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Established and New Mouse Models Reveal E2f1 and Cdk2 Dependency of Retinoblastoma and Expose Strategies to Block Tumor Initiation

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Abstract

RB $^{+/-}$ individuals develop retinoblastoma and, subsequently, many other tumors. The Rb relatives p107 and p130 protect the tumor-resistant *Rb*$^{-/-}$ mouse retina. Determining the mechanism underlying this tumor suppressor function may expose novel strategies to block Rb-pathway cancers. p107/p130 are best known as E2f inhibitors, but here we implicate E2f-independent Cdk2 inhibition as the critical p107 tumor suppressor function *in vivo*. Like *p107* loss, deleting *p27* or inactivating its Cdk inhibitor (CKI) function (*p27*$^{CK-}$) cooperated with *Rb* loss to induce retinoblastoma. Genetically, p107 behaved like a CKI because inactivating *Rb* and one allele each of *p27* and *p107* was tumorigenic. While *Rb* loss induced canonical E2f targets, unexpectedly *p107* loss did not further induce these genes but instead caused post-transcriptional Skp2-induction and Cdk2 activation. Strikingly, Cdk2 activity correlated with tumor penetrance across all the retinoblastoma models. Therefore, Rb restrains E2f, but p107 inhibits cross-talk to Cdk. While removing either *E2f2* or *E2f3* genes had little effect, removing only one *E2f1* allele blocked tumorigenesis. More importantly, exposing retinoblastoma-prone fetuses to small molecule E2f or Cdk inhibitors for merely one week dramatically inhibited subsequent tumorigenesis in adult mice. Protection was achieved without disrupting normal proliferation. Thus, exquisite sensitivity of the cell-of-origin to E2f and Cdk activity can be exploited to prevent Rb pathway-induced cancer *in vivo* without perturbing normal cell division. These data suggest that E2f inhibitors, never before tested *in vivo*, or Cdk inhibitors, largely disappointing as therapeutics, may be effective preventive agents.

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Keywords

Chemoprevention; Retinoblastoma; Cdk; E2f; p107

INTRODUCTION

The Rb-E2f and Cdk inhibitor (CKI)-Cdk2/1 interactions regulate cell cycle progression (Figure 1A). The Rb family (Rb, p107 and p130) bind and form repressor complexes with E2f family proteins. Activating E2fs, (E2f1/2/3) induce factors required for DNA replication, drive proliferation of quiescent cells (reviewed in (1, 2)) and, although not required for normal progenitor division (3), are essential for abnormal division of differentiating *Rb* null cells (4–8). Cip/Kip inhibitors (p21/p27/p57) on the other hand bind and inhibit the kinase activity of Cyclin A/E-Cdk2/1 complexes. Cdk2 fires replication origins, and feeds back to promote E2f activity by phosphorylating Rb family proteins (9).

To prevent uncontrolled G1-S progression, there are extensive controls limiting positive cross talk between E2f and Cdk2 (Figure 1A). For example, by preventing Rb family phosphorylation, CKIs inhibit Cdk2-mediated activation of E2f. Equally, by preventing Cyclin E/A gene induction the Rb family blocks E2f-mediated activation of Cdk2. Some work has emerged suggesting E2f-independent ways in which Rb proteins limit Cdk2 activity. For example, Rb promotes Skp2 degradation through APC and thus stabilizes CKIs (10, 11). Subsequent studies validated this link, as Skp2 is required in Rb-deficient pituitary tumors (12). p107/p130 do not bind APC, but p107 reduces Skp2 post-transcriptionally *in vitro* (13); whether this occurs *in vivo* is unclear. p107/p130 bind and inhibit Cdk2 *in vitro* (14), but a p107-Cdk2 complex in cells has only been detected in the absence of p21 and p27 (15). Thus, the extent to which p107 may utilize E2f-independent mechanisms to regulate Cdk2 *in vivo* and its relevance, if any, to tumorigenesis is unclear.

The ocular cancer retinoblastoma generated fundamental discoveries with broad relevance to cell cycle regulation and cancer, including the classic two-hit hypothesis and *RB*, the first recognized tumor suppressor. RB protein turned out to have universal relevance to cancer, and 50% of $RB^{+/-}$ survivors develop secondary tumors by the age of 50 (reviewed in (16)). The unique sensitivity of the human retina to *RB* loss implies that other human tissues and the retina in other species have extra protection. Indeed, *p107* and *p130* protect mouse retina, and current retinoblastoma models utilize loss of *Rb* and one relative (17–21). How *p107*/*p130* protect the $Rb^{-/-}$ retina is unclear, but elucidating the mechanism could expose strategies to prevent tumors initiated by RB pathway defects in humans. One explanation for the quantum difference between the tumor-resistant $Rb^{-/-}$ and tumor-prone *Rb/p107* null retina is that E2f targets become super-induced in the latter. This occurs in keratinocytes (22, 23), but in fibroblasts Rb and p107/p130 appear to regulate distinct targets (24, 25). Here, we show that the major function of p107 in the *Rb* null retina is not to regulate canonical E2f-regulated genes. Genetic, biochemical and pharmacological studies instead show that p107 prevents E2f-independent cross-talk to Cdk2 and that combined activation of E2f and Cdk2, through loss of *Rb*, and *p107* or *p27*, respectively, underpins tumor susceptibility in the mouse retina. Strikingly exposure of the fetal retina to either E2f or Cdk small molecule

inhibitors for *merely one week* blocked retinoblastoma without perturbing normal division. We suggest that the sensitivity of the human retina to *RB* loss not only reflects E2F activation, but poor buffering of feedback regulation of CDKs. Given the universal role of the RB pathway in cancer, further studies are needed to assess the potential clinical relevance of our findings to multiple cancers.

