Deletions of Retinoblastoma 1 (Rb1) and Its Repressing Target S Phase Kinase-associated protein 2 (Skp2) Are Synthetic Lethal in Mouse Embryogenesis*

Received for publication, January 26, 2016, and in revised form, March 8, 2016 Published, JBC Papers in Press, March 10, 2016, DOI 10.1074/jbc.M116.718049

Hongling Zhao, Hongbo Wang, Frederick Bauzon, Zhonglei Lu, Hao Fu, Jinhua Cui, and Liang Zhu
From the Department of Developmental and Molecular Biology, and Ophthalmology & Visual Sciences, and Medicine, The Albert Einstein Comprehensive Cancer Center and Liver Research Center, Albert Einstein College of Medicine, Bronx, New York 10461

Tumor suppressor pRb represses Skp2, a substrate-recruiting subunit of the SCFSkp2 ubiquitin ligase. Rb1+/− mice incur “two-hit” pituitary tumorigenesis; Skp2−/−;Rb1+/− mice do not. Rb1−/− embryos die on embryonic day (E) 14.5–15.5. Here, we report that Skp2−/−;Rb1−/− embryos died on E11.5, establishing an organismal level synthetic lethal relationship between Rb1 and Skp2. On E10.5, Rb1−/− placentas showed similarly active proliferation and similarly inactive apoptosis as WT placentas, whereas Rb1−/− embryos showed ectopic proliferation without increased apoptosis in the brain. Combining Skp2−/− did not reduce proliferation or increase apoptosis in the placentas but induced extensive apoptosis in the brain. We conditionally deleted Rb1 in neuronal lineage with Nes-Cre and reproduced the brain apoptosis in E13.5 Nes-Cre;Rb1lox/lox;Skp2−/− embryos, demonstrating their synthetic lethal relationship at a cell autonomous level. Nes-Cre-mediated Rb1 deletion increased expression of proliferative E2F target genes in the brains of Skp2+/+ embryos; the increases rose higher with activation of expression of apoptotic E2F target genes in Skp2−/− embryos. The brain apoptosis was independent of p53 but coincident with proliferation. The highly activated expression of proliferative and apoptotic E2F target genes subsided with gradually reduced roles of Skp2 in preventing p27 protein accumulation in the brain in late gestation, allowing the embryos to reach full term with normally sized brains. These findings establish that Rb1 and Skp2 deletions are synthetic lethal and suggest how this lethal relationship might be circumvented, which could help design better therapies for pRb-deficient cancer.

Children who inherit one null allele of the retinoblastoma 1 gene (Rb1) are Rb1+/− in all cells and develop retinoblastoma with full penetrance, and the retinoblastoma cells are invariably Rb1−/−. These cases represent the classic “two-hit” Rb1-deficient tumorigenesis. Mice that inherited one knock-out allele of Rb1 (the mouse homolog of Rb1) are Rb1+/− in all cells and develop pituitary melanotroph tumors with full penetrance, and the tumor cells are invariably Rb1−/−. Rb1−/− mice therefore model the two-hit Rb1-deficient tumorigenesis. Biochemical studies have identified many repressing targets of pRb (encoded by Rb1), with E2F1, E2F2, and E2F3 being the best studied (1). It is hypothesized that Rb1-deficient tumorigenesis is caused by abnormal activation of pRb repressing targets following the inactivation of pRb. When Rb1−/− mice were used to test this hypothesis, the combined deletion of E2F1 (2) or E2F3 (3) inhibited pituitary tumorigenesis in Rb1−/− mice to various degrees.

pRb represses Skp2 by inhibiting its binding to p27 (4), reducing its mRNA expression via E2F5 (5, 6), and promoting its degradation via APC/C (7). Combined deletion of Skp2 inhibited pituitary melanotroph tumorigenesis in Rb1+/− mice, establishing Skp2 as a functionally significant pRb repressing target in two-hit Rb1-deficient tumorigenesis. Remarkably, whereas combined deletion of pRb repressing targets E2F1 (2), E2F3 (3), and Id2 (8) slowed pituitary tumorigenesis and extended the survival of Rb1−/− mice by a few months, pituitary of Skp2−/−;Rb1+/− mice were free of oncogenic lesions at 17 months of age when littermate Skp2−/−;Rb1−/− mice all died of pituitary tumors before 13 months of age. Artificial deletion of Rb1 in melanotrophs by Pomc-Cre revealed apoptosis in pituitary intermediate lobe in 10-day-old Skp2−/−;Pomc-Cre; Rb1lox/lox mice and reduction of the intermediate lobe to a layer one cell thick by 7 weeks of age. These observations suggest a survival role of Skp2 in Rb1-deficient melanotroph tumorigenesis, shedding light on how Skp2−/− prevents pituitary tumorigenesis in Skp2−/−;Rb1−/− mice.

