

Human Arrest Defective 1 Acetylates and Activates β -Catenin, Promoting Lung Cancer Cell Proliferation

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Abstract

Arrest defective 1 (ARD1), an acetyltransferase, is essential for the yeast life cycle. Although its human homologue (hARD1) has been identified, its biological functions in human cells remain unclear. In the present study, we examined the biological function of hARD1. In H1299 and A549 lung cancer cells, hARD1-silencing RNA inhibited cell proliferation and induced G₁ arrest. Cyclin D1 was also found to be down-regulated in these growth-arrested cells, and the ectopic expression of cyclin D1 rescued cell growth. hARD1 knockdown repressed the promoter activity of the *cyclin D1* gene, which inhibited the transcription of cyclin D1. Moreover, hARD1 knockdown reduced the binding of β -catenin/TCF4 transcription factor to cyclin D1 promoter and repressed its transcriptional activity. Inversely, hARD1 expression increased the transcriptional activity of β -catenin. Both endogenous and ectopically expressed hARD1 was coimmunoprecipitated with β -catenin. hARD1 knockdown did not affect β -catenin expression or degradation but noticeably reduced acetylated β -catenin. The β -catenin binding and acetylation by hARD1 were observed *in vitro*. Therefore, it is suggested that hARD1 participates in proliferation of lung cancer cells via the activation of β -catenin. (Cancer Res 2006; 66(22): 10677-82)

Introduction

Acetylation is a common means of protein modification in eukaryotes, and protein NH₂-terminal α -acetylation is the most frequent of these protein modifications (1). ϵ -Amino lysine acetylation, unlike NH₂-terminal acetylation, reversibly regulates the function and/or stabilities of many proteins (2). Of mammalian protein acetyltransferases, homologues of yeast arrest defective 1 protein (Ard1p) show both α -protein and ϵ -protein acetylation activities. Ard1p was originally described in *Saccharomyces cerevisiae* (3), where it functions as a catalytic subunit of the NH₂-terminal acetyltransferase complex with N-acetyltransferase 1 protein (Nat1p; ref. 4). Similarly, both mouse and human Ard1p homologues (mARD1 and hARD1), in complexes with homologues of yeast Nat1p, express α -protein acetylation activity (5, 6). In addition, ARD1 has been reported to show ϵ -protein acetylation activity in mammalian cells; for example, it acetylates Lys⁵³² in hypoxia-inducible factor 1 α (HIF-1 α) and thereby degrades HIF-1 α

via the ubiquitin-proteasome system (7). However, two research groups recently posed questions about the involvement of ARD1 in HIF-1 α regulation (8, 9).

In terms of its biological functions, Ard1p has been linked with G₀ entry, growth, and sporulation in yeast cells (3, 10, 11). However, its function in mammalian cells has not been defined. Recently, Fisher et al. (12) showed that hARD1 knockdown is correlated with the down-regulation of proliferative genes and up-regulation of antiproliferative genes. Because the expressions of HIF-1-regulated genes were not found to be affected by the small interfering RNA (siRNA), hARD1 was suggested to be directly linked to cell proliferation, regardless of HIF-1. However, the molecular entities that mediate gene expressions by hARD1 remain unidentified.

In the present study, we evaluated the biological functions of hARD1 and identified the transcription factor regulated by hARD1. In H1299 and A549 lung cancer cells, hARD1-silencing RNA inhibited cell proliferation and induced G₁ arrest, and this was attributed to the transcriptional repression of the *cyclin D1* gene. hARD1 knockdown inhibited the activity of β -catenin/TCF, which is a transcription factor for cyclin D1. We also showed that hARD1 associated with and acetylated β -catenin, which in turn induced the binding of TCF4 to cyclin D1 promoter. We conclude that hARD1 may participate in cell proliferation as an activator of β -catenin.

Materials and Methods

Cell culture and proliferation analysis. Two non-small cell lung carcinoma cell lines, H1299 and A549, were obtained from the American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 supplemented with 10% FCS in a 5% CO₂ humidified atmosphere at 37°C. To analyze cell proliferation, bromodeoxyuridine (BrdUrd) incorporation assays were done using a FITC BrdUrd Flow kit purchased from BD PharMingen (San Diego, CA). Total DNAs were stained with 7-amino-actinomycin D (7-AAD). To analyze cell cycle, cells were fixed in 75% ethanol and labeled with propidium iodide (0.05 mg/mL) for 30 minutes. FITC, 7-AAD, and propidium iodide were detected using a FACStar flow cytometer (BD Biosciences, San Jose, CA).

