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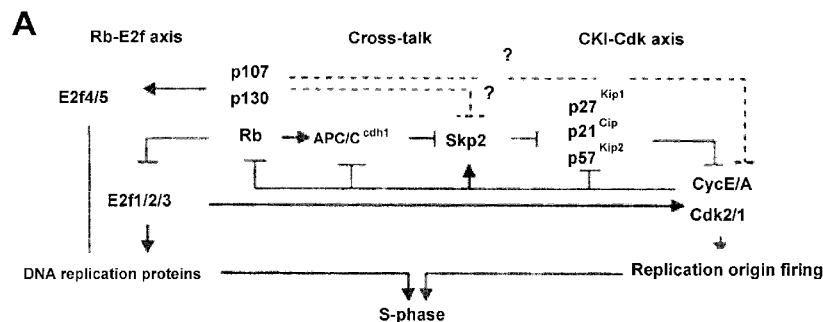
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(54) Title: TARGETING THE RB PATHWAY FOR THE PREVENTION OF CANCER

Figure 1

(57) **Abstract:** Method of preventing retinoblastoma in a cancer prone subject with RB pathway inhibitors. Preferred inhibitors are Cdk inhibitors, E2f inhibitors, atypical protein kinase C inhibitors and Skp2 inhibitors. Also methods to determine the likelihood of a population of cells to be cancer-prone by measuring the activity levels of Cdk and E2f, aPKC and SKP2.

TARGETING THE RB PATHWAY FOR THE PREVENTION OF CANCER

RELATED APPLICATIONS

This application claims priority to U.S. Provisional Application No. 61/559,729 filed on
5 November 15, 2011 and U.S. Provisional Application No. 61/560,299 filed on
November 16, 2011.

FIELD OF THE INVENTION

The invention relates to the prevention of cancer by targeting proteins in the RB
10 pathway.

BACKGROUND OF THE INVENTION

Cancer is a multi-step process that arises from continuous selection of more
aggressive clones through sporadic mutagenesis. Abnormal proliferation is a hallmark
15 of cancer cells. Most human cancers begin decades before they are detected,
providing a long yet under-exploited latency period for prevention. In the premalignant
phase initiated cells can expand considerably, but remain contained either as benign
tumors or as indistinct precancerous fields (Deng et al., 1996; Jonason et al., 1996;
Moreno, 2008). The number and type of oncogenic events required to reach this
20 dangerous yet non-transformed "cancer-prone" state varies from tissue to tissue.
Irrespective, a common principal is that millions of susceptible cells are generated,
from which a tiny fraction of clones emerge with more aggressive properties. Other
than the oncogene and tumor-suppressor mutations that create the pre-malignant
condition, we know surprisingly little regarding the molecular networks that underpin
25 the development of the cancer-prone state *in vivo*. Regardless, understanding the
cancer-prone state is critical to impede tumorigenesis. The notion of prevention as a
viable therapeutic goal has gained considerable ground in recent years, in particular
for familial cancers of which >50 have been identified (Lindor et al., 2008). However,
chemopreventative strategies are also critical for cancer survivors, who are at higher

risk of secondary tumors due to pre-existing susceptibility and/or exposure to mutagenic treatments (Ng et al., 2010). For example, long-term exposure to anti-inflammatory drugs such as aspirin and non-steroidal anti-inflammatory drugs (NSAID) reduces cancer incidence (Kelloff et al., 2006). However, there have also been
5 important failures, such as the lack of protection afforded by statins (Kelloff et al., 2006), highlighting the critical need to define the mechanisms and optimal targets underlying cancer prone states.

Abnormal proliferation is a hallmark of cancer cells. However, whether the core cell cycle machinery is engaged to different degrees during distinct stages of tumor
10 development is unclear. The Rb-E2f and Cdk inhibitor (CKI)-Cdk2/1 interactions regulate cell cycle progression (**Figure 1A**) and are routinely disrupted in cancer cells (Malumbres and Barbacid, 2001; Sherr, 1996). The Rb family (Rb, p107 and p130) bind and form repressor complexes with E2f family proteins. Activating E2fs, (E2f1, 2 and 3) induce factors required for DNA replication, drive proliferation of quiescent cells
15 (reviewed in (Cobrinik, 2005; Dimova and Dyson, 2005) and, although not required for normal progenitor division (Chen et al., 2009), are essential for abnormal division of differentiating *Rb*-null cells in multiple tissues (Chen et al., 2007; Chong et al., 2009; McClellan et al., 2007; Tsai et al., 1998; Ziebold et al., 2001). Notably, the Rb-E2F pathway is a bistable switch that converts graded growth factor levels into all-or-none
20 E2F responses that regulate progression into S-phase (Yao et al., 2008). Cip/Kip inhibitors (p21, p27 and p57) on the other hand bind and inhibit the kinase activity of Cyclin A/E-Cdk2/1 complexes. Cdk2 inactivates the Rb family, and is essential to fire replication origins (Malumbres and Barbacid, 2009). As well as driving G1-S progression, E2f and Cdk2 also work together to promote centrosome duplication
25 (Meraldi et al., 1999). Inactivation of the Rb and CKI pathways and concomitant activation of E2f and Cdk occur together almost universally in human cancer (Malumbres and Barbacid, 2001).

The human retinoblastoma gene (*RB*) encodes a protein (RB) that is the pivotal component in a molecular pathway, which is defective in essentially all human cancers
30 (Weinberg, 1995). *RB* was the first recognized tumor suppressor gene, and was discovered through analysis of the childhood retinal cancer it is named after, retinoblastoma (DiCiommo et al. 2000). 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.

$RB1$ mutations are associated with >95% risk of retinal cancer (retinoblastoma) and 50% of $RB^{+/-}$ survivors develop secondary tumors by the age of 50 (reviewed in Balmer et al., 2006). In sporadic cases, one tumor arises from a developing retinal cell that spontaneously acquires defects in both copies of RB . In inheritable cases one
 5 gene copy is mutated in every cell ($RB^{+/-}$ individuals), and the high probability of sustaining a defect in the second copy means that these children develop 5-12 tumors affecting both eyes. 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 the mouse retina, and current retinoblastoma models utilize the
 10 loss of Rb and one relative (Robanus-Maandag et al., 1998; Chen et al., 2004; MacPherson et al., 2004; Dannenberg et al., 2004; Zhang et al., 2004). 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 the E2f targets become super-induced in the latter. This occurs in keratinocytes (Lara et al., 2008a and 2008b), but in fibroblasts Rb and $p107/p130$ appear to regulate distinct targets (Hurford et al., 1997; Black et al., 2003). However, whether elevated E2f or Cdk activity is required separately or together, and whether distinct quanta of E2f and/or Cdk activity contribute to different stages of
 15 either tumor initiation or tumor progression is unclear.

To prevent uncontrolled G1-S progression, there are extensive controls limiting the 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
 25 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 (Binne et al., 2007; Ji et al., 2004). Subsequent studies validated this link, as Skp2 is required in Rb -deficient pituitary tumors (Wang et al. 2010). $p107/p130$ do not bind APC, but $p107$ reduces Skp2 post-transcriptionally *in vitro* (Rodier et al., 2005); whether this occurs *in vivo* is unclear. Skp2 is involved in the ubiquitin-dependent degradation of the CKI, p27, which binds to and inhibits Cyclin E-Cdk2 complexes thus halting cell cycle progression (Carrano et al., 1999). Because it degrades a key regulator of the cell cycle, Skp2 overexpression has been linked to progression of tumors. $p107/p130$ bind and inhibit Cdk2 *in vitro* (Castano et al., 1998)

but a p107–Cdk2 complex in cells has only been detected in the absence of p21 and p27 (Chibazakura et al., 2004). Skp2 is required for *Rb*-initiated mouse pituitary but not thymic tumors (Wang et al., 2010), thus the general relevance of the Rb-Skp2 link remains to be defined.

- 5 Retinoblastoma patients usually survive in the West, but are often partially or wholly blind due to eye removal (enucleation) or treatments that damage the retina, and in developing countries mortality is ~70% (Dimaras et al., 2010). Moreover, *RB*^{+/-} patients are at high risk of secondary tumors and 50% will develop osteosarcoma, soft tissue sarcoma, lung cancer, melanoma, brain cancer or other tumors by the age of 50
10 (Fletcher et al., 2004; Abramson et al., 2001; Yu et al. 2009; Shields and Shields, 2004). In cases where there is an affected parent, fetal DNA can be screened for susceptibility. In the case of new germ line mutations (e.g. that arise during spermatogenesis) genetic screening follows presentation with retinoblastoma, usually in the first year of life. Positive identification of a germ line defect triggers intensive
15 monitoring for new tumors. Effective strategies are required to prevent new tumors, either in the eye or in other organs later in life, or treat existing retinoblastoma tumors.

SUMMARY OF THE INVENTION

- According to an aspect, there is provided a method of preventing retinoblastoma in a
20 subject comprising inhibiting the RB pathway.

According to an aspect, there is provided a use of an RB pathway inhibitor for the prevention of retinoblastoma in a subject.

According to an aspect, there is provided a use of an RB pathway inhibitor in the preparation of a medicament for the prevention of retinoblastoma in a subject.

- 25 According to an aspect, there is provided an RB pathway inhibitor for use in the prevention of retinoblastoma in a subject.

According to an aspect, there is provided a method of determining the likelihood of a population of cells to be cancer-prone, comprising: determining the level of E2f and Cdk activity in the population of cells, wherein a high level of both E2f and Cdk activity

in the population compared to a control population is indicative of the population being cancer-prone.

According to an aspect, there is provided a method of determining the likelihood of a population of cells to be cancer-prone, comprising: determining the level of atypical
 5 protein kinase C activity in the population of cells, wherein a high or abnormal level of atypical protein kinase C in the population compared to a control population is indicative of the population being cancer-prone.

According to an aspect, there is provided a method of determining the likelihood of a population of cells to be cancer-prone, comprising: determining the level of Skp2
 10 activity in the population of cells, wherein a high or abnormal level of both Skp2 activity in the population compared to a control population is indicative of the population being cancer-prone.

BRIEF DESCRIPTION OF FIGURES

15 These and other features of the preferred embodiments of the invention will become more apparent in the following detailed description in which reference is made to the appended drawings wherein:

Figure 1 shows that both Rb-E2f and CKI-Cdk axes contribute to retinoblastoma initiation. (A) The Rb-E2f and Cip/Kip-Cdk2 dual axes regulate G1/S progression.
 20 There are many feed forward and feedback links between these axes. Feed forward effects include blockade of Skp2-mediated degradation of p27 by Rb-Cdh1, and E2f-mediated induction of CycE/A. Feedback effects include inhibition of Rb by Cdk2 thus further activating both E2fs and Skp2. The figure does not include all regulators and links. (B) Loss of *Rb* together with inactivation of *p107* and/or *p27* (by deletion or
 25 inactivation of its CKI activity) initiates the growth of protruding retinoblastoma (a-e) that fills the vitreous (f-j). Hematoxylin and Eosin (H&E) sections showed rosettes (k-t). Ki67 staining (u-y) reveals many dividing cells in tumors. For simplicity "*Rb*^{-/-}" represents *αCre;Rb*^{fl/fl}. Scale bars are 500 μm (f-j), 150 μm (k-o), 50 μm (p-t) and 25 μm (u-y). See also Figure 7.

30 **Figure 2** shows that dividing Ap2α⁺ amacrine like cells in multiple mouse models of retinoblastoma. (A) Tumors of the indicated genotypes and ages were stained for

Ap2 α (green) and DAPI (blue). Scale bar 100 μ m. (B-D). Tumors of the indicated genotypes and ages were stained for the cell cycle markers Ki67 (B), PH3 (C) and BrdU (D) (red), and the amacrine cell marker Ap2 α (green). Scale bar 20 μ m. For simplicity "*Rb*^{-/-}" represents α Cre;*Rb*^{fl/fl}. See also Figure 8.

5 **Figure 3** shows that Cdk activity correlates with tumor penetrance. (A) Reverse transcriptase-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 and 5 are not known E2f targets but
10 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*-
15 test for Pearson's product-moment correlational coefficient, *r*. All assays were carried out 3-6 times and the mean $\pm$ SD is shown. For simplicity "*Rb*^{-/-}" represents α Cre;*Rb*^{fl/fl}.

Figure 4 shows that p27, but not p21, 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
20 indicated on the left. Values below input blots represent protein expression as a percentage of *Rb* KO retina in a representative blot. (B-C) Cdk2 kinase activity (from Figure 3) was plotted against the amount of bound p27 (B) or p21 (C) as determined in (A). "*Rb*^{-/-}" is used to indicate α Cre;*Rb*^{fl/fl}. *p* values were calculated using a one-sample *t*-test for Pearson's product-moment correlational coefficient, *r*. All assays were run a
25 minimum of 3 times.

Figure 5 shows that heterozygosity for *E2f1* is sufficient to block tumorigenesis in both *Rb/p107* and *Rb/p27* null retina. (A) P0 and adult (P30–P60) retinal sections of the indicated genotypes were stained for mitotic cells (PH3, red) and nuclei (DAPI, blue), or Hematoxylin and Eosin (H&E) (lower panels). Apical mitoses (white arrows)
30 represent normal progenitors whereas 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=50 μ m. 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 $\pm$ s.d. and asterisks indicate significant difference from WT (* P <0.05, ** P <0.01. Students t -test For simplicity ' $Rb^{-/-}$ ' represents $\alpha Cre;Rbflf$ in (a, b). GCL, ganglion cell layer; NBL, neuroblastic layer.

- 5 **Figure 6** shows chemoprevention of retinoblastoma through brief Cdk or E2f inhibition. (A) Summary of chemoprevention strategy. (B) Hematoxylin and Eosin (H&E) stain of P45 retina in $\alpha Cre;Rbflf;p27^{-/-}$ mice treated with either vehicle ($n=8$) or pan-CKI R547 ($n=10$) with quantification of tumor volume (bottom panel). (C) H&E stain of P45 retina in $Chx10Cre;Rbflf;p107^{-/-}$ mice treated with vehicle ($n=10$), R547 ($n=8$) or E2f inhibitor
- 10 HLM006474 ($n=4$) with tumor volume quantified (lower panel). (B,C) T, tumor; R, retina; L, lens; ON, optic nerve (scale bar=1 μ m). (D) P0 retina of $Chx10Cre;Rbflf;p107^{-/-}$ 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 μ m). (E) Quantification
- 15 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 with vehicle using an unpaired Student's t -test (B) or one-way analysis of variance followed by Bonferroni multiple comparisons posthoc test (C,F). Data represented as mean $\pm$ s.e.m. (n represented per eye, $n4$ for each condition).
- 20 **Figure 7** supplements Figure 1 and shows the expression of p21 and p27 at P8 in Wild Type (WT) and Rb null retina. (A) Overlay of DAPI (blue) and anti-p21 (upper panel) or anti-p27 (lower panel) immunofluorescence (red) on horizontal sections from WT and Rb null retinas (O, ONL; I, INL; G, GCL). Strong p21 staining is specific to a few cells in Rb null retina whereas p27 is expressed extensively in WT and $Rb^{-/-}$
- 25 retinas. Scale bar is 50 μ m. (B) Real-time RT-PCR analysis of $Cdkn1a$ and $Cdkn1b$ genes in WT and Rb null retina at P8. Error bar represents SD of measurements from three independent experiments.

- Figure 8** supplements Figure 2 and shows that emerging $Rb^{-/-};p27^{-/-}$ tumors contain dividing cells positive for amacrine cell markers. (A) Marker analysis of P30 WT and
- 30 $Rb^{-/-};p27^{-/-}$ retina. Low magnification views are shown on the left (scale bar 100 μ m) and high magnification views of the boxed regions are shown on the right (scale bar 20 μ m). Emerging tumors stained primarily for Ap2 α (green), which specifically marks amacrine cells, Pax6 (red), which marks amacrine cells as well as other cell types, and

Ki67 (green) which marks dividing cells. Tumors had no or very few cells that stained for markers of cone, bipolar, ganglion or rod cells. Note also the absence of rods and cones in the thin untransformed KO retina (arrows), thus resembling the *Rb/p107* null retina. All sections in A were also stained with DAPI to mark nuclei (blue). For simplicity "*Rb^{-/-}*" represents *αCre;R^{ff}*. (B) Tumors at P30 were labeled with Ki67 (green) to mark dividing cells, Prox1 (red) which marks horizontal, bipolar and a subset of amacrine cells in normal adult retina, and DAPI to label nuclei (blue). (C) Prox1⁺ cells (red) were amacrine-like as they co-stained for Ap2α (green). Scale bar 100μm upper panel and 20μm lower panel. (D) Tumors were stained for Ki67 and glutamine synthase (GS, green), which marks Müller glia. These cells, unlike those with amacrine markers were not dividing. Scale bar 20μm. For simplicity "*Rb^{-/-}*" represents *αCre;Rb^{ff}*.

Figure 9 supplements Table 1 and shows Loss of Heterozygosity (LOH) analysis in heterozygotes. (A) Genotyping of tail (Tl) and tumor (Tm) DNA for *p27^{CK-}* allele in *αCre;Rb^{ff};p27^{+/CK-}* mice showing LOH as indicated by single 600bp band for *p27^{CK-}* knock-in allele and loss of 520bp band for Wild Type (WT) allele in tumor DNA for all three tumor samples. The *p27^{CK-}* diagnostic primers also often generate a background band between the 600 bp and 520 bp fragments. (B) Genotyping of four *αCre;Rb^{ff};p107^{+/+};p27^{+/+}* mice showing no LOH for *p27* (upper panel; WT allele 400bp and KO allele 550bp) and *p107* (lower panel; WT allele 386bp and KO allele 513bp). (C) *αCre;R^{ff};p107^{+/+};p27^{+/CK-}* tumor showing LOH for *p27^{CK-}* knock-in allele for a single tumor sample (Tm4; upper panel) and no LOH for *p107* allele (lower panel). * Tumor samples showing LOH.

Figure 10 supplements Figure 3 and shows that at P8 Cdk2 activity does not correlate with cell cycle index. (A) P8 retinas of the indicated genotypes were stained for Ki67 (green) and DAPI (blue). Scale bar 50 μm. (B) Quantification Ki67 positive cells in indicated genotypes. Data are mean ± SD. (C) Cdk2 kinase activity (percent of that in the *Rb* null retina) was plotted against percentage of Ki67+ cells at P8. p value was determined using a one-sample t-test for Pearson's product-moment correlational coefficient, r. All assays were carried out at least 3 times. For simplicity "*Rb^{-/-}*" represents *αCre;Rb^{ff}*.

Figure 11 supplements Figure 4 and shows that p21 levels bound to Cdk2 do not correlate with kinase activity. Cdk2 kinase activity (from Figure 3) was plotted against the amount of p21 bound to Cdk2. "*Rb^{-/-}*" is used to indicate *αCre;Rb^{ff}*. p values were

calculated using a one-sample t-test for Pearson's product-moment correlational coefficient, r .

Figure 12 shows Skp2 is essential for retinoblastoma initiation. (A) Adult retinal sections stained with Hematoxylin and Eosin (H&E) at 10x and 4x of the indicated genotypes. Deletion of *Skp2* completely blocked tumor initiation in the *Rb/p107* DKO retina. Furthermore, Skp2 binds to T187-phosphorylated p27 and facilitates its polyubiquitylation by the SCF^{skp2} complex and subsequent proteosomal degradation. We bred a knock-in strain expressing a Skp2 resistant version of p27 (p27^{T187A}) with the mouse knockout model of retinoblastoma (*αCre;Rb^{fl}p107^{-/-}*) and found tumor initiation was also blocked. (B) Western blot analysis of P8 retinal lysates showed that p27 protein levels were induced upon *Skp2* deletion. Additionally, p21 and Cyclin E – also targets of Skp2 – were stabilized in Skp2 null retinas. (C) A table summarizing tumor penetrance of the indicated genotypes as a percentage of affected animals of the total animals. Deletion of a single allele of *Skp2* was sufficient to inhibit tumor growth, and similarly, tumor penetrance in *Rb^{-/-};p107^{-/-};p27^{+/T187A}* retinas also dropped to 0%. *Skp2* deletion had no effect on the 100% tumor penetrance in *Rb/p27* DKO retina, suggesting that Skp2 acts upstream of p27. (D and E) Abnormally positioned mitoses (yellow arrows pointing to red nuclei, white arrows are normal apical mitoses) in the newborn tumour-prone retina disappeared when E2f1 was removed, but Skp2 deletion had virtually no effect. (F and G). Rather than driving abnormal division, Skp2 disrupted polarity/adhesion (phalloidin stains filamentous actin, green). Breaks in the apical domain quantified in (G).

Figure 13 shows polarity/adhesion defect in the tumor-prone *Rb/p107* null retina. (A) E14 *Rb/p107* null retina was stained with phalloidin to mark filamentous actin (green) and DAPI (blue). The discontinuous apical actin stain and escape of cells outside the normal apical boundary is indicated with a white arrow. A blow up of the break in another *Rb/p107* null retina stained with phalloidin is shown below (compressed Z-stack of confocal images). (B) N-cadherin is part of the apical adherens junction that anchor filamentous actin. The N-cadherin stain is continuous in tumor-resistant Wild Type (WT) or *Rb* null retina, but aberrant breaks are detected in the tumor-prone *Rb/p107* null retina (bottom picture). (C) Additional markers of adherens junction or regulators associated with adherens junction also reveal the phenotype. (D) Two examples of *p107* null E15 retinas in which the apical barrier is disrupted (arrows). This phenotype is never seen in the WT or *Rb* null retina. (E) Summary of genotype/phenotype

correlations. Loss of *Rb* but not *p107* causes ectopic division. Loss of *p107* but not *Rb* causes polarity/AJ defects (~20% in mixed background and 100% in C57 pure). Loss of *Rb* and *p107* accentuates both phenotypes and is associated with a third, tumorigenesis.

5 **Figure 14** shows the key polarity players. The partitioning defective (PAR), Crumbs, and Scribble complexes control many polarization processes in different organisms. PAR-3 and PAR-6 are PDZ domain proteins and bind the Ser/Thr kinase, atypical Pkc (aPkc) to form the PAR complex. aPkc kinase activity is essential for the function of this complex. The transmembrane protein Crumbs and the cytoplasmic scaffolding
10 molecules PALS1 ((protein associated with LIN-7)-1) and PTJ (PALS1-associated tight junction protein) form the Crumbs complex. The Crumbs and PAR6 complexes repel Scribble complex to the basolateral surface. The latter contains the tumor suppressors lethal giant larval (LGL), Scribble and DLG1. In polarized mammalian epithelial cells, the PAR3 and Crumbs-3 complexes localize predominantly to tight junctions. aPkc
15 phosphorylates Lgl to maintain the asymmetric distribution shown. From Nature Reviews Molecular Cell Biology 9, 846-849, 2008.

Figure 15 shows aPkc is required for retinoblastoma in the *Rb/p107* null retina. Hematoxylin and Eosin (H&E) stained 2.5x (a), 10x (b) or 40x (c) magnification of horizontal sections of P30 retinas of indicated phenotypes. Note the emerging tumor in
20 *Chx10Cre;Rb^{fl};p107^{-/-}* retina. All retinas of this genotype form tumors. In contrast, the *Chx10Cre;Rb^{fl};p107^{-/-};Pkcλ^{fl}* retina is very thin and lacks tumor. Deletion of both alleles of *Pkcλ* blocked tumor formation and removing one allele reduced tumor frequency by 10-fold.

Figure 16 shows a *Pkcλ* is an essential prosurvival molecule in tumor-prone *Rb/p107*
25 null cells. (A) P8 retinas from mice of the indicated genotypes were stained with DAPI and TUNEL or anti activated Caspase 3 (AC3). A few apoptotic cells in Wild Type (WT) and *p107* null retinas represent normal pruning of retinal neurons. *Rb* loss induces E2f1-dependent death in a subset of differentiating neurons, which is elevated when both *Rb* and *p107* are missing (top and bottom panels). aPkcλ removal triggered
30 massive apoptosis of tumor prone *Rb/p107* null cells at P8 (bottom panel). This effect was dependent on the tumor-initiated state as it did not occur in the *Rb/Pkcλ* null retina (bottom panel). For simplicity, retina specific or germline null alleles are mentioned as knockout or KO; *Pkcl* denotes *Pkcλ*. All conditional knockouts used in this experiment

are Chx10-Cre specific. (B) Quantification of TUNEL and AC3 +ve cells in retinas of indicated genotypes at P0 (upper panel). Quantification of TUNEL and AC3 +ve cells in P8 retinas of indicated genotypes. Level of cell death in Rb/p107/Pkcl TKO retinas at P8 was ~10 times higher than the tumor prone Rb/p107 DKO retinas.

