Tumor and Stem Cell Biology

RB Family TumorSuppressor Activity May Not Relate to Active Silencing of E2F Target Genes

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Abstract

The retinoblastoma protein pRB and its two homologs p130 and p107 form the family of pocket proteins and play a major role in cell-cycle regulation and suppression of human and mouse tumorigenesis. Pocket proteins regulate the activity of E2F transcription factors during G1–S transition. Two mechanisms have been described: (i) pocket protein binding blocks the transactivation domain of activator E2Fs, inhibiting E2F-dependent transcription and (ii) E2F-bound pocket proteins can recruit chromatin remodeling proteins containing an LxCxE motif (x encoding any amino acid), resulting in active repression of E2F target genes. To investigate the importance of pRB's LxCxE-interacting motif in cell-cycle control and tumor suppression, we generated mouse embryonic fibroblasts and mice expressing a mutant pRB protein carrying an asparagine for phenylalanine substitution at position 750, abrogating LxCxE binding. Because p130 may compensate for loss of pRB, we studied pRBN750F activity in the presence and absence of p130. The pRB–LxCxE interaction was not required for cell-cycle arrest upon mitogen deprivation and cell-cell contact, but did contribute to RASV12+ and radiation-induced cell-cycle arrest. Remarkably, the pRB–LxCxE interaction was not required for suppression of in vitro and in vivo transformation, even in the absence of p130. These results indicate that pRB's tumor suppressor activity is not effectuated by active silencing of E2F target genes, but rather by regulation of activator E2Fs or another unidentified mechanism. Furthermore, the in vitro response of pocket protein–perturbed cells to mitogen deprivation and cell–cell contact seems a better predictor of tumor development than the response to ectopic RASV12+ expression. Cancer Res; 74(18): 1–11. ©2014 AACR.

Introduction

Loss of the retinoblastoma suppressor gene, RB1, is a frequent event in human cancer. pRB and its homologs p130 and p107 form the family of pocket proteins and play a key role in cell-cycle regulation. They collectively regulate the family of E2F transcription factors, whose activity is essential for the G1–S transition. Eleven E2F transcription factors have been identified, classified as "activator E2Fs" (E2F1, E2F2, and E2F3a) and "repressor E2Fs" (E2F3b, E2F4, E2F5, E2F6a, E2F6b, E2F7a, E2F7b, and E2F8). Whereas pRB can interact with E2F1-4, p130 and p107 interact with E2F4 and E2F5. E2F6-8 do not bind pocket proteins (1–3).

Pocket protein–E2F complex formation inhibits transcription of E2F target genes via several mechanisms. First, pocket protein binding blocks E2F's transactivation domain, preventing E2F-mediated transcription. The balance between pocket protein–bound and free E2Fs is regulated by cyclin-dependent kinases that phosphorylate pocket proteins, causing release of E2Fs. This enables activating E2Fs to promote transcription and cell-cycle progression (3). Second, pocket protein "repressor" complexes pRB–E2F3b, pRB–E2F4, p130/p107–E2F4, and p130/p107–E2F5 can recruit chromatin remodeling proteins to E2F target genes by the capacity of pocket proteins to bind proteins containing an Lx(CxE)-like motif (x encoding any amino acid; ref. 4). The LxCxE motif, originally identified in viral oncoproteins, is present in a variety of chromatin remodeling proteins that favor a chromatin state incompatible with transcription (4). These include histone deacetylase 1 (HDAC1), HDAC2, heterochromatin protein 1 (HP1), the histone methyltransferase Suv39h1, and the CtBP-interacting protein (1H1P; refs. 3–7).

Consistent with their role in cell-cycle control, loss of pocket proteins initiates tumorigenesis. In mice, Rb loss induces pituitary and thyroid tumors, and the tumor spectrum is extended by the additional loss of p130 or p107 (8–10). However, it is unclear which of the E2F-regulating activities of

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pocket proteins is critical for tumor suppression. One view is that regulation of "activator," rather than "repressor" E2Fs, mediates pRB's tumor suppressor activity (11). However, pocket protein–chromatin remodeling complexes have been implicated in (irreversible) cell-cycle arrest and senescence, which are considered critical for tumor suppression. The recruitment of p130–E2F4 to silenced promoters in G0–G1-arrested mouse and human cells suggests active repression (12, 13). Furthermore, pRB and LxCxE-containing proteins have been implicated in repression of E2F target genes upon serum starvation (14, 15), RASV12 expression (16), and ectopic expression of p16INK4A (17).

Although LxCxE-containing proteins have thus been implicated in processes counteracting transformation, the requirement for these proteins to suppress tumorigenesis in vivo remains elusive. To address this issue, we generated mouse embryonic fibroblasts (MEF) and mice expressing a pRB variant in which asparagine at position 750 was replaced for phenylalanine. pRB\(^{N750F}\) protein still inhibited E2F transactivation activity, but was impaired in binding an LxCxE-containing protein and therefore likely unable to recruit chromatin remodelers to E2F-regulated promoters. Because of functional compensation within the pocket protein family (this communication; ref. 8), the essential role of p130-repressor complexes in G0–G1 (12, 13), and the ability of p130 to recruit chromatin remodeling proteins via its LxCxE binding site (18, 19), we anticipated the phenotypic consequences of pRB\(^{N750F}\) to be masked by p130 activity. Therefore, we also generated Rb\(^{N750F/N750F}\) p130\(^{wt/wt}\) MEFs and Rb\(^{N750F/p130}\) mutant mice. Our results show that the pRB–LxCxE interaction contributes to cell-cycle arrest upon RASV12 expression or γ-irradiation. Remarkably, attenuation of LxCxE-dependent interactions did not promote tumor formation, even in the absence of p130, suggesting that pRB's tumor suppressor activity mainly operates via inhibition of activator E2Fs or an LxCxE-independent repressor mechanism.

