E2F transcriptional repressor complexes are critical downstream targets of p19^{ARF}/p53-induced proliferative arrest

Benjamin D. Rowland,[1] Serguei G. Denissov,[2] Sirith Douma,[1] Hendrik G. Stunnenberg,[2] René Bernards,[1,3] and Daniel S. Peeper[1]

[1]Division of Molecular Carcinogenesis, Center for Biomedical Genetics, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands
[2]Department of Molecular Biology, NCMLS 191, University of Nijmegen, PO Box 9101, 6500 HB Nijmegen, The Netherlands
[3]Correspondence: r.bernards@nki.nl

Summary

The p16^{INK4a}/pRB/E2F and p19^{ARF}/p53 tumor suppressor pathways are disrupted in most human cancers. Both p19^{ARF} and p53 are required for the induction of senescence in primary mouse embryonic fibroblasts (MEFs), but little is known about their downstream targets. Disruption of E2F-mediated transcriptional repression in MEFs caused a general increase in the expression of E2F target genes, including *p19ARF*. We detected no contribution of E2F-mediated transactivation in this setting, indicating that a predominant role of endogenous E2F in asynchronously growing primary MEFs is to repress its target genes. Moreover, relief of transcriptional repression by E2F rendered MEFs resistant to senescence induced by either p19^{ARF}, p53, or RAS^{V12}. Thus, E2F transcriptional repressor complexes are critical downstream targets of antiproliferative p19^{ARF}/p53 signaling.

Introduction

Upon explantation, cultured primary murine embryonic fibroblasts (MEFs) divide only a limited number of times before they undergo replicative senescence (Hayflick, 1965; reviewed in Sherr and DePinho, 2000). This cell cycle arrest is accompanied by increased levels of the p16^{INK4a}, p19^{ARF} (both encoded by the *INK4a* locus), p53, and p21^{CIP1} proteins. Both p16^{INK4a} and p19^{ARF} are induced during in vitro propagation of primary MEFs. p16^{INK4a} inhibits cellular proliferation in a manner that requires the function of either the retinoblastoma protein pRB or, as shown recently, both pRB-related "pocket" proteins, p107 and p130 (Bruce et al., 2000; Koh et al., 1995; Lukas et al., 1995; Medema et al., 1995). *p19ARF*, too, is a tumor suppressor gene that has been proposed to protect cells against excessive mitogenic or oncogenic signaling (Sherr, 1998). p19^{ARF} neutralizes the E3-ubiquitin ligase for p53, MDM-2, and thereby stabilizes p53 (Pomerantz et al., 1998; Zhang et al., 1998). As a result of p19^{ARF} expression, p53 transcriptionally induces its target genes, including *MDM-2* and the cell cycle inhibitor *p21^{CIP1}* (Vogelstein et al., 2000; Vousden, 2000). Although p53 is a regulatory target of p19^{ARF}, the latter can interfere with cell cycle progression in both p53-dependent and p53-independent manners (Carnero et al., 2000; Martelli et al., 2001; Weber et al., 2000).

When a constitutively active mutant allele of the *Ha-RAS* gene (encoding RAS^{V12}) is introduced into primary MEFs, they also enter replicative senescence, but now prematurely (Serrano et al., 1997). Both spontaneous and RAS^{V12}-induced senescence depend on the presence of functional *p19ARF* and *p53*, as primary fibroblasts deficient for either of these genes can be cultured indefinitely, irrespective of RAS^{V12} expression (Kamijo et al., 1997; Serrano et al., 1997; Tanaka et al., 1994). However, how activation of the p19^{ARF}/p53 pathway by (RAS^{V12}-induced) senescence signaling eventually results in an irreversible cell cycle arrest is not well understood. As MEFs deficient for *p21^{CIP1}* are not immortal and still undergo RAS^{V12}- or p19^{ARF}-induced arrest, *p21^{CIP1}* likely is not a critical p19^{ARF}/p53 target gene in this setting (Groth et al., 2000; Pantoja and Serrano, 1999). PML is required for the regulation of p53 (Ferbeyre et al., 2000; Pearson et al., 2000), but which target genes act downstream of p53 in the senescence response remains unknown.

When primary cells enter either RAS^{V12}-induced or spontaneous senescence, pRB accumulates in its active, hypophosphorylated form (Serrano et al., 1997; Stein et al., 1990). We and others recently reported that both spontaneous and RAS^{V12}-induced senescence are dependent on the retinoblastoma gene family. Fibroblasts deficient for all three pocket proteins (pRB, p107, and p130) were shown to be immortal and failed to se-

SIGNIFICANCE

E2F transcriptional repressor complexes are considered to be important downstream components of the p16^{INK4a}/pRB tumor suppressor pathway. We find that E2F repressors are also critical targets for the ARF and p53 tumor suppressors during induction of replicative senescence and cell cycle arrest. Hence, our finding suggests that p16^{INK4a} and ARF/p53 converge at the level of E2F repressor complexes. This is unexpected, because the pRB and p53 pathways were thought to communicate to different downstream targets. As E2F repressors are controlled by these two major tumor suppressor pathways, our model predicts that E2F-dependent transcriptional repression is deregulated not only by a mutant p16^{INK4a}/pRB pathway, but also upon mutation of the ARF/p53 pathway, i.e., in the vast majority of human tumors.

nesce upon expression of either RAS^{V12} or $p19^{ARF}$ (Dannenberg et al., 2000; Peeper et al., 2001; Sage et al., 2000). pRB interacts with a number of proteins involved in cell cycle regulation, including MDM-2 (Xiao et al., 1995), PML (Alcalay et al., 1998), the tyrosine kinase c-ABL (Welch and Wang, 1993), and E2F transcription factors (Helin et al., 1992; Kaelin et al., 1992), but which of these act in the senescence response is unclear.

The E2F transcription factor family consists of six structurally related members, five of which (E2F-1 through E2F-5) contain a transactivation domain that is inhibited by binding to a pocket protein. E2F-1, -2, and -3 preferentially associate with pRB, E2F-4 with p107 or p130, and E2F-5 with p130 (Muller and Helin, 2000). The pocket proteins not only interfere with transactivation, but form, upon association with E2F and histone deacytelases (HDACs), active transcriptional repressor complexes (Harbour and Dean, 2000; Zhang et al., 2000). E2F-6 is unique in that it has a pocket protein-independent repression motif (Cartwright et al., 1998; Gaubatz et al., 1998; Trimarchi et al., 1998). The pRB family controls cell cycle progression by transiently associating with E2Fs. During G1, D type CYCLINS activate CDK4 and CDK6, which in turn phosphorylate pRB (Weinberg, 1995). pRB and HDAC subsequently dissociate, which results in derepression of E2F target genes (Zhang et al., 2000). Further phosphorylation of pRB by CYCLIN E-CDK2 causes pRB to dissociate from E2F, thereby actively inducing E2F-dependent transactivation and stimulating the cell to enter S phase (Harbour et al., 1999).

Pocket proteins are not the only communicative link between senescence signaling and the E2F transcription factors. A number of additional observations suggest a possible role for E2F in the regulation of senescence: the levels of various E2Fs decrease during the onset of senescence (Dimri et al., 1994; Haddad et al., 1999), and overexpressed E2F-1 induces both *ARF* (Bates et al., 1998; DeGregori et al., 1997; Dimri et al., 2000) and premature senescence (Dimri et al., 2000). To analyze in more detail its role in senescence, we disrupted the transcription-regulating function of E2F and analyzed the ($p19^{ARF}$/p53-dependent) senescence response in primary murine fibroblasts. As E2Fs have the ability to mediate active transcriptional repression, as well as activation, we also addressed in primary MEFs which of these functions is required for the transcriptional regulation of endogenous E2F target genes, including *p19ARF*, and for an adequate response to antiproliferative $p19^{ARF}$/p53 signaling.

