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Report

E2F2 represses cell cycle regulators to maintain quiescence

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E2F transcription factors control diverse biological processes through regulation of target gene expression. However, the mechanism by which this regulation is established, and the relative contribution of each E2F member are still poorly defined. We have investigated the role of E2F2 in regulating cellular proliferation. We show that E2F2 is required for the normal G_0/G_1 phase because targeted disruption of the E2F2 gene causes T cells to enter S phase early and to undergo accelerated cell division. A large set of E2F target genes involved in DNA replication and cell cycle progression (such as Mcm's, cyclins and Cdc2a) that are silent in G_0 and typically transcribed late in G_1 phase are already actively expressed in quiescent T cells and MEFs lacking E2F2. The classic E2F activators, E2F1 and E2F3, are largely dispensable for this process because compound loss of $E2F1^{-/-}$ and $E2F2^{-/-}$ produces a comparably shortened G_0/G_1 phase, with early S phase entry. Likewise, shRNA knockdown of E2F3 does not alter significantly the $E2F2^{-/-}$ phenotype. Chromatin immunoprecipitation analysis indicates that in wild-type cells the promoters of the aberrantly early-transcribed genes are occupied by E2F2 in G_0, suggesting a direct role for E2F2 in transcriptional repression. We conclude that E2F2 functions to transcriptionally repress cell cycle genes to establish the G_0 state.

Introduction

The E2F transcription factors (E2F1-8) are known to play a central role in regulating gene expression during cellular proliferation. The function of E2F as a transcriptional regulator is linked to its association with pocket proteins, consisting of the retinoblastoma tumor suppressor protein, pRB, and its relatives p107 and p130. The finding that pRB interacts with E2F and blocks its transcriptional activity before the G_1/S transition established an early model for cell cycle control. According to this model, unphosphorylated pRB binds to E2F in G_0/G_1, leading to the repression of E2F target genes. The subsequent phosphorylation of pRB by cyclin-dependent kinases in late G_1 inactivates pRB, liberating free E2F, which then functions to activate the expression of target genes required for S-phase entry and cell cycle progression.[1-3]

Subsequent work has complicated the model, suggesting that different E2F family members function differently as positive or negative regulators. Thus, E2F family members have been divided into positive regulators of the cell cycle (E2F activators) and negative regulators of the cell cycle (E2F repressors) based on their transcriptional roles in vitro and conserved structural features.[1-3] In tissue culture studies, overexpression of the so-called E2F activators (E2F1-3) is sufficient to activate transcription of target genes involved in the G_1/S transition, and to promote DNA replication in immortalized, quiescent rodent fibroblasts in the absence of growth factors.[4,5] By contrast, E2Fs 4 and 5 are considered to possess predominantly repressive activity. They are mainly nuclear in G_0/G_1 cells, where they are bound to members of the pRB family,[6,7] and their overexpression in serum-starved fibroblasts does not induce S phase.[5] The newest members of the family (E2F6-8) diverge considerably from the other E2F proteins, since they lack sequences that mediate transcriptional activation and the pocket protein-binding domain. They are considered to function as transcriptional repressors of certain E2F-responsive genes, by mechanisms that differ from the conventional mode of E2F-mediated regulation.[8,9]

A considerable amount of evidence has accumulated indicating that this division of E2F members into activators and repressors may be too simplistic. Indeed, microarray analyses have shown that ectopic expression of E2F1-3 not only leads to transcriptional activation, but also to the transcriptional repression of a large number of genes,[10,11] although, of course, many of these effects may be indirect. However, there is good evidence that E2F3 can bind directly to the $p19^{ARF}$ promoter and thereby repress its expression.[12] The underlying mechanisms remain to be defined, although some of them could be independent of the pocket-proteins.[1] On the other hand, E2F4 has been detected on certain mouse promoters in late G_1/S phase, and ectopically expressed E2F4 has been able to induce cellular proliferation,[13,14] suggesting a transcriptional activator function for this E2F member. Thus, the relative importance of the E2F members as transcriptional activators or repressors in cell cycle control remains unresolved.

The physiological roles of the E2F transcription factors have begun to be determined with the generation of mouse strains carrying

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targeted mutations for individual E2F genes. Mice lacking E2F1-8 have been generated. The analysis of single and compound mutant E2F mice has provided a series of unexpected results, revealing overlapping roles in the control of cellular proliferation, but also unique functions during development, tissue homeostatic maintenance, and tumor formation, implying the existence of specific target genes for each E2F.[3,15] E2F1 appears to have a unique function in the induction of apoptosis and tumor suppression, whereas E2F3 is required to promote normal cell cycle progression. E2F4 and E2F5 are thought to be involved in cell cycle exit and terminal differentiation, but are not required for cell cycle re-entry from G_0 or for normal cellular proliferation.[16]

The biological function of E2F2 remains unresolved. Transgenic mice overexpressing E2F2 in thymic epithelial cells develop thymomas,[17] and ectopic expression of E2F2 in cultured cells leads to induction of target gene expression and entry into S phase,[15] implying that E2F2 promotes cell cycle progression. However, targeted disruption of E2F2 gives rise to lymphoproliferation and autoimmunity[18] as well as tumors,[19] which are further increased by the expression of a myc transgene,[20] suggesting a tumor suppressor role for E2F2. Moreover, E2F2$^{-/-}$ lymphoid cells and pancreatic cells exhibit an increased capacity to replicate their DNA,[18,21] also implying a negative role for E2F2 in cellular proliferation. Furthermore, E2F2 has been shown to have a tissue-restricted role in erythropoiesis and in neuronal differentiation.[22,23] Although structurally very similar to E2F2, E2F1 and E2F3 do not appear to inhibit DNA synthesis,[18,19,24,25] suggesting that this is a specific function of E2F2, not shared by the other members of the E2F subfamily. We have now investigated the role of E2F2 in regulating cellular proliferation. We show that E2F2 functions to maintain the quiescent, G_0, state through direct transcriptional repression of cell cycle target genes.

Results

Role of E2F1 and E2F2 in cell cycle entry. Primary T lymphocytes are arrested in G_0 and undergo cell cycle entry and subsequent cell division in response to ligation of the T cell receptor complex, providing a good model system to study cell cycle entry. Initial entry into the cell cycle is delayed by a lengthy G_0/G_1, followed by several rapid cell cycles over the subsequent ~96 hours, and then a return to quiescence.[26] To determine the role of E2F1 and E2F2 in this process, we studied the response of T cells from mice in which we had knocked-out E2F1, E2F2 or both together, and examined the kinetics of cell cycle entry and cell proliferation in response to stimulation with an activating antibody to CD3. Cell cycle distribution was measured by pulsing with BrdU to label cells in S phase, detected by immunofluorescence with an antibody to BrdU, and counter-staining with propidium iodide to measure DNA content to distinguish G_0/G_1 from G_2/M. Cell populations were analyzed by flow cytometry. As shown in Figure 1A, wild-type T lymphocytes undergo a lengthy delay before the onset of S phase, which begins approximately 24 hours after stimulation and peaks after 42 hours.

In a parallel experiment, we examined the rate of cell proliferation by labeling the cells with the vital fluorescent dye CFSE, which is diluted approximately two-fold with each cell division. The cell division rate was analyzed by flow cytometry. By 72 hr after stimulation by CD3, approximately 74% of the cells had divided at least once, and progressed variably through two to six cell divisions. However, the remaining 26% of the wild-type lymphocytes had not undergone a single cell division after 72 hours (Fig. 1B). By 96 hr most of the cells had divided at least once, and half had divided more than 3 times (data not shown).

