E2F4 loss suppresses tumorigenesis in *Rb* mutant mice

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Summary

The E2F transcription factors mediate the activation or repression of key cell cycle regulatory genes under the control of the retinoblastoma protein (pRB) tumor suppressor and its relatives, p107 and p130. Here we investigate how E2F4, the major "repressive" E2F, contributes to pRB's tumor-suppressive properties. Remarkably, E2F4 loss suppresses the development of both pituitary and thyroid tumors in $Rb^{+/-}$ mice. Importantly, E2F4 loss also suppresses the inappropriate gene expression and proliferation of pRB-deficient cells. Biochemical analyses suggest that this tumor suppression occurs via a novel mechanism: E2F4 loss allows p107 and p130 to regulate the pRB-specific, activator E2Fs. We also detect these novel E2F complexes in pRB-deficient cells, suggesting that they play a significant role in the regulation of tumorigenesis in vivo.

Introduction

The retinoblastoma protein (pRB) was the first identified tumor suppressor, and it is mutated in approximately one third of all human tumors. pRB blocks cells in G1 by inhibiting the activity of a cellular transcription factor, E2F, that controls the expression of key components of the cell cycle and DNA replication machinery (reviewed in Dyson, 1998; Trimarchi and Lees, 2002). pRB regulates E2F through two distinct mechanisms. First, its association is sufficient to block E2F transcriptional activity. Second, the pRB-E2F complex can recruit histone deacetylases (HDACs) to the promoters of E2F-responsive genes and thereby actively repress their transcription. Cell cycle entry requires the phosphorylation of pRB and its subsequent dissociation from E2F. This phosphorylation is mediated by cell cycle-dependent kinase complexes, cyclin D-CDK4/6, and cyclin E-CDK2. Importantly, tumors that retain wild-type pRB almost always carry activating mutations in *cyclin D1* or *CDK4* or inactivating mutations in the cdk4 inhibitor, *p16* (reviewed by Bartek et al., 1996; Sherr, 1996). This suggests that the functional inactivation of pRB, and the resulting deregulation of E2F, is an essential step in tumorigenesis.

pRB belongs to a family of proteins, called the pocket proteins, that also includes p107 and p130 (reviewed by Dyson, 1998; Trimarchi and Lees, 2002). p107 and p130 share many properties with pRB: they bind to E2F in vivo, inhibit E2F transcriptional activity, and recruit HDACs to mediate the active repression of E2F-responsive genes. However, there are dramatic differences in the tumor-suppressive properties of the individual pocket proteins (reviewed by Mulligan and Jacks, 1998). Inheritance of a single *Rb* mutant allele predisposes both mice and humans to tumors with 100% penetrance. The tumors consistently lose the wild-type *Rb* allele, confirming that pRB behaves as a classical tumor suppressor. In contrast, the loss of p107 and/or p130 does not appear to promote tumorigenicity in mice or cells (Cobrinik et al., 1996; Lee et al., 1996). Yet there is growing evidence that mutation of *p107* and/or *p130* promotes tumor formation when pRB is also inactivated. This is exemplified by $Rb^{-/-};p107^{-/-}$ chimeric mice, which develop an additional tumor type, retinoblastoma, compared to $Rb^{-/-}$ chimeras (Robanus-Maandag et al., 1998), and $Rb^{-/-};p107^{-/-};p130^{-/-}$ mouse embryonic fibroblasts (MEFs), which are more tumorigenic than $Rb^{-/-}$ controls (Dannenberg et al., 2000; Sage et al., 2000). Biochemical and mechanistic studies in cells deficient for different pocket protein family mem-

SIGNIFICANCE

Understanding how the E2F and pRB family members contribute to the regulation of tumorigenesis is a key goal. Our finding of tumor suppression in the $Rb^{+/-};E2f4^{-/-}$ mice through the formation of novel E2F complexes in $Rb^{+/-};E2f4^{-/-}$, $Rb^{-/-};E2f4^{-/-}$, and $Rb^{-/-}$ cells strongly suggests that tumor formation is critically and exclusively dependent upon the inactivation of pRB, rather than p107 or p130, because it triggers the release of the normally pRB-specific, activator E2Fs. However, p107 and p130 assume significant tumor-suppressive properties in pRB-deficient cells because they can substitute for pRB in the regulation of these activator E2Fs. This model suggests a novel strategy for the generation of chemotherapeutics that would act by increasing the available pools of p107 and p130.

bers should help to identify the critical, tumor suppressive function(s) of pRB.

To date, eight genes have been identified as components of the E2F transcriptional activity (reviewed by Dyson, 1998; Helin, 1998). These genes have been divided into two distinct groups: the *E2fs* (*E2f1* through *E2f6*) and the *DPs* (*DP1* and *DP2*). The protein products from these two groups heterodimerize to give rise to functional E2F activity (Bandara et al., 1993; Helin et al., 1993; Krek et al., 1993). The functional specificity of the E2F-DP complex is primarily determined by the identity of the E2F subunit. The pocket protein binding E2Fs can be divided into two subgroups that appear to have opposing roles in vivo (reviewed by Trimarchi and Lees, 2002).

The first E2F subgroup includes E2F1, 2, and 3. These E2Fs play a key role in promoting the activation of E2F-responsive genes, and thereby cell cycle entry. Chromatin immunoprecipitation (ChIP) experiments confirm that these E2Fs associate with the promoters of known target genes coincident with their activation in late G_1 (Rayman et al., 2002; Takahashi et al., 2000). MEFs lacking E2F3 or E2F1, E2F2, and E2F3 exhibit reduced E2F target gene expression and significant proliferative defects (Humbert et al., 2000b; Wu et al., 2001). Furthermore, the ectopic expression of E2F1, 2, or 3 is sufficient to induce quiescent cells to initiate E2F-responsive gene expression and cell cycle re-entry (DeGregori et al., 1997; Lukas et al., 1996). Importantly, these so-called "activator" E2Fs are specifically regulated by pRB but not by p107 or p130 in vivo (Moberg et al., 1996).

E2F4 and E2F5 represent the second E2F subgroup. The transcriptional properties of these E2Fs are largely determined by their subcellular localization (Gaubatz et al., 2001; Magae et al., 1996; Muller et al., 1997; Verona et al., 1997). The endogenous E2F4-DP and E2F5-DP complexes are localized in the cytoplasm and are therefore unable to contribute to the activation of E2F-responsive genes. However, pocket protein binding enables the nuclear localization of E2F4 and E2F5. As a result, E2F4 and E2F5 appear to be primarily involved in the active repression of E2F-responsive genes. E2F4 associates with pRB, p107, and p130 in vivo and accounts for the majority of the repressive pocket protein complexes (Moberg et al., 1996). E2F5 is expressed in G_0 cells and is primarily regulated by p130 (Hijmans et al., 1995; Sardet et al., 1995). ChIP assays confirm that E2F4, p107, p130, and HDAC specifically associate with E2F-responsive promoters in G_0/G_1 cells under physiological conditions (Rayman et al., 2002; Takahashi et al., 2000). Importantly, MEFs deficient for E2F4 and E2F5 are unable to arrest in G_1 in response to a variety of growth arrest signals, suggesting that the repressive E2Fs promote cell cycle arrest (Gaubatz et al., 2000).