Results

Ectopic expression of an E2F-1 C-terminal deletion mutant displaces endogenous E2Fs from DNA

Overexpression of E2F-1 has been reported to induce *ARF* transcription (Bates et al., 1998; DeGregori et al., 1997; Dimri et al., 2000). To address whether *p19ARF* is also subjected to transcriptional repression by endogenous E2Fs, we interfered with E2F signaling by the use of a mutant of E2F-1 (E2F-DB; Figure 1A) in primary MEFs. E2F-DB lacks both the C-terminal transactivation and the pRB binding domains, but can still bind to DNA in heterodimeric complex with DP-1. E2F-DB has been used previously to rescue pocket protein-mediated transcriptional repression (Johnson, 1995; Qin et al., 1995) and to show that cell cycle arrest induced by either $p16^{INK4a}$, TGFβ, or contact inhibition requires active E2F-mediated transcriptional repression (Zhang et al., 1999).

The mechanism of action of E2F-DB has previously been shown to involve binding to E2F sites and subsequent displacement of endogenous E2Fs, as demonstrated by electrophoretic mobility shift assays (EMSAs) in both fibroblasts and epithelial cells (Krek et al., 1995; Zhang et al., 1999). First, we wished to establish whether E2F-DB displaces endogenous E2F from E2F sites also in MEFs. Indeed, upon infection of MEFs with E2F-DB-encoding retroviruses, we observed that the DNA binding activity of endogenous E2F was almost completely abolished and replaced by an E2F-DB/DNA complex (Figure 1B). This suggests that E2F-DB occupies the E2F sites in E2F-responsive promoters in vivo. To address this, we performed chromatin immune precipitation (ChIP) on E2F-DB, followed by real-time PCR on three different E2F-responsive promoters. Consistent with the results obtained by EMSA, we observed that in E2F-DB-expressing MEFs, significant amounts of E2F-DB occupy the *p19ARF, CYCLIN A*, and *CYCLIN E* promoters (Figure 1C). Together, these data provide strong evidence that E2F-DB acts to displace endogenous E2Fs from E2F-responsive promoters.

Endogenous E2Fs act as repressors in cycling primary murine fibroblasts

The E2F transcription factors have the ability to mediate either transcriptional repression or activation. In order to determine the relative contribution of these two functions on the regulation of endogenous target genes of E2F, we compared wild-type E2F-1 to mutants of E2F-1, which lack either the repression function only (E2F-1 (Y411C)) or both the repression and transactivation functions (E2F-DB). Upon retroviral expression in primary MEFs, wild-type E2F-1 differentially induced its transcriptional targets $p19^{ARF}$, PCNA, p107, MCM3, and CYCLINS E1 and A (Figure 2A). E2F-DB, on the other hand, markedly induced all of these targets (Figure 2A). This was not a global effect of E2F-DB, as the expression of $p16^{INK4a}$, CYCLIN D1, and CDK4 remained unaffected (Figure 2E). As expected, a DNA binding-deficient mutant of E2F-DB (E2F-DB (E132)) (Hsieh et al., 1997) failed to induce the expression of the E2F target genes (Figure 2B).

If endogenous E2Fs act predominantly to activate transcriptional targets, one would expect that E2F-DB would interfere with this activation, something we clearly did not observe. We therefore conclude that in cycling primary MEFs, endogenous E2F controls target gene expression predominantly by means of active repression. Restoration of specifically E2F's transactivation function (i.e., expression of E2F-1 (Y411C)), which transactivates but does not repress (Helin et al., 1993), failed to give rise to additional induction on top of that achieved by E2F-DB, indicating that the transactivating function of E2F-1 is dispensable for the induction of at least this set of E2F target genes. As E2F-1 controls its own transcription (Hsiao et al., 1994; Johnson et al., 1994; Neuman et al., 1994), we wished to exclude that E2F-DB induces the levels of transcriptionally competent endogenous E2F-1, which in turn switches on expression of E2F target genes. Indeed, the levels of endogenous E2F-1 remained unaltered in the presence of E2F-DB (Figure 2E), excluding this possibility.

E2F-mediated transcriptional repression occurs through association with pocket proteins. Hence, it seemed likely that the E2F-DB-mediated derepression is pocket protein dependent.

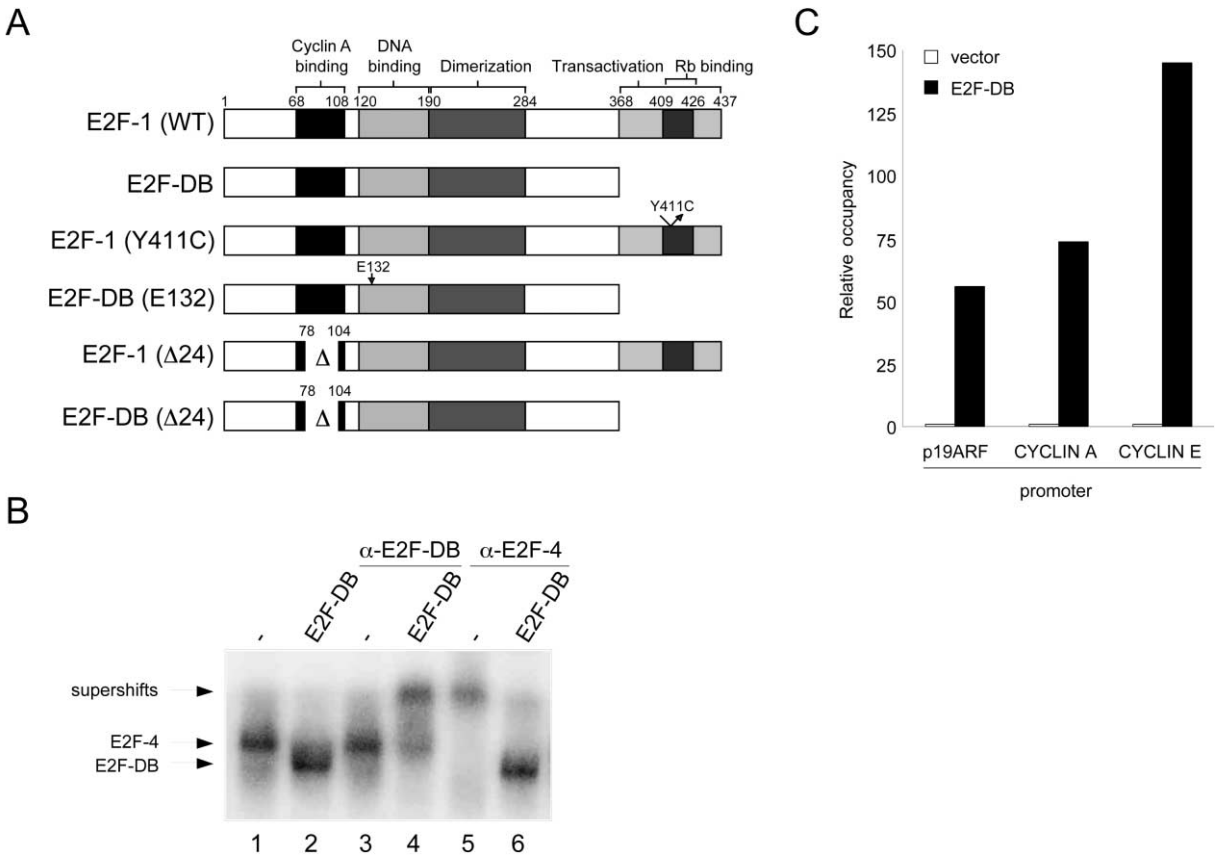

Figure 1. Ectopic expression of an E2F-1 C-terminal deletion mutant displaces endogenous E2Fs from DNA

A: Schematic representation of the E2F-1 mutants used in this study. **B:** E2F-DB displaces endogenous E2F from the DNA. Nuclear extracts were prepared from primary p53$^{-/-}$ MEFs infected with retroviruses encoding E2F-DB or control viruses and analyzed by EMSA for E2F DNA binding activity in the absence or presence of antibodies for E2F-DB or E2F-4. **C:** E2F-DB binds to E2F-responsive promoters in vivo. ChIP assays performed in wild-type primary MEFs for E2F-DB on the *p19ARF*, *CYCLIN A*, and *CYCLIN E* promoters. Data are represented as real-time PCR signals from *p19ARF*, *CYCLIN A*, or *CYCLIN E*, relative to a γ-*ACTIN* control PCR performed on the same ChIP.

