Distinct and overlapping roles of ARID3A and ARID3B in regulating E2F-dependent transcription via direct binding to E2F target genes

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Abstract. The AT-rich interacting domain (ARID) family of DNA-binding proteins is involved in various biological processes, including the regulation of gene expression during cell proliferation, differentiation and development. ARID3A and ARID3B are involved in chromatin remodeling and can bind to E2F1 and retinoblastoma tumor suppressor protein (RB), respectively. However, their role in regulating E2F target gene expression remains poorly understood. E2F transcription factors are critical regulators of cell cycle progression and are modulated by RB. Herein, putative ARID3-binding sites (BSs) in E2F target genes were identified, including *Cdc2*, *cyclin E1* and *p107*, and it was found that ARID3A and ARID3B bound to these BSs in living cells. The mutation of ARID3 BSs reduced *Cdc2* promoter activity, while ARID3A and ARID3B overexpression increased the promoter activity, depending on both ARID3 and E2F BSs. ARID3B knockdown blocked the transcription of *Cdc2*, *cyclin E1* and *p107* in normal human dermal fibroblasts (NHDFs), whereas the effects of ARID3A knockdown varied depending on the target genes. ARID3B overexpression, but not that of ARID3A, upregulated the transcription of E2F target genes, and activated *cyclin E1* transcription and induced cell death with E2F1 assistance. Finally, ARID3A and ARID3B knockdown attenuated the cell cycle progression of NHDFs and T98G cells, and suppressed tumor cell growth. On the whole, these results indicate that ARID3A and ARID3B play distinct and overlapping roles in E2F-dependent transcription by directly binding to the E2F target genes. The present study provides novel insight into the mechanisms underlying the E2F dysregulation caused by ARID3A and ARID3B overexpression, which may have a significant influence on the progression of tumorigenesis.

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Abbreviations: ARID, AT-rich interacting domain; BSs, binding sites; NHDFs, normal human dermal fibroblasts; RB, retinoblastoma tumor suppressor protein; MEFs, mouse embryonic fibroblasts; MAR, matrix attachment region; siRNA, small interfering RNA; shRNA, short hairpin RNA; MOI, multiplicity of infection; TSS, transcription start site; EdU, 5-ethynyl-2'-deoxyuridine

Key words: AT-rich interacting domain, ARID3A, ARID3B, E2F, transcriptional regulation, E2F target genes, cell proliferation, tumorigenesis

Introduction

The E2F transcription factors are involved in cell cycle progression, DNA synthesis and cellular proliferation, and are key regulators of cell cycle-dependent gene expression. The E2F family contains 8 different genes encoding transcriptional activators and repressors, and their activity is regulated by the pathway that controls the activity of the retinoblastoma tumor suppressor protein (RB), an essential regulator of cell cycle progression, apoptosis, senescence and differentiation. E2F activity is frequently altered in human malignancies, due to the dysregulation of the RB/E2F pathway, i.e., the overexpression of cyclins or cyclin-dependent kinases (CDKs) and the inactivation of RB or CDK inhibitors (1-4).

AT-rich interacting domain 3A (ARID3A/Bright/DRIL1/E2FBP1, hereafter referred to as ARID3A) (5-8) was identified as a B cell-specific transcription factor that transactivates the immunoglobulin heavy chain (IgH) enhancer. ARID3A was also identified as a protein that interacts with E2F1. ARID3B/Bdp/DRIL2 (hereafter referred to as ARID3B) is closely related to ARID3A and was initially identified as a protein that physically interacts with RB (9). ARID3A and ARID3B share a highly conserved ARID DNA-binding domain and REKLES, a common domain required for self-association and tetramerization (6,10-13). ARID3A forms heteromeric complexes with ARID3B through its REKLESb domain. ARID3B predominantly localizes in the nucleus,

while its heterodimerization with ARID3A increases in the nuclear retention of ARID3A that shuttles between the nucleus and cytoplasm (12,13). ARID3A rescues the oncogenic RAS-induced premature senescence in mouse embryonic fibroblasts (MEFs) (8) and immortalizes MEFs, and collaborates with oncogenic RAS to transform MEFs. Furthermore, ARID3A can increase the transcriptional activity of E2F1 (7) and induce cyclin E1, a target of E2F, which in turn triggers escape from senescence and induces malignant transformation. A recent study reported that ARID3A binds to RB in chromatin and sequesters the RB-HDAC1 complex from the E2F1 promoter (14). Similar to ARID3A, ARID3B has also been shown to immortalize MEFs and confer malignancy to MEFs in collaboration with N-Myc oncogene (15). ARID3B expression is increased in human malignancies, including neuroblastoma, ovarian cancer and thyroid cancer (15-17). Furthermore, accumulating evidence has indicated that ARID3A and ARID3B are involved in the regulation of stem cell genes in embryonic stem cells (ESCs) and cancer stem cells (17-21). ARID3B regulates the expression of stemness genes to promote cancer stemness in ovarian and head and neck squamous carcinoma cells (21-26).

ARID3A and ARID3B have similar DNA-binding properties and recognize specific AT-rich DNA sequences (ARID3-binding sites, ARID3 BSs) in the matrix attachment region (MAR) located in the IgH enhancer region (5,9). The authors have previously reported that both ARID3A and ARID3B bind to ARID3 consensus BSs in p53 target genes and activate transcription of p53 target genes (27,28). Although ARID3A and ARID3B have been shown to bind to E2F1 and RB, respectively, their role in regulating E2F target gene expression remains largely unknown. The present study identified putative ARID3 BSs in E2F target genes, including *Cdc2*, *cyclin E1* and *p107*. In addition, the role of ARID3A and ARID3B in E2F-dependent gene expression and cell cycle progression was examined by using normal human cells, normal human dermal fibroblasts (NHDFs).

Materials and methods

Cells and cell culture. Neonatal NHDFs (CC-2511) and 293A (R70507) cells were purchased from Lonza Group AG and Invitrogen; Thermo Fisher Scientific, Inc., respectively. T98G (CRL-1690), H1299 (CRL-5803), and Saos-2 (HTB-85) cells were obtained from the American Type Culture Collection (ATCC). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich; Merck KGaA) supplemented with 10% fetal bovine serum (FBS) (Gibco; Thermo Fisher Scientific, Inc.) and 50 μg/ml gentamicin (Gibco; Thermo Fisher Scientific, Inc.), and incubated at 37˚C in a humidified atmosphere containing 5% CO_2. To induce quiescence in NHDFs the cells were cultured in DMEM containing 0.1% FBS for 48 h, and then either left unstimulated or stimulated with DMEM containing 10% FBS and cultured for an additional 24 h.

Plasmids and transfection. To generate *Cdc2*-Luc reporter plasmids, the *Cdc2* promoter fragments (-848 to +30) containing either wild-type, mutated ARID3 (mARID3), or mutated E2F (mE2F) BSs were synthesized (Genscript Japan Inc.) and then cloned into pGL2 (Promega Corp.). To generate expression vectors for ARID3A and ARID3B short hairpin RNA (shRNA) (pshARID3A and pshARID3B, respectively), double-stranded oligonucleotides targeting ARID3A and ARID3B were cloned into the pcPURU6β (Takara Bio Inc.) vector downstream of the human U6 promoter. The recombinant plasmids were confirmed by DNA sequencing. The shRNA sequences are available upon request. A control shRNA (shCon) expression vector targeting the luciferase gene was obtained from Takara Bio Inc. Expression vectors for Xpress-ARID3A, V5-ARID3B and E2F1 were previously as described (28). T98G, 293A, H1299 and Saos-2 cells were transfected using the Gene Juice transfection reagent (Sigma-Aldrich; Merck KGaA) according to the manufacturer's instructions.

Small interfering RNA (siRNA). Stealth small interfering RNA (siRNA) duplexes targeting ARID3A (5'-AACAGAACUCCUG UGUACAUGAUGC-3') and ARID3B (5'-UUUCCUUUCAGG AUCACCGUCCAGU-3') were purchased from Invitrogen, Thermo Fisher Scientific, Inc. MISSION siRNA universal negative control (Sigma-Aldrich; Merck KGaA) was used for negative control duplexes. NHDFs and T98G cells were reverse transfected with 15 nM siRNA using Lipofectamine RNAiMAX (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions.

