A Functional Genetic Screen Identifies *TFE3* as a Gene That Confers Resistance to the Anti-proliferative Effects of the Retinoblastoma Protein and Transforming Growth Factor-*β**

Sebastian M. B. Nijman[‡]**, E. Marielle Hijmans**[‡]**, Selma El Messaoudi**[§]**, Miranda M. W. van Dongen**[‡]**, Claude Sardet**[§]**, and René Bernards**[‡1]

From the [‡]*Division of Molecular Carcinogenesis and Centre for Biomedical Genetics, The Netherlands Cancer Institute, 121 Plesmanlaan, 1066 CX Amsterdam, The Netherlands and* [§]*Institut de Genetique Moleculaire, Unité Mixte de Recherche 5535/IFR24 CNRS, 1919 Route de Mende 34293, Montpellier Cedex 5, France*

The helix-loop-helix transcription factor TFE3 has been suggested to play a role in the control of cell growth by acting as a binding partner of transcriptional regulators such as E2F3, SMAD3, and LEF-1 (1–4). Furthermore, translocations/TFE3 fusions have been directly implicated in tumorigenesis (5–7). Surprisingly, however, a direct functional role for TFE3 in the regulation of proliferation has not been reported. By screening retroviral cDNA expression libraries to identify cDNAs that confer resistance to a pRB-induced proliferation arrest, we have found that TFE3 overrides a growth arrest in Rat1 cells induced by pRB and its upstream regulator p16[*INK4A*]. In addition, TFE3 expression blocks the anti-mitogenic effects of TGF-*β* in rodent and human cells. We provide data supporting a role for endogenous TFE3 in the direct regulation of *CYCLIN E* expression in an E2F3-dependent manner. These observations establish TFE3 as a functional regulator of proliferation and offer a potential mechanism for its involvement in cancer.

During cell cycle progression, the retinoblastoma pocket protein (pRB)[2] is phosphorylated by upstream cyclin-CDK complexes, ultimately leading to activation of genes critical for cell division. Extensive study of pRB has indicated that it serves as a nodal point for the integration of various signaling pathways, including TGF-β and p16[*INK4A*] signaling, in controlling cell growth (8, 9). Numerous mutations in the retinoblastoma gene itself have been reported, and somatic ablation in mouse embryo fibroblasts is sufficient for cell cycle entry (10). In addition, in many tumors, the *INK4A* locus is found inactivated, leading to uninhibited phosphorylation of pRB due to loss of the p16[*INK4A*] CDK inhibitor. Alternatively, cancer cells overexpress upstream inhibitors of the growth-suppressive effects of pRB, such as D-type cyclins. Indeed, detailed analysis of the pRB pathway indicates that functional inactivation of pRB is a requirement for the multistep process toward tumorigenesis. Furthermore, it is likely that other genetic aberrations resulting in defects in pRB function remain to be discovered.

The activity of the transcription factors E2F1, -2, and -3 is actively repressed by pRB, and this is thought to represent the most important role of pRB in S-phase entry, DNA replication, and apoptosis (11, 12). The central part that these E2Fs play in cell cycle regulation is emphasized by the finding that enforced expression of E2F1 alone is sufficient to mediate S-phase entry, whereas genetic ablation of all three pRB-interacting E2Fs leads to a proliferation arrest (13, 14). Nonetheless, no strong evidence supports a direct oncogenic role for E2Fs.

Repression of E2F1–3 target genes by pRB is achieved in a number of distinct ways (12). First, pRB binds directly to the transactivation domains of the E2Fs, blocking their activity. In addition, pRB promoter binding prevents the assembly of pre-initiation factors, thereby inhibiting the action of adjacent transcription factors. Finally, pRB recruits chromatin modification and remodeling factors, such as HDACs, BRG1, and hBRM, which promote heterochromatin formation and promote long term gene silencing (15, 16).

