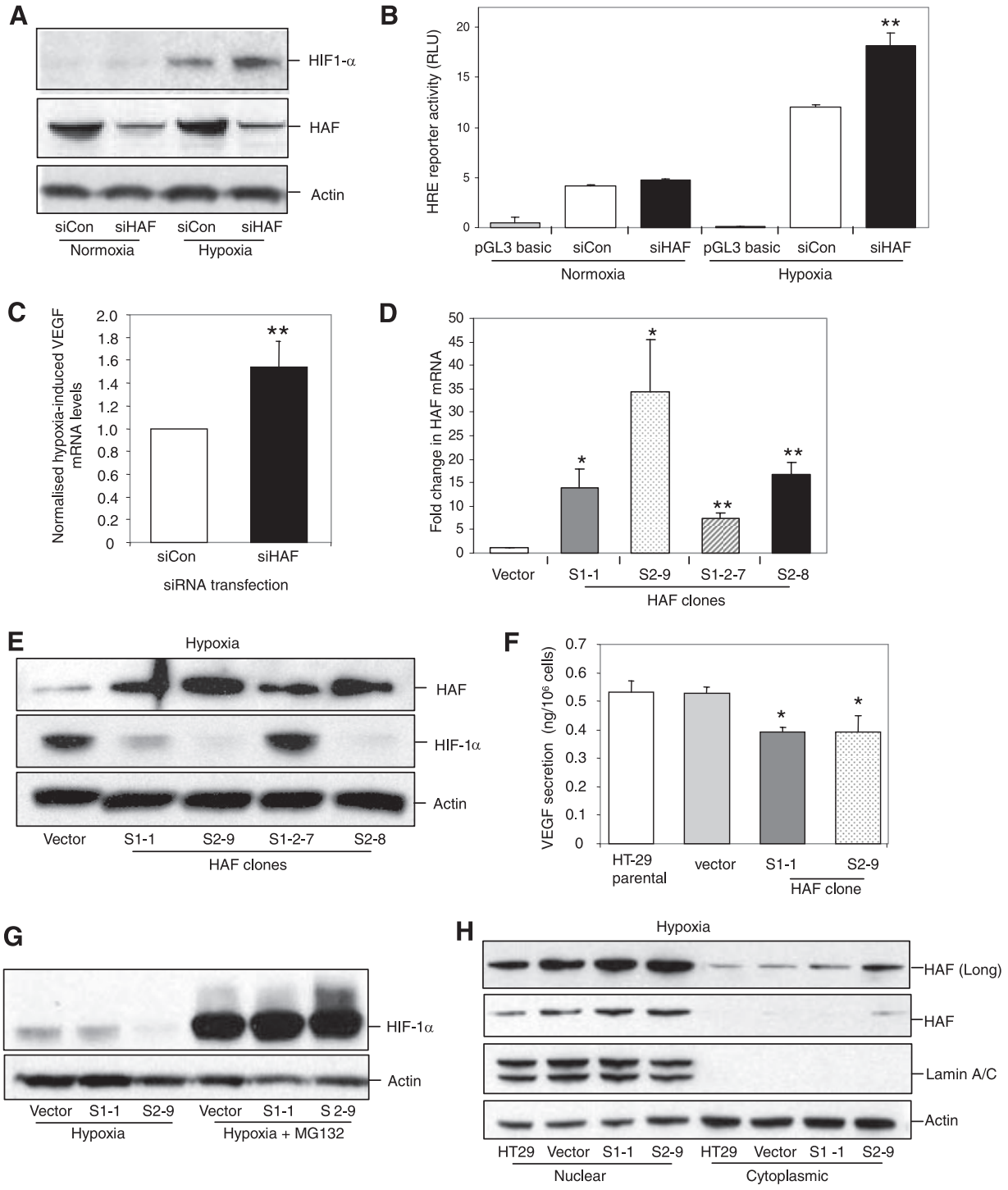


FIG. 2. Effect of HAF knockdown or overexpression on HIF-1α levels and activity in HT29 cells. (A) Effect of HAF or control siRNA (siHAF or siCon, respectively) transfection on HIF-1α levels in normoxia and hypoxia in HT29 cells. (B) Effect of HAF knockdown on HIF-1 transactivation. Cells were cotransfected with siRNA and HRE-firefly luciferase vector (or pGL3 basic vector only) and pTK-Renilla vector as the control. Dual-luciferase activity was measured using the ratio of firefly/*Renilla* luciferase in relative light units (RLU). The data shown are from a representative experiment performed in triplicate (± standard error). (C) Effect of HAF knockdown on hypoxia-induced *VEGF* transcription. HT29 cells were transiently transfected with HAF siRNA and then incubated under normoxic or hypoxic conditions for 16 h. *VEGF* mRNA levels in hypoxia were determined by TaqMan quantitative RT-PCR and normalized to *VEGF* mRNA levels in normoxia. Data are the means of four independent experiments performed in triplicate, and bars represent ± standard error. **, $P = 0.004$. (D) Relative increase in *HAF* mRNA in the HAF-overexpressing stable clones compared to vector control cells determined by quantitative RT-PCR. The data shown are for cells in normoxia, with similar results obtained for cells in hypoxia. (E) Western blots showing the levels of HAF and HIF-1α in the HAF-overexpressing stable clones. (F) Hypoxia-induced VEGF secretion in parental, empty vector, and HAF clones. VEGF concentrations measured by ELISA were determined by subtracting VEGF concentrations in cells grown in normoxia from the corresponding VEGF concentration of cells grown in hypoxia. *, $P < 0.1$. (G) Western blot showing the effect of proteasome inhibition (5 μM MG132 prior to hypoxic incubation) on the HAF-associated decrease of HIF-1α levels in HAF clones S1-1 and S2-9. (H) Western blots of cell fractionation showing HAF localization in HT29 parental, vector, and HAF clones. "HAF (Long)" indicates increased exposure showing cytoplasmic HAF.