RESULTS

CKI Activity Suppresses Mouse Retinoblastoma

Current mouse knockout models of retinoblastoma require deletion of *Rb* plus either *p107* or *p130* (18, 17, 19–21). p107 or p130 could protect $Rb^{-/-}$ retina by repressing E2f targets, but we wondered if cross-talk to Cdk may also be important (Figure 1A). When we deleted floxed Rb (Rb^{f}) in peripheral retina at embryonic day 10 (E10) using the *αCre* transgene as before (18, 5), we observed elevated p21 and p27 mRNAs, with p27 protein detected in virtually all cells at postnatal day 8 (P8), and sporadic p21 expression (Figure S1 and data not shown). To test whether CKI loss mimics *p107* or *p130* loss we therefore removed *p27*. Strikingly, $\alpha Cre;Rb^{f/f};p27^{-/-}$ mice developed retinoblastoma with 100% penetrance (Figure 1B and Table 1), double that of $\alpha Cre;Rb^{f/f};p107^{-/-}$ mice and similar to $\alpha Cre;Rb^{f/f};p130^{-/-}$ mice (18, 19). As in other double knockout (DKO) models of retinoblastoma (18, 17, 19–21), *Rb/p27* DKO tumor cells expressed the amacrine cell marker Ap2α as well as markers in this and other cells such as Pax6, and Prox1, but lacked other cell type markers (Figure 2 and Figure S2). P30 tumor cells were positive for markers that label all cell cycle phases (Ki67), or S-phase (BrdU). In $Rb^{-/-};p107^{-/-}$, $Rb^{-/-};p27^{-/-}$, $Rb^{-/-};p27^{CK-/CK-}$, $Rb^{-/-};p107^{+/-};p27^{+/-}$ and $Rb^{-/-};p107^{+/-};p27^{+/CK-}$ tumors, the fraction of $Ki67^{+}$ cells that were also $Ap2\alpha^{+}$ was 92±4%, 91±1%, 92±5%, 92±2%, and 90±1% respectively, and the fraction of $BrdU^{+}$ cells that were $Ap2\alpha^{+}$ was 89±11%, 91±10%, 85±4%, 84±5%, and 87±4%, respectively (Figure 2B–2C). The contaminating glutamine synthase (GS)-labelled Müller glia were quiescent (Figure S2). Thus, in the $Rb^{-/-}$ mouse retina, p27 is a potent tumor suppressor suggesting that low CKI activity in the human retina may contribute to the sensitivity of this tissue to *RB* loss.

p27 binds and regulates proteins other than Cyclin/Cdk2 (reviewed in (26)). To define the critical tumor suppressor activity we assessed the $p27^{CK-}$ allele in which four substitutions specifically disrupt CKI activity (27). $\alpha Cre;Rb^{f/f};p27^{CK-/CK-}$ mice developed retinoblastoma with 100% penetrance (Figure 1B and Table 1). $p27^{CK-/CK-}$ animals exhibit retinal dysplasia (27), which we confirmed, but they never developed retinoblastoma (data not shown). Furthermore, of 28 eyes from $\alpha Cre;Rb^{f/f};p27^{+/CK-}$ animals only three had tumors, and strikingly all showed loss of heterozygosity (LOH) (Table 1 and Figure S3). Thus, $p27^{CK-}$ is not a dominant oncoprotein either in normal or $Rb^{-/-}$ retina, contrasting lung where it causes tumors (27). Collectively, our results demonstrate that retinoblastoma requires loss of p27 CKI activity.

These data suggest a role for Cdk in retinoblastoma initiation. Conceivably p107 could, like p27, suppress tumorigenesis by limiting Cdk activity. To test this model, we first searched for genetic interaction between *p107* and *p27*. In stark contrast to $\alpha Cre;Rb^{f/f};p107^{+/-}$ or $\alpha Cre;Rb^{f/f};p27^{+/-}$ mice, which never developed retinoblastoma, $\alpha Cre;Rb^{f/f};p107^{+/-};p27^{+/-}$

compound heterozygotes developed tumors (Figure 1B, Table 1). Importantly, none of five tumors analyzed showed LOH for *p107* or *p27* (Figure S3 and data not shown). We also analyzed compound heterozygotes harboring the $p27^{CK-}$ allele ($\alpha Cre;Rb^{f/f};p107^{+/-};p27^{+/CK-}$) and similarly observed retinoblastoma with only 1/7 tumors displaying LOH (Figure 1B, Table 1, Figure S3C and data not shown). Previously, the Rb-E2f axis has been the focus in retinoblastoma, but these genetic data expose a new role for the CKI-Cdk axis.

Cdk2 Activity Correlates with Tumor Penetrance

Quantifying tumor penetrance revealed various tumor frequencies across genetic models (Table 1). This variability might reflect differences in the activity of E2fs and/or Cdks. Normal retinal progenitor division, which is unaffected by *Rb* loss, ceases at post natal day 8 (P8) but ectopic division of differentiating *Rb* null neurons continues (18, 19). Thus E2f targets and Cdk activity were assessed at P8 to focus specifically on ectopically dividing differentiating cells. We assessed 24 E2f targets most of which were elevated in $Rb^{-/-}$ retinas (Figure 3A). Surprisingly, in four tumor-prone genetic models, expression of these targets was remarkably similar to $Rb^{-/-}$ levels (Figure 3A). We next analyzed Cdk pathway activity and found that Cdk2 protein was negligible in WT retina but induced similarly in $Rb^{-/-}$ and all tumor-prone models (Figure 3B). Strikingly, Cdk activity was strongly elevated in the tumor-prone state relative to *Rb* null tumor-resistant retinas, and quantification revealed an excellent correlation with tumor penetrance (Figure 3B–3C). However, we did not observe any correlation between Cdk2 kinase activity and cell cycle index (Figure S4), excluding the possibility that higher Cdk activity is a result of increased cell proliferation in tumor-prone genotypes. Thus, while E2f is deregulated similarly in the tumor-resistant *Rb* KO versus tumor-prone states, Cdk2 activity predicts susceptibility to sporadic transformation. This result, together with our new models of retinoblastoma, suggested that rather than constraining E2f target induction, p107 may protect the $Rb^{-/-}$ retina by blocking cross-talk to Cdk2.

p107 Affects Skp2 and p27 Levels *In Vivo*

Typically, p107 is thought to influence Cdk activity through E2f-regulation of Cyclins. However, as noted above, Cyclin A/E mRNA induction was already maximal after *Rb* loss (Figure 3A). Cyclin E protein also showed similar or lower levels in tumor-prone retinas (Figure 4A). *In vitro* data link p107 to Cdk either directly by binding and inhibiting Cdk2 (14), or indirectly by decreasing Skp2 the substrate binding component of the SCF^{Skp2} E3 ubiquitin ligase which stimulates p27 degradation (Figure 1A) (13). Although, Cdk2 immunoprecipitates from all six of the genotypes described, showed interaction with p27 and p21 (see below), no interaction were observed between Cdk2 and p107 or p130, arguing against a direct CKI function *in vivo* (Figure 4A), These results are consistent with *in vitro* data showing that p107 only binds CyclinA-Cdk2 in MEFs lacking both p21 and p27 (15). p57 was also not detected in Cdk2 IPs, and as expected p27 and $p27^{CK-}$ were not associated with Cdk2 in the $Rb^{-/-};p27^{-/-}$ or $Rb^{-/-};p27^{CK-/CK-}$ retina, respectively (Figure 4A). However, p27 bound Cdk2 in the $Rb^{-/-}$ tumor-free retina and, consistent with a role in tumor suppression, the amount was reduced in tumor-prone $Rb^{-/-};p107^{-/-}$ and $Rb^{-/-};p107^{+/-};p27^{+/-}$ retinas (Figure 4A). In $Rb^{-/-};p107^{-/-}$ retina the total amount of p27 was

