Genetic mosaics reveal both cell-autonomous and cell-nonautonomous function of murine $p27^{Kip1}$

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Loss of the cyclin-dependent kinase inhibitor $p27^{Kip1}$ leads to an overall increase in animal growth, pituitary tumors, and hyperplasia of hematopoietic organs, yet it is unknown whether all cells function autonomously in response to $p27^{Kip1}$ activity or whether certain cells take cues from their neighbors. In addition, there is currently no genetic evidence that tumor suppression by $p27^{Kip1}$ is cell-autonomous because biallelic gene inactivation is absent from tumors arising in $p27^{Kip1}$ hemizygous mice. We have addressed these questions with tissue-specific targeted mouse mutants and radiation chimeras. Our results indicate that the suppression of pars intermedia pituitary tumors by $p27^{Kip1}$ is cell-autonomous and does not contribute to overgrowth or infertility phenotypes. In contrast, suppression of spleen growth and hematopoietic progenitor expansion is a consequence of $p27^{Kip1}$ function external to the hematopoietic compartment. Likewise, $p27^{Kip1}$ suppresses thymocyte hyperplasia through a cell-nonautonomous mechanism. The interaction of $p27^{Kip1}$ loss with epithelial cell-specific cyclin-dependent kinase 4 overexpression identifies the thymic epithelium as a relevant site of $p27^{Kip1}$ activity for the regulation of thymus growth.

cell cycle | cyclin-dependent kinase inhibitor | growth genetics | pituitary tumor | thymus development

In the mouse, loss of the $p27^{Kip1}$ cyclin-dependent kinase (CDK) inhibitor induces increased growth of the whole animal, tumors of the pituitary intermediate lobe (pars intermedia), and a disproportionate degree of hyperplasia of the thymus and spleen (1–3). A similar phenotype is observed with disruption of $p18^{Ink4c}$, a CDK inhibitor specific for the D-type cyclins (4). Because these molecules modify the intracellular activity of CDKs, it is commonly presumed that their mechanism of growth control is chiefly cell-autonomous. However, it is not known how loss of these inhibitors causes proportionate growth in some tissues but not in others. One hypothesis is that loss of $p27^{Kip1}$ alters an internal counting mechanism of cells and thus all tissues undergo additional cell divisions during their growth phase and then exit from the cell cycle (5). However, not all cell lineages contribute equally to the growth process. In hematopoietic tissues, for example, loss of $p27^{Kip1}$ causes disproportionate expansion of hematopoietic progenitors and stem cells (6–8). Lymphocytes display increased *in vitro* sensitivity to IL-2 and other cytokines (1, 9).

Alternatively, loss of CDK inhibitors could alter growth by cell-nonautonomous mechanisms such as an endocrine disturbance. The fact that $p27^{Kip1}$ knockout mice develop pituitary tumors raised suspicion that hormone alterations could account for the growth and female infertility phenotypes. Growth hormone or IGF-1 also causes hyperplasia of the thymus relative to other organs (10). Yet marked differences in growth hormone, IGF-1, and IGF-2 were not seen in $p27^{Kip1}$ knockout mice (1, 2). In addition, spontaneous tumors arise chiefly in α-melanocyte-stimulating hormone-producing melanotroph cells, not in growth hormone and gonadotropin-producing cells of the adjacent pars distalis (homologue of the human anterior lobe). It is possible that α-melanocyte-stimulating hormone contributes to infertility considering that, administered exogenously, it alters estrous cycle of rats and decreases progesterone production of ovarian granulosa cells (11). Other forms of cell-nonautonomous mechanisms of growth control include local production of paracrine factors and cellular contact. For example, forced expression of cyclin D1 in the thymic epithelial cells with the keratin 5 (K5) promoter leads to marked thymic lymphocyte hyperplasia and overgrowth of the entire organ (12). It is conceivable that spleen and thymus hyperplasia induced by loss of $p27^{Kip1}$ is also due to its loss of function outside of the hematopoietic cell compartment.

In addition to these developmental phenotypes, there is reason to question whether tumor suppression by $p27^{Kip1}$ is cell-autonomous. Hemizygous $p27^{+/-}$ mice develop tumors of the pars intermedia and other tissues after γ-irradiation (6). However, unlike the classic tumor suppressor gene Rb, whose loss also causes tumors in the pars intermedia, tumors arising in mice lacking one copy of $p27^{Kip1}$ or $p18^{Ink4c}$ do not acquire bialleleic gene mutations (6, 13). Thus, there is genetic evidence that tumor suppression by $p27^{Kip1}$ is haploinsufficient but no evidence that it is cell-autonomous. Cell-nonautonomous mechanisms of tumor suppression include intracellular altered immune response, paracrine factors, and the suppression of angiogenesis. Our studies indicate that, depending on the context, $p27^{Kip1}$ suppresses growth by both cell-autonomous and cell-nonautonomous mechanisms.

Results

p27loxP and p27stop Mouse Models. We have produced two types of Cre-inducible targeted mutations of $p27^{Kip1}$. The first strain, p27loxP, harbors the (L+) mutation with *loxP* sites flanking $p27^{Kip1}$ (Fig. 1*A*). The introduction of the Cre recombinase into p27loxP mice deletes the entire coding region of $p27^{Kip1}$ and produces a null allele (L−). The second mouse model, p27stop, is a mouse strain carrying a "Cre-on" targeted mutation of $p27^{Kip1}$. A transcriptional STOP cassette, which is flanked by loxP sites, was targeted to a critical region of the $p27^{Kip1}$ 5′ UTR, thereby producing a null allele (S−). However, the STOP cassette can be excised by Cre, resulting in an activated allele (S+) that expresses wild-type $p27^{Kip1}$ under control of its endogenous promoter (Fig. 1*B*).