To address this directly, we infected MEFs deficient for all three pocket proteins (TKO MEFs) (Dannenberg et al., 2000) with E2F-DB-encoding retrovirus. Whereas E2F-DB derepressed p19^{ARF}, PCNA, CDK1, MCM3, and CYCLINS E1 and A in wild-type MEFs, this regulation was completely absent in TKO MEFs (although E2F-DB was expressed slightly less in TKO MEFs than in wild-type MEFs; Figure 2C). These results demonstrate that in MEFs, the observed E2F-dependent repression of target genes is mediated mainly, if not exclusively, by pocket proteins. Importantly, in TKO MEFs, we also failed to observe an E2F-DB-mediated decrease in the levels of E2F targets, which, as E2Fs in these cells are thought to be free and transactivation competent, supports our notion that in proliferating MEFs, E2Fs serve mainly as repressors of transcription.

Recently, p19^{ARF} was shown to bind to E2F-1 and target it for degradation (Martelli et al., 2001). Possibly, E2F-DB sequesters p19^{ARF} and thereby protects endogenous E2F from degradation, leading to the induction of E2F target genes. However, E2F-DB-dependent derepression of all E2F targets tested occurred similarly (albeit to different extents) in wild-type and *p19ARF*$^{-/-}$ MEFs, excluding an important role for p19^{ARF} in this respect (Figure 2D).

Expression of *p19ARF*, an E2F target gene, is induced by RAS^{V12} and required for RAS^{V12}-induced senescence (Palmero et al., 1998), raising the possibility that p19^{ARF} induction by RAS^{V12} requires E2F activity. However, E2F-DB did not inhibit p19^{ARF} induction by RAS^{V12}, but rather enforced it (Figure 2E). The RAS^{V12}-dependent induction of p19^{ARF} in the presence of E2F-DB was functional, as it led to an increase in p53 levels (Figure 2E). This result strongly suggests that RAS^{V12}-dependent induction of p19^{ARF} and p53 does not require E2F-mediated transactivation.

Disruption of E2F-dependent repression results in immortalization

Increased p19^{ARF} levels cause cell cycle arrest or senescence (Kamijo et al., 1997; Quelle et al., 1995). As E2F-DB derepressed p19^{ARF} expression in MEFs, we expected that E2F-DB would cause premature senescence. Surprisingly, whereas control-infected MEFs lost their replicative potential, at least initially (see below), cells expressing E2F-DB completely failed to undergo senescence (Figure 3A). This effect of E2F-DB was independent of its CYCLIN A binding domain (Figure 3B), which is required to appropriately inactivate the DNA binding function of E2F in S phase (Krek et al., 1994). Moreover, this bypass of senescence was not observed for wild-type E2F-1, which caused both cell

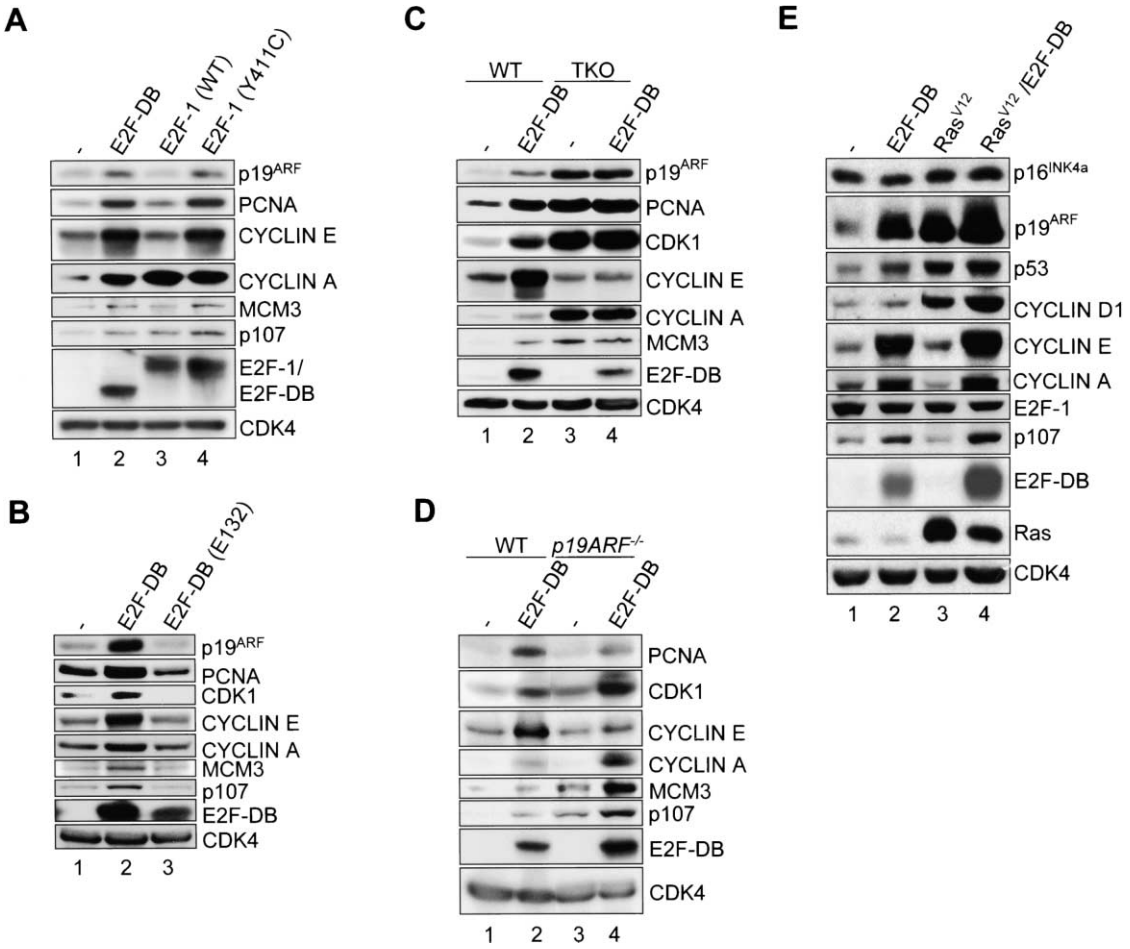

Figure 2. Endogenous E2Fs act as repressors in primary murine fibroblasts

A: Disruption of E2F function results in derepression of various E2F targets. Young primary wild-type MEFs were infected with retroviruses encoding E2F-DB, E2F-1, or E2F-1 (Y411C). At 5 days after infection, cell extracts were prepared and subjected to Western blotting with the indicated antibodies. **B:** E2F-DB-mediated derepression requires its DNA binding function. MEFs were infected with E2F-DB, E2F-DB (E132), or control viruses and processed as in **A**. **C:** E2F-dependent repression is mediated by pocket proteins. Wild-type and $Rb^{-/-}/p107^{-/-}/p130^{-/-}$ (TKO) MEFs were infected with E2F-DB or control viruses and processed as in **A**. **D:** E2F-dependent repression is independent of *p19ARF* status. Wild-type and $p19ARF^{-/-}$ MEFs were infected and processed as in **A**. **E:** RAS^{V12} induces $p19^{ARF}$ independent of E2F transactivation. Wild-type MEFs were infected with retroviruses encoding E2F-DB, RAS^{V12}, or both. Cell extracts were prepared at 6 days after infection. Similar observations were made for extracts prepared at 3, 12, and 24 days postinfection (not shown). In all panels, CDK4 served as a loading control.

