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Active Transcriptional Repression by the Rb–E2F Complex Mediates G1 Arrest Triggered by $p16^{INK4a}$, TGFβ, and Contact Inhibition

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Summary

Rb inhibits progression from G1 to S phase of the cell cycle. It associates with a number of cellular proteins; however, the nature of these interactions and their relative significance in cell cycle regulation are still unclear. We present evidence that Rb must normally interact with the E2F family of transcription factors to arrest cells in G1, and that this arrest results from active transcriptional repression by the Rb–E2F complex, not from inactivation of E2F. Thus, a major role of E2F in cell cycle regulation is assembly of this repressor complex. We demonstrate that active repression by Rb–E2F mediates the G1 arrest triggered by TGFβ, $p16^{INK4a}$, and contact inhibition.

Introduction

The retinoblastoma protein (Rb) is an important regulator of the G1 to S phase transition (Ewen, 1994; Weinberg, 1995). Rb has been shown to interact with a number of cellular proteins; however, it is still unclear which of these interactions are important for Rb function in vivo. The best studied of these Rb-binding proteins is the E2F family of transcription factors (Chellappan et al., 1991; Nevins, 1992; La Thangue, 1994; Lam and La Thangue, 1994; Adams and Kaelin, 1996; Slansky and Farnham, 1996). Interaction with Rb not only blocks transcriptional activation by E2F; the Rb–E2F complex that forms at the promoter actively represses transcription of cell cycle genes (Hamel et al., 1992; Weintraub et al., 1992, 1995; Adnane et al., 1995; Bremner et al., 1995; Sellers et al., 1995; Luo et al., 1998). However, it has not been determined whether the most important role of Rb is blocking transcriptional activation by E2F, or the formation of the active Rb–E2F repressor complex.

The gene for E2F-1 has been knocked out in mice, and the mice develop relatively normally and are fertile (Field et al., 1996; Yamasaki et al., 1996). However, they later exhibit testicular atrophy and exocrine gland dysplasia, and they develop a variety of tumors. These results suggest that E2F-1 may be necessary to maintain some tissues, but it can also act as a tumor suppressor in several other tissues. However, even though E2F-1 is not required for proliferation of most normal cells, tumor cell proliferation is inhibited in *E2F-1*(−/−) mice (Pan et al., 1998; Tsai et al., 1998). Therefore, E2F-1 can act as both an oncogene and a tumor suppressor (Weinberg, 1996). However, these complex biological effects of E2F say little about how these proteins work to regulate the cell cycle.

The activity of Rb is regulated by G1 cyclin-dependent kinases (CDKs), which phosphorylate Rb during G1 (Mihara et al., 1989; Ludlow et al., 1990; Hinds et al., 1992; Roberts et al., 1994; Sherr and Roberts, 1995; Sherr, 1996; Lundberg and Weinberg, 1997). Initially, CDK4 and 6 interact with D family cyclins to form active kinases that phosphorylate Rb during mid G1. Subsequently, cyclin E is expressed, and it forms an active kinase with CDK2 that phosphorylates Rb near the end of G1. This hyperphosphorylation of Rb disrupts its association to E2F and allows transcription of S phase genes and movement of cells from G1 into S phase. CDK4/6 activity is regulated by the INK family of inhibitors ($p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, and $p19^{INK4d}$), which block their activity (Sherr and Roberts, 1995). Like Rb, $p16^{INK4a}$ has been shown to be a tumor suppressor and is mutated in a high percentage of tumor cells (Palmero and Peters, 1996).

One regulator of the G1 to S phase transition in epithelial cells is transforming growth factor β (TGFβ) (Massague, 1996). TGFβ appears to regulate cell cycle progression at several different levels. It can decrease the activity of the G1/S phosphatase cdc25A (Iavarone and Massague, 1997), which may be required for activation of CDK4/6, and it can decrease expression of CDK4 (Ewen et al., 1993, 1995). Additionally, TGFβ induces the accumulation of the CDK inhibitors $p15^{INK4b}$ and $p27^{Kip1}$ (Reynisdottir et al., 1995). While $p15^{INK4b}$ selectively blocks the activity of CDK4/6, $p27^{Kip1}$ can inhibit the activity of CDK2, which is also required for transition of cells from G1 to S phase and for progression through S phase (Roberts et al., 1994; Sherr, 1996).

Proliferation of epithelial cells is also regulated by cell–cell contact. One consequence of cell–cell contact is an increase in the CDK inhibitor $p27^{Kip1}$ and a decrease in the level of cyclin D1 (Polyak et al., 1994; St. Croix et al., 1998). As with overexpression of $p16^{INK4a}$ and TGFβ treatment, the result is accumulation of hypophosphorylated Rb and G1 arrest. This phenomenon is known as contact inhibition, and mutations that overcome contact inhibition are common in tumor cells.

Here, we provide evidence that Rb must normally interact with E2F to arrest cells in G1. We also show that it is repression by the Rb–E2F complex, not an Rb-mediated block in transcriptional activation by E2F, that is responsible for this arrest. We show that active repression by Rb–E2F mediates the G1 arrest caused by TGFβ, $p16^{INK4a}$, and contact inhibition.

Results

An E2F Mutant Containing Only the DNA-Binding Domain Blocks E2F Site Activity

E2F family members share a similar DNA-binding domain and bind a similar DNA sequence. Thus, it appeared that overexpression of an E2F DNA-binding

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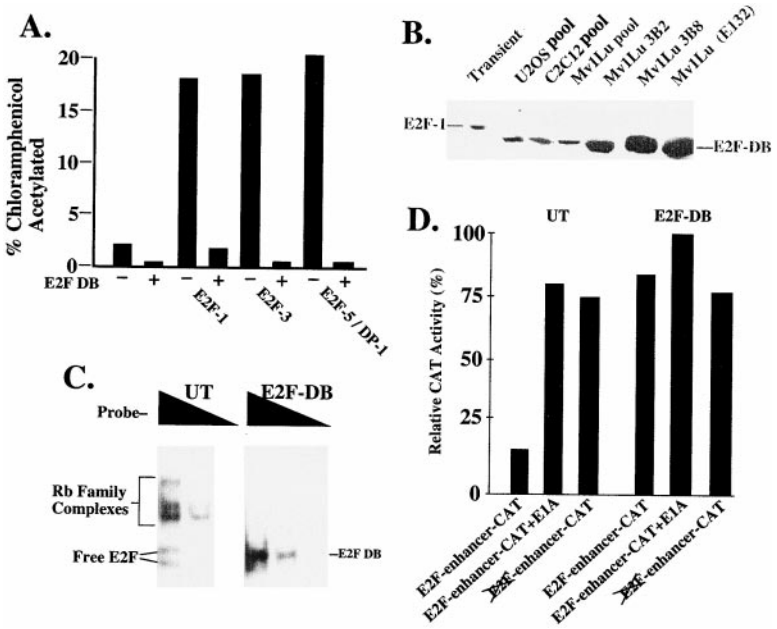

Figure 1. E2F-DB Displaces Wild-Type E2F and Rb–E2F Complexes from E2F Sites and Blocks E2F Site Function

(A) E2F-DB blocks E2F activity in transfection assays. The indicated E2F family or DP-1 expression vectors (0.5 μg) were cotransfected into 60 mm plates of U2OS cells along with 2.0 μg of the minimal E2F site reporter construct E2F-CAT (Weintraub et al., 1992). Similar results were also obtained with the Rb(−) C33A cell line (results not shown). Where indicated, cells were also cotransfected with 1 μg of the E2F-DB expression vector. One microgram of empty vector was cotransfected in plates that did not receive the E2F-DB expression vector. Results are representative of at least three separate assays, each in duplicate.