Baseline Characteristics of p27loxP and p27stop Mice. We validated the new $p27^{Kip1}$ mutations by comparing uninduced mutants ($p27^{L+/L+}$ and $p27^{S-/S-}$) versus germ-line Cre-induced alleles ($p27^{L-/L-}$ and $p27^{S+/S+}$). As predicted, $p27^{Kip1}$ protein is present in $p27^{L+/L+}$ mice and is undetectable after Cre-mediated excision in the $p27^{L-/L-}$ animals (Fig. 2*A*). Unexpectedly, quantitative Western blots of $p27^{L+/L+}$ tissues showed a moderate reduction of

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Abbreviations: CDK, cyclin-dependent kinase; POMC, proopiomelanocortin; K5, keratin 5; Neo, neomycin.

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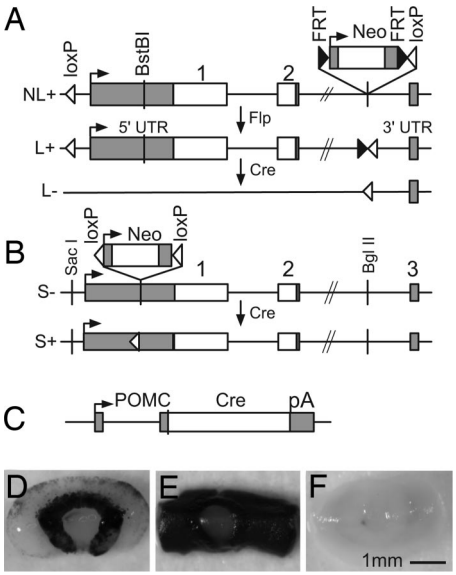

Fig. 1. p27loxP and p27stop targeted mutations and POMC–Cre transgenics (+ and − designate the presence and absence of $p27^{Kip1}$ expression from each allele). (*A*) Schematic of p27LoxP mutations. The NL+ allele is preliminary the result of targeting with the pKNFL vector. It includes two loxP sites (left triangle) and Neo flanked by FRT sites (right triangle). The p27loxP mouse carries the L+ allele with a pair of LoxP sites flanking $p27^{Kip1}$. Cre recombinase activity results in the L− null allele. (*B*) p27stop mice harbor the S− null allele, which contains a Neo-STOP cassette in the 5′ UTR (shaded) of $p27^{Kip1}$. Cre recombinase deletes the STOP cassette, resulting in the active S+ allele. (*C*) Structure of the POMC–Cre transgene. X-Gal-stained pituitaries show the location of Cre recombinase activity from POMC–Cre × R26R mice (*D*) compared with R26-positive controls (*E*) and wild-type controls (*F*).

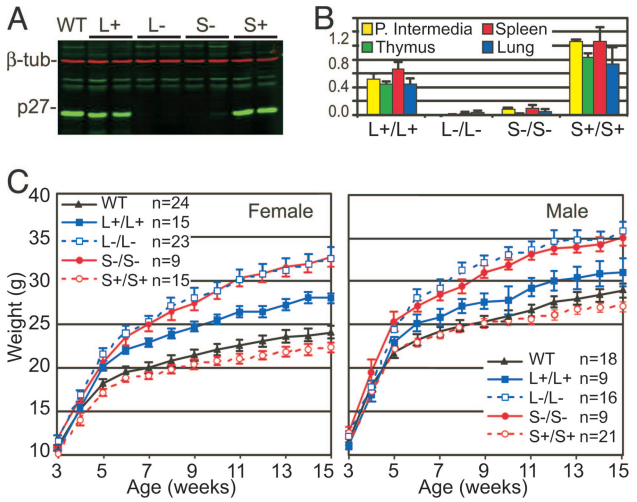

Fig. 2. Baseline assessments of p27loxP and p27stop mouse models. (*A*) Western blot using fluorescent antibodies to p27 and β-tubulin from extracts. (*B*) Quantification of p27 protein levels in various tissues relative to that of wild-type littermates of mice with constitutive p27loxP and p27stop mutations (mean ± SEM). Tissues from $p27^{L+/L+}$ mice show a significant decrease in p27 protein relative to wild type ($n = 8$, $P = 0.00015$, Wilcoxon test). $p27^{L-/L-}$ ($n = 4$) and $p27^{S-/S-}$ ($n = 4$) tissues do not show appreciable expression of p27. (*C*) Growth curves. $p27^{S-/S-}$ mice are significantly larger than wild-type (WT) and $p27^{S+/S+}$ mice and are comparable to constitutive $p27^{L-/L-}$ knockouts. $p27^{L+/L+}$ mice show intermediate growth rates.

$p27^{Kip1}$ protein (Fig. 2*B*) and RNA levels (Fig. 7, which is published as supporting information on the PNAS web site). Expression of $p27^{Kip1}$ is nearly undetectable in $p27^{S-/S-}$ mice, whereas $p27^{Kip1}$ levels are normal in mice with the reactivated p27stop mutation ($p27^{S+/S+}$). Uninduced and constitutively induced p27loxP and p27stop mice displayed the expected size (Fig. 2*C*) and organ hyperplasia phenotypes (data not shown). However, uninduced $p27^{L+/L+}$ mice demonstrated increased growth rates that were intermediate between that of wild-type and constitutive knockouts, consistent with their reduced protein expression.

