Hypoxia-Inducible Factor-1α Obstructs a Wnt Signaling Pathway by Inhibiting the hARD1-Mediated Activation of β-Catenin

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Abstract

Although a splice variant of mouse mARD1s was found to acetylate and destabilize hypoxia-inducible factor-1α (HIF-1α), human hARD1 has no such activities. Nonetheless, hARD1 has been reported to bind directly with HIF-1α. Here, we addressed the functional significance of the hARD1–HIF-1α interaction. Because hARD1 acetylates and activates β-catenin, we examined whether HIF-1α regulates the hARD1-mediated activation of Wnt signaling. It was found that HIF-1α binds hARD1 through the oxygen-dependent degradation domain and, in so doing, dissociates hARD1 from β-catenin, which prevents β-catenin acetylation. In LiCl-stimulated HEK293 or cancer cell lines with active Wnt signaling, β-catenin acetylation and activity were suppressed in hypoxia, and these suppressions were mediated by HIF-1α. Moreover, HIF-1α disruption of hARD1/β-catenin repressed TCF4 activity, resulting in c-Myc suppression and $p21^{cip1}$ induction. In addition, we confirmed that the HIF-1α NH_2 terminal inactivates TCF4 by directly binding β-catenin. In conclusion, HIF-1α was found to inactivate the Wnt signaling by binding to hARD1 or β-catenin, which may contribute to the hypoxia-induced growth arrest of tumor cells. [Cancer Res 2008;68(13):5177–84]

Introduction

Hypoxia drives malignant tumor phenotypes and initiates angiogenesis and cell migration (1–3). Hypoxia is also associated with restrained proliferation and development of resistance to cancer therapy (4). Furthermore, hypoxia-inducible factor-1α (HIF-1α) plays central roles in these hypoxia-driven tumor phenotypic changes (5–7). HIF-1α functions as a transactivating factor for HIF-1, whereas HIF-1β (or named ARNT) acts as a partner of HIF-1α to bind DNA (8). In normoxia, HIF-1–prolyl hydroxylases (PHD1–PHD3) hydroxylate P402 and P564 within the oxygen-dependent degradation domain (ODDD) of HIF-1α, which leads to the recruitment of von Hippel-Lindau tumor suppressor protein (pVHL)/E3 components (9, 10) and to the ubiquitination and degradation of HIF-1α (11, 12). In addition, HIF-1α acetylation by arrest defective 1 (ARD1) has been suggested to participate in HIF-1α degradation (13). Functionally, HIF-1α is oxygen-dependently regulated by factor inhibiting HIF-1, which hydroxylates N803 in the transactivation domain of HIF-1α, and thus blocks p300/CBP recruitment and deactivates HIF-1α (14). However, under hypoxic conditions, these HIF-1α modifications are limited, and as a result, HIF-1α is stabilized and activated.

β-Catenin participates in cell-cell adhesion by interacting with cadherin, also functions as a transcription factor in the canonical Wnt signaling, and, hence, plays important roles in cell proliferation, differentiation, and survival (15, 16). In the absence of Wnt, cytoplasmic β-catenin is phosphorylated by Axin/APC/GSK3β complex and then degraded by the ubiquitin/proteasome system. When Wnt binds to Frizzled, Disheveled (Dsh) inhibits GSK3β and, in turn, stabilizes β-catenin. Accumulated β-catenin then associates with TCF transcription factors in the nucleus, which transactivate proliferation-related genes, such as, Myc and cyclin D1 (17). Furthermore, β-catenin has been found to be aberrantly activated and to function as a proto-oncogene in various tumors (18), and it has also been reported that HIF-1α dissociates β-catenin/TCF complex by directly binding β-catenin and, thus, suppresses Wnt signaling, which may contribute to hypoxia-induced quiescence (19).

ARD1 catalyzes the NH_2 terminal α-acetylation of proteins in yeast and mammalian cells (20–23), and mammalian ARD1 has also been reported to catalyze the ε-acetylation of HIF-1α (13). However, several research groups have posed questions concerning HIF-1α regulation by ARD1 (24). Recently, several splice variants of mammalian ARD1 mRNAs were identified, i.e., three mouse variants ($mARD1^{235}$, $mARD1^{225}$, and $mARD1^{198}$) and two human variants ($hARD1^{235}$ and $hARD1^{131}$). Furthermore, $mARD1^{225}$ was found to acetylate K532 of HIF-1α, but $mARD1^{235}$ and $hARD1^{235}$ did not (25). Thus, because no equivalent of $mARD1^{225}$ exists in human cells, the mechanism of HIF-1α acetylation remains unclear (25, 26). However, it was interesting to learn that $hARD1^{235}$ physically associates with HIF-1α in human cells (27) because this begs the question, what is the functional significance of the $hARD1^{235}$–HIF-1α interaction?

Recently, we showed that hARD1 (which, from here, we use as a synonym for $hARD1^{235}$) binds and acetylates β-catenin, which in turn activates β-catenin/TCF4 and leads to the Wnt signaling–dependent proliferation of non–small cell lung cancer cells (28). However, given that hARD1 and HIF-1α are associated, it is surprising that investigations to date have focused exclusively on the possibility that ARD1 regulates HIF-1α, i.e., *ARD1→ HIF-1α*. Furthermore, in addition to acting as a transcription factor, HIF-1α can regulate other proteins by directly binding with them (19, 29, 30). Therefore, we hypothesized that, instead, HIF-1α regulates hARD1, i.e., *HIF-1α→ hARD1*, and undertook this study to determine whether HIF-1α regulates the hARD1-mediated activation of Wnt signaling.

Materials and Methods

Reagents and antibodies. Culture media and FCS were purchased from Invitrogen and other chemicals from Sigma-Aldrich. Anti–HIF-1α

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

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and anti-hARD1 antisera were generated, as previously described (31, 32). Antibodies against β-catenin, c-Myc, p21, His-tag, glutathione *S*-transferase (GST)–tag, GFP, β-tubulin, and horseradish peroxidase–conjugated secondary antibodies were purchased from SantaCruz Biotech. Anti–Ac-Lys (Cell Signaling Tech.), anti–HA-tag (Roche Applied Science), and anti–Flag-tag (Sigma-Aldrich) antibodies were obtained from the indicated companies.

Cell culture. H1299 (non–small cell lung cancer), A549 (non–small cell lung cancer), HCT116 (colon cancer), and HEK293 (human embryonic kidney) cell lines were obtained from American Type Culture Collection, and VHL null and VHL wild-type RCC4 (kidney cancer) cell lines from European Collection of Animal Cell Cultures. Lung cancer cells were cultured in RPMI 1640 and the others in DMEM supplemented with 10% FCS. Cells were incubated in either 5% CO_2/20% (normoxic) or 1% (hypoxic) O_2 atmospheres.