(B) Stable expression of E2F-DB and E2F-DB(E132) in cells. Colonies from stable transfection of Mv1Lu cells, C2C12 myoblasts, and U2OS cells with E2F-DB were analyzed for expression of E2F-DB. Western blots from pooled clones are shown for the different cell lines. A low level of E2F-1 is evident in each of the cell lines upon a long exposure. The left-hand lane shows the position of migration of full-length E2F-1 from cells transiently transfected with an E2F-1 expression vector. A number of individual clones expressing E2F-DB were obtained, two such clones for Mv1Lu cells are shown (clones from U2OS cells are shown in Figure 2C).

(C) E2F-DB displaces free E2F and complexes between E2F and Rb family members from E2F sites. Gel retardation assay using extracts from untransfected (UT) Mv1Lu cells and cells stably expressing E2F-DB (3B2 clone in [C]). The triangles indicate decreasing concentration of probe in the assays. Note the absence of binding of free E2F and complexes between E2F and Rb family members in the cells expressing E2F-DB as the probe becomes limiting.

(D) E2F-DB blocks the silencer activity of E2F sites. A reporter construct containing E2F sites and an ATF enhancer (pE2F-ATF-CAT) or a reporter construct where the E2F sites are mutated (pE2Fm-ATF-CAT) (Weintraub et al., 1992) was transfected into wild-type Mv1Lu cells or cells expressing E2F-DB. Note that the E2F sites are silencers in wild-type Mv1Lu cells, but not in cells expressing E2F-DB. As a control, cells were cotransfected with an expression vector for adenovirus protein E1a (Weintraub et al., 1992), which binds Rb and blocks its interaction with E2F.

domain (E2F-DB) would displace wild-type E2F and Rb–E2F complexes from E2F-binding sites. In fact, it has been demonstrated previously that the E2F-DB will block transactivation by wild-type E2F (Fan and Bertino, 1997). We used a mutant form of E2F-1 (amino acids 1–374) containing the DNA-binding domain but lacking the transactivation domain and Rb-binding site to determine the consequence of displacing wild-type E2F and Rb–E2F complexes from promoters.

To demonstrate that E2F-DB indeed blocks E2F function, we cotransfected an expression vector for E2F-DB along with a reporter containing E2F sites in the context of a minimal promoter. E2F-DB blocked the enhancer activity of E2F sites in the minimal promoter setting, even when expression vectors for different E2F family members were cotransfected into Rb(−) C33A cells or Rb(+) U2OS cells (Figure 1A and results not shown). E2F-DB(E132), which contains a mutation that blocks DNA binding (but does not prevent interaction with DP proteins or CDK2/cyclin A) (Cress et al., 1993), had no effect on E2F site activity in these transfection assays (results not shown). Thus, DNA binding of E2F-DB is required for its function.

Stable Expression of E2F-DB in Cells

Based on the above results, expression of E2F-DB would be expected to displace endogenous wild-type E2F from the promoter of cell cycle genes. If the transactivation domain of E2F is required for progression of cells from G1 to S phase, then expression of E2F-DB should arrest cells in G1. Alternatively, if the major role of E2F in the cell cycle is to form an active repressor complex with Rb at the promoter, then displacement of wild-type E2F (by E2F-DB) would not block the G1/S transition. In colony formation assays performed with various Rb(+)/p53(+) cell lines, we did not observe growth suppression when an expression vector for E2F-DB was transfected (results not shown). Western blots of pooled colonies from these assays showed a high level of expression of E2F-DB (Figure 1B). When individual clones were examined for expression of the E2F-DB, we found levels much greater than that of endogenous E2F-1 (Figure 1B, Figure 2C, and results not shown). However, even though these clones stably overexpress E2F-DB, we had not yet demonstrated that E2F-DB actually displaces wild-type E2F from E2F sites in these cells.

E2F-DB Displaces Wild-Type E2F in Stable Cell Lines

To determine whether E2F-DB was actually displacing wild-type E2F from promoter sites in the stable transfectants, gel retardation assays were done. We found a predominant complex with E2F-DB in the cell extracts,

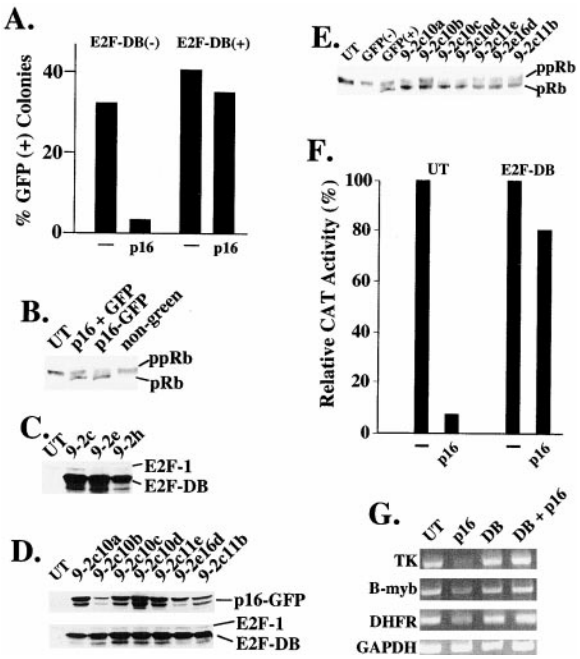

Figure 2. E2F-DB Prevents Growth Arrest by p16^{INK4a}

(A) E2F-DB blocks growth suppression by p16^{INK4a}. p16^{INK4a}(−) U2OS cells on 10 cm plates were cotransfected with PuroBabe vector and expression vectors for p16^{INK4a} and GFP. Transfected cells were selected with puromycin for 2 weeks, and the percentage of GFP(+) colonies was determined.

(B) p16^{INK4a}–GFP and wild-type p16^{INK4a} cause a similar accumulation of hypophosphorylated Rb in U2OS cells. Cells transfected with the indicated expression vectors were sorted for GFP expression and analyzed by Western blot for phosphorylation of Rb. "ppRb" indicates hyperphosphorylated Rb, and "pRb" indicates hypophosphorylated protein.

(C) Stable expression of E2F-DB in U2OS cells. Western blots of several subclones of cells expressing E2F-DB are shown.

(D) Coexpression of p16^{INK4a}–GFP and E2F-DB in U2OS cells. Expression of E2F-DB and p16^{INK4a}–GFP is shown by Western blot.

(E) p16^{INK4a}–GFP causes accumulation of hypophosphorylated Rb in U2OS cells stably expressing E2F-DB. A Western blot of Rb in cells stably expressing E2F-DB and p16^{INK4a}–GFP is shown. As a control, wild-type U2OS cells transiently transfected with the p16^{INK4a} expression vector (and GFP on a separate plasmid) were sorted by flow cytometry for GFP, and the GFP(+) and GFP(−) cells were also Western blotted for Rb. "UT" indicates untransfected U2OS cells.

(F) E2F-DB displaces the Rb–E2F repressor complex from E2F sites in U2OS cells transfected with the p16^{INK4a} expression vector. Wild-type and E2F-DB-expressing U2OS cells were transfected with an expression vector for p16^{INK4a} and the reporter construct pE2F-ATF-CAT, which contains E2F sites upstream of the ATF enhancer (Weintraub et al., 1992).