Pituitary Gene Mutation by POMC–Cre in p27loxP and p27stop Mice. To create pars intermedia pituitary-specific gene mutations, we generated POMC–Cre transgenics that express the Cre recombinase by the proopiomelanocortin (POMC) promoter (Fig. 1*C*). The specificity of Cre was initially assessed by X-gal-staining tissues from POMC–Cre transgenics bred to Rosa26 reporter mice (R26R). Two founder lines showed the expected pattern for the POMC promoter, i.e., a high level of β-gal activity in the pituitary pars intermedia, patchy activity in the pituitary pars distalis, and no activity in other organs (Fig. 1 *D–F*). POMC–Cre transgenics were then bred to both p27loxP and p27stop mouse strains. In tissues where POMC–Cre should not be expressed deletion of the $p27^{Kip1}$ gene was not detectable by PCR (Fig. 3*A*). Likewise, deletion of the STOP cassette was always detectable in the pars intermedia of p27stop mice carrying POMC–Cre (Fig. 3*B*). Quantitative Western blots of pituitaries from $p27^{L+/L+}$;POMC–Cre^{+} animals demonstrate loss of $p27^{Kip1}$ expression in the pars intermedia compared with wild-type pituitaries (Fig. 3*C*), becoming complete between 6 and 15 weeks of age. Likewise, POMC–Cre restored expression of $p27^{Kip1}$ protein in $p27^{S-/S-}$ pituitary pars intermedia at 6 weeks, but it did not further increase with time. The fact that the restoration of p27 is only partial at 6 weeks of age is partly because of the presence of pars nervosa or other POMC-negative cells in the tissue preps. For males and females, the growth curves of mice carrying POMC–Cre is nearly identical to the curves of mice without this transgene. In other words, deletion of $p27^{Kip1}$ from the pars intermedia of p27loxP mice did not increase animal growth, and restricting $p27^{Kip1}$ expression to the pars intermedia in p27stop mice did not suppress the overgrowth phenotype. Likewise, the presence of POMC–Cre did not diminish fertility of p27loxP females, nor did it restore fertility to p27stop females (see Table 1, which is published as supporting information on the PNAS web site).

Autonomy of Pars Intermedia Pituitary Tumors. We assayed pituitary tumor development to determine whether it requires mutation of $p27^{Kip1}$ outside the pars intermedia melanotrophs. p27loxP ($p27^{L+/L+}$) mice did not develop pituitary tumors (Fig. 4 *A*, *E*, and *F*) in the absence of Cre. However, $p27^{L+/L+}$;POMC–Cre^{+} mice developed tumors of a size and appearance similar to those seen in constitutive ($p27^{L-/L-}$) null animals (Fig. 4 *B* and *E*). Thus, deletion of $p27^{Kip1}$ in the pituitary melanotroph cell is necessary and sufficient for tumor development. In contrast, p27stop mice ($p27^{S-/S-}$) developed pituitary tumors similar in size to those of constitutive null animals (Fig. 4 *C* and *E*) in the absence of Cre. Activation of $p27^{Kip1}$ with POMC–Cre in these mice (Fig. 4 *D* and *E*) inhibited tumor development and prolonged their survival (Fig. 4*G*). Before 1 year of age, 32% of mice succumbed to nonmalignant skin ulcers, but only two mice had large (>15 mg) pituitary tumors (Table 2, which is published as supporting information on the PNAS web site). Southern blots and quantitative PCR (data not shown), using these two pituitary tumors, demonstrated retention of the p27(S−) allele, indicating that they had arisen from a subset of cells that failed to undergo POMC–Cre-mediated $p27^{Kip1}$ reactivation. In comparison to normal pars intermedia tissue, cyclin A-associated kinase activity was modestly increased in tumor tissue (≈2.5-fold, similar to other normal organs), yet not nearly as much as $p27^{Kip1}$ knockout thymus (Fig. 3*D*).

Nonautonomous Spleen and Thymus Hyperplasia in Radiation Chimeras. We transplanted marrow from either wild-type or $p27^{-/-}$ mice into lethally irradiated knockout and wild-type recipients (Fig. 5*A*)

