E2f3 is critical for normal cellular proliferation

Patrick O. Humbert,[1,2] Raluca Verona,[1] Jeffrey M. Trimarchi, Catherine Rogers, Savita Dandapani, and Jacqueline A. Lees[3]

Center for Cancer Research and Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139 USA

E2F is a family of transcription factors that regulate both cellular proliferation and differentiation. To establish the role of E2F3 in vivo, we generated an *E2f3* mutant mouse strain. E2F3-deficient mice arise at one-quarter of the expected frequency, demonstrating that E2F3 is important for normal development. To determine the molecular consequences of E2F3 deficiency, we analyzed the properties of embryonic fibroblasts derived from *E2f3* mutant mice. Mutation of *E2f3* dramatically impairs the mitogen-induced, transcriptional activation of numerous E2F-responsive genes. We have been able to identify a number of genes, including *B-myb*, *cyclin A*, *cdc2*, *cdc6*, and *DHFR*, whose expression is dependent on the presence of E2F3 but not E2F1. We further show that a critical threshold level of one or more of the E2F3-regulated genes determines the timing of the G_1/S transition, the rate of DNA synthesis, and thereby the rate of cellular proliferation. Finally, we show that E2F3 is not required for cellular immortalization but is rate limiting for the proliferation of the resulting tumor cell lines. We conclude that E2F3 is critical for the transcriptional activation of genes that control the rate of proliferation of both primary and tumor cells.

[*Key Words*: *E2f3*; cellular proliferation; transcription]

Received December 17, 1999; revised version accepted February 9, 2000.

The E2F transcription factors control the cell cycle-dependent expression of genes that are essential for cellular proliferation (for review, see Dyson 1998; Helin 1998). E2F activity is regulated by the retinoblastoma protein (pRB), a tumor suppressor that is functionally inactivated in most, if not all, human tumors (for review, see Weinberg 1992; Dyson 1998). pRB binds to E2F during the G_1 phase of the cell cycle. This association inhibits the transcriptional activity of E2F and the resulting complex actively represses E2F-responsive genes by recruiting histone deacetylases to the promoter (for review, see Dyson 1998; Brehm and Kouzarides 1999). At the G_1/S transition, pRB is phosphorylated by the cyclin-dependent kinases and the released E2F now activates transcription. In this manner, E2F participates in both the repression and activation of E2F responsive genes.

pRB belongs to a family of proteins, called the pocket proteins, which includes two additional members, p107 and p130 (for review, see Dyson 1998). Like pRB, p107 and p130 can bind to E2F complexes, inhibit E2F-mediated transactivation and enforce the active transcriptional repression of E2F-responsive genes (Starostik et al. 1996; Zwicker et al. 1996; Iavarone and Massagué 1999). However, the biological properties of p107 and p130 clearly differ from those of pRB (for review, see Mulligan and Jacks 1998). Mutations within *p107* or *p130* are rarely detected in human tumors and they do not increase the tumor predisposition of mutant mouse strains. Moreover, the homozygous mutation of *Rb* causes developmental defects that are distinct from those resulting from the combined loss of *p107* and *p130*. It is widely believed that the differential developmental and tumor suppressive roles of pRB, p107, and p130 arise from differences in the way in which they regulate E2F.

To date, eight genes encoding components of E2F have been cloned (for review, see Dyson 1998). Their protein products can be subdivided into two groups, the E2Fs (1–6) and the DPs (1,2). Overexpression studies indicate that E2F and DP must heterodimerize to generate functional E2F activity. Although the DP subunit is critical for activity, the functional specificity of the E2F · DP complex is determined by the E2F subunit (for review, see Dyson 1998). The E2F family can be divided into three distinct subgroups, on the basis of both sequence homology and functional properties.

The first subclass contains E2F1, E2F2, and E2F3. When complexed with DP, these E2Fs each have high transcriptional activity and are sufficient to induce quiescent cells to enter S phase (DeGregori et al. 1997; Lukas et al. 1997; Verona et al. 1997). DP · E2F1, DP · E2F2, and DP · E2F3 complexes are specifically

[1]These authors contributed equally to this work.
[2]Present address: Department of Research, Peter McCallum Cancer Institute, Melbourne 3002, Australia.
[3]Corresponding author.
E-MAIL jalees@mit.edu; FAX (617) 253-9863.

regulated by pRB, and not p107 or p130, and the timing of their release from pRB correlates with the timing of activation of E2F-responsive genes (Lees et al. 1993; Moberg et al. 1996). E2F4 and E2F5 represent the second E2F subclass. The DP · E2F4 and DP · E2F5 species are very poor transcriptional activators and they are unable to induce quiescent cells to enter S phase (Lukas et al. 1996; Muller et al. 1997; Verona et al. 1997). Instead, E2F4 and E2F5 are thought to be important in the repression of E2F-responsive genes through their ability to recruit pRB, p107, and p130 and the associated histone deacetylases (for review, see Dyson 1998; Helin 1998). E2F6 represents the final E2F subclass (Cartwright et al. 1998; Gaubatz et al. 1998; Trimarchi et al. 1998). E2F6 lacks the sequences required for transcriptional activation or pRB family binding and it can inhibit the transcription of E2F-responsive genes. From this point on, we will use E2F1, E2F2, E2F3, etc., to refer to individual E2F proteins, free E2F to refer to the E2F · DP complexes, and E2F activity to refer to the total pool of the endogenous free and pocket protein-containing E2F · DP complexes.

The individual E2F proteins are thought to have different target gene specificities that will account for the different biological properties of pRB, p107, and p130. Potential specificity has been investigated by three different approaches (for review, see Helin 1998). First, a combination of classic promoter mapping and in vivo footprinting have been used to compare the relative contribution of repression (by pocket protein · E2F complexes) and activation (by free E2F complexes) in regulating the activity of individual promoters. These studies concluded that many E2F-responsive genes, including *B-myb*, *cdc2*, *cyclin E*, *cyclin A*, and *E2F-1*, are regulated primarily by repressive E2F complexes (Dalton 1992; Lam and Watson 1993; Neuman et al. 1994; Tommasi and Pfeifer 1995; Huet et al. 1996; Zwicker et al. 1996; Le Cam et al. 1999). In contrast, the cell cycle regulation of other E2F-responsive genes (e.g., *DHFR*) seems to be largely dependent on the presence of activating E2F species (Means et al. 1992; Wade et al. 1992).

In the second approach, a variety of overexpression systems have been used to compare the ability of individual E2F family members to activate the transcription of either endogenous or coexpressed E2F-responsive genes (DeGregori et al. 1997; Vigo et al. 1999). These studies have revealed significant differences in the specificity of target gene activation. However, the identity of the E2F-specific targets varies considerably from one study to the next, suggesting that it is highly system dependent.

The third approach has utilized mutant mouse embryonic fibroblasts (MEFs) to determine how loss of pRB, p107, and/or p130 affects the regulation of known E2F-responsive genes (Herrera et al. 1996; Hurford et al. 1997). These studies demonstrated that p107 and p130 have overlapping functions and together regulate a subset of E2F-responsive genes that are distinct from the pRB-regulated targets. This specificity directly supports the notion that pRB, p107, and p130 regulate E2F in distinct ways. This is presumed dependent on the ability of the pocket proteins to bind to different E2F family members.

To date, mutant mouse models have been generated for two of the E2F family members. *E2f5*$^{-/-}$ mice die from hydrocephalus caused by excessive secretion of cerebral spinal fluid (Lindeman et al. 1998). This phenotype appears due to a defect in differentiation rather than proliferation. *E2f1*$^{-/-}$ mice are viable and fertile but they develop tissue abnormalities, including testicular atrophy, exocrine gland dysplasia, and a defect in thymus apoptosis (Field et al. 1996; Yamasaki et al. 1996). In addition, these mice also develop a broad spectrum of late onset tumors, suggesting that E2F1 can act as a tumor suppressor in vivo (Yamasaki et al. 1996). Analyses of *Rb*$^{-/-}$;*E2f1*$^{-/-}$ mice suggest that E2F1 accounts for much of the inappropriate p53-dependent apoptosis and approximately one-half of the ectopic S-phase entry in *Rb*$^{-/-}$ embryos (Tsai et al. 1998). Consistent with these observations, the absence of E2F1 significantly reduces formation of tumors in *Rb*$^{+/-}$ mice (Yamasaki et al. 1998). These mouse models confirm that individual members of the E2F subclasses have very different biological properties. However, it is unclear how these differences relate to the target specificity of the different E2Fs in vivo.

In this study we have used *E2f3* mutant mouse strains and the resulting *E2f3* mutant cell lines to investigate the role of E2F3 in normal cell cycle regulation. We show that E2F3 plays a crucial role in mediating the normal cell cycle-dependent activation of most known E2F-responsive genes and the reduced expression of one or more of these genes in E2F3-deficient cells causes specific defects in the initiation and progression of DNA synthesis. As a result, E2F3 acts in a dose-dependent manner to control the rate of proliferation of both primary and immortalized cells.

Results

E2f3 *is critical for full neonatal viability*

To establish the role of E2F3 in cell cycle control, we used standard gene-targeting techniques to generate *E2f3*-deficient mice. We functionally inactivated the *E2f3* gene in ES cells by introducing an in-frame termination codon immediately prior to the nuclear localization signal (NLS), and replacing the genomic sequences encoding the NLS, cyclin A binding, DNA binding, and the leucine zipper domains (amino acids 134–294) with a neomycin resistance marker (Fig. 1A). After electroporation and G418 selection, correctly targeted *E2f3*$^{+/-}$ ES cell lines were used to generate chimeric animals. Two independent cell lines (F3-1-1 and F3-2-13) were used to transmit the mutation into the germ line. The following data was obtained from the analysis of mice and cells derived from ES clone F3-1-1, although both lines showed identical phenotypes.

