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Tissue-specific tumor suppressor activity of retinoblastoma gene homologs $p_{107}$ and $p_{130}$

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The retinoblastoma gene family consists of three genes: $RB$, $p_{107}$, and $p_{130}$. While loss of pRB causes retinoblastoma in humans and pituitary gland tumors in mice, tumorigenesis in other tissues may be suppressed by $p_{107}$ and $p_{130}$. To test this hypothesis, we have generated chimeric mice from embryonic stem cells carrying compound loss-of-function mutations in the $Rb$ gene family. We found that $Rb/p_{107}$- and $Rb/p_{130}$-deficient mice were highly cancer prone. We conclude that in a variety of tissues tumor development by loss of pRB is suppressed by its homologs $p_{107}$ and $p_{130}$. The redundancy of the retinoblastoma proteins in vivo is reflected by the behavior of $Rb$-family-defective mouse embryonic fibroblasts in vitro.

[Keywords: Retinoblastoma, cancer, mouse model, pocket proteins]

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Inactivation of the retinoblastoma suppressor pRB has been found in many human cancers, including hereditary retinoblastoma and sporadic breast, bladder, prostate, and small cell lung carcinoma (Friend et al. 1986, 1987; Harbour et al. 1988; Lee et al. 1988; T’Ang et al. 1988; Bookstein et al. 1990). pRB controls the G1/S transition of the cell cycle by modulating the activity of E2F transcription factors. Hypophosphorylated pRB binds to E2Fs and forms complexes harboring chromatin remodeling proteins like histone deacetylases, SWI/SNF and histone methyltransferases, which actively repress genes that are essential for cell cycle regulation, DNA replication, DNA repair, G3/M checkpoints and differentiation (Harbour and Dean 2000; Muller et al. 2001; Ishida et al. 2001; Kalma et al. 2001; Kalma et al. 2001; Rayman et al. 2002; Ren et al. 2002). Upon sequential phosphorylation by Cyclin D/CDK4 and Cyclin E/CDK2 kinases, pRB undergoes a conformational change leading to the release of E2Fs. Derepression and activation of E2F target genes then allows cell cycle progression (Trimarchi and Lees 2002). Conversely, down regulation of Cyclin/CDK activity by the INK4A and CIP/KIP family of cyclin dependent kinase inhibitors (CDKIs), promotes cell cycle arrest (Ruas and Peters 1998, Sherr and Roberts 1999, Sherr 2001].

Thus, inactivation of the G1/S checkpoint can occur at different levels, and indeed, besides loss of $RB$ in many cancers, loss of $p_{16}^{N_{k}A}$ function has been found in melanoma, pancreatic, and bladder carcinomas, amplification of $Cyclin D1$ in breast, oesophagus, and head-and-neck cancer, and $CDK4$ amplification or mutational activation in melanoma (for review, see Sherr 1996).

$RB$ is a member of the retinoblastoma gene family that encodes the so-called pocket proteins pRB, $p_{107}$, and $p_{130}$ [Ewen et al. 1991; Hannon et al. 1993; Li et al. 1993; Chow and Dean 1996]. All three can repress transcription from E2F-responsive promoters (Zamanian and La 1993, Bremner et al. 1996; Ren et al.2002. Over-expression of each of the pocket proteins results in growth suppression, although not every (tumor) cell type is equally sensitive to each family member [Zhu et al. 1996; Claudio et al. 1994; Beijersbergen et al. 1995]. Whereas pRB predominantly binds E2F1, E2F2, and E2F3, $p_{107}$ and $p_{130}$ specifically bind E2F4 and E2F5 (Dyson 1998; Trimarchi and Lees 2002) and each of these complexes appears at different stages of the cell cycle: p130/E2F4 is mainly found in $G_{0}$, pRB/E2F in $G_{0}$ and $G_{1}$, and $p_{107}$/E2F in S-phase [Dyson 1998]. Finally, $p_{107}$/E2F and $p_{130}$/E2F complexes act as transcriptional repressors of a set of genes different from that regulated by Rb/E2F complexes [Hurford et al. 1997].
In primary mouse embryonic fibroblasts (MEFs), pocket proteins collectively regulate the response of cells to growth-inhibiting signals (Dannenberg et al. 2000; Sage et al. 2000). Triple knockout cells were refractory to replicative and oncogenic RAS-induced senescence despite accumulating levels of the cell cycle inhibitors p16\textsuperscript{INK4A}, p19\textsuperscript{ARF}, p53, and p21\textsuperscript{CIP}, indicating that both the p16\textsuperscript{INK4A}–Cyclin D1/CDK4 and p19\textsuperscript{ARF}–MDM2–p53 tumor surveillance pathways converge on the retinoblastoma protein family. By comparing the growth behavior of double knockout MEFs, we now show that p107 and p130 together can compensate for loss of pRb in mediating growth arrest following activation of the p19ARF–MDM2–p53 pathway.