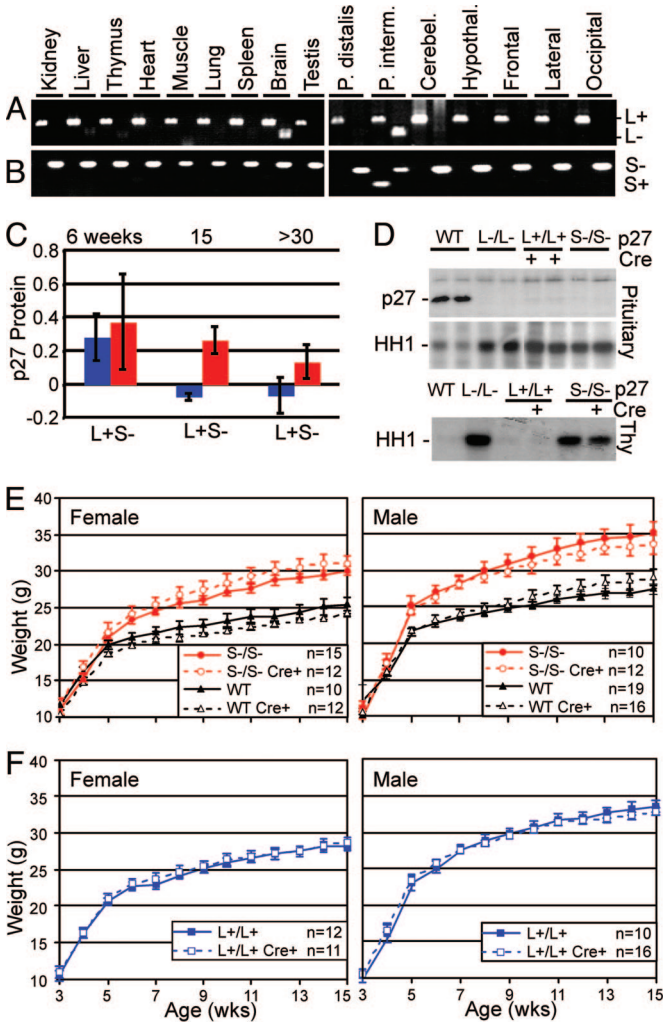

Fig. 3. Effects of POMC–Cre expression in p27loxP and p27stop mice. (*A*) PCR of tissues from $p27^{L+/L+}$;POMC–Cre^{+} mice for the uninduced (L+) and induced (L−) null version of the p27loxP allele. (*B*) PCR of similar tissues from a $p27^{S-/S-}$;POMC–Cre^{+} mouse for the uninduced null (S−) and activated (S+) alleles. (*C*) Mean levels of $p27^{Kip1}$ protein in the pituitary pars intermedia of $p27^{L+/L+}$ (L+) and $p27^{S-/S-}$ (S−) (all POMC-Cre^{+}) mice plotted relative to wild type is shown for mice at 6, 15, and 30 weeks of age ($n = 6 \pm$SEM). (*D*) Cyclin A-associated histone H1 kinase activity (HH1) from pooled wild-type (WT) pars intermedia tissue and individual pars intermedia tumors arising in $p27^{L-/L-}$, $p27^{L+/L+}$;POMC–Cre^{+}, and $p27^{S-/S-}$ mice is shown in comparison to thymus extracts. (*E*) Growth of $p27^{+/+}$ and $p27^{S-/S-}$ mice is shown both with (Cre^{+}) and without (Cre^{-}) POMC–Cre. (*F*) Growth curves of $p27^{L+/L+}$ mice compared with $p27^{L+/L+}$;POMC–Cre^{+} mice.

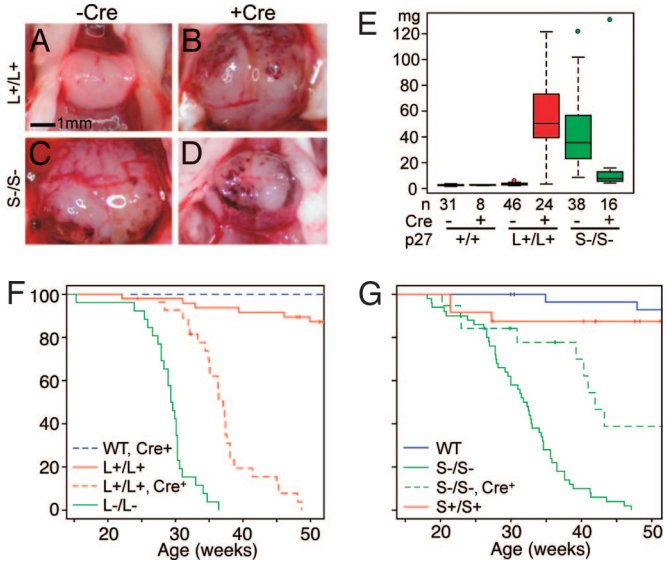

Fig. 4. Pituitary tumors and overall animal survival. *In situ* appearance of pituitaries from $p27^{L+/L+}$ (*A*) and $p27^{L+/L+}$;POMC–Cre^{+} (*B*) mice shows that POMC–Cre induces pituitary tumor formation. (*C*) In contrast, $p27^{S-/S-}$ mice develop spontaneous tumors. (*D*) POMC–Cre largely suppressed tumors in p27stop mice, but some $p27^{S-/S-}$. POMC–Cre^{+} mice developed smaller tumors at 1 year of age. (*E*) Box plot of pars intermedia weights at 1 year of age or when morbid. Median values (horizontal bar) are plotted with the first and third quartiles (box), ranges (whiskers), and outliers (circles). (*F*) Percent survival of $p27^{L+/L+}$ mice with POMC–Cre is shortened compared with $p27^{L+/L+}$ mice without Cre ($P = 3 \times 10^{-5}$, log-rank test) and is shown in comparison to $p27^{L-/L-}$ and $p27^{+/+}$;POMC–Cre^{+} controls. (*G*) Survival of $p27^{S-/S-}$;POMC–Cre^{+} mice is significantly prolonged compared with $p27^{S-/S-}$ mice without Cre ($P = 3 \times 10^{-16}$) and is shown in comparison with mice with constitutively reactivated allele ($p27^{S+/S+}$) and wild-type p27 (WT).

to test whether $p27^{Kip1}$ suppresses hematopoietic cell growth by a cell-autonomous mechanism. Recipients received transplants at 3 weeks of age, before the pubertal growth phase, and were necropsied after full donor engraftment, 4–5 weeks later. Flow cytometry using CD45 cell surface markers, which distinguished donor from recipient hematopoietic cells, confirmed that the recipients had achieved ≥98% donor chimerism. The spleens of $p27^{-/-}$ recipient animals were enlarged and hypercellular compared with $p27^{+/+}$ recipients, regardless of the genotype of the donor cells with which they had been transplanted (Fig. 5*B*). In addition, the spleens of $p27^{-/-}$ recipients harbored increased proportions of hematopoietic progenitor cells, even if reconstituted with $p27^{+/+}$ hematopoietic cells (Fig. 5*C*). This result indicates that loss of $p27^{Kip1}$ causes expansion of hematopoietic progenitors by a mechanism involving cells outside the hematopoietic cell compartment.

Thymuses of $p27^{-/-}$ recipient animals were hyperplastic compared with wild-type recipients regardless of the genotype of the transplanted hematopoietic cells (Fig. 5*D*). The presence or absence of p27 protein in the thymuses was a function of the genotype of the donor mice, which is expected because lymphocytes are the predominant cell type in this organ. In addition, both cyclin A (Fig. 5*E*) and Cdk2-associated histone H1 kinase activity are increased in the mice transplanted with $p27^{-/-}$ donor cells. Flow cytometry of transplanted animals demonstrated that all four groups had similar profiles of lymphocyte differentiation as determined by CD3, CD4, and CD8 subsets (data not shown). However, mice that received $p27^{-/-}$ donor marrow had a 2-fold relative increase in cells harboring the γ/δ T cell receptor (Fig. 5*F*), which others have shown to be characteristic of $p27^{Kip1}$ knockout mice (14).