Materials and Methods

Constructs

The 6xE2F-luciferase reporter, CMV-HA-E2F1, CMV-HA-E2F4, CMV-Renilla, and p3-mRbb were provided by Dr. R. Bernards (Division of Molecular Carcinogenesis, The Netherlands Cancer Institute, Amsterdam, The Netherlands); GAL4-TK-luciferase by Dr. R. Medema (Division of Cell Biology, The Netherlands Cancer Institute); and eCE-ePBhAc8rHA by Dr. J. Lukas (Novo Nordisk Foundation Center for Protein Research, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark). Addition of an N-terminal HA-tag to pRB, mutation of the Rb cDNA and cloning into pcDNA3.1(−) is described in Supplementary Materials and Methods. GAL4-pRbb vectors were generated by cloning PCR-amplified Rb fragments (wild-type or mutant) into pM (Clontech; Supplementary Materials and Methods), pSG5-Tag was provided by Dr. J. J. DeCaprio (Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA), pBABE-RASV12-puro by Dr. T. Brummelkamp (Division of Biochemistry, The Netherlands Cancer Institute), and pCL-Eco by Dr. D. Peeper (Division of Molecular Oncology, The Netherlands Cancer Institute). The pEYK-TBX2 vector was previously isolated from the pEYK-MCF7 library (20) obtained from Dr. G. Q. Daley (Department of Pediatric Hematology/Oncology, Children's Hospital Boston and Dana-Farber Cancer Institute, Boston, MA).

Reporter assays

Rb\(^{−/−}\) 3T9 cells were seeded onto a 12-well plate and transfected the next day with 0.4 μg DNA per well using FuGENE6 Transfection Reagent (Roche) with a DNA-FuGENE ratio of 1 μg to 3 μL. All transfections included either 6xE2F-luciferase or GAL4-TK-luciferase reporter plasmid, Renilla luciferase expression plasmid and pcDNA3.1(−)-HA-mRbb or pM-GAL4-pRB plasmids to a total of 0.4 μg DNA/well. For detailed concentrations, see Supplementary Materials and Methods. Twenty-four hours after transfection, cells were washed in PBS, lysed, and measured for Luciferase and Renilla luminescence activity using the Dual-Luciferase Reporter Assay System (Promega E1910). Luciferase luminescence was divided by Renilla luminescence.

MEF isolation

Crossings of Rb\(^{N750F/wt}\) animals (21) and Rb\(^{wt/wt}\) animals (22) with p130\(^{wt/wt}\) mice, and intercrossings of the resulting offspring, enabled us to isolate Rb\(^{N750F/N750F}\) p130\(^{+/−}\), Rb\(^{N750F/N750F}\) p130\(^{−/−}\), Rb\(^{−/−}\) p130\(^{−/−}\), Rb\(^{−/−}\) p130\(^{+/−}\), and Rb\(^{−/−}\) p130\(^{−/−}\) MEFs at embryonic day (E) 14.5 and Rb\(^{−/−}\) p130\(^{−/−}\) at E13.5. Rb\(^{−/−}\) p130\(^{−/−}\) p107\(^{−/−}\) MEFs were previously generated (22).

Cell culture

MEFs were cultured with 100 units/mL penicillin (Invitrogen/Gibco) and 100 μg/mL streptomycin (Invitrogen/Gibco; ref. 22). Cells were plated and treated as follows: Mitogen deprivation: 8 × 10⁴ cells/10-cm dish; 3 hours after plating, cells were washed in PBS and medium without FCS was added. Contact inhibition: 8 × 10⁴ cells/10-cm dish; cells were analyzed 10 days after plating. γ-irradiation: 5 × 10⁶ cells/10-cm dish; cells were irradiated the next day with 5.5, 10, or 15 Gy and analyzed 16 hours after irradiation. All assays: cells were harvested for FACS at the indicated time points and, if applicable, 10 μmol/L bromodeoxyuridine (BrdUrd) was added 1 hour before harvesting. BrdUrd labeling and FACS: See Supplementary Materials and Methods.

Production of retroviruses, retroviral infections, and soft agar plating was performed as described (20). For serial infections, MEFs were cultured in nonvirus-containing media for at least 36 hours between infections and reseeded before infection to obtain optimal cell density.