(G) E2F-DB prevents downregulation of thymidine kinase (TK), B-myb, and dihydrofolate reductase (DHFR) mRNA when p16^{INK4a}–GFP is expressed in U2OS cells. We have described the RT–PCR analysis for these RNAs in detail previously (Luo et al., 1998). "p16" indicates wild-type cells transiently expressing p16^{INK4a}–GFP, obtained by cell sorting for GFP. "DB" and "DB+p16" indicate cells expressing E2F-DB (9-2c, [D]) and E2F-DB + p16^{INK4a}–GFP (9-2c10a, [E and F]), respectively.

and there was little evidence of complexes with either free wild-type E2F or with wild-type E2F complexed to Rb family proteins (Figure 1C and results not shown). These results suggest that in the nucleus (where the ratio of E2F-DB to E2F sites is even greater than that in these gel shift assays) these high levels of E2F-DB are likely to be displacing wild-type E2F and Rb–E2F complexes from E2F sites in the stable cell lines.

E2F-DB Blocks Recruitment of the Rb–E2F Repressor Complex to Promoters

We and others have found that Rb can function as an active transcriptional repressor while tethered to a promoter through binding to E2F (Hamel et al., 1992; Weintraub et al., 1992, 1995; Adnane et al., 1995; Bremner et al., 1995; Sellers et al., 1995). There appears to be two mechanisms for this repression. First, Rb can interact with the transactivation domains of surrounding transcription factors on the promoter (through a site distinct from the E2F-binding site) and block their interaction with the basal transcription complex (Weintraub et al., 1992, 1995). Second, while bound to E2F, Rb can recruit histone deacetylase, which induces nucleosome assembly, thereby blocking access of transcription factors to the promoter (Brehm et al., 1998; Luo et al., 1998; Magnaghi-Jaulin et al., 1998). The result is that E2F sites can function as transcriptional silencers when the Rb–E2F complex forms at these sites (Figure 1D and Weintraub et al., 1992).

We wondered whether stable expression of E2F-DB would block interaction of Rb–E2F with E2F sites, thereby preventing them from acting as transcriptional silencers. A reporter containing E2F sites and an adjacent enhancer was transfected into wild-type cells and cells stably expressing E2F-DB. In wild-type cells, E2F sites acted as silencers, inhibiting the activity of surrounding enhancers on the promoter (Figure 1D). This activity was blocked when the adenovirus E1a protein, which binds to Rb and prevents formation of the Rb–E2F complex, was coexpressed. However, in cells expressing E2F-DB, the E2F sites were not silencers. We conclude from these results that the high level of E2F-DB stably expressed in the cells is blocking interaction of the Rb–E2F repressor complex with the E2F sites, thereby preventing the E2F sites from acting as silencers.

Stable Expression of E2F-DB Does Not Prolong G1

Even though cells stably overexpressing E2F-DB were proliferating, we wondered whether E2F-DB might be delaying entry into S phase. A flow cytometry cell cycle profile of wild-type and E2F-DB-expressing Mv1Lu cells showed that E2F-DB does not increase the percentage of cells in G1 (Table 1). Similar results were seen with U2OS cells stably expressing E2F-DB (results not shown).

It has been demonstrated previously that overexpression of E2F-1 can drive cells arrested in G_0 by serum starvation into S phase, and that the transactivation domain of E2F-1 is required for this activity (Johnson et al., 1993; Qin et al., 1994; Shan and Lee, 1994; DeGregori, et al., 1995; Krek et al., 1995; Adams and Kaelin, 1996; Lukas et al., 1996). Indeed, we found that cells expressing E2F-DB (which lacks the transactivation domain) were still arrested in G_0/G1 by serum starvation (Table 1). In fact the cells consistently arrested more rapidly and completely than wild-type cells when serum growth factors were deprived.

Table 1. TGFβ Arrests Cells at Two Different Stages in the Cell Cycle

	Untreated			− Serum		24 hr + T		48 hr + T			72 hr + T		
	UT	E132	DB	UT	DB	UT	DB	UT	E132	DB	UT	E132	DB
G1	47.2	51.0	47.9	68.8	81.2	72.6	71.5	79.7	86.2	60.0	87.1	86.2	53.1
S	31.8	27.6	28.0	19.9	10.6	15.3	15.6	7.7	4.5	13.9	4.5	3.7	13.8
G2/M	21.4	21.4	23.8	11.4	7.7	12.0	12.2	12.5	9.0	25.8	8.2	9.8	32.7

Untransfected Mv1Lu cells (UT), cells expressing E2F-DB (DB), or cells expressing E2F-DB with a mutation in the DNA-binding domain (E132) were subjected to serum starvation for 24 hr, or TGFβ treatment (+T) (50 pM) for the indicated time. Cells were then harvested, and the cell cycle profile was analyzed by flow cytometry. Results are representative of at least three independent experiments, each in duplicate.

E2F-DB Overcomes Growth Suppression by p16^{INK4a}

We wondered whether expression of E2F-DB would be sufficient to prevent G1 arrest by Rb. Rb's growth inhibitory activity is thought to be related to its ability to interact with a number of cellular proteins in addition to E2F, but the relative significance of most of these interactions is still unclear. Initially, we transfected an Rb expression vector into cells stably expressing E2F-DB, and we found Rb indeed caused growth arrest in colony formation assays (results not shown). These results suggest that Rb has targets in addition to E2F in the cells when it is overexpressed. However, we were concerned that overexpression of Rb might lead to nonphysiologic interactions between Rb and cellular proteins, and thus it was important to maintain a wild-type level of Rb in order to examine physiologically significant interactions.

One way to specifically trigger accumulation of hypophosphorylated Rb and thus G1 arrest without overexpression of Rb is expression of p16^{INK4a} (Serrano et al., 1993). p16^{INK4a} binds specifically to CDK4 and 6, blocking their activity and thereby leading to accumulation of hypophosphorylated Rb and growth arrest in G1 (Koh et al., 1995; Lukas et al., 1995). It is functionally linked to Rb and does not suppress growth in Rb(−) cells (Palmero and Peters, 1996), suggesting that the only target of p16^{INK4a} is the Rb pathway. We reasoned that if active repression of cell cycle genes by Rb–E2F is a primary mechanism through which Rb normally suppresses cell proliferation, then E2F-DB should displace this repressor complex from promoters of cell cycle genes and overcome the growth suppression by Rb (triggered by p16^{INK4a}).

It has been demonstrated previously that expression of p16^{INK4a} causes growth arrest of the p16^{INK4a}(−) cell line U2OS (Koh et al., 1995; Lukas et al., 1995). Expression vectors for p16^{INK4a}, the green fluorescent protein (GFP), and puromycin resistance were all cotransfected into wild-type U2OS cells and into cells stably expressing E2F-DB (Figure 2A; see Western blot for E2F-DB in Figure 2C). We reasoned that cells expressing both GFP and the puromycin resistance gene would be more likely to coexpress p16^{INK4a}. As expected, p16^{INK4a} blocked formation of puromycin-resistant/GFP(+) colonies in the wild-type cells, but it had little effect on such colony formation in cells expressing E2F-DB.

To assist in the isolation of colonies expressing p16^{INK4a}, a fusion protein between p16^{INK4a} and GFP was created. GFP was fused to the C-terminal region of p16^{INK4a} to ensure that cells expressing GFP would also express p16^{INK4a}. We found that p16^{INK4a}–GFP suppressed growth of U2OS cells in the same fashion as wild-type p16^{INK4a} (results not shown). Cells from transfection assays were sorted for GFP expression and analyzed for the level of hypophosphorylated (active) Rb by Western blot. Cells expressing p16^{INK4a}–GFP or wild-type p16^{INK4a} showed similar accumulation of hypophosphorylated Rb (Figure 2B). In contrast, the untransfected cells in these assays had no hypophosphorylated Rb.