- 5 **Figure 17** shows aPkc λ is required for tumor development in *Rb/p107* null retinas. P30 retinas from mice of the indicated genotypes were stained with DAPI, cell division markers PH3 and Ki67, and cell death markers TUNEL and activated Caspase 3. Division is completed in WT retinas by P8 and therefore no dividing cell was visible at P30 in Wild Type (WT) retinas (data not shown). In contrast, extensive division was
- 10 observed in the emerging tumors of Rb/p107 double knock-out (DKO) retinas (middle panel). Removal of aPkc λ from tumor prone retina led to a marked decrease in ectopic division at P30. No apoptosis was observed in the normal WT retina (data not shown) whereas many cells were undergoing apoptosis in the tumor prone DKO retina (middle panel). The number of cells undergoing apoptosis in the Rb/p107/Pkcl TKO retina was
- 15 less than that in the Rb/p107 DKO retina (bottom panel) as majority of tumor prone cells in the total knock-out (TKO) retinas are already dead by this time point (Figure 15). As a result, TKO retina was also much thinner in comparison to the DKO retina. Remaining dividing cells in the TKO retinas (bottom panel) would also eventually die by P60 (data not shown). (B) Quantification of Ki67 and PH3 +ve cells in retinas of
- 20 indicated genotypes at P30. Pkcl denotes aPkc λ .

- Figure 18** shows aPkc also promotes survival in human RB cell lines. Effect of aPKC co-knock down in WERI-RB1 (A, B, C) and Y79 (D, E, F). RB lines were co infected with two different lentiviruses carrying a resistant gene for either puromycin (*) or blasticidin (+) overnight. Coinfected cells were then selected by puromycin (7 μ g/ml)
- 25 and blasticidin (7 μ g/ml) cotreatment for 7 days. aPkc λ is the human counterpart of mouse aPkc λ . (A and D): Knock down efficacy of coinfecting cells for aPKC isoenzymes was assessed by Western Blotting after puromycin and blasticidin coselection. Cell lysates were made and when possible, 50 μ g of proteins were loaded to assess aPKC expression (N/A, no proteins because of massive cell death). (B and
- 30 E) Effect on cell viability. 10,000 of remaining viable cells were plated per well in 96 well plates and viability was evaluated every day over a week post selection using CellTiterGlo® luminescent kit. Day 5 post selection is shown. Values of luminescence were averaged, normalized to day 0 post-selection and converted to percentage of control uninfected cells. Errors bars represent standard deviations of triplicates. (C and

F) Positive correlation between aPKC expression and cell viability. Two time points are shown: day 0 (red) and day 5 (blue) post selection *P<0.05, **P<0.01.

Figure 19 shows the gold compound aurothiomalate (ATM), which inhibits aPKC interaction with the polarity regulator Par6, reaches the adult retina. Adult mice were treated with a single intraperitoneal injection of 15, 30, or 60 mg/kg ATM for 1 hour. Mice were then sacrificed by CO₂ inhalation and Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES) was used to measure gold levels in (A) serum and (B) eyes. n: denotes number of mice. Data are presented as average ± mean difference.

Figure 20 shows ATM kills tumor-prone, but not tumor-resistant retinal cells. (A) P8 retinal explants were treated from P0-P8. P0 retinal explants of the indicated phenotypes were exposed to the indicated amounts of ATM for 8 days then fixed and stained with DAPI (blue) to mark nuclei and TUNEL (red), activated caspase-3 staining shows similar results (data not shown), to label late or early stage apoptosis, respectively. (B) Quantification of TUNEL data in (A).

Figure 21 shows ATM kills retinoblastoma cells *in vitro*. (A) Effect of ATM on Y79 cell viability. Cells were treated with the indicated concentrations of ATM for 7 days and cell viability was assessed using the CellTiterGlo® luminescent kit. Average luminescence of triplicates ± SD were plotted as fold change relative to day 0. (B, C) ATM induces apoptosis. Y79 cells were treated with 50 μM ATM for 5 days and were either stained live for Annexin V/PI (B), or fixed and stained for PI (cell cycle analysis), and analyzed by flow cytometry (C). (D) Effect of ATM on caspase 3/7 activity. Cells were treated with 50 μM ATM for 7 days and caspase activity was assessed using Caspase 3/7 Glo® luminescent assay kit. Average luminescence of triplicates ± SD was normalized to the number of viable cells.

Figure 22 shows aPKC kinase activity and interaction with the polarity complex Par6 is essential for the prosurvival function of aPKC. (A and B): Genetic loss of Pkcl reveals unique strategy to block RB initiation. Loss of Pkcl triggers massive apoptosis in P8 tumor prone retina, uncovering a novel synthetic lethal interaction. To test whether aPKC is acting through the Par complex, we transduced Rb/p107-null cells with (A) eGFP or (B) a Par6K19A dominant negative, which abolishes polarity complex function. Transfected retinas were stained with apoptosis marker activated caspase-3 (AC3). Strikingly, the Par6K19A dominant negative recapitulates substantial apoptosis

(B) as seen in Rb/p107/Pkcl TKO retina (Figure 15), suggesting that the synthetic lethal interaction is acting through the Par complex. For simplicity Rb^{-/-} represents α Cre;Rb^{ff}; Pkcl denotes α Pkc λ (C) Quantification of activated caspase-3 data in A and B.

- 5 **Figure 23** shows E2f inhibitor 6474 blocks nascent retinoblastoma growth *in vivo*. (A) α Cre;Rb^{ff}/p107^{-/-} mice were treated orally every week from P28 to P56 with either vehicle or a E2f small molecule inhibitor, 6474. The mice were harvested a day after the last injection (P57). (B) Quantification of tumor volumes (mm³) normalized to body weight (kg) from animals treated in (A). 6474 reduces tumor volume by ~14-fold and is
- 10 approaching significance ($p=0.09$ using Kruskal-Wallis ANOVA). Each dot represents one eye from the study (Vehicle: n=6; 6474: n=16) and the solid bar represents the median with respective numerical label above.

Figure 24 shows a model summarizing critical molecular steps to the tumor-prone state. In the mouse retina, Rb loss activates E2f1 and triggers ectopic division, but

15 additional genetic events are required to activate Cdk2 and thus create tumor susceptibility. Sporadic mutations permit progression to cancer. Rb loss delays cell cycle exit. Ectopic E2f1-dependent division stops eventually by RB-independent means. Other components of the dual axes (Skp2 and Cdk2) are also modestly elevated. These cells never form tumors. p107 loss lowers Rb pathway activity even

20 further, resulting in elevated Skp2 levels and Cdk2 activity. E2f activity is essentially the same as in Rb null cells. Polarity and fate are disrupted. α Pkc is engaged to promote survival. Most of these cells also exit the cell cycle eventually. But, this constellation of molecular events together creates an initiation network that confers the potential for sporadic tumorigenesis. This step can be reversed by modestly reducing

25 E2f, Cdk2 or α Pkc activity, or by removing Skp2. An additional sporadic event generates retinoblastoma.

DETAILED DESCRIPTION

In the following description, numerous specific details are set forth to provide a thorough understanding of the invention. However, it is understood that the invention may be practiced without these specific details.

- 5 Retinoblastoma is an uncommon cancer, but rarity should not be confused with irrelevance. Several fundamental discoveries with broad relevance to cell cycle regulation and cancer have emerged from studying this disease, including the classic two hit hypothesis, and the discovery of *Rb*, which was the first recognized tumor suppressor and turned out to have universal relevance to multiple cancers (Friend et al., 1986; Knudson, 1971). More recently, the long-standing notion that E2fs are essential for cell cycle progression, based mainly on study of fibroblasts, was countered by the finding that retinal progenitors divide in their absence (Chen et al., 2009). Concomitant findings in multiple other tissues indicated that fibroblasts, not retinal progenitors, are the exception (Chong et al., 2009).
- 15 Mouse models of retinoblastoma are ideal to study initiation because the generation of tumor-susceptibility can be separated into two distinct genetic steps. *Rb* loss alone triggers E2f1-dependent ectopic division of differentiating retinal cells (Chen et al., 2007), but the retina remains tumor-resistant with the cancer-prone state also requiring removal of at least one *Rb* relative, *p107* or *p130* (Chen et al., 2004; Dannenberg et al., 2004; MacPherson et al., 2004; Robanus-Maandag et al., 1998; Zhang et al., 2004). The separation of ectopically dividing yet tumor-resistant from tumor-prone conditions exposes an opportunity to define the molecular networks that create the latter state. However, the downstream molecular targets associated with this distinct condition are unclear. *Rb* family proteins are best known as E2f inhibitors and no CKI-
- 25 Cdk axis mutations have been described in human or mouse retinoblastoma. Superficially the disease thus fits a model in which enhanced E2f activity is sufficient to create tumor-prone cells. However, there is extensive cross-talk between E2f and Cdk axes (**Figure 1A**) (Binne et al., 2007; Buttitta et al., 2010; Buttitta et al., 2007; Ji et al., 2004; Rodier et al., 2005; Wang et al., 2010) which could lead to elevated Cdk activity
- 30 when the tumor-prone state arises upon mutation of both *Rb* and either *p107* or *p130*. We asked whether removing the Cdk inhibitor (CKI) *p27* could cooperate with *Rb* loss to trigger retinoblastoma. Strikingly, we found that this CKI was an effective tumor suppressors in the retina. Moreover, through the generation of multiple new

- retinoblastoma models, we found that while E2f activity was not substantially different in tumor-resistant versus tumor-prone retinas, Cdk2 activity strongly correlated with cancer susceptibility. Thus, partially inhibiting either the E2f or Cdk axis prevented retinoblastoma, and was achieved without altering normal division. These data expose
- 5 three quantum states of dual E2f and Cdk activity in the retina that mediate normal division (low E2f and Cdk), ectopic division (High E2f) or, critically, cancer susceptibility (High E2f and Cdk). Thus, deregulation of cell cycle regulators during tumorigenesis is not required simply to drive abnormal division, but has an essential and separate role in creating the unique tumor-prone state.
- 10 E2f and Cdk are commonly deregulated in cancer, but it was unclear whether they have roles beyond simply disrupting proliferation. Using mouse genetics we show that a quantum difference in E2f and Cdk dual axes activity not only distinguishes normal vs ectopically dividing retinal cells, but a third level demarcates the unique cancer-prone condition. E2f was similarly high in both abnormal states, but elevated Cdk
- 15 activity was specific to tumor-prone cells. Brief pharmaceutical E2f or Cdk inhibition did not perturb normal division, and only partially impaired ectopic division, yet dramatically curtailed tumorigenesis. Thus, beyond perturbing division, specific dual axes levels engender tumor susceptibility. E2f and Cdk inhibitors, untested or largely unproven as therapeutics, could be potent chemopreventive agents.
- 20 Chemoprevention, the blockade of neoplasia, is particularly important for high risk cancer-prone individuals. Deducing the perturbations that underlie the tumor-prone state is critical to develop effective chemoprevention strategies. Cell cycle regulation in cancer is typically viewed as normal or abnormal, but here we now define a third cell cycle state that is tumor-prone and is uniquely marked by elevated activity of E2f and
- 25 Cdk cell cycle regulatory axes as well as Skp2 and aPKC. Remarkably, exposing the cell-of-origin briefly to small molecule antagonists prevented subsequent tumorigenesis in cancer-prone mice without affecting normal division. Our *in vivo* findings demonstrate that targeting a cell cycle state unique to the cancer cell-of-origin achieves effective and safe chemoprevention. It is proposed, therefore, that
- 30 suppressing/inhibiting the members of the tumour initiation network is a feasible strategy to prevent retinoblastoma.

According to an aspect, there is provided a method of preventing retinoblastoma in a subject comprising inhibiting the RB pathway. Preferably, the method comprises inhibiting at least one of Cdk, E2f, atypical protein kinase C, and Skp2.

By "RB pathway" it is meant the entire pathway of molecular signaling that includes retinoblastoma protein (RB), and other protein/protein families in the pathway, including but not limited to Cdk, E2f, atypical protein kinase C, and Skp2. Cdk, E2f, atypical protein kinase C, and Skp2 as used herein, refers individually and collectively to proteins, protein isoforms and protein families, unless indicated otherwise.

The term "preventing" includes any and all of primary, secondary, tertiary and quaternary prevention levels, for example, methods to avoid occurrence of disease, methods to diagnose and treat existent disease in early stages before it causes significant morbidity, methods to reduce negative impact of extant disease by restoring function and reducing disease-related complications, and methods to mitigate or avoid results of unnecessary or excessive interventions in the health system, respectively. Without limiting the foregoing, with respect to cancer specifically, prevention includes any avoiding or mitigation of a cancerous state, including the slowing or halting of cancer progression to a more detrimental state or stage.

The term "downregulate" or "inhibit" is used herein to refer to at least partial inhibition of the activity or expression of a gene or protein.

In some embodiments, the inhibiting comprises administering to the subject an effective amount of an RB pathway inhibitor, preferably selected from the group consisting of a Cdk inhibitor, an E2f inhibitor, an atypical protein kinase C inhibitor, and a Skp2 inhibitor.

By "effective" amount is meant a nontoxic but sufficient amount of an active compound or composition to provide the desired therapeutic or preventative effect. In the present case, that dose of a Cdk inhibitor, an E2f inhibitor, an atypical protein kinase C inhibitor, or a Skp2 inhibitor compound effective to relieve, ameliorate, or prevent symptoms of the condition or disease being treated, e.g. cancer, such as retinoblastoma.

Various methods of suppressing proteins/protein families are generally known in the art (Oncogene suppression by small interfering RNAs. Heidenrich O., Curr Pharm Biotechnol. 2004, 5(4): 349-54) and also specifically with respect to:

- 5 E2f (Resveratrol induces apoptosis in breast cancer cells by E2F1-mediated up-regulation of ASPP1 Shi et al. Oncology reports 25: 1713-1719, 2011; Lentivirus-mediated RNA interference of E2F-1 suppresses Tca8113 cell proliferation. Yuan et al.. Molecular Medicine Reports. 5: 420-426, 2012; Lentivirus-mediated RNA interference targeting E2F-1 inhibits human gastric cancer MGC-803 cell growth in vivo. Wang et al. Experimental and Molecular
- 10 Medicine, 43: 638-645, 2011; Selective inhibition of rRNA transcription downregulates E2F-1: a new p53-independent mechanism linking cell growth to cell proliferation. Donati et al. Journal of Cell Science 124, 3017–3028, 2011.);
- 15 Cdk (Dual blockade of lipid and cyclin-dependent kinases induces synthetic lethality in malignant glioma. Cheng et al. Proc Natl Acad Sci U S A. 2012 Jul 31;109(31):12722-7.; Bioluminescent imaging of Cdk2 inhibition in vivo. blocking Cdk2 activity with inhibitory proteins, peptides or small interfering RNA (siRNA). Zhang et al. Nature Medicine, Vol. 10, No. 6, June 2004 643 – 648; siRNA agents inhibit oncogene expression and attenuate human tumor cell
- 20 growth. Yin et al. Journal of Experimental Therapeutics and Oncology 3: 194–204, 2003; Cyclin D1 and CDK4 Activity Contribute to the Undifferentiated Phenotype in Neuroblastoma. Molenaar et al. Cancer Res 2008;68(8):2599–609; Combined Depletion of Cell Cycle and Transcriptional Cyclin-Dependent Kinase Activities Induces Apoptosis in Cancer Cells. Cai et al. Cancer Res
- 25 2006; 66(18): 9270-80).
- 30 aPKC: atypical (aPKC; aPKC ζ and aPKC ι/λ) PKCs (Coordination of glioblastoma cell motility by PKC ζ . Baldwin et al. Mol Cancer. 2010 Sep 3;9:233.; The Proto-oncogene PKC ζ regulates the alternative splicing of Bcl-x pre-mRNA. Shultz et al. Mol Cancer Res. 2012 May;10(5):660-9.; Regulation of TGF β receptor trafficking and signaling by atypical protein kinase C. Gunaratne et al. Cell Signal. 2012 Jan;24(1):119-30.; Tumor necrosis factor alpha and inflammation disrupt the polarity complex in intestinal epithelial cells by a posttranslational mechanism. Mashukova et al. Mol Cell Biol. 2011

Feb;31(4):756-65.; Protein Kinase C/ Is Required for Pancreatic Cancer Cell Transformed Growth and Tumorigenesis. Scotti et al. Cancer Res 2010;70:2064-2074); and

5 Skp2 (Is exclusive Skp2 targeting always beneficial in cancer therapy? Koutsami et al. Blood, 2008; Vol. 112, No. 12, 4777-4779; Selective inhibition of rRNA transcription downregulates E2F-1: a new p53-independent mechanism linking cell growth to cell proliferation. Donati et al. Journal of Cell Science 124, 3017–3028, 2011.; Targeting the p27 E3 ligase SCFSkp2 results in p27- and Skp2-mediated cell-cycle arrest and activation of autophagy. Chen
10 et al. Blood. 2008;111: 4690-4699 ; Skp2 is required for survival of aberrantly proliferating Rb1-deficient cells and for tumorigenesis in Rb1+/- mice. Wang et al. Nat Genet. 2010 January ; 42(1): 83–88. (skp2 siRNA); Inhibition of S-phase kinase-associated protein 2 (Skp2) reprograms and converts diabetogenic T cells to Foxp3+ regulatory T cells; Wanga et al. PNAS 109: 9493–9498).

15 In some embodiments, the subject has cells with mutated Rb or cyclin-dependent kinase inhibitor, preferably p27, and the RB pathway inhibitor is a Cdk inhibitor or an E2f inhibitor.

In some embodiments, the Cdk inhibitor inhibits at least one of Cdk1 and Cdk2.

In some embodiments, the Cdk inhibitor inhibits both Cdk1 and Cdk2.

20 In some embodiments, the E2f inhibitor inhibits at least one of E2f1, E2f2 and E2f3.

In some embodiments, the E2f inhibitor inhibits E2f1.

In some embodiments, the E2f inhibitor is selected from the group consisting of 4-hydroxynonenal, E2f aptamer, Eugenol, thymoquinone, HLM006474, and Lithium; preferably Eugenol, thymoquinone, HLM006474 and Lithium; further preferably
25 HLM006474 and Lithium.

In some embodiments, the Cdk inhibitor is selected from the group consisting of Flavopiridol, SNS-032 (derivative 4), BS-181 hydrochloride, Indirubin, PHA-703887, [Ala92]-p16 (84-103), P1446A-05, SNS-032, AZD5438, BAY80-3000, JNJ7706621, SCH 727965, CDKI-71, Bohemine, RO-3306, (R)-DRF053 dihydrochloride, SU 9516,
30 WHI-P180, Hydrochloride, CVT-313, GW8510, (R)-DRF053, NU6140, Cdc2-Like

Kinase Inhibitor, TG003, Hymenidin, Iso-olomoucine, Indirubin-3'-monoxime-5-sulphonic Acid, Butyrolactone I, Indirubin-3'-monoxime, Purvalanol A, BML-259, PNU 112455A hydrochloride, N9-Isopropyl-olomoucine, WR 216174, Olomoucine, Dimethylamino-, Olomoucine II, Olumoucine, NU6027, NU6102, NSC 625987, 5 NU2058, N-6-(δ 2-Isopentenyl)-adenine, Isogranulatimide, Benfluorene, Kenpauellone, Arcyriaflavin A, Aminopurvalanol A, Alsterpauellone, 2-Cyanoethyl, Aloisine, RP106, Aloisine A, 9-Cyanopauellone, 5-Iodo-indirubin-3'-monoxime, 2-Hydroxyboheminine, NSC43042, NSC63002, 3-ATA, 2-Bromo-12,13-dihydro-5H-indolo[2,3-a]pyrrolo[3,4-c]carbazole-5,7(6H)-dione, xylocydine, Ibulocydine, pyrazolo[1,5-a]pyrimidine derived 10 compound, 4k (BS-194), AT7519, CYC202 (roscovitine), R547, P276-00, AG-025322, PD-0332991, CYC065, SNS-032, AT7519, CYC202 (roscovitine) and R547; preferably CYC065, SNS-032, AT7519, CYC202 (roscovitine) and R547; further preferably CYC202 (roscovitine) and R547.

In some embodiments, the subject has cells with mutated Rb or p107, and the RB 15 pathway inhibitor is an atypical protein kinase C inhibitor or a Skp2 inhibitor.

In some embodiments, the atypical protein kinase C inhibitor is aurothiomalate or ICA-1.

According to an aspect, there is provided a use of an RB pathway inhibitor for the prevention of retinoblastoma in a subject.

20 According to an aspect, there is provided a use of an RB pathway inhibitor in the preparation of a medicament for the prevention of retinoblastoma in a subject.

According to an aspect, there is provided an RB pathway inhibitor for use in the prevention of retinoblastoma in a subject.

25 According to an aspect, there is provided a method of determining the likelihood of a population of cells to be cancer-prone, comprising: determining the level of E2f and Cdk activity in the population of cells, wherein a high level of both E2f and Cdk activity in the population compared to a control population is indicative of the population being cancer-prone.

30 According to an aspect, there is provided a method of determining the likelihood of a population of cells to be cancer-prone, comprising: determining the level of atypical protein kinase C activity in the population of cells, wherein a high or abnormal level of

atypical protein kinase C in the population compared to a control population is indicative of the population being cancer-prone.

According to an aspect, there is provided a method of determining the likelihood of a population of cells to be cancer-prone, comprising: determining the level of Skp2
5 activity in the population of cells, wherein a high or abnormal level of both Skp2 activity in the population compared to a control population is indicative of the population being cancer-prone.

The level of protein activity is preferably determined using one of Western blot, realtime PCR or RT PCR and kinase assay. Preferably, if there is a likelihood of
10 retinoblastoma, the methods further comprise preventing retinoblastoma by administration of a Cdk inhibitor, an E2f inhibitor, an atypical protein kinase C inhibitor or a Skp2 inhibitor.

Advantages of the present invention are further illustrated by the following examples. The examples and their particular details set forth herein are presented for illustration
15 only and should not be construed as a limitation on the claims of the present invention.

EXAMPLES

Example 1. Materials and Methods

Mouse strains and genotyping.

20 Mice were treated according to institutional and national guidelines. α Cre mice, Rb^{flf} mice, $p107^{-/-}$ mice, $p27^{-/-}$ mice, and $p27^{Ck-/Ck-}$ mice, were maintained on a mixed background. $p107$ and $E2f1$ are in close proximity on chromosome 2, thus $E2f1^{+/-};p107^{-/-}$ mice were interbred and after analysis of > 150 pups one was identified in which a crossover had occurred to generate an $E2f1^{-};p107$ chromosome. Mice of
25 different genotypes were compared within the same litter and across a minimum of three litters. We have not noted any phenotypic differences in separate litters. Genotyping was performed as before (Besson et al., 2007; Chen et al., 2004).

Histology and immunofluorescence.

BrdU labelling, fixation and immunostaining were essentially as described before (Chen et al., 2007; Chen et al., 2009). For p27, p21 and Ki67 immunostaining, antigen retrieval was performed by boiling sections in citric acid solution (H-3300, Vector Lab Inc.) for 15 min.

RNA extraction, reverse transcription and PCR.

RT and qPCR for E2f targets were run in duplicate on at least three separate biological samples as described (Chen et al., 2009). Values obtained for test RNAs were normalized to Hprt mRNA levels.

10 Western blots.

Mouse retinas were homogenized by passing them through 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 (Chen et al., 2007). Blots were scanned using ODYSSEY Infrared Imaging System (LI-COR Biosciences).

Immunoprecipitation and kinase assays.

Complete RIPA was prepared by combining 10 μ l PMSF solution, 10 μ l sodium orthovanadate solution, 10 μ l protease inhibitor cocktail solution per ml of 1X RIPA lysis buffer (Santa Cruz Biotechnology Inc.). Mouse retinas were lysed by incubating in ice cold RIPA for 10 min. Cellular debris was removed by centrifugation at 10,000xg for 10 min at 4 °C and protein concentration of supernatants determined by Bradford assay. Retina lysate was pre-cleared by adding 1.0 μ g of rabbit IgG, together with 20 μ l resuspended volume of Protein A/G Plus-Agarose (Santa Cruz Biotechnology Inc.) at 4°C for 30 min. Agarose beads were removed by centrifugation at approximately 1,000xg for 5 min at 4 °C. Supernatant containing 250 μ g total cellular protein was incubated with 1 μ g of primary antibody at 4°C for 2hrs. 20 μ l of resuspended volume of Protein A/G Plus-Agarose was added and incubated at 4 °C on a rocker platform for 1hr to overnight to pull down primary antibodies. Immunoprecipitates were collected by centrifugation at approximately 1,000xg for 5 min at 4 °C. Pellets were washed three times with PBS and once with 1x kinase buffer (Cell Signaling). After the final wash pellets were resuspended in 40 μ l of 1x kinase buffer. The Cdk2 kinase reactions were

performed at 30°C for 15 min in kinase buffer containing 2.5 µg histone H1 (Upstate Biotechnology) as substrate, 2 µCi of ³²P-γ-ATP, and 20 µM ATP. The reaction was stopped by adding 2x Laemmli buffer and boiled for 5 min before loading on a 10% SDS-PAGE. The gel was then dried on a Whatman filter paper and exposed on a
5 phosphoimager for quantification.