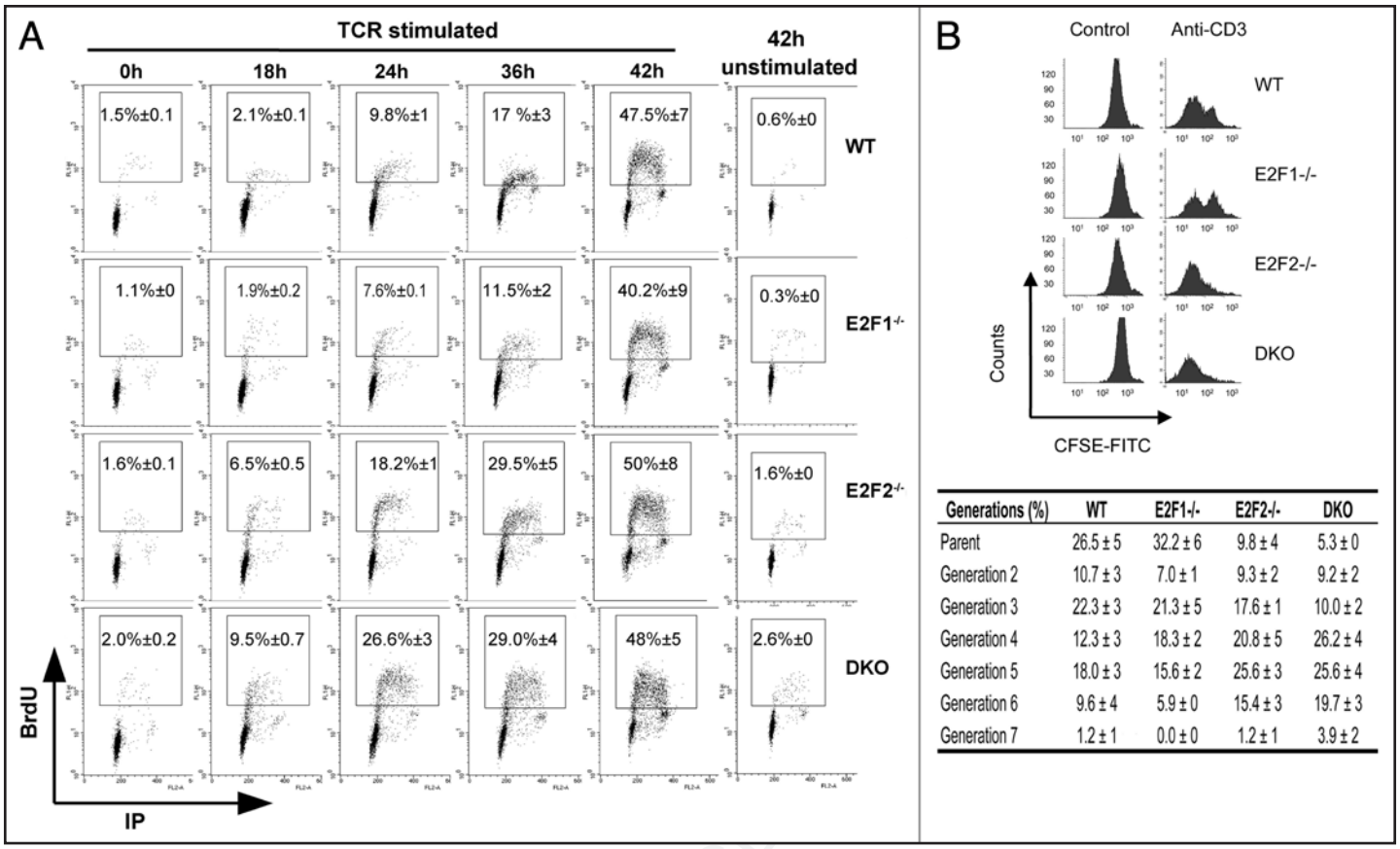
In these same assays T lymphocytes harvested from E2F1$^{-/-}$ mice demonstrated a modest quantitative decrease in the fraction of cells that enter the cell cycle (Fig. 1A). Likewise, the E2F1$^{-/-}$ T lymphocytes exhibited modestly impaired proliferation in response to stimulation through CD3 (Fig. 1B). These data are consistent with previously published results indicating that E2F1 normally plays a positive role in driving cell cycle entry.[1,3,15]

Remarkably, T lymphocytes harvested from E2F2$^{-/-}$ mice exhibited accelerated entry into S phase (Fig. 1A), beginning approximately 18 hr after anti-CD3 stimulation, that is, 6–12 hr earlier than wild-type cells. Likewise, they showed earlier and enhanced proliferation in response to signaling through CD3 (Fig. 1B). By 72 hr, more than 90% of the cells had undergone at least one cell division cycle, and more than half of them had divided at least 3 times. In contrast to E2F1, these results clearly demonstrate that E2F2 normally plays a negative role to restrain cell cycle progression, consistent with previously published results.[18,19,21]

It is possible that, in part, the accelerated cell cycle entry and enhanced proliferation observed in E2F2$^{-/-}$ cells reflects a switch in E2F activity from E2F2, a negative regulator, to E2F1, a positive regulator. Therefore we also examined T lymphocytes from mice deficient in both E2F1 and E2F2. These double knock-out (DKO) T lymphocytes demonstrated accelerated cell cycle entry and enhanced proliferation comparable to the E2F2$^{-/-}$ cells (Fig. 1A and B). Therefore E2F1 is not required for the effects of E2F2 deficiency. From this analysis it remains possible, however, that another E2F is functioning to replace E2F2 in its absence, and serves to positively regulate cell cycle entry, a possibility examined more closely below.

Transcriptional targets of E2F1 and E2F2. To begin to define the mechanism by which E2F1 and E2F2 contribute positively and negatively, respectively, to cell cycle entry we sought to determine the identity of their target genes. In wild-type lymphocytes we observed that levels of E2F1 and E2F2 are low (but detectable) in quiescence, and rise significantly 24–48 hours after stimulation by anti-CD3 (Fig. 2A). Therefore we isolated RNA at two time points, 0 and 36 hours, representing cells in G_0 and near the G_1/S border, to study gene expression in wild-type, E2F1$^{-/-}$, E2F2$^{-/-}$ and DKO mice. To quantify gene expression, Cy3 and Cy5 labeled complementary DNA pools were prepared and hybridized to mouse microarrays containing probes for ~16,000 murine genes and ESTs, and binding was observed by detection of fluorescence (see Materials and Methods section). A total of six hybridization experiments were performed for each mutant genotype and time-point (corresponding to three biological replicates with two technical replicates each). Wild-type RNA obtained from three independent experiments and a total of 9 mice was combined for each time-point, and served as a control in all the hybridization experiments. Changes in gene expression between mutant genotypes relative to wild-type were analyzed using one-sample t-tests, and scored as significant when $p < 0.01$.[27-30]

Microarray analysis showed many genes with altered expression in E2F mutant T lymphocytes, both in quiescence, as well as in the G_1/S phase (Fig. 2B). Loss of E2F1 led to significantly deregulated



Generations (%)	WT	E2F1-/-	E2F2-/-	DKO
Parent	26.5 ± 5	32.2 ± 6	9.8 ± 4	5.3 ± 0
Generation 2	10.7 ± 3	7.0 ± 1	9.3 ± 2	9.2 ± 2
Generation 3	22.3 ± 3	21.3 ± 5	17.6 ± 1	10.0 ± 2
Generation 4	12.3 ± 3	18.3 ± 2	20.8 ± 5	26.2 ± 4
Generation 5	18.0 ± 3	15.6 ± 2	25.6 ± 3	25.6 ± 4
Generation 6	9.6 ± 4	5.9 ± 0	15.4 ± 3	19.7 ± 3
Generation 7	1.2 ± 1	0.0 ± 0	1.2 ± 1	3.9 ± 2

Figure 1. Enhanced cell cycle entry and proliferation in E2F2$^{-/-}$ and DKO T lymphocytes. (A) Cell cycle analysis of representative WT, E2F1$^{-/-}$, E2F2$^{-/-}$ and DKO T lymphocyte cultures after anti-CD3 treatment. To assay cell cycle distribution T lymphocytes were purified from lymph nodes, cultured in vitro with anti-CD3 for the indicated times, pulse labeled with BrdU for 20 min, harvested and fixed. DNA content was assessed by staining with propidium iodide, and DNA synthesis was assessed by staining with an antibody to BrdU, and measured by flow cytometry. The percentage of cells in S phase is indicated, and corresponds to the average of three independent experiments with standard deviations. (B) Analysis of proliferation of representative wild-type and mutant T lymphocyte cultures stimulated with anti-CD3. Lymphocytes were purified from lymph nodes, stained with CFSE and then cultured with plate-bound anti-CD3 for 72 h. Cells were harvested and CFSE fluorescence was determined by flow cytometry. Proliferation Wizard software was used to identify cells in different generations as indicated in the table. Note that the parent population is the brightest population, with subsequent cell divisions showing reduced CFSE signal. The percentage of cells in each cellular generation at the 72 hr time-point is indicated in the table, and corresponds to the average of three independent experiments.

expression of close to 400 genes, both in quiescence as well as after stimulation. The proportion of upregulated genes was similar to that of downregulated genes. E2F2-deficient cells showed a higher number of significantly deregulated genes than E2F1-deficient cells. This was particularly evident in the subset of upregulated genes (≈500 genes in quiescence and ≈400 genes upon activation). Concurrent loss of E2F1 and E2F2 resulted in a larger set of deregulated genes, especially in proliferating cells, implying that the expression of many of these genes is controlled redundantly by E2F1 and E2F2. The level of deregulated expression for most of the genes was generally modest, but highly significant ($p < 0.01$) (see Suppl. Tables 1 and 2).

We used Gene Ontology (GO) annotations to cluster E2F1- and E2F2-regulated genes according to function. Inspection of these clusters revealed that the E2F regulated genes are involved in a wide spectrum of pathways. To determine whether gene enrichment in a given functional category was statistically significant (relative to the representation of genes within that functional category in our cDNA microarray) we used the EASE algorithm,[31] considering separately the two time points (quiescence and G_1/S) and up versus downregulated genes. Importantly, in resting T cells, but not in proliferating cells, loss of E2F2 was associated with upregulation of genes clustered into functional groups related to cell cycle regulation, mitosis and DNA metabolism and repair (Table 1 and Suppl. Tables 1 and 2). Included in these categories were *mcm2*, *mcm6 cdc6*, *cycA*, *cycB*, *cdc2a* and *chk1*, among others. A similar clustering of upregulated genes was observed in DKO cells, although the number of genes upregulated in each cluster in DKO samples was higher. By contrast, no overrepresented categories were present in the transcriptome of quiescent E2F1$^{-/-}$ cells.

Thus, E2F2, but not E2F1, negatively regulates the expression of a set of genes that are involved in cell cycle regulation and DNA replication and repair in quiescent, G_0, T cells. The aberrantly elevated expression of these genes in E2F2$^{-/-}$ and DKO cells might account for the accelerated S-phase entry and hyperproliferation of these cells. Promoter sequence analysis using the TELiS (Transcription Element Listening System) database (www.telis.ucla.edu) indicated that many genes displaying aberrantly increased expression in E2F2-deficient quiescent cells contain E2F binding sites, and a subset have been

previously identified as targets of regulation by the Rb/E2F pathway. This raises the possibility that E2F2 acts to directly repress their expression during quiescence.

GO analysis also showed a battery of overrepresented categories related to signaling, regulation of transcription and regulation of cellular metabolic processes in E2F2$^{-/-}$ and DKO cells, both in quiescence as well as in G_1/S (Table 1). However, promoter search analysis using the TELiS database carried out with the deregulated genes indicated that only a minor fraction of these carried E2F sites in their promoters (data not shown), suggesting an indirect effect of the loss of E2F1 and E2F2 in their transcriptional regulation.