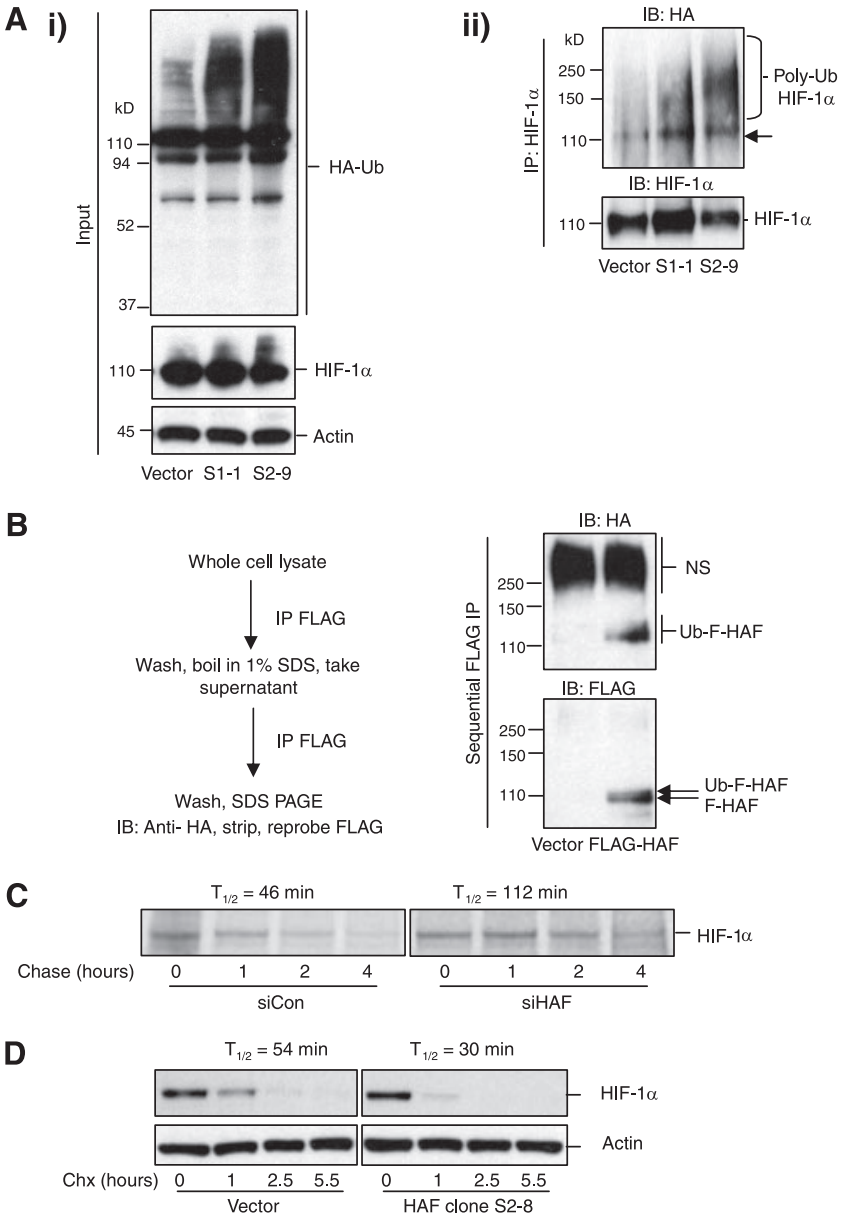


FIG. 3. HAF promotes the ubiquitination of HIF-1α and decreases HIF-1α half-life. (A) Effect of HAF overexpression on HIF-1α ubiquitination. HAF clones and vector control cells were transiently transfected with HA-ubiquitin (HA-Ub) and then incubated in hypoxia with MG132. (Ai) Western blot of whole-cell lysates used for IP. (Aii) IP of HIF-1α from the lysates in panel Ai probed with anti-HA showing levels of ubiquitinated HIF-1α. The arrow indicates the expected migration for unmodified HIF-1α. (B) Sequential IP to demonstrate specific ubiquitination of F-HAF. HT29 cells were transiently transfected with HA-Ub and F-HAF or vector, incubated in hypoxia with MG132, and then subjected to sequential IP as outlined in the schema. IB, immunoblot; NS, nonspecific. (C) Pulse-chase experiment showing the effects of HAF knockdown on HIF-1α half-life in RCC4 cells in normoxia. RCC4 cells were transiently transfected with HAF or control siRNA for 72 h, pulsed with [^{35}S]methionine/cysteine, and chased for 0 to 4 h. Immunoprecipitated HIF-1α was detected by autoradiography and quantified by densitometry, and the HIF-1α half-life ($T_{1/2}$) was calculated. (D) Effects of HAF overexpression on HIF-1α half-life. The vector control or HAF-overexpressing stable HT29 clone S2-8 was incubated in hypoxia for 6 h and then treated with 25 μg/ml cycloheximide (Chx), after which the decay of HIF-1α was monitored at the indicated times by Western blotting. HIF-1α intensity was quantified by densitometry and normalized to actin.

In vitro growth studies. Cells were seeded in triplicate at 10^5 cells/well of six-well tissue culture plates and then trypsinized and counted at daily intervals. Viability was determined by trypan blue exclusion.

In vivo growth studies. Nude mice (four per group) were injected subcutaneously with 10^7 HT29 cells. Tumor diameters were measured twice weekly at right angles (d_{short} and d_{long}) using electronic calipers, and tumor volumes were calculated by the formula volume = $(d_{short})^2 \times (d_{long})^2$ (35). Mice were euthanized when the tumors reached 1,500 mm^3, and the tumors were photographed and split in half, with one-half stored in RNAlater (Ambion) for RNA isolation and the other half snap-frozen in liquid nitrogen for protein analyses.

Tumor vasculature was visualized by staining with CD105 antibody (R&D), and nuclei were visualized by staining with hematoxylin. Five randomly acquired fields per tumor were analyzed for CD105-positive objects using Compix SimplePCI (Compix, Inc., Sewickley, PA) image analysis software. Mature vasculature was excluded based on the area contained by a structure.

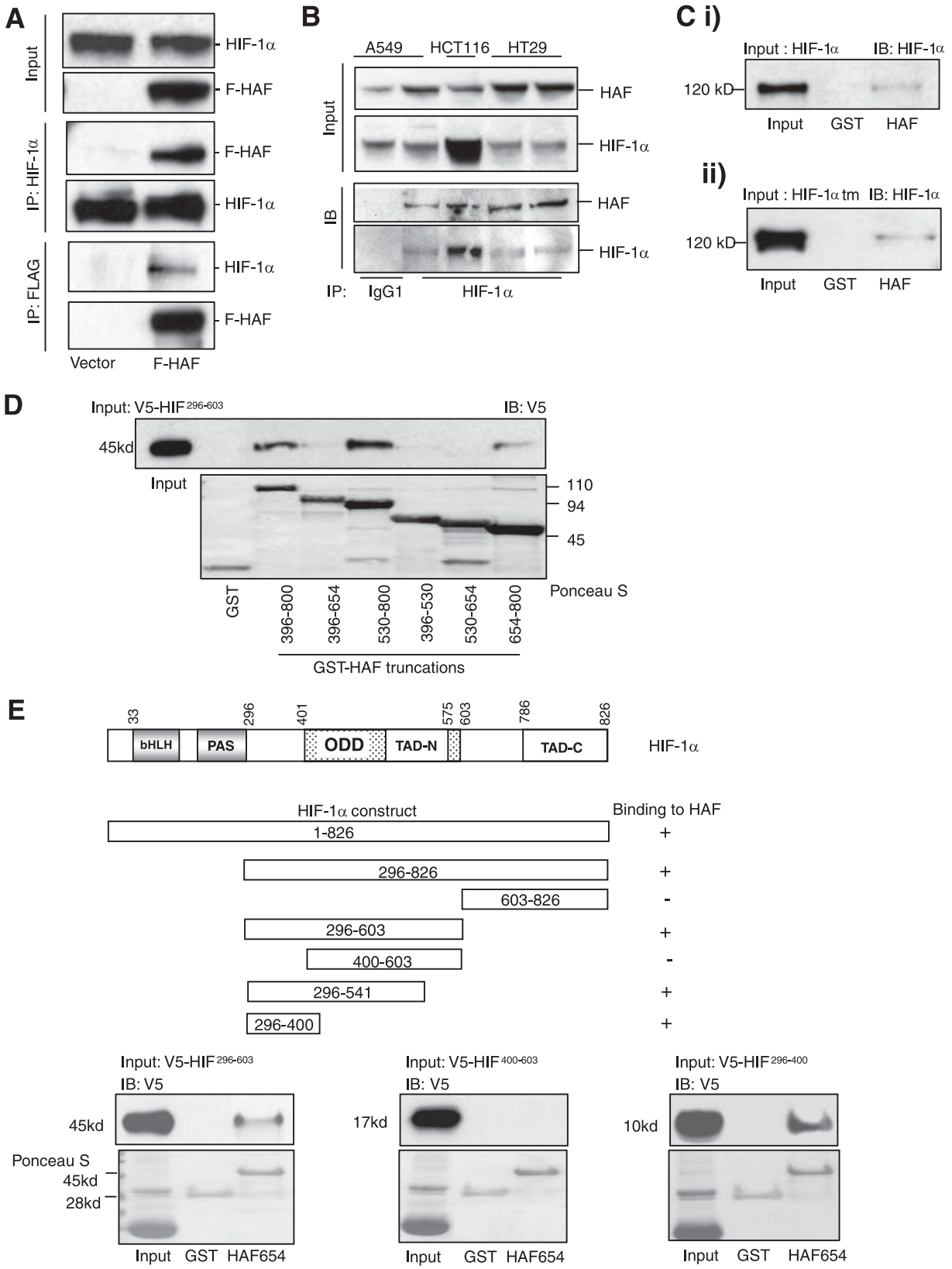


FIG. 4. Characterization of the HAF–HIF-1α interaction. (A) Coimmunoprecipitation of HAF with HIF-1α. HT29 cells were cotransfected with F-HAF or empty vector and HIF-1α and incubated in hypoxia with MG132. Lysates were immunoprecipitated with either anti-FLAG or anti-HIF-1α antibodies and then probed for HIF-1α or FLAG, respectively. (B) Co-IP of endogenous HAF with HIF-1α from a panel of human tumor xenografts in vivo. Flash-frozen xenografts were lysed and immunoprecipitated with HIF-1α or control IgG1 antibodies as indicated and

RESULTS

HAF regulates oxygen-independent degradation of HIF-1α. Since HAF has been shown to promote the hypoxia-induced activation of the HIF-1α target genes *VEGF* and *EPO* (16), we initially investigated whether HAF overexpression could increase HIF-1α transactivation activity monitored by a cotransfected HRE-luciferase reporter construct. HAF overexpression in pVHL-deficient RCC4 cells resulted in a significant decrease in HIF-1 reporter activity in both normoxia and hypoxia (Fig. 1A). It should be noted that increased HRE activity in hypoxia observed in cells transfected with HRE and both HRE plus HAF may be a result of decreased *Renilla* luciferase activity (normalization control) as part of the reduced transcription/translation rates that normally occur in hypoxia. Further investigation showed that F-HAF overexpression in two pVHL-deficient cell lines, RCC4 and UMRC6, resulted in a proteasome-dependent decrease in HIF-1α protein levels (Fig. 1B). HAF overexpression also decreased hypoxic HIF-1α protein levels in a proteasome dependent manner and decreased HIF-1 reporter activity in RCC4 cells in which pVHL was stably reintroduced (RCC4/VHL cells [Fig. 1C and D]). Consistent with the presence of functional pVHL, the RCC4/VHL cells do not express detectable HIF-1α and demonstrate only baseline levels of HIF-1 reporter activity in normoxia. HAF overexpression did not affect the levels of HIF-2α expressed in the RCC4 or RCC4/VHL cells (Fig. 1B and C), while the UMRC6 cells do not express detectable levels of HIF-2α.

