Cell lineage-specific interactions between \textit{Men1} and \textit{Rb} in neuroendocrine neoplasia

Andres Matoso\textsuperscript{1}, Zongxiang Zhou\textsuperscript{1}, Ryo Hayama, Andrea Flesken-Nikitin and Alexander Yu Nikitin\textsuperscript{*}

Department of Biomedical Sciences, Cornell University, Ithaca, NY 14853, USA

To whom correspondence should be addressed. Tel: +1 607 253 4347; Fax: +1 607 253 4212. Email: an58@cornell.edu

Introduction

The \textit{retinoblastoma 1} (\textit{Rb}) gene is a prototypical tumor suppressor gene (reviewed in ref. 1). The protein encoded by \textit{Rb} is a 928-amino acid nuclear phosphoprotein with a nuclear localization signal at the C-terminus. Along with p107 and p130, \textit{Rb} is a member of protein family characterized by A/B domain (2). During the G\textsubscript{1}–S phase transition, phosphorylation of \textit{Rb} by cyclin/cyclin-dependent kinase complex (3,20). Loss of function of p18 and p19 inhibits which enhance \textit{Rb} activity by suppressing cyclin/kinase inhibitors which enhance \textit{Rb} activity by suppressing cyclin/kinase inhibitors. p18\textsubscript{ink-4a} and p27\textsubscript{kip1} (18,19), indicate existence of interactions between \textit{Rb} and \textit{Men1} pathways. p18 and p27 are cyclin-dependent kinase inhibitors which enhance \textit{Rb} activity by suppressing cyclin/kinase inhibitors which enhance \textit{Rb} activity by suppressing cyclin/kinase inhibitors (3,20). Loss of function of p18 and p27 has been implicated in neuroendocrine carcinogenesis in mice (21), and cooperation between \textit{Men1} and p18 in suppression of neuroendocrine neoplasms has been recently reported (22).

In humans, \textit{Men1} loss of function leads to the development of \textit{Men} type 1 syndrome, a dominant hereditary disease characterized by occurrence of tumors in the pituitary anterior lobe, the parathyroid gland and pancreatic islets of Langerhans. To lesser extent, \textit{Men1} patients often develop other tumors, including gastrinomas, angiofibromas, collagenomas, lipomas, meningoimomas and smooth muscle tumors (23). \textit{Men1} mice develop neuroendocrine neoplasms analogous to human disease after loss of the wild-type \textit{Men1} allele (24,25). Accordingly, conditional deletion of \textit{Men1} in cells of the pituitary and parathyroid glands and pancreatic islets leads to development of tumors in these tissues (26,27).

The product of \textit{Men1}, menin, is a 67 kDa protein found predominantly in the nucleus and possesses three nuclear localization signals near the C-terminus (28,29). Menin has diverse functions. It participates in cell-cycle regulation as it represses proliferation of insulinoma cells and human endocrine tumor cells (30,31). In addition to transcriptional enhancement of p18 and p27 (18,19), menin is a direct co-activator of the estrogen receptor \alpha-mediated transcription (32). It also mediates apoptosis through induction of expression of caspase 8 (33), and ectopic expression of menin in an insulinoma cell line increases the number of cells stained for annexin V (31). Menin also participates in genome stability, as peripheral lymphocytes from \textit{Men1} patients display increased chromosome breakage (34). Additionally, menin functionally interacts with the activator of the S-phase kinase, which is essential for DNA replication (35). The wide range of functions associated with menin indicates that its relevance stands beyond suppression of \textit{Men1} syndrome and its interactions with other genes merit investigation.

To test for interactions between \textit{Rb} and \textit{Men1} pathways, we intercrossed \textit{Rb}\textsuperscript{−/−} and \textit{Men1}\textsuperscript{−/−} mice and analyzed resulting \textit{Rb}\textsuperscript{−/−}/\textit{Men1}\textsuperscript{−/−} compounds in parallel with their littermates of parental genotypes.