Inactivation of E2F2 deregulates expression of cell cycle control proteins. Several important cell cycle regulatory genes had altered mRNA levels in the mutant cells. We examined these by Western blot to determine whether the changes in mRNA were reflected by changes in protein levels. We examined the expression of cyclins (D2, D3, E1, A2 and B1), CDKs (CDK4, CDK2, CDK1) and CKIs (p18, p19, p21 and p27) in quiescent T cells or T cells stimulated with anti-CD3 for 18 h or 36 h, that is, when the bulk of the cells are in G_0, G_1 or G_1/S, respectively, and found a tight correlation between RNA expression data and protein expression data.

We observed the deregulated expression of several cell cycle regulators which are normally not expressed in G_0 but whose levels raise upon entry into the cell cycle (Fig. 3A). Remarkably, in E2F2-deficient or E2F1/E2F2-compound deficient T cells these proteins are expressed at elevated levels even in quiescent, G_0, T cells. These proteins include cyclins D2 (absent in the microarray), D3, A2 and B1, cdk1 and chk1 (Fig. 3A). Levels of these rise higher after stimulation of the TCR, but importantly, unlike wild-type, all are detectably expressed in G_0 in T cells lacking E2F2.

We next examined the expression of cyclin-dependent kinase inhibitors. The expression of CKIs was difficult to detect by Western analysis, due to their low levels. We therefore measured their levels by real-time RT-PCR. Knockout of E2F1 or E2F2 had no effect on p19 or p27 levels in quiescent or stimulated cells (Fig. 3B). The levels of p21 (absent in the microarray) were also unchanged by loss of E2F1 or E2F2 in quiescent cells. However, after TCR stimulation, cells deficient for E2F1 and/or E2F2 expressed aberrantly high levels of p21 mRNA.

The INK4 family member p18 has emerged as a cyclin-dependent kinase inhibitor with an essential function in T lymphocyte homeostasis. p18 induction has been shown to lead to G_1 arrest. Loss of p18 confers a proliferative advantage to lymphocytes, and p18$^{-/-}$ mice develop a lymphoproliferative disorder and T cell lymphomas.[32] Loss of E2F1, E2F2 or both resulted in reduced levels of p18 mRNA in resting T cells (Fig. 3B). After stimulation through the TCR E2F1$^{-/-}$ cells achieved normal levels of p18 expression. However, E2F2$^{-/-}$ and DKO cells had significantly reduced levels of p18 even after TCR-mediated stimulation. Thus, p18 provides an example of a negative regulator of the cell cycle whose activation of expression depends on E2F2. Importantly, the expression of p18 was also downregulated in the transcriptome of E2F2$^{-/-}$ and DKO cells compared to WT cells, both at quiescence and after stimulation.

Loss of E2F2 results in premature assembly of the MCM complex on chromatin in quiescent cells. We examined the expression of MCM complex origin of replication proteins by Western blot of protein extracts obtained from T cells that were quiescent or stimulated for 18 h or 36 h with anti-CD3. These showed that

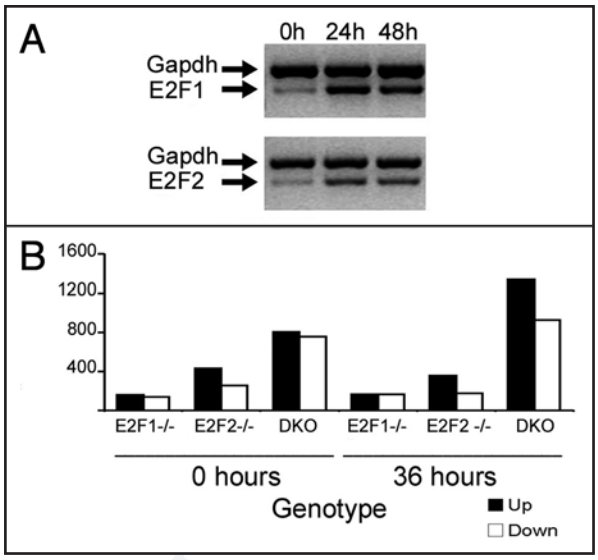


Figure 2. Changes in gene expression resulting from loss of E2F1 and/or E2F2. (A) Representative RT-PCR analysis of RNA samples from wild-type mice showing expression of E2F1 and E2F2 in T lymphocytes stimulated with anti-CD3 for the indicated times. The internal standard (*Gapdh*) was amplified in the same reaction as *E2F1* and *E2F2*. (B) Total number of deregulated genes identified in microarray expression analyses of E2F1$^{-/-}$, E2F2$^{-/-}$ and DKO cells compared to wild-type controls. Three independent hybridization experiments were performed for each mutant and time-point. Changes in gene expression between mutant phenotypes compared to wild-type were considered significant when $p < 0.01$ (one sample t-test). (C and D) Functional (gene ontology, GO) categories overrepresented in T lymphocytes lacking E2F1, E2F2 or both, either in G_0 (C) or in G_1/S (D). GO analysis was used to classify the genes differentially expressed in mutant genotypes on the basis of their functional roles. Subsequently, Expression Analysis System Software (EASE) analysis was performed to identify the overrepresented functional categories.

in wild-type T cells Mcm2, Mcm6 and Cdc6 are present in very low amounts in G_0, begin to accumulate in G_1, and reach a peak at G_1/S at the initiation of DNA replication, (Fig. 3C). Strikingly, loss of E2F2 led to significantly increased levels of Mcm2, Mcm6 and Cdc6, even in unstimulated cells, with a further increase in levels upon TCR stimulation (Fig. 3C). A similar, but even more accentuated increase in the levels of Mcm2, Mcm6 and Cdc6 was observed in DKO cells.

Numerous studies have demonstrated that Cdc6 is necessary for the Mcm complex to associate with origins of DNA replication and that Mcm complex assembly on chromatin is required for cells to enter S-phase.[33] Given the fact that quiescent DKO cells exhibit increased levels of Cdc6 and Mcm protein we asked whether the Mcm proteins are associated with chromatin in these cells even during quiescence. Cells were lysed and fractionated into a chromatin enriched pellet and a soluble supernatant. MEK2 was used as a control to demonstrate that the chromatin fraction is not contaminated by cytosolic proteins. As expected, Mcm2 is not associated with chromatin in quiescent cells derived from wild-type animals. Strikingly, loss of E2F2 caused some Mcm2 to become chromatin-associated even in quiescent cells, and the remaining Mcm2 was found in the soluble fraction. More dramatically, concomitant loss of E2F1 and E2F2 led to the majority of Mcm2 to become chromatin-bound even in quiescent cells (Fig. 4).

Table 1 **Overrepresented GO categories in E2F2$^{-/-}$ and DKO cells relative to WT controls**

0 hours Upregulated		**DKO**			**E2F2$^{-/-}$**	
	Count[a]	**%[b]**	**p-Value[c]**	**Count[a]**	**%[b]**	**p-Value[c]**
cell cycle phase	67	6.7	2.1E-11	39	9.1	4.5E-10
mitosis	48	4.8	5.3E-10	30	7.0	1.1E-9
DNA metabolic process	91	9.0	6.4E-7	52	12.1	1.1E-7
DNA repair	37	3.7	7.2E-5	20	4.7	5.0E-4
lymphocyte activation	26	2.6	1.2E-3			
nucleobase, nucleoside and nucleotide metabolic process	28	2.8	4.1E-3			
establishment of protein localization	78	7.8	4.7E-3			
carboxylic acid metabolic process	51	5.1	5.8E-3			
macromolecular complex assembly				25	5.8	2.0E-3
0 hours Downregulated		**DKO**			**E2F2$^{-/-}$**	
	Count	**%**	**p-Value**	**Count**	**%**	**p-Value**
regulation of transcription,	132	17.5	5.0E-3	40	15.8	9.9E-3
regulation of cellular metabolic process	153	20.3	6.9E-4	43	17	4.8E-2*
biopolymer modification	133	17.6	2.9E-5			
chromosome organization and biogenesis	35	4.6	8.5E-3			
36 hours Upregulated		**DKO**			**E2F2$^{-/-}$**	
	Count	**%**	**p-Value**	**Count**	**%**	**p-Value**
carboxylic acid metabolic process	63	4.7	9.9E-3	29	8.6	6.8E-6
amino acid metabolic process	36	2.7	4.0E-2*	19	5.6	5.4E-5
establishment of protein localization	92	6.9	4.4E-2*	31	9.2	6.3E-3
protein transport	91	6.8	2.4E-2*	30	8.9	6.8E-3
lipid catabolic process	20	1.5	6.1E-5			
regulation of transferase activity	38	2.8	6.5E-5			
vesicle-mediated transport	69	5.2	1.0E-4			
cell morphogenesis	69	5.2	1.2E-4			
regulation of signal transduction	56	4.2	3.6E-4			
cellular carbohydrate metabolic process	42	3.1	5.4E-4			
protein complex assembly	31	2.3	1.1E-3			
intracellular signaling cascade 136	10.2	1.8E-3				
regulation of cell activation	16	1.2	4.7E-3			
positive regulation of catalytic activity	30	2.2	6.0E-3			
chemical homeostasis	25	1.9	6.9E-3			
cell migration	41	3.1	7.1E-3			
36 hours Downregulated		**DKO**			**E2F2$^{-/-}$**	
	Count	**%**	**p-Value**	**Count**	**%**	**p-Value**
DNA metabolic process	87	9.4	3.3E-9	22	12.6	9.2E-5
regulation of cell cycle	41	4.4	8.1E-3	14	8.0	1.5E-3
mitosis	24	2.6	4.2E-2*	11	6.3	6.2E-4
chromosome organization and biogenesis	48	5.2	2.1E-5	10	5.7	4.9E-2*
RNA metabolic process	222	23.9	7.6E-12			
transcription	172	18.5	1.0E-5			
regulation of cellular metabolic process	179	19.3	5.6E-5			
macromolecular complex assembly	41	4.4	1.4E-3			
ribosome biogenesis and assembly	20	2.2	9.9E-3			
cell cycle phase				14	8.0	5.2E-4

[a]Refers to the number of genes included in each overrepresented functional category. [b]Refers to the percentage of genes detected in each functional category relative to the total number of genes included in that particular category. [c]Refers to the EASE score threshold, a modified Fisher Exact p-value, for gene-enrichment analysis. Shown are categories with a p-value of <0.01, except in the cases indicated with an asterisk, where the p-value is <0.05.