In mice, homozygous inactivation of Rb through the germ-line resulted in embryonic lethality and Rb heterozygosity led to development of pituitary gland tumors (Clarke et al. 1992; Jacks et al. 1992; Lee et al. 1992; Wu et al. 2003). In contrast, p107−/− or p130−/− mice in a C57BL/6 background did not show an overt phenotype nor a higher incidence of tumors compared with wild-type mice (Cobrinik et al. 1996; Lee et al. 1996). However, Rb−/−p107−/− and Rb−/−p130−/− mice showed a strongly reduced viability (Lee et al. 1996; J.-H. Dannenberg and H. te Riele, unpubl.).

While these results clearly point to redundant functions of pocket proteins in development, it is unclear whether this also extends to suppression of tumorigenesis. Embryonic lethality associated with pRB deficiency and the reduced viability caused by combinations of knockout alleles of Rb gene family members, precludes studying this issue by combining knockout alleles in conventional crosses. Embryonic lethality might be circumvented by using conditional knockout alleles; however, this approach heavily relies on pre-existing knowledge of the spatial and temporal requirements for expression of Cre recombinase. We have therefore taken a more unbiased approach based on our previous observation that embryonic lethality caused by Rb deficiency could be circumvented in chimeric mice generated from Rb−/− ES cells (Robanus Maandag et al. 1994). In these animals Rb−/− deficient cells were present in virtually all tissues including the pituitary gland where they developed tumors (Robanus Maandag et al. 1994, Williams et al. 1994a). By this approach we also found that combined inactivation of Rb and p107 resulted in development of retinoblastoma (Robanus Maandag et al. 1998). To explore the role of p107 and p130 in tumor suppression we have generated Rb+/−p107−/−, Rb+/−p130−/−, and Rb−/−p130−/− chimeras. The broad spectrum of tumors found in these mice but not in pure Rb−/− and chimeric Rb−/−, p130−/− mice identifies both p107 and p130 as tumor suppressors in a variety of different tissues.

Results
Pocket protein requirements for replicative and Ras-induced senescence

We have generated ES cells carrying different combinations of genetically inactivated alleles of Rb, p107, and p130 and used these to derive an isogenic set of MEF cultures (Fig. 1A) as described before (Dannenberg et al. 2000). Compared with wild-type MEFs, Rb−/− and Rb−/−p130−/− MEFs showed elevated levels of p107 and Rb−/− and Rb−/−p130−/− MEFs showed up-regulation of p130.
indicative for a functional p53 protein (Fig. 1C). Furthermore, both Rb−/−p107−/− and Rb−/−p130−/− MEFs showed increased levels of Cyclin E and A and the E2F1 target DHFR [Fig. 1A]. We compared the growth behavior of the three double knockout combinations to that of wild-type and Rb−/− cells. Figure 1B shows that upon serial passaging, wild-type, Rb−/−, and p107−/−p130−/− cells rapidly lost proliferative potential and entered a senescent state, albeit that the arrest of Rb−/− cells was somewhat less stringent. In these experiments, all three genotypes escaped senescence after ~13–20 passages. As shown for six p107−/−p130−/−/3T9 clones in Figure 1C, these cells had either lost p19ARF (clones 1,3) or p53 (clones 2,4,5,6). In contrast, Rb−/−p107−/− and Rb−/−p130−/− cells never entered a senescent state but continued to proliferate despite accumulating levels of p19ARF and the presence of active p53 function. Thus, p19ARF was still expressed in six independent Rb−/−p130−/−/3T9 clones obtained from six different MEF isolates [Fig. 1C]. Furthermore, treatment of Rb−/−p130−/−/3T9 clones with the DNA damaging agent cisplatin for 24 h induced p53 concomitantly with its target p21CIP, indicative for a functional p53 protein [Fig. 1C].

Activation of the p19ARF–Mdm2–p53 pathway not only restricts proliferation of cells during prolonged culturing, but also provides a mechanism to block proliferation of cells in the presence of oncogenic signals. Infection of early passage wild-type, Rb−/−, Rb−/−p107−/−, Rb−/−p130−/−, p107−/−p130−/−, and TR+ MEFs with RasV12, expressing retroviruses resulted in elevated levels of RasV12 and induction of p19ARF and p53 [data not shown]. Thus, the pathway normally required by RasV12 to induce a cell cycle arrest was intact in all genotypes. Figure 1D shows that expression of RasV12 suppressed the growth of wild-type and p107−/−p130−/− MEFs and after a brief latency period also of Rb−/− MEFs. In contrast, Rb−/−p107−/− and Rb−/−p130−/− cells were resistant to RasV12-induced cell cycle arrest and, similar to triple knockout MEFs, showed increased proliferative capacity.

