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c-Jun Protects Hypoxia-Inducible Factor-1α from Degradation via Its Oxygen-Dependent Degradation Domain in a Nontranscriptional Manner

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Abstract

Although hypoxia-inducible factor-1α (HIF-1α) has long been intensively investigated as a drug target by interfering with its expression or transcriptional function, the regulatory mechanisms of HIF-1α remain to be further clarified. We report here that c-Jun associates with HIF-1α via its oxygen-dependent degradation domain, masks the sites for ubiquitination, and thus protects HIF-1α from proteasome-executing degradation. All of these together resulted in the stabilization and accumulation of HIF-1α, consequently promoting the transcription of its target gene and driving angiogenesis-related events. The stabilization of HIF-1α was dependent on the domains of c-Jun for DNA binding and heterodimerization but independent of the $Ser^{63/73}$ phosphorylation that is critical for transcriptional function. These findings highlight a previously unrecognized nontranscriptional function of c-Jun on the one hand and a distinct regulatory mechanism of HIF-1α activity on the other, consequently offering profound mechanistic insights into multiple events simultaneously involving both c-Jun and HIF-1α in tumor progression. [Cancer Res 2009;69(19):OF1–9]

Introduction

Hypoxia-inducible factor-1 (HIF-1) is a transcription factor that drives neoangiogenesis in response to hypoxia in the progression of solid tumors (1, 2). It is a heterodimer composed of an α subunit (HIF-1α) and a constitutively expressed β subunit (HIF-1β; ref. 3). The transcription activity of HIF-1 is dominantly determined by HIF-1α in response to microenvironmental oxygenation (4). More than 70 bona fide HIF-1α–regulated genes have been identified that are widely involved in the malignant features of tumors, including angiogenesis (5), invasion, metastasis (6), and drug resistance (7). HIF-1α is overexpressed in the majority of malignant solid tumors and is highly associated with low drug responsiveness and poor clinical prognosis (8).

HIF-1α is regulated by various pathways at different levels (9). Except for its own expression controlled by both the phosphatidylinositol 3-kinase/AKT (10, 11) and the mitogen-activated protein kinase pathways (12), a ubiquitination-mediated proteasome-executing degradation pathway is the most important regulator of the cellular HIF-1α level (13). The central event is that an oxygen-sensitive prolyl hydroxylase triggers the hydroxylation of HIF-1α at its Pro^{402} and Pro^{564} residues (14, 15), facilitating its pVHL-mediated ubiquitination at Lys^{532} (16). Then, the 26S proteasome takes up the ubiquitinated HIF-1α for degradation (17, 18). In addition to pVHL, several other factors have been reported to affect HIF-1α ubiquitination and stability, such as p53, HSP90, RACK1, and Jab1 (19–22).

c-Jun is a well-known transcription factor that functions as a homodimer with itself or a heterodimer with other activator protein-1 (AP-1) family members. It is involved in many cellular processes, including the proliferation, transformation, differentiation, apoptosis, and stress-adaptive responses. c-Jun reportedly cooperates with HIF-1α to activate the transcription of vascular endothelial growth factor (VEGF) and NDRG-1/Cap43 in a c-Jun NH_2-terminal kinase (JNK)–phosphorylated fashion (23, 24) and to associate with HIF-1α to promote the transcription of the multidrug resistance *mdr1* gene under hypoxia (25). On the other hand, c-Jun is also reported to be activated by HIF-1 under hypoxia (26, 27). Despite recent rapid advances in understanding the molecular contribution of c-Jun to the HIF-1α pathway in response to hypoxia, important questions remain to be answered, for example, the possible links between HIF-1α activation and the c-Jun pathways, as well as the distinct mechanisms by which c-Jun contributes to HIF-1α–mediated tumor responses.

In this report, we show for the first time that c-Jun associates with HIF-1α via its oxygen-dependent degradation (ODD) domain, thus potentiating its transcriptional activity. This effect is mediated by stabilization and hence protection of HIF-1α from proteasome-executed degradation, which, distinct from its well-recognized transcriptional function, depends on the nontranscriptional function of c-Jun.

Materials and Methods

Cells and reagents. Human breast cancer cells MDA-MB-468 and MDA-MB-435, human cervix cancer cells HeLa, and human microvascular endothelial cells HMEC-1 were obtained from the American Type Culture Collection. The MDA-MB-468, MDA-MB-435, and HeLa cells were cultured in RPMI 1640, whereas HMEC-1 cells were cultured in MCDB131 supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere containing 5% CO_2 (referred to as the normoxic conditions). Hypoxia treatment was performed by placing cells in a CO_2 Water Jacketed Incubator (model 3110 series; Thermo Forma) flushed with a mixture of 1% O_2, 5% CO_2, and 94% N_2.

Transfection reagents Oligofectamine and Lipofectamine were from Invitrogen; the JNK inhibitor SP600125 was from Tocris; and the 26S proteasome inhibitor MG132 and epoxomicin, the calpain inhibitor ALLN, and the protein synthesis inhibitor cycloheximide were from Sigma. Antibodies against HIF-1α, ubiquitin, β-actin, phosphorylated c-Jun, and c-Jun were from Becton Dickinson, Cell Signaling Technology, Sigma, and

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Santa Cruz Biotechnology, respectively. c-Jun small interfering RNA (siRNA)-1 was in the form of c-Jun SMARTpool from Dharmacon; c-Jun siRNA-2 targeting 5′-GAUGGAAACGACCUUCUAUTT-3′, c-Jun siRNA-3 targeting 5′-CCUCAGCAACUUCAACCCATT-3′, HIF-1α siRNA targeting 5′-CUGAUGACCAGCAACUUGATT-3′, and mock (scrambled) siRNA targeting 5′-UUCUCCGAACGUGUCACGUTT-3′ were from GenePharma. Rabbit IgG and mouse IgG were from Dingguo. Protein A/G agarose beads were from Santa Cruz Biotechnology. Fluorescent secondary antibodies Alexa Fluor were from Invitrogen. The VEGF-ELISA kit was from Jingmei. Matrigel was from Becton Dickinson. All the expression plasmids of the wild-type and mutated c-Jun were provided by Prof. Dirk Bohmann (University of Rochester, Rochester, NY). The constructs of hemagglutinin (HA)-HIF-1α (plasmid 18949; Addgene) and HA-HIF-1α (401Δ603) were kindly offered by Prof. William G. Kaelin (Harvard Medical School, Boston, MA) and Prof. H. Franklin Bunn (Harvard Medical School), respectively.