To assess the role of E2F3 in normal development, we intercrossed the *E2f3*$^{+/-}$ animals. In this mixed (C57BL/6 × 129/sv) strain background, we were able to detect vi-

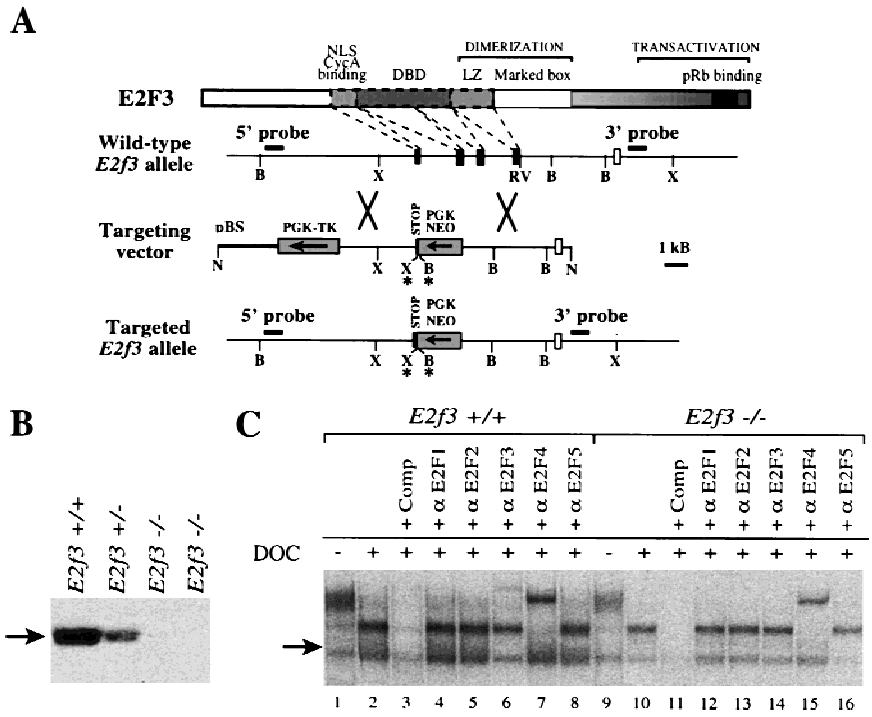

Figure 1. E2F3 does not affect the expression of other E2F species. (*A*) The *E2f3* gene was inactivated in ES cells using the targeting construct shown. This strategy introduces an in-frame termination codon upstream of the NLS of E2F3 and replaces the exons encoding the NLS, DNA-binding domain (DBD), and leucine zipper (LZ) domain (shown in black) with a neomycin resistance gene. (*B*) Western blot analysis was performed on whole cell extracts generated from MEFs derived from the progeny of $E2f3^{+/-}$ crosses. E2F3 is indicated (arrow). (*C*) To examine how E2F3 loss affects other E2F species, gel shift analysis was performed in the absence or presence of sodium deoxycholate (DOC), which releases the free E2F · DP complexes from the associated pRB family members. The free DP · E2F3 complex is indicated (arrow).

able $E2f3^{-/-}$ animals at weaning, however, these were not present at the expected frequency (Table 1; $\chi^2 = 47.8$, $P = 0.005$). Instead, viable $E2f3^{-/-}$ animals arose at approximately one-quarter of the predicted number. Preliminary backcrosses suggest that the partial penetrance of this phenotype is due to the presence of one or more strain-specific modifiers (J.E. Cloud, R.L. Landsberg, and J.A. Lees, unpubl.). We are still investigating the phenotypes of the $E2f3^{-/-}$ animals and the nature of the modifier effect, but these studies indicate that E2F3 is critical for full viability.

Loss of E2F3 does not affect other E2F species

We have used the *E2f3* mutant animals to investigate the molecular consequences of E2F3 deficiency and the role of E2F3 in cell cycle control. MEFs were isolated from the progeny of $E2f3^{+/-}$ crosses at embryonic day 13.5. Initially, we examined how the mutation of E2F3 affects the endogenous E2F species. Western blot analysis showed that the homozygous mutation of *E2f3* completely abolishes expression of the E2F3 protein, confirming that this mutation is a null (Fig. 1B). We then compared the relative levels and composition of the other E2F complexes using gel shift analysis (EMSA) of whole cell extracts. In wild-type cells, the majority of E2F activity (~70%–80%) was generated by pocket protein-bound rather than free E2F species (Fig. 1C, lane 1). To facilitate the detection of the individual E2F family members, we treated the whole cell extracts with sodium deoxycholate (DOC) to dissociate the pocket proteins from the E2F · DP complexes. In wild-type MEFs, addition of anti-E2F-4 antibodies shifts >70% of the released E2F activity. E2F1, E2F2, and E2F5 were present at low to undetectable levels (Fig. 1C; data not shown). In contrast, the anti-E2F-3 antibodies recognized a minor species (representing ~10% of total E2F activity) in the wild-type MEF extracts (Fig. 1C, cf. lanes 2 and 6). Supershift analysis of non-DOC treated extracts showed that this was largely derived from the pRb · E2F complex (data not shown).

Consistent with the complete absence of E2F3 protein, we observed no E2F3 species in the $E2f3^{-/-}$ MEFs in either the absence (data not shown) or the presence of DOC. Apart from this change, we did not detect any significant alteration in the relative levels of the other E2F complexes. Thus, at least at a qualitative level, the homozygous mutation of *E2f3* completely disrupts the relevant E2F3 complexes without any apparent compensation by the other E2F family members.

Table 1. E2f3 *is essential for full viability*

	$E2f3^{+/+}$	$E2f3^{+/-}$	$E2f3^{-/-}$
Number of pups[a]	80	174	19
Exepected ratio	1	2	1
Observed ratio	1	2.2	0.24

[a]Progeny arising from an intercross of *E2f3* heterozygous mutant mice in the mixed (C57BL/6 × 129/sv) genetic background.

$E2f3^{-/-}$ *cells have a proliferation defect*

We next wished to determine whether the loss of E2F3 affected the rate of cellular proliferation. For these experiments, passage 4 MEFs, derived from wild-type,

E2f3$^{+/-}$, and *E2f3*$^{-/-}$ littermates, were cultured under either high or low density conditions. At high density, the *E2f3*$^{-/-}$ MEFs grew considerably less well than their wild-type counterparts (Fig. 2A). The severity of the proliferation defect varied from one preparation of *E2f3*$^{-/-}$ MEFs to the next, but the average doubling time was approximately twice that of wild-type littermate controls. The *E2f3*$^{+/-}$ MEFs also exhibited a range in their growth rates; some grew at rates indistinguishable from wild type (Fig. 2A), whereas others grew slightly slower (data not shown). This phenotypic variation only occurred between mutant MEF lines isolated from different embryos and never the same embryo (data not shown), arguing that it results from genetic variation in the individual mixed (C57BL/6 × 129/sv) background embryos. As described below, we have exploited this variation to dissect the molecular basis of the proliferation defect.

The proliferation defect of the *E2f3*$^{-/-}$ cells was more apparent under low-density culture conditions (Fig. 2B). Whereas some of the *E2f3*$^{-/-}$ MEF cell lines divided at a greatly reduced rate, a significant proportion did not proliferate at all. There was little or no difference in the level of apoptosis observed in wild-type and *E2f3*$^{-/-}$ MEFs and there was also no evidence to suggest that the *E2f3*$^{-/-}$ cells reach the end of their proliferative capacity sooner than the wild-type controls (data not shown). This suggests that the proliferation defect of the *E2f3* mutant cells is due to a defect in cell division rather than the induction of apoptosis or premature senescence. As in the high-density experiments, some of the *E2f3*$^{+/-}$ MEFs grew as well as wild-type cells, whereas others have a phenotype that is intermediate between that of the wild-type and *E2f3*$^{-/-}$ cells. Thus, E2F3 plays a key role in controlling the rate of proliferation of MEFs in a dose-dependent manner.

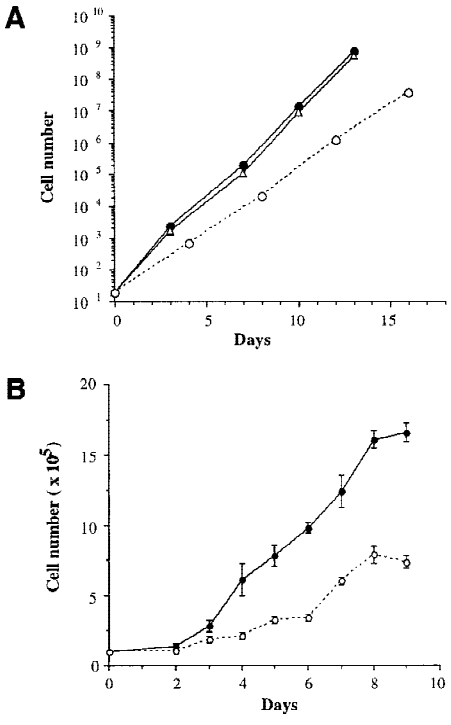

Figure 2. *E2f3*$^{-/-}$ cells have a proliferation defect. Passage 4 MEFs derived from wild-type and *E2f3* mutant littermates were cultured under high (*A*) or low (*B*) density conditions as described in the methods. (● *E2f3*$^{+/+}$; △ *E2f3*$^{+/-}$; ○ *E2f3*$^{-/-}$).

E2f3$^{-/-}$ *cells have a cell cycle defect*

To understand the nature of the proliferation defect, we compared the cell cycle progression of the wild-type, *E2f3*$^{+/-}$, and *E2f3*$^{-/-}$ MEFs. The cells were serum starved for 72 hr and then stimulated to re-enter the cell cycle by the readdition of serum. Cells were harvested at regular intervals and labeled for 1 hr with [^{3}H]thymidine to monitor DNA synthesis. Figure 3A shows the analysis of MEFs derived from two different sets of littermate embryos (H and E). The wild-type MEFs (H1 and E1) began incorporating [^{3}H]thymidine 8–12 hr after serum stimulation and showed maximal levels of incorporation at 16–20 hr. The incorporation of [^{3}H]thymidine by the *E2f3*$^{+/-}$ cell lines, H2 and E2, was similar. In contrast, the *E2f3*$^{-/-}$ cell lines showed significantly reduced levels and slower kinetics of [^{3}H]thymidine incorporation. Consistent with our asynchronous studies, some of the *E2f3*$^{-/-}$ cell lines (e.g., H8 and E4) were significantly more impaired than others (e.g., H6 and E5). In each case, there was a direct correlation between the rates of proliferation and [^{3}H]thymidine incorporation (data not shown). We therefore conclude that the impaired proliferation of the *E2f3*$^{-/-}$ MEFs results from a defect in cell cycle progression.