reduced by 26±4%, and the amount bound to Cdk2 was reduced by 52±7%, suggesting negative effects of p107 loss on both the level and function of this CKI. The level of p27-Cdk2 complexes across multiple models correlated inversely with both Cdk2 activity and tumor penetrance (Figure 4B, C). In addition, Skp2 showed increased protein levels when p107 was reduced or absent, suggesting that the p107 --| Skp2 --| p27 pathway described *in vitro* may be relevant *in vivo* (Figure 1A) (13). Skp2 induction was post-transcriptional as Skp2 mRNA levels were similar in tumor-resistant $Rb^{-/-}$ retina versus tumor-prone retinas (Figure 3A). Total p21 levels were negligible in WT retina, low in $Rb^{-/-}$ and $Rb^{-/-};p107^{+/-};p27^{+/-}$ retinas and induced in $Rb^{-/-};p107^{-/-}$, $Rb^{-/-};p27^{-/-}$ or $Rb^{-/-};p27^{CK-/CK-}$ retina (Figure 4A). p21 associated with Cdk2 in five genotypes (Figure 4A). Unlike p27, p21 levels rose with increasing Cdk2 activity, although this positive correlation was poor (Figure S5). Thus, p27 is the major CKI tumor suppressor in the $Rb^{-/-}$ retina, and when it is missing ($p27^{-/-}$), unable to bind Cdk2 ($p27^{CK-/CK-}$), or reduced ($p107^{-/-}$), p21 is induced, but at insufficient levels to compensate for p27. Altogether, our data suggest that in *Rb/p107* mutant retinas Rb loss enhances E2f activity, whereas p107 loss elevates Skp2, reduces p27 and elevates Cdk2 activity, yielding the tumor-prone state.

Inhibiting Either of the Dual Axes Blocks Tumorigenesis

Our results suggest that E2f and Cdk2 form dual axes that can generate three states: WT, ectopically dividing (*Rb* null), and tumor-prone (*Rb* + *p107* or *p27*-depleted), with only the latter being exquisitely dependent on elevated activity of both axes. These findings led us to hypothesize that lowering the activity of either axis could prevent sporadic retinoblastoma arising from cancer-prone cells. Removing E2f2 or E2f3 did not block retinoblastoma (Figure 5A, Table 2), concurring with the prior observation that E2f1, but not E2f2 or E2f3, drives ectopic division of differentiating neurons in the $Rb^{-/-}$ retina (5). *p107* and *E2f1* genes are in close proximity, hence to study E2f1 function in *Rb/p107* DKO retina, we screened >150 pups to isolate a crossover event generating linked *p107* and *E2f1* null alleles. Although tumors occur in 54% or 100% of $\alpha Cre;Rb^{f/f};p107^{-/-}$ or $\alpha Cre;Rb^{f/f};p27^{-/-}$ retinas, respectively (Table 1), homozygous deletion of *E2f1* completely blocked tumorigenesis in both models (Table 2). Notably, even reduction to heterozygosity completely blocked retinoblastoma in the $\alpha Cre;Rb^{f/f};p107^{-/-}$ retina, and reduced penetrance from 100% to 10% in the $\alpha Cre;Rb^{f/f};p27^{-/-}$ retina (Figure 5A, Table 2). Therefore, unlike normal cells which proliferate in the absence of E2f1–3 (3, 4), the tumor-prone state requires full E2f1 activity. Moreover, *E2f1* heterozygosity did not affect progenitor division, but specifically reduced ectopic division in *Rb/p107* deficient cells (Figure 5A and 5B). Thus, a therapeutic window of E2f activity exists that can be exploited to prevent abnormal pre-cancerous events without perturbing normal division.

Next, we examined whether lowering Cdk activity might also inhibit retinoblastoma initiation. Cdk1 can functionally substitute for Cdk2 *in vivo* (28), thus we exploited a pharmaceutical approach to inhibit both and to test a novel chemoprevention strategy. Newborn neurons that survive *Rb/p107* loss divide ectopically, but the vast majority (millions) of neurons escape tumorigenesis by eventually exiting the cell cycle (18). We hypothesized that - assuming drug crossed the placental barrier - mild and brief Cdk2-inhibition during this dangerous period of ectopic division would reduce sporadic

transformation (Figure 6A). In contrast, if elevated Cdk activity is required only after transformation, this chemoprevention strategy would fail. For these assays we utilized R547, a potent Cdk inhibitor that passed preclinical evaluation (29) and is in phase I trials for solid tumors (30). It does not inhibit 113 other kinases, and requires >100 fold higher doses to inhibit Gsk3α/β versus Cdk1/2 (29). *αCre;Rb*$^{f/f}$*;p27*$^{-/-}$ males were bred to *αCre;Rb*$^{f/f}$*;p27*$^{+/-}$ females and pregnant dams received daily I.P. injections of vehicle or R547 (20 mg/kg) from embryonic day 12.5 (E12.5) to parturition and tumors were assessed at P45. Of 8 eyes examined in the treatment group, 6 eyes were tumor-free, and tumor volume in the affected eyes was considerably reduced, also consistent with reduced tumor frequency (Figure 6B). The failure to block all tumor formation may be due to some late stage amacrine cell birth that occurs in the far periphery up to ~P3, beyond the period of R547 exposure.

We also examined models of retinoblastoma involving *p107* instead of *p27* loss, and to test a lower dose of R547. In *αCre;Rb*$^{-/-}$*;p107*$^{-/-}$ mice, *Rb* knockout and tumorigenesis is limited to the periphery with 54% penetrance (Table 1) (18), whereas in *Chx10Cre;Rb*$^{f/f}$*;p107*$^{-/-}$ mice, Cre is expressed across the entire retina, there is considerable dysplasia, and tumors emerge in multiple locations with 100% penetrance (21). This pattern was observed in P45 mice born to dams exposed to vehicle (Figure 6C). However, following brief exposure of fetuses to R547, 2/8 eyes in the resultant adult mice were tumor-free and the remainder showed much reduced tumor volume, most noticeably in the central retina, again consistent with the idea that Cdk inhibition in the embryonic retina blocks transformation of early-mid-born amacrine cells (Figure 6C, **bottom panel**). An appealing aspect of reduced *E2f1* gene dosage was that it blocked tumorigenesis without perturbing normal division (Figure 5A–5B). We further examined *Chx10Cre;Rb*$^{f/f}$*;p107*$^{-/-}$ retinas at P0, prior to tumor formation, for effects on division and the extensive dysplasia in this model. Strikingly, R547 reduced dysplasia (Figure 6D) and also modestly reduced ectopic mitoses but had no effect on progenitor mitoses that are distant or adjacent to phalloidin-marked apical membranes, respectively (Figure 6E), thus resembling the effect of lowered E2f activity (Table 2 and Figure 5). Thus only one week of pharmacological Cdk antagonism in fetuses is sufficient to inhibit the subsequent appearance of retinoblastoma in either *Rb/p107* or *Rb/p27* null cells, without perturbing normal progenitor division.