Proliferation curves

Cells were seeded in triplicate at a density of 2.5 × 10⁴ cells per well of a 12-well plate (Corning Incorporated; 3512). At the indicated time points (time point 0: 3 hours after seeding), cells were fixed in 4% formaldehyde/PBS, washed in demineralized water, stained with 0.1% crystal violet solution, washed and dried (dark area). The dye was extracted in 1 mL 10% acetic acid per well, and the optical density at 590 nm was measured using a microplate reader (M200 Tecxan).
Protein isolation, immunoprecipitation, and immunoblot

Protein isolation, separation on 3% to 8% Tris-Acetate and 4% to 12% Bis-Tris NuPage gradient gels (Invitrogen), and immunoblotting were performed according to standard protocols. Cells were lysed in 150 mmol/L NaCl, 50 mmol/L Hepes pH 7.5, 5 mmol/L EDTA, 0.1% NP-40, and 1 tablet complete protease inhibitor cocktail (Roche) per 50 mL. Protein concentrations were determined using Bio-Rad protein assay (Bio-Rad).

For immunoprecipitation experiments, C33A cells cotransfected with pSG5-TAg plus pcDNA3.1(−) encoding HA-tagged wild-type or mutant pRB proteins and coimmunoprecipitated SV40 TAg using anti-TAg and anti-pRB antibodies. Bottom, total protein extracts (input), B–E, transcriptional repression by wild-type and mutant pRB in luciferase reporter assays. Rb−/− 3T3 cells were transfected with the indicated constructs using the concentrations described in Supplementary Materials and Methods. Luciferase/Renilla luminescence is plotted in B–D and set to 100% in lane 2 (B and D) or lane 1 (C). Fold repression, defined as (Luciferase/Renilla luminescence)GAL4/Luciferase/Renilla luminescence)GAL4-pRB wildtype or mutant, is plotted in E. For each analysis, two independent experiments were performed and carried out in triplicate (B, C, and E) or in duplicate (D); a representative experiment is shown.

Mice

RbN750F/N750F p130wt−, RbN750F/p130+−, and RbN750F/N750F p107/w− mice plus appropriate control genotypes were inspected twice a week and sacrificed when ill. Mice were examined histologically; blood was collected into heparinized tubes and diluted 2× in PBS. Red- and white blood cell counts were determined using a Coulter counter.

Cellular preparations and FACS analysis

Cell populations from spleen, bone marrow, blood (leukocytes), and thymus were stained in antibody cocktails and analyzed by FACS as described in Supplementary Materials and Methods.

Results

Mutating the LxCxE binding site of pRB impairs transcriptional repression

Binding of pRB to LxCxE-containing proteins involves four conserved residues in the B domain: Asn757, Tyr756, Lys713, and Tyr709 (23). Substituting asparagine at position 757 for phenylalanine disrupted LxCxE binding, whereas binding to E2F1 remained intact (24, 25). We introduced the corresponding N750F substitution in murine pRB and found this to fully abrogate interaction with the LxCxE-containing protein SV40 large T antigen (TAg; Fig. 1A). As controls, we used pRBK699F.
luciferase reporter, whereas pRBC699F did not (Fig. 1C). These penetrance pRB mutant defective in binding E2Fs and LxCxE—likely by forming E2F4-pocket protein transactivation activity.

pRBN750F only partly repressed the E2F-luciferase reporter transcriptional activator (Fig. 1D, compare lanes 1 and 2; ref. 31). These experiments demonstrate pRBN750F to effectively block E2F transcription (Fig. 1E).

To test the ability of pRB N750F to reduce transcription, we introduced a luciferase gene preceded by six E2F binding sites in mouse Rb−/− 3T3 cells. Luciferase activity induced by ectopic expression of E2F1 (Fig. 1B, lanes 1 and 2) was efficiently suppressed by coexpression of wild-type pRB (pRB wt) or pRB N750F (Fig. 1B, compare lanes 3 to 5 with lane 6 to 8), but not by pRB C700F (Fig. 1B, lanes 9 to 11). Also, without ectopically expressed E2F1, pRB wt and pRB N750F repressed the luciferase reporter, whereas pRB C700F did not (Fig. 1C). These experiments demonstrate pRB N750F to effectively block E2F transactivation activity.

E2F4 generally acts as a transcriptional repressor (12, 13), likely by forming E2F4-pocket protein–chromatin remodeling complexes. However, E2F4 can also function as a transcriptional activator (Fig. 1D, compare lanes 1 and 2; ref. 31). pRB N750F only partly repressed the E2F-luciferase reporter induced by E2F4 (Fig. 1D, compare lanes 2, 3, and 4). We propose that pRB wt suppressed E2F4-mediated transcription by simultaneously inhibiting transactivation and forming E2F4–pRB–chromatin remodeling complexes and that pRB N750F was impaired in the latter. Consistently, when pRB was fused to a GAL4-DNA binding motif, targeting pRB to a cotransfected GAL4-TK-luciferase reporter pRB N750F was severely, but perhaps not completely, impaired in repressing transcription (Fig. 1E).

We conclude that pRB N750F is able to bind E2F transcription factors but unable to establish complete transcriptional repression, likely because it is impaired in recruiting chromatin remodeling complexes.