death and premature senescence (consistent with previous reports; DeGregori et al., 1997; Dimri et al., 2000; Qin et al., 1994; Shan et al., 1994; Wu and Levine, 1994; Figures 3A and 3D), nor was it observed for E2F-1 (Δ24) lacking the CYCLIN A binding domain (Figure 3B). The pRB binding-deficient E2F-1 (Y411C) mutant caused cell death (Figure 3B), although especially at higher cell density, a substantial amount of the cells did continue to proliferate (Figure 3D). As expected, the DNA binding-deficient point mutant of E2F-DB failed to yield any proliferative advantage (Figure 3C).

We next determined whether the E2F-DB-mediated immortalization requires mutations in the genes that are frequently mutated during spontaneous immortalization, namely *p19ARF* and *p53*. We propagated four independent E2F-DB-expressing MEF populations for 20 passages and then analyzed $p16^{INK4a}$, $p19^{ARF}$, p53, and $p21^{CIP1}$ levels, as well as p53 function. Compared to control MEFs at passage 8, E2F-DB MEFs at passage 20 continued to abundantly express both $p19^{ARF}$ and $p16^{INK4a}$ (Figure 3E). By contrast, spontaneously immortalized ("3T3") mouse fibroblasts had lost expression of $p19^{ARF}$. We then used the DNA damage-induced stabilization of p53 and concomitant transcriptional activation of $p21^{CIP1}$ to determine whether p53 was still functional in the E2F-DB-immortalized MEFs. *cis*-platin treatment led to stabilization of p53 in all four late-passage E2F-DB-expressing lines (Figure 3F). Moreover, p53 was functional, as DNA damage increased the levels of its transcriptional target $p21^{CIP1}$ (Figure 3F).

Disruption of E2F-dependent repression results in bypass of RAS^{V12}-induced senescence

RAS^{V12}-induced premature senescence much resembles spontaneous senescence of primary cells. Therefore, we tested whether disruption of E2F transcriptional repression not only leads to immortalization of primary MEFs, but also rescues

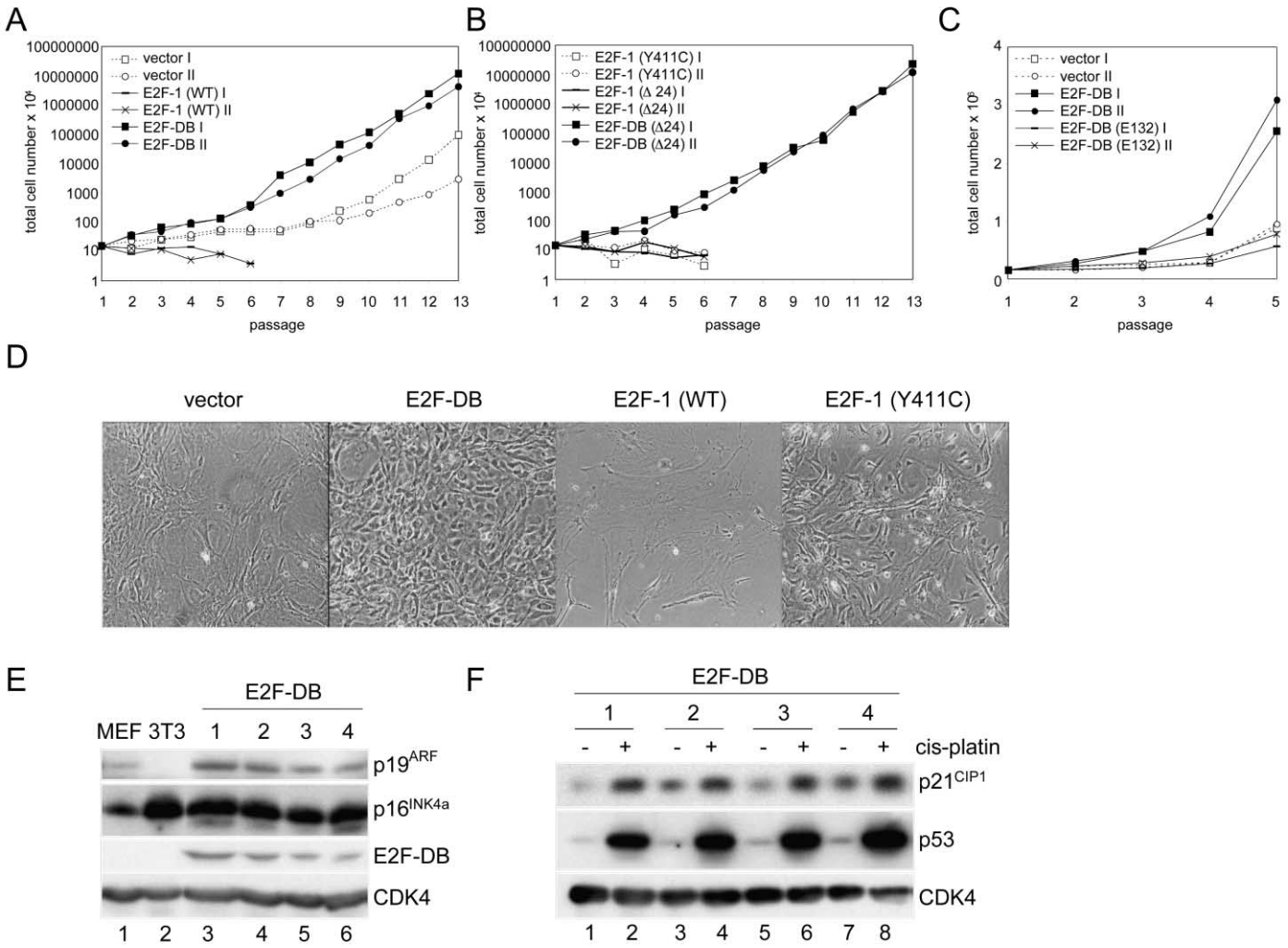

Figure 3. Disruption of E2F-dependent repression results in immortalization

A: E2F-DB immortalizes primary MEFs. Wild-type, primary MEFs at passage 3 were infected with pBABE-puro retroviruses encoding wild-type or mutant E2F-1, as indicated. At 2 days postinfection, infected cells were selected for expression of the puromycin-selectable marker for 5 days and used in a proliferation curve performed in duplicate. **B:** Immortalization by E2F-DB does not require a CYCLIN A binding domain. MEFs were infected with retroviruses encoding E2F-1 (Δ24), E2F-DB (Δ24), or E2F-1 (Y411C). Analysis was performed as in **A**. **C:** Immortalization by E2F-DB requires its DNA binding function. MEFs were infected with retroviruses encoding E2F-DB, E2F-DB (E132), or control viruses. Analysis was performed as in **A**. **D:** E2F-1 (Y411C) can stimulate MEF proliferation. Low-passage MEFs were infected with either E2F-DB, E2F-1 (wild-type), or E2F-1 (Y411C) and cultured under subconfluent conditions. Photographs were taken at passage 7. **E:** Immortalization by E2F-DB occurs in the presence of $p19^{ARF}$ and $p16^{INK4a}$. Western blot analysis for the indicated proteins of immortal E2F-DB-expressing MEF clones at passage 20. 3T3 indicates a spontaneously immortalized MEF clone (Vector I from the growth curve in **A**) that apparently had lost $p19^{ARF}$ expression. E2F-DB lines 3 and 4 represent cell extracts taken from E2F-DB lines I and II, respectively, also taken from the growth curve in **A**. E2F-DB lines 1 and 2 are independently propagated MEFs. MEF indicates passage 8 primary MEFs. CDK4 served as a loading control. **F:** Immortalization by E2F-DB occurs in the presence of functional p53. Western blot analysis of the indicated proteins of immortal E2F-DB-expressing MEF clones at passage 20, after 16 hr treatment with *cis*-platin (50 μM). CDK4 served as a loading control.

