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p14ARF Interacts With E2F Factors to Form p14ARF–E2F/Partner-DNA Complexes Repressing E2F-Dependent Transcription

Hai-Jun Zhang,[1] Wen-Juan Li,[2] Yan-Yan Gu,[1] Shu-Yan Li,[1] Guo-Shun An,[1] Ju-Hua Ni,[1] and Hong-Ti Jia[1,2*]

[1]*Department of Biochemistry and Molecular Biology, Peking University Health Science Center, Beijing 100191, PR China*

[2]*Department of Biochemistry and Molecular Biology, Capital Medical University, Beijing 100069, PR China*

ABSTRACT

Primarily, E2F factors such as E2F1, -2, and -3 stimulate cell-cycle progression, while ARF tumor suppressor mediates growth suppression. The *ARF* gene can be induced by oncogenic signal through activating E2F-dependent transcription. In turn, ARF may target E2F for its degradation via a p53-dependent mechanism. However, it remains unclear how the cell keeps the balance between the functional opposites of E2F and ARF. In this study, we demonstrate that p14ARF interacts with E2F1–3 factors to directly repress their transcriptional activities through forming p14ARF–E2F/partner-DNA super complexes, regardless of E2F protein degradation. The inhibition of E2F transcriptional activities by p14ARF in this manner occurs commonly in a variety of cell types, including p53-deficient and p53-wild type cells. Thus, E2F-mediated activation of the ARF gene and ARF-mediated functional inhibition of E2F compose a feedback loop, by which the two opposites act in concert to regulate cell proliferation and apoptosis, depending on the cellular context and the environment. J. Cell. Biochem. 109: 693–701, 2010.

KEY WORDS: p14ARF; E2F; TRANSCRIPTIONAL ACTIVATION; PROTEIN INTERACTION; DNA BINDING

Human tumor suppressor p14ARF (p19ARF in mouse) is an alternative transcript of the INK4a–ARF tumor suppressor locus that encodes the p16INK4a inhibitor of cyclin-dependent kinases [Sherr, 2001]. ARF inhibits cell growth by binding Mdm2 and stabilizing p53 that triggers cell growth arrest and apoptosis [Kamijo et al., 1998; Stott et al., 1998]. However, ARF functions are not confined to the ARF–Mdm2–p53 pathway. For instance, p19ARF can suppress colony formation in $p53^{-/-}$ cells by activating Rb pathway [Carnero et al., 2000]. Reintroduction of p19ARF in mouse embryonic fibroblasts lacking p53, Mdm2, and p19ARF stops cell proliferation [Weber et al., 2000]. Similarly, p14ARF can induce p53-independent cell growth arrest [Eymin et al., 2003]. In addition, ARF is involved in apoptosis [Zindy et al., 1998; Eymin et al., 2003] and senescence [Dimri et al., 2000; Randle et al., 2001].

E2F family has eight members that exhibit distinct cell cycle and apoptotic activities. The E2F1, -2, and -3 proteins promote cell growth, while E2F4, -5, -6, -7, and -8 act as negative regulators [Dyson, 1998; Gaubatz et al., 2000; Christensen et al., 2005]. E2F1 stimulates cell-cycle progression by controlling the expression of a large spectrum of genes required for DNA synthesis and more generally proliferation [Qin et al., 1994; Kamijo et al., 1998; La Thangue, 2003]. On the other hand, E2F1 can play a role in cell-cycle arrest and apoptosis [La Thangue, 2003; Qin et al., 1994]. Similar to E2F1, expression of E2F2 or E2F3 can induce quiescent cells to enter S phase [DeGregori et al., 1997]. In some circumstances, however,

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Hai-Jun Zhang's present address is Masonic cancer center, University of Minnesota, Minneapolis, Minnesota 55455.

Wen-Juan Li's present address is Graduate Center for Toxicology, College of Medicine, University of Kentucky, Lexington, Kentucky 40536.

Yan-Yan Gu's present address is Department of Hematology and Oncology, Emory University School of Medicine, Atlanta, Georgia 30322.

*Correspondence to: Hong-Ti Jia, Department of Biochemistry and Molecular Biology, Peking University Health Science Center, Xue Yuan Road 38, Beijing 100191, PR China. E-mail: jiahongti@bjmu.edu.cn

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the three members have divergent effects on cell cycle and apoptosis [Dyson, 1998].

The regulation of E2F1 activity is closely linked to the cell cycle [Dyson, 1998; Nevins, 1998; Martelli et al., 2001] and to the cellular responses to genotoxic stress [Stevens and La Thangue, 2004], which includes multiple steps, such as chemical modification/stabilization, DNA binding activity, and transcriptional activation. Generally, the levels of E2F1 protein may influence its effects on the cell. For instance, a threshold E2F1 level, such as the high level reached in response to DNA damage, may determine a particular outcome by activating a different spectrum of genes. Similar to p53, the DNA damage-induced phosphorylation of E2F1 by ATM/ATR and Chk2 kinases may promote the subsequent acetylation of lysine residues within its C-terminal region, enhancing E2F1 DNA binding and transcriptional activities [Pediconi et al., 2003]. The degradation of E2F via the ubiquitin–proteasome pathway can be initiated by binding either pRb to the C-terminal [Hateboer et al., 1996; Hofmann et al., 1996] or Skp2 to the N-terminal region of E2F1 [Marti et al., 1999]. When E2F1 interacts with a pocket protein (pRb, p107, and p130), the ability of E2F1 activating certain genes is suppressed. DNA-binding activity of E2F1 can also be regulated by cyclin A/Cdk2 [Krek et al., 1994].