Cell transfection. The transfection with siRNA was conducted with Oligofectamine according to the manufacturer's instructions. Then, the cells were cultured in full medium for 48 h before being treated under hypoxic or normoxic conditions.

To reexpress the wild-type or mutated c-Jun (Figs. 1 and 3), we first knocked down c-Jun with c-Jun siRNA-2. Twenty-four hours after transfection with the siRNA, the cells were transcultured in Opti-MEM with no serum. Lipofectamine and 1 μg of c-Jun plasmids were used according to the manufacturer's instructions. Then, the cells were cultured in full medium for another 24-h period before being further treated.

Lipofectamine was also used to transfect the construction of HIF-1α and HIF-1α (401Δ603) according to the manufacturer's instructions 24 h before being further treated.

Western blotting analyses. Cells were lysed in 1× SDS lysis buffer [50 mmol/L Tris-HCl (pH 6.8), 100 mmol/L DTT, 2% SDS, 0.1% bromphenol blue, 10% glycerol] and then boiled for 5 to 10 min. Standard Western blotting analyses were performed to measure the levels of cellular c-Jun and HIF-1α.

Tube formation assays. Tube formation assays were conducted to examine the effect of c-Jun silencing on the *in vitro* angiogenesis of HMEC-1 cells (28). Briefly, a 96-well plate was coated with 55 μL of Matrigel, which was allowed to solidify at 37°C for 1 h. HMEC-1 cells (1.8×10^4 per well) were seeded into Matrigel-coated 96-well plates and cultured in MCDB131 medium with 10% FBS under normoxic or hypoxic conditions for the indicated times. The tube-like networks were photographed under a microscope (IX70, Olympus). The perimeters of all the tubes were measured for semiquantitative analyses.

Reverse transcription-PCR analyses. Cells were lysed with Trizol, and total RNA was isolated with chloroform and isopropyl alcohol. RNA (1 μg) was subjected to reverse transcription with the RT Ace kit (Toyobo) according to the manufacturer's instructions. The cDNA was amplified via 26-cycle PCR with Taq DNA polymerase and primers for VEGF (sense, 5′-TCGGGCCTCCGAAACCATG-3′; antisense, 5′-CCTGGAGAGA-GATCTGGTTC-3′), c-Jun (sense, 5′-AACGACCTTCTATGACGATGCCCTC-3′; antisense, 5′-GCGAACCCCTCCTGCTCATCTGTC-3′), HIF-1α (sense,

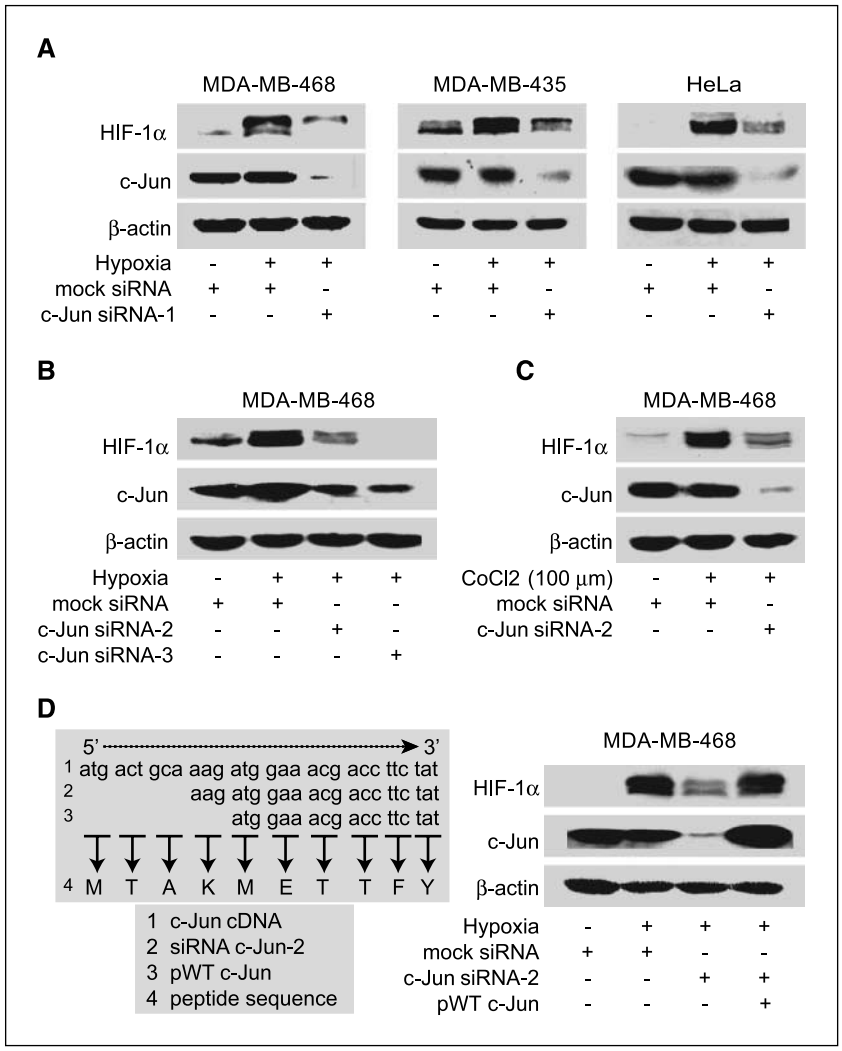

Figure 1. c-Jun knockdown leads to a decrease in HIF-1α levels under persistent hypoxia. *A,* levels of HIF-1α protein in c-Jun siRNA-1–transfected MDA-MB-468, MDA-MB-435, and HeLa cells decreased significantly under hypoxia for 6 h. Cells were transfected with c-Jun siRNA-1 or mock siRNA and cultured for 48 h before hypoxia treatment. *B,* treatments with both c-Jun siRNA-2 and c-Jun siRNA-3 led to HIF-1α decrease in MDA-MB-468 cells under hypoxia for 6 h. *C,* HIF-1α in c-Jun siRNA-2–transfected MDA-MB-468 cells also decreased in response to the treatment with $CoCl_2$ for 6 h. *D,* complementation with wild-type c-Jun rescued HIF-1α decrease under hypoxia for 6 h. MDA-MB-468 cells were transfected with the wild-type c-Jun plasmid 24 h after c-Jun siRNA treatment. All the experiments above were repeated at least thrice.

