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ATP6V0C Competes with Von Hippel-Lindau Protein in Hypoxia-Inducible Factor 1α (HIF-1α) Binding and Mediates HIF-1α Expression by Bafilomycin A1

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ABSTRACT

HIF-1α not only enables cells to survive under hypoxic conditions but also promotes cell cycle arrest and apoptosis. Therefore, its expression should be controlled at optimal levels in growing tumors. We recently reported that bafilomycin A1 exorbitantly expressed HIF-1α and induced the p21$^{WAF1/Cip1}$-mediated growth arrest of tumors (*Mol Pharmacol* **70:**1856–1865, 2006). In the present study, we addressed the mechanism underlying bafilomycin-induced HIF-1α expression. Bafilomycin stabilized HIF-1α under normoxic conditions without changes in intracellular pH. However, when ATP6V0C, the target protein of bafilomycin, was knocked down, this bafilomycin effect was significantly attenuated. Inversely, ATP6V0C expression increased HIF-1α levels in a gene dose-dependent manner. ATP6V0C competed with Von Hippel-Lindau protein in HIF-1α binding by directly interacting with HIF-1α, which was stimulated by bafilomycin. In confocal images, ATP6V0C was normally present in the cytoplasm but was translocated in company with HIF-1α to the nucleus by bafilomycin. The N-terminal end (amino acids 1–16) of HIF-1α was identified as the ATP6V0C-interacting motif. These results suggest that ATP6V0C, a novel regulator of HIF-1α, mediates HIF-1α expression by bafilomycin.

HIF-1α contains the basic helix-loop-helix/periodicity–aryl hydrocarbon receptor nuclear translocator (ARNT)–simple-minded domains at the N terminus (Jiang et al., 1996) and two transactivation domains at the C terminus (Jiang et al., 1997). It also has the Pro-Ser-Thr-rich oxygen-dependent degradation domain (ODDD, aa. 401–603) in the middle part, which is responsible for destabilization of HIF-1α protein (Huang et al., 1998). Under normoxic conditions, HIF-1-prolyl hydroxylases (PHD1–3) (Bruick and McKnight, 2001; Epstein et al., 2001) hydroxylate two proline residues (Pro402 and Pro564) within the ODDD (Ivan et al., 2001; Jaakkola et al., 2001; Masson et al., 2001) and, in turn, the von Hippel-Lindau tumor suppressor protein (pVHL) binds to the modified HIF-1α, resulting in ubiquitination and proteasomal degradation of HIF-1α (Maxwell et al., 1999; Kamura et al., 2000). Because PHDs require molecular oxygen as a substrate, the proline hydroxylation is limited under hypoxic conditions, and thereby HIF-1α is stabilized. In the nucleus, HIF-1α binds ARNT to form a HIF-1 complex, which up-regulates many genes essential for cellular adaptation to hypoxia (Semenza, 2000).

HIF-1α is frequently found to be overexpressed in human tumors (Zhong et al., 1999), and its levels in tumor specimens are correlated with tumor hypervascularity, aggressiveness, and poor prognosis (Birner et al., 2000, Zagzag et al., 2000). The essential roles of HIF-1α in tumor growth and angiogenesis have also been demonstrated in

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ABBREVIATIONS: HIF-1α, hypoxia-inducible factor 1α; ARNT, aryl hydrocarbon receptor nuclear translocator; ODDD, oxygen-dependent degradation domain; PHD, prolyl hydroxylase; pVHL, von Hippel-Lindau tumor suppressor protein; ATP6V0C, subunit c in V0 sector of V-ATPase; Vc, ATP6V0C; BM, bafilomycin A1; V-ATPase, vacuolar H^+ ATPase; HEK, human embryonic kidney; HA, hemagglutinin; PCR, polymerase chain reaction; RT, reverse transcription/transcriptase; siRNA, small interfering RNA; pH_i, intracellular pH; BCECF, 2,7-bis(2-carboxyethyl)-5(6)-carboxyfluorescein; RFP, red fluorescent protein; GFP, green fluorescent protein; mHIF-1α, HIF-1α mutant; MG132, *N*-benzoyloxycarbonyl (*Z*)-Leu-Leu-leucinal.

tumor xenograft studies (Ryan et al., 2000). Therefore, HIF-1α is indicated as a tumor promoting factor and a most compelling target for cancer therapy. However, in stark contrast with the above, HIF-1α has also tumor-inhibiting properties. Its overexpression inhibits cell proliferation by expressing cell cycle inhibitors such as p21, p27, and p53. Moreover, HIF-1α promotes apoptosis by inducing proapoptotic molecules such as p53, Nip3, Noxa, and HGTD-P (Greijer and van der Wall, 2004; Koshiji and Huang, 2004). Indeed, tumors xenografted with *HIF-1α*(−/−) cells grew faster than those with *HIF-1α*(+/+) cells (Carmeliet et al., 1998). It was also demonstrated that the *HIF-1α*(−/−) tumors were more proliferative and less apoptotic than the *HIF-1α*(+/+) tumors. When HIF-1α was overexpressed by knocking-out *VHL*, the grafted tumor growth decreased, rather than increased, despite the induction of HIF-1 downstream genes (Mack et al., 2003). Thus, HIF-1α at optimal levels is required for tumor growth and angiogenesis, but under some circumstances, its overexpression may provide a reverse effect on tumor growth. Therefore, HIF-1α overexpression could be a strategy for cancer therapy.

Bafilomycin A1, a V-ATPase inhibitor, has been viewed as a potential anticancer agent because it inhibits tumor growth both in vitro (Ohkuma et al., 1993) and in vivo (Ohta et al., 1998). These anticancer effects of bafilomycin have been considered to be attributable to the intracellular acidosis by V-ATPase inhibition. However, we recently proposed a novel mechanism underlying tumor inhibition by bafilomycin. Bafilomycin up-regulated HIF-1α in eight human cancer cell lines, which caused a robust induction of p21 and cell cycle arrest. Although it showed a marginal activity in transcription of HIF-1 target genes such as *VEGF*, *PGK1*, and *ENO1*, bafilomycin-induced HIF-1α dissociated c-Myc repressor from p21 promoter, and thereby de-repressed p21. In grafted fibrosarcomas, bafilomycin also showed the HIF-1α-dependent anticancer effect (Lim et al., 2006). Based on these results, we suggested that the anticancer action of bafilomycin is attributable to HIF-1α overexpression. However, we did not clarify the mechanism whereby bafilomycin inhibited HIF-1α protein degradation.