RAS^{V12}-induced premature senescence. MEFs expressing only RAS^{V12} prematurely senesced, although after prolonged culturing one of these populations ("RAS^{V12} II") regained proliferative potential (Figure 4A; see below). By contrast, MEFs expressing both RAS^{V12} and E2F-DB, in spite of overexpressing RAS^{V12} (not shown), efficiently bypassed RAS^{V12}-induced senescence (Figure 4A).

Like E2F-DB-immortalized MEFs, both RAS^{V12}/E2F-DB MEF lines had retained normal expression of both $p19^{ARF}$ and p53, as well as of $p16^{INK4a}$ (Figure 4B). This was in contrast to a spontaneously immortalized RAS^{V12}-expressing cell line (RAS^{V12} II) that had lost expression of both $p16^{INK4a}$ and $p19^{ARF}$ (Figure 4B). Thus, disruption of E2F function results in derepression of *p19ARF*, but in spite of this, it also concomitantly causes a bypass of both spontaneous and RAS^{V12}-induced senescence, thereby allowing cells to proliferate indefinitely while ignoring the sharp elevation in endogenous $p19^{ARF}$ levels.

Disruption of E2F-dependent repression rescues cell cycle arrest induced by ectopically expressed $p19^{ARF}$ and p53

The results shown above strongly argue that endogenous E2F repressor function is required for cells to respond to senescence-associated induction of $p19^{ARF}$. To test whether E2F-DB

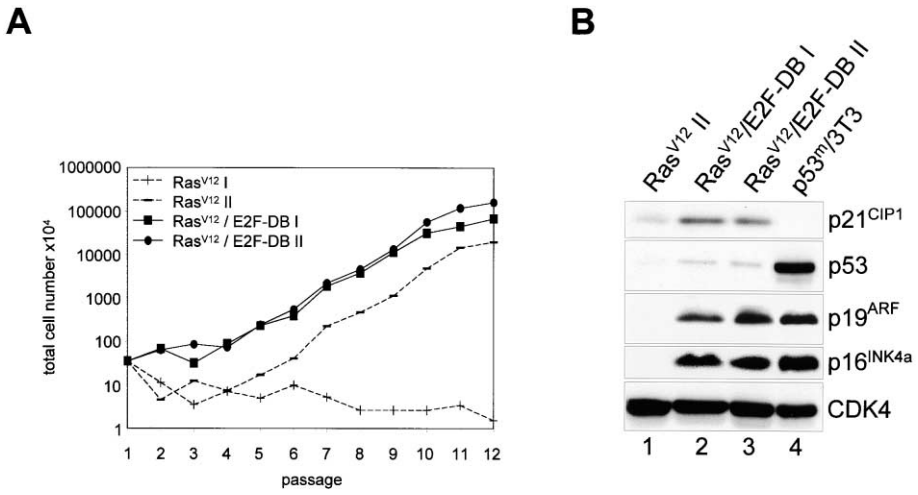

Figure 4. Disruption of E2F-dependent repression results in bypass of RAS^{V12}-induced senescence

A: E2F-DB expression allows primary MEFs to bypass RAS^{V12}-induced senescence. Wild-type, primary MEFs at passage 3 were (co-) infected with retroviruses encoding RAS^{V12} and either control or E2F-DB-encoding retroviruses, selected for puromycin and hygromycin, and processed as described for Figure 3A. **B:** Bypass of RAS^{V12}-induced senescence by E2F-DB occurs in the presence of p19^{ARF}, p16^{INK4a}, and p53. Western blot analysis of passage 15 clones, after completion of the growth curves described in **A**. p53^m/3T3 (lane 4) indicates a spontaneously immortalized MEF clone expressing high levels of mutant p53, which failed to transcriptionally activate *p21^{CIP1}*. CDK4 served as a loading control.

can bypass also an ectopic p19^{ARF}-induced cell cycle arrest, we generated NIH 3T3 cell lines stably expressing E2F-DB or empty vector. These cell lines retained functional p53, as judged by its DNA damage-induced stabilization and concomitant induction of p21^{CIP1} (data not shown). We infected both cell populations with a p19^{ARF}-encoding retrovirus. As expected, p19^{ARF} induced a robust G1 arrest in the control cells (Figure 5A). By contrast, this arrest was reduced by half in E2F-DB-expressing cells. This observation was supported by a colony formation assay: upon infection with a retrovirus producing a p19^{ARF}-RFP chimeric protein, the control population showed only few proliferating, p19^{ARF}-positive cells (Figure 5B). In fact, the small number of cells that did express p19^{ARF} (localized in the nucleoli, in agreement with previous observations; Weber et al., 1999;

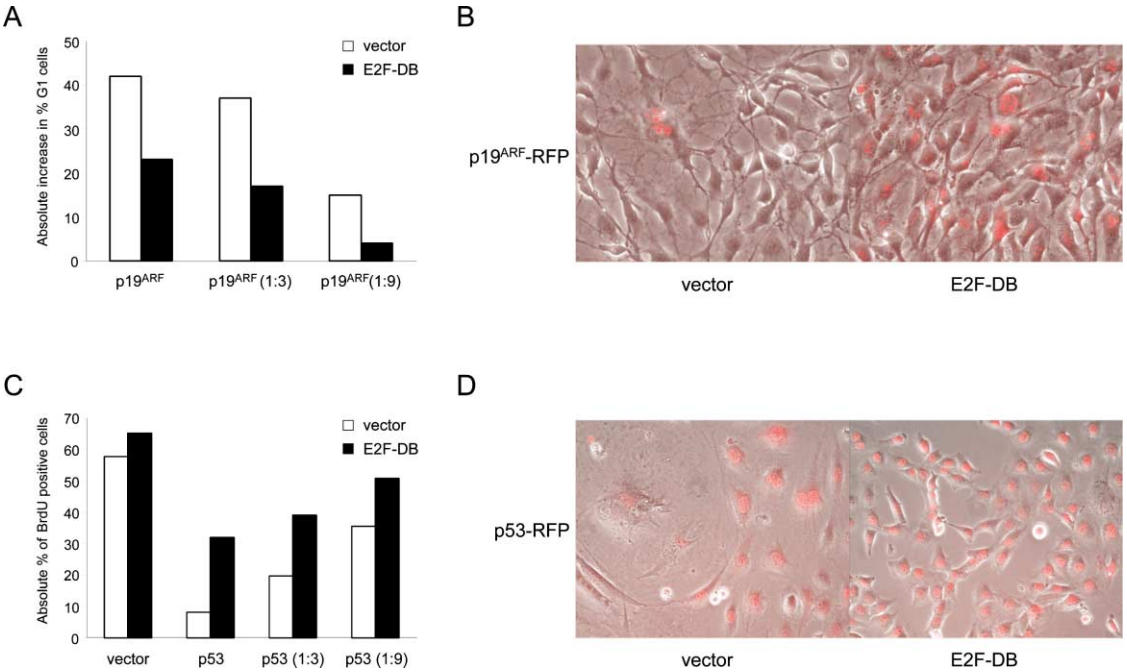

Figure 5. Disruption of E2F-dependent repression rescues cell cycle arrest induced by ectopically expressed p19^{ARF} and p53