Recombinant adenoviruses. Ad-ARID3A, Ad-E2F1 and Ad-con viruses have been previously described (29,30). To generate the Ad-ARID3B virus, pEF-ARID3B was recombined with pDONR221 vector (Invitrogen; Thermo Fisher Scientific, Inc.) using BP clonase enzyme mix (Invitrogen; Thermo Fisher Scientific, Inc.). The resulting ARID3B entry vector was recombined with the destination vector pAd/CMV/V5-DEST (Invitrogen; Thermo Fisher Scientific, Inc.) using the ViraPower Adenoviral Gateway Expression kit (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Amplification, purification and titer determination of the recombinant adenoviruses were performed as previously described (29). Cells were infected at the indicated multiplicity of infection (MOI) in OPTI-MEM (Invitrogen; Thermo Fisher Scientific, Inc.) for 1 h at 37˚C with brief agitation every 5 min, after which the cells were cultured in DMEM supplemented with either 0.1 or 10% FBS.

Reverse transcription-quantitative PCR (RTq-PCR). Total RNA was prepared using the the Direct-zol RNA miniprep kit (Zymo Research Corp.) according to the manufacturer's instructions. Complementary DNA was synthesized from 0.5 μg of total RNA with the ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo Life Science). qPCR was performed on the LightCycler 480 real-time PCR instrument (Roche Applied Science) in triplicate using TaqMan GeneExpression Assays for *Cdc2*, *cyclin E1*, *p107* and 18S rRNA (Hs00938778_m1, Hs01026536_m1, Hs00765700_m1, and Hs03003631_g1, respectively) (Applied Biosystems; Thermo Fisher Scientific, Inc.) and the LightCycler 480 Probes Master kit (Roche Applied Science) under the following conditions: Initial denaturation at 95˚C for 2 min, followed by 50 cycles at 95˚C for 15 sec and 60˚C for 60 sec, according to the manufacturer's instructions. The relative expression of mRNA, normalized to 18S rRNA, was calculated using the $\Delta\Delta$Cq method (31).

Semiquantitative RT-PCR. PCR was performed with GoTaq Hot Start Green Master Mix (Promega Corp.) under the following conditions: Initial denaturation at 95˚C for 1 min, followed by 28 cycles for GAPDH, 30 cycles for ARID3A, 34 cycles for ARID3B at 95˚C for 40 sec, 55˚C for 40 sec, 72˚C for 10 sec, with a final extension at 72˚C for 3 min. PCR products were resolved on 2% agarose gels, stained with ethidium bromide, and visualized by UV transillumination. The primer sequences used for RT-PCR are presented in Table SI.

Electrophoretic mobility shift assays (EMSAs). Reticulocyte lysate was programmed with Xpress-ARID3A or V5-ARID3B cDNAs using the TNT Coupled Reticulocyte Lysate System (Promega Corp.) and then incubated with ^{32}P-labeled double-stranded oligonucleotides in 10 μl of a reaction mixture containing 20 mM HEPES (pH 7.9), 40 mM KCl, 6 mM $MgCl_2$, 1 mM EGTA, 0.1% Nonidet P-40, 0.2 mM DTT, 10% glycerol, 1 μg of Poly(dI-dC), and 30 μg of bovine serum albumin, and resolved using electrophoresis as described previously (32). The reaction mixtures were incubated for 20 min at 25˚C and resolved in a 5% polyacrylamide gel containing 5% glycerol in 0.5X TBE (50 mM Tris-borate, 1 mM EGTA) for 2 h at 300 V at 4˚C.

Chromatin immunoprecipitation (ChIP) assays. T98G cells were cross-linked with 1% formaldehyde for 10 min in PBS (pH 7.4). Nuclei preparation and chromatin digestion were performed using the SimpleChIP Enzymatic Chromatin IP kit (Cell Signaling Technology, Inc.) according to the manufacturer's instructions. Nuclei that were digested with micrococcal nuclease were sonicated with a sonicator (VP-5S; Taitec Corp.) in wet ice (3 cycles of a 20-sec pulse with a 30-sec interval). DNA concentration of soluble chromatin was measured using the Qubit Fluorometer (Invitrogen; Thermo Fisher Scientific, Inc.). Chromatin immunoprecipitation was performed using the ChIP Kit Magnetic-One Step kit (Abcam) according to the manufacturer's instructions. Briefly, soluble chromatin (5 mg) was incubated with an immunoglobulin G (IgG), anti-ARID3A, or anti-ARID3B antibodies. qPCR analysis was performed using the BRYT Green and GoTaq qPCR Master Mix (Promega Corp.) on the LightCycler 480 real-time PCR instrument. For input control, 2% of the chromatin sample was amplified. Data were calculated using the ΔΔCq method (31) and presented as fold enrichment relative to the input.

Luciferase assay. For transient reporter assays, T98G cells in 12-well plates were co-transfected with 50 ng of reporter constructs and 10 ng of pRL-TK (Promega Corp.), along with 10 ng of E2F1, or 100 ng of ARID3A or ARID3B expression vectors by GeneJuice Transfection Reagent (Sigma-Aldrich; Merck KGaA). The total amount of transfected DNA was adjusted to 500 ng per well by adding control vectors. Cells were cultured for 48 h in DMEM supplemented with either 0.1 or 10% FBS before reporter assays. According to the manufacturer's instructions, luciferase and *Renilla* luciferase activities were measured using the Dual-Luciferase reporter assay system (Promega Corp.). The relative Firefly luciferase activities were normalized to those of the control *Renilla* luciferase.

Western blot analysis and immunoprecipitation. Whole-cell extract preparation and immunoprecipitation and the western blot analysis procedures were performed as previously described (32). T98G cells were collected, lysed in 5 times packed cell volume of whole cell extraction buffer [50 mM Tris Cl (pH 8.0)/350 mM NaCl/0.5% Nonidet P-40/10% (vol/vol) glycerol/1 mM dithiothreitol/proteinase inhibitors (Complete; Roche Biochemicals,), kept on ice for 60 min, and centrifuged at 14,000 x g for 10 min at 4˚C. The protein concentration of the supernatant was measured using Bio-Rad protein assay kit (Bio-Rad Laboratories, Inc.). A total of 80 μg of the lysates was loaded onto an SDS-PAGE gel (7.5 or 10%), electrophoresed, transferred onto a polyvinylidene difluoride membrane, blocked with 5% skimmed milk in TBS-0.1% Tween-20 buffer (TBST) at 4˚C overnight, and incubated with the following primary antibodies at 4˚C overnight: Rabbit polyclonal anti-ARID3A (1:3,000; ref. 29;), anti-ARID3B (1:3,000; A302-564A; Bethyl Laboratories), anti-E2F-1 (1:3,000; sc-193; Santa Cruz Biotechnology, Inc.) antibodies, and mouse monoclonal anti-Xpress (1:5,000; R910-25; Invitrogen, Thermo Fisher Scientific, Inc.), anti-V5 (1:5,000; 37-7500; Invitrogen, Thermo Fisher Scientific, Inc.), anti-Cdc2 p34 (1:2,000; sc-54; Santa Cruz Biotechnology, Inc.), anti-cyclin E (1:2,000; sc-247; Santa Cruz Biotechnology, Inc.), anti-p107 (1:3,000; sc-250; Santa Cruz Biotechnology, Inc.) and anti-β-actin (1:1,5,000; A1978; Sigma-Aldrich; Merck KGaA) antibodies. The membrane was washed with TBST 6 times, reacted with Amersham ECL HRP-Linked anti-rabbit (1:50,000; NA934; GE Healthcare) or anti-mouse (1:50,000; NA931; GE Healthcare) antibody at room temperature for 60 min, washed with TBST buffer 6 times, and visualized by Amersham ECL Prime Western blotting detection reagent (GE Healthcare).

Caspase-3/7 assays. Cells were infected with adenoviruses and then cultured in DMEM containing 0.1% FBS for 48 h, after which caspase-3/7 activity was determined using the Caspase-Glo 3/7 assay kit (Promega Corp.) according to the manufacturer's instructions.