Antibodies. The rat polyclonal anti-hARD1 antiserum was generated against full-length hARD1 peptide (13). Antibodies against cyclin A, cyclin E, β -catenin, TCF4, p300, GFP, β -tubulin, and horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-cyclin D1 (Cell Signaling Technology, Beverly, MA), anti-hemagglutinin (HA; Roche Applied Science, Indianapolis, IN), and anti-acetyl-lysine (Upstate Biotechnology, Lake Placid, NY) antibodies were obtained from the indicated companies.

siRNAs and plasmids. The siRNA (Invitrogen, Carlsbad, CA) sequence corresponds to nucleotides (the coding region) 311 to 335 of hARD1 (Genbank no. NM_003491). A verified siRNA of p300 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). cDNAs of hARD1, cyclin D1 (NM_053056), Wnt3a (NM_033131), and β -catenin (NM_001904) were cloned by reverse transcription-PCR (RT-PCR) using Pfu DNA polymerase and inserted into pcDNA, pcDNA-HA, or pEGFP expression vector by blunt-end ligation (13). The cyclin D1 promoter/5'-untranslated region from -403

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

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doi:10.1158/0008-5472.CAN-06-3171

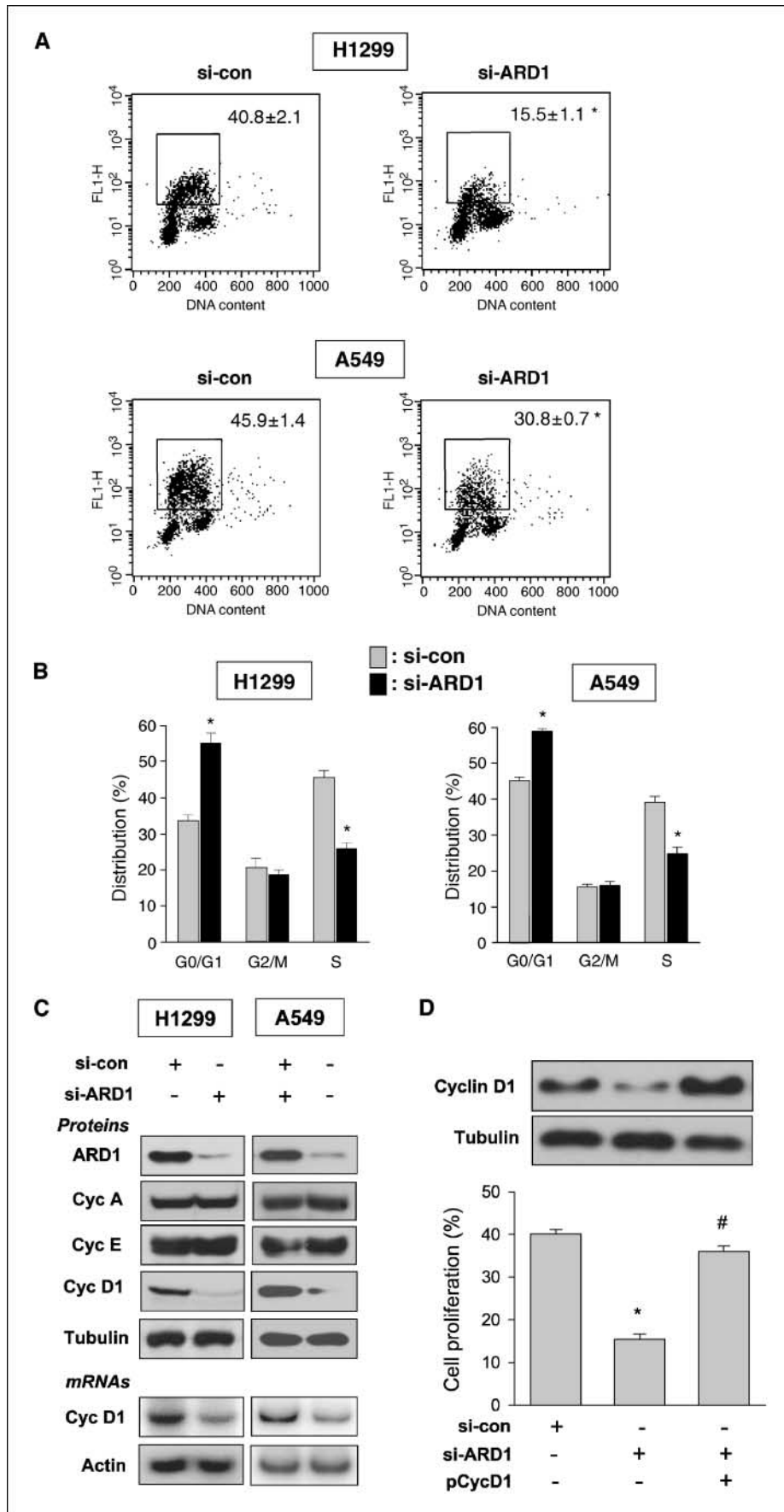


Figure 1. hARD1 inhibition induces G₁ arrest by down-regulating cyclin D1. **A**, si-ARD1 reduced lung cancer cell proliferation. H1299 or A549 cells were twice transfected with 20 nmol/L of hARD1 siRNA (*si-ARD1*) or scrambled RNA (*si-con*). Cell proliferation was analyzed by assaying BrdUrd incorporation (Y axis) to DNA (X axis). Small boxes, BrdUrd-incorporated cells in the S phase, and the numbers are mean ± SD (n = 4). *, P < 0.05 versus the si-con group. **B**, si-ARD1 induced G₁ arrest. Cell cycle was analyzed by propidium iodide labeling and flow cytometry. Columns, mean (n = 4) of proportions in the G₀-G₁, S, and G₂-M phases; bars, SD. *, P < 0.05 versus the si-con group. **C**, si-ARD1 down-regulated cyclin D1. hARD1, cyclin (Cyc) A/E/D1, and β-tubulin expressions were analyzed by immunoblotting. Cyclin D1 and β-actin mRNA levels were semiquantified by RT-PCR. **D**, cyclin D1 expression rescued cell proliferation inhibited by si-ARD1. After cotransfecting H1299 cells with 40 nmol/L si-ARD1 and 1.5 μg of the plasmid cyclin D1 (*pCycD1*), cell proliferation was analyzed by determining BrdUrd incorporation. Top, cyclin D1 levels were analyzed by immunoblotting. Columns, mean (n = 4) of the populations of proliferating cells; bars, SD. *, P < 0.05 versus the si-con group; #, P < 0.05 versus the si-ARD1 group.