A: E2F-DB interferes with cell cycle arrest induced by overexpression of p19^{ARF}. Parental or E2F-DB-expressing NIH-3T3 cells were infected with either control or p19^{ARF}-ires-GFP-encoding retroviruses (either undiluted or diluted into medium, as indicated) and treated with nocodazole (50 ng/ml) to specifically analyze the cell fraction in G1. At least 90% of the cells were infected, as judged by the number of GFP-positive cells. At 48 hr after infection, the cell cycle profile was determined by FACS analysis. **B:** E2F-DB interferes with inhibition of colony formation by overexpression of p19^{ARF}. Parental or E2F-DB-expressing NIH-3T3 cells were infected with either control or LZRS-p19^{ARF}-RFP-ires-zeo retrovirus. At 6 days after infection, fluorescence microscopy photographs were taken. Representative examples are shown. **C:** E2F-DB interferes with cell cycle arrest induced by overexpression of p53. Parental or E2F-DB-expressing *p53$^{-/-}$* cells were infected with either control or LZRS-p53-RFP-ires-zeo retrovirus (either undiluted or diluted into medium, as indicated). BrdU incorporation was measured 48 hr after infection. **D:** E2F-DB interferes with inhibition of colony formation by overexpression of p53. Parental or E2F-DB-expressing *p53$^{-/-}$* cells were infected as in **C**. At 6 days after infection, fluorescence microscopy photographs were taken. Representative examples are shown.

Zhang and Xiong, 1999) had a large and flattened morphology, typical of senescent cells. Apparently, strong selection occurred against maintaining $p19^{ARF}$ expression. By contrast, the vast majority of the E2F-DB-expressing cells produced clearly detectable levels of $p19^{ARF}$ without displaying a senescent morphology (Figure 5B), reinforcing our notion that ectopically expressed $p19^{ARF}$ blocks cellular proliferation in an E2F-dependent manner.

E2F-DB rescued $p19^{ARF}$-induced cell cycle arrest, a p53-dependent event (at least in part), raising the possibility that E2F-DB acts downstream of p53. To test this, we established *p53*$^{-/-}$ MEFs stably expressing E2F-DB, which we subsequently infected with a p53-encoding retrovirus. As expected, control cells ceased to undergo DNA replication almost completely upon expression of p53 (Figure 5C). By contrast, in the presence of E2F-DB, p53 decreased the number of cells undergoing DNA replication only by roughly half. In support of this finding, we observed that in a colony formation assay, E2F-DB-expressing MEFs continued to proliferate despite clearly detectable levels of retrovirally expressed p53-RFP fusion protein, which was localized in the nucleus (Figure 5D). In control cells, neither abundant expression nor nuclear localization of p53-RFP was compatible with proliferation, and cells that did express p53-RFP again displayed a "flat-cell" phenotype (Figure 5D). Thus, E2F-DB not only rescues spontaneous senescence (a $p19^{ARF}$- and p53-dependent process), but also significantly interferes with a cell cycle arrest imposed by ectopic overexpression of $p19^{ARF}$ and p53. These results further support our notion that E2F acts downstream of both $p19^{ARF}$- and p53-dependent antiproliferative signaling.

E2F activity is required for cell cycle reentry from quiescence

The data above show that E2F's transactivation function is dispensable for the induction of (at least a number of) E2F target genes. Moreover, they strongly suggest that the absence of E2F-mediated transactivation is compatible with proliferation of primary murine cells, something that has been reported previously for other cell types (Sellers et al., 1995; Zhang et al., 1999; He et al., 2000). However, most E2Fs possess a transactivation domain, arguing that specific situations should exist where transactivation is required. Indeed, ectopic E2F-1-mediated transactivation, but not repression, is sufficient to induce S phase entry in the absence of mitogens (Johnson et al., 1993; Kowalik et al., 1995; Qin et al., 1994; Shan et al., 1996). Moreover, *E2F1*$^{-/-}$ fibroblasts display a delayed G0-S transition in response to mitogen stimulation (Wang et al., 1998). We therefore investigated whether endogenous E2F transactivation function is not only sufficient, but also required for mitogen-induced cell cycle reentry.

We deprived E2F-DB expressing cells of serum, which caused them to exit the cell cycle as efficiently as control cells (Figure 6). Apparently, in immortal murine fibroblasts, no E2F function is required for G0 entry, consistent with previous observations in other cell types (Zhang et al., 1999). By contrast, upon serum refeeding, the control cells readily reentered the cell cycle, whereas the E2F-DB-expressing cells were significantly impaired in their DNA replication (Figure 6). Taken together, this suggests that loss of E2F transcriptional activity prevents mitogen-induced cell cycle reentry, indicating that in this specific situation, E2F-mediated transactivation is required.

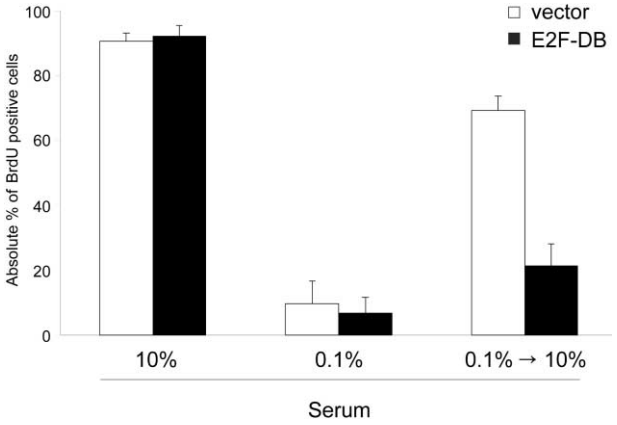

Figure 6. E2F activity is required for cell cycle reentry from quiescence

NIH 3T3 cell lines stably expressing either E2F-DB or empty vector were propagated in 10% serum and subsequently deprived of serum for 72 hr. Then, the cells were refed with 10% serum for 24 hr. Represented is the proportion of cells incorporating BrdU, as a measure of DNA synthesis, in each situation. Average and standard deviations are based on three independent experiments.

Discussion

E2F-mediated transcriptional repression and $p19^{ARF}$-dependent regulation of senescence

We demonstrate that in primary murine fibroblasts, various E2F targets, including *p19ARF*, are repressed rather than transactivated by endogenous E2F. More importantly, disruption of E2F-mediated repression allowed MEFs to bypass both spontaneous and RAS^{V12}-induced senescence and to proliferate indefinitely, in the face of high levels of $p19^{ARF}$. We therefore conclude that $p19^{ARF}$ promotes proliferative arrest in an E2F transcriptional repression-dependent manner, a notion that is reinforced by our observation that disruption of E2F repression interfered also with the induction of cell cycle arrest by ectopically expressed $p19^{ARF}$ or p53.

