

Distinct E2F-mediated transcriptional program regulates $p14^{ARF}$ gene expression

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The tumor suppressor $p14^{ARF}$ gene is induced by ectopically expressed E2F, a positive regulator of the cell cycle. The gene is expressed at low levels in normally growing cells in contrast to high levels in varieties of tumors. How $p14^{ARF}$ gene is regulated by E2F in normally growing cells and tumor cells remains obscure. Here we show that regulation of $p14^{ARF}$ gene by E2F is distinct from that of classical E2F targets. It is directly mediated by E2F through a novel E2F-responsive element that varies from the typical E2F site. The element responds to E2F activity resulting from ectopic E2F1 expression, inactivation of pRb by adenovirus E1a or shRNA, but not to phosphorylation of pRb by serum stimulation or ectopic cyclin D1/cyclin-dependent kinase-4 expression in normal human fibroblasts. The element has activity in various tumor cells with defective pRb, but not in normally growing cells. These results indicate that the distinct regulation constitutes the basis of $p14^{ARF}$ function as a tumor suppressor, discriminating abnormal growth signals caused by defects in pRb function from normal growth signals.

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Introduction

In many forms of human cancer, $p14^{ARF}$ ($p19^{ARF}$ in mouse), an alternative reading frame of $p16^{INK4a}$ that is encoded on the *Ink4a-Arf* locus, is mutated, deleted, or silenced. The loss of $p19^{ARF}$ as well as $p16^{INK4a}$ function renders mice susceptible to tumor formation, indicating that $p14/19^{ARF}$ and $p16^{INK4a}$ play roles as tumor suppressor proteins (Serrano *et al*, 1996; Kamijo *et al*, 1997; Krimpenfort *et al*, 2001; Sharpless *et al*, 2001). $p14/19^{ARF}$ induces apoptosis or cell cycle arrest via p53-dependent and -independent pathways (Lowe and Sherr, 2003). $p14/p19^{ARF}$ is believed to play a crucial role in protecting cells from latent growth induced by aberrant oncogene activation because expression of $p19^{ARF}$ is induced by the ectopic expression of cellular and

viral oncogenes such as E2F1, c-myc, Ras, and adenovirus E1a, which inactivates pRb family members (Lowe and Sherr, 2003). In response to the ectopic expression of Ras and c-Myc, the responsiveness of $p14^{ARF}$ is distinct from that of $p19^{ARF}$ because neither Ras nor c-Myc alone induces $p14^{ARF}$ in normal human fibroblasts (Ferbeyre *et al*, 2000; Wei *et al*, 2001; Lindstrom and Wiman, 2003; Voorhoeve and Agami, 2003). In contrast, both $p14^{ARF}$ and 19^{ARF} show similar responses to the ectopic expression of E2F1 (Lowe and Sherr, 2003), suggesting that $p14^{ARF}$ does play a crucial role in protecting cells from aberrant E2F1 activation in human. Therefore, it is primarily important to elucidate the regulatory mechanism of $p14^{ARF}$ gene expression by E2F to understand the role of $p14^{ARF}$ as a tumor suppressor protein.

E2F, together with the retinoblastoma tumor suppressor family members pRb, p107, and p130, plays a central role in cell cycle regulation by coordinating expression of genes required for cell cycle progression (called the classical E2F target genes in this study) (Dyson, 1998; Nevins, 1998; Trimarchi and Lees, 2002). During quiescence, pRb families with members of the E2F family negatively affect expression of the classical E2F target genes either by preventing them from activating transcription or by recruiting transcriptional corepressor complexes on DNA (Dyson, 1998; Nevins, 1998; Trimarchi and Lees, 2002). As mitogen signals stimulate quiescent cells, they progress through G₁- to S-phases through principal events including the activation of cyclin-dependent kinases (cdks), phosphorylation of the pRb family by cyclin D/cdk4, 6 and cyclin E/cdk2 complexes, subsequent release of E2Fs from the pRb family, accumulation of free E2F activity, and the induction of classical E2F target gene expression (Dyson, 1998; Nevins, 1998; Trimarchi and Lees, 2002). During the normal cell cycle, functional balance between pRb/E2F complex-mediated transcriptional repression and E2F-mediated transcriptional activation of the classical E2F target genes is controlled by cdk activity. In contrast, in a vast majority of human cancers, the balance is inappropriately inclined to E2F-mediated transcriptional activation by disruption of the pRb pathway, including upstream regulators of pRb, such as loss of $p16^{INK4a}$ gene or amplification of *cyclin D* or *cdk4/6* genes (Sherr and McCormick, 2002). Disruption of the pRb pathway is a hallmark of human cancers and causes deregulated E2F activity (Weinberg, 1995; Sherr and McCormick, 2002).

Deregulated E2F resulting from an *RB* deficiency causes not only hyperproliferation but also apoptosis, the latter being mainly dependent on the p53-dependent pathway in the mouse model (Morgenbesser *et al*, 1994; Macleod *et al*, 1996; Tsai *et al*, 1998; Ziebold *et al*, 2001). The ectopic expression of E2F1 activates p53 through $p14/19^{ARF}$ -dependent and -independent pathways (DeGregori *et al*, 1997; Bates *et al*, 1998; Robertson and Jones, 1998; Dimri *et al*, 2000; Lomazzi *et al*, 2002; Parisi *et al*, 2002; Rogoff *et al*, 2002). Loss of $p19^{ARF}$ function allows ectopically expressed E2F1 to efficiently induce S-phase progression, reduces apop-

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tosis by the ectopic expression of E1a in primary fibroblasts, overcomes p107-dependent cell cycle arrest in mouse embryonic fibroblasts (MEFs) from the $RB^{-/-}$ mouse, and accelerates the development of pituitary tumors in $RB^{-/+}$ mice (de Stanchina *et al*, 1998; Lomazzi *et al*, 2002; Tsai *et al*, 2002; Sage *et al*, 2003). These facts imply that p14/19^{ARF} induced by deregulated E2F activity safeguards against latent cell growth.

However, induction of the $p14/19^{ARF}$ gene by physiological E2F activity is apparently inconvenient for normal cell cycle progression, as p14/19^{ARF} induced in this manner would prevent this process. In normal tissues, p19^{ARF} is expressed at low levels and has no effect on Mdm2 regulation (Zindy *et al*, 1997; O'Leary *et al*, 2004). The promoter activity of p19^{ARF} is high in tumor cells, but low or undetectable in normal adult tissues of $Arf^{GFP/GFP}$ mice (Zindy *et al*, 2003). p14^{ARF} is also expressed at high levels in many human tumor cells, particularly in tumors with defective pRb, but not in normally growing cells (Stott *et al*, 1998). These observations led to the notion that regulation of the $p14/19^{ARF}$ gene by E2F is not coupled with the cell cycle in normal cells although the $p14/19^{ARF}$ gene is induced by ectopically expressed E2F1 (Lowe and Sherr, 2003). If it is the case, the regulatory mechanism of the $p14^{ARF}$ gene by E2F should be distinct from that of the classical E2F target genes. Elucidation of the distinct mechanism would provide one answer to the challenging issue of how cells distinguish abnormal from normal growth signals, especially those induced by deregulation of E2F, and prevent aberrant growth by activating the $p14^{ARF}$ gene.

We examined regulation of the $p14^{ARF}$ gene by E2F during the cell cycle and by aberrant growth stimulation such as the ectopic expression of E2F1, cyclin D1/cdk4, and inactivation of pRb by adenovirus E1a or shRNA against RB mRNA in normal human fibroblasts. Our results show that the regulation of the $p14^{ARF}$ gene by E2F is distinct from that of the classical E2F target genes and independent of the cell cycle. Moreover, the distinct transcriptional program is active in tumor cell lines with defective pRb, but not in normally growing cells. These observations indicate that cells are endowed with a distinct E2F-mediated transcriptional program that monitors deregulation of E2F activity originating from defects in pRb function and thus prevents aberrant cell growth.

Results

Distinct regulation of $p14^{ARF}$ gene expression by E2F in normal human fibroblasts

Expression of the classical E2F target genes is regulated in a cell cycle-dependent manner through phosphorylation of the pRb family by G₁-cdks (Dyson, 1998; Nevins, 1998; Trimarchi and Lees, 2002). To verify that the induction of human $p14^{ARF}$ gene expression is dependent on the cell cycle, we examined whether expression of the $p14^{ARF}$ gene is induced by serum stimulation in normal human fibroblasts. We monitored levels of expression of the $p14^{ARF}$ gene and of the classical E2F target $CDC6$ gene (Hateboer *et al*, 1998; Ohtani *et al*, 1998; Yan *et al*, 1998) by reverse transcription (RT)-PCR after serum stimulation in quiescent WI-38 normal human fibroblasts. WI-38 cells synchronously progressed into S-phase around 16 h after serum stimulation (Figure 1A). Whereas expression of the $CDC6$ gene was obviously induced at the

G₁/S-phase boundary, expression of the $p14^{ARF}$ gene did not significantly change at any point during the cell cycle (Figure 1A). This is consistent with the recent finding that the $p19^{ARF}$ gene is not significantly induced during the MEF cell cycle (Aslanian *et al*, 2004). These results suggest that endogenous E2F activity induced by serum stimulation has little, if any, effect on $p14^{ARF}$ gene expression during the cell cycle of normal human fibroblasts, although the $p14^{ARF}$ gene is believed as a direct target of ectopic E2F1 (Lomazzi *et al*, 2002).