V-ATPase is a large multisubunit enzyme and plays an important role in pH homeostasis by lowering lumen pH and raising cytoplasmic pH (Forgac, 1989). V-ATPase consists of two sectors, a catalytic V1 sector and a membrane-bound V0 sector. The V1 sector, which is composed of eight different subunits, hydrolyzes ATP to ADP to abstract energy for H^+ transportation. The V0 sector, which is composed of five different subunits, forms the H^+ translocating channel (Forgac, 1989). Of these subunits, subunit c in V0 (ATP6V0C), which is the target protein of bafilomycin, has been found to interact with other structures independently of V-ATPase, including gap junction complexes and mediatophores (Finbow et al., 1994). It also forms a complex with papillomavirus E5 oncoprotein plus platelet-derived growth factor-β receptor (Goldstein et al., 1992) or with β1 integrin (Lee et al., 2004). Thus, ATP6V0C is likely to participate in diverse cell signaling via protein-protein interactions. In the present study, we tested the possibility that ATP6V0C is involved in the HIF-1α induction by bafilomycin.

Materials and Methods

Reagents and Antibodies. Bafilomycin A1 and MG132 were purchased from Alexis Biochemicals (Lausanne, Switzerland), and other chemicals from Sigma-Aldrich (St. Louis, MO). Culture media and fetal calf serum were purchased from Invitrogen (Carlsbad, CA). Anti-HIF-1α antiserum was generated in rabbits, as described previously (Chun et al., 2001). ARNT and β-actin antibodies were purchased from SantaCruz Biotechnology (Santa Cruz, CA). pVHL and ATP6V0C antibodies were purchased from BD Biosciences PharMingen (San Diego, CA) and Chemicon (Temecula, CA), respectively. Hemagglutinin and Flag antibodies were obtained from Roche (Basel, Switzerland) and Sigma-Aldrich, respectively.

Cell Culture. Human prostate cancer PC3 and human embryonic kidney (HEK) 293 cell lines were obtained from the American Type Culture Collection (Manassas, VA), and VHL-null and VHL wild-type RCC4 cell-lines were from the European Collection of Cell Cultures (London, UK). These cells were cultured in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum, in a 5% CO_2 humidified atmosphere at 37°C.

Preparation of Expression Plasmids and Transfection. Hemagglutinin (HA)-tagged HIF-1α expression plasmid (pcDNA3) was constructed as described previously (Chun et al., 2001). The plasmid for pVHL-independent HIF-1α mutant (P402A/P564A), *m*HIF-1α was made by substituting Ala402 and Ala564 for Pro402 and Pro564, respectively, using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Truncated HIF-1α mutants were made by deleting specific domains, using a PCR-based mutagenesis kit (Stratagene). cDNA of V-ATPase V0 part subunit c (ATP6V0C; GenBank number NM_001694) was cloned by RT-PCR using Pfu DNA polymerase, and inserted into pcDNA-Flag or pcDNA by blunt-end ligation. To make fluorescent proteins, cDNA of HIF-1α or ATP6V0C was inserted into a green fluorescence protein vector, pEGFP-C2 (Clontech, Mountain View, CA) or a red fluorescence protein vector (pDsRed2-C1), respectively. cDNA of pVHL (GenBank number NM_000551) was cloned by RT-PCR and inserted into pcDNA-Flag or pcDNA-HA. The nucleotide sequences of the primers used for PCR were 5′-ATGTCCGAGTCCAAGAGC-3′ and 5′-CTACTTTGTGGAGAGGATGAG-3′ for ATP6V0C, and 5′-ATGCCCCGGAGGGCGGAG-3′ and 5′-TCAATCTCCCATCCGTTGATGTGC-3′ for pVHL. All constructs were verified by DNA sequencing. For transient transfection, approximately 40% of confluent cells in 60-mm cell culture dishes were transfected with plasmids, using the calcium phosphate method. Cells were allowed to stabilize for 48 h before being used in experiments.

Preparation of siRNAs and Transfection. Synthesized siRNA duplexes were obtained from Invitrogen (Carlsbad, CA). The sequence targeting ATP6V0C corresponded to nucleotides 91 to 115 (5′-GCCTATGGCACAGCCAAGAGCGGTA-3′, Vc-I) or to nucleotides 229 to 253 (5′-GCCAACTCCCTGAATGACGACATCA-3′, Vc-II) of the coding region. The sequence targeting ATP6V1A corresponded to nucleotides 394 to 417 (5′-AAATGGGACTTTACACCTTGCAAA-3′, Va) of the coding region (GenBank number NM_001690). HEK293 cells were transfected with siRNAs with the use of the calcium phosphate method. Cells were allowed to stabilize for 48 h before being used in experiments. To verify mRNA knock-down by siRNAs, mRNA levels were analyzed by RT-PCR using specific primers (forward and reverse): 5′-AGCAGATCATGAAGTCCATC-3′ and 5′-GACGATGAGACCGTAGAGG-3′ for ATP6V0C, and 5′-GGCAGAAACAGATAAAATCA-3′ and 5′-CTGCATGTCTTCAAGAAGTT-3′ for ATP6V1A.

Immunoblotting, Immunoprecipitation, and in Vitro Binding Assay. To quantify protein levels, proteins were separated on SDS/polyacrylamide gels, and transferred to an Immobilon-P membrane (Millipore, Billerica, MA). Membranes were blocked with 5% nonfat milk and incubated overnight at 4°C with a primary antibody diluted 1:1000. Membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (diluted 1:5000) for 2 h, and

antigen-antibody complexes were visualized using an Enhanced Chemiluminescence Plus kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK). β-Actin protein was used as an internal standard.

For the immunoprecipitation of HA-tagged HIF-1α, HIF-1α, or ATP6V0C, cell lysates (150 μg of protein) were incubated with 10 μl of anti-HA-conjugated Sepharose beads (Roche, Basel, Switzerland), anti-HIF-1α, or anti-ATP6V0C antibody at 4°C for 4 h, respectively. The immune complex of HIF-1α or ATP6V0C was further incubated with protein A/G-Sepharose beads (GE Healthcare) at 4°C for 4 h. The antigen-bead complexes obtained were washed extensively with lysis buffer. Immunocomplexes were eluted by boiling for 3 min in a sample buffer containing 2% SDS and 10 mM dithiothreitol, subjected to SDS-polyacrylamide gel electrophoresis, and then immunoblotted using anti-HIF-1α, anti-HA, anti-Flag, or anti-pVHL antibody.

For in vitro binding assay, HA-HIF-1α, Flag-ATP6V0C, or Flag-pVHL protein was overexpressed in HEK293 cells. The cell lysate (500 μg of protein) containing HA–HIF-1α was incubated with the Flag-ATP6V0C cell lysate at 4°C for 1 h, and then further incubated with the Flag-pVHL cell lysate at 4°C for 1 h. After immunoprecipitating HA-HIF-1α with anti-HA-conjugated Sepharose beads, HIF-1α-binding proteins were identified by immunoblot assay using anti-Flag antibody.