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to +209 was amplified by PCR with primers 5'-GGCTGGATATGGGGTGC-3' and 5'-GGCTGGGGCTCTTCCTGGG-3' and inserted into pGL3basic (Promega, Madison, WI), which is designated cycD1-Luc. TCF reporter plasmid containing wild-type (5'-CCTTTGATC-3'; TOP-FLASH) or mutated (5'-CCTTTGGCC-3'; FOP-FLASH) β-catenin/TCF-binding site was obtained from Upstate Biotechnology.

Reporter assays. Cells were cotransfected with reporter plasmids and cytomegalovirus-β-gal plasmid or siRNAs using LipofectAMINE (Invitrogen). The transfected cells were then allowed to stabilize for 48 hours before being used in experiments. The cells were lysed and assayed for luciferase activity, and β-gal assays were done for normalization of transfection efficiency.

Immunoblotting and immunoprecipitation. Proteins were separated on SDS/polyacrylamide gels and transferred to Immobilon-P membranes. Membranes were blocked with 5% nonfat milk, incubated overnight at 4°C

with primary antibodies (1:1,000), incubated with a HRP-conjugated secondary antibodies (1:5,000) for 2 hours, and visualized using an enhanced chemiluminescence kit (GE Healthcare Bio-Sciences, Piscataway, NJ). For immunoprecipitation, cell lysates (1 mg protein) were incubated with 10 μL of antiserum and further incubated with 10 μL of protein A/G-Sepharose beads (GE Healthcare Bio-Sciences) for 4 hours. Immuno-complexes were eluted in a sample buffer containing 2% SDS and 10 mmol/L DTT and then subjected to immunoblotting.

Chromatin immunoprecipitation and PCR. Cells were fixed with formaldehyde, and soluble chromatin samples were immunoprecipitated with anti-ARD1, anti-β-catenin, anti-TCF4, or preimmune serum at 4°C overnight (14). DNA isolated from immunoprecipitated material was amplified by semiquantitative PCR with [α -³²P]dCTP. The PCR primer sequences used were 5'-TTCCTAGTTGTCCCTACTG-3' and 5'-TTGCAACTTCAACAAACTC-3', which produced a 362-bp fragment,

