Transcription, Chromatin, and Epigenetics:

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J. Biol. Chem. 2010, 285:3651-3663.
doi: 10.1074/jbc.M109.068577 originally published online November 23, 2009

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 285, NO. 6, pp. 3651–3663, February 5, 2010

Hsp70 and CHIP Selectively Mediate Ubiquitination and Degradation of Hypoxia-inducible Factor (HIF)-1α but Not HIF-2α*[S]

Received for publication, September 21, 2009, and in revised form, October 30, 2009 Published, JBC Papers in Press, November 23, 2009, DOI 10.1074/jbc.M109.068577

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Hypoxia-inducible factors (HIFs) are transcription factors that mediate adaptive responses to reduced oxygen availability. HIF-α subunits are stabilized under conditions of acute hypoxia. However, prolonged hypoxia leads to decay of HIF-1α but not HIF-2α protein levels by unknown mechanisms. Here, we identify Hsp70 and CHIP (carboxyl terminus of Hsc70-interacting protein) as HIF-1α-interacting proteins. Hsp70, through recruiting the ubiquitin ligase CHIP, promotes the ubiquitination and proteasomal degradation of HIF-1α but not HIF-2α, thereby inhibiting HIF-1-dependent gene expression. Disruption of Hsp70-CHIP interaction blocks HIF-1α degradation mediated by Hsp70 and CHIP. Inhibition of Hsp70 or CHIP synthesis by RNA interference increases protein levels of HIF-1α but not HIF-2α and attenuates the decay of HIF-1α levels during prolonged hypoxia. Thus, Hsp70- and CHIP-dependent ubiquitination represents a molecular mechanism by which prolonged hypoxia selectively reduces the levels of HIF-1α but not HIF-2α protein.

Hypoxia-inducible factors (HIFs)[2] are transcription factors that regulate adaptive responses to low O_2 concentrations in metazoans. HIFs are heterodimers that consist of α and β subunits (1). Three α subunits, HIF-1α, HIF-2α, and HIF-3α, and one β subunit, HIF-1β, have been identified (2–4). HIF-1 activates transcription of genes encoding proteins that mediate angiogenesis, erythropoiesis, metabolism, and cell survival (5, 6). HIF-1-dependent responses play essential roles in development, maintenance of oxygen homeostasis, and the pathophysiology of cancer and ischemic cardiovascular disease (5, 7, 8). Understanding the fundamental mechanisms regulating HIF-1 activity may lead to novel therapies for these diseases.

The regulation of HIF-1α protein levels is a critical determinant of HIF-1 activity. Several major mechanisms underlying regulation of HIF-1α protein stability have been delineated. Under normoxic conditions, HIF-1α is hydroxylated at proline residues 402 and 564 by prolyl hydroxylases (PHDs) that utilize O_2 as a substrate (9, 10). Hydroxylated HIF-1α is bound by the von Hippel-Lindau protein (VHL), which recruits the Elongin-C-Elongin-B-Cullin-2-E3-ubiquitin ligase complex, thereby targeting HIF-1α for degradation by the 26 S proteasome (11, 12). The degradation of PHDs, which is mediated by the E3 ubiquitin ligases Siah1a and Siah2, promotes HIF-1α stabilization (13). HIF-1α stability is also regulated by O_2/PHD/VHL-independent mechanisms. RACK1 (receptor of activated protein kinase C) competes with heat shock protein (Hsp) 90 for binding to HIF-1α and promotes the ubiquitination and degradation of HIF-1α in cells exposed to the Hsp90 inhibitor 17-allylaminogeldanamycin (17-AAG) (14) or the calcineurin inhibitor cyclosporine A (15). It also has been reported that hypoxia-associated factor is an E3 ubiquitin ligase, which binds to HIF-1α and regulates its degradation in nonhypoxic and hypoxic cells (16).

In contrast to rapid degradation under aerobic conditions, HIF-1α is resistant to prolyl hydroxylation and translocated to the nucleus under hypoxic conditions (1, 17). The accumulated nuclear HIF-1α dimerizes with HIF-1β, recruits the co-activators p300/CBP, and binds to *cis*-acting hypoxia-response elements (HREs) in target genes, leading to the transcriptional activation of genes encoding proteins that mediate adaptive responses to hypoxia (18, 19). Although HIF-1α protein is stabilized in response to acute hypoxia, HIF-1α protein levels are attenuated during prolonged hypoxia (20).

HIF-2α has a similar structure to HIF-1α but is only expressed in certain tissues (21). HIF-2α protein stability is also O_2-regulated and degraded through the PHD/VHL/Elongin pathway in well oxygenated cells. HIF-2α is stabilized during hypoxia, and protein levels increase with prolonged hypoxia (22). Like HIF-1α, HIF-2α dimerizes with HIF-1β and regulates gene transcription in response to hypoxia. In addition to common target genes regulated by both HIF-1 and HIF-2, HIF-2 mediates transcription of some unique genes, such as *POU5F1*, *CCND1*, and *TGFA* (23, 24). The amino-terminal transactivation domains of HIF-1α and HIF-2α are essential for target gene

* This work was supported, in whole or in part, by National Institutes of Health Grant N01-HV28180 from NHLBI and Roadmap Grant U54 RR020839.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S3 and Table S1.

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[2] The abbreviations used are: HIF, hypoxia-inducible factor; 17-AAG, 17-allylaminogeldanamycin; GST, glutathione *S*-transferase; HRE, hypoxia-response element; Hsp, heat shock protein; PHD, prolyl hydroxylase; shRNA, short hairpin RNA; SILAC, stable isotope labeling by amino acids in cell culture; VHL, von Hippel-Lindau protein; MS, mass spectrometry; WCL, whole cell lysate; Ni-NTA, nickel-nitrilotriacetic acid; shSC, scrambled control shRNA; EV, empty vector.

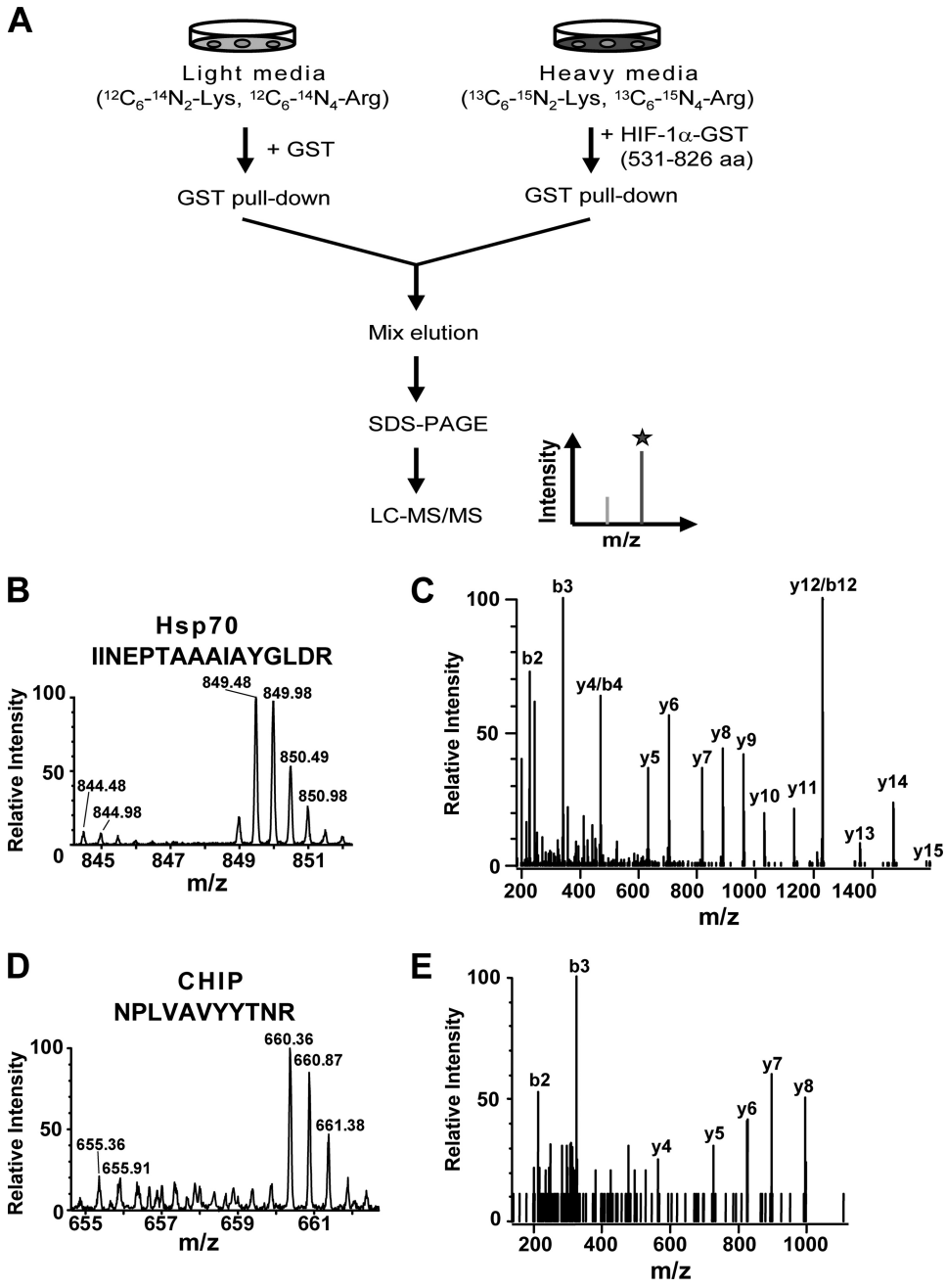


FIGURE 1. **Identification of Hsp70 and CHIP as HIF-1α-interacting proteins.** *A,* proteomic screen was performed to identify HIF-1α-interacting proteins. *B,* mass spectrum of a tryptic peptide of Hsp70 identified by SILAC was determined. *C,* fragmentation spectrum of a representative Hsp70 peptide shown in *B* was determined. *D,* mass spectrum of a tryptic peptide of CHIP identified by SILAC was determined. *E,* fragmentation spectrum of a representative CHIP peptide shown in *D* was determined. For the peptides shown in *B* and *D*, the heavy and light forms differ by *m/z* 5. *LC-MS/MS*, liquid chromatography-tandem mass spectrometry; *aa,* amino acids.

specificity (25). In this study, using a stable isotope labeling by amino acids in cell culture (SILAC)-based quantitative proteomic screen, we identified Hsp70 and CHIP (carboxyl terminus of Hsc70-interacting protein) as HIF-1α-interacting proteins that selectively regulate ubiquitination and degradation of HIF-1α but not HIF-2α.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—The complete coding sequence of human Hsp70 or CHIP was amplified from HEK293 cell cDNA by PCR and inserted into pcDNA3.1-V5-His vector (Invitrogen). Hsp70-V5 or CHIP-V5 cDNA was excised with BamHI and PmeI, polished by Klenow, and ligated into lentiviral vector EF.v-CMV.GFP. Full-length HIF-1α cDNA was subcloned to pGex-6P-1 vector (GE Healthcare). Deletion mutants of Hsp70 or CHIP were generated by PCR and cloned into pcDNA3.1-V5-His or pGex-6P-1 vectors. The CHIP(H260Q) and CHIP(K30A) mutations were generated using QuikChange site-directed mutagenesis kit (Stratagene). Other constructs have been described previously (14). The DNA sequences of plasmid constructs were confirmed by nucleotide sequencing.