Rb1 is an essential gene in mouse embryogenesis, because Rb1−/− embryogenesis becomes lethal on embryonic day 14.5–15.5 (E14.5–15.4) (9–11). Rb1−/− embryogenesis provides another experimental system to test the functional significance of biochemically identified pRb repressing targets. In previous studies, when deletion of a pRb repressing target inhibited pituitary tumorigenesis in Rb1+/+ mice, the same deletion extended survival of Rb1−/− embryos (12–14). A

*This work was supported by National Institutes of Health Grants RO1CA127901 and RO1CA131421 (to L. Z.), Albert Einstein Comprehensive Cancer Research Center Grant SP30CA13330, and Liver Research Center Grant SP30DK061153. The authors declare that they have no conflicts of interest with the contents of this article.

1Recipient of Department of Defense Prostate Cancer Research Program (PCRP) Postdoctoral Fellowship PC121837.

2Present address: Dept. of Pathology, University of Texas Health Science Center, San Antonio, TX 78229.

3Irma T. Hirschl Career Scientist Award recipient. To whom correspondence should be addressed: Dept. of Developmental and Molecular Biology, Albert Einstein College of Medicine, 1300 Morris Park Ave., Rm. U-521, Bronx, NY 10461. Tel.: 718-430-3320; Fax: 718-430-8975; E-mail: liang.zhu@einstein.yu.edu.

4The abbreviations used are: En, embryonic day; n, IHC, immunohistochemistry; IF, immunofluorescence; PCNA, proliferating cell nuclear antigen; qPCR, quantitative PCR; H&E, hematoxylin and eosin.
**Skp2 and Rb1 Deletions Are Synthetic Lethal**

straightforward paradigm therefore emerged: if a biochemically identified pRb repressing target is functionally significant, its deletion would lessen the effects of Rb1 inactivation, manifested as inhibition of pituitary tumorigenesis to extend survival of Rb1−/− mice and correction of defects in Rb1−/− embryogenesis to extend survival of Rb1−/− embryos. In the current study, we determined how Skp2, whose deletion more effectively inhibited pituitary tumorigenesis in Rb1−/− mice than all other pRb repressing targets tested so far, fits into this paradigm.

**Experimental Procedures**

Mice—Nes-Cre mice were obtained from The Jackson Laboratory. Rb1+/− (9), Rb1lox/lox (15), Skp2−/− (16), and Trp53lox/lox mice (17) have been described. Nes-Cre was genotyped using primers Cre F (5′-ATGCCCAAGAAGAGGAAAGGT-3′) and Cre R (5′-GAAAATCAGTCGTCAGCCTAGA-3′). Other genotyping details were previously described (18, 19).

The mice used in this study were on mixed C57BL/6J×129Sv strain background. The mice were maintained under pathogen-free conditions in the Albert Einstein College of Medicine animal facility. All procedures were reviewed and approved by Einstein Animal Care Committee, conforming to accepted standards of humane animal care.

**Embryos Gestation Determination and Analyses**—Gestation and embryo age were determined by timed mating. Male and female mice were put in the same cage in late evening on day 1 and were separated in early morning on day 2. The embryo age was counted as E0.5 on day 2 if the female mice were found pregnant later. Pregnant females were intraperitoneally injected with BrdU (0.1 mg/g) 2 h before harvest. Embryos at various ages were harvested and were fixed in 10% formalin (Fisher Scientific, SF 100-4), embedded in paraffin wax, and sectioned. For embryos older than E15.5, decalcification was performed before routine processing to paraffin embedding. Embryo histology studies were conducted together with Albert Einstein Cancer Center mouse pathology core. Embryo brains used for RT-PCR and Western blots were dissected, snap frozen, and kept in −80 °C freezer.

**Immunohistochemistry (IHC), Immunofluorescence (IF) Staining, and Microscopy**—For IHC and IF staining, embryonic brains were sectional at 5-μm thickness. The slides were deparaffinized, hydrated, and incubated in a steamer for 20 min in sodium citrate buffer (Vector Labs, H3301) for antigen retrieval. Sections were first treated with 3% H2O2 to quench endogenous peroxidase, washed several times, blocked with 10% normal goat serum, and then incubated in primary antibodies at 4 °C overnight. The following antibodies were used: BrdU (CalBiochem, NA61, 1:100 dilution), PCNA (Santa Cruz Biotechnology, sc-56, 1:500 dilution), E2F1 (Santa Cruz Biotechnology, sc-193, 1:200 dilution), Cyclin A (Santa Cruz Biotechnology, sc-751, 1:200 dilution), Cyclin E (Santa Cruz Biotechnology, sc-481, 1:200 dilution), Skp2 (Santa Cruz Biotechnology, sc-7164, 1:200 dilution), p73 (Abcam, ab40658, 1:1000 dilution), phosphorylated-Histon H3 (Cell Signaling, 9701L, 1:1000 dilution), aCasp3 (Cell Signaling Technology, 9664S, 1:1000 dilution), p27 (BD Transduction Lab, 610242, 1:1000 dilution), aCasp3 (Cell Signaling Technology, 9664S, 1:1000 dilution), p27 (BD Transduction Lab, 610242, 1:1000 dilution), and Tubulin (Sigma-Aldrich, T6074, 1:5000 dilution). 8% SDS-PAGE gel was used for pRb protein analysis, 12% gels were used for pH3 and aCasp3 analyses, and 10% gels were used for other protein analyses. The protein marker for Western blot was from Crystalgen Inc. (catalog no. 65-0671). At least three embryo brains of indicated genotypes at different embryonic stages were analyzed with highly reproducible results.