Intracellular pH Assay. For the measurement of intracellular pH (pH_i), PC-3 cells were exposed to acetoxymethyl ester form of 2,7-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF; 2 μM) for 15 min at room temperature to load the cells with the fluorescent dye. Unloaded dye was washed twice with fresh Tyrode's solution after 3 min of spinning (800 rpm). BCECF-loaded cells were allowed to adhere to the base of a superfusion chamber mounted on a Eclipse-2000 microscope (Nikon, Tokyo, Japan) interfaced with DeltaRAM ratiometry system (Photon Technology International, Lawrenceville, NJ). Cells were excited at 488 and 440 nm, and emitted fluorescence was measured at 530 nm. Intracellular pH was estimated by in situ calibration of the ration of fluorescence at 488 nm to that at 440 nm (F_{488}/F_{440}) according to the nigericin-high K^+ method (Silver, 1998). Because the fluorescence intensity affects the emission ratio of BCECF (Hegyi et al., 2004), calibration curves were obtained for every individual cell in this experiment. For the cells treated with bafilomycin, the same concentration of drug (10 or 100 nM) was included through the procedure of dye loading and pH_i measurement.

Confocal Imaging. HEK293 cells were transfected with plasmid RFP, RFP-Vc, GFP-HIF-1α, GFP-dN_HIF-1α, or RFP-Vc. The expressed fluorescent proteins were imaged with a confocal microscope (TCS SP2; Leica, Wetzlar, Germany) equipped with an argon ion laser. GFP or RFP fluorescence was excited with 488 or 543 nm, and confocal sections were collected using 500 to 535 nm or 555 to 700 nm emission setting, respectively. The nuclei of the cells were counterstained with 0.1 μg/ml 4,6-diamidino-2-phenylindole.

Statistical Analysis. All data were analyzed using Excel 2000 (Microsoft, Redmond, WA), and results are expressed as means and standard deviations. The Mann-Whitney *U* test (SPSS 10.0 for Windows; SPSS, Inc., Chicago, IL) was used to compare pH or protein levels. Differences were considered statistically significant at the $P < 0.05$ level. All statistical tests were two-sided.

Results

HIF-1α Expression by Bafilomycin A1 Is Independent of pH. As previously reported (Lim et al., 2006), 10 nM bafilomycin A1 noticeably induced HIF-1α under normoxic conditions in both PC3 and HEK293 cells but did not affect ARNT expression (Fig. 1A). To rule out the possibility that the bafilomycin effect is secondary to intracellular acidification, we measured intracellular pH in situ. Figure 1B showed the verification and the calibration of the pH measurement. Intracellular pH was not changed by 10 nM bafilomycin but was reduced by 100 nM BM (Fig. 1C). Therefore, HIF-1α induction by 10 nM bafilomycin is likely to occur irrelevantly of intracellular acidification.

ATP6V0C Is Required for Bafilomycin-Induced HIF-1α Expression. Of at least 13 different proteins composing the V-ATPase complex, ATP6V0C, alternatively named subunit c in the V0 sector of V-ATPase, has been suggested to be targeted by bafilomycin (Huss et al., 2002; Bowman et al., 2004). Therefore, we hypothesized that bafilomycin stabilizes HIF-1α by directly targeting ATP6V0C. When the *ATP6V0C* gene was knocked-down using siRNAs,

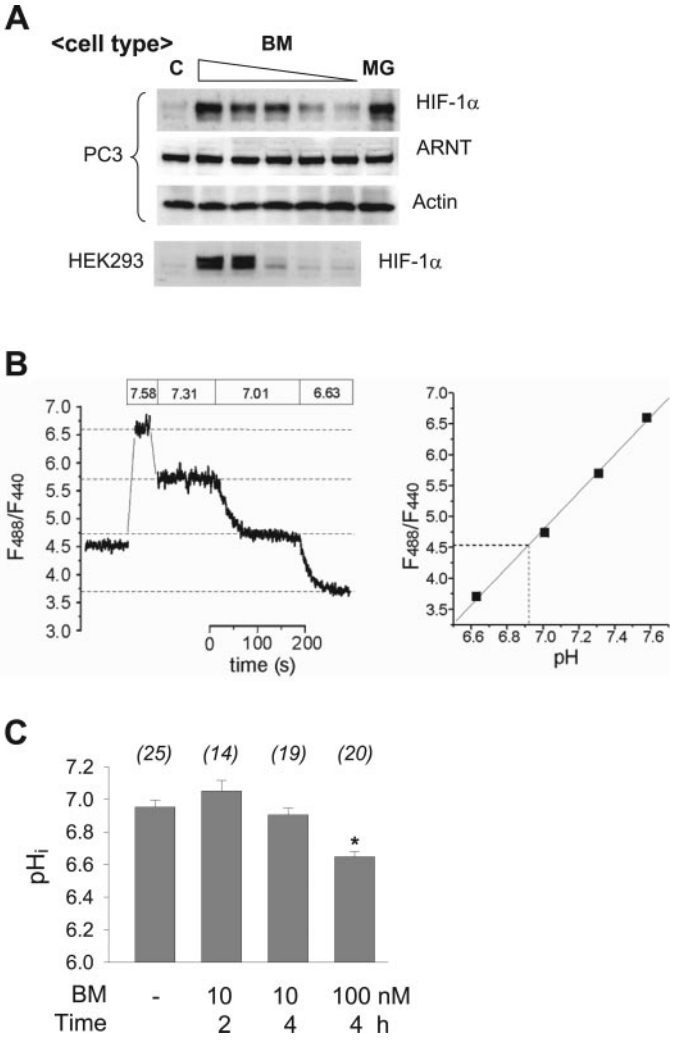

Fig. 1. Bafilomycin induces HIF-1α without significant changes in intracellular pH. A, HIF-1α induction by bafilomycin. PC3 or HEK293 cells were incubated with bafilomycin A1 (BM) for 4 h and harvested. The concentrations of BM used were 10, 5, 2, 1, and 0.2 nM. Protein levels of HIF-1α, ARNT, and β-actin were evaluated by immunoblotting. A proteasome inhibitor MG132 (MG) at 10 μM was used as a reference for HIF-1α expression. B, calibration of intracellular pH (pH_i). A representative trace of BCECF emission ration (F_{488}/F_{440}) measured in a PC-3 cell. The titrated pH of calibration solution containing 145 mM KCl and 10 μM nigericin is indicated above (left). Emission ratios (F_{488}/F_{490}) in the calibration solutions are plotted at the pH indicated and fit to a linear function (thread line). The pHi was measured from the emission ratio obtained in the control cell (dotted line). C, effect of bafilomycin on pH_i. PC3 cells were treated with 10 nM bafilomycin for 2 or 4 h, or with 100 nM bafilomycin for 4 h. pHi was calculated in the reference of the standard curve shown in B. Numbers in parentheses indicate the numbers of cells measured. *, $p < 0.05$ versus the control group.

both the basal and bafilomycin-induced levels of HIF-1α were significantly reduced (Fig. 2A). However, the knocking down of *ATP6V1A* (subunit A in the V1 sector) did not affect the HIF-1α levels (Fig. 2B), which further supports the specific involvement of ATP6V0C in HIF-1α stabilization. The overexpression of ATP6V0C up-regulated HIF-1α under both normoxic and hypoxic conditions, but ATP6V0C did not do so in the presence of a proteasome inhibitor, MG132 (Fig. 2C).