Materials and methods

Animals

All mice were maintained under identical conditions recommended by the Institutional Laboratory Animal Use and Care Committee. C57BL/6J/129-Rb\textsuperscript{−/−}/C0 mice are heterozygous for mutation in the third exon of the \textit{Rb} gene (36). FVB/N129-Men1\textsuperscript{−/−}/C0 mice are heterozygous for exon 3–8 deletion in the \textit{Men1} gene (24). Mice heterozygous for both genes were prepared by crossing \textit{Rb}\textsuperscript{−/−} and \textit{Men1}\textsuperscript{−/−} mice. Primer sequences used for detection of \textit{Rb} locus were 5'-TTCAGGTGCCCATGTGCCCTCA-3' and 5'-AGAACGGAGTACGGCAGCTCTGTC-3'. These primers discriminate between wild-type and mutant \textit{Rb} alleles resulting in 122 and 175 bp fragments, respectively. For \textit{Men1} gene, primers were 5'-ACAGCTCCTACGCGGCCCCTTCTC-3' and 5'-AGAACGGAGTACGGCAGCTCTGTC-3'. These primers yield 334 and 272 bp fragments specific for wild-type and mutant.

Abbreviations: \textit{GSH}, \textit{GSH}-catalase subunit; H&E, hematoxylin and eosin; MEN, multiple endocrine neoplasia; PALT, pituitary anterior lobe tumor; PCR, polymerase chain reaction; PRL, prolactin; \textit{Rb}, \textit{retinoblastoma 1}; RNAi, RNA Interference; siRNA, small interfering RNA.
sequences, respectively. All genotyping procedures were performed as described previously (14).

**Histologic analyses**

Moribund mice were anesthetized with avertin and, if necessary, subjected to cardiac perfusion at 90 mmHg with phosphate-buffered saline followed by phosphate-buffered 4% paraformaldehyde. After macroscopic evaluation during necropsy, tissues were embedded in paraffin, sectioned at 4 μm thickness and stained with hematoxylin and eosin (H&E). Serial sectioning was done as described previously (17,37). To determine mitotic and apoptotic rates of tumor cells, different tumor areas were recorded for mitotic figures and apoptotic bodies using H&E-stained sections under magnification ×40 (17,37).

**Immunohistochemistry**

Paraffin sections of paraformaldehyde-fixed tissue were stained according to the modified avidin–biotin–peroxidase technique (37). The immunostaining for detecting pituitary hormones (National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD) on parallel sections and calcitonin was performed as described previously (14). The antibody used for detection of p18 (dilution 1:300) and p27 antibodies (dilution 1:400) were from Santa Cruz Biotechnology (Santa Cruz, CA), phosphorylated-Rb antibody (Ser801/811; dilution 1:100) and activated caspase-3 (Asp175; dilution 1:200) were from Cell Signaling Technology (Danvers, MA) and menin antibody (dilution 1:200) was from Abcam (Cambridge, MA).

**Microdissection–polymerase chain reaction**

For laser microdissection, 4 μm thick paraffin sections were prepared on glass slides covered by plastic foil, stained with H&E and evaluated under the microscope. Tumor cells were microdissected using a blue laser (Laser Microdissection System, Leica AS, Heidelberg, Germany) and collected into caps of 0.6 ml Eppendorf tubes filled with lysis buffer, digested in proteinase K, divided equally between tubes for detection of Men1 and Rb mutations and used for sequencing.

---

**Fig. 1.** Endocrine neoplasms in Men1<sup>+/−</sup>-Rb<sup>+/−</sup> mice. (A) Survival curves of Men1<sup>+/−</sup> (median 546 days, n = 29), Men1<sup>+/−</sup>-Rb<sup>+/−</sup> (median 402 days, n = 29) and Rb<sup>+/−</sup> (median 372 days, n = 15) mice. (B) Spectrum of endocrine neoplasms in Men1<sup>+/−</sup>-Rb<sup>+/−</sup> mice. Top left, PALT is characterized by pronounced vascularization (arrow); top right, monomorphic solid melanotroph tumor with central necroses (arrow) in the pituitary intermediate lobe; middle left, medullary carcinoma of the thyroid with central necroses (arrow) and vascular invasion (arrowhead); middle right, parathyroid adenomas composed of uniform chief cells (arrow) with areas of cystic formation (arrowhead); bottom left, pancreatic islet adenoma characterized by solid packets of cells (arrow) and abundant vascularization (arrowhead) and bottom right, adrenal pheochromocytoma with disorganized architecture of densely packed atypical cells (arrow), H&E. Bar, 100 μm for all images.
for subsequent polymerase chain reaction (PCR) amplification following previously described protocol (37). For manual microdissection, 4 μm thick paraffin sections were placed on glass slides, stained with H&E and evaluated under microdissection microscope. Tumor cells were collected using a 30G1/2 needle and processed as described above. PCR product was run on non-denaturing 12% polyacrylamide gel stained with silver (17).