There is strong evidence to suggest that E2F1 also plays a key role in the control of cellular proliferation in vivo (Field et al. 1996; Yamasaki et al. 1996; Pan et al. 1998; Tsai et al. 1998). We therefore examined the effects of E2F1 loss on cell cycle regulation. Strikingly, there was no detectable difference in either the level or timing of [^{3}H]thymidine incorporation between wild-type, *E2f1*$^{+/-}$, or *E2f1*$^{-/-}$ MEFs in serum starvation/restimulation experiments (Fig. 3B). This was true of multiple MEF preparations (data not shown). Consistent with this observation, we did not observe any differences in the rate of proliferation of asynchronous wild-type or *E2f1* mutant populations (data not shown). Thus, E2F1 is fully dispensable for the normal cell cycle regulation of mouse embryo fibroblasts, whereas E2F3 is rate limiting for correct cell cycle progression in response to mitogenic factors.

The reduced thymidine incorporation observed in the *E2f3*$^{-/-}$ MEFs could result from a defect in passage through the G_1/S transition and/or a reduction in the rate of DNA synthesis. To distinguish between these two models, we followed the cell cycle re-entry of either wild-type or *E2f3*$^{-/-}$ cells at the single cell level by scoring for BrdU incorporation by immunofluorescence (Fig. 3C). In the wild-type cells, BrdU incorporation was first detected 10 hr after serum re-addition. The intensity of BrdU staining continued to increase during subsequent

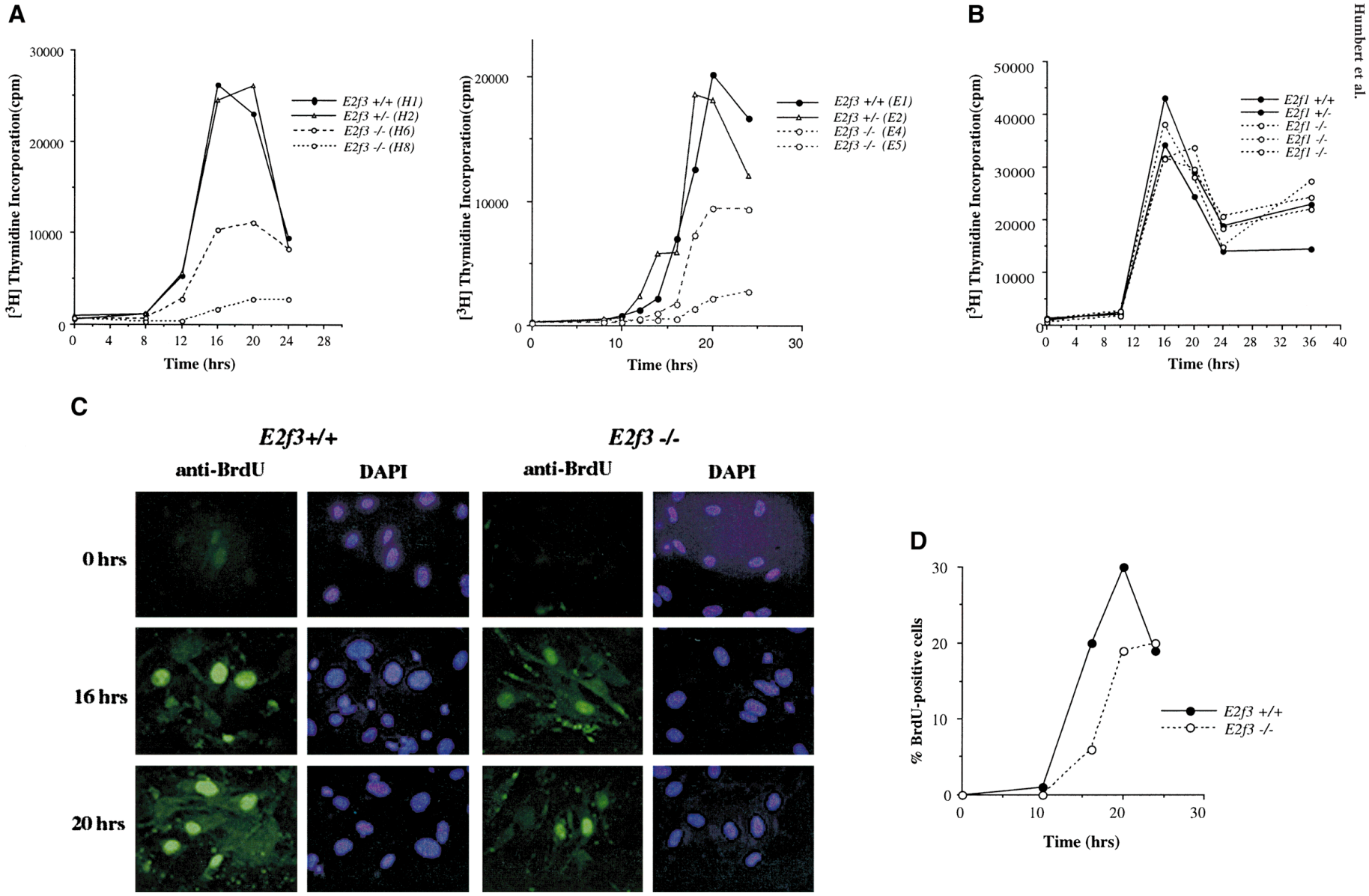

Figure 3. *E2f3*$^{-/-}$ cells have defects in the initiation and progression of DNA synthesis. (*A*) Wild-type and *E2f3* mutant MEFs from two different MEF preparations (H and E) were synchronized by serum starvation and their cell cycle re-entry properties assayed using [^{3}H]thymidine incorporation. (*B*) MEFs derived from a wild-type, an *E2f1*$^{+/-}$, and three *E2f1*$^{-/-}$ littermate embryos were analyzed as in *A*. (*C*,*D*) Wild-type and *E2f3*$^{-/-}$ cells were plated on coverslips and synchronized by serum starvation. At each time point after serum addition, DNA synthesis was monitored by assaying for BrdU incorporation.

time points, peaking at 20 hr. We detected two clear differences in the *E2f3*$^{-/-}$ cells. First, the intensity of the BrdU signal was significantly reduced, indicating a substantial reduction in the rate of BrdU incorporation and therefore of DNA synthesis. In addition, the timing of appearance of BrdU-positive cells seemed to be delayed in the mutants relative to the wild-type cells. To quantitate this difference, we counted the number of BrdU-positive cells at each time point without scoring for the intensity of the signal (Fig. 3D). At 16 hr, only 5% of the *E2f3*$^{-/-}$ cells had incorporated BrdU, compared with 20% of the wild-type cells. Even 20 hr after serum addition, the proportion of BrdU-positive cells was still lower in the *E2f3*$^{-/-}$ (19%) than in the wild-type (30%) population. These data indicate that E2F3 loss delays the initiation of DNA synthesis and dramatically reduces the rate at which this process occurs. Together, these two defects increase the time necessary to complete S phase in a manner that is consistent with the increased doubling time of the *E2f3*$^{-/-}$ cells.

The majority of E2F-responsive genes are down-regulated in the E2f3$^{-/-}$ *MEFs*

The timing of the cell cycle defect is consistent with the known timing of action of E2F-responsive genes. We therefore wished to determine whether the loss of E2F3 altered the expression of E2F-responsive genes and whether or not there was any correlation between the severity of the transcriptional changes and the degree of the proliferative defect. To address this issue, we compared the expression of E2F-responsive genes in a wild-type control (H1), an *E2f3*$^{+/-}$ cell line (H2) whose proliferative properties were indistinguishable from wild type, and two *E2f3*$^{-/-}$ cell lines, one of which had a moderate cell cycle defect (H6) and one of which was dramatically impaired (H8). Parallel cell cycle fractions were used to assess [^{3}H]thymidine incorporation (Fig. 3A) or to generate RNA for Northern blot analysis. The blots were normalized according to the levels of *ARPP PO*, a gene whose expression does not vary in quiescent or cycling cells (Hurford et al. 1997), and then probed for the known E2F-responsive gene transcripts, *cyclin E*, *cyclin A2*, *cdc2*, *B-myb*, *cdc6*, *PCNA*, *RRM2, TS*, *DHFR*, and *E2f1* (Fig. 4A; data not shown). For representative genes, we quantitated the expression level relative to that of the internal *ARPP PO* control (Fig. 4B).

In the wild-type cells, we detected a significant cell cycle-dependent induction of most of the genes, including *cyclin E*, *cyclin A2*, *cdc2*, *B-myb*, *cdc6*, *PCNA*, and *RRM2* (Fig. 4A). (The one exception, *E2f1*, will be described in the following section). These cell cycle-regulated genes could be divided into three groups, on the basis of whether peak expression occurred at earlier (16 hr, *cyclin E*, *B-myb*, *cdc6*, and *PCNA*), intermediate (16–20 hr, *RRM2*) or later (20–24 hr, *cyclin A2* and *cdc2*) timepoints.