EdU (5-ethynyl-2'-deoxyuridine) incorporation. NHDFs were reverse transfected with siRNA on 4-chamber glass slides (BD Falcon, Thermo Fisher Scientific, Inc.) and cultured in DMEM containing 0.1% FBS for 48 h. The cells were induced to re-enter cell cycle by adding DMEM containing 10% FBS. The cells were cultured for 24 h, and EdU was added at a 10 μM final concentration 4 h before harvesting the cells. The detection of incorporated EdU and Hoechst 33342 staining was performed using the Click-iT EdU Alexa Fluor 488 Imaging Kit (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions.

Flow cytometric analysis. T98G cells reverse transfected with siRNA were subjected to serum starvation in DMEM/F-12 medium (Sigma-Aldrich; Merck KGaA) without FBS for 60 h, then stimulated with fresh DMEM medium containing 10% FBS. After 24 h, the cells were fixed in 70% ethanol on ice, re-suspended in PBS containing RNase A (100 μg/ml; Sigma-Aldrich; Merck KGaA), propidium iodide (50 μg/ml) (Sigma-Aldrich; Merck KGaA) and 0.05% Triton X-100, and then incubated for an additional 15 min at 37˚C. Cellular DNA content was detected using a MoFlo XDPflow cytometer

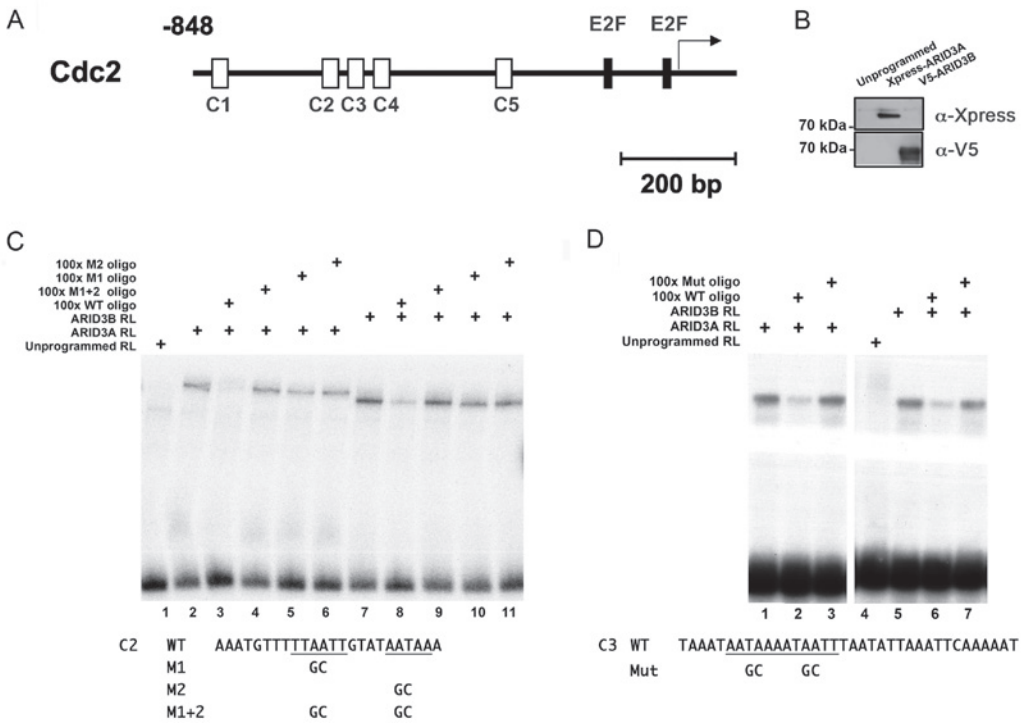

Figure 1. Binding of ARID3A and ARID3B to the AT-rich sequences corresponding to ARID3 BSs on the *Cdc2* promoter. (A) Schematic of the human *Cdc2* promoter. Open and filled boxes represent putative ARID3 (C1-C5) and E2F BSs, respectively. The bent arrow indicates the TSS. (B) Western blot analysis of the unprogrammed and programmed reticulocyte lysates with anti-Xpress or anti-V5 antibodies. (C and D) Binding of ARID3A and ARID3B to ARID3 BSs on the *Cdc2* promoter. Unprogrammed reticulocyte lysate (unprog. RL) or lysates programmed with cDNAs for either Xpress-tagged ARID3A (Xpress-ARID3A RL) or V5-tagged ARID3B (V5-ARID3B RL) were incubated with ^{32}P-labeled oligonucleotides containing putative ARID3 BSs (C2 and C3) (C and D, respectively). Competitive EMSA was performed by adding a 100-fold molar excess of the indicated unlabeled WT or mutant oligonucleotides to reaction mixtures. Sequences of WT probes and the substituted nucleotides in mutated ARID3 BSs are shown below the images. ARID, AT-rich interacting domain; TSS, transcription start site; BSs, binding sites; WT, wild-type.

(Beckman Coulter Inc.) and analyzed using FCS Express 7 software (De Novo Software Inc.).

Colony formation assay. Colony formation was assayed as previously described (33). Briefly, since puromycin susceptibility varies between cell lines, its optimal concentration of puromycin for 293A and Saos-2 cells were initially determined (2.5 μg/ml for the 293A cells and 1 μg/ml for the Saos-2 cells). The cells were transfected with 5 μg plasmid DNA consisting of either a control, pshARID3A, or pshARID3B vectors, along with a vector expressing the puromycin resistance gene at a ratio of 3:1, passaged at a split ratio of 1:3 48 h following transfection, and then selected with puromycin for 3 weeks. Puromycin-resistant colonies were stained with Giemsa for 10 min at room temperature, and then visualized using a GF-X900 scanner (Epson Inc.).

Statistical analysis. Results are presented as the means ± standard deviation. Statistical tests were analyzed using both one-way and two-way ANOVA followed by Tukey's multiple comparisons test, using GraphPad Prism7 software (GraphPad Software, Inc.). P-values <0.05 were considered to indicate statistically significant differences.

Results

Binding of ARID3A and ARID3B proteins to ARID3 BSs on the Cdc2 promoter in vitro. To investigate whether ARID3A and ARID3B are directly involved in E2F target gene expression, the present study sought to identify BSs for ARID3A and ARID3B in E2F target genes. Both ARID3 proteins have been shown to bind to the consensus ARID3A/Bright DNA-binding sites (ARID3 BSs) that contain a core hexamer ((G/A)AT(T/A)AA) within a region of over 12 bp of AT/ATC-rich sequences, together with a second AT dimer located near the hexamer (5,9,27,28). Additionally, ARID3A has been implicated in spatially distant interactions with regulatory elements (5). Herein, a cluster of ARID3 consensus BSs (C1-C5, containing 7 core hexamers) was found between -833 and -303 upstream the transcription start site (TSS) of the human *Cdc2* gene (Table I and Fig. 1A).

To determine whether ARID3A and ARID3B bind to these sequences, we performed EMSAs using oligonucleotide probes corresponding to C2 and C3 (-620 to -597 and -589 to -554, respectively) with recombinant proteins produced by rabbit reticulocyte lysates programmed with Xpress-ARID3A and V5-ARID3B cDNAs (Fig. 1B). The incubation of ARID3A and ARID3B lysates with C2 and C3 probes, each containing 2 core hexamers, generated shifted bands, whereas the unprogrammed lysate did not (Fig. 1C and D, respectively). Competitive EMSA experiments revealed that the addition of a 100-fold molar excess of unlabeled oligonucleotides corresponding to the wild-type ARID3 BS inhibited the interaction between ARID3A/ARID3B proteins and both C2 and C3 sites. By contrast, competition with unlabeled oligonucleotides in which both hexamers had been mutated did not affect ARID3A/ARID3B binding to both sites. Furthermore,