Cell proliferation assays. BrdUrd incorporation assays were performed using FITC BrdUrd flow kits purchased from BD PharMingen. Total DNAs were also stained with 7-amino-actinomycin D (7-AAD). FITC and 7-AAD were excited using an argon laser at 488 nm and detected at 515 to 565 nm and 630 to 660 nm, respectively, using a FACStar flow cytometer (BD Biosciences). After cells had been detached in a trypsin/EDTA solution, cell numbers were counted using a hemocytometer.

Small interfering RNAs, plasmids, and transfection. Small interfering RNA (siRNA; Invitrogen) sequences corresponded to the following nucleotides (the coding regions); 311-335 of hARD1 (Genbank NM_003491), 360-384 of HIF-1α (NM_001530), and 393-411 of β-catenin (NM_001904). Plasmids of HIF-1α, stable HIF-1α (lacking pVHL-binding motifs), the NH_2 terminal of HIF-1α(amino acids 1-400), ODDD of HIF-1α(amino acids 393-595), and the COOH terminal of HIF-1α (amino acids 577-826) were constructed as described previously (33, 34). Plasmids of hARD1 and β-catenin were constructed by reverse transcription–PCR (RT-PCR) and blunt-end ligation (28). TCF reporter plasmids containing wild-type (TOP-FLASH) or mutated (FOP-FLASH) TCF4 binding sites were obtained from Millipore. For transient transfection, ~40% confluent cells in 60-mm cell culture dishes were transfected with siRNAs or plasmids using Lipofectamine (Invitrogen). Cells were allowed to stabilize for 48 h before being used in the experiments.

Immunoblotting and immunoprecipitation. Cell lysates were electrophoresed in SDS/polyacrylamide gels and transferred to Immobilon-P membranes (Millipore). Membranes were incubated with a primary antibody diluted at 1:1,000 to 5,000 and then with horseradish peroxidase–conjugated secondary antibody (diluted 1:5,000), and blots were visualized by enhanced chemiluminescence (GE Healthcare Bio-Sciences). For immunoprecipitation, cell lysates (1 mg of protein) were incubated with 10 μL of antiserum and then further incubated with 10 μL of protein A/G-Sepharose beads (GE Healthcare Bio-Sciences) for 4 h. Immunocomplexes were eluted using the sample buffer for SDS PAGE.

RT-PCR. Total RNAs were isolated from cultured cells using TRIZOL (Invitrogen) and reverse-transcribed at 48°C for 30 min. cDNAs were amplified over 23 PCR cycles (94°C for 30 s, 52°C for 30 s, and 70°C for 30 s), electrophoresed on 2% agarose gels, and visualized with ethidium bromide. Primers for vascular endothelial growth factor (VEGF), aldolase, and β-actin were constructed as previously described (31).

GST pull-down assay. His-hARD1 and GST–HIF-1α proteins were purified from *Escherichia coli* BL21 cells by nickel and glutathione affinity chromatography (28); purified GST–β-catenin was obtained from Millipore. One-microgram quantities of GST, GST–HIF-1α, or GST–β-catenin immobilized on glutathione-Sepharose beads were incubated with 1 μg of His-hARD1 at 4°C for 3 h, and bound proteins were identified by SDS-PAGE and Coomassie staining.

***In vitro* acetylation assays.** To assay hARD1 acetyltransferase activity, 500 ng of GST–β-catenin and 500 ng of His-hARD1 were incubated in a reaction mixture [50 mmol/L Tris-HCl (pH 8.0), 0.1 mmol/L EDTA, 1 mmol/L