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5′-CCCCAGATTCAGGATCAGACA-3′; antisense, 5′-CCATCATGTTC-CATTTTTCGC-3′), and β-actin (sense, 5′-TCTACAATGAGCTGCGTGTG-3′; antisense, 5′-GGTGAGGATCTTCATGAGGT-3′). The PCR products were analyzed with 1% agarose gel and stained with ethidium bromide for visualization.

ELISA assays for VEGF secretion. To test the secreted VEGF, the medium was replaced with 1 mL/well of fresh medium, and the cells were subjected to hypoxia or normoxia for 12 h. Then, the cell supernatants were collected, clarified by centrifugation at 1,000 × *g* for 5 min, and stored at −20°C. The amount of VEGF in the supernatant was determined with a VEGF-ELISA kit according to the manufacturer's instructions.

HIF-1α half-life assays. The MDA-MB-468 cells at 48 h after transfection with c-Jun siRNA were preincubated under hypoxic conditions for 2 h. Then, the cells were exposed to 100 μmol/L cycloheximide under hypoxic conditions for the indicated time to block protein synthesis. The cells were collected for Western blotting analyses.

Immunoprecipitation. Cells treated under hypoxic conditions for 6 h were collected for immunoprecipitation. Briefly, cells were lysed with NP40 lysis buffer (Beyotime) for coimmunoprecipitation or with 0.1% SDS lysis buffer (Beyotime) for immunoprecipitation. Followed by centrifugation, the supernatant was precleared with 20 μL protein A/G agarose beads coupled with mouse or rabbit IgG for >2 h and then was exposed to 20 μL protein A/G agarose beads coupled with the indicated antibodies for >6 h. The beads were washed thrice with 1 mL NP40 lysis buffer or PBS for 20 min each. The precipitants were dissolved with the SDS loading buffer for Western blotting analyses.

Cell immunofluorescence. Cells (0.5×10^5) were seeded onto coverslips in 24-well plates and exposed to hypoxia for 6 h. Then, the cells were rinsed with PBS, fixed with 4% formaldehyde for 10 min, and permeabilized with 0.2% Triton X-100 in PBS for 10 min. Next, the cells were washed with PBS and 3% bovine serum albumin (blocking reagent) for 20 min. After that, the cells were sequentially incubated with diluted primary antibodies for 1 h, washed thrice with PBS, and incubated with fluorescent secondary antibodies for 30 min. Finally, the coverslips were washed with PBS for examination under Leica TCS SP2 confocal microscope.

Sequence-based protein partner searches. The probabilities of the direct interactions between c-Jun and HIF-1α or its deletion mutants were analyzed with the program sequence-based protein partner search (SPPS) supported by the Drug Discovery and Design Center, Shanghai Institute of Materia Medica (29). The system is open to the public.[1] Click "Query Two," choose "Human Being" for database, and submit the sequences of the protein partners (Fig. 4). The results were sent to the submitter by email.

Densitometry and statistical analyses. The densitometry analyses for Western blotting and reverse transcription-PCR (RT-PCR) were conducted using Adobe Photoshop CS2 version 9.0.2. The integrated absorbance value (defined as each pixel value − background value) was determined for the equal-sized boxes drawn around the bands of HIF-1α and β-actin, respectively, with the background values shown below each band of interest. The protein levels of HIF-1α were calculated as the integrated absorbance values of HIF-1α over those of β-actin and normalized as the values of each group over that of the mock group. The Student's *t* test was used to assess the difference of the secreted VEGF or the tube formation of HMEC-1 cells. $P < 0.05$ was defined as the level of statistical significance.

Results

c-Jun regulates cellular HIF-1α protein levels. We used a specific c-Jun siRNA pool to knock down c-Jun expression in human tumor cell lines, including MDA-MB-468, MDA-MB-435, and HeLa. When these c-Jun–silenced cells were exposed to 1% O_2 for 6 hours, the levels of cellular HIF-1α protein were noted to be far lower than those in the hypoxic control cells (Fig. 1*A*). Similar results were obtained by transfecting two additional siRNAs targeting distinct sequences of c-Jun into MDA-MB-468 cells (Fig. 1*B*). Moreover, the same scenario has been observed in a $CoCl_2$-induced hypoxia-mimic system (Fig. 1*C*).

To test for a causal link between the reduction of c-Jun expression and the decrease in cellular HIF-1α protein levels, we conducted c-Jun complementation experiments by transfecting a plasmid carrying a 5′-end truncated wild-type c-Jun sequence (Fig. 1*D*; ref. 30) into the c-Jun–silenced MDA-MB-468 cells. This plasmid expresses functional c-Jun protein (30), but its transcripts could not be disrupted by c-Jun siRNA-2 due to their 5′-terminal unpairing (Fig. 1*D*). c-Jun complementation effectively restored the responsiveness of HIF-1α to hypoxia (Fig. 1*D*), indicating that c-Jun plays a causal role in the control of cellular HIF-1α protein levels.

c-Jun protects HIF-1α from 26S proteasome–dependent degradation. To examine whether c-Jun affects the transcription of HIF-1α, we detected the levels of HIF-1α mRNA in the c-Jun–silenced MDA-MB-468 cells. However, c-Jun knockdown did not change the level of HIF-1α mRNA, indicating that c-Jun does not contribute to the transcription of the *HIF-1α* gene (Fig. 2*A*). Furthermore, c-Jun silencing failed to alter the levels of AKT (and) or p42/44, which reportedly regulate the transcription and translation of HIF-1α (Supplementary Fig. S1*A*; refs. 10–12).

Next, we characterized the alteration kinetics of the HIF-1α protein levels in MDA-MB-468 cells transfected with mock or c-Jun siRNA responding to hypoxia. In the control cells, the levels of HIF-1α protein went up rapidly on exposure to 1% O_2, peaked at the 4-hour time point, stayed there for ∼2 hours, and then went down slowly. In contrast, in the c-Jun–silenced cells, the levels of HIF-1α protein reached the maximal value at the 2-hour time point, then rapidly decreased, and went down to the basal level by 10 hours (Fig. 2*B*). Notably, the highest level of the HIF-1α protein was not only much lower but it was lowered much earlier and faster in the c-Jun–silenced cells than in the control cells. The data suggest that a reduction in c-Jun is likely to impair the stability of the HIF-1α protein.

