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-/-;p130-/-/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 Trimmer 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 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 G1 (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 (Hubert 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 G0 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 G0/G1 cells under physiological conditions (Rayman et al., 2002; Takahashi et al., 2000). Importantly, MEFs deficient for E2f4 and E2f5 are unable to arrest in G0 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.
early pituitary tumors, (v) normal pituitaries, and (vi) medium intermediate Rb and E2F4 relative to wild-type MEFs (see Supplemental mice remained alive and healthy. Indeed, a significant fraction could account for the suppression of tumors in the Rb+/−;E2f4−/− mice (Table 1). However, the incidence of pituitary tumors was significantly lower than in the Rb+/− controls (p = 0.0000034). 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+/− mice (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

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
the wild-type and Rb<sup>+/−</sup> mutant mice (Figures 3A, 3B, and 3D). In contrast, p107 bound at least three distinct E2F complexes in the tissues derived from the Rb<sup>+/−;E2f4<sup>−/−</sup> 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<sup>−/−</sup> mice is dependent upon the inactivation of the wild-type Rb allele. Therefore, we also determined the nature of the E2F complexes in Rb<sup>−/−</sup> versus Rb<sup>+/−;E2f4<sup>−/−</sup></sup> cells. Since the Rb<sup>−/−</sup> and Rb<sup>+/−;E2f4<sup>−/−</sup></sup> 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<sup>+/−;E2f4<sup>−/−</sup></sup> samples, some of the p107- and p130-associated E2F species in Rb<sup>−/−;E2f4<sup>−/−</sup></sup> MEFs were unaffected by anti-E2F4 and anti-E2F5 antibodies. The remaining complexes corresponded to E2F1, E2F3a, and E2F5 (Figure 4A). In contrast, p107 bound at least three distinct E2F complexes in the tissues derived from the Rb<sup>−/−;E2f4<sup>−/−</sup></sup> MEFs was a composite of those of the wild-type and Rb<sup>+/−;E2f4<sup>−/−</sup></sup> MEFs. Specifically, whereas E2F4 accounted for either all or a large fraction of the p107-associated E2F activity in wild-type and Rb<sup>−/−</sup> cells, respectively, p107 bound significant quantities of activator E2Fs in Rb<sup>−/−</sup> and Rb<sup>+/−;E2f4<sup>−/−</sup></sup> MEFs. Further, p130 associated almost exclusively with E2F4 in Rb<sup>−/−</sup> cells, but in the doubly deficient cells, it associated to a large extent with E2F1 and E2F5 (Figure 4A and see below).

This analysis raised the possibility that p107 and p130 might act in Rb<sup>−/−</sup>-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<sup>−/−</sup> and Rb<sup>+/−;E2f4<sup>−/−</sup></sup> MEFs by immunoprecipititating 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. Furthermore, the absence of both E2F4 and pRB strikingly increased the level of E2F1-associated p130 when compared to Rb<sup>−/−</sup> cells. Taken together, these data yield two important conclusions. First, in Rb<sup>−/−</sup>-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

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**Table 1. Histological analysis of Rb<sup>+/−;E2f4<sup>−/−</sup></sup> mice**