To confirm the responsiveness of the $p14^{ARF}$ gene to E2F under our experimental conditions and to further explore E2F regulation of the gene, we examined the induction of $p14^{ARF}$ and $CDC6$ gene expression via the ectopic expression of E2F1 in WI-38 cells during quiescence or S-phase. We infected quiescent WI-38 cells with a recombinant adenovirus expressing E2F1 or a control virus, cultured in the presence or absence of serum, and examined $p14^{ARF}$ gene expression at 21 h post-infection and after adding serum. The ectopic expression of E2F1 induced obvious $CDC6$ gene expression in quiescence and significantly less during S-phase, possibly because the gene was already induced by endogenous E2F that was activated by serum stimulation (Figure 1B). Thus, the ectopic expression of E2F1 has more powerful effect on the classical E2F target gene during quiescence than S-phase. In contrast, the level of $p14^{ARF}$ gene expression did not significantly change between quiescence and S-phase, and the ectopic expression of E2F1 similarly induced the $p14^{ARF}$ gene under both conditions (Figure 1B). This result indicates that ectopic E2F1 expression induces $p14^{ARF}$ gene expression independently of endogenous E2F activity induced by serum stimulation. We therefore concluded that regulation of the $p14^{ARF}$ gene by E2F1 is distinct from that of the classical E2F target genes in normal human fibroblasts.

Based on the above observation, we tested whether induction of the $p14^{ARF}$ gene by E2F1 would induce endogenous p14^{ARF} protein expression. As well as induction of the $p14^{ARF}$ gene expression, p14^{ARF} protein was induced by ectopic E2F1 expression in asynchronously growing WI-38 cells (Supplementary Figure S1). This result suggests that the distinct regulation of the $p14^{ARF}$ gene by E2F1 contributes to the basis of the p14^{ARF} action as a tumor suppressor.

To explore the distinct regulatory mechanism of $p14^{ARF}$ gene expression by E2F, we examined whether promoter activity of the $p14^{ARF}$ gene is responsible for expression. We performed reporter assays using the p14^{ARF} and $CDC6$ promoters linked to the *luciferase* gene (Figure 1C). We transfected the reporters into WI-38 cells, starved the cells of serum, re-stimulated them with serum and then monitored promoter activities. Serum stimulation induced obvious $CDC6$ promoter activity and mutation of the two E2F sites in the promoter almost totally abolished the responsiveness to serum stimulation (Figure 1D). In contrast, p14^{ARF} promoter activity did not significantly change during the cell cycle (Figure 1D). Conversely, the ectopic expression of p16^{INK4a}, which inhibits cdk4/6 activity and subsequently represses endogenous E2F activity, had little, if any, effect on p14^{ARF} promoter activity in asynchronously growing WI-38 cells (data not shown). We also examined the responsiveness of the p14^{ARF} and $CDC6$ promoters to ectopically expressed E2F1 in WI-38 cells cultured with or without serum. Like expression of the endogenous $CDC6$ gene, the $CDC6$ pro-

motor was more activated by the ectopic expression of E2F1 during quiescence than S-phase (Figure 1E). In contrast, the *p14^{ARF}* promoter was activated by ectopic E2F1 expres-

sion during both quiescence and S-phase to a similar extent (Figure 1E). The *p14^{ARF}* promoter was also activated by the ectopic expression of E2F2 and E2F3 in quiescent

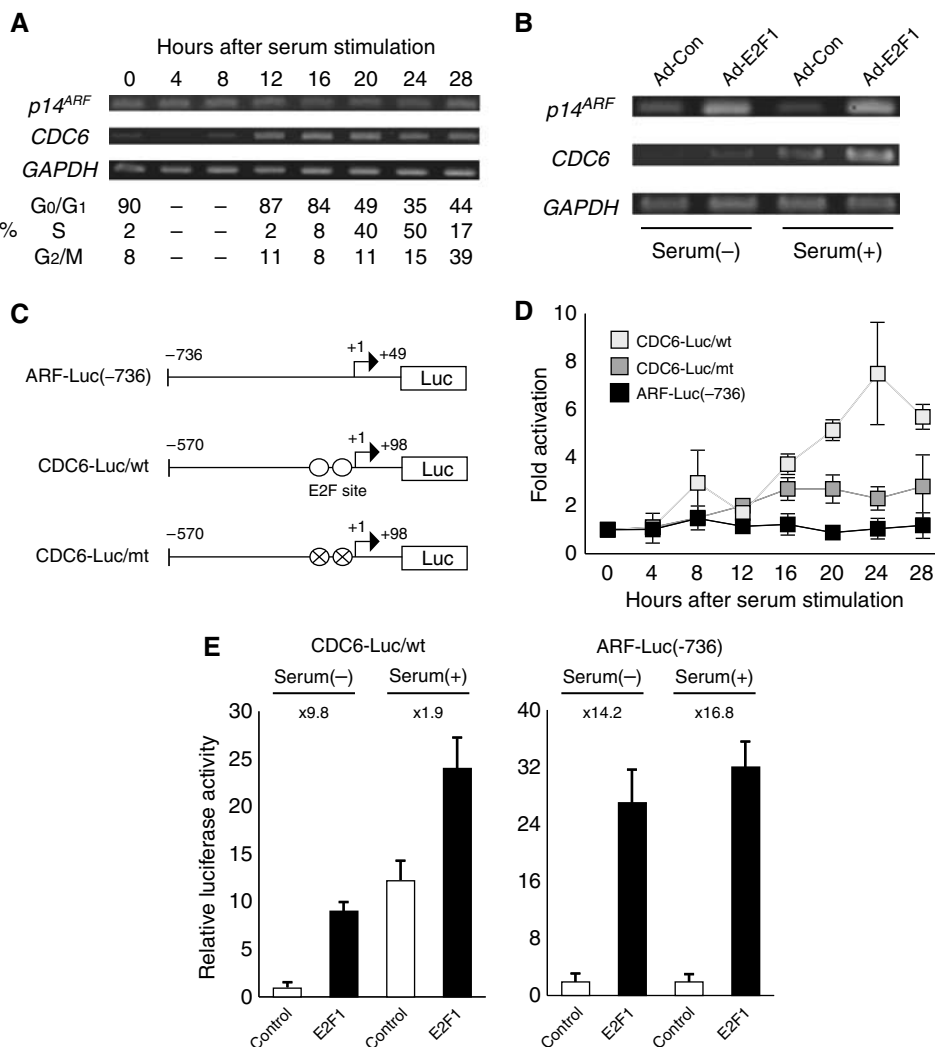
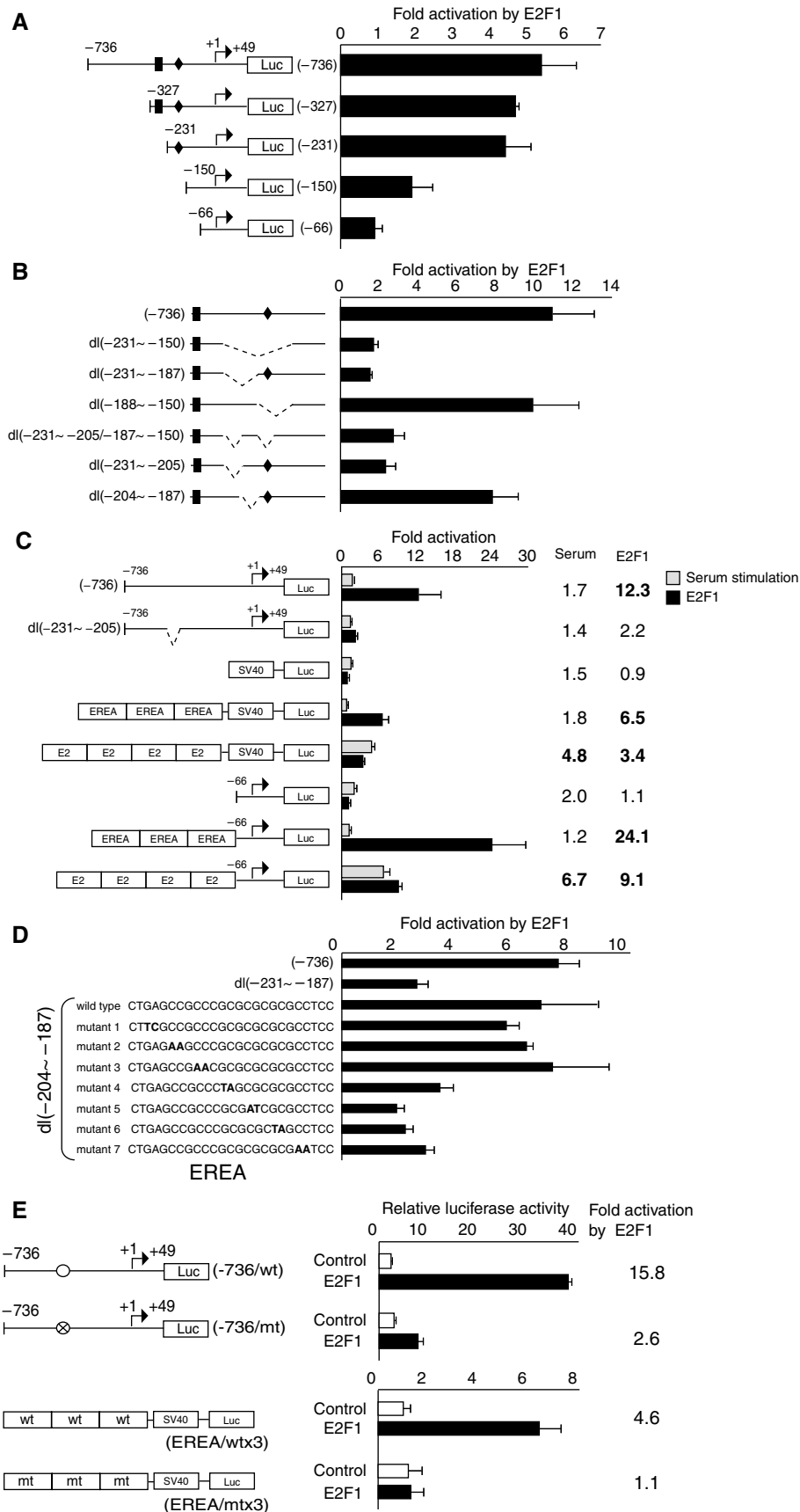