Next, we transfected an expression vector for p16^{INK4a}–GFP into clones of U2OS cells stably expressing E2F-DB (Figures 2C and 2D). As with wild-type p16^{INK4a}, p16^{INK4a}–GFP did not arrest the cells expressing E2F-DB, and we isolated a number of green fluorescent clones. Every clone that we tested expressed p16^{INK4a}–GFP (Figure 2D). We wondered whether p16^{INK4a}–GFP expression had actually triggered accumulation of hypophosphorylated Rb in the E2F-DB-expressing clones. We found that stable expression of p16^{INK4a}–GFP in E2F-DB cells triggered accumulation of at least as much hypophosphorylated Rb as wild-type cells transiently overexpressing p16^{INK4a} (Figure 2E), but these cells continued to proliferate.

The Rb–E2F complex can bind to E2F sites and repress transcription by inhibiting activity of surrounding enhancers on the promoter. Expression of p16^{INK4a} in U2OS cells leads to accumulation of hypophosphorylated Rb, promoting formation of the Rb–E2F and association of this complex with the promoter. We transfected a reporter plasmid containing E2F sites and an enhancer (as in Figure 1D) into U2OS cells. The U2OS cells are p16^{INK4a}(−) and contain only hyperphosphorylated Rb, so an Rb–E2F complex does not form and E2F sites do not act as silencers (Figure 2F). However, when p16^{INK4a} was coexpressed in the transfection assays, hypophosphorylated Rb accumulated, and the E2F sites acted as silencers. In contrast, coexpression of p16^{INK4a} did not cause E2F sites to act as silencers in cells stably expressing E2F-DB. Additionally, we found that E2F-DB prevented the downregulation of endogenous genes containing E2F sites when p16^{INK4a}–GFP was expressed in U2OS cells (Figure 2G). We conclude that E2F-DB is displacing the Rb–E2F complex from promoters.

Our results demonstrate that (following p16^{INK4a} expression) accumulation of hypophosphorylated Rb arrests cells in G1 by inducing formation of the Rb–E2F repressor complex at the promoter of cell cycle genes, and not by blocking transcriptional activation by E2F. Additionally, the results indicate that (with endogenous levels of Rb) Rb must interact with E2F to arrest cells in G1 (as opposed to when Rb is overexpressed). Otherwise, E2F-DB would not be able to relieve the p16^{INK4a}-induced growth arrest.

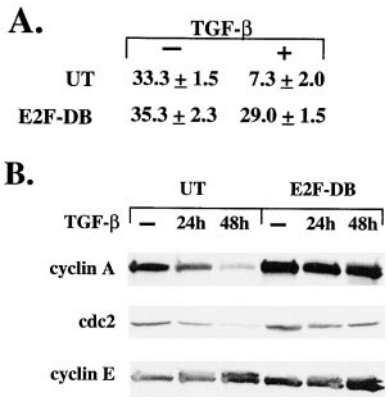

Figure 3. Expression of E2F-DB Prevents the G1 Arrest and the Downregulation of Cyclin A and cdc2 in Mv1Lu Epithelial Cells Treated with TGFβ

(A) Wild-type Mv1Lu cells and cells expressing E2F-DB were treated for 48 hr with 50 pM TGFβ1, and percent BrdU(+) nuclei is presented with standard deviations.

(B) E2F-DB prevents TGFβ from inhibiting expression of cyclin A and cdc2. Untransfected (UT) Mv1Lu cells and cells expressing E2F-DB were treated for the indicated times with TGFβ, and extracts were Western blotted. As controls, TGFβ and E2F-DB do not affect expression of cyclin E (Koff et al., 1993) or CDK2 (see Figure 4B) in Mv1Lu cells.

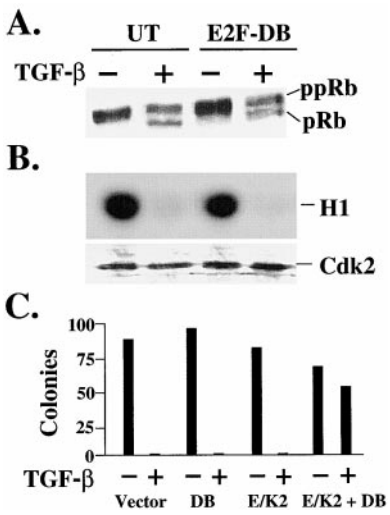

Figure 4. Blocking Interaction of Rb–E2F with the Promoter and Restoring CDK2 Activity Allow TGFβ-Treated Mv1Lu Cells to Proliferate

(A) Rb Western blots of wild-type Mv1Lu cells and cells expressing E2F-DB. Cells were treated for 48 hr with TGFβ as in Figure 3A.

(B) TGFβ inhibits CDK2 activity in wild-type Mv1Lu cells and in cells expressing E2F-DB. An immunoprecipitation-kinase assay for CDK2 is shown. Histone H1 was used as a substrate for CDK2. A Western blot for CDK2 is shown below.

(C) Colony formation assays were used to analyze the effect of E2F-DB and CDK2 activity on proliferation of Mv1Lu cells treated with TGFβ. Cells were transfected with the indicated expression vectors encoding a *neo* resistance gene, and G418-resistant colonies were selected for 2 weeks. Empty vector controls were included in transfections when expression vectors were absent. "DB" is E2F-DB, and "E/K2" is cyclin E plus CDK2.

E2F-DB Prevents G1 Arrest by TGFβ

TGFβ treatment of epithelial cells is another way to trigger accumulation of hypophosphorylated Rb and arrest of cells in G1 without overexpression of Rb. We asked whether blocking interaction of the Rb–E2F repressor complex with E2F sites by expression of the E2F-DB would overcome the G1 arrest imposed by TGFβ. Untransfected mink lung epithelial cells (Mv1Lu) (classically used for TGFβ studies [Massague, 1996]) and cells stably expressing E2F-DB were treated with TGFβ, and incorporation of BrdU was analyzed as an indication of DNA synthesis and entry into S phase. As expected, TGFβ inhibited incorporation of BrdU into wild-type cells; however, it had no effect on BrdU incorporation in the cells stably expressing E2F-DB (Figure 3A). Western blot analysis indicated that E2F-DB blocked the TGFβ-mediated downregulation of cyclin A and cdc2, which both contain E2F sites in their promoters (Figure 3B). Together, the results suggest that preventing interaction of Rb–E2F with cell cycle genes is sufficient to overcome the G1 block imposed by TGFβ. As with wild-type Mv1Lu cells, TGFβ treatment triggered accumulation of hypophosphorylated Rb in cells stably expressing E2F-DB (Figure 4A). We also immunoprecipitated CDK4 and CDK2 and analyzed their activity in in vitro kinase assays. Treatment with TGFβ resulted in inhibition of both CDK4 and CDK2 activity in wild-type cells and cells stably expressing E2F-DB (Ewen et al., 1993; Figure 4B and results not shown). Thus, expression of E2F-DB does not prevent TGFβ from inhibiting CDK activity and causing accumulation of hypophosphorylated Rb. Instead, it appears that E2F-DB is functioning by displacing Rb–E2F from the promoter of cell cycle genes.

As with growth suppression by $p16^{INK4a}$, TGFβ appears to arrest cells in G1 by promoting interaction of the Rb–E2F repressor complex with the promoter of cell cycle genes and not through blocking transactivation by E2F. Also, as with $p16^{INK4a}$, the endogenous hypophosphorylated Rb that accumulates in response to TGFβ treatment does not appear to arrest cells in G1 by directly targeting proteins other than E2F.