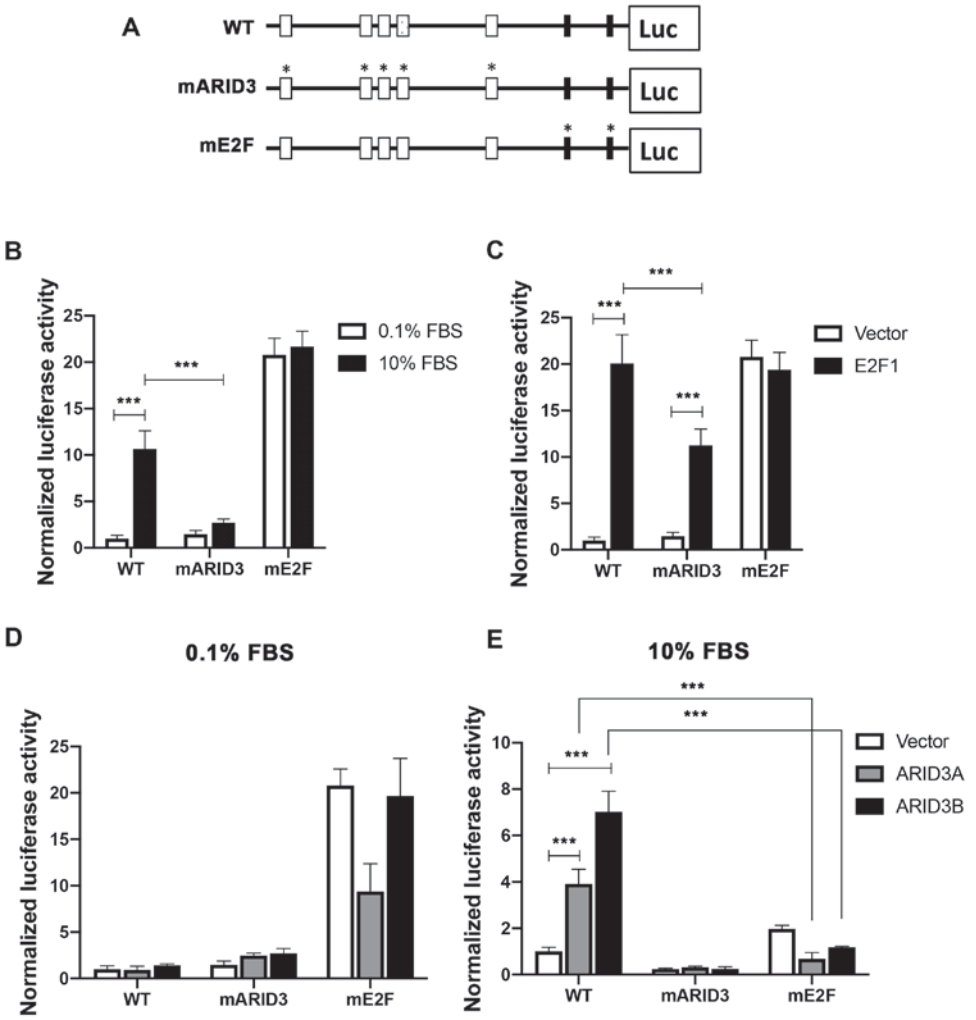

Figure 2. ARID3A and ARID3B activate the *Cdc2* promoter depending on both ARID3 and E2F BSs in transient reporter assays. (A) Schematic representation of the *Cdc2* promoter-reporter construct. Open and filled boxes represent ARID3 and E2F BSs, respectively. Asterisks indicate the mutated ARID3 or E2F BSs. (B and C) Luciferase activity assay of T98G cells transiently transfected with the indicated *Cdc2* reporter constructs. (D and E) T98G cells transiently transfected with the indicated *Cdc2* reporter constructs were subjected to reporter assays. Data represent the average of three independent experiments, each performed in duplicate and are shown as the means ± standard deviation (SD). ***P<0.001. ARID, AT-rich interacting domain; BSs, binding sites.

ARID3A/ARID3B failed to bind to the C2 probe in which only either one of the hexamers had been mutated (M1 and M2), indicating that the binding to one hexamer depended on the interactions with the other one (Fig. 1C). Consistent with previous studies, these results indicate that ARID3A and ARID3B bind to ARID3 BSs in the Cdc2 promoter with similar DNA-binding properties *in vitro* (5,9,27,28).

ARID3A and ARID3B activate the Cdc2 promoter depending on both ARID3 and E2F BSs in transient reporter assays. To investigate the role of ARID3 BSs in regulating the *Cdc2* promoter activity, a luciferase reporter gene driven by the Cdc2 promoter (-848 relative to the transcription start site, WT) was constructed and mutations were introduced into core hexamers in ARID3 BSs in the reporter construct (mARID3) (Fig. 2A). Mutations were also introduced to the 2 E2F-binding sites (E2F-BSs), corresponding to positive- and negative-acting E2F elements (mE2F) (34,35). The T98G cells transfected with the WT or mutant reporter constructs were incubated under serum-starved conditions, and then either left unstimulated or stimulated with serum prior to the reporter assays. The WT *Cdc2* reporter activity increased following serum stimulation, whereas the mutations in the ARID3 BSs impaired the reporter activity (a 74.8% reduction relative to the WT control) (Fig. 2B). To examine the effects of E2F1 overexpression on the mutations of ARID3 BSs in the *Cdc2* promoter, the reporter constructs were co-transfected with either an empty or E2F1 expression vector under serum-starved conditions to reduce endogenous E2F activity. E2F1 overexpression led to a 20- and 10-fold activation of the reporter activity of WT and mARID3 reporters, respectively, indicating that ARID3 BSs are not essential for E2F1 to transactivate the *Cdc2* promoter; however, ARID3 BS mutations (mARID3) resulted in a reduction (43.9%) in the reporter activity relative to the WT (Fig. 2C). These results indicate that ARID3 BSs play an important role in activating the *Cdc2* promoter in response to endogenous and ectopic E2F activity. By contrast, the E2F BS mutation increased the *Cdc2* promoter activity in quiescent cells, which was not altered in either serum-stimulated or E2F1-transfected cells, in line with previous findings demonstrating that the E2F BSs can mediate both the activation and repression of the *Cdc2* promoter (34,35).

