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Different subcellular localizations and functions of human ARD1 variants

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Abstract. ARD1 is present in various species and has several variants derived from alternative splicing of mRNA. Previously, we reported differential biological functions and cellular distributions of mouse ARD1 (mARD1) variants. However, in comparison to mARD1 variants, human ARD1 (hARD1) variants have been rarely studied. In this study, we characterized a hARD1 variant, hARD1^{131} and investigated its cellular activities. hARD1^{131} mRNA was isolated from HeLa cells and sequenced. Sequence alignment revealed that, compared to hARD1^{235}, the most common form of hARD1, the mRNA sequence encoding hARD1^{131} possesses an altered reading frame due to a 46-bp deletion. Thus, hARD1^{131} and hARD1^{235} differ in their C-terminal regions with a partially deleted acetyltransferase domain at the C-terminus of hARD1^{131}. Moreover, hARD1^{131} and hARD1^{235} showed different subcellular localizations and biological functions. hARD1^{131} was mostly localized in the cell nucleus, whereas hARD1^{235} was primarily localized in the cytoplasm. In addition, hARD1^{235} stimulated cell proliferation by upregulation of cyclin D1, however hARD1^{131} had no influence on cyclin D1 expression and cell growth. Because hARD1^{235} enhances cell proliferation by its autoacetylation activity, we examined the autoacetylation activity of hARD1^{131} and observed that this function was absent in hARD1^{131}. These results suggest that human ARD1 variants have different effects on cell proliferation, which may result from distinct subcellular localizations and autoacetylation activities.

Acknowledgements

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Materials and methods

Reagents and antibodies. Anti-GFP and cyclin D1 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-acetyl-lysine antibody was purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-tubulin was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture. HeLa and 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37˚C and 5% CO_2 in a humid atmosphere.

Plasmid constructions and transfection. To construct expression vectors for human ARD1 variants, ARD1 cDNA was amplified by PCR and sub-cloned into a GFP-tagged pCS2+ vector for cell expression, and a pGEX-4T vector for bacterial induction of the recombinant protein. Transfection was carried out using Lipofectamine (Life Technology, Carlsbad, CA, USA) or Polyfect (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions.

Immunoblotting and immunoprecipitation. Cells were harvested and proteins were extracted using protein lysis buffer (10 mM HEPES at pH 7.9, 40 mM NaCl, 0.1 mM EDTA, 5% glycerol, 1 mM DTT and protease inhibitors). The concentration of extracted protein was measured using a BCA assay. Total cell lysates were resolved by SDS-PAGE and transferred onto nitrocellulose membranes (Amersham Pharmacia Bioscience, Piscataway, NJ, USA). The membrane was probed with a primary antibody followed by a secondary antibody conjugated to horseradish peroxidase, and protein was visualized using the ECL system (Intron Biotechnology, Gyeonggi-do, Korea).

In vitro acetylation assay. Recombinants of GST-hARD1 variants were freshly prepared as previously described (21). ARD1 recombinants were incubated in reaction mixture (50 mM Tris-HCl at pH 8.0, 0.1 mM EDTA, 1 mM DTT, 10% glycerol and 10 mM acetyl-CoA) at 37˚C for 1 h.

Immunofluorescence staining and microscopy. Cells were placed on cover slips then incubated with Hoechst 33342 (Molecular Probes, Eugene, OR, USA) for nucleus staining. Axiovert M200 microscopes (Carl Zeiss, Jena, Germany) were used for immunofluorescence imaging.