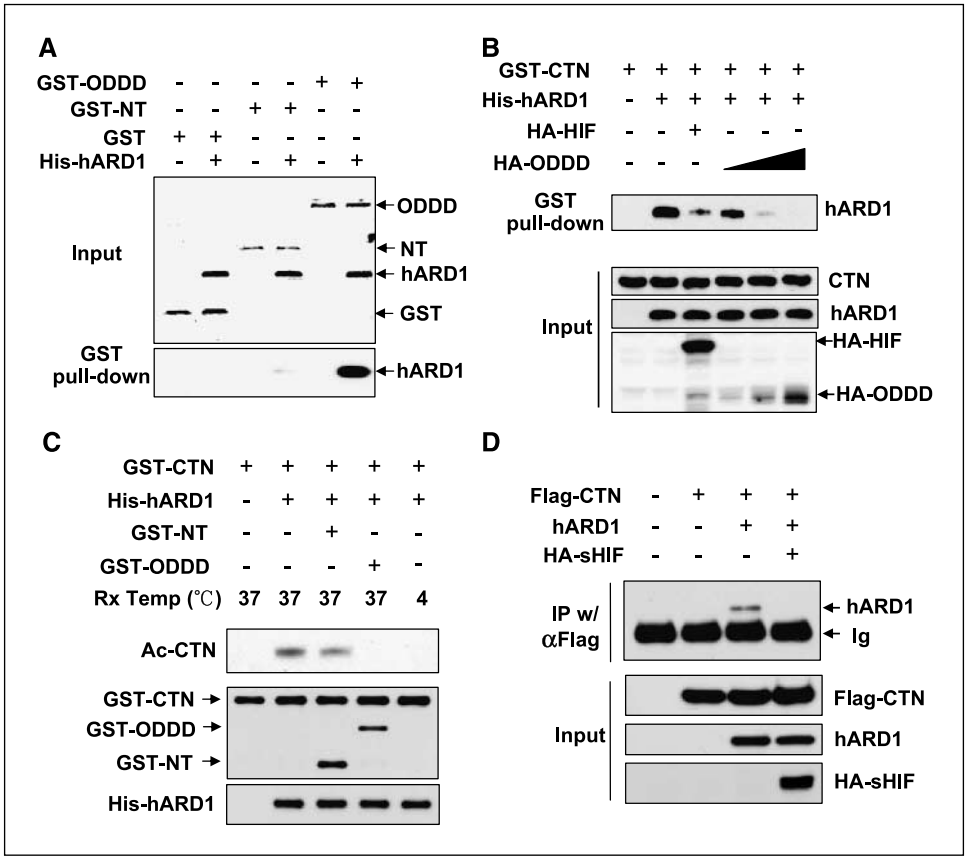

Figure 1. HIF-1α ODDD inhibited β-catenin acetylation by interacting with hARD1. *A, in vitro* binding of HIF-1α ODDD to hARD1. Purified recombinant His-hARD1 and GST fusion peptides, HIF-1α ODDD or NH_2 terminal (*NT*), were coincubated (0.5 μg each), and then His-hARD1 was pulled-down using glutathione beads. Input proteins were analyzed by SDS-PAGE and Coomassie staining, and pulled-down hARD1 was analyzed by Western blotting. *B,* HIF-1α ODDD dissociated the hARD1/β-catenin complex. Purified His-hARD1 and GST–β-catenin (*CTN*) peptides were incubated with HA bead–purified HA–HIF-1α (5 μL)/or ODDD peptides (3, 5, and 10 μL), and then His-hARD1 was pulled-down using glutathione beads. Input and pulled-down proteins were analyzed by Western blotting. *C,* HIF-1α ODDD inhibited the hARD1-mediated acetylation of β-catenin. GST–β-catenin (*CTN*; 0.7 μg) and His-ARD (0.1 μg) were incubated with GST-fusion peptides (0.3 μg) in acetylation reaction mixtures at 37°C or 4°C (the latter as a negative control to verify enzymatic reaction) for 1 h; lysine acetylation was identified using anti–Ac-Lys antibody (*top*). Western blotting (*bottom*) shows proteins present in reaction mixtures. *D,* HIF-1α expression dissociated the hARD1–β-catenin complex. HEK293 cells were cotransfected with pFlag–β-catenin (4 μg), phARD1 (2 μg), and/or pHA–sHIF-1α (stable mutant; 7 μg). Flag–β-catenin was immunoprecipitated using anti-Flag antibody and coprecipitated hARD1 was analyzed using Western blotting with anti-hARD1 antibody.

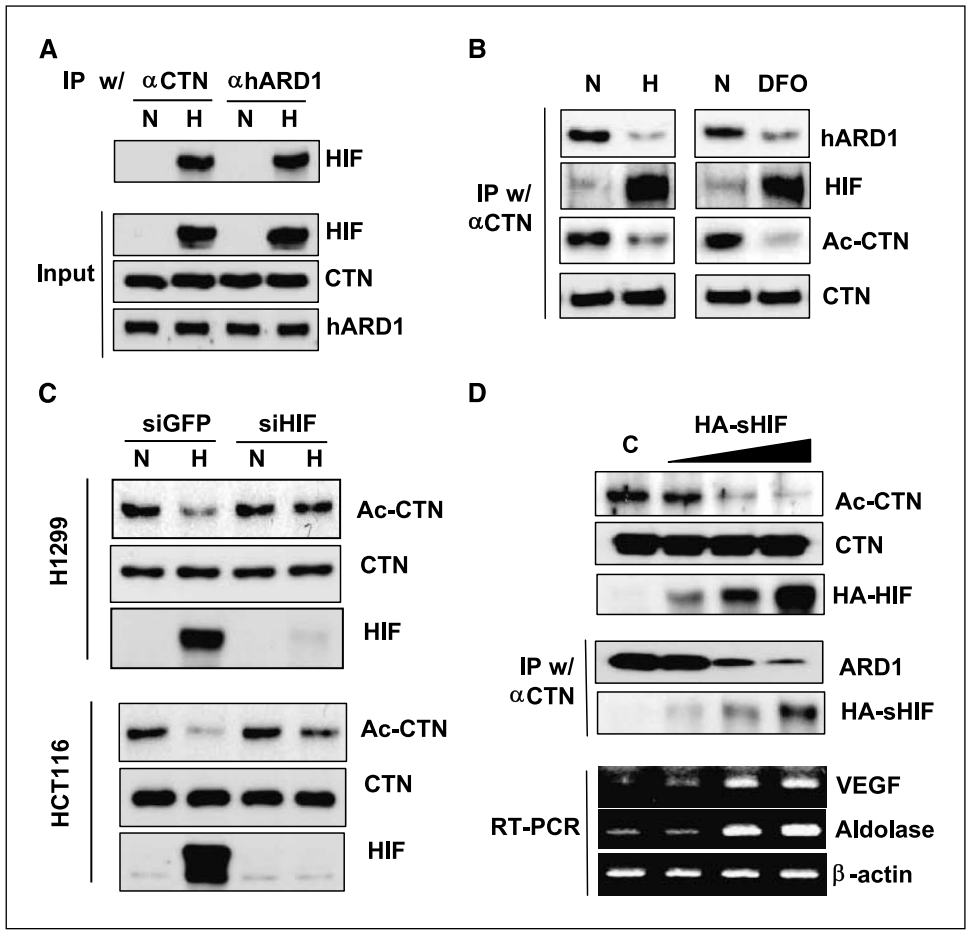

Figure 2. HIF-1α inhibited β-catenin acetylation under hypoxic conditions. *A,* HIF-1α binding to hARD1 or β-catenin under hypoxic conditions. H1299 cells were incubated under normoxic (*N*) or hypoxic (*H*) conditions for 24 h, and hARD1 and β-catenin were immunoprecipitated with specific antisera. The coprecipitation of HIF-1α was analyzed using anti–HIF-1α antibody. *B,* hARD1–HIF-1α dissociation and β-catenin deacetylation under hypoxic conditions. H1299 cells were exposed to hypoxia or 130 μmol/L desferrioxamine (*DFO*) for 24 h. β-Catenin was immunoprecipitated with its antibody, and coprecipitations of hARD1 and HIF-1α were analyzed with Western blotting. Acetylated β-catenin was identified using acetyl-lysine (*Ac-Lys*) antibody. *C,* HIF-1α mediated the hypoxic inhibition of β-catenin acetylation. After being transfected with siRNAs (40 nmol/L), H1299 and HCT116 cells were subjected to hypoxia, and acetylated and total β-catenins were identified using immunoprecipitation and immunoblotting. *D,* HIF-1α expression inhibited β-catenin acetylation by causing the dissociation of hARD1 from β-catenin. After H1299 cells had been transfected with pHA–sHIF-1α (2, 5, and 10 μg), acetylation (*Ac-CTN, top*), and hARD1 or HIF-1α binding to β-catenin (*middle*) were analyzed using immunoprecipitation and immunoblotting. Expressions of HIF-1–induced mRNAs (VEGF-A and aldolase-A) were analyzed by RT-PCR (*bottom*) to verify functional activation of expressed HA–sHIF-1α.

DTT, 10 mmol/L sodium butylate, 20 μmol/L acetyl-CoA, 10% glycerol] for 1.5 h. Acetylated GST–β-catenin was identified by immunoblotting with anti–Ac-Lys antibody. Protein loadings were verified by immunoblotting with anti-GST and anti-His antibodies.