Chemoprevention.

R547 and 6474 were synthesized by University Health Network, Shanghai and purity confirmed at >98% according to published methods (DePinto et al., 2006; Ma et al., 2008). Male and female mice were mixed in the early afternoon, checked the following
10 morning and dams with vaginal plugs were 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 20mg/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 (I.P.) until birth. At P0, half the litter was harvested for assessment of
15 ectopic division and the other half remained until P45 for tumor assessment.

Tumor volume and stereology.

Eyes were sectioned horizontally at 14 µm. Every 8th section was stained with H&E and scanned on a Leica DMRB. Once the tumor-positive areas were scanned and selected, the tumor volume was estimated using the Cavalieri Estimator in Stereo
20 Investigator (MBF Bioscience).

Statistical analysis.

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

Example 2. CKI Activity Suppresses Mouse Retinoblastoma

25 Current mouse knockout models of retinoblastoma require the deletion of *Rb* plus either *p107* or *p130* (Chen et al., 2004; Dannenberg et al., 2004; MacPherson et al., 2004; Robanus-Maandag et al., 1998; Zhang et al., 2004). Thus, compared to humans, where *Rb* loss alone is sufficient to render cells tumor-prone, the mouse retina has extra Rb-like activity. The additional Rb activity embodied in *p107* or *p130*
30 could simply protect the mouse retina through added repression of E2f targets.

However, given the existence of extensive inhibitory cross-talk to CKIs, they might also be acting to suppress Cdk activity (**Figure 1A**). Indeed, when we deleted floxed Rb (*Rb^f*) in peripheral retina at embryonic day 10 (E10) using the α Cre transgene as before (Chen et al., 2004; Chen et al., 2007), we observed elevated p21 and p27 mRNAs, with p27 detected in virtually all cells at postnatal day 8 (P8), and sporadic p21 expression (**Figure 7**). To test whether CKI loss mimics *p107* or *p130* loss, we therefore removed *p27*, the most abundant CKI in the retina (Dyer and Cepko, 2001; Levine et al., 2000). Strikingly, α Cre;*Rb^{ff}*;*p27^{-/-}* mice developed retinoblastoma with 100% penetrance (**Figure 1B and Table 1**), double that of α Cre;*Rb^{ff}*;*p107^{-/-}* mice and similar to α Cre;*Rb^{ff}*;*p130^{-/-}* mice (Chen et al., 2004; MacPherson et al., 2004). As in other double knockout (DKO) models of retinoblastoma (Chen et al., 2004; Dannenberg et al., 2004; MacPherson et al., 2004; Robanus-Maandag et al., 1998; Zhang et al., 2004), *Rb/p27* DKO tumor cells expressed the amacrine cell marker *Ap2a*, as well as markers found in this and other cells such as *Pax6*, and *Prox1*, but lacked markers for other cell types (**Figure 2 and Figure 8**). P30 tumor cells were positive for markers that label all cell cycle phases (*Ki67*), M-phase (phosphohistone H3; *PH3*), 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 *Ap2a⁺* was 92±4%, 91±1%, 92±5%, 92±2% and 90±1%, respectively, and the fraction of *BrdU⁺* cells that were *Ap2a⁺* was 89±11%, 91±10%, 85±4%, 84±5% and 87±4%, respectively (**Figure 2B-2D and Figure 8**). The contaminating glutamine synthase-labelled Müller glia were quiescent. 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.

To date, all mouse knockout models of retinoblastoma require deletion of *Rb* plus either *p107* or *p130* (Chen et al., 2004; Dannenberg et al., 2004; MacPherson et al., 2004; Robanus-Maandag et al., 1998; Zhang et al., 2004). Thus, compared to humans, where *Rb* loss alone is sufficient to render cells tumor-prone, the mouse retina has extra Rb-like activity. The additional Rb activity embodied in p107 or p130 could simply protect the mouse retina through added repression of E2f targets. However, given the existence of extensive inhibitory cross-talk to CKIs, they might also be acting to suppress Cdk activity (**Figure 1A**). Indeed, when we deleted floxed Rb (*Rb^f*) in peripheral retina at embryonic day 10 (E10) using the α Cre transgene as before (Chen et al., 2004; Chen et al., 2007), we observed elevated p21 and p27

mRNAs with p27 detected in virtually all cells at postnatal day 8 (P8), while p21 expression was sporadic (**Figure 7**). To test whether removing a CKI mimics *p107* or *p130* loss we therefore removed *p27*, the most abundant CKI in the retina (Dyer and Cepko, 2001; Levine et al., 2000). Strikingly, $\alpha\text{Cre};Rb^{ff};p27^{-/-}$ mice developed

5 retinoblastoma with 100% penetrance (**Figure 1B and Table 1**), double that of $\alpha\text{Cre};Rb^{ff};p107^{-/-}$ mice and similar to $\alpha\text{Cre};Rb^{ff};p130^{-/-}$ mice (Chen et al., 2004; MacPherson et al., 2004). As in other double knockout (DKO) models of retinoblastoma (Chen et al., 2004; Dannenberg et al., 2004; MacPherson et al., 2004; Robanus-Maandag et al., 1998; Zhang et al., 2004), the vast majority of *Rb/p27* DKO

10 tumor cells expressed the amacrine cell marker *Ap2 α* , as well as markers found in this and other cells such as *Pax6*, and *Prox1*, but lacked markers for other cell types (**Figure 2 and Figure 8**). P30 tumor cells were positive for markers that label all cell cycle phases (*Ki67*), M-phase (phosphohistone H3; *PH3*), or S-phase (*BrdU*), whereas contaminating glutamine synthase (*GS*)-labelled Müller glia were quiescent (**Figure**

15 **2B-2D and Figure 8**). Thus, in the *Rb*^{-/-} mouse retina, p27 is a potent tumor suppressor.

Table 1 Tumor penetrance in *Rb* null retinas with compromised p107 and/or CKI status

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

Abbreviations: CKI, Cdk inhibitor; LOH, loss of heterozygosity.

20 *p27* binds and regulates proteins other than Cyclin/Cdk2 (Baldassarre et al., 2005; Besson et al., 2004; Nguyen et al., 2006). To define the critical tumor suppressor activity, we assessed the *p27*^{CK-} allele in which four amino acid substitutions

- specifically disrupt CKI activity (Besson et al., 2007). $\alpha\text{Cre};\text{Rb}^{\text{flf}};\text{p27}^{\text{CK-}/\text{CK-}}$ mice developed retinoblastoma with 100% penetrance (**Figure 1B** and **Table 1**). $\text{p27}^{\text{CK-}/\text{CK-}}$ animals exhibit retinal dysplasia (Besson et al., 2007), which we confirmed, but they never developed retinoblastoma (data not shown). Furthermore, of the 28 eyes from
- 5 $\alpha\text{Cre};\text{Rb}^{\text{flf}};\text{p27}^{\text{+/CK-}}$ animals only three had tumors, and strikingly all showed loss of heterozygosity (LOH) (**Table 1** and **Figure 9**). Thus, $\text{p27}^{\text{CK-}}$ is not a dominant oncogene in either in normal or $\text{Rb}^{-/-}$ retina, contrasting lung where it causes tumors (Besson et al., 2007). Collectively, our results demonstrate that retinoblastoma requires loss of p27 CKI activity.
- 10 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 $\alpha\text{Cre};\text{Rbflfp107}^{+/+}$ or $\alpha\text{Cre};\text{Rbflfp27}^{+/+}$ mice, which never developed retinoblastoma, $\alpha\text{Cre};\text{Rbflfp107}^{+/+};\text{p27}^{+/+}$ compound heterozygotes developed tumors (**Figure 1B**,
- 15 **Table 1**). Importantly, none of the five tumors analyzed showed loss of heterozygosity for p107 or p27 (**Figure 9** and data not shown). We also analyzed compound heterozygotes harboring the $\text{p27}^{\text{CK-}}$ allele ($\alpha\text{Cre};\text{Rb}^{\text{flf}};\text{p107}^{+/+};\text{p27}^{\text{+/CK-}}$) and similarly observed retinoblastoma with only 1/7 tumors displaying loss of heterozygosity (**Figure 1B**, **Table 1**, **Figure 9** and data not shown). Previously, the Rb–E2f axis has been the
- 20 focus in retinoblastoma, but these genetic data expose a new role for the CKI–Cdk axis.

Example 3. 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
- 25 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 (Chen et al., 2004; MacPherson et al., 2004). Thus E2f targets and Cdk activity were assessed at P8 to focus specifically on ectopically dividing differentiating cells. We assessed 24 E2f targets almost all of which were elevated in $\text{Rb}^{-/-}$ retinas (**Figure 3A**).
- 30 Surprisingly, in four tumor-prone genetic models, expression of these targets was remarkably similar to $\text{Rb}^{-/-}$ levels and none correlated with tumor penetrance (**Figure 3A**). We next analyzed Cdk pathway activity and found that Cdk2 protein was negligible in wild type (WT) retina, but induced similarly in $\text{Rb}^{-/-}$ 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 10**),
 5 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* knock out (KO) versus tumor-prone states, Cdk2 activity predicts the susceptibility to sporadic transformation. This result, together with our new models of retinoblastoma, suggested that rather than constraining E2f target induction, p107
 10 may protect the *Rb*^{-/-} retina by blocking cross talk to Cdk2.

Example 4. p107 Affects Cyclin, Skp2 and p27 Levels *In Vivo*.

Typically, p107 is thought to influence Cdk activity through E2f regulation of Cyclins (**Figure 1A**). However, as noted above, Cyclin A and E induction was already maximal after *Rb* loss (**Figure 3A**). Cyclin E protein also showed similar or lower levels in
 15 tumor-prone retinas (**Figure 4A**). *In vitro* data link p107 to Cdk either directly by binding and inhibiting Cdk2 or indirectly, by decreasing Skp2 the substrate binding component of the SCF^{Skp2} E3 ubiquitin ligase, which stimulates p27 degradation (**Figure 1A**) (Rodier et al., 2005). Furthermore, our genetic and biochemical results suggested that p107 tumor suppressor activity might be manifested through
 20 antagonism of Cdk. Therefore, we sought to define how p107 regulates Cdk activity by first assessing their physical interaction. Cdk2 was complexed with p21 and p27 in genotypes expressing these CKIs. However, no interactions 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 (Chibazakura et al., 2004). p57 was also not
 25 detected in Cdk2 immunoprecipitates (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 an important role in tumor suppression, the amount was modestly, but
 30 consistently, 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 and 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**) (Rodier et al., 2005). Skp2 induction was post-transcriptional as Skp2 mRNA levels were similar in tumor-resistant *Rb*^{-/-} retina versus tumor-prone retinas (**Figure 3A**).

To complete the analysis of CKIs we also studied p21. Total p21 levels were negligible in the 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 11**). 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 following loss of p107 (*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.

Example 5. Inhibiting Either of the Dual Axes Blocks Tumorigenesis

Our results suggest that E2f and Cdk2 form dual axes that can generate three states in the retina: WT, ectopically dividing (*Rb* null) and a 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 (Chen et al., 2007). *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 *αCre;Rb*^{fl/fl};*p107*^{-/-} or *αCre;Rb*^{fl/fl};*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 *αCre;Rb*^{fl/fl};*p107*^{-/-} retina, and reduced penetrance from 100% to 10% in the *αCre;Rb*^{fl/fl};*p27*^{-/-} retina (**Figure 5A, Table 2**). Therefore, unlike normal cells, which proliferate in the absence of E2f1-3, the tumor-prone state requires E2f1 activity. Moreover, *E2f1* heterozygosity did not affect progenitor division, but

specifically reduced ectopic division in *Rb/p107*-deficient cells (**Figure 5B and 5C**). Thus, a therapeutic window of E2f activity exists that can be exploited to prevent abnormal pre-cancerous events without perturbing normal division.

Table 2. Tumor frequency in retinas lacking *E2f* alleles

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

5

Next, we examined whether lowering Cdk activity might also inhibit retinoblastoma initiation. Cdk1 can functionally substitute for Cdk2 *in vivo* (Santamaria et al., 2007), 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 (Chen et al., 2004). 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 CKI that passed preclinical evaluation (DePinto et al., 2006) and is in Phase I trials for solid tumor (Malumbres et al., 2008). It does not inhibit 113 other kinases, and requires >100 fold higher doses to inhibit Gsk3 α/β versus Cdk1/2 (DePinto et al., 2006). *α Cre;Rb^{fl/fl};p27^{-/-}* males were bred to *α Cre;Rb^{fl/fl};p27^{+/-}* females and pregnant dams received daily intraperitoneal injections of vehicle or R547 (20 mg/kg) from embryonic day 12.5 (E12.5) to parturition and tumors were assessed at P45. Of eight eyes examined in the treatment group, six were tumor free, and tumor volume in the

20

affected eyes was considerably reduced, also consistent with reduced tumor frequency (**Figure 6B**). The failure to block all tumor formation may be because of some late stage amacrine cell birth that occurs in the far periphery up to ~P3, beyond the period of R547 exposure.

- 5 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**) (Chen et al., 2004), whereas in *Chx10Cre;Rb^{fl/fl};p107^{-/-}* mice, Cre is expressed across the entire retina, there is considerable dysplasia, and tumors emerge in multiple locations with 100%
 10 penetrance (Zhang et al., 2004). This pattern was observed in P45 mice born to dams exposed to vehicle (**Figure 6C**). However, following a brief exposure of fetuses to R547, two of the eight 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
 15 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 (**Figures 5A and B**). We further examined *Chx10Cre;Rb^{fl/fl};p107^{-/-}* retinas at P0, before tumor formation, for effects on division and the extensive dysplasia in this model. Strikingly, R547 reduced dysplasia (**Figure 6D**)
 20 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 1 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,
 25 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 Bandara et al., 1997; Fabrizio et al., 1999; Montigiani et al., 2003) and a small molecule inhibitor (HLM006474, abbreviated here to 6474) (Ma et al., 2008). To our knowledge, none
 30 have been tested *in vivo*. 6474 was designed in a computer-based virtual screen of E2f and blocks DNA binding, thus down-regulating multiple E2f target genes and inhibiting proliferation (Ma et al., 2008). Although all four drugs inhibit division *in vitro*, only 6474 was tested on tissue, stalling tumor growth in a 3D skin model. It can induce apoptosis of cancer lines *in vitro*, but in the tissue assay mentioned above, no

apoptosis was observed (Ma et al., 2008). So far, no E2f inhibitors have been tested *in vivo* on primary tumors. Strikingly, short exposure of *Chx10Cre;Rb^{fl};p107^{-/-}* embryos to 6474 had a dramatic effect on tumorigenesis (**Figure 6C**). Thus, modest and temporary pharmaceutical E2f inhibition blocks retinoblastoma initiation. Collectively,
 5 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**).

Example 6. Skp2 is essential for retinoblastoma initiation

10 Traditional mouse models of RB delete *Rb1* and its relative, *p107*. It has been assumed that p107 simply substitutes for Rb1, quenching the activity of E2f transcriptional activators that induce genes required for division. We showed that the critical function of p107 is actually to regulate Cdk2, which it does by suppressing Skp2, part of a complex that degrades the Cdk2 inhibitor, p27 (Sangwan et al., 2012). Most
 15 importantly, our data suggest that the primary tumor suppressive function of p107 in an *Rb*-null tissue is to prevent the activation of Cdk2 by E2f-independent and possibly Skp2-dependent means. Indeed, we show that removing one allele of either E2f1 or Skp2 prevented retinoblastoma (**Figure 12**). Furthermore, Skp2 binds to T187-phosphorylated p27 and facilitates its polyubiquitylation by the SCF^{Skp2} complex and
 20 subsequent proteosomal degradation. We bred a knockin strain expressing a Skp2 resistant version of p27 (p27^{T187A}) with the mouse knockout model of retinoblastoma (*αCre;Rb^{fl}p107^{-/-}*) and found tumor initiation was also blocked (**Figure 12**). Western blot analysis of P8 retinal lysates showed that p27 protein levels were induced upon
 25 *Skp2* deletion. Additionally, p21 and Cyclin E—also targets of Skp2—were stabilized in *Skp2* null retinas (**Figure 12B**). Deletion of a single allele of *Skp2* was sufficient to inhibit tumor growth, and similarly, tumor penetrance in *Rb^{-/-};p107^{-/-};p27^{T187A}* retinas also dropped to 0% (**Figure 12C**). *Skp2* deletion had no effect on the 100% tumor penetrance in *Rb/p27* DKO retina, suggesting that Skp2 acts upstream of p27 (**Figure 12C**). Abnormally positioned mitoses in the newborn tumor-prone retina disappeared
 30 when E2f1 was removed, but Skp2 deletion had virtually no effect (**Figure 12D and E**). Rather than driving abnormal division, we show that Skp2 disrupted polarity/adhesion (**Figure 12 F and G**).

**Example 6. Atypical Pkc and the polarity components
are linked to tumor susceptibility**

The *p107* null mouse retina does not show a cell cycle defect, but we have discovered that tissue polarity is disrupted (**Figure 13**). We hypothesized, therefore, that in addition to the *E2f/Cdk2* requirement detailed above, polarity components may be linked to tumor susceptibility. We focused on the polarity enzyme atypical protein kinase C (*aPkc*)—Pkc lambda (λ), termed Pkc iota (ι) in humans and Pkc zeta (ζ) (**Figure 14**). In the *Rb* null mouse retina differentiating neurons exhibit delayed cell cycle exit, but never develop into tumors (**Figure 13**). Remarkably, *Rb/p107/aPkcλ* mice were tumor-free, and even removing one copy of *aPkcλ* had a dramatic protective effect (**Figure 15**). Tumour formation was completely blocked in *Rb/p107/aPkcλ* total knockout and even removal of just one allele of *aPkcλ* led to a 90% reduction in tumorigenesis (**Figure 15, also see Table**). Thus, like *E2f1* loss, reducing *aPkc* levels by removing one or both alleles of its gene, *aPkcλ* prevented RB.

To understand the cellular mechanisms at play, we analyzed early stages of development. While *aPkcλ* deletion had little effect on survival in the post-natal day 8 (P8) normal or *Rb* null retina, there was a high level of cell death in the *Rb/p107/aPkcλ* null retina (**Figure 16**). Thus, removing *aPkc* prevented RB because tumour-prone cells died. Additionally, we show that *aPkc* is required for tumor development in *Rb/p107* null retinas (**Figure 17**). When *aPkcλ* was removed from tumor-prone retina, a marked decrease in ectopic division was observed at P30. While no cell death was observed in normal wild type cells, apoptosis was enhanced in tumor-prone knockout retina (**Figure 17**). Furthermore, *aPKC* promotes survival in human RB cell lines (**Figure 18B and E**). When various strategies were used to knockdown *aPkc* levels in human RB cell lines, cell viability correlated with *aPkc* protein levels (**Figure 18 C, F**). Thus, like *E2f1* loss, reducing *aPkc* levels by removing one or both alleles of its genes prevented RB. Additionally, loss of *aPkc* triggered apoptosis specifically in tumor-prone retina, i.e., synthetic lethality.

Example 7. ATM kills tumor-prone, but not tumor-resistant retinal cells

The gold compound aurothiomalate (ATM) was recently identified in a screen for molecules that inhibit interaction between *aPkc* and the polarity regulator Par6 (Erdogan et al., 2006). To test ATM levels we used Inductively Coupled Plasma Atomic Emission Spectrometry (ICP AES), as described elsewhere (Xiao-quan et al.,

1987) and showed significant amounts of ATM in both serum and the retina after intraperitoneal delivery to adult mice (**Figure 19**).

To understand the mechanism underlying the prosurvival function of aPKC in retinoblastoma, we assessed apoptotic cell death. We show that ATM specifically enhanced cell death in *Rb/p107* tumor-prone retinal explants, but had little effect on survival in normal or tumor-resistant *Rb* null retina (**Figure 20**). ATM also induced death of human retinoblastoma cell lines *in vitro* (**Figure 21A-C**) and activated caspase 3/7 activity (**Figure 21D**). ATM was used for years in the clinic as a treatment for arthritis and is thus already approved for use in humans (Kean and Kean, 2008). These data suggest that the Par6-aPkc λ complex is critical for survival of tumor-prone or transformed but not normal cells, making it an ideal therapeutic target. In other words, aPkc is likely pivotal for the initiation and/or sustenance of RB-pathway tumors and thus are ideal targets to prevent these cancers. ATM is not a potent drug as μ M amounts are required, but its prior use in humans is an important advantage. A new aPkc λ inhibitor ICA-1, which is ~1000x more potent than ATM has been previously described (Pillai et al., 2011). The aPkc inhibitor ATM specifically kills tumor-prone explanted retinal cells and retinoblastoma cell lines *in vitro*, suggesting that *in vivo* studies and examination of more potent aPkc antagonists would yield similar conclusions.

Example 8. aPkc kinase activity and interaction with Par6 is essential for the prosurvival function of aPkc

We have shown that loss of aPKC triggers massive apoptosis in tumor-prone retina (**Figure 21**), uncovering a novel synthetic lethal interaction. To test whether aPkc is acting through the Par complex, we electroporated *Rb/p107*-null cells with either eGFP or a Par6K19A dominant negative mutant, which abolishes polarity complex function (**Figure 22A and B**). Strikingly, the Par6K19A dominant negative recapitulates substantial apoptosis (**Figure 16C**) as seen in *Rb/p107/Pkcl* total knockout (TKO) retina, suggesting that the synthetic lethal interaction is acting through the Par complex. Moreover, aPkc knockdown blocked growth of orthotopic human RB xenografts. Thus, aPkc is a prevention target for RB.

Example 9. Prevention and nascent tumor therapy

We tested the possibility that inhibiting E2f could prevent nascent retinoblastoma growth. *αCre;Rb^{fl}/p107^{-/-}* mice were treated orally every week from P28 to P56 with either vehicle or the E2f inhibitor, 6474. The mice were harvested a day after the last
 5 injection (P57). We show that 6474 reduces tumor volume by ~14-fold, thus providing evidence that inhibiting E2f halts nascent retinoblastoma growth *in vivo*.

Example 10. Tumor initiation network that leads to a tumor-prone state (Figure 24)

Rb loss delays cell cycle exit. Ectopic E2f1-dependent division stops eventually by RB-independent means. Other components of the dual axes (Skp2 and Cdk2) are also
 10 modestly elevated. These cells never form tumors. p107 loss lowers Rb pathway activity even further, resulting in elevated Skp2 levels and Cdk2 activity. E2f activity is essentially the same as in *Rb* null cells. Polarity and fate are disrupted. aPkc is engaged to promote survival. Most of these cells also exit the cell cycle eventually.
 15 But, this constellation of molecular events together create an initiation network that confers the potential for sporadic tumorigenesis. This step can be reversed by modestly reducing E2f, Cdk2 or aPkc activity, or by removing Skp2. An additional sporadic event generates retinoblastoma. We have compelling evidence that these four factors in the "tumor initiation network" are essential to drive retinoblastoma
 20 initiation that is critical for the tumor-prone state, thus uncovering a therapeutic window in the retinoblastoma pathway that can be exploited to block transformation.

