nature genetics

Skp2 is required for survival of aberrantly proliferating *Rb1*-deficient cells and for tumorigenesis in $Rb1^{+/-}$ mice

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Heterozygosity of the retinoblastoma gene *Rb1* elicits tumorigenesis in susceptible tissues following spontaneous loss of the remaining functional allele. Inactivation of previously studied retinoblastoma protein (pRb) targets partially inhibited tumorigenesis in $Rb1^{+/-}$ mice[1–6]. Here we report that inactivation of pRb target Skp2 (refs. 7,8) completely prevents spontaneous tumorigenesis in $Rb1^{+/-}$ mice. Targeted *Rb1* deletion in melanotrophs ablates the entire pituitary intermediate lobe when Skp2 is inactivated. Skp2 inactivation does not inhibit aberrant proliferation of *Rb1*-deleted melanotrophs but induces their apoptotic death. Eliminating p27 phosphorylation on T187 in p27T187A knock-in mice reproduces the effects of *Skp2* knockout, identifying p27 ubiquitination by SCF^{Skp2} ubiquitin ligase as the underlying mechanism for Skp2's essential tumorigenic role in this setting. *RB1*-deficient human retinoblastoma cells also undergo apoptosis after Skp2 knockdown; and ectopic expression of p27, especially the p27T187A mutant, induces apoptosis. These results reveal that *Skp2* becomes an essential survival gene when susceptible cells incur *Rb1* deficiency.

Skp2 binds T187-phosphorylated p27 for the SCF^{Skp2} ubiquitin ligase to ubiquitinate p27 (ref. 9). pRb binds Skp2 to interfere this binding and ubiquitination[7]. pRb-Skp2 binding also bridges Skp2 to the APC-Cdh1 ubiquitin ligase for Skp2 ubiquitination[8]. Because Skp2 is a target for the transcription factor E2F (refs. 10,11), pRb could repress Skp2 mRNA expression via E2F. Consistent with the above findings, $Rb1^{+/-}$ mice developed $Rb1^{-/-}$ pituitary tumors that had substantially increased amounts of Skp2 mRNA and protein along with decreased amounts of p27 protein (**Fig. 1a**,**b**).

To define the role of Skp2 in tumorigenesis in $Rb1^{+/-}$ mice, we generated cohorts of $Rb1^{+/-}Skp2^{+/+}$ and $Rb1^{+/-}Skp2^{-/-}$ mice. Skp2 is not required for pituitary gland development (see **Supplementary Fig. 1**). $Rb1^{+/-}$ mice develop pituitary intermediate lobe melanotroph tumors with a well-defined course, from early atypical proliferates to foci, microscopic tumors and gross tumors (**Supplementary Fig. 2a**), resulting in death around 1 year of age[12]. At 6 months more than half of $Rb1^{+/-}Skp2^{+/+}$ mice had early atypical proliferates and foci (**Fig. 1c**). By 9 months one pituitary had a gross tumor, and most had foci and microscopic tumors. Later, all 27 $Rb1^{+/-}Skp2^{+/+}$ mice died between 10 and 15 months of age (**Fig. 1d**), all but one with gross pituitary tumors (**Fig. 1c**). In contrast, none of the $Rb1^{+/-}Skp2^{-/-}$ mice had any sign of pituitary tumorigenesis at 6, 9 and 17 months, when healthy $Rb1^{+/-}Skp2^{-/-}$ mice were killed.

Thyroid C-cell tumors develop with 50–70% penetrance in $Rb1^{+/-}$ mice. Among the same 27 $Rb1^{+/-}Skp2^{+/+}$ mice, 16 had gross thyroid tumors at death, and the dead mouse that lacked a pituitary tumor had an especially large thyroid tumor (**Fig. 1c**). About half of the remaining dead mice had microscopic thyroid tumors (**Fig. 1c** and **Supplementary Fig. 2b**). In contrast, all 29 $Rb1^{+/-}Skp2^{-/-}$ mice had normal-appearing, tumor-free thyroid glands (**Fig. 1c**). Together with the lack of pituitary tumors, these results identify Skp2 as the first pRb target that is required for spontaneous tumorigenesis in $Rb1^{+/-}$ mice.

The above findings could reflect the fact that Skp2 plays a required role in the development of *Rb1* mutant tumors or that Skp2 is generally required for tumorigenesis. To begin to investigate these possibilities, we treated $Skp2^{+/+}$ and $Skp2^{-/-}$ mice with a tumorigenesis protocol using *N*-ethyl-*N*-nitrosurea (ENU) induction. This experiment demonstrated no difference in tumor development in the two genotypes, including survival (**Fig. 1e**) and tumor types and burdens (**Supplementary Fig. 3a**). Although Skp2 was frequently overexpressed in the tumors, its expression levels did not correlate with p27 protein levels (**Supplementary Fig. 3b**). Thus, Skp2 is not required for ENU-induced tumorigenesis.

Because spontaneous tumorigenesis in $Rb1^{+/-}$ mice requires the loss of the remaining *Rb1* allele, it was possible that *Skp2* inactivation prevented the second *Rb1* mutation, rather than the growth of *Rb1*-deficient tumors. We next used a tissue-specific *Rb1* deletion

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Received 19 June; accepted 28 October; published online 6 December 2009; doi:10.1038/ng.498