Together with our previous observations [Dannenberg et al. 2000], these results define the minimal requirement for pocket proteins in the p19ARF–Mdm2–p53 growth suppression pathway. In particular, p107 plus p130 can substitute for loss of pRb to suppress proliferation of MEFs in the presence of oncogenic signaling. These results prompted us to study whether p107 and p130 can also substitute for loss of pRb in suppressing proliferation in vivo.

Rb−/−p107−/− chimeric mice develop a wide spectrum of tumors

In pure Rb−/− and chimeric Rb−/− mice, tumor development is restricted to the intermediate lobe of the pituitary gland [Robanus Maandag et al. 1994; Williams et al. 1994a]. To study whether tumor development in other tissues was suppressed by p107, we have generated chimeric mice from Rb−/−p107−/− and Rb−/−p130−/− ES cells. The latter genotype resulted in development of retinoblastoma at early age; however, the poor viability of these mice precluded the assessment of tumor suscepti-

bility later in life [Robanus-Maandag et al. 1998]. In contrast, Rb−/−p107−/− chimeras were readily obtained and showed high ES cell contribution despite the poor viability of pure Rb−/−p107−/− mice [Lee et al. 1996; J.-H. Dannenberg and H. te Riele, unpubl.].

In a cohort of 53 Rb−/−p107−/− chimeric mice, tumors started to develop after 6 mo, most frequently in the pituitary gland, the cocum, the bone, and lymphoid tissue [Table 1]. Primary and metastasized osteosarcoma and leiomyosarcoma are shown in Figure 2A–D. These animals did not develop retinoblastoma but occasionally showed retinal dysplasia [data not shown]. A polymorphism in the Rb allele allowed us to distinguish by Southern blotting the wild-type Rb alleles in blastocyst (C57BL/6-) and ES cell [129OLA]-derived DNA as well as the Rb knockout allele. This showed that all tumors were of ES cell origin and that loss of the wild-type Rb allele had occurred in all pituitary gland tumors and in 70% of the other tumors [Fig. 2E, Table 1]. We conclude that the limited tumor spectrum in Rb-deficient mice is caused by tumor suppressor activity of p107 in a variety of tissues.

Tumorigenesis in Rbp130-deficient chimeras

To investigate whether p130 can also compensate for loss of pRB in tumor suppression, we followed cohorts of Rb−/−p130−/− and Rb−/−p130−/− chimeric mice. Both genotypes were readily obtained [e.g., Rb−/−p130−/− chimeras at a frequency of 11 out of 28 births as opposed to only seven Rb−/−p107−/− chimeras out of 56 births; Robanus-Maandag et al. 1998]. In 15 Rb−/−p130−/− chimeras, we found a thymoma, a hepatoma, a Leydig cell tumor, an insulinoma, and an adrenal gland tumor. However, none of these tumors was found in more than one animal. In contrast, Rb−/−p130−/− chimeric mice consistently developed retinoblastoma, pheochromocytoma, and hyperplasia of neuro-endocrine epithelial cells of the

| Table 1. Tumor incidence in 53 Rb−/−p107−/− chimeric mice and frequency of Rb LOH |
|-----------------|-----------------|
| Tumor                  | Number | Loss of Rb′ allele |
|-----------------|-----------------|
| pituitary gland tumor | 22       | 20/20       |
| adenocarcinoma coecum | 8        | 4/6          |
| osteosarcoma          | 8        | 3/5          |
| lymphosarcoma         | 8        | 4/5          |
| leiomyosarcoma        | 4        | 1/1          |
| thymoma               | 3        | 0/3          |
| ovary tumor           | 2        | 2/2          |
| thyroid tumor         | 2        | 1/1          |
| adrenal gland tumor   | 2        | 1/1          |
| intestinal tumor      | 2        | 1/1          |
| lung tumor            | 1        | 1/1          |
| testis tumor          | 1        | 1/1          |
| Total                 | 41       | 20/28        |

*Number of tumors showing loss of the Rb wild-type allele/number of tumors tested.
eyes that were sacrificed at 2 wk of age due to tumors at other sites (see below), retinal dysplasia was found in the outer plexiform layer in the peripheral region of the retina. The four remaining animals were poorly chimeric and showed no retinal abnormalities.