Considerable attention has focused on understanding how the growth-suppressive properties of pRB relate to its role in the inhibition of the activating E2Fs versus its participation in repressive pRB-E2F complexes. The analysis of *Rb;E2f1* and *Rb;E2f3* compound mutant mice has shown that the absence of E2F1 or E2F3 is sufficient to suppress both the ectopic S phase entry and p53-dependent apoptosis arising in pRB-deficient embryos (Tsai et al., 1998; Ziebold et al., 2001). Moreover, E2F1 deficiency significantly diminishes the development of tumors in $Rb^{+/-}$ mice (Yamasaki et al., 1998). These data suggest that the inappropriate release of the activator E2Fs makes a significant contribution to the phenotypic consequences of pRB deficiency. However, these experiments do not rule out a role for the repressive pRB-E2F complexes in tumor suppression. Indeed, numerous overexpression studies have led to the conclusion that regulation of E2F-responsive genes, and therefore cell cycle entry, is largely controlled by the repressive, and not activating, E2Fs (Dahiya et al., 2001; Zhang et al., 1999, 2000). In this study, we use *Rb;E2f4* compound mutant mice to investigate whether repressive E2F complexes contribute to tumor suppression. This analysis shows that the absence of E2F4 suppresses the formation of pRB-deficient tumors by promoting the formation of novel complexes between the activating E2Fs and p107 and p130 as well as correcting inappropriate target gene expression and cell growth. Most significantly, these data provide support for a model in which pocket proteins function as tumor suppressors by controlling activator E2Fs rather than by forming repressive E2F complexes.

Results

Loss of E2F4 extends lifespan and alters tumorigenesis in *Rb* mutant mice

Overexpression studies strongly suggest that the repressive E2F-pocket protein complexes play a critical role in controlling the expression of E2F-responsive genes. Given this finding, we wished to establish whether these repressive E2F-pocket protein complexes contribute to tumor suppression. E2F4 is the major repressive E2F in vivo, accounting for the majority of the endogenous pRB-, p107- and p130-associated E2F activity. Thus, if the repressive E2F complexes are important, E2F4 loss should exacerbate the formation of pRB-deficient tumors. To test this hypothesis, we intercrossed *Rb* and *E2f4* mutant mouse strains with the same C57BL/6 X 129/Sv mixed background. We then compared the lifespan and tumor phenotype of $Rb^{+/-}$, $Rb^{+/-}$;$E2f4^{+/-}$ and $Rb^{+/-}$;$E2f4^{-/-}$ littermates.

The phenotype of the $Rb^{+/-}$ mice was entirely consistent with previous studies (reviewed by Mulligan and Jacks, 1998). All mice died between 8.5 and 13.9 months of age (Figure 1A). Histological examination confirmed that the cause of death was intermediate lobe pituitary tumors and that the vast majority of the $Rb^{+/-}$ animals (23/27) also displayed c-cell thyroid tumors (Figures 1A and 1B; data not shown). Mutation of a single *E2f4* allele did not significantly alter the lifespan of $Rb^{+/-}$ animals (Figure 1A). Moreover, the $Rb^{+/-}$;$E2f4^{+/-}$ mice developed pituitary (55/57) and thyroid (47/57) tumors that were comparable to those arising in the $Rb^{+/-}$ controls with respect to both incidence and size (Figure 1B; data not shown). Thus, a reduction in the levels of E2F4 had no notable effect on tumorigenicity in the *Rb* mutant mice.

Remarkably, the phenotype of $Rb^{+/-}$;$E2f4^{-/-}$ animals diverged considerably from those of their littermate controls. First, there was a significant difference ($p = 0.0033$) in lifespan of the $Rb^{+/-}$;$E2f4^{-/-}$ versus the $Rb^{+/-}$ animals (Figure 1A; Table 1). Two of the $Rb^{+/-}$;$E2f4^{-/-}$ mice died at early ages (2.7 and 5.4 months) as a result of an increased susceptibility to infections. This is a characteristic phenotype of the $E2f4^{-/-}$ mice and was therefore an anticipated outcome for a fraction of the $Rb^{+/-}$;$E2f4^{-/-}$ mice. However, we unexpectedly found that neither of these animals had any evidence of tumorigenic lesions (data not shown), even though such lesions are routinely observed in the pituitaries of $Rb^{+/-}$ mice by 3 months of age (Nikitin and Lee, 1996). Most importantly, the majority of the $Rb^{+/-}$;$E2f4^{-/-}$ mice (17/19) survived at least until the window of lethality of the $Rb^{+/-}$ littermate

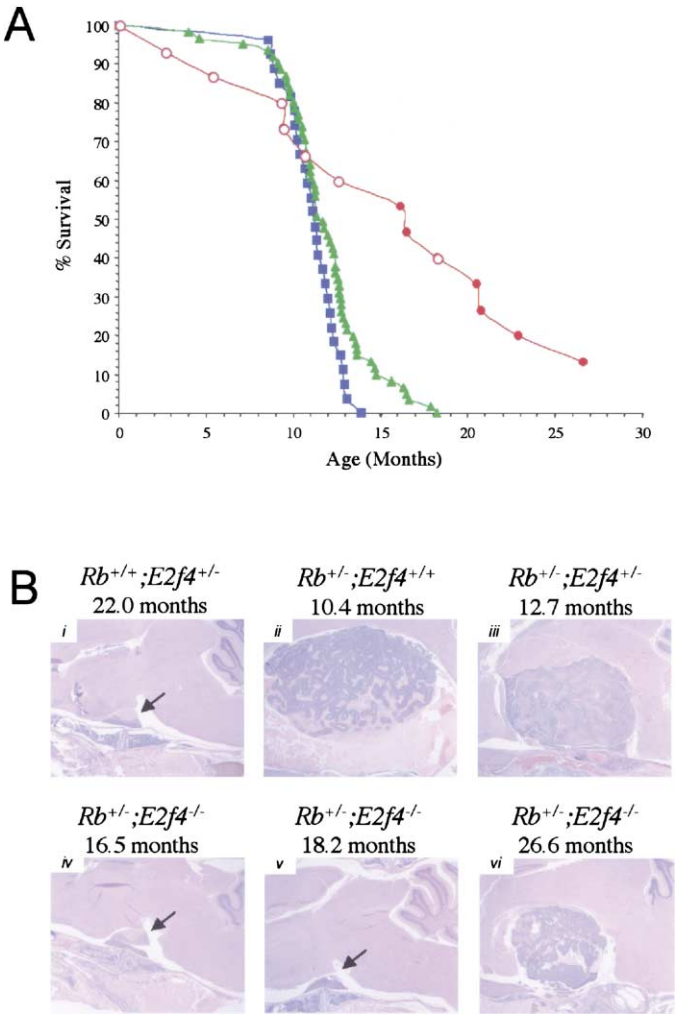

Figure 1. Loss of E2F4 extends the lifespan of $Rb^{+/-}$ adults by reducing the tumor incidence