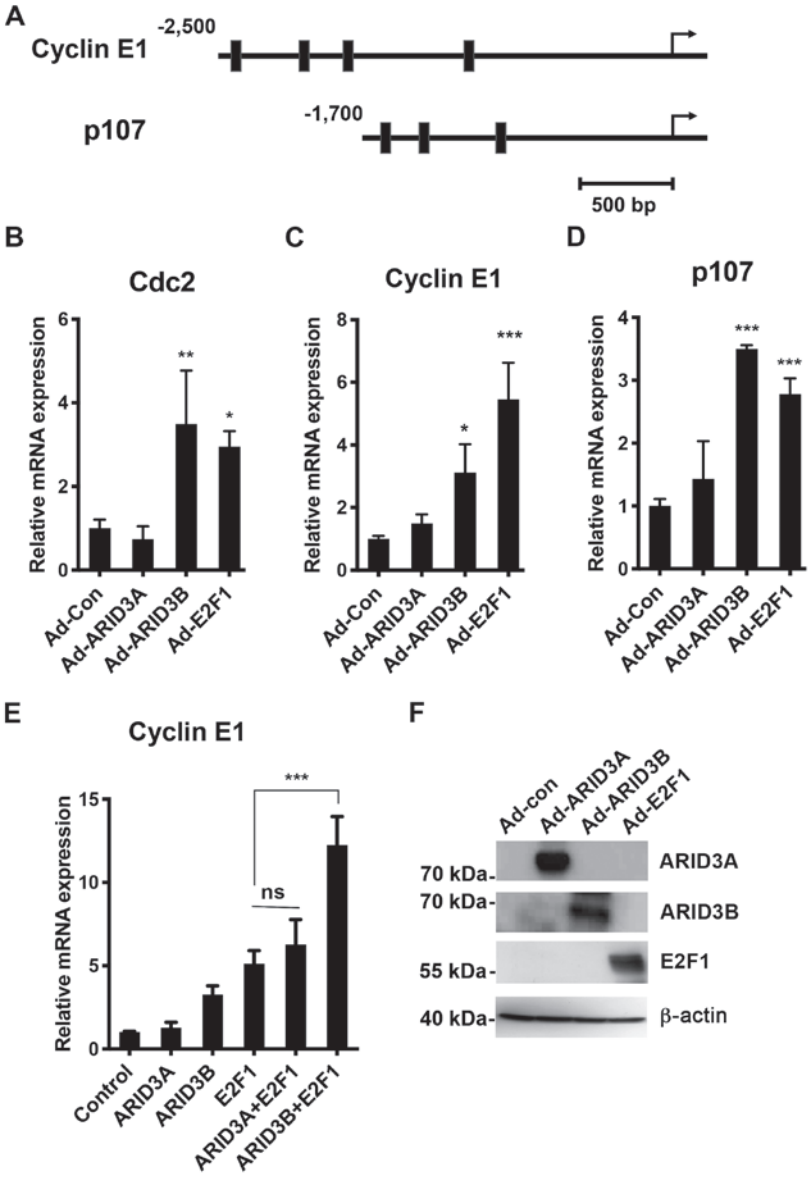

Figure 3. ARID3B overexpression induces the transcription of E2F-responsive genes. (A) Schematic representation of the *cyclin E1* and *p107* promoter. Filled boxes represent ARID3 BSs. (B-F) NHDFs were cultured in DMEM containing 0.1% FBS for 48 h to induce quiescence. (B-D) NHDFs were infected with the indicated viruses at 80 MOI (Ad-Con, Ad-ARID3A and Ad-ARID3B) or 30 MOI (Ad-E2F1). (E) NHDFs were infected with the indicated combination of the viruses at 60 MOI (Ad-Con, Ad-ARID3A and Ad-ARID3B) and 15 MOI (Ad-E2F1). (B-E) RT-qPCR analysis for the indicated transcripts at 24 h post-infection. Data are shown as the means ± SD (n=3). *P<0.05, **P<0.01, ***P<0.001 compared with the control group (Ad-Con). (F) Western blot analysis for Xpress-tagged ARID3A, V5-tagged ARID3B and E2F1 at 48 h post-infection. β-actin was used as the loading control. ARID, AT-rich interacting domain; NHDFs, normal human dermal fibroblasts; MOI, multiplicity of infection.

To examine the effects of the ectopic expression of ARID3A and ARID3B on the *Cdc2* promoter, cells were co-transfected with the reporter constructs, along with empty, ARID3A, or ARID3B expression vector, under either quiescent or growing conditions. Ectopic ARID3A and ARID3B increased the WT promoter activity (3.8- and 7.2-fold, respectively) in growing cells, but not in quiescent cells, and mutations in ARID3 BSs abolished their transactivation activities (Fig. 2D and E). Of note, the ectopic expression of ARID3A and ARID3B did not transactivate the mE2F promoter in both growing and quiescent cells. Furthermore, they failed to activate the ARID3 BS-containing Cdc2 promoters (WT and mARID3) under quiescent conditions, where endogenous E2F activities are low, indicating that ARID3A and ARID3B activate the Cdc2 promoter in an E2F-dependent manner.

ARID3B overexpression induce the transcription of E2F target genes. The present study then determined whether ARID3A and ARID3B can transactivate *Cdc2* expression in NHDFs using recombinant adenoviruses expressing ARID3A, ARID3B and E2F1 (Ad-ARID3A, Ad-ARID3B and Ad-E2F1, respectively). The infection of Ad-ARID3A or Ad-ARID3B exerted no or minimal effects on *Cdc2* expression in exponentially growing NHDFs with a high endogenous E2F activity (data not shown). Therefore, serum-starved quiescent NHDFs were infected with these adenoviruses and subjected to RT-qPCR analysis at 24 h post-infection. As shown in Fig. 3, the overexpression of ARID3B and E2F1 activated *Cdc2* expression. The *cyclin E1* and *p107* genes were further examined, which have been shown to be regulated by E2F via E2F BSs on these genes (36-38). Clusters of ARID3 BSs were found on the *cyclin E1* and *p107* promoters