To ascertain that the retinoblastomas were of 129OLA ES cell origin, tumor tissue recovered from paraffin sections was subjected to a PCR amplifying the polymorphic CA-repeat sequence D2mit94. 129OLA and C57BL/6 DNA gave 190 and 160 base pair products, respectively. The predominant amplification of a 190 base pair fragment indicates that all three retinoblastomas were of ES cell origin [Fig. 3E].

**Rb⁻/⁻p130⁻/⁻ retinoblastoma has inner nuclear layer characteristics**

Similar to Rb⁻/⁻p107⁻/⁻ retinoblastomas (Robanus-Maandag et al. 1998), we found that Rb⁻/⁻p130⁻/⁻ retinoblastomas did not express IRBP [Fig. 4, cf. A and B,C] but showed positive staining for syntaxin that labels all mature neuronal amacrine cells in the inner nuclear layer and their synaptic processes in the inner plexiform layer [Barnstable et al. 1985] and γ-amino butyric acid (GABA) that identifies GABAergic amacrine cells [Haverkamp and Wässle 2000] [Fig. 4D,H]. In most tumors, staining for these two amacrine markers was local [Fig. 4F,J]; one early retinoblastoma stained homogeneously for GABA [Fig. 4I]. The tumors occasionally showed expression of glutamine synthethase, which identifies Müller glia cells [Bjorklund et al. 1985; Haverkamp and Wässle 2000] and glial fibrillary acidic protein (GFAP), which predominantly recognizes reactive Müller glia cells [Eisenfield et al. 1984] [Fig. 4F-H]. Finally, the DNA staining pattern of tumor cells closely resembled that of inner nuclear layer cells [Fig. 4E]. Thus, both Rb⁻/⁻p130⁻/⁻ and Rb⁻/⁻p107⁻/⁻ retinoblastomas show characteristics of amacrine cells in the inner nuclear layer.

**Early stages of Rb⁻/⁻p130⁻/⁻ retinoblastoma**

To identify early retinal lesions in Rb⁻/⁻p130⁻/⁻ chimeras, we have provided mutant ES cells with a β-galactosidase marker gene, placed under control of the ubiquitously active Rosa26 promoter [Fig. 5, panel I]. X-gal staining of the retina of two 2-wk-old Rb⁻/⁻p130⁻/⁻-LacZ chimeric mice readily identified ES cell-derived hyperplastic lesions in the outer plexiform layer where nor-
nally no nuclei are present [Fig. 5, panel II, A–C]. No obvious retinal abnormalities were observed in $Rb^{-/-}$p130$^{-/-}$LacZ chimeric embryos at day 18 p.c. (post-coitum) [Fig. 5, panel II, D]. The nuclear morphology of lesions in newborn $Rb^{-/-}$p130$^{-/-}$LacZ chimeras resembled the inner rather than the outer nuclear layer (Fig. 5, panel II, F). Furthermore, in contrast to wild-type cells, which all had left the cell cycle by postnatal day 10 (Young 1985), $Rb^{-/-}$p130$^{-/-}$ cells in the lesions were actively synthesizing DNA as evidenced by BrdU incorporation (Fig. 5, panel II, E,F). These data indicate that only a subset of Rbp130-deficient retinoblasts committed to the inner nuclear layer can escape cell cycle control and form hyperplastic lesions, which are likely the precursors of retinoblastoma later in life.

Figure 3. ES cell-derived retinoblastoma in $Rb^{-/-}$p130$^{-/-}$ chimeric mice. (A) Retinoblastoma in a 3-mo-old $Rb^{-/-}$p130$^{-/-}$ chimera. The lens shows cataracts. (B) The tumor has invaded the posterior eye chamber. (C,D) Rosette-like structures present throughout the tumor. (D) Two independent retinoblastomas in the peripheries of the retina in a 1-mo-old $Rb^{-/-}$p130$^{-/-}$ chimera. One tumor grew between the ONL and the RPE, while the other was growing between the INL and the GCL. (E) PCR amplification of the CA-repeat sequence D2Mit94 in DNA from three retinoblastomas [1–3] and two pheochromocytomas [1,2], giving 190-bp [129OLA] and 160-bp [C57BL/6] fragments. DNA from a F1 [129OLAxC57BL/6] mouse was used as a control. (GCL) Ganglion cell layer; (INL) inner nuclear layer; (L) lens; (ONL) outer nuclear layer; (RPE) retinal pigment epithelium; (R) rosette-like structures; (T) tumor. Magnification: A, 2.5×; B, 10×; C, 20×; D, 5×.