To test this possibility, we determined the half-life of the HIF-1α protein by the introduction of cycloheximide, a known protein synthesis inhibitor. In c-Jun–silenced cells, the HIF-1α protein had a 50% shorter half-life (15.7 minutes) than that (31.0 minutes) of the control (Fig. 2*C*). To further examine whether the faster reduction of HIF-1α protein in c-Jun–silenced cells is associated with its proteasome-dependent degradation, we exposed the cells to the 26S proteasome inhibitors MG132 and epoxomicin and the calpain inhibitor ALLN as a control. Both proteasome inhibitors rescued the decline of HIF-1α levels induced by c-Jun silencing, whereas the calpain inhibitor ALLN failed (Fig. 2*D*). All these findings collectively indicate that c-Jun protected HIF-1α from proteasome-executed degradation.

c-Jun interacts with and stabilizes HIF-1α protein independent of its transcriptional function. We next addressed whether the effect of c-Jun on the stability of HIF-1α is due to their functional cooperation. We first conducted coimmunoprecipitation and then confocal microscopy analyses. c-Jun was found to be coimmunoprecipitated with HIF-1α protein in both of the tested cell lines MDA-MB-468 and HeLa cells (Fig. 3*A, top*). Consistently, c-Jun was also observed to be colocalized in the nuclei with the HIF-1α protein in both cell lines (Fig. 3*A, bottom*).

c-Jun contains several critical functional regions, including the $Ser^{63/73}$ sites for phosphorylation, which is closely related to its transcriptional activity (31), and also regions for DNA binding, Jun-Fos dimerization, and homodimerization (Fig. 3*B*). To detect which

[1] http://www.dddc.ac.cn/spps/index.php

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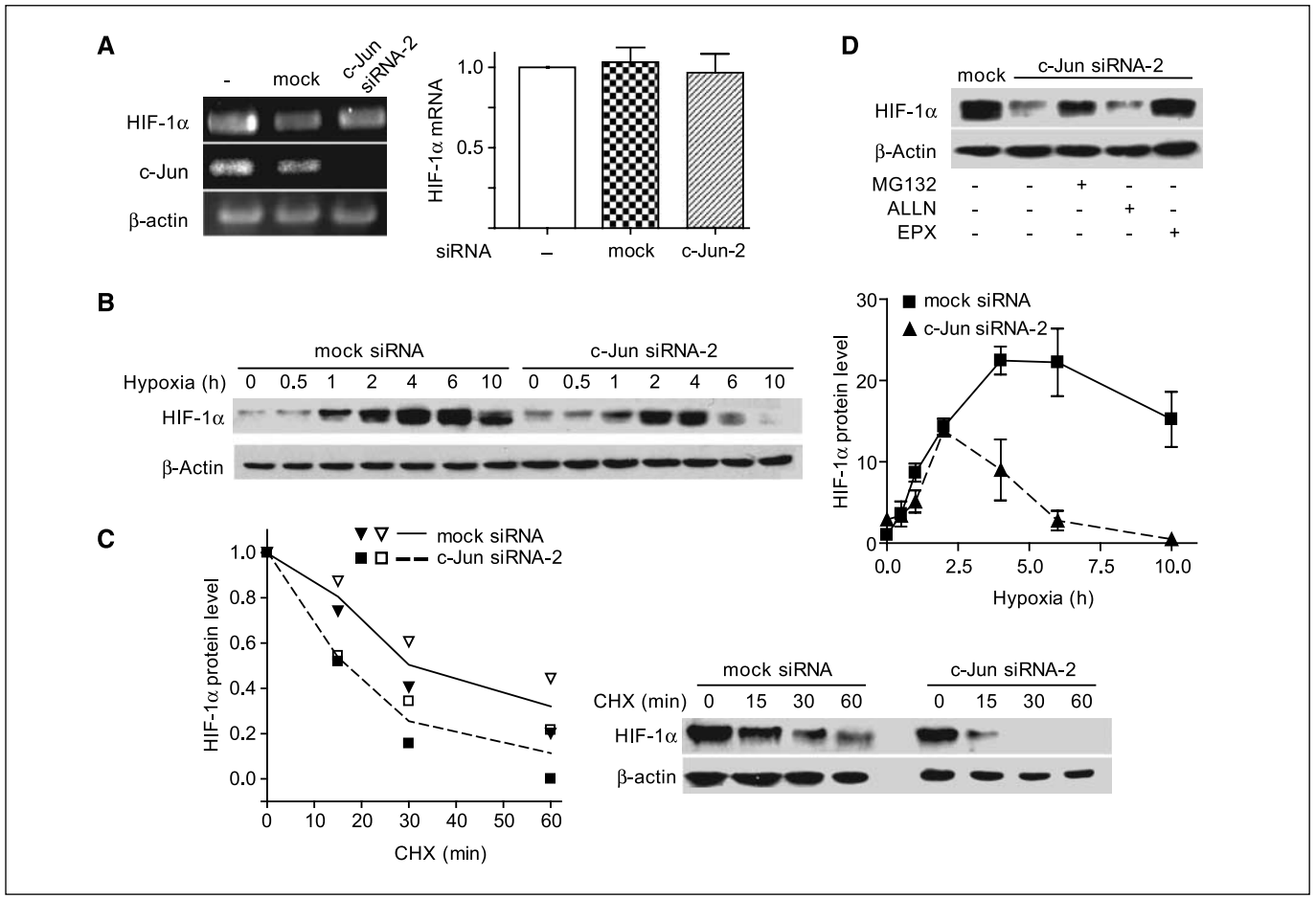

Figure 2. c-Jun prevents HIF-1α from ubiquitin-mediated degradation. *A,* RT-PCR analyses revealed that c-Jun silencing in MDA-MB-468 cells did not lower the levels of HIF-1α mRNA under hypoxia for 6 h. *B,* silencing c-Jun with c-Jun siRNA-2 in MDA-MB-468 cells affected the kinetics of the HIF-1α protein when the cells were exposed to hypoxic conditions. *C,* c-Jun silencing led to faster degradation of HIF-1α protein under hypoxia. Forty-eight hours after transfection with c-Jun siRNA-2 or mock siRNA, MDA-MB-468 cells were pretreated under hypoxia for 2 h followed by treatment with 100 μg/mL cycloheximide (*CHX*) to block protein synthesis for the indicated times. The values of two siRNA treatment groups are represented by the triangles and squares, and the corresponding values in two independent experiments are indicated by the solid and hollow symbols, respectively. The mean values from two experiments are connected by the lines. *D,* the 26S proteasome inhibitors MG132 (10 μmol/L) and epoxomicin (*EPX*; 1 μmol/L) rescued the down-regulation of HIF-1α in the c-Jun siRNA-2–transfected MDA-MB-468 cells after 6-h exposure to hypoxia, whereas the calpain protease inhibitor ALLN (10 μmol/L) did not. All the experiments above were conducted thrice, except *C*. Data are the mean ± SD.