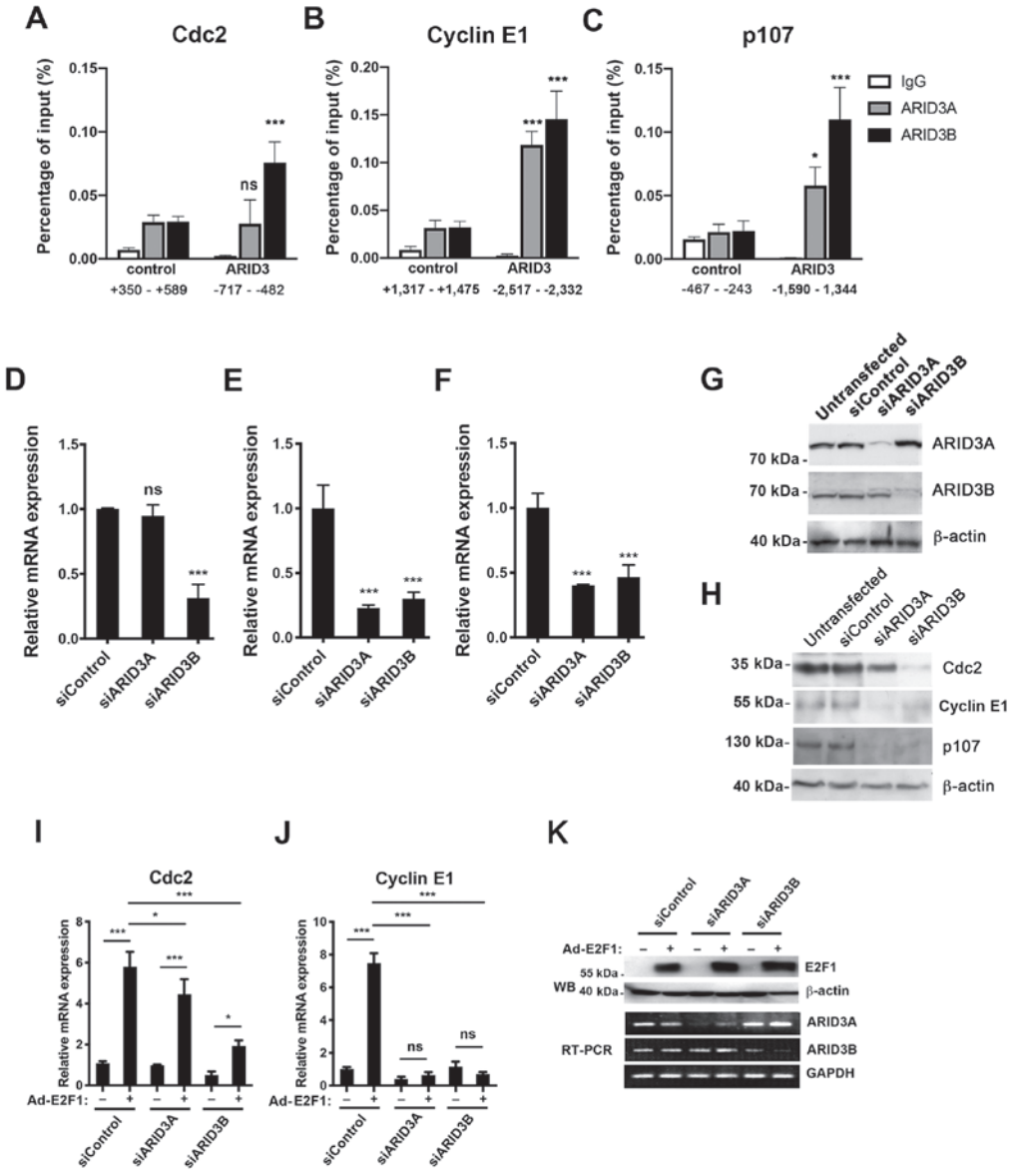

Figure 4. Roles of ARID3A and ARID3B in E2F target gene expression. (A-C) Binding of ARID3A and ARID3B to E2F target genes in living cells. T98G cells were fixed and processed for ChIP assay using control IgG, anti-ARID3A, or anti-ARID3B antibodies. Signals amplified by the control and ARID3 primers that cover the putative ARID3 BSs were measured using qPCR. (D-G and I-K) The knockdown of ARID3A and ARID3B inhibited transcription of E2F target genes. NHDFs were transfected with either control, ARID3A, or ARID3B siRNA (siControl, siARID3A, and siARID3B, respectively) (D-F). RT-qPCR analysis of the expression levels of the indicated transcripts at 48 h post-transfection (D-F). (G) Lysates were immunoprecipitated with ARID3A or ARID3B antibodies, followed by western blot analysis with the same antibodies. β-actin was used as a control. (H) The ARID3A and ARID3B knockdown inhibited the expression of E2F target gene products. T98G cells were transfected with the indicated siRNA, followed by western blot analysis to determine the expression levels of the indicated proteins at 48 h post-transfection. (I-K) ARID3B and ARID3A knockdown inhibited *Cdc2* and *cyclin E* expression induced by E2F1. NHDFs were transfected with either control, ARID3A, or ARID3B siRNA. At 48 h following transfection, the cells were infected with either Ad-Con or Ad-E2F1 and cultured in DMEM containing 0.1% FBS for an additional 24 h. (I and J) RT-qPCR analysis for expression levels of *Cdc2* and *cyclin E1* and transcripts. (K) Western blot analysis for E2F1 at 24 h post-infection. β-actin was used as the loading control (upper panel). Semiquantitative RT-PCR analysis of ARID3A and ARID3B transcripts at 24 h post-infection. GAPDH was used as an internal control (lower panel). Data represent the average of 3 independent experiments, each performed in duplicate and are shown as mean ± SD. *P<0.05, ***P<0.001; ns, not significant. ARID, AT-rich interacting domain; NHDFs, normal human dermal fibroblasts.

(-2,481 to -1,105 and -1,568 to -931, respectively). Similar to that observed in *Cdc2*, the ectopic expression of ARID3B and E2F1 transactivated *cyclin E1* and *p107* expression under quiescent conditions. Furthermore, ARID3B cooperated with E2F1 to synergistically activate *cyclin E1* transcription (Fig. 3E), indicating that ARID3B can also enhance E2F1 transcriptional activity. Under quiescent conditions, ARID3B activated endogenous *Cdc2* gene expression, but not in transient reporter assays (Fig. 2E), suggesting that ARID3B may play an important role in expression of genes integrated into chromatin structure, as previously reported for ARID3A (39). By contrast, no apparent upregulation was detected following Ad-ARID3A infection in quiescent NHDFs, which may be due to cytoplasmic sequestration of exogenous ARID3A in quiescent cells (12).

Binding of ARID3A and ARID3B to E2F-responsive genes in living cells. To confirm the implication of ARID3A and ARID3B in the regulation of E2F-responsive genes in living cells, ChIP assays we performed using the T98G cells. As shown in Fig. 4A, the cross-linked *Cdc2* promoter-ARID3B

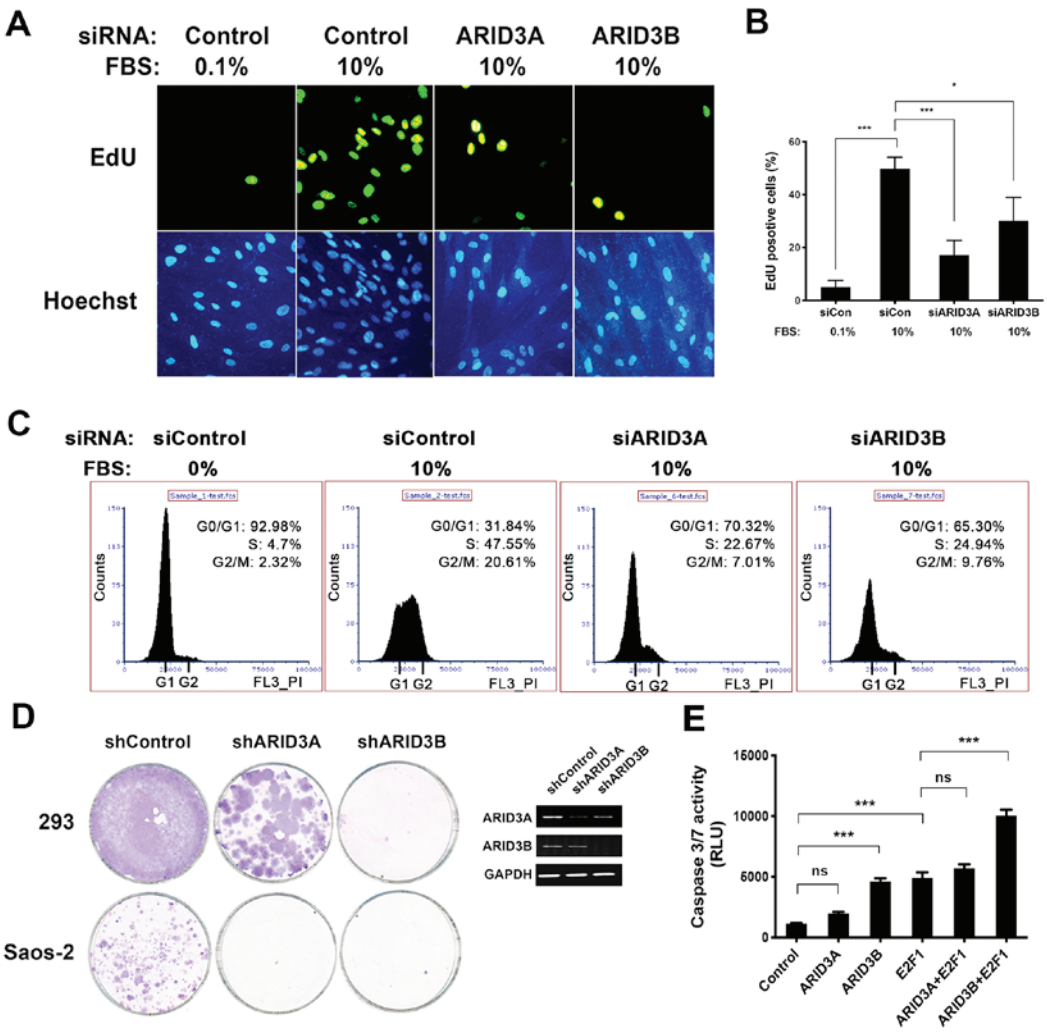

Figure 5. Role of ARID3A and ARID3B in cell proliferation and death. (A and B) The knockdown of ARID3A and ARID3B inhibited DNA synthesis. NHDFs were reverse transfected with the indicated siRNA. At 16 h following transfection, the cells were cultured in DMEM containing 0.1% FBS for 48 h, after which the cells were either left unstimulated or stimulated with 10% FBS and cultured for an additional 24 h. EdU was added 4 h before harvesting the cells. (A) Cells were visualized for EdU incorporation or DNA staining with Hoechst 33342 using fluorescence microscopy. (B) Quantification of EdU incorporation. More than 200 Hoechst 33342-stained nuclei per sample were scored. Data represent the mean percentages of EdU-positive cells obtained from three independent experiments. Data are shown as means ± SD. (C) The ARID3A and ARID3B knockdown inhibited cell cycle progression. T98G cells reverse transfected with the indicated siRNA were serum-starved for 60 h, after which they were either left unstimulated or stimulated with 10% FBS and cultured for an additional 24 h, followed by flow cytometric analysis. (D) ARID3A and ARID3B knockdown inhibited tumor cell growth. 293 and Saos-2 cells were transfected with vectors expressing either control shCFF, shARID3A, or shARID3B and then cultured in the presence of puromycin for three weeks. Images of plates showing colony formation assays in 293 and Saos-2 cells. Puromycin-resistant colonies were visualized using Giemsa staining (left panel). Semi-quantitative RT-PCR for ARID3A and ARID3B in H1299 cells 48 h following transfection. GAPDH was used as an internal control (right panel). (E) NHDFs were infected with the indicated combination of adenoviruses at 80 MOI and then cultured in DMEM containing 0.1% FBS. At 48 h post-transfection, cells were subjected to caspase-3/7 analysis. Data present the average of 3 independent experiments, each performed in duplicate, and are shown as the means ± SD. *P<0.05, ***P<0.001; ns, not significant. ARID, AT-rich interacting domain; NHDFs, normal human dermal fibroblasts.

complexes immunoprecipitated with the antibody against ARID3B, but not with the control IgG, were detected by qPCR amplification with primers spanning the ARID3 BSs (-717 to -482) on the *Cdc2* gene. The levels of ARID3B complexes were significantly higher than those of control ChIPs using primers spanning a region of the *Cdc2* gene which recognizably lacks ARID3 BSs (+350 to +589). However, significantly higher ARID3A complexes were not detected with the ARID3 BS primers than the control primers. A significantly higher ARID3A and ARID3B binding to the *cyclin E1* and *p107* promoters was detected with primers spanning the ARID3 BSs (*cyclin E1*; -2,517 to -2,332, *p107*; -1,590 to -1,344, respectively), compared to the control primers (Fig. 4B and C). Lower levels of ARID3A binding were detected on the *p107* promoters compared to that of ARID3B. These results suggested that the role of ARID3A and ARID3B in controlling gene expression may differ depending on the E2F target genes.