The *ARF* gene can be induced by oncoproteins through activating E2F-dependent transcription [DeGregori et al., 1997; Bates et al., 1998]. Indeed, overexpressed [Robertson and Jones, 1998; Parisi et al., 2002; Komori et al., 2005] or stimulated [del Arroyo et al., 2007; Zhang et al., 2009] E2F1 can activate the p14ARF gene. In turn, p14ARF may suppress the activity of E2F1 factor through binding E2F1, for which Mdm2 is required [Eymin et al., 2001]. It is also suggested that the interaction of ARF with E2F1 leads to its degradation via proteasome pathways [Martelli et al., 2001; Rizos et al., 2007], which, however, occurs only in the presence of functional p53 [Rizos et al., 2007]. In this study, we demonstrate that p14ARF interacts with E2F1, -2, and -3 factors to partially inhibit their transcriptional activities through forming p14ARF–E2F/partner-DNA super complexes.

MATERIALS AND METHODS

CELL CULTURE AND CHEMICAL REAGENT

Human lung cancer H1299 (p53-null) and A549 (p53-wild-type), liver cancer HepG2 (p53-wild-type), cervix cancer HeLa (low p53 expression), human embryonic kidney 293T (p53-disabled) (ATCC, Rockville, MD), and human diploid fibroblast 2BS (p53-wild-type) (a gift from Yun-Biao Lu) cells were cultured in RPMI 1640 or DMEM medium supplemented with 10% fetal bovine serum (GIBCO BRL, Carlsbad, CA), 100 U/ml penicillin and 100 mg/ml streptomycin, and grown at 37°C with 5% CO_2. *N*-acetyl-L-leucyl-L-leucyl-L-norleucinal (LLnL) was purchased from Sigma (St. Louis, MO).

CONSTRUCTS AND TRANSFECTION

The p14ARF gene was released from pcDNA3p14ARF plasmid (a gift from Sonia Laín) by digestion with *Bam*H I and *Eco*R I, and inserted into pGEX-4T-2 (Amersham Biosciences, Uppsala, Sweden) between the *Bam*H I and *Eco*R I sites to generate GST fusion protein expression plasmid pGEXp14ARF. The pcDNA3p14ARF(1–65) and pcDNA3p14ARF(66–132) were constructed by amplifying the sequence coding for the 1–65 or 66–132 amino acid residues of p14ARF using pcDNA3p14ARF as a template, following by insertion of the two fragments into the *Bam*H I/*Eco*R I sites in pcDNA3 (Invitrogen, Carlsbad, CA), respectively. pCMV–HA–E2F1, pCMV–HA–E2F2, and pCMV–HA–E2F3 were kindly provided by Chan Tian. Transfection was performed by Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's protocol. Cells were also harvested for expression efficiency test.

WESTERN BLOTTING

Fifty micrograms of proteins was subjected to SDS–PAGE, and transferred onto nitrocellulose membranes and probed with specific antibodies (1:1,000) for p14ARF (Chemicon International, Temecula, CA), E2F1, HA, Actin (Santa Cruz Biotechnology, Santa Cruz, CA) or p53 (Cell Signaling Technology, Danvers, MA). Blots were developed with a secondary antibody coupled to horseradish peroxidase. Chemiluminescence signals were visualized using SuperSignal® West Pico Chemiluminescent Substrate (Pierce, Rockford, IL).

GST PULL-DOWN

HA-tagged E2F1, E2F2, or E2F3 was expressed in 293T cells, and the cells were lysed by RIPA buffer (50 mM Tris–HCl, pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, 1 mM NaF, and protease inhibitor cocktail). GST or GST–p14ARF fusion protein was expressed in bacteria and purified using Glutathione Sepharose 4B (Amersham Biosciences). The beads bound to GST fusion proteins were washed with PBS three times and incubated with cell lysates overnight at 4°C.

CO-IMMUNOPRECIPITATION

HA-tagged E2F1, E2F2, or E2F3 and recombinant p14ARF were expressed in 293T, and the cells were lysed with RIPA buffer. The supernatants were cleared by centrifugation. Equal amounts of protein (400 μg) were precleared using protein G PLUS agarose (Santa Cruz Biotechnology) beads and immunoprecipitated by standard procedures. Anit-HA antibody (Santa Cruz Biotechnology) was used for immunoprecipitation.

LUCIFERASE REPORTER ASSAY

Firefly and *Renilla* luciferase activities were assayed using Dual-Luciferase® Reporter Assay System (Promega, Madison, WI). *Firefly* luciferase activities were verified for transfection efficiencies as computed relative to Renilla luciferase activities.

ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

Nuclear extracts (NE) were prepared, and EMSA was performed as described [Zhang et al., 2009]. Briefly, prior to the addition of biotin-labeled probe, 2 μg NE was incubated for 10 min at room temperature in binding buffer. Then 2 μl (20 fmol) of probe was added. To identify E2F1, p14ARF and Sp1 in DNA–protein complexes, anti-E2F1 (C-20) (Santa Cruz Biotechnology), anti-p14ARF (Chemicon), and anti-Sp1 (PEP2) antibodies (2 μg) (Santa Cruz Biotechnology) were added. Protein–DNA complexes were

separated on 6% PAGE and visualized by LightShift® Chemiluminescent EMSA Kit (Pierce).

STATISTICAL ANALYSIS

The Student's *t*-test and Wilcoxon's rank-sum test were used for statistical analysis. Statistical significance was defined by a two-tailed *P*-value of 0.05.

RESULTS

p14ARF INTERACTS WITH E2F1, E2F2, AND E2F3

Mouse [Martelli et al., 2001] and human [Eymin et al., 2001] ARF interacts with E2F1 to inhibit cell growth. To confirm the interaction of p14ARF with E2F1, -2, and -3, we examined the in vitro interaction of p14ARF with the three E2F factors using GST pull-down assay. Three expression plasmids, pCMV–HA–E2F1, pCMV–HA–E2F2, and pCMV–HA–E2F3, were transfected into 293T cells, and the HA–E2F proteins in cell lysates were pulled down by incubating with GST–p14ARF fusion protein. Western blotting showed that HA-tagged E2F1, E2F2, and E2F3 were associated with GST–p14ARF, however, no signal associated with the affinity-purified GST could be detected (Fig. 1A).