The loss of E2F3 has a profound effect on the expression of all of these cell cycle-regulated, E2F-responsive genes. The severity of the transcriptional defect was most pronounced in the cell line (H8) with the severest cell cycle and proliferation defect (Figs. 3A and 4A). In the H8 cells, we saw a dramatic reduction in the maximal transcript levels and peak expression was significantly delayed compared with the wild-type control (Fig. 4A,B). Thus, the cell cycle-dependent induction of these genes was almost completely ablated. Similar results were observed with other E2F-responsive genes including *TS* and *TK* (data not shown). The transcriptional defects were less severe in the second *E2f3*$^{-/-}$ cell line, H6. In this cell line, there was little or no change in the timing of peak expression, but the maximal induction of these target genes was greatly reduced. We were also able to detect some variation in the degree of down-regulation of individual target genes (Fig. 4B). In some cases (e.g., *cyclin A2*, *cdc2*, *B-myb*, and *RRM2*), the mRNA levels were intermediate between those observed in the wild-type and the H8, *E2f3*$^{-/-}$ cell line. In others (e.g., *cyclin E* and *cdc6*), the degree of transcriptional impairment approached that observed in the H8 cells. Significantly, there did not appear to be any correlation between the degree of the transcriptional defect and whether or not the gene was normally expressed at earlier, intermediate, or later timepoints. Taken together, these data indicate that the loss of E2F3 significantly impairs the cell cycle-dependent induction of most E2F-responsive genes and the severity of this defect correlates with the severity of the cell cycle and proliferation defect.

We also detected a significant reduction in the expression of most E2F-responsive genes in the *E2f3*$^{+/-}$ cell line, H2 (Fig. 4A,B). In most cases (e.g., *cyclin A2*, *cdc2*, *B-myb*, and *RRM2*), the level of expression seemed to be intermediate between that observed in the wild-type and the *E2f3*$^{-/-}$ cell lines. In contrast, expression of *PCNA* was only slightly lower than that observed in the wild-type cells, whereas the expression of *cyclin E* and *cdc6* much more closely resembled that seen in the *E2f3*$^{-/-}$ cell lines. These data indicate that E2F3 contributes to the correct transcriptional activation of most E2F-responsive genes in a dose-dependent manner. Importantly, the cell cycle regulation and proliferative properties of the H2, *E2f3*$^{+/-}$ cell line are indistinguishable from those of the wild-type control, H1 (Fig. 3A; data not shown). Thus, changes in the levels of E2F3 can impair the transcriptional activation of most E2F-responsive genes without causing any detectable cell cycle defect. Similar results were observed in several other *E2f3*$^{+/-}$ cell lines (data not shown). This strongly suggests that the defects in cell cycle progression are a consequence, and not a cause, of the failure to induce the appropriate activation of one, or more, of these E2F-responsive genes.

E2F1 and E2F3 play distinct roles in the transcriptional regulation of MEFs

Our transcriptional analysis detected only one known E2F-responsive gene, *E2f1*, whose expression was unaltered in the *E2f3* mutant MEFs (Fig. 4A). However, contrary to the literature, the expression of this gene did not alter significantly across the cell cycle. We therefore ex-

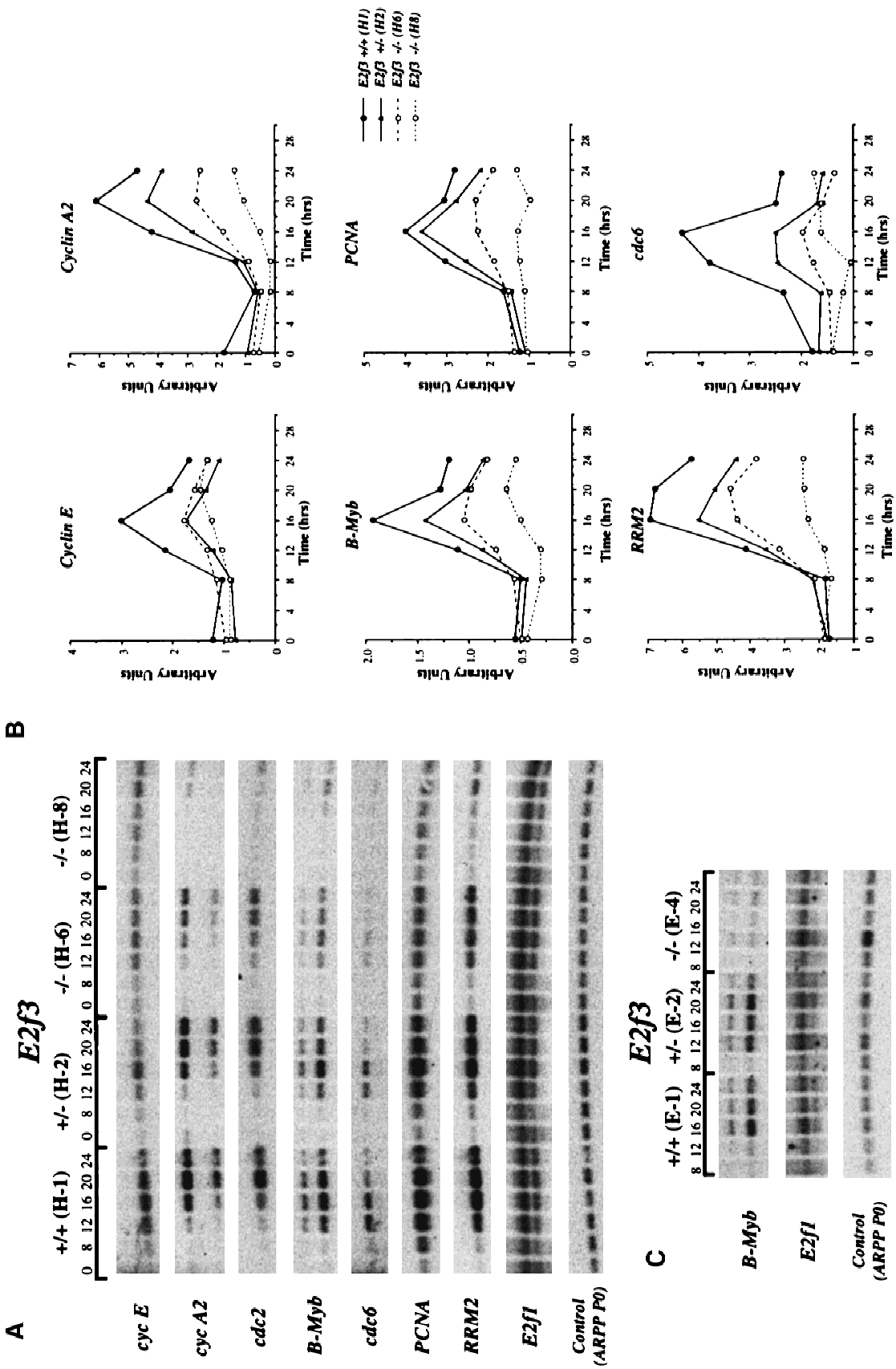

Figure 4. E2F3 is required for the mitogen-induced, transcriptional activation of most known E2F-responsive genes. Wild-type, *E2f3* mutant MEFs were synchronized by serum starvation. At each time point, RNA was isolated and equal amounts of RNA were subjected to Northern blot analysis to determine the pattern of expression of E2F-responsive genes (*A*,*C*). (*B*) The expression level of selected genes shown in *A* was quantitated by PhosphorImager analysis and normalized to the *ARPP PO* control.

amined the expression pattern of *E2f1* in wild-type (E1), *E2f3*$^{+/-}$ (E2), and *E2f3*$^{-/-}$ (E4) MEFs from a second set of littermate embryos (see Fig. 3A). We were able to detect a significant cell cycle dependence in the expression of *E2f1* in these wild-type MEFs (Fig. 4C). Consistent with our previous studies, the expression of *B-myb* was partially impaired in the *E2f3*$^{+/-}$ (E2) cell line and was dramatically down-regulated in the *E2f3*$^{-/-}$ (E4) cell line (Fig. 4C). We also observed a dramatic down-regulation of *cyclin E*, *cyclin A2*, *cdc2*, *cdc6*, *PCNA*, and *RRM2* (data not shown). In contrast, we did not detect any substantive difference in the expression pattern of *E2f1* between wild-type, *E2f3*$^{+/-}$ or *E2f3*$^{-/-}$ MEFs. We therefore conclude that E2F3 is not required to maintain the normal cell cycle regulation of *E2f1* in mouse embryonic fibroblasts. This strongly suggests that the deregulation of *cyclin E*, *cyclin A2*, *cdc2*, *cdc6*, *B-myb*, *PCNA*, *RRM2*, *TS*, and *TK* arising from the loss of E2F3 is not an indirect consequence of changes in the level of the *E2f1* mRNA.

Considerable emphasis has been placed on understanding the specificity of target gene expression by the individual E2F family members. Therefore, we wished to establish how the loss of E2F1, the other major pRB-specific E2F, would affect the expression patterns of E2F-responsive genes. To address this issue, we conducted Northern blot analysis of cell cycle fractions derived from serum starved/restimulated wild-type, *E2f1*$^{+/-}$ and *E2f1*$^{-/-}$ MEFs (Fig. 5). The loss of E2F1 had no detectable effect on the cell cycle-dependent expression of *cyclin A2*, *cdc2*, *cdc6*, *B-myb*, *PCNA*, *TS*, or *RRM2*. However, the expression of *cyclin E* was consistently down-regulated in the *E2f1* mutant cells. This suggests that E2F1 and E2F3 both contribute to the transcriptional regulation of the *cyclin E* gene. However, there appears to be significant specificity in the regulation of other targets. E2F3 acts, in a dose-dependent manner, to determine both the timing and maximal activation of the majority of E2F-responsive genes, including *cyclin A2*, *cdc2*, *B-myb*, *cdc6*, *PCNA*, *TS*, *TK*, *DHFR*, and *RRM2*. In contrast, E2F1 is fully dispensable for the correct regulation of these targets. Significantly, E2F3 is not required for the correct transcriptional regulation of the *E2f1* gene and its cell cycle-dependent expression can be uncoupled from that of other E2F-responsive genes and from the G_1/S transition.