These results encouraged us to intervene pharmacologically with the E2f axis. Four inhibitors have been described of which three are peptides (31–33) and one is a small molecule inhibitor (HLM006474, abbreviated here to 6474) (34). To our knowledge, none have been tested *in vivo*. Although all four drugs inhibit division *in vitro*, only 6474 was tested on tissue, stalling tumor growth in a 3D skin model (34). Strikingly, short exposure of *Chx10Cre;Rb*$^{f/f}$*;p107*$^{-/-}$ embryos to 6474 had a dramatic effect on tumorigenesis (Figure 6C). Thus, modest and temporary pharmaceutical E2f inhibition blocks retinoblastoma initiation.

Collectively, our results reveal greater dependency on E2f and Cdk activity for transformation than for normal progenitor proliferation (Figure 6F), creating a convenient therapeutic window in the cell-of-origin that, when targeted, dramatically impedes the subsequent emergence of cancer cells (Figure 6G).

DISCUSSION

Cross-talk to Cdk2 as the primary tumor suppressor function of p107

A simple explanation for p107 tumor suppressor function is that it replaces the E2f repressor role of Rb. However, we found that multiple E2f-regulated genes, including canonical targets, were expressed at similar levels in *Rb* and Rb/*p107* null retina, contrasting the situation in keratinocytes (22, 23). We acknowledge that while deletion of *p107* did not result in further induction of well known E2f target genes in *Rb* null retina, we can not rule out the possibility that the tumor-promoting effects of *p107* loss may be mediated by E2f targets other than those tested in Fig 3A. However, akin to our *in vivo* findings, E2f-responsive reporter vectors show comparable activity in *Rb* or *Rb/p107* null MEFs (35). Notably, Rb, but not p107/p130, inhibits E2f target expression during senescence (36), again mimicking our findings in terminally differentiating retinal neurons. Potentially, *p107* cannot affect E2f targets in some *Rb* null contexts because it is not recruited to these genes (36), it is redundant with p130 (24), there is feedback inhibition of E2f by Cdk2-mediated phosphorylation (37, 38), and/or it is already sequestered in other complexes (39).

In stark contrast to E2f, we observed a marked increase in Cdk2 activity in the tumor-prone *Rb/p107* null retina relative to the tumor-resistant $Rb^{-/-}$ tissue. E2f-induction of Cyclins did not explain elevated Cdk2 activity, but we observed post-transcriptional induction of Skp2, and reduced p27-Cdk2 binding that correlated with kinase activity and tumorigenesis. New retinoblastoma models developed here coupled with biochemical and pharmacological data strongly support the notion that this cross-talk to Cdk2 is central to tumorigenesis: i. Like the *Rb/p107* null tissue the *Rb/p27* deficient retina developed retinoblastoma, and *p27* or $p27^{CK-}$ alleles behaved identically; ii. Compound heterozygosity for *p107* and either *p27* or $p27^{CK-}$ cooperated with *Rb* loss to drive retinoblastoma; iii. Cdk2 activity correlated with tumor penetrance across these multiple models; and iv. Short-term exposure to a Cdk inhibitor prevented tumorigenesis in *Rb/p107* and *Rb/p27* null retinas. The ability of Rb pathway and CKI defects to cooperate is well known (40–42), and we now extend this pattern to the retina where the focus had been primarily on Rb-E2f regulation. Our data are the first to prove unequivocally that it is the CKI function of p27 that cooperates with Rb to block tumorigenesis. Most importantly, they suggest that the primary tumor suppressive function of p107 in an *Rb* null tissue is to prevent activation of Cdk2 by E2f-independent and possibly Skp2-dependent means. Indeed, Skp2 is essential for retinoblastoma in the *Rb/p107* null retina (M.S and R.B., unpublished data). These data justify further examination of the mechanism by which p107 might regulate Skp2 and cross-talk to Cdk2, and expose potent chemopreventive strategies for Rb pathway-initiated tumors. It is interesting to note that the kinetics and penetrance of tumorigenesis in the *Rb/p27* null retina are similar to that observed in the *Rb/p130* null retina (Macpherson et al., 2004). In theory, p27 loss could stimulate phosphorylation and inactivation of p130. However, we did not observe an increase in the slower migrating hyperphosphorylated form of p130 in the *Rb/p27* null retina (Fig 4A). Further work would be required to comprehensively assess individual p130 phosphorylation sites. An alternate possibility is that p130 affects p27 levels, either through Skp2 or another mechanism. It would be interesting to assess Skp2 and p27 levels in the *Rb/p130* null retina.

A Model to Explain Variable Sensitivity to *RB* Inactivation

The human retina is exquisitely sensitive to *RB* defects, but the underlying reason is unclear. Our data show that p107 protects mouse retina by preventing cross-activation of Cdk2 and thus cooperation with elevated E2f to create tumor susceptibility. We suggest, therefore, that low or negligible p107/p130/CKIs levels or activity in the human retinoblastoma cell-of-origin strengthens positive feedback regulation between E2Fs and CDKs. *Rb* loss alone in this case would be sufficient to raise Skp2 levels, reduce p27 further and thus efficiently activate Cdk2 (Figure 1A). Mouse pituitary shows the same sensitivity to *Rb* loss as the human retina (43), suggesting it also has unusually higher level of E2F and CDK activity. Finally, while inducing E2f or Cdk activity drives temporary ectopic proliferation in *Drosophila*, extended abnormal division requires both (44). Thus, a buffer that limits E2f to Cdk positive feedback regulation may be critical to avoid tumorigenesis in all animals.

Clinical Relevance

Chemoprevention is a growing field with important successes (45). Long-term exposure to anti-inflammatory drugs such as aspirin and non-steroidal anti-inflammatory drugs reduces cancer incidence, but failures, such as the lack of protection afforded by statins, highlight the need to define optimal targets (45). The notion of prevention as a viable goal has gained considerable ground in recent years, in particular for familial cancers of which >50 have been identified (46). In addition, effective chemopreventative strategies could also benefit cancer survivors, who are at higher risk of secondary tumors (47).