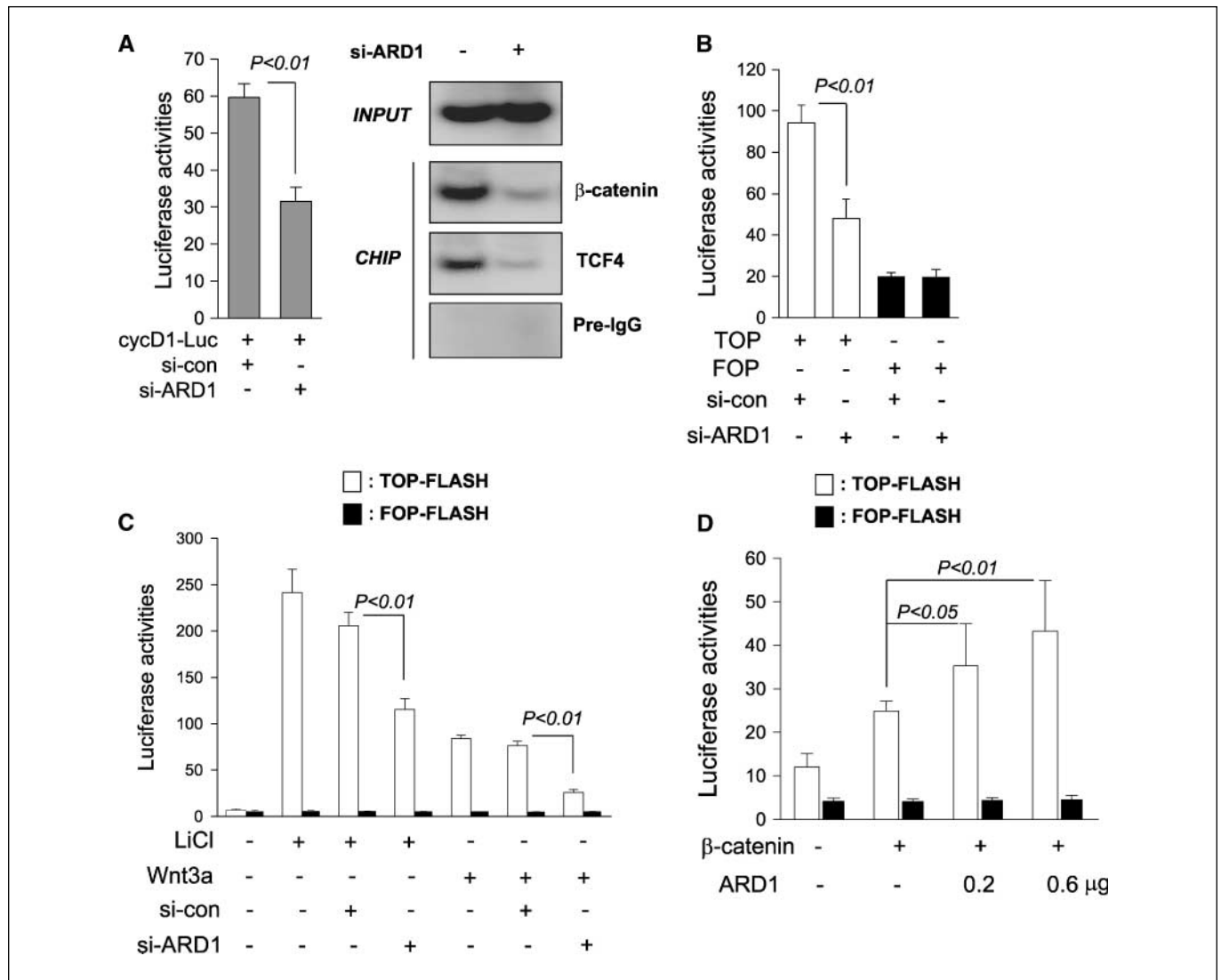


Figure 2. hARD1 inhibition represses β-catenin/TCF activity. *A, Left*, si-ARD1 repressed the cyclin D1 promoter activity. After transfected with 40 nmol/L of si-ARD1 or si-con, H1299 cells were cotransfected with 0.25 μg of cycD1-Luc and 0.25 μg of β-gal plasmids. Luciferase activities were measured using a luminometer, and β-gal assays were done to normalize transfection efficiencies. *Right*, hARD1 inhibition prevented the binding of β-catenin/TCF to cyclin D1 promoter. Soluble chromatin was prepared from cells transfected with 40 nmol/L siRNA and immunoprecipitated with anti-catenin, anti-TCF4, or preimmune serum (*Pre-IgG*). Final DNA extractions were amplified by primers spanning cyclin D1 promoter. *B*, si-ARD1 reduced the transcriptional activity of β-catenin/TCF. H1299 cells were cotransfected with 0.25 μg of TCF reporter plasmid, 0.25 μg of β-gal plasmid, and 20 nmol/L of siRNAs. *C*, si-ARD1 prevented β-catenin/TCF activation by LiCl or Wnt3a. HEK293 cells were cotransfected with TCF reporter, β-gal plasmid, and siRNAs and then treated for 16 hours with 30 mmol/L LiCl or 10 μL of Wnt3a extract. *D*, hARD1 expression enhanced β-catenin activity. HEK293 cells were cotransfected with 0.25 μg of TCF reporter plasmid, 0.25 μg of β-gal plasmid, 0.1 μg of β-catenin plasmid, and 0.2/0.6 μg of hARD1 plasmid. *Columns*, mean of eight experiments; *bars*, SD.

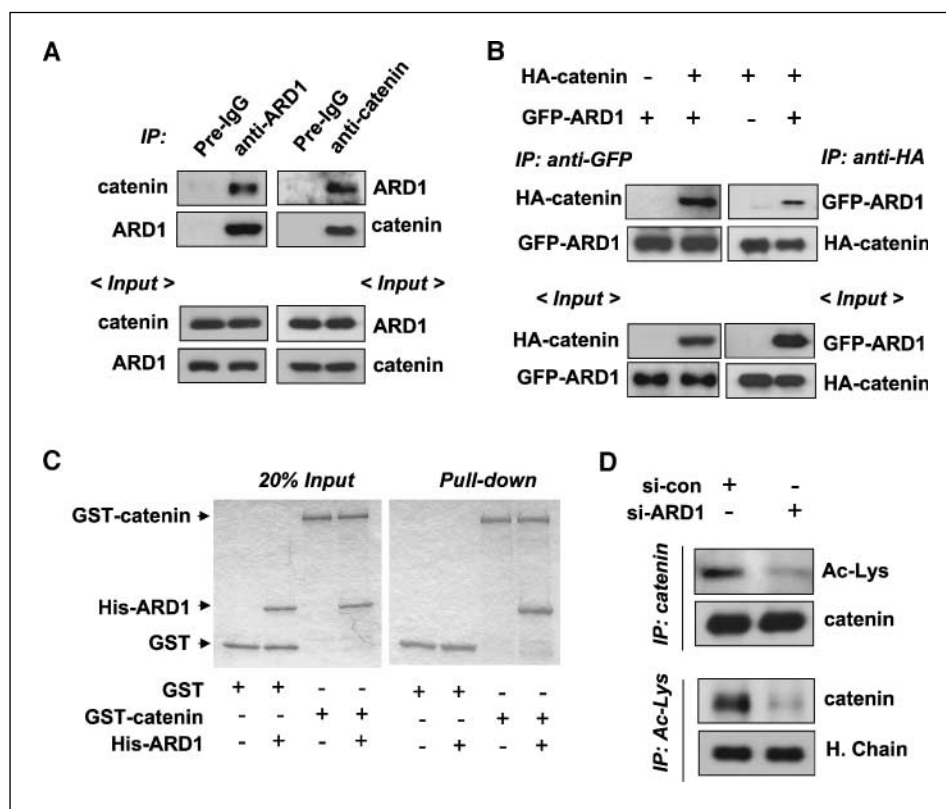