A: Survival curves for $Rb^{+/-}$ (blue, n = 27), $Rb^{+/-}$;$E2f4^{+/-}$ (green, n = 61), and $Rb^{+/-}$;$E2f4^{-/-}$ (red, n = 15) mice. $Rb^{+/-}$;$E2f4^{-/-}$ mice with no evidence of tumor formation (open circles) and those with tumors (closed circles) are shown, but the animals that were prematurely sacrificed are not included. **B:** Representative H&E stained median sections of adult heads including the pituitary (marked with an arrow) from (i) control $Rb^{+/+}$;$E2f4^{+/-}$ animal, (ii) $Rb^{+/-}$;$E2f4^{+/+}$, (iii) $Rb^{+/-}$;$E2f4^{+/-}$, and (iv–vi) $Rb^{+/-}$;$E2f4^{-/-}$ mice. The older $Rb^{+/-}$;$E2f4^{-/-}$ mice have a variety of pituitary phenotypes including (iv) small, early pituitary tumors, (v) normal pituitaries, and (vi) medium intermediate lobe pituitary tumors. Magnification, 20×.

controls (8.5–13.9 months). Moreover, 4 months after the death of the oldest surviving $Rb^{+/-}$ animal, half of the $Rb^{+/-}$;$E2f4^{-/-}$ mice remained alive and healthy. Indeed, a significant fraction of the $Rb^{+/-}$;$E2f4^{-/-}$ animals lived to an age (20–27 months) comparable to wild-type controls (Figure 1A; Table 1). Thus, the absence of E2F4 actually extended the lifespan of the $Rb^{+/-}$ mice.

Consistent with the prolonged lifespan, E2F4 loss greatly suppressed the formation of tumors in the $Rb^{+/-}$ mice (Table 1). Histological examination showed that the vast majority of the $Rb^{+/-}$;$E2f4^{-/-}$ animals died as a result of defects typical of the $E2f4^{-/-}$ mice. Indeed, prior to 16 months of age, none of the $Rb^{+/-}$;$E2f4^{-/-}$ mice displayed any evidence of pituitary tumors, although they were detected in some of the older $Rb^{+/-}$;$E2f4^{-/-}$ mice (Table 1). However, the incidence of pituitary tumors was significantly lower than in the $Rb^{+/-}$ controls ($p = 0.000092$), and there was a considerable range in size in the tumors that did arise in the $Rb^{+/-}$;$E2f4^{-/-}$ animals (Table 1; Figure 1B; data not shown). Three of the older $Rb^{+/-}$;$E2f4^{-/-}$ animals (16.2, 20.5, and 23 months) eventually developed tumors comparable to those seen in the $Rb^{+/-}$ mice (8.5–13.9 months), but others developed very early lesions or mid-sized tumors (16.5 and 26.6 months), and two animals had completely normal pituitaries (18.2 and 18.5 months).

E2F4 loss had an even greater effect on the development of thyroid tumors in $Rb^{+/-}$ mice ($p = 0.00000034$). Despite the extremely high incidence of c-cell thyroid tumors in the $Rb^{+/-}$ (23/27) and $Rb^{+/-}$;$E2f4^{+/-}$ (47/57) animals, only 1/17 of the $Rb^{+/-}$;$E2f4^{-/-}$ mice developed a thyroid tumor (Table 1; data not shown). Indeed, there was no evidence of thyroid hyperplasia in the remaining 16/17 $Rb^{+/-}$;$E2f4^{-/-}$ animals. Thus, we conclude that the absence of E2F4 dramatically suppresses the development of both pituitary and thyroid tumors in the $Rb^{+/-}$ mice and thereby greatly extends their lifespan.

Loss of E2F4 induces profound rearrangement of E2F-pocket protein complexes

We initiated the tumor studies with the expectation that E2F4 loss would either have no effect on or would exacerbate the formation of pRB-deficient tumors depending on whether or not the repressive E2F-pocket protein complexes were important for tumor suppression. Instead, our data clearly show that E2F4 loss inhibits the formation of tumors. To establish the underlying mechanism, we characterized the effect that E2F4 loss had on the remaining E2F-pocket protein complexes. Initially, we compared the E2F complexes present in extracts from wild-type, $Rb^{+/-}$, and $Rb^{+/-}$;$E2f4^{-/-}$ MEFs by immunoprecipitating specific E2Fs and then Western blotting to identify the associated pocket proteins. Consistent with previous studies, E2F1 and E2F3 bound specifically to pRB in wild-type and $Rb^{+/-}$ cells (Figure 2; data not shown). In contrast, in $Rb^{+/-}$;$E2f4^{-/-}$ MEFs, activating E2Fs participated in novel pocket protein complexes in addition to binding to pRB. Specifically, E2F1 bound to p130, and E2F3 associated with p107. This was not due to an alteration of E2F1 or E2F3 levels since steady-state amounts of these proteins were not affected in cells deficient for either pRB or pRB and E2F4 relative to wild-type MEFs (see Supplemental Figure S1C at http://www.cancercell.org/cgi/content/full/2/6/463/DC1; data not shown). Thus, E2F4 loss allows p107 and p130 to substitute for pRB by binding E2F1 and E2F3.

Since the activating E2Fs are known to be important downstream targets of the pRB tumor suppressor, the formation of novel complexes between activating E2Fs and p107 and p130 could account for the suppression of tumors in the $Rb^{+/-}$;$E2f4^{-/-}$ mice. To address this issue, we used electrophoretic mobility shift assays to establish whether these novel complexes were present in $Rb^{+/-}$;$E2f4^{-/-}$ tissues. For these experiments, we immunoprecipitated p107 from extracts derived from several tissues, including the pituitary, which is prone to tumors in $Rb^{+/-}$ animals. The associated E2F species were released by the addition of the detergent deoxycholate (DOC) and then identified in electrophoretic mobility shift assays (Figure 3). Regardless of the tissue examined, p107 associated specifically with E2F4 in

Table 1. Histological analysis of *Rb*$^{+/-}$;*E2f4*$^{-/-}$ mice

Age (months)	Pituitary tumor	Thyroid tumor	Cause of death
2.7	—	—	Sinusitis
5.4	—	—	Pylonephritis
8.7	—	—	Sacrificed early
9.3	—	—	Unknown
9.4	—	—	Severe dermatitis
10.7	—	—	Dermatitis, Aspiration
12.6	—	—	Severe dermatitis
15.6	—	—	Sacrificed early
16.2	++++	—	Pituitary tumor
16.5	+	++++	Thyroid tumor and metastasis
18.2	—	—	Histiocytic sarcoma
18.5	—	—	Sacrificed early
18.6	++	—	Sacrificed early
20.5	++++	—	Pituitary tumor
20.8	++	—	Hemangiosarcoma
23.0	++++	—	Pituitary tumor and pheochromocytoma
26.0	ND	ND	Alive
26.6	+++	—	Infection of reproductive organs
27.0	ND	ND	Alive

ND—not determined.
Tumor size is indicated as follows: "+" indicates very early tumor growth and "++++" indicates tumors comparable to those of *Rb*$^{+/-}$ mice.

the wild-type and *Rb*$^{+/-}$ mutant mice (Figures 3A, 3B, and 3D). In contrast, p107 bound at least three distinct E2F complexes in the tissues derived from the *Rb*$^{+/-}$;*E2f4*$^{-/-}$ mice (Figures 3C and 3D). An anti-E2F5 antibody recognized one of these species, and the other E2F complexes were completely retarded by a combination of antibodies against E2F1 and E2F3. E2F1 and E2F5 were also observed when the immunoprecipitations were conducted with anti-p130 antibodies (data not shown). Thus, the absence of E2F4 enables p107 and p130 to bind activator E2Fs in a variety of tissues, including the tumor-prone pituitary.