Cell Culture and Transfection—HEK293, HEK293T, and RCC4 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a 5% CO_2, 95% air incubator. For SILAC labeling, HEK293 cells were grown in Dulbecco's modified Eagle's medium (AthenaES) supplemented with $^{13}C_6$-$^{15}N_2$-lysine and $^{13}C_6$-$^{15}N_4$-arginine (Sigma) for at least six doublings. For hypoxic exposure, cells were placed in a modular incubator chamber (Billups-Rothenberg) flushed with 1% O_2, 5% CO_2, balance N_2, and incubated at 37 °C. Cells were transfected using FuGENE 6 reagent, according to the manufacturer's protocol (Roche Applied Science).

Lentivirus Production—Recombinant lentivirus was generated by transfection of HEK293T cells with the transducing vector containing Hsp70-V5 or CHIP-V5 cDNA and packaging vectors pMD.G and pCMVR8.91 (26). After 48 h, lentivirus was harvested and transduced into RCC4 cells.

SILAC-based Proteomic Screen—HEK293 cells cultured in Dulbecco's modified Eagle's medium containing $^{12}C_6$-$^{14}N_2$-lysine/$^{12}C_6$-$^{14}N_4$-arginine (light isotope media) or $^{13}C_6$-$^{15}N_2$-lysine/$^{13}C_6$-$^{15}N_4$-arginine (heavy isotope media) were lysed in modified RIPA buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM β-mercaptoethanol, 150 mM NaCl, 1 mM Na_3VO_4, 1 mM NaF, 1 mM EDTA, 0.25% sodium deoxycholate, 1% Igepal, and protease inhibitor mixture (Roche Applied Science). 72 mg

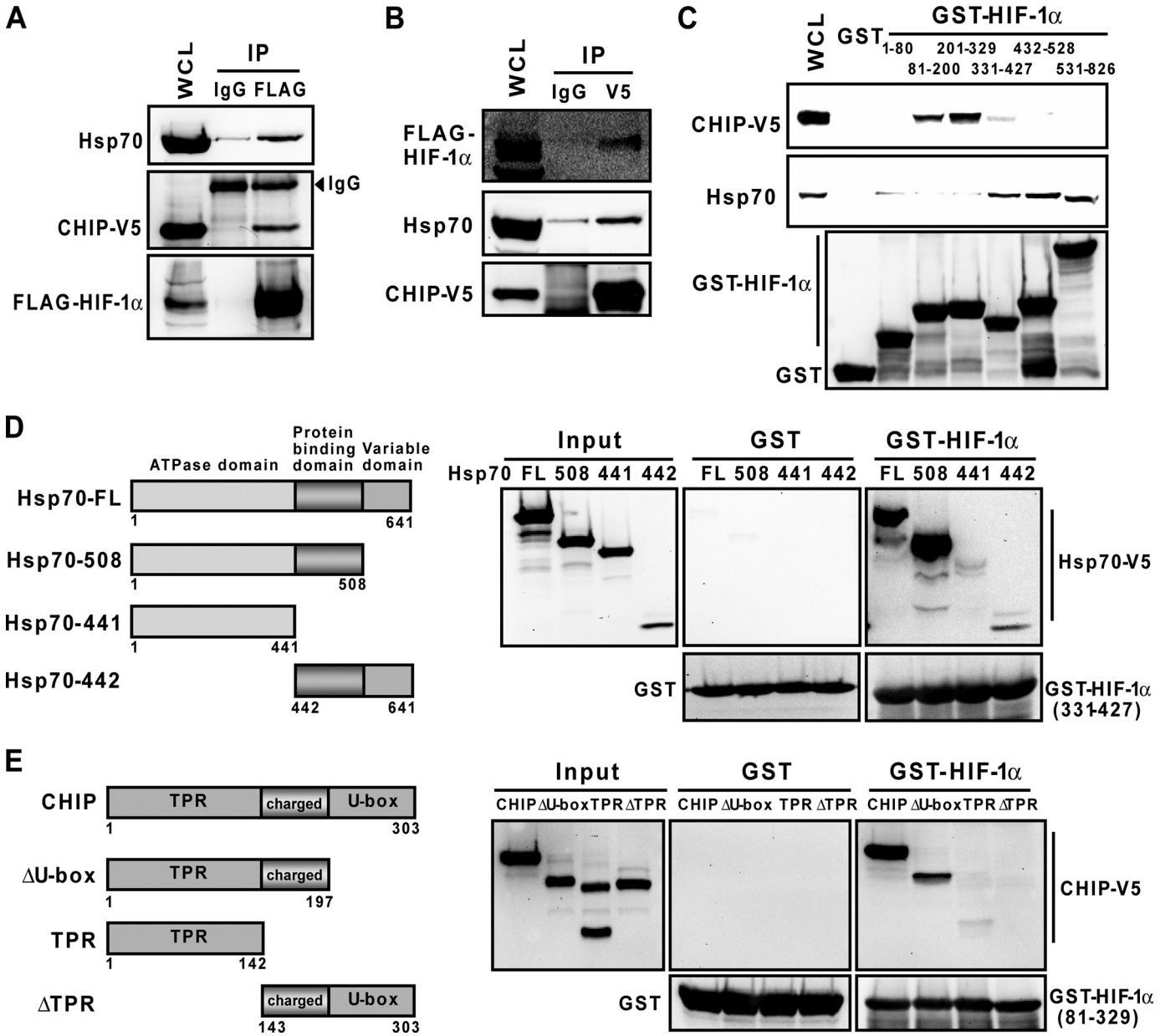


FIGURE 2. **Hsp70-CHIP complex binds to HIF-1α *in vitro* and in human cells.** *A* and *B*, co-immunoprecipitation (*IP*) of FLAG-HIF-1α and CHIP-V5 or Hsp70 from transfected HEK293T cells treated with 10 μM MG132 for 8 h was performed. *C*, GST pulldown assays were performed with purified GST or GST fusion protein containing the indicated amino acid residues of HIF-1α and whole cell lysates (*WCLs*) from HEK293T cells expressing CHIP-V5. *D*, GST pulldown assays were performed with purified GST or GST-HIF-1α-(331–427) and WCLs from HEK293T cells expressing full-length (*FL*) or truncated Hsp70-V5 polypeptides that are illustrated in the *left panel*. *E*, GST pulldown assays were performed with purified GST or GST-HIF-1α-(81–329) and WCLs from HEK293T cells expressing full-length or truncated CHIP-V5 polypeptides that are illustrated in the *left panel*. *TPR*, tetratricopeptide repeat.

of whole cell lysates were incubated overnight with 200 μg of GST or GST-HIF-1α-(531–826) immobilized on glutathione-Sepharose beads. The bound proteins were eluted, mixed, fractionated by SDS-PAGE, and stained with colloidal blue staining kit (Invitrogen). Stained protein bands were excised and digested with trypsin. After in-gel digestion, the tryptic peptides were extracted, dried, and reconstituted in 0.1% formic acid. The peptide mixture was analyzed by reverse phase liquid chromatography-tandem MS. MS spectra were acquired on a quadrupole time-of-flight MS (Q-TOF US-API, micromass) in a survey scan (m/z range from 350 to 1200) in a data-dependent mode selecting the four most abundant ions for tandem MS (m/z range from 100 to 1800). The acquired data were processed using MassLynx (version 4.02) and searched against NCBI Protein Database (released on March 5, 2007) using Mascot search engine (version 2.2.0, Matrixscience). Proteins with at least two reliable peptides (rank 1; unique; individual score higher than or equal to 30) were considered as positively identified proteins. Relative quantitation of stable isotope-labeled peptides was performed using MSQuant (version 1.4.3a39) and manually verified (27).

GST Pulldown Assays—GST and GST fusion proteins were expressed in *Escherichia coli* BL21-Gold (DE3) and purified (14, 28). His-tagged proteins were expressed in HEK293 or HEK293T cells, captured by Ni-NTA beads (Qiagen) from