**Statistical Analysis**—Differences in BrdU, TUNEL labeling, and RT-qPCR between indicated samples were analyzed by two-sided Student’s t test. p < 0.05 is considered as statistically significant. Embryo survival analyses were analyzed by Fisher’s exact test (*, p < 0.05; **, p < 0.01; ***, p < 0.005).

**Results**

Skp2−/−;Rb1−/− Embryogenesis Is Lethal on E11.5—To define functional relationships between Skp2−/− and Rb1−/− in embryogenesis, we mated Skp2+/−;Rb1+/− mice to combine Skp2−/− and Rb1−/− in embryogenesis. Skp2−/− mice are viable with normal life span (16), whereas Rb1−/− embryos die on E14.5-E15.5 (9–11). Table 1 shows the survival and death (presence or absence of a beating heart) of three key genotypes (WT, Rb1−/−, and Skp2−/−;Rb1−/−) between E10.5 and E13.5. We
found dead Rb1−/− embryos at low frequencies on E10.5 (1 of 19), E11.5 (2 of 19), E12.5 (0 of 9), and E13.5 (1 of 7). These low frequencies increased to 1 of 8, 9 of 16, 3 of 4, and 5 of 5, respectively, in Skp2−/−;Rb1−/− embryos. The increases are statistically significant on E11.5 (p = 0.0088), E12.5 (p = 0.014), and E13.5 (p = 0.015). Thus, Skp2−/− and Rb1−/− are synthetic lethal at the organismal level in mouse embryogenesis.

**TABLE 1**

Skp2−/−;Rb1−/− embryogenesis becomes lethal on E11.5

| Age | Total | Skp2+/+; Rb1−/− | Skp2+/−; Rb1−/− | Skp2−/−; Rb1−/− | p values |
|-----|-------|----------------|----------------|----------------|----------|
| E10.5 | 148   | 10 (0)         | 19 (1)         | 8 (1)          | p = 1.000 |
| E11.5 | 197   | 16 (0)         | 19 (2)         | 16 (9)         | p = 0.009 |
| E12.5 | 116   | 3 (0)          | 9 (0)          | 4 (3)          | p = 0.014 |
| E13.5 | 120   | 5 (0)          | 7 (1)          | 5 (5)          | p = 0.015 |

**Figure 1.** Indistinguishable high proliferation and low apoptosis in Skp2+/−;Rb1−/−, Skp2+/−;Rb1−/−, Skp2−/−;Rb1−/−, and Skp2−/−;Rb1−/− placentas on E10.5. A–D, BrdU labeling (A) and TUNEL staining (B) of E10.5 placentas of the indicated genotypes, and their quantification (C and D). E, representative H&E staining of the indicated E10.5 placentas showing both the spongiotrophoblast layer (sp) and labyrinth layer (lb) separated by dashed lines (upper row) and trophoblast clumps in the labyrinth layer (lower row).

Skp2−/− induces apoptosis in brains of E10.5 Skp2−/−; Rb1−/− embryos—Lethality in Rb1−/− embryogenesis on E14.5–E15.5 is caused by placental defects. Leone and co-workers (11, 21) showed that WT and Rb1−/− placentas were similarly highly proliferative on E11.5, but proliferation failed to decrease in Rb1−/− placentas compared with WT placentas on E13.5, leading to overexpansion of trophoblasts in labyrinth layer, which clogs mother-fetus exchange. Because 60% of Skp2−/−;Rb1−/− embryos were dead on E11.5, we examined proliferation and apoptosis of placentas of the four combinations of Skp2 and Rb1 genotypes (Skp2+/+; Rb1+/−, Skp2+/−; Rb1−/−, Skp2−/−; Rb1+/−, and Skp2−/−; Rb1−/−) on E10.5. We found indistinguishable high proliferation (Fig. 1, A and C) and low apoptosis (Fig. 1, B and D) among these four genotypes. The structural defect of Rb1−/− placentas on E13.5 is clumps of trophoblasts in the labyrinth layer to the extent that restricted mother-fetus exchange space. On E10.5, trophoblast clumps can be found in the labyrinth layer of WT and Skp2−/− placentas, likely because of the high proliferation in this location at
Skp2 and Rb1 Deletions Are Synthetic Lethal