Cell culture and RNA Interference experiments
Rh-deficient cell line CTN3 established from thyroid medullary C-cell carcinoma (14) was used for small interfering RNA (siRNA) experiments. Cells were transfected using DharmaFECT 3 (Dharmacon, Lafayette, CO) with a set of four oligonucleotides for Men1 RNA interference (Dharmacon). The sequences of the RNAi used are (a) sense sequence 5’-CGCUAGUAACCGCAUUU-3’ and anti-sense sequence 5’-PUUGC-GACUAGAAACACCUCUU-3’, (b) sense sequence 5’-AGGUGUUCUCAGUGCGCAAUU-3’ and anti-sense sequence 5’-PAAUGCAGACUGAAACACCCCUU-3’, (c) sense sequence 5’-ACUAUUGCUCCGCAAUUU-3’ and anti-sense sequence 5’-PAUAUCCGGCAGAAUAUGUUU-3’ and (d) sense 5’-GUAAGUAGGUGGGCUUU-3’ and anti-sense sequence 5’-PAAGCCACCCUCAUUCAUU-3’. Non-targeting RNAi 5’-UGUUCAUGUGCCAGAUU-3’ (siCONTROL from Dharmacon) was used as a control. Cells were collected after 24 h after transfection for quantitative real-time reverse transcription PCR studies and 48 h after transfection for apoptotic and proliferation analyses. Transfection with all oligonucleotides together (a-d) was most effective in Men1 down-regulation, followed by (a), (b) and (c) (supplementary Figure 1 is available at Carcinogenesis online). Thus, (a-d) mix was used for subsequent experiments. Estimation of apoptotic and proliferation indices was performed as described previously (14). Antibody used for proliferation assay was anti-Ki67 (NCL-Ki67p from Novoceastra Laboratories Ltd, Newcastle upon Tyne, UK, dilution 1:1000) and for apoptosis anti-cytoeinstin-1 aspartic protease-3 (cleaved caspase-3, Cell Signalling Technology; dilution 1:200).

Quantitative real-time reverse transcription–PCR
Total RNA was isolated using RNAeasy kit (Qiagen, Valencia, CA) according to the manufacturer’s protocol. A 2 μg aliquot of the total RNA was reverse transcribed using SuperScript III (Invitrogen, Carlsbad, CA) and random hexamer primers. The reactions were done in a 30 μl volume in a 96-well plate using pre-developed FAM Taqman probes (Gene expression assay Mm 1310760_g1 from Applied Biosystems, Foster City, CA). Mouse Glyceraldehyde 3-phosphate dehydrogenase was used as an endogenous reference control (pre-developed FAM TaqMan probes (Gene expression assay Mm 1332578_g1 from Applied Biosystems). Cycling parameters were as follows: 95°C for 1 min followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. Increase in real-time fluorescence was measured and relative fold changes were calculated using the 2^(-ΔΔCt) method (38).

Western analysis
CTN3 cell lysates were prepared using RIPA (Tris–HCL 50 mM, pH 7.4; NP-40 1%; Na-deoxicholate 0.25%; NaCl 150 mM; ethylenediaminetetraacetic acid 1 mM; phenylmethylsulfonyl fluoride 1 mM; aprotinin, leupeptin and pepstatin, 1 μg/ml each; NaVO3, 1 mM and NaF 1 mM) buffer and 80 μg of lysates were separated by 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electrophoretically transferred to polyvinylidene difluoride membrane (Millipore, Billerica, MA). The membrane was incubated overnight at 4°C with antibodies to detect p18 and p27 (both from Santa Cruz Biotechnology; 1:200 dilution) and Glyceraldehyde 3-phosphate dehydrogenase (Advanced Immunohistochemical, Long Beach, CA; 1:400 dilution) followed by incubation for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) and developed using chemiluminescence substrate (SuperSignal West Pico from Pierce, Rockford, IL).

Statistical analyses
Statistical analyses were performed with InStat 3.03 and Prism 4.02 software (GraphPad, San Diego, CA).