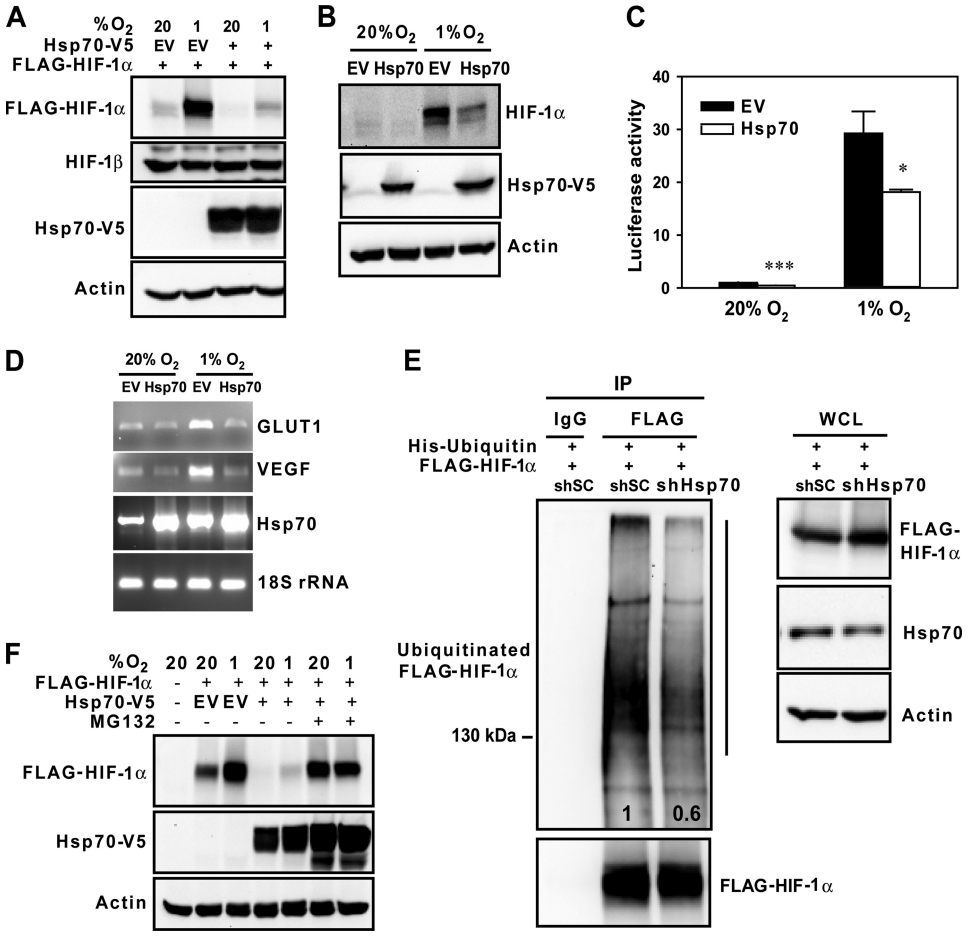


FIGURE 3. **Hsp70 overexpression induces ubiquitination and proteasomal degradation of HIF-1α.** *A,* immunoblot assays were performed to determine FLAG-HIF-1α, HIF-1β, Hsp70-V5, and actin protein levels in co-transfected HEK293T cells exposed to 20% or 1% O_2 for 4 h. *EV*, empty vector. *B,* immunoblot assays were performed to determine HIF-1α, Hsp70-V5, and actin protein levels in transfected HEK293T cells exposed to 20% or 1% O_2 for 4 h. *C,* HEK293 cells were co-transfected with HIF-1-dependent firefly luciferase reporter p2.1, control reporter pSV-Renilla, and EV or Hsp70-V5 expression vector and exposed to 20% or 1% O_2 for 24 h. The ratio of firefly/*Renilla* luciferase activity was determined and normalized to the nonhypoxic EV condition (mean ± S.E., $n = 5$). *, $p < 0.05$; ***, $p < 0.001$ compared with EV. *D*, expression of GLUT1, vascular endothelial growth factor (*VEGF*), and Hsp70 mRNA and 18 S rRNA was analyzed by reverse transcription-PCR in transfected HEK293T cells exposed to 20% or 1% O_2 for 24 h. *E*, HEK293T cells were co-transfected with vectors encoding His-ubiquitin, FLAG-HIF-1α, and Hsp70 shRNA (*shHsp70*) or scrambled control shRNA (*shSC*) and treated with 10 μM MG132 for 8 h. Whole cell lysates were used for co-immunoprecipitation (*IP*). Band intensities of the ubiquitinated HIF-1α species (indicated by *vertical bar* at *right*) were quantified by densitometry and normalized to shSC. *F*, immunoblot assays were performed to determine FLAG-HIF-1α, Hsp70-V5, and actin protein levels in co-transfected HEK293T cells exposed to 20% or 1% O_2 for 4 h in the presence or absence of MG132.

whole cell lysates, and eluted with 250 mM imidazole. Equal amounts of GST and GST fusion proteins immobilized on glutathione-Sepharose beads were incubated overnight with whole cell lysates or purified Hsp70-V5-His. After washing three times, the bound proteins were fractionated by SDS-PAGE, followed by immunoblot assays.

Co-immunoprecipitation and Immunoblot Assays—Whole cell lysates prepared in modified RIPA buffer were incubated overnight with anti-FLAG antibody (Sigma) in the presence of protein A-Sepharose beads (GE Healthcare) or anti-V5-agarose affinity gel (Sigma). After washing three times, the bound proteins were fractionated by SDS-PAGE, followed by immunoblot assays using the following antibodies: HIF-1α (BD Biosciences); HIF-2α, HIF-1β, and CHIP (Novus Biologicals); V5 epitope (Invitrogen); Hsp70, GST, and actin (Santa Cruz Biotechnology); His (Qiagen); and FLAG (Sigma).

Reverse Transcription-PCR—Total RNA was isolated from cells and reverse-transcribed (14). cDNA was amplified by PCR and analyzed by 1.5% agarose/ethidium bromide gel electrophoresis.

Luciferase Reporter Assays—HEK293 cells were seeded onto 48-well plates and transfected with firefly luciferase HRE reporter p2.1, control reporter pSV-Renilla, pcDNA3.1-V5-His, pcDNA3.1-Hsp70-V5-His, scrambled short hairpin RNA (shRNA), or shRNA against Hsp70 or CHIP. Four independent transfections were performed. Cells were exposed to 20% or 1% O_2 for 24 h. Firefly and *Renilla* luciferase activities were determined using the Dual-Luciferase assay system (Promega).

shRNA Assays—The 19-nucleotide sequences targeting Hsp70 or CHIP are shown in supplemental Table S1. Oligonucleotides encoding shRNAs were annealed and ligated into BglII/HindIII-digested pSUPER.retro.neo.GFP vector (OligoEngine). A scrambled shRNA with no significant homology to any mammalian gene sequence was also prepared (28). Cells were transfected with shRNA expression vectors for 72 h prior to analysis.

Statistical Analysis—Data were expressed as mean ± S.E. Differences were examined by Student's *t* test between two groups or one-way analysis of variance within multiple groups. $p < 0.05$ was considered significant.

RESULTS

Identification of HIF-1α-interacting Proteins—To study novel molecular mechanisms that regulate HIF-1α protein stability during hypoxia, we screened for HIF-1α-interacting proteins using SILAC combined with a GST pulldown approach. HEK293 cells were cultured in standard culture media (light media) or media supplemented with heavy isotope-labeled lysine/arginine (heavy media), so that the cellular proteomes incorporated light or heavy lysine and arginine. The heavy amino acids introduce a mass shift, which is distinguishable and quantifiable by MS (29). The cell lysates containing light isotope- or heavy isotope-labeled proteins were incubated with GST or a GST fusion protein containing residues 531–826 of

human HIF-1α, respectively, immobilized on glutathione-Sepharose beads. After extensive washing, proteins that bound to GST or GST-HIF-1α were boiled in sample buffer, mixed, and fractionated by SDS-PAGE. Following in-gel trypsin digestion and peptide extraction, the tryptic peptide mixtures were analyzed by liquid chromatography-tandem mass spectrometry (Fig. 1*A*). The peptide spectra were searched against the NCBI Protein Database by Mascot search engine, verified, and quantified by MSQuant software. Among the identified proteins that showed a ratio of heavy to light peak ≥2, we found known HIF-1α-binding proteins, including the VHL-Elongin-C-Elongin-B-Cullin-2 complex, factor inhibiting HIF-1, and p300 (data not shown), which validated our approach. Hsp70 and CHIP exhibited SILAC ratios of 10 and 4.5, respectively (Fig. 1, *B* and *D*). The tandem MS spectrum demonstrated the accurate assignment of each representative peptide of Hsp70 or CHIP (Fig. 1, *C* and *E*). Together, MS data indicate that Hsp70 and CHIP are HIF-1α-interacting proteins. Hsp70 is a widely expressed, inducible heat shock protein, which is involved in protein folding and unfolding, signal transduction, cell survival, and inflammation (30). CHIP is an Hsp70-associated E3 ubiquitin ligase that promotes protein degradation by the 26 S proteasome (31).

To confirm the proteomic finding, we performed a co-immunoprecipitation assay. HEK293T cells were co-transfected with FLAG-tagged HIF-1α and V5-tagged CHIP expression vectors and treated with the proteasome inhibitor MG132. CHIP-V5 was specifically precipitated from cell lysates by anti-FLAG antibody but not by control IgG (Fig. 2*A*). Endogenous Hsp70 was also detected in immunoprecipitates of FLAG-HIF-1α (Fig. 2*A*). Conversely, FLAG-HIF-1α was pulled down with CHIP-V5 from lysates of co-transfected HEK293T cells by anti-V5 antibody (Fig. 2*B*). Endogenous Hsp70 was also precipitated by anti-V5 antibody (Fig. 2*B*). These data indicate that Hsp70/CHIP and HIF-1α form a protein complex in human cells. *In vitro* GST pulldown assays further revealed that V5/His-tagged Hsp70 purified by Ni-NTA beads from transfected HEK293 cell lysates associated with purified GST-HIF-1α but not with GST indicating that Hsp70-HIF-1α interaction is direct (supplemental Fig. S1).

To map the HIF-1α domains that are involved in Hsp70/CHIP binding, we performed *in vitro* GST pulldown assays. GST-HIF-1α fusion proteins were purified from bacteria and incubated with lysates from HEK293T cells expressing CHIP-V5 and endogenous Hsp70. As shown in Fig. 2*C*, Hsp70 bound strongly to GST fusion proteins containing HIF-1α amino acid residues 331–427, 432–528, and 531–826 but not to GST. CHIP-V5 bound strongly to HIF-1α residues 81–200 and 201–329. These results suggest that the initial identification of CHIP based on interaction with HIF-1α-(531–826) in the proteomic screen was probably due to dual interaction of Hsp70 with both HIF-1α-(531–826) and CHIP.