Figure 3. hARD1 binds β -catenin and is required for β -catenin acetylation. **A**, association of hARD1 and β -catenin. hARD1 of H1299 cells was immunoprecipitated (IP) with anti-ARD1 antiserum or nonimmunized rat serum (*Pre-IgG*). *Left*, coprecipitation of β -catenin with hARD1 was analyzed by immunoblotting (IB) using anti-catenin antibody; *right*, coprecipitation of hARD1 with β -catenin was also analyzed. **B**, association of expressed hARD1 and β -catenin. HEK293 cells were cotransfected with 1 μ g of pGFP-hARD1 and 1.5 μ g of pHA-catenin. *Left*, GFP-hARD1 was immunoprecipitated with anti-GFP antibody, and HA-catenin coprecipitates were analyzed using anti-HA antibody; *right*, HA-catenin was also immunoprecipitated, and the coprecipitation of GFP-hARD1 was identified. **C**, *in vitro* binding of hARD1 to β -catenin. Purified recombinant His-hARD1 and GST- β -catenin (or GST) peptides were coincubated, and then His-hARD1 was pulled down using glutathione beads. Input and pull-down proteins were analyzed by SDS-PAGE and Coomassie staining. **D**, hARD1 was required for β -catenin acetylation. After transfected with siRNAs (20 nmol/L), H1299 cells were treated with 10 mmol/L sodium butyrate to prevent protein deacetylation. *Top*, after cell lysates were immunoprecipitated with β -catenin antibody, acetylated and total (acetylated plus unacetylated) β -catenins were identified using acetyl-lysine (*Ac-Lys*) and β -catenin antibodies, respectively; *bottom*, this immunoprecipitation was cross-checked by changing the sequence of antibody treatment. The heavy chain of IgG (*H. Chain*) was used as a loading control.