Chromatin immunoprecipitation. Cells were homogenized in a lysis buffer containing 0.2% Nonidet P-40 and briefly spun at 3,000 rpm. Pellets were cross-linked with 1% formaldehyde, resuspended in 1% SDS buffer, and sonicated to break DNAs. Chromatin solutions were incubated with 10 μL of antiserum at 4°C overnight and then treated with salmon sperm DNA-pretreated protein G-Sepharose beads. After sequentially washing with NaCl and LiCl solutions, immunocomplexes were extracted from the beads using 1% SDS and 100 mmol/L $NaHCO_3$, incubated at 65°C overnight, and digested with RNase A and proteinase K. DNAs were extracted using phenol-chloroform-isoamyl alcohol and further purified by ethanol precipitation. They were then amplified over 30 PCR cycles in the presence of 5 μCi of [α-^{32}P]dCTP and electrophoresed in 4% polyacrylamide gels. The PCR primer sequences used for human c-Myc promoter were 5-GCTCTCCACTTGCCCCTTTTA-3 and 5-GTTCCCAATTTCTCAGCC-3.

Statistical analysis. Results are expressed as means and SDs and were calculated using Microsoft Excel 2002 software. The Mann-Whitney *U* test was used to compare reporter activities, cell numbers, and BrdUrd incorporation. All statistical tests were two-sided, and *P* values of <0.05 were considered significant.

Results

HIF-1α binds hARD1 and inhibits β-catenin acetylation. In an *in vitro* GST pull-down assay using recombinant proteins, GST-ODDD strongly bound His-hARD1 (Fig. 1*A*), and HA–HIF-1α or HA-ODDD caused β-catenin to dissociate from hARD1 (Fig. 1*B*). Moreover, ODDD inhibited the hARD1-mediated lysine acetylation of β-catenin *in vitro*, whereas HIF-1α NH_2 terminal did not (Fig. 1*C*). In H1299, hARD1 coprecipitated with Flag–β-catenin, and this was disrupted by HIF-1α (Fig. 1*D*). These results suggest that HIF-1α functions as a negative regulator of hARD1-mediated β-catenin acetylation.

β-Catenin acetylation is inhibited by HIF-1α under hypoxic conditions. To identify interactions between endogenous proteins, HIF-1α was induced under hypoxic conditions in H1299 and was found to associate with hARD1 (Fig. 2*A, fourth lane*). As previously reported (19), we also found that β-catenin and HIF-1α interact (Fig. 2*A*, second lane), and consequently, we examined the effect of hypoxia on the hARD1–β-catenin interaction. In H1299, which constitutively expresses active β-catenin (Supplementary Fig. S1), endogenous hARD1 and β-catenin were present as a complex under normoxic conditions. However, this complex dissociated under hypoxic conditions or after desferrioxamine (a HIF-1α inducer) treatment (Fig. 2*B*), and β-catenin then associated with HIF-1α. Moreover, β-catenin acetylation was inhibited by these two treatments (Fig. 2*B*). Furthermore, β-catenin acetylation was enhanced by HIF-1α knockdown (Fig. 2*C* and reduced by HIF-1α overexpression Fig. 2*D, top*), and hARD1 binding to β-catenin was reduced by HIF-1α, but HIF-1α binding to β-catenin increased (Fig. 2*D, middle*). In addition, increased VEGF-A and aldolase-A mRNA levels indicated that expressed HA–sHIF-1α is transcriptionally

functional (Fig. 2*D, bottom*). These results suggest that under hypoxic conditions β-catenin is deacetylated due to HIF-1α competition with it for hARD1 binding.

hARD1 is involved in the HIF-1α–mediated, hypoxic inactivation of TCF4. To evaluate β-catenin/TCF4 activity, TOP-FLASH with a TCF binding site or FOP-FLASH lacking a TCF binding site were used as reporters. In both LiCl-treated HEK293 and H1299 cells, TOP-FLASH activity was inhibited when HIF-1α was overexpressed or during hypoxia, and this was significantly rescued by knocking-down HIF-1α (Fig. 3*A* and *B* and Supplementary Fig. S2). Hypoxia response element reporter activity was also analyzed to confirm HIF-1α expression efficacy. Under hypoxic conditions, TOP-FLASH activity was further inhibited by hARD1 knockdown, and this was recovered by hARD1 expression (Fig. 3*B*). These results suggest that hARD1 participates in the HIF-1α–mediated, hypoxic inhibition of TCF4.

TCF4 inactivation by HIF-1α is accompanied by c-Myc suppression and p21 induction. During Wnt signaling, β-catenin/TCF4 expresses c-Myc, which in turn represses $p21^{cip1}$ transcription (35). This process is viewed as being critically responsible for the proliferations of many cancer cells (36). Because TCF4 was found to be inactivated by HIF-1α, we examined whether the expressions of c-Myc and p21 are altered under hypoxic conditions. In HCT116 and A549 cells having active Wnt signaling (Supplementary Fig. S1), c-Myc and p21 were respectively down-regulated and up-regulated by hypoxia (Fig. 4*A*). Moreover, these modulations returned to normoxic levels when HIF-1α was knocked-down, and even under normoxic conditions, HIF-1α expression induced these changes in c-Myc and p21 levels (Fig. 4*B*). Compared with VHL(+) RCC4, VHL(−) RCC4, which expresses constitutive HIF-1α, did not show hypoxic alterations of c-Myc and p21 levels (Supplementary Fig. S3). It was also found that c-Myc expression and p21 repression were β-catenin dependent (Supplementary Fig. S4).

hARD1 mediates c-Myc down-regulation and p21 up-regulation by HIF-1α. hARD1 rescued c-Myc expression and reduced p21 expression under hypoxic conditions and also recovered the β-catenin acetylation inhibited by hypoxia (Fig. 4*C*). In hARD1 knocked-down cells, the normoxic level of c-Myc decreased, and more importantly, the hypoxic suppression of c-Myc did not occur (Supplementary Fig. S5). In HEK293 cells, active β-catenin induced c-Myc and repressed p21, and these effects were augmented by hARD1. However, stable HIF-1α attenuated the hARD1-induced activation of β-catenin/TCF4 (Supplementary Fig. S6). By chromatin immunoprecipitation (CHIP) analyses, β-catenin, and hARD1 were recruited to c-Myc promoter under normoxic conditions in H1299 cells (Fig. 4*D*), and as was expected, HIF-1α expression and hypoxia promoted the dissociation of β-catenin and hARD1 from the *c-Myc* gene. However, in HIF-1α knocked-down cells, their DNA bindings were maintained even under hypoxic conditions. These results suggest that HIF-1α inhibits TCF4-mediated c-Myc expression by interacting with hARD1.