To determine the role of endogenous HAF in HIF-1α regulation, UMRC6 and RCC4 cells were transfected with HAF siRNA. HAF knockdown increased HIF-1α levels in both the RCC4 and UMRC6 cells to levels comparable to that achieved by the proteasomal inhibitor MG132 but did not affect the levels of HIF-2α (Fig. 1E). The increase in HIF-1α levels by HAF siRNA was accompanied by a significant increase in HIF-1 transactivation (Fig. 1F). Together, these data show that endogenous HAF is an important HIF-1α-specific regulator in the absence of pVHL function.

To confirm that these observations were not specific to renal cancer cells, we transfected HT29 human colon carcinoma cells with HAF siRNA and found that, similar to the renal cancer cells, HAF knockdown increased hypoxia-induced HIF-1α protein levels, HIF-1 transactivation, and hypoxia-induced VEGF transcription (Fig. 2A to C). As the HT29 cells have functional pVHL and hence have undetectable levels of HIF-1α protein and activity in normoxia, we could not detect any effect of HAF knockdown on normoxic HIF-1α levels and transactivation.

To further characterize the effect of HAF on HIF-1α levels, we stably overexpressed HAF in HT29 cells. Selected clones showed a 7- to 34-fold increase in HAF mRNA (Fig. 2D) accompanied by a proportional increase in HAF protein that was associated with decreased HIF-1α levels and VEGF secretion (Fig. 2E and F). The decrease in HIF-1α was proteasome dependent as it could be blocked by treatment with MG132 (Fig. 2G). Endogenous and overexpressed HAF protein was almost exclusively nuclear (Fig. 2H). However, increased exposure demonstrates that a proportion of HAF is cytoplasmic, raising the possibility of nuclear and cytoplasmic shuttling of the protein. No differences were observed in the levels of HIF-1β or of HIF-1α mRNA (data not shown).

Taken together, these data and the data in Fig. 1 provide evidence that (i) HAF overexpression mediates the proteasome-dependent degradation of HIF-1α independently of oxygen and without requiring pVHL and (ii) endogenous HAF is a repressor of HIF-1α levels in multiple cell lines.

HAF promotes ubiquitination of HIF-1α. To investigate the mechanism by which HAF increases HIF-1α degradation, we studied the effect of HAF on HIF-1α ubiquitination. The HAF and vector control clones were transfected with HA-ubiquitin and incubated under hypoxic conditions in the presence of MG132 (Fig. 3Ai). IP of HIF-1α showed increased levels of ubiquitinated HIF-1α in the HAF clones compared to the vector control cells (Fig. 3Aii), confirming that HAF overexpression increases the levels of HIF-1α ubiquitination. We also observed that HAF overexpression increased the overall ubiquitination of proteins larger than 110 kDa, although it is unclear whether this is due to increased ubiquitination of HIF-1α alone, HAF, or other potential HAF substrates. To determine whether HAF is ubiquitinated, sequential IPs were performed in HT29 cells transfected with F-HAF and HA-ubiquitin, whereby F-HAF was immunoprecipitated from the cell lysates, boiled in 1% SDS to dissociate HIF-1α and other binding proteins, and then reimmunoprecipitated with FLAG antibody. We found that F-HAF itself was also ubiquitinated in these cells, although the tight band observed is suggestive of monoubiquitination rather than the polyubiquitination obtained with HIF-1α (Fig. 3B).

To investigate the effect of HAF on HIF-1α half-life, we performed pulse-chase experiments with the pVHL-deficient RCC4 cells transfected with control or HAF siRNA and then labeled with [^{35}S]methionine/cysteine followed by incubation (chase) for the indicated time in normal media. Immunoprecipitated HIF-1α was detected by autoradiography and quantified by densitometry (Fig. 3C). We found that HAF siRNA increased HIF-1α half-life from 46 min to 112 min, showing that endogenous HAF reduces HIF-1α half-life. We could not use this technique to examine the effect of HAF overexpres-

probed for HAF. Membranes were stripped and reprobed for HIF-1α. (C) Pull down of in vitro-transcribed/translated HIF-1α (Ci) and P402A P564A N803A HIF-1α (HIF-1α tm) (Cii) by GST-HAF but not GST alone. Recombinant GST-HAF or GST bound to glutathione-agarose beads was incubated with HIF-1α or HIF-1α tm, and then beads were pelleted and bound HIF-1α was detected with anti-HIF-1α antibody. (D) Mapping of the HIF-1α minimal binding domain of HAF. Pull-down assays were performed as in panel C using the indicated GST-HAF truncations and in vitro-transcribed/translated V5-HIF$^{296–603}$. Pull down of HIF-1α was detected by immunoblotting (IB) with anti-V5 antibody. Input of GST and GST-HAF truncations are indicated by Ponceau S staining. (E) Mapping of the HAF minimal binding domain of HIF-1α. Pull-down assays were performed as in panel D using GST-HAF$^{654–800}$ and HIF-1α–V5 truncations generated based on the known HIF-1α functional domains indicated. The results of the pull-down assays are summarized schematically. Representative blots confirming interaction between HAF$^{654–800}$ and HIF-1α$^{296–603}$ and HIF-1α$^{400–603}$ and HIF-1α$^{296–400}$ are depicted.

sion as we could not obtain sufficient labeling of the decreased HIF-1α levels in the HAF-overexpressing cells (not shown). Hence, to address the question, we exposed the HT29 HAF clone S2-8 and vector control to hypoxia for 6 h to allow HIF-1α accumulation and then treated them with 25 μg/ml cycloheximide and monitored the rate of HIF-1α decay. We found that HIF-1α half-life in hypoxia was decreased from 54 min in the vector control cells to 30 min in the HAF-overexpressing S2-8 clone (Fig. 3D).

Collectively, the data show that HAF overexpression promotes the ubiquitination of HIF-1α under hypoxic conditions and that HAF itself is ubiquitinated in cells. Furthermore, HAF is an important determinant of HIF-1α stability as knockdown of endogenous HAF increases HIF-1α half-life in pVHL-deficient cells while HAF overexpression decreases HIF-1α half-life in HT29 cells.

HAF binds to HIF-1α in vitro and in vivo. We next investigated whether HAF and HIF-1α are capable of forming a complex. Western blotting of hypoxic HT29 cells revealed co-IP of exogenously expressed HIF-1α and F-HAF (Fig. 4A). Significantly, HAF was detected in xenograft lysates immunoprecipitated with HIF-1α antibody, showing that the HAF–HIF-1α interaction also occurs in vivo between endogenous proteins in human tumor xenografts (Fig. 4B).

To characterize the interaction between HIF-1α and HAF, HIF-1α was in vitro transcribed and translated and used in pull-down assays with bacterially expressed GST-HAF or GST alone. We found that GST-HAF but not GST alone pulled down in vitro-transcribed/translated HIF-1α (Fig. 4Ci), confirming the interaction between HAF and HIF-1α that we previously observed in cells. In contrast to the hydroxyproline-dependent interaction of pVHL with HIF-1α, HAF also interacted with a P402A P564A N803A triple mutant of HIF-1α (HIF-1α tm), showing that the binding of HAF to HIF-1α does not require proline hydroxylation (Fig. 4Cii).

To map the regions of HAF–HIF-1α interaction, we generated a series of HIF-1α–V5 and GST-HAF deletion mutants. Experiments using full length HIF-1α (Fig. 4Ci) and deletion mutants HIF-1α$^{296–826}$, HIF-1α$^{296–603}$, and HIF-1α$^{603–826}$ (see Fig. S1 in the supplemental material) suggest that HAF binds to HIF-1α within amino acids 296 to 603 and that this binding is mediated by the HAF C terminus (HAF$^{396–800}$). Next, we generated additional HAF C-terminal deletion mutants to map the HIF-1α binding domain of HAF using HIF-1α$^{296–603}$ as the input. Hence, we found that the minimal binding domain of HAF is HAF$^{654–800}$ (Fig. 4D). Similarly, using GST-HAF$^{654–800}$ to pull down in vitro-transcribed/translated truncations within HIF-1α$^{296–603}$, we mapped the minimal HAF binding domain of HIF-1α to HIF-1α$^{296–400}$ (Fig. 4E).

Taken together, these data show that (i) HAF and HIF-1α interact in cells and in tumor xenografts in vivo, (ii) HAF interacts with HIF-1α in vitro and does not require HIF-1α prolyl hydroxylation, and (iii) the C terminus of HAF (HAF$^{654–800}$) interacts with HIF-1α specifically within amino acids 296 to 400.

HAF is a novel E3-ubiquitin ligase for HIF-1α. The ability of HAF to both bind and increase the ubiquitination of HIF-1α suggests that HAF could be a novel ubiquitin ligase. To test this hypothesis, we performed in vitro ubiquitination assays with recombinant proteins expressed in bacteria, which lack the ubiquitin system (20). GST-HAF was incubated with ubiquitin in the presence of E1, ATP, and a panel of E2 enzymes to assay the autoubiquitination activity of HAF. HAF displayed significant autopolyubiquitination activity in an E2-dependent manner with the E2s UbcH5 (particularly UbcH5a) and Ubc13/Uev1a (Fig. 5A). In addition, the C terminus of HAF containing the HIF-1α binding domain was sufficient for autopolyubiquitination activity (Fig. 5B).