Analysis of single and compound *E2F1–3* knock-out mice has revealed redundant (but also unique) roles for the various E2Fs. For instance, E2F3 appears to play a critical role in maintaining proliferation by repressing p19[*ARF*] transcription, a potent activator of p53 (17).
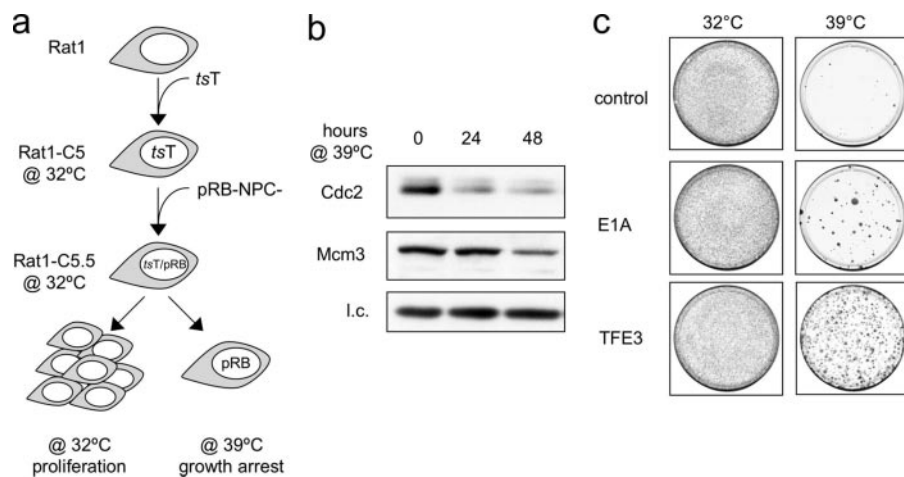
Because E2F1–3 regulate overlapping (but also distinct) genes, these functional differences are likely because of differences in target gene specificity. The mechanisms for E2F target specificity are not well understood but may involve recruitment of specific co-factors. For example, E2F1 (but not E2F2/3) binds to the ETS-related transcription factor GABP-γ1, whereas YY1 specifically interacts with E2F2/3 (18, 19).

The basic helix-loop-helix transcription factor TFE3 and its close family member USF1 were recently found to specifically interact with E2F3, thereby regulating target promoters containing adjacent E2F and TFE3 binding sites (3, 4). TFE3 was originally identified by its affinity for an E-box in the immunoglobulin heavy chain enhancer element and later as a co-factor in a number of signal transduction pathways (20). For instance, TFE3 and its family member, MitF, bind to the Wnt transcription factor LEF-1 and cooperate to induce dopachrome tautomerase transcription (2). In addition, TFE3 cooperates with

FIGURE 1. **TFE3 rescues Rat1 cells from pRB-NPC-induced cell cycle arrest.** *a*, schematic outline of the generation of the cell line to perform the functional genetic screen. Rat1 fibroblasts were transfected with temperature-sensitive SV40 T antigen and subsequently with pRB-NPC. The resulting Rat1-C5.5 clone grew normally at 32 °C but at 39 °C goes into a pRB-NPC-dependent growth arrest (see "Experimental Procedures"). *b*, Western blots of Rat1-C5.5 cells shifted to 39 °C for the indicated time (hours). Blots were probed as indicated. *l.c.*, loading control. *c*, colony formation of Rat1-C5.5 at 32 or 39 °C infected with the indicated retrovirus-encoded cDNAs (*e.g.* pLIB-GFP (*control*), pLIB-TFE3, and pLIB-12S-E1A). The cells were fixed and stained with Coomassie Blue stain after 2 weeks.

ETS-1, and TFE3 has been found to bind SMAD3 and SMAD4 and synergistically transactivate *PAI-1* transcription in response to TGF-β, a feature that may be shared with some of the TFE3 fusions found in cancer (1, 21–23). Importantly, however, these studies have been mostly limited to biochemical and molecular studies and consequently have failed to define a role for TFE3 in proliferation. Indeed, despite the observation that many of the binding partners of TFE3 play a critical role in proliferation, a cellular function of TFE3 has not been determined.