To determine the Hsp70 domains required for binding to HIF-1α, the cell lysates of HEK293T cells expressing truncated Hsp70-V5 were incubated with either GST or GST-HIF-1α-(331–427) immobilized on glutathione-Sepharose beads. Full-length Hsp70 (Hsp70-FL, amino acids 1–641) and Hsp70–508 (amino acids 1–508) bound strongly to GST-HIF-1α-(331–427) (Fig. 2*D*). However, Hsp70–441 (amino acids 1–441) failed to interact with GST-HIF-1α-(331–427). The lack of interaction of truncated Hsp70 proteins with GST confirmed

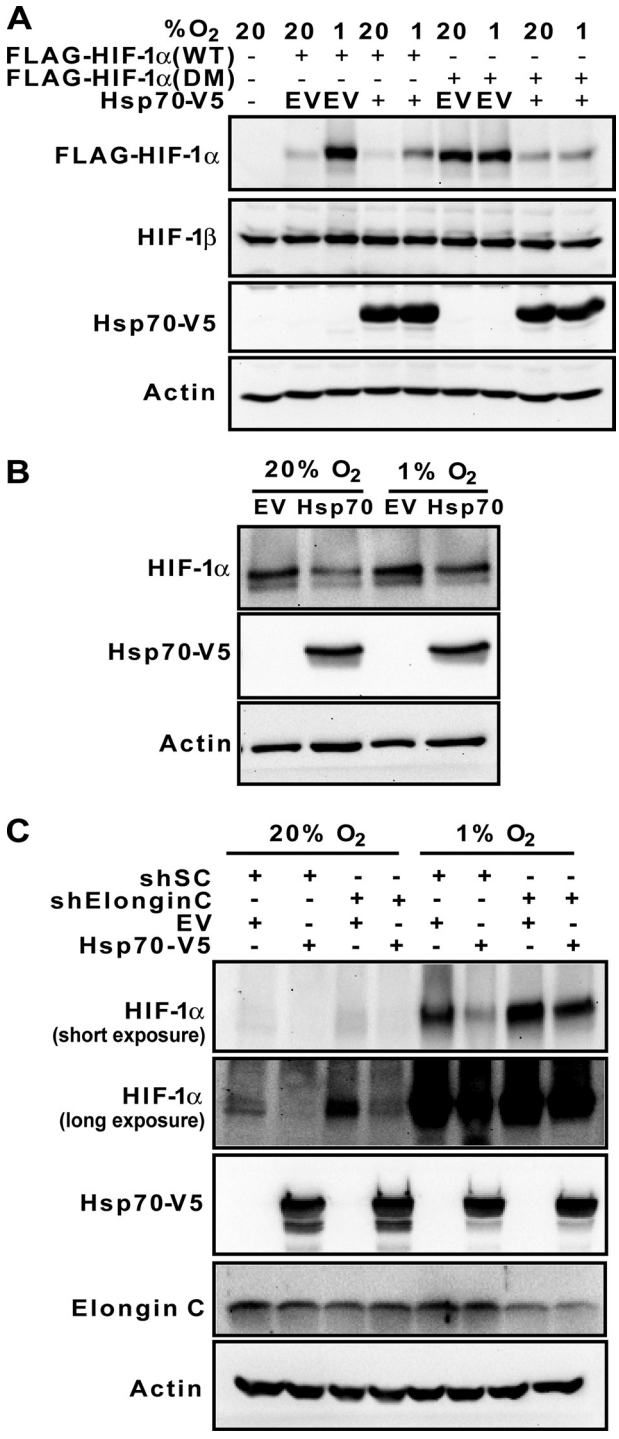


FIGURE 4. **PHD/VHL/Elongin-C pathway is not required for Hsp70-mediated HIF-1α degradation.** *A,* immunoblot assays were performed to determine double mutant (*DM*) FLAG-HIF-1α (P402A/P564A), wild-type (*WT*) FLAG-HIF-1α, HIF-1β, Hsp70-V5, and actin protein levels in co-transfected HEK293T cells exposed to 20% or 1% O_2 for 4 h. *B,* immunoblot assays were performed to determine HIF-1α, Hsp70-V5, and actin protein levels in transduced RCC4 cells exposed to 20% or 1% O_2 for 4 h. *C,* immunoblot assays were performed to determine HIF-1α, Hsp70-V5, Elongin-C, and actin protein levels in transfected HEK293T cells exposed to 20% or 1% O_2 for 4 h. Representative blots from three experiments are shown. *shSC*, scrambled control shRNA; *shElonginC*, Elongin-C shRNA.

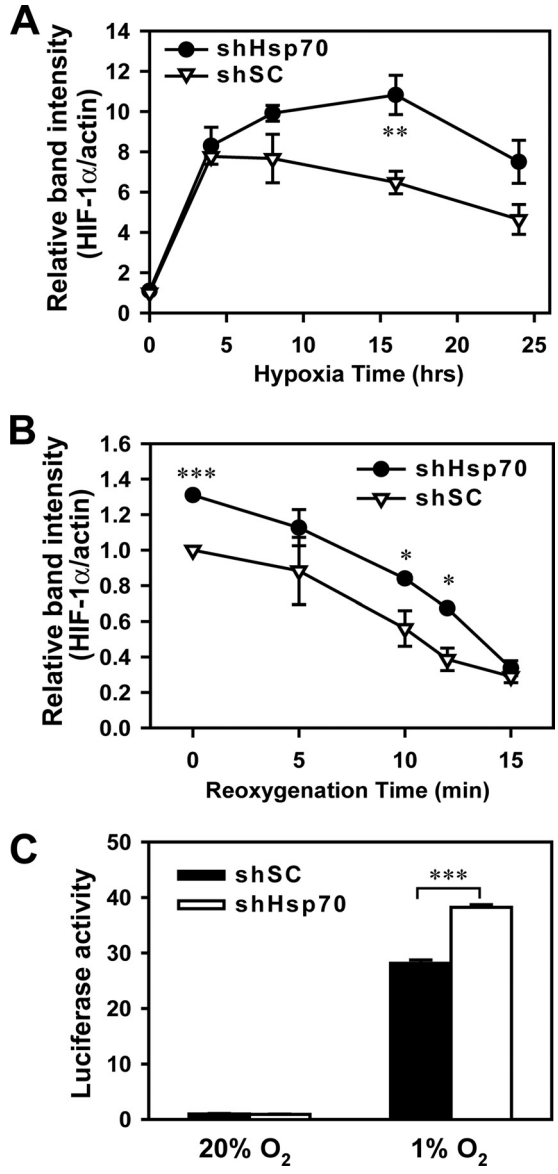


FIGURE 5. **Hsp70 knockdown increases HIF-1α protein levels and HIF-1-dependent gene transcription during prolonged hypoxia.** *A,* HEK293T cells were transfected with expression vector encoding shRNA against Hsp70 (*shHsp70*) or a scrambled control shRNA (*shSC*) and exposed to 20% or 1% O_2 for the indicated time. HIF-1α and actin levels were determined by immunoblot assays and quantified by densitometry. The ratio of HIF-1α/actin was normalized to the nonhypoxic shSC condition (mean ± S.E., $n = 3$). **, $p < 0.01$, relative to shSC. *B,* HEK293T cells were transfected with shHsp70 or shSC and exposed to 20% or 1% O_2 for 24 h, followed by exposure to 20% O_2 for the indicated time. HIF-1α and actin levels were determined by immunoblot assays and quantified by densitometry. The ratio of HIF-1α/actin was normalized to hypoxic shSC group (mean ± S.E., $n = 3$). *, $p < 0.05$; ***, $p < 0.001$, relative to shSC. *C,* HEK293 cells were co-transfected with p2.1, pSV-Renilla, and expression vector encoding shSC or shHsp70 and exposed to 20% or 1% O_2 for 24 h. The ratio of firefly/*Renilla* luciferase activity was determined and normalized to nonhypoxic shSC condition (mean ± S.E., $n = 4$). ***, $p < 0.001$.

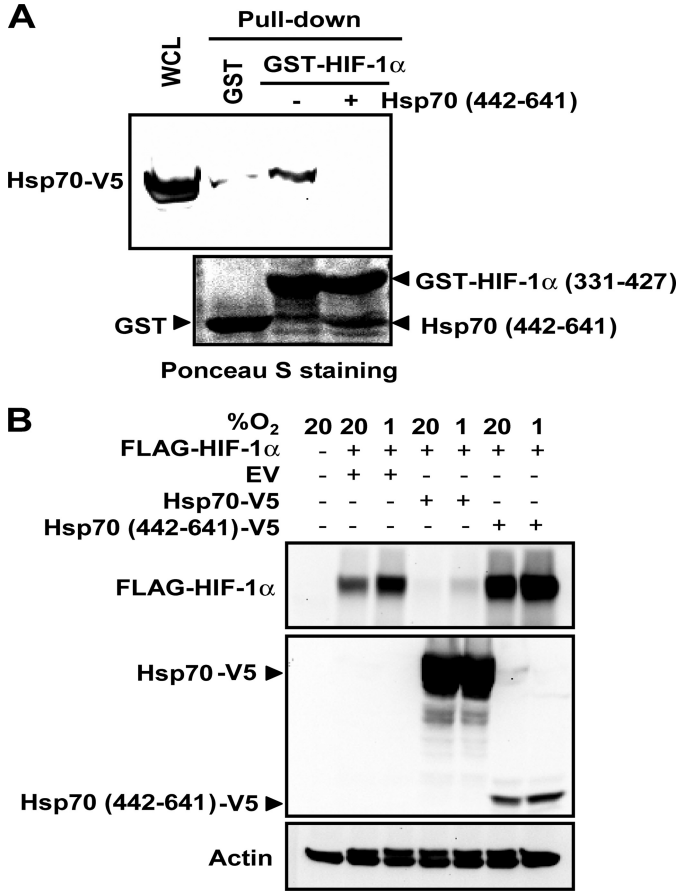


FIGURE 6. **Disruption of Hsp70-HIF-1α interaction prevents HIF-1α degradation.** *A,* GST pulldown assays were performed with purified GST or GST-HIF-1α-(331–427) and WCLs from HEK293 cells expressing Hsp70-V5 in the presence or absence of purified Hsp70-(442–641). *B,* HEK293T cells were co-transfected with vector encoding FLAG-HIF-1α and EV, Hsp70-V5, or Hsp70-(442–641)-V5 and exposed to 20% or 1% O_2 for 4 h. WCLs were subjected to immunoblot assays.

the specificity of binding (Fig. 2*D*). These data suggest that residues 442–508 of Hsp70 are involved in interaction with HIF-1α, which was confirmed by the subsequent finding that Hsp70–442 (amino acids 442–641) was able to bind to GST-HIF-1α-(331–427), even though this truncated protein was expressed weakly in cells (Fig. 2*D*).

Analysis of binding between GST-HIF-1α and truncated CHIP proteins demonstrated that deletion of the U-box domain of CHIP (ΔU-box) did not affect interaction with GST-HIF-1α-(81–329) (Fig. 2*E*). The tetratricopeptide repeat domain was necessary but not sufficient for interaction with GST-HIF-1α-(81–329) (Fig. 2*E*). These data indicate that residues 1–197 of CHIP are required for interaction with HIF-1α *in vitro*.

Hsp70 Promotes the Proteasomal Degradation of HIF-1α—To test whether Hsp70 regulates HIF-1α protein turnover, HEK293T cells were co-transfected with expression vector encoding FLAG-HIF-1α and empty vector (EV) or vector encoding Hsp70-V5 and exposed to 20% or 1% O_2. Hsp70-V5 significantly reduced FLAG-HIF-1α protein levels by 53% at 20% O_2 and by 68% at 1% O_2 but did not affect protein levels of HIF-1β or actin (Fig. 3*A* and supplemental Fig. S2*A*). Hsp70-V5 also decreased levels of endogenous HIF-1α protein induced by exposure of HEK293T cells to 1% O_2 (Fig. 3*B* and supplemental Fig. S2*B*).