Ectopic expression of E2F3 or E2F1 rescues the proliferation defect of E2f3$^{-/-}$ *cells*

Given these findings, we wished to establish whether we could rescue the proliferation defect of the *E2f3* mutant cells by ectopic expression of E2F3 or E2F1. To address this issue, we used recombinant replication-deficient retroviruses to reintroduce the human E2F3 and E2F1 genes into wild-type or *E2f3*$^{-/-}$ cells and then compared the growth rate of large pools of drug-resistant clones (Fig. 6). The control virus had no effect on the growth rate of the *E2f3*$^{-/-}$ cells. In contrast, the expression of either E2F3 or E2F1 was sufficient to rescue the proliferation defect of the *E2f3*$^{-/-}$ cells. This confirms that the reduction in the rate of proliferation of the *E2f3*$^{-/-}$ cells is caused by the absence of E2F3 and this defect is fully reversible. At least when overexpressed, E2F1 can substitute for the loss of E2F3.

E2F3 is rate limiting for the proliferation of transformed cells

The tumor-suppressive properties of pRB are thought to be largely dependent on its ability to inhibit the transcriptional activity of the E2F transcription factors. Our data indicate that the loss of E2F3 significantly impairs the proliferation of primary cell lines. Given these findings, we wished to establish whether the absence of E2F3 would affect either the generation or proliferation of tumor cells.

Initially, we tested whether E2F3 is essential for generation of immortalized cell lines. An activated *ras* allele (*H-rasV12*) was introduced into wild-type and *E2f3*$^{-/-}$ MEFs with either E1A or a dominant-negative p53 allele (*p53R175H*) by use of recombinant replication-deficient retroviruses. We were able to select pure populations of *E2f3*$^{-/-}$ cells that expressed either E1A plus H-rasV12 or p53R175H plus H-rasV12, albeit at reduced efficiency compared with the wild-type controls (data not shown). The selected wild-type and *E2f3*$^{-/-}$ populations exhibited characteristic morphologies of transformed cells (data not shown), indicating that E2F3 is not essential for the immortalization of primary mouse cells.

We next asked whether the absence of E2F3 would affect the rate of proliferation of these transformed cells. The growth rate of the wild-type and *E2f3*$^{-/-}$ transformants was compared under low density conditions, as described previously for the parental primary MEFs (Fig. 2B). The expression of these oncogenes did improve the ability of the *E2f3*$^{-/-}$ cells to grow at low density (cf. Figs. 2B and 7). However, the E2F3-deficient cells still grew at a considerably reduced rate compared with the wild-type controls (Fig. 7).

Anchorage-independent growth of transformed cells correlates with tumorigenic potential in vivo. To examine the requirements for E2F3 in tumor cell proliferation, we assessed the ability of the wild-type and E2F3-deficient transformants to grow in soft agar. After 5 days in the semisolid medium, the wild-type cells formed discrete foci that increased in size over time (Fig. 7; data not shown). Significantly, the *E2f3*$^{-/-}$ cells formed far fewer foci that each contained significantly fewer cells than their wild-type controls (Fig. 6; data not shown). This was true regardless of whether the cells were transformed with E1A and H-rasV12 or p53R175H and H-rasV12. On the basis of these findings, we conclude that E2F3 deficiency does not prevent the transformation of primary murine cells and the subsequent ability of these cells to grow in soft agar. However, the absence of E2F3 compromises the ability of these cells to proliferate. Taken together, our data indicate that E2F3 is rate limiting for the proliferation of both primary and tumor cells.

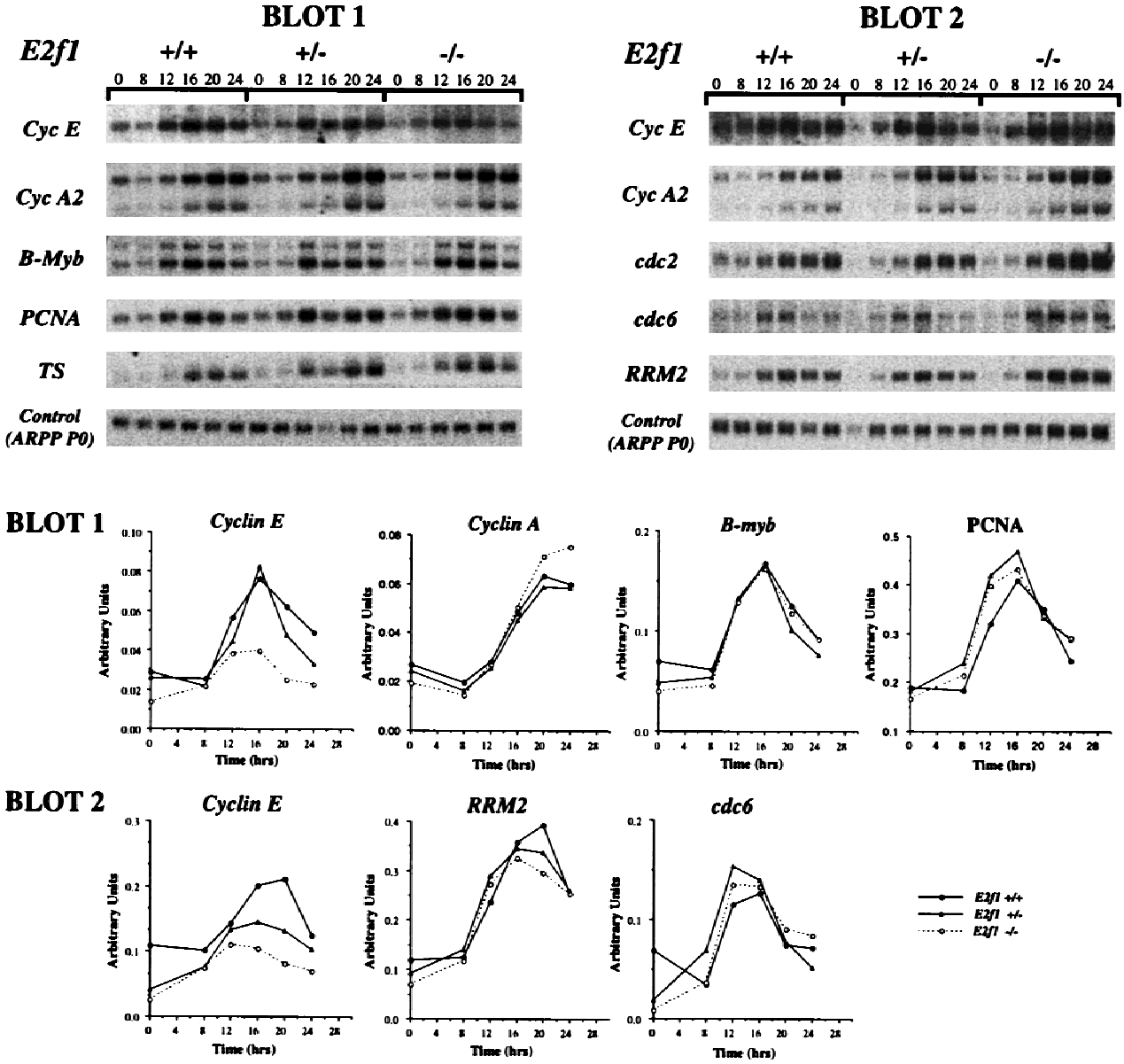

Figure 5. The loss of E2F1 does not affect the expression of most E2F-responsive genes. Wild-type, heterozygous, or homozygous *E2f1* mutant MEFs were synchronized by serum starvation/readdition and Northern blot analysis was performed at various cell cycle stages. The expression levels of selected genes was quantitated by PhosphorImager analysis and normalized to the *ARPP PO* control.

Discussion

The role of E2F3 in the control of cellular proliferation

We have used cell lines derived from *E2f3* mutant mice to investigate the role of E2F3 in cell cycle regulation. These studies show that the loss of E2F3 significantly reduces the rate of cellular proliferation of both primary and transformed cell lines. This is caused by an increase in doubling time that results from defects in the initiation and rate of progression of DNA synthesis. This observation is highly consistent with the prior report that anti-E2F3 antibodies can inhibit rat embryonic fibroblasts from entering S phase (Leone et al. 1998). In addition, our E2F3 mutant cells have a major defect in the regulation of E2F-responsive gene transcription. In the $E2f3^{-/-}$ cells, we see a dramatic impairment of the transcriptional activation of many E2F-responsive genes. The degree of this transcriptional defect correlates closely with the severity of the proliferation defect, indicating that these two phenotypes are closely linked. In contrast, in the majority of the $E2f3^{+/-}$ cell lines, we see a reduction in the maximal activation of the same panel of E2F-responsive genes without any detectable cell cycle or proliferation defects. This observation yields two important conclusions. First, E2F3 contributes to the transcriptional regulation of many E2F-responsive genes in a dose-dependent manner. Second, cells can tolerate limited reduction in the expression of these targets

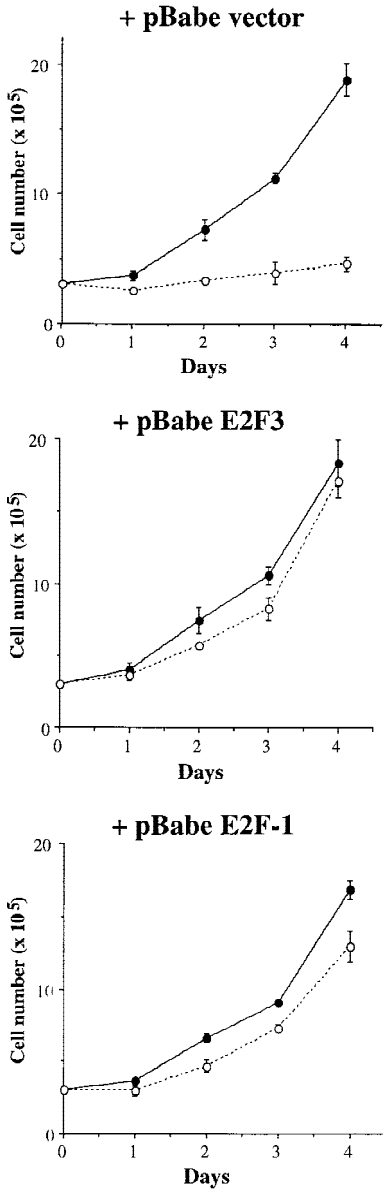

Figure 6. The proliferation defect of the *E2f3*$^{-/-}$ cells can be rescued by the ectopic expression of E2F3 or E2F1. Wild-type or *E2f3* mutant MEFs were infected with either control or E2F3- or E2F1-expressing retroviruses. (●) *E2f3*$^{+/+}$; (○) *E2f3*$^{-/-}$. After 4 days in selection, the cells were plated at equal densities and their growth rates monitored.

without any deleterious consequences but, once expression drops below a critical threshold, there is a direct correlation between the level of expression and the rate of proliferation. By extension of this logic, we conclude that E2F3 plays a key role in regulating the expression of one, or more, target genes that determine the rate of initiation and progression of DNA synthesis.