In the present study, we set out to find new mechanisms by which cells can become insensitive to the p16-cyclin D-pRB pathway. More specifically, we looked for genes that allow proliferation in the presence of hypophosphorylated pRB by enforcing a strong growth-inhibitory block using a dominant active pRB mutant. We now have established TFE3 as a gene that can uncouple cell cycle progression from pRB phosphorylation and TGF-β signaling and identify a functional role for TFE3 in regulating *CYCLIN E* expression.

EXPERIMENTAL PROCEDURES

Generation of Cells to Screen for cDNAs That Confer Resistance to pRB-induced Growth Arrest—Rat1 fibroblasts were transfected at 32 °C with pcDNA3.1-*ts*T, expressing *ts*T antigen (see Fig. 1*a*) (24). G418-resistant colonies were assayed for the expression of the large T antigen by Western blot analysis. Clone C5 was then transfected with the pRB-NPC expression vector or with the p16-GFP expression vector. The pRB-NPC protein harbors an amino-terminal GFP tag to allow continuous monitoring of the presence of the growth-inhibitory RB protein in individual cells. A stable colony of pRB-NPC-transfected cells having the lowest number of spontaneous background colonies at the non-permissive temperature (39 °C) was chosen for the functional genetic screens (named Rat1 C5.5). Similarly, a stable colony from the p16-GFP transfection was selected with low spontaneous background (termed Rat1 C5A).

Retroviral Rescue Screens—High titer retroviral library supernatants derived from human brain (Clontech) were used to infect $4–5 \times 10^6$ Rat1 C5.5 cells at 32 °C as described previously (25). Two days post-infection, the cells were split 1 in 12, allowed to attach at 32 °C, and then transferred to 39 °C. Several individual colonies were visible as soon as 8 days after the temperature shift. The colonies were picked and expanded. Recovery of integrated proviruses for the second round selection has been described previously (26). After the second round selection, retrovirally encoded cDNAs were recovered by PCR with retrovirus-specific primers and identified by sequence analysis (25).

Western Blotting—Cell extracts were prepared in E1A lysis buffer (ELB), and 40–60 μg of extract was resolved on 10–12% SDS-polyacrylamide gels. Proteins were transferred to polyvinylidene difluoride membranes. Primary antibodies used for Western blotting were M-20 for cyclin E, C15 for pRB, C18 for p107, C19 for cyclin A, PC-10 for PCNA, C19 for CDC2, C19 for MCM3, and C22 for CDK4, all from Santa Cruz Biotechnology. The p419 antibody was used for the detection of T antigen. Enhanced chemiluminescence (Amersham Biosciences) was used for the detection of proteins.

Chromatin Immunoprecipitation—Wild-type or E2F3 knock-out MEFs were immortalized by infection with a retrovirus encoding the SV40 large T antigen (27). Exponentially growing cells were used for chromatin immunoprecipitation and processed essentially as already described (28, 29). *Cyclin E* and *Gapdh* primers were as described; CE12 (−47, +46 + 1/start site) (28). Anti-mouse TFE3 was purchased from Pharmingen.

RESULTS

A Screening System for Bypass of a pRB-dependent Growth Arrest—To search for genes that, when overexpressed, confer resistance to the growth suppressing activity of pRB, we used a functional genetic approach. We first transfected the Rat1 fibroblast cell line with a temperature-sensitive mutant of SV40 T antigen (Fig. 1*a*). The *ts*T antigen binds and functionally inactivates pRB at the permissive temperature (32 °C) but is inactive and degraded at 39 °C (see Fig. 3*a*, *second panel from top*) (24). A clone stably expressing relatively high levels of *ts*T was selected (Rat1-C5) and subsequently transfected at the permissive temperature with a constitutively active form of pRB (pRB-NPC) (31). This pRB mutant, in which 14 of the 15 conserved CDK phosphorylation sites have been mutated to alanines, has strong anti-proliferative effects, even in the presence of high CDK activity, thus imposing a stringent cell cycle block when overexpressed. However, in the Rat1-C5 cells at the permissive

temperature (32 °C), pRB-NPC is inactivated due to its binding to the T antigen.