Results
Men1 and Rb do not cooperate in suppression of neuroendocrine neoplasia
By crossing Men1+/- and Rb+/- mice, we obtained Men1+/-Rb+/- mice. Consistent with death during embryogenesis of Rb+/- (36) and Men1+/- mice (24), no litter contained mice that were homozygous for the mutant allele of either Men1 or Rb genes. Our backcross experiments demonstrated no significant effect of the various proportions of the original backgrounds on the survival (A.Matoso, Z.Zhou, R.Hayama, A. Flesken-Nikitin and A. Yu. Nikitin, unpublished observations) and all mice were evaluated together. Survival curves of Rb+/- and Men1+/-Rb+/- mice were not significantly different from each other (median 372 and 402, respectively; P = 0.84; Figure 1A), whereas Men1+/- strain lived significantly longer than the others (median 546 days; P < 0.0001 for both). Thus, Men1 heterozygosity does not affect the lifespan of Rb+/- mice.

To determine whether the Men1+/-Rb+/- compound mice had altered tumor spectrum and to establish the reasons for their death, Men1+/-Rb+/- and Men1+/-Rb+/- mice were subjected to careful pathological evaluation (Table I and Figure 1B). Rb+/- mice exhibited the syndrome of multiple neuroendocrine neoplasia, including tumors of pituitary anterior and intermediate lobes, metastatic thyroid C-cell carcinomas and pheochromocytomas in agreement with previous studies (14,17). Parathyroid adenomas previously reported in the Rb-deficient mice (17) were absent in our Rb+/- group, probably due to genetic background variations and the presence of large thyroid C-cell carcinomas that prevented their identification. Men1+/- mice also exhibited neoplasms reported previously, including PALTs, parathyroid and pancreatic islet adenomas and pheochromocytomas (24,25). Pulmonary metastasis of an adrenal cortical carcinoma was found in a single case.

Men1+/-Rb+/+ compound mice developed neoplasms typical for either Rb or Men1 deficiency and died from fast-growing pituitary tumors or thyroid C-cell carcinomas. Metastases in the lung were confirmed to be of thyroid C-cell origin by calcitonin immunohistochemistry. No novel neoplastic phenotype was observed in Men1+/-Rb+/- mice.

No significant difference was found in the frequencies of neoplasms typical for Rb deficiency between Rb+/+ and Men1+/-Rb+/- mice. Frequencies of neoplasms typical for Men1 deficiency were also similar between Men1+/- mice and Men1+/-Rb+/- mice, except for a higher incidence of PALTs and adrenal cortical tumors in the Men1+/- group. The higher frequency of PALTs in Men1+/- mice is probably due to their longer lifespan, as compared with Men1+/-Rb+/-, because the incidence at a matched age range (from 360 to 490 days survival) was not significantly different between the two genotypes (6 of 8 Men1+/- versus 10 of 19 Men1+/-Rb+/-) (Fishier’s exact P = 0.18). Since some of the Men1+/- mice at that age have small PALTs (less than six), it is also possible that the overgrowth of intermedial lobe tumor in pituitaries of Men1+/-Rb+/- mice hindered the identification of these early lesions. Similarly, the frequency of adrenal cortical tumor in age-matched mice was not significantly different

<table>
<thead>
<tr>
<th>Neoplasm</th>
<th>Men1+/-</th>
<th>Men1+/-Rb+/-</th>
<th>Men1+/-Rb+/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>PALT</td>
<td>81 (13/16)</td>
<td>40 (11/27)</td>
<td>42 (5/12)</td>
</tr>
<tr>
<td>Pituitary intermediate lobe tumor</td>
<td>0 (0/16)</td>
<td>96 (26/27)</td>
<td>100 (11/11)</td>
</tr>
<tr>
<td>Thyroid C-cell carcinoma</td>
<td>0 (0/16)</td>
<td>96 (26/27)</td>
<td>100 (14/14)</td>
</tr>
<tr>
<td>Metastasis in the lungs#</td>
<td>6 (1/15)</td>
<td>70 (14/20)</td>
<td>60 (9/15)</td>
</tr>
<tr>
<td>Parathyroid gland tumor</td>
<td>61 (8/13)</td>
<td>50 (7/14)</td>
<td>0 (0/8)</td>
</tr>
<tr>
<td>Adrenal pheochromocytoma</td>
<td>44 (11/25)</td>
<td>45 (13/29)</td>
<td>40 (6/15)</td>
</tr>
<tr>
<td>Adrenal cortical tumor</td>
<td>28 (7/25)</td>
<td>10 (3/29)</td>
<td>0 (0/5)</td>
</tr>
<tr>
<td>Pancreatic islet tumor</td>
<td>57 (8/14)</td>
<td>55 (11/20)</td>
<td>0 (0/14)</td>
</tr>
</tbody>
</table>

All specimens were serially sectioned and both neoplasia and foci of early atypical proliferation were counted as tumor incidences. Inside the parentheses are the numbers of tumor incidences out of the total number of mice examined. There is a variation in the total number of mice because some tissues were lost during necropsy or histological processing.