Expression of E2F-DB led to induction (i.e., derepression), as opposed to reduction (i.e., loss of transactivation), of various E2F targets, including *p19ARF*. Moreover, similar to E2F-DB-expressing MEFs, pRB family-deficient MEFs, which lack E2F-repressor complexes, are immortal despite high levels of $p19^{ARF}$. These observations indicate that in primary MEFs, it is specifically E2F's transcriptional repression function, but not its transactivation function, which is required for the senescence checkpoint. What, then, is the function of E2F's transactivation domain? We observed neither an effect of E2F-DB on the levels of $p19^{ARF}$ (or other E2F targets) in pRB family-deficient cells, nor a contribution of E2F's transactivation domain on top of the induction of E2F target genes by E2F-DB in wild-type MEFs. Although we cannot exclude that in proliferating cells some residual E2F transactivator function remains despite the presence of E2F-DB, our results argue that in cycling primary MEFs, E2F-dependent transactivation is dispensable. By contrast, E2F-DB did efficiently prevent mitogen-stimulated cell cycle reentry. Our data therefore suggest that E2F-dependent transactivation is required only in specific circumstances, like in the presence of apoptosis-inducing signals or during cell cycle reentry (as shown schematically in Figure 7). Furthermore, we show

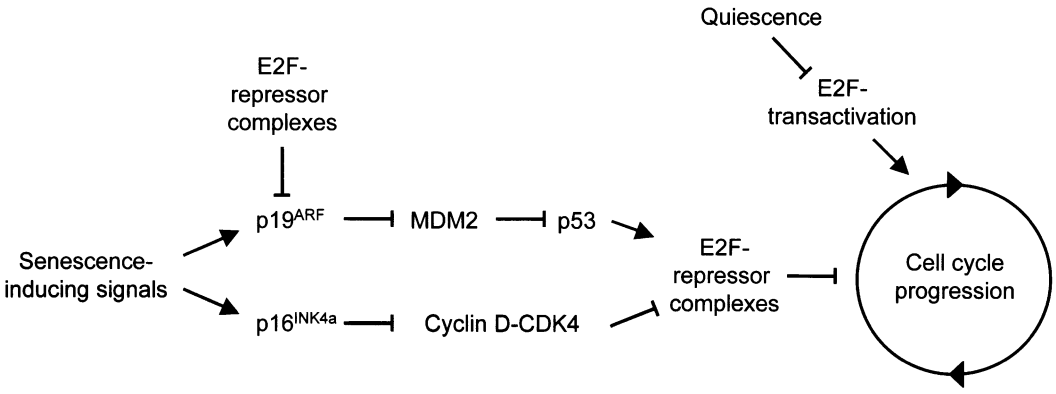

Figure 7. Hypothetical model for E2F function in cell cycle regulation
See text for details.

that it is specifically E2F-dependent repression that is required for cell cycle exit in response to activation of the p19^{ARF}/p53 pathway.

Although various E2F target genes were derepressed both in E2F-DB-expressing wild-type MEFs and in pRB family-deficient MEFs, the extent of derepression was greater (with the exception of CYCLIN E) in the latter cell type. This can be explained by the fact that E2F-DB acts in a dominant-negative manner and displaces most, but not all, endogenous E2F complexes from the DNA (see Figure 1B). By contrast, pRB family-deficient MEFs completely lack E2F/pocket protein complexes and as a result show maximal derepression of E2F targets. Importantly, ectopic expression of E2F-DB and genetic ablation of pocket proteins do result in a similar biological phenotype: both interfere with the senescence checkpoint and lead to immortalization.

Recent data show that the simultaneous absence of E2Fs 1, 2, and 3 causes defects in G1/S target gene activation and inhibition of proliferation (Wu et al., 2001). This may seem at odds with our finding that MEFs proliferate well in the presence of E2F-DB. However, whereas in E2F-1-3 triple knockout cells E2F-4 and E2F-5 are free to repress transcription and thereby may inhibit proliferation, this is compromised by E2F-DB, as this displaces endogenous E2Fs from E2F sites (Krek et al., 1995; Zhang et al., 1999).

Previous studies (Bates et al., 1998; Dimri et al., 2000) showed that wild-type E2F-1 induced the human *p14ARF* gene, whereas E2F-DB did not. This seemingly is in contrast with our present observations with E2F-DB. However, Bates et al. made their observations in SAOS-2 cells, which lack functional pRB and therefore contain only a limited amount of E2F repressor complexes. On the other hand, Dimri et al. used primary human WI-38 fibroblasts, which suggests that E2F-dependent *ARF* regulation varies depending on species or cell type, possibly as a result of differences in the composition and/or quantity of E2F complexes. For some targets we did observe induction by wild-type E2F-1, consistent with previous reports (Muller and Helin, 2000). However, we propose that some effects of overexpressed wild-type E2F may be caused by titrating cellular factors like pocket proteins away from E2F sites in promoters. In this case, the net effect would be similar to what we describe here for the transcription-deficient E2F-DB mutant, namely an increase in the levels of E2F targets, like *ARF*.

Primary MEFs expressing E2F-DB produce elevated levels of CYCLIN E1, which can contribute to immortalization (Peeper et al., 2002). In fact, overexpression of CYCLIN E1 has been observed in, and most likely contributes to the emergence of, many human tumors (Keyomarsi and Herliczek, 1997). However, it clearly is not the sole target in this respect, as also *Rb*-deficient MEFs produce high levels of CYCLIN E1 (Herrera et al., 1996), yet undergo senescence normally. Indeed, numerous genes are regulated by the pRB/E2F pathway, strongly suggesting that immortalization requires derepression of a number of E2F targets. An important question that remains to be addressed is how p19^{ARF}/p53 signaling leads to a requirement for E2F repressor complexes in a pathway leading to proliferative arrest. In this respect, we note that our data do not discriminate between a linear pathway between p19^{ARF}/p53 and E2F and a more indirect mechanism of communication with intermediary signals.

Communication between p19^{ARF}, p16^{INK4a}, p53, and E2F

It has been suggested previously that p19^{ARF} requires functional p16^{INK4a} to induce cell cycle arrest (Carnero et al., 2000). As E2F-DB rescues not only p19^{ARF}-induced arrest, as we show here, but also a p16^{INK4a}-induced arrest (Zhang et al., 1999), and since E2F-DB-expressing MEFs proliferate in the presence of normal p16^{INK4a} levels, it is formally possible that E2F-DB immortalizes by acting downstream of p16^{INK4a}. Recent data, however, reveal that *p16^{INK4a}* is dispensable both for spontaneous and RAS^{V12}-induced senescence in MEFs (Krimpenfort et al., 2001; Sharpless et al., 2001). This makes it highly unlikely that disruption of E2F-dependent repression renders MEFs immortal through interference with p16^{INK4a} signaling and argues in favor of our model that in senescence signaling, the p19^{ARF}/p53 pathway acts in an E2F-dependent manner.

In conclusion, we show that in primary MEFs, E2F-mediated repression is linked to antiproliferative signaling by p19^{ARF} in at least two ways (Figure 7). Upstream, E2F represses *p19ARF*, in a pocket protein-dependent manner. Downstream, functional E2F is required for both p19^{ARF} and p53 to induce cell cycle arrest and for the appropriate execution of the senescence program. Interestingly, the *INK4a* locus encodes two unrelated proteins, p16^{INK4a} and p19^{ARF}, proposed to act in independent pathways, namely pRB- and p53-dependent, respectively. The Dean and Livingston laboratories have previously demonstrated that E2F

repressor complexes are required for an appropriate cellular response to $p16^{INK4a}$ (Gaubatz et al., 2000; Zhang et al., 1999). Quite unexpectedly, however, we find that E2F repressor complexes are also essential for cell cycle exit in response to the other *INK4a* product, $p19^{ARF}$. Thus, together these findings suggest that $p16^{INK4a}$ and $p19^{ARF}$/p53 do not operate independently, but converge on E2F repressor complexes. This model would predict that tumors with mutations in the *INK4a* locus (as well as in *p53*) share deregulation of E2F-controlled target genes, irrespective of which *INK4a* gene is affected. As the E2F repressor complex is controlled by these two major tumor suppressor pathways, our model predicts that E2F-dependent transcriptional repression is deregulated in the vast majority of human tumors.