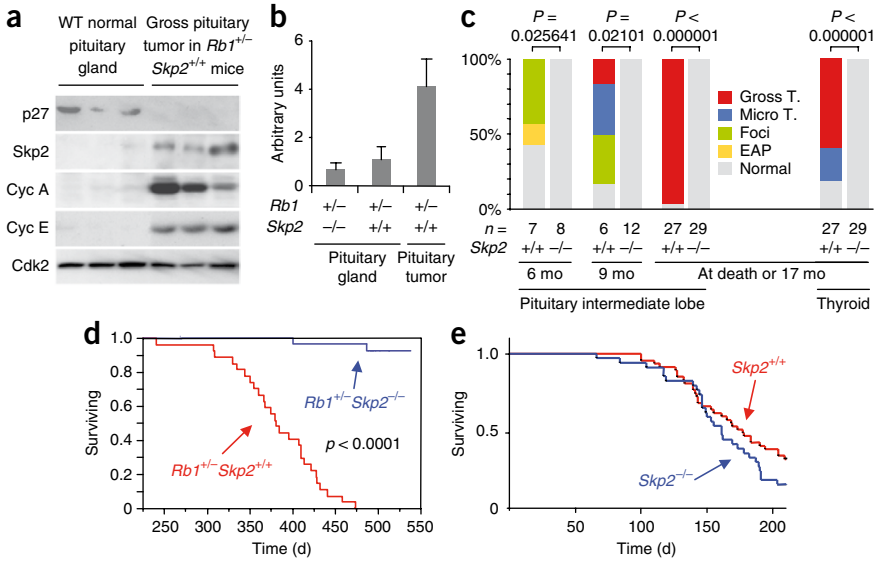

Figure 1 Roles of *Skp2* in spontaneous tumorigenesis in $Rb1^{+/-}$ mice and in ENU-induced tumorigenesis. (**a**) Expression of the indicated proteins in wild-type (WT) normal pituitary glands and pituitary tumors developed in $Rb1^{+/-}Skp2^{+/+}$ mice, determined by protein blot. (**b**) Amounts of Skp2 mRNA in pituitary glands and pituitary tumors (developed in $Rb1^{+/-}$ mice), determined by quantitative PCR (Q-PCR) normalized to the abundance of the enzyme GAPDH. (**c**) Incidence of pituitary and thyroid tumors at various stages in $Rb1^{+/-}Skp2^{+/+}$ and $Rb1^{+/-}Skp2^{-/-}$ mice. *P* values are by Fisher's exact tests (various lesions were combined for analyses). (**d**) Kaplan-Meier survival analysis for the indicated mice. *P* value is by log rank test. One $Rb1^{+/-}Skp2^{-/-}$ mouse died at 13 months, and one died at 16 months with macroscopically normal pituitary and thyroid glands. The causes of death were unclear, with a possible association with eye and skin lesions. (**e**) Kaplan-Meier survival analysis for the indicated mice treated with ENU.

scheme involving a proopiomelanocortin promoter–Cre recombinase (POMC-Cre) fusion and *loxP* sites to artificially generate $Rb1^{-/-}$ pituitary intermediate lobe melanotrophs[13]. To determine whether *Skp2* inactivation affects the efficiency of POMC-Cre–*loxP*-mediated recombination, we generated *POMC-Cre*;*Rosa26R*;$Skp2^{+/+}$ and *POMC-Cre*;*Rosa26R*;$Skp2^{-/-}$ mice. We found that the *POMC-Cre* strain could induce Cre-*loxP*–mediated deletion in most of the intermediate lobe melanotrophs in both $Skp2^{+/+}$ and $Skp2^{-/-}$ mice (**Fig. 2a**). Because the POMC promoter is also active in corticotrophs in the anterior lobe, scattered anterior lobe recombination events were detected in both strains of mice as well (**Fig. 2a**, **v**,**vi**).

We then generated *POMC-Cre*;$Rb1^{lox/lox}Skp2^{+/+}$ and *POMC-Cre*;$Rb1^{lox/lox}Skp2^{-/-}$ mice and examined their pituitary glands at 7 weeks of age. As expected[13], *POMC-Cre*;$Rb1^{lox/lox}Skp2^{+/+}$ mice harbored dysplastic nodular lesions across the entire intermediate lobes (**Fig. 2b**, **ii**,**vi**). Unexpectedly, *POMC-Cre*;$Rb1^{lox/lox}Skp2^{-/-}$ mice did not contain normal-appearing intermediate lobes as we predicted based on the lack of pituitary tumorigenesis in $Rb1^{+/-}Skp2^{-/-}$ mice. Instead, the intermediate lobes of these mice were essentially absent, with only a single layer of lining cells separating the anterior and posterior lobes (**Fig. 2b**, **iii**,**vii**). The intermediate lobes of *POMC-Cre*;$Rb1^{lox/lox}Skp2^{+/-}$ mice were also considerably thinner than normal (**Fig. 2b**, **iv**,**viii**). These results confirm that *Skp2* inactivation blocks tumorigenesis and demonstrate that this effect was achieved not by reverting *Rb1*-deficient melanotrophs to normal cells but by eliminating them.

We traced the fate of *Rb1* and *Skp2* doubly deficient melanotrophs by generating *POMC-Cre*;*Rosa26R*;$Rb1^{lox/lox}Skp2^{+/+}$ and *POMC-Cre*;*Rosa26R*;$Rb1^{lox/lox}Skp2^{-/-}$ mice and allowing them to age to 10–13 weeks. The intermediate lobes of *POMC-Cre*;*Rosa26R*;$Rb1^{lox/lox}Skp2^{+/+}$ mice, observed with hematoxylin stain and enhanced yellow fluorescent protein (EYFP) fluorescence (**Fig. 2c**, **i**,**iii**), were in more advanced stages of tumorigenesis than those at 7 weeks (compare with **Fig. 2b**, **ii**), whereas the intermediate lobes of *POMC-Cre*;*Rosa26R*;$Rb1^{lox/lox}Skp2^{-/-}$ mice remained a single-cell layer (**Fig. 2c**, **ii**). Notably, the cells in this layer were EYFP positive (**Fig. 2c**, **iv**), suggesting that this single-cell layer environment could prevent death of *Rb1* and *Skp2* doubly deficient cells or that these cells escaped