Reverse transcription-PCR analysis. Total RNA was extracted using an RNA extraction kit (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized from 2 μg of RNA using an oligo(dt)

primer. Primers used for PCR reactions were as follows: human ARD1, 5'-ATGAACATCCGCAATGCGAG-3' (forward) and 5'-CTCATATCATGGCTCGAGAGG-3' (reverse); cyclin D1, 5'-CTGGCCATGAACTACCTGGA-3' (forward) and 5'-GTC ACACTTGATCACTCTGG-3' (reverse); GAPDH, 5'-ACCAC AGTCCATGCCATCAC-3' (forward) and 5'-TCCACCACCCT GTTGCTGTA-3' (reverse). The PCR reaction was performed for 25 cycles to allow ARD1, cyclin D1 and GAPDH amplification.

Cell proliferation assay. The rate of cell proliferation was measured using a Non-Radioactive Proliferation Assay kit (Promega) according to the manufacturer's instructions. Briefly, cells were seeded into 96-well plates and cultured for three days. Subsequently, 20 μl of substrate solution was added and the cells were incubated for 1 h to allow color development. The absorbance at 492 nm was measured to determine the number of proliferating cells.

Table I. Design of the primer sets for RT-PCR.

Primer	Sequence (5'-3')	Region (nucleotide)
P1 sense	ATGAACATCCGCAATGCCAGG	1-21
P1 antisense	CTAGGAGGCTGAGTCGGAGGC	688-708
P2 sense	AACTTCAATGCCAAATATGTC	301-321
P2 antisense	TCATGGCATAGGCGTCCTCCC	422-442
P3 sense	AGCGGGACCTCACTCAGATGG	443-463
P3 antisense	CTAGGAGGCTGAGTCGGAGGC	688-708

The sequences of P1, P2 and P3 are conserved in $hARD1^{235}$ and $hARD1^{131}$ mRNA sequences. P1 sense and antisense primers correspond to the regions containing the start and stop codons of $hARD1^{235}$, respectively. P2 antisense primer corresponds to the region containing the stop codon of $hARD1^{131}$.