ARID3B knockdown reduces the transcription of E2F-responsive genes. To examine the role of ARID3A and ARID3B in the expression of E2F target genes, the effects of the siRNA-mediated knockdown of endogenous ARID3A or ARID3B on the expression of E2F target genes were examined. The NHDFs were transfected with either control siRNA (siCon), specific siRNA against ARID3A (siARID3A), or

Table I. Locations of the AT-rich sequences that matched consensus ARID3-binding sites in humans.

Cdc2 promoter designation	5'-Sequence-3'	Position
C1	CTTAAATAT**AATTAA**AACACAAAAATTCACAATTCTAT	-796 to -833
C2	AAATGTTT**TTAATT**GTAT**AATAAA**	-620 to -597
C3	TAAAT**AATAAAATAATT**TAATATTAAATTCAAAAAT	-589 to -554
C4	ATATAAAT**AATAAA**TTTTCCTTTACATTTTT	-531 to -501
C5	TATTTAGAGTAT**AATAAA**TTTGAA	-326 to -303

The core hexamers and AT dimers are indicated in bold and underlined font, respectively. Numbers indicate nucleotide positions relative to the transcription start site.

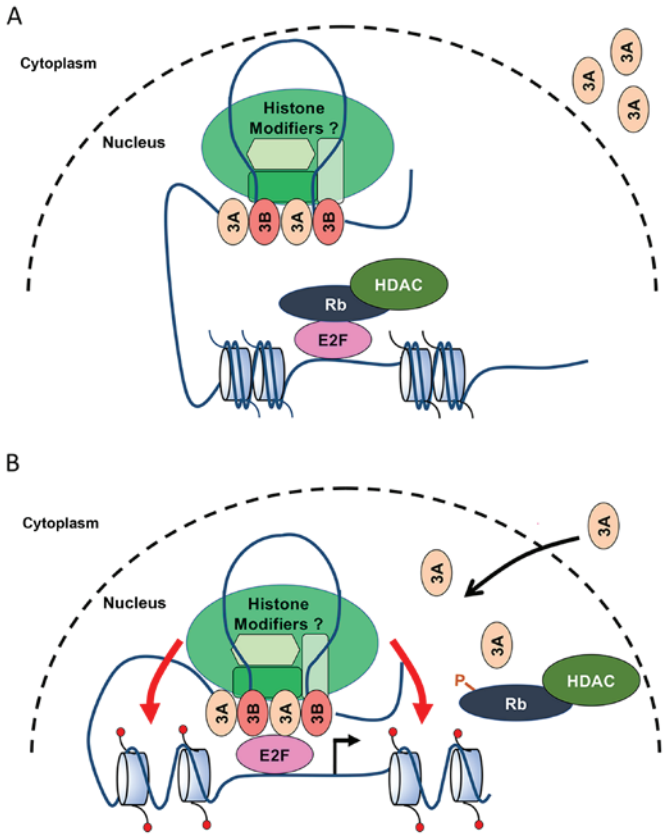

Figure 6. Model illustrating the possible role of ARID3A and ARID3B in regulating E2F-dependent transcription. ARID3A and ARID3B form a hetero-tetramer and bind to clusters of ARID3 BSs in the E2F target genes. (A) Quiescent cells. ARID3 BSs are dispensable for Rb-dependent transcriptional repression, and their role in the repression remains unclear. (B) Proliferating cells. ARID3A and ARID3B may promote E2F target gene expression by bringing ARID3 BSs close to E2F BSs thereby allowing E2Fs to interact with histone-modifying complexes. While ARID3B is localized in the nucleus, ARID3A shuttles between the nucleus and the cytoplasm during the S phase but not quiescence. See text for details. 3A, ARID3A; 3B, ARID3B. ARID, AT-rich interacting domain; BSs, binding sites; Rb, retinoblastoma; HDAC, histone deacetylase.

ARID3B (siARID3B). The RNA levels of these genes were examined by RT-qPCR. As shown in Fig. 4D-G, ARID3B knockdown suppressed the transcription of all tested E2F target genes, whereas that of ARID3A inhibited the transcription of *cyclin E1* and *p107*, but not that of *Cdc2*, in line with the results of the ChIP assays (Fig. 4A-C), and western blot analysis of the E2F target gene products following ARID3A or ARID3B knockdown in T98G cells (Fig. 4H).

To further examine the difference between ARID3A and ARID3B in controlling the expression of E2F target genes, the effects of ARID3A and ARID3B knockdown on the E2F1-induced expression of *Cdc2* and *cyclin E1* were compared, the latter of which is a crucial target for ARID3A to rescue cellular senescence in MEFs (8). NHDFs that had been transfected with siCon, siARID3A, or siARID3B for 48 h were infected with Ad-con or Ad-E2F1 and then cultured for an additional 24 h under serum-starved conditions (Fig. 4J). Both ARID3A and ARID3B knockdown (Fig. 4K) blocked cyclin E1 transcription following Ad-E2F1 infection, whereas ARID3A knockdown inhibited Cdc2 transcription to a lesser extent than ARID3B knockdown did (Fig. 4I and J). These results suggest that ARID3A and ARID3B are directly involved in E2F1-mediated transcriptional regulation and that the roles of ARID3A and ARID3B may differ depending on the E2F target genes.

Role of ARID3A and ARID3B in cell proliferation and cell death. ARID3A knockdown has been shown to cause premature senescence in normal human fibroblasts (40). The data presented above suggested that ARID3B also plays a critical role in cell cycle progression. To examine this possibility, quiescent NHDFs transfected with siCon, siARID3A or siARID3B for 48 h were stimulated to re-enter the cell cycle by the addition of 10% FBS. At 24 h following growth stimulation, DNA synthesis was determined by EdU incorporation assay. Growth stimulation of siCon-transfected quiescent NHDFs resulted in 49.8% of the cells becoming EdU-positive (Fig. 5A and B). siARID3A and siARID3B transfection reduced the number of EdU-positive cells to 17.2 and 30.1%, respectively, indicating that ARID3A and ARID3B knockdown attenuated the ability of quiescent NHDFs to enter the S phase. Furthermore, cell cycle analysis revealed that the growth stimulation of siCon-transfected quiescent T98G cells increased the population of cells in the S and G2/M phases (9.7-fold). However, the knockdown of ARID3A and ARID3B reduced the S and G2/M cell population by approximately half (Fig. 5C), indicating that ARID3A and ARID3B knockdown attenuated the cell cycle entry of T98G cells.

To further confirm the role of ARID3A and ARID3B in cell proliferation, colony formation assays were performed

using shRNA expression vectors that target ARID3A and ARID3B sequences (shARID3A and shARID3B, respectively). Both ARID3A and ARID3B have been shown to be involved in transcriptional regulation of the Rb and p53 tumor suppressor pathways (7-7,14,27-29). To determine RB- and p53-independent effects of ARID3A and ARID3B knockdown on cell proliferation, the Saos-2 and 293A cell lines were used, in which both Rb and p53 pathways are inactivated due to gene deletions and adenovirus EIA/EIB expression, respectively. The cells were transfected with plasmids expressing shARID3B and shARID3B and then cultured in the presence of puromycin for 3 weeks. A control shRNA vector targeting the luciferase gene (CFF) was used as a negative control. As shown in Fig. 5D, ARID3A and ARID3B knockdown resulted in a substantial reduction in the colony formation of both cell lines.