**Rb1 Deletion in Neuronal Lineage Defines the Skp2-Rb1 Synthetic Lethal Neuronal Apoptosis as Cell Autonomous**—To determine whether the neuroepithelium apoptosis in E10.5 Skp2−/−;Rb1−/− embryos implicated a cell autonomous Skp2-Rb1 synthetic lethal relationship, we next conditionally deleted Rb1 in neuronal lineage with Nes-Cre and Rb1lox/lox. Nes-Cre-mediated deletion of Rb1 did not result in embryonic lethality on Skp2+/− (22) or Skp2−/− background (Table 2). When genotyping was performed at weaning age (21 days or older), we have not found a Skp2−/−;Nes-Cre;Rb1lox/lox mouse, indicating that Skp2−/− did not rescue the neonatal lethality of Nes-Cre;Rb1lox/lox mice (22). E13.5 Skp2+/−;Nes-Cre;Rb1lox/lox embryos did not contain apoptosis in the neuroepithelium of the brain (Fig. 3, A and B, panel b), which confirmed a previous report (22). In Skp2−/− E13.5 embryos, the same Rb1 deletion induced massive apoptosis in the brain, detectable by TUNEL staining (Fig. 3, B, panel d, and D, panel d; quantified Fig. 3E), H&E staining (Fig. 3C, panel d), and staining (see Fig. 5G) and Western blotting (Fig. 5D) for cleaved (activated) Caspase 3 (aCasP3 for short). In Rb1−/− embryogenesis, E13.5 Rb1−/− embryos also showed similar neuronal apoptosis, but the apoptosis was prevented when Rb1−/− embryos were supplied with Rb1+/+ placentas. Nes-Cre;Rb1lox/lox does not delete Rb1 in placentas nor in non-neuronal tissues in embryo proper.

Apoptosis in the brain of Rb1−/− embryos was prevented by additional deletion of Trp53 (23, 24). When we additionally

FIGURE 2. Apoptosis in developing brains of E10.5 Skp2−/−;Rb1−/− embryos. A, scan images of H&E-stained whole embryo sections of indicated genotypes. Certain organogenesis landmarks are marked: E (encephalic vesicles), R (roof of hindbrain), 4 (fourth ventricle), and H (heart). B and D, BrdU labeling shows ectopic proliferation in the intermediate zone of neuroepithelium of the developing brains in Skp2−/−;Rb1−/− and Skp2−/−;Rb1−/− E10.5 embryos (B), quantified in D, C and E. TUNEL staining of neuroepithelium in the same set of embryos (C) and quantified in E. Quantification included data from three sets of embryos. Statistical analyses were performed by Student’s t test. **, p = 0.002.

TABLE 2

Nes-Cre;Rb1lox/lox;Skp2−/− embryos develop to full term

Pregnant females were sacrificed to obtain the embryos on three different gestational days (Age column). All embryos were alive; total and expected (in parentheses) numbers for the two key genotypes are shown. These two genotypes also generated similar numbers of live newborns. Live E13.5 and E15.5 embryos are indicated by the presence of a heartbeat. Presence of movement indicate live E17.5 embryos and live newborns.

| Age | Nes-Cre;Rb1lox/lox;Skp2−/− | Nes-Cre;Rb1lox/lox;Skp2−/− |
|-----|--------------------------|---------------------------|
| E13.5 | 22 (15) | 14 (8) |
| E15.5 | 15 (10) | 12 (9) |
| E17.5 | 13 (8) | 4 (3) |

this gestational stage. In comparison to WT and Skp2−/− placentas, Rb1−/− and Skp2−/−;Rb1−/− placentas showed noticeably larger trophoblast clumps (Fig. 1). In the embryo proper, the four genotypes showed comparable organogenesis at E10.5 of heart (Fig. 2A, marked as H) and the central nervous system showing encephalic vesicles (Fig. 2A, marked as E), roof of hindbrain (Fig. 2A, marked as R), and fourth ventricle (Fig. 2A, marked as 4). Upon BrdU labeling, Rb1−/− embryos (Skp2+/+ or Skp2−/−) showed ectopic proliferation in the intermediate zone of the neuroepithelium in the developing brain (Fig. 2, B and D), and these brain regions in Skp2−/−;Rb1−/− embryos further contained significant TUNEL staining (Fig. 2, C and E). Thus, the Skp2-Rb1 synthetic lethal relationship is present at a cellular level as neuronal apoptosis in E10.5 Skp2−/−;Rb1−/− embryos, but not in E10.5 Rb1−/− or E10.5 Skp2−/− embryos.
deleted Trp53 in neuronal lineage together with Rb1 in Skp2<sup>−/−</sup>; Nes-Cre;Rb1<sup>lox/lox</sup>;Trp53<sup>lox/lox</sup> embryos, the brain apoptosis remained, as detected by TUNEL staining (Fig. 4, B, panel b, and D, panel b) and H&E staining (Fig. 4C, panel b). These findings demonstrate that combined deletion of Rb1 and Skp2 can cell autonomously induce apoptosis in neuroepithelium of the developing brain and that the underlying mechanism for this synthetic lethal apoptosis is fundamentally different from that for the non-cell autonomously induced brain apoptosis in Rb1<sup>−/−</sup> embryos.