region(s) contributes to the formation of the c-Jun–HIF-1α complexes, we transfected a series of plasmids carrying different c-Jun mutations (kindly gifted by Dr. Dirk Bohmann; Fig. 3*B*; refs. 30, 32, 33) into the c-Jun–silenced MDA-MB-468 cells. Unexpectedly, mutations at both the $Ser^{63/73}$ sites (the plasmid Mut111) and the sites for homodimerization (the plasmid Mut22-23) were noted to fail to combat the reversability of exogenous c-Jun against the c-Jun knockdown-induced decrement in HIF-1α (Fig. 3*C* and *D*). However, mutations at the sites for DNA binding (the plasmids Mut12 and Mut14) and for heterodimerization (the plasmid Mut17) caused a dramatic impairment (Fig. 3*D*). Moreover, the JNK inhibitor SP600125, which inhibits c-Jun phosphorylation, was also noted to not cause any change in the levels of the HIF-1α protein (Fig. 3*C*). The data collectively indicate that the regions of c-Jun for DNA binding and heterodimerization, but not those for phosphorylation or homodimerization, contribute to the protection on HIF-1α, which in turn highlights the nontranscriptional function of c-Jun.

c-Jun associates with the ODD domain of HIF-1α and blocks its ubiquitination. HIF-1α protein contains several functional domains, including bHLH, PAS-A, PAS-B, ODD, and TAD (Fig. 4*A*; refs. 20, 34). To find out which domain(s) mediates the formation between c-Jun and HIF-1α, we first used the SPPS program to analyze the probability of protein interaction based on their primary sequences (29). The interaction probability of c-Jun with HIF-1α in either the wild-type or any of the deletion mutations of bHLH, PAS-A, PAS-B, and TAD was calculated to be >99%. In contrast, the deletion of the ODD domain dramatically reduced the interaction probability of c-Jun with HIF-1α to 71% (Fig. 4*A*), strongly suggesting the indispensability of the ODD domain for the formation of c-Jun–HIF-1α complexes.

To validate the role of the ODD domain, the plasmids HA-HIF-1α (35) and HA-HIF-1α (401Δ603), which lack the ODD domain (36), were transfected into both MDA-MB-468 and HeLa cells. Coimmunoprecipitation assays showed that c-Jun formed complexes only with HIF-1α containing the ODD domain (Fig. 4*B*; Supplementary Fig. S2). The data substantiate that the ODD domain is responsible for the interaction of c-Jun with HIF-1α.

Given the ODD domain is well known for HIF-1α ubiquitination (36, 37), we thus addressed whether this is the case in this setting.

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We introduced MG132, a blocker of proteasome-executing HIF-1α degradation, into our experimental system. The results revealed that a much greater quantity of HIF-1α was modified with ubiquitin in the c-Jun–silenced cells than the control MDA-MB-468 and HeLa cells (Fig. 4*C*), indicating that the complexing with c-Jun blocks HIF-1α ubiquitination and thus any subsequent degradation.

c-Jun knockdown disrupts HIF-1α–mediated angiogenesis-related events. *VEGF* is one of the most important target genes of HIF-1α. HIF-1α promotes VEGF transcription and angiogenesis (38, 39). To detect whether c-Jun knockdown impairs HIF-1α biological function, we first examined the mRNA levels and the protein secretion of VEGF in the c-Jun–silenced MDA-MB-468 cells. Silencing c-Jun did not cause any detectable change in either the mRNA level or the protein secretion of VEGF under normoxic conditions (Fig. 5*A*); in contrast, however, there were dramatic drops in both the mRNA level and protein secretion of VEGF under hypoxic conditions (Fig. 5*A*), indicating that a reduction in the c-Jun level diminishes the transcriptional activity of HIF-1α.

To validate the role of c-Jun and HIF-1α in angiogenesis-related events, c-Jun and HIF-1α siRNAs were transfected into human microvascular endothelial cells (HMEC-1). Silencing both c-Jun and HIF-1α (Fig. 5*B*) reduced the tube formation of HMEC-1 cells when exposed to both normoxia and hypoxia for 12 hours (Fig. 5*B*; Supplementary Fig. S3*A*). Careful kinetic analyses further revealed the time dependence. Although both mock and c-Jun siRNA-transfected HMEC-1 cells formed similar numbers of net-like tubes when exposed to hypoxia for 4 hours, the net-like tubes broke down much faster in the c-Jun siRNA groups than in the mock siRNA groups as the exposure time was prolonged (Fig. 5*C*; Supplementary Fig. S3*B*). The expression of HIF-1α (401Δ603) partially rescued the degradation of tubes induced by the c-Jun knockdown (Fig. 5*D*; Supplementary Fig. S3*C*), supporting a functional link between c-Jun and HIF-1α in tube formation.

Discussion

SPPS and coimmunoprecipitation assays revealed c-Jun to be directly complexed with HIF-1α protein via its ODD domain in MDA-MB-468 cells. Similar results were replicated in another human cancer HeLa cell line. Of note, such cooperation between c-Jun and HIF-1α was further observed to lead to the stabilization and cellular accumulation of HIF-1α by protecting the HIF-1α protein from degradation.