#Metastasis of adrenal cortical carcinoma (synchronized by calcitonin negative) was detected in a Men1+/- mouse. All other metastases are derived from thyroid C-cell carcinomas according to positive staining for calcitonin.
between Men1+/− and Men1+/−/Rb+/− mice (2 of 12 Men1+/− versus 1 of 18 Men1+/−/Rb+/−; Fisher’s exact P = 0.54).

In agreement with absence of significant differences in tumor frequencies, mitotic and apoptotic rates of tumor cells were similar among the different genotypes (P = 0.33 for both).

**Mutations of Men1 and Rb are mutually exclusive**

In agreement with absence of significant differences in tumor frequencies, mitotic and apoptotic rates of tumor cells were similar among the different genotypes (P = 0.33 for both).

Organ sites with neoplasms shared by Rb- and Men1-deficient phenotypes are the pituitary anterior lobe and adrenal medulla. To determine if all PALTs are phenotypically identical, neoplasms were subjected to immunohistochemical profiling for anterior pituitary hormones. In agreement with previous studies (14,17), PALTs from Rb+/− mice were predominantly positive for α-glycoprotein subunit (α-GSU) and had a substantial number of prolactin (PRL)-positive cells, whereas they were mostly negative for other hormones (Figure 3B and Table II). On the other hand, similarly to previous report (26), all the PALTs from Men1+/− mice were positive for PRL. Furthermore, PALTs from Men1+/− mice were all negative for α-GSU (Figure 3B and Table II). Thus, the PALTs from Men1+/− mice were prolactinomas, the most common PALT type among human MEN1 patients (23). PALTs were present in 11 Men1+/−/Rb+/− mice, four of them...
Fig. 3. Immunohistochemical characterization of pancreatic and pituitary anterior lobe tumors. (A) Detection of menin in nuclei of cells of pancreatic Langerhans islets of \textit{Men1}^{+/−} mice. Left: pancreas of \textit{Men1}^{+/−} mouse with an islet of Langerhans containing a focus of early atypical proliferation (arrow) characterized by densely packed atypical cells and disorganized architecture. Right: neoplastic cells (arrow) lack menin as compared with untransformed cells (arrowhead). (B) Detection of α-GSU and PRL in ALT. Upper panel, tumors from \textit{Men1}^{+/−} mice contain PRL-positive cells (left, arrow) and negative for α-GSU (right, arrow). Posterior lobe does not contain either α-GSU or PRL (arrowheads). Middle panel, ALT contains areas of cells positive for either PRL (left, arrow) or α-GSU (right, arrow) in \textit{Men1}^{+/−}\textit{Rb}^{+/−} mice. Lower panel, ALT stains mainly for α-GSU (right, arrow), whereas some cells are positive for PRL (left, arrow) in \textit{Rb}^{−/−} mice. \textit{Men1}^{+/−}\textit{Rb}^{+/−} and \textit{Rb}^{−/−} mice share the presence of intermediate lobe tumor (ILT) (middle and lower panels). ABC Elite method, hematoxylin (A and B, upper and middle panels) and methyl green (B, lower panel) counterstaining. Bar A, 200 μm (left) and 50 μm (right). B, 50 μm (all large images) and 500 μm (inserts).