DISCUSSION

Dual Axes Activation Creates Tumor-Prone Cells

Typically, cell cycle activity is loosely defined as normal or abnormal, with the latter
 25 being associated with cancer initiation. However, here we show that distinct quanta of E2f and Cdk dual axes activity are not limited to binary states, but that beyond deregulated proliferation, core cell cycle components engage at another previously unrecognized level to create the tumor-prone state (**Figure 6F, G**). WT retinal progenitors had the lowest dual axes activity, while ectopically dividing *Rb* null cells
 30 exhibited elevated E2f activity that was required for abnormal division, but was insufficient to create cancer susceptibility (this work and (Chen et al., 2007)). Previous

work showed that also removing p107 or p130 creates tumor susceptibility, and logically one would assume that this might function by enhancing E2f activity. However, we found that E2f target levels were similar in *Rb* or *Rb/p107* null cells, but in stark contrast Cdk2 activity was markedly induced in the latter tumor-prone scenario. Moreover, *Rb* null tumor-resistant cells could also be converted to the cancer-prone state by removing *p27*, the CKI function of *p27*, or disabling one allele each of *p107* and *p27*, and the degree of susceptibility to transformation in all these retinoblastoma models correlated tightly with Cdk2 activity. Thus, high E2f and Cdk activity are specific characteristics of tumor-prone retinal cells. It is well known that these factors are deregulated in multiple human cancers, but our data are the first to show that discrete quanta of E2f and Cdk create dual axes that distinguish three quantal states: normal division, ectopic division and an unexpected tumor-prone state. The surprising finding that the latter two states can be molecularly separated on the basis of E2f and Cdk activity led us to the hypothesis that lowering either axis might prevent tumor initiation but not affect normal division. Indeed, only brief (one week) exposure of tumor-prone fetuses to either E2f or Cdk inhibitors dramatically curtailed the subsequent appearance of retinoblastoma tumors without affecting normal progenitor proliferation. Such a stunning effect with only short-term drug exposure is, to our knowledge, unprecedented in the field of chemoprevention. The fact that this beneficial effect on tumorigenesis was observed despite only a modest drop in ectopic division and no reduction of normal division further indicates the existence of separate thresholds of dual axes activity. The concept of a third molecularly and phenotypically unique state of E2f and Cdk activity is unanticipated and provides a mechanistic explanation as to why they are often both deregulated in cancer (Malumbres and Barbacid, 2001).

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 (Lara et al., 2008a and 2008b). Akin to our *in vivo* findings, E2f-responsive reporter vectors show comparable activity in *Rb* or *Rb/p107*-null MEFs (Classon et al., 2000). Notably, Rb, but not p107/p130, inhibits E2f target expression during senescence (Chicas et al., 2010) 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 (Chicas et al., 2010), it is redundant with p130 (Hurford et al., 1997) there is feedback inhibition of E2f by Cdk2-mediated phosphorylation (Xu et al., 1994; Dynlacht et al., 1994) and/or it is already sequestered in other complexes (Lee et al., 2002).

- 5 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
- 10 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 *p27CK*– alleles behaved identically; (ii) Compound heterozygosity for *p107* and either *p27* or *p27CK*– cooperated with *Rb* loss to drive retinoblastoma; (iii) Cdk2 activity correlated with
- 15 tumor penetrance across these multiple models and (iv) Short-term exposure to a CKI prevented tumorigenesis in *Rb/p107* and *Rb/p27*-null retinas. The ability of Rb pathway and CKI defects to cooperate is well known (Brugarolas et al., 1998; Park et al., 1999; Franklin et al., 1998) and we now extend this pattern to the retina where the focus had been primarily on Rb-E2f regulation.
- 20 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 the activation of Cdk2 by E2f-independent and possibly Skp2-dependent means. These data justify further examination of the mechanism by which p107 might regulate Skp2
- 25 and cross talk to Cdk2, and expose potent chemopreventive strategies for Rb pathway-initiated tumors. Cdk2 is an important cell cycle regulator that fires DNA replication origins. Skp2 is required for *Rb*-initiated mouse pituitary but not thymic tumors (Wang et al., 2010). In support of *in vivo* relevance for aPkc beyond retinoblastoma, this kinase is also required for lung tumors initiated by K-ras activation
- 30 (Regala et al., 2009). Traditional mouse models of RB delete *Rb1* and its relative, *p107*. It has been assumed that p107 simply substitutes for Rb1, quenching the activity of E2f transcriptional activators that induce genes required for division. We showed that the critical function of p107 is actually to regulate Cdk2, which it does by suppressing Skp2, part of a complex that degrades the Cdk2 inhibitor, p27 (Sangwan

et al., 2012). We could also induce mouse RB by combining *Rb1* loss with inactivation of the Cdk inhibitor *p27* or one allele each of *p27* and *p107*. Moreover, removing only one allele of *E2f1* or *Skp2* prevented RB, but whereas *E2f1* was required for abnormal division, *Skp2* was essential for the polarity defect. Furthermore, *Skp2* binds to T187-phosphorylated *p27* and facilitates its polyubiquitylation by the SCF^{skp2} complex and subsequent proteosomal degradation. We bred a knockin strain expressing a *Skp2* resistant version of *p27* (*p27*^{T187A}) with the mouse knockout model of retinoblastoma (*αCre;Rb^{fl/fl}p107^{-/-}*) and found tumor initiation was also blocked. Moreover, deletion of a single allele of *Skp2* was sufficient to inhibit tumor growth. *Skp2* deletion had no effect on the 100% tumor penetrance in *Rb/p27* DKO retina, suggesting that *Skp2* acts upstream of *p27*. Finally, rather than driving abnormal division, we show that *Skp2* disrupted polarity/adhesion. Thus, our data suggest that *Skp2* is essential for retinoblastoma.

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 as shown by 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 (**Figure 4A**). An alternate possibility is that *p130* affects *p27* levels, either through *Skp2* or another mechanism.

20 **Cross-talk inhibition buffers tumor susceptibility**

Our work shows that the organisation of *E2f* and Cdk activity into quantal states creates an important buffer to prevent the emergence of tumor-prone cells. Thus, while *Rb* loss in the mouse retina activated *E2f*, *p107* constrained cross-activation of *Cdk2*, apparently by lowering *Skp2* levels. Unlike the mouse retina, the human retina is exquisitely sensitive to *Rb* defects, and *Rb*^{+/-} individuals inevitably develop retinoblastoma following inactivation of the wild type allele during fetal development. The underlying reason for this unusual sensitivity has been a long-standing puzzle in the field. Our data raise the new possibility that negligible levels and/or activity of *p107* or *p130* in the human retinoblastoma cell-of-origin may weaken the buffer that separates *E2f* and Cdk axes. In addition, CKI levels/activity may be dangerously low in the human retinoblastoma cell-of-origin as we showed that *Rb* + *p27* loss mimics *Rb* + *p107* loss in the mouse retina. In cells with intrinsically low levels or activity of *p107/p130* and/or CKIs, *Rb* loss alone would be sufficient to raise *Skp2* levels, reduce

p27 further and thus efficiently cross-activate Cdk2 (**Figure 1A**). In this regard, it is intriguing that the mouse pituitary shows the same sensitivity to *Rb* loss as the human retina (Jacks et al., 1992) and we propose that the vulnerability in both these mammalian tissues reflects an unusually weak inhibitory buffer between the E2f and Cdk axes. In support of this model, Skp2 is essential for the development of pituitary tumors in the *Rb*^{+/-} mouse, and for the growth of human retinoblastoma cell lines (Wang et al., 2010). Moreover, Skp2 is required for the development of retinoblastoma in the *Rb/p107* null mouse retina. Skp2 however is not required for all cancers, including thymic tumors seen in *Rb*^{+/-} mice, ENU induced tumors, and Myc induced lymphomas (Old et al., 2010; Wang et al., 2010). Finally, it is notable that studies in *Drosophila* showed that while inducing either E2f or Cdk activity drives temporary ectopic proliferation, extended abnormal division requires activation of both, and can be achieved by interrupting negative regulation by cross-talk inhibitors (Buttitta et al., 2010; Buttitta et al., 2007). Furthermore, while strong buffering between the E2f-Cdk dual axes would be most critical to prevent the acquisition of a tumor-prone state in tissues that divide throughout life, tissues that exhibit little or no division after embryogenesis may have evolved weak buffering, thus making them much more susceptible to cancer. Notably, the retina and pituitary fit the latter description perfectly. It is likely that inducing the dual axes is an early requirement to engender cancer susceptibility in many tissues, and that poor cross-talk inhibition makes it unusually easy to exceed the necessary thresholds for both axes in the human 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 (Jacks et al., 1992) suggesting it also has unusually higher level of E2F and CDK activity. Finally, although inducing E2f or Cdk activity drives temporary ectopic proliferation in *Drosophila*, extended abnormal division requires both (Buttitta et al., 2007). Thus, a buffer that limits E2f to Cdk positive feedback regulation may be critical to avoid tumorigenesis in

all animals.

Synthetic lethality of defects in the RB pathway: aPKC, Skp2 and polarity complex in the initiation of retinoblastoma

If disrupting gene A and B together causes cell death but disrupting either alone does not this effect is termed "synthetic lethality". Oncogenic defects drive cancer, and thus a major goal in therapeutics is to find targets that are synthetically lethal with these mutations. The purpose is to kill tumor cells without affecting their normal neighbours. We showed that in addition to the E2f/Cdk2 requirement detailed above, polarity components are linked to tumor susceptibility. In the *Rb* null mouse retina differentiating neurons exhibit delayed cell cycle exit, but never develop into tumors. The *p107* null mouse retina does not show a cell cycle defect, but we have discovered that tissue polarity is disrupted (**Figure 13**). Abnormal division and defective polarity occur together only in the tumor prone *Rb/p107* null retina, not in tumor-resistant *Rb* or *p107* null retinas. We generated microarray data from 21 human and 18 mouse RB tumours (from 6 different genetic models) and profiles from normal human and mouse fetal retina mRNA. One of the hits was atypical protein kinase C, and we obtained striking parallel evidence that this is a critical hub for retinoblastoma initiation. aPkc regulates epithelial cell polarity and we noted a polarity defect specifically in mouse retinas prone to RB. Removing aPkc prevented retinoblastoma because tumor-prone cells died, and this synthetic lethality was phenocopied by interfering with the aPkc polarity partner Par6. Moreover, aPkc knockdown blocked growth of orthotopic human RB xenografts. Thus, aPkc is a prevention target for retinoblastoma. Aurothiomalate (ATM), formerly used to treat arthritis, inhibits aPkc-Par6 binding¹³, and notably this drug also caused synthetic lethality specifically in tumor-prone but not tumor-resistant retina. Indeed, *Rb/p107/aPkc1* mice were tumor-free, and even removing one copy of *aPkc* had a dramatic protective effect. Moreover, we found that ATM specifically enhanced death in *Rb/p107* tumor-prone retinal explants, but had little effect on survival in normal or tumor-resistant *Rb* null retina. Finally, we found that ATM also induced death of human retinoblastoma cell lines *in vitro*. We therefore show that the aPkc-Par6 complex is critical for survival of tumor-prone or transformed but not normal cells, making it an ideal target for prevention of retinoblastoma.

Clinical Relevance

Fully transformed cancer cells are complex, heterogeneous, and adaptable, making

drug resistance a major hurdle to durable cure. The ideal solution would be to prevent cancer at its inception, before cells become highly adaptable. Chemoprevention is a growing field with important successes (Kelloff et al., 2006). It has broad relevance, but is particularly critical in the case of high risk, cancer prone populations. 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 (Kelloff et al., 2006). 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 (Lindor et al., 2008). In addition, effective chemopreventative strategies could also benefit cancer survivors, who are at higher risk of secondary tumors (Lindor et al., 2008). The systematic assessment of small molecules for chemoprevention lags far behind that for therapy. Hopes that some drugs already in the clinic for other indications (e.g. Statins) might lower cancer incidence have remained largely unfulfilled. These epidemiological studies were not based on mechanistic dissection of cancer initiation, nor were the drugs tested in genetic models of cancer initiation. We assert that a genomics perspective on the early stages of cancer *in vivo* is pivotal to discover targets to prevent neoplastic transformation.

We exploited our discovery of a tumor-prone state to prevent cancer in an *in vivo* genetic model of tumorigenesis. We prevented cancer in an *in vivo* model of tumorigenesis using various approaches, including distinct small molecule therapies, and with only 1 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 immunodeficient hosts. Most obviously, they raise the exciting notion that *Rb*^{+/-} patients, many of whom die of secondary tumors (Yu et al., 2009), may benefit from the preventive therapy we show is so potent in retina. Moreover, because the Rb pathway is disrupted in many cancers and because elevated E2f and Cdk activity is essentially a universal feature of human tumors, our chemopreventive strategies may be relevant to many familial cancer syndromes, of which >50 are known (Lindor et al., 2008). Although Cdk2 is dispensable for tumorigenesis in *p27*, *p21* or *p53*-null mice (Padmakumar et al., 2009; Martin et al., 2005; Tetsu et al., 2003), it acts redundantly with Cdk1 (Santamaria et al., 2007), thus the success of our chemotherapeutic strategy likely reflects the inhibition of both kinases. 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. 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
5 transformation versus normal cell cycle progression. Furthermore, we report the first successful application of any E2f inhibitor *in vivo* and the remarkable efficacy achieved indicates that, like Cdk, E2f is an important chemopreventative target. Therapeutic trials with Cdk2 inhibitors in human cancer have been largely unsuccessful (Malumbres and Barbacid, 2009), but we suggest that their real benefit may lie in
10 preventive strategies in individuals with genetic susceptibility to a range of cancers. Indeed, recent work using Cdk inhibitors in genetic models of colon cancer support this conclusion (Boquoi et al., 2009).

Using a sporadic mutation strategy that mimics human cancer, we have shown the general relevance of Cdk2, aPkc and Skp2 to retinoblastoma pathway tumors. The
15 implications of these studies for the prevention of human cancer are considerable given the frequency of RB pathway defects in multiple types of malignancy (Malumbres and Barbacid, 2001). Notably there are strikingly distinct cellular effects of lowering Cdk, E2f1 (division), Skp2 (apical polarity) or aPkc (synthetic lethality) in tumour-prone retina. We have compelling evidence that these four factors in the
20 "tumor initiation network" are essential to drive retinoblastoma initiation and represent a therapeutic window in the Rb pathway that can be exploited to block transformation (**Figure 24**). Cdk, E2f, Skp2 and/or aPkc are pivotal for the initiation and/or sustenance of RB pathway tumors and are ideal targets to prevent these cancers.

Currently, there are no targeted treatments for Rb pathway tumors. We identify four
25 oncogenes critical for retinoblastoma, all of which can be targeted pharmaceutically. Suppressing or abolishing one of these four factors in this network in the RB pathway thus represents a feasible strategy to prevent retinoblastoma.

Although preferred embodiments of the invention have been described herein, it will be understood by those skilled in the art that variations may be made thereto without
30 departing from the spirit of the invention or the scope of the appended claims. All documents disclosed herein, including those in the following reference list, are incorporated by reference.

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5 *Dev* 15: 386-391

CLAIMS:

1. A method of preventing retinoblastoma in a subject comprising inhibiting the RB pathway.
2. The method of claim 1, wherein the inhibiting the RB pathway comprises
5 inhibiting at least one of Cdk, E2f, atypical protein kinase C, and Skp2.
3. The method of claim 1, wherein the inhibiting comprises administering to the subject an effective amount of an RB pathway inhibitor.
4. The method of claim 3, wherein the RB pathway inhibitor is selected from the group consisting of a Cdk inhibitor, an E2f inhibitor, an atypical protein kinase C
10 inhibitor, and a Skp2 inhibitor.
5. The method of claim 4, wherein the subject has cells with mutated Rb or cyclin-dependent kinase inhibitor, preferably p27, and the RB pathway inhibitor is a Cdk inhibitor or an E2f inhibitor.
6. The method of claim 4, wherein the Cdk inhibitor inhibits at least one of Cdk1
15 and Cdk2.
7. The method of claim 6, wherein the Cdk inhibitor inhibits both Cdk1 and Cdk2.
8. The method of method of claim 4, wherein the E2f inhibitor inhibits at least one of E2f1, E2f2 and E2f3.
9. The method of claim 8, wherein the E2f inhibitor inhibits E2f1.
- 20 10. The method of claim 4, wherein the E2f inhibitor is selected from the group consisting of 4-hydroxynonenal, E2f aptamer, Eugenol, thymoquinone, HLM006474, and Lithium; preferably Eugenol, thymoquinone, HLM006474 and Lithium; further preferably HLM006474 and Lithium.
- 25 11. The method of claim 4, wherein the Cdk inhibitor is selected from the group consisting of Flavopiridol, SNS-032 (derivative 4), BS-181 hydrochloride, Indirubin, PHA-703887, [Ala92]-p16 (84-103), P1446A-05, SNS-032, AZD5438, BAY80-3000, JNJ7706621, SCH 727965, CDKI-71, Bohemine, RO-3306, (R)-DRF053 dihydrochloride, SU 9516, WHI-P180, Hydrochloride, CVT-313,

- GW8510, (R)-DRF053, NU6140, Cdc2-Like Kinase Inhibitor, TG003, Hymenidin, Iso-olomoucine, Indirubin-3'-monoxime-5-sulphonic Acid, Butyrolactone I, Indirubin-3'-monoxime, Purvalanol A, BML-259, PNU 112455A hydrochloride, N9-Isopropyl-olomoucine, WR 216174, Olomoucine, 5 Dimethylamino-, Olomoucine II, Olumoucine, NU6027, NU6102, NSC 625987, NU2058, N-6-(δ 2-Isopentenyl)-adenine, Isogranulatimide, Benfluorene, Kenpauillone, Arcyriaflavin A, Aminopurvalanol A, Alsterpauillone, 2-Cyanoethyl, Aloisine, RP106, Aloisine A, 9-Cyanopauillone, 5-Iodo-indirubin-3'-monoxime, 2-Hydroxybohemine, NSC43042, NSC63002, 3-ATA, 2-Bromo-12,13-dihydro- 10 5H-indolo[2,3-a]pyrrolo[3,4-c]carbazole-5,7(6H)-dione, xylocydine, Ibulocydine, pyrazolo[1,5-a]pyrimidine derived compound, 4k (BS-194), AT7519, CYC202 (roscovitine), R547, P276-00, AG-025322, PD-0332991, CYC065, SNS-032, AT7519, CYC202 (roscovitine) and R547; preferably CYC065, SNS-032, AT7519, CYC202 (roscovitine) and R547; further preferably CYC202 15 (roscovitine) and R547.
12. The method of claim 4, wherein the subject has cells with mutated or otherwise inactivated Rb or Rb family protein, preferably p130 or p107, and the RB pathway inhibitor is an atypical protein kinase C inhibitor or a Skp2 inhibitor.
13. The method of claim 12, wherein the atypical protein kinase C inhibitor is 20 aurothiomalate.
14. The method of claim 12, wherein the atypical protein kinase C inhibitor is ICA-1.
15. Use of an RB pathway inhibitor for the prevention of retinoblastoma in a subject.
- 25 16. Use of an RB pathway inhibitor in the preparation of a medicament for the prevention of retinoblastoma in a subject.
17. An RB pathway inhibitor for use in the prevention of retinoblastoma in a subject.
18. A method of determining the likelihood of a population of cells to be cancer- 30 prone, comprising:

determining the level of E2f and Cdk activity in the population of cells,

wherein a high level of both E2f and Cdk activity in the population compared to a control population is indicative of the population being cancer-prone.

19. The method of claim 17, wherein the level of E2f and Cdk activity is determined
5 using one of Western blot, realtime PCR or RT PCR and kinase assay.
20. The method of claim 17, further comprising preventing retinoblastoma by administration of a Cdk inhibitor or an E2f inhibitor.
21. A method of determining the likelihood of a population of cells to be cancer-prone, comprising:
- 10 determining the level of atypical protein kinase C activity in the population of cells,
- wherein a high or abnormal level of atypical protein kinase C in the population compared to a control population is indicative of the population being cancer-prone.
- 15 22. A method of determining the likelihood of a population of cells to be cancer-prone, comprising:
- determining the level of Skp2 activity in the population of cells,
- wherein a high or abnormal level of both Skp2 activity in the population compared to a control population is indicative of the population being cancer-prone.
- 20

Figure 1

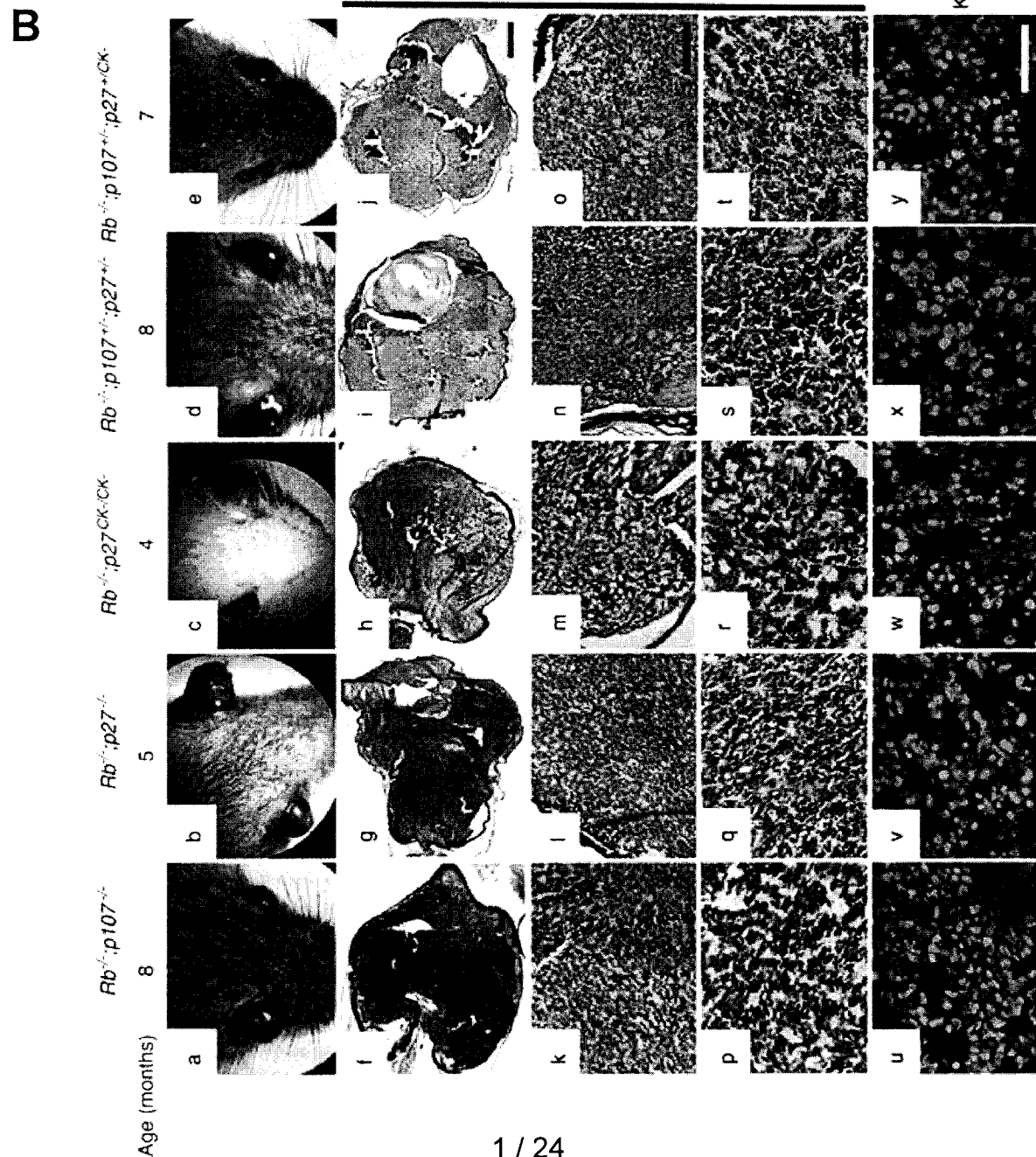
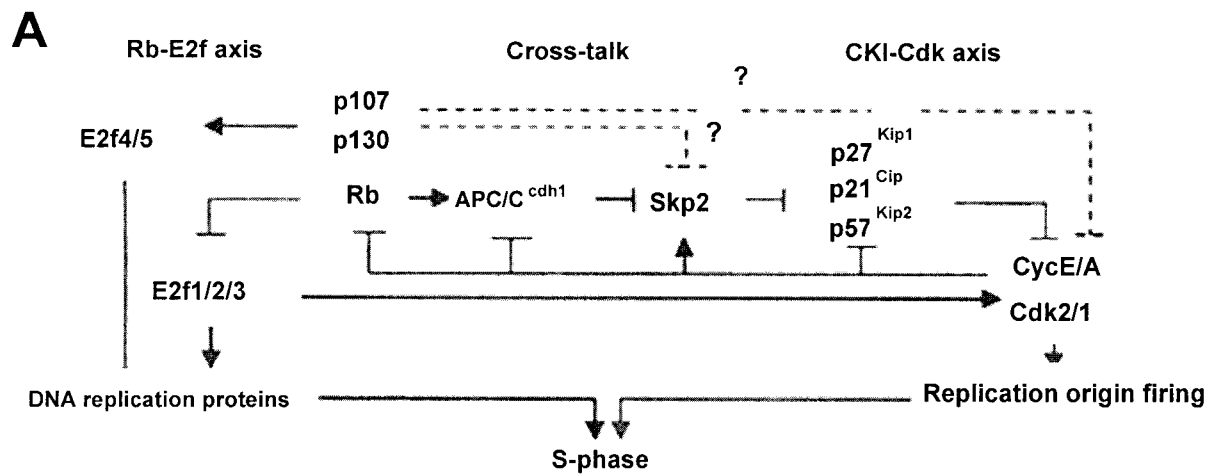