ATP6V0C Binds HIF-1α and Interferes with pVHL Binding to HIF-1α. To examine whether ATP6V0C is associated with HIF-1α in the presence of bafilomycin, ATP6V0C was precipitated with ATP6V0C antiserum and was found to be associated with HIF-1α (Fig. 3A). The ATP6V0C-HIF-1α binding was confirmed using expressed proteins. Expressed Flag-ATP6V0C was coprecipitated with expressed HA-HIF-1α (Fig. 3B). Because proteasomal degradation is preceded by the binding of pVHL and HIF-1α (Maxwell et al., 1999), we examined the effect of ATP6V0C on the pVHL-HIF-1α interaction using immunoprecipitation, and found that pVHL was dissociated from HIF-1α by ATP6V0C expression (Fig. 3C). To understand how ATP6V0C dissociates pVHL from HIF-1α, the in vitro protein binding assay was performed. ATP6V0C could bind HIF-1α and inhibited the pVHL-HIF-1α interaction (Fig. 3D). This suggests that ATP6V0C competes with pVHL in HIF-1α binding.

Bafilomycin Dissociates pVHL from HIF-1α by Stimulating ATP6V0C Binding. To examine the effect of bafilomycin on the pVHL-HIF-1α interaction, HIF-1α binding of ATP6V0C or pVHL was examined in transfected HEK293 cells. The Flag-ATP6V0C binding to HIF-1α was increased by bafilomycin in a dose-dependent manner, whereas the Flag-VHL binding to HIF-1α was decreased (Fig. 4A). To confirm that HIF-1α stabilization by ATP6V0C and bafilomycin is linked with the pVHL-dependent HIF-1α degradation pro-

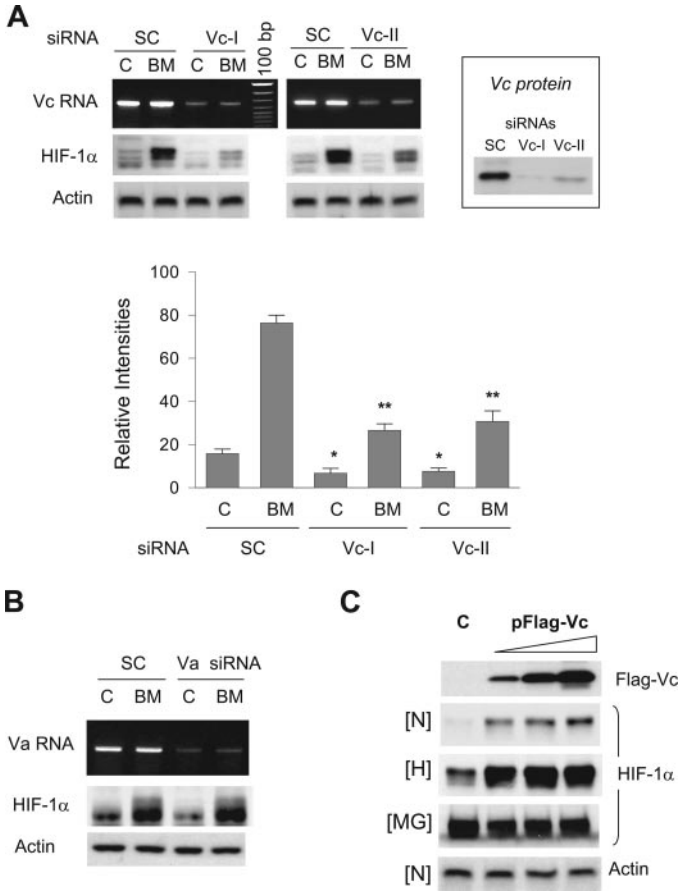

Fig. 2. ATP6V0C mediates bafilomycin-induced HIF-1α expression. A, HIF-1α is down-regulated by siRNAs of ATP6V0C. HEK293 cells were transfected with 25 nM siRNAs of ATP6V0C (Vc-I or Vc-II) or a scrambled control (SC), and incubated with vehicle (C) or 10 nM BM. The ATP6V0C-silencing effect of its siRNA was confirmed by sequential RT-PCR, electrophoresis on 2% agarose gel, and ethidium bromide staining (top left). To confirm ATP6V0C knock-down, ATP6V0C proteins were immunoprecipitated with anti-ATP6V0C serum and were then identified by immunoblotting with the ATP6V0C antibody (top right). HIF-1α levels were measured by immunoblotting. Band intensities were quantified using a MCID-M4 image analysis system and are plotted at bottom. Each bar represents the mean and S.D. of four experiments. *, $p < 0.05$ versus the control group of SC; **, $p < 0.01$ versus the BM-treated group of SC. B, HIF-1α expression is not affected by siRNAs of ATP6V1A. HEK293 cells were transfected with 25 nM of siRNAs of ATP6V1A (Va) or a scrambled control (SC), and incubated with vehicle (C) or 10 nM BM. The ATP6V1A-silencing effect was confirmed by RT-PCR (top), and HIF-1α levels were measured (bottom). Results are representative of four separate experiments. C, ATP6V0C expression induces HIF-1α. HEK293 cells were transfected with various doses of the plasmid Flag-tagged ATP6V0C (pFlag-Vc: 0.5, 1, 2 μg). The transfected cells were then incubated under normoxic (N) or hypoxic conditions (H), or in the presence of 10 μM MG132 (MG). Flag-Vc and HIF-1α levels were determined by immunoblotting.