HIF-1 regulates the transcription of numerous hypoxia-adaptive genes (5). To test the effect of Hsp70 on HIF-1 transcriptional activity, HEK293 cells were co-transfected with the following: HIF-1-dependent reporter plasmid p2.1, which contains a 68-bp HRE from the human *ENO1* gene upstream of SV40 promoter and firefly luciferase coding sequences (32);

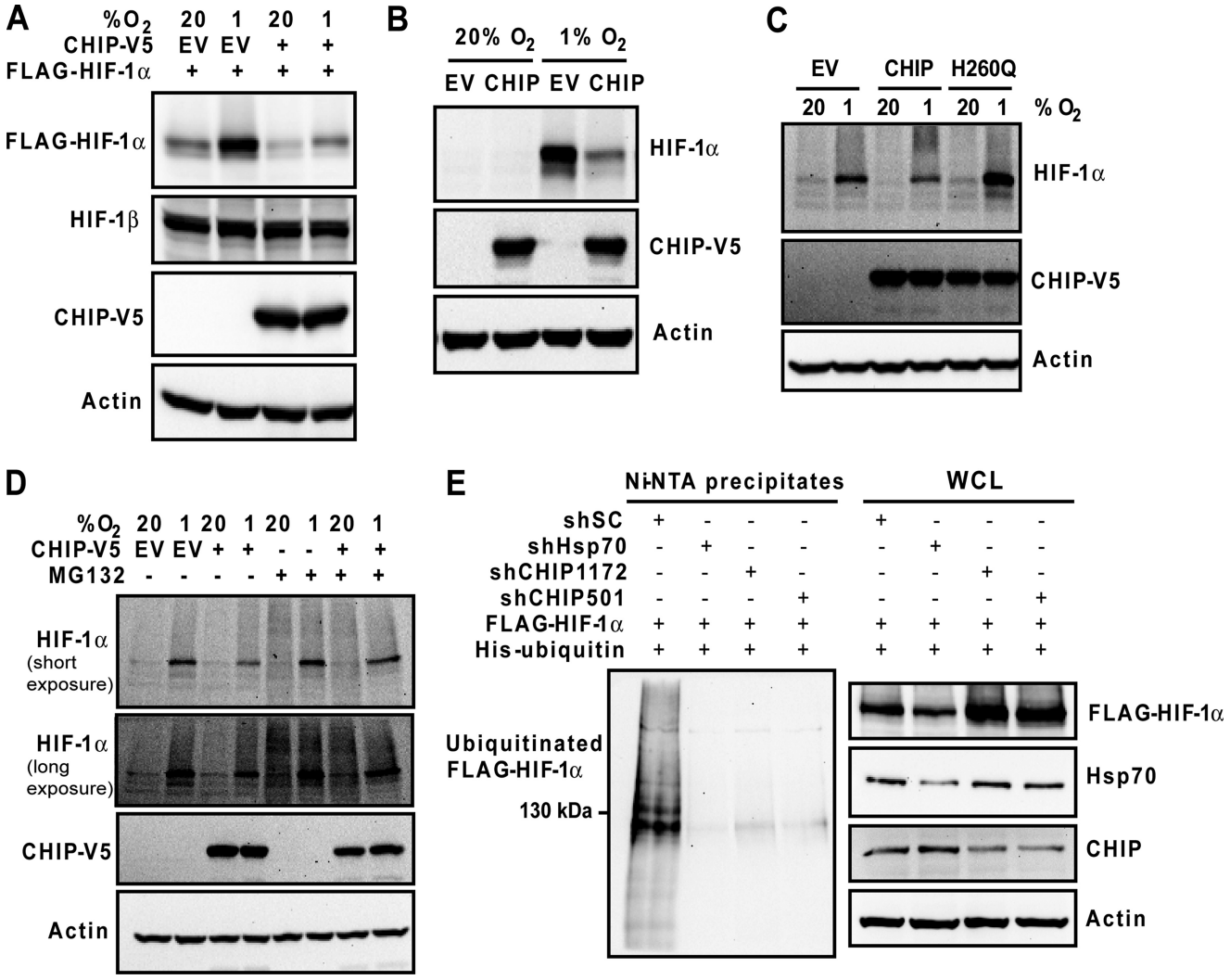


FIGURE 7. **CHIP promotes ubiquitination and degradation of HIF-1α.** *A,* immunoblot assays were performed to determine FLAG-HIF-1α, HIF-1β, CHIP-V5, and actin protein levels in co-transfected HEK293T cells exposed to 20% or 1% O_2 for 8 h. *B,* immunoblot assays were performed to determine HIF-1α, CHIP-V5, and actin protein levels in transfected HEK293T cells exposed to 20% or 1% O_2 for 8 h. *C,* immunoblot assays were performed to determine HIF-1α, CHIP-V5, or CHIP (H260Q)-V5 and actin protein levels in transfected HEK293T cells exposed to 20% or 1% O_2 for 8 h. *D,* immunoblot assays were performed to determine HIF-1α, CHIP-V5, and actin protein levels in transfected HEK293T cells exposed to 20% or 1% O_2 for 8 h in the presence or absence of 10 μM MG132. *E,* HEK293T cells were co-transfected with vector encoding His-ubiquitin, FLAG-HIF-1α, shSC, shHsp70, shCHIP1172, or shCHIP501 and treated with MG132 for 8 h. Total ubiquitinated proteins were precipitated from WCLs by Ni-NTA beads and analyzed by immunoblot assay with anti-FLAG antibody.

control plasmid pSV-Renilla, where *Renilla* luciferase expression is driven by the SV40 promoter alone; and EV or Hsp70-V5 expression vector. The transcriptional activity of HIF-1 was significantly decreased, at both 20% and 1% O_2 when Hsp70-V5 was overexpressed in HEK293 cells (Fig. 3*C*). Analysis of HIF-1 target gene expression by reverse transcription-PCR demonstrated that Hsp70-V5 reduced expression of GLUT1 and vascular endothelial growth factor mRNA, but it had no effect on 18 S rRNA levels in HEK293T cells exposed to 20% or 1% O_2 (Fig. 3*D*). Interestingly, Hsp70 mRNA levels were also increased in hypoxic cells (Fig. 3*D*), suggesting that Hsp70 may be encoded by a HIF-1 target gene. Taken together, these data indicate that Hsp70 reduces HIF-1α protein levels, thereby decreasing HIF-1 transcriptional activity and downstream target gene expression.

To investigate the mechanism underlying Hsp70-mediated HIF-1α down-regulation, we first studied the effect of Hsp70 on HIF-1α ubiquitination using an shRNA targeting both the Hsp70-1A and Hsp70-1B isoforms (shHsp70) or a scrambled control shRNA (shSC) that does not target any known human gene (28). shHsp70, but not shSC, markedly reduced levels of Hsp70 mRNA and protein in HEK293T cells (supplemental Fig. S2*E*). The levels of 18 S rRNA and actin protein were not affected by shHsp70. HEK293T cells were co-transfected with vectors encoding His-tagged ubiquitin, FLAG-HIF-1α, and shHsp70 or shSC and treated with the proteasome inhibitor MG132 to block degradation of ubiquitinated HIF-1α. Analysis of immunoprecipitated FLAG-HIF-1α by anti-His antibody demonstrated that ubiquitination of FLAG-HIF-1α was inhibited by 40% in Hsp70 knockdown cells, compared with cells transfected with shSC, whereas total FLAG-HIF-1α levels were similar (Fig. 3*E*). Further experiments revealed that MG132 treatment inhibited FLAG-HIF-1α protein degradation mediated by Hsp70-V5 in HEK293T cells at both 20% and 1% O_2 (Fig. 3*F* and supplemental Fig. S2*C*). Taken together, our gain-of-function data indicate that Hsp70 pro-

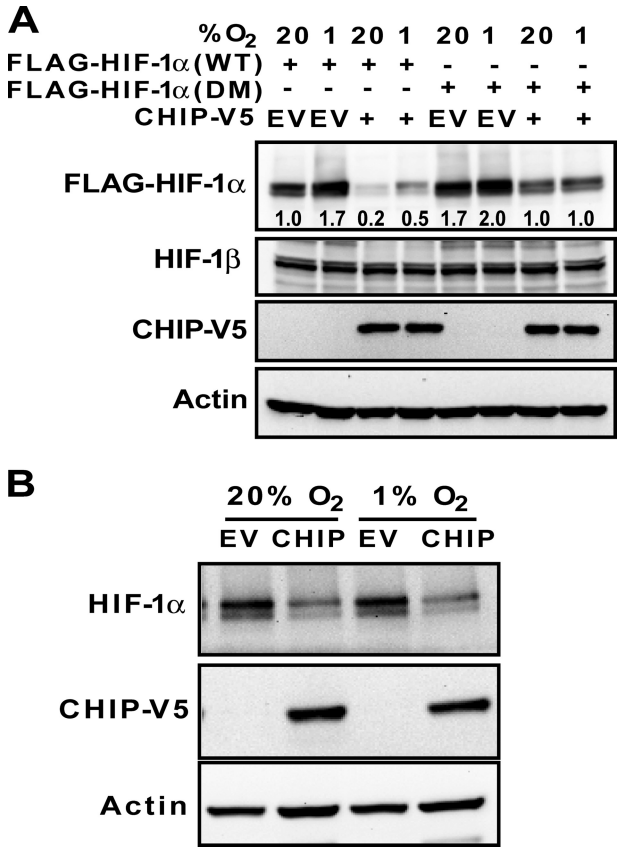


FIGURE 8. **PHD/VHL pathway is not involved in CHIP-mediated HIF-1α degradation.** *A,* immunoblot assays were performed to determine double mutant (*DM*) FLAG-HIF-1α(P402A/P564A), wild-type (*WT*) FLAG-HIF-1α, HIF-1β, CHIP-V5, and actin protein levels in co-transfected HEK293T cells exposed to 20% or 1% O_2 for 8 h. Band intensities of FLAG-HIF-1α and actin were quantified by densitometry. The ratio of FLAG-HIF-1α/actin was normalized to nonhypoxic EV condition, as indicated on the blot. *B,* immunoblot assays were performed to determine HIF-1α, CHIP-V5, and actin protein levels in RCC4 cells that were transduced with lentivirus encoding CHIP or EV and exposed to 20% or 1% O_2 for 8 h. Representative blots from three experiments are shown.

motes ubiquitination and degradation of HIF-1α through a 26 S proteasome-dependent pathway.