Table II. PALT immunohistochemistry

<table>
<thead>
<tr>
<th>Hormones</th>
<th>\textit{Men1}^{+/−} mice</th>
<th>\textit{Men1}^{+/−}\textit{Rb}^{+/−} mice</th>
<th>\textit{Rb}^{−/−} mice$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Frequency of positive PALTs</td>
<td>Percentage of positive cells</td>
<td>Frequency of positive PALTs</td>
</tr>
<tr>
<td>α-GSU</td>
<td>0/8</td>
<td>0</td>
<td>10/15</td>
</tr>
<tr>
<td>GH</td>
<td>3/8</td>
<td>10 ± 5</td>
<td>6/15</td>
</tr>
<tr>
<td>FSH</td>
<td>0/8</td>
<td>0</td>
<td>0/15</td>
</tr>
<tr>
<td>ACTH</td>
<td>0/8</td>
<td>0</td>
<td>0/15</td>
</tr>
<tr>
<td>β-LH</td>
<td>3/8</td>
<td>13 ± 3</td>
<td>1/15</td>
</tr>
<tr>
<td>β-TSH</td>
<td>1/8</td>
<td>5</td>
<td>5/15</td>
</tr>
<tr>
<td>PRL</td>
<td>8/8</td>
<td>80 ± 6</td>
<td>9/15</td>
</tr>
</tbody>
</table>

PALTs were stained with anterior lobe hormones: α-GSU, growth hormone (GH), follicle-stimulating hormone (FSH), adrenocorticotropic hormone (ACTH), β subunit of luteinizing hormone (β-LH), β subunit of thyroid-stimulating hormone (β-TSH) and PRL. 

$^a$\textit{Rb}^{−/−} mice from other experiments were included in this group to increase the number of PALTs studied.
were positive only for α-GSU, one was positive only for PRL and six were positive for both hormones. Notably, four tumors had well-defined separated areas, positive either for PRL or α-GSU (Figure 3B).

Microdissection–PCR showed loss of the wild-type copy of Rb or Men1 in areas of tumors positive for α-GSU (three of three; Figure 2B and data not shown) or PRL (three of three; Figure 2B), respectively. Four additional microdissected α-GSU-positive tumors also had loss of the wild-type copy of Rb and not Men1 including those with a higher proportion of PRL-positive cells. Intriguingly, the only case of PRL-positive and α-GSU-negative PALTs in the Men1+/−/Rb+/− mice was also shown to be Rb deficient by loss of heterozygosity study. Overall, the tumor was homozygous for mutant alleles of both genes simultaneously.

Interestingly, the frequency of Men1-deficient PALTs in Men1+/−/ Rb+/− mice was significantly lower than expected if compared with their parental Men1+/− mice of the same age range (28% in Men1+/−/Rb+/− mice versus 75% in Men1+/− mice, P < 0.05) and that Rb mutation was predominant over that of Men1 in the carcinogenesis of PALT of the compound mutant mice. Since α-GSU expression occurs in all anterior lobe progenitors and precedes PRL expression during mouse pituitary development (39,40), formation of α-GSU-positive Rb-deficient tumors may precede PRL-positive Men1-deficient tumors.

Since cells of the adenalt medulla represent a homogeneous population, it was of particular interest to evaluate potential interactions between Men1 and Rb in these cells. Frequencies of adrenal pheochromocytomas were not significantly different among the three genotypes, indicating that susceptibility to these lesions was not altered by combining Men1 and Rb heterozygosity. All pheochromocytomas lost the remaining copy of the gene in Rb+/− (17 and current results) and Men1+/− (24 and current results) mice. At the same time, in compound mice, all mutations of Men1 and Rb were mutually exclusive. Majority (five of six) of pheochromocytomas showed loss of the wild-type Men1 but not Rb, whereas one pheochromocytoma had inverse genotype (Figure 2B).

Down-regulation of Men1 targets p18 and p27 and increased presence of phosphorylated-Rb in Men1-deficient neoplasms

To further test interaction between Rb and menin pathways, we evaluated expression of p18, p27 and phosphorylated-Rb in Men1-deficient pheochromocytomas. In a good agreement with previous reports on transcriptional enhancement of p18 and p27 expression by menin (18), expression for both proteins was down-regulated in menin-deficient pheochromocytomas of Men1+/−/ (positive cells, mean ± SD, p18: 8 ± 5%, n = 3, P = 0.01 and p27: 5 ± 3%, n = 3, P = 0.01) and Men1+/−/Rb+/− mice (positive cells, mean ± SD, p18: 18 ± 16%, n = 3, P = 0.06 and p27: 16 ± 12%, n = 3, P = 0.05) compared with Rb+/− (positive cells, mean ± SD, p18: 82 ± 13%, n = 3 and p27: 77.5 ± 17%, n = 3). As expected from p18- and p27-mediated control of Rb phosphorylation by cyclin/cyclin-dependent kinases (3.20) coincidentally with p18 and p27 down-regulation, menin-deficient pheochromocytomas of Men1+/−/ and Men1+/−/Rb+/− mice and not those of Rb+/− mice showed positive staining for phosphorylated-Rb, the inactive form of the protein (positive cells, mean ± SD, 6.5 ± 1.5% Men1+/−/, n = 3 and 4 ± 1% Men1+/−/Rb+/−, n = 3; Figure 4A). Detection of apoptotic cells using activated caspase-3 staining shows no significant difference among the pheochromocytomas of the three genotypes (supplementary Figure 2 is available at Carcinogenesis online).