**E2F target gene expression in Rb N750F/N750F and Rb N750F/N750F p130−/− MEFs**

To study E2F target gene expression in a physiologic setting, we generated Rb N750F/N750F primary embryonic fibroblasts (MEFs) carrying the N750F substitution at the endogenous Rb gene (Supplementary Fig. S1A and ref. 21). As the consequences of abrogating pRB’s LxCxE-binding capacity may be masked by p130 and/or p107 (8, 22) and p130 is the main pocket protein recruited to repressed promoters in G0–G1, we also generated Rb N750F/N750F p130−/− MEFs and appropriate controls (Rb−/−, p130−/−, and Rb−/− p130−/−). Unfortunately, we have not obtained Rb N750F/N750F p107−/− MEFs as in our mouse background p107 deficiency alone already affected embryonic development.

pRB N750F protein level was similar to wild-type pRB (Supplementary Fig. S1B and S1C). Rb N750F/N750F MEFs displayed elevated expression of the E2F target genes Cyclin E and p107 during asynchronous proliferation (Supplementary Fig. S1B, lanes 1 and 3), upon expression of RASV12 (Supplementary Fig. S1B, lanes 4 and 6) and after serum starvation (Supplementary Fig. S1B, lanes 7 and 9), indicative for similar derepression as in Rb−/− cells. The effect on Cyclin A seemed less prominent. Strikingly, derepression was not enhanced by the absence of p130 (Supplementary Fig. S1C). Thus, the pRB–LxCxE interaction contributes to repression of E2F target genes in a physiologic setting.
Cell-cycle regulation in Rb<sup>N750F/N750F</sup> and Rb<sup>N750F/N750F<p130<sup>+/−</sup></p130−/−> MEFs

We tested the ability of Rb<sup>N750F/N750F</sup> and Rb<sup>N750F/N750F<p130<sup>+/−</sup></p130−/−> MEFs to arrest in response to antiproliferative signals.

Mitogen deprivation and confluency. Bypass of cell-cycle arrest in response to mitogen deprivation or cell–cell contact only occurred upon the combined loss of pRB and p130 (Table 1). Rb<sup>N750F/N750F</sup>p130<sup>+/−</sup> MEFs arrested as efficiently as wild-type and p130<sup>−/−</sup> MEFs (Table 1), showing that the LxCxE interaction of pRB did not contribute to cell-cycle arrest.

Ionizing radiation. G<sub>1</sub> arrest in response to γ-irradiation relies on pRB (32). Indeed, Rb<sup>−/−</sup> MEFs were severely impaired in arresting the cell cycle in response to γ-irradiation: at 5.5 Gy, wild-type and p130<sup>−/−</sup> MEFs displayed a 45% to 50% reduction in S phase cells, opposed to only 25% in Rb<sup>−/−</sup> MEFs (Fig. 2A, lanes 1–6). This effect was not aggravated upon concomitant ablation of p130 (Fig. 2A, lanes 7–8). Also the fold increase in (G<sub>1</sub> + G<sub>2</sub>)S ratio of irradiated versus untreated cells revealed an impaired arrest in Rb<sup>−/−</sup> MEFs, irrespective of p130 (Fig. 2B, lanes 1–4). Similarly irradiated Rb<sup>N750F/N750F</sup> MEFs displayed a reduction in S phase cells of 35% (Fig. 2A, lanes 9 and 10) and the fold increase in (G<sub>1</sub> + G<sub>2</sub>)S ratio was similar as in Rb<sup>−/−</sup> MEFs (Fig. 2B, lanes 2 and 5). Again, this effect was not modulated by p130 (Fig. 2A, lanes 9–12; Fig. 2B, lanes 5 and 6).

At higher doses of irradiation, Rb<sup>N750F/N750F</sup> MEFs displayed an intermediate phenotype: at 10 or 15 Gy, Rb<sup>N750F/N750F</sup> MEFs arrested less efficiently than wild-type MEFs, but more efficiently than Rb<sup>−/−</sup> MEFs (Supplementary Fig. S2A, lanes 4–6 and 7–9). Moreover, irradiation with 15 Gy induced a shift from G<sub>1</sub> toward G<sub>2</sub> arrest in both Rb<sup>N750F/N750F</sup> MEFs and Rb<sup>−/−</sup> MEFs (Supplementary Fig. S3).

To measure the stringency of radiation-induced cell-cycle arrest, serum-starved Rb<sup>N750F/N750F</sup>, Rb<sup>−/−</sup>, and Rb<sup>+/−</sup> MEFs were restimulated with serum and irradiated 7 hours later. Rb<sup>−/−</sup> MEFs entered S phase earlier than Rb<sup>+/−</sup> MEFs, with Rb<sup>N750F/N750F</sup> MEFs showing an intermediate phenotype (Supplementary Fig. S2B). Consistent with earlier findings (33), these results indicate that the pRB-LxCxE interaction contributes to DNA-damage–induced G<sub>1</sub> arrest.

Expression of RAS<sup>V12</sup>. Whereas pRB alone is the central player in the cellular response to ionizing radiation, bypass of RAS<sup>V12</sup>-induced growth inhibition required concomitant loss of pRB and p130, pRB and p107, or all three proteins (8, 34). We therefore studied the effect of RAS<sup>V12</sup> expression in a p130-defective background. Similar to Rb<sup>−/−</sup>p130<sup>−/−</sup> MEFs, Rb<sup>N750F/N750F</sup>p130<sup>−/−</sup> MEFs maintained a high proliferation rate upon RAS<sup>V12</sup> expression, whereas Rb<sup>+/−</sup>p130<sup>−/−</sup> MEFs decelerated proliferation (Fig. 3). This result indicates that the pRB–LxCxE interaction is critical for RAS<sup>V12</sup>-induced inhibition of proliferation.