TGFβ Arrests Cells at Two Different Stages in the Cell Cycle

It is thought that CDK4/6 and CDK2 progressively phosphorylate Rb during G1 (Lundberg and Weinberg, 1997). CDK4/6 is activated through interaction with D type cyclins initially in G1; then near the end of G1, cyclin E is expressed, and it forms an active complex with CDK2. The activity of both CDK4/6 and CDK2 is required for progression of cells from G1 to S phase, and CDK4/6 and CDK2 activity is inhibited in TGFβ-treated cells (Ewen et al., 1993; Reynisdottir et al., 1995). In addition to hyperphosphorylation of Rb, CDK2 seems to have other targets that are important regulators of the G1/S transition. Additionally, CDK2 has been shown to be necessary for assembly of origins of DNA replication and thus for progression through S phase (Hua and Newport, 1998). Therefore, we were surprised that E2F-DB-expressing cells treated with TGFβ were able to incorporate BrdU because CDK2 activity was inhibited (Reynisdottir et al., 1995; Figures 3A and 4B).

We examined the cell cycle profile of wild-type and E2F-DB-expressing cells treated with TGFβ. Wild-type

cells were arrested in G1 after 24 hr of treatment with TGFβ, and at longer incubation times the G1 arrest became even more pronounced (Table 1). As with wild-type cells, treatment of the E2F-DB-expressing cells with TGFβ for 24 hr resulted in an initial arrest in G1 (Table 1). However, when we examined the E2F-DB-expressing cells after incubation with TGFβ for 48 hr (this 48 hr time point was used for the BrdU studies in Figure 3A above), we found that they were moving from G1 to G2/M, and this exit from G1 continued after 72 hr of treatment with TGFβ. The cells remained arrested in G2/M, which is consistent with the findings that CDK2 activity is required for activation of cdc2 and onset of mitosis in *Xenopus* (Guadagno and Newport, 1996), and that cyclin E-CDK2 is required for reproduction of centrosomes (Hinchcliffe et al., 1999). As a control in the above experiments, cells expressing E2F-DB(E132) arrested like wild-type cells when treated with TGFβ (Table 1). We conclude that DNA binding of E2F-DB, and therefore the displacement of the Rb–E2F repressor complex from promoters, is required for S phase entry when cells are treated with TGFβ. Furthermore, it is likely that there is enough residual CDK2 activity in the TGFβ-treated cells for assembling origins of replication and moving through S phase, although movement from G1 to S phase is significantly delayed. However, apparently this low level of CDK2 activity is not sufficient for the cells to move through G2/M.

Next, we examined proliferation of TGFβ-treated cells using colony formation assay. Overexpression of cyclin E or CDK2 alone or in combination did not lead to proliferation of TGFβ-treated cells; however, when E2F-DB was coexpressed with cyclin E/CDK2, the cells did indeed proliferate in the presence of TGFβ (Figure 4C). These results imply that TGFβ arrests cells at two distinct stages of the cell cycle. The arrest in G1 is due primarily to induction of the Rb–E2F repressor, whereas arrest in G2/M may be a result of decreased CDK2 activity.

Contact Inhibition in Epithelial Cells Is Dependent upon Active Repression by Rb–E2F

Epithelial cells arrest in G1 in response to cell–cell contact, and as with overexpression of $p16^{INK4a}$ or treatment with TGFβ, this cell–cell contact triggers accumulation of hypophosphorylated Rb in Mv1Lu cells (Polyak et al., 1994). We wondered whether E2F-DB would also prevent the G1 arrest imposed by contact inhibition. Therefore, we cultured wild-type Mv1Lu cells and cells expressing E2F-DB at confluence for 3 days. The wild-type cells were plated at higher density and actually reached confluence before the E2F-DB-expressing cells. However, while the wild-type cells arrested in response to contact inhibition and maintained a monolayer on the culture dishes, cells expressing E2F-DB continued to proliferate, causing them to pile on top of each other (Figures 5A and 5B). Both cells showed similar accumulation of hypophosphorylated Rb after culture at confluence (results not shown), indicating that the pathway leading to accumulation of hypophosphorylated Rb in response to cell–cell contact is intact in cells expressing E2F-DB. These results suggest that E2F-DB significantly decreases contact inhibition in the epithelial cells. Thus, we conclude that—as with $p16^{INK4a}$ and TGFβ—contact inhibition in epithelial cells is also mediated through transcriptional repression by Rb–E2F, and E2F-DB functions by displacing this repressor complex from promoters.

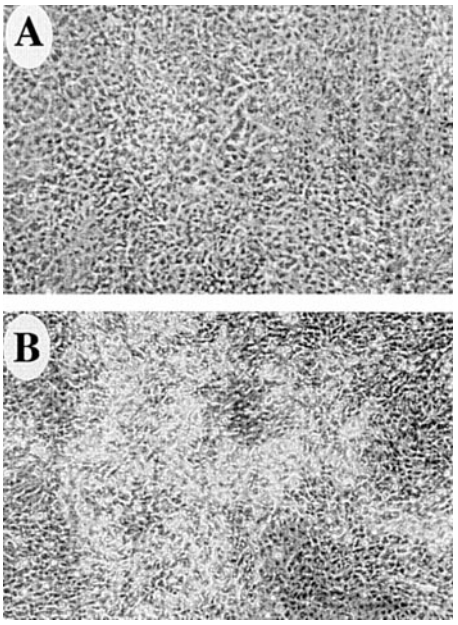

Figure 5. E2F-DB Overcomes Contact Inhibition in Mv1Lu Epithelial Cells

(A and B) Mv1Lu cells become contact inhibited as they reach confluence, causing them to remain as a monolayer (A). In contrast, expression of E2F-DB decreased contact inhibition. The cells continue to proliferate after reaching confluence, causing them to pile on top of each other. The photographs are of cells 3 days after reaching confluence (wild-type cells were plated at a higher density and reached confluence before cells expressing E2F-DB).

Discussion

Rb-Mediated Inhibition of E2F Transactivation versus Active Repression by Rb–E2F

We and others have found that the Rb–E2F complex can function as an active transcriptional repressor (Hamel et al., 1992; Weintraub et al., 1992, 1995; Adnane et al., 1995; Bremner et al., 1995; Sellers et al., 1995; Luo et al., 1998). Nevertheless, the relative contribution of this repressor activity to cell cycle regulation remained unclear. Indeed, it is still thought that transcriptional activation by E2F is essential for progression from G1 to S phase, and that a major function of Rb is to block this transcriptional activation. Moreover, Rb can interact with a number of other cellular proteins in addition to E2F, and it is thought that at least some of these other interactions must be important for Rb's role in regulating the G1/S transition. Thus, not only is the functional relationship between Rb and E2F unresolved, the overall significance of the Rb–E2F interaction (in the face of multiple Rb-binding proteins) is still undetermined.

Our results suggest that Rb must normally (in the absence of Rb overexpression) interact with E2F to arrest cells in G1, and it appears that Rb arrests cells in

G1 by forming a repressor complex with E2F, not by blocking the transcriptional activity of E2F. We provide evidence that $p16^{INK4a}$, TGFβ, and contact inhibition arrest cells in G1 through this active transcriptional repression by Rb–E2F.

It appears that the transactivation domain of E2F-1 is important in triggering p53-mediated growth arrest or apoptosis when Rb function is lost and free E2F-1 accumulates (Bates et al., 1998). E2F-1 activates expression of the alternate reading frame gene (ARF) at the *INK4a* locus, whose product inhibits the MDM2-mediated turnover of p53 (Pomerantz et al., 1998; Zhang et al., 1998). This leads to accumulation of p53 and growth arrest or apoptosis. Thus, transactivation by E2F-1 may function as an additional checkpoint that triggers growth arrest or apoptosis when Rb function is lost.