Figure 1 Distinct regulation of *p14^{ARF}* gene expression by E2F in normal human fibroblasts. (A) Kinetics of *p14^{ARF}* gene expression during the cell cycle in normal human fibroblasts. WI-38 cells were rendered quiescent, stimulated with serum, and harvested at indicated time points. mRNA levels were analyzed by RT-PCR. Internal control was the *GAPDH* gene. Cell cycle distribution of cells at individual time points was determined by measuring DNA content by FACS. (B) Cell cycle-independent induction of the *p14^{ARF}* gene by the ectopic expression of E2F1. mRNA levels were analyzed by RT-PCR. Quiescent WI-38 cells were infected with either Ad-E2F1 or Ad-Con, cultured with 0.1% FCS {Serum(-)} or with 10% FCS {Serum(+)}, and harvested at 21 h after infection and serum stimulation. (C) Schematic view of the *p14^{ARF}* {ARF-Luc(-736)} and CDC6 {(CDC6-Luc/wt) and (CDC6-Luc/mt)} promoters. Wild-type and E2F site mutant were described as wt and mt, respectively. (D) Kinetics of *p14^{ARF}* promoter activity during the cell cycle in normal human fibroblasts. WI-38 cells were transfected with indicated reporter plasmids (4 μ g) and pCMV- β -gal (1 μ g) as internal control. Transfected cells were rendered quiescent, restimulated with serum, and harvested at indicated time points. (E) Cell cycle-independent activation of the *p14^{ARF}* promoter by the ectopic expression of E2F1 in normal human fibroblasts. WI-38 cells were transfected with indicated reporter plasmid (4 μ g), expression vector for E2F1 (10 ng) or control vector, and pCMV- β -gal (1 μ g) as internal control. Transfected cells were rendered quiescent, restimulated with serum, and harvested at 0 and 21 h after serum stimulation. Fold activation by the ectopic expression of E2F1 during quiescence and S phase is indicated.

Figure 2 Identification of EREA. WI-38 cells were transfected as described in the legend to Figure 1E. Transfected cells were rendered quiescent and harvested (A-E), or restimulated with serum and harvested at 21 h after serum stimulation (C). Left panel: schematic view of reporter constructs. The square and rhombus indicate a typical E2F site (TTTCCCGC) and an E2F-like site (CTTCCCGC), respectively. (A) 5' deletion analysis of the *p14^{ARF}* promoter by ectopic E2F1 expression. (B) Internal deletion analysis of the *p14^{ARF}* promoter. (C) Responsiveness of EREA to ectopically expressed E2F1 and serum stimulation. E2 indicates the enhancer element of adenovirus E2 promoter. Right: fold activation of individual promoters by serum stimulation and the ectopic expression of E2F1. (D) Point mutation analysis of EREA using pARF-Luc/dl(-204 ~ -187) in response to the ectopic expression of E2F1. (E) Point mutation analysis of the wild-type *p14^{ARF}* promoter and EREA linked to the SV40 core promoter.

WI-38 cells (data not shown), indicating that the responsiveness of the *p14^{ARF}* promoter was not specific to E2F1 and independent of endogenous E2F1-E2F3 during the cell

cycle. These results demonstrate that the distinct regulation of the *p14^{ARF}* gene by E2F is mediated essentially by *p14^{ARF}* promoter activity.



Identification of the responsive element for the distinct regulation of $p14^{ARF}$ gene expression by E2F1

To investigate the distinct regulatory mechanism of $p14^{ARF}$ gene expression by E2F, we aimed to identify the element in the $p14^{ARF}$ promoter that was responsive to the ectopic expression of E2F1. We initially explored the responsive region by analyzing a series of 5' truncated promoters (Figure 2A). The results indicated that the $p14^{ARF}$ promoter had two regions that responded to ectopic E2F1 expression in WI-38 cells: a primary region located at -231 to -150 , and a secondary region at -150 to -66 (Figure 2A).

We focused on the region between -231 and -150 , since deletion of this region caused a more obvious reduction in $p14^{ARF}$ promoter activation by the ectopic expression of E2F1 in WI-38 cells. To precisely define the element within the region, we made internal deletion constructs between -231 and -150 in the $p14^{ARF}$ promoter (Figure 2B). We identified the region between -231 and -205 in the promoter that plays a major role in activation by ectopic E2F1 expression (Figure 2B). We named this element EREA for E2F-responsive element of the $p14^{ARF}$ promoter. It is important to note that the region does not contain a typical E2F-binding sequence (TTT^C/G^G/CGC), and, although the $p14^{ARF}$ promoter contains a typical E2F site (-272 to -265) and an E2F-like site (-184 to -177), they did not respond to the ectopic expression of E2F1 in WI-38 cells (Figure 2A and B). These results support the notion that the regulatory mechanism of the $p14^{ARF}$ gene by E2F is distinct from that of the classical E2F target genes.

To examine whether EREA is sufficient for responsiveness to ectopically expressed E2F1, we inserted three copies of EREA upstream of the SV40 and $p14^{ARF}$ core (-66) promoters that did not significantly respond to the ectopic expression of E2F1 (Figure 2C). Inserting three copies of EREA generated a response to ectopically expressed E2F1 by both core promoters, rendering the homologous core promoter more sensitive than the wild-type promoter (Figure 2C). Importantly, both promoters remained unresponsive to serum stimulation (Figure 2C). In contrast, inserting typical E2F sites from the adenovirus E2 promoter into both the SV40 and $p14^{ARF}$ (-66) core promoters rendered them responsive to both the ectopic expression of E2F1 and serum stimulation (Figure 2C). We conclude that EREA is sufficient for the distinct regulation by E2F.

To define the E2F1 responsive site in EREA, we generated a series of two-base EREA mutants based on the internal deletion reporter ARF-Luc/dl($-204 \sim -187$) (Figure 2D). The activation of mutants 4, 5, 6, and 7 by ectopic E2F1

expression was reduced almost to the same level as that obtained by a deletion of -231 to -187 (Figure 2D). The same mutation as in mutant 5 in both the wild-type promoter and EREA linked to SV40 core promoter dramatically abolished responsiveness to ectopically expressed E2F1 (Figure 2E), demonstrating that the GC repeat in EREA plays a major role in the distinct regulation of the $p14^{ARF}$ promoter by E2F1 in WI-38 cells. It is noteworthy that no mutations or deletions of EREA significantly affected the basal activity of the $p14^{ARF}$ promoter in quiescent WI-38 cells (Figure 2E and data not shown). These findings suggest that the $p14^{ARF}$ promoter is not under repression by the pRb/E2F family complexes through EREA unlike most classical E2F targets.