Two mechanisms are responsible for the HIF-1α–mediated repression of TCF4. Another mechanism was recently suggested for the hypoxic repression of TCF4. Kaidi and colleagues (19) found that HIF-1α interacts with β-catenin via its NH_2 terminal domain and that this interferes with the β-catenin–TCF4 association. To distinguish between the two modes of action of HIF-1α, we separately expressed three HIF-1α peptides fused with GFP (Fig. 5*A, top*). As reported above, the NH_2 terminal and ODDD of HIF-1α were found to associate with β-catenin and hARD1, respectively, whereas the COOH terminal and free GFP did not (Fig. 5*A, middle*). Interestingly, both the NH_2 terminal and ODDD

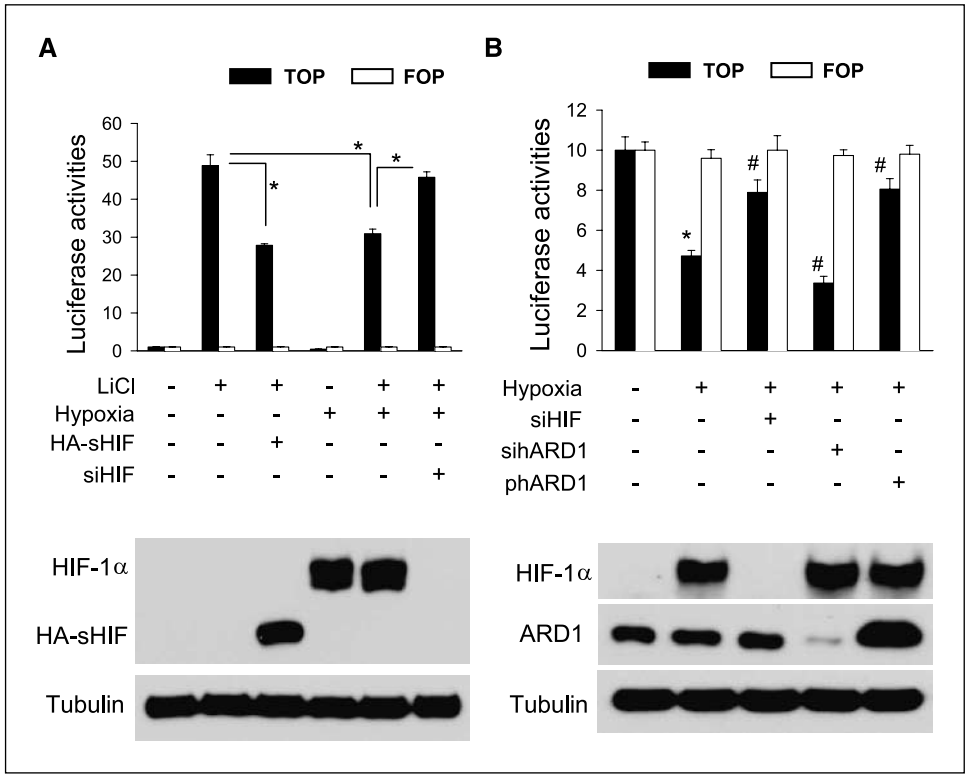

Figure 3. HIF-1α and hARD1 were both involved in the hypoxic inactivation of TCF4. *A,* HIF-1α prevented the LiCl-stimulated activation of β-catenin/TCF4. HEK293 cells were transfected with TCF reporter (0.25 μg), β-gal plasmid (0.25 μg), pHA–HIF-1α (0.2 μg), or HIF-1α siRNA (40 nmol/L) and then treated for 24 h with 30 mmol/L LiCl under normoxic or hypoxic conditions. Luciferase activities were measured using a luminometer, and β-gal assays were performed to normalize transfection efficiencies. *, $P < 0.05$ (*top*). HIF-1α and expressed HA–sHIF-1α were analyzed by immunoblotting with anti–HIF-1α antibody (*bottom*). *B,* hARD1 mediated HIF-1α–mediated TCF4 inactivation under hypoxic conditions. TCF reporter plasmid was cotransfected into H1299 cells with siRNAs (40 nmol/L) or phARD1 (0.2 μg). After cells had been incubated in a hypoxic environment for 24 h, luciferase activities were measured. *, $P < 0.05$ versus the normoxic control; #, $P < 0.05$ versus the hypoxic control (*top*). HIF-1α and ARD1 levels were measured by immunoblot analyses (*bottom*). *Columns,* means of four experiments; *bars,* SD.