To further demonstrate the interaction of E2F proteins with p14ARF, we performed co-immunoprecipitation assays. 293T cells were co-transfected with pcDNA3p14ARF and pCMV–HA–E2F1, pCMV–HA–E2F2, or pCMV–HA–E2F3. Proteins were extracted and immunoprecipitated with anti-HA antibody. The immunoblot with anti-p14ARF antibody showed that p14ARF was present in anti-HA antibody precipitates (Fig. 1B, upper), and HA-tagged E2F1, E2F2, and E2F3 were present in anti-HA antibody precipitates (Fig. 1B, down). Taken the results from GST-pull down and co-immunoprecipitation, p14ARF can interact with E2F1, E2F2, and E2F3 specifically, which supports previous observation [Eymin et al., 2001; Martelli et al., 2001].

p14ARF INHIBITS E2F1-3 TRANSACTIVATION DEPENDENT OF ITS 1–65 RESIDUES AND REGARDLESS OF THE p53 STATUS

To determine the effects of p14ARF–E2F interactions on the transcriptional activity of these E2Fs, 293T (p53-disabled) (Fig. 2A), and A549 (p53-wild-type) (Fig. 2B) cells were co-transfected by luciferase reporter plasmid 3 × E2Fwt-Luc (in which the reporter is driven by a promoter baring three tandem artificial E2F binding sites) with pCMV–HA–E2Fs and/or pcDNA3p14ARF. Evident activation of the luciferase gene was detected after E2F1, E2F2, or E2F3 transfection, while overexpressed p14ARF significantly decreased E2F-dependent reporter activation.

To determine which exon-encoded product of the p14ARF was responsible for inhibiting E2F transcriptional activities, we performed luciferase reporter assay using 293T and A549 cells transfected with the specific exon truncate of the p14ARF gene. Like the full length product of p14ARF, expression of exon 1β coding for amino acid residues 1–65 of the protein showed a marked inhibition of E2F transcriptional activities, while expression of exon 2 coding for amino acid residues 66–132 did not show any effect on reporter activity (Fig. 2A,B), indicating that partial inhibition of E2F transcriptional activities is attributed to the N-terminal of p14ARF.

Similar experiments were repeated using cancer cell lines HeLa (low p53 expression), HepG2 (p53-wild-type), H1299 (p53-null), and human diploid fibroblast 2BS cells (p53-wild-type). Inhibition of E2F-dependent activation with variant extents by p14ARF was observed in all tested cells (Fig. 2C–F, Fig. 3A), although p53 status was different in these cells (Fig. 3B). These results indicate that the decrease of E2F1, E2F2, and E2F3 transcriptional activities by p14ARF is not restricted to specific cell type, regardless of the p53 status.

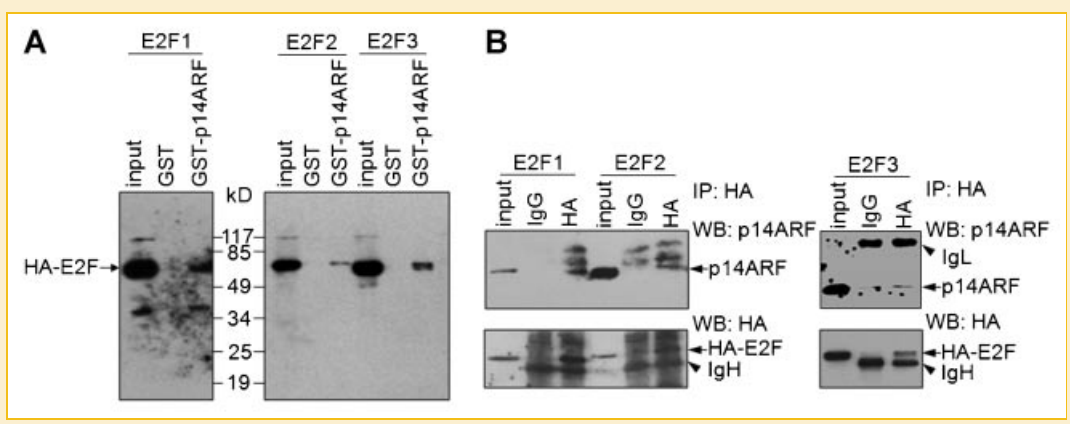

Fig. 1. p14ARF interacts with E2F1, E2F2, and E2F3 in GST pull-down and co-immunoprecipitation assays. A: E2Fs interaction with GST–p14ARF in the GST pull-down assay. This assay was performed as described in the Materials and Methods Section. 293T cells expressed HA-tagged E2F1, E2F2, or E2F3 were lysed, and the cell lysates were incubated with beads bound to GST or GST–p14ARF fusion protein (expressed by pGEX-4T-2 or pGEXp14ARF in bacteria). Protein complexes from all bead fractions were analyzed by Western blotting with anti-HA antibody. Ten percent of the input was loaded. B: Co-immunoprecipitation of HA-tagged E2Fs and p14ARF. 293T cells were co-transfected by HA-tagged E2F1, E2F2, or E2F3 plasmid with pcDNA3p14ARF. After lysed, the whole-cell extracts were cleared by centrifugation. Equal amounts of proteins (400 μg) were precleared using protein G PLUS agarose beads, and immunoprecipitated by anti-HA antibody. The p14ARF (upper panel) and HA-tagged E2Fs (lower panel) proteins in the precipitates were immunoblotted by anti-p14ARF or anti-HA antibodies. Forty percent of the proteins inputs were loaded.