It is well documented that the formation of tumors in *Rb*$^{+/-}$ mice is dependent upon the inactivation of the wild-type *Rb* allele. Therefore, we also determined the nature of the E2F complexes in *Rb*$^{-/-}$ versus *Rb*$^{-/-}$;*E2f4*$^{-/-}$ cells. Since the *Rb*$^{-/-}$ and *Rb*$^{-/-}$;*E2f4*$^{-/-}$ animals both die in utero (reviewed by Mulligan and Jacks, 1998; E.Y.L. and J.A.L., unpublished observations), these experiments were conducted using MEFs (Figure 4A). DOC release and electrophoretic mobility shift assays confirmed that E2F4 and E2F5 accounted for all of the p107- and p130-associated E2F activity in wild-type cells (Figure 4A). In agreement with our analysis of *Rb*$^{+/-}$;*E2f4*$^{-/-}$ samples, some of the p107- and p130-associated E2F species in *Rb*$^{-/-}$;*E2f4*$^{-/-}$ MEFs were unaffected by anti-E2F4 and anti-E2F5 antibodies. The remaining complexes corresponded to E2F1, E2F3a, and E2F3b (data not shown). Importantly, we found that the spectrum of E2F complexes in the *Rb*$^{-/-}$ MEFs was a composite of those of the wild-type and *Rb*$^{-/-}$;*E2f4*$^{-/-}$ MEFs. Specifically, whereas E2F4 accounted for either all or a large fraction of the p107-associated E2F activity in wild-type and *Rb*$^{-/-}$ cells, respectively, p107 bound significant quantities of activator E2Fs in *Rb*$^{-/-}$ and *Rb*$^{-/-}$;*E2f4*$^{-/-}$ MEFs. Further, p130 associated almost exclusively with E2F4 in *Rb*$^{-/-}$ cells, but in the doubly deficient cells, it associated to a large extent with E2F1 and E2F5 (Figure 4A and see below).

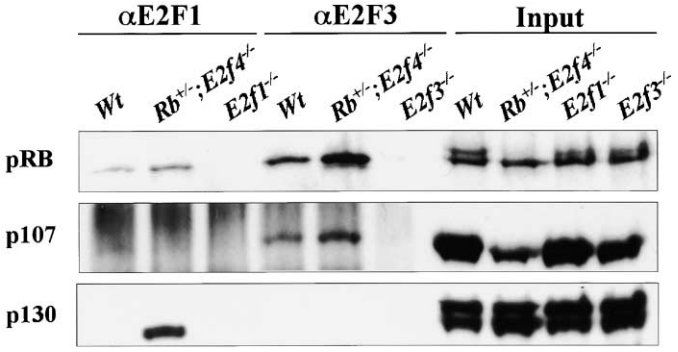

Figure 2. E2F complex rearrangement in *Rb*$^{+/-}$; *E2f4*$^{-/-}$ MEFs

Western blot detection of pRB, p107, and p130 after immunoprecipitation of lysates from wild-type and *Rb*$^{+/-}$; *E2f4*$^{-/-}$ MEFs with anti-E2F1 and anti-E2F3 antibodies. Input lysate (10% of total) is shown at right.

This analysis raised the possibility that p107 and p130 might act in pRB-deficient cells to bind to E2F1 and E2F3 even in the presence of physiological levels of E2F4. To further test this hypothesis, we examined the pocket protein binding properties of E2F1 and E2F3 in *Rb*$^{-/-}$ and *Rb*$^{-/-}$;*E2f4*$^{-/-}$ MEFs by immunoprecipitating the activator E2Fs and Western blotting for associated pocket proteins (Figure 4B). These experiments confirmed that there was a robust association between the activating E2Fs and p107 and p130 in *Rb*$^{-/-}$ cells. Furthermore, the absence of both E2F4 and pRB strikingly increased the level of E2F1-associated p130 when compared to *Rb*$^{-/-}$ cells. Taken together, these data yield two important conclusions. First, in pRB-deficient cells, p107 and p130 appear to substitute for pRB in the regulation of the activating E2Fs. Second, E2F4 loss enhances the formation of these novel complexes, presumably by increasing the levels of the free pools of p107 and p130.

Loss of E2F4 suppresses inappropriate E2F target gene expression and cell proliferation in pRB-deficient cells

Our experiments indicated that tumor suppression in animals lacking both pRB and E2F4 resulted from the reassortment