**Skp2-Rb1 Synthetic Lethal Neuronal Apoptosis Coexists with Further Enhanced Proliferation**—To investigate the mechanisms of Skp2-Rb1 synthetic lethal brain apoptosis, we determined how Skp2<sup>−/−</sup> affected the effects of Rb1 deletion in the brain. Nes-Cre-mediated deletion of Rb1 in embryos afforded us brain tissues to quantitatively determine E2F target gene expression by RT-qPCR and Western blot. pRb represses the activating E2F subfamily E2F1, E2F2, and E2F3, whose target genes are mostly proliferative, with E2F1 being able to additionally activate expression of apoptotic gene PUMA via ARF-p53 and p73 directly as an E2F1 target gene. Following loss of Rb1, expression of typical proliferative E2F target genes is increased comparing brain tissues of Rb1<sup>lox/lox</sup> and Nes-Cre;Rb1<sup>lox/lox</sup> embryos (Fig. 5A). Consistent with the lack of brain apoptosis in Nes-Cre;Rb1<sup>lox/lox</sup> embryos, expression of E2F1 apoptotic target genes was not increased (Fig. 5B). When we determined expression of proliferative E2F target genes in brains of Skp2<sup>−/−</sup>; Nes-Cre;Rb1<sup>lox/lox</sup> embryos, we found that Skp2<sup>−/−</sup> did not correct the increased expression but instead increased the expression further and activated expression of E2F1 apoptotic target genes (Fig. 5, A and B). The further increased expression of proliferative E2F target genes and activation of expression of apoptotic E2F1 target genes in the brains of Skp2<sup>−/−</sup>; Nes-Cre; Rb1<sup>lox/lox</sup> embryos were more dramatic at protein levels (Fig. 5D). We next show that ectopic proliferation in the intermediate zone, as measured by BrdU labeling (Fig. 5, E and F) and Western blot for pH3 (Fig. 5D), is increased following Rb1 deletion in brains of Skp2<sup>+/+</sup> embryos and increased further in brains of Skp2<sup>−/−</sup> embryos. Western blot for aCasp 3 more dramatically demonstrated Skp2-Rb1 synthetic lethal brain apoptosis (Fig. 5D). These brain tissue findings indicate the cause of

**FIGURE 3.** **Cell autonomous Skp2-Rb1 synthetic lethal neuronal apoptosis on E13.5.** A–D, sections of E13.5 brains of the indicated genotypes were stained with H&E (A and C) or TUNEL (B and D) and imaged at low (A and B) and high (C and D) magnifications. E, quantification of TUNEL staining. 400–500 cells were counted on high magnification pictures from three sets of embryos. The bar graphs show averages ± S.E. Statistical analyses were by Student’s t test. ***, p < 0.001.
**Skp2 and Rb1 Deletions Are Synthetic Lethal**

Skp2-Rb1 synthetic lethal neuronal apoptosis to be the further activation of E2F1.

When we additionally deleted Trp53, expression of its target gene PUMA was significantly reduced, whereas expression of E2F1 target gene p73 remained (Fig. 5C). Because the synthetic lethal neuronal apoptosis in embryonic brains is independent of p53 (Fig. 4), these findings further identify E2F1 p53-independent apoptotic activity as the underlying mechanism. Finally, most clusters of TUNEL or aCasp3-positive apoptotic cells in Skp2−/−;Nes-Cre;Rb1lox/lox brains were doubly positive for PCNA, product of the proliferative E2F target gene (Fig. 5G), directly linking ectopic proliferation to the apoptosis.

**Skp2-Rb1 Synthetic Lethal Neuronal Apoptosis Subsides in Late Gestation**—When we determined the impact of Skp2-Rb1 synthetic lethal brain apoptosis on brain development in Skp2−/−;Nes-Cre;Rb1lox/lox embryos, it quickly became evident that Skp2−/−;Nes-Cre;Rb1lox/lox embryos had normally sized brains (Fig. 6A) without gross structural abnormalities (Fig. 6B) on E18.5. To investigate the molecular basis for this lack of structural impact by the massive apoptosis seen on E13.5, we measured the expression of proliferative and apoptotic E2F target genes in E13.5, E15.5, and E17.5. The sets of PCNA, E2F1, cyclin A, cyclin E (proliferative E2F target genes), p73 (apoptotic E2F1 target gene), pHH3, and aCasp3 (mitosis and apoptosis markers) that were highly expressed in the brains of Skp2−/−;Nes-Cre;Rb1lox/lox on E13.5 became less highly expressed on E15.5 and reduced to WT levels on E17.5 (Fig. 6C). These findings provided a biochemical explanation for the development of the brain to normal size and structure when Skp2−/−;Nes-Cre;Rb1lox/lox embryogenesis progressed to late gestation.

To investigate how these biochemical changes took place, we determined mRNA expression of proliferative E2F target genes as WT embryogenesis progressed to late gestation. Fig. 6D shows that levels of PCNA, E2F1, cyclin A, and cyclin E mRNA quickly reduced in the WT brains from E13.5. These findings suggest that mitotic exit for neuronal differentiation begins in the last 5 gestational days.