E2F1 binds to EREA *in vitro*

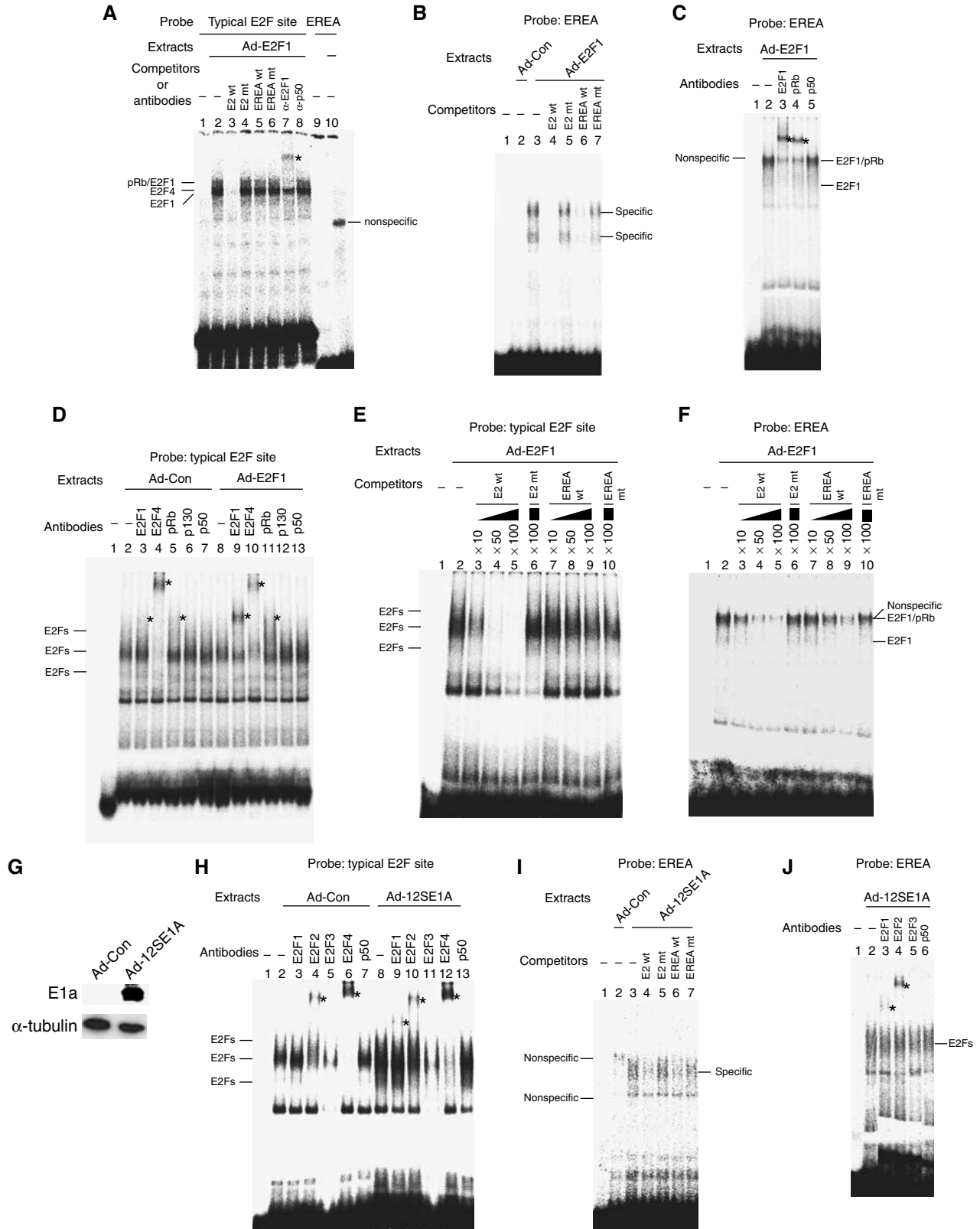
To investigate whether E2F1 could bind to EREA, we performed gel mobility shift assay according to standard E2F shift condition (Ikeda *et al*, 1996) using extracts from WI-38 cells ectopically expressing E2F1. In the standard condition, overexpressed E2F1 and pRb/E2F1 as well as endogenous E2F4 complexes were observed with the radioisotope-labeled DHFR promoter fragment containing typical E2F sites (Figure 3A, lane 2). The E2F complexes were competed out by the cold adenovirus E2 enhancer fragment containing typical E2F sites (E2 wt), but not by its mutant form (E2 mt) (Figure 3A, lanes 3 and 4). Molar excess (100-fold) of the cold EREA fragment did not have significant effect on the E2F complexes with the DHFR promoter fragment (Figure 3A, lane 5). In addition, we could not detect any specific factor bound to the radioisotope-labeled EREA fragment when used as a probe (Figure 3A, lane 10). These results, together with the fact that the responsiveness to E2F and sequence of EREA are different from those of the typical E2F target sequence, suggest two possibilities. One is that the binding affinity of E2F to EREA is too low to detect in this shift condition. The other is that there is another factor which binds to EREA, that is able to mediate the activation of EREA by ectopically expressed E2F1 but cannot be detected in this shift condition.

To address these possibilities, we modified E2F shift condition to be able to detect a specific binding factor for EREA (see Materials and methods for details). In the new shift condition, when the labeled EREA fragment was used as a probe, we did not see any specific signal in WI-38 cells infected with control virus (Figure 3B, lane 2). In contrast, we observed two bands in WI-38 cells infected with Ad-E2F1

Figure 3 E2F binds to EREA *in vitro*. E2F gel shift assays were performed in standard (A) and in new (B–F and H–J) conditions. Probes were the DHFR promoter fragment containing typical E2F sites (A, D, E, and H) and the EREA fragment (A–C, F, I, and J). Cell extracts were from asynchronously growing WI-38 cells infected with Ad-E2F1, Ad-12SE1A, or Ad-Con. Competitors were the adenovirus E2 enhancer fragment containing typical E2F sites (E2 wt), its mutant (E2 mt), EREA fragment (EREA wt), and its mutant (EREA mt). Super shifts are shown by asterisks. (A) E2F-binding activity in WI-38 cell extracts ectopically expressing E2F1 in the standard shift condition. The probes were the DHFR promoter fragment in lanes 1–8 and the EREA fragment in lanes 9 and 10. (B) Identification of factors that specifically bind to EREA in the new shift condition. Cell extracts from WI-38 cells infected with control virus were used in lane 2, and those infected with Ad-E2F1 were used in lanes 3–7. (C) Ectopically expressed E2F1 and endogenous pRb can bind to EREA. Cell extracts from WI-38 cells infected with Ad-E2F1 were used. Nonspecific band was observed at the same location of E2F/pRb complex. (D) The new shift condition essentially reproduces E2F binding to the typical E2F site in the standard shift condition. Cell extracts from WI-38 cells infected with control virus were used in lanes 2–7, those infected with Ad-E2F1 were used in lanes 8–13. (E, F) EREA shows weaker binding activity to E2F1 than the typical E2F site. Competition assays were performed with varying ratios (10, 50, 100-fold molar excess) of competitors. (G) Detection of E1a protein in cell extracts. Expression of adenovirus 12S E1a protein was confirmed by immunoblotting in WI-38 cells infected with Ad-12SE1A. (H) Expression of E1a induces general enhancement of endogenous E2F-binding activity to the typical E2F site in WI-38 cells. Cell extracts from WI-38 cells infected with control virus were used in lanes 2–7 and those infected with Ad-12SE1A were used in lanes 8–13. (I) Expression of E1a newly induces endogenous E2F-binding activity to EREA in WI-38 cells. (J) Supershift assay with antibodies against E2F1, E2F2, and E2F3.

(Figure 3B, lane 3). They were competed out by the cold EREA fragment, but not by its mutant (Figure 3B, lanes 6 and 7). These results suggest that the complexes are specific for EREA and originated by ectopic E2F1 expression. The complexes were also competed out by the cold E2 wt fragment,

but not by the E2 mt fragment, suggesting that these complexes contain E2F (Figure 3B, lanes 4 and 5). The antibody for E2F1 supershifted the complexes and reduced the original bands, demonstrating that E2F1 is contained in both the complexes (Figure 3C, lane 3). The antibody for pRb super-



shifted and reduced the upper complex but not the lower complex, suggesting that pRb is contained in the upper complex and the lower one is a free E2F1 complex with the probe (Figure 3C, lane 4). Taken together, these results show that E2F1/pRb complex and free E2F1 can specifically bind to EREA in the new shift condition when E2F1 is ectopically expressed. These results also indicate that the binding affinity of E2F1 to EREA is too low to detect in the standard shift condition.

It is remarkable that endogenous E2F4 could not be observed with EREA although the new shift condition made it possible to detect ectopically expressed E2F1 with EREA (Figure 3B and C). In the new gel shift condition, the labeled DHFR promoter fragment formed a complex with mainly endogenous E2F4 in WI-38 cells infected with control virus (Figure 3D, lane 4). Even in WI-38 cells infected with Ad-E2F1, endogenous E2F4 could still be observed along with ectopically expressed E2F1 (Figure 3D, lanes 9 and 10). These results indicate that E2F4 bound to the typical E2F site can be seen in the new shift condition as well as in the standard shift condition. In contrast, endogenous E2F4 bound to EREA could not be detected in both standard and modified shift conditions (Figure 3A–C). Additionally, the cold EREA fragment did not significantly compete out E2F4 complexes with the labeled DHFR promoter fragment in WI-38 cells infected with control virus (data not shown). These results suggest that EREA binds more preferentially to E2F1 than typical E2F site, which binds to both E2F1 and E2F4 with similar affinity. This observation points out that the property of EREA is distinct from that of the typical E2F site in the binding preference with E2Fs.