Figure 4. Inner nuclear layer characteristics of $Rb^{-/-}$p130$^{-/-}$ retinoblastomas. Immunohistochemical staining for retinal markers in a normal retina [A,D,H] and different $Rb^{-/-}$p130$^{-/-}$ retinoblastomas [B,C,E–G,I,J]. (A) Normal retina showing IRBP in the outer segments (OS) of the photoreceptor cells. (B) IRBP staining was absent in retinoblastomas in a 1-mo-old [B] or a 3-mo-old [C] chimera. (D) Normal retina showing Syntaxin-positive amacrine cells in the inner nuclear layer (INL) and their synaptic processes in the inner plexiform layer. (E) Topro-3 DNA-stained tumor nuclei resemble the INL. A rosette-like structure is indicated with an asterisk. (F) Syntaxin+ as well as GFAP+ [Müller glia cells] cells were identified locally in retinoblastoma. (G) Syntaxin+ and GFAP+ cells in retinoblastoma close to remnants of amacrine synaptic processes. (H) Normal retina showing GABAergic amacrine cells in the INL (arrows) and Müller glia cells and their synaptic processes, identified by glutamine synthetase expression, in the GCL, INL, and ONL. (I) Extensive positive staining for GABA but not for glutamine synthetase in a retinoblastoma in a 1-mo-old chimera. (J) Local staining for GABA and glutamine synthetase in a retinoblastoma in a 3-mo-old chimera. (B,E,F) Tumor borders are indicated with dotted lines. Magnification: A,D,E,H, 20×; B,C,F,I,J, 10×; G, 40×.
Absence of pRb and p130 predisposes to pheochromocytoma and lung hyperplasia

Six out of 11 Rb−/−p130−/− chimeras developed bilateral adrenal medullary tumors (Fig. 6A,B; Table 2), some already at 2 wk of age (Fig. 6C). The ES cell origin of these tumors was confirmed by PCR amplification of the D2Mit94 CA-repeat marker from tumor DNA (Fig. 3) and whole mount X-gal staining taking advantage of /H9252-galactosidase-marked ES cells (Fig. 6F). Positive staining for the neuro-endocrine tissue markers chromogranin A and synaptophysin and negative staining for S-100 (Fig. 6D,E; data not shown), identified the tumors as pheochromocytomas (PCC) (Lloyd et al. 1985). The tumors were actively proliferating as evidenced by the presence of mitotic figures and incorporation of BrdU (Fig. 6G). The adrenal medulla of Rb+/−p130−/− chimeric animals up to 9 mo of age did not display hyperplasia or tumors, despite the presence of ES-cell-derived cells (Fig. 6H). However, one out of 15 Rb+/−p130−/− chimeric animals developed a unilateral ES-cell-derived PCC at 14 mo of age [Fig. 6I]. Since Rb−/− chimeric mice showed hyperplastic nodules in the adrenal medulla, but never full blown tumors [Robanus Maandag et al. 1994, Williams et al. 1994a], we conclude that p130 suppresses oncogenic transformation of pRB-deficient adrenal medulla cells.

Histological examination of the lungs of Rb−/−p130−/− chimeric mice showed that seven mice contained hyperplastic lesions in the bronchial epithelium (Fig. 7A–C) and, occasionally, in alveolar parts (Fig. 7B). All lesions were of ES-cell origin as evidenced by blue staining [Fig. 7E–G]. The presence of blue patches in the epithelial lining of the bronchia that were histologically normal, suggests that inactivation of Rb and p130 is not sufficient to induce bronchial hyperplasia (Fig. 7E). Consistently, we did not observe bronchial abnormalities in Rb+/−p130−/− chimeric mice, although ES cell descendants were abundantly present (Fig. 7H). Positive staining for the neuro-endocrine-specific marker synaptophysin showed that all hyperplastic lesions were of...
Nevertheless, this approach has identified p107 and p130 as genuine tumor suppressors in a number of tissues. Thus, p107 alone collaborates with pRb in suppressing development of coecal adenocarcinomas, osteosarcomas, lymphomasarcomas, leiomyosarcomas, and tumors located to the thyroid, adrenal gland, and ovary. Interestingly, osteosarcoma and leiomyosarcoma, are well-documented secondary malignancies in retinoblastoma patients [Moll et al. 1997; Ryan et al. 2003]. p130 compensates for loss of pRb in suppressing pheochromocytomas and small cell lung cancer. Remarkably, p107 and p130 are both required to suppress transformation of pRb-deficient retinoblasts.