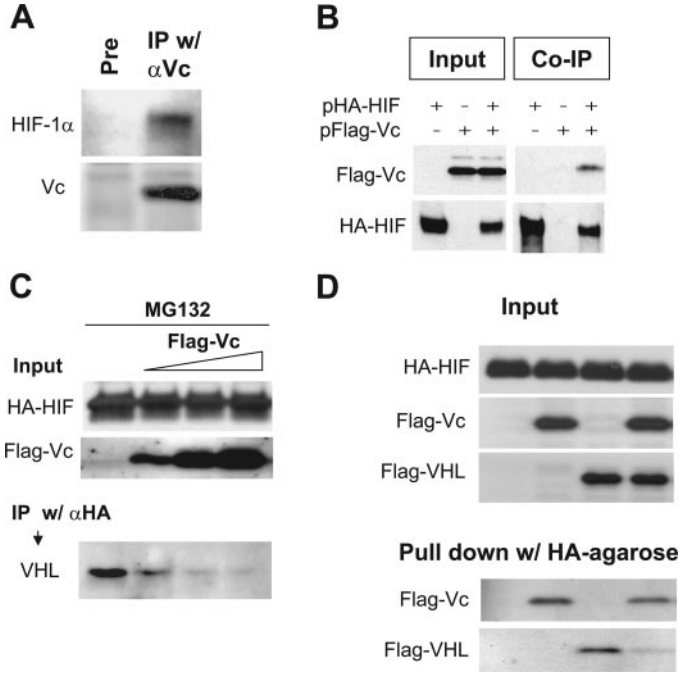

Fig. 3. ATP6V0C binds HIF-1α and competes with pVHL for HIF-1α binding. A, association of ATP6V0C and HIF-1α. PC3 cells were treated with 10 nM BM for 4 h. ATP6V0C was immunoprecipitated with ATP6V0C antiserum (αVc) or nonimmunized rabbit serum (Pre), and the coprecipitation of HIF-1α was analyzed with HIF-1α antiserum. B, association of expressed ATP6V0C and HIF-1α. In HEK293 cells cotransfected with pHA-HIF-1a (1 μg) and pFlag-Vc (1 μg), HA-HIF-1α was immunoprecipitated with HA-affinity beads, and the coprecipitation of Flag-Vc and HA-HIF-1α was analyzed with anti-Flag and anti-HA antibodies. C, pVHL is dissociated from HIF-1α by ATP6V0C expression. HEK293 cells were cotransfected with 1 μg of pHA-HIF-1α and various doses of pFlag-Vc (0.5, 1, and 2 μg). HA-HIF-1α was immunoprecipitated using HA-affinity beads, and the coprecipitation of pVHL was analyzed with anti-pVHL antibody. D, in vitro binding assay for pVHL and HIF-1α. HA-HIF-1α protein was incubated with Flag-ATP6V0C and/or Flag-pVHL in vitro. Flag-ATP6V0C and Flag-pVHL were pulled-down with anti-HA-conjugated Sepharose beads and identified using anti-Flag antibody.

cess, we used an HIF-1α mutant (*m*HIF-1α) lacking two pVHL-targeted proline residues and found that *m*HIF-1α expression was not affected by ATP6V0C expression (Fig. 4B). This suggests that HIF-1α stabilization by ATPV0C is linked with pVHL. Conversely, pVHL expression diminished HIF-1α stabilization by ATP6V0C (Fig. 4C), suggesting that ATP6V0C and pVHL are functionally counterbalanced with respect to HIF-1α regulation. Moreover, HIF-1α expression was not induced by bafilomycin in RCC4 cells having defective a *VHL* gene. However, in RCC4 cells expressing wild-type VHL, the HIF-1α induction by bafilomycin was shown again (Fig. 4D). All the results support that ATP6V0C and bafilomycin inhibit the pVHL-dependent degradation of HIF-1α.

Subcellular Localization of ATP6V0C and HIF-1α. ATP6V0C, a highly hydrophobic protein, is normally localized to lipid membranes, such as vesicles and the plasma membrane. In contrast, HIF-1α is a soluble protein that mainly locates in the nucleus. Then, how do these proteins associate together? To answer this question, we examined their localizations using GFP and RFP tags. Localization of expressed ATP6V0C or HIF-1α was achieved by tagging the gene with RFP or GFP, respectively. RFP-ATP6V0C was shown as aggregated particles in the cytoplasm (Fig. 5, column II), whereas free RFP was expressed diffusely (Fig. 5, column I). Presumably, expressed ATP6V0C is mainly localized to the endoplasmic reticulum or the endosomes. We next expressed RFP-ATP6V0C together with GFP-HIF-1α. In the absence of bafilomycin, HIF-1α was very weakly expressed and distributed diffusely (Fig. 5, column III). The location and distribution pattern of ATP6V0C were not changed particularly. However, after bafilomycin treatment, both HIF-1α and ATP6V0C were localized in the nucleus, as shown in column IV of Fig. 5. These results suggest that bafilomycin stimulates the binding of HIF-1α and ATP6V0C and in turn stabilized HIF-1α drags ATP6V0C into the nucleus.

Identification of the HIF-1α Motif Interacting with ATP6V0C. To identify the ATP6V0C-binding site of HIF-1α, various HIF-1α mutants were immunoprecipitated with ATP6V0C; immunoprecipitates were cross-checked using anti-HA and anti-Flag antibodies. The N-terminal portion of HIF-1α was coimmunoprecipitated with ATP6V0C, but the ODDD and C-terminal portions were not (Fig. 6A, left). We next undertook to identify the ATP6V0C binding site in the HIF-1α N-terminal. Removing amino acids 1 to 16 (dN_HIF-1α) prevented ATP6V0C binding (Fig. 6A, right), and also abolished the HIF-1α-stabilizing effect of bafilomycin (Fig. 6B). Moreover, both dN_HIF-1α and ATP6V0C did not translocate to the nucleus after bafilomycin treatment (Fig. 5, column VI). These results indicate that ATP6V0C interacts with the N terminus of HIF-1α and this interaction is essential for HIF-1α stabilization by bafilomycin. Taken together, we here propose a new hypothesis regarding the ATP6V0C-mediated regulation of HIF-1α, as summarized in Fig. 7.

Fig. 4

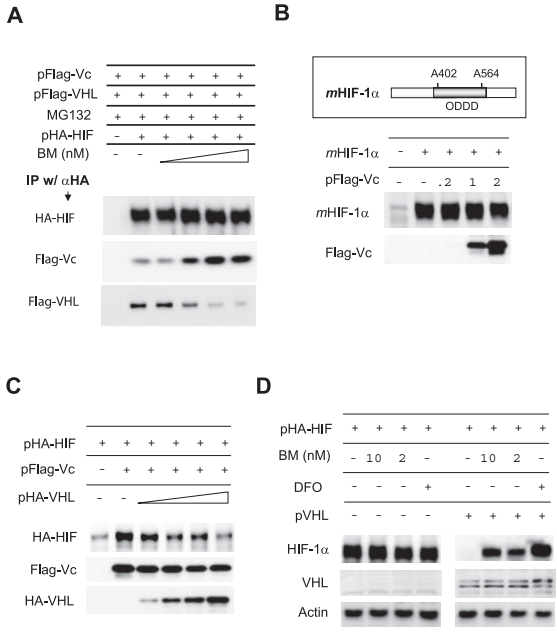

Fig. 4. Bafilomycin inhibits the pVHL-dependent degradation of HIF-1α. A, bafilomycin enhances the ATP6V0C-HIF-1α interaction, but dissociates pVHL from HIF-1α. HEK293 cells were cotransfected with 1 μg of pHA-HIF-1α, 0.5 μg of pFlag-Vc, and 0.5 μg of pFlag-VHL and then treated with BM (0.5, 1, 5, or 10 nM) and MG132 (10 μM) for 6 h. HA-HIF-1α was immunoprecipitated using HA-affinity beads, the coprecipitation of Flag-Vc or Flag-VHL was analyzed with anti-Flag antibody. B, the HIF-1α stabilizing effect of ATP6V0C is associated with the pVHL-mediated regulation of HIF-1α. HEK293 cells were cotransfected with 1 μg of plasmid HIF-1α mutant (*m*HIF-1α: P402A/P564A) and pFlag-Vc (0.2, 1, or 2 μg). HA-mutated HIF-1α and Flag-Vc levels were analyzed by immunoblotting. The structure of mutant is illustrated at the top. C, pVHL reverses HIF-1α induction by ATP6V0C. HEK293 cells were cotransfected with 1 μg of pHA-HIF-1α, 0.5 μg of pFlag-Vc and 0.1, 0.2, 0.5, or 1 μg of pHA-VHL. D, bafilomycin does not induce HIF-1α in the absence of pVHL. VHL-null or VHL wild-type RCC4 cells were treated with 10 or 2 nM bafilomycin. The lysate from 130 μM desferrioxamine-treated cells was loaded as a HIF-1α-positive control.