Here, we prevented cancer in an *in vivo* model of tumorigenesis using two distinct small molecule therapies, and with only one week of drug treatment. These striking observations have implicit clinical relevance, especially as they were obtained using genetic models that mimic human cancer, rather than cell-line derived xenografts in immune-deficient hosts. Our data raise the exciting notion that $RB^{+/-}$ patients, who often die of secondary tumors (48), may benefit from the preventive therapy we show is so potent in retina. Moreover, because the RB pathway is disrupted in many cancers, and elevated E2F and CDK activity is a universal feature of human tumors, our chemopreventive strategies may be broadly relevant. Although Cdk2 is dispensable for tumorigenesis in *p27*, *p21* or *p53* null mice (49–51) it acts redundantly with Cdk1 (28), thus the success of our chemotherapeutic strategy likely reflects inhibition of both kinases. Our data provide the first successful application of any E2f inhibitor *in vivo* and the remarkable efficacy indicates that like Cdk, E2f is an important chemopreventative target, although further work is required to confirm if this inhibitor lowers E2F target levels *in vivo*. Therapeutic trials with Cdk2 inhibitors in human cancer have been largely unsuccessful (9), but we suggest that their real benefit may lie in prevention. Recent work with Cdk inhibitors in genetic models of colon cancer support this notion (52). Future studies should examine whether the cell-of-origin in this and other tumor types also require elevated E2f activity. We found that E2f or Cdk inhibition could prevent retinoblastoma without perturbing normal retinal progenitor cell division. These data indicate a unique role for E2f and Cdk in supporting transformation versus normal cell cycle progression.

Note added in Proof

As we were preparing our manuscript for submission David Macpherson's lab published work showing that microRNAs that down-regulate the CKI p21 are amplified or over-expressed in mouse and human retinoblastoma (53). These data support the notion that Rb and CKI inactivation cooperate to transform the human retina.

MATERIALS AND METHODS

Mouse strains and genotyping

Mice were treated according to institutional and national guidelines. αCre mice (P. Gruss), $Rb^{f/f}$ mice (A. Berns), $p107^{-/-}$ mice (M. Rudnicki), $p27^{-/-}$ mice (J. Roberts), and $p27^{Ck-/Ck-}$ mice (J. Roberts), were maintained on a mixed background. Different genotypes were compared within the same litter and across at least three litters. Genotyping was performed as before (18, 27).

Histology and immunofluorescence

BrdU-labelling, fixation and immunostaining were performed as before (5, 3). For p27, p21 and Ki67 antigen retrieval was performed by boiling sections in citric acid (H-3300, Vector Lab Inc.).

RNA-extraction, RT- PCR

RT-qPCR for E2f targets were run in duplicate on at least three separate biological samples as described (3).

Western blots

Mouse retinas were homogenized with a 30-gauge needle (BD) 5–10 times in lysis buffer. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane. Blots were blocked and probed as described (5). Blots were scanned using ODYSSEY Infrared Imaging System (LI-COR).

Immunoprecipitation and kinase assays

Complete RIPA was prepared by combining PMSF, sodium orthovanadate, protease inhibitor cocktail (SantaCruz, CA, USA). Mouse retinas were lysed in RIPA and pre-cleared with 1.0 μg of rabbit IgG. Supernatant containing 250 μg total protein was incubated with 1μg primary antibody at 4°C for 2 hrs. 20 μl of resuspended Protein A/G Plus-Agarose was added and rocked at 4°C for 1hr to overnight. Kinase reactions were performed at 30°C for 15 min in kinase buffer containing 2.5 μg histone H1 (Upstate Biotechnology, NY, USA), 2 μCi of ^{32}P-γ-ATP, and 20 μM ATP. Reactions were stopped with 2x Laemmli-buffer and boiled for 5 min before loading on 10% SDS-PAGE gels. Gels were dried and quantified using phosphoimager.

Chemoprevention

R547 and 6474 were synthesized by University Health Network Shanghai, and purity confirmed at >98% according to published methods (29, 34). Male and female mice were

mixed in the early afternoon, checked the following morning and dams with vaginal plugs considered to be 0.5 days post-coitus (E0.5). After twelve days, pregnant dams were treated with either vehicle (2.5% v/v DMSO (5% for 20 mg/kg dose), 28% w/v 2-hydroxypropyl-β-cyclodextrin, 10% v/v PEG400 in distilled water), or R547 (5–20 mg/kg;) or 6474 (100 mg/kg) daily intraperitoneally until birth.

Tumor volume and stereology

Eyes were sectioned horizontally at 14 μm. Every 8th section was stained with H&E and scanned on Leica DMRB. Tumor volume was estimated using a Cavalieri Estimator in Stereo Investigator (MBF Bioscience, CA, USA).

Statistical analysis

Statistical analysis was performed using Prism software (Version 5.0a, GraphPad Software, LaJolla, CA, USA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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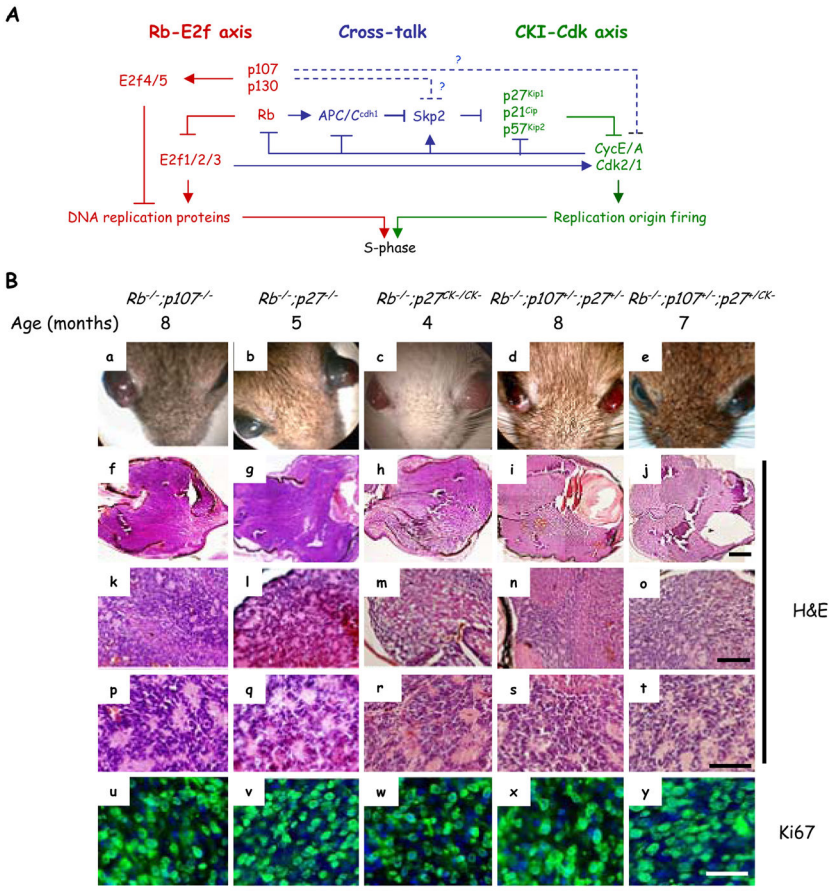

Figure 1. Both Rb-E2f and CKI-Cdk axes contribute to retinoblastoma initiation

(A) The Rb-E2f (red) and Cip/Kip-Cdk2 (green) dual axes regulate G1/S progression. There are also links that affect cross-talk between the axes (blue). p107 is thought to inhibit cross-talk by regulating E2f targets, but *in vitro* data also suggest E2f-independent roles in controlling Skp2 stability and in binding Cdk2 (dotted blue lines). The relative contribution of these pathways to p107 function *in vivo*, especially to tumor suppression, is unclear. The figure does not include all regulators and links. (B) Loss of *Rb* together with inactivation of *p107* and/or *p27*, either by deletion or inactivating the latter's CKI activity, initiates the growth of protruding retinoblastoma (a–e) that fills the vitreous (f–J). H&E sections showed rosettes (k–t). Ki67 staining (u–y, green) reveals many dividing cells in tumors. For simplicity "$Rb^{-/-}$" represents *αCre;Rb* $^{f/f}$. Scale bars are 500 μm (f–j), 150 μm (k–o), 50 μm (p–t) and 25 μm (u–y). See also Figure S1.