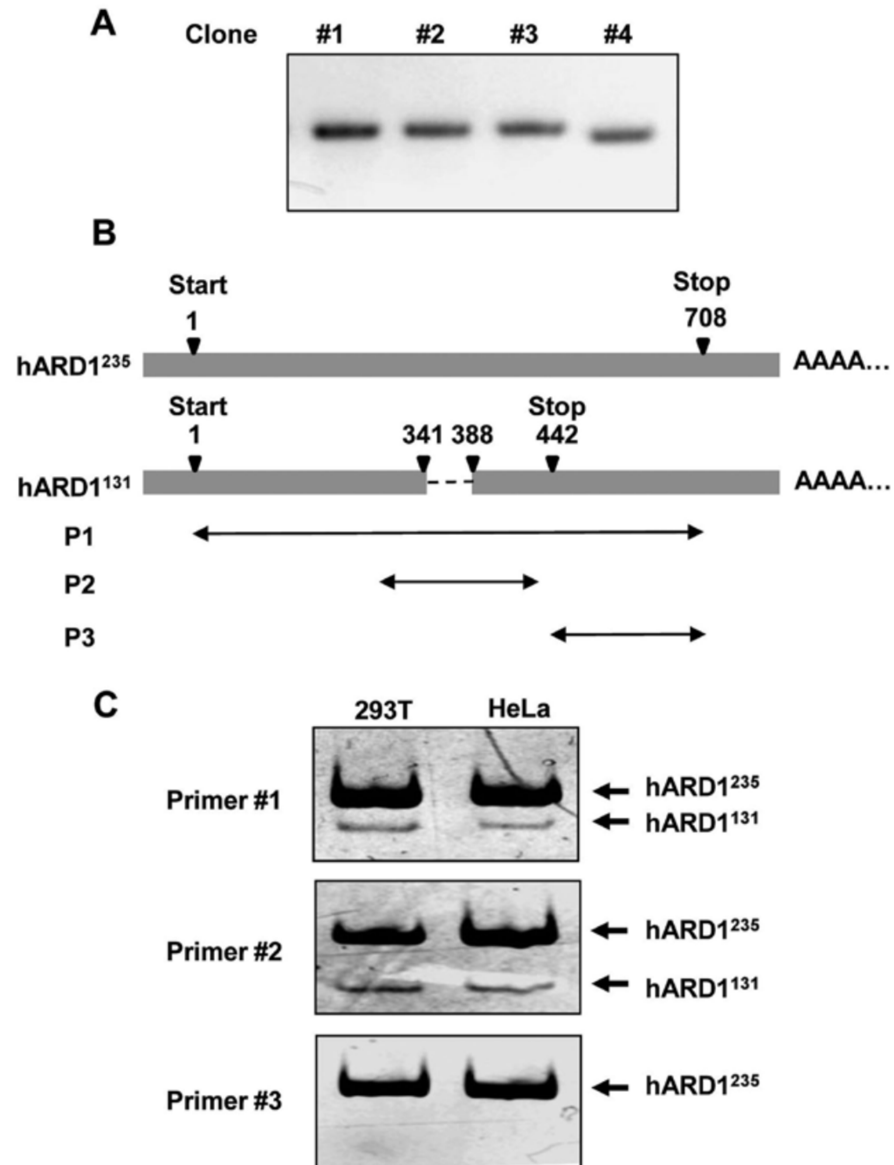

Figure 1. Expression of hARD1 variants in human cell lines. (A) hARD1 sequence was amplified from cDNA reverse transcribed from HeLa cell mRNA. Four clones were analyzed by electrophoresis on agarose gels. (B) cDNA alignment of $hARD1^{235}$ and $hARD1^{131}$. Primers sets P1, P2 and P3 were designed for the detection of $hARD1^{235}$ and $hARD1^{131}$. (C) Total RNA was exacted from 293T and HeLa cells and RT-PCR was performed using primer sets P1, P2 and P3. Expression of $hARD1^{235}$ and $hARD1^{131}$ was analyzed by electrophoresis on DNA polyacrylamide gels.

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h235   ATGAACATCCGCAATGCGAGGCCAGAGGACCTAATGAACATGCAGCACTGCAACCTCCTC 60
h131   ATGAACATCCGCAATGCGAGGCCAGAGGACCTAATGAACATGCAGCACTGCAACCTCCTC 60
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h235   TGCCTGCCCGAGAACTACCAGATGAAATACTACTTCTACCATGGCCTTTCCTGGCCCCAG 120
h131   TGCCTGCCCGAGAACTACCAGATGAAATACTACTTCTACCATGGCCTTTCCTGGCCCCAG 120
       ************************************************************

h235   CTCTCTTACATTGCTGAGGACGAGAATGGGAAGATTGTGGGGTATGTCCTGGCCAAAATG 180
h131   CTCTCTTACATTGCTGAGGACGAGAATGGGAAGATTGTGGGGTATGTCCTGGCCAAAATG 180
       ************************************************************

h235   GAAGAGGACCCAGATGATGTGCCCCATGGACATATCACCTCATTGGCTGTGAAGCGTTCC 240
h131   GAAGAGGACCCAGATGATGTGCCCCATGGACATATCACCTCATTGGCTGTGAAGCGTTCC 240
       ************************************************************

h235   CACCGGCGCCTCGGTCTGGCTCAGAAACTGATGGACCAGGCCTCTCGAGCCATGATAGAG 300
h131   CACCGGCGCCTCGGTCTGGCTCAGAAACTGATGGACCAGGCCTCTCGAGCCATGATAGAG 300
       ************************************************************

h235   AACTTCAATGCCAAATATGTCTCCCTGCATGTCAGGAAGAGTAACCGGGCCGCCCTGCAC 360
h131   AACTTCAATGCCAAATATGTCTCCCTGCATGTCAGGAAGAG------------------- 341
       *****************************************

h235   CTCTATTCCAACACCCTCAACTTTCAGATCAGTGAAGTGGAGCCCAAATACTATGCAGAT 420
h131   ---------------------------ATCAGTGAAGTGGAGCCCAAATACTATGCAGAT 374
                                  *********************************