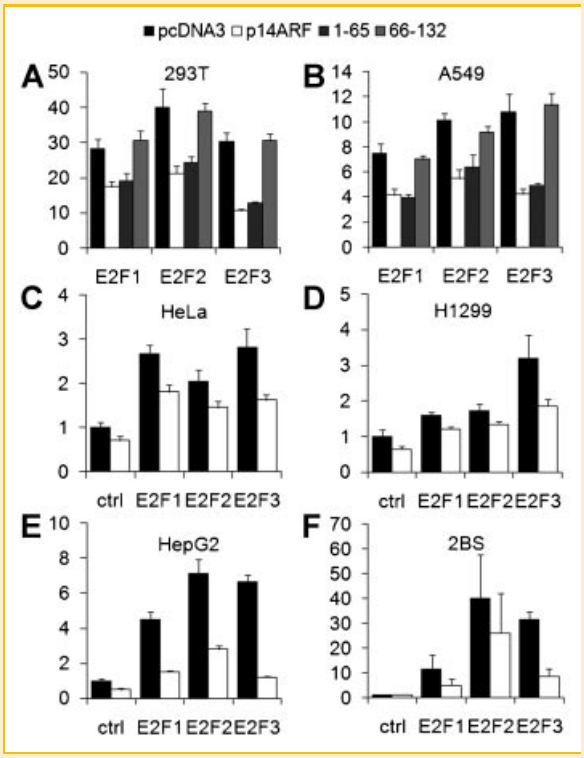

Fig. 2. p14ARF inhibits E2F-dependent transcriptional activation. A,B: p14ARF 1–65 region encoded by exon 1β is responsible for inhibiting E2F-dependent transcriptional activation. 293T (p53-disabled) (Panel A) and A549 (p53-wild-type) (Panel B) cells were co-transfected with 0.2 μg 3 × E2Fwt-Luc reporter vector (in which the reporter is driven by a promoter baring three tandem artificial E2F binding sites) with or without 0.01 μg pCMV–HA–E2F1, pCMV–HA–E2F2, pCMV–HA–E2F3, and/or 0.1 μg pcDNA3-p14ARF, pcDNA3p14ARF(1–65), pcDNA3p14ARF(66–132). Empty vector was added in each transfection to ensure equal amount of DNA. After transfection for 24 h, the luciferase enzyme activities were assayed. The enzyme activities of 3 × E2Fwt-Luc reporter vector-transfected cells were normalized to 1, and the activity in each sample was expressed as relative luciferase activity. Data represent mean ± SD of three independent experiments performed in triplicate. C–F: Repression of E2F-dependent transcriptional activation by p14ARF is common in a variety of cell types. The cells, including HeLa (low p53 expression) (C), H1299 (p53-null) (D), HepG2 (p53-wild-type) (E), and 2BS (p53-wild-type) (F), were co-transfected as in the figure legends to Panels (A) and (B), except that pcDNA3p14ARF(1–65) and pcDNA3p14ARF(66–132) transfection was omitted. After transfection, the cell treatment and the reporter enzyme assay were performed, and relative luciferase activities in each sample were calculated as in (A) and (B). All data represent mean ± SD of three independent experiments performed in triplicate. Transfection with the plasmids of p14ARF and its mutants are indicated by black, white, dark and light gray squares shown on the top.

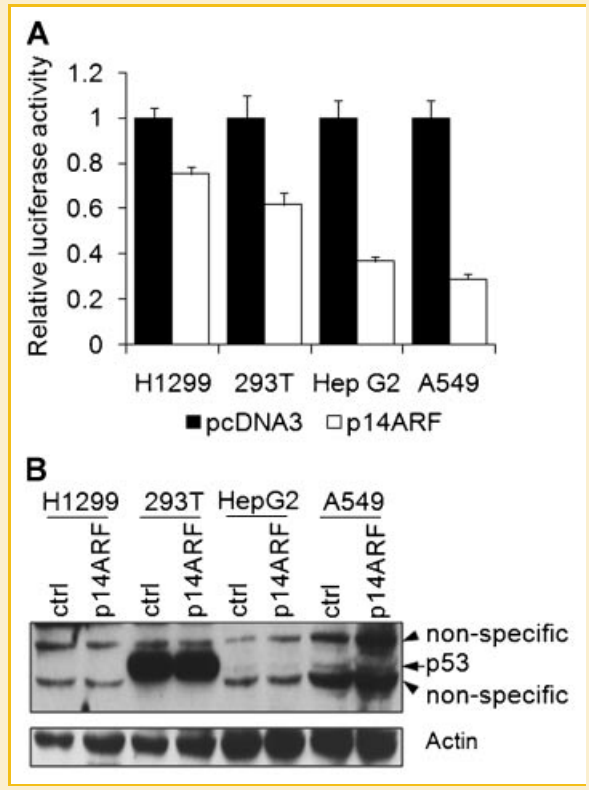

Fig. 3. Repressive effect of p14ARF on E2F-dependent activation is variated in the cells with different p53 status. A: Variation of repressive effect of p14ARF on E2F-dependent activation in the cells with different p53 status. Transfection of H1299, 293T, HepG2, and A549 cells and reporter enzyme activity analyses were performed as same as described in Figure 2. The enzyme activities of empty vector-transfected cells were normalized to 1 (black square). B: The endogenous p53 levels in Western blotting. Cell lysates were prepared and the proteins of lysates were extracted with Triton X-100 buffer. Fifty micrograms of proteins was subjected to SDS–PAGE, and transferred onto nitrocellulose membranes and probed with anti-p53 antibody. The p53 protein in HepG2 and A549 is indicated. Actin was used as a loading control.

p14ARF INHIBITS E2F1-, E2F2-, AND E2F3-DEPENDENT PROMOTER ACTIVATION

Next we examined the inhibitory effects of p14ARF on the activation of the cyclin E and p14ARF promoters that are E2F-dependent [Bates et al., 1998]. The cyclin E-Luc or pGL3ARF(−735/+54) plasmid, containing a functional *Luc* gene under the control of the cyclin E or p14ARF promoter [Zhang et al., 2009], was co-transfected with expression vectors pCMV–HA–E2F1, pCMV–HA–E2F2, or pCMV–HA–E2F3 and/or pcDNA3p14ARF into 293T and HepG2, and the reporter activities were measured. Compared with control vector pcDNA3 transfection, pCMV–HA–E2F1, pCMV–HA–E2F2, or pCMV–HA–E2F3 transfection significantly activated cyclin E promoter- and p14ARF promoter-driven-reporter enzyme activities (Fig. 4A–D). However, the enzyme activities were significantly decreased, when the pcDNA3p14ARF was co-transfected. These results indicate that p14ARF can inhibit E2F-dependent gene activation.