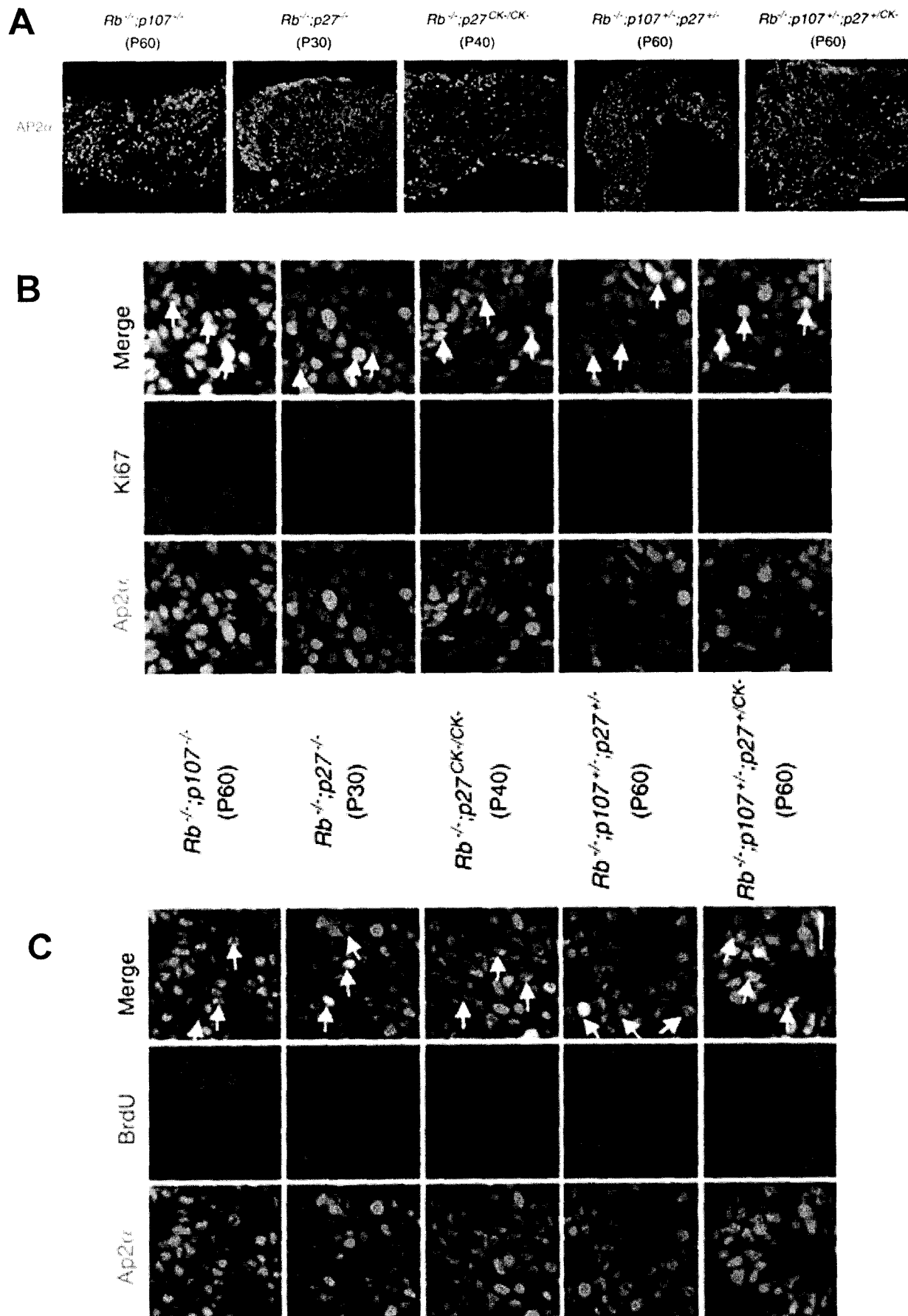
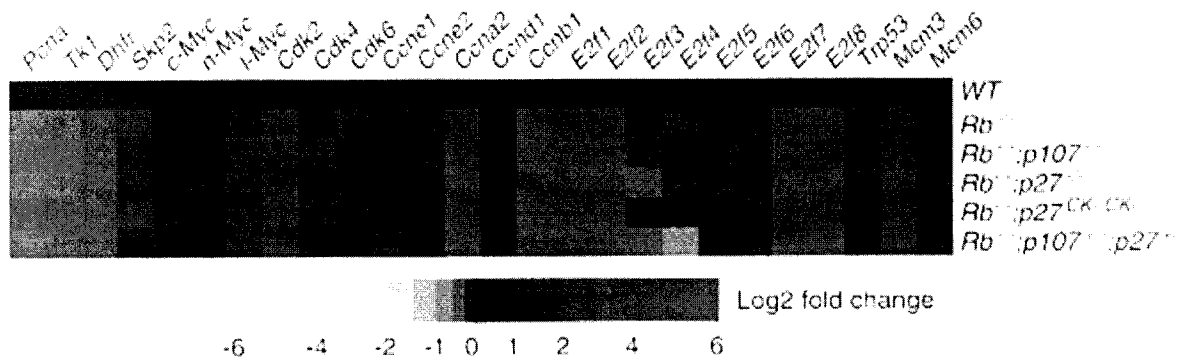
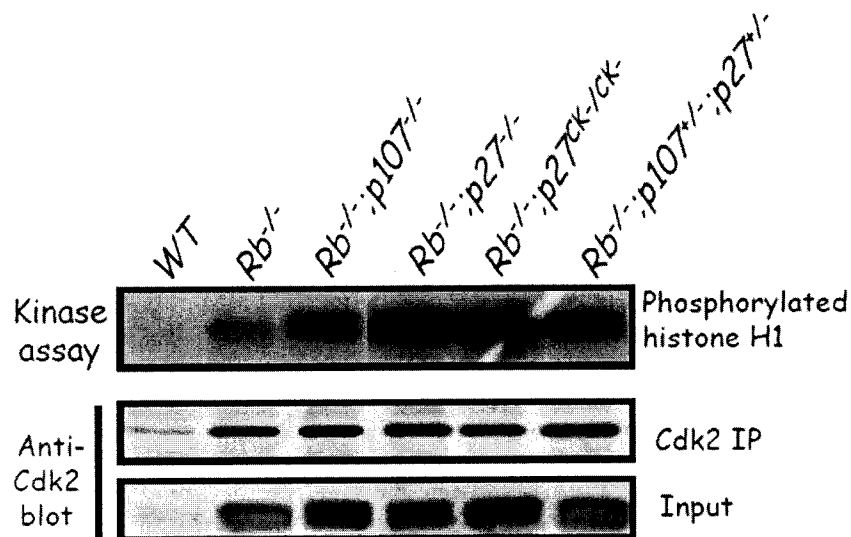
Figure 2

Figure 3

A



B



C

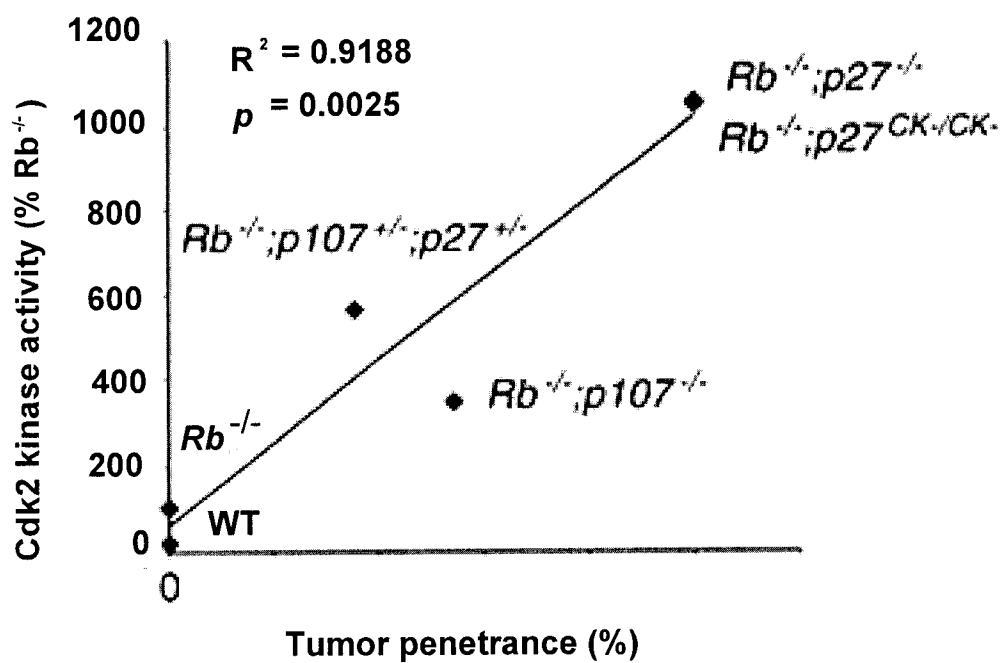
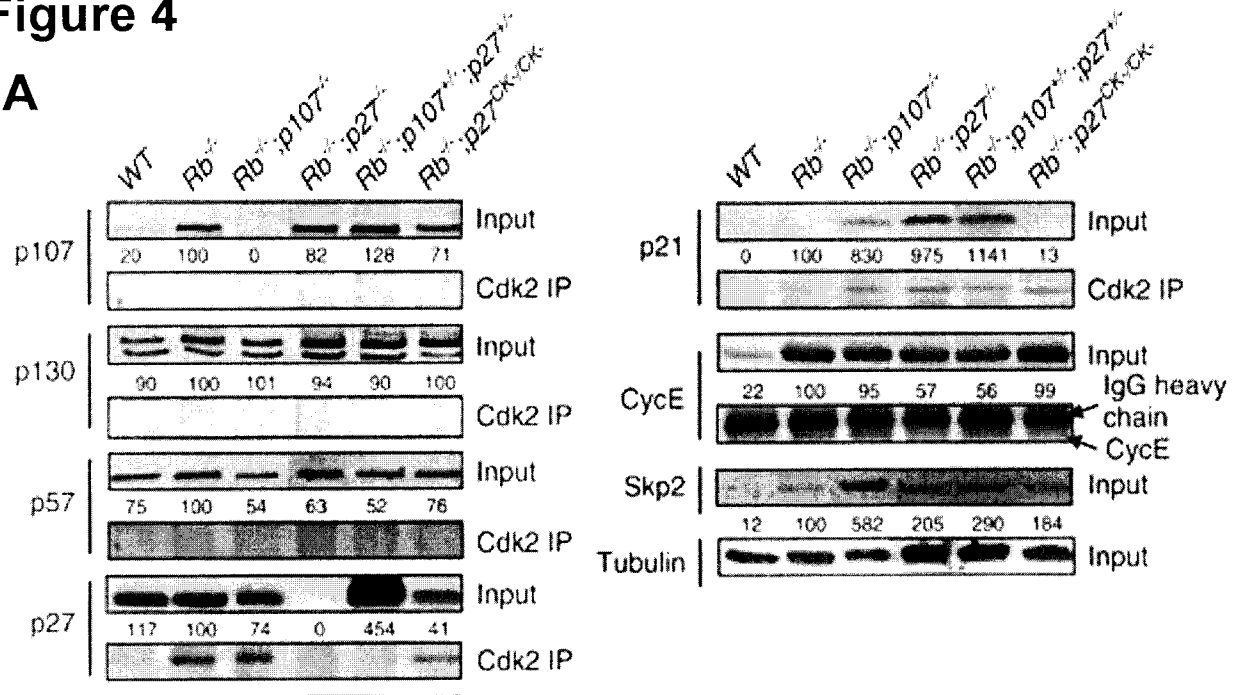
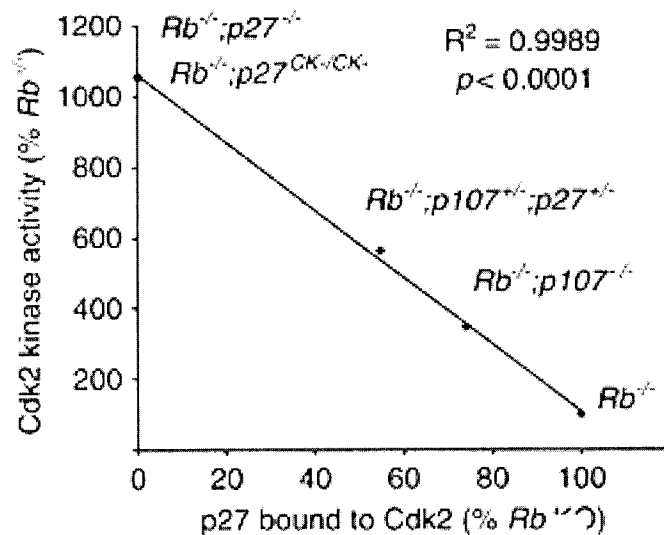


Figure 4

A



B



C

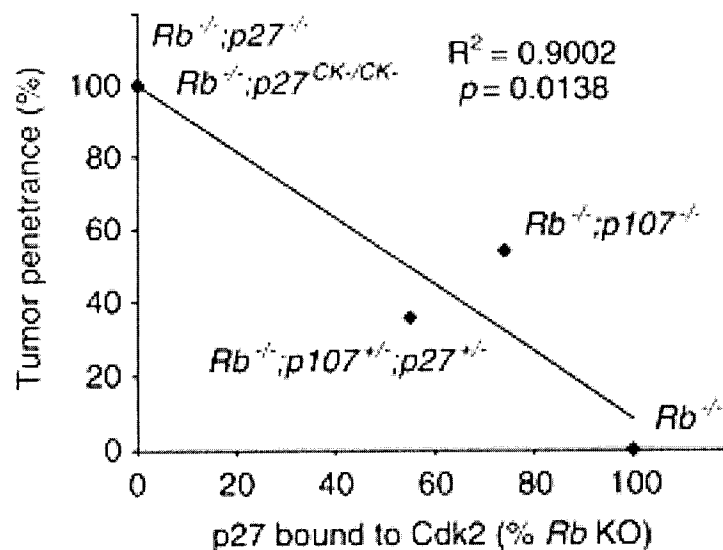
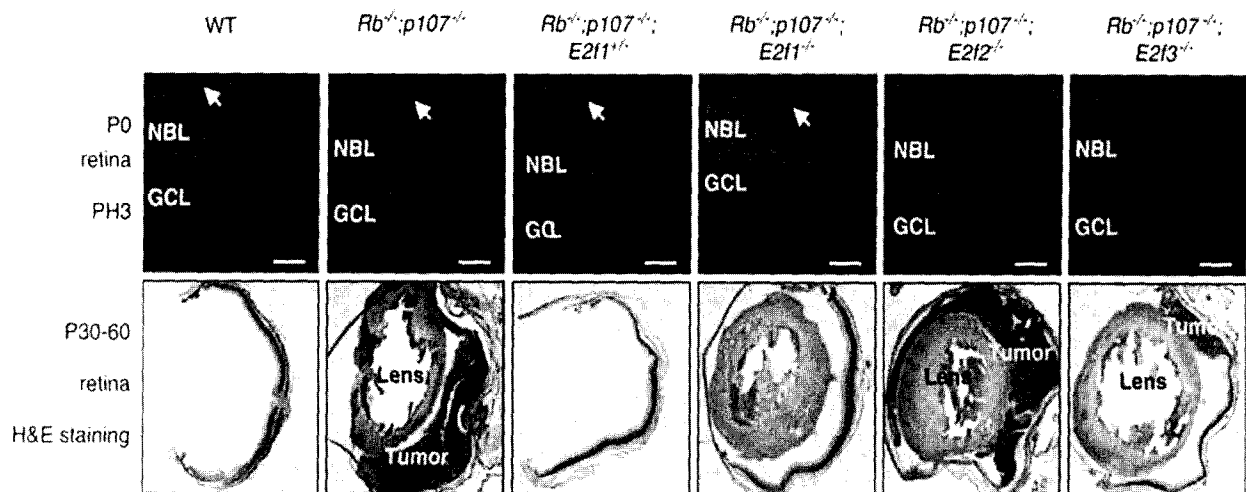


Figure 5

A



B

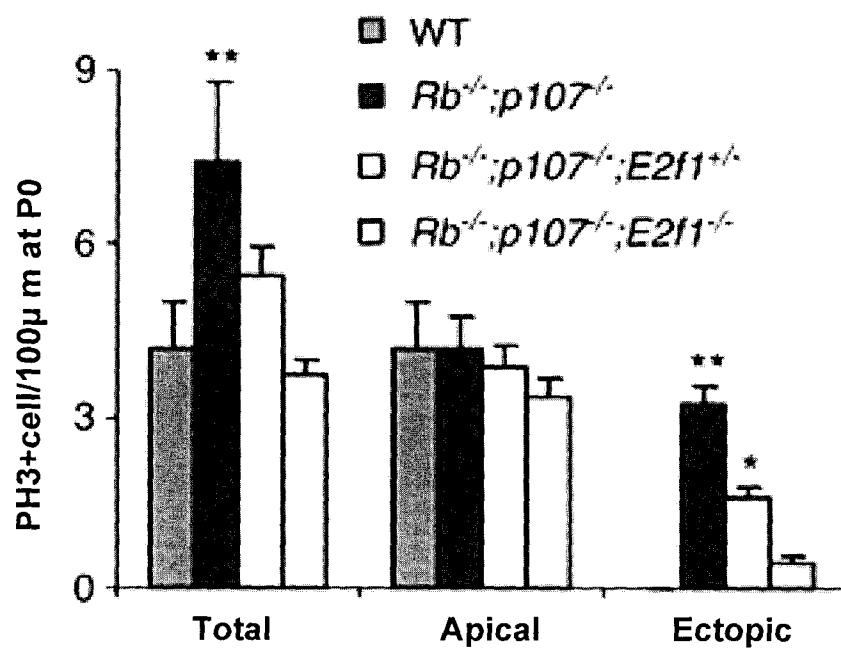
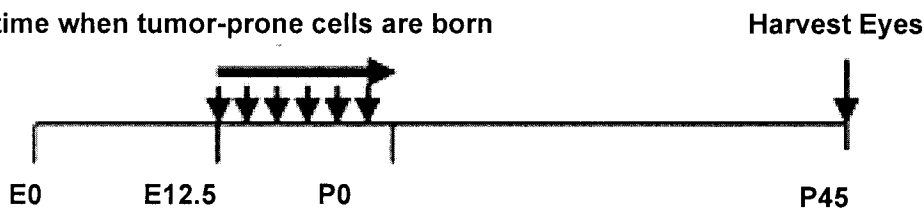
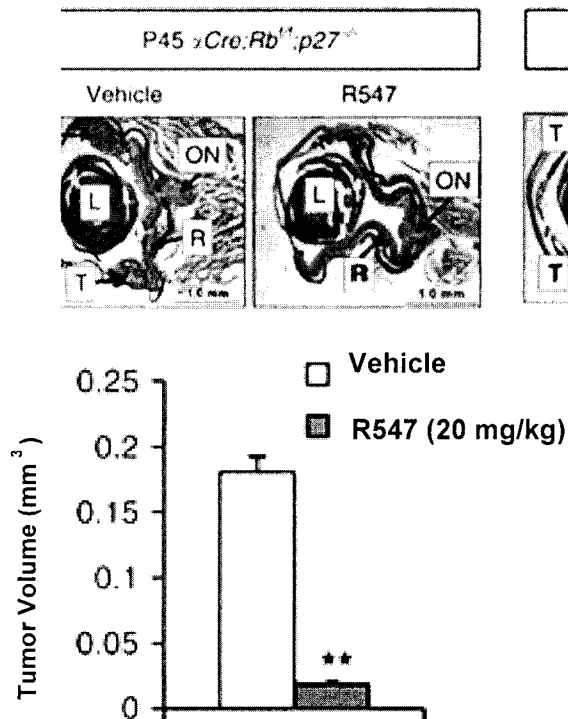


Figure 6

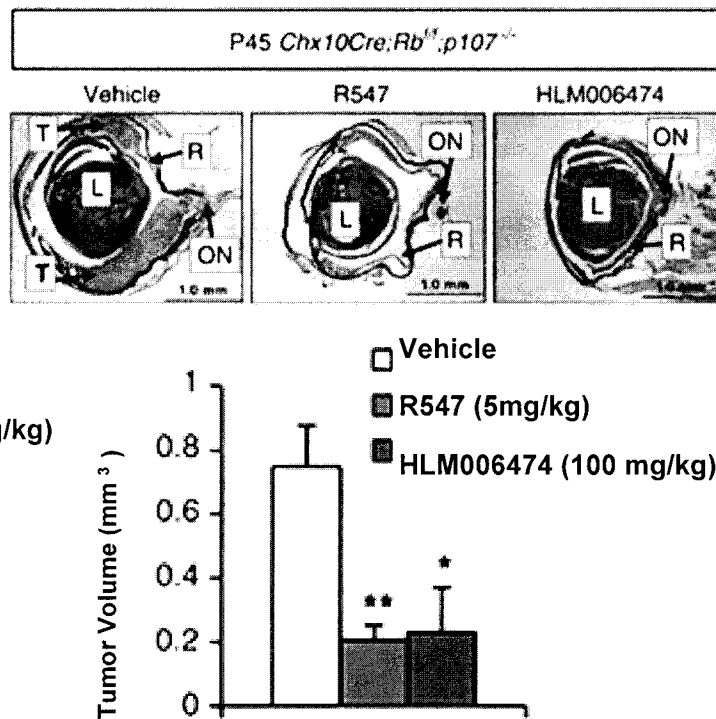
A Daily IP injection to pregnant dam during time when tumor-prone cells are born