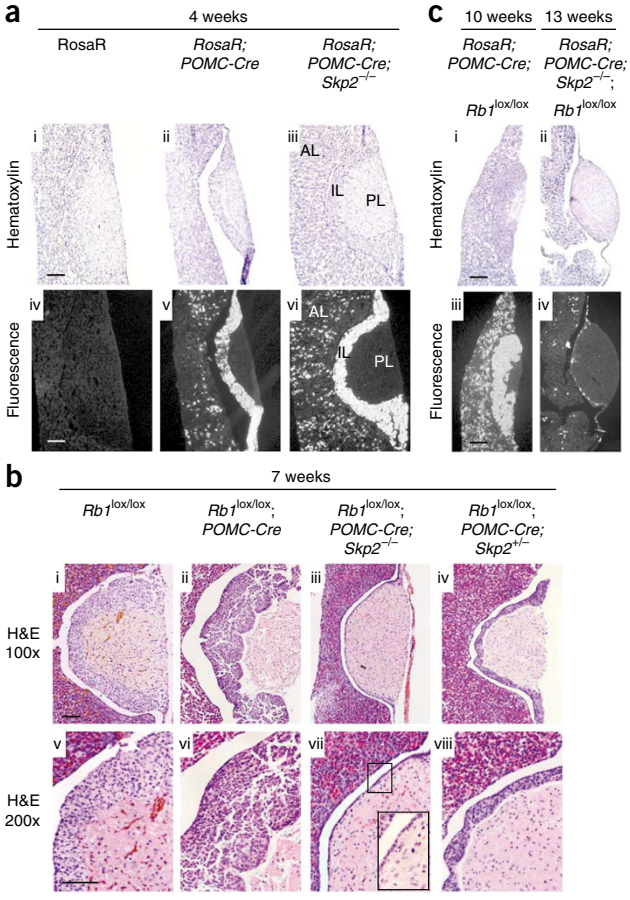

Figure 2 Effects of targeted deletion of *Rb1* in pituitary intermediate and anterior lobes of $Skp2^{+/+}$ and $Skp2^{-/-}$ mice. (**a**) The *POMC-Cre* strain induced Cre-*loxP*–mediated excision in posterior and anterior lobes of $Skp2^{+/+}$ and $Skp2^{-/-}$ mice. '*Rosa26R*' indicates *Rosa26-loxP*-STOP-*loxP*-*EYFP*. Mice were examined at 4 weeks of age. EYFP expression was by fluorescence of frozen-sectioned samples. (**b**) Pituitary intermediate lobes of indicated mice at 7 weeks of age. Hematoxylin and eosin (H&E)-stained sections of various pituitaries are shown. Large inset in **vii** is enlarged view of areas marked by the small box. (**c**) Pituitary glands of the indicated mice at the indicated ages, examined as in **a**. Scale bar, 200 μm.