Experimental procedures

Cell culture

MEFs of OLA and FVB origin were isolated as described (Peeper et al., 2001) and cultured in DMEM medium supplemented with 10% FBS, L-Glutamine, and penicillin/streptomycin (all GIBCO) and 0.1 mM β-mercaptoethanol. NIH-3T3 cells were cultured as MEFs, but with 10% NCS (GIBCO) and without β-mercaptoethanol. The Phoenix packaging cell line was used for the generation of ecotropic retroviruses (Serrano et al., 1997). MEFs were infected for 8 hr with filtered (0.45 μm) virus supernatant supplemented with 8 μg/ml polybrene.

Proliferation curves were performed by infection of low-passage MEFs with pBABE-puro retroviral vectors containing HA-E2F-1 (wild-type or mutant, as described in Figure 1) and pBABE-hygro retroviral vectors containing RAS^{V12}. Cells were subsequently selected for puromycin and hygromycin resistance until all uninfected cells had died. Then, 1.5×10^5 cells were seeded in a 6 cm dish and counted and split every 3.5 days. For *cis*-platin treatment, E2F-DB immortalized MEFs were cultured for 20 passages and incubated in 50 μM *cis*-platin for 16 hr.

Plasmids

pBABE-puro vectors containing HA-tagged E2F-1 wild-type, E2F-1 (1–368), E2F-1 (Δ24), and E2F-1 (1–368 Δ24) were kind gifts from W. Krek (Krek et al., 1995). E2F-1 mutants (1–374) (Helin and Harlow, 1994), E2F-1 (1–374, E132) (Hsieh et al., 1997), and E2F-1 (Y411C) (Helin et al., 1993) were subcloned into pBABE-puro. LZRS-$p19^{ARF}$-RFP-ires-zeo and LZRS-p53-RFP-ires-zeo retroviral vectors were kindly provided by T. Brummelkamp. MSCV-$p19^{ARF}$-ires-GFP and MSCV were kind gifts from C. Sherr.

Western blot analysis and EMSA

Preparation of protein extracts and Western blotting were performed as described (Peeper et al., 2001). Primary antibodies were M-156 for $p16^{INK4a}$, C19 for $p21^{CIP1}$, C22 for CDK4, PC-10 for PCNA, H-295 for Cyclin D1, M-20 for Cyclin E, C-19 for Cyclin A, C-19 for CDK1, G-19 for MCM3, C-18 for p107, KH95 for E2F-1 and E2F-DB (all Santa Cruz), KH-20 for E2F-DB (used in Figure 2E) (Neomarkers), R02120 for RAS (Transduction Laboratories), R562 for $p19^{ARF}$ (Abcam), and Ab-7 for p53 (Calbiochem). Mobility shift assays were performed as described (Beijersbergen et al., 1995). Antibodies used for supershifts were KH-95 for E2F-DB and C-20 for E2F-4 (both Santa Cruz).

ChIP and real-time PCR

Primary MEFs were infected at passage 2 with pBABE-puro-HA-E2F-DB or parental pBABE-puro retroviruses and selected for puromycin resistance. At passage 6, chromatin was isolated from approximately 5×10^7 E2F-DB or control MEFs in essence as described (Botquin et al., 1998; Orlando et al., 1997), but excluding CsCl-gradient purification. Chromatin was sonicated to an average size of 1000 bp and subsequently precleared with protein A/G sepharose beads (Santa Cruz) in incubation buffer (0.1% SDS, 1% Triton X-100, 0.1 M NaCl, 1 mM EDTA, 1 mM Tris [pH 8.0], 0.5 mM EGTA, 1 mg/ml BSA, and protease inhibitors). Antibody incubation (using 2 μg of KH95 for E2F-DB [Santa Cruz]) of precleared chromatin was performed overnight in incubation buffer at 4°C, and immunocomplexes were recovered with protein A/G-Sepharose beads. Immunoprecipitates were washed sequentially with 0.1% SDS, 1% Triton X-100, 0.1% deoxycholate, 0.15 M NaCl, 1 mM EDTA, 10 mM Tris (pH 8.0), 0.5 mM EGTA; 0.1% SDS, 1% Triton X-100, 0.1% deoxycholate, 0.5 M NaCl, 1 mM EDTA, 10 mM Tris (pH 8.0), 0.5 mM EGTA; 0.25 M LiCl, 0.5% deoxycholate, 0.5% NP-40, 1 mM EDTA, 10 mM Tris (pH 8.0), 0.5 mM EGTA, and 1 mM EDTA, 10 mM Tris (pH 8.0), 0.5 mM EGTA. Immunocomplexes were eluted twice from the beads in 1% SDS, 0.1 M $NaHCO_3$ at room temperature for 15 min. Protein-DNA crosslinks were reversed in 0.2 M NaCl at 65°C for 4 hr, after which DNA was isolated by phenol-chloroform extraction. Real-time PCR was performed using the GeneAmp 5700 Sequence Detection System (PE Biosystems) using the SYBR Green I kit (PE Biosystems). Primers used for real-time PCR were for *p19ARF* (E2F binding sites region), (forward) TTTTTATA GATGGACTCGGAGCAA and (reverse) GTCCCGAAACTTTCGTCTATGC; for *CYCLIN A* (E2F binding sites region), (forward) CCGGCGCTTCTGGTGAC and (reverse) CAAGTAGCCCGCGACTATTGA; for *CYCLIN E* (E2F binding sites region), (forward) GGGCGTGTTCTTTTACGGG and (reverse) GCC CTGACATCTAGCCCCA; and for γ-*ACTIN* (exon 5), (forward) TCCGCAAAGA CCTGTATGCC and (reverse) CTCCTTCTGCATCCTGTCAGC.

Cell cycle analysis and fluorescence microscopy

NIH-3T3 cells were infected with pBABE-puro-HA-E2F-DB or parental pBABE-puro retroviruses, and polyclonal pools were selected for puromycin resistance. For FACS analysis, cells were infected with MSCV-$p19^{ARF}$-ires-GFP or MSCV control virus. After 32 hr, 50 ng/ml nocodazole was added to the medium for 16 hr. Then, cells were permeabilized and stained with propidium iodide, and cell cycle profiles were determined by FACS analysis and analyzed using CellQuest software. For fluorescence microscopy, cells were infected with LZRS-$p19^{ARF}$-RFP-ires-zeo or parental pLZRS-ires-zeo retroviruses. Cells were plated at 10^5 cells/10 cm dish. Photographs were taken after 6 days of zeocin selection using fluorescence microscopy, at 60× magnification.

$p53^{-/-}$ MEFs were infected with pBABE-puro-HA-E2F-DB or parental pBABE-puro retroviruses, and polyclonal pools were selected for puromycin resistance. Subsequently, cells were infected with pLZRS-p53-RFP-ires-zeo or parental pLZRS-ires-zeo retroviruses. At 48 hr postinfection, 7.5 μg/ml bromo-deoxyuridine (BrdU) was added to the medium for 1 hr. Then, cells were processed for staining with anti-BrdU antibodies. FACS analysis and fluorescence microscopy were performed as described above. For serum starvation experiments, NIH-3T3 cells expressing E2F-DB or control cells were propagated in medium containing 10% NCS. Hereafter, cells were starved in 0.1% serum for 72 hr and then refed with 10% serum for 24 hr in the presence of 7.5 μg/ml BrdU.

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