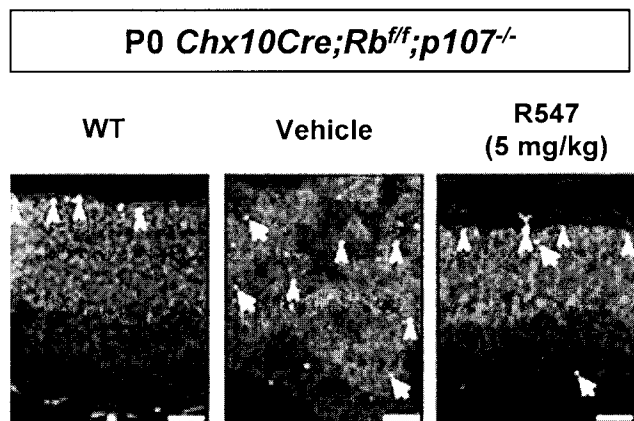
B



C



D



E

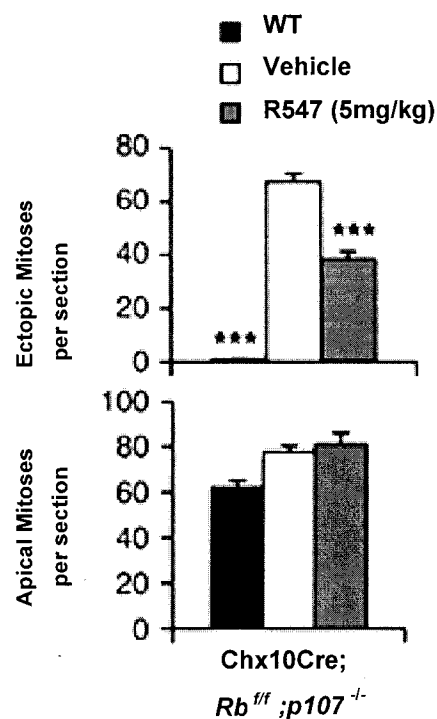


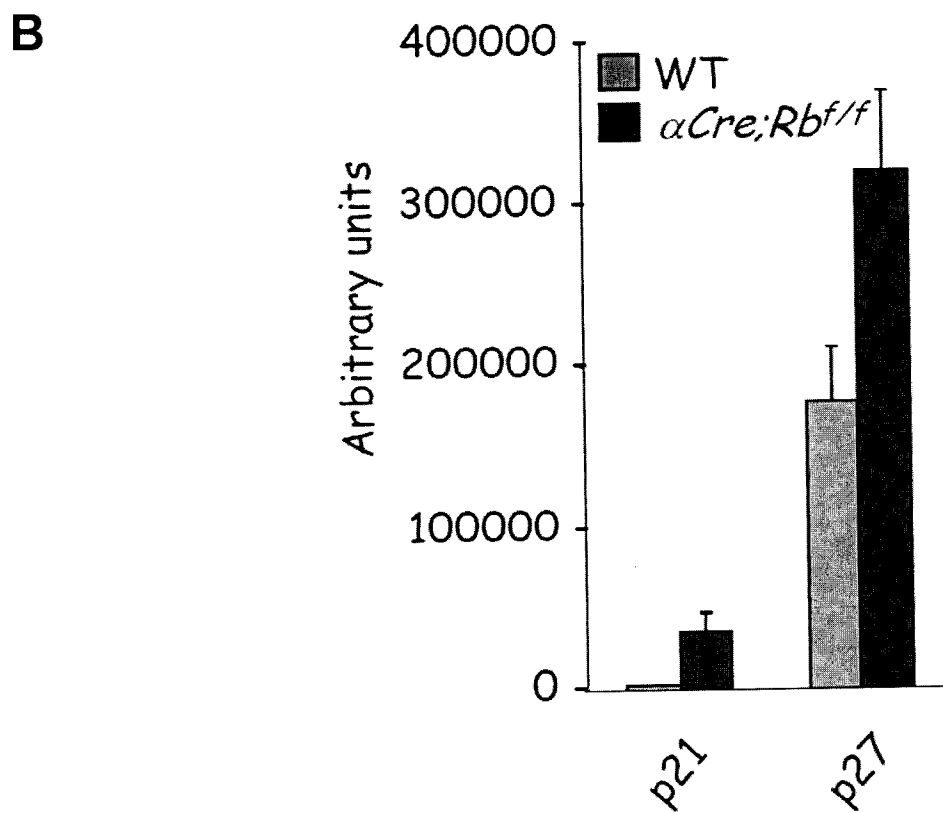
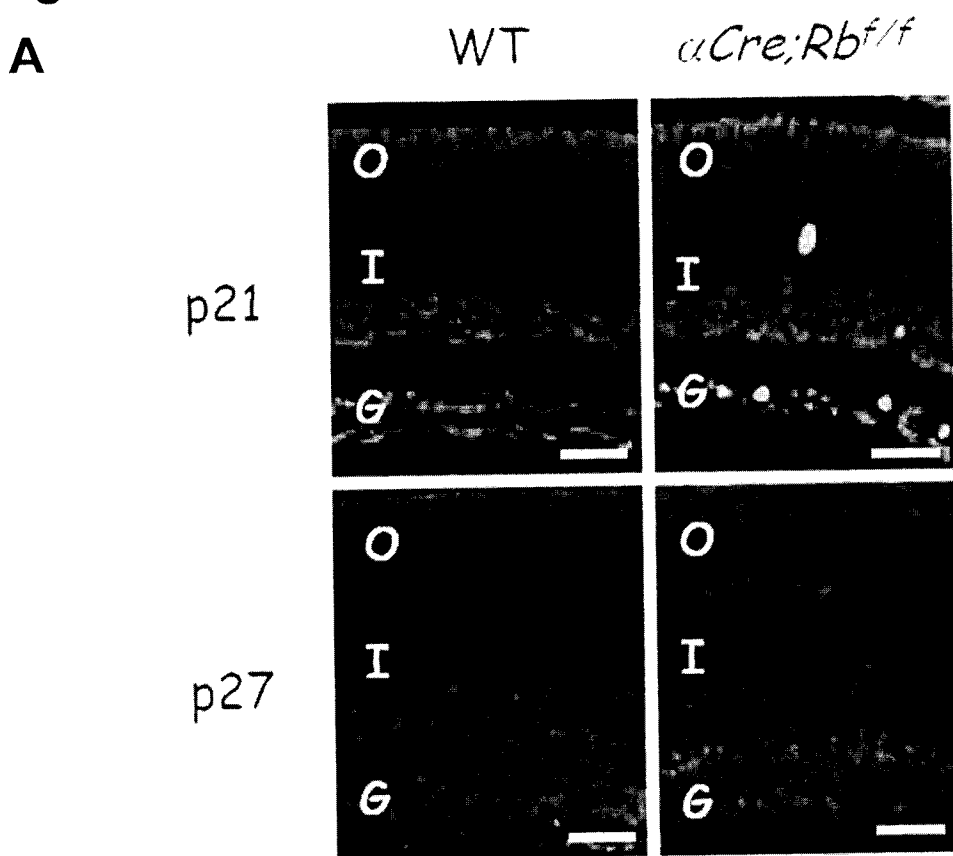
Figure 7

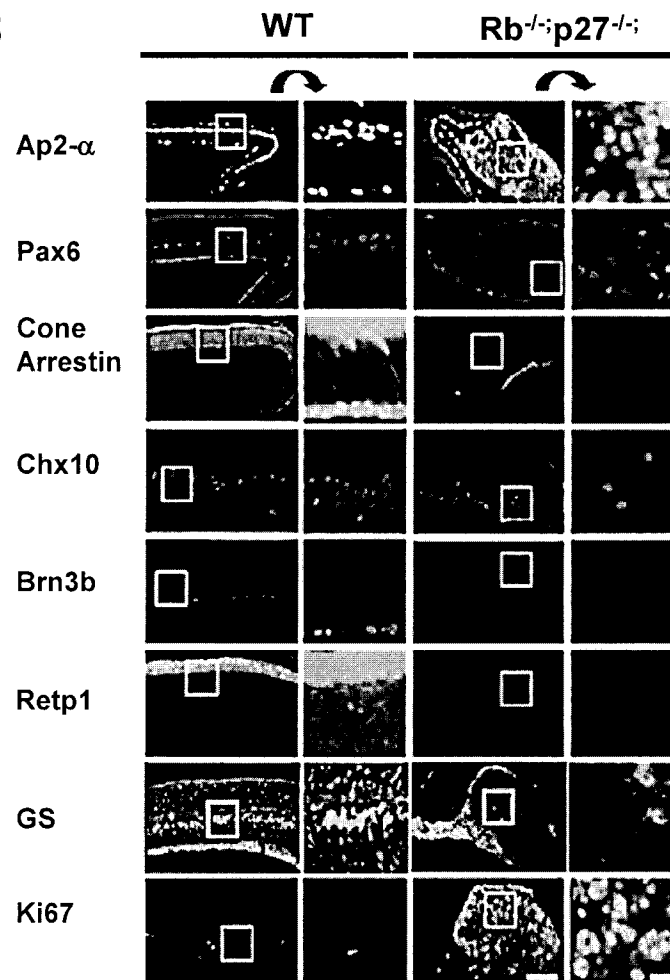
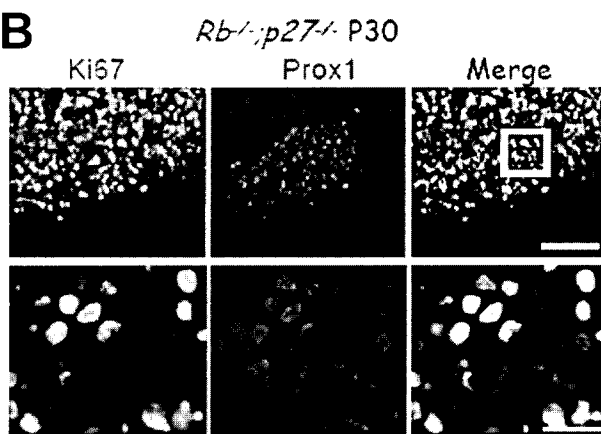
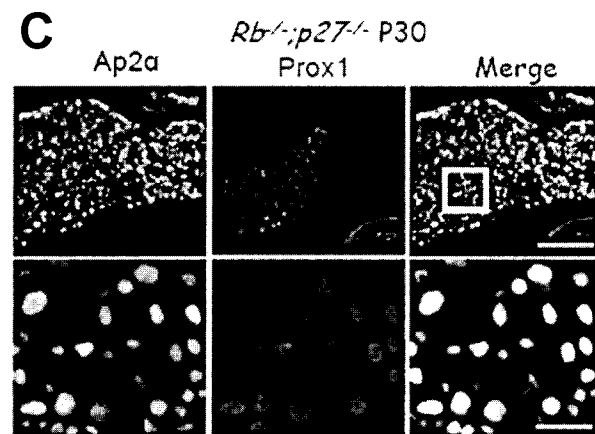
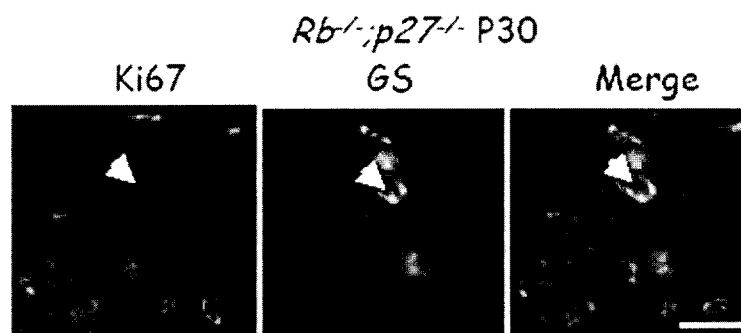
Figure 8**A****B****C****D**

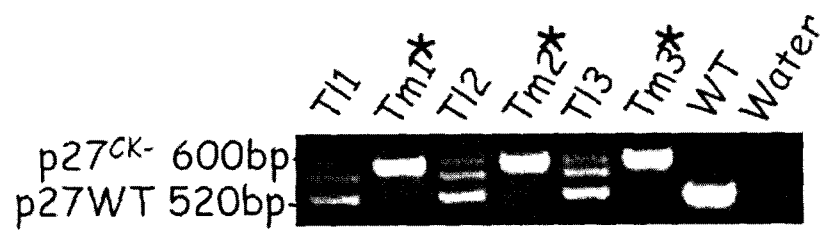
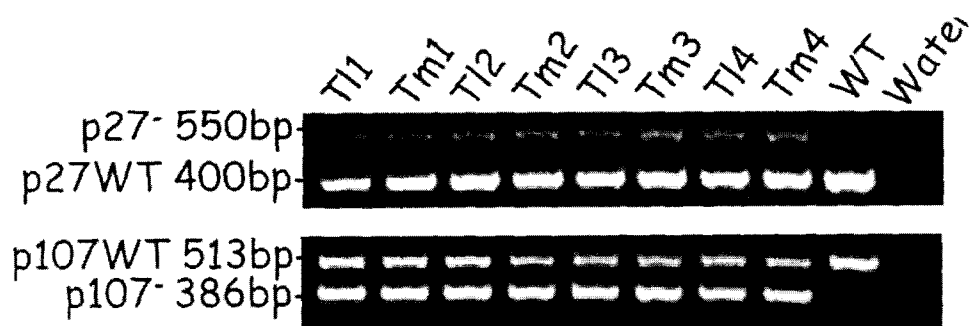
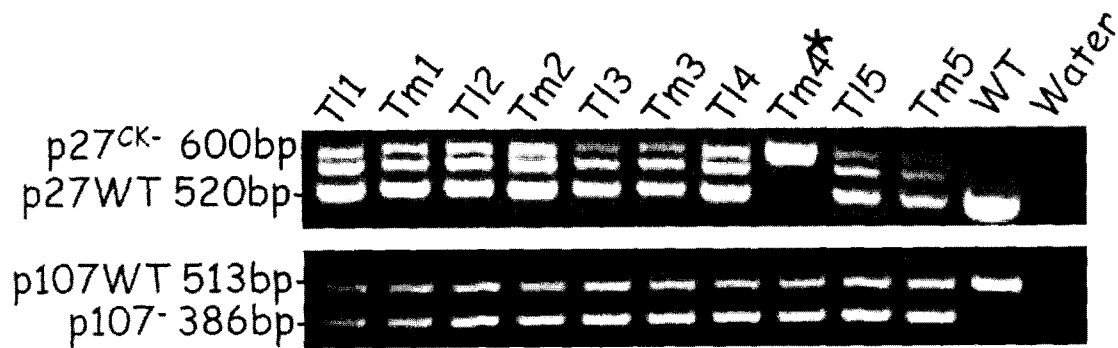
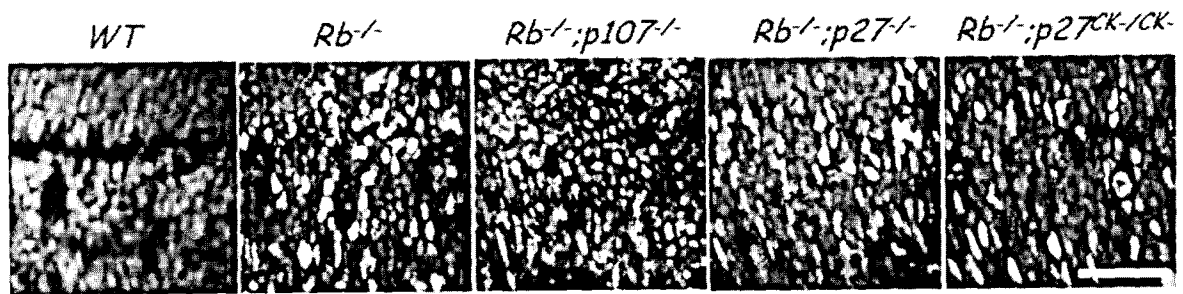
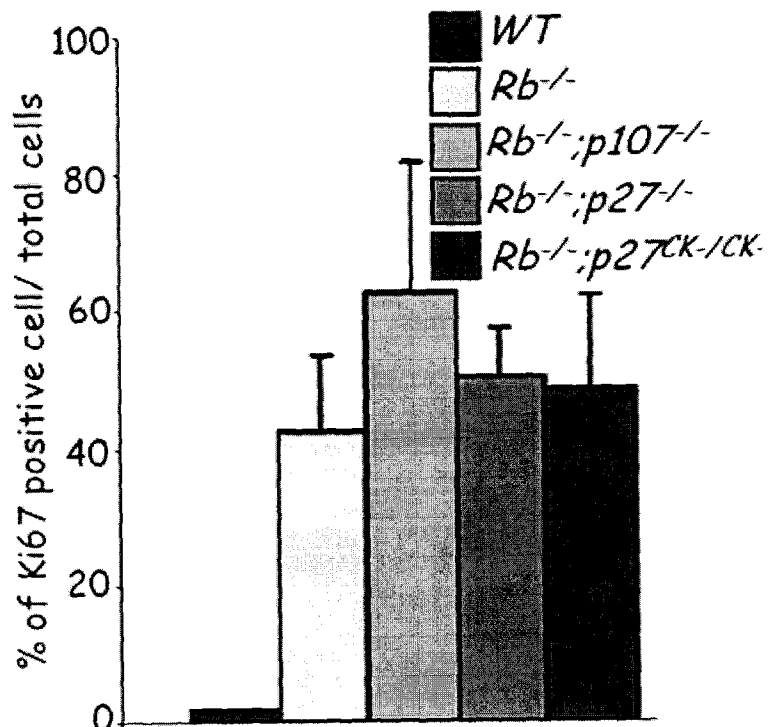
Figure 9**A****B****C**

Figure 10

A



B



C

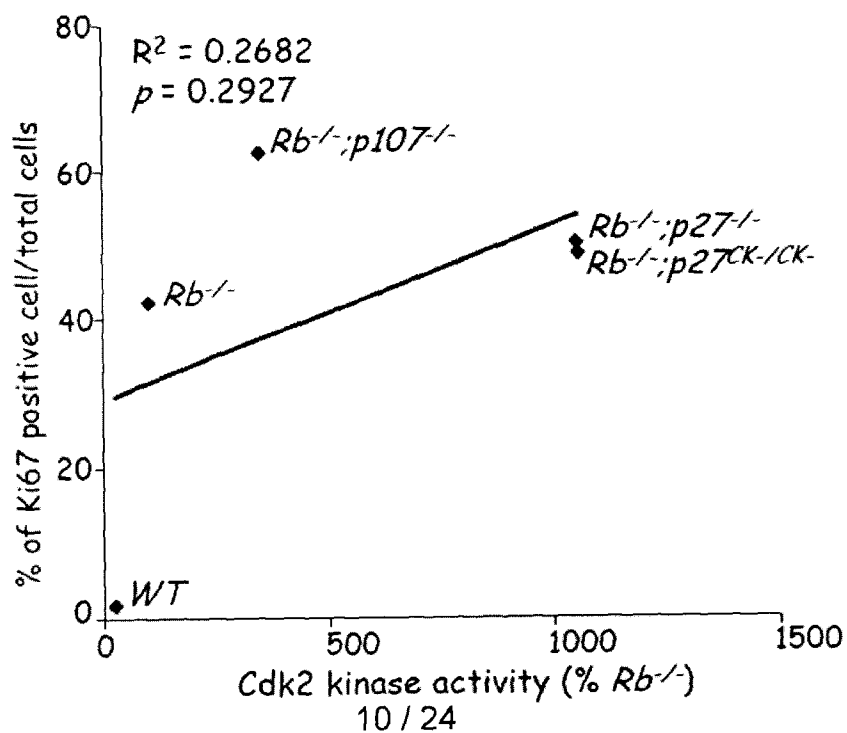


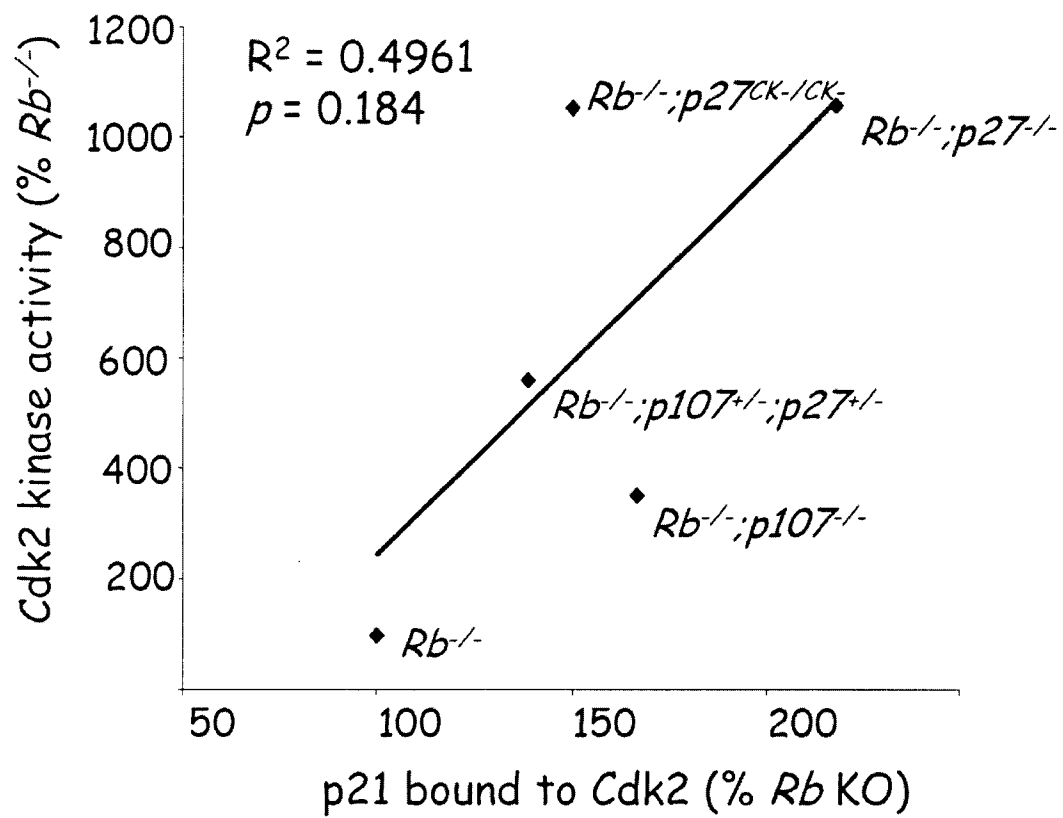
Figure 11

Figure 12

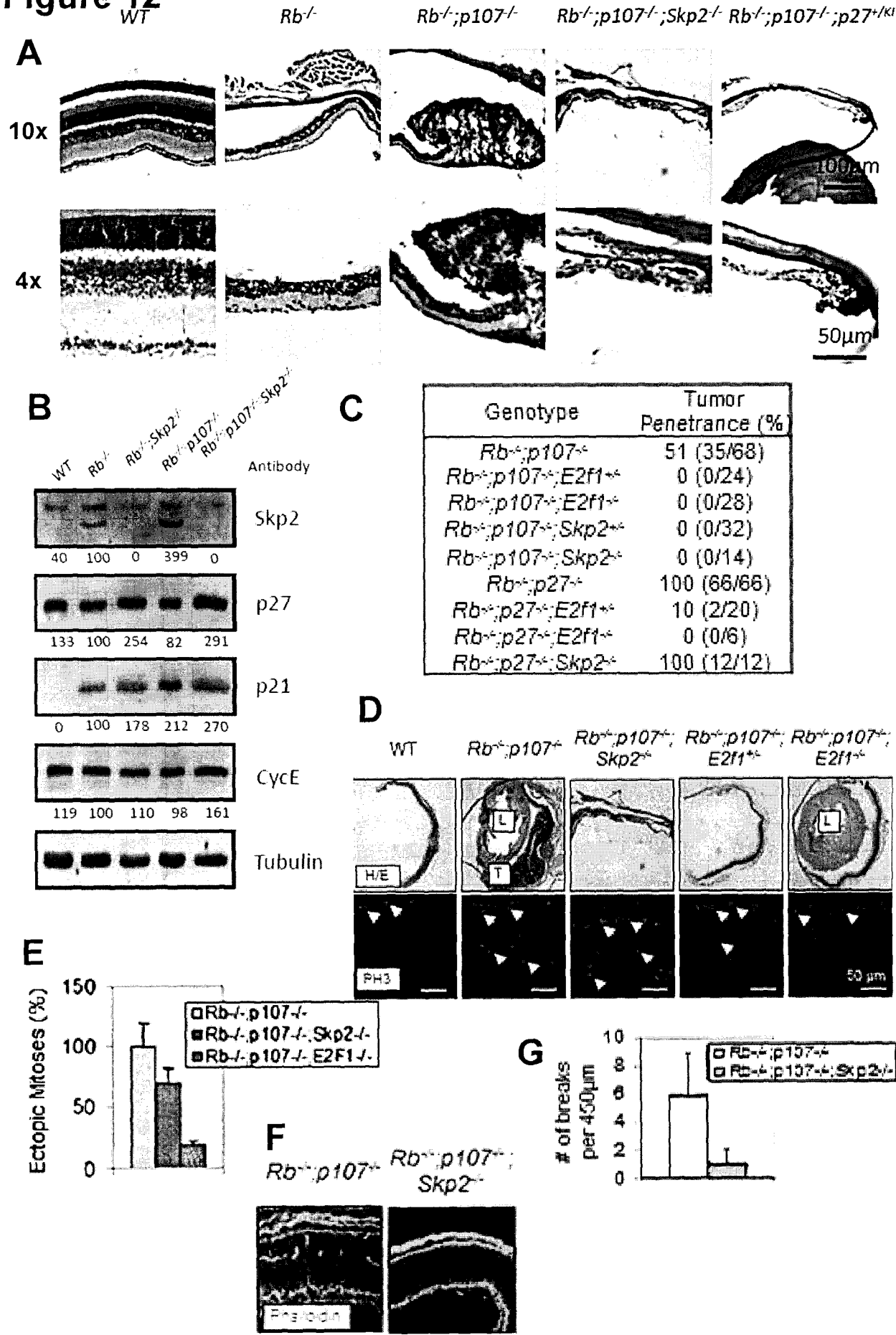
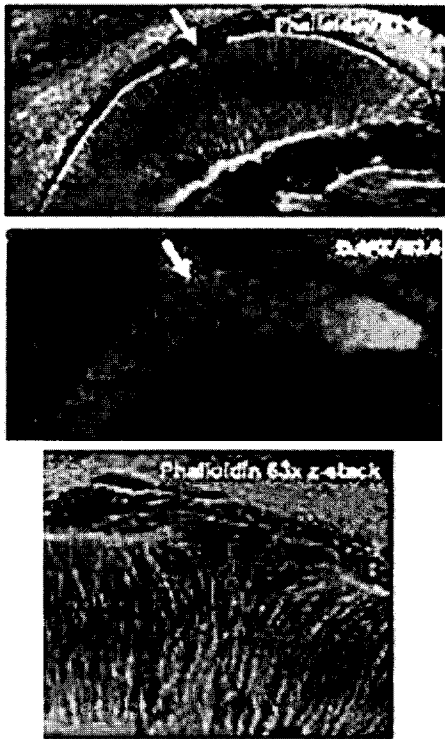
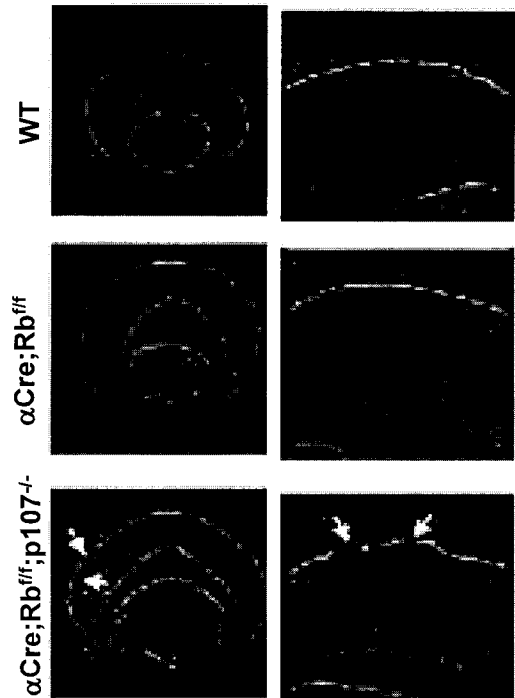