PROTEASOME INHIBITOR LLNL CANNOT ABOLISH p14ARF-INHIBITED E2F TRANSCRIPTIONAL ACTIVITIES

It shows that ARF targets E2F1 for degradation [Martelli et al., 2001; Rizos et al., 2007] via a p53-dependent mechanism [Rizos et al., 2007]. To compare the effects of p14ARF on E2F1 protein stability in different p53 status, the 293T (in which p53 is disabled by E1B and large T antigen [Martelli et al., 2001]) and HepG2 (wide-type p53) cells were co-transfected with pcDNA3p14ARF and pCMV–HA–E2F.

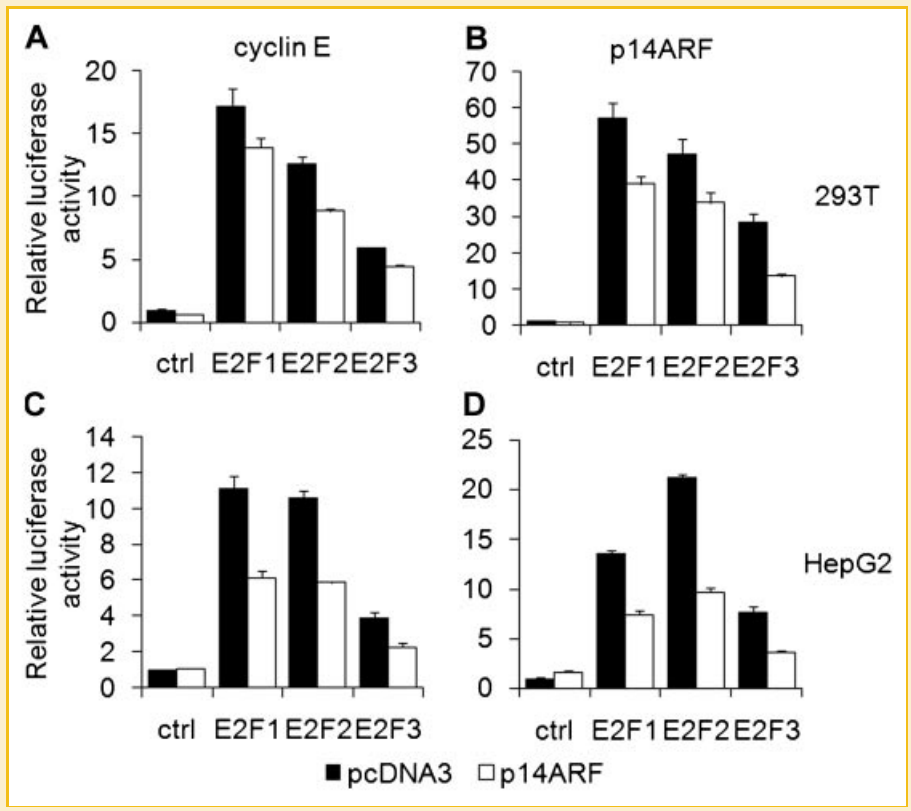

Fig. 4. p14ARF represses activation of E2F-dependent cyclin E and p14ARF genes. 293T (A,B) and HepG2 (C,D) cells were co-transfected by cyclin E promoter-driven Luc gene (cyclin E-Luc reporter vector) (A,C) or p14ARF promoter-driven Luc gene [pGL3ARF(−735/+54) reporter vector] (B,D) with pCMV-HA-E2F1, pCMV-HA-E2F2, or pCMV-HA-E2F3 and pcDNA3p14ARF (white square) or pcDNA3 (black square). Empty vector was added in each transfection to ensure equal amount of DNA. After transfection for 24 h, the enzyme activities were assayed. The enzyme activities of simple reporter transfected cells were normalized to 1. Data represent mean ± SD of three independent experiments performed in triplicate. p14ARF versus pcDNA3: $P < 0.05$ in E2F1, E2F2, and E2F3 transfections.

Western blotting revealed that compared with control transfection, overexpressed p14ARF did not lead to down-regulation of HA-tagged E2F1, E2F2, and E2F3 proteins in 293T, (Fig. 5A,B), while p14ARF-targeted E2F1 degradation was observed in HepG2 cells (Fig. 5C). To further determine that p14ARF binding to E2F did not accelerate proteasome-depended E2F turnover in 293T, the cells were co-transfected with pCMV-HA-E2F expression vectors, pcDNA3p14ARF expression vector and 3 × E2Fwt-Luc or cyclin E-Luc plasmid. After 6 h of transfection, the cells were exposed to the proteasome inhibitor LLnL (50 mM). As shown in Figure 5D,E, exposure to LLnL did not deliver the inhibitory effect of p14ARF on E2F1, E2F2, and E2F3 transcriptional activities in 293T cells. These results indicate that the repression of E2F-dependent transcription activation by p14ARF may not be correlated to proteasome-mediated degradation of E2Fs in p53-deficient cells.

p14ARF INTERACTS WITH E2F1 TO FORM p14ARF–E2F1/PARTNER–DNA SUPER COMPLEXES

To test the effect of p14ARF–E2F interaction on the activity of E2F binding to DNA, we performed EMSA using an 27-bp oligonucleotide baring an E2F1 consensus (Fig. 6A) as probe, and incubated with the NEs from 293T cells overexpressed E2F1 and p14ARF (Fig. 6B). Compared with free probe, three major protein–DNA complexes (bands A–C) and a very thin band D occurred, when the probe was incubated with E2F1-expressed NEs (Fig. 6C, lane 2). Interestingly, the band C was expanded with the increase of p14ARF (lanes 3–5), whereas band D was increased in the presence of moderate amounts of p14ARF but shifted to band D′ with a little high migration on the gel in the presence of a large amount of p14ARF (lane 5). These results indicate that p14ARF joins the complexes C and D.