VHL binds to HIF-1α that is hydroxylated at Pro-402/Pro-564 and recruits the Elongin-C-Elongin-B-Cullin-2-E3-ubiquitin ligase complex, which promotes proteasomal degradation of HIF-1α (9–11). To test whether prolyl hydroxylation is required for Hsp70-induced HIF-1α degradation, HEK293T cells were co-transfected with vector encoding Hsp70-V5 and wild-type FLAG-HIF-1α or double mutant FLAG-HIF-1α (P402A/P564A), which is resistant to PHD2-mediated hydroxylation and subsequent VHL-dependent ubiquitination. Hsp70-V5 efficiently decreased protein levels of double mutant FLAG-HIF-1α by 72% in cells exposed to 20% or 1% O_2, similar to its effect on wild-type FLAG-HIF-1α (Fig. 4*A* and supplemental Fig. S2*D*). Hsp70-mediated HIF-1α degradation was also studied in VHL-null RCC4 cells transduced with lentiviral Hsp70-V5. Hsp70-V5 reduced HIF-1α protein levels in both nonhypoxic and hypoxic RCC4 cells (Fig. 4*B*). Similarly, Elongin-C knockdown did not inhibit HIF-1α degradation mediated by Hsp70-V5 in nonhypoxic and hypoxic HEK293T cells (Fig.

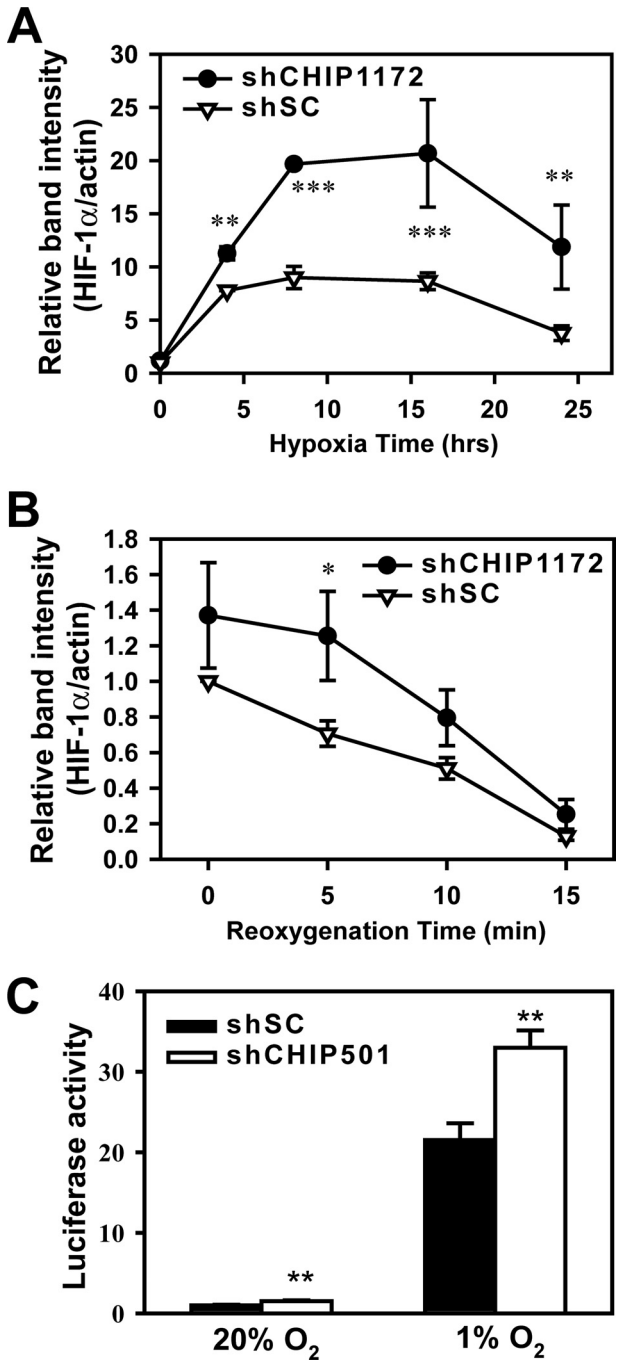


FIGURE 9. **CHIP knockdown increases HIF-1α protein levels and HIF-1-dependent gene transcription during prolonged hypoxia.** *A,* HEK293T cells were transfected with expression vector encoding shCHIP1172 or shSC and exposed to 20% or 1% O_2 for the indicated time. HIF-1α and actin levels were determined by immunoblot assays and quantified by densitometry. The ratio of HIF-1α/actin was normalized to the nonhypoxic shSC condition (mean ± S.E., $n = 3$). **, $p < 0.01$; ***, $p < 0.001$, relative to shSC. *B,* HEK293T cells were transfected with expression vector encoding shCHIP1172 or shSC and exposed to 20% or 1% O_2 for 24 h, followed by exposure to 20% O_2 for the indicated time. HIF-1α and actin levels were determined by immunoblot assays. The ratio of HIF-1α/actin was normalized to the hypoxic shSC condition (mean ± S.E., $n = 4$). *, $p < 0.05$, compared with shSC. *C,* HEK293 cells were co-transfected with HIF-1-dependent firefly luciferase reporter p2.1, control reporter pSV-Renilla, and expression vector encoding shSC or shCHIP501 and exposed to 20% or 1% O_2 for 24 h. The ratio of firefly/*Renilla* luciferase activity was determined and normalized to the nonhypoxic shSC condition (mean ± S.E., $n = 4$). **, $p < 0.01$, compared with shSC.

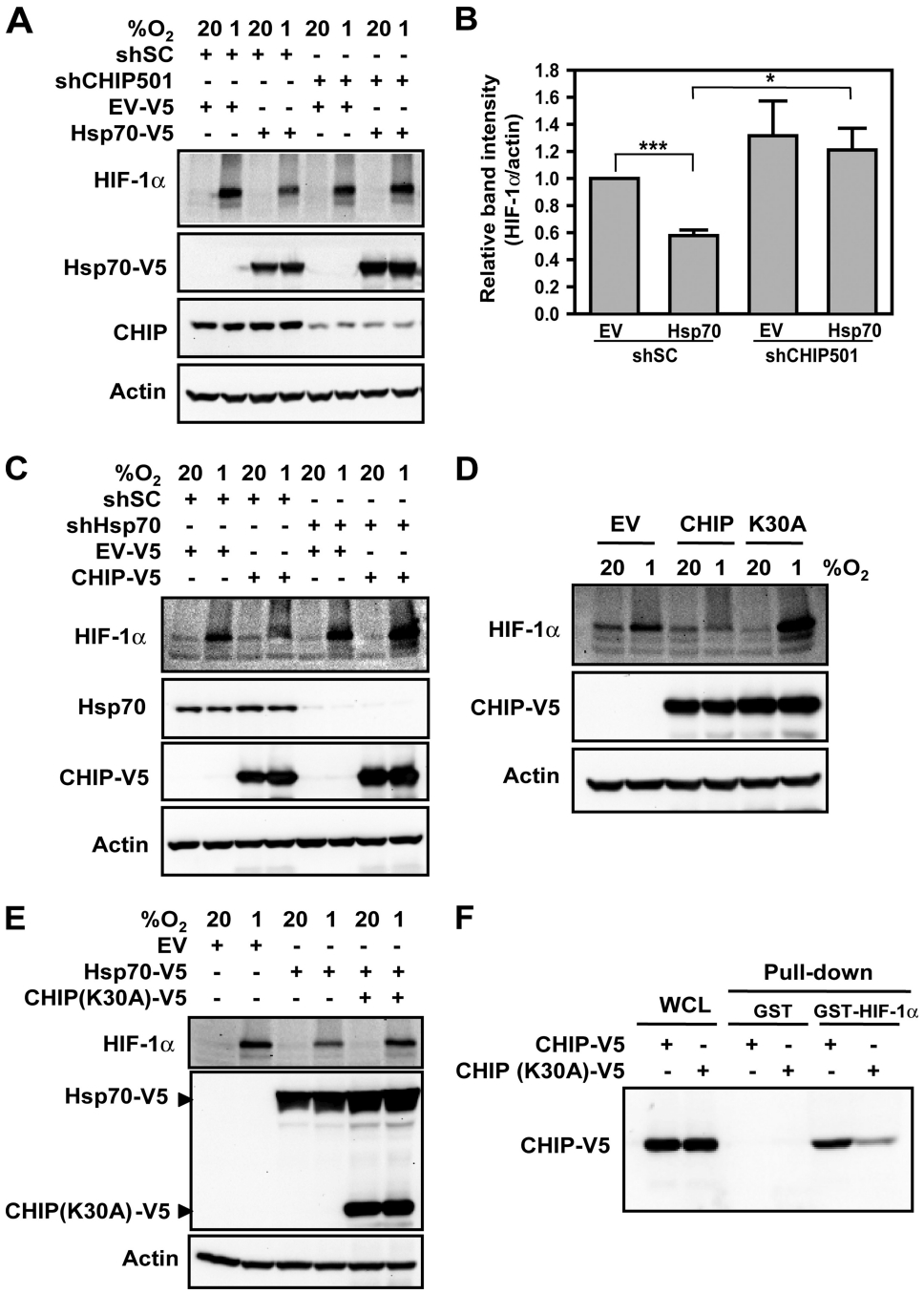


FIGURE 10. **Hsp70 recruits CHIP and regulates HIF-1α degradation.** *A* and *B,* immunoblot assays were performed to determine HIF-1α, Hsp70-V5, CHIP, and actin protein levels in transfected HEK293T cells exposed to 20% or 1% O_2 for 8 h (*A*). Band intensities of HIF-1α and actin were quantified by densitometry (*B*). The ratio of HIF-1α/actin was normalized to hypoxic EV group (mean ± S.E., $n = 3$). *, $p < 0.05$; ***, $p < 0.001$. *C,* immunoblot assays were performed to determine HIF-1α, Hsp70, CHIP-V5, and actin protein levels in transfected HEK293T cells exposed to 20% or 1% O_2 for 8 h. *D,* immunoblot assays were performed to determine HIF-1α, CHIP-V5, or CHIP(K30A)-V5, and actin protein levels in transfected HEK293T cells exposed to 20% or 1% O_2 for 8 h. *E,* immunoblot assays were performed to determine HIF-1α, Hsp70-V5, CHIP(K30A)-V5, and actin protein levels in transfected HEK293T cells exposed to 20% or 1% O_2 for 8 h. *F,* GST pulldown assays were performed with purified GST or GST-HIF-1α and WCLs from HEK293T cells expressing CHIP-V5 or CHIP(K30A)-V5.