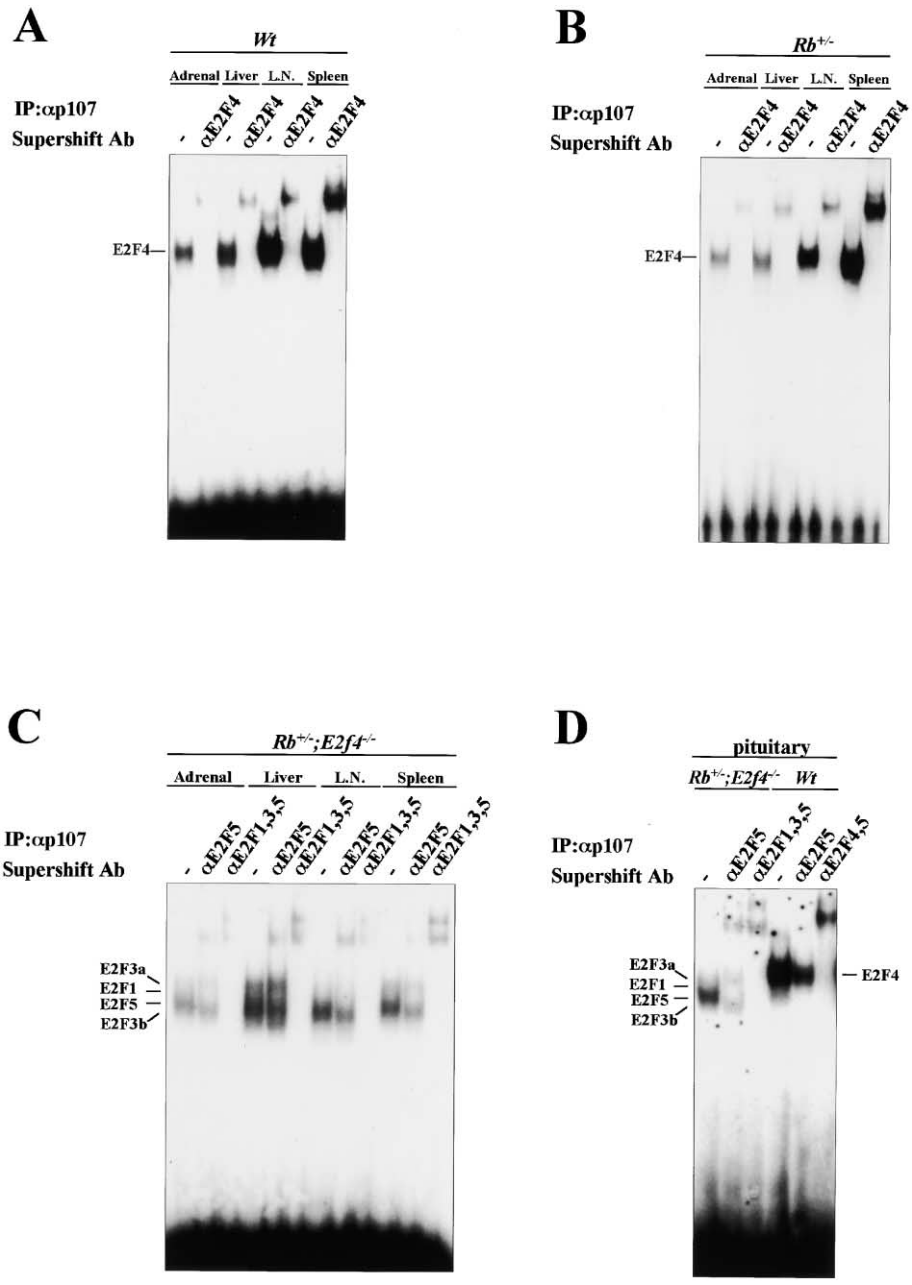

Figure 3. p107 associates exclusively with E2F4 in organs of wild-type and $Rb^{+/-}$ mice but associates with E2F1, 3, and 5 in $Rb^{+/-};E2f4^{-/-}$ mice

A–C: Immunoprecipitation, DOC release, and EMSA were performed with anti-p107 antibody, using homogenates of adrenal glands (**A** and **C**, 500 μg; **B**, 300 μg), liver (**A–C**, 2 mg), lymph nodes (L.N.) (**A** and **C**, 500 μg; B, 250 μg), and spleen (**A** and **C**, 200 μg; **B**, 100 μg) of wild-type, $Rb^{+/-}$, and $Rb^{+/-};E2f4^{-/-}$ mice.

D: Coupled immunoprecipitation-DOC release of E2F proteins in pituitary homogenates of wild-type (130 μg) and $Rb^{+/-};E2f4^{-/-}$ (200 μg) mice with anti-p107 antibody. Specific E2F-DNA complexes in the absence of antibody retardation are indicated. The identities of each of the distinct p107/E2F complexes in $Rb^{+/-};E2f4^{-/-}$ tissues were deduced by performing EMSA assays on MEFs deficient for individual E2F family members as well as compound E2F mutant cells.

of complexes such that p107/p130 associated with activating E2Fs. Given these findings, we investigated whether loss of E2F4 had an impact on the proliferative capacity of *Rb*-deficient MEFs. To address this issue, we compared the levels of proliferation in wild-type, $Rb^{-/-}$, and $Rb^{-/-};E2f4^{-/-}$ MEFs grown to confluence. Wild-type cells incorporated BrdU at low levels, as expected for a quiescent population (Figures 5A and 5B). In contrast, $Rb^{-/-}$ cells largely failed to arrest in response to confluent growth, and approximately 40% of the cells entered S phase. Remarkably, loss of *E2f4* completely suppressed this inappropriate proliferation and restored the low levels of BrdU incorporation observed in wild-type cells.

The abnormal proliferation observed in confluent $Rb^{-/-}$ MEFs has been shown to correlate with the inappropriate expression of known E2F-responsive genes, cyclin E and p107 (Herrera et al., 1996; Hurford et al., 1997). Given the apparent rescue of the $Rb^{-/-}$ proliferation defect in $Rb^{-/-};E2f4^{-/-}$ MEFs, we hypothesized that the loss of E2F4 might also modulate the expression of E2F-responsive genes. We investigated this possibility by examining expression of the *cyclin E* gene in wild-type, $Rb^{-/-}$, and $Rb^{-/-};E2f4^{-/-}$ MEFs grown to confluence. As expected from previous studies (Herrera et al., 1996; Hurford et al., 1997), cyclin E was expressed at very low levels in confluent, wild-type cells but was markedly elevated in cells deficient for *Rb* (Figure 5C). In striking contrast, cyclin E RNA levels were dramatically and consistently reduced in cells deficient for both *Rb* and *E2f4* to levels that approximated those observed in wild-type cells. We demonstrated that each of these effects was

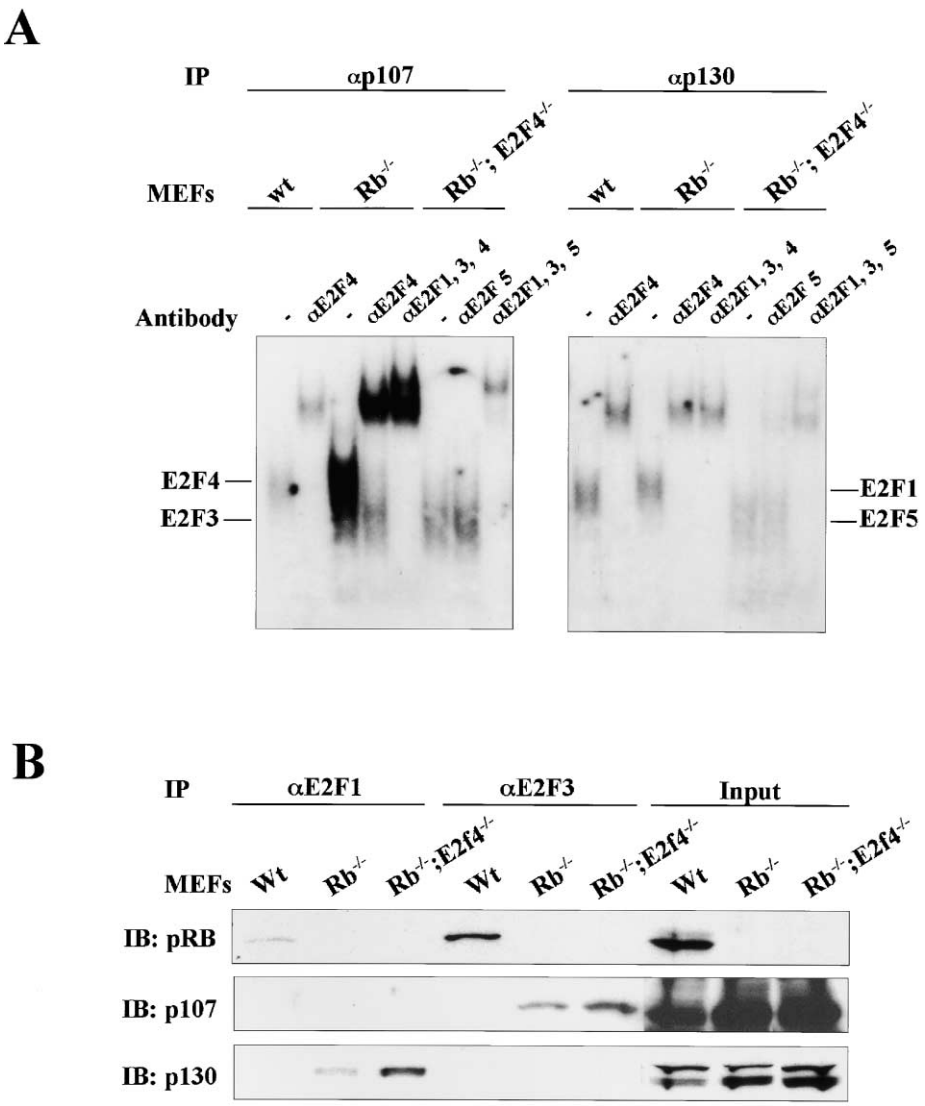

Figure 4. E2F complex rearrangement in $Rb^{-/-}$ MEFs and enhanced by further loss of E2F4