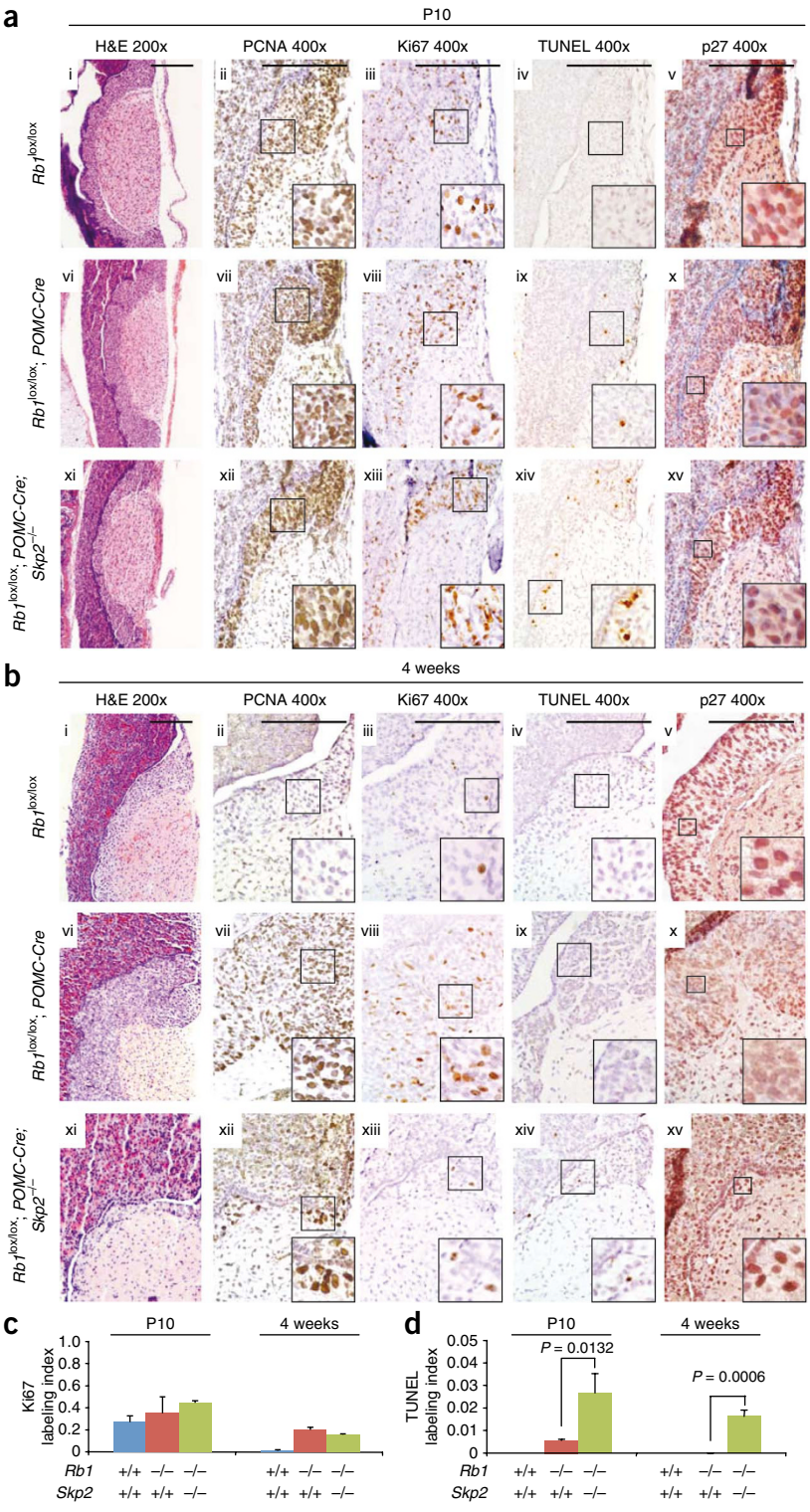

Figure 3 Effects of Skp2 inactivation on E2F deregulation, aberrant proliferation and apoptosis, and p27 expression in pituitary intermediate lobes following *Rb1* deletion. (**a**,**b**) Various indicated mice at the ages of P10 (**a**) and 4 weeks (**b**) are presented. E2F deregulation is examined by PCNA expression, proliferation by Ki67 expression and apoptosis by TUNEL labeling. (**c**,**d**) Quantification of Ki67 (**c**) and TUNEL labeling (**d**) in intermediate lobes was performed with three pituitaries of each indicated genotype at the indicated ages. *Rb1* genotypes indicate the outcome of Cre-*loxP*–mediated deletion in intermediate lobe. *P* values are by Student's *t*-test. Error bars, s.d. Scale bar, 200 μm.

Rb1 deletion. We also found that *Rb1* deletion in corticotrophs induced the presence of more corticotrophs in the anterior lobe, and combined deletion of *Rb1*and *Skp2* markedly reduced their numbers (**Fig. 2a**, **v**,**vi**, and **c**, **iii**,**iv**). This indicates that combined *Rb1* and *Skp2* deletion could eliminate corticotrophs as well as melanotrophs.

We next harvested the mice at earlier ages to investigate how the intermediate lobes were eliminated (**Fig. 3**). At postnatal day (P) 10 the intermediate lobes of both *POMC-Cre*;$Rb1^{lox/lox}$ $Skp2^{+/+}$ and *POMC-Cre*;$Rb1^{lox/lox}Skp2^{-/-}$ mice showed slightly higher cellularity than those of $Rb1^{lox/lox}$ mice (**Fig. 3a**, **i**,**vi**,**xi**, and data not shown). Expression of PCNA (encoded by an E2F target gene) and Ki67 (a proliferation marker) was readily observed in $Rb1^{lox/lox}$ melanotrophs, indicating the proliferative status of these cells at this age (**Fig. 3a**, **ii**,**iii**, and **c**). Deletion of *Rb1* increased PCNA and Ki67 expression, consistent with deregulation of E2F and proliferation caused by pRb inactivation (**Fig. 3a**, **vii**,**viii**, and **c**). *Skp2* inactivation reduced neither PCNA expression nor the aberrant proliferation of the *Rb1*-deficient cells (**Fig. 3a**, **xii**,**xiii**, and **c**), but it significantly increased TUNEL-positive intermediate lobe cells compared with $Rb1^{lox/lox}$ and *POMC-Cre*;$Rb1^{lox/lox}$ $Skp2^{+/+}$ controls (**Fig. 3a**, **iv**,**ix**,**xiv**, and **d**).

At 4 weeks of age the cells in the intermediate lobes of *POMC-Cre*;$Rb1^{lox/lox}Skp2^{-/-}$ mice maintained deregulated PCNA expression and proliferation and increased apoptosis (**Fig. 3b–d**). Whereas the aberrantly proliferating intermediate lobes of 4-week-old *POMC-Cre*;$Rb1^{lox/lox}Skp2^{+/+}$ mice had become more than twofold thicker than those of the $Rb1^{lox/lox}$ controls (**Fig. 3b**, **i**,**vi**), the proliferating yet apoptotic intermediate lobes of 4-week-old *POMC-Cre*;$Rb1^{lox/lox}$ $Skp2^{-/-}$ mice had become more than twofold thinner than normal (**Fig. 3b**, **xi**). Together, these findings indicate that *Skp2* is required for the survival of aberrantly proliferating *Rb1*-deficient melanotrophs and that $Rb1^{-/-}Skp2^{-/-}$ melanotroph apoptosis caused the elimination of the intermediate lobes in *POMC-Cre*;$Rb1^{lox/lox}Skp2^{-/-}$ mice.

POMC-Cre;$Rb1^{lox/lox}$ mice allowed us to evaluate the effect of Skp2 on p27 expression during melanotroph tumorigenesis using immunohistochemical staining. Melanotrophs of $Rb1^{lox/lox}$, *POMC-Cre*;$Rb1^{lox/lox}$ and *POMC-Cre*;$Rb1^{lox/lox}Skp2^{-/-}$ mice at P10 had comparable nuclear p27 protein stains (**Fig. 3a**, **v**,**x**,**xv**). However, by 4 weeks p27 concentrations clearly decreased in melanotrophs of *POMC-Cre*;$Rb1^{lox/lox}$ mice (**Fig. 3b**, **x**) but were maintained in the melanotrophs of *POMC-Cre*;$Rb1^{lox/lox}Skp2^{-/-}$ mice (**Fig. 3b**, **xv**),