Tumor surveillance by the pRB protein family

One explanation for tumor development upon concomitant ablation of pRB and p107 or p130 may be derived from the critical role of pocket proteins in the p19ARF-p53 tumor surveillance pathway [Sherr 1998]. Our present and previous results show that in MEFs, concomitant ablation of Rb and p107 or Rb and p130 disabled the p19ARF-p53 tumor surveillance mechanism causing immortality and allowing sustained proliferation upon expression of oncogenic Ras120 (see also Dannenberg et al. 2000; Peep et al. 2001). Consistently, development of

Figure 6. Neuro-endocrine adrenal medullary tumors in Rb−/− p130−/− chimeras. Hematoxilin-eosin-stained (A–E,G) and X-gal-stained/nuclear fast red-counterstained (F,H,I) histological sections of adrenal gland and adrenal medullary tumors. (A) Normal adrenal gland in an Rb−/− p130−/− chimeric mouse. (B) Large adrenal medullary tumor invading the retroperitoneal fat (arrow). (C) Small adrenal medullary gland tumor in a 2-wk-old chimera. (D,E) Adrenal medullary tumors stain positive for neuro-endocrine markers chromogranin A (D) and synaptophysin (E). (F) An X-gal-stained Rb−/− p130−/− adrenal tumor, counterstained with nuclear fast red. In contrast to the adrenal cortex, the tumor entirely consists of β-galactosidase+ cells. (G) Extensive proliferation in an adrenal medullary tumor indicated by anti-BrdU staining. (H) No tumor development in the adrenal medulla of a 9-mo-old Rb−/− p130−/− chimera despite the presence of β-galactosidase+ cells. (I) Unilateral adrenal medullary tumor in a 14-mo-old Rb−/− p130−/− chimera showing β-galactosidase+ cells. (C) Adrenal cortex; (M) adrenal medulla; (T) tumor; (K) kidney. Magnification: A,C,E, 5×; B, 2.5×; D,F,G,H, 10×; I, 20×.

Figure 7. Pulmonary neuro-endocrine epithelial hyperplasias in Rb−/− p130−/− chimeric mice. (A) Normal lung. (B) Alveolar hyperplastic nodules and bronchial epithelial hyperplasia (arrows) in a 5-mo-old Rb−/− p130−/− chimera. (C,D) Bronchial epithelial hyperplasia in a 2.5-mo-old (C) and a 5-mo-old (D) Rb−/− p130−/− chimera. (E) X-gal-stained/nuclear-fast-red-counterstained Rb−/− p130−/− chimeric lung, containing multiple bronchial epithelial hyperplasias (arrows). (F,G) Only β-galactosidase+ Rb−/− p130−/− ES cell-derived cells are present in bronchial epithelial hyperplasias. (H) Rb−/− p130−/− ES-cells contributed to the lung of a 9-mo-old Rb−/− p130−/− chimeric mouse but did not form hyperplastic lesions. (I) Bronchial epithelial hyperplasia staining positive for the neuro-endocrine marker synaptophysin. Magnification: A,D,H, 20×; B,E, 10×; C,G, 40×; F,I, 20×.
Retinoblastoma or PCC in pocket protein deficient mice did not require mutation of p53 [Supplementary Fig. 1]. These results may suggest that in certain cell types p19Arf/p53 and pocket proteins are part of the same tumor surveillance mechanism and that loss of this pathway contributes to tumor development.

It should be noted, however, that the tumor spectrum in Rb/p107- and Rb/p130-defective chimeras did not entirely overlap. Thus, the requirement for pocket proteins in tumor suppression is cell-type dependent. For example, in the retina, similar as in MEF cultures, p107 and p130 are both required to suppress proliferation of pRb-deficient cells, while in the adrenal gland p130 alone can compensate for loss of pRb. This may indicate that the suppression of tumorigenesis by p107 and p130 involves other, tissue-specific functions besides regulation of E2Fs. For example, the occurrence of osteosarcomas in Rb+/−p107−/− chimeras but not in Rb+/−p130−/− may be related to a specific role of p107 in bone development (Thomas et al. 2001; Laplantine et al. 2002).

Retinoblastoma origin

By LacZ tagging we showed that only a subset of Rb−/−p130−/− retinoblasts, displaying features of inner nuclear layer nuclei, sustained continuous proliferation. Additionally, retinoblastomas in both Rb−/−p107−/− and Rb−/−p130−/− chimeras expressed amacrine-specific markers. Based on immunohistochemical analyses and morphological characteristics, human retinoblastoma was hypothesized to originate from photoreceptor cells in the outer nuclear layer [Bogenmann et al. 1988; Tajima et al. 1994]. However, analyses of early, small retinoblastomas pointed to an inner nuclear layer origin, although they showed Flexner-Wintersteiner rosettes [Gallie et al. 1999]. Thus, murine retinoblastomas resemble at least a subset of human retinoblastoma.