h235   GGGGAGGACGCCTATGCCATGAAGCGGGACCTCACTCAGATGGCCGACGAGCTGAGGCGG 480
h131   GGGGAGGACGCCTATGCCATGA-------------------------------------- 396
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h235   CACCTGGAGCTGAAAGAGAAGGGCAGGCACGTGGTGCTGGGTGCCATCGAGAACAAGGTG 540
h131   ------------------------------------------------------------

h235   GAGAGCAAAGGCAATTCACCTCCGAGCTCAGGAGAGGCCTGTCGCGAGGAGAAGGGCCTG 600
h131   ------------------------------------------------------------

h235   GCTGCCGAGGATAGTGGTGGGGACAGCAAGGACCTCAGCGAGGTCAGCGAGACCACAGAG 660
h131   ------------------------------------------------------------

h235   AGCACAGATGTCAAGGACAGCTCAGAGGCCTCCGACTCAGCCTCCTAG 708
h131   ------------------------------------------------
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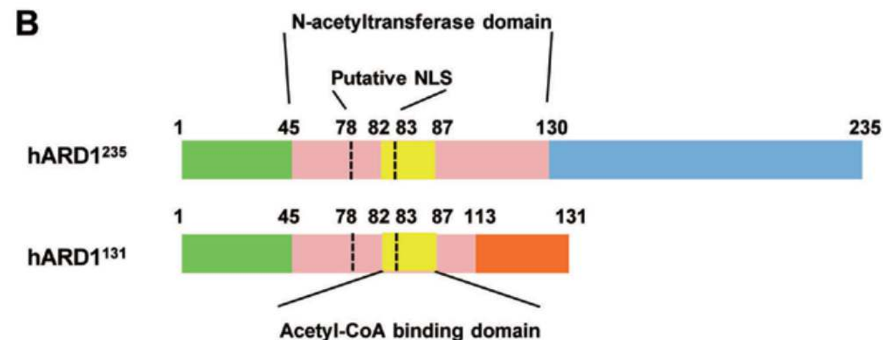

Figure 2. Sequence comparison of human ARD1 (hARD1) variants. (A) Sequence alignment of $hARD1^{235}$ and $hARD1^{131}$. Identical residues are indicated by asterisk. (B) Schematic representation of $hARD1^{235}$ and $hARD1^{131}$ amino acid sequences. The acetyltransferase domain at amino acid 45-130, the putative NLS at amino acid 78-83 and the acetyl-CoA binding domain at amino acid 82-87 are indicated.