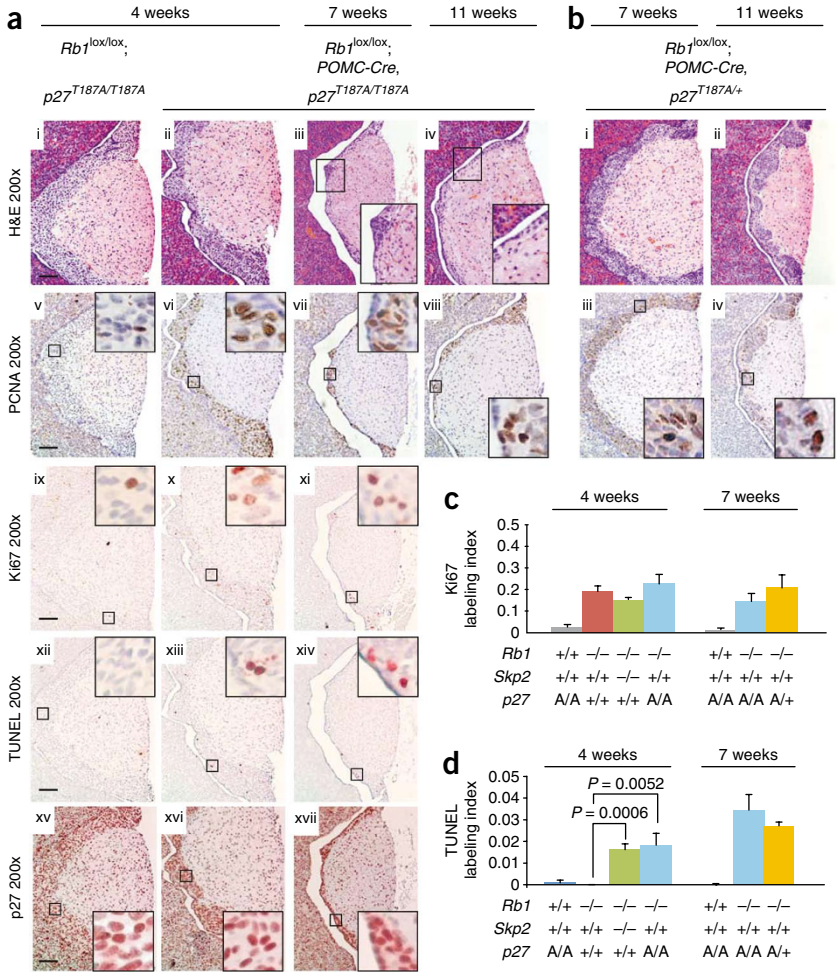

Figure 4 Effects of targeted deletion of *Rb1* in pituitary intermediate lobe of p27T187A knock-in mice. (**a**) Intermediate lobe morphology, PCNA expression, Ki67 and TUNEL labeling, and p27 expression were examined at the indicated ages. (**b**) Intermediate lobe morphology and PCNA expression after *Rb1* deletion in $p27^{T187A/+}$ mice at 7 and 11 weeks of age. (**c**,**d**) Quantification of Ki67 (**c**) and TUNEL labeling (**d**) in **a**. *P* values are by Student's *t*-test. Error bars, s.d. Scale bar, 200 μm.

suggesting that *Skp2* is required for the downregulation of p27 during melanotroph tumorigenesis following *Rb1* deletion.

We next investigated how Skp2 inactivation led to the failure of p27 downregulation and whether this failure was responsible for the tumor-blocking effects of Skp2 inactivation. *In vitro* studies have established that Skp2 mediates p27 ubiquitination in the SCF^{Skp2} ubiquitin ligase after p27 is phosphorylated on T187. However, the *in vivo* role of this Skp2 function has remained unclear because of divergent findings from Skp2-null mice (in which all Skp2 functions are absent) and p27T187A knock-in mice (in which only Skp2's ability to mediate ubiquitination of T187-phosphorylated p27 is absent). Skp2-null mice showed p27 protein accumulation in certain tissues and smaller body sizes[14], but p27T187A knock-in mice neither showed p27 protein accumulation nor phenocopied Skp2-null mice[15]. Thus, *in vivo*, Skp2's ability to mediate ubiquitination of T187-phosphorylated p27 does not figure importantly in its ability to regulate p27. Our previous finding that pRb inhibits Skp2-mediated p27 ubiquitination by interfering with Skp2 binding to T187-phosphorylated p27 (ref. 7) suggested that this Skp2 function may be deregulated and contribute to p27 protein reduction and tumorigenesis following *Rb1* loss. To evaluate this prediction, we generated *POMC-Cre*,$Rb1^{lox/lox}p27^{T187A/T187A}$ and the control $Rb1^{lox/lox}p27^{T187A/T187A}$ mice and examined their pituitary intermediate lobes at 4, 7 and 11 weeks of age.

The intermediate lobes of $Rb1^{lox/lox}$ $p27^{T187A/T187A}$ mice appeared normal (**Fig. 4a**,**i**), consistent with the general lack of abnormality in $p27^{T187A/T187A}$ mice. Following *POMC-Cre*–mediated *Rb1* deletion, intermediate lobes of *POMC-Cre*,$Rb1^{lox/lox}p27^{T187A/T187A}$ mice at 4 weeks of age did not show the hyperplastic thickening observed in *POMC-Cre*,$Rb1^{lox/lox}$ mice (**Fig. 3b**, **vi**), but instead had regional thinning (**Fig. 4a**, **ii**). The thinning of the intermediate lobe became more widespread by 7 weeks of age (**Fig. 4a**, **iii**), and by the age of 11 weeks the entire intermediate lobes were only two to three cell layers thick (**Fig. 4a**, **iv**). The nature of the T187A knock-in mutation (blocking T187 phosphorylation–dependent ubiquitination of p27 by SCF^{Skp2}) predicted that the tumor-blocking effects observed in $p27^{T187A/T187A}$ homozygous mice should also occur in $p27^{T187A/+}$ heterozygous mice, though potentially to a smaller extent. Results shown in **Figure 4b** confirm this prediction.