A–B: MEFs were lysed with ELB buffer and were subjected to immunoprecipitation, DOC release, and EMSA with p107 or p130 antibodies (**A**) or were immunoprecipitated with anti-E2F1 or anti-E2F3 antibodies and Western blotted with pocket protein antibodies (**B**). Specific E2F-DNA complexes in the absence of antibody retardation are indicated.

specific, since expression of a second E2F target gene, *B-myb*, known to be under the control of p107/p130 but not pRB (Hurford et al., 1997; Rayman et al., 2002), was not affected by mutation of *Rb* or *Rb* and *E2f4* (Figure 5C; data not shown).

To extend these findings, we performed Western blotting on extracts derived from wild-type and mutant MEFs and examined expression of several E2F target genes. These experiments confirmed our RT-PCR studies and showed that expression of cyclin E and a second established pRB target, p107, was markedly elevated in *Rb*-deficient cells. Furthermore, simultaneous loss of *E2f4* largely reversed this deregulation in two independent preparations of doubly null MEFs (Figure 5D). These findings strongly suggest that loss of *E2f4* suppresses tumorigenic growth of *Rb*-deficient cells by restoring both appropriate levels of expression of critical E2F target genes and a normal response to cues that limit cell proliferation.

Discussion

The goal of these studies was to establish whether the formation of repressive E2F complexes contributes to the tumor-suppressive properties of pRB. Since E2F4 cooperates with the pocket proteins in gene repression, we anticipated that E2F4 loss would either exacerbate or have no effect on the tumor phenotype of the $Rb^{+/-}$ mice depending upon whether or not repression was important. Instead, we found that the absence of E2F4 greatly inhibited the formation of both pituitary and thyroid tumors, enabling a significant fraction of the $Rb^{+/-}$;*E2f4*$^{-/-}$ mice to live as long as wild-type controls. Indeed, the degree of tumor suppression significantly exceeded that resulting from the loss of the activating E2Fs, E2F1 or E2F3, in an $Rb^{+/-}$ background (Yamasaki et al., 1998; U.Z. and J.A.L., unpublished observations). Furthermore, we demonstrated that loss of *E2f4* in *Rb*-deficient cells restored the control of E2F-responsive genes and the inhibition of DNA synthesis characteristic of wild-type, confluence-arrested cells. Since the loss of contact inhibition is one of the hallmarks of a cancer cell, we suggest that this finding could explain the tumor suppression we observe in pituitaries and thyroids of $Rb^{+/-}$;*E2f4*$^{-/-}$ mice. Thus, this study provides direct evidence for a critical role of E2F4 in pRB function.

E2F4 loss could be exerting its tumor-suppressive effects

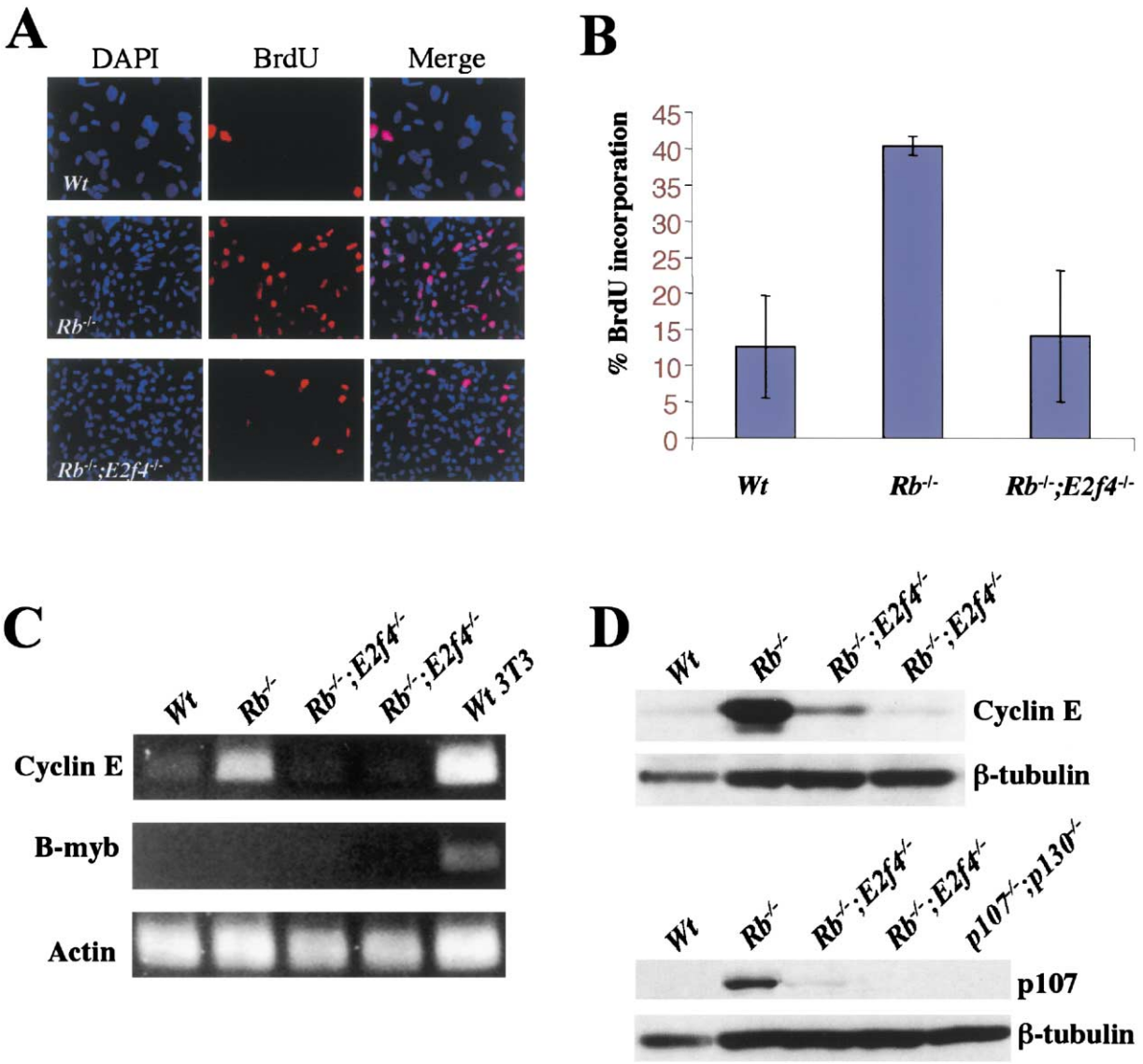

Figure 5. Loss of E2F4 restores confluence arrest and regulation of target genes in $Rb^{-/-}$ MEFs