Previously, it was shown that E2F-DB decreased the number of cells in S phase and inhibited transcription of some genes containing E2F sites in Rb(−)/p53(−) cells, resulting in a slower growth rate (Fan and Bertino, 1997). E2F-1 is not required for cell cycle progression of normal cells in *E2F-1*(−/−) mice, but tumor cells in these mice were inhibited from entering S phase (Pan et al., 1998). These results suggest that E2F-1 transcriptional activity may be required for the high rate of cell cycle progression seen in certain tumors, but not in more normal cells. Additionally, it has been demonstrated that expression of E2F-DB in functional Rb(−)/p53(−) cells can lead to a p53-independent form of apoptosis (Hsieh et al., 1997; Phillips et al., 1997); however, this does not occur in Rb(+)/p53(+) cells (Qin et al., 1994; Shan and Lee, 1994; Wu and Levine, 1994, and results not shown). Such apoptosis itself could lead to an apparent decrease in the growth rate of Rb(−)/p53(−) cells in the presence of E2F-DB.

Is E2F the Only Functional Target of Rb?

E2F-DB is not able to prevent G1 arrest when Rb is overexpressed in transfection assays. However, in contrast to E2F-DB, coexpression of wild-type E2F-1 did overcome the G1 arrest resulting from overexpression of Rb (Qin et al., 1995; Sellers et al., 1995). Why would E2F-DB overcome G1 arrest by $p16^{INK4a}$, TGFβ, and contact inhibition in cells with a wild-type level of Rb, yet be unable to overcome the G1 arrest when Rb is overexpressed in transfection assays? It appears that with an endogenous level of Rb, G1 arrest is mediated through Rb–E2F, and overexpression of either E2F-DB or wild-type E2F-1 would squelch binding of this repressor complex to promoters. However, when Rb is overexpressed in transfection assays, the high concentration of the protein leads to nonphysiologic interactions between Rb and non-E2F proteins. Because wild-type E2F-1 contains an Rb-binding domain (which E2F-DB lacks), it may be able to directly titrate Rb in addition to squelching the binding of the Rb–E2F to promoters.

Our results do not rule out a function for Rb binding to non-E2F proteins. In contrast, this is the mechanism that makes Rb an active transcription repressor; it can bind to and inactivate surrounding transcription factors while it is concentrated at the promoter of cell cycle genes through interaction with E2F (Weintraub et al., 1995). Additionally, we and others have found that Rb can interact with histone deacetylase, while bound to a promoter through E2F, to remodel chromatin and repress transcription (Brehm et al., 1998; Luo et al., 1998; Magnaghi-Jaulin et al., 1998). The binding site for E2F on Rb is distinct from the site(s) that Rb uses to bind these other cellular proteins, allowing Rb to interact simultaneously with E2F and these other cellular proteins at the promoter. However, both repressor mechanisms are based on the idea that Rb is first recruited to promoters through interaction with E2F. It is only after Rb is selectively targeted to and concentrated at promoters via E2F that interaction with these other proteins occurs. Obviously, overexpression of Rb in transfection assays may facilitate direct interactions with these non-E2F proteins, resulting in a loss of specificity and eliminating the need for Rb to be concentrated initially at promoters through E2F before interacting with the non-E2F proteins.

Interestingly, Qin et al. found that expression of a fusion protein between E2F-DB and the VP16 activation domain could overcome growth suppression, even when Rb is overexpressed (1995). As outlined above, we propose that non-E2F transcription factors directly targeted by Rb when the protein is overexpressed are factors that bind to the promoters of cell cycle genes and are normally (in the absence of overexpression of Rb) only inhibited after Rb is concentrated at the promoter through interaction with E2F. E2F-DB alone cannot affect these types of direct interactions between Rb and non-E2F interactions. However, we demonstrated previously that transcriptional activation by VP16 is resistant to repression by Rb (Weintraub et al., 1995), and we suggest that binding of E2F-DB-VP16 to E2F sites of such cell cycle genes can overcome repression, even if Rb is directly targeted to the promoter through a non-E2F protein. Taken together, our studies and the studies of Qin et al. (1995) suggest that even when overexpressed Rb may function in cell cycle regulation by repressing genes with E2F sites. Indeed, we suggest that overexpression of Rb leads to short circuiting (binding of Rb directly to non-E2F transcription factors that normally require Rb to first be concentrated at the promoter through interaction with E2F) of a pathway already in place to repress genes with E2F sites.

Experimental Procedures

Cell Culture and Transfections

Mv1Lu epithelial cells, U2OS cells, C2C12 cells, and C33A cells were cultured as described previously (Weintraub et al., 1995). Cells were transfected using the calcium phosphate method, and CAT activity was determined as described (Weintraub et al., 1995). To generate stable clones that overexpress E2F-DB, cells were transfected with CMVneoE2F1(1–374) (Lukas et al., 1996), selected with G418 (1 mg/ml for Mv1Lu cells, 500 mg/ml for U2OS cells, and 800 mg/ml for C2C12 myoblasts) for 2 weeks. Western blot with the mAb KH20 (Lukas et al., 1996) was used to identify clones expressing E2F-DB. To generate U2OS clones that overexpress $p16^{INK4a}$–GFP, U2OS clones that express E2F-DB (clones 9-2c and 9-2e) were transfected with pEGFP$p16^{INK4a}$ and puroBabe vector at a ratio of 10:1, and cells were selected with 1 μg/ml of puromycin for 2 weeks. Colonies with green fluorescence were cloned out and expanded.

Plasmids

CMV E2F1 and CMV E2F1(1–374) were gifts from K. Helin (Lukas et al., 1996), Rc-CMV cyclin E was a gift from R. Weinberg (Hinds et

al., 1992), CMV-CDK2 was a gift from E. Harlow (van den Heuvel et al., 1993), CMV-DP-1 was a gift from D. Livingston (Krek et al., 1993), CMV-E2F3 was a gift from J. Lees (Lees et al., 1993), CMV-E2F5 was a gift from R. Bernards (Hijmans et al., 1995), and $p16^{INK4a}$ expression vectors were gifts from Y. Xiong (Guan et al., 1994). $p16^{INK4a}$ cDNA was amplified by PCR and cloned into the HindIII and BamHI sites of pEGFPN1 (Clontech). E2F-CAT, E2F-ATF-CAT, and mut-E2F-ATF-CAT were described previously (Weintraub et al., 1992).

BrdU Labeling

Mv1Lu cells were plated onto coverslips 1 day before adding TGFβ1 (50 pM). After 48 hr of TGFβ1 treatment, cells were labeled with BrdU (Amersham) (1:500 dilution) for 3 hr. Cells on coverslips were washed twice with PBS, fixed with 70% ethanol at −20°C, rinsed with PBS, denatured with 2N HCL/0.5% NP40 for 45 min at room temperature, then neutralized with 0.1 M sodium borate at pH 8.5. Cells were blocked with 2% BSA/0.1% Tween 20 in PBS for 1 hr at room temperature, then incubated with fluorescein-conjugated anti-BrdU antibody (Caltag, 1:100 dilution) for 30 min. After three washes, cells were incubated with DAPI (Boehringer Mannheim) for 1 hr. Coverslips were washed with PBS and mounted.

Cell Cycle Analysis

For flow cytometry, cells were harvested and fixed in 70% ethanol and then treated with 50 μg/ml of propidium iodide and 100 U/ml of RNase A. At least 10,000 cells were scored using a FACScan flow cytometer. Cell cycle distribution was analyzed with CellQuest software.

Gel Shifts

Gel shift assays were performed as described (Helin et al., 1993) with nuclear extract from wild-type Mv1Lu cells or cells expressing E2F-DB. The E2F site from the dihydrofolate reductase gene promoter was used as a probe: 5′-ATTTAAGTTTCGCGCCCTTTCT CAA-3′. The mutant probe sequence was 5′-ATTTAAGTTTCG**AT**CC CTTTCTCAA-3′.