RAS<sup>V12</sup>-induced transformation. We previously showed that Rb<sup>−/−</sup>p130<sup>−/−</sup> MEFs, but not Rb<sup>−/−</sup> or p130<sup>−/−</sup> MEFs, could proliferate anchorage independently upon expression of RAS<sup>V12</sup> and TBX2 (20). We therefore studied the transforming capacity of RAS<sup>V12</sup> in Rb<sup>N750F/N750F</sup>p130<sup>−/−</sup> MEFs rather than in Rb<sup>N750F/N750F</sup> MEFs. Surprisingly, Rb<sup>N750F/N750F</sup>p130<sup>−/−</sup> MEFs did not grow anchorage independently upon expression of RAS<sup>V12</sup> and TBX2 (Fig. 4). Thus, ablation of the pRB–LxCxE interaction alleviated RAS<sup>V12</sup>-induced growth inhibition (in the absence of p130) but was not sufficient to support RAS<sup>V12</sup>/TBX2-induced transformation.

p130 compensates for loss of pRB–LxCxE interactions in embryonic development

To investigate whether the pRB–LxCxE interaction is critical for pRB’s role in embryonic development, we intercrossed Rb<sup>N750F/wt</sup> mice. Although full ablation of Rb was embryonic lethal (35), Rb<sup>N750F</sup> animals were born according to Mendelian ratio and survived into adulthood.

In contrast, in crosses with p130<sup>−/−</sup> animals, life-born Rb<sup>N750F</sup>p130<sup>−/−</sup> animals were not obtained (Supplementary Table S1). Rb<sup>N750F</sup>p130<sup>−/−</sup> embryos were detected at embryonic day 18 but died prenatally. We could not test the effect of p107 disruption on the viability of Rb<sup>N750F</sup> mice as p107<sup>−/−</sup> mice died in utero. However, combination of Rb<sup>N750F</sup>p130<sup>−/−</sup>, Rb<sup>N750F</sup>p130<sup>−/−</sup>, and Rb<sup>N750F</sup>p107<sup>−/−</sup> allowed normal embryonic development and survival into adulthood.
Concomitant loss of pRB–LxCxE interactions and p130/p107 is not sufficient to drive tumorigenesis

Ablation of the pRB LxCxE-interacting domain did not affect lifespan or tumorigenesis (Fig. 5A). The absence of tumor development in Rb<sup>N750F/N750F</sup> mice sharply contrasted with the high incidence of pituitary and thyroid tumors in Rb<sup>+/-</sup> mice (<sup>9</sup>, <sup>10</sup>). We previously showed that Rb<sup>N750F/wtp130</sup> mice were not predisposed to tumor-ogenesis (<sup>9</sup>, <sup>10</sup>), reduced the survival of Rb<sup>N750F/N750F</sup> mice (<sup>9</sup>, <sup>10</sup>), and included retinoblastoma, osteosarcoma, lymphosarcoma, pheochromocytoma, and coecal adenocarcinoma (<sup>8</sup>, <sup>36</sup>). We therefore wondered whether tumor development in Rb<sup>N750F/wtp130</sup> mice was suppressed by compensatory activities of p130 or p107. Because Rb<sup>N750F/wtp130</sup> animals were not viable in the absence of p130 or p107 (Supplementary Table S1), we studied tumor incidence in mice with three mutant alleles, i.e., Rb<sup>N750F/N750F</sup>, p130<sup>-/-</sup>, or Rb<sup>N750F/N750F</sup>, p107<sup>-/-</sup>, anticipating that similar to p130<sup>-/-</sup> or p107<sup>-/-</sup> chimeric mice, tumor development may ensue from loss of the respective wild-type alleles (<sup>10</sup>). Although hemizygosity for p107, and perhaps also for p130, reduced the survival of Rb<sup>N750F/N750F</sup> mice (Fig. 5B and D, respectively), Rb<sup>N750F/N750F</sup>, p107<sup>-/-</sup> animals were not predisposed to tumorigenesis (Supplementary Table S2).

Also, Rb<sup>N750F/wt</sup>, p130<sup>-/-</sup> mice were not tumor prone, although they showed a severe reduction in life span (Fig. 5C), their median survival being reduced from >70 weeks in control littersmates to 32 weeks. Rb<sup>N750F/wt</sup>, p130<sup>-/-</sup> animals were smaller and skinnier than control littersmates and had an irregular fur. Of 37 animals, 24 had to be sacrificed due to sudden illness and 8 suddenly died before 60 weeks of age.

Figure 3. The pRB–LxCxE interaction is involved in RAS<sup>V12</sup>-induced senescence. p130<sup>-/-</sup>, Rb<sup>N750F/N750F</sup>, p130<sup>-/-</sup>, and Rb<sup>-/-</sup>, p130<sup>-/-</sup> MEFs were infected with pBABE-RAS<sup>V12</sup>-puro (+RAS<sup>V12</sup>) or pBABE-puro (–RAS<sup>V12</sup>) and plated for proliferation assays 4 days after infection. Cells were fixed and stained with crystal violet at the indicated time points. The relative cell number was determined by dividing OD<sub>590</sub> at time point x by OD<sub>590</sub> at time point 0. Two independent experiments (top and bottom), performed in triplicate, are shown.