E2F3 plays a key role in mediating the transcriptional activation of most E2F-responsive genes

Our studies yield considerable insight into the general mechanisms of regulation of individual E2F-responsive genes. On the basis of a combination of in vivo footprinting and promoter mapping experiments, other studies have concluded that many E2F-responsive genes, including *B-myb*, *cdc2*, *cyclin A*, and *E2f1*, will be primarily regulated by the binding of repressive, pocket protein · E2F complexes during the G_0/G_1 stage of the cell cycle (Dalton 1992; Lam and Watson 1993; Neuman et al. 1994; Tommasi and Pfeifer 1995; Huet et al. 1996; Zwicker et al. 1996; Le Cam et al. 1999). In this work, we have shown that the mutation of E2F3 has no effect on the regulation of E2F-responsive genes during G_0/G_1, but it inhibits the normal, cell cycle-dependent induction of these targets in a dose-dependent manner. This indicates that activating E2F complexes must play a key role in mediating the induction of these genes at the G_1/S transition. It is unclear why previous approaches have failed to appreciate the importance of activating E2F complexes. Because in vivo footprinting requires site occupancy within a high proportion of the cell population, it is biased toward the detection of stable complexes. It is therefore possible that the transcriptionally active E2F-3 complexes bind to the promoter in a narrow window of time that cannot be detected by existing cell synchronization methods. It is less easy to explain why the promoter mapping studies detect repressive and not activating E2F complexes, but this could be attributed to differences in chromatin assembly on transiently transfected reporters versus the endogenous promoter. Clearly, our data do not refute the importance of repression in the regulation of E2F-responsive genes but they provide strong genetic evidence that activation by E2F3 plays a major role in mediating the cell cycle-dependent induction of these targets.

Identification of E2F3 downstream target genes

It is widely believed that the different biological properties of pRB family members are mediated through their ability to regulate different E2F family members with distinct biological properties. This is supported by the finding that different subsets of E2F-responsive genes are deregulated in Rb$^{-/-}$ or *p107*$^{-/-}$; *p130*$^{-/-}$ mutant MEFs (Herrera et al. 1996; Hurford et al. 1997). This has raised considerable interest in establishing the target specificity of the individual E2F complexes. Our studies provide strong genetic evidence that the normal cell cycle-dependent activation of many known E2F-responsive genes is dependent on E2F3. In particular, we have identified a number of genes, including *cyclin E*, *cyclin A2*, *cdc2*, *cdc6*, *B-myb*, and *RRM2*, whose transcription is downregulated in *E2f3*$^{+/-}$ cell lines that have no detectable cell cycle defect. This strongly suggests that the altered expression of these genes occurs independently of any changes in the timing of the G_1/S transition.

We can subdivide these target genes into two distinct subgroups. The expression of genes in the first subgroup, which includes *cyclin A2*, *cdc2*, *B-myb*, and *RRM2*, is directly proportional to the *E2f3* gene dosage. In contrast, genes in the second subgroup, *cyclin E* and *cdc6*, appear

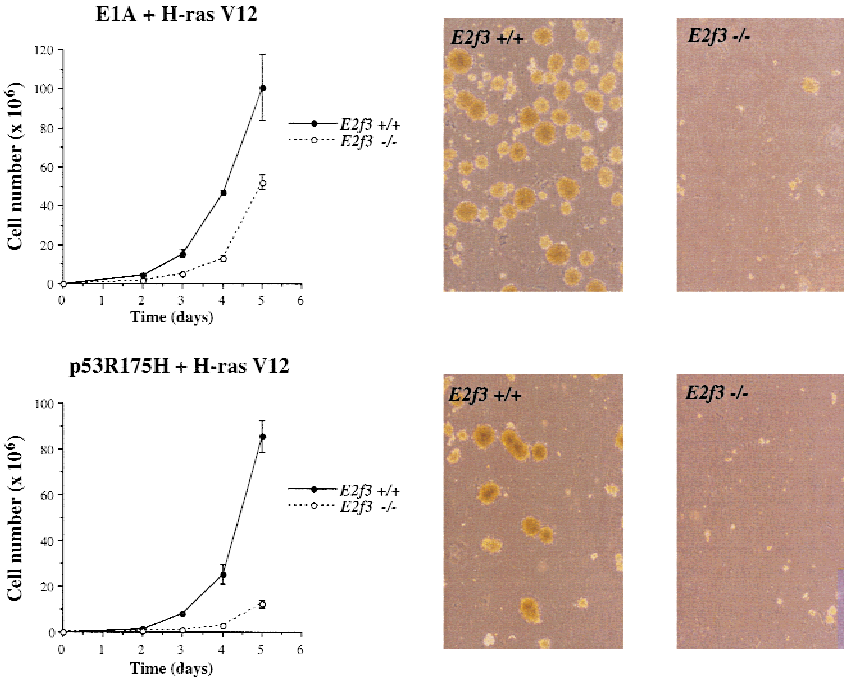

Figure 7. E2F3 deficiency impairs the proliferation of transformed cell lines. Wild-type or *E2f3*$^{-/-}$ MEFs were infected with retroviruses expressing an activated *ras* allele (*H-rasV12*) and either *E1A* or a dominant-negative *p53* allele, *p53 R175H*. After selection, cells were plated at equal densities and their proliferation rates were monitored by daily counting. To assess anchorage-independent growth, equal numbers of transformed cells were plated in 0.3% low melting point agarose. Representative wild-type and *E2f3* mutant fields are shown.

particularly sensitive to any change in the levels of E2F3. Mutation of a single *E2f3* allele impairs the cell cycle-dependent expression of these genes almost as efficiently as the complete loss of E2F3. Thus, expression of *cyclin E* and *cdc6* seems to require a critical threshold level of free E2F activity that is close to the maximal levels present in these cells and higher than the levels required to activate expression of *cyclin A2*, *cdc2*, *B-myb*, and *RRM2*. Significantly, the peak expression of *cyclin E* and *cdc6* occurs earlier in the cell cycle than that of many of the other E2F-responsive targets. This suggests that accumulation of critical threshold levels of E2F3 cannot fully account for the differential timing of expression of these genes.

Our data strongly suggest that the loss of expression of one, or more, of the E2F3-regulated genes impairs the ability of the cells to proliferate. It is clearly important that we identify the rate-limiting gene(s). Because the absence of E2F3 significantly reduces the rate of DNA synthesis, it is tempting to speculate that at least one of the critical targets may be directly involved in the DNA replication process. Several of the E2F3-dependent genes are known to be required for the initiation of DNA replication (*cdc6*; Stillman 1996) or the maintenance of the nucleotide pools (*RRM2* and *TS*), and there are many other candidates, including *DNA polymerase α*, *orc1*, and *mcm 2-7* (Stillman 1996), whose expression we have yet to analyze.

We have also identified E2F-responsive genes that do not appear to be directly regulated by E2F3. Because we detect little difference in the expression of PCNA between the wild-type and the *E2f3*$^{+/-}$ cell lines, it is unclear whether the reduced PCNA expression in *E2f3*$^{-/-}$ cells is a direct consequence of E2F3 loss or an indirect consequence of changes in cell cycle regulation. More striking is the regulation of *E2f1*. Our studies indicate that the expression pattern of this gene is not altered in any of the *E2f3* mutant cell lines despite the presence of a well-documented E2F site in the *E2f1* promoter (Hsiao et al. 1994; Johnson et al. 1994; Neuman et al. 1994). This suggests that E2F3 is not required to maintain the correct cell cycle regulation of *E2f1*. Consequently, at least in the absence of E2F3, the expression of this gene must be mediated by other E2F family members or in an E2F-independent manner. Most importantly, our data indicate that *E2f1* expression continues to be activated normally despite a substantial delay in the timing of the initiation of S phase. This result indicates that *E2f1* expression can be uncoupled from the G_1/S transition and from the induction of most other E2F-responsive genes. These data strongly suggest that E2F1 and E2F3 function independently of one another.

Target specificity of individual E2F family members

We have shown that E2F3 plays a key role in mediating the cell cycle-dependent induction of most E2F-responsive genes in mouse embryonic fibroblasts. Clearly, the remaining E2F family members are unable to substitute for the loss of E2F3. This is true even in the presence of E1A, which mediates the release of all E2F · DP complexes through the sequestration of pRB family members. These observations raise clear questions about the role of other E2F family members. Do they have distinct transcriptional targets or is there some degree of functional redundancy? To address this question, we directly compared the consequences of E2F3 and E2F1 deficiency. We selected E2F1 for a number of reasons. First, E2F1 and E2F3 are the major pRB-specific E2Fs. Second, the analyses of mutant mouse strains show that E2F1 makes

a significant contribution to the inappropriate proliferation arising from the functional inactivation of pRb (Pan et al. 1998; Tsai et al. 1998; Yamasaki et al. 1998). Third, the availability of *E2f1* mutant mice (Yamasaki et al. 1996) allowed us to compare the roles of E2F1 and E2F3 within a common cell type. Finally, our data indicate that the *E2f1* gene is regulated independently of E2F3. Our analyses of *E2f1* mutant MEFs indicate that the loss of E2F1 has no detectable effect on either the cell cycle regulation or the proliferative capacity of primary murine fibroblasts. Similarly, E2F1 is not required for the correct cell cycle expression of many E2F-responsive genes, including most of those affected by E2F3 loss (*cyclin A2*, *cdc2*, *cdc6*, *B-myb*, and *RRM2*). However, the loss of E2F1 causes a down-regulation in the levels of *cyclin E* that are comparable with that observed in the E2F3 mutant cells. Consistent with this finding, Wang et al. (1998) have also reported that the cell cycle-dependent expression of cyclin E is impaired in E2F1-deficient cells. Because there is no proliferation defect in the *E2f1* mutant MEFs, the down-regulation of the *cyclin E* mRNA levels cannot fully account for the cell cycle defects arising in the *E2f3*$^{-/-}$ MEFs.