Similar to certain recently described E3s (28, 29), inspection of the primary sequence of HAF revealed no significant similarities to any known E3-ubiquitin ligase motifs (such as RING, HECT, or U box). In addition, mutation of each of the three Cys residues in HAF$^{396–800}$ had no effect on its autopolyubiquitination activity (Fig. 5C) suggesting that HAF is a novel, atypical E3-ubiquitin ligase that, in contrast to the RING- and HECT-type E3 ligases, does not require conserved cysteine residues for functional activity.

To map the HAF ligase domain, in vitro ubiquitination assays were performed using GST-HAF C-terminal truncations. We found that HAF$^{654–800}$, which is also the HIF-1α binding region of HAF (Fig. 4D), was necessary for HAF autopolyubiquitination activity (Fig. 5D).

To determine if the GST-HAF truncations can also ubiquitinate HIF-1α, bacterially expressed GST–HIF-1α–V5 (HIF$^{296–826}$) was included in the assays. HIF-1α produced in *Escherichia coli* is unhydroxylated as bacterial cells lack this capability (22). Hence, we found that only the GST-HAF truncations containing the HAF$^{654–800}$ region were able to generate the higher-molecular-weight forms of HIF-1α (polyubiquitinated HIF-1α), whereas reaction mixtures containing UbcH5a alone or UbcH5a and HAF$^{396–654}$ did not result in the polyubiquitination of HIF-1α (Fig. 5E).

To confirm the specific ubiquitination of HIF-1α, ubiquitination assays using full-length GST-HAF and GST–HIF-1α–V5 were performed with beads washed and boiled to dissociate all binding proteins and the supernatant immunoprecipitated with V5 antibody as outlined (Fig. 5F). We found that HIF-1α is polyubiquitinated by HAF only in reaction mixtures containing both HAF and UbcH5, whereas UbcH5 in the absence of HAF or vice versa is unable to polyubiquitinate HIF-1α (Fig. 5F). Similar results were obtained using HAF$^{396–800}$ (see Fig. S2 in the supplemental material).

These results provide clear evidence that (i) HAF is a novel, atypical E3-ubiquitin ligase that ubiquitinates non-prolyl hydroxylated HIF-1α in an E2-dependent manner, and (ii) the HAF E3 ligase activity is within amino acids 654 to 800.

HAF overexpression in tumor xenografts decreases HIF-1α levels and inhibits angiogenesis and tumor growth. HIF-1α is believed to promote tumor growth and angiogenesis (42). Therefore, HAF, as an E3 ligase that mediates HIF-1α degradation, would be expected to have tumor suppressor properties. To evaluate this possibility, we studied the effect of HAF on in vivo tumor growth. Indeed, the HAF-overexpressing clones S1-1 and S2-9 showed reductions in growth by 36% ± 4% and 48% ± 5%, respectively, compared with the vector control tumors when grown as xenografts in nude mice (Fig. 6A). In addition, tumors from both HAF clones were of a markedly paler appearance than the vector tumors, suggesting decreased vascularization (Fig. 6B). These observations were

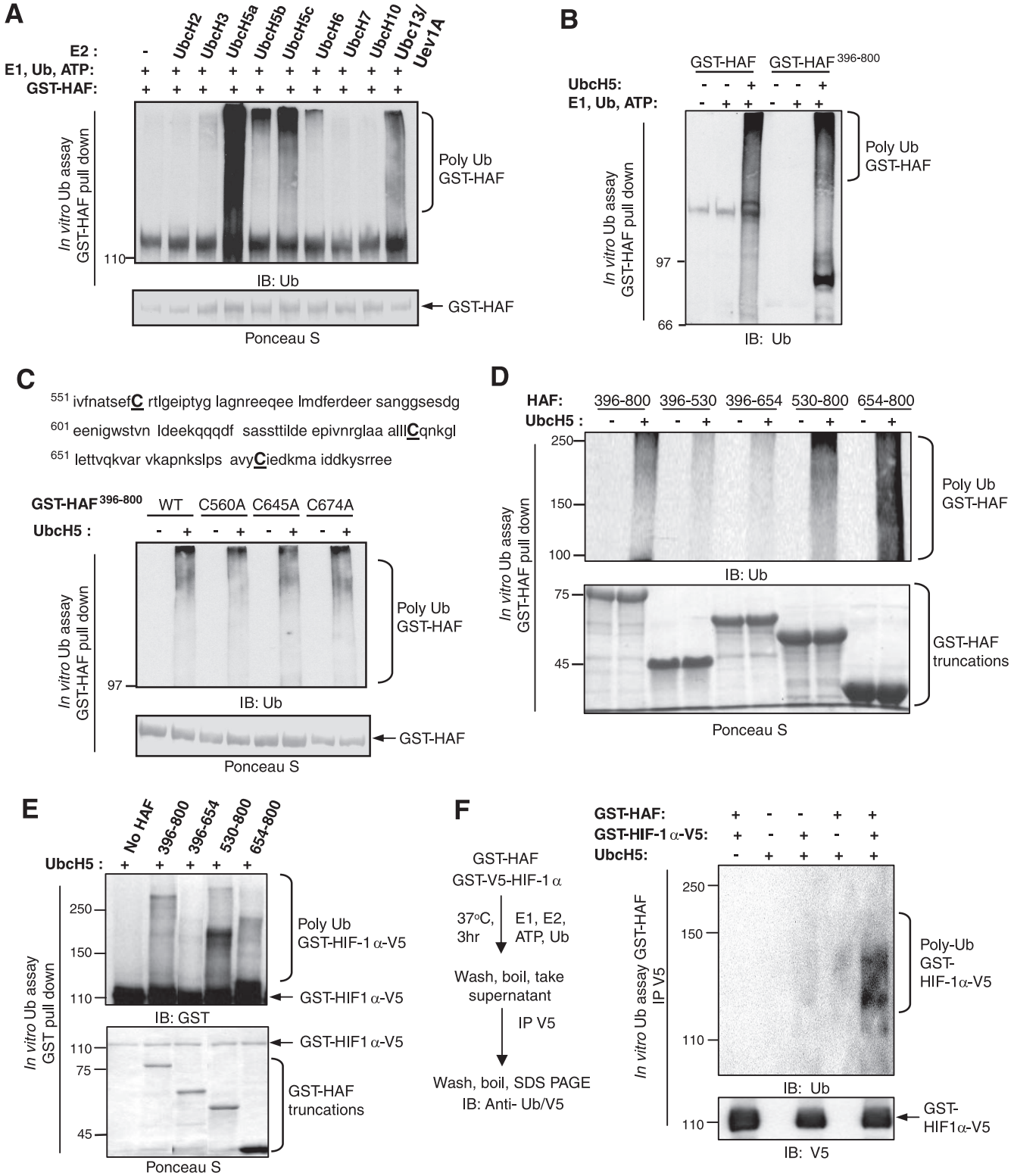
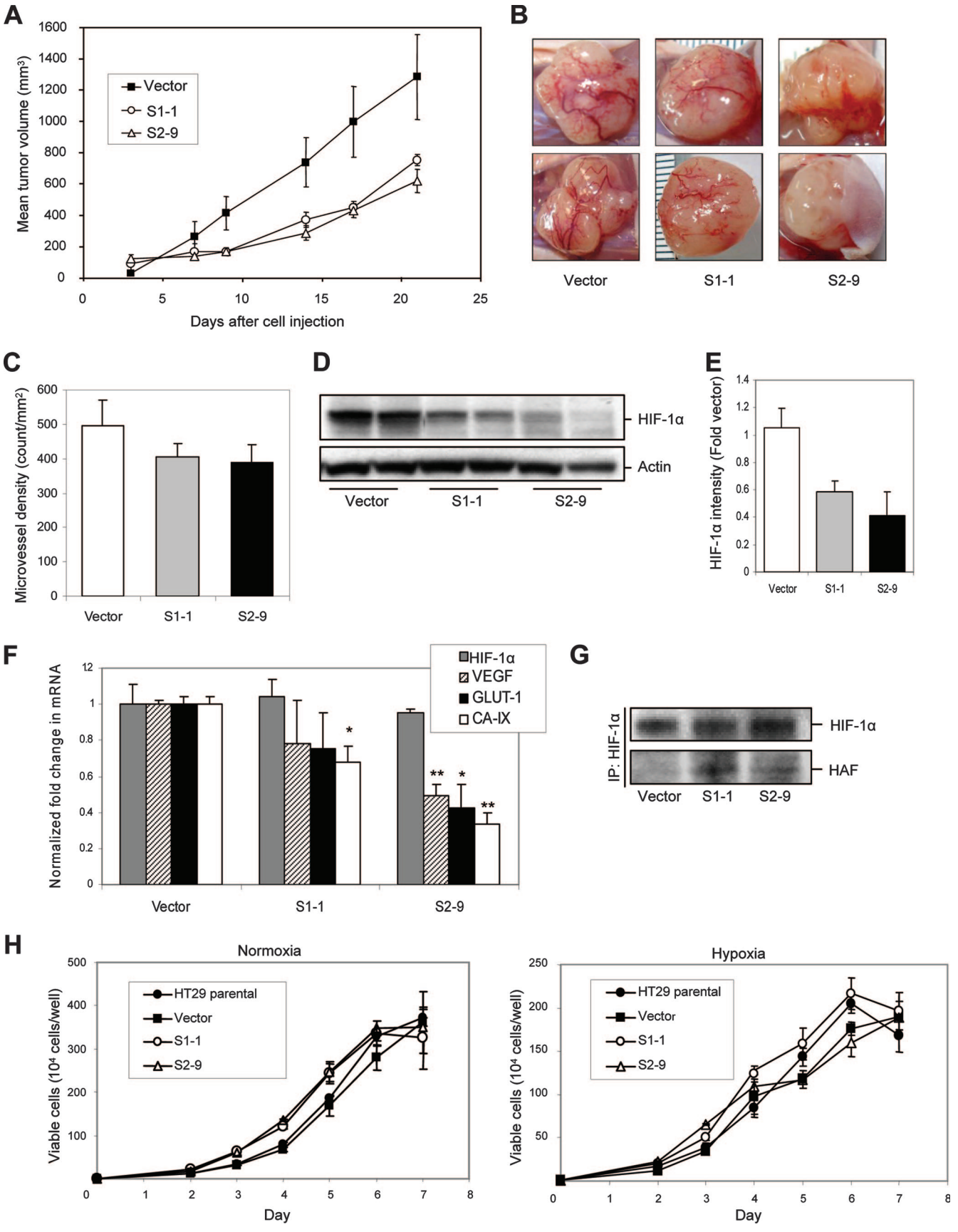