including the TCF-binding site of the *cyclin D1* gene. To quantify mRNA expressions, highly sensitive semiquantitative RT-PCR was done (13). Total RNA (1 μ g) was reverse transcribed, and the cDNA obtained was amplified over 18 to 20 PCR cycles with [α - 32 P]dCTP. PCR products were electrophoresed in a 4% polyacrylamide gel, and dried gels were autoradiographed. The sequences of the cyclin D1 primers were 5'-CGGTGCTCACTTCAAATGT-3' and 5'-TTGGAGAGGAAGTGTCAAT-3' and those of β -actin primers were 5'-ACACCTTCTACAATGAGCTG-3' and 5'-CATGATGGAGTTGAAGGTAG-3'.

Glutathione S-transferase pull-down assay. His-tagged full-length hARD1 (His-hARD1) and glutathione S-transferase (GST) proteins were purified from *Escherichia coli* BL21 cells using nickel-affinity and glutathione-affinity chromatography (13), and purified GST- β -catenin was obtained from Upstate Biotechnology. The purities (>95%) of recombinant proteins were checked by SDS-PAGE and Coomassie Blue R-250 staining (Supplementary Fig. S1). GST (1 μ g) or GST- β -catenin immobilized on glutathione-Sepharose beads was incubated with 1 μ g His-hARD1 in the reaction buffer [25 mmol/L HEPES (pH 7.5), 150 mmol/L KCl, 12.5 mmol/L MgCl₂, 20 μ mol/L ZnCl₂, 5 mmol/L DTT, 0.1% NP40, 10% glycerol] at 4°C for 3 hours. After washing, the bound proteins were eluted with sample buffer and identified by SDS-PAGE and Coomassie staining.

In vitro acetylation assays. To assay the activity of hARD1 expressed from HEK293 cells, cells were transfected separately with pARD1 or pHA-catenin. The HA-catenin lysates were incubated with the hARD1 lysates. Acetylated β -catenin was identified using immunoprecipitation with anti- β -catenin and immunoblotting with anti-acetyl-lysine. To assay the activity

of bacterially expressed hARD1, 500 ng GST- β -catenin and 400 ng His-hARD1 were incubated in the reaction mixture [50 mmol/L Tris-HCl (pH 8.0), 0.1 mmol/L EDTA, 1 mmol/L DTT, 10 mmol/L sodium butyrate, 20 μ mol/L acetyl-CoA, 10% glycerol] for 1.5 hours at 37°C or 4°C. Acetylated GST- β -catenin was identified by immunoblotting with anti-acetyl-lysine, and protein loading was verified by Coomassie staining.

Statistical analysis. Results are expressed as mean and SD using Microsoft Excel 2002 software. The Mann-Whitney *U* test (SPSS, Chicago, IL) was used to compare BrdUrd incorporations, cell cycle populations, and reporter activities. All statistical tests were two sided, and *P*s < 0.05 were considered to be significant.

Results and Discussion

hARD1 promotes cell proliferation by regulating cyclin D1.

In hARD1 knockdown cells, BrdUrd incorporation to DNA was significantly reduced by 62% in H1299 or by 33% in A549 (Fig. 1A). In addition, the G₀-G₁ population significantly increased by 21.5% in H1299 or by 13.8% in A549 at the expense of the S population (Fig. 1B). Of cyclins, cyclin D1 was markedly reduced by si-ARD1 at the protein and mRNA levels (Fig. 1C). Moreover, cyclin D1 expression rescued growth arrest induced by si-ARD1 (Fig. 1D). These results suggest that hARD1 is required for the proliferation of non-small cell lung cancer cells by expressing cyclin D1.