It has been reported that the K5 promoter is active in the thymic epithelium and that forced expression of cyclin D1 by the K5 promoter causes marked thymus hyperplasia. However, the thymic lymphocyte expansion was cell-nonautonomous because cyclin D was not overexpressed in T cells (12). To determine whether forced expression of Cdk4, a catalytic partner of cyclin D1, has a similar effect, we assessed thymuses of K5–Cdk4 transgenic mice (15). Western blots for Cdk4 (Fig. 6*A*) indicate that it is overexpressed in thymic stromal tissues but not in thymocyte suspensions. However, unlike K5–cyclin D1 or K5–cyclin D2 transgenics, K5–Cdk4 mice did not develop progressive thymus hyperplasia (Fig. 6*B*) (12, 16). This finding is consistent with the hypothesis that cyclin D is limiting in the thymic epithelium and that forced expression of Cdk4 in this tissue is not sufficient to induce organ hyperplasia. To test the hypothesis that $p27^{Kip1}$ loss cooperates with overexpression of Cdk4 in the thymic epithelium, we bred K5–Cdk4 mice to $p27^{Kip1}$ knockouts. Remarkably, $p27^{-/-}$;K5–$Cdk4^{+}$ compound mutant an-

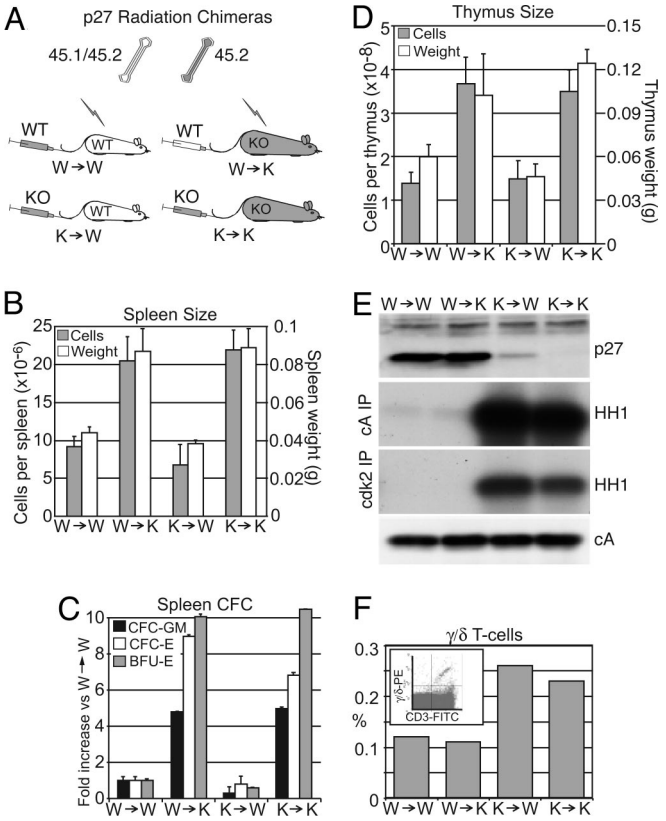

Fig. 5. Cell-nonautonomous growth of the spleen and thymus. (*A*) $p27^{+/+}$ bone marrow was transplanted into lethally irradiated $p27^{+/+}$ (W→W) and $p27^{-/-}$ (W→K) recipients. Also, $p27^{-/-}$ marrow was transplanted into $p27^{+/+}$ (K→W) and $p27^{-/-}$ (K→K) mice. Donor vs. recipient cells were distinguished by flow cytometry with CD45 cell-surface markers. W, wild type; K, knockout. (*B*) The spleens of $p27^{-/-}$ recipient mice are heavier and hypercellular compared with wild-type recipients. (*C*) $p27^{-/-}$ recipient mice have increased numbers of hematopoietic progenitor cells including CFC-GM, CFC-E, and BFU-E compared with wild-type recipients. (*D*) $p27^{-/-}$ recipient mice also develop heavier thymuses with increased lymphocytes compared to $p27^{+/+}$ recipients. (*E*) $p27^{Kip1}$ protein is abundant in thymuses of mice transplanted from $p27^{+/+}$ donors. Cyclin A and Cdk2-associated histone H1 (HH1) kinase activity is increased in thymuses from $p27^{-/-}$ donors, but cyclin A levels are unchanged. (*F*) Flow cytometry demonstrates that mice receiving transplants from $p27^{-/-}$ donors have a relative increase in γ/δ T cells.

imals developed massive thymic hyperplasia (Fig. 6*D*). $p27^{-/-}$;K5–$Cdk4^{+}$ thymuses weighed 745 ± 280 mg (mean ± range) by day 100, which is 4-fold larger than $p27^{-/-}$ and 6-fold larger than $Cdk4^{-/-}$ mice. Histological sections from these animals demonstrated normal thymocyte cell density but a marked increase in thymic lymphocyte cellularity. Areas of medullary and cortical maturation were present (Fig. 6*G*), and thymic expansion did not occur because of infiltration by monomorphic lymphoblasts. Flow cytometry (data not shown) showed normal proportions of CD4/CD8 subsets in p27-null mice with or without K5–Cdk4, and K5–Cdk4 did not lead to further increases of γ/δ T cells.