Kinase Assays

Immunoprecipitation-kinase assays were performed as described (Ewen et al., 1993). For CDK2 kinase assays, a Sepharose-conjugated anti-CDK2 antibody (Santa Cruz) was incubated with 200 μg of Mv1Lu cell lysate, and immunocomplexes were washed with lysis buffer followed by kinase buffer. Five micrograms of histone H1 (Boehringer Mannheim), 10 μCi of γ-ATP[^{32}P], and 10 μM ATP were added to the immunocomplex in a final volume of 50 μl, and the mixture was incubated for 30 min at 30°C.

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References

Adams, P.D., and Kaelin, W.G., Jr. (1996). The cellular effects of E2F overexpression. Curr. Top. Microbiol. Immunol. *208*, 79–93.

Adnane, J., Shao, Z., and Robbins, P.D. (1995). The retinoblastoma susceptibility gene product represses transcription when directly bound to the promoter. J. Biol. Chem. *270*, 8837–8843.

Bates, S., Phillips, A.C., Clark, P.A., Stott, F., Peters, G., Ludwig, R.L., and Vousden, K.H. (1998). $p14^{ARF}$ links the tumour suppressors RB and p53. Nature *395*, 124–125.

Brehm, A., Miska, E.A., McCance, D.J., Reid, J.L., Bannister, A.J., and Kouzarides, T. (1998). Retinoblastoma protein recruits histone deacetylase to repress transcription. Nature *391*, 597–601.

Bremner, R., Cohen, B.L., Sopta, M., Hamel, P.A., Ingles, C.J., Gallie, B.L., and Phillips, R.A. (1995). Direct transcriptional repression by pRb and its reversal by specific cyclins. Mol. Cell. Biol. *15*, 3256–3265.

Chellappan, S.P., Hiebert, S., Mudryj, M., Horowitz, J.M., and Nevins, J.R. (1991). The E2F transcription factor is a cellular target for the RB protein. Cell *65*, 1053–1061.

Cress, W.D., Johnson, D.G., and Nevins, J.R. (1993). A genetic analysis of the E2F1 gene distinguishes regulation by Rb, p107, and adenovirus E4. Mol. Cell. Biol. *13*, 6314–6325.

DeGregori, J., Leone, G., Ohtani, K., Miron, A., and Nevins, J.R. (1995). E2F-1 accumulation bypasses a G1 arrest resulting from the inhibition of G1 cyclin-dependent kinase activity. Genes Dev. *9*, 2873–2887.

Ewen, M.E. (1994). The cell cycle and the retinoblastoma protein family. Cancer Metastasis Rev. *13*, 45–66.

Ewen, M.E., Sluss, H.K., Whitehouse, L.L., and Livingston, D.M. (1993). TGF-beta inhibition of Cdk4 synthesis is linked to cell cycle arrest. Cell *74*, 1009–1020.

Ewen, M.E., Oliver, C.J., Sluss, H.K., Miller, S.J., and Peeper, D.S. (1995). p53-dependent repression of cdk4 translation in TGF-beta induced cell-cycle arrest. Genes Dev. *9*, 204–217.

Fan, J., and Bertino, J.R. (1997). Functional roles of E2F in cell cycle regulation. Oncogene *14*, 1191–1200.

Field, S.J., Tsai, F.Y., Kuo, F., Zubiaga, A.M., Kaelin, W.J., Livingston, D.M., Orkin, S.H., and Greenberg, M.E. (1996). E2F-1 functions in mice to promote apoptosis and suppress proliferation. Cell *85*, 549–561.

Guadagno, T.M., and Newport, J.W. (1996). Cdk2 kinase is required for entry into mitosis as a positive regulator of Cdc2-cyclin B kinase activity. Cell *84*, 73–82.

Guan, K.L., Jenkins, C.W., Li, Y., Nichols, M.A., Wu, X.Y., Okeefe, C.L., Matera, A.G., and Xiong, Y. (1994). Growth suppression by p18, a p16(ink4/mts1)- and p14(ink4b/mts2)-related cdk6 inhibitor, correlates with wild-type pRb function. Genes Dev. *8*, 2939–2952.

Hamel, P.A., Gill, R.M., Phillips, R.A., and, Gallie, B.L. (1992). Transcriptional repression of the E2-containing promoters EIIaE, c-myc, and RB1 by the product of the RB1 gene. Mol. Cell. Biol. *12*, 3431–3438.

Helin, K., Wu, C.L., Fattaey, A.R., Lees, J.A., Dynlacht, B.D., Ngwu, C., and Harlow, E. (1993). Heterodimerization of the transcription factors E2F-1 and DP-1 leads to cooperative trans-activation. Genes Dev. *7*, 1850–1861.

Hijmans, E.M., Voorhoeve, P.M., Beijersbergen, R.L., Vantveer, L.J., and Bernards, R. (1995). E2f-5, a new e2f family member that interacts with p130 in vivo. Mol. Cell. Biol. *15*, 3082–3089.

Hinchcliffe, E.H., Li, C., Thompson, E.A., Maller, J.L., and Sluder, G. (1999). Requirement of cdk2-cyclin E activity for repeated centrosome reproduction in *Xenopus* egg extracts. Science *283*, 851–854.

Hinds, P.W., Mittnacht, S., Dulic, V., Arnold, A., Reed, S.I., and Weinberg, R.A. (1992). Regulation of retinoblastoma protein functions by ectopic expression of human cyclins. Cell *70*, 993–1006.

Hsieh, J.K., Fredersdorf, S., Kouzarides, T., Martin, K., and Lu, X. (1997). E2F1-induced apoptosis requires DNA binding but not transactivation and is inhibited by the retinoblastoma protein through direct interaction. Genes Dev. *11*, 1840–1852.

Hua, X.H., and Newport, J. (1998). Identification of a preinitiation step in DNA replication that is independent of origin recognition complex and cdc6, but dependent on cdk2. J. Cell Biol. *140*, 271–281.

Iavarone, A., and Massague, J. (1997). Repression of the CDK activator cdc25A and cell-cycle arrest by cytokine TGF-beta in cells lacking the cdk inhibitor p15. Nature *387*, 417–422.

Johnson, D.G., Schwarz, J.K., Cress, W.D., and Nevins, J.R. (1993). Expression of transcription factor E2F1 induces quiescent cells to enter S phase. Nature *365*, 349–352.

Koff, A., Ohtsuki, M., Polyak, K., Roberts, J.M., and Massague, J. (1993). Negative regulation of G1 in mammalian cells: inhibition of cyclin E-dependent kinase by TGF-beta. Science *260*, 536–539.

Koh, J., Enders, G.H., Dynlacht, B.D., and Harlow, E. (1995). Tumour-derived p16 alleles encoding proteins defective in cell cycle inhibition. Nature *375*, 506–510.

Krek, W., Livingston, D.M., and Shirodkar, S. (1993). Binding to DNA and the retinoblastoma gene product promoted by complex formation of different E2F family members. Science *262*, 1557–1560.

Krek, W., Xu, G., and Livingston, D.M. (1995). Cyclin A kinase regulation of E2F-1 DNA binding function underlies suppression of an S-phase checkpoint. Cell *83*, 1149–1158.

Lam, E.W., and La Thangue, N.B. (1994). Dp and E2F proteins: coordinating transcription with cell cycle progression. Curr. Opin. Cell Biol. *6*, 859–866.

La Thangue, N.B. (1994). DRTF1/E2F: an expanding family of heterodimeric transcription factors implicated in cell-cycle control. Trends Biochem. Sci. *19*, 108–114.

Lees, J.A., Saito, M., Vidal, M., Valentine, M., Look, T., Harlow, E., Dyson, N., and Helin, K. (1993). The retinoblastoma protein binds to a family of E2F transcription factors. Mol. Cell. Biol. *13*, 7813–7825.