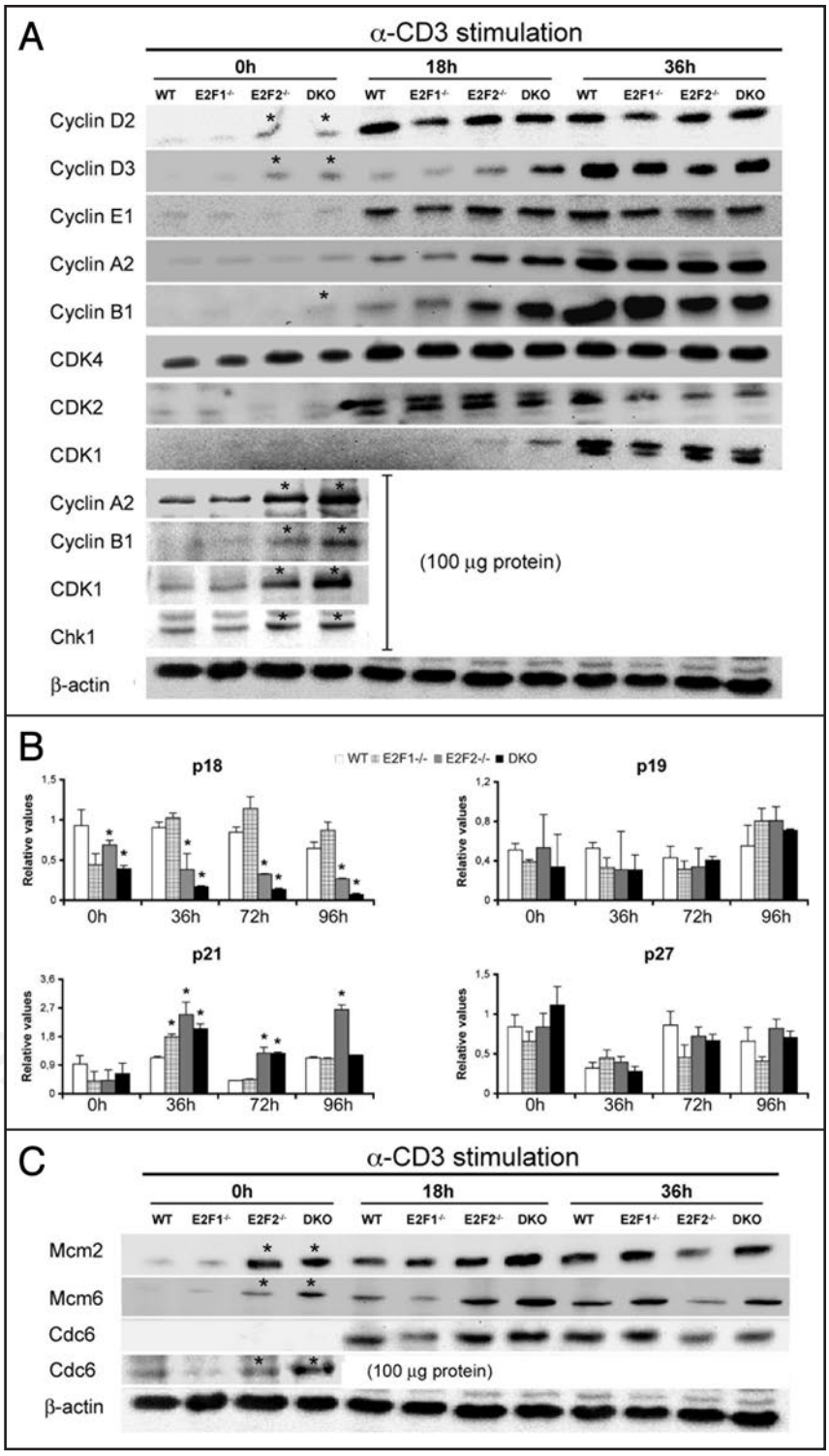


Figure 3. Altered expression kinetics of cell cycle regulators and DNA replication proteins in T cells lacking E2F2. (A) Western blot analysis of cyclins and cdks in extracts prepared from WT, E2F1$^{-/-}$, E2F2$^{-/-}$ and DKO T lymphocytes treated with plate-bound anti-CD3. Cells were harvested 0 h, 18 h and 36 h after stimulation. In each lane, 20 μg of total protein extract was loaded, except when indicated as 100 μg. Expression of β-actin served as loading control. These data are representative of greater than three experiments. * Denotes elevated expression in G_0 relative to WT. (B) Real-time PCR analysis of p18^{Ink4c}, p19^{Arf}, p21^{Cip1} and p27^{Kip1} expression. Total RNA was harvested from the indicated genotypes (n = 3 for each genotype), and used to produce cDNA. In each case, expression values are normalized to the expression of Eef1a1, used as a standard control. Error bars: SEM. Asterisk (*) indicates significant differences ($p < 0.05$) from WT controls using one-way analysis of variance. (C) Western blot analysis of DNA replication proteins Mcm2, Mcm6 and Cdc6 in T cell extracts prepared from WT and E2F1/E2F2 mutant genotypes treated with anti-CD3 for the indicated times. In each lane, 20 μg of total cell extract was loaded, except when indicated as 100 μg. These data are representative of greater than three experiments. Asterisk (*) denotes elevated expression in G_0 relative to WT.

Thus, loss of E2F2 results in increased expression of cyclins, cdk1, and replication proteins, and the increased association of replication proteins with chromatin in resting cells, leaving the cells poised to rapidly initiate DNA synthesis. This aberrant pattern suggests that these cells are not truly in G_0, but exhibit some features of G_1 or G_1/S. Nevertheless, the mutant T cells do not enter the cell cycle in the absence of stimulation (Fig. 1A), indicating that they remain functionally quiescent.

Abnormal expression of G_1/S-specific genes in quiescent E2F2-deficient fibroblasts. We wondered whether the abnormal gene expression that we observed in G_0 in E2F2$^{-/-}$ T lymphocytes was also seen in other cell types. Therefore we examined the expression of MCM complex and cell cycle genes in primary mouse embryonic fibroblasts (MEFs) obtained from wild-type or E2F2$^{-/-}$ mice. As shown in Figure 5A, in wild-type MEFs rendered quiescent by serum-starvation the level of Mcm2, Mcm6 and CDK1 was very low, near the limit of detection. Stimulation by serum led to a significant increase in expression. By contrast, E2F2$^{-/-}$ MEFs expressed high levels of these proteins even when quiescent, and these levels rose even higher after stimulation by serum. Thus, the abnormally increased gene expression observed in E2F2$^{-/-}$ T lymphocytes is also observed in E2F2$^{-/-}$ MEFs.

E2F1 and E2F3 are not required for the enhanced expression of G_1/S-specific genes in quiescent E2F2-deficient cells. We considered two potential explanations for the increase in expression of putative E2F target genes upon loss of E2F2. First, E2F2 itself may function to repress gene transcription of these genes. Alternatively, E2F2 may be transcriptionally neutral or weakly positive, but upon its loss another E2F with stronger transcriptional activating function could replace it, leading to a net increase in gene expression. To test the latter model, we asked if other E2Fs are required for the increases in gene expression that are observed in E2F2-deficient cells. As discussed in the Introduction, E2F1 and E2F3 would be the best candidates, since they have been associated with strong transcriptional activation.

As described above, knockout of E2F1 in addition to E2F2, i.e., the DKO cells, results in similarly enhanced gene expression and accelerated cell cycle entry as loss of E2F2 alone (see Figs. 1–4). Therefore replacement of E2F2 by E2F1 is not responsible for the enhanced gene expression observed in E2F2-deficient cells.

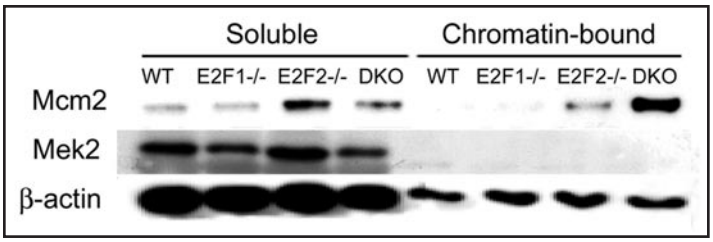


Figure 4. Chromatin binding by DNA replication proteins is increased in quiescent E2F2$^{-/-}$ and DKO cells. T lymphocytes purified from lymph nodes of WT and E2F1/E2F2 mutant mice were fractionated to recover chromatin-bound and soluble fractions. Fractions were resolved by SDS-PAGE, transferred to nitrocellulose membrane and probed with anti-Mcm2 (upper), anti-Mek2 (middle) or β-actin (lower) antibodies. These data are representative of two experiments.