Clones stably expressing pRB-NPC were picked and selected for efficient growth arrest at 39 °C. One of the clones tested grew normally at 32 °C but efficiently ceased proliferation at 39 °C (Fig. 1*c*, clone *Rat1-C5.5*). The observed decrease in cell proliferation at 39 °C correlated with a marked decrease in protein abundance of the E2F target genes *Mcm3* and *Cdc2* (Fig. 1*b*). Retroviral introduction of adenovirus E1A, a viral protein capable of inactivating pRB, allowed some of the cells to continue proliferation at 39 °C (Fig. 1*c*). The relatively inefficient rescue by E1A of the pRB growth arrest may be due to the induction of apoptosis by E1A. We conclude that, at 39 °C, the Rat1-C5.5 cells enter a robust cell cycle arrest that is dependent on pRB.

Next, we infected the Rat1-C5.5 cells at 32 °C with a high complexity retroviral cDNA expression library derived from human brain or a control virus expressing GFP. Forty-eight hours after infection, the cells were shifted and selected for colony outgrowth at 39 °C. Several colonies of proliferating cells became visible after 2 weeks in the cDNA library-infected dishes. To determine whether the growing colonies on the library-infected plates were rescued because of a retrovirally encoded cDNA, the colonies were infected with wild-type Moloney virus to recover integrated proviruses (26). Two days later, supernatant containing mobilized retroviruses was harvested from the colonies and used to infect fresh Rat1-C5.5 cells. Mobilized virus derived from one of the primary clones yielded a significant number of colonies in second round infection, and these colonies were studied further (not shown). Retrovirally encoded cDNAs were recovered using PCR and identified by DNA sequence analysis (25). All colonies were found to contain a full-length cDNA for the basic helix-loop-helix transcription factor TFE3 (20). To provide conclusive evidence that TFE3 indeed rescued the pRB-induced proliferation arrest, we recloned the TFE3 cDNA in a retroviral vector and again infected Rat1-C5.5 cells. As shown, TFE3 efficiently rescued the arrest at 39 °C (Fig. 1*c*). We conclude that TFE3 overexpression renders Rat1 cells insensitive for the growth-inhibitory effects of pRB-NPC.

TFE3 Renders Cells Insensitive for p16^{INK4A} and TGF-β— Next, we investigated whether TFE3 could also rescue cells from a growth arrest dependent on endogenous pRB. First, we determined whether TFE3 could prevent growth arrest induced by p16^{INK4A}, as this requires the presence of functional pRB (32, 33). To test this, we used a second Rat1-C5 clone that, instead of pRB-NPC, expresses high levels of a p16^{INK4A}-GFP fusion protein. Similar to the Rat1-C5.5 cells, this clone grows normally at 32 °C but ceases proliferation at 39 °C (Fig. 2*a*). Retroviral transduction of these cells with TFE3 and subsequent selection for colony outgrowth at 39 °C yielded many proliferating colonies expressing p16-GFP, as judged by fluorescence microscopy (Fig. 2*a* and data not shown).