Knockdown of Men1 is detrimental to Rb-deficient medullary thyroid carcinoma cells

To test if inactivation of both Men1 and Rb may be disadvantageous for neoplastic cell growth, Men1 expression was knocked down by siRNA in Rb-deficient medullary thyroid carcinoma cell line CTN3 (14). Two-fold down-regulation of Men1 expression resulted in decreased expression of p18 and p27 and a significant increase in apoptotic cells with no change in proliferation as assessed by caspase-3 and Ki67 assays, respectively (Figure 4B). These results indicate that combination of Men1 and Rb deficiency might be lethal at least in some cell lineages.

Discussion

The results obtained from the addition of Men1 heterozygosity to Rb+/− mice demonstrate a lack of cooperation between these tumor suppressor genes, based on unaltered lifespans of mice, lack of changes in frequencies of tumors and metastasis, as well as absence of novel phenotypes. Our results are in agreement with a recently published study reporting a lack of enhancement in carcinogenesis in Men1+/−/Rb+/− compound mice (41). However, Loffler et al. did not find any types of neoplasm that would be shared between Men1+/−/ and Rb+/− mice. Thus, instead of suggested possibility that menin and Rb function in a common pathway of tumor suppression, it could not be excluded that observed lack of cooperation in suppressing neuroendocrine neoplasms is due to independent functions of Men1 and Rb in non-overlapping cell lineages. Probably due to differences between genetic backgrounds of Loffler’s and our mice, our pathological evaluation identified cells of the adrenal medulla and PRL-producing cells of the pituitary anterior lobe as targets for both Men1 and Rb mutations, giving rise to pheochromocytomas and prolactinomas, respectively. Notably, none of studied neoplasms of either origin carried deletion of both genes simultaneously in Men1+/−/Rb+/− genotypes, thereby demonstrating mutually exclusive character of Men1 and Rb mutations in the same cell lineage settings.

Importantly, a similar genetic interplay between Men1 and Rb genes might take place in humans, where mutations for each gene are described in different sets of neuroendocrine tumors of the lung. MEN1 and not RB mutations have been reported in typical and atypical carcinoid tumors, whereas inactivating mutations of RB and not MEN1 gene are common in large cell neuroendocrine tumors and small cell lung cancers (42,43). It remains to be determined how frequent are mutually exclusive mutations of RB and MEN1 in other types of human tumors and if presence of either gene is required for carcinogenesis associated with deficiency for another.

Down-regulation of p18 and p27 and expression of phosphorylated-Rb in pheochromocytomas of Men1+/−/ and Men1+/−/Rb+/− mice indicates that menin and Rb share a common pathway, with Rb being downstream of menin, and Rb mutation becomes redundant due to functional inactivation of pRb by active cyclin-dependent kinases. These results are in a good agreement with our observation of similar proliferation and apoptosis rates in matched neoplasms of all tested genotypes.

At the same time, our experiments demonstrate that down-regulation of Men1 expression in Rb-deficient medullary thyroid carcinoma cells leads to their death, and, therefore, inactivation of both Men1 and Rb may be disadvantageous for carcinogenesis in some neuroendocrine cell lineages. It is also possible that menin down-regulation is more harmful to cells with complete inactivation of Men1 followed by Rb down-regulation. This possibility would be in a good agreement with our observation that loss of the remaining wild-type copy of Men1 followed by Rb functional inactivation by phosphorylation was more common than Rb loss of heterozygosity in pheochromocytomas of Men1+/−/Rb+/− mice. In either case, our observations indicate an attractive prospect of treating Rb-deficient neoplastic cells with Men1 siRNA. Future work will test to what extent functional redundancy of menin and Rb pathways and detrimental effects of their combined deficiency are specific for other cell lineages, thereby establishing the applicability of Men1 siRNA treatment to a broad variety of neoplasms associated with Rb deficiency.