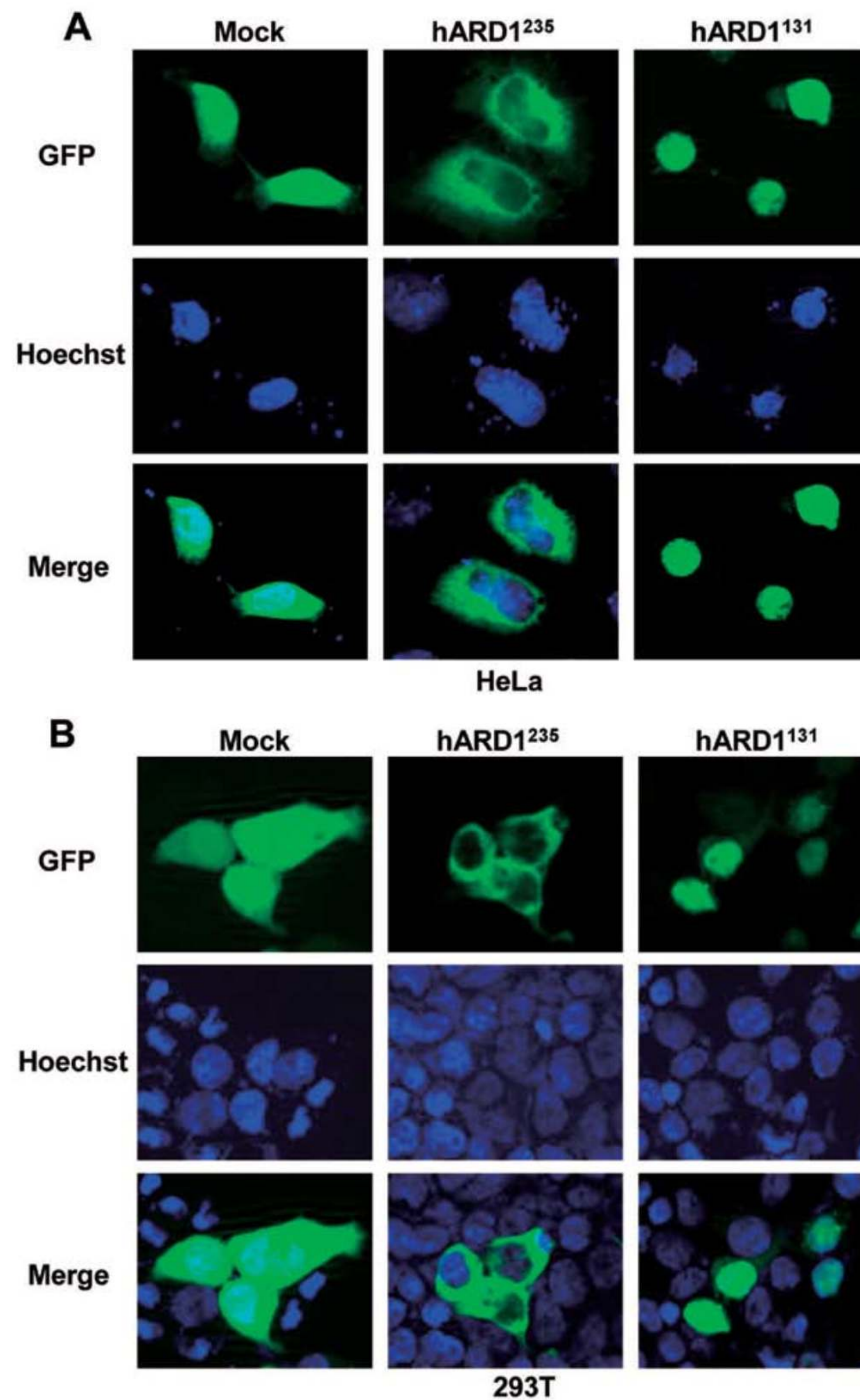

Figure 3. Subcellular localization of hARD1 variants in human cell lines. GFP-tagged plasmids for hARD1 variants were transfected into HeLa cells (A) and 293T cells (B). Nucleus-staining was performed with Hoechst. Localization of ARD1 variants was analyzed and photographed by a fluorescence microscope.