Notably, expression of Skp2 mRNA also reduced significantly in this period (Fig. 6D), consistent with Skp2 being an E2F target gene (5, 6). We next found that Skp2 protein levels in WT embryos also gradually reduced from E13.5 to E17.5 (Fig. 6E). To determine the functional impact of these reductions, we determine the protein levels of p27, because mediating p27 degradation is the best known function of Skp2. Unexpectedly, the gradual decrease in Skp2 protein levels did not induce gradual accumulation of p27 protein. Furthermore, Skp2−/− induced accumulation of p27 in the brain, and combined deletion of Rb1 further increased p27 accumulation on E13.5. This two-step p27 accumulation was much reduced on E15.5 and became nonexistent on E17.5. These findings suggest that, with reduced expression, Skp2 roles in the degradation of p27 gradually became dispensable for preventing p27 protein accumulation. The reduced role of Skp2 in preventing p27 protein accumulation could be due to its reduced expression, activation of other ubiquitin ligases for p27, or, more likely, a combination of these. These findings suggest that naturally reduced expression of Skp2 and naturally increased Skp2-independent mechanisms to prevent p27 protein accumulation weakened the Skp2-Rb1 synthetic lethal relationship when embryogenesis progressed to late gestation.

**Discussion**

A key strategy to study pRb is to identify its repressing targets, defined as pRb binding proteins whose functions are repressed when bound by pRb. The best established pRb repressing targets are E2F1, E2F2, and E2F3, the activator members of the E2F family. pRb binds these activator E2Fs to inhibit their transactivating functions and to recruit repressive chromatin modifiers onto the promoters of their target genes to achieve long term repression. When a pRb repressing target is functionally significant, the effects of Rb1 deletion should be reduced when this pRb repressing target is inactivated. Indeed, pituitary tumorigenesis in Rb1−/− mice is inhibited in E2f1−/−; Rb1−/− mice (2) or E2f3−/−;Rb1−/− mice (3) and combining deletion of E2f1 (12), E2f2 (25), or E2f3 (11, 13) corrected certain defects in Rb1−/− embryos and placentas to improve Rb1−/− embryogenesis. A weakness shared by these three pRb repressing targets is that their individual deletion only partially inhibited pituitary tumorigenesis in Rb1−/− mice. Deleting E2f1, E2f2, and E2f3 together is embryonic lethal (26); its
effects on pituitary tumorigenesis in \( Rb1^{+/−} \) mice remain to be tested by their conditional deletion in pituitary melanotrophs.

Skp2 is the first pRb repressing target whose deletion prevented pituitary tumorigenesis in \( Rb1^{+/−} \) mice. Pomc-Cre-mediated deletion of \( Rb1 \) in pituitary melanotrophs in \( Skp2^{−/−} \) mice suggested apoptotic elimination of \( Skp2^{−/−};Rb1^{+/−} \) melanotrophs that lost the remaining WT allele of \( Rb1 \). However, Pomc-Cre-mediated deletion of \( Rb1 \) in pituitary melanotrophs is not a physiologically relevant mechanism of \( Rb1 \) inactivation, and the pituitary intermediate lobe is too minis-
We next used cultured MEFs to study the mechanisms of apoptosis by co-deletion of Skp2 and Rb1 (20). As expected, acute deletion of Rb1 in WT MEFs induced increased expression of E2F target genes. Unexpectedly, E2F target gene expression increased further when Rb1 was deleted in Skp2−/− MEFs. Cyclin A binds E2F1 to repress its transactivating activity, and cyclin A is a E2F target gene; activated E2F1 following Skp2−/− MEFS accumulation a significant amount of p27 protein, which competed against E2F1 for binding to cyclin A. The freed E2F1 is therefore “superactivated” to further increase expression of proliferation and apoptotic target genes. Remarkably, E2F1 deletion reduced oncogenic expansion of pituitary intermediate lobe in Skp2+/+; Pomc-Cre;Rb1lox/lox mice but increased the thickness of the pituitary intermediate lobe in Skp2−/−;Pomc-Cre;Rb1lox/lox mice. However, important questions remained. Skp2−/− MEFs in culture accumulated p27 to higher degrees than most tissues in Skp2−/− mice, and the role of p53 in apoptosis is unclear.

In the present study, we used embryogenesis to study the functional relationship between Skp2−/− and Rb1−/−. By mating Skp2+/−;Rb1+/− mice, we have now established that Skp2−/− and Rb1−/− are synthetic lethal at the organismal level. We demonstrate that Skp2−/− did not inhibit ectopic proliferation in Rb1−/− neuroepithelium intermediate zone but induced apoptosis specifically in the neuroepithelium of Rb1−/− embryos. These findings provide in vivo evidence for a synthetic lethal relationship that is currently unique among the growing list of pRb repressing targets. By generating Skp2−/−; Nes-Cre;Rb1lox/lox embryos, we have further established the Skp2-Rb1 synthetic lethal relationship in vivo at a cell autonomous level with a biochemical understanding. In this cell autonomous setting, Rb1 deletion activated E2Fs to increase expression of proliferative genes but not apoptotic genes; co-deletion of Skp2 and Rb1 not only further activated E2F proliferative target genes but activated expression of E2F apoptotic target genes. The role of a further activated E2F1 in this Skp2-Rb1 synthetic lethal apoptosis is dramatically demonstrated by the findings that (i) ectopic proliferation in the neuroepithelium is further increased, (ii) most apoptotic cells expressed the proliferative marker PCNA, and (iii) the apoptosis is independent of p53.