Next we asked whether E2F3 might be required for the effect of loss of E2F2. First, we examined E2F3 expression by quantitative RT-PCR. This analysis showed that the expression level of E2F3 was low in wild-type cells at rest, and increased significantly after 36 h of TCR stimulation (Fig. 5B). E2F3 mRNA levels in E2F2$^{-/-}$ or DKO cells were found to trend slightly higher, but this trend did not reach statistical significance compared to the levels in WT or E2F1$^{-/-}$ cells.

To more definitively test the requirement for E2F3 for the abnormal gene expression observed in E2F2$^{-/-}$ cells we sought to perform experiments to knockdown E2F3. Because of difficulties achieving efficient transfection and high knockdown efficiency in T lymphocytes we chose to pursue these experiments in MEFs.

Silencing of E2F3 in E2F2-deficient MEFs led to a drastic reduction in the levels of Mcm2, Mcm6 and Cdk1 at the 18 hr time-point (Fig. 5C). However, the expression of Mcm2 or Mcm6 did not change in quiescent MEFs after E2F3 silencing, despite a virtually complete reduction in the expression of E2F3. In the case of Cdk1 we observed a 10-to 30% reduction of expression after E2F3 depletion. Therefore E2F3 appears to play an important role in gene expression after growth factor stimulation, but does not contribute significantly to the enhanced expression of genes in quiescence that results from loss of E2F2. Taken together, replacement of E2F2 by E2F1 or E2F3 in E2F2$^{-/-}$ cells is not required for the aberrantly elevated gene expression that results from loss of E2F2. Instead, the data support a direct role for E2F2 as a transcriptional repressor.

E2F2 is bound to target promoters in quiescent cells. As a test for the ability of E2F2 to directly repress the expression of genes which show enhanced expression in E2F2$^{-/-}$ cells we asked whether E2F2 is directly bound to the promoters of these genes during quiescence. Therefore we chose several candidate genes to examine which E2Fs are bound to their promoters in quiescence and at the G_1/S border.

It has been shown that E2F target promoters are bound by multiple E2Fs in vivo.[14] As cells progress through the cell cycle, E2F binding patterns on target cells change, and these changes in binding patterns appear to correlate with changes in expression level of the target genes. Therefore we also examined promoter binding by the other E2Fs (E2F3a, E2F3b, E2F4 and E2F5) and the Rb family members (pRb, p107 and p130) comparing wild-type and E2F-mutant T lymphocytes in quiescence or 36 hours after TCR stimulation, near the G_1/S border.

We performed chromatin immunoprecipitations (ChIP) followed by PCR with primers to detect E2F binding within the promoters of four genes (Mcm2, Cdc6, Chk1, Cdc2a), whose expression was found to be upregulated in G_0 upon E2F2 ablation (see Fig. 3 and Suppl. Tables 1 and 2). As a control for specificity, we showed that an irrelevant antibody (anti-SV40 large T antigen) was unable to immunoprecipitate any of the various E2F target sequences in these experiments (mock lanes). As can be seen in Figure 6, ChIP analyses carried out with wild-type T cells revealed robust binding by E2F3b, E2F4 and p130 to all E2F-responsive promoters in quiescent cells (G_0) as well as after TCR stimulation, consistent with previous reports.[34,35] By contrast, E2F3a, E2F5 and p107 were absent or at low levels on G_0 promoters, but were recruited to E2F sites upon entering the cell cycle (Fig. 6B), suggesting a role for these factors in E2F-mediated gene regulation during the G_1/S transition. pRB binding to the target promoters was detectable in both G_0 and G_1/S, although it required more PCR cycles to detect. We could not detect E2F1 bound to these targets either in G_0 or after TCR stimulation. By contrast, E2F2 was efficiently recruited to target promoters in quiescence (Fig. 6A). Thus, we could observe binding of E2F2 to the promoters of the genes that are activated by loss of E2F2, implying a direct role for E2F2 in the transcriptional repression of these genes in G_0. Quantitative analysis of immunoprecipitated DNA carried out by real-time PCR on duplicate samples confirmed results obtained by gel electrophoresis (Fig. 6C).

Next, we determined the changes that occur in promoter occupancy after loss of E2F1 and/or E2F2. For this purpose, parallel ChIP analyses were performed using T cells derived from E2F1$^{-/-}$, E2F2$^{-/-}$ and DKO mice. As a control, we showed that the antibodies against E2F1 and E2F2 used in the ChIP experiments were specific for E2F1 and E2F2 since they did not immunoprecipitate any detectable promoter sequences from the E2F1$^{-/-}$ and E2F2$^{-/-}$ cells, respectively (Fig. 6). Thus, the specificity of the antibodies was confirmed. The pattern of promoter occupancy was altered by knockout of E2F1 or E2F2. E2F1$^{-/-}$ T cells in quiescence exhibited modestly increased binding of E2F5 and the pocket protein p107. By contrast the quiescent E2F2$^{-/-}$ T cells exhibited increased promoter binding by E2F3a and p107. The DKO cells were similar to the E2F2$^{-/-}$ cells, but with further increased binding by p107. Interestingly, the enhanced binding of E2F5 and p107 observed in the quiescent E2F1$^{-/-}$ cells recapitulates some of the findings in wild-type cells at the G_1/S transition. Similarly, the enhanced binding of E2F3a and p107 observed in the quiescent E2F2$^{-/-}$ or DKO cells recapitulates some of the findings in wild-type cells at the G_1/S transition. However, it is striking that these similar findings in E2F1$^{-/-}$ and E2F2$^{-/-}$ cells produce dramatically different effects on gene expression (slightly decreased expression in E2F1$^{-/-}$ cells but dramatically increased expression in E2F2$^{-/-}$ cells). We conclude that these changes in binding by other E2F family members or pocket proteins are not causal. Indeed, the change in E2F3a clearly cannot be causal since knockdown of E2F3 does not alter significantly the enhanced gene expression observed in E2F2$^{-/-}$ cells. Instead, we argue that the most significant change in promoter occupancy in the E2F2$^{-/-}$ (or DKO) cells is loss of E2F2 itself, strongly supporting the model that E2F2 functions to repress gene expression in G_0 to maintain the quiescent state.

Discussion

Previous work has revealed that E2F2 suppresses cellular proliferation and mediates immunological tolerance.[18] Here we have shown