FIG. 5. Characterization of HAF E3-ubiquitin ligase activity. (A) Western blot of in vitro assay showing E2-dependent autoubiquitination activity of HAF. The assay was performed using GST-HAF on glutathione-agarose beads, E1, ubiquitin (Ub), ATP, and a panel of recombinant E2s. After incubation, beads were washed and polyubiquitinated HAF was detected by SDS-PAGE using anti-Ub. (B) The in vitro autoubiquitination assay was performed as in panel A with UbcH5a and GST-HAF or GST-HAF$^{396–800}$. (C) The top portion shows that the HAF C terminus (140-amino-acid region depicted) contains only three cysteine residues, which are shown in boldface capital letters. Below is shown the in vitro autoubiquitination assay of GST-HAF$^{396–800}$ (wild type [WT]) and GST-HAF$^{396–800}$ C-A mutants with UbcH5a. (D) Mapping of the HAF E3 ligase domain. In vitro ubiquitination assays as in panel A were performed using GST-HAF C-terminal truncations, as indicated using UbcH5a. (E) In vitro ubiquitination assays as in panel D with the addition of GST–HIF-1α–V5 to GST-HAF C-terminal truncations. The presence of higher-molecular-weight polyubiquitinated GST–HIF-1α–V5 was detected using GST antibody. (F) Confirmation of HAF-mediated E2-dependent ubiquitination of HIF-1α. In vitro ubiquitination assays were performed as outlined in the schema, and then GST–HIF-1α–V5 was immunoprecipitated and analyzed by SDS-PAGE using anti-Ub.



supported by immunohistochemical staining with the endothelial marker CD105, which revealed a trend toward decreased microvessel density in the HAF clones (Fig. 6C). Furthermore, xenograft tumors from the HAF clones (two representative tumors depicted for each clone) showed significantly lower expression levels of HIF-1α protein (Fig. 6D and E) and of the HIF-regulated genes *VEGF*, *GLUT-1* (coding for glucose transporter 1), and *CA-IX* (coding for carbonic anhydrase IX) compared to vector-derived xenograft tumors, whereas HIF-1α mRNA was unchanged (Fig. 6F). HAF also coimmunoprecipitated with HIF-1α, confirming the interaction between HIF-1α and HAF in the stable clones (Fig. 6G). In contrast to the effects of HAF overexpression on tumor growth in vivo, no significant difference was observed in the in vitro rate of proliferation of the HAF clones compared to that of the vector or parental cells under both normoxic and hypoxic conditions (Fig. 6H). This is not surprising as the effect of HIF-1α on proliferation is frequently only observed in vivo (5), as cells in culture are minimally stressed and increased production of proangiogenic factors mediated by HIF-1 provides them with little proliferative advantage. In summary, HAF overexpression decreases HIF-1α levels in vivo, which is accompanied by decreased angiogenesis and inhibition of tumor growth.

Endogenous HAF is a key regulator of HIF-1α. To determine whether the HAF-mediated HIF-1α degradation pathway is a general mechanism, we studied the effect of HAF knockdown in a panel of cell lines. HAF siRNA transfection resulted in a clear increase in HIF-1α levels in PANC-1, DU-145, and PC-3 cells in both normoxia and hypoxia (Fig. 7A), supporting our initial observations in the renal carcinoma and HT29 cells and indicating that HAF mediates a general effect as an important, oxygen-independent regulator of HIF-1α.

To evaluate the significance of HAF in HIF-1α regulation, we compared the effects of HAF to that of the best-studied regulator of HIF-1α, pVHL. In normoxia, treatment of PANC-1 or PC-3 cells with siRNA to either HAF or pVHL induced comparable levels of HIF-1α, suggesting that in these cells, the HAF pathway is of equal importance to the pVHL pathway in regulating HIF-1α (Fig. 7B). However, unlike the pVHL pathway, HAF-mediated HIF-1α degradation is maintained in hypoxia. Transfection of the PANC-1 and PC-3 cells with HAF siRNA resulted in 1.5- and 2.1-fold increases in the already elevated hypoxic HIF-1α levels, whereas transfection with pVHL siRNA resulted in more modest changes of 1.2- to 1.3-fold, respectively (Fig. 7B). Thus, HAF regulates HIF-1α together with pVHL in normoxia but plays an additional role in HIF-1α regulation in hypoxia, when pVHL cannot bind HIF-1α.

It is notable that knockdown of pVHL or HAF in normoxia did not result in an induction of HIF-1α protein to a similar magnitude as that obtained in hypoxia, as would be expected if the hypoxia-induced increase in HIF-1α was solely attributed to decreased degradation. In this regard, the PANC-1 and PC-3 cells have elevated HIF-1α levels that have been linked to Src or phosphatidylinositol 3-kinase (PI-3K) activation (13). As Src or PI-3K activity can be modulated by hypoxia (3, 25), it is possible that these factors in addition to the inhibition of degradation are important for the induction of HIF-1α in hypoxia in these cells.

The expression of HIF-1α in normoxia in the presence of functional pVHL can be induced in prostate cancer cells such as PC-3 and DU-145 cells by EGF stimulation (24). To evaluate the role of endogenous HAF in the regulation of EGF-induced HIF-1α in normoxia, we transfected these cells with HAF or pVHL siRNA prior to EGF stimulation and then monitored the levels of HIF-1α. Transfection with HAF siRNA resulted in a comparable or greater induction of HIF-1α than pVHL siRNA under serum starvation or EGF stimulation in PC-3 and DU-145 cells (Fig. 7C). This indicates that HAF plays an important role together with pVHL in degrading EGF-induced HIF-1α in normoxia.

Hence, we show that the HAF-mediated HIF-1α degradation pathway is important in regulating HIF-1α levels in normoxia, hypoxia, and under EGF stimulation in a panel of cell lines, suggesting that HAF is a general, oxygen-independent regulator of HIF-1α levels. The HAF pathway functions in parallel to the pVHL pathway in normoxia but plays a pivotal role in HIF-1α degradation during hypoxia, when pVHL cannot degrade HIF-1α.

HAF degrades HIF-1α independently of the RACK1-elongin C pathway. RACK1 has been shown to recruit elongin C and other components of an E3 ligase complex in a manner mechanistically similar to pVHL for oxygen-independent degradation of HIF-1α (30). To determine if elongin C or RACK1 is required for HAF-mediated HIF-1α degradation, we overexpressed HAF in PANC-1 cells where either elongin C or RACK1 was knocked down by siRNA. We found that HAF overexpression decreased HIF-1α levels in the absence of either elongin C or RACK1 (Fig. 7D and E), suggesting that neither is required for HAF-mediated HIF-1α degradation (Fig. 7E). Hence, HAF degrades HIF-1α through a mechanism distinct from the RACK1-elongin C pathway.

DISCUSSION

HAF degrades HIF-1α independently of oxygen and pVHL. The most extensively studied mechanism for the regulation of HIF-1α protein levels is its ubiquitin-proteasome degradation

FIG. 6. Effects of HAF overexpression on tumor growth. (A) In vivo growth curves of the HAF or vector control HT29 xenografts in nude mice. Values are the means of four mice per group (± standard error). (B) Photographs of representative tumors demonstrating the differences in vascularization between the HAF and vector clones. (C) Xenograft microvessel density determined via staining with anti-CD105 antibody (± standard error). (D) Western blot showing HIF-1α expression in HT29 xenografts. Samples depicted are from two representative tumor lysates derived from each HAF or vector clone. (E) Densitometric analyses of the Western blot shown in panel D normalized to actin (± standard deviation). (F) Transcription of *HIF-1α* and key HIF-1α-regulated genes *VEGF*, *GLUT-1*, and *CA-IX* (coding for VEGF, glucose transporter 1, and carbonic anhydrase IX, respectively) in HAF xenografts determined by quantitative RT-PCR normalized to values from pooled vector xenografts RNA (± standard error). *, $P < 0.05$; **, $P < 0.01$. (G) Co-IP of HAF with endogenous HIF-1α from xenograft lysates depicted in panel D. (H) In vitro growth curves of HAF clones in normoxia and hypoxia ($n = 3$ [± standard error]).