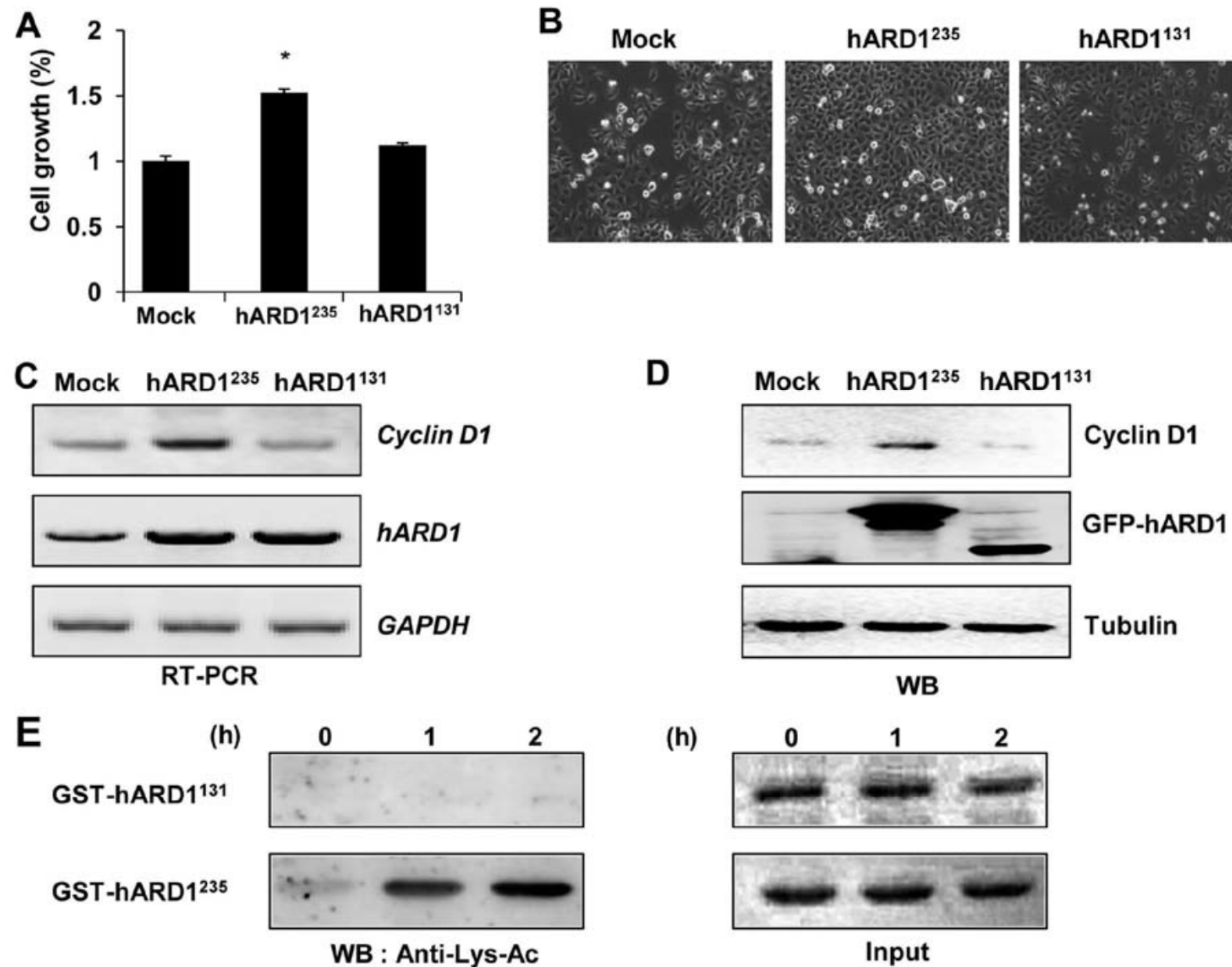

Figure 4. Differential functions of human ARD1 (hARD1) variants in the regulation of cell proliferation. (A) HeLa cells were transfected with GFP-tagged hARD1^{235} and hARD1^{131}, then cell growth was analyzed $^{*}P<0.05$ vs. control. (B) After transfecting cells with plasmids containing hARD1^{235} and hARD1^{131}, cells were grown for 3 days and photographed. (C, D) GFP-tagged hARD1^{235} and hARD1^{131} plasmids were transfected into HeLa cells. Cyclin D1 mRNA and protein levels were analyzed by RT-PCR and western blot, respectively. (E) Purified recombinants for GST-hARD1^{235} and GST-hARD1^{131} were each subjected to the *in vitro* acetylation assay for 1 and 2 h. Acetylated proteins were detected using an anti-acetyl lysine (Lys-Ac) antibody. Total proteins were stained with Coomassie Brilliant Blue (Input).