4*C*). These data indicate that the PHD2/VHL/Elongin-C system is not required for Hsp70-induced degradation of HIF-1α.

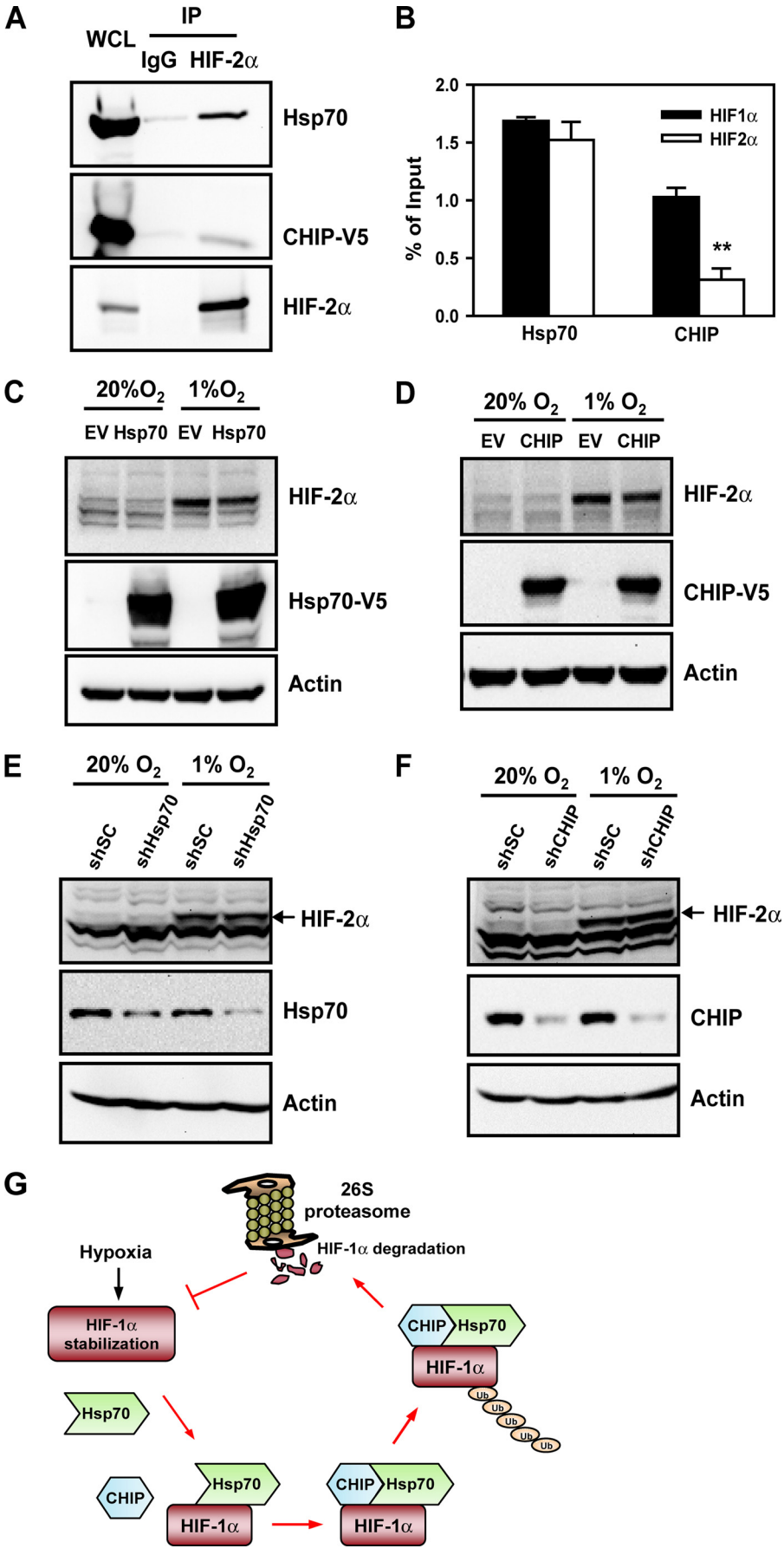
To determine whether endogenous Hsp70 physiologically regulates HIF-1α protein turnover, we next performed loss-of-function studies using shHsp70. HIF-1α protein levels were increased after 4 h of hypoxia and then progressively decreased in shSC-transfected HEK293T cells (Fig. 5*A*). However, Hsp70 knockdown led to a delay in, and reduced magnitude of, the down-regulation of HIF-1α protein levels during prolonged hypoxia (Fig. 5*A*). Reoxygenation time course experiments showed that Hsp70 knockdown did not affect the rate of post-hypoxic decay of HIF-1α protein in HEK293T cells, as compared with that in shSC-transfected cells (Fig. 5*B*). The increased HIF-1α levels at time points up to 12 min were due to the higher starting levels of HIF-1α in shHsp70 cells. The p2.1 HRE reporter assay revealed that Hsp70 knockdown by shHsp70 significantly increased the transcriptional activity of HIF-1 in HEK293 cells that were subjected to hypoxia for 24 h (Fig. 5*C*). Taken together, the loss-of-function data indicate that Hsp70 physiologically regulates HIF-1α protein stability and transcriptional activity in hypoxic human cells but does not affect post-hypoxic decay of HIF-1α.

Disruption of Hsp70-HIF-1α Interaction Prevents HIF-1α Degradation—Binding domain mapping studies (Fig. 2*D*) showed that HIF-1α associated with residues 442–641 of Hsp70. Thus, we tested whether Hsp70-(442–641) could competitively interfere with the interaction between HIF-1α and Hsp70. To this end, GST-Hsp70-(442–641) was expressed and purified from bacteria, and the GST moiety was removed by PreScission protease cleavage. Purified Hsp70-(442–641) completely inhibited the interaction of GST-HIF-1α-(331–427) with Hsp70-V5 present in transfected HEK293 cell lysates (Fig. 6*A*), thus providing a means to study the biological significance of Hsp70-HIF-1α interaction. Remarkably, expression of Hsp70-(442–641)-V5 greatly increased FLAG-HIF-1α protein levels in co-transfected HEK293T cells at both 20% and 1% O_2, exerting a completely opposite effect from full-length Hsp70, which induced degradation of FLAG-HIF-1α (Fig. 6*B*). Thus, disruption of

Hsp70-HIF-1α interaction abolishes Hsp70-induced degradation of HIF-1α, suggesting that their interaction is required for the physiological regulation of HIF-1α by Hsp70.

CHIP Promotes the Proteasomal Degradation of HIF-1α—To test the effect of CHIP on HIF-1α protein stability, HEK293T cells were co-transfected with FLAG-tagged HIF-1α and V5-tagged CHIP vectors and exposed to 20% or 1% O_2. CHIP-V5 significantly decreased protein levels of FLAG-HIF-1α by 86% at 20% O_2 and by 76% at 1% O_2, respectively, but did not alter levels of HIF-1β or actin (Fig. 7*A* and supplemental Fig. S3*A*). CHIP-V5 also markedly reduced endogenous HIF-1α protein levels in hypoxic HEK293T cells (Fig. 7*B* and supplemental Fig. S3*B*).

CHIP is a U-box-dependent E3 ubiquitin ligase (31). To determine whether the ubiquitin ligase activity is required for CHIP to promote HIF-1α degradation, we used the dominant-negative CHIP(H260Q), a U-box mutant that does not bind to the E2 ubiquitin-conjugating enzyme and lacks E3 activity (33). Expression of CHIP(H260Q)-V5 led to increased HIF-1α protein levels in hypoxic HEK293T cells (Fig. 7*C*). Similar results were also observed when U-box domain-deleted CHIP-V5 was expressed in HEK293T cells (data not shown). Thus, the ubiquitin ligase activity of CHIP regulates HIF-1α degradation. MG132 treatment also blocked CHIP-induced HIF-1α degradation in hypoxic HEK293T cells (Fig. 7*D* and supplemental Fig. S3*C*). We further investigated HIF-1α ubiquitination by CHIP loss-of-function using two shRNAs targeting different CHIP mRNA sequences. In HEK293T cells, shCHIP1172 and shCHIP501 both efficiently knocked down levels of CHIP mRNA and protein, compared with the negative control shSC and the untransfected control (supplemental Fig. S3*D*). Next, HEK293T cells were co-transfected with vectors encoding His ubiquitin, FLAG-HIF-1α, shSC, shCHIP1172, or shCHIP501 and then treated with



MG132. Ubiquitinated proteins were precipitated by Ni-NTA beads from whole cell lysates. Analysis of Ni-NTA precipitates with anti-FLAG antibody revealed that FLAG-HIF-1α was highly ubiquitinated in nonhypoxic HEK293T cells transfected with shSC. However, CHIP knockdown markedly reduced FLAG-HIF-1α ubiquitination, similar to the effect of Hsp70 knockdown (Fig. 7*E*). Taken together, these results indicate that CHIP promotes degradation of HIF-1α by a ubiquitin- and proteasome-dependent mechanism.

To rule out an effect of the PHD/VHL pathway on CHIP-mediated HIF-1α degradation, HEK293T cells were co-transfected with vector encoding double mutant FLAG-HIF-1α (P402A/P564A) and EV or CHIP-V5 vector. CHIP-V5 expression led to reduced levels of double mutant FLAG-HIF-1α at both 20% and 1% O_2, similar to its effect on wild-type FLAG-HIF-1α (Fig. 8*A*). Endogenous HIF-1α levels were also decreased in nonhypoxic and hypoxic RCC4 cells transduced with lentiviral CHIP-V5 (Fig. 8*B*). Thus, CHIP-mediated HIF-1α degradation is independent of the PHD/VHL pathway.

To complement the CHIP gain-of-function data, we performed loss-of-function analysis. Relative to shSC, transfection of shCHIP1172 increased levels of HIF-1α and impaired HIF-1α protein decay during prolonged hypoxia (Fig. 9*A*). Although HIF-1α protein levels were increased in CHIP knockdown cells during the first 10 min of reoxygenation following 24 h of hypoxia, loss of CHIP did not attenuate the rate of HIF-1α degradation (Fig. 9*B*). Similar results were also observed in HEK293T cells transfected with shCHIP501 (supplemental Fig. S3, *E–H*), which indicates that the observed effects were specifically due to CHIP loss of function. The p2.1 HRE reporter assay demonstrated that CHIP knockdown by shCHIP501 increased HIF-1 transcriptional activity in HEK293 cells at 20% and 1% O_2 (Fig. 9*C*). Therefore, as in the case for Hsp70 (Fig. 5), CHIP knockdown increases HIF-1α protein levels and HIF-1 transcriptional activity.