Ludlow, J.W., Shon, J., Pipas, J.M., Livingston, D.M., and DeCaprio, J.A. (1990). The retinoblastoma susceptibility gene product undergoes cell cycle-dependent dephosphorylation and binding to and release from SV40 large T. Cell *60*, 387–396.

Lukas, J., Parry, D., Aagaard, L., Mann, D.J., Bartkova, J., Strauss, M., Peters, G., and Bartek, J. (1995). Retinoblastoma protein-dependent cell cycle inhibition by the tumor suppressor p16. Nature *375*, 503–506.

Lukas, J., Petersen, B.O., Holm, K., Bartek, J., and Helin, K. (1996). Deregulated expression of E2F family members induces S-phase entry and overcomes $p16^{INK4a}$-mediated growth suppression. Mol. Cell. Biol. *16*, 1047–1057.

Lundberg, A.S., and Weinberg, R.A. (1997). Functional inactivation of the retinoblastoma protein requires sequential modification by at least two distinct cyclin-cdk complexes. Mol. Cell. Biol. *18*, 753–761.

Luo, R.X., Postigo, A.A., and Dean, D.C. (1998). Rb interacts with histone deacetylase to repress transcription. Cell *92*, 463–473.

Magnaghi-Jaulin, L., Groisman, R., Naguibneva, I., Robin, P., Lorain, S., Le Villain, J.P., Troalen, F., Trouche, D., and Harel-Bellan, A. (1998). Retinoblastoma protein represses transcription by recruiting a histone deacetylase. Nature *391*, 601–605.

Massague, J. (1996). TGF-beta signaling: receptors, transducers, and MAD proteins. Cell *85*, 947–950.

Mihara, K., Cao, X.R., Yen, A., Chandler, S., Driscoll, B., Murphree, A.L., T'Ang, A., and Fung, Y.K. (1989). Cell cycle-dependent regulation of phosphorylation of the human retinoblastoma gene product. Science *246*, 1300–1303.

Nevins, J.R. (1992). E2F: a link between the Rb tumor suppressor protein and viral oncoproteins. Science *258*, 424–429.

Palmero, I., and Peters, G. (1996). Perturbation of cell cycle regulators in human cancer. Cancer Surv. *27*, 351–367.

Pan, H., Yin, C., Dyson, N.J., Harlow, E., Yamasaki, L., and Van Dyke, T. (1998). Key roles for E2F1 in signaling p53-dependent apoptosis and in cell division within developing tumors. Mol. Cell *2*, 283–292.

Phillips, A.C., Bates, S., Ryan, K.M., Helin, K., and Vousden, K.H. (1997). Induction of DNA synthesis and apoptosis are separable functions of E2F-1. Genes Dev. *11*, 1853–1863.

Polyak, K., Kato, J.Y., Solomon, M.J., Sherr, C.J., Massague, J., Roberts, J.M., and Koff, A. (1994). $p27^{Kip1}$, a cyclin-Cdk inhibitor, links transforming growth factor-beta and contact inhibition to cell cycle arrest. Genes Dev. *8*, 9–22.

Pomerantz, J., Schreiber, A.N., Liegeois, N.J., Silverman, A., Alland, L., Chin, L., Potes, J., Chen, K., Orlow, I., Lee, H.W., Cordon, C.C., and DePinho, R.A. (1998). The *Ink4a* tumor suppressor gene product, p19Arf, interacts with MDM2 and neutralizes MDM2's inhibition of p53. Cell *92*, 713–723.

Qin, X.Q., Livingston, D.M., Kaelin, W.J., and Adams, P.D. (1994). Deregulated transcription factor E2F-1 expression leads to S-phase entry and p53-mediated apoptosis. Proc. Natl. Acad. Sci. USA *91*, 10918–10922.

Qin, X.Q., Livingston, D.M., Ewen, M., Sellers, W.R., Arany, Z., and Kaelin, W.G. (1995). The transcription factor E2F-1 is a downstream target of Rb action. Mol. Cell. Biol. *15*, 742–755.

Reynisdottir, I., Polyak, K., Iavarone, A., and Massague, J. (1995). Kip/Cip and Ink4 Cdk inhibitors cooperate to induce cell cycle arrest in response to TGF-beta. Genes Dev. *9*, 1831–1845.

Roberts, J.M., Koff, A., Polyak, K., Firpo, E., Collins, S., Ohtsubo, M., and Massague, J. (1994). Cyclins, Cdks, and cyclin kinase inhibitors. Cold Spring Harb. Symb. Quant. Biol. *59*, 31–38.

Sellers, W.R., Rodgers, J.W., and Kaelin, W.G., Jr. (1995). A potent transrepression domain in the retinoblastoma protein induces a cell cycle arrest when bound to E2F sites. Proc. Natl. Acad. Sci. USA *92*, 11544–11548.

Serrano, M., Hannon, G.J., and Beach, D. (1993). A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. Nature *366*, 704–707.

Shan, B., and Lee, W.H. (1994). Deregulated expression of E2F-1 induces S-phase entry and leads to apoptosis. Mol. Cell. Biol. *14*, 8166–8173.

Sherr, C.J. (1996). Cancer cell cycles. Science *274*, 1672–1677.

Sherr, C.J., and Roberts, J.M. (1995). Inhibitors of mammalian G1 cyclin-dependent kinases. Genes Dev. *9*, 1149–1163.

Slansky, J.E., and Farnham, P.J. (1996). Introduction to the E2F family: protein structure and gene regulation. Curr. Top. Microbiol. Immunol. *208*, 1–30.

St. Croix, B., Sheehan, C., Rak, J.W., Florenes, V.A., Slingerland, J.M., and Kerbel, R.S. (1998). E-Cadherin-dependent growth suppression is mediated by the cyclin-dependent kinase inhibitor p27(KIP1). J. Cell Biol. *142*, 557–571.

Tsai, K.Y., Hu, Y., Macleod, K.F., Crowley, D., Yamasaki, L., and Jacks, T. (1998). Mutation of E2f-1 suppresses apoptosis and inappropriate S phase entry and extends survival of Rb-deficient mouse embryos. Mol. Cell *2*, 293–304.

van den Heuvel, S., and Harlow, E. (1993). Distinct roles for cyclin-dependent kinases in cell cycle control. Science *262*, 2050–2054.

Weinberg, R.A. (1995). The retinoblastoma protein and cell cycle control. Cell *81*, 323–330.

Weinberg, R.A. (1996). E2F and cell proliferation: a world turned upside down. Cell *85*, 457–459.

Weintraub, S.J., Prater, C.A., and Dean, D.C. (1992). Retinoblastoma protein switches the E2F site from positive to negative element. Nature *358*, 259–261.

Weintraub, S.J., Chow, K., Luo, R.X., Zhang, S.H., He, S., and Dean, D.C. (1995). Mechanism of active transcriptional repression by the retinoblastoma protein. Nature *375*, 812–815.

Wu, X., and Levine, A.J. (1994). p53 and E2F-1 cooperate to mediate apoptosis. Proc. Natl. Acad. Sci. USA *91*, 3602–3606.

Yamasaki, L., Jacks, T., Bronson, R., Goillot, E., Harlow, E., and Dyson, N.J. (1996). Tumor induction and tissue atrophy in mice lacking E2F-1. Cell *85*, 537–548.

Zhang, Y., Xiong, Y., and Yarbrough, W.G. (1998). ARF promotes MDM2 degradation and stabilizes p53: *ARF-INK4a* locus deletion impairs both the Rb and p53 tumor suppression pathways. Cell *92*, 725–734.