Figure 4. Ablation of the pRB–LxCxE interaction in a p130 null background is not sufficient for anchorage-independent growth upon expression of TBX2 and RAS<sup>V12</sup>. Rb<sup>N750F/N750F</sup>, p130<sup>-/-</sup> and Rb<sup>-/-</sup>, p130<sup>-/-</sup> MEFs were infected with pEYK-TBX2 and subsequently with pBABE-RAS<sup>V12</sup>-puro and then plated in soft agar. Pictures were taken after 2 weeks using a non-phase-contrast lens (magnification, ×2.5) in A and a phase-contrast lens (magnification, ×5) in B. RAS<sup>V12</sup> and TBX2 were expressed at comparable levels in the two cell lines (not shown).
Surprisingly, microscopic examination of pituitary (n = 10), thyroid (n = 16), brain (n = 24), eyes (n = 23), digestive system (stomach, intestines, liver, pancreas; n = 22), testes (n = 7), ovary (n = 15), heart (n = 15), lungs (n = 22), adrenals (n = 16), spleen (n = 22), thymus (n = 19), and bone marrow (sternum, hind leg, head; n = 16) did not reveal abnormalities, except in the spleen (see below). Only three animals, older than 50 weeks, were diagnosed with tumor(s): one alveolar-bronchiolar adenoma, two adenomas in the pituitary gland, and one lymphoma (Supplementary Table S2). For comparison, RbN750F/wt p130+/– animals developed pituitary tumors between 32 and 36 weeks (10), and RbN750F/wt p107+/– mice, older than 50 weeks, had a reduced life span but were not predisposed to tumorigenesis.

Hematopoiesis in RbN750F/wt p130−/− animals

Twenty-four percent (n = 21) of RbN750F/wt p130−/− animals showed a reduced number of hematopoietic cells in the red pulp of the spleen ("reduced cellularity"), a phenomenon not seen in RbN750F/wt p130+/– and Rbwt/wt p130−/– animals (both n = 6). Of the five cases, only two showed reduced hematopoiesis in the bone marrow (not shown). Furthermore, red and white blood cell counts were sometimes reduced in ill RbN750F/wt p130−/– animals (data not shown), but this did not correlate with the reduced cellularity in the spleen. Therefore, the cause and significance of this observation remain elusive.

FACS analysis revealed normal percentages of Lin− Sca1+c-kit+ cells, long-term hematopoietic stem cells, short-term hematopoietic stem cells, myeloid progenitors, lymphoid progenitors, erythroid progenitors, monocytes, and granulocytes in the bone marrow of RbN750F−/− p130−/− mice (not shown). However, 2 of 4 RbN750F/wt p130−/− animals had a decreased population of cells with intermediate expression of CD19 and B220, possibly reflecting a deficit in pre-B-cell population (mice a and c in Supplementary Table S3; 0.75% vs. on average 6.6% in control animals, n = 10). Kaplan–Meier survival analyses argue against a B-cell defect in RbN750F/wt p130−/− animals. However, mature B cells, characterized by high CD19/B220 expression, were only mildly reduced (1.7% and 2.1%, vs. on average 3.3% in control animals, n = 16) did not reveal abnormalities, except in the spleen (see below). Only three animals, older than 50 weeks, were diagnosed with tumor(s): one alveolar-bronchiolar adenoma, two adenomas in the pituitary gland, and one lymphoma (Supplementary Table S2). For comparison, RbN750F/wt p130+/– animals developed pituitary tumors between 32 and 36 weeks (10), and RbN750F/wt p107+/– mice, older than 50 weeks, had a reduced life span but were not predisposed to tumorigenesis.

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FACS analysis revealed normal percentages of Lin− Sca1+c-kit+ cells, long-term hematopoietic stem cells, short-term hematopoietic stem cells, myeloid progenitors, lymphoid progenitors, erythroid progenitors, monocytes, and granulocytes in the bone marrow of RbN750F−/− p130−/− mice (not shown). However, 2 of 4 RbN750F/wt p130−/− animals had a decreased population of cells with intermediate expression of CD19 and B220, possibly reflecting a deficit in pre-B-cell population (mice a and c in Supplementary Table S3; 0.75% vs. on average 6.6% in control animals, n = 10). Kaplan–Meier survival analyses argue against a B-cell defect in RbN750F/wt p130−/− animals. However, mature B cells, characterized by high CD19/B220 expression, were only mildly reduced (1.7% and 2.1%, vs. on average 3.3% in control animals, n = 16) did not reveal abnormalities, except in the spleen (see below). Only three animals, older than 50 weeks, were diagnosed with tumor(s): one alveolar-bronchiolar adenoma, two adenomas in the pituitary gland, and one lymphoma (Supplementary Table S2). For comparison, RbN750F/wt p130+/– animals developed pituitary tumors between 32 and 36 weeks (10), and RbN750F/wt p107+/– mice, older than 50 weeks, had a reduced life span but were not predisposed to tumorigenesis.