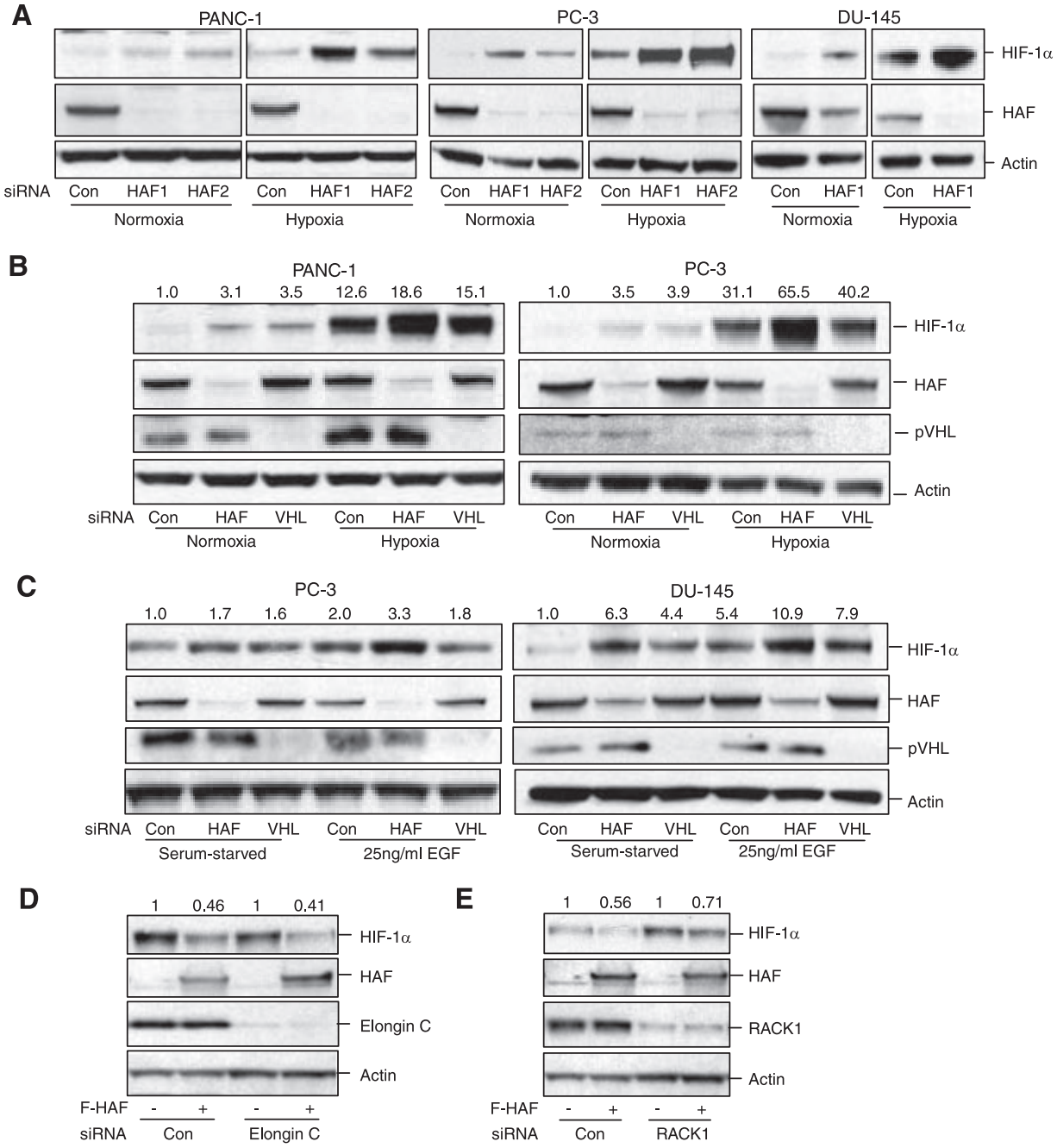


FIG. 7. The oxygen-independent HAF pathway complements the oxygen-dependent pVHL pathway to determine total levels of HIF-1α and is independent of the RACK1-elongin C pathway. (A) HAF siRNA increases HIF-1α levels in normoxia and hypoxia. Cells were transfected with a nontargeting siRNA control (Con) or two different HAF siRNA constructs (HAF1 and HAF2) for 48 h and then incubated in normoxia or hypoxia for a further 16 h and analyzed by Western blotting. (B) Comparison of the effects of HAF and pVHL knockdown on HIF-1α levels. PANC-1 or PC-3 cells were transfected with HAF (HAF1), pVHL, or Con siRNA and then treated as in panel A. Quantitations of HIF-1α intensities normalized to actin are depicted above the respective bands. The results are representative of at least two independent experiments. (C) Endogenous HAF regulates EGF-induced HIF-1α. Cells were transfected with siRNA as in panel A, serum starved for 24 h, and then induced with EGF for 7 h. (D and E) Effect of knockdown of elongin C (D) or RACK1 (E) on the HAF-mediated decrease in HIF-1α. PANC-1 cells were transfected with control, elongin C, or RACK1 siRNA as indicated and with F-HAF or the vector control. Seventy-two hours later, cells were placed into hypoxia for 16 h and analyzed by Western blotting. HIF-1α intensities normalized to actin are shown above the respective bands.

through the pVHL-E3 ligase complex, a process contingent upon the oxygen-dependent hydroxylation of HIF-1α. However, many investigators have observed pVHL- and oxygen-independent degradation of HIF-1α through unknown mechanisms. Our data suggest that HAF is the mediator of a new mechanism for the degradation of HIF-1α that is not limited by the presence of oxygen.

HAF and HIF-1α interact in vitro and in vivo through bind-

ing of HAF residues 654 to 800 to HIF-1α residues 296 to 400. Unlike pVHL, the binding of HAF to HIF-1α is oxygen independent and does not require prolyl hydroxylation. Our studies have also shown that HAF and pVHL do not interact (not shown). HAF displays E2-dependent autopolyubiquitination activity and ubiquitinates HIF-1α in cell-free assays using recombinant proteins, mediated by the $HAF^{654–800}$ domain. In addition, HAF is monoubiquitinated in vitro, which may provide an avenue for self-regulation (18). HAF does not contain a prototypical E3 ligase motif, nor does it require catalytic cysteines for its activity, and thus HAF joins a growing list of atypical E3 ligases whose catalytic domains cannot be defined as either HECT, RING, or U-box motifs (28, 29).

HAF is a multifunctional DNA binding ubiquitin ligase. HAF is evolutionarily conserved, and both HAF and its yeast homolog, Snu66p, are components of the human and yeast U4/U6 · U5 tri-snRNP (small nuclear ribonucleoprotein) complexes, respectively, and are essential for the assembly of mature spliceosomes (32). HAF has also been identified in a genomic RNA interference screen as a gene essential for cell division, the silencing of which results in mitotic arrest (26).

The N terminus of murine HAF has previously been shown to bind DNA and induce the transcription of *EPO* and *VEGF* (16). Here we describe for the first time, E3-ubiquitin ligase activity within the C terminus of HAF. This indicates that HAF may be a modular protein with DNA binding activity within its N terminus and E3 ligase activity within its C terminus. HAF does not require DNA in order to associate with or ubiquitinate HIF-1α (our in vitro data), but the ability of both HIF-1α and HAF to bind to DNA regulatory regions of common genes raises the intriguing possibility of increased interaction between DNA-bound HIF-1α and HAF, which may result in increased degradation of HIF-1α.

The dual functionality of DNA binding and E3 ligase activity is not unique to HAF. The tumor suppressor BRCA1 is a RING E3 ligase that binds directly to DNA independently of its RING domain (36), while p300 acts as both a transcriptional coactivator and ubiquitin ligase for p53 (14). It has been suggested that the apparent self-antagonism and multifunctionality of these proteins may allow a single molecule to integrate multiple diverse signals into a single highly sophisticated outcome, thus forming critical nodes where mutation or loss may invariably result in disease (9).

Importance of HAF in HIF-1α regulation in vivo. HAF has been detected in all cell lines (both normal and tumor derived) and in proliferating tissue but is undetectable in normal, nonproliferating tissue (47). As tumors are almost invariably highly proliferative, HAF has been detected at higher levels in some tumors compared with the surrounding, nonproliferating normal tissue (41).