TFE3 has been implicated in TGF-β signaling by synergizing with SMAD3 and SMAD4 to activate target genes (1, 34). However, the functional consequences of this potential TGF-β signal-enhancing role of TFE3 have remained unaddressed. Furthermore, the anti-proliferative action of TGF-β depends on
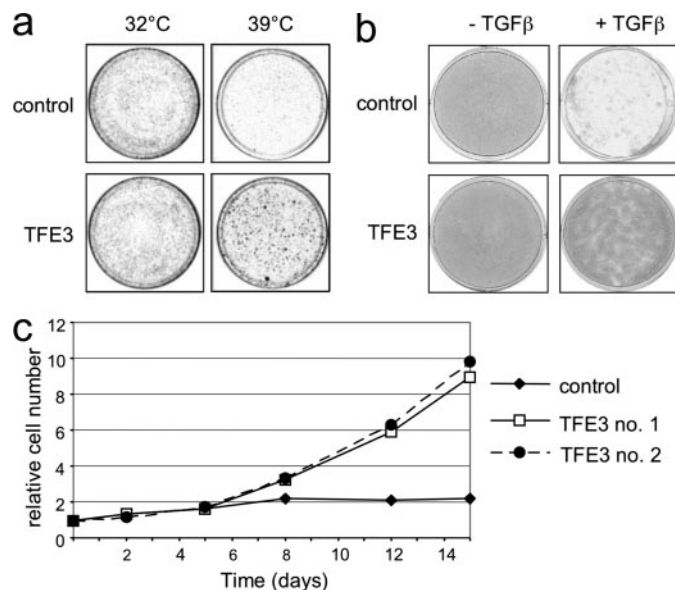
FIGURE 2. **TFE3 rescues cells from p16INK4A and TGFβ-induced proliferation arrest.** *a*, colony formation of Rat1-C5A cells (expressing p16INK4A-GFP) at 32 or 39 °C, infected with the indicated retrovirus-encoded cDNAs (*e.g.* pLIB-GFP (*control*), pLIB-TFE3). The cells were fixed and stained with Coomassie Blue stain after 2 weeks. *b*, MV-1 mink lung epithelial cells, expressing the murine ecotropic retroviral receptor, were infected with TFE3-encoding retrovirus (pLIB-TFE3) or control retrovirus (pLIB-GFP) and, 48 h after infection, cultured in the presence of TGF-β (12.5 pM). Plates were fixed and stained to visualize cells 14 days after exposure to TGF-β. *c*, growth curve of two independent batches of MV-1 cells infected with TFE3 virus (pLIB-TFE3) compared with GFP-infected cells (*control*) in the presence of 12.5 pM of TGF-β.

the presence of functional pRB (35–38). Therefore, our results also open the possibility that, in addition to enhancing the expression of certain TGF-β target genes, TFE3 might confer resistance to TGF-β signaling. To investigate this, we used MV-1 mink lung epithelial cells, as these cells are exquisitely sensitive to the anti-proliferative effects of TGF-β. MV-1 cells were infected with TFE3-expressing retrovirus or control retrovirus, exposed to TGF-β 2 days post-infection, and proliferation was followed in time. TFE3 conferred strong resistance to TGF-β-mediated growth inhibition, whereas proliferation of mock-infected MV-1 cells was efficiently inhibited by TGFβ (Fig. 2, *b* and *c*). Similar results were obtained in NMuMG cells and human HaCaT cells, two other TGF-β-sensitive cell lines (data not shown). Together, the results suggest that TFE3 can render cells insensitive to growth-inhibitory signals that depend on endogenous pRB.