A: Immunofluorescence for BrdU (red) and DAPI (blue) on wild-type, $Rb^{-/-}$, and $Rb^{-/-};E2f4^{-/-}$ MEFs treated with BrdU 2 days after reaching confluence.
B: Quantification of BrdU incorporation. For each genotype, the percentage of BrdU-positive nuclei was calculated. The graph depicts the average of two experiments with standard deviation.
C: RT-PCR analysis of E2F target genes, *cyclin E* and *B-myb* (not deregulated in $Rb^{-/-}$ MEFs), and actin (loading control) on day 2 confluent cells. Wild-type 3T3 cells were used as a positive control.
D: Western blot analysis of E2F target genes, *cyclin E* and *p107*, and β-tubulin (loading control) on day 2 confluent cells. Asynchronously growing $p107^{-/-};p130^{-/-}$ MEFs were used as a negative control for the p107 blot.

via several possible mechanisms. The simplest model is that E2F4 contributes to the activation of E2F-responsive genes and is therefore a key downstream target of pRB in a similar manner to E2F1 and E2F3. This conclusion is supported by early studies that showed that E2F4 has significant transcriptional activity in overexpression experiments (Beijersbergen et al., 1994; Ginsberg et al., 1994). However, analysis of the endogenous E2F4 protein does not support this conclusion. First, the predominant cytoplasmic localization of the free E2F4-DP complexes is inconsistent with their role in transcriptional activation (Gaubatz et al., 2001; Magae et al., 1996; Muller et al., 1997; Verona et al., 1997). Second, ChIP assays strongly suggest that E2F4 specifically occupies E2F-responsive promoters in association with p107 and p130 during the G_0/G_1 stages of the cell cycle when these targets are transcriptionally repressed (Rayman et al., 2002; Takahashi et al., 2000). Finally, primary cells that are deficient for E2F4 and E2F5 are defective in cell cycle arrest but not proliferative functions (Gaubatz et al., 2000). Clearly, these data do not rule out the possibility that E2F4 could contribute to the activation of E2F-responsive genes in pRB-deficient tumor cells, and experiments that investigate both expression profiles and promoter occupancy of target genes will be needed to address this issue further. Moreover, it is important to note that although it is widely assumed that E2F1 and E2F3 contribute

to the formation of tumors through this mechanism, this has not yet been demonstrated. Therefore, experiments with *Rb;E2f* compound mutant cells will be critical in testing this hypothesis as well.

An alternative model arising from our data suggests that E2F4 loss could increase the apoptotic potential of pRB-deficient cells. Under these conditions, cells in the $Rb^{+/-};E2f4^{-/-}$ mice that lose the wild-type *Rb* allele might be eliminated by apoptosis rather than become tumorigenic. This is a reasonable concern because there is considerable evidence supporting a role for the E2F proteins in the regulation of many apoptosis genes (reviewed by Trimarchi and Lees, 2002). We have not observed any obvious difference in the apoptotic potential of $Rb^{-/-};E2f4^{-/-}$ versus $Rb^{-/-}$ MEFs (our unpublished observations). However, since this does not address the consequences of E2F4 loss in the adult pituitary and thyroid, we are attempting to establish $Rb^{-/-};E2f4^{-/-}$ ES cell lines that can be used to generate chimeric mutant mice. Such mutant animals will be invaluable because they will allow us to establish whether $Rb^{-/-};E2f4^{-/-}$ cells can contribute to adult tissues. Since it is well established that the formation of tumors in $Rb^{+/-}$ mice depends upon the inactivation of the wild-type *Rb* allele, it is also possible that the rearrangement in pocket protein complexes in the $Rb^{+/-};E2f4^{-/-}$ tissues somehow diminishes the selective pressure for loss of heterozygosity. In addition, our data do not rule out the possibility that the observed tumor-suppressive effect of E2F4 loss is cell non-autonomous. Thus, the generation of both conditional and chimeric mice will also be essential in allowing us to address these two issues.

A final model suggests that E2F4 loss suppresses tumors by simply altering the spectrum of the remaining E2F complexes. We currently favor this hypothesis, based on our biochemical analysis. Specifically, our data show that E2F4 loss promotes the formation of novel E2F complexes in which p107 and p130 associate with the normally pRB-specific E2Fs, E2F1 and 3 (Figure 6). Previous studies have shown that inappropriate release of the activating E2Fs makes a major contribution to the phenotypic consequences of pRB loss (Tsai et al., 1998; Yamasaki et al., 1998; Ziebold et al., 2001). We therefore propose that E2F4 loss suppresses tumorigenesis by increasing the free pools of p107 and p130 and thereby enabling them to substitute for pRB in the inhibition of the activating E2Fs (Figure 6). This could also account for the observed suppression of inappropriate E2F-responsive gene expression and cell cycle entry of confluence-arrested $Rb^{-/-}$ MEFs (Figures 5B and 5C). Additional tumor studies will be required to distinguish between these models. However, regardless of the precise mechanism by which E2F4 loss is operating, our studies do not provide any support for a role of repressive E2F-pocket protein complexes in tumor suppression. Instead, they strongly suggest that the critical tumor suppressive role of pRB is to inhibit E2F family members that mediate the activation of E2F-responsive genes.

Importantly, we also detected p130-E2F1 and p107-E2F3 complexes in cells that had physiological levels of E2F4, but lacked the pRB tumor suppressor. Since the generation of $Rb^{-/-}$ cells is a key step in the development of many naturally occurring tumors, we believe that the formation of novel E2F-pocket protein complexes has significant in vivo relevance. There is extensive evidence from both human tumors and mutant mouse models that the pocket proteins play non-overlapping roles in the suppression of tumors (Cobrinik et al., 1996; Dannenberg et al., 2000; Lee et al., 1996; Robanus-Maandag et al., 1998; Sage et al., 2000). Specifically, pRB is a classical tumor suppressor, but mutation of *p107* and/or *p130* promotes tumor formation only when pRB is also inactivated. We believe that our observations can account for these differential properties. First, we propose that tumor formation is dependent upon the inappropriate release of the activating E2Fs. Since these E2Fs are specifically regulated by pRB in normal cells, their release can only be triggered by the loss of pRB and not p107 and/or p130, explaining why pRB is the key tumor suppressor in vivo. Second, our data suggest that pRB loss causes p107 and p130 to substitute for pRB in the regulation of the activating E2Fs. In this manner, p107 and p130 become significant tumor suppressors in pRB-deficient cells. Consistent with this hypothesis, $Rb^{-/-};p107^{-/-}$ chimeric mice develop an additional tumor type, retinoblastoma, compared to $Rb^{-/-}$ chimeras (Robanus-Maan-