To compare the affinity of E2F1 to the probes, we performed competition assay using titrated cold competitors and extracts from WI-38 cells infected with Ad-E2F1. When the labeled DHFR promoter fragment was used as a probe, 100-fold molar excess of the cold E2 wt fragment completely competed out the E2F complexes with the probe, whereas 100-fold molar excess of the cold EREA fragment had little effect (Figure 3E). When EREA was used as a probe, the cold E2 wt fragment more strongly competed out the E2F1/pRb and E2F1 complexes with EREA than the same amount of cold EREA (Figure 3F). These results suggest that the affinity of E2F1 to the typical E2F site is higher than that of E2F1 to EREA *in vitro*.

To verify whether the endogenous E2F proteins, when deregulated, bind to EREA, we performed the new gel shift assay using extracts from WI-38 cells infected with Ad-12SE1A, which expressed adenovirus 12S E1a protein (Figure 3G). E1a inactivates pRb and activates the $p14^{ARF}$ promoter and EREA (Figure 5A). In E1a-expressing WI-38 cells, the amount of endogenous E2F released from pRb family proteins complexed with the labeled DHFR promoter fragment was a little increased, but still mainly E2F4 formed complex with the probe (Figure 3H). This result indicates that expression of E1a, in asynchronously growing WI-38 cells, does not have dramatic effect on the binding activity of endogenous E2F family proteins to the typical E2F site *in vitro*. When EREA was used as a probe, expression of E1a newly induced a complex, which was observed as a broad band (Figure 3I). These complexes were competed out by the cold E2 wt and EREA fragments, but not by their mutant fragments, indicating that the binding is specific to EREA fragment (Figure 3I). It should be noted that the antibodies

for E2F1 and E2F2 originated supershifted bands. Although the antibodies for E2F3 did not originate supershifted band, they reduced the intensity of the broad band (Figure 3J), suggesting that the antibodies rather inhibited the binding of E2F3 to the probe. These results suggest that the complexes formed with the labeled EREA fragment contain at least endogenous E2F1, E2F2, and possibly E2F3 *in vitro*.

E2F1 directly binds to the $p14^{ARF}$ promoter through EREA *in vivo*

Next, to investigate the binding of E2F to the $p14^{ARF}$ promoter *in vivo*, we performed chromatin immunoprecipitation (ChIP) assay. In quiescent WI-38 cells infected with Ad-E2F1, E2F1 was detectable on both the $p14^{ARF}$ promoter including EREA and the CDC6 promoter including two E2F-binding sites but not on the β -actin promoter, an E2F-unrelated target (Figure 4A). In contrast, E2F1 was undetectable on any promoters in quiescent WI-38 cells infected with the control virus (Figure 4A). These results indicate that ectopically expressed E2F1 associates with the $p14^{ARF}$ promoter, suggesting that regulation of the $p14^{ARF}$ promoter by E2F1 is directly mediated *in vivo*. Although E2F4 and p130 were detected on the CDC6 promoter in quiescent WI-38 cells infected with the control virus, they were undetectable on the $p14^{ARF}$ promoter (Figure 4A and data not shown). This observation, together with the finding that a mutation in EREA did not elevate the basal activity of the $p14^{ARF}$ promoter, suggests that the $p14^{ARF}$ promoter is not repressed by the p130/E2F4 repressor complex through EREA in quiescent WI-38 cells.

As shown in Figure 4A, the amplified region of the $p14^{ARF}$ promoter for PCR in our ChIP assay contained not only EREA but also the E2F-like site (–184 to –177). This leaves a question whether EREA actually contributes to binding of E2F1 to the $p14^{ARF}$ promoter *in vivo*. To address this issue, we generated two cell lines integrated with the $p14^{ARF}$ promoter or its EREA mutant (Figure 4B) using REF52 cells, in which the responsiveness of the $p14^{ARF}$ promoter to the ectopic expression of E2F1 and serum stimulation is quite similar to that of WI-38 cells (data not shown). The integration of the $p14^{ARF}$ promoter constructs was confirmed by Southern blotting using luciferase cDNA as a probe (Figure 4C). We performed ChIP assay using these cell lines. Ectopically expressed E2F1 bound to the endogenous DHFR promoter, one of the classical E2F targets, but not to the β -actin promoter in both cell lines (Figure 4D). In this condition, ectopically expressed E2F1 was seen on the integrated wild-type $p14^{ARF}$ promoter but not on the EREA mutant (Figure 4D). These results show that integrity of EREA is required to recruit E2F1 to the $p14^{ARF}$ promoter *in vivo*.

To further verify the direct effect of E2F on EREA *in vivo*, we examined whether ectopically expressed E2F1 can activate EREA dependently on the DNA-binding ability of E2F1 without additional protein synthesis. We used the estrogen receptor (ER)–E2F system, in which E2F1 fused to the ER ligand-binding domain localizes in the cytoplasm and can be transported into the nucleus by adding 4-hydroxytamoxifen (OHT) to activate E2F-mediated transcription (Muller *et al*, 2001). We examined whether the nuclear localization of ER–E2F1 activates transcription of the *luciferase* gene driven by the $p14^{ARF}$ core promoter linked with three copies of EREA (Figure 2C). In this experiment, we used REF52 cells again. Luciferase assays showed that OHT activated EREA reporter

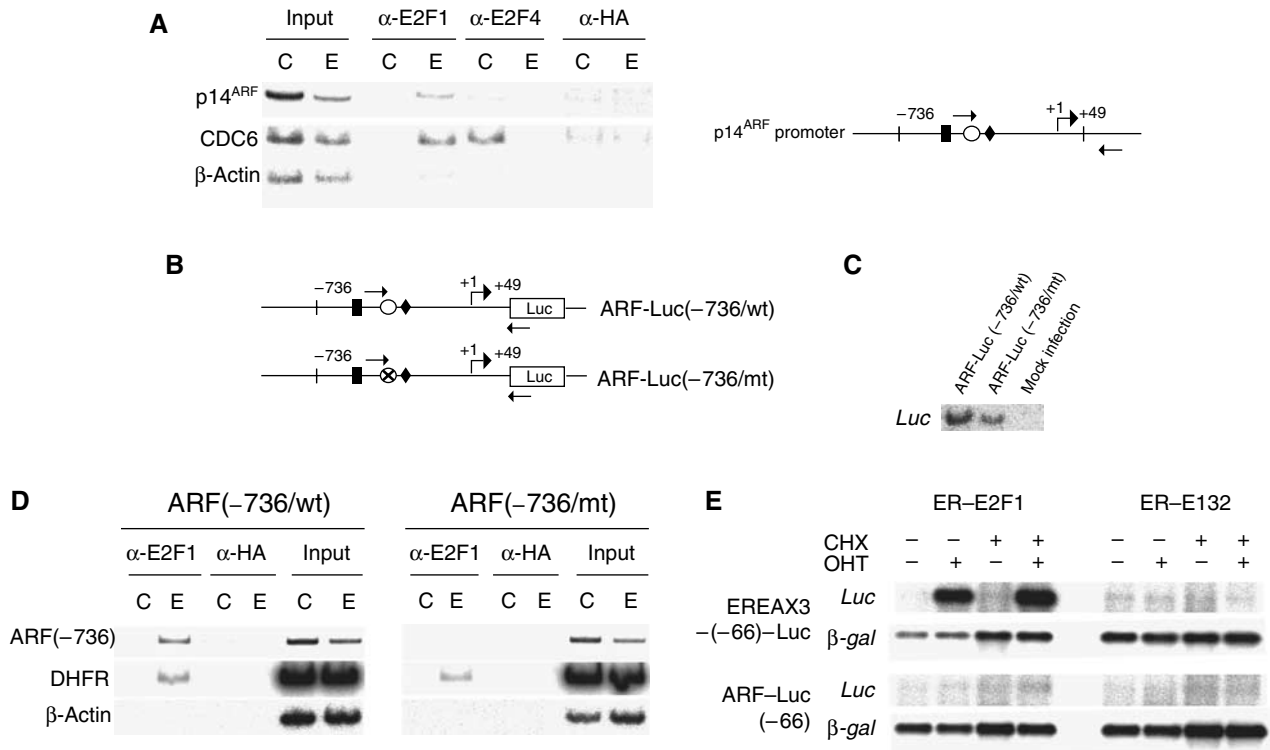


Figure 4 Direct binding of E2F1 to the p14^{ARF} promoter through EREA *in vivo*. (A) Left panel: ChIP analysis of the p14^{ARF} promoter in quiescent WI-38 cells ectopically expressing E2F1. Quiescent WI-38 cells were infected with recombinant adenovirus expressing E2F1 (E) or control virus (C) and chromatin was immunoprecipitated using indicated antibodies. Control contained anti-HA antibody. Immunoprecipitated DNA was amplified by PCR with primer pairs specific to indicated promoters. Right panel: schematic view of primer locations. Small arrows indicate primer locations. Square, rhombus, and circle indicate a typical E2F site, an E2F-like site, and EREA, respectively. (B) Schematic view of promoter constructs used for integration into the genome of REF52 cells and primer locations (small arrows) for ChIP assay. (C) Integration of the reporter constructs into the genome of REF52 cells. Integrated luciferase gene was detected by Southern blotting. (D) ChIP analysis of the p14^{ARF} promoter constructs in quiescent REF52 cells ectopically expressing E2F1. (E) E2F1 directly activates EREA dependently on DNA-binding ability. REF52 cells were transfected, cultured for 2 days, incubated with OHT and/or CHX for 8 h and harvested. Induction of *luciferase* gene expression by either ER-E2F1 or ER-E132 was analyzed by Northern blotting.