Discussion

We analyzed genetic mosaic mouse models generated through Cre-Lox technology and through stem cell transplantation and show that, depending on the tissue type, $p27^{Kip1}$ regulates growth by both cell-autonomous and cell-nonautonomous mechanisms. Our results support the model of a cell-autonomous mechanism of tumor suppression in the pituitary. p27loxP mice developed a high incidence of fatal tumors after tissue-specific deletion of the gene in the pituitary pars intermedia. This finding shows that $p27^{Kip1}$ is

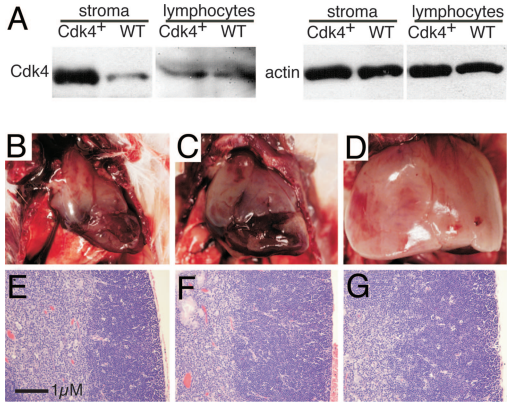

Fig. 6. Cooperation of K5–Cdk4 and p27 deletion in thymic hyperplasia. (*A*) Western blots show increased Cdk4 protein expression in thymic stroma but not in thymocytes from K5–Cdk4 transgenics compared with wild-type controls, with actin immunostaining as a loading control. (*B–D*) Appearance of the thymus at 10 weeks in mice with $p27^{Kip1}$ deletion (*B*), the K5–Cdk4 transgene (*C*), or both $p27^{Kip1}$ deletion and K5–Cdk4 (*D*). (*E–G*) Hematoxylin and eosin-stained sections of 7-week-old thymuses show both medullary and cortical thymic maturation in $p27^{-/-}$ mice (*E*), K5–$Cdk4^{+}$ transgenics (*F*), and compound $p27^{-/-}$;K5–$Cdk4^{+}$ mutants (*G*).

required in the melanotroph cell itself for tumor suppressor function. In a corollary set of experiments, expression of $p27^{Kip1}$ expression in the pars intermedia of p27stop mice suppressed pituitary tumor development but did not prevent the mice from developing skin ulcers. Despite an outwardly normal appearance, two $p27^{S-/S-}$;POMC–Cre^{+} mice had tumors of >15 mg at the time of necropsy. However, these tumors arose in a subset of melanotroph cells that had failed to excise the Stop cassette. This finding implies that $p27^{Kip1}$-deficient melanotroph cells are capable of forming tumors even when surrounded by melanotroph cells with normal $p27^{Kip1}$ expression. We conclude that loss of $p27^{Kip1}$ expression outside of the pars intermedia is not sufficient to induce pituitary tumors and that tumor suppression by $p27^{Kip1}$ may be autonomous at the level of individual melanotroph cells. Others have demonstrated increased rates of pituitary tumor development in mice lacking both $p27^{Kip1}$ and either $p18^{Ink4c}$ or one copy of Rb (4, 17, 18). We show that, despite the propensity to develop spontaneous tumors, loss of $p27^{Kip1}$ in the pars intermedia does not induce extraordinary increases in cyclin A/Cdk2 kinase activity. This finding is consistent with the hypothesis that this tissue is more sensitive to modest changes in cyclin/Cdk activity or that its mechanism of tumor suppression is independent of Cdk activity.

Although loss of $p27^{Kip1}$ from POMC-producing cells was sufficient to induce pituitary tumors in $p27^{L+/L+}$;POMC–Cre^{+} mice, it had no discernable effect on female fertility. This finding indicates that loss of $p27^{Kip1}$ from cells outside the pars intermedia is required for the expression of this phenotype. In contrast, p27stop females ($p27^{S-/S-}$) rarely produced viable offspring. It is possible that low-level $p27^{Kip1}$ protein expression exists in certain cells in the p27stop animals. However, tissue-specific expression of $p27^{Kip1}$ expression in the pars intermedia (of $p27^{S-/S-}$;POMC–Cre^{+} mice) did not improve breeder productivity, so the pars intermedia does not appear to play a significant role in the fertility defect. Similar to female fertility, loss or gain of $p27^{Kip1}$ expression in the pars intermedia had no discernable effect on the overall animal growth in the p27loxP and p27stop models. These data indicate that $p27^{Kip1}$ gene disruption in the pars intermedia pituitary is sufficient to induce tumors but does not cause overgrowth or infertility. However, this finding does not rule out functional defects in other pituitary cell types such as the gonadotropin or growth hormone-producing cells of the pars distalis. Indeed, $p27^{Kip1}$;$p18^{Ink4c}$ double knockouts demonstrate increased pars distalis proliferation in

response to growth hormone-releasing hormone administration, and ovary transplants suggest that female infertility in $p27^{Kip1}$-deficient mice is due to a combination of ovarian and pituitary defects (19, 20). Our p27loxP and p27stop mouse models in combination with other cell-type-specific Cre transgenes may be useful to determine whether other pituitary cells contribute to these phenotypes.

In contrast to the pituitary, we found that $p27^{Kip1}$ suppresses hyperplasia of hematopoietic cells of the thymus and spleen by a cell-nonautonomous mechanism. In radiation chimeras, thymus and spleen overgrowth occurred in only $p27^{-/-}$ recipient animals, but it was not affected by the genotype of the donor cells. Likewise, expansion of hematopoietic progenitors occurred by a cell-nonautonomous mechanism, but we have not identified the cells responsible for this phenotype. We examined cyclin/CDK activity from the thymuses of radiation chimeras because $p27^{-/-}$ mice display the most dramatic increase in cyclin/CDK activity in this tissue, perhaps because of a lack of compensatory function of $p21^{Cip1}$ (21). Interestingly, $p27^{-/-}$ lymphocytes continue to display marked increases in cyclin A and Cdk2 kinase activity even if transplanted into wild-type recipients. These data indicate that loss of $p27^{Kip1}$ within thymocytes is sufficient to activate cyclin/CDK complexes but does not account for the overgrowth of the organ. The fact that loss of $p27^{Kip1}$ increases thymic lymphocyte numbers by a cell-nonautonomous mechanism does not mean that $p27^{Kip1}$ has no direct effects in T cells. In fact, we observed increased γ/δ T cells in wild-type mice transplanted $p27^{-/-}$ T cells, as reported previously in untransplanted $p27^{-/-}$ mice, implying that $p27^{Kip1}$ directly increases the proportions of these cells (14). In addition, a number of studies have shown enhanced responsiveness of $p27^{-/-}$ T lymphocytes to *in vitro* stimulation (1, 9, 22). Thus it appears that $p27^{Kip1}$ expression within the lymphocyte itself controls the response of T cells to IL-2 and other proliferative stimuli but does not determine homeostatic thymocyte cell number.