Figure 13

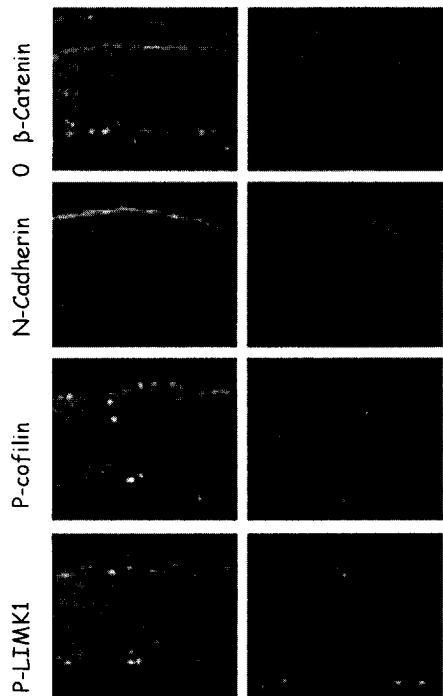
A Polarity/adhesion defect in Rb/p107 null retina



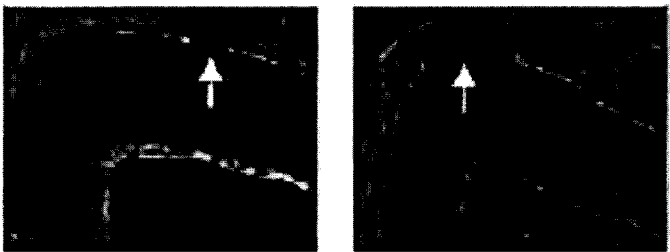
B N-cadherin at E14



C αCre-Rb



D Partial penetrance of polarity in p107 null retina



E

Genotype	Phenotype		
	Ectopic division	Polarity/AJ defect	Tumors
Rb null	Yes	No	No
p107 null mixed bckgd	No	Yes - 20%	No
p107 null C57BL inbred	No	Yes - 100%	No
Rb/p107 null	Yes	Yes - 100%	Yes

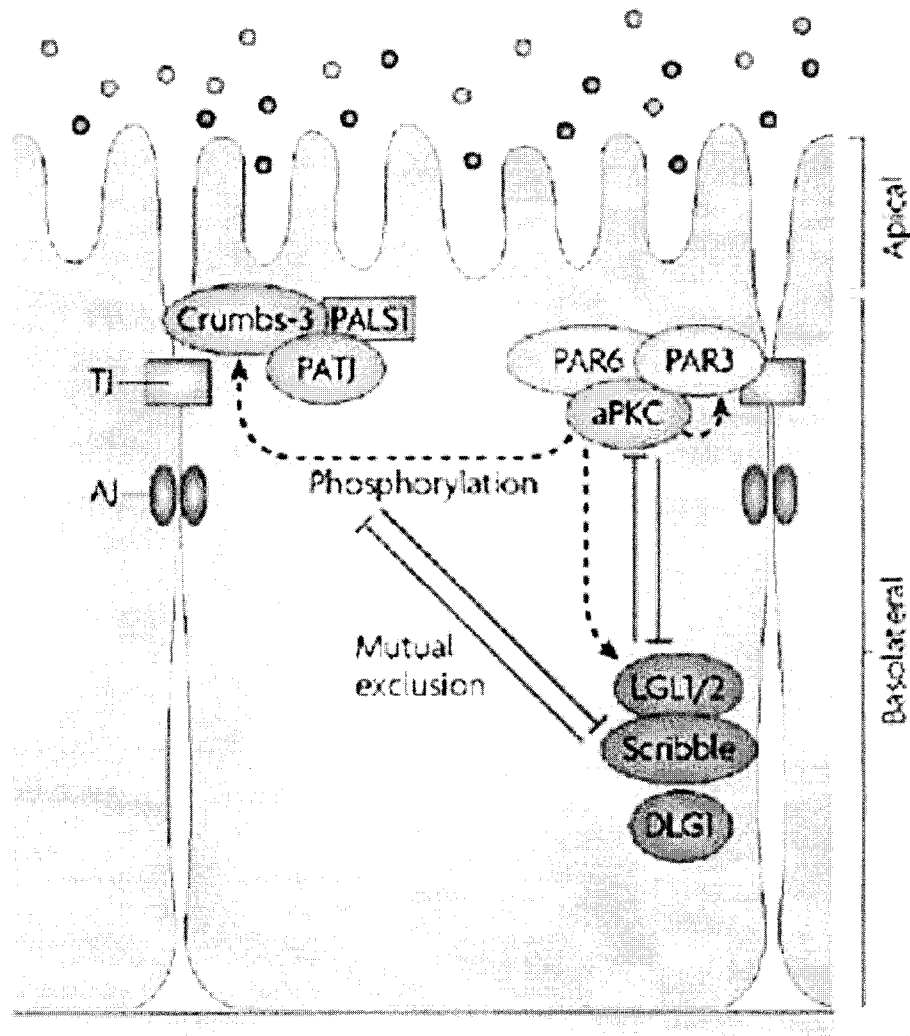
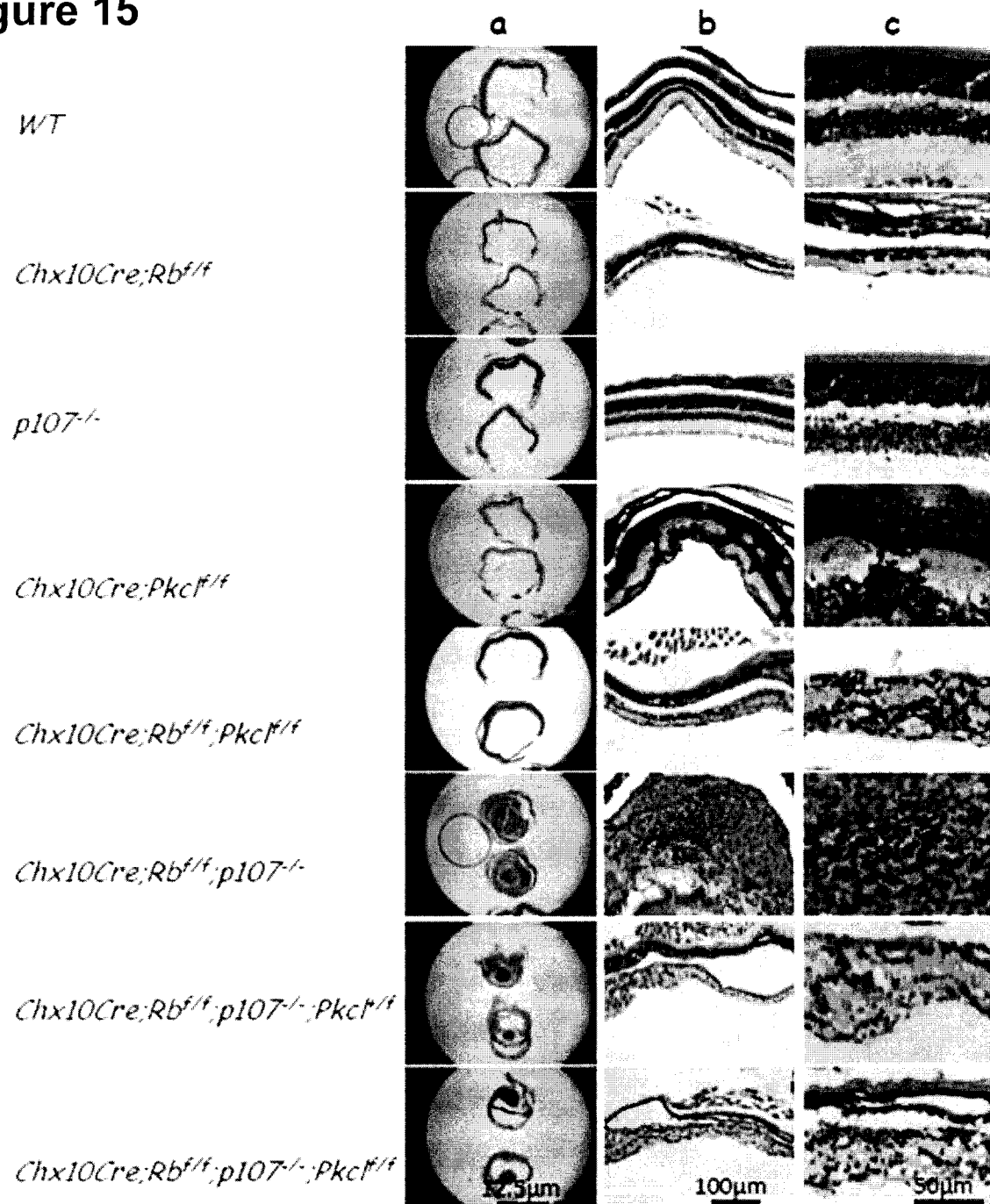
Figure 14**Polarity Regulators**

Figure 15

Genotype	Eyes with tumor/ Total eyes	Penetrance
<i>Chx10Cre;Rb^{f/f};p107^{-/-}</i>	40/40	100%
<i>Chx10Cre;Rb^{f/f};p107^{-/-};Pkc^{f/f}</i>	4/45	10%
<i>Chx10Cre;Rb^{f/f};p107^{-/-};Pkc^{f/f}</i>	0/48	0%

Figure 16

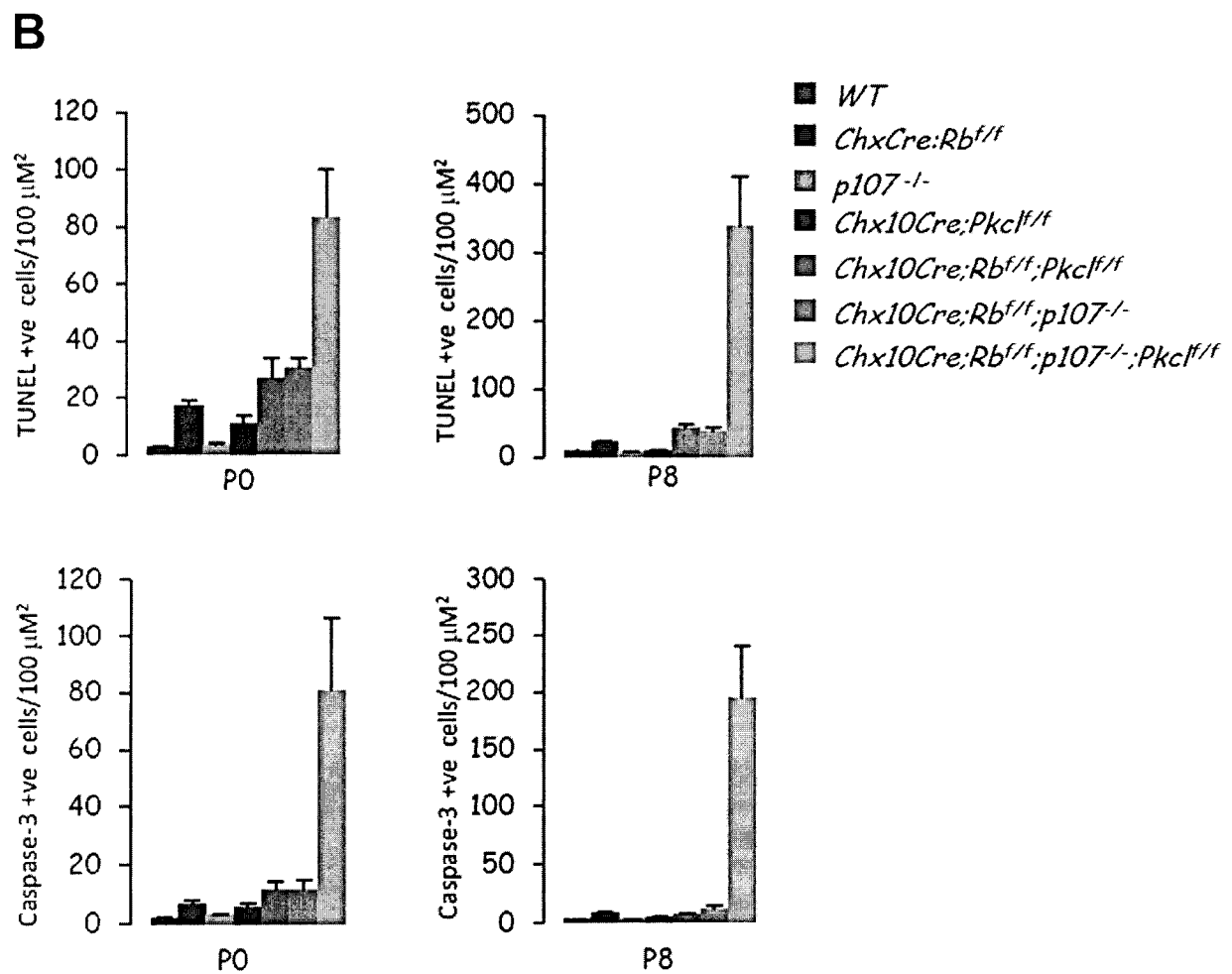
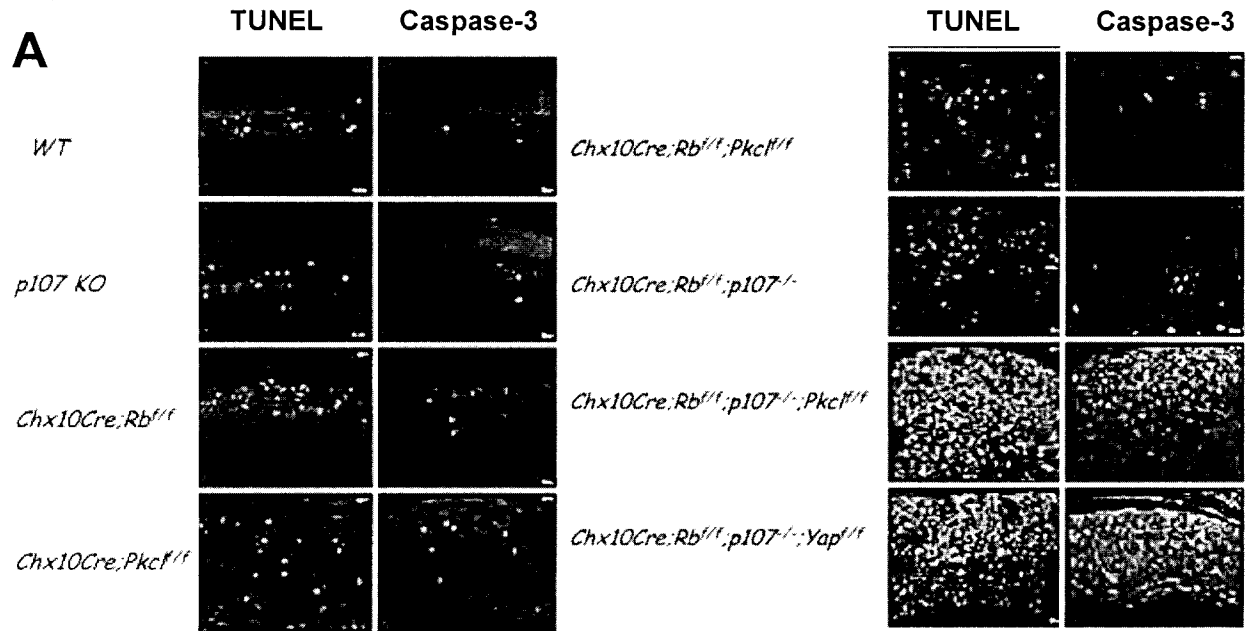


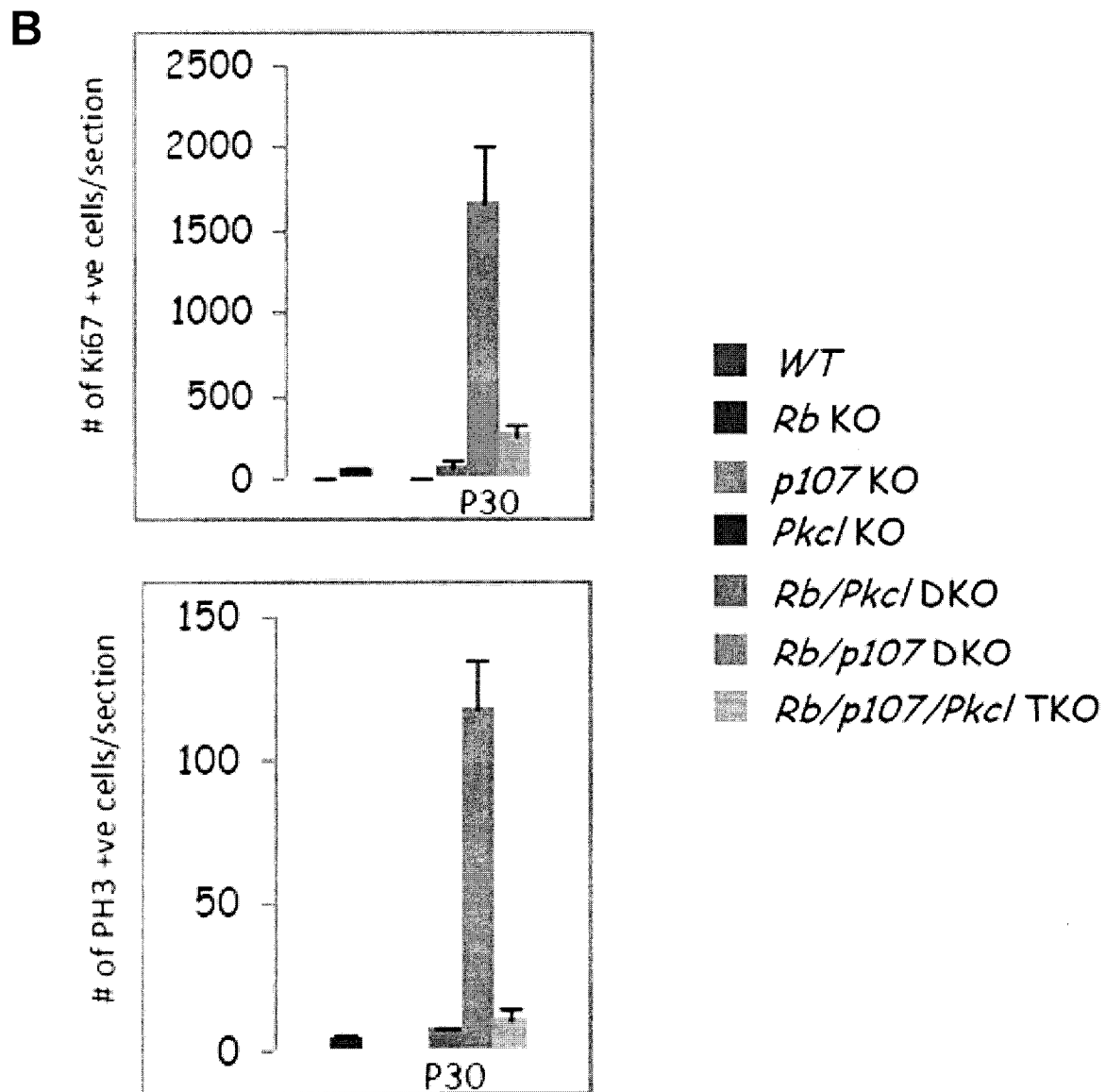
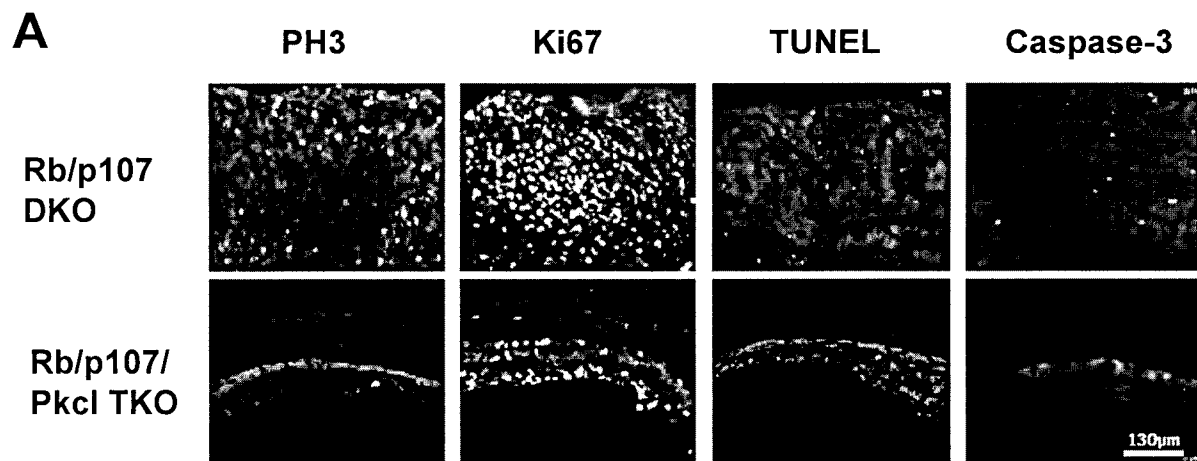
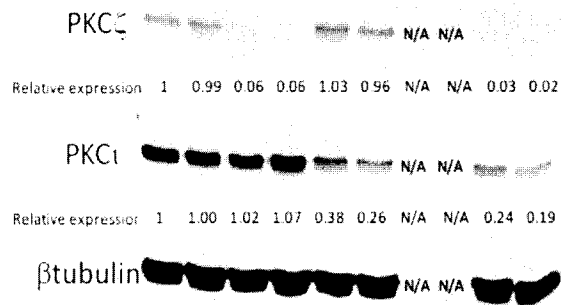
Figure 17

Figure 18

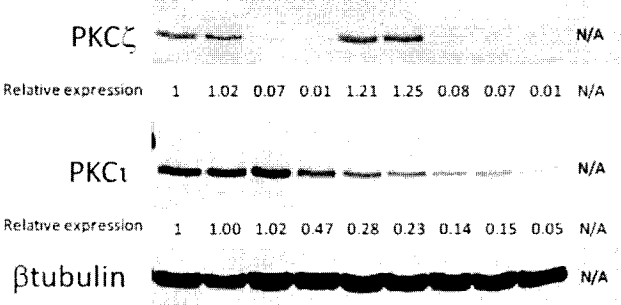
A

shRNA target	control	single KD				double KD			
sh-control*									
sh-control*									
sh-PKC ζ a*									
sh-PKC ζ b*									
sh-PKC ζ a*									
sh-PKC ζ b*									

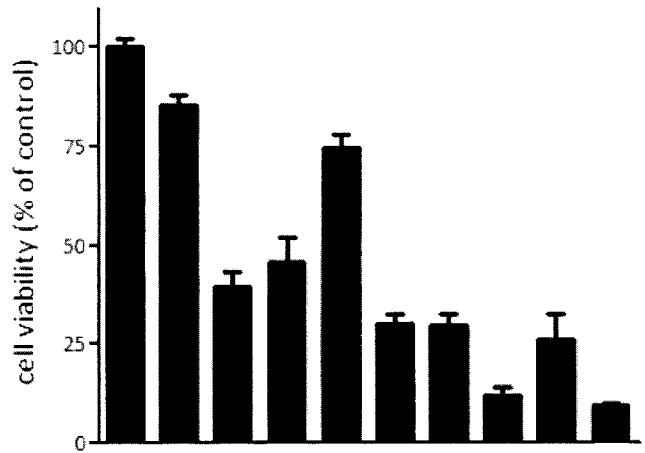


D

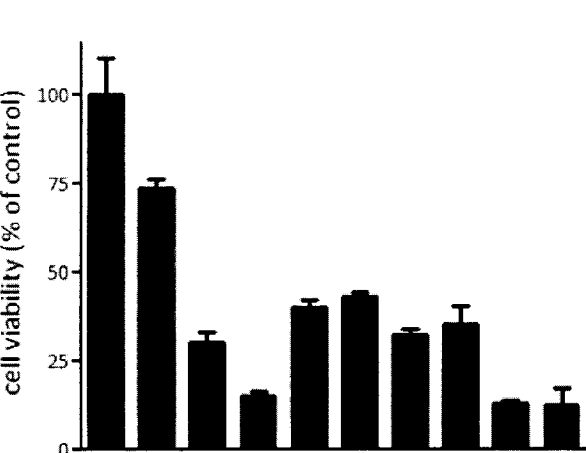
shRNA target	control	single KD				double KD			
sh-control*									
sh-control*									
sh-PKC ζ a*									
sh-PKC ζ b*									
sh-PKC ζ a*									
sh-PKC ζ b*									