Similar to the effects of *Skp2* knockout in *Rb1*-deficient melanotrophs, p27T187A knock-in did not reduce the deregulated expression of PCNA and proliferation (**Fig. 4a**, **v–vii**,**ix–xi**, and **c**), but it increased apoptosis (**Fig. 4a**, **xii–xiv**, and **b**). These effects were also observed in the presence of one allele of p27T187A (**Fig. 4b–d** and data not shown). Finally, the reduced p27 expression in melanotrophs in 4-week-old *POMC-Cre*,$Rb1^{lox/lox}$ mice (**Fig. 3b**, **x**) occurred neither in melanotrophs in either 4- or 7-week-old *POMC-Cre*,$Rb1^{lox/lox}p27^{T187A/T187A}$ mice (**Fig. 4a**, **xv–xvii**) nor in 7-week-old *POMC-Cre*,$Rb1^{lox/lox}p27^{T187A/+}$ mice (data not shown). Together these results suggest that the T187 phosphorylation–dependent ubiquitination of p27 by the SCF^{Skp2} ubiquitin ligase underlies Skp2's essential role in pituitary tumorigenesis following *Rb1* loss, and that the apoptotic ablation of melanotrophs in *POMC-Cre*;$Rb1^{lox/lox}Skp2^{-/-}$ mice could be explained by a proapoptotic effect of p27 in these cells[16].

Notably, p27T187A knock-in is not equivalent to Skp2 knockout, because the intermediate lobes of *POMC-Cre*,$Rb1^{lox/lox}Skp2^{-/-}$ mice thinned to a greater degree and with faster kinetics than those in *POMC-Cre*,$Rb1^{lox/lox}p27^{T187A/T187A}$ mice (for example, compare **Fig. 2b**, **iii**,**vii** with **Fig. 4a**, **iii**,**iv**). Skp2 has a growing list of potential substrates in addition to p27 and can support cancer cell survival by protecting cyclin A from inhibition by p27 and p21 (ref. 17) and by blocking p53 activation by p300 (ref. 18). Further studies will be required to determine the roles of these additional mechanisms.

We next investigated whether the survival function of Skp2 revealed with mouse models was applicable to human tumors that develop as a result of *Rb1* mutations. Because retinoblastoma is the main tumor that is associated with *Rb1* deficiency in humans, we examined the effect of Skp2 knockdown in retinoblastoma cells. We found that

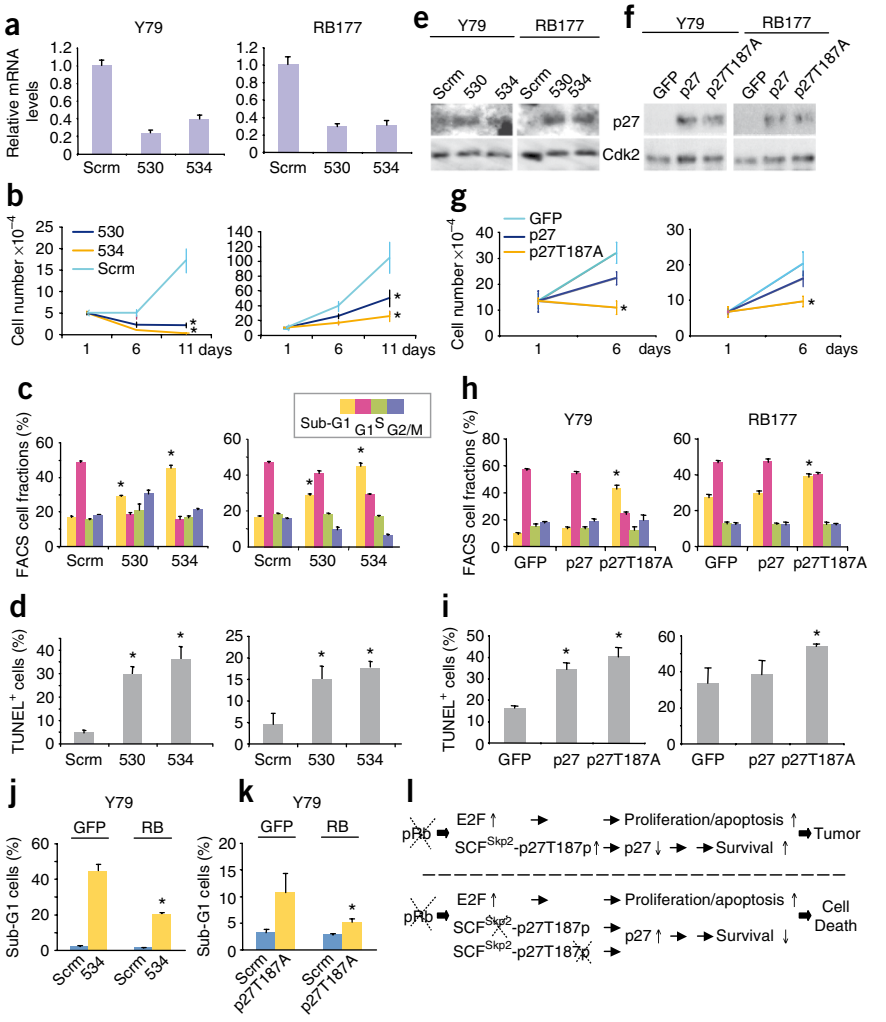

Figure 5 Effects of Skp2 knockdown and stabilized p27 expression on established Y79 cells and early passage RB177 retinoblastoma cells. (**a**–**e**) Y79 and RB177 cells infected with lentiviruses expressing short hairpin RNAs (shRNAs) targeting Skp2. Two independent Skp2 shRNAs and a scrambled shRNA control (Scrm) were used as indicated. After drug selection, infected cells were evaluated for Skp2 mRNA by quantitative reverse transcriptase PCR (**a**), cell proliferation by counting live cells (**b**), cell cycle profile by FACS (**c**), apoptosis by TUNEL staining (**d**) and p27 expression by protein immunoblotting, with Cdk2 as a loading control (**e**). (**f**–**i**) Y79 and RB177 cells infected with BE-GFP lentiviral vector encoding *p27* or *p27T187A*. Infected cells were evaluated for p27 expression (**f**), cell proliferation (**g**), cell cycle profile (**h**) and TUNEL staining (**i**). (**j**,**k**) Y79 cells transduced with BE-GFP vector or BE-GFP-RB, followed 2 d later by transduction with *Skp2* shRNA or scrambled shRNA control (**j**) or with BE-GFP or BE-GFP-p27T187A (**k**), and evaluated cells with sub-G1 DNA content. Averages with s.d. are shown. Asterisks indicate $P < 0.05$ relative to applicable controls. (**l**) A new model of tumorigenesis after *Rb1* loss. Two consecutive arrows suggest the presence of multiple steps between them.