Finally, it is evident that most other tissues in E10.5 Skp2−/−; Rb1−/− embryos do not contain significantly increased apoptosis, thereby documenting that the Skp2-Rb1 synthetic lethal relationship is context-dependent. Our study of brains of Skp2−/−;Nes-Cre;Rb1lox/lox embryos in late gestation further demonstrated a number of mechanisms that could nullify Skp2-Rb1 synthetic lethal neuronal apoptosis, including lower expression of Skp2 and developing additional mechanisms of
p27 degradation to render Skp2 dispensable in preventing p27 accumulation.

Inactivation of pRb is a highly recurrent feature of cancer cells, and inhibiting pRb repressing targets is a logical and intensively pursued therapeutic strategy for pRb-deficient cancer. The identification of Skp2-Rb1 synthetic lethal relationship illuminated a highly attractive new strategy for pRb-deficient cancer. It is increasingly clear that cancer as an heterogeneous disease cannot be cured by a single treatment, no matter how effective it might be for certain cases. It is further evident that no matter how effective a treatment is at the beginning, cancer always evolves to resist it. The Skp2-Rb1 synthetic lethal apoptosis in embryogenesis show these two features, and our findings suggest the cancer characteristics that are best suited for the Skp2-Rb1 synthetic lethal treatment strategy (genetic inactivation of pRb with high expression of Skp2), how these cancers might develop resistance to this treatment (select for additional abilities to degrade p27), and how the resistant cancer might be treated (combining targeting the other p27 ubiquitin ligases).

Author Contributions—H. Z., H. W., and L. Z. conceived and coordinated the study. H. Z., H. W., F. B., Z. L., H. F., and J. C. performed the experiments. H. Z. and L. Z. wrote the paper.

Acknowledgments—We thank Dr. Keiko Nakayama and Dr. Keichi Nakayama for providing the Skp2 KO mice.