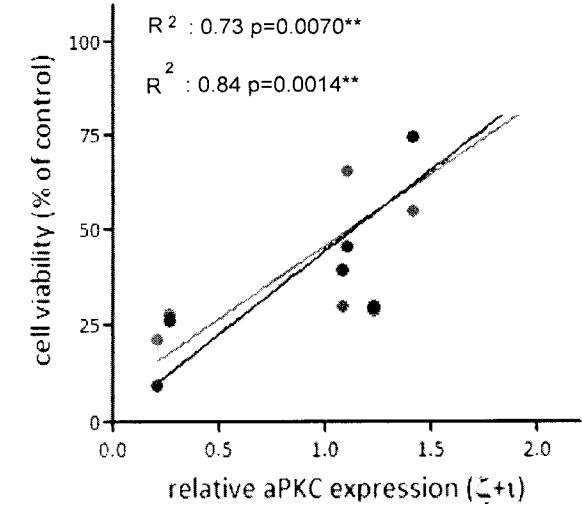
B



E



C



F

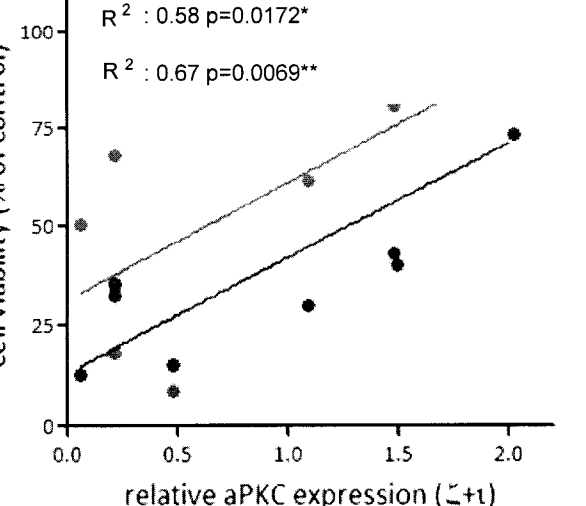


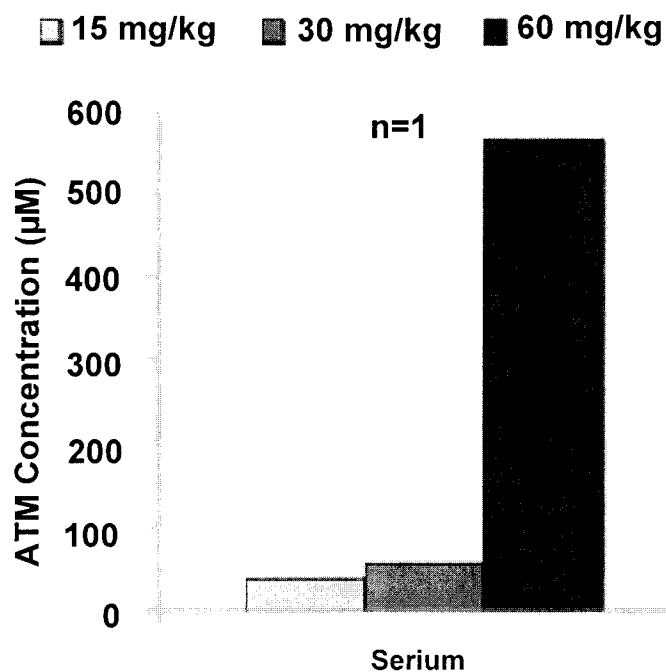
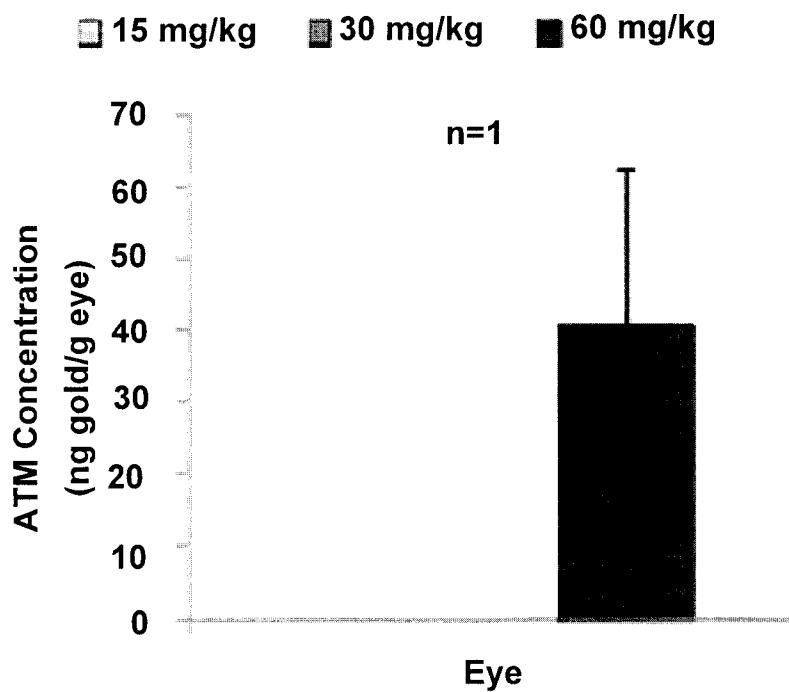
Figure 19**A****B**

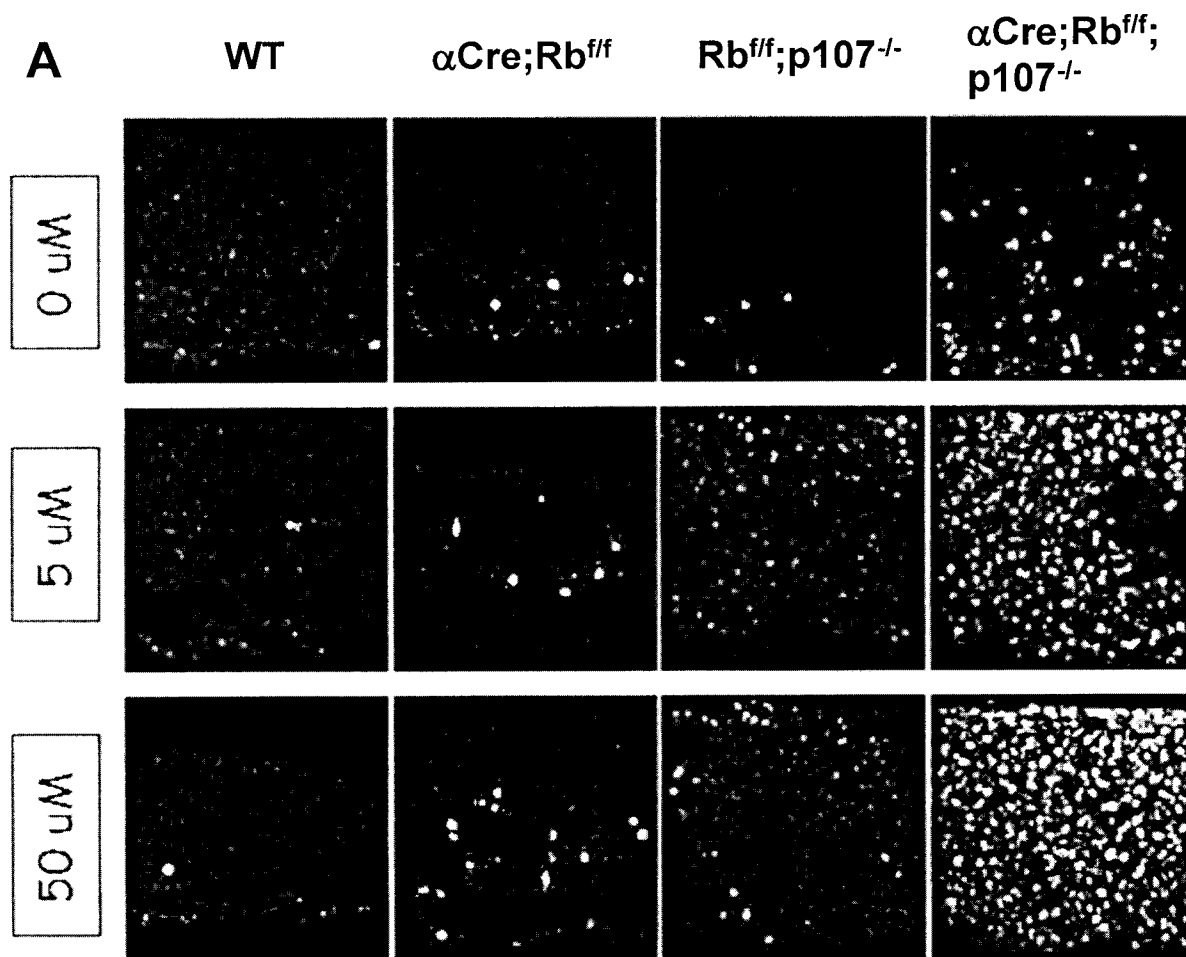
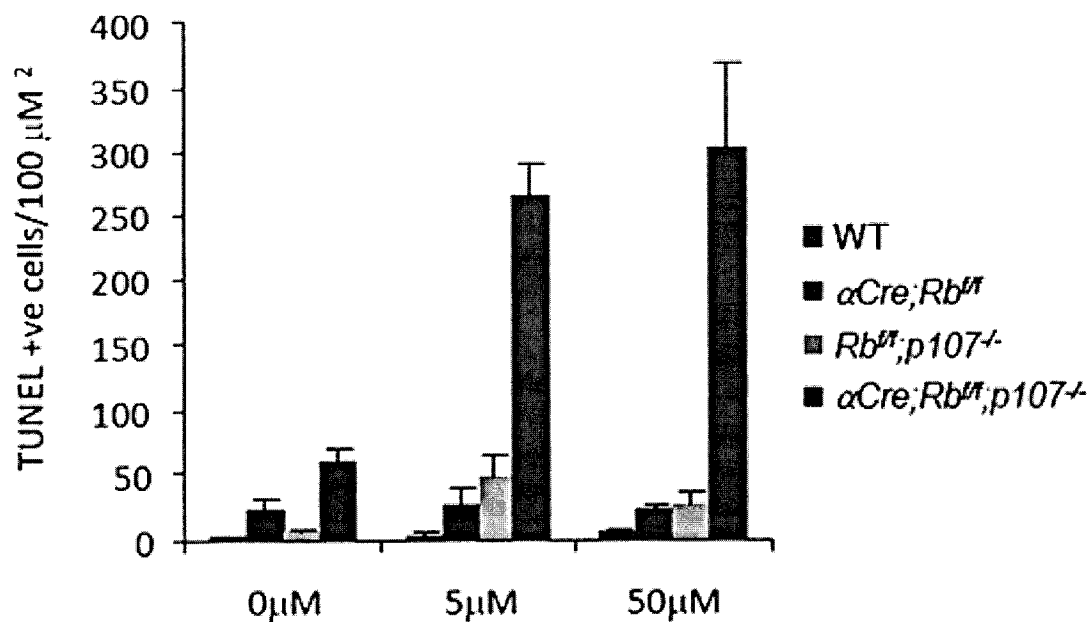
Figure 20**B**

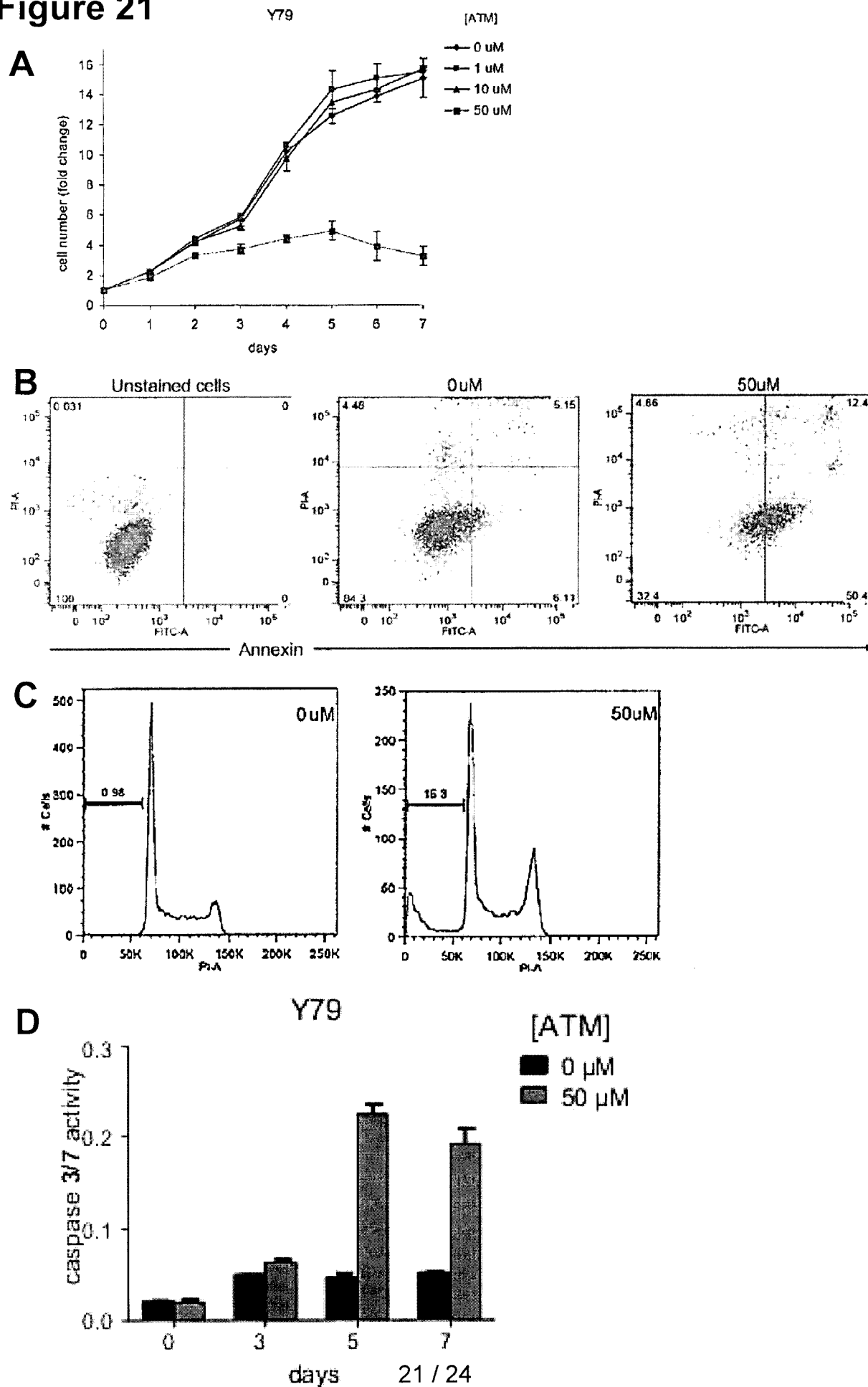
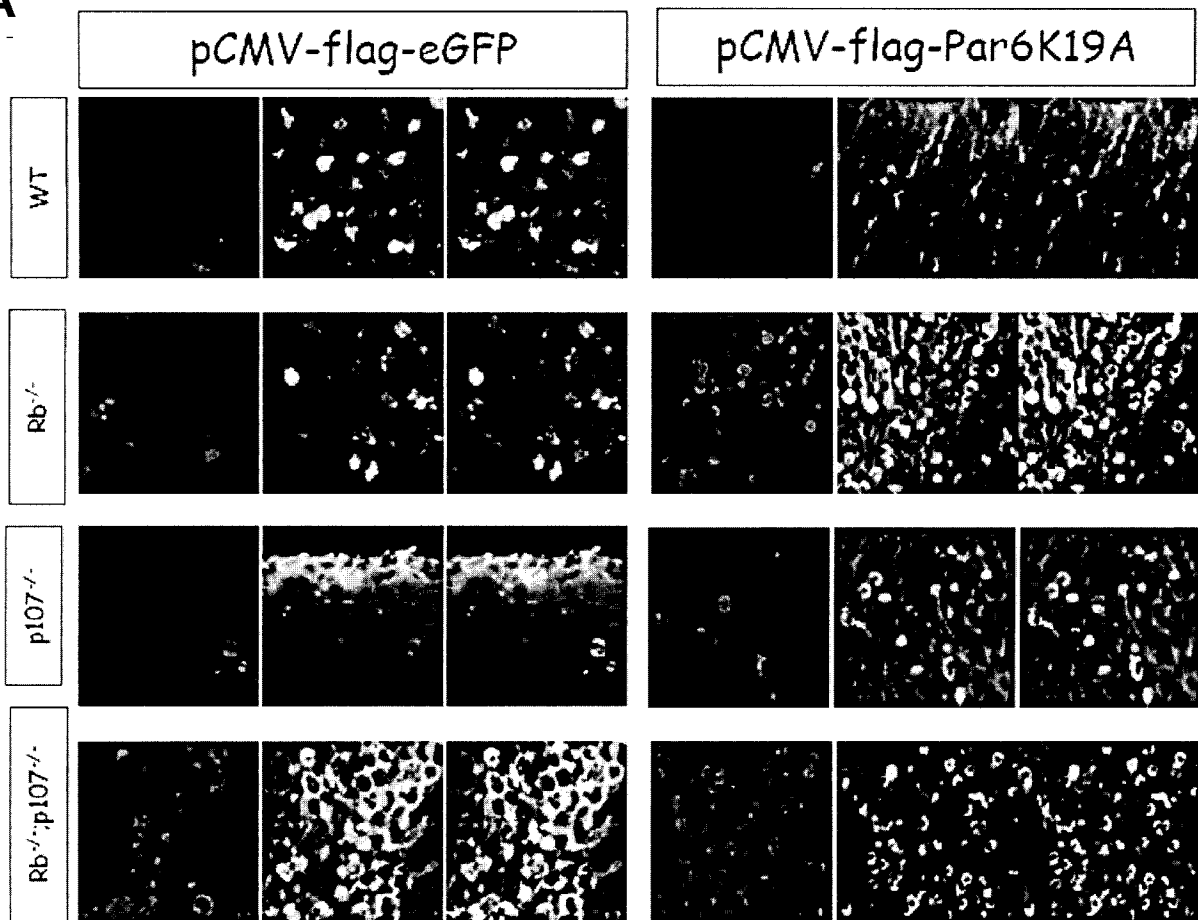
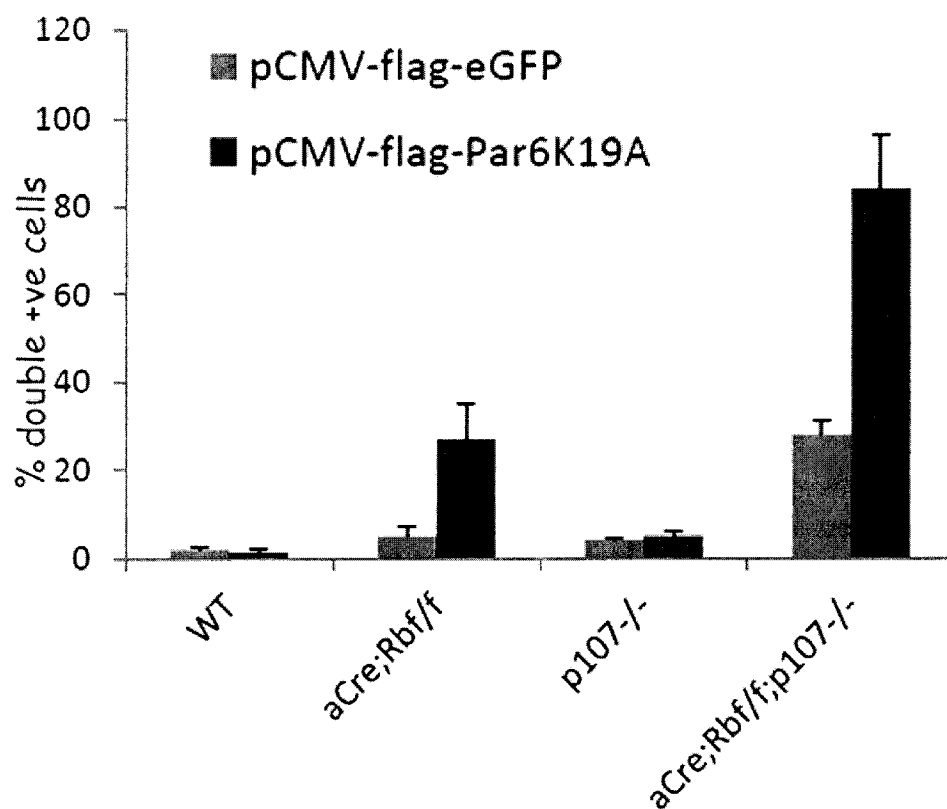
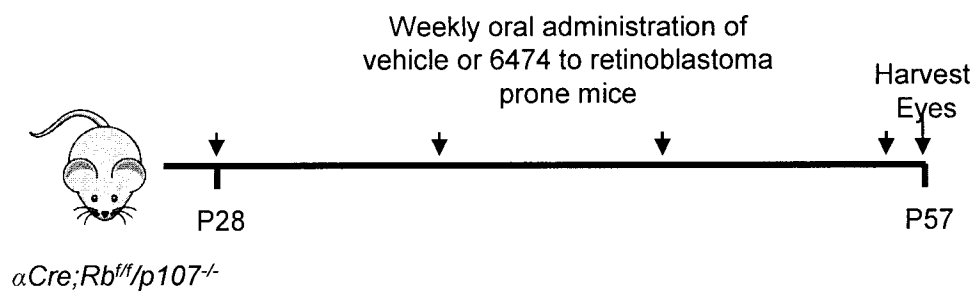
Figure 21

Figure 22**A****B**

A



28 day treatment

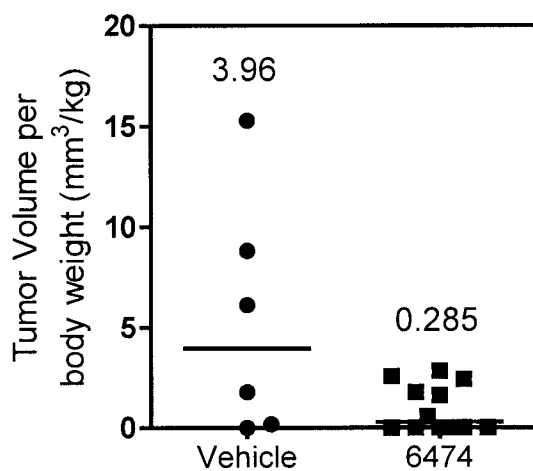
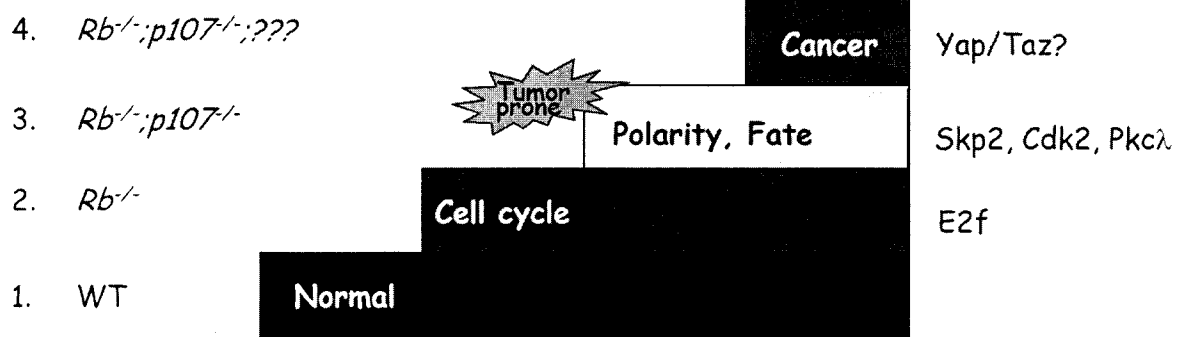