knockdown of Skp2 (**Fig. 5a**) significantly inhibited retinoblastoma cell proliferation (**Fig. 5b**). Skp2 knockdown induced apoptosis, as measured by sub-G1 DNA content and TUNEL staining, but did not diminish S-phase population, as measured by FACS (**Fig. 5c**,**d**). The apoptotic effects of Skp2 knockdown were evident both in the established Y79 cell line and in early-passage RB177 cells.

As expected, Skp2 knockdown induced accumulation of p27 in these human retinoblastoma cells (**Fig. 5e**). Moreover, ectopic expression of p27 was able to inhibit proliferation and induce apoptosis (**Fig. 5f**–**i**) similar to the effects of Skp2 knockdown. Notably, the mutant p27T187A was considerably more potent in inhibiting proliferation and inducing apoptosis, consistent with our findings from p27T187A knock-in mice. Restoration of pRb function largely prevented apoptosis induced either by Skp2 knockdown or by ectopic p27 expression (**Fig. 5j**,**k**), even though the modest pRb concentrations slowed but did not entirely block cell proliferation (data not shown), suggesting that lack of pRb rendered the retinoblastoma cells dependent on Skp2 and sensitive to aberrantly expressed p27.

We recently showed that MDM2 has essential roles in proliferation and survival of retinoblastoma cells and that knockdown of p14Arf diminished the requirement for MDM2 (ref. 19). In similar experiments we found that knockdown of p14Arf did not mitigate the effects of Skp2 knockdown, suggesting that p14Arf is not a critical target of Skp2 in these cells (**Supplementary Fig. 4**).

Before the current study, inactivation of previously studied pRb targets delayed tumorigenesis in $Rb1^{+/-}$ mice accompanied by reduced tumor cell proliferation[1–6]. In contrast, our study reveals that inactivation of Skp2 did not reduce deregulated proliferation of $Rb1^{-/-}$ cells but induced apoptosis, which completely prevented tumorigenesis. Our findings add a survival arm to the pRb-E2F model of pRb function, in which pRb loss not only deregulates E2F to result in aberrant proliferation and apoptosis through various E2F target genes but also deregulates the SCF^{Skp2}-p27T187p p27 ubiquitination mechanism to downregulate p27 to provide survival support for the aberrantly proliferating pRb-deficient cells (**Fig. 5l**). When this mechanism is disrupted, either by inactivation of Skp2 or by blocking of p27 T187 phosphorylation, the outcome of pRb loss becomes cell death, revealing that *Rb1* and *Skp2* mutations are synthetically lethal to susceptible cells. The above model predicts that Skp2 is a potentially effective drug target to prevent and treat pRb-deficient tumors. Because our data imply that the p27T187 phosphorylation–dependent function of Skp2 is required for tumorigenesis following pRb loss, yet is not needed for normal development[15], therapeutic targeting of Skp2 can focus on the p27T187-dependent function of Skp2 or p27T187 phosphorylation.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturegenetics/.

Note: Supplementary information is available on the Nature Genetics website.

ACKNOWLEDGMENTS

We thank J. Cui for expert technical assistance, R. Mahmood for help with tissue preparation and histological analysis, D. Abramson and S. Jhanwar for support of retinoblastoma cell analysis, A. Koff for comments on the manuscript and A. Burns and W. Zhang for encouragement. We are grateful to T. Jacks for providing $Rb1^{+/-}$

mice (from L. Yamasaki and A. Iavarone) and $Rb1^{lox/lox}$ mice, B. Lowell and S. Chua for *POMC-Cre* mice, J. Roberts for p27T187A knock-in mice and F. Costantini for Rosa26YFP mice (from J. Pollard). This work was supported by grants from the National Institute of Diabetes and Digestive and Kidney Diseases and National Cancer Institute of the US National Institutes of Health to L.Z. Albert Einstein Comprehensive Cancer Research Center and Liver Research Center provided core facility support. F.B. was supported by the Training Program in Cellular and Molecular Biology and Genetics (T32 GM007491) at the Albert Einstein College of Medicine. L.Z. is a recipient of the Irma T. Hirschl Career Scientist Award.

AUTHOR CONTRIBUTIONS

H.W., P.J., F.B., D.S. and L.Z. designed and performed experiments with mice mutant for *Rb1*, *Skp2*, *p27* or targeted deletion of *Rb1*. J.L. and R.S.S. performed pathology studies. D.C. and X.X. designed and performed analyses of retinoblastoma cells; H.W. performed protein blot experiments. K.N. and K.I.N. provided $Skp2^{+/-}$ mice. H.W., J.L., D.C. and L.Z. wrote the paper.