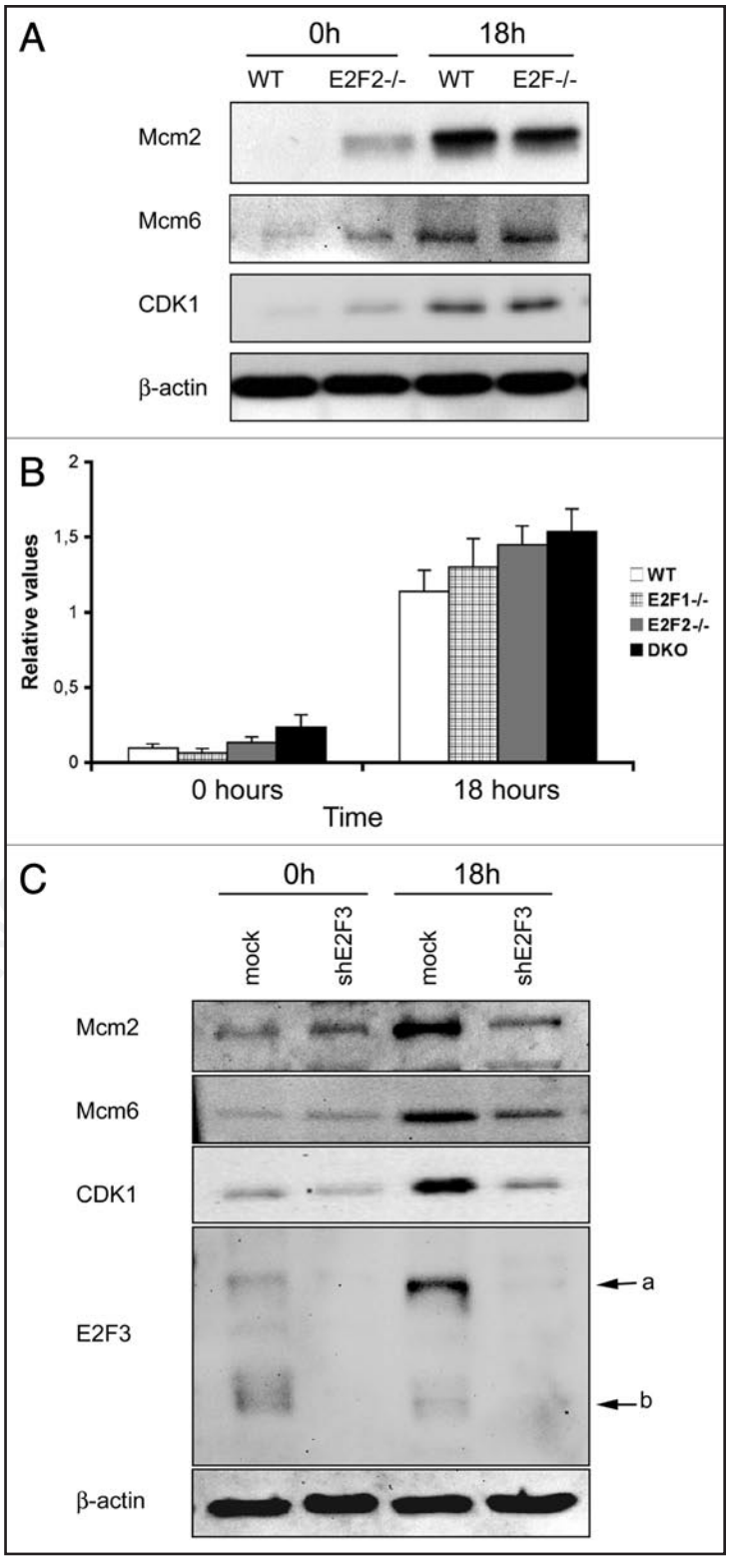


Figure 5. E2F3 is dispensable for aberrant E2F target gene expression in G_0 upon loss of E2F2. (A) E2F2 is required for repression of gene expression in quiescent MEFs. Western blot analysis of Mcm2, Mcm6 and Cdk1 in primary MEFs prepared from WT and E2F2$^{-/-}$ mice. Cells were brought to a quiescent state by serum starvation for 48 h, and then stimulated to grow by the addition of serum for 18 h. Proteins were detected by Western blot. The blot was subsequently reprobed with β-actin, demonstrating equal loading of the cell lysates. (B) Real-time PCR analysis of E2F3a expression. Total RNA was harvested from the indicated genotypes (n = 3 per genotype) and used to produce cDNA. A TaqMan probe specific for E2F3a was used for PCR amplifications. Expression values are normalized to the expression of Eef1a1, used as a standard control. Error bars: SEM. (C) Effect of E2F3 knockdown on E2F-target gene expression. Primary E2F2$^{-/-}$ MEFs were infected with mock or with siE2F3 viruses. Cells were brought to quiescence by serum starvation for 48 h after virus infection, and then stimulated to grow by the addition of 20% serum for 18 h. Cells were harvested 0h and 18 h after stimulation. Mcm2, Mcm6 and Cdk1 protein levels were evaluated by Western blot. A parallel gel was probed with β-actin to show equal loading of the cell lysates. These data are representative of three experiments.

that a large set of E2F target genes involved in cell cycle regulation is aberrantly activated by loss of E2F2 in resting T cells. Regulation of gene expression by E2F2 has been proposed to be restricted mainly to G_1/S, coinciding with an increase in E2F2 protein levels.[1-3] Thus, we were surprised to discover that in cells in G_0, hundreds of genes are deregulated in response to the loss of E2F2. Our results argue that the low steady-state levels of E2F2 found in G_0 are sufficient for promoter binding and for transcriptional regulation. Indeed, our chromatin immunoprecipitation experiments clearly demonstrate that E2F2 is bound to target promoters in cells in G_0 and disruption of the E2F2 gene results in an increase in expression of these same genes.

We have analyzed the contribution of other E2F members to the hyperproliferative phenotype and aberrant gene expression exhibited by E2F2$^{-/-}$ cells. Previous work would argue that E2F1 and E2F3 would be the most likely candidates to contribute to enhanced gene expression and cell cycle entry since they are strong transcriptional activators that when overexpressed can drive cell cycle entry.[4,5] Indeed, proliferating E2F2$^{-/-}$ cells do, in fact, show increased expression of E2F1.[18] Despite this correlation, our data clearly demonstrate that E2F1 is not required for the hyperproliferation and aberrant gene expression observed in E2F2$^{-/-}$ cells, as knockout of the E2F1 gene actually slightly enhances the effect of loss of E2F2. In the case of E2F3, it is intriguing that the increased occupancy of E2F target promoters by E2F3a in quiescent cells lacking E2F2 does not seem to play a significant role in upregulated gene expression. Indeed, knockdown of E2F3 by RNA interference does not restore normal gene expression in quiescent E2F2$^{-/-}$ MEFs, although we cannot rule out the possibility that the contribution of E2F3a to the elevated gene expression detected in quiescent E2F2$^{-/-}$ cells is bigger that what was revealed in our knockdown experiments, since the RNAi sequence depleted both the activator E2F3a and the repressor E2F3b isoforms. Taken together, our results support a direct role for E2F2 in repression of cell cycle genes in resting cells.

Unlike E2F2, loss of E2F3 is reported to result in lower levels of target gene expression and to reduced proliferation.[25] Concomitant inactivation of E2Fs 1, 2 and 3 results in a complete block of the cell cycle.[36] However, published data indicate that the mechanism is not due to a loss of E2F-dependent transcriptional activation of cell cycle genes. Instead, the mechanism appears to involve a loss of E2F3-dependent repression of the p19^{ARF} protein[12] or E2F3-dependent repression of p53-regulated genes.[37,38] In fact, activation of the p53 pathway has been proposed to be a primary consequence of E2F1-3 ablation.[37,38] By contrast, deregulation of the p53 pathway does not appear to contribute to E2F2-mediated cell cycle gene derepression,

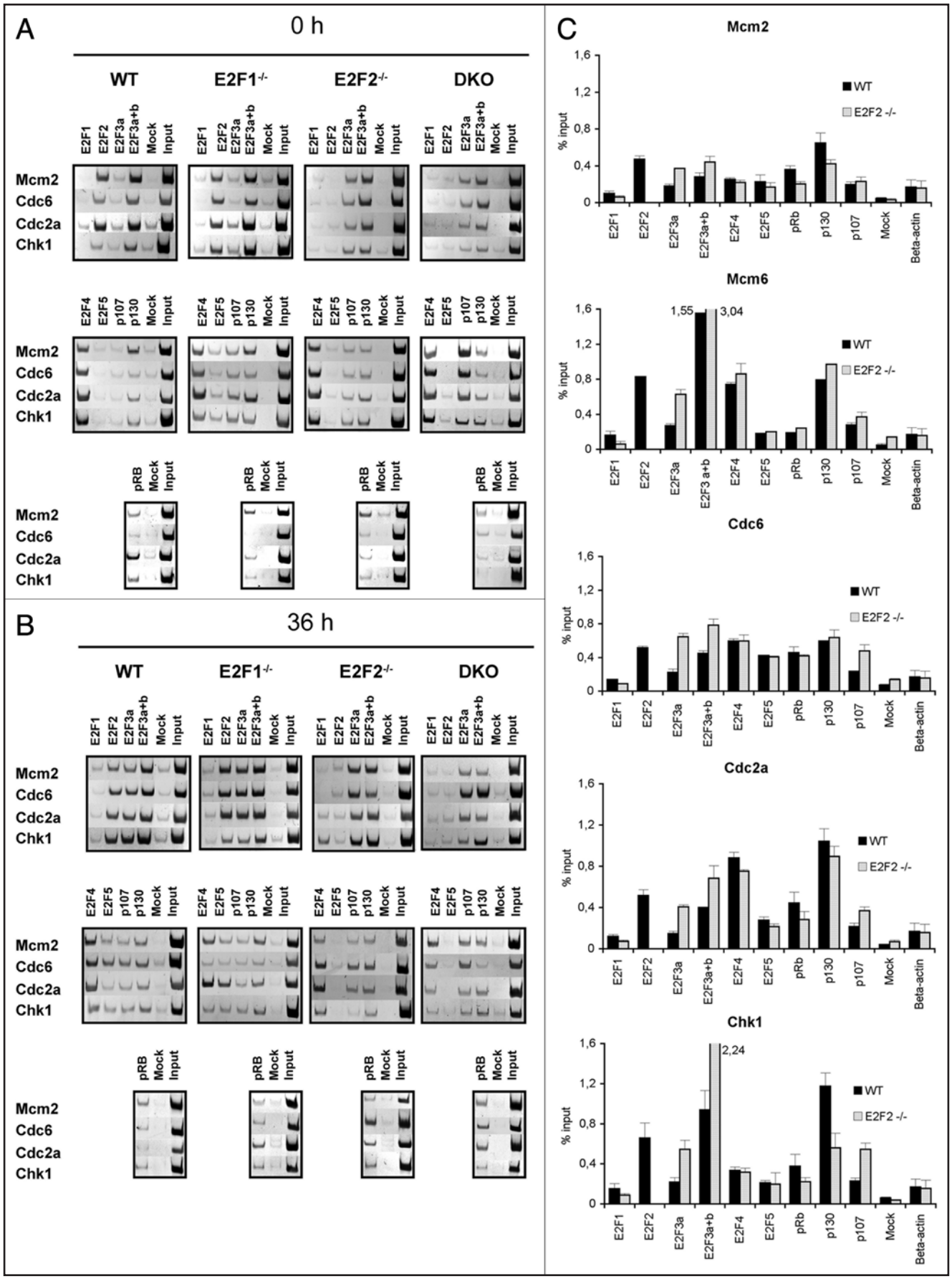


Figure 6. Figure legend on page 3924.

Figure 6. E2F2 binds to promoters in quiescence. Cell lysates from WT, E2F1$^{-/-}$, E2F2$^{-/-}$ and DKO T lymphocytes in quiescence (A) or stimulated with plate-bound anti-CD3 (B) were harvested and then used for chromatin immunoprecipitation assays with the indicated antibodies. Bound DNA was analyzed by PCR using primers around or near the E2F sites in the *Mcm2, Cdc6, Cdc2a* and *Chk1* promoters. Antibodies to large T-antigen were used as irrelevant antibody control (mock lanes). Input lanes correspond to PCR reactions with 0.5% of the amount of total chromatin used in the specific immunoprecipitation reactions. These data are representative of three experiments. (C) Quantification of precipitated DNA retrieved from chromatin IP experiments of quiescent WT and E2F2$^{-/-}$ T lymphocytes was carried out by real-time PCR where the amount of immunoprecipitated promoter DNA was expressed as a percentage of the total input chromatin. Analysis of the *Mcm6* promoter was also included in the Q-PCR experiments. The promoter of β-*actin*, a gene that is not an E2F target but is highly expressed in T lymphocytes, was used as a negative control.

based on our finding that p19^{ARF} and p21 levels in the absence of E2F2 are unchanged relative to wild-type cells in quiescence. In proliferating cells we did observe an increase in p21 levels in E2F2$^{-/-}$ and DKO cells that could be associated with the activation of the p53 pathway and the reduced expression of cell cycle genes. However, the activation of this pathway clearly temporally follows the aberrant gene expression observed in quiescent E2F2$^{-/-}$ cells. The model proposed for E2F3 function suggests that it allows cell cycle progression by serving to repress the p53 pathway. By contrast, our results suggest that E2F2 functions to allow quiescence by repressing the expression of genes in the classical Rb-E2F pathway.