TFE3 Regulates Cyclin E Expression in an E2F3-dependent Manner—To start to investigate the mechanism whereby TFE3 uncouples cell cycle progression from pRB phosphorylation, we asked whether TFE3 might interfere with the pRB-NPC-mediated repression of specific E2F target genes. We cultured Rat1-C5.5 cells and TFE3-infected derivatives at 32 °C and prepared cell lysates both before and 2 days after shifting to the nonpermissive temperature (39 °C). As expected, temperature shift of control cells caused a significant decrease in the protein abundance of the E2F target genes *Cyclin E*, *Cdc2*, and *Mcm3*, and to a lesser extent *Cyclin A* (Fig. 3*a*). Some other targets (*PCNA* and *p107*) appeared unaffected under these conditions. Strikingly, in the TFE3-infected cells, *Cyclin E* levels were significantly increased, even at 32 °C. At 39 °C, expression of
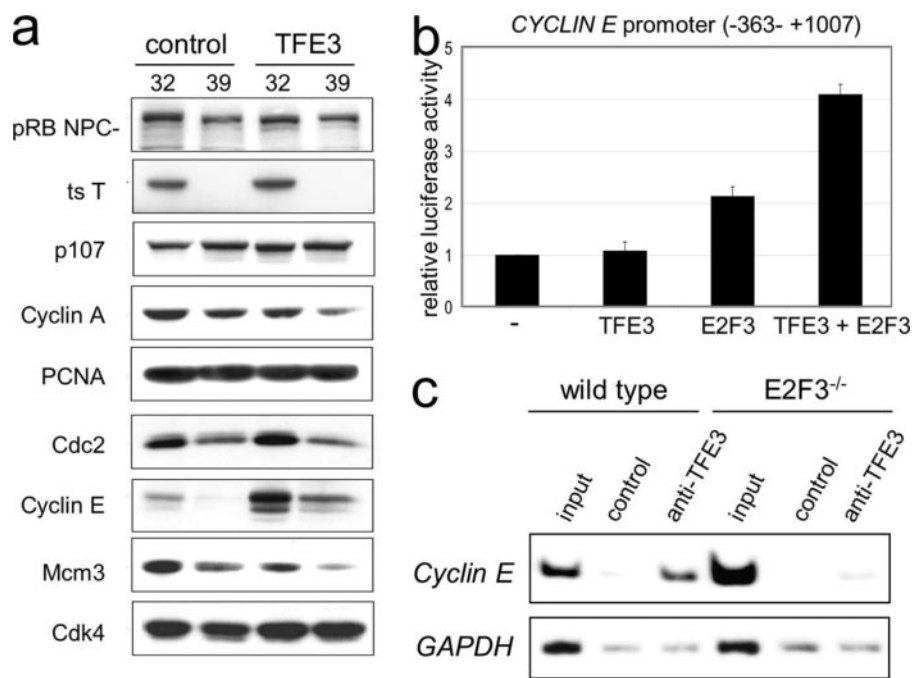
FIGURE 3. **TFE3 regulates Cyclin E expression in an E2F3-dependent manner.** *a*, Western blots of lysates from pLIB-TFE3-infected Rat1-C5.5 cells selected for growth at 39 °C or control cells (pLIB-GFP-infected) at both 32 and 39 °C (2 days after shift). The blots were probed with the indicated antibodies, and Cdk4 served as a loading control. *b*, *CYCLIN E*-promoter-reporter activity. Human U2-OS osteosarcoma cells were transfected with a human *CYCLIN E*-promoter-luciferase-reporter gene construct (−363 to +1007) in the presence and absence of transfected E2F3, hemagglutinin-TFE3, or both. Firefly luciferase was normalized to co-transfected SV40 *Renilla* luciferase activity. Shown are the averages of three independent transfections, including standard deviations. *c*, chromatin immunoprecipitation analysis of *Cyclin E* promoter. Formaldehyde-cross-linked chromatin from exponentially growing wild-type or E2F3−/− cells was immunoprecipitated with TFE3 antiserum or control antibody (anti-FLAG epitope). One-tenth of the input chromatin and the immunoprecipitated chromatin was analyzed by PCR for mouse *Cyclin E* promoter fragments or *GAPDH*.

dependent on the presence of E2F3, because significantly less TFE3 was bound to the *Cyclin E* promoter in E2F3−/− cells (Fig. 3*c*). We conclude that TFE3 enhances Cyclin E expression and that E2F3 is required for endogenous TFE3 binding to the *Cyclin E* promoter.