Similar findings were recently reported using heritable mouse models for retinoblastoma in which Rb was ablated in a p107- or p130-deficient background [Chen et al. 2004; MacPherson et al. 2004]. Also in these models, amacrine cells were identified as the major component of retinoblastomas. These reports also showed that loss of Rb in the developing retina extended the proliferative capacity of all retinal precursors, which was exacerbated by concomitant loss of p107. Our results show that also combined loss of Rb and p130 caused ectopic proliferation which, at day 14 after birth, was confined to the inner nuclear layer and caused severe dysplasia. As amacrine cells ultimately arrest, it was suggested that retinoblastoma in mice requires an extra genetic alteration that overcomes cell cycle arrest rather than apoptotic cell death [Chen et al. 2004]. Enhanced proliferation of double knockout cells may facilitate the acquisition of such an alteration. Alternatively, conforming to our in vitro data, the delayed cell cycle exit of double knockout cells may indicate that oncogenic signals can induce a bypass of differentiation more easily in cells that lack both Rb and p107 or Rb and p130. In cells lacking only Rb this would lead to p107- and p130-mediated cell cycle arrest.

Loss of p130 in adrenal gland and small cell lung cancer

While Rb+/− mice and Rb−/− chimeras display adrenal medulla hyperplasia [Robanus Maandag et al. 1994; Williams et al. 1994a; Yamasaki et al. 1998], Rb+/−p130+/− chimeric mice developed ES cell-derived bilateral adrenal medulary tumours displaying characteristics of pheochromocytomas (PCC). Rb+/−p130+/− chimeras developed the same tumors with a much longer latency, suggesting that loss of pRB is a rate-limiting step in the onset of this tumor type. In humans, this tumor of neuro-endocrine origin frequently shows loss of pRB expression [Gupta et al. 2000]. Our observations indicate that also p130 plays a role in suppressing PCC.

Other neuro-endocrine cells that are affected by ablation of pRb and p130 are pulmonary neuro-endocrine epithelial cells. Rb−/−p130−/− chimeric mice readily developed hyperplasia originating from this cell type. Although these hyperplastic lesions did not progress into full tumors before the appearance of other tumors, it is possible that they are precursors of small cell lung cancer. Small cell lung cancer (SCLC) in humans shows in more than 90% of the cases loss of heterozygosity (LOH) of chromosome region 13q14, harboring RB, and in more than 70% of the cases also LOH of the p130 containing chromosome arm 16q [Stanton et al. 2000], suggesting that a large fraction of these tumors has lost both pRB and p130 expression. Furthermore, inactivating p130 mutations were found in a pRB-deficient SCLC cell line [Helen et al. 1997] and in primary SCLCs [Claudio et al. 2000]. In mice combined loss of pRb and p53 also resulted in bronchial epithelial hyperplasia and SCLC [Williams et al. 1994b; Meuwissen et al. 2003]. Together with our data, this may again suggest that p53 and p130 act in a similar tumor suppression pathway. Analysis of mice with conditional inactivation of Rb and p130 in the bronchial epithelium will be required to determine whether loss of p130 can indeed substitute for loss of p53 in development of SCLC.

Materials and methods

Generation of Rb gene family mutant ES cells, chimeras and MEFs

Knockout alleles of Rb, p107 and p130 were generated in 129OLA (E14) embryonic stem cells as described (te Biele et al. 1992; Robanus-Maandag et al. 1998; Dannenberg et al. 2000). The Rosa26geo targeting construct was generated by inserting a splice-acceptor-site-geo fragment from plasmid p8geo, into a Neo site of a 10-kb genomic Rosa26 DNA fragment [Friedrich and Soriano 1991]. Genomic DNA isolated from G418 resistant colonies was digested with EcoRI and subjected to Southern blot analysis, using a 5′ external PstI-Sall 800 base pair Rosa26 genomic probe to identify correctly targeted clones. These were obtained with a frequency of 97%. Chimeras were generated by...
injecting mutant ES cells into C57Bl/6 blastocysts. The derivation of MEF cultures from chimeric embryos and the 3T9 protocol are described in Dannenberg et al. [2000].