Tissue-specific deletion of $p27^{Kip1}$ in other cell types may be necessary to positively identify the cells that drive thymic hyperplasia. However, our data from K5–Cdk4 transgenics strongly suggest that the thymic epithelium is responsible. Although K5–Cdk4 transgenics themselves have only minimal thymic enlargement, the combination of $p27^{Kip1}$ deletion and K5–Cdk4 expression has a dramatic synergistic effect on thymus size and cellularity. Because the K5 promoter is active in the thymic epithelium but not in thymocytes, it is likely that $p27^{Kip1}$ interacts directly with cyclin D/Cdk4 complexes in this cell type. The thymic epithelium plays an essential role in thymus development and T cell maturation. It mediates thymocyte responses to both local and systemic factors such as IFNγ, androgen, and keratinocyte growth factor (23, 24). The thymic epithelium, in turn, produces cytokines such as IL-7, which regulates growth and differentiation of $CD4^{-}CD8^{-}$ thymocytes (25). A similar mechanism may mediate the response to $p27^{Kip1}$ deficiency. It is also possible that $p27^{Kip1}$ loss in the thymic epithelium causes thymocyte expansion by interacting with molecules other than cyclin D/Cdk4. For example, $p27^{Kip1}$ inhibits Cdk2 hyperphosphorylation of the retinoblastoma protein (pRb), which might synergize with cyclin D/Cdk4 phosphorylation of pRb. Elucidation of the biochemical nature of the interaction between $p27^{Kip1}$ and Cdk4 will require additional studies. Our results highlight the importance of cell-cycle gene function in the epithelial cell compartment for the regulation of thymic lymphocyte growth (12, 16).

It is noteworthy that $p27^{Kip1}$ knockout mice are prone to developing T cell lymphomas, e.g., after exposure to the Moloney murine leukemia virus or in conjunction with transgenic c-Myc expression (26, 27). One intriguing idea is that inhibition of CDK activity in nonneoplastic epithelial cells has tumor-suppressive action on nearby lymphocytes harboring oncogene mutations just as it does in normal thymocytes. There is growing interest in cell-nonautonomous mechanisms of growth control and tumor suppression such as through stromal–epithelial interactions and through regulation of angiogenesis. Because they lack genetic evidence of cellular autonomy, genes that display haploinsufficiency in knockout mouse models may be more likely to suppress tumor development through such unconventional mechanisms (28).

Methods

POMC–Cre Transgenics. A 5.5-kb segment of the POMC gene promoter and 5′ UTR was PCR-amplified from pHAL* (courtesy of Malcolm Low, Oregon Health and Science University, Portland) to include EcoRI and SalI ends and was ligated to pBS–Cre (GIBCO) to include CreMT and poly(A) sequences. Transgenic animals were created by pronuclear injection into CD-1 embryos. Resultant progeny were screened by PCR using primers Cre1 (ACCTGATGGACATGTTCAGG) and Cre2 (CTACACCTGCGGTGCTAAC) for a 0.5-kb product and backcrossed 10 generations to 129S4. To verify the pattern of Cre expression, POMC–Cre animals were bred to R26R reporter mice. Brain, pituitary, and other organs were fixed in 4% paraformaldehyde and stained with X-gal both *in situ* and in histologic sections.

p27loxP and p27stop Targeted $p27^{Kip1}$ Mutants. The pKNFL targeting vector contains a 12.5-kb EcoRI fragment from a 129S4 genomic clone of $p27^{Kip1}$. A neomycin (Neo)-selectable marker flanked by *FRT* sequences and a single *loxP* sequence was inserted into the BglII site 1.25 kb 3′ of the stop codon. A second loxP cassette was inserted into SacI 0.54 kb upstream of the initiation codon. The $p27^{NL+}$ mutation was introduced by homologous recombination in AK7 mouse ES cells (also substrain 129S4) and screened by PCR and Southern blots as described (1). Positive clones were further screened by PCR for inclusion of the 5′ *loxP* site with primers K40 (TGGCAAACAGTCGGAGCGTAGG) and Lox2 (AGCATACATTATACGAAGTTATATTAAGGGTT). Chimeric mice were bred to 129S4 FlpeR mice to produce coisogenic animals without Neo (29). PCR-based genotyping was performed with primers K53 (GGTATAATACGGAAAGTGACTCTAATGGCC) and K52 (TAGGGG AAATGGATAGTAGATGTTAGGACC) for wild-type ($p27^{+}$) and $p27^{L+}$ alleles or with K53 and K57 (AGCGGCTCCCGGCCCGAGAC) for the $p27^{L-}$ allele.

p27stop mice were generated by using the pKSNL targeting vector, which includes a Neo-selectable marker and three transcription termination signals inserted into the BstBI site located 120 bp upstream of the Start codon (30). ES cells were screened by genomic Southern blots by using the 5′ $p27^{Kip1}$ probe as described (see Fig. 7) (1). p27stop mouse genotyping was accomplished by PCR with primers K55 (CGCCTGGCTCTGCT CCATTTGAC) and K56 (GACACTCTCACGTTTGACATCTTCC) for wild-type and $p27^{S+}$ alleles or K55 and N5 (CTACCCGCTTCCATTGCTCAG) for the $p27^{S-}$ allele. To demonstrate that the $p27^{L-}$ and $p27^{S+}$ alleles produce the expected phenotypes, we induced germline mutations by breeding both strains to 129S4 Mox2–Cre. The resulting $p27^{L-}$ and $p27^{S+}$ alleles were bred to homozygosity in the absence of Cre.