activity in the absence of the protein synthesis inhibitor cycloheximide (CHX), but not in the presence of both OHT and CHX (Supplementary Figure S2). These findings demonstrate that OHT activated ER-E2F1 and that CHX blocked new protein synthesis in REF52 cells. We performed Northern blotting under the same conditions. Adding OHT induced *luciferase* mRNA from the EREA reporter in the presence of CHX, indicating that the effect of E2F1 upon EREA is independent of *de novo* protein synthesis (Figure 4E). Furthermore, a DNA-binding mutant of E2F1 (ER-E132) could not induce *luciferase* gene expression from the EREA reporter, indicating that DNA-binding activity is necessary for an effect on EREA (Figure 4E). Collectively with the data of ChIP assays, these results indicate that E2F1 directly binds to and activates EREA *in vivo*.

EREA responds to endogenous E2F activity resulting from pRb inactivation by E1a and shRNA, but not by phosphorylation through cdk activity

The p14^{ARF} promoter was directly activated by the ectopic expression of E2F1 through EREA, but not by physiological E2F activity induced by serum stimulation. This indicates that there is a mechanism associated with EREA that discriminates deregulated E2F activity from physiological E2F activity during the cell cycle to activate the p14^{ARF} promoter. To

address this issue, we focused on the manner of inactivation of pRb, in particular by phosphorylation of pRb, to activate the p14^{ARF} promoter through endogenous E2F, since serum stimulation phosphorylates the pRb family through cdk activation and subsequently activates endogenous E2F during the cell cycle. In contrast, deregulated E2F activity is generated from aberrant inactivation of pRb, including mutation and deletion of *RB* gene.

We investigated the responsiveness of the p14^{ARF} promoter to several manners of pRb inactivation as follows. We first questioned whether the p14^{ARF} promoter responds to deregulation of endogenous E2F through inactivation of pRb family proteins by adenovirus E1a in normal human fibroblasts, since E1a directly binds and inactivates the pRb family, leading to latent cell growth. We examined the responsiveness of the CDC6 and p14^{ARF} promoters to wild-type E1a, a p300-binding domain mutant, and a pRb family-binding domain mutant by reporter analysis in WI-38 cells. While the wild-type and p300 interaction mutant activated both the CDC6 and p14^{ARF} promoters, the pRb-binding domain mutant did not significantly affect either of them (Figure 5A). These results are consistent with previous report that E1a induces p19^{ARF} gene expression (de Stanchina *et al*, 1998). Essentially, the results were similar with EREA and a typical E2F site linked to the SV40 core promoter (EREAX3-Luc and

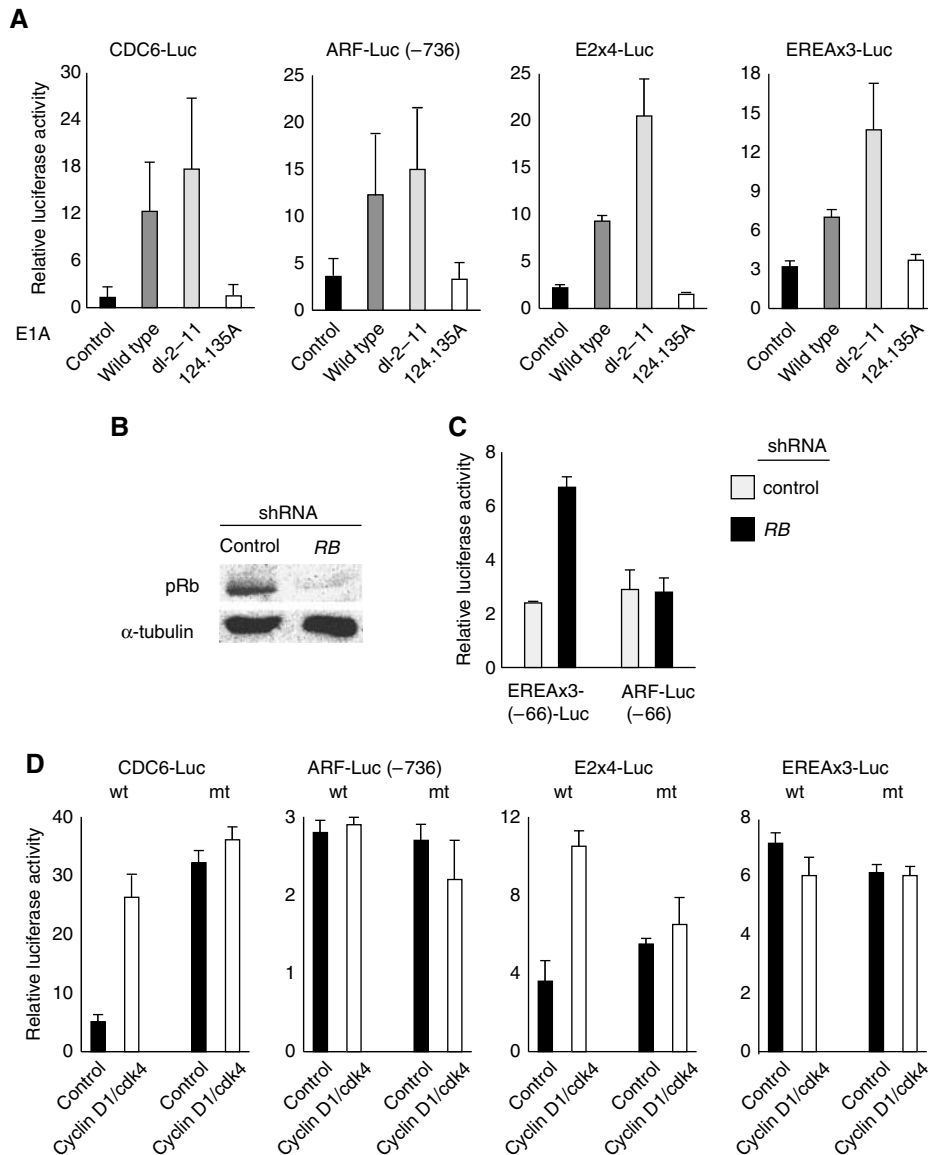


Figure 5 EREA is activated by endogenous E2F activity resulting from inactivation of pRb by E1a and shRNA, but not from pRb phosphorylation by cdk activity in normal human fibroblasts. (A) Activation of the $p14^{ARF}$ promoter and EREA by the ectopic expression of E1a in normal human fibroblasts. WI-38 cells were transfected with indicated reporter constructs (4 μ g), expression vector for E1a (100 ng) or its mutants and pCMV- β -gal (900 ng) as internal control. Transfected cells were cultured under serum starvation and harvested at 72 h later. (B) Reduction of pRb protein level in 293 cells by the expression of shRNA against *RB* mRNA. Level of pRb protein was examined by immunoblotting in 293 cells transfected with pshRB or control vector. Internal control was α -tubulin. (C) Activation of EREA by the expression of shRNA against *RB* mRNA in normal human fibroblasts. WI-38 cells were transfected with indicated reporter plasmid (1 μ g), pshRB or control vector (3 μ g), and pCMV- β -gal (1 μ g) as internal control. Cells were rendered quiescent, restimulated with serum and harvested at 21 h thereafter. (D) The $p14^{ARF}$ promoter and EREA do not respond to the ectopic expression of cyclin D1 and cdk4 in normal human fibroblasts. WI-38 cells were transfected with indicated reporter constructs (1 μ g), expression vector for Cyclin D1 (2.5 μ g), cdk4 (500 ng), and pCMV- β -gal (1 μ g) as internal control. Transfected WI-38 cells were cultured under serum starvation and harvested at 72 h later.

E2x4-Luc) in response to E1a (Figure 5A). These results, together with the observation that, when E1a is expressed, E2F forms the complex with EREA *in vitro*, indicate that EREA is sufficient in response to deregulated endogenous E2F activity induced by E1a independently of pRb phosphorylation.

To further understand that EREA responds to loss of pRb function independently of phosphorylation, we examined whether a forced reduction in pRb protein expression also activates EREA using the shRNA expression vector, pshRB, against human *RB* mRNA. The pRb level was significantly decreased by the expression of shRNA in 293 cells

(Figure 5B). The activity of the EREA reporter was significantly increased by pshRB, but not control reporter, in WI-38 cells (Figure 5C). We thus concluded that EREA responds to deregulated endogenous E2F activity resulting from the pRb level being reduced by shRNA in WI-38 cells.