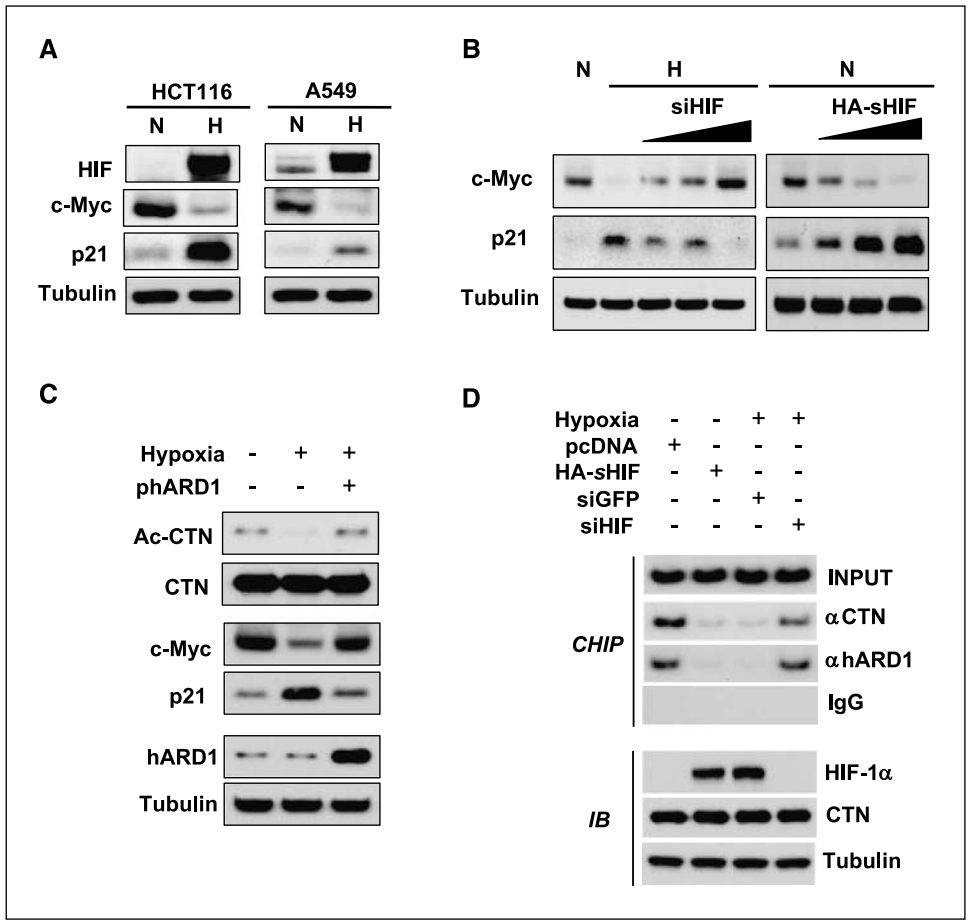

Figure 4. hARD1 mediated c-Myc and p21 regulation by HIF-1α. *A,* oxygen-dependent regulation of c-Myc or p21 in cancer cells with activated Wnt signaling. HCT116 or A549 cells were incubated under normoxic or hypoxic conditions for 24 h, and then c-Myc and p21 levels were analyzed by Western blotting. *B,* HIF-1α–dependent regulations of c-Myc and p21. H1299 cells were transfected with HIF-1α siRNA (10, 20, and 40 nmol/L) or pHA–sHIF-1α (0.2, 0.5, and 1 μg) and then exposed to normoxia or hypoxia for 24 h. *C,* hARD1 prevented hypoxic changes in acetylated β-catenin, c-Myc, and p21 levels. H1299 cells were transfected with 4 μg of phARD1 and exposed to normoxia or hypoxia for 24 h. Acetylated β-catenin was analyzed by immunoprecipitation/immunoblotting, and other proteins were analyzed by Western blotting. *D,* HIF-1α inhibited the recruitments of β-catenin and hARD1 to c-Myc promoter. Bindings of β-catenin and hARD1 to c-Myc promoter were analyzed by CHIP. For H1299 cells transfected with 10 μg of pHA-sHIF or 40 nmol/L of siRNAs, fixed chromatin samples were precipitated with anti–β-catenin, anti-hARD1, or nonimmunized serum. Precipitated DNAs were amplified by PCR with [α-^{32}P]dCTP.

inhibited c-Myc expression and induced p21 (Fig. 5*A, bottom*). Moreover, in TOP-FLASH reporter, both of these parts of HIF-1α significantly inhibited TCF4 activity (Fig. 5*B*). These results suggest that HIF-1α inhibits TCF4 via two distinct mechanisms.

Cell proliferation control by HIF-1α, hARD1, and β-catenin. We next examined whether proliferation inhibition by hypoxia is mediated by interactions between HIF-1α, hARD1, and β-catenin. Initially, H1299 cell growth was assessed by counting cells after 2 days of culture. HIF-1α expression and ARD1 knockdown both inhibited cell growth under normoxic conditions. Moreover, cell growth was retarded under hypoxic conditions, and this retardation was noticeably recovered by HIF-1α knockdown or by hARD1 or β-catenin expression (Fig. 6*A*). We rechecked cell proliferation under these conditions by determining BrdUrd-positive cell populations in the S phase, as shown in Supplementary Fig. S7, and obtained similar results (Fig. 6*B*). These findings suggest that in terms of β-catenin–dependent cell proliferation, hARD1 and HIF-1α function as positive and negative regulators, respectively. Interestingly, hARD1 knockdown did not noticeably inhibit cell proliferation under hypoxic conditions, whereas it did under normoxic conditions (Fig. 6*B*). Because hARD1 is already inhibited by HIF-1α induced by hypoxia, redundant inhibition of hARD1 using siRNA may not be effective in cell growth arrest. Summarizing, hARD1 seems to participate in Wnt signaling as a β-catenin activator under normoxic conditions, but under hypoxic conditions HIF-1α is stabilized and transactivates the genes essential for cellular adaptation to hypoxia and concomitantly inhibits cell proliferation by interacting with hARD1 and β-catenin (Fig. 6*C*).

Discussion

Hypoxia alters cell cycle distribution and increases the population of quiescent cells (4), which causes the acquisition of resistance to radiotherapy and chemotherapy (37). Moreover, as c-Myc plays a key role in G_1-S phase transition, its inhibition is regarded as a primary event in the cascade leading to G_1 arrest in tumor cells (38, 39). With regard to c-Myc inhibition by hypoxia, two molecular mechanisms have been recently proposed, i.e., (*a*) HIF-1α inhibits gene targeting of c-Myc by directly binding it (30) and (*b*) HIF-1α binds β-catenin and down-regulates c-Myc by disrupting β-catenin/TCF4 complexes (19). In the present study, we propose a novel mechanism for the hypoxia-induced inhibition of c-Myc, namely, that HIF-1α inhibits the hARD1-mediated activation of β-catenin/TCF4 by binding and inactivating hARD1. Moreover, this regulation of c-Myc may be associated with hypoxia-induced growth arrest in tumor cells with active Wnt signaling.

Kaidi and colleagues (19) recently proposed a mechanism for the hypoxia-induced down-regulation of c-Myc, i.e., that HIF-1α dissociates β-catenin/TCF4 complex in hypoxia by directly interacting with β-catenin, which releases p21 transcription repressed by c-Myc. They also showed that HIF-1α NH_2 terminal (amino acids 1–344) is necessary for the interaction between

HIF-1αand β-catenin. In the present study, we also confirmed that full-length HIF-1α and its NH_2 terminal (amino acids 1–400) associate with β-catenin (Figs. 2*A* and 5*A*). Furthermore, we found that the NH_2 terminal is sufficient to inactivate TCF4 (Fig. 5*B*). Thus, TCF4 inactivation by the interaction between HIF-1α and β-catenin seems obvious. Under the same conditions, as were used in the above experiments, it was also found that HIF-1α interacts with hARD1 and that its ODDD region (amino acids 393–595) is sufficient for hARD1 binding and TCF4 inactivation. Based on these results, we propose that the hypoxic repression of TCF4 is subject to double-checking by two distinct mechanisms.