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Decreased fat deposition in \( \text{Rb}^{N750F/\text{wt}}/p130^{-/-} \) animals

A defect that could relate to early death of \( \text{Rb}^{N750F/\text{wt}}/p130^{-/-} \) mice was suggested by their skinny appearance. Indeed, at the time they became ill, \( \text{Rb}^{N750F/\text{wt}}/p130^{-/-} \) mice showed strongly reduced or even absent subcutaneous adipose depots (Supplementary Fig. S4A) that lacked unilocular lipid droplet-containing adipocytes (Fig. 6A). Fat deposits, if present, looked like brown rather than white fat. The back skin of \( \text{Rb}^{N750F/\text{wt}}/p130^{-/-} \) animals was thinner and more transparent than in control mice (Supplementary Fig. S4A); in particular, the adipose tissue layer in the lower dermis was thin and in some \( \text{Rb}^{N750F/\text{wt}}/p130^{-/-} \) animals totally absent (Fig. 6B). Four to seven weeks old \( \text{Rb}^{N750F/\text{wt}}/p130^{-/-} \) animals already showed reduced thickness of the dermal adipose layer containing fewer adipocytes and smaller lipid droplets (Supplementary Fig. S4B). Anterior and posterior subcutaneous fat deposits were present, but again the size of the lipid droplets was smaller than in control littermates (Supplementary Fig. S4C). These observations indicate a shift of white to brown fat in \( \text{Rb}^{N750F/\text{wt}}/p130^{-/-} \) animals, which may impact on thermoregulation and viability of the animals.

Discussion

Given the proposed role of the pRB–LxCxE interaction in silencing of E2F target genes (4, 14), we asked whether this interaction is essential for pRB’s role in cell-cycle regulation, development, and suppression of tumorigenesis. To this aim, we abrogated the LxCxE binding site of mouse pRB by substituting asparagine at position 750 for phenylalanine. pRBN750F retained the ability to inhibit E2F1 transcriptional activity, but was unable to bind an LxCxE-containing protein and to effectively mediate E2F4-dependent transcriptional repression. We introduced the N750F substitution at the endogenous \( \text{Rb} \) gene and studied its consequences in the absence of p130.

The role of pRB–LxCxE interactions in cell-cycle regulation

We found the pRB–LxCxE interaction to contribute to growth inhibition in response to \( \gamma \)-irradiation and expression of RAS\(_{V12} \), but not after growth factor deprivation or contact inhibition.
Growth factor deprivation and irradiation. Although serum starvation was found to induce pRB- and HDAC1-dependent deacetylation and silencing of the Cyclin E promoter (15) and a mutation in the LxCxE binding site of pRB increased Cyclin E expression during serum starvation (our experiments; ref. 14), Rb<sup>N750F/N750F</sup>p130<sup>−/−</sup> MEFs arrested normally in response to serum starvation, whereas Rb<sup>−/−</sup>p130<sup>−/−</sup> MEFs did not. Therefore, cell-cycle arrest after serum withdrawal does not rely on pRB’s LxCxE binding site but is effectuated by other pocket protein functions, possibly inhibition of E2F transactivation or another activity of pRB.

In contrast, pRB’s critical role in cycle-cell arrest after γ-irradiation partially relied on its interaction with LxCxE proteins. Similar observations were made in response to UV irradiation and cisplatin exposure (33, 39). Cycle arrest upon γ-irradiation coincided with reduced CDK2 kinase activity, indicative for pRB-HDAC-mediated repression of the CDK2 regulator Cyclin E (32). However, other LxCxE proteins may be involved as well, such as the LxCxE-containing protein FB-Cp145, which enhanced survival after DNA damage in the presence of pRB (39); LxCxE members of the p200 family, which may stabilize the hypo-phosphorylated form of pRB upon DNA damage (40); the APC–Cdh1 complex that binds pRB in an LxCxE-dependent manner causing downregulation of Skp2 and stabilization of p27<sup>Kip1</sup> (41).

RAS<sup>V12</sup>-induced senescence and transformation. RAS<sup>V12</sup>-induced growth inhibition also required pRB’s LxCxE-interacting domain. Consistently, expression of the DNA binding domain of E2F1 (E2F-DB) could bypass RAS<sup>V12</sup>-induced senescence (42). Because E2F-DB cannot activate transcription but only displace endogenous E2Fs from promoter regions, these results implied that relieve of E2F-mediated transcriptional repression can overcome RAS<sup>V12</sup>-induced senescence. In addition, Narita and colleagues (16) detected recruitment of HIP1γ and the presence of methylated H3K9 to promoter regions in RAS<sup>V12</sup>-induced senescent cells, but not in confluent quiescent cells.

Surprisingly, although loss of pRB’s LxCxE-binding activity in Rb<sup>N750F/N750F</sup>p130<sup>−/−</sup> MEFs allowed proliferation in the presence of RAS<sup>V12</sup>, it did not allow transformation by RAS<sup>V12</sup> and TBX2. Possibly, derepression of Cyclin E in Rb<sup>N750F/N750F</sup>p130<sup>−/−</sup> MEFs induced CDK2 activity to a level sufficient to bypass RAS<sup>V12</sup>-induced senescence, but insufficient to counteract the dramatic drop in kinase activities that normally arrest cells as a response to serum starvation, whereas Rb<sup>−/−</sup>p130<sup>−/−</sup> MEFs did not. Therefore, cell-cycle arrest after serum withdrawal does not rely on pRB’s LxCxE binding site but is effectuated by other pocket protein functions, possibly inhibition of E2F transactivation or another activity of pRB.