<table>
<thead>
<tr>
<th>Age (months)</th>
<th>Pituitary tumor</th>
<th>Thyroid tumor</th>
<th>Cause of death</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.7</td>
<td>—</td>
<td>—</td>
<td>Sinusitis</td>
</tr>
<tr>
<td>5.4</td>
<td>—</td>
<td>—</td>
<td>Pylonephritis</td>
</tr>
<tr>
<td>8.7</td>
<td>—</td>
<td>—</td>
<td>Sacrificed early</td>
</tr>
<tr>
<td>9.3</td>
<td>—</td>
<td>—</td>
<td>Unknown</td>
</tr>
<tr>
<td>9.4</td>
<td>—</td>
<td>—</td>
<td>Severe dermatitis</td>
</tr>
<tr>
<td>10.7</td>
<td>—</td>
<td>—</td>
<td>Dermatitis, Aspiration</td>
</tr>
<tr>
<td>12.8</td>
<td>—</td>
<td>—</td>
<td>Severe dermatitis</td>
</tr>
<tr>
<td>15.6</td>
<td>—</td>
<td>—</td>
<td>Sacrificed early</td>
</tr>
<tr>
<td>16.2</td>
<td>+ + +</td>
<td>—</td>
<td>Pituitary tumor</td>
</tr>
<tr>
<td>16.5</td>
<td>+ + +</td>
<td>—</td>
<td>Thyroid tumor and metastasis</td>
</tr>
<tr>
<td>16.5</td>
<td>+ + +</td>
<td>—</td>
<td>Histiocytic sarcoma</td>
</tr>
<tr>
<td>18.2</td>
<td>—</td>
<td>—</td>
<td>Sacrificed early</td>
</tr>
<tr>
<td>18.5</td>
<td>+ +</td>
<td>—</td>
<td>Sacrificed early</td>
</tr>
<tr>
<td>20.5</td>
<td>+ + +</td>
<td>—</td>
<td>Pituitary tumor</td>
</tr>
<tr>
<td>20.8</td>
<td>++</td>
<td>—</td>
<td>Hemangiosarcoma</td>
</tr>
<tr>
<td>23.0</td>
<td>+ + +</td>
<td>—</td>
<td>Pituitary tumor and pheochromocytoma</td>
</tr>
<tr>
<td>26.0</td>
<td>ND</td>
<td>ND</td>
<td>Alive</td>
</tr>
<tr>
<td>26.6</td>
<td>++</td>
<td>—</td>
<td>Infection of reproductive organs</td>
</tr>
<tr>
<td>27.0</td>
<td>ND</td>
<td>ND</td>
<td>Alive</td>
</tr>
</tbody>
</table>

ND—not determined.

Tumor size is indicated as follows: “+” indicates very early tumor growth and “++++” indicates tumors comparable to those of Rb<sup>+/−</sup> mice.
Figure 3. p107 associates exclusively with E2F4 in organs of wild-type and Rb<sup>−/−</sup> mice but associates with E2F1, 3, and 5 in Rb<sup>−/−</sup>;E2f4<sup>−/−</sup> 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<sup>−/−</sup>, and Rb<sup>−/−</sup>;E2f4<sup>−/−</sup> mice.

D: Coupled immunoprecipitation–DOC release of E2F proteins in pituitary homogenates of wild-type (130 μg) and Rb<sup>−/−</sup>;E2f4<sup>−/−</sup> (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<sup>−/−</sup>;E2f4<sup>−/−</sup> 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<sup>−/−</sup>, and Rb<sup>−/−</sup>;E2f4<sup>−/−</sup> 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<sup>−/−</sup> cells largely failed to arrest in response to confluence 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<sup>−/−</sup> 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<sup>−/−</sup> proliferation defect in Rb<sup>−/−</sup>;E2f4<sup>−/−</sup> 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<sup>−/−</sup>, and Rb<sup>−/−</sup>;E2f4<sup>−/−</sup> 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
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 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, DOG 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.
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 G1/S 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

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.
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\textsuperscript{+/+};E2f4\textsuperscript{-/-} 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\textsuperscript{+/+};E2f4\textsuperscript{-/-} versus Rb\textsuperscript{-/-} 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\textsuperscript{+/+};E2f4\textsuperscript{-/-} 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\textsuperscript{+/+};E2f4\textsuperscript{-/-} cells can contribute to adult tissues. Since it is well established that the formation of tumors in Rb\textsuperscript{-/-} 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\textsuperscript{+/+};E2f4\textsuperscript{-/-} 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; Yamashaki 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\textsuperscript{+/+} 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 p107-pRB1 and p107-pE2F3 complexes in cells that had physiological levels of E2F4, but lacked the pRB tumor suppressor. Since the generation of Rb\textsuperscript{-/-} 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\textsuperscript{-/-};p107\textsuperscript{-/-} chimeric mice develop an additional tumor type, retinoblastoma, compared to Rb\textsuperscript{-/-} chimeras (Robanus-Maan-

![Figure 6. Model for tumor suppression resulting from simultaneous deficiency of E2f4 and Rb](image-url)
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 (Hubert 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, E.W., Caputi, M., and Giordano, A. (2000a). Mutations in the retinoblastoma-related gene RB2/p130 in lung tumors and suppression of tumor growth in vivo by retrovirus-mediated gene transfer. Cancer Res. 60, 2245–2262.


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-p130 (sc-317) 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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