DISCUSSION

We have identified here TFE3 as a potent inhibitor of anti-proliferative signals emanating from the p16INK4A-pRB and TGF-β pathways. Although in Rat1 cells, TFE3 conferred resistance to both p16INK4A and pRB-NPC, we were unable to show similar activity in human BJ fibroblasts (data not shown). Nonetheless, TFE3 conferred resistance to TGF-β-induced growth arrest in human and rodent cells, indicating that at least some of the growth-stimulating properties of TFE3 are evolutionarily conserved.

Because a TGF-β growth arrest depends on the presence of pRB, it is tempting to speculate that TFE3 confers resistance to TGF-β signaling by interfering with the repression of pRB target genes (35–38). However, TFE3 was also shown to

Cyclin E was still repressed but remained higher than observed in cycling Rat1-C5.5 cells at 32 °C. This effect was specific for TFE3, because E1A-infected cells did not display elevated levels of *Cyclin E* (data not shown).

Recently, it was shown that TFE3 and E2F3 cooperatively bind to a subset of E2F target genes, thereby synergistically activating transcription (3, 4). Therefore, we wanted to test whether E2F3 and TFE3 can cooperate to activate the human *CYCLIN E* promoter. For this purpose, we measured the effects of these two transcription factors on a −363 to +1007 *CYCLIN E*-promoter-luciferase reporter construct. At the concentrations of expression vector used, TFE3 alone hardly activated the *CYCLIN E* reporter gene, whereas expression of E2F3 (E2F3A) activated the reporter ~2-fold (Fig. 3*b*). Importantly, co-transfection of TFE3 and E2F3 led to a 4-fold activation of the *CYCLIN E* reporter gene construct, suggesting that TFE3 and E2F3 can indeed cooperate to activate the *CYCLIN E* promoter.

Finally, we investigated whether TFE3 can bind to the *Cyclin E* promoter *in vivo* and whether this interaction is dependent on the presence of E2F3. We performed chromatin immunoprecipitation analysis with TFE3 antibodies, both in cells expressing and lacking E2F3 protein. PCR analysis of chromatin immunoprecipitates from exponentially growing immortalized wild-type MEFs revealed that endogenous TFE3 is bound to the *Cyclin E* promoter but not to a control promoter (GAPDH). Importantly, the binding of endogenous TFE3 appeared to be

synergize with TGF-β in activating certain SMAD target genes, notably the negative feedback protein SMAD7 (34). Interestingly, this suggests that TFE3 can be both an enhancer of TGF-β signaling and an inhibitor of TGF-β-induced cell cycle arrest.

It was recently found that TFE3 and its close family member, USF1, bind specifically to E2F3, resulting in synergistic activation of certain E2F target promoters (3, 4). A model was proposed in which adjacent E2F binding sites and E-boxes in a promoter allow the cooperative binding of TFE3/E2F3 dimers, resulting in transcriptional activation. In agreement with a similar role for TFE3 in regulating *CYCLIN E* transcription, we found that 1) TFE3-overexpressing Rat1 cells express high levels of *CYCLIN E*, 2) TFE3 and E2F3 synergistically activate a *CYCLIN E* reporter construct, and 3) endogenous TFE3 binds to the *Cyclin E* promoter in an E2F3-dependent manner. Possibly, enhanced recruitment of TFE3/E2F3 dimers renders the *CYCLIN E* promoter relatively resistant to pRB-mediated repression, explaining the resistance to pRB-NPC, p16INK4A, and TGF-β (Fig. 4).