In vitro significance of pRB–LxCxE interaction

Our <em>in vitro</em> experiments show that ablation of the pRB–LxCxE interaction attenuated cell-cycle arrest upon γ-irradiation and expression of RAS<sup>V12</sup>, but did not alleviate cell-cycle arrest upon serum starvation or cell–cell contact and was not sufficient to support anchorage-independent growth. It was therefore of interest to determine whether recruitment of LxCxE-containing proteins by pRB is critical for mouse development and survival and for tumor suppression, in particular in the absence of p130 or p107.

Development. The perinatal death of Rb<sup>N750F/N750F</sup>p130<sup>−/−</sup> animals as opposed Rb<sup>N750F/N750F</sup>p107<sup>−/−</sup> mice suggests that recruitment of chromatin remodeling complexes via LxCxE-mediated interactions is essential for completion of embryonic development. This is consistent with embryonic failure of p130<sup>−/−</sup>p107<sup>−/−</sup> and E2F4<sup>−/−</sup>E2F5<sup>−/−</sup> mice (45, 46).

Triple Rb<sup>N750F/N750F</sup>p130<sup>−/−</sup> and Rb<sup>N750F/wtp130<sup>−/−</sup> mice developed to term and survived into adulthood, although the lifespan of the latter was severely reduced. This may be related to strongly reduced dermal and subcutaneous fat deposits, with white adipose tissue having acquired a morphology resembling brown fat. Previous reports have shown the involvement of pocket proteins in the control of white versus brown fat cell differentiation, the latter predominating in the absence of Rb or p107 (47, 48). Perhaps Rb<sup>N750F/wtp130<sup>−/−</sup> mice suffered from excessive heat loss due to the reduced presence of dermal white adipocytes and thin skin. Heat loss may be compensated for by “browning” of residual subcutaneous white fat deposits through recruitment of thermogenic adipocytes in normally white adipose depots. This may result in excessive energy consumption in adipose tissue, possibly depleting energy-rich substrates required for maintaining normal thermoregulation. However, further experiments are required to support this scenario.

Tumorigenesis. Our previous studies demonstrated that Rb<sup>−/−</sup>p107<sup>−/−</sup> and Rb<sup>−/−</sup>p130<sup>−/−</sup> chimeric mice developed a wide spectrum of tumors following loss of the wild-type Rb allele (8). Surprisingly, not only Rb<sup>N750F/N750F</sup> mice but also Rb<sup>N750F/N750F</sup>p130<sup>−/−</sup>, Rb<sup>N750F/wtp130<sup>−/−</sup>, and Rb<sup>N750F/N750F</sup>p107<sup>−/−</sup> mice were not tumor prone. Although one may argue that the wasting syndrome in Rb<sup>N750F/wtp130<sup>−/−</sup> mice may have precluded tumor development, this cannot explain the absence of tumor development in Rb<sup>N750F/N750F</sup>p130<sup>−/−</sup> and Rb<sup>N750F/N750F</sup>p107<sup>−/−</sup> mice that did not show this syndrome. Therefore, the absence of tumor susceptibility in these triple allele mutant mice implies that Rb<sup>N750F/N750F</sup>p130<sup>−/−</sup> and Rb<sup>N750F/N750F</sup>p107<sup>−/−</sup> cells, which are expected to frequently arise by spontaneous loss of heterozygosity, do not readily undergo oncogenic transformation. Our results therefore argue against a major role for LxCxE-containing proteins in tumor suppression.

It remains possible that loss of the pRB–LxCxE interaction stimulates tumor formation only under specific conditions for example, mice with an ablated pRB–LxCxE interaction were prone to genotoxin-induced liver tumorigenesis, consistent with the role of pRB–LxCxE interactions in DNA damage response (33). Also, abrogation of the pRB–LxCxE interaction enhanced tumorigenesis in combination with mutant or...
inactive p53 (43, 49), an effect that may be related to the involvement of pRB’s LxCxE-binding activity in chromosome condensation and cohesion (49, 50).

The absence of tumor development in Rb$^{N750F/p130}$- and Rb$^{N750F/p107}$-mutant mice indicates that disturbance of transcriptional regulation by loss of pRB–LxCxE interactions is insufficient to promote spontaneous tumor development. Thus, pRB’s tumor suppressor activity may be effecuated through regulation of activator E2Fs rather than by active silencing of E2F target genes. However, in some tissues, activator E2F activity was dispensable for proliferation (51–53). It may therefore be possible that pRB–LxCxE and pRB-activator E2F interactions are each dispensable for tumor suppression but not both together. Alternatively, a different function of pRB is critical for tumor suppression, such as LxCxE-independent transcriptional repression or a function of pRB in S-phase during DNA replication. Irrespective of the mechanism, our results suggest that the in vitro cellular response to mitogen deprivation or cell–cell contact is a better predictor of tumorigenesis than RAS$^{\text{V12}}$-induced growth inhibition.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References

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RB Family Tumor Suppressor Activity May Not Relate to Active Silencing of E2F Target Genes

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