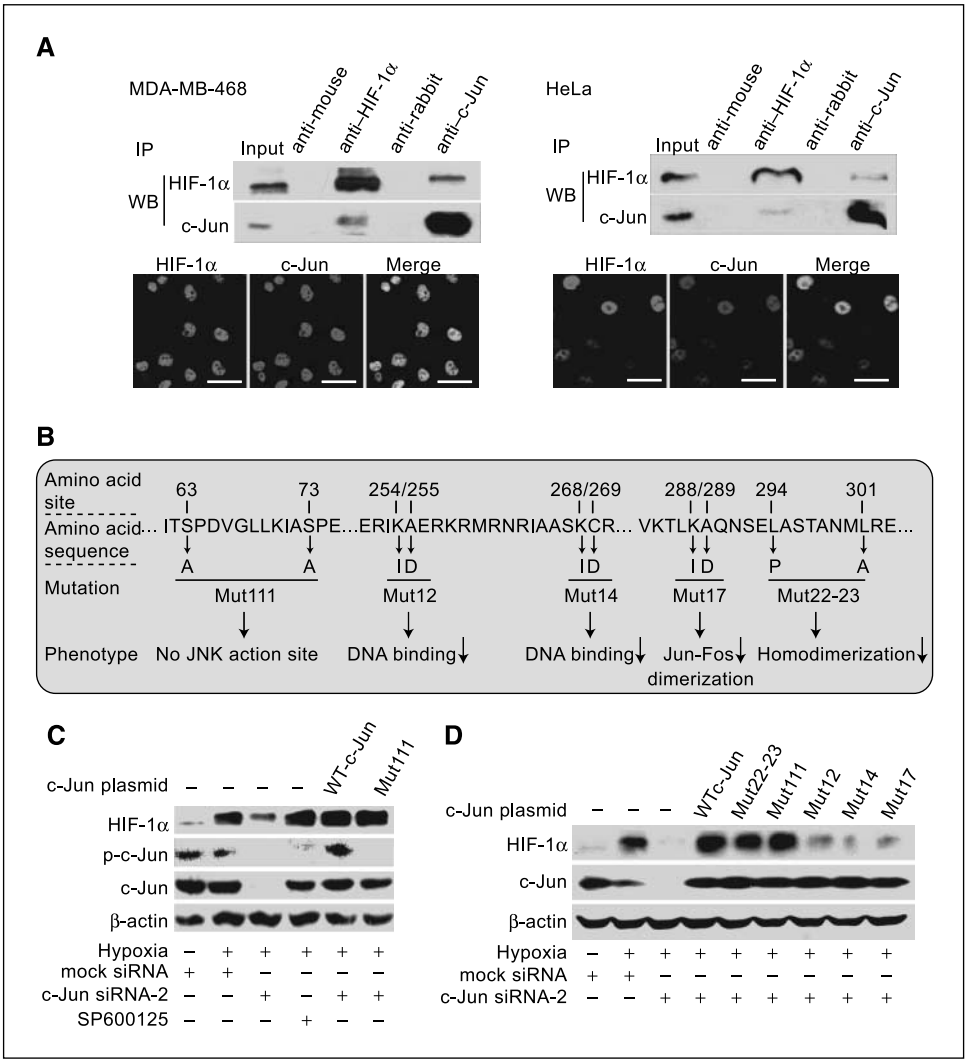

Figure 3. c-Jun stabilizes HIF-1α independent of its transcription factor activity. *A,* coimmunoprecipitation and immunofluorescence of c-Jun and HIF-1α in MDA-MB-468 and HeLa cells exposed to hypoxia for 6 h. *Scale bar,* 5 μm. *B,* effect of different point mutations on the function of c-Jun. *C,* inhibition of c-Jun phosphorylation by the JNK inhibitor SP600125 and reexpression of Mut111 did not lead to HIF-1α decrease in MDA-MB-468 cells under hypoxia. *D,* wild-type c-Jun and mutated Mut22-23 and Mut111 constructs rescued the HIF-1α decrease in MDA-MB-468 cells exposed to hypoxia for 6 h, whereas mutated Mut12, Mut14, and Mut17 constructs did not. The experiments above were performed thrice.

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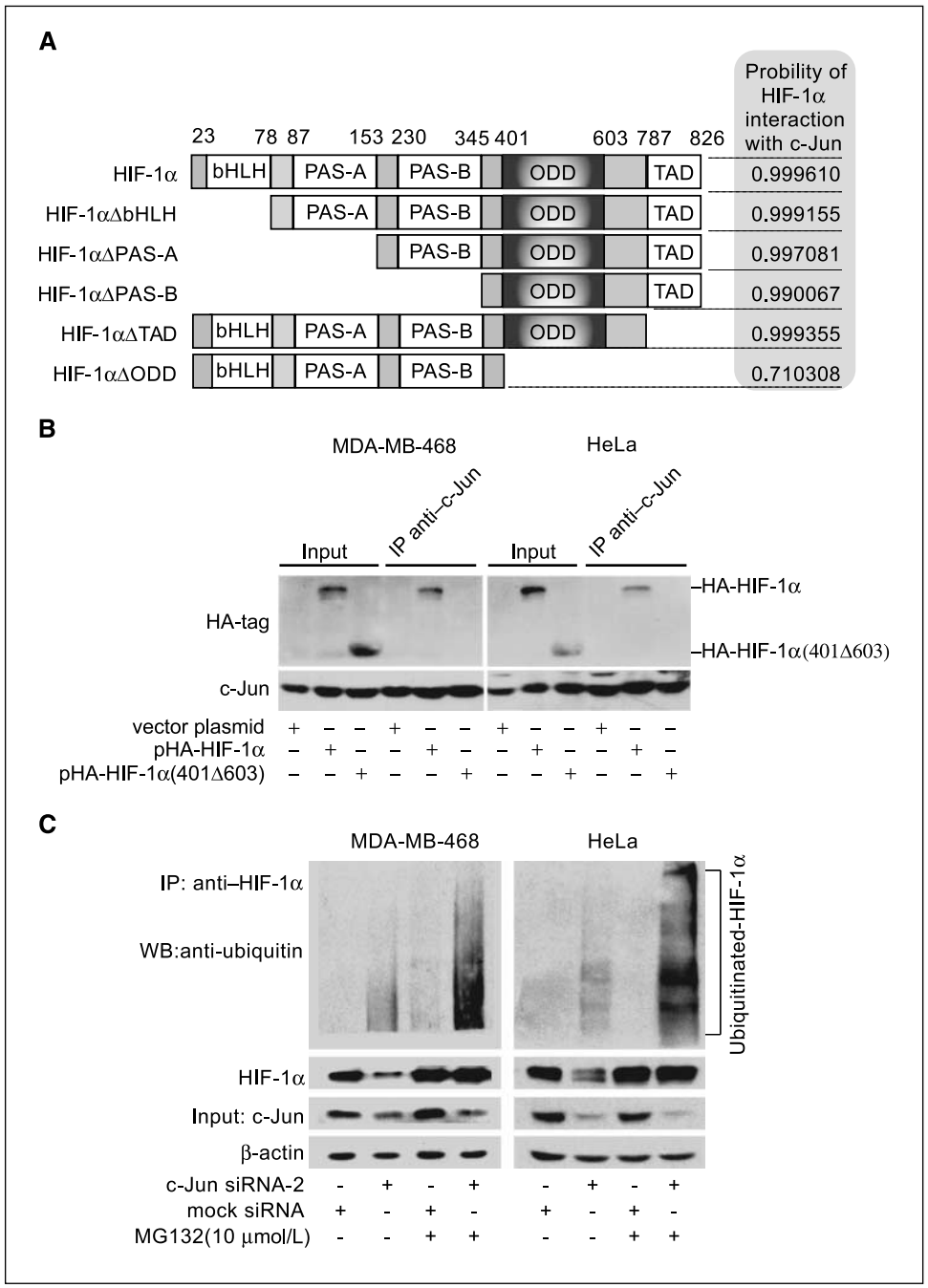