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ONLINE METHODS

Mice. *Rb1*$^{+/-}$ mice and *Skp2*$^{+/-}$ mice have been described elsewhere[12,14]. Mouse strain background is as follows. *Skp2*$^{+/-}$ mice on mixed C57BL/6J×129Sv strain background were backcrossed to C57BL/6J strain mice four times, and *Rb1*$^{+/-}$ mice on mixed C57BL/6J×129Sv strain background were backcrossed to C57BL/6J mice once. *Rb1*$^{+/-}$*Skp2*$^{+/-}$ mice were then generated from these mice and were used to generate littermate *Rb1*$^{+/-}$*Skp2*$^{+/+}$ and *Rb1*$^{+/-}$*Skp2*$^{-/-}$ mice. Our *Rb1*$^{+/-}$ mice may therefore show a slower tumor development kinetics than *Rb1*$^{+/-}$ mice with equal contributions from C57BL/6J and 129Sv strain background[20]. *Rb1*-heterozygous mice were genotyped according to a published protocol[12]. *POMC-Cre* transgenic mice were genotyped as described[21]. Primers for genotyping *Skp2*$^{+/-}$ mice, *Rb1*$^{lox/lox}$ mice[22], *Rosa26R*(YFP) mice[23] and p27T187A knock-in mice[15] are listed in **Supplementary Table 1**.

The animals studied for ENU mutagenesis were C57BL/6J×129Sv hybrid strain littermate mice from Skp2 heterozygous crosses. *Skp2*$^{+/+}$ and *Skp2*$^{-/-}$ mice were injected intraperitoneallywith ENU (0.5 mmol per g body weight) at P15 ± 2 d as described[24]. Mice were killed at the first sign of morbidity, which included abdominal swelling, hunched posture and rapid breathing. Complete necropsies of all internal organs were performed including size measurement of tumors.

All mouse study protocols were approved by the Albert Einstein College of Medicine Animal Institute.

Protein blot and reverse transcriptase–PCR analyses. Normal pituitaries, fractions of gross pituitary tumors and fractions of ENU-induced tumors were snap-frozen in ethanol–dry ice and stored in −80 °C. For protein blot, frozen tissues were homogenized with Dounce glass homogenizer in tissue lysis buffer (50 mM HEPES, pH 7.2, 150 mM NaCl, 1 mM EDTA, 0.1% Tween-20, 1 mM dithiothreitol and standard protease inhibitors). Tissue debris was removed by centrifugation for 10 min at 14,000 r.p.m. in an Eppendorf Centrifuge 5415C (F-45-18-11 rotor) at 4 °C. Protein concentrations of the extracts were determined by Bio-Rad protein assay kit, and equal amounts of protein samples were loaded on 10% SDS gels and blotted onto polyvinylidene fluoride membrane. Antibodies to Skp2 (H435), p27 (C-19), cyclin A (C-19), cyclin E (M-20) and Cdk2 (C-19) were from Santa Cruz Biotechnology.

For quantitative PCR (Q-PCR), tissue RNA was extracted by Trizol reagent (Invitrogen). Total RNA was treated with RQ1 DNase (Promega) at 37 °C for 30 min, and RQ1 was denatured at 65 °C for 20 min. T7 oligonucleotides and SuperScript II (Invitrogen) were used for the synthesis of the first-strand cDNA at 42 °C for 60 min. The PCR primers for mSkp2 and mGAPDH are listed in **Supplementary Table 1**. SYBR Green PCR Master Mix (4309155; ABI) and the standard program of ABI Prism 7000 were used for Q-PCR amplification.

Immunohistochemistry staining and frozen sectioning for fluorescence detection. Paraffin sections were stained with Histomouse-plus kit (Zymed) with antibodies to PCNA (PC10) and p27 (C-19) from Santa Cruz Biotechnology, and to BrdU (Ab-2) from Calbiochem, and Ki67 as primary antibody (1 μg/ml). TUNEL staining was performed with the reagents and instructions of Apoptosis Detection Kit (S7101) from Chemicon.

Pituitaries were fixed in 4% paraformaldehyde, 10% glucose in PBS for 30 min and embedded in Tissue Freezing Medium (H-TFM; Triangle Biomedical Sciences) on dry ice for frozen sectioning. After fluorescence photography, slides were counterstained by hematoxylin.

Lentivirus infection and analysis of human retinoblastoma cells. Y79 cells were purchased from the American Type Culture Collection, and RB177 cells were derived from a human retinoblastoma and passaged for approximately 2 months, with no evidence of a crisis phase, before the knockdown analyses[19]. Skp2 shRNAs were delivered by pLKO constructs TRCN0000007530 and TRCN0000007534 (Open Biosystems) and were compared to a pLKO encoding a nonsilencing control shRNA (Addgene). RB177 cells with constitutive *CDKN2A*^{ARF}-null and pLKO-transduced controls were as described[19]. pRb, p27 and p27T187A were delivered using the bidirectional BE-GFP vector[25]. BE-GFP-p27+3′ and BE-GFP-p27T187A+3′ were produced by inserting a XmaI-XbaI fragment of pCS+p27 and pCS+p27(T187A)[26] extending from the p27 coding region to the 3′ UTR between the corresponding XmaI site and a vector XbaI site of BE-GFP-p27 (ref. 25). BE-GFP-Rb was as described[25]. Cells were cultured, infected and analyzed as described[19].

Statistical analysis. In the survival analysis, difference in Kaplan-Meier survival curves was analyzed by log-rank test (JMP Software). Differences in gross tumor incidence and incidence of microscopic lesions in macroscopically normal pituitary and thyroid glands were analyzed by Fisher's exact test (MedCalc Software). Differences in TUNEL-labeling indices between *Rb1*$^{lox/lox}$;*POMC-Cre*;*Skp2*$^{+/+}$ and *Rb1*$^{lox/lox}$;*POMC-Cre*;*Skp2*$^{-/-}$ intermediate lobes and between *Rb1*$^{lox/lox}$;*POMC-Cre*;*p27*$^{+/+}$ and *Rb1*$^{lox/lox}$;*POMC-Cre*;*p27*$^{T187A/T187A}$ intermediate lobes were analyzed by Student's *t*-test (MedCalc Software).

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