Discussion

In the present study, the most important thing is the discovery of a novel HIF-1α regulation mechanism. We found that ATP6V0C interacts with HIF-1α, which in turn stabilizes HIF-1α. Under normoxic conditions, HIF-1α is hydroxylated by PHDs and then undergoes pVHL binding, ubiquiti-

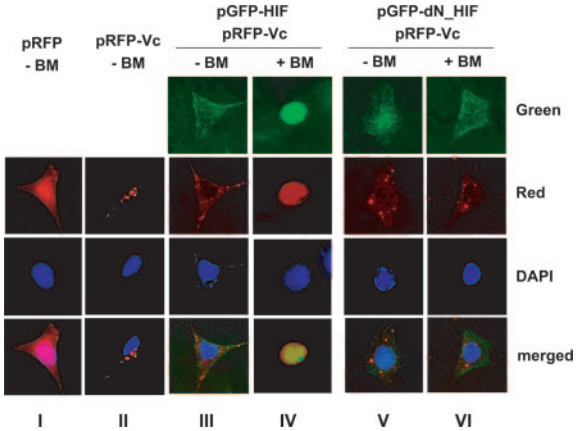

Fig. 5. Localization of HIF-1α and ATP6V0C. pRFP (1 μg of DNA) or pRFP-Vc (1 μg of DNA) was transfected into HEK293 cells. For coexpression, 2 μg of pGFP-HIF-1α or pGFP-dN_HIF-1α were cotransfected with pRFP-Vc. After stabilization for 36 h, the transfected cells were treated with DMSO (−BM) or 10 nM bafilomycin (+BM) for 8 h. The nuclei were stained with DAPI, and the subcellular localizations of the expressed proteins were examined by confocal microscopy. The images in the 1st, 2nd, and 3rd rows were captured using green, red, and blue fluorescence filters, respectively, and those in the 4th row were merged.

nation, and proteasomal degradation. However, if one of these degradation steps is inhibited, HIF-1α could be stabilized. Then, which step is inhibited by ATP6V0C? It is possible that ATP6V0C inhibits HIF-1α proteolysis per se. If so, HIF-1α induced by bafilomycin and ATP6V0C would remain as polyubiquitinated forms, which can retard the electrophoretic mobility of HIF-1α on a SDS/polyacrylamide gel. However, HIF-1α with a higher molecular weight was not produced by bafilomycin and ATP6V0C. Moreover, although dN-HIF-1α with intact ODDD can be easily destructed by the proteasome, it was not stabilized by bafilomycin (Fig. 6B). This also supports the idea that the HIF-1α stabilization is not due to nonspecific inhibition of proteolysis. We next examined the possibility that ATP6V0C directly inhibits the pVHL binding to HIF-1α, using the in vitro binding assay. In this experiment, the MG132 treatment under normoxic conditions may produce hydroxylated HIF-1α, which associated with both pVHL and ATP6V0C directly. However, ATP6V0C dissociated pVHL from HIF-1α (Fig. 3D). This suggests that ATP6V0C inhibits the pVHL binding step after the HIF-1α hydroxylation step.

In addition to ATP6V0C, several proteins are known to modulate the HIF-1α degradation process. For instance, HIF-1α degradation is enhanced by p53 or OS-9 (Ravi et al., 2000; Baek et al., 2005) and inhibited by hsp90, ARNT, Jab1/CSN5, or HBx (Bae et al., 2002; Isaacs et al., 2004; Katschinski et al., 2004; Moon et al., 2004). It is noteworthy that HIF-1α-stabilizing proteins interact with different HIF-1α motifs, whereas HIF-1α-destabilizing proteins interact with motifs near the pVHL-binding site. Of HIF-1α stabilizers, Jab1/CSN5 competes directly with pVHL at the ODD domain. However, hsp90, ARNT, or HBx indirectly inhibits pVHL binding by interacting with the N-terminal portion of HIF-1α. Likewise, ATP6V0C also interfered with pVHL binding by interacting with the N terminus of HIF-1α. We speculate that protein interaction at the N-terminal side alters HIF-1α conformation and thus inhibits pVHL binding to the ODD domain. However, the exact mechanisms involved remain to be elucidated.

Bafilomycin A1 was first isolated from *Streptomyces* species culture media in 1980s. It has since been developed as an antimicrobial agent because it exhibits activity against fungi and bacteria (Drose and Altendorf, 1997). Some years later, however, Bowman et al. (1988) discovered that it inhibits V-ATPase. It has recently been revealed that bafilomycin binds to a pocket formed by helices 1, 2, and 4 of ATP6V0C and that it may thus inhibit H^+ translocation by preventing the rotation of the ATP6V0C multimer (Bowman et al., 2004). Although it acts as an inhibitor of ATP6V0C for H^+ translocation, it acts as an activator of ATP6V0C for HIF-1α regulation. Moreover, at concentrations of a few nanomoles, bafilomycin stimulated ATP6V0C binding to HIF-1α and stabilized HIF-1α. At such the concentrations, intracellular pH was not altered, which suggests that V-ATPase activity is not inhibited very much. Apparently, the interaction between