Hsp70 Recruits CHIP and Cooperates with CHIP to Regulate HIF-1α Degradation—The experiments described thus far demonstrate that both Hsp70 and CHIP mediate HIF-1α degradation. CHIP associates with Hsp70 and functions as a chaperone-dependent E3 ubiquitin ligase (30, 31). Therefore, we hypothesized that CHIP mediates Hsp70-induced proteasomal degradation of HIF-1α. To test this hypothesis, we investigated the effect of Hsp70 on HIF-1α stability in CHIP-deficient cells. Hsp70 significantly inhibited HIF-1α protein induction by hypoxia in HEK293T cells transfected with shSC (Fig. 10, *A* and *B*), consistent with data presented above. However, CHIP knockdown by shCHIP501 prevented Hsp70-induced HIF-1α degradation in hypoxic HEK293T cells (Fig. 10, *A* and *B*). Similar results were obtained using shCHIP1172 (data not shown). These results indicate that CHIP is required for Hsp70-induced degradation of HIF-1α.

To test whether CHIP is sufficient to mediate HIF-1α degradation in the absence of Hsp70, HEK293T cells were co-transfected with vector encoding shHsp70 and CHIP-V5 and exposed to 20% or 1% O_2. CHIP-V5 was able to promote degradation of HIF-1α in HEK293T cells transfected with shSC at 1% O_2 (Fig. 10*C*, compare *2nd* with *4th lane*). However, CHIP-induced HIF-1α degradation was not observed in the presence of shHsp70 (Fig. 10*C*, compare *6th* with *8th lane*), indicating that the effect of CHIP on HIF-1α protein stability depends on Hsp70 activity. To further support this conclusion, we tested CHIP(K30A), which fails to dock with Hsp70, but still possesses E3 ligase activity (33). CHIP(K30A)-V5 expression did not reduce HIF-1α protein levels in hypoxic HEK293T cells, in contrast to wild-type CHIP-V5 (Fig. 10*D*). Moreover, CHIP(K30A)-V5 also blocked degradation of HIF-1α mediated by Hsp70-V5 (Fig. 10*E*). These data indicate that Hsp70 and CHIP cooperatively regulate HIF-1α protein stability. Disruption of Hsp70-CHIP interaction prevents degradation of HIF-1α mediated by Hsp70 and CHIP.

The finding that Hsp70 is required for CHIP-mediated HIF-1α degradation suggested that CHIP-HIF-1α interaction may depend on Hsp70. As shown in Fig. 10*F*, the interaction between CHIP-V5 and GST-HIF-1α-(1–826) was dramatically reduced by the K30A mutation, which disrupts the binding of CHIP to Hsp70 (33). These data demonstrate that stable CHIP-HIF-1α interaction in human cells requires CHIP-Hsp70 interaction, indicating that Hsp70 recruits CHIP to HIF-1α.

Hsp70 and CHIP Fail to Regulate HIF-2α Stability—To investigate whether Hsp70 and CHIP have a similar effect on HIF-2α stability, we first performed a co-immunoprecipitation assay. HEK293T cells were co-transfected with HIF-2α and CHIP-V5 expression vectors and treated with MG132. Hsp70 co-immunoprecipitated with HIF-2α (Fig. 11*A*), and its binding to HIF-2α was comparable with its binding to HIF-1α (Fig. 11*B*). However, co-immunoprecipitation of CHIP-V5 with HIF-2α was markedly reduced (Fig. 11, *A* and *B*) compared with its interaction with HIF-1α (Fig. 2*A*). Western blot analysis demonstrated that expression of Hsp70-V5 or CHIP-V5 did not reduce HIF-2α protein levels in hypoxic HEK293T cells (Fig. 11, *C* and *D*). Loss-of-function studies revealed that knockdown of either Hsp70 (Fig. 11*E*) or CHIP (Fig. 11*F*) also failed to affect HIF-2α protein levels in nonhypoxic and hypoxic HEK293T cells. These results indicate that Hsp70 and CHIP selectively regulate the protein stability of HIF-1α but not HIF-2α.

FIGURE 11. **Hsp70 and CHIP fail to regulate HIF-2α protein stability.** *A*, co-immunoprecipitation (*IP*) of HIF-2α and CHIP-V5 or Hsp70 from transfected HEK293T cells treated with 10 μM MG132 for 8 h was performed. *B*, binding of Hsp70 and CHIP to HIF-2α (as shown in *A*) or to HIF-1α (as shown in Fig. 2*A*) was quantified by densitometric analysis of the immunoblots. The band intensity for the precipitated protein was normalized to the band density for the total protein in WCL used for immunoprecipitation (mean ± S.E., $n = 3$). **, $p < 0.01$, compared with HIF-1α. *C*, immunoblot assays were performed to determine HIF-2α, Hsp70-V5, and actin protein levels in transfected HEK293T cells exposed to 20% or 1% O_2 for 4 h. *D*, immunoblot assays were performed to determine HIF-2α, CHIP-V5, and actin protein levels in transfected HEK293T cells exposed to 20% or 1% O_2 for 8 h. *E*, immunoblot assays were performed to determine HIF-2α, Hsp70, and actin protein levels in transfected HEK293T cells exposed to 20% or 1% O_2 for 16 h. *F*, immunoblot assays were performed to determine HIF-2α, CHIP, and actin protein levels in transfected HEK293T cells exposed to 20% or 1% O_2 for 16 h. Representative blots from three experiments are shown. *G*, mechanism by which Hsp70 induces the ubiquitination and degradation of HIF-1α through recruitment of CHIP is shown.

DISCUSSION

In this study, we demonstrate that Hsp70 protein directly interacts with HIF-1α and mediates selective HIF-1α degradation by recruiting the ubiquitin ligase CHIP (Fig. 11*G*). Hsp70 knockdown impaired the decay of HIF-1α protein levels during prolonged hypoxia, but not during reoxygenation, when HIF-1α is rapidly degraded through the PHD/VHL/Elongin pathway. Therefore, Hsp70 plays a predominant role in HIF-1α degradation specifically under conditions of prolonged hypoxia. PHD2 expression is also induced by hypoxia, and an earlier study concluded it does not promote HIF-1α degradation until cells are reoxygenated (34), whereas a recent study implicated PHDs in HIF-1α degradation during prolonged hypoxia (35). However, this mechanism cannot explain the differential stability of HIF-1α and HIF-2α, as both proteins are negatively regulated by PHD2.

CHIP, a chaperone-dependent E3 ubiquitin ligase (31), mediates Hsp70-induced HIF-1α ubiquitination. To date, two different E3 ubiquitin ligases, Elongin-C/Elongin-B/Cullin-2 and hypoxia-associated factor, have been shown to regulate HIF-1α protein stability (12, 14, 16). In this study, we found that CHIP was not sufficient to mediate HIF-1α degradation in the absence of Hsp70. CHIP recruitment by Hsp70 was a prerequisite for HIF-1α degradation, because mutant CHIP(K30A) failed to bind to GST-HIF-1α and to promote HIF-1α degradation, suggesting that the interaction between CHIP and intact HIF-1α in human cells is only stable in the presence of CHIP-Hsp70 association. *In vitro* binding of CHIP to GST-HIF-1α-(81–329), which has no Hsp70-binding sites, suggests that the CHIP-binding site is masked in full-length HIF-1α, because CHIP(K30A), which cannot interact with Hsp70, did not bind efficiently to GST-HIF-1α-(1–826).

We also found that Hsp70 and CHIP did not regulate HIF-2α protein stability. Although Hsp70 had a comparable binding capacity to HIF-1α and HIF-2α, CHIP-HIF-2α interaction was very weak, suggesting that CHIP cannot be recruited to HIF-2α by Hsp70. This result provides a molecular mechanism underlying the preferential induction of HIF-2α under conditions of prolonged hypoxia (20, 22). HIF-1α and HIF-2α both exert adaptive responses to hypoxia by activating transcription of overlapping but distinct batteries of target genes, thereby playing distinct roles in many developmental, physiological, and pathological processes (5, 8, 24, 25, 36). Differences in the amino-terminal transactivation domains of HIF-1α and HIF-2α are responsible for target gene specificity (25). This study indicates that differences in the expression of Hsp70 or CHIP in different cell types may alter the relative ratio of HIF-1α and HIF-2α that accumulates in response to chronic hypoxia and thereby influence target gene selection. The finding that Hsp70 mRNA levels were increased under hypoxic conditions suggests the existence of a negative feedback loop that is specifically designed to decrease HIF-1α levels under conditions of chronic hypoxia.

RACK1 is responsible for HIF-1α degradation induced by the Hsp90 inhibitor 17-AAG. RACK1 competes with Hsp90 for binding to HIF-1α when Hsp90 binding is inhibited by 17-AAG, and then it recruits the Elongin-C-ubiquitin ligase complex, leading to HIF-1α degradation (14). We found that loss of function of Hsp70 or CHIP did not block 17-AAG-induced HIF-1α degradation (data not shown). Therefore, Hsp70/CHIP and RACK1/Elongin-C independently regulate HIF-1α degradation pathways.

Hsp70 was previously implicated in the degradation of HIF-1α in cells exposed to trichostatin A, a histone deacetylase inhibitor that is currently in clinical trials as an anti-cancer agent. Trichostatin A-induced HIF-1α degradation was reported to occur independently of ubiquitination, but an alternative mechanism for recruitment of HIF-1α to the proteasome was not identified (37). Further studies are indicated to determine whether CHIP is required for proteasomal degradation of HIF-1α in response to trichostatin A treatment.

In conclusion, our data demonstrate that Hsp70 interacts with HIF-1α and promotes its degradation during prolonged hypoxia through recruitment of the E3 ubiquitin ligase CHIP (Fig. 11*G*). These findings reveal a molecular mechanism by which prolonged hypoxia leads to the selective decay of HIF-1α but not HIF-2α protein levels.

Acknowledgments—We thank Karen Padgett (Novus Biologicals, Littleton, CO) for providing antibodies against HIF-1β, HIF-2α, and CHIP. We are also grateful to Linzhao Cheng (The Johns Hopkins University) for providing lentiviral vectors.

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