We have shown that HAF is an important oxygen-independent regulator of HIF-1α in normoxia, hypoxia, and under EGF stimulation and thus dictates the basal levels of HIF-1α in each setting. Given its association with proliferation, it is possible that HAF mediates the oxygen-independent, proliferation-dependent degradation of HIF-1α, whereas the pVHL-mediated pathway specifically modulates the levels of HIF-1α in the presence of oxygen. Further studies are required to determine whether specific physiological stimuli can lead to changes in HAF levels or activity, thus modifying levels of HIF-1α. Significantly, HAF and not pVHL is the critical determinant of the levels of hypoxia-induced HIF-1α, whereas in normoxia, both the HAF- and pVHL-mediated pathways function in parallel (Fig. 8). Hence, HAF provides an additional layer of control allowing the precise regulation of HIF-1α levels, possibly under diverse conditions.

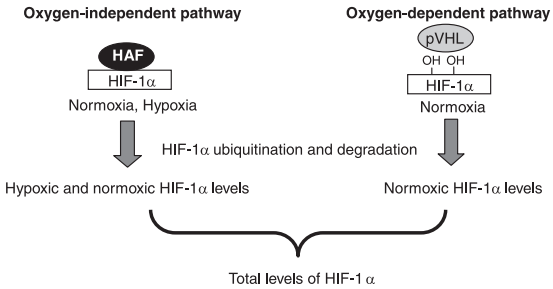


FIG. 8. HAF and pVHL participate in two overlapping but distinct HIF-1α regulatory pathways. HAF regulates the basal levels of HIF-1α in an oxygen-independent pathway and degrades HIF-1α in normoxia and hypoxia, whereas pVHL maintains low HIF-1α levels only in normoxia in an oxygen-dependent pathway. Together the two parallel but complementary pathways determine the total levels of HIF-1α.

It is intriguing that HAF does not appear to regulate the levels of HIF-2α in the RCC4 cells. It has recently become clear that HIF-1α and HIF-2α regulate unique target genes and that the relative importance of the different HIF-α isoforms differs according to cell or tissue type (12). Additionally, HIF-1α and HIF-2α have been shown to demonstrate a reciprocal relationship in which overexpression of one isoform results in the downregulation of the remaining isoform (39). This may explain our conflicting results with the study showing that HAF knockdown decreases hypoxia-dependent *EPO* and *VEGF* transcription (15). HIF-2α (and not HIF-1α) is the key regulator of *EPO* in the HEP3B cells used in that study (50), and it is possible that HAF knockdown increases HIF-1α levels, hence resulting in the decreased HIF-2α-dependent transcriptional activity reported. In contrast, our study focuses on cells such as the HT29 cells in which HIF-1α is the dominant isoform.

HIF-1α, although promoting the cellular adaptation to hypoxia, also induces apoptosis under certain settings (6). Furthermore, overexpression of stable HIF-1α inhibits tumor growth, while HIF-2α overexpression promotes tumor growth in renal carcinoma xenograft models (39). Therefore, HAF may play a more important role in cells that express both HIF-1α and HIF-2α by selectively degrading HIF-1α while simultaneously allowing HIF-2α to drive tumorigenesis.

Conclusions. This study introduces a new mechanism for HIF-1α regulation by HAF, a novel HIF-1α E3 ligase that binds to HIF-1α causing its ubiquitination and proteasomal degradation. This is a unique mechanism that occurs independently of oxygen and HIF-1α prolyl hydroxylation and is therefore capable of regulating HIF-1α levels under conditions in which the pVHL-E3 ligase complex is inactive (such as during hypoxia). Intriguingly, HAF overexpression does not result in the degradation of HIF-2α but is the mediator of a new degradation pathway specific to HIF-1α. Consequently, we believe that HAF determines the basal levels of HIF-1α in an oxygen-independent manner, hence setting a baseline level of HIF-1α

upon which the oxygen-regulated component of HIF-1α degradation regulated by pVHL can be mediated.