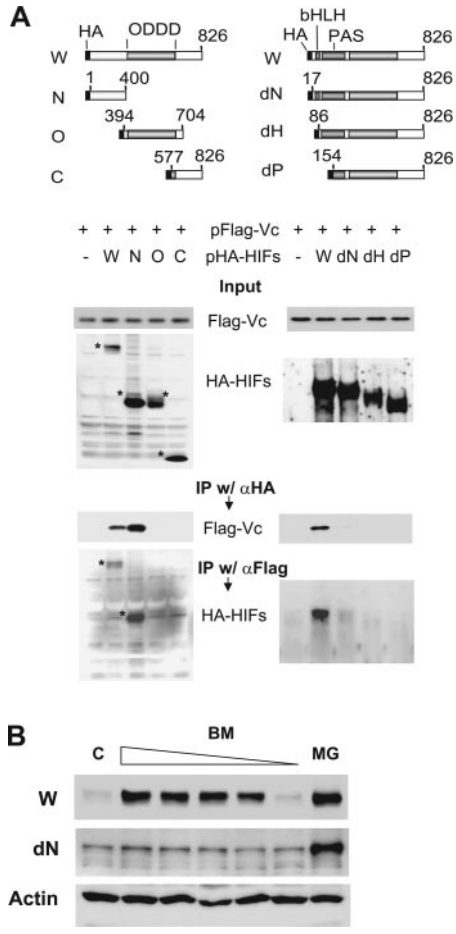

Fig. 6. Identification of the HIF-1α motif responsible for ATP6V0C binding. A, HEK293 cells were cotransfected with 0.5 μg of pFlag-Vc and 1 μg of a plasmid HIF-1α mutant. The structures of the HIF-1α mutants used are illustrated in the upper . HIF-1α and its mutants were immunoprecipitated with anti-HA, and Flag-Vc coprecipitation was analyzed by immunoblotting using anti-Flag. In addition, Flag-Vc was immunoprecipitated with anti-Flag, and coprecipitated HIF-1α and its mutants were analyzed by immunoblotting using anti-HA antibody. *, HIF-1α or its mutants. B, effect of bafilomycin on N-deleted HIF-1α (dN) expression. HEK293 cells were transfected with pHA-HIF-1α (W) or pHA-dN and then treated with bafilomycin (10, 5, 2, 1, and 0.2 nM). Expressed proteins and β-actin were detected using anti-HA and anti-actin antibodies, respectively. MG132 (10 μM) was treated as a positive control.

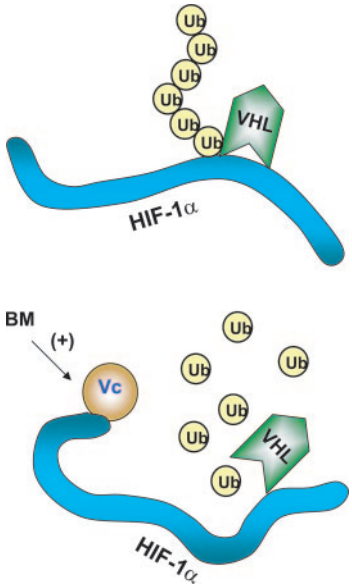

Fig. 7. Hypothetical ATP6V0C-mediated regulation of HIF-1α. ATP6V0C binds to the N-terminal end of HIF-1α, which in turn perturbs the HIF-1α structure to a form unfavorable for pVHL binding, thus resulting in HIF-1α stabilization. Bafilomycin stimulates ATP6V0C binding to HIF-1α. Ub, ubiquitin; +, stimulation of Vc-HIF-1α interaction.

ATP6V0C and HIF-1α seems to be as sensitive to bafilomycin as the V-ATPase inhibition.

In summary, bafilomycin induced HIF-1α by blocking protein degradation. Although bafilomycin is known as a V-ATPase inhibitor, its HIF-1α stabilizing effect was not due to V-ATPase inhibition or intracellular acidification. Of V-ATPase subunits, ATP6V0C is responsible for the HIF-1α stabilization. siRNAs targeting ATP6V0C reduced the basal levels of HIF-1α and inhibited the HIF-1α stabilization by bafilomycin. ATP6V0C was coimmunoprecipitated with HIF-1α and inhibited the pVHL binding to HIF-1α. The in vitro binding assay showed that ATP6V0C dissociated pVHL from HIF-1α by directly binding HIF-1α. Bafilomycin enhanced the ATP6V0C–HIF-1α interaction and inhibited the pVHL-HIF-1α interaction. The confocal imaging showed that ATP6V0C in company with HIF-1α was translocated from the cytoplasm to the nucleus by bafilomycin. HIF-1α mutants lacking the N-terminal end failed to associate with ATP6V0C. We here propose a novel mechanism of HIF-1α regulation, as follows, ATP6V0C binding to the N terminus of HIF-1α inhibits pVHL binding, and stabilizes HIF-1α by preventing its ubiquitination, which is stimulated by bafilomycin.

References

Bae MK, Ahn MY, Jeong JW, Bae MH, Lee YM, Bae SK, Park JW, Kim KR, and Kim KW (2002) Jab1 interacts directly with HIF-1α and regulates its stability. *J Biol Chem* **277:**9–12.

Baek JH, Mahon PC, Oh J, Kelly B, Krishnamachary B, Pearson M, Chan DA, Giaccia AJ, and Semenza GL (2005) OS-9 interacts with hypoxia-inducible factor 1alpha and prolyl hydroxylases to promote oxygen-dependent degradation of HIF-1alpha. *Mol Cell* **17:**503–512.

Birner P, Schindl M, Obermair A, Plank C, Breitenecker G, and Oberhuber G (2000) Overexpression of hypoxia-inducible factor 1alpha is a marker for an unfavorable prognosis in early-stage invasive cervical cancer. *Cancer Res* **60:**4693–4696.

Bowman EJ, Graham LA, Stevens TH, and Bowman BJ (2004) The bafilomycin/concanamycin binding site in subunit c of the V-ATPases from *Neurospora crassa* and *Saccharomyces cerevisiae*. *J Biol Chem* **279:**33131–33138.

Bowman EJ, Siebers A, and Altendorf K (1988) Bafilomycins: a class of inhibitors of membrane ATPases from microorganisms, animal cells, and plant cells. *Proc Natl Acad Sci USA* **85:**7972–7976.

Bruick RK and McKnight SL (2001) A conserved family of prolyl-4-hydroxylases that modify HIF. *Science (Wash DC)* **294:**1337–1340.

Carmeliet P, Dor Y, Herbert JM, Fukumura D, Brusselmans K, Dewerchin M, Neeman M, Bono F, Abramovitch R, Maxwell P, et al. (1998) Role of HIF-1alpha in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. *Nature (Lond)* **394:**485–490.

Chun YS, Choi E, Yeo EJ, Lee JH, Kim MS, and Park JW (2001) A new HIF-1 alpha variant induced by zinc ion suppresses HIF-1-mediated hypoxic responses. *J Cell Sci* **114:**4051–4061.

Drose S and Altendorf K (1997) Bafilomycins and concanamycins as inhibitors of V-ATPases and P-ATPases. *J Exp Biol* **200:**1–8.

Epstein AC, Gleadle JM, McNeill LA, Hewitson KS, O'Rourke J, Mole DR, Mukherji M, Metzen E, Wilson MI, Dhanda A, et al. (2001) *C. elegans* EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. *Cell* **107:**43–54.

Finbow ME, Goodwin SF, Meagher L, Lane NJ, Keen J, Findlay JB, and Kaiser K (1994) Evidence that the 16 kDa proteolipid (subunit c) of the vacuolar H(+)-ATPase and ductin from gap junctions are the same polypeptide in *Drosophila* and *Manduca*: molecular cloning of the Vha16k gene from *Drosophila*. *J Cell Sci* **107:**1817–1824.

Forgac M (1989) Structure and function of vacuolar class of ATP-driven proton pumps. *Physiol Rev* **69:**765–796.