In the absence of β-catenin, TCFs act as transcriptional repressors by recruiting Groucho/TLE, CtBP, and histone deacetylase. Moreover, when Wnt is activated, β-catenin binds TCF and changes its role from repressor to activator, during which β-catenin provides the transactivation domain (16). β-Catenin/TCF4 complex formation and the transcriptional activity of the complex are further stimulated by β-catenin acetylation, which is mediated by lysyl acetyltransferases, namely, p300 and hARD1 (28, 40). Moreover, when even one of these acetyltransferases is knocked down, deacetylated β-catenin loses its transcriptional activity, and thus, its downstream genes are transcriptionally repressed, which suggests that both p300 and hARD1 are required for the full activation of β-catenin (28). In the present study, we also confirmed that the hARD1 acetylation of β-catenin is necessary for Wnt signaling-dependent c-Myc expression and cell proliferation. Furthermore, the hypoxic inactivation of β-catenin/TCF4 was found to be mediated by hARD1 inactivation by HIF-1α.

HIF-1α is currently believed to play both positive and negative roles in tumor growth. In many xenografts, HIF-1α knockout (41) or treatment with HIF-1α inhibitors (42) were found to significantly retard tumor growth. On the contrary, cancer cells in HIF-1α(−/−) tumors, despite impaired vascularization, were more proliferative and less apoptotic than those of HIF-1α(+/+) tumors (43). Moreover, in VHL(−/−) tumors, HIF-1α expression and its downstream gene transcripts increased, but tumor growth was retarded (44). Therefore, the role of HIF-1α in tumor growth may be determined by the balance between the tumor-promoting and tumor-inhibiting effects of HIF-1α. Moreover, it is suggested that this tumor-promoting effect depends on the transcriptional activity of HIF-1α, whereas the tumor-inhibiting effect is provided by HIF-1αbinding to proteins. In the present study, it was found that the NH_2 terminal and ODDD regions of HIF-1α inhibited Wnt signaling but the COOH terminal region, which contains the transactivation domain, did not. Koshiji and colleagues (30) also showed that HIF-1α NH_2 terminal is sufficient to antagonize c-Myc and derepress p21. Thus, the forced up-regulation of HIF-1α with low transcriptional activity offers a potential strategy for cancer treatment. Indeed, bortezomib (a proteasome inhibitor) and bafilomycin A (a V-ATPase inhibitor) both induce the overexpression of nonfunctional HIF-1α under hypoxic conditions, which reenforces their inhibitory effects on cell proliferation (45, 46).

In the present study, cell proliferation was reduced by hARD1 knockdown in normoxia and rescued by hARD1 expression in hypoxia (Fig. 6*A* and *B*). Based on these results, we propose that hARD1 functions as a proproliferative factor in cancer cells with an active Wnt signaling pathway. However, contradictory findings have been reported concerning the effect of hARD1 on cell survival. Recently, Yi and colleagues showed that hARD1 knockdown prevents caspase-3 activation and cell death in response to doxorubicin-induced DNA damage (47), thus implying a proapoptotic role for hARD1. On the other hand, Arnesen and colleagues reported that hARD1 knockdown induced apoptosis and enhanced

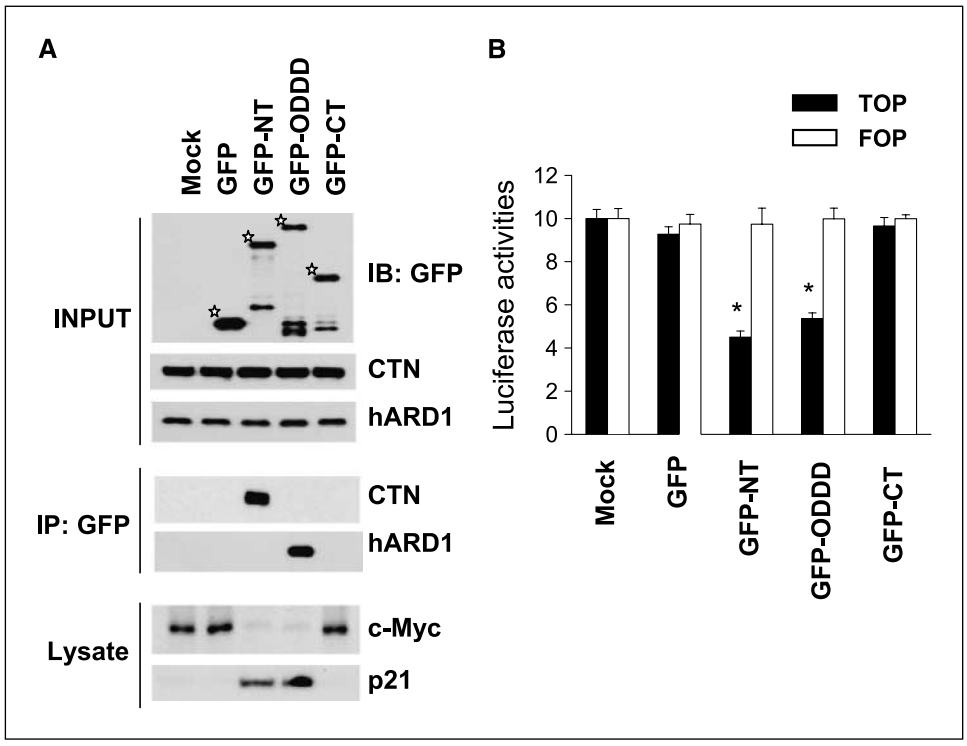

Figure 5. Two domains of HIF-1α independently inactivated β-catenin/TCF4. *A,* HIF-1α domains binding β-catenin or hARD1 and their roles on the expressions of c-Myc and p21. H1299 cells were transfected with 3 μg of pGFP, 3 μg of pGFP–NH_2 terminal HIF-1α (*NT*), 12 μg of pGFP-ODDD, or 7 μg of pGFP-COOH terminal HIF-1α (*CT*). GFP fusion proteins were immunoprecipitated with anti-GFP antibody, and coprecipitated β-catenin or hARD1 was identified by Western blotting. c-Myc and p21 levels were analyzed by Western blotting in H1299 lysates (*INPUT* samples). *B,* the NH_2 terminal and ODDD regions of HIF-1α independently repressed the transcriptional activity of TCF4. In HEK293 cells, TCF reporter (0.05 μg) and pFlag-aCTN (0.1 μg) were cotransfected with 0.05 μg of pGFP, 0.05 μg of pGFP-NT, 0.3 μg of pGFP-ODDD, or 0.15 μg of pGFP-CT. After cells had stabilized for 24 h, luciferase activities were measured. *Columns,* means of four experiments; *bars,* SD. *, $P < 0.05$ versus the Mock control.