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REFERENCES

1. **Baek, J. H., Y. V. Liu, K. R. McDonald, J. B. Wesley, M. E. Hubbi, H. Byun, and G. L. Semenza.** 2007. Spermidine/spermine-N1-acetyltransferase 2 is an essential component of the ubiquitin ligase complex that regulates hypoxia-inducible factor 1alpha. J. Biol. Chem. **282:**23572–23580.
2. **Baek, J. H., P. C. Mahon, J. Oh, B. Kelly, B. Krishnamachary, M. Pearson, D. Chan, A. J. Giaccia, and G. L. Semenza.** 2005. OS-9 interacts with hypoxia-inducible factor 1α and prolyl hydroxylases to promote oxygen-dependent degradation of HIF-1α. Mol. Cell **17:**503–512.
3. **Belaiba, R. S., S. Bonello, C. Zahringer, S. Schmidt, J. Hess, T. Kietzmann, and A. Gorlach.** 2007. Hypoxia up-regulates hypoxia-inducible factor-1alpha transcription by involving phosphatidylinositol 3-kinase and nuclear factor kappaB in pulmonary artery smooth muscle cells. Mol. Biol. Cell **18:**4691–4697.
4. **Besse, A., A. D. Campos, W. K. Webster, and B. G. Darnay.** 2007. TRAF-interacting protein (TRIP) is a RING-dependent ubiquitin ligase. Biochem. Biophys. Res. Commun. **359:**660–664.
5. **Blouw, B., H. Song, T. Tihan, J. Bosze, N. Ferrara, H. P. Gerber, R. S. Johnson, and G. Bergers.** 2003. The hypoxic response of tumors is dependent on their microenvironment. Cancer Cell **4:**133–146.
6. **Carmeliet, P., Y. Dor, J. M. Herbert, D. Fukumura, K. Brusselmans, M. Dewerchin, M. Neeman, F. Bono, R. Abramovitch, P. Maxwell, C. J. Koch, P. Ratcliffe, L. Moons, R. K. Jain, D. Collen, and E. Keshert.** 1998. Role of HIF-1alpha in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. Nature **394:**485–490.
7. **Cheng, J., X. Kang, S. Zhang, and E. T. Yeh.** 2007. SUMO-specific protease 1 is essential for stabilization of HIF1alpha during hypoxia. Cell **131:**584–595.
8. **Ciechanover, A., A. Orian, and A. L. Schwartz.** 2000. Ubiquitin-mediated proteolysis: biological regulation via destruction. Bioessays **22:**442–451.
9. **Crosetto, N., M. Bienko, and I. Dikic.** 2006. Ubiquitin hubs in oncogenic networks. Mol. Cancer Res. **4:**899–904.
10. **Demidenko, Z. N., A. Rapisarda, M. Garayoa, P. Giannakakou, G. Melillo, and M. V. Blagosklonny.** 2005. Accumulation of hypoxia-inducible factor-1alpha is limited by transcription-dependent depletion. Oncogene **24:**4829–4838.
11. **Flügel, D., A. Gorlach, C. Michiels, and T. Kietzmann.** 2007. Glycogen synthase kinase-3 phosphorylates hypoxia-inducible factor 1α and mediates its destabilization in a VHL-independent manner. Mol. Cell. Biol. **27:**3253–3265.
12. **Gordan, J. D., and M. C. Simon.** 2007. Hypoxia-inducible factors: central regulators of the tumor phenotype. Curr. Opin. Genet. Dev. **17:**71–77.
13. **Gray, M. J., J. Zhang, L. M. Ellis, G. L. Semenza, D. B. Evans, S. S. Watowich, and G. E. Gallick.** 2005. HIF-1alpha, STAT3, CBP/p300 and Ref-1/APE are components of a transcriptional complex that regulates Src-dependent hypoxia-induced expression of VEGF in pancreatic and prostate carcinomas. Oncogene **24:**3110–3120.
14. **Grossman, S. R., M. E. Deato, C. Brignone, H. M. Chan, A. L. Kung, H. Tagami, Y. Nakatani, and D. M. Livingston.** 2003. Polyubiquitination of p53 by a ubiquitin ligase activity of p300. Science **300:**342–344.
15. **Gupta, M., and E. Goldwasser.** 1996. The role of the near upstream sequence in hypoxia-induced expression of the erythropoietin gene. Nucleic Acids Res. **24:**4768–4774.
16. **Gupta, M., P. T. Mungai, and E. Goldwasser.** 2000. A new transacting factor that modulates hypoxia-induced expression of the erythropoietin gene. Blood **96:**491–497.
17. **Haase, V. H.** 2006. Hypoxia-inducible factors in the kidney. Am. J. Physiol. Renal Physiol. **291:**F271–F281.
18. **Hicke, L.** 2001. Protein regulation by monoubiquitin. Nat. Rev. Mol. Cell Biol. **2:**195–201.
19. **Hickey, M. M., and M. C. Simon.** 2006. Regulation of angiogenesis by hypoxia and hypoxia-inducible factors. Curr. Top. Dev. Biol. **76:**217–257.
20. **Hochstrasser, M.** 2000. Evolution and function of ubiquitin-like protein-conjugation systems. Nat. Cell Biol. **2:**E153–E157.
21. **Isaacs, J. S., Y. J. Jung, E. G. Mimnaugh, A. Martinez, F. Cuttitta, and L. M. Neckers.** 2002. Hsp90 regulates a von Hippel Lindau-independent hypoxia-inducible factor-1 alpha-degradative pathway. J. Biol. Chem. **277:**29936–29944.
22. **Ivan, M., K. Kondo, H. Yang, W. Kim, J. Valiando, M. Ohh, A. Salic, J. M. Asara, W. S. Lane, and W. G. Kaelin.** 2001. HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O_2 sensing. Science **292:**464–468.
23. **Jaakkola, P., D. R. Mole, Y. M. Tian, M. I. Wilson, J. Gielbert, S. J. Gaskell, A. Kriegsheim, H. F. Hebestreit, M. Mukherji, C. J. Schofield, P. H. Maxwell, C. W. Pugh, and P. J. Ratcliffe.** 2001. Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O_2-regulated prolyl hydroxylation. Science **292:**468–472.
24. **Jiang, B. H., G. Jiang, J. Z. Zheng, Z. Lu, T. Hunter, and P. K. Vogt.** 2001. Phosphatidylinositol 3-kinase signaling controls levels of hypoxia-inducible factor 1. Cell Growth Differ. **12:**363–369.
25. **Karni, R., Y. Dor, E. Keshet, O. Meyuhas, and A. Levitzki.** 2002. Activated pp60c-Src leads to elevated hypoxia-inducible factor (HIF)-1alpha expression under normoxia. J. Biol. Chem. **277:**42919–42925.
26. **Kittler, R., G. Putz, L. Pelletier, I. Poser, A. K. Heninger, D. Drechsel, S. Fischer, I. Konstantinova, B. Habermann, H. Grabner, M. L. Yaspo, H. Himmelbauer, B. Korn, K. Neugebauer, M. T. Pisabarro, and F. Buchholz.** 2004. An endoribonuclease-prepared siRNA screen in human cells identifies genes essential for cell division. Nature **432:**1036–1040.
27. **Kong, X., B. Alvarez-Castelao, Z. Lin, J. G. Castano, and J. Caro.** 2007. Constitutive/hypoxic degradation of HIF-alpha proteins by the proteasome is independent of von Hippel Lindau protein ubiquitylation and the transactivation activity of the protein. J. Biol. Chem. **282:**15498–15505.
28. **Le Cam, L., L. K. Linares, C. Paul, E. Julien, M. Lacroix, E. Hatchi, R. Triboulet, G. Bossis, A. Shmueli, M. S. Rodriguez, O. Coux, and C. Sardet.** 2006. E4F1 is an atypical ubiquitin ligase that modulates p53 effector functions independently of degradation. Cell **127:**775–788.
29. **Linares, L. K., R. Kiernan, R. Triboulet, C. Chable-Bessia, D. Latreille, O. Cuvier, M. Lacroix, L. Le Cam, O. Coux, and M. Benkirane.** 2007. Intrinsic ubiquitination activity of PCAF controls the stability of the oncoprotein Hdm2. Nat. Cell Biol. **9:**331–338.
30. **Liu, Y. V., J. H. Baek, H. Zhang, R. Diez, R. N. Cole, and G. L. Semenza.** 2007. RACK1 competes with HSP90 for binding to HIF-1alpha and is required for O_2-independent and HSP90 inhibitor-induced degradation of HIF-1alpha. Mol. Cell **25:**207–217.
31. **Livak, K. J., and T. D. Schmittgen.** 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods **25:**402–408.
32. **Makarova, O. V., E. M. Makarov, and R. Luhrmann.** 2001. The 65 and 110 kDa SR-related proteins of the U4/U6 · U5 tri-snRNP are essential for the assembly of mature spliceosomes. EMBO J. **20:**2553–2563.
33. **Nakayama, K., and Z. Ronai.** 2004. Siah: new players in the cellular response to hypoxia. Cell Cycle **3:**1345–1347.
34. **Ohh, M., C. W. Park, M. Ivan, M. A. Hoffman, T. Y. Kim, L. E. Huang, N. Pavletich, V. Chau, and W. G. Kaelin.** 2000. Ubiquitination of hypoxia-inducible factor requires direct binding to the beta-domain of the von Hippel-Lindau protein. Nat. Cell Biol. **2:**423–427.
35. **Paine-Murrieta, G. D., C. W. Taylor, R. A. Curtis, M. H. Lopez, R. T. Dorr, C. S. Johnson, C. Y. Funk, F. Thompson, and E. M. Hersh.** 1997. Human tumor models in the severe combined immune deficient (scid) mouse. Cancer Chemother. Pharmacol. **40:**209–214.
36. **Paull, T. T., D. Cortez, B. Bowers, S. J. Elledge, and M. Gellert.** 2001. Direct DNA binding by Brca1. Proc. Natl. Acad. Sci. USA **98:**6086–6091.
37. **Polek, T. C., M. Talpaz, B. G. Darnay, and T. Spivak-Kroizman.** 2003. TWEAK mediates signal transduction and differentiation of RAW264.7 cells in the absence of Fn14/TweakR. Evidence for a second TWEAK receptor. J. Biol. Chem. **278:**32317–32323.
38. **Qian, D. Z., S. K. Kachhap, S. J. Collis, H. M. Verheul, M. A. Carducci, P. Atadja, and R. Pili.** 2006. Class II histone deacetylases are associated with VHL-independent regulation of hypoxia-inducible factor 1α. Cancer Res. **66:**8814–8821.
39. **Raval, R. R., K. W. Lau, M. G. B. Tran, H. M. Sowter, S. J. Mandriota, J.-L. Li, C. W. Pugh, P. H. Maxwell, A. L. Harris, and P. J. Ratcliffe.** 2005. Contrasting properties of hypoxia-inducible factor 1 (HIF-1) and HIF-2 in von Hippel-Lindau-associated renal cell carcinoma. Mol. Cell. Biol. **25:**5675–5686.
40. **Ravi, R., B. Mookerjee, Z. M. Bhujwalla, C. H. Sutter, D. Artemov, Q. Zeng, L. E. Dillehay, A. Madan, G. L. Semenza, and A. Bedi.** 2000. Regulation of tumor angiogenesis by p53-induced degradation of hypoxia-inducible factor 1alpha. Genes Dev. **14:**34–44.
41. **Sasatomi, T., H. Yamana, S. Shichijo, S. Tanaka, T. Okamura, Y. Ogata, K. Itoh, and K. Shirouzu.** 2000. Expression of the SART1 tumor-rejection antigens in colorectal cancers. Dis. Colon Rectum **43:**1754–1758.
42. **Semenza, G. L.** 2000. HIF-1: using two hands to flip the angiogenic switch. Cancer Metastasis Rev. **19:**59–65.
43. **Semenza, G. L.** 2004. Intratumoral hypoxia, radiation resistance, and HIF-1. Cancer Cell **5:**405–406.
44. **Semenza, G. L.** 2003. Targeting HIF-1 for cancer therapy. Nat. Rev. Cancer **3:**721–732.
45. **Semenza, G. L., and G. L. Wang.** 1992. A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene en-

hancer at a site required for transcriptional activation. Mol. Cell. Biol. **12:**5447–5454.
46. **Shibata, T., A. J. Giaccia, and J. M. Brown.** 2000. Development of a hypoxia-responsive vector for tumor-specific gene therapy. Gene Ther. **7:**493–498.
47. **Shichijo, S., M. Nakao, Y. Imai, H. Takasu, M. Kawamoto, F. Niiya, D. Yang, Y. Toh, H. Yamana, and K. Itoh.** 1998. A gene encoding antigenic peptides of human squamous cell carcinoma recognized by cytotoxic T lymphocytes. J. Exp. Med. **187:**277–288.
48. **Tang, T. T., and L. A. Lasky.** 2003. The forkhead transcription factor FOXO4 induces the down-regulation of hypoxia-inducible factor 1 alpha by a von Hippel-Lindau protein-independent mechanism. J. Biol. Chem. **278:** 30125–30135.
49. **Vertegaal, A. C., S. C. Ogg, E. Jaffray, M. S. Rodriguez, R. T. Hay, J. S. Andersen, M. Mann, and A. I. Lamond.** 2004. A proteomic study of SUMO-2 target proteins. J. Biol. Chem. **279:**33791–33798.
50. **Warnecke, C., Z. Zaborowska, J. Kurreck, V. A. Erdmann, U. Frei, M. Wiesener, and K. U. Eckardt.** 2004. Differentiating the functional role of hypoxia-inducible factor (HIF)-1alpha and HIF-2alpha (EPAS-1) by the use of RNA interference: erythropoietin is a HIF-2alpha target gene in Hep3B and Kelly cells. FASEB J. **18:**1462–1464.
51. **Wiesener, M. S., J. S. Jurgensen, C. Rosenberger, C. K. Scholze, J. H. Horstrup, C. Warnecke, S. Mandriota, I. Bechmann, U. A. Frei, C. W. Pugh, P. J. Ratcliffe, S. Bachmann, P. H. Maxwell, and K. U. Eckardt.** 2003. Widespread hypoxia-inducible expression of HIF-2alpha in distinct cell populations of different organs. FASEB J. **17:**271–273.