Goldstein DJ, Andresson T, Sparkowski JJ, and Schlegel R (1992) The BPV-1 E5 protein, the 16 kDa membrane pore-forming protein and the PDGF receptor exist in a complex that is dependent on hydrophobic transmembrane interactions. *EMBO (Eur Mol Biol Organ) J* **11:**4851–4859.

Greijer AE and van der Wall E (2004) The role of hypoxia inducible factor 1 (HIF-1) in hypoxia induced apoptosis. *J Clin Pathol* **57:**1009–1014.

Hegyi P, Rakonczay Z Jr, Gray MA, and Argent BE (2004) Measurement of intracellular pH in pancreatic duct cells: a new method for calibrating the fluorescence data. *Pancreas* **28:**427–434.

Huang LE, Gu J, Schau M, and Bunn HF (1998) Regulation of hypoxia-inducible factor 1alpha is mediated by an O_2-dependent degradation domain via the ubiquitin-proteasome pathway. *Proc Natl Acad Sci USA* **95:**7987–7992.

Huss M, Ingenhorst G, Konig S, Gassel M, Drose S, Zeeck A, Altendorf K, and Wieczorek H (2002) Concanamycin A, the specific inhibitor of V-ATPases, binds to the V_o subunit c. *J Biol Chem* **277**:40544–40548.

Isaacs JS, Jung YJ, and Neckers L (2004) Aryl hydrocarbon nuclear translocator (ARNT) promotes oxygen-independent stabilization of hypoxia-inducible factor-1α by modulating an Hsp90-dependent regulatory pathway. *J Biol Chem* **279:**16128–16135.

Ivan M, Kondo K, Yang H, Kim W, Valiando J, Ohh M, Salic A, Asara JM, Lane WS, and Kaelin WG Jr (2001) HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing. *Science (Wash DC)* **292:**464–468.

Jaakkola P, Mole DR, Tian YM, Wilson MI, Gielbert J, Gaskell SJ, Kriegsheim Av, Hebestreit HF, Mukherji M, Schofield CJ, et al. (2001) Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O_2-regulated prolyl hydroxylation. *Science (Wash DC)* **292:**468–472.

Jiang BH, Rue E, Wang GL, Roe R, and Semenza GL (1996) Dimerization, DNA binding, and transactivation properties of hypoxia-inducible factor 1. *J Biol Chem* **271:**17771–17778.

Jiang BH, Zheng JZ, Leung SW, Roe R, and Semenza GL (1997) Transactivation and inhibitory domains of hypoxia-inducible factor 1alpha. Modulation of transcriptional activity by oxygen tension. *J Biol Chem* **272:**19253–19260.

Kamura T, Sato S, Iwai K, Czyzyk-Krzeska M, Conaway RC, and Conaway JW (2000) Activation of HIF1alpha ubiquitination by a reconstituted von Hippel-Lindau (VHL) tumor suppressor complex. *Proc Natl Acad Sci USA* **97:**10430–10435.

Katschinski DM, Le L, Schindler SG, Thomas T, Voss AK, and Wenger RH (2004) Interaction of the PAS B domain with HSP90 accelerates hypoxia-inducible factor-1alpha stabilization. *Cell Physiol Biochem* **14:**351–360.

Koshiji M and Huang LE (2004) Dynamic balancing of the dual nature of HIF-1alpha for cell survival. *Cell Cycle* **3:**853–854.

Lee I, Skinner MA, Guo HB, Sujan A, and Pierce M (2004) Expression of the vacuolar H^+-ATPase 16-kDa subunit results in the Triton X-100-insoluble aggregation of β1 integrin and reduction of its cell surface expression. *J Biol Chem* **279:**53007–53014.

Lim JH, Park JW, Kim MS, Park SK, Johnson RS, and Chun YS (2006) Bafilomycin induces the p21-mediated growth inhibition of cancer cells under hypoxic conditions by expressing HIF-1α. *Mol Pharmacol* **70:**1856–1865.

Mack FA, Rathmell WK, Arsham AM, Gnarra J, Keith B, and Simon MC (2003) Loss of pVHL is sufficient to cause HIF dysregulation in primary cells but does not promote tumor growth. *Cancer Cell* **3:**75–88.

Masson N, Willam C, Maxwell PH, Pugh CW, and Ratcliffe PJ (2001) Independent function of two destruction domains in hypoxia-inducible factor-alpha chains activated by prolyl hydroxylation. *EMBO (Eur Mol Biol Organ) J* **20:**5197–5206.

Maxwell PH, Wiesener MS, Chang GW, Clifford SC, Vaux EC, Cockman ME, Wykoff CC, Pugh CW, Maher ER, and Ratcliffe PJ (1999) The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. *Nature (Lond)* **399:**271–275.

Moon EJ, Jeong CH, Jeong JW, Kim KR, Yu DY, Murakami S, Kim CW, and Kim KW (2004) Hepatitis B virus X protein induces angiogenesis by stabilizing hypoxia-inducible factor-1alpha. *FASEB J* **18:**382–384.

Ohkuma S, Shimizu S, Noto M, Sai Y, Kinoshita K, and Tamura H (1993) Inhibition of cell growth by bafilomycin A1, a selective inhibitor of vacuolar H(+)-ATPase. *In Vitro Cell Dev Biol Anim* **29:**862–866.

Ohta T, Arakawa H, Futagami F, Fushida S, Kitagawa H, Kayahara M, Nagakawa T, Miwa K, Kurashima K, Numata M, et al. (1998) Bafilomycin A1 induces apoptosis in the human pancreatic cancer cell line Capan-1. *J Pathol* **185:**324–330.

Ravi R, Mookerjee B, Bhujwalla ZM, Sutter CH, Artemov D, Zeng Q, Dillehay LE, Madan A, Semenza GL, and Bedi A (2000) Regulation of tumor angiogenesis by p53-induced degradation of hypoxia-inducible factor 1alpha. *Genes Dev* **14:**34–44.

Ryan HE, Poloni M, McNulty W, Elson D, Gassmann M, Arbeit JM, and Johnson RS (2000) Hypoxia-inducible factor-1alpha is a positive factor in solid tumor growth. *Cancer Res* **60:**4010–4015.

Semenza GL (2000) HIF-1: mediator of physiological and pathophysiological responses to hypoxia. *J Appl Physiol* **88:**1474–1480.

Silver RB (1998) Ratio imaging: practical considerations for measuring intracellular calcium and pH in living tissue. *Methods Cell Biol* **56:**237–251.

Zagzag D, Zhong H, Scalzitti JM, Laughner E, Simons JW, and Semenza GL (2000) Expression of hypoxia-inducible factor 1alpha in brain tumors: association with angiogenesis, invasion, and progression. *Cancer* **88:**2606–2618.

Zhong H, De Marzo AM, Laughner E, Lim M, Hilton DA, Zagzag D, Buechler P, Isaacs WB, Semenza GL, and Simons JW (1999) Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. *Cancer Res* **59:**5830–5835.

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