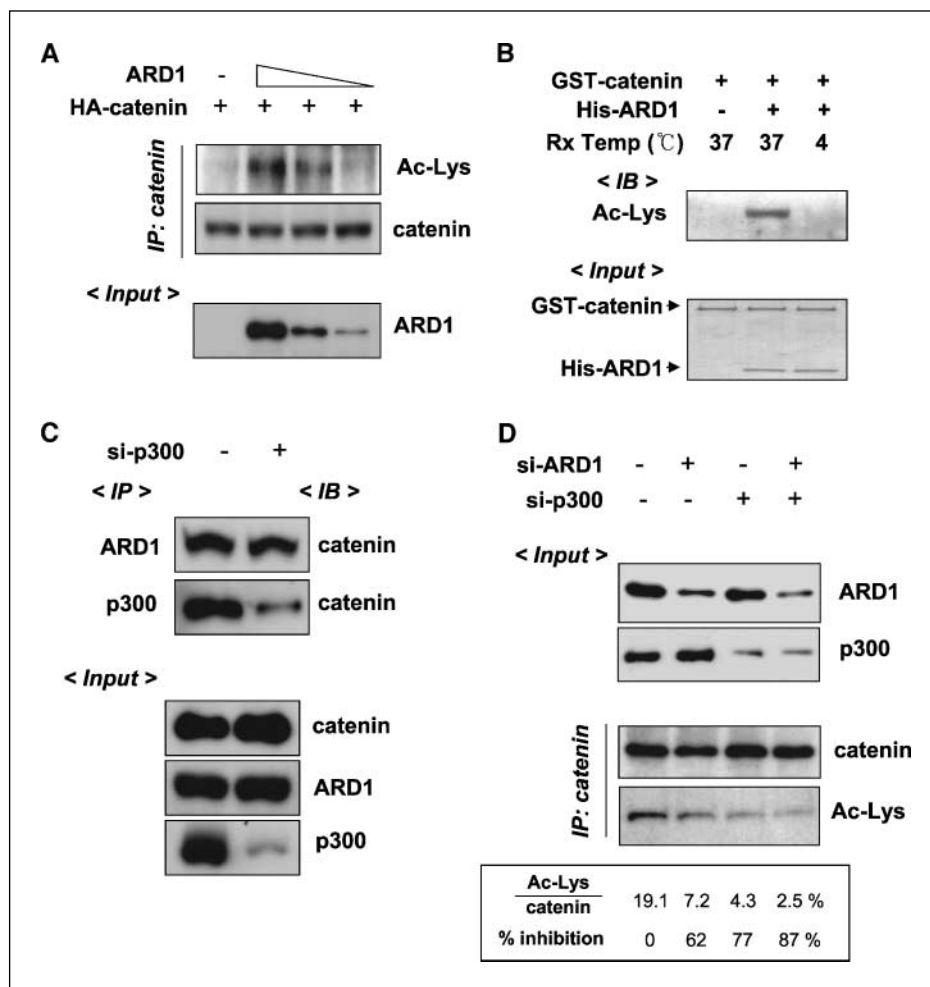
hARD1 is required for the activation of β-catenin/TCF. In the cycD1-Luc reporter system, the cyclin D1 promoter activity was significantly repressed by si-ARD1 (Fig. 2A, left). We next examined whether hARD1 activates β-catenin/TCF, which is known to regulate cyclin D1 (15). A chromatin immunoprecipitation analysis showed that si-ARD1 inhibited the recruitment of β-catenin/TCF on the cyclin D1 promoter (Fig. 2A, right). Interestingly, the β-catenin/TCF complex was shown to bind the cyclin D1 promoter (Fig. 2A) and β-catenin was translocated into the nucleus (Supplementary Fig. S2) without additional Wnt signaling stimulants, which suggest that H1299 and A549 cells have the activated β-catenin. Indeed, many non-small cell lung cancer cells, including H1299 and A549, have been reported to show basal activation of Wnt signaling by overexpressing Wnt ligands (16, 17). The activity of a TCF reporter plasmid, TOP-FLASH, was significantly reduced by si-ARD1 in H1299 cells but that of FOP-FLASH lacking TCF-binding site was not (Fig. 2B). In HEK293 cells stimulated by LiCl and Wnt3a, TOP-FLASH activity was also inhibited by si-ARD1 (Fig. 2C). Moreover, hARD1 expression augmented the TOP-FLASH activation by β-catenin (Fig. 2D). These results suggest that hARD1 induces cyclin D1 by activating β-catenin/TCF.