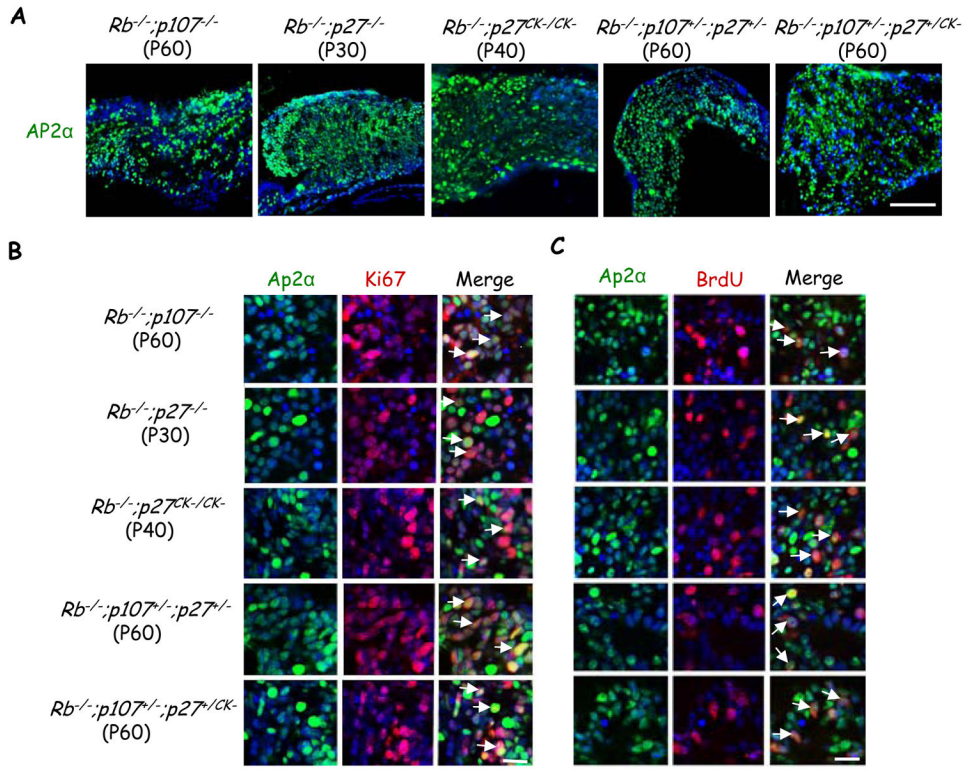

Figure 2. Dividing Ap2α^{+} amacrine like cells in multiple mouse models of retinoblastoma

(A) Tumors of the indicated genotypes and ages were stained for Ap2α (green) and DAPI (blue). Scale bar 100 μm. (B–C) Tumors of the indicated genotypes and ages were stained for the cell cycle markers Ki67 (B), and BrdU (C) (red), and the amacrine cell marker Ap2α (green). White arrows indicate double positive cells. Scale bar 20 μm. For simplicity "*Rb*$^{-/-}$" represents α*Cre;Rb*$^{f/f}$. See also Figure S2.

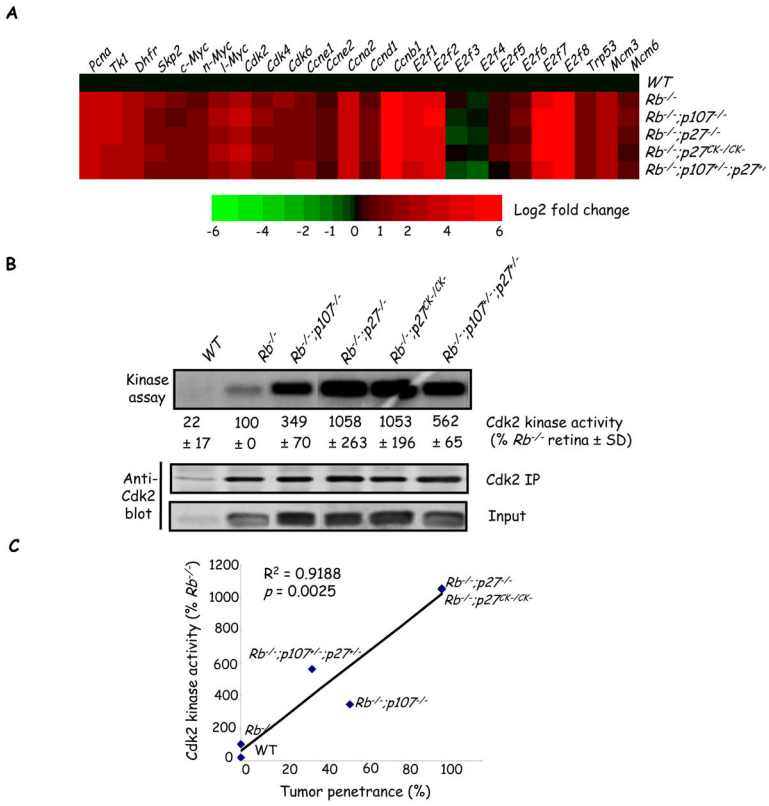

Figure 3. Cdk activity correlates with tumor penetrance

(A) RT-qPCR was used to measure the mRNA level of the indicated E2f target genes in P8 retinas of the indicated genotypes. Heat map shows the log2-fold changes of gene expression relative to WT. Red and green colors represent positive and negative expression changes, respectively. E2f4 & 5 are not known E2f targets but are included to show expression of the entire E2f family. (B) Cdk2 was immunoprecipitated from P8 retinas of the indicated genotypes. Kinase activity was determined using histone H1 as a substrate and the amount of Cdk2 in the IP was determined by Western blotting. (C) Cdk activity (percent of that in the *Rb* null retina) was plotted against tumor penetrance. *p* value was determined using a one-sample *t*-test for Pearson's product-moment correlational coefficient, *r*. All assays were carried out 3–6 times and the mean ± SD is shown. For simplicity "$Rb^{-/-}$" represents $\alpha Cre;Rb^{f/f}$.