All E2F members are able to bind a similar consensus as a homodimer [Roussel et al., 1994] and heterodimer with a member (45–55 KD) of the DP family [Wu et al., 1995]. Also, E2F1 and Sp1 (105 KD) can bind to each other [Karlseder et al., 1996], and both can recognize the E2F1 consensus [Zhang et al., 2009]. According to the above-mentioned and the previous binding analyses [Wu et al., 1995], the bands A and B in Figure 6C should be E2Fs–DNA complexes (without exclusion of p14ARF joining them), band C might be attributed to p14ARF–E2F–DP trimer binding to DNA, and band D was formed by p14ARF–E2F–Sp1 binding. If this is true, the shift of complex D to D′ might be due to partial remove of E2Fs by p14ARF–E2F interaction from the binding reaction, allowing Sp1 to dominantly bind to DNA. To demonstrate this speculation, specific antibodies were employed in EMSA assays under the reaction conditions of lane 4 in Figure 6C. Addition of anti-Sp1 antibody completely abolished band D (lane 5 in Fig. 6D left and right panels

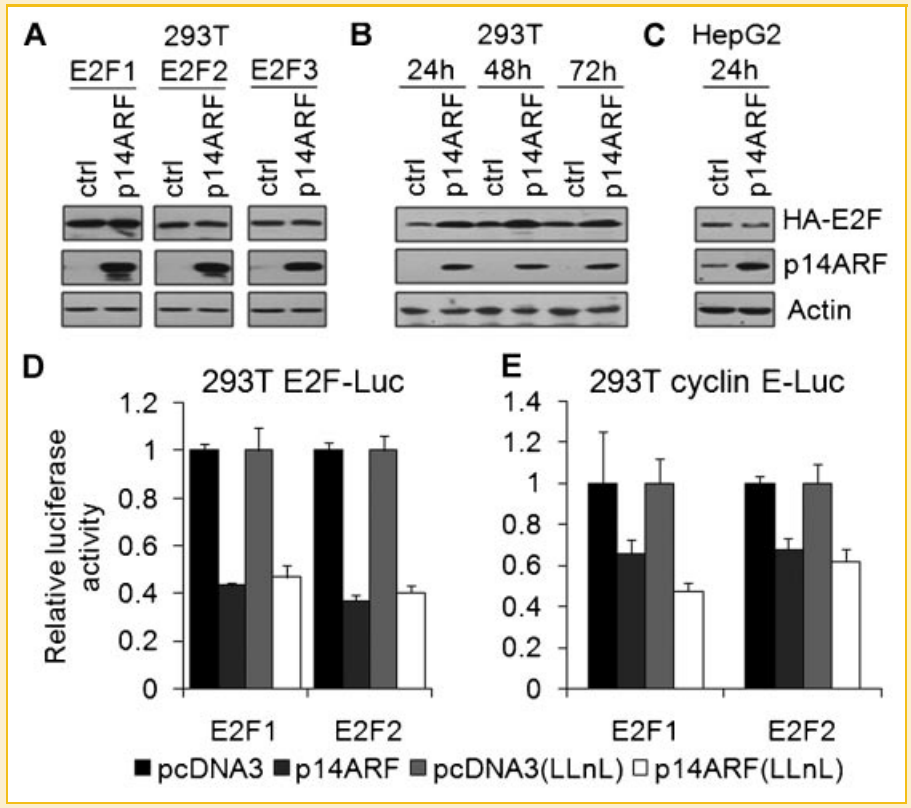

Fig. 5. Repression of E2F-dependent transcription by p14ARF is not correlated to E2F degradation in p53-deficient cells. 293T (p53-disable) cells were co-transfected with pCMV–HA–E2F1, pCMV–HA–E2F2, or pCMV–HA–E2F3 expression vector with pcDNA3p14ARF or pcDNA3 (control). HepG2 (p53-wild-type) cells were co-transfected with pCMV–HA–E2F1 expression vector with pcDNA3p14ARF or pcDNA3 (control). A: Overexpression of p14ARF does not decrease the levels of E2F1, E2F2, and E2F3 proteins in 293T cells. After 24 h transfection, the cells were harvested and lysed. The proteins in the lysates were extracted, and the levels of HA-tagged E2F1, E2F2, and E2F3 proteins were analyzed by Western blotting with anti-HA antibody; the levels of p14ARF protein was analyzed with anti-p14ARF antibody. Actin was used as a loading control. B: Overexpression of p14ARF does not decrease the levels of E2F1 protein in long-term cultured 293T cells. After 24, 48, or 72 h transfection, the transfected 293T cells were harvested and lysed. The levels of E2F1 and p14ARF proteins were analyzed by Western blotting as described in (A). Actin was used as a loading control. C: Overexpression of p14ARF decreases the levels of E2F1 protein in HepG2 cells. After 24 h transfection, the cells transfected with pCMV–HA–E2F1 expression vector and pcDNA3p14ARF were harvested, and the levels of E2F1 and p14ARF proteins were analyzed by Western blotting as described in (A). Actin was used as a loading control. D,E: Ubiquitin/proteasome inhibitor cannot deliver the repressive effect of p14ARF on E2F dependent transcription activation in 293T cells. The cells were transfected by 3 × E2Fwt-Luc vector (E2F-Luc) (D) or cyclin E-Luc vector (E) with pCMV–HA–E2F1 or pCMV–HA–E2F2 with pcDNA3p14ARF or pcDNA3. After 6 h transfection, the cells were exposed to DMSO or 50 μM LLnL/DMSO for 18 h, the enzyme activities were assayed. The enzyme activities of pcDNA3 transfection (black) and pcDNA3 transfection plus LLnL exposure (light gray) were normalized to 1. Data represent mean ± SD of three independent experiments performed in triplicate.