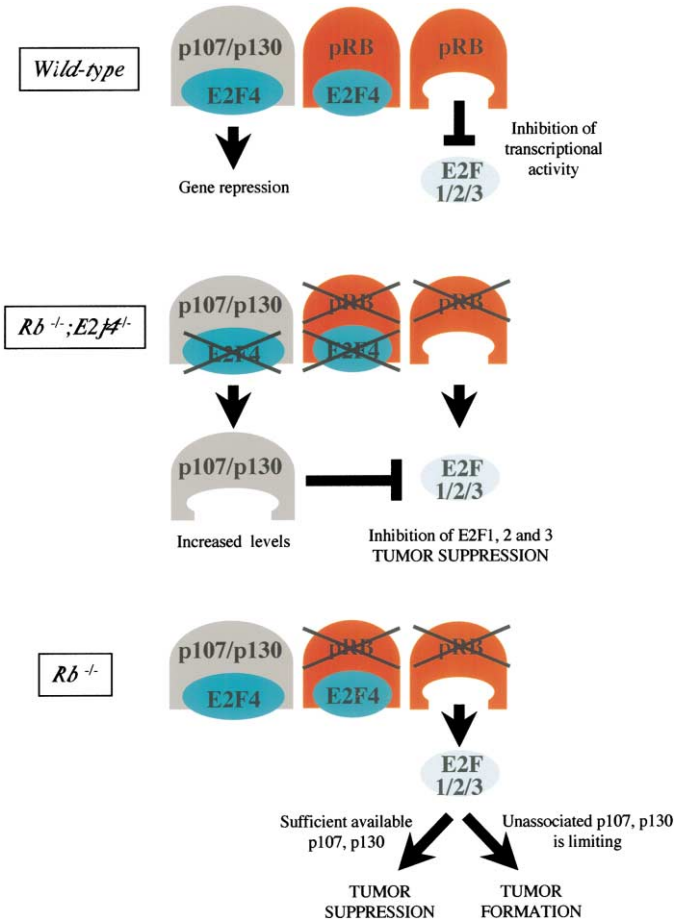

Figure 6. Model for tumor suppression resulting from simultaneous deficiency of *E2f4* and *Rb*

In wild-type cells, the activator E2Fs are specifically regulated by pRB while E2F4 associates with pRB, p107, and p130. In the absence of *Rb,* E2F1 and E2F3 activators are released, activating inappropriate S phase target gene expression and thereby promoting uncontrolled proliferation and tumor formation. The simultaneous deficiency of *Rb* and *E2f4* in $Rb^{+/-};E2f4^{-/-}$ mice results in the association of p107 and p130 with the activator E2Fs, conferring tumor-suppressive functions on p107 and p130. Loss of pRB alone also promotes some binding of p107 and p130 to E2F1 and E2F3. Thus, the levels of available p107 and p130 in individual tissues may account for the tissue specificity of $Rb^{-/-}$ tumor formation.

dag et al., 1998), and the combined mutation of pRB, p107, and p130 has been shown to be highly tumorigenic (Dannenberg et al., 2000; Sage et al., 2000). Moreover, *p107* and/or *p130* mutations have been detected at a low frequency in certain pRB-deficient human tumors (Claudio et al., 2000a, 2000b; Helin et al., 1997).

It is important to note that mutation of *p107* and/or *p130* is not required for the formation of most pRB-deficient tumors. We must therefore conclude that p107 and p130 are unable to compensate for the loss of pRB in tumor-prone tissues. Inheritance of germline *Rb* mutations results in a highly tissue-specific tumor spectrum in both humans (retinoblastoma) and mice (pituitary and thyroid tumors). Since pRB is believed to play a key role in all tissue types, the underlying basis for this tissue-specific spectrum is not understood. We believe that our observations could also explain this phenomenon. Our data show that the absence of E2F4 increases the levels of p107 and p130 that associate with the activating E2Fs in $Rb^{-/-}$ cells, suppressing the formation of both pituitary and thyroid tumors in $Rb^{-/-}$ mice. This raises the possibility that the tumor-prone tissues may simply be those where the levels of available p107 and p130 are insufficient to substitute fully for pRB in the inhibition of the activating E2Fs (Figure 6). By extension of this logic, a relatively modest increase in the free pools of p107 and p130 may be sufficient to prevent the formation of tumors. This suggests a novel strategy for the generation of chemotherapeutic agents that would either release free pools of p107 and p130 by depleting cells of E2F4 (as in this study) or increasing intracellular p107/p130 levels.

Experimental procedures

Generation, genotyping, and analysis of mice and MEFs

The $Rb^{+/-}$;$E2f4^{+/-}$ strain was generated by intercrossing 129/Sv x C57BL/6 mice carrying germline mutations in *Rb* or *E2f4*. Genotyping was conducted as previously described (Humbert et al., 2000a; Jacks et al., 1992). Soft tissues were fixed in 10% formalin, stained with hematoxylin and eosin, and scored for tumors histologically and/or macroscopically. For comparison, the tumors were measured at the widest diameter. MEFs were generated using embryos between 10.5 and 13.5 days post coitum from $Rb^{+/-}$;$E2f4^{+/-}$ intercrosses as previously described (Humbert et al., 2000b). MEFs were used before passage 7. The statistical significance of the differential lifespan and tumor incidence of the $Rb^{+/-}$ versus the $Rb^{+/-}$;$E2f4^{-/-}$ animals was determined by the log-rank test and two-tailed Fisher's exact test (as previously described by Yamasaki et al., 1998), respectively.

Immunoprecipitation, deoxycholate release, and electrophoretic mobility shift assays

Asynchronously growing MEFs were lysed in ELB buffer (50 mM Hepes [pH 7.0], 250 mM NaCl, 5 mM EDTA, 0.1% NP-40, and 10% glycerol containing a protease inhibitor cocktail). Immunoprecipitation using ELB buffer and Western blotting were conducted using anti-E2F1 (sc-193, Santa Cruz Biotech), anti-E2F3 (AB) (sc-878), anti-pRB (G3-245, PharMingen), anti-p107 (sc-318), and anti-p130 (sc-317) antibodies. Whole organs were lysed in E2F extraction buffer (20 mM Hepes [pH 7.8], 450 mM NaCl, 0.2 mM EDTA [pH 8.0], 0.1% NP-40, 25% glycerol) by three rounds of freeze-thaw and then homogenized in 1.5 ml Eppendorf tubes with a tight-fitting pestle. Deoxycholate (DOC) release and electrophoretic mobility shift assays were conducted as described (Woo et al., 1997) using anti-E2F1 (KH129, Neomarkers), anti-E2F3 (sc-878), anti-E2F4 (sc-1082), and anti-E2F5 (sc-1083) antibodies. The specificity of these antibodies was confirmed by performing electrophoretic mobility shift assays with extracts from various E2F-deficient MEFs (Supplemental Figures S1A and S1B on *Cancer Cell* website).

Confluence arrest, BrdU incorporation, and target gene expression assays

MEFs were grown to confluence, and two days later, cells were labeled with 10 μM 5-bromo-2′-deoxyuridine (BrdU; Sigma) for 8 hr. Incorporation was quantified by indirect immunofluorescence with anti-BrdU (347580, Becton Dickinson) antibodies and DAPI. The percentage of BrdU-positive cells was determined by counting more than 875 cells per genotype. Whole-cell extracts from day 2 confluent MEFs were prepared as previously described (Moberg et al., 1996), and Western blotting was performed using anti-cyclin E (sc-481), anti-p107 (sc-318) (each from Santa Cruz Biotech), and anti-β-tubulin (T-4026, Sigma) antibodies. RT-PCR assays were carried out as described in Ren et al. (2002) using an Invitrogen RT-PCR Superscript One Step kit.

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