Interestingly, Men1 and cyclin kinase inhibitor p27Kip1, but not p18ink4c, also failed to cooperate in tumor suppression in compound mice (22), even so that both p18 and p27 expressions are regulated by Men1 (18,22 and current observation). Thus, it is likely that functions
Fig. 4. Immunohistochemical characterization of pheochromocytomas and effect of Men1 knockdown in CTN3 cells. (A) Detection of p18, p27 and phosphorylated-Rb in adrenal pheochromocytomas of Men1<sup>+/−</sup>, Men1<sup>/−</sup>/Rb<sup>+/−</sup> and Rb<sup>/−</sup>/ mice. Neoplastic cells (arrows) show decreased staining for p18 and p27 as compared with normal cells (arrowheads) in Men1<sup>+/−</sup> and Men1<sup>/−</sup>/Rb<sup>+/−</sup> mice. Levels of p18 and p27 are similar in neoplastic and normal cells of Rb<sup>/−</sup>/ mice. Phosphorylated-Rb is detected in tumor cells of Men1<sup>+/−</sup> and Men1<sup>/−</sup>/Rb<sup>+/−</sup> (arrow) mice but not in those of Rb<sup>/−</sup>/ mice. Bar, 50 µm (all large images) and 500 µm (inserts). (B) Left top: efficiency of Men1 knockdown by siRNA (siMen1) in CTN3 cells as compared with untransfected cells and those transfected with control siRNA. Expression of Men1 was detected by quantitative real-time reverse transcription–PCR and normalized to mouse Glyceraldehyde 3-phosphate dehydrogenase. Left bottom: western blot showing decreased expression of p18 and p27 after knockdown of Men1 in CTN3 cells. Center: detection of menin, activated caspase-3 and Ki67 in CTN3 cells treated either with control or Men1-specific siRNA. Note decreased menin expression, increased number of cells with...
of Rb are closer aligned with those of p27, as compared with p18. Lack of complete Men1 inactivation in Men1+/−p18−/− compound mice (22) indicates cooperation between Men1 haploinsufficiency and lack of p18 might be sufficient for carcinogenesis. However, mice with conventional gene knockouts may have adaptive mechanisms to compensate for ubiquitous permanent loss of gene function. Given that mice heterozygous for both Men1 and p18 or p27 have not been studied, it remains uncertain whether there is any selective advantage for the loss of the remaining copy of p18 or p27 in case of Men1 complete loss of function. Conversely, there is a possibility that acute loss of Men1 and p18 or p27 in individual cells could be detrimental similar to our observations. Men1 and Rb are expressed in normal cells of target organs (44,45), indicating that Men1 may play role even in those tissues that typically give rise to MEN2-type tumors. At the same time, it should be noted that the spectrum of neoplasms associated with Rb deficiency overlaps with those of both MEN1 and MEN2 syndromes. MEN2 syndromes A and B are associated with mutations of receptor protein tyrosine kinase Ret1 and are mainly represented by medullary thyroid carcinomas and pheochromocytomas. Interestingly, activating mutations of the ret gene have been reported in neoplasms of p53+/−Rb+/− mice (46). However, direct sequencing of the ret gene from 19 samples of the thyroid C-cell tumors of Rb+/− mice did not demonstrate similar mutations in our study (A.Flesken-Nikitin and A.Yu. Nikitin, unpublished observations), indicating an important role of p53 deficiency in pathogenesis of MEN2 tumors. Further studies of potential roles of Rb in MEN2 syndromes should address this issue.

Taken together, our results demonstrate that Men1 and Rb do not cooperate in suppressing neoplasms with neuroendocrine differentiation. Instead, their combined mutations may disadvantage carcinogenesis in cell lineage-specific context. This possibility could be of particular importance for rational design of individual therapy of neuroendocrine neoplasms, as well as other cancers.

Supplementary material
Supplementary Figures 1 and 2 can be found at http://carcin.oxfordjournals.org/

Funding
National Institutes of Health (R01 CA96823 and K26 RR017595) to A.Y.N.

Acknowledgements
We thank Dr Lee Burns and David C. Corney for critical review of the manuscript.

Conflict of Interest Statement: None declared.

References
A.Matoso et al.


Received May 18, 2007; revised September 5, 2007; accepted September 12, 2007.