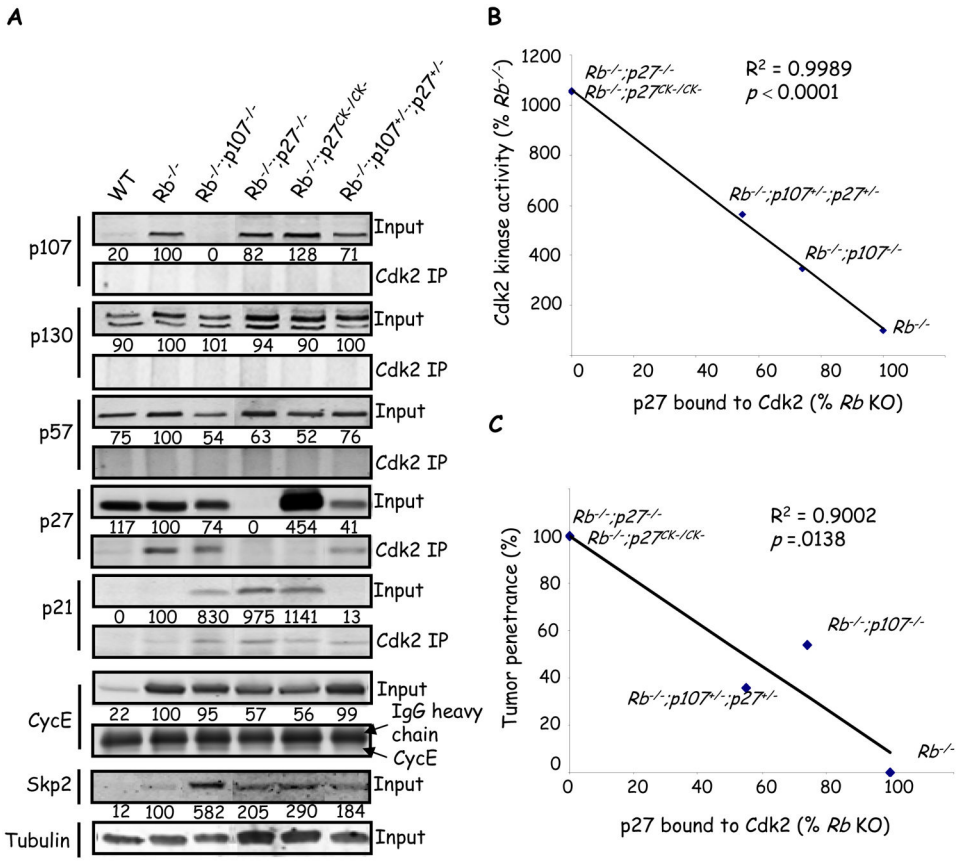

Figure 4. p27 bound to Cdk2 correlates inversely with kinase activity and tumor penetrance

(A) Anti-Cdk2 immunoprecipitates or straight lysates (input) from P8 retina of the indicated genotypes were immunoblotted for the proteins indicated on the left. Values below input blots represent protein expression as a percentage of *Rb* KO retina in a representative blot. (B–C) Average amount of p27 bound to Cdk2 from 3 independent experiments was plotted against Cdk2 kinase activity (from Fig. 3) (B) or tumor penetrance (C). "$Rb^{-/-}$" is used to indicate $\alpha Cre;Rb^{f/f}$. *p* values were calculated using a one-sample *t*-test for Pearson's product-moment correlational coefficient, *r*. All assays were run a minimum of 3 times. See also Fig S4 and S5.

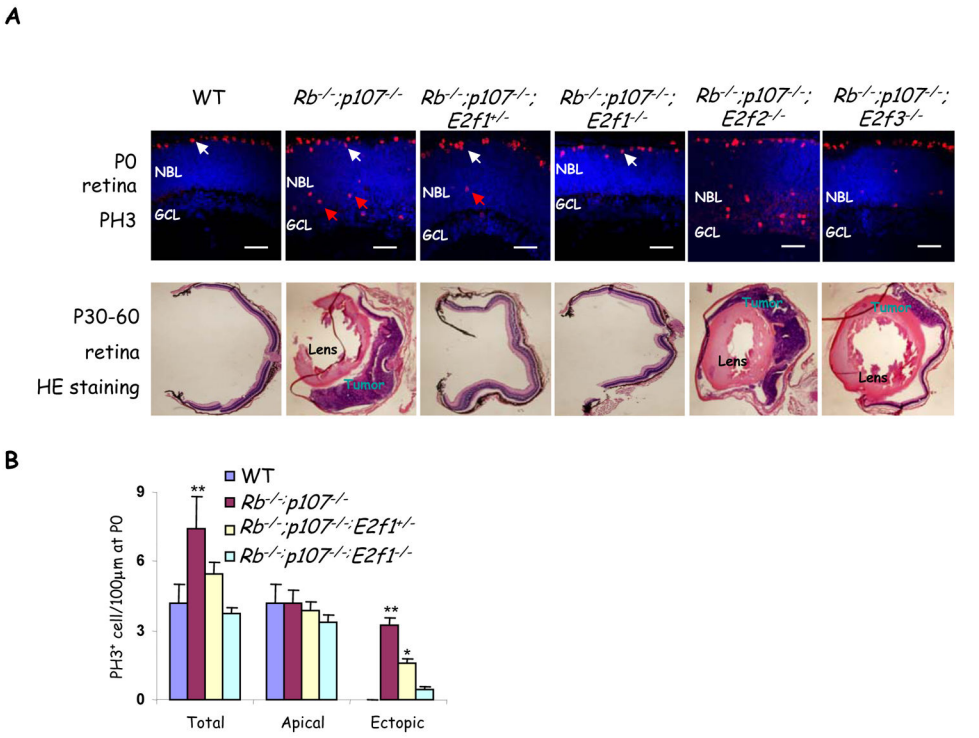

Figure 5. Heterozygosity for *E2f1* is sufficient to block retinoblastoma

(A) P0 and adult (P30–P60) retinal sections of the indicated genotypes were stained for mitotic cells (PH3, red) and nuclei (DAPI, blue), or H&E (lower panels). Apical mitoses (white arrows) represent normal progenitors while ectopic mitoses (red arrows) represent abnormally dividing differentiating neurons that are abundant in the *Rb/p107* null retina, and reduced or virtually absent when one or two *E2f1* alleles are removed, respectively. Scale bar, 50μm. The lens or tumors in adult H&E sections are indicated. For quantification of tumor frequency see Table 2. (B) Quantification of mitoses in indicated genotypes. Data are mean ± SD and asterisks indicate significant difference from WT (* P<0.05, ** P<0.01. Students t-test For simplicity "*Rb−/−*" represents *αCre;Rb*$^{f/f}$ in (A & B). NBL: neuroblastic layer, GCL: ganglion cell layer.