showing long- and short-term exposed film, respectively), indicating complex D containing Sp1. In this reaction, Sp1 binding to DNA might not be excluded, although it was weak (D′ band in lane 5 in Fig. 6D, left panel). Similarly, addition of anti-E2F1 antibody partly inhibited the formation of complexes C and D (lane 3 in both panels), indicating E2F1 present in the complexes. Markedly, addition of 4 μg anti-p14ARF antibody resulted in disappearance of complexes C and D (lane 4), possibly due to removing E2F1 by its association with p14ARF/anti-p14ARF antibody. Notably, the disappearance of complexes C and D was antibody dose-dependent (Fig. 6E), in which a moderate amount of anti-p14ARF antibody (0.5 or 1.0 μg) led to a supershift band (C′ band in lanes 3 and 4 in Fig. 6E) and reduction of C band, while a high amount of antibodies (2.0 or 4.0 μg) inhibited the formation of C complex completely (lanes 5 and 6). Together, these results suggest that p14ARF may physically interact with E2F1 to form p14ARF–E2F1/partner-DNA super complexes.

DISCUSSION

Primarily, E2F1 stimulates cell-cycle progression by controlling the expression of a large spectrum of genes required for DNA synthesis and cell proliferation. On the other hand, E2F1 can play a role in cell-cycle arrest and apoptosis through the transcriptional activation of checkpoint control and apoptotic target genes including the ARF gene [Stevens and La Thangue, 2004]. Recent studies suggest that human ARF may target E2Fs for its degradation via a p53-dependent mechanism, thereby inhibiting E2F-dependent transcription [Eymin et al., 2001; Martelli et al., 2001; Rizos et al., 2007]. It remains unclear how the cell keeps the balance between the functional opposites of E2F and ARF. In this study, we demonstrate that p14ARF interacts with E2F1-3 factors to partially and directly repress their transcriptional activities through forming p14ARF–E2F/partner-DNA super complexes. Thus, E2F-mediated activation

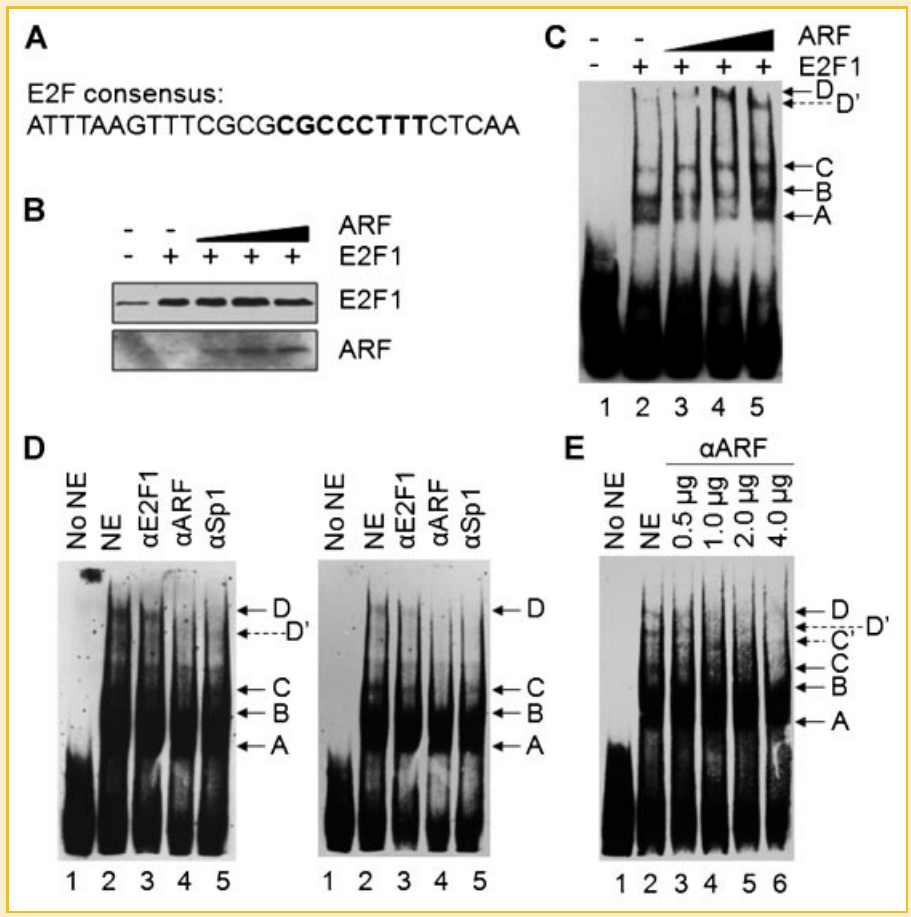

Fig. 6. p14ARF interacts with E2F1 to form p14ARF–E2F1/partner-DNA complexes. A: The sequence of a biotin-labeled synthesized oligonucleotide containing E2F1 consensus used as probe in EMSA assays. The E2F1 consensus sequence is in boldface letter. B: The levels of E2F1 and p14ARF proteins in E2F1- and/or p14ARF-transfected cells. 293T cells were co-transfected with HA-tagged E2F1 (0.2 μg) with 0, 0.2, 1, or 2 μg pcDNA3p14ARF. After 24 h transfection, the cells were harvested and the nuclear extracts were prepared. The levels of E2F1 and p14ARF proteins were analyzed by Western blotting. C: p14ARF joining p14ARF–E2F1/partner-DNA complexes in EMSA assay. The EMSA experiments were performed using a biotin-labeled synthesized oligonucleotide as a probe (see Panel A) that was incubated with 2 μg nuclear extracts (NE) from the transfected 293T cell (the same as in B) by HA-tagged E2F1 (0.2 μg) with pcDNA3p14ARF of 0 (lane 2), 0.2 (lane 3), 1 (lane 4), or 2 (lane 5) μg. The nucleoprotein–DNA complexes were separated by 6% PAGE. Protein–DNA complexes are indicated by capital letters. D: p14ARF co-existing with E2F1 and Sp1 in nucleoprotein–DNA complexes. EMSA was performed, in which binding reaction conditions are the same as in Panel C, lane 4, except that special antibodies (4 μg) were used. αE2F1, anti-E2F1 antibody; αARF, anti-p14ARF antibody; αSp1, anti-Sp1 antibody. Left panel showing long-term (5 min) exposed film, right panel showing short-term (1 min) exposed film. E: Effects of different amounts of anti-p14ARF antibody on p14ARF–E2F1/partner-DNA super complexes. EMSA assays are the same as in (D), except that different amounts of anti-p14ARF antibody (0.5–4 μg) were used, respectively. Anti-p14ARF antibody-induced supershift band is labeled by "C'."