Figure 4. c-Jun binds to the ODD domain of HIF-1α protein and blocks its ubiquitin modification. *A,* analyses with the program SPPS revealed that the ODD domain of HIF-1α was indispensable for the interaction with c-Jun. *B,* coimmunoprecipitation of c-Jun with ectopically expressed HIF-1α or HIF-1α(401Δ603) in MDA-MB-468 and HeLa cells exposed to hypoxia for 6 h. *HA-tag,* Western blotting with the primary antibody against HA-tag; *pHA-HIF-1α,* plasmids carrying the wild-type *HIF-1α* gene with a HA-tag; *pHA-HIF-1α(401Δ603),* plasmids carrying mutated *HIF-1α* (401Δ603)gene with a HA-tag. *C,* immunoprecipitation with anti–HIF-1α IgG showed that HIF-1α was modified with ubiquitins in c-Jun–silenced MDA-MB-468 and HeLa cells exposed to hypoxia for 6 h. The proteasome inhibitor MG132 was used to block HIF-1α degradation. The experiments above were repeated at least thrice.

c-Jun is a well-established transcription factor known exclusively for its transcriptional activity (40), owing to its phosphorylation at $Ser^{63/73}$ (41). However, in the current study, we show that c-Jun stabilizes the HIF-1α protein and protects it from ubiquitination-mediated degradation, independent of its well-accepted transcriptional activity. This is substantiated by the fact that neither specific blockade of the JNK pathway nor mutation of c-Jun at Ser^{63} and Ser^{73} impairs its functional cooperation with HIF-1α. Rather, the mutations in the regions critical for DNA binding and heterodimerization disrupt the cellular accumulation of HIF-1α. The contribution of c-Jun to HIF-1α protein stability rather than mRNA transcription indicates c-Jun functions as a protector, instead of a transcriptional factor in this case. Given that c-Jun is able to regulate the stability of another transcriptional factor, it is necessary to address whether c-Jun–associated partners are also under such regulation. The expression of neither c-Jun–associated partners from the AP-1 family, such as c-Fos (42) and JunB (4), nor non–AP-1 transcription factors, such as SP1 (43) and signal transducer and activator of transcription 3 (44), was affected by c-Jun knockdown (Supplementary Fig. S1*B*), indicating that the nontranscriptional function of c-Jun is unique in stabilizing HIF-1α.

The ODD domain is well established as being critical for the stability of the cellular HIF-1α protein and is controlled by an oxygen-sensitive prolyl hydroxylase (45). Under normoxic conditions, the prolyl hydroxylase hydroxylates two prolyl residues at 402 and 564 in the ODD domain of HIF-1α. The hydroxylated

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HIF-1α then interacts with pVHL and thus enables itself to be ubiquitinated, which triggers degradation via the proteasome pathway. In contrast, hypoxia suppresses the enzyme, reduces the ubiquitination of HIF-1α, and results in the rapid accumulation of the latter in cells (46). In our study, we showed that the ODD domain mediates the association of c-Jun with HIF-1α under hypoxia, as evidenced by both SPPS analysis and coimmunoprecipitation assay. The details for the precise mechanism of this novel regulatory process remain open to further investigation. Nevertheless, these results establish an indispensable link and highlight the contribution of the ODD domain to the stabilization of HIF-1α by c-Jun. In parallel, the ODD domain is reported to be necessary for an oxygen-independent degradation pathway (47) besides its known oxygen-dependent event. This account fits in principle with our results and supports the outcome that the ODD domain is essential for this novel regulatory mode of HIF-1α. Together with the data presented here, a model is proposed (Fig. 6). c-Jun, under hypoxia, first complexes with and then masks the ODD domain of HIF-1α. c-Jun in the complexes in turn acts as a molecular barrier to prevent HIF-1α from being attacked by ubiquitin E3 ligase complexes.

The effect of c-Jun on the stability of HIF-1α was further related to VEGF expression and endothelial tube formation. Silencing c-Jun