hARD1 binds β-catenin and is required for β-catenin acetylation. β-Catenin was coimmunoprecipitated with hARD1 and vice versa (Fig. 3A), and expressed HA-catenin and GFP-hARD1

were also coimmunoprecipitated (Fig. 3B). In an *in vitro* GST pull-down assay using recombinant proteins, His-hARD1 directly interacted with GST-β-catenin (Fig. 3C). To examine the mechanism by which hARD1 activates β-catenin, we tested the possibility that β-catenin is activated by hARD1-mediated acetylation and found that the β-catenin acetylation was attenuated by si-ARD1 (Fig. 3D). However, hARD1 did not affect the cellular level or the ubiquitination of β-catenin (Supplementary Fig. S3).

hARD1 directly acetylates β-catenin. hARD1 acetylated β-catenin in HEK293 lysates (Fig. 4A). Moreover, recombinant β-catenin was acetylated by 37°C incubation with recombinant hARD1 but not by 4°C incubation (Fig. 4B), suggesting that hARD1 directly acetylates lysine residues of β-catenin by enzymatic reaction without other cellular components (e.g., p300). Because p300 is also known to activate β-catenin via acetylation (18–20), we test the possibility that p300 is linked with the β-catenin acetylation by hARD1. p300 knockdown attenuated p300 binding to β-catenin but did not affect the association of hARD1 and β-catenin (Fig. 4C), suggesting that p300 is not necessary for hARD1-β-catenin interaction. However, β-catenin acetylation was inhibited noticeably (>60%) by si-ARD1 or si-p300 each and further suppressed by si-ARD1 and si-p300 combination (Fig. 4D). Although hARD1 and p300 bind β-catenin independently, it cannot be ruled out that they cooperatively acetylate β-catenin *in vivo*.

Figure 4. hARD1 acetylates β-catenin independently of p300. **A**, hARD1 acetylated β-catenin in cell extracts. HEK293 cells were transfected separately with 1 μg pARD1 or 1 μg pHA-catenin. Lysates (200 ng protein) containing HA-catenin were incubated with lysates (500, 250, or 100 ng protein) containing expressed hARD1 at 30°C for 2 hours and then precipitated with β-catenin antibody. Total and acetylated β-catenins were identified by immunoblotting with β-catenin and acetyl-lysine antibodies, respectively. *Bottom*, the amounts of hARD1 in the reaction mixture were analyzed by immunoblotting with hARD1 antibody. **B**, hARD1 directly acetylated β-catenin. *Top*, GST-β-catenin was incubated with His-hARD1 and acetyl-CoA at 37°C or 4°C (as a negative control to verify enzymatic reaction), and then the lysine acetylation was identified using anti-acetyl-lysine antibody; *bottom*, Coomassie staining showed proteins present in reaction mixtures. **C**, p300 was not required for hARD1 binding to β-catenin. HEK293 cells were transfected with 40 nmol/L of si-p300 or si-con. hARD1 or p300 was immunoprecipitated with anti-ARD1 or anti-p300 antiserum, and coprecipitation of β-catenin was analyzed using anti-catenin antibody. **D**, hARD1 and p300 both were responsible for β-catenin acetylation. After transfected with hARD1 or/and p300 siRNAs (20 nmol/L each), H1299 cells were treated with 10 mmol/L sodium butyrate. Cell lysates were immunoprecipitated with anti-catenin antibody and immunoblotted with anti-acetyl-lysine or anti-catenin antibody. Protein band intensities were quantified using ImageJ 1.36b image analysis software (NIH, Bethesda, MD). *Bottom*, ratios of acetylated form to total β-catenin and the percentages of β-catenin acetylation inhibited by siRNAs.



Implication and speculation. Wnt signaling plays crucial roles in normal lung development and in lung cancer promotion, similar to its roles in other organs and tumors. In lung cancers, Wnt pathway is found to be aberrantly activated at multiple steps: overexpression of Wnt ligands, up-regulation of β -catenin, and down-regulation of Wnt inhibitors, including Wnt inhibitory factor-1, secreted frizzled-related proteins, and Dickkopf proteins (17). Therefore, each component of the Wnt pathway is reviewed as a potential target for development of new cancer therapy. In the present study, it is suggested that hARD1, as a component of the Wnt pathway to activate β -catenin, is involved in the Wnt signaling-dependent

proliferation of non-small cell lung cancer cells. Indeed, hARD1 siRNA showed antiproliferative activities in lung cancer cells. We thus propose that hARD1 be a novel target for cancer therapy.

Acknowledgments

Received 8/25/2006; revised 9/18/2006; accepted 10/5/2006.

Grant support: Seoul National University College of Medicine Research Fund 2003 and National R&D Program for Cancer Control, Ministry of Health & Welfare Research Fund grant 0520260-2.

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