The differential regulation of E2F-responsive genes in *E2f1* and *E2f3* mutant cells supports two alternative models of E2F function. First, E2F1 and E2F3 could have very different biological properties that result from differences in target gene regulation. In this model, E2F3 acts as the work-horse to mediate the cell cycle-dependent activation of the key components of the cell cycle control and DNA replication machinery in response to mitogenic signals. In contrast, E2F1 acts primarily in response to inappropriate signals, such as DNA damage or uncontrolled proliferation, as a surveillance mechanism. This model is supported by the finding that E2F3 is critical for the normal proliferation of cell lines and the normal development and viability of E2F3-deficient mice. In contrast, E2F1 seems largely dispensable for normal cellular proliferation and development, but there is strong evidence to support its role in apoptosis. First, E2F1, but not the other E2Fs, induces apoptosis when overexpressed in quiescent cells (DeGregori et al. 1997). Second, *E2f1*$^{-/-}$ mice exhibit a defect in thymocyte apoptosis and are tumor prone (Field et al. 1996; Yamasaki et al. 1996). Finally, loss of E2F1 causes a dramatic reduction in the level of apoptosis arising from the functional inactivation of pRB (Pan et al. 1998; Tsai et al. 1998).

The second model proposes that E2F1 and E2F3 regulate common target genes, but their differential biological properties result from differences in their relative expression levels. Because E2F3 is expressed at higher levels than E2F1 in MEFs, the loss of this protein brings the levels of free transcriptionally active E2F below the critical threshold that is required for the correct regulation of most E2F-responsive genes. In contrast, the reduction in free E2F activity arising from the loss of E2F1 is only sufficient to impair the expression of a single gene, *cyclin E*. This model is entirely consistent with our conclusion that *cyclin E* is extremely sensitive to the levels of activating E2F. Moreover, at least when overexpressed, E2F1 can rescue the proliferation defect in the E2F3$^{-/-}$ MEFs in a similar manner to E2F3.

Understanding the role of E2F3 in tumorigenesis

The retinoblastoma protein is functionally inactivated in most, if not all, human tumors (Weinberg et al. 1992). E2F3 is one of three E2F family members that are specifically regulated by this tumor suppressor (Lees et al. 1993). We have now shown that E2F-3 regulates the expression of genes that determine the rate of proliferation of both primary and tumor cell lines. These observations suggest that E2F3 will make a major contribution to the inappropriate proliferation resulting from the loss of pRB. Given this hypothesis, it will be important to establish whether the loss of E2F3 alters the viability of *Rb* homozygous mutant embryos or the rate of tumor formation in *Rb* heterozygous mutant mice. This will allow us to establish how E2F3 contributes to tumorigenesis in vivo and will yield critical insight into the relative roles of E2F1 and E2F3.

Materials and methods

Construction of E2f3 *targeting vector*

Overlapping mouse *E2f3* genomic clones containing the *E2f3* cyclin A-binding domain, DNA-binding domain, and the dimerization domain exons were isolated from a 129/Sv mouse library by standard techniques. A 0.9-kb *Hin*dIII fragment containing the cyclin A-binding domain was subcloned into pBKS. An in-frame STOP codon was inserted after the third codon of the *E2f3* cyclin A-binding domain by inserting an engineered *Xba*I–*Pvu*II linker. A 750-bp *Kpn*I–*Xba*I fragment was then transferred into pPNT (Tybulewicz et al. 1991) and a 3.1-kb *Kpn*I genomic fragment containing additional 5′ sequences was added. The targeting vector, *E2f3–neo*, was completed by subcloning a 3.4-kb *Eco*RI–*Eco*RV 3′ genomic fragment into the *Not*I and *Xho*I sites using linkers.

Generation of targeted ES cells and E2f3*-deficient mice*

D3 ES cells were electroporated with 50 μg of *Not*I linearized *E2f3–neo* and selected for resistance to G418 (300 μg/ml) and Gancyclovir (0.5 μg/ml). DNA from double-resistant ES cell clones was digested with *Bgl*II and analyzed by Southern blotting using a 720-bp *Rsa*I DNA fragment as the 5′ probe. Two independent electroporations yielded 29 clones with a novel 6.5-kb band corresponding to a correctly targeted 5′ end (wild type, 9.5 kb). DNA from these clones was digested with *Xba*I and probed with a 650-bp *Eco*RI–*Kpn*I 3′ fragment (mutant, 9 kb vs. wild type, 11 kb) and then a 450-bp *Pst*I–*Hin*dIII *neo* fragment. A total of 22/29 ES clones contained a single integration of the *E2f3* targeting vector that had undergone correct homologous recombination on each side of the *neo* cassette. These ES cell clones were injected into 3.5-day C57BL/6 blastocysts and the resulting chimerics were mated to C57BL/6 females. One clone from each electroporation (ES clones F3-1-1 and F3-2-13) transmitted the mutation through the germ line. The targeted *E2f3* allele was detected in agouti pups by Southern blotting of tail DNA as described above. PCR of mouse ear punch DNA was then used for subsequent genotyping using the common primer 5′-GTATCTGGGAAACACAAGGAGGTG, the wild-

type *E2f3*-specific primer 5′-GGTACTGATGCCACTCTC-GCC, and the targeting vector specific primer, 5′-GCT-CATTCCTCCCACTCATGATC.

MEF preparation

E2f3$^{+/-}$ females were crossed with *E2f3*$^{+/-}$ males and embryos were dissected 13.5 days after detection of vaginal plugs. The head and internal organs were removed and the embryos were minced and incubated in trypsin for 30 min at 37°C. The cells were resuspended in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FCS, 50 U/ml penicillin, 50 μg/ml streptomycin, and 2 mM L-glutamine. Fetal livers and/or yolk sacs were used for PCR genotyping.

High- and low-density growth experiments

For the high-density experiments, the MEFs were plated at 2×10^5/6-cm dish. Cells were counted as they reached confluence and replated at 2×10^5 cells/6-cm dish. For low density experiments, MEFs were plated at a density of 1×10^5 cells/10-cm dish and their growth rate was monitored by daily counting for 10 days. For the E2F3 and E2F1 rescue experiments, transduced wild-type or *E2f3* mutant cells were plated at 2×10^5/6-cm dish, and their growth rate was monitored for 4 days. Transformed cells were plated at 5×10^4/10-cm dish and counted daily for 6 days.

Serum starvation and release experiments

Passage 4 MEFs were plated in triplicate at 2×10^5/3.5-cm dish. After 48 hr, the cells were washed twice with PBS and then incubated in DMEM containing 0.1% FCS for 72 hr. The cells were then fed with DMEM containing 10% FCS. For each time point, the cells were incubated with 5 μCi [^{3}H]thymidine for 1 hr at 37°C, washed with PBS and harvested. [^{3}H]thymidine incorporation was quantitated as described (Moberg et al. 1996). For BrdU incorporation experiments, cells were plated onto coverslips. At each timepoint, the cells were incubated in medium containing 3 mg/ml BrdU and 0.3 mg/ml fluorodeoxyuridine for 2 hr at 37°C (Sigma). The cells were fixed for 15 min in 2% paraformaldehyde and permeabilized with PBS/0.25% Triton X-100. After denaturing the DNA for 10 min in 1.5 N HCl, the cells were incubated with mouse anti-BrdU antibodies (Beckton-Dickinson, 1:50) for 30 min and then with FITC-anti-mouse antibodies (Capel, 1:1000) for 30 min. The coverslips were washed four times, incubated with DAPI (0.1 mg/ml) for 5 min, washed, and mounted on glass slides with Vectashield (Vector).

Northern blot analysis

Passage 4 MEFs were plated onto 15-cm dishes at 3×10^6 cells/dish and then serum starved as described above. At each time point, the cells were pelleted and RNA purified using the Ultraspec RNA isolation system (Biotex Laboratories, Inc). The RNA was denatured and separated on gels containing 1% agarose, 6% formaldehyde, and 1xMOPS buffer (pH 7.0). The RNA was transferred to Hybond-N nylon membranes (Amersham), hybridized in ExpressHyb solution (Clontech) and washed twice in 2× SSC, 0.1% SDS for 30 min at 65°C. The cDNA probes were labeled using the Prime-It II-kit (Stratagene) with 100 μCi of [α-^{32}P]dCTP. The amount of RNA used for each timepoint was determined by probing a test Northern with the *ARPP PO* control. Subsequent Northerns were then probed with full-length cDNAs for *B-myb*, *cdc2*, *cdc6*, *cyclin A2*, *cyclin E*, *RRM2*, *PCNA*, or *cyclin D1* or a partial *E2F-1* cDNA fragment (nucleotides 524–1388) and then reprobed for *ARPP PO*. The expression level of each gene was quantitated by PhosphorImager analysis and normalized to the levels of *ARPP PO*.

Western blot and gel retardation assays

Western blotting and gel retardation assays were performed as described previously (Moberg et al. 1996) using 100 or 30 μg of whole cell lysates, respectively. Western blotting was conducted using anti-E2F3 (Santa Cruz sc-878, 1:1000) and an HRP-coupled anti-rabbit antibody (Amersham, 1:5000). Gel retardation assays were performed in the absence or presence of sodium DOC as described (Moberg et al. 1996) using antibodies against E2F1 (KH95 and KH20), E2F2 (LLF2-1), E2F3 (Santa Cruz sc-878x), E2F4 (LLF4-1), or E2F5 (Santa Cruz, sc-1083x).