Several studies have suggested that CYCLIN E expression can control S-phase entry independently of the pRB phosphorylation status (30, 39). However, overexpression of CYCLIN E did not prevent cell cycle arrest by the pRB-NPC mutant used here (31). In agreement with this, we were unable to override pRB-NPC growth arrest in Rat1-C5.5 cells through ectopic
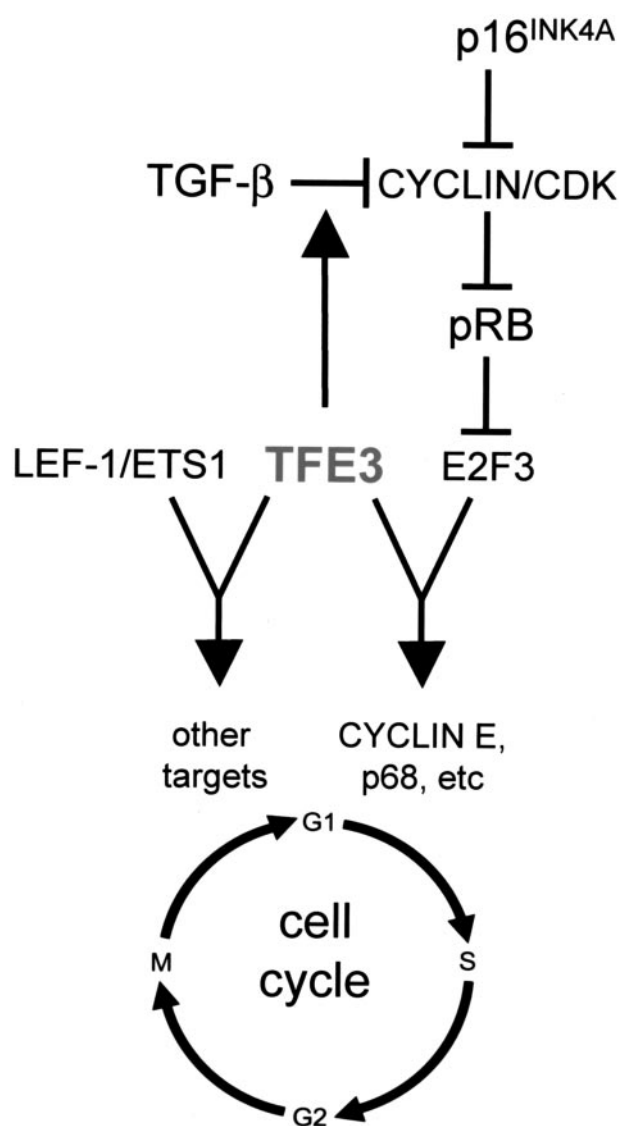
FIGURE 4. **Model for the uncoupling of pRB function from cell cycle progression by TFE3.** TFE3 acts as a co-factor for E2F3 to regulate genes, such as CYCLIN E (this study) and p68 DNA polymerase (3, 4). In addition, TFE3 cooperates with other transcription factors known to play a role in proliferation, such as LEF-1 and ETS1 (2, 21). As a consequence, overexpression of TFE3 desensitizes the cell for pRB-dependent growth-inhibitory signals. Because TFE3 is known to also cooperate with SMADs to enhance TGF-β signaling, TFE3 may act both upstream and downstream of pRB.

expression of CYCLIN E (data not shown). More likely, TFE3 acts on additional transcriptional targets to render cells insensitive to pRB-dependent inhibitory signals, and some of these targets may be regulated by TFE3/E2F3. Indeed, because TFE3 functions as a co-factor for a number of transcription factors, it is possible that E2F3-independent functions of TFE3 contribute to the bypass of a pRB-induced growth arrest. For instance, TFE3 (but not its close family member USF1) binds to the ETS-1 transcription factor, suggesting a USF1-independent role of TFE3 in the cell cycle (21). In agreement with a nonredundant function of TFE3 in regulating proliferation, $TFE3^{-/-}$ MEFs display a delay in S-phase entry after serum stimulation.[3]

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[3] P. H. Giangrande and J. R. Nevins, personal communication.

The exact mechanisms whereby TFE3 bypasses a pRB-dependent cell cycle arrest are undoubtedly complex and require further investigation (Fig. 4). Nonetheless, this study establishes TFE3 as a protein that can uncouple cell cycle progression from pRB phosphorylation and provides a potential mechanism for its role in tumorigenesis.

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