Virus production and infection of MEFs

Ecotropic retroviral supernatants were produced by transfecting 293T [Phoenix] packaging cells with 50 µg viral DNA using calcium-phosphate precipitation. The medium was collected and filtered through a 0.45-µm filter 48, 56, and 72 h post-transfection. Low-density cultures of MEFs were infected at least three times for 3 h with viral supernatant containing 4 µg/mL polybrene (Sigma) yielding infection efficiencies of 95% or more. After the last infection, the viral supernatant was exchanged for fresh medium and the cells were allowed to recover for 48 h.

Protein analyses

Protein levels were determined by Western blot analyses following established protocols. Antibodies against p107 [C-18], p130 [C-20], CDK4 [C-22], Cyclin E [M-20], Cyclin A, E2F-1 [C-20], p21Ca [C-19], were obtained from Santa Cruz, the pRB antibody [G3-245] from BD Pharmingen; RAS [R02120] and DHFR antibodies from BD Transduction Laboratories; the p19ARF antibody [R562] from Abcam; the p53 antibody [Ab-7] from Oncogene Science. Peroxidase conjugated goat anti-rabbit and goat anti-mouse secondary antibodies were from Biosource. To detect p53 antibodies we used peroxidase conjugated rabbit anti-goat antibodies from DAKO.

Southern Blot analysis of tumor DNA

Tumors were minced and incubated overnight in 50 mM Tris [pH 8.5], 1 mM EDTA, 0.5% Tween-20, and 200 µg/mL proteinase K at 37°C for 10 min and subsequently centrifuged at 14,000 × g for 5 min. Five microliters of the DNA extraction was digested with Stul and analyzed by Southern blotting using a 5′ external probe (te Riele et al. 1992).

Isolation of DNA from paraffin-embedded tumors and PCR analysis

Tumor tissue was removed from paraffin section using a laser capture microscope. Tumor samples were incubated overnight in 20 µL of 50 mM KCl, 10 mM Tris [pH 8], 2.5 mM MgCl₂, 0.1 mg/mL gelatin, 0.46% Tween-80, 0.1 mg/mL proteinase K at 37°C. To inactivate proteinase K, samples were incubated at 80°C for 10 min and subsequently centrifuged at 14,000 × g for 5 min. Five microliters of the DNA solution was used in a PCR using D2mit94 5′ FAM labeled primer set (Mouse MapPairs, Research Genetics). PCR products were separated on an ABI Prism 3700 DNA Analyzer.

X-gal staining

Tissues and embryos were fixed in freshly made 0.1 M sodium phosphate [pH 7.3], 1.5% formaldehyde, 0.2% glutaraldehyde, 2 mM MgCl₂, and 5 mM EGTA for 30–90 min. The samples were rinsed for 3 × 10 min in 0.1 M sodium phosphate [pH 7.3], 0.1% sodium deoxycholate 0.2% NP40, 2 mM MgCl₂. Staining with X-gal [1 mg/mL; Roche] was performed in the rinse buffer containing 5 mM K₃Fe(CN)₆ and 5 mM K₄Fe(CN)₆ and 0.2 mM Tris [pH 7.5] overnight at room temperature. After an additional 6 h of staining in fresh solution, samples were rinsed 3 × 10 min, fixed overnight in 4% phosphate buffered formalin, and processed for paraffin embedding. Four-micrometer sections were stained either with hematoxylin or nuclear fast red.

BrdU/FUUr labeling, histology and immunohistochemistry

Mice received an i.p. injection of 5-bromo-2′-deoxyuridine [BrdU, Sigma] and 5-fluoro-2′-deoxyuridine [FUUr, Sigma] in PBS [50 and 10 mg/kg body weight] 2 h before being sacrificed. Tissues were processed for X-gal staining or directly fixed in phosphate-buffered formalin. Five-micron sections of paraffin embedded tissues were stained with hematoxylin and eosin. For immunohistochemistry, sections were dehydrated, heated for 15 min in a citrate buffer [pH 6.0] using a microwave for antigen retrieval, and blocked in 1% normal goat serum at room temperature. Antibodies detecting BrdU (DAKO), Chromogranin A (Chemicon), GABA [Sigma], GFAP (Chemicon), glutamine synthetase (BD Transduction Laboratories), IRBP (gift of Y. De Kok, U450 INSERM, Paris, France), p53 [pAb421; Oncogene Science], p75 [Chemicon], synapthophysin [Chemicon], and syntaxin [HPC-1; Sigma] were diluted in 1% normal goat serum. Goat anti-mouse [Alexa 488] and goat anti-rabbit [Alexa 568] antibodies were obtained from Molecular Probes. Nuclear staining was performed using Topro-3 stain diluted 1:1000 in mounting medium ( Vectashield).

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