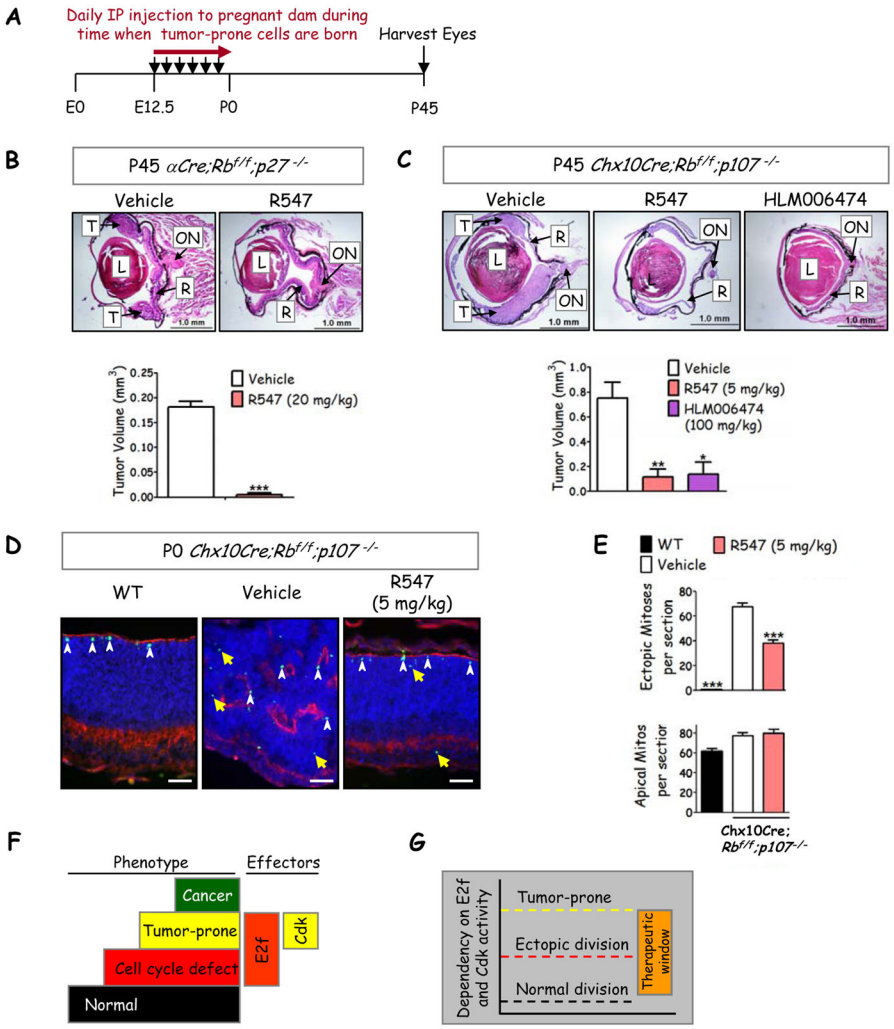

Figure 6. Chemoprevention of retinoblastoma through brief Cdk or E2f inhibition

(A) Summary of chemoprevention strategy. (B) H&E stain of P45 retina in *αCre;Rb f/f;p27−/−* mice treated with either vehicle (n=8) or pan-Cdk inhibitor R547 (n=10) with quantification of tumor volume (bottom panel). (C) H&E stain of P45 retina in *Chx10Cre;Rbf/f;p107−/−* mice treated with vehicle (n=10), R547 (n=8), or E2f inhibitor HLM006474 (n=4) with tumor volume quantified (lower panel). (B,C) T: Tumor, R: retina, L: lens, ON: optic nerve (scale bar, 1 mm). (D) P0 retina of *Chx10Cre;Rb f/f;p107−/−* mice treated with vehicle or R547 were stained with PH3 (green) and the F-actin marker, phalloidin (red). Yellow arrows indicate ectopic $PH3^+$ cells and white arrowheads represent apical mitotic progenitors (scale bar, 50 μm). (E) Quantification of ectopic (upper panel) or apical (lower panel) mitoses per section shows that the drug inhibits abnormal but not normal division. *= $p<0.05$; ** = $p<0.01$; *** = $p<0.0001$ compared to vehicle using an unpaired Student's *t*-test (B) or One-way ANOVA followed by Bonferroni multiple comparisons posthoc test (C, F). Data represented as mean ± SEM (n represented per eye, n ≥ 4 for each condition). (F) Model summarizing critical molecular steps to the tumor-prone state. In the mouse retina *Rb* loss activates E2f1 and triggers ectopic division (red step), but additional genetic events are required to activate Cdk2 and thus create tumor susceptibility

(yellow step). Sporadic mutations permit progression to cancer (green step). (G) The data suggest distinct dependence on E2f and Cdk activity for normal division, ectopic division and tumor susceptibility, and thus expose a therapeutic window of dual axes activity which can be exploited to block transformation.

Table 1

Tumor penetrance in *Rb* null retinas with compromised p107 and/or CKI status

p107 **or** ***p27*** **status of** ***Rb*** **null retina**	**Eyes with tumor/ Total eyes**	**Penetrance**
$p107^{-/-}$	12/22	54%
$p107^{+/-}$	0/60	0%
$p27^{-/-}$	28/28	100%
$p27^{+/-}$	0/24	0%
$p27^{CK-/CK-}$	20/20	100%
$p27^{+/CK-}$	3/28 (3/3 LOH)	10.7% (all LOH)
$p107^{+/-};p27^{+/-}$	10/28 (0/5 LOH)	35.7% (no LOH)
$p107^{+/-};p27^{+/CK-}$	7/18 (1/7 LOH)	38.9% (14% LOH)

LOH: loss of heterozygosity

See also Figure S3.

Table 2

Tumor frequency in retinas lacking *E2f* alleles

Genotype	Age analyzed	No. of tumors/No. of eyes analyzed	Tumor penetrance
$Rb^{-/-};p107^{-/-}$	4 months	26/48	54%
$Rb^{-/-};p107^{-/-};E2f1^{+/-}$	4 months	0/24	0%
$Rb^{-/-};p107^{-/-};E2f1^{-/-}$	2 months	0/28	0%
$Rb^{-/-};p107^{-/-};E2f2^{-/-}$	4 months	22/48	46%
$Rb^{-/-};p107^{-/-};E2f3^{-/-}$	4 months	4/8	50%
$Rb^{-/-};p27^{-/-}$	2 months	24/24	100%
$Rb^{-/-};p27^{-/-};E2f1^{+/-}$	4 months	2/20	10%
$Rb^{-/-};p27^{-/-};E2f1^{-/-}$	2 months	0/6	0%