Growth, Fertility, and Pituitary Tumor Development. Weekly weights were obtained from p27loxP mice ($p27^{L+/L+}$) and p27stop mice ($p27^{S-/S-}$) and compared with wild-type littermates and with mice with homozygous induced $p27^{Kip1}$ mutations ($p27^{+/+}$, $p27^{L-/L-}$, and $p27^{S+/S+}$) until 15 weeks of age. Weekly weights were also obtained from a second group of mice, designated as the "observation group" at the time of weaning, whose genotypes were either $p27^{L+/L+}$ or $p27^{S-/S-}$ in combination with the POMC–Cre transgene, and compared with littermates without Cre. Organ weights were obtained from an additional 10 mice from each genotype at 6 weeks of age. Female fertility was assessed by recording the production of offspring from a separate group of adult virgin females of various genotypes (Table 1) paired with fertile males for

an average of 3 months. Necropsies were performed in the observational cohort after development of morbid behavior or at 1 year of age to determine causes of death and morbidity. Survival was calculated with the R program (v.2.1; www.r-project.org) using the survdiff function, pituitary weights were plotted with BOXPLOT, and χ^2 tests used CHISQ.TEST. Intact pituitaries were photographed *in situ* on the skull base after excision of the brain and meninges. Pars intermedia together with pars nervosa tissue was dissected free of the pars distalis under a stereomicroscope, weighed, and then fixed in 4% paraformaldehyde for histology, frozen for RNA and DNA, or homogenized in RIPA buffer with protease and phosphatase inhibitors for protein studies.

p27^{Kip1} Expression and CDK Activity. Western blots were stained with rabbit anti-p27^{Kip1} antibody (1:2,000) and anti-β-tubulin monoclonal antibody (1:10,000, T-0198, Sigma) followed by fluorescent goat anti-mouse IgG–Alexa Fluor 680 (Molecular Probes) or goat anti-rabbit IgG-IRDye800 (Rockland) and scanning on a Li-Cor Odyssey. For qualitative assessment, horseradish peroxidase-conjugated secondary antibodies were detected by ECL (Amersham Pharmacia). p27^{Kip1} protein standards were generated from admixtures of p27$^{+/+}$ and p27$^{-/-}$ tissue extracts. Protein loading was normalized by adjustment to β-tubulin staining in the same lane. CDK activity using histone H1 substrate was determined after immunoprecipitation with cyclin A or Cdk2 antibodies (Santa Cruz Biotechnology) from 100 μg of protein extract on Protein-A Sepharose beads (Amersham Pharmacia) as described (31). RNA from mouse tissues ($n = 4$ for each genotype) was purified with TRIzol (Invitrogen), and p27^{Kip1} quantitative RT-PCR was performed by using SYBR green (Applied Biosystems) with primers K25 (GCTGTTTACGTCTGGCGTCGA) and K66 (AGGAGAGCCAGGATGTCAGCG). Each sample was run in triplicate and normalized to mouse ribosomal protein S16 (AGGAGCGATTTGCTGGTGTGGA and GCTACCAGGCCTTTGAGATGGA).

p27^{Kip1} Radiation Chimeras. p27^{Kip1} knockout mice, previously described, were backcrossed (N15) to C57BL/6J and then bred to p27$^{-/-}$ mice coisogenic to 129S4 to create p27$^{-/-}$ F_1 hybrids (1). Control mice were F_1 hybrids of 129S4 mice crossed to C57BL/6J mice or to C57 mice with the CD45.1 cell-surface polymorphism (The Jackson Laboratory, stock catalog no. 2014, B6.SJL-*Ptprc*^{a} *Pep3*^{b}/BoyJ). p27$^{-/-}$ and p27$^{+/+}$ F_1 hybrid recipients (3.5 weeks old, $n = 5$ per group) were lethally irradiated (11 Gy, Linac) and then transplanted with bone marrow pooled from either p27$^{-/-}$ or p27$^{+/+}$ F_1 hybrid donors ($n = 2$–3) to create four groups as in Fig. 5*A*. Five weeks later mice were killed, and donor cell engraftment of the blood, thymus, and splenocytes was assessed by flow cytometry by using CD45.1 and CD45.2 antibodies (Pharmingen). Organs were weighed and cell counts were obtained after suspension in Iscove's modified Dulbecco's medium or RPMI medium 1640. Splenic hematopoietic progenitor cells were quantified with colony-forming assays for granulocyte-monocyte colony-forming cells (CFC-GM), erythroid colony-forming cells (CFC-E), and erythroid burst-forming units (BFU-E) as described (32). Plates were incubated in 5% O_2 plus 5% CO_2, and colonies were scored on day 2 (CFU-E), days 5–6 (BFU-E), and day 7 (CFU-GM) by a researcher blinded to the experimental group. The transplant procedure was replicated to confirm the splenic colony-forming assays and thymus flow cytometry/kinase assays. FITC- or phycoerythrin-conjugated rat monoclonal antibodies (CD4, CD8, CD3, TCR γ/δ, Pharmingen) were diluted 1:100 and incubated for 30 min with 10^5 (CD4 and CD8) or 10^6 (CD3 and TCR γ/δ) thymocytes. Live cells were gated by propidium iodide (10 μg/ml) exclusion.

K5–Cdk4 Transgenics. The K5–Cdk4 transgene combines human Cdk4 with the bovine K5 promoter (15). K5–Cdk4 transgenics were backcrossed five generations to the SENCAR strain and then bred for two generations to p27$^{-/-}$ mice (C57BL/6J) to generate p27$^{-/-}$;K5–Cdk4$^+$ compound mutants. Thymuses from young adult animals (6–7 weeks) were minced and suspended in RPMI medium 1640. Lymphocytes were separated from stromal material by differential sedimentation. Four mice carrying various combinations of p27$^{-/-}$ and K5–Cdk4 were killed at 7 weeks of age for histology to determine thymus size and for flow cytometry. An additional four p27$^{-/-}$;K5Cdk4$^+$ mice were assessed at 14–15 weeks.

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