of the ARF gene and ARF-mediated functional inhibition of E2F factors compose a feedback loop, by which the two opposites act in concert to regulate cell proliferation and apoptosis, depending on the cellular context and the environment.

In mammals, E2F1, -2, and -3 function primarily as transactivators, which are required for gene activation and cell-cycle entry [Taubert et al., 2004]. The activities of E2F1–3 are regulated in a cell cycle-dependent manner, principally through its temporal association with pocket-protein family members, the prototype being the retinoblastoma tumor suppressor protein (pRb), which counteracts E2F-dependent gene activation [Stevens and La Thangue, 2004]. In addition, overexpressed ARF can suppress cell proliferation by inhibiting E2F1 transcriptional activity [Eymin et al., 2001; Rizos et al., 2007] and destabilizing E2F1 protein [Martelli et al., 2001; Rizos et al., 2007]. For instance, mouse p19ARF targets E2F1 for its degradation through a p53-independent mechanism [Martelli et al., 2001]. Human p14ARF, however, induces E2F1 degradation only in the presence of functional p53 [Rizos et al., 2007]. In fact, p14ARF may also function as a tumor repressor in a p53-independent way [Eymin et al., 2003]. This raises the question of how p14ARF functions in the absence of p53, and whether p14ARF-suppressed E2F transcription activity must be linked to E2F degradation.

Supporting previous observation [Eymin et al., 2001; Rizos et al., 2007], we found that overexpressed p14ARF significantly inhibited E2F-dependent activation of the artificial E2F binding site-containing promoter in six cell lines with different p53 status (Fig. 2). In addition, we did find a detectable decrease of E2F1 in HepG2 (p53-wild-type) but not in 293T (p53-disabled) cells (Fig. 5A–C). Consistently, the proteasome inhibitor LLnL could

not rescue p14ARF-inhibited E2F transcriptional activity in 293T cells (Fig. 5D,E). Since ARF-targeted E2F1 degradation via a proteasome pathway occurs only in the presence of functional p53 [Rizos et al., 2007], and 293T is p53-disabled by E1B and large T antigen [Martelli et al., 2001], E2F degradation may not be involved in the inhibition of E2F transcriptional activity by p14ARF in this cell. This inference is also supported by our previous observation that E2F1 keeps a steady increase, following p14ARF activation during 8-chloro-adenosine exposure [Zhang et al., 2009]. An explanation of un-degradation of E2F could be that E2F1 interacts with ARF through its N-terminal domain, which might hide the binding site for the Skp2, a component of the ubiquitin protein ligase SCF^{Skp2}. We therefore suggest that the inhibition of E2F transcriptional activity by p14ARF can be directly attributed to the interaction of p14ARF with E2F1, -2, and -3 factors in p53-deficient cells. Previous studies have demonstrated that E2F1 interacts with ARF through its N-terminal domain [Eymin et al., 2001], and targeting of N-terminal domain by Cyclin A/Cdk2 kinase leads to inhibition of DNA binding capacity [Krek et al., 1994]. Interestingly, the analyses of E2F1–DNA complexes showed that p14ARF binding to E2F1 did not decrease E2F1 binding activity, but altered the binding pattern, in which p14ARF–E2F1/partner-DNA super complexes (bands C and D in Fig. 6C) were increased in the presence of p14ARF. The formation of super complexes joined by p14ARF might repress the E2F1-dependent genes. However, how the ARF kinetically targets E2F/partner-DNA complex and the precise mechanism of E2F-dependent transcription repression remain to be addressed.

Although the inhibition of E2F target activation by p14ARF could be seen in a variety of cell types (Fig. 2), the inhibition extents were greatly varied in these cells: H1299 (p53-null), 293T (p53-disabled) < HepG2 (p53-wild-type), A549 (p53-wild-type), which was correlated to the p53 status (Fig. 3). Therefore, our data support the notion that ARF targeting E2F for its ubiquitination/degradation via a p53-dependent mechanism [Rizos et al., 2007].

Previous works [Eymin et al., 2001; Martelli et al., 2001; Rizos et al., 2007] and present data demonstrate that ARF can bind E2F factors to repress their transcriptional activities with or without targeting protein degradation. Thus, E2F-mediated positive regulation of ARF and ARF-mediated negative regulation of E2F compose a feedback loop, where the two opposites act in concert to regulate cell proliferation and apoptosis, depending on the cellular context and the environment. Increased evidence suggests that E2F1 plays a central role in response to DNA damage [Stevens and La Thangue, 2004; Eymin et al., 2006]. We have recently shown that E2F1 is upregulated in the cells exposed to 8-chloro-adenosine (8-Cl-Ado) [Li et al., 2009; Zhang et al., 2009] that can damage DNA [Jia et al., 2009; Yang et al., 2009]. Induction of E2F1 by the agent may promote ARF gene expression [Zhang et al., 2009], thereby leading to 8-Cl-Ado-induced late apoptosis [Li et al., 2009]. Our previous works indicate that E2F1-ARF loop is operational during DNA damage, which might play distinct roles in the different stages of cellular response to DNA damage. Answers to such questions would provide aid in better understanding of DNA repair, cell-cycle checkpoint and apoptosis during genotoxic stress.

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