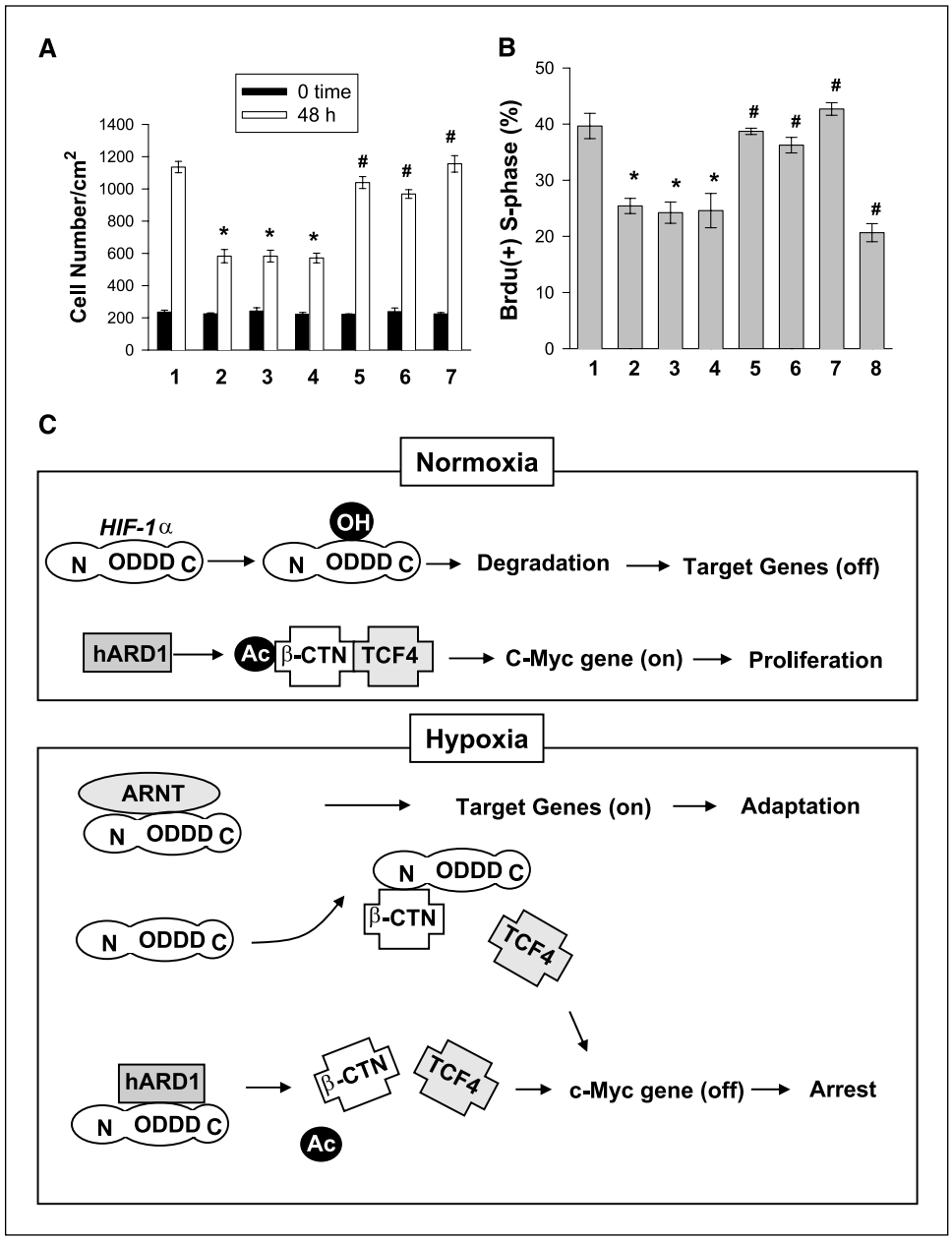

Figure 6. Cell proliferation was regulated by the HIF-1α–hARD1–β-catenin axis. *A,* cell numbers. H1299 cells were transfected with pHA–HIF-1α (7 μg), phARD1 (5 μg), pFlag-aCTN (6 μg), or 40 nmol/L of the siRNAs and then incubated under normoxic or hypoxic conditions for 48 h. Cells were then detached and counted in a hemocytometer. *Columns,* means of four experiments; *bars,* SD. *B,* populations of proliferating cells. After transfected cells had been incubated for 48 h, proliferations were analyzed by counting BrdUrd-positive cells in the S-phase (Supplementary Fig. S3). BrdUrd-positive cell populations are plotted as bar graphs. *Columns,* means (n = 3); *bars,* SD. *1,* Mock/normoxia; *2,* pHA–HIF-1α/normoxia; *3,* hARD1 siRNA/normoxia; *4,* Mock/hypoxia; *5,* HIF-1α siRNA/hypoxia; *6,* phARD1/hypoxia; *7,* pFlag-*a*CTN/hypoxia; *8,* hARD1 siRNA/hypoxia. *, $P < 0.05$ versus *1*; #, $P < 0.05$ versus *4*. *C,* schematic representation of cell growth under the control of the HIF-1α–hARD1–β-catenin axis. Under normoxic conditions, hARD1 activates β-catenin/TCF4 and promotes cell proliferation. When HIF-1α is stabilized under hypoxic conditions, HIF-1α removes β-catenin from TCF4, inactivates β-catenin by binding hARD1, and thus causes cell growth arrest. *OH,* hydroxyl moiety; *Ac,* acetyl moiety.

the cytotoxicity of daunorubicin (48). To examine the roles of hARD1 and HIF-1α in apoptosis, we transfected H1299 cells with pHA–sHIF-1α or ARD1 siRNA and then treated the cells with doxorubicin. Cell death and caspase-3 activation were found to be significantly augmented by either HIF-1α expression or hARD1 knockdown (Supplementary Fig. S8). Thus, in our experimental settings, hARD1 seems to play either a proproliferative or antiapoptotic role in cancer cells.

In summary, we tested the possibility that HIF-1α regulates β-catenin by binding to hARD1. It was found that HIF-1α deacetylated β-catenin by removing hARD1 from β-catenin and, in so doing, repressed the transcriptional activity of the β-catenin/TCF4 complex. We also found that the HIF-1α–hARD1 interaction in hypoxia were responsible for changes in c-Myc and p21 expression and for restrained proliferation. Based on these results, we propose that the Wnt signaling inhibition by HIF-1α–hARD1 binding contribute to tumor growth arrest in hypoxia. However, in cancer cells with inactive Wnt signaling, the HIF-1α–hARD1 interaction may play a minor role in the hypoxia-induced growth arrest.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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