Retrovirus-mediated gene transfer and soft agar assay

pBabe–E2F3 and pBabe–E2F1 were generated by subcloning human E2F3 and E2F1 into the pBabe vector. The retrovirus-mediated transfer was conducted as described by Serrano et al. (1997), except that the infected cells were grown for 2 days prior to selection with 2 μg/ml puromycin (pBABE–E2F3, pBABE–E2F1, and pBABE–H-rasV12) or 75 μg/ml hygromycin (pWZL–E1A and pWZL–p53R175H). For soft agar assays, 6-cm dishes were coated with 0.5% low melting point agarose (GIBCO BRL) in DME containing 10% FCS. Cells (5×10^4) were resuspended in 0.3% LMP agarose plus DME with 10% FCS and grown on the coated dishes for 1–2 weeks.

Acknowledgments

We thank Jessica Wen for plasmid construction and Tyler Jacks and his laboratory for advice in generating the *E2f3* mutant mice and for providing the *E2f1* mutant mice. Also, Scott Lowe for providing pBABE–H-ras(V12), pWZL–E1A, and pWZL–p53(R175H). We thank Steve Bell, Nick Dyson, and Terry Orr-Weaver for helpful discussions and critical comments on the manuscript. P.H. was supported by an Anna Fuller fellowship. This work was supported by a grant from the American Cancer Society (RPG MGO-97549) to J.A.L.

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact.

References

Brehm, A. and T. Kouzarides. 1999. The retinoblastoma protein meets chromatin. *Trends Biochem. Sci.* **24:** 142–145.

Cartwright, P., H. Muller, C. Wagener, K. Holm, and K. Helin. 1998. E2F-6: A novel member of the E2F family is an inhibitor of E2F-dependent transcription. *Oncogene* **17:** 611–623.

Dalton, S. 1992. Cell cycle regulation of the human *cdc2* gene. *EMBO J.* **11:** 1797–1804.

DeGregori, J., G. Leone, A. Miron, L. Jakoi, and J.R. Nevins. 1997. Distinct roles for E2F proteins in cell growth control and apoptosis. *Proc. Natl. Acad. Sci.* **94:** 7245–7250.

Dyson, N. 1998. The regulation of E2F by pRB-family proteins. *Genes & Dev.* **12:** 2245–2262.

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

Gaubatz, S., J. Wood, and D.M. Livingston. 1998. Unusual proliferation arrest and transcriptional control properties of a newly discovered E2F family member, E2F-6. *Proc. Natl. Acad. Sci.* **95:** 9190–9195.

Helin, K. 1998. Regulation of cell proliferation by the E2F transcription factors. *Curr. Opin. Genet. Dev.* **8:** 28–35.

Herrera, R.E., V.P. Sah, B.O. Williams, T.P. Makela, R.A. Weinberg, and T. Jacks. 1996. Altered cell cycle kinetics, gene expression, and G1 restriction point regulation in *Rb*-deficient fibroblasts. *Mol. Cell. Biol.* **16:** 2401–2407.

Hsiao, K.-M., S.L. McMahon, and P.J. Farnham. 1994. Multiple DNA elements are required for the growth regulation of the mouse *E2F1* promoter. *Genes & Dev.* **8:** 1526–1537.

Huet, X., J. Rech, A. Vie, and J.M. Blanchard. 1996. Cyclin A is under negative transcriptional control during the cell cycle. *Mol. Cell. Biol.* **16:** 3789–3798.

Hurford, R.K., Jr., D. Cobrinik, M.-H. Lee, and N. Dyson. 1997. pRB and p107/p130 are required for the regulated expression of different sets of E2F responsive genes. *Genes & Dev.* **11:** 1447–1463.

Iavarone, A. and J. Massagué. 1999. E2F and histone deacetylase mediate transforming growth factor beta repression of cdc25A during keratinocyte cell cycle arrest. *Mol. Cell. Biol.* **19:** 916–922.

Johnson, D.G., K. Ohtani, and J.R. Nevins. 1994. Autoregulatory control of *E2F1* expression in response to positive and negative regulators of cell cycle progression. *Genes & Dev.* **8:** 1514–1525.

Lam, E.W.-F. and R.J. Watson. 1993. An E2F-binding site mediates cell-cycle regulated repression of mouse B-myb transcription. *EMBO J.* **12:** 2705–2713.

Le Cam, L., J. Polanowska, E. Fabbrizio, M. Olivier, A. Philips, E.N. Eaton, M. Classon, Y. Geng, and C. Sardet. 1999 Timing of *cyclin E* gene expression depends on the regulated association of a bipartite repressor element with a novel E2F complex. *EMBO J.* **18:** 1878–1890.

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

Leone, G., J. DeGregori, Z. Yan, L. Jakoi, S. Ishida, R.S. Williams, and J.R. Nevins. 1998. E2F3 activity is regulated during the cell cycle and is required for the induction of S phase. *Genes & Dev.* **12:** 2120–2130.

Lindeman, G.J., L. Dagnino, S. Gaubatz, Y. Xu, R. Bronson, H.B. Warren, and D.M. Livingston. 1998. A specific, nonproliferative role for E2F-5 in choroid plexus function revealed by gene targeting. *Genes & Dev.* **12:** 1092–1098.

Lukas, J., B.O. Petersen, K. Holm, J. Bartek, and K. Helin. 1996. Deregulated expression of E2F family members induces S-phase entry and overcomes $p16^{INK4A}$-mediated growth suppression. *Mol. Cell. Biol.* **16:** 1047–1057.

Lukas, J., T. Herzinger, K. Hansen, M.C. Moroni, D. Resnitzky, K. Helin, S.I. Reed, and J. Bartek. 1997. Cyclin E-induced S phase without activation of the pRB/E2F pathway. *Genes & Dev.* **11:** 1479–1492.

Means, A.L., J.E. Slansky, S.L. McMahon, M.W. Knuth, and P.J. Farnham. 1992. The HIP binding site is required for growth regulation of the dihydrofolate reductase promoter. *Mol. Cell. Biol.* **12:** 1054–1063.

Moberg, K., M.A. Starz, and J.A. Lees. 1996. E2F-4 switches from p130 to p107 and pRB in response to cell cycle reentry. *Mol. Cell. Biol.* **16:** 1436–1449.

Muller, H., M.C. Moroni, E. Vigo, B.O. Petersen, J. Bartek, and K. Helin. 1997. Induction of S-phase entry by E2F transcription factors depends on their nuclear localization. *Mol. Cell. Biol.* **17:** 5508–5520.

Mulligan, G. and T. Jacks. 1998. The retinoblastoma gene family: Cousins with overlapping interests. *Trends Genet.* **14:** 223–229.

Neuman, E., E.K. Flemington, W.R. Sellers, and W.G. Kaelin. 1994. Transcription of the E2F1 gene is rendered cell cycle-dependent by E2F DNA binding sites within its promoter. *Mol. Cell. Biol.* **14:** 6607–6615.

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

Serrano, M., A.W. Lin, M.E. McCurrach, D. Beach, and S.W. Lowe. 1997. Oncogenic *ras* provokes premature cell senescence associated with accumulation of p53 and $p16^{INK4a}$. *Cell* **88:** 593–602.

Starostik, P., K.N.B. Chow, and D.C. Dean. 1996. Transcriptional repression and growth suppression by the p107 pocket protein. *Mol. Cell. Biol.* **16:** 3606–3614.

Stillman, B. 1996. Cell cycle control of DNA replication. *Science* **274:** 1659–1664.

Tommasi, G. and G.P. Pfeifer. 1995. In vivo structure of the human cdc2 promoter: Release of a p130-E2F-4 complex from sequences immediately upstream of the transcription initiation site coincides with the induction of cdc2 expression. *Mol. Cell. Biol.* **15:** 6901–6913.

Trimarchi, J.M., B. Fairchild, R. Verona, K. Moberg, N. Andon, and J.A. Lees. 1998. E2F-6, a member of the E2F family that can behave as a transcriptional repressor. *Proc. Natl. Acad. Sci.* **95:** 2850–2855.

Tsai, K., Y. Hu, K.F. Macleod, D. Crowley, L. Yamasaki, and T. Jacks. 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.

Tybulewicz, V.L., C.E. Crawford, P.K. Jackson, R.T. Bronson, and R.C. Mulligan. 1991. Neonatal lethality and lymphopenia in mice with a homozygous disruption of the c-abl proto-oncogene. *Cell* **65:** 1153–1163.

Verona, R., K. Moberg, S. Estes, M. Starz, J.P. Vernon, and J.A. Lees. 1997. E2F activity is regulated by cell cycle-dependent changes in subcellular localization. *Mol. Cell. Biol.* **17:** 7268–7282.

Vigo, E., H. Muller, E. Prosperini, G. Hateboer, P. Cartwright, M.C. Moroni, and K. Helin. 1999. CDC25A phosphatase is a target of E2F and is required for efficient E2F-induced S phase. *Mol. Cell. Biol.* **19:** 6379–6395.

Wade, M., T.F. Kowalik, M. Mudryj, E.S. Huang, and J.C. Azizkhan. 1992. E2F mediates dihydrofolate reductase promoter activation and multiprotein complex formation in human cytomegalovirus infection. *Mol. Cell. Biol.* **12:** 4364–4374.

Wang, Z.M., H. Yang, and D.H. Livingston. 1998. Endogenous E2F-1 promotes timely G0 exit of resting mouse embryo fibroblasts. *Proc. Natl. Acad. Sci.* **22:** 15583–15586.

Weinberg, R.A. 1992. Tumor suppressor genes. *Science* **254:** 1138–1146.

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

Yamasaki, L., R. Bronson, B.O. Williams, N.J. Dyson, E. Harlow, and T. Jacks. 1998. Loss of E2F-1 reduces tumorigenesis and extends lifespan of Rb1(+/–) mice. *Nature Genet.* **18:** 360–364.

Zwicker, J., N. Liu, K. Engeland, F.C. Lucibello, and R. Muller. 1996. Cell cycle regulation of E2F site occupation in vivo. *Science* **271:** 1595–1597.