References

1. Trimarchi, J. M., and Lees, J. A. (2002) Sibling rivalry in the E2F family. Nat. Rev. Mol. Cell Biol. 3, 11–20
2. Yamasaki, L., Bronson, R., Williams, B. O., Dyson, N. J., Harlow, E., and Jacks, T. (1998) Loss of E2F-1 reduces tumorigenesis and extends the lifespan of Rb1−/− mice. Nat. Genet. 18, 360–364
3. Ziebold, U., Lee, E. Y., Bronson, R. T., and Lees, J. A. (2003) E2F3 loss has opposing effects on different pRb-deficient tumors, resulting in suppression of pituitary tumors but metastasis of medullary thyroid carcinomas. Mol. Cell. Biol. 23, 6542–6552
4. Ji, P., Jiang, H., Rekhtman, K., Bloom, J., Ichetovkin, M., Pagano, M., and Zhu, L. (2004) An Rb-Skp2-p27 pathway mediates acute cell cycle inhibition by Rb and is retained in a partial-penetrant Rb mutant. Mol. Cell 16, 47–58
5. Zhang, L., and Wang, C. (2006) F-box protein Skp2: a novel transcriptional target of E2F. Oncogene 25, 2615–2627
6. Yung, Y., Walker, J. L., Roberts, J. M., and Asooja, R. K. (2007) A Skp2 autoinduction loop and restriction point control. J. Cell Biol. 178, 741–747
7. Binné, U. K., Classon, M. K., Dick, F. A., Wei, W., R ape, M., Kaelin, W. G., Jr., Nähr, A. M., and Dyson, N. J. (2007) Retinoblastoma protein and anaphase-promoting complex physically interact and functionally cooperate during cell-cycle exit. Nat. Cell Biol. 9, 225–232
8. Losa rella, A., Rothchild, G., Yokota, Y., Russell, R. G., and Iavarone, A. (2005) Id2 mediates tumor initiation, proliferation, and angiogenesis in Rb mutant mice. Mol. Cell. Biol. 25, 3563–3574
9. Jacks, T., Fazeli, A., Schmitt, E. M., Bronson, R. T., Goodell, M. A., and Weinberg, R. A. (1992) Effects of an Rb mutation in the mouse. Nature 359, 295–300
10. Lee, E. Y., Chang, C.-Y., Hu, N., Wang, Y.-C., Lai, C.-C., Herrup, K., Lee, W.-H., and Bradley, A. (1992) Mice deficient for Rb are nonviable and show defects in neurogenesis and hematopoiesis. Nature 359, 288–294
11. Wenzel, P. L., Wu, L., de Bruin, A., Chong, J. L., Chen, W. Y., Dureska, G., Sites, E., Pan, T., Sharma, A., Huang, K., Ridgway, R., Mosaliganti, K., Sharp, R., Machiraju, R., Sultz, J., Yamamoto, H., Cross, J. C., Robinson, M. L., and Leone, G. (2007) Rb is critical in a mammalian tissue stem cell population. Genes Dev. 21, 85–97
12. Tsai, K. Y., Hu, Y., Macleod, K. F., Crowley, D., Yamasaki, L., and Jacks, T. (1998) Mutation of E2f-1 suppresses apoptosis and inappropriate S phase entry and extends survival of Rb-deficient mouse embryos. Mol. Cell 2, 293–304
13. Ziebold, U., Reza, T., Caron, A., and Lees, J. A. (2001) E2F3 contributes both to the inappropriate proliferation and to the apoptosis arising in Rb mutant embryos. Genes Dev. 15, 386–391
14. Losa rella, A., Nosed a, M., Beyma, M., Yokota, Y., and lavarone, A. (2000) Id2 is a retinoblastoma protein target and mediates signalling by Myc oncoproteins. Nature 407, 592–598
15. Sage, J., Miller, A. L., Pérez-Mancera, P. A., Wysocki, J. M., and Jacks, T. (2003) Acute mutation of retinoblastoma gene function is sufficient for cell cycle re-entry. Nature 424, 223–228
16. Nakayama, K., Nagahama, H., Minamishima, Y. A., Matsumoto, M., Nakamichi, I., Kitagawa, K., Shirane, M., Tsunematsu, R., Tsukiyama, T., Ishida, N., Kitagawa, M., Nakayama, K., and Hatakeyama, S. (2000) Targeted disruption of Skp2 results in accumulation of cyclin E and p27Kip1, polyplody and centrosome overduplication. EMBO J. 19, 2069–2081
17. Marino, S., Vooijs, M., van Der Gulden, H., Jonkers, J., and Berns, A. (2000) Induction of medulloblastomas in p53-null mouse mutants by somatic inactivation of Rb in the external granular layer cells of the cerebellum. Genes Dev. 14, 994–1004
18. Wang, H., Bauzon, F., Li, P., Xu, X., Sun, D., Locker, J., Sellers, R. S., Nakayama, K., Nakayama, K. I., Cobrinik, D., and Zhu, L. (2010) Skp2 is required for survival of aberrantly proliferating Rb1-deficient cells and for tumorigenesis in Rb1−/− mice. Nat. Genet. 42, 83–88
19. Zhao, H., Bauzon, F., Fu, H., Lu, Z., Cui, J., Nakayama, K., Nakayama, K. I., Locker, J., and Zhu, L. (2013) Skp2 deletion unmasks a p27 safeguard that blocks tumorigenesis in the absence of pRb and p53 tumor suppressors. Cancer Cell 24, 645–659
20. Lu, Z., Bauzon, F., Fu, H., Cui, J., Zhao, H., Nakayama, K., Nakayama, K. I., and Zhu, L. (2014) Skp2 suppresses apoptosis in Rb1-deficient tumours by limiting E2F1 activity. Nat. Commun. 5, 3463
21. Wu, L., de Bruin, A., Saavedra, H. I., Starovic, M., Trimboli, A., Yang, Y., Opavská, J., Wilson, P., Thompson, J. C., Ostrowski, M. C., Rosol, T. J., Woollett, L. A., Weinstein, M., Cross, J. C., Robinson, M. L., and Leone, G. (2003) Extra-embryonic function of Rb is essential for embryonic development and viability. Nature 421, 942–947
22. MacPherson, D., Sage, J., Crowley, D., Trump, A., Bronson, R. T., and Jacks, T. (2003) Conditional mutation of Rb causes cell cycle defects without apoptosis in the central nervous system. Mol. Cell. Biol. 23, 1044–1053
23. Morgenbesser, S. D., Williams, B. O., Jacks, T., and DePinho, R. A. (1994) p53-dependent apoptosis produced by Rb-deficiency in the developing mouse lens. Nature 371, 72–74
24. Macleod, K. F., Hu, Y., and Jacks, T. (1996) Loss of Rb activates both p53-dependent and independent cell death pathways in the developing mouse nervous system. EMBO J. 15, 6178–6188
25. Dirlam, A., Woollett, L. A., Weinstein, M., Cross, J. C., Robinson, M. L., and Leone, G. (2003) Extra-embryonic function of Rb is essential for embryonic development and viability. Nature 421, 942–947
26. MacPherson, D., Sage, J., Crowley, D., Trump, A., Bronson, R. T., and Jacks, T. (2003) Conditional mutation of Rb causes cell cycle defects without apoptosis in the central nervous system. Mol. Cell. Biol. 23, 1044–1053
27. Morgenbesser, S. D., Williams, B. O., Jacks, T., and DePinho, R. A. (1994) p53-dependent apoptosis produced by Rb-deficiency in the developing mouse lens. Nature 371, 72–74