Previous studies have demonstrated that ARID3B has a unique function in inducing cell death (28,44). The present study thus analyzed the effects of ARID3A and ARID3B expression on E2F1-induced cell death. As shown in Fig. 5E, infection with Ad-E2F1 and Ad-ARID3B, but not Ad-ARID3A, induced caspase-3/7 activity. Furthermore, ARID3B and E2F1 co-operatively induced caspase-3/7 activity. These results demonstrate the overlapping and distinct roles of these ARID3 proteins in promoting cell proliferation and inducing cell death, respectively.

Discussion

The cooperative interactions of E2F with other transcription factors regulates the expression of E2F-dependent genes, facilitating the tighter regulation of gene expression (34,35,41,42). ARID3A and ARID3B have been originally identified as E2F1 and RB binding proteins, respectively. However, their direct involvement in controlling the E2F target gene has not been clarified. The present study demonstrated the cooperation of E2F with ARID3A and ARID3B to activate E2F-dependent transcription via the direct binding to E2F target genes. Clusters of ARID3 BSs upstream of the transcription start sites of certain E2F target genes were found, including *Cdc2*, *cyclin E1* and *p107*. Both ARID3 proteins activated the *Cdc2* promoter depending on not only on ARID3 BSs, but also on E2F BSs, indicating that ARID3A and ARID3B require cooperation with E2F to activate the *Cdc2* promoter. The requirement of their interaction with specific transcription factors to activate transcription is supported by a previous study reporting that ARID3A was unable to activate a reporter gene driven by tandemly repeated ARID3 consensus BSs (39). In B lymphocytes, ARID3A functions as a tether to bring TFII-I and Btk adjacent to the IgH promoter thereby activating transcription. By contrast, E2F1 activated the *Cdc2* promoter without ARID3 BSs, since E2F BSs alone are sufficient for E2F1 to activate transcription. In the absence of ARID3 BSs, however, the promoter activity was significantly reduced, indicating that ARID3 BSs play a vital role in the complete activation of the *Cdc2* promoter in response to E2F activity.

ARID3B induced endogenous E2F target genes, but not the transiently transfected *Cdc2* promoter under quiescent conditions, suggesting that ARID3B is involved in controlling gene expression by altering chromatin structure. ARID3A is activated only when the MAR-containing IgH S107 promoter is integrated into chromatin (39). Similarly, the authors have previously reported that ARID3A did not activate the transiently transfected promoter of *p21*, a p53 target gene, but activated the stably transfected *p21* promoter integrated into the chromatin (27). ARID3A and ARID3B have been shown to bind to histone-modifying enzymes, including histone deacetylase (HDAC1 and HDAC2) and histone demethylase 4C (KDM4C) (Fig. 6) (14,21,43). ARID3A also binds to histone deacetylase HDAC1 and sequester the RB-HDAC1 complexes from the *E2F1* promoter (14). RB controls local promoter activity and also chromatin structure through physical interactions between histone modifiers and chromatin-bound proteins. Further investigations are warranted for determining whether ARID3B regulates E2F target genes by disrupting the RB-E2F repressor complexes or by hitherto unrecognized epigenetic functions in chromatin remodeling.

It was also found that ARID3 consensus BSs in the E2F target genes are clustered >300 bp upstream of TSS (Cdc2, -303 to -833; cyclin E1, -1,105 to -2,481; p107, -931 to -1,568), which is in contrast to the previously reported mechanism of E2F-dependent gene activation and repression that involve other transcription factor BSs located adjacent to E2F BSs (34,40-42). It has been reported that ARID3A distorts the DNA structure by bending DNA up to 90˚, allowing it to interact with spatially distant regulatory elements of the *IgH* gene (5,39). Moreover, ARID3A exists as a stable tetramer that could bind to 2 ARID3 BSs. These findings suggest that ARID3A can interact with spatially separated BSs and bring the enhancer close to the promoter by interacting with additional DNA-binding proteins (Fig. 6). Considering the highly conserved functional domains and heterodimerization between ARID3A and ARID3B, both ARID3 proteins may promote E2F target gene expression by bringing ARID3 BSs close to E2F BSs by interacting with E2Fs and other chromatin modifiers.

The present study demonstrated that the roles of ARID3A and ARID3B in controlling E2F target gene expression differed, depending on the E2F target gene. It appears that ARID3B may play a more important role in the expression of *Cdc2* compared to ARID3A. By contrast, both ARID3A and ARID3B were indispensable for *cyclin E1* expression, a critical target for ARID3A to rescue MEFs from replicative senescence or RAS-induced premature senescence (8,14). The fact that ARID3B cooperates with E2F1 to activate *cyclin E1* transcription and immortalizes MEFs (15), suggests that ARID3B may have a similar function in senescence rescue. The unique functions of ARID3B are indicated by the fact that ARID3B, but not ARID3A, cooperates with E2F1 to induce cell death, which is consistent with the findings of previous studies (28,44). In line with this result, phenotypes of ARID3A and ARID3B knockout mice differ. Furthermore, ARID3A is strongly expressed in the lung and spleen and at lower levels in various other tissues, whereas ARID3B is expressed ubiquitously (9,17). These observations support the notion that ARID3A and ARID3B have overlapping and distinct functions.

Additionally, the regulation of subcellular localization of ARID3A and ARID3B may contribute to the differences in their function. Although stably transfected ARID3A induces E2F1 transcriptional activity (8,14), transient ARID3A

overexpression failed to activate cyclin E1, as well as Cdc2 and p107 genes under quiescent conditions. Unlike ARID3B, which is localized in the nucleus, ARID3A actively shuttles between the nucleus and the cytoplasm in the S phase. Thus, overexpressed ARID3A protein may be retained in the cytoplasm under quiescent conditions. The regulation of ARID3A nucleocytoplasmic shuttling may regulate E2F target gene expression during the cell cycle. Alternatively, epigenetic changes induced by ARID3A, such as remodeling of chromatin structures, may require to activate gene expression, as suggested by previous studies (8,14).

ARID3A and ARID3B have been shown to regulate stem cell genes in ovarian cancer and head and neck squamous carcinoma cells (18,20-26). ARID3A binds to the OCT4/SOX2 binding site and an A/T-rich sequence, in addition to ARID3 BSs in the promoter/enhancer regions of pluripotency factors, Oct4, Sox2, and Nanog in MEFs (20). In ARID3B-overexpressing ovarian cancer cells, ChIP-seq experiments have shown that ARID3B binds to a consensus binding motif of 5'-TGGGATTACAG-3' (23). This differs from the previously established ARID3 consensus BSs, possibly due to differences in cell types or ARID3A/ARID3B binding partners. Furthermore, both ARID3A and ARID3B are regulated by lethal-7 (let-7), a tumor-suppressor microRNA, and recruit KDM4C onto target genes, contributing to H3K9 demethylation and promotion of transcription of stemness genes (21). These studies, together with the results presented herein, indicate that the dysregulation of ARID3A and ARID3B in ARID3B-overexpressing or let-7-deficient cancer cells may disrupt the regulation of the RB-E2F and stem cell pathways, which contributes to their oncogenic activities in human malignancies.

There are some limitations as regards the number of E2F target genes examined in the present study that must be addressed in future research. The authors observed the presence of consensus BSs for ARID3 proteins at a distance from TSS in many E2F target genes, even though their functional roles in transcriptional regulation of E2F target genes remain undetermined. Given the implication of ARID3A and ARID3B in spatially distant interactions with regulatory elements, further studies are warranted to analyze the formation of tertiary structures of both ARID3 proteins and E2F target genes, along with other chromatin modifiers, and elucidate the extent to which ARID3A and ARID3B are involved in the regulation of E2F target genes. Furthermore, the present study highlights the need for investigations directed at a comprehensive understanding of the mechanisms underlying epigenetic regulation by the ARID3, E2F and RB family of proteins, which could provide deeper insight into the regulation of cell proliferation, differentiation and tumorigenesis.

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Availability of data and materials

The data used and/or analyzed during the current study are presented in the manuscript or are available from the corresponding author on reasonable request.

Authors' contributions

KASMS and MAI conceived and designed the experiments. KASMS, TM, MT and MAI performed the experiments. KASMS, WL, EP and MAI analyzed the data. WL and SI interpreted the data. MAI wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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