E2F2 loss has been shown to induce cell cycle arrest in differentiating erythroblasts, which was associated with increased DNA double-stranded breaks.[22] This phenotype was not observed in T lymphocytes, perhaps owing to differences in cell types, although the elevated levels of p21 (see Fig. 3B), as well as the increased apoptotic rate detected after several days of TCR-stimulated proliferation in E2F2$^{-/-}$ and DKO samples (ref. 18; Infante A and Zubiaga AM, unpublished observations) is consistent with some type of damage operating in proliferating lymphocytes. Overexpression of the DNA replication licensing proteins has been shown to significantly increase the over-replication of chromosomal DNA, leading to accumulation of double strand DNA breaks and activation of the DNA damage response.[39] The upregulated levels of replication proteins, the premature assembly of Mcm2 on chromatin, and the inappropriate DNA replication activity that E2F2-deficient naïve T cells exhibit during the initial cell cycle rounds after stimulation could also induce DNA breaks in these cells. We did not observe the increased γH2AX levels that characterizes a DNA damage response, although it is possible that damaged T cells are instructed to activate apoptosis rapidly under these conditions, which could hinder the detection of markers of a typical DNA damage response.

In the absence of an additional genetic abnormality, loss of E2F2 in T cells does not inhibit apoptosis.[18] By contrast, in T cells that overexpress Myc it leads to an accelerated lymphomagenesis that is associated with an increased resistance to apoptosis.[20] Thus, the specific cellular context may determine the fate of a cell that lacks E2F2. Aberrant E2F2$^{-/-}$ T lymphocytes that would normally be eliminated could exhibit a higher survival rate in a pro-malignant environment, such as a deregulated Myc pathway, suggesting that E2F2 is a risk factor for tumor development.

Interestingly, there is a striking resemblance between the phenotype of E2F2-deficient cells and the phenotype of cells lacking pocket proteins. Like E2F2-deficient cells, Rb$^{-/-}$ cells and Rb/p107/p130 triple knockout (TKO) cells show an abbreviated G_1 phase with accelerated entry into S-phase.[40-43] Similarly, E2F2$^{-/-}$ and both Rb$^{-/-}$ or Rb/p107/p130 TKO cells demonstrate elevated expression of E2F target genes in G_0 and G_1.[40,44,45] Most of the genes that are reported to be aberrantly activated in pRb$^{-/-}$ or in p107/p130$^{-/-}$ cells are also aberrantly activated in E2F2-deficient cells (e.g., p107, cdc2, cyclinA). The similarity in phenotypes suggests that the maintenance of the G_0 state may be regulated primarily by repressor complexes containing E2F2 and pocket proteins.

Published results have argued that E2F2 interacts primarily with pRb.[46] Recently, it has been shown that defects in erythroblast differentiation observed in Rb$^{-/-}$ cells stem from deregulated E2F2 activity, suggesting that the intracellular concentration of E2F2 may be of critical importance for terminal differentiation.[22] Moreover, other reports, and our own work, show detectable binding of pRb and of p130 to E2F target promoters in quiescent T lymphocytes.[45,47] Furthermore, we observe a slight decrease in promoter occupancy by pRb and by p130 upon loss of E2F2, perhaps arguing for a role for E2F2-pRb and/or E2F2-p130 complexes in gene repression in G_0. Although our data hint at this possibility, further experiments will be required to identify the nature of the E2F2 and pocket protein complexes that are bound to promoters that are repressed by E2F2 in G_0. Alternatively, the transcriptional repression mediated by E2F2 could be independent of the pocket proteins, similar to the mechanism proposed for E2F3-mediated repression of p19^{ARF}.[12]

Recently, an E2F complex has been described that functions to repress gene expression involving E2F4 and p130. This complex, termed DREAM, has been reported to interact with chromatin modifying complexes of the MuvB group to carry out transcriptional repression mediated by E2F in G_0.[48-50] E2F2 has not been observed in these complexes, but this could reflect levels below the detection limits in their assays. Alternatively, E2F2 may function independently of the DREAM complex. It remains possible that in different cell types different E2F family members are involved in repression of gene expression in G_0. However, our observation that E2F2 is required for G_0-specific gene repression in both T lymphocytes and embryonic fibroblasts, together with published data that E2F4 is dispensable for this role,[16] suggest that E2F2 plays a unique role in maintaining cell cycle quiescence. The loss of E2F2 produces cells that are functionally quiescent, but have characteristics of cells at the G_1/S border, including increased expression of cyclins and cdks, and assembly of origin of replication complexes on chromatin. The E2F2$^{-/-}$ cells are poised to rapidly enter S phase in response to a signal. It is surprising that cells can essentially skip much of G_1 and still proliferate normally. Future studies of E2F2$^{-/-}$ cells may help to identify the evolutionary advantage of the normal prolonged delay between receipt of a proliferative signal and commencement of DNA replication.

Materials and Methods

Mouse strains and MEF preparation. Colonies of E2F1$^{-/-}$,[24] E2F2$^{-/-}$,[18] and double null E2F1/2$^{-/-}$ mice were maintained in a C57Bl6:129Sv background on a normal light/dark cycle in cages with microisolator lids, and were genotyped by standard PCR technology, as previously described.[18,51] All procedures were approved by the University of the Basque Country Animal Care and Use Committee.

Mouse embryonic fibroblasts (MEFs) were prepared from embryos of the different genotypes at day 13.5 of gestation as previously described.[52] After disaggregation of embryos and brief expansion, MEFs from individual embryos were stored in liquid nitrogen until use. Plating of MEFs after thawing was considered passage 1. Cells were maintained in DMEM medium containing 10% FCS and a cocktail of antibiotics in a CO_2 incubator at 37°C.

Harvest, purification and activation of T lymphocytes. Complete medium (RPMI 1640 supplemented with 10% FCS, 2 mM l-glutamine, 50 U/ml penicillin and 50 ug/ml streptomycin) was used for cell preparation and culture. Lymph nodes were harvested from 4-to-6 week-old wild-type, E2F1$^{-/-}$, E2F2$^{-/-}$ and DKO mice as previously described,[24] with minor modifications. Lymph node T cells were purified by negative enrichment, consisting of B lymphocyte depletion with magnetic beads coated with biotinylated anti-B220 antibodies, followed by separation with a magnetic particle concentrator (IMagnet, Becton-Dickinson). More than 95% of the remaining cells were T lymphocytes.

For analysis of TCR-mediated responses, purified T lymphocytes (10^6/ml) were stimulated for the indicated times with immobilized antibodies against CD3 (145.2C11, 0.1–1.5 μg/well; BD).

Cell cycle and proliferation analyses. Cells were pulse-labeled with 10 μM BrdU for 20 min, washed in ice-cold phosphate-buffered saline (PBS), and fixed in ice-cold 70% ethanol. One to ten days later, cells were processed for flow cytometric analysis of DNA content (staining with propidium iodide) and DNA synthesis (staining with fluorescein isothiocyanate-conjugated antibody to BrdU) according to a protocol from the supplier of the antibody (BD).

For carboxyfluorescein diacetate succinimidyl ester (CFSE) staining, 10^7 cells/ml were incubated with 3 μM CFSE (Molecular Probes) in PBS for 15 min at 37°C, washed with PBS and with RPMI for 30 min, and then cultured in complete medium for 3 days in the presence of anti-CD3. Fluorescence was detected and analyzed using a FACSCalibur (BD) flow cytometer.

Western blotting. Cells were lysed in lysis buffer containing 10 mM $NaPO_4H$ pH 7.2; 1 mM EDTA; 1 mM EGTA; 150 mM NaCl; 1% NP-40, and a cocktail of protease and phosphatase inhibitors (Roche). Protein concentrations in supernatants were determined using a commercially available kit (CD Protein Assay from Bio-Rad). Twenty μg were loaded per lane, fractionated by SDS-PAGE in 10–12% polyacrylamide gels, and transferred onto nitrocellulose membranes (Bio-Rad). Antibodies against the following proteins were used: MCM2 (559542, BD), MCM6 (sc-22781, Santa Cruz), CDC6 (sc-9964, Santa Cruz), Cyclin D2 (sc-593, Santa Cruz), Cyclin D3 (sc-182, Santa Cruz), Cyclin E1 (sc-481, Santa Cruz), Cyclin A2 (sc-596, Santa Cruz), cyclin B1 (4135, Cell Signalling), CDK2 (558896, BD), CDK4 (sc-260, Santa Cruz), CDK1 (sc-54, Santa Cruz), MEK2 (554098, BD), E2F3 (sc-878, Santa Cruz), β-Actin (A5441, Sigma). Immunocomplexes were visualized with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibodies (Amersham), followed by chemiluminiscence detection (ECL, Amersham) with a ChemiDoc camera (Bio-Rad). Western analyses were performed at least two, but most often three times.