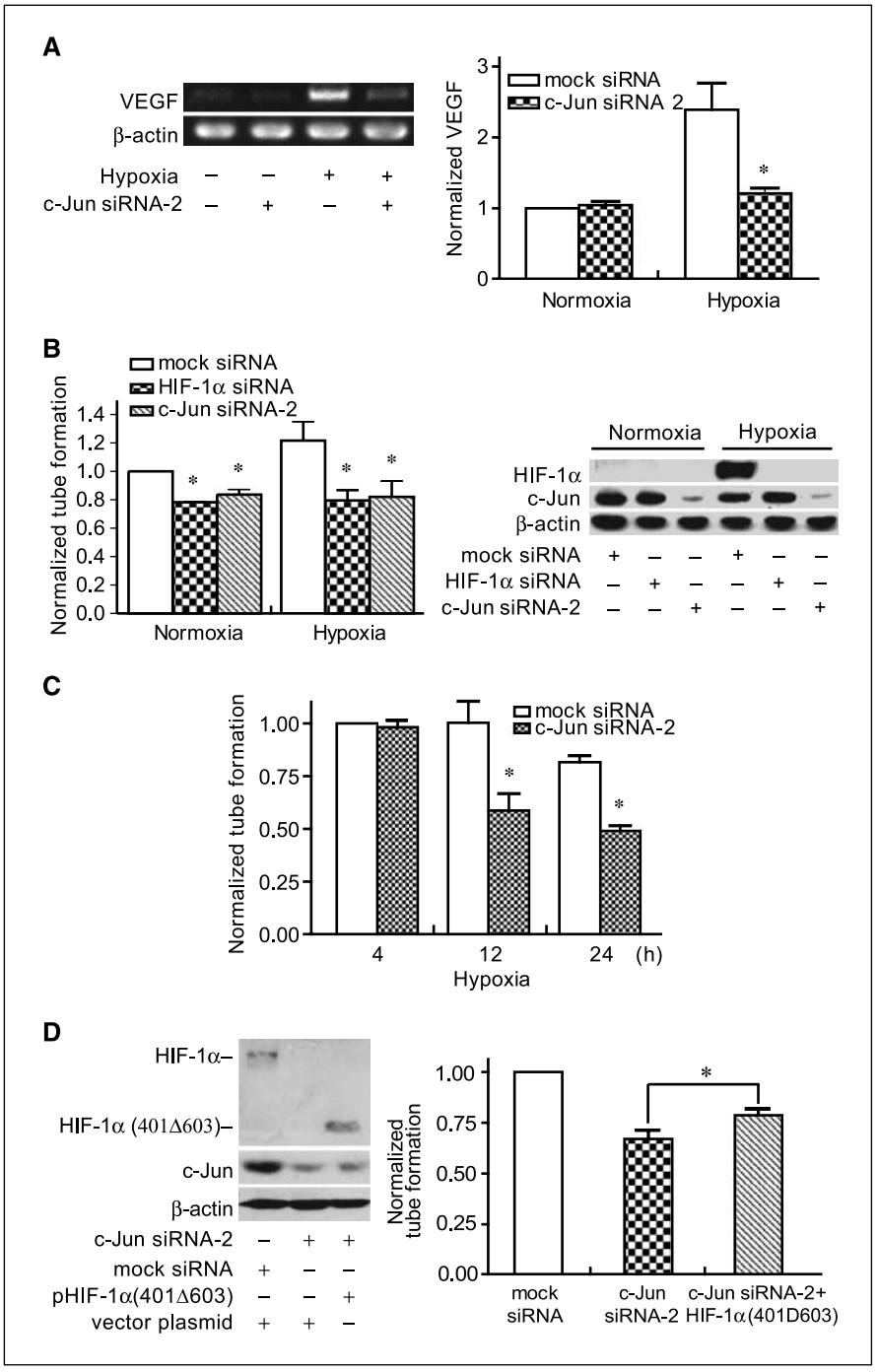

Figure 5. Down-regulation of HIF-1α after c-Jun silencing attenuates angiogenesis-related events. *A,* RT-PCR and ELISA assays showed that c-Jun siRNA-2 led to a decrease in VEGF mRNA and in the secretion of VEGF in MDA-MB-468 cells exposed to hypoxia for 12 h. *B,* c-Jun or HIF-1α silencing led to reduced tube formation more clearly under hypoxia than normoxia for 12 h. *C,* c-Jun silencing caused a more prominently decreased HMEC-1 tube formation of HMEC-1 cells as hypoxia was prolonged over a period of 12 h. *D,* the expression of HIF-1α (401Δ603) partially rescued the degradation of the tubes induced by c-Jun knockdown under hypoxia for 12 h. The experiments above were repeated thrice. *Columns,* mean; *bars,* SD. The representative images of tube formation in *B* to *D* can be found in Supplementary Fig. S3.

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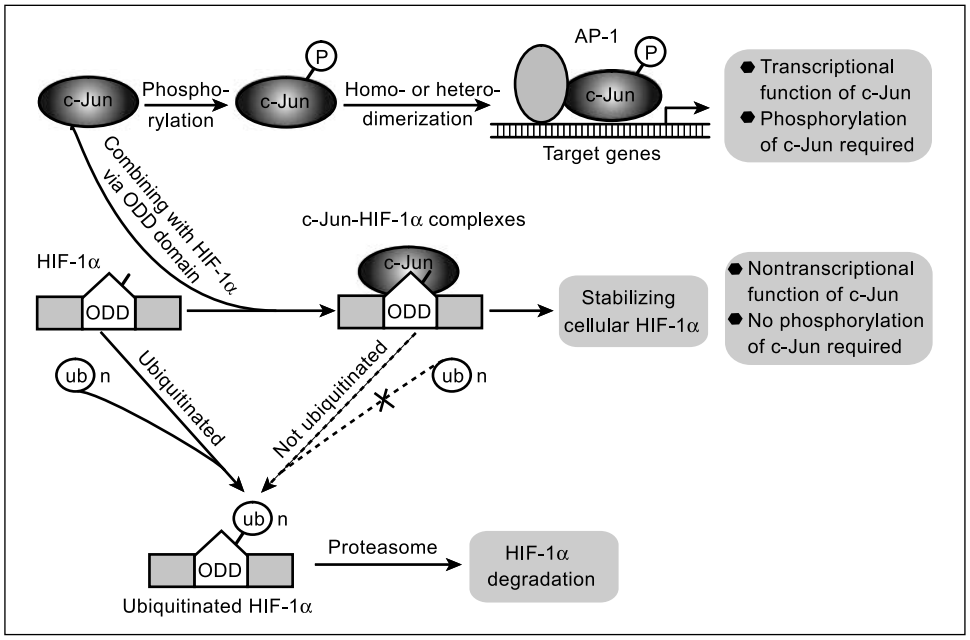

Figure 6. A schematic presentation of the functions of c-Jun and of the regulation of HIF-1αstability. The classic function of c-Jun is to regulate the transcription of target genes in the form of homodimers or heterodimers (AP-1) following its own phosphorylation. HIF-1α is ubiquitinated within its ODD domain followed by destruction via the proteasome. Our present study proposes a novel regulatory mode of HIF-1α such that c-Jun binds to the ODD domain of HIF-1α, functions as a molecular barrier that prevents HIF-1α from ubiquitination, and thus stabilizes cellular HIF-1α. In this case, c-Jun does not function as a transcription factor, and its $Ser^{63/73}$ phosphorylation is not required.

led not only to a decrease in VEGF gene transcription and the secretion of VEGF protein but also to an accelerated degradation of the endothelial tube–like network. As previously reported (48), we further confirm the contribution of HIF-1α to tube formation by the silencing of HIF-1α. In particular, the ectopic expression of the ODD domain–lacking HIF-1α partially rescues the degradation of tube formation in c-Jun–deficient HMEC-1 cells. Although c-Jun has been shown to induce VEGF expression in a HIF-1–independent manner (49, 50), our data indeed provide strong support that c-Jun is mediated by HIF-1α to regulate VEGF and tube formation, thus establishing the functional significance of the stabilization of HIF-1α by c-Jun, at least in this experimental setting.

Thus, far it has been shown that both HIF-1α and c-Jun are concurrently engaged in multiple malignant behaviors and have a functional cooperation in transcription activation (23–25). Moreover, the activation of c-Jun by HIF-1 under hypoxia has also been reported (26, 27). This study offers a novel mechanistic insight into their distinct contributions to tumor progression: a novel regulatory mode of HIF-1α activity and a nontranscriptional function of c-Jun. Further research will reveal how c-Jun precisely modulates HIF-1α, and this will advance the understanding of the previous HIF-1α stabilization program under hypoxia. In turn, this will allow a probing of the complicated pathophysiologic events in tumor progression as well as new therapeutic approaches.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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c-Jun Protects Hypoxia-Inducible Factor-1α from Degradation via Its Oxygen-Dependent Degradation Domain in a Nontranscriptional Manner

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