Next, to determine whether the $p14^{ARF}$ promoter responds to endogenous E2F activity induced by pRb phosphorylation, we examined whether the ectopic expression of cyclin D1/cdk4 could activate the $p14^{ARF}$ and CDC6 promoters in WI-38 cells. The CDC6 promoter was activated by the ectopic expression of cyclin D1/cdk4, dependently on the E2F-bind-

ing site (Figure 5D), demonstrating that ectopically expressed cyclin D1/cdk4 phosphorylated the pRb family and activated endogenous E2F, which in turn activated the classical target promoter. In contrast, the $p14^{ARF}$ promoter was not significantly activated in response to the ectopic expression of cyclin D1/cdk4 (Figure 5D), as well as to serum stimulation. These results are consistent with those of another report, in which the ectopic expression of cyclin E did not induce $p14^{ARF}$ gene expression in normal human fibroblasts (Minella *et al*, 2002). The results were essentially identical with EREAx3-Luc and E2x4-Luc in response to the ectopic expression of cyclin D1/cdk4 (Figure 5D). These results, together with the activation of EREA by E1a and shRNA, indicate that EREA responds to deregulated endogenous E2F activity resulting from pRb inactivation by E1a and shRNA but not from pRb phosphorylation in WI-38 cells.

Distinct E2F-mediated transcriptional program associated with EREA senses loss of pRb function

The $p14^{ARF}$ gene is expressed at low levels in normally growing cells, but at high levels in many types of cancer cells, including those from tumors lacking pRb function (Stott

et al, 1998). This observation, together with our finding that EREA is activated when the pRb function is lost by shRNA and E1a in WI-38 cells, suggests that the $p14^{ARF}$ gene is induced by deregulation of endogenous E2F activity resulting from loss of pRb function through EREA in tumor cells that have defective pRb. If so, the deregulated E2F activities should be detected by determining whether or not EREA has activity.

We verified that the distinct and active E2F-mediated transcriptional program is associated with EREA in tumor cells lacking pRb function. We examined whether the ectopic expression of pRb represses EREA reporter activity in the tumor cell lines, Saos-2 osteosarcoma, 5637 bladder carcinoma, and C33-A cervical carcinoma, which lack both pRb and p53 functions and express high levels of $p14^{ARF}$ (Stott *et al*, 1998). The ectopic expression of the constitutive active mutant form of human pRb repressed EREA reporter activities to control levels in all tumor cell lines (Figure 6A). It is noted that EREA reporter activity was repressed by wild-type pRb but not by a mutant pRb, which lacks exon 21 and cannot bind to E2F (Heibert *et al*, 1992), in these tumor cell lines (data not shown). This result shows that pRb represses EREA

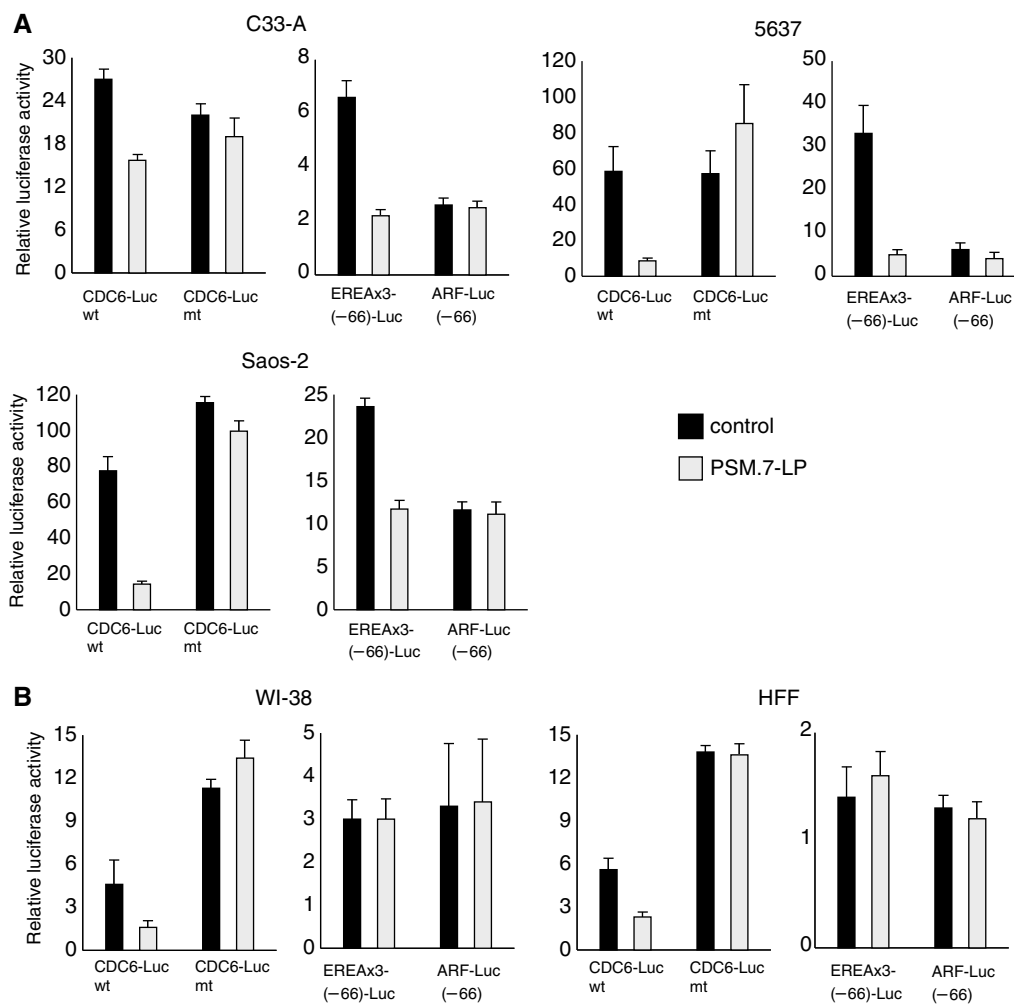


Figure 6 EREA is activated in tumor cells with defective pRb. (A) EREA has activity in pRb-defective tumor cell lines. Cells were transfected with indicated reporter plasmid (1 µg) together with expression vector for constitutive active mutant of human pRb, pPSM.7-LP, or control vector (100 ng) and pCMV-β-gal (1 µg). (B) EREA does not have activity in normally growing cells. WI-38 cells and HFFs were transfected as above, cells were rendered quiescent, stimulated with serum, and harvested at 21 h later.

reporter activity through inhibition of deregulated E2F activity. In contrast, constitutive active pRb repressed the CDC6 promoter in normally growing cells, but did not significantly affect EREA reporter activities (Figure 6B). These data confirm that the unique mechanism associated with EREA is not active in normally growing cells. These results, together with the observation that EREA responds to loss of pRb function in normally growing cells, suggest that EREA senses the deregulated E2F activity caused by loss of pRb function in physiological circumstances. This observation implies that the distinct E2F-mediated transcriptional program associated with EREA allows the discrimination of malignant cells with defective pRb from normally growing cells.

Discussion

The $p14^{ARF}$ protein is thought to play a crucial role in tumor suppression against oncogenic cell growth induced by ectopically expressed E2F (Lowe and Sherr, 2003). Although E2F constitutes an integral part of normal cell growth signaling, $p14^{ARF}$ is expressed at low levels in normally growing cells in contrast to many tumor varieties (Stott *et al*, 1998). These observations raise the enigmatic issue of how E2F regulates $p14^{ARF}$ gene expression in normal and tumor cells. We discovered that regulation of the $p14^{ARF}$ gene by E2F is mediated through a novel E2F-responsive element that differs from the typical E2F site. This regulation enables the $p14^{ARF}$ promoter to distinguish E2F activity that arises due to defective pRb function from that in normally growing cells. This explains why the $p14^{ARF}$ gene is expressed at low levels in normally growing cells, as well as how the $p14^{ARF}$ promoter monitors abnormal growth signals that originate from defects in pRb function and plays a role as a tumor suppressor in cells with defective pRb.

Regulation of the classical E2F target genes by E2F is dependent on the cell cycle because the pRb family is under the control of cdk during the cell cycle (Dyson, 1998; Nevins, 1998; Trimarchi and Lees, 2002). However, we showed that regulation of the $p14^{ARF}$ gene by E2F is distinct from that of the classical E2F target genes and is independent of the cell cycle. This distinct regulation was mediated through EREA. These findings raise the question as to the aspect of E2F activity that determines whether or not to activate EREA. A comparison of EREA responsiveness to the signals that activate the classical E2F target genes revealed a unique property. EREA responded to deregulated E2F activity caused by pRb inactivation independently of G1-cyclin/cdk-dependent phosphorylation, such as E1a and shRNA against *RB* mRNA, but not to E2F activity induced by pRb phosphorylation, such as through serum stimulation or ectopic cyclin D1/cdk4 expression in normal human fibroblasts. Consistent with these observations, EREA had activity in several tumor cell lines that have defective pRb, but not in normally growing cells. We propose the following model based on these results. Deregulated E2F that arises through defective pRb function gains the ability to activate the $p14^{ARF}$ promoter through EREA independently of the cell cycle, whereas physiological E2F activity induced during the normal cell cycle cannot activate the $p14^{ARF}$ promoter (Figure 7).

Our observations are consistent with those of others, who have shown that $p19^{ARF}$ is expressed in mouse cells with

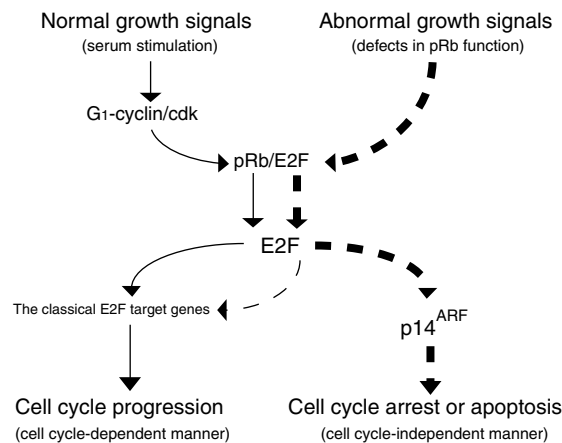


Figure 7 Model for unique transcriptional regulation of $p14^{ARF}$ gene expression by E2F. Solid arrows indicate normal growth signals mediated through cdk activity. Thick broken arrows indicate abnormal growth signals resulting from loss of pRb function.

defective pRb but not in normal mouse tissues (Zindy *et al*, 1997, 2003; de Stanchina *et al*, 1998; Sage *et al*, 2003). Together with the fact that $p14/19^{ARF}$ induced by deregulated E2F activity functions in the suppression of aberrant cell growth (de Stanchina *et al*, 1998; Lomazzi *et al*, 2002; Tsai *et al*, 2002; Sage *et al*, 2003), these findings suggest that the distinct E2F-mediated transcriptional program of the $p14^{ARF}$ gene forms an important basis of $p14/19^{ARF}$ action as a tumor suppressor by sensing defects in pRb function.

EREA could activate transcription when linked to both homologous and heterologous core promoters. It contains a novel GC repeat sequence and does not include a T stretch, which is typical of the E2F-binding sequence ($\text{TTT}^{\text{C}}/\text{C}^{\text{C}}/\text{C}^{\text{C}}\text{GC}$). Although the $p14^{ARF}$ promoter possesses a typical E2F site and an E2F-like site, they did not activate the $p14^{ARF}$ promoter through ectopic E2F1 expression. The most highly conserved region of the mouse $p19^{ARF}$ promoter involves the sequence aligned with EREA and the behavior of the mouse sequence, and human EREA in response to E2F was consistent in REF52 and in WI-38 cells (data not shown). These observations suggest that the distinct E2F-mediated transcriptional program associated with EREA is conserved from rodents to humans. It is noteworthy that any specific binding activity to EREA could not be detected with extracts from cells cultured in the presence of serum although endogenous E2Fs were physiologically activated. In contrast, binding of endogenous E2F1, E2F2, and possibly E2F3 to EREA was observed, when they were deregulated by expression of E1a. Taken together, these observations suggest that endogenous E2F1, E2F2, and E2F3 may gain ability to bind to EREA and activate transcription upon inactivation of pRb family members by E1a. In contrast, physiologically activated E2F through phosphorylation of pRb by cdks remains unable to bind to EREA or activate transcription. This notion is consistent with previous results that showed induction of endogenous E2F1 binding to the mouse $p19^{ARF}$ promoter upon E1a expression that was not detected in normally growing cells by ChIP assay (Aslanian *et al*, 2004). It remains unknown why E2F physiologically activated by cdks does not have the ability to bind to EREA. It might obtain some modification, which blocks its binding to EREA, when

bound to or upon release from pRb through phosphorylation by cdks. Difference of EREA sequence from typical E2F site sequence also suggests that E2F is modified and/or forms a novel complex with another factor to bind to EREA. Further studies are required to clarify what determines the ability of E2F to bind to EREA.

This study shows another point of distinct regulation of EREA by E2F at the molecular level. Mutations in EREA did not significantly affect the basal activity of the $p14^{ARF}$ promoter and neither E2F4 nor p130 (components of the p130/E2F4 repressor complex) could not be detected on the $p14^{ARF}$ promoter by both gel shift and CHIP assays. These observations suggest that the $p14^{ARF}$ promoter is not under the control of the p130/E2F4 repressor complex through EREA in quiescent cells. This is consistent with a recent report showing that the p130/E2F4 repressor complex does not bind to the $p19^{ARF}$ promoter in quiescence (Aslanian *et al*, 2004). In the report, it was suggested that E2F3 might repress the mouse $p19^{ARF}$ promoter in quiescent MEFs (Aslanian *et al*, 2004). This observation seems not to be consistent with regulation of EREA identified in our study. EREA was not repressed in quiescent cells, and deregulated E2Fs had the affinity to bind to EREA and activate the $p14^{ARF}$ promoter. In addition, previous reports show that E2F1 and E2F3 are positive players in the apoptosis induced by loss of pRb function (Tsai *et al*, 1998; Ziebold *et al*, 2001). It may be reasonable to predict that E2F3 plays an activating role in the regulation of the $p14/19^{ARF}$ promoter. Our results are consistent with this notion, suggesting that regulation of the $p14^{ARF}$ promoter through EREA reflects regulation of the $p14^{ARF}$ gene in the physiological situation. In CHIP assays, although E2F3 was detected on the $p14^{ARF}$ promoter integrated in REF52 cells, mutation in EREA did not significantly affect binding of E2F3 to the $p14^{ARF}$ promoter (data not shown). These results suggest that E2F3 may have another function in regulation of the $p14/19^{ARF}$ promoter independently of EREA. Further studies are required to determine whether E2F3 is involved in the regulation of the human $p14^{ARF}$ promoter in quiescent cells.

The question remains whether the $p14^{ARF}$ promoter can respond to oncogenic growth signals that arise upstream of the pRb pathway, such as the increased cyclin D1 expression caused by amplification or translocation of the gene that is a frequent feature of human cancers (Sherr and McCormick, 2002). Under our experimental conditions, the ectopic expression of cyclin D1/cdk4 activated the classical E2F target promoters, but not EREA. This suggests that EREA senses dysfunction of pRb by itself and might be unable to monitor defects upstream of pRb. This notion is consistent with previous report which showed ectopic expression of cyclin

E did not induce $p14^{ARF}$ gene expression in normal human fibroblasts (Minella *et al*, 2002). Alternatively, the $p14^{ARF}$ promoter might respond to defects upstream of pRb through EREA or another element(s) under different cellular circumstances or mechanisms. Further studies are required to clarify these issues.

The present study discovered a distinct E2F-mediated transcriptional program of $p14^{ARF}$ gene associated with EREA. This novel program can discriminate deregulated E2F activity caused by defects in pRb function from physiological E2F activity during the normal cell cycle. To specifically kill cancer cells with therapies such as irradiation and chemotherapy is difficult, as these strategies are targeted towards cells undergoing rapid proliferation, including normally growing cells. This distinct mechanism enables the discrimination of tumor cells with defective pRb from normally growing cells by monitoring deregulated E2F activity. Thus, this program will be a useful means to generate novel strategies which are able to specifically target and attack tumors with defective pRb, that account for about 30% of all human cancers (Weinberg, 1995), without affecting normal cells.

Materials and methods

Gel mobility shift assay

The standard E2F gel mobility shifts were performed as described (Ikeda *et al*, 1996). The modified E2F gel shifts were performed as follows. Cell extracts were incubated with radiolabeled probes for 20 min at room temperature in 10 mM Tris-HCl, pH 7.9/25 mM KCl/25 mM NaCl/1 mM EDTA/10 mM dithiothreitol/5% glycerol/1 μ g of sonicated herring sperm DNA (average length <200 bp), and then resolved by electrophoresis on 5% polyacrylamide gels in TAE buffer (6.7 mM Tris-HCl, pH 7.5/3.3 mM sodium acetate/1 mM EDTA). It is important to use highly sonicated DNA, for which we used BD Yeastmaker Carrier DNA (Clontech). The adenovirus E2 enhancer, EREA fragment, and their mutants were used as competitors. Supershift experiments included anti-E2F1 (sc-251 X; Santa Cruz), anti-E2F2 (sc-633 X; Santa Cruz), anti-E2F3 (sc-878 X; Santa Cruz), anti-E2F4 (sc-512 X; Santa Cruz), anti-pRb, anti-p130 (sd-317 X; Santa Cruz), and anti-p50 (sc-848 X; Santa Cruz) antibodies.

See Supplementary data regarding other Materials and methods.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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