Chromatin-binding and immunoprecipitation analyses. For chromatin binding analyses, chromatin was isolated as described[53] with minor modifications. Cells (1.5 x 10^6) were lysed in CSK buffer containing 10 mM PIPES pH 7.0, 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 0.5% Triton X-100, 0.5 mM PMSF and 1X of protease inhibitor cocktail (Roche). Lysates were incubated on ice for 20 min and then centrifuged at 300 xg for 5 min at 4°C. The supernatant (soluble fraction) was further clarified by high-speed centrifugation (10 min, 20,000 xg, 4°C) to remove cell debris and insoluble aggregates. Chromatin pellets were washed with lysis buffer for 5 min on ice and centrifuged again. The final chromatin pellet was resuspended in lysis buffer, sonicated in an ultrasonic cell disruptor (Misonix) and resuspended in Laemli buffer. The samples were boiled for 5 min and analyzed by SDS-PAGE and Western bloting.

For chromatin immunoprecipitation analyses, T lymphocytes were crosslinked by addition of formaldehyde to 1.1% final concentration. Crosslinking was allowed to proceed at room temperature for 10 min and was stopped with glycine. Cells were washed with PBS, collected by centrifugation and lysed on ice for 10 min with a buffer containing 5 mM PIPES pH 8.0, 85 mM KCl, 0.5% NP-40, 1 mM PMSF and 1X of protease inhibitor cocktail (Roche). Nuclei were collected by centrifugation, resuspended in 50 mM Tris-Cl pH 8, 10 mM EDTA pH 8.0, 1% SDS, 1 mM PMSF and 1X protease inhibitor cocktail, and incubated for 10 min on ice. Chromatin was sonicated on a Misonix sonicator to an average length of 300–400 bp. After microcentrifugation, the supernatant was diluted 10-fold in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-Cl pH 8.0, 167 mM NaCl, 1 mM PMSF and protease inhibitor cocktail) and pre-cleared with protein A-sepharose (Amersham, 50% slurry, blocked with salmon sperm DNA and BSA) at 4°C for 3 h. 100–120 μg of precleared chromatin were incubated overnight at 4°C with 4–5 μg of each antibody: E2F1 (sc-251), E2F2 (sc-633), E2F3 (sc-878), E2F3 (sc-879), E2F4 (sc-1082), E2F5 (sc-999), p107 (sc-318), p130 (sc-317), pRb (sc-50), SV40Tag (sc-147), all from Santa Cruz. Next, samples were incubated with protein A-sepharose at 4°C for 2 h. Immune complexes were recovered and washed once with Low Salt Wash Buffer (20 mM Tris-Cl pH 8.0, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl), High Salt Wash Buffer (20 mM Tris-Cl pH 8.0, 0.1% SDS, 1% Triton X-100, 2 mM EDTA pH 8, 500 mM NaCl) and LiCl Wash Buffer (10 mM Tris-Cl pH 8.0, 0.25 M LiCl, 1% NP-40, 1% Na-deoxycholate, 1 mM EDTA pH 8.0) and three times with TE buffer. The elution of the immunecomplexes was carried out with a buffer containing 0.1 M $NaHCO_3$ and 1% SDS. Crosslinking was reversed by addition of NaCl to a final concentration of 200 mM followed by an overnight incubation at 65°C and RNA was removed with 10 μg of RNAse A. Proteins were digested with 80 μg of proteinase K at 42°C for 2 h, and the DNA extracted with phenol-chloroform and ethanol precipitation.

The DNA was amplified with primers complementary to the Chk1, Cdc2, Mcm2 and Cdc6 promoters (The PCR primers are shown in Table 3 of Suppl. Material). The PCR reaction conditions were as follows: 94°C 30 s, 60°C 30 s, 72°C 45 s. We ensured linear amplification in all cases, and reactions were performed a minimum of two, but most often three, times. The PCR products were resolved on 12% polyacrilamide gels, stained with ethidium bromide and photographed under UV illumination.

Quantification of immunoprecipitate-enriched DNA sequences was performed by real-time PCR with Power SYBR Green PCR

Master Mix and the Applied Biosystems 7900 Fast Real-Time PCR System, and analysis was done with the SDS 2.2.1 software. Samples were analyzed in triplicate. Data are represented as percentages of the input, calculated by $2^{\Delta CT}$ x 20, where ΔCT is determined by $CT_{a\ dilution\ of\ the\ input}$ - $CT_{IP\ sample}$ and 20 refers to the total input being 20% of the chromatin amount exposed to IP.

shRNA virus construction and infection. Second-generation self-inactivating lentiviral plasmid pLVTHM, and packaging plasmids pCMVΔR8.91 and pMD2.G, were kindly provided by Dr. D. Trono (University of Geneva, Switzerland) and have been previously described.[54] The sequence for knocking down mouse E2F3,[55] was first cloned into pSUPER and subcloned subsequently into pLVTHM.

For lentivirus production, HEK293T cells were transfected simultaneously with pLVTHM, pCMVΔR8.91 and pMD2.G, by the calcium phosphate method. After incubating plates for 36 h in OptiMEM (Invitrogen Inc.,), supernatants containing lentivirus at approximately 5 x 10^6 TU/ml were collected.

MEFs were trypsinized and infected with empty control or siE2F3 viruses at relative MOI of 20. For each infection, 2 x 10^6 cells were incubated with virus for 48 h, and plated into DMEM supplemented with 0.1% FBS for 4 days, and then stimulated by the addition of 20% serum. Cells were harvested 16 h after stimulation. Cells infected with virus were identified with GFP signal by flow cytometry (~90% of the transduced cells yielded a positive signal).

RNA isolation and RT-PCR. Total RNA was prepared from cells using TRIzol reagent (Invitrogen), according to the manufacturer's instructions, purified using RNeasy (Quiagen), and electrophoresed on a denaturing agarose gel to examine for RNA integrity. cDNA was synthesized from 2.5 μg of total RNA using Superscript First-Strand Synthesis System for RT-PCR (Invitrogen). Real-time PCR was performed on several cDNA dilutions plus 1x SYBR green PCR Master Mix (Applied Biosystems) and 300 nM of primers (sequences available upon request). E2F3a expression was determined with a custom-made TaqMan gene expression assay (sequences available upon request) following the manufacturer's instructions (Applied Biosystems). Reactions were carried out at the Genomics Facility of the University of the Basque Country using an ABI Prism 7900 SDS (Applied Biosystems) for 40 cycles (95°C for 15 s and 60°C for 1 min) after an initial 10-min incubation at 95°C. Each PCR generated only the expected amplicon as shown by the melting-temperature profiles of the final products. Standard curves were calculated using cDNA to determine the linear range and PCR efficiency of each primer pair. Reactions were done in triplicate, and relative amounts of cDNA were normalized to the internal control *Eef1a1*. Statistical analyses were performed using one-way ANOVA simple contrasts to carry out comparisons among groups at a significance level of $p < 0.05$ with SPSS 14.0 program.

cDNA microarrays. RNA samples from purified T lymphocytes (n = 9 for each genotype) were obtained, before or after 36 h of anti-CD3 stimulation. Three pools were made from either $E2F1^{-/-}$, $E2F2^{-/-}$ or E2F1/E2F2 double-mutant mice. Each pool was a mix of RNA samples from three mice of the same genotype. A unique pool was made with RNA samples from nine WT mice at each time-point and was used as control in all hybridizations. Each pool of RNA samples was used to generate a cDNA with the Superscript First-Strand Synthesis System for RT-PCR (Invitrogen). Then, the aRNA was again retrotranscribed into cDNA and this cDNA was labeled with Cy3-dUTP or Cy5-dUTP with the SuperscriptTM II RNase H Reverse Transcriptase kit (Invitrogen), purified with GFX columns (Amersham) and fragmented. Target cRNA was hybridized overnight to each cDNA microarray containing probes for approximately 16,000 murine genes and expressed sequence tags (ESTs). Microarrays were fabricated with a set of mouse cDNA clones of the National Institute of Aging (http://lgsun.grc.nia.gov/cDNA/15K.htlm) containing approximately 15,000 unique clones derived from ESTs isolated from mouse embryos and female gonads. Additionally, a set of approximately 600 clones representing genes related to the cell cycle that were not present in the NIA set (obtained from Research Genetics Arrays) were included in the microarray. A total of six hybridizations were performed for each time-point and genotype (one hybridization for each mutant pool vs. control pool, and its corresponding dye-swap hybridization). Thus, each biological replicate has two technical replicates. After hybridization, microarrays were washed and scanned on a DNA microarray scanner (Agilent Technologies). Microarray gene annotations were stored in Array Express (http://www.ebi.ac.uk/arrayexpress/) (accession number E-MEXP-945), which complies with MIAME (Minimal Information About a Microarray Experiment) guidelines.

Analysis of microarray data. Fluorescence intensities were analyzed with GenePix 4.0 software (Axon Instruments). For background we used the median spot intensity, and for foreground we used the mean spot intensity, as indicated by GenePix software. Average intensity and red and green foregrounds showed print order effect patterns, similar across the 38 arrays that were corrected using loess on the print order trend.[27] Next, we subtracted median background from corrected foreground, and used print-tip loess normalization[28,29] to correct for possible systematic variations in log-ratio with mean intensity.

Whereas normalization was based on all the spots, analyses were restricted to the relevant clones. First, we excluded all control spots, including human (non-mouse) genes, negative controls, and no-DNA spots. Second, we excluded all the spots that had two, or more, missing values per treatment condition (i.e., we excluded all spots with more than 1/3 missing values per condition).

Fluorescence intensity data of each spot were analyzed using one-sample t-tests,[27] after computing the mean over two technical and three biological replicates for each genotype and time-point (n = 6), and $p < 0.01$ values were considered as significant. To minimize the number of false positives we applied the False Discovery Rate method of Benjamini & Hochberg.[30]

All analyses were carried out in R (http://cran.r-project.org). For the microarray-specific parts we used the limma package from Bioconductor (http://www.bioconductor.org).

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Note

Supplementary materials can be found at:
www.landesbioscience.com/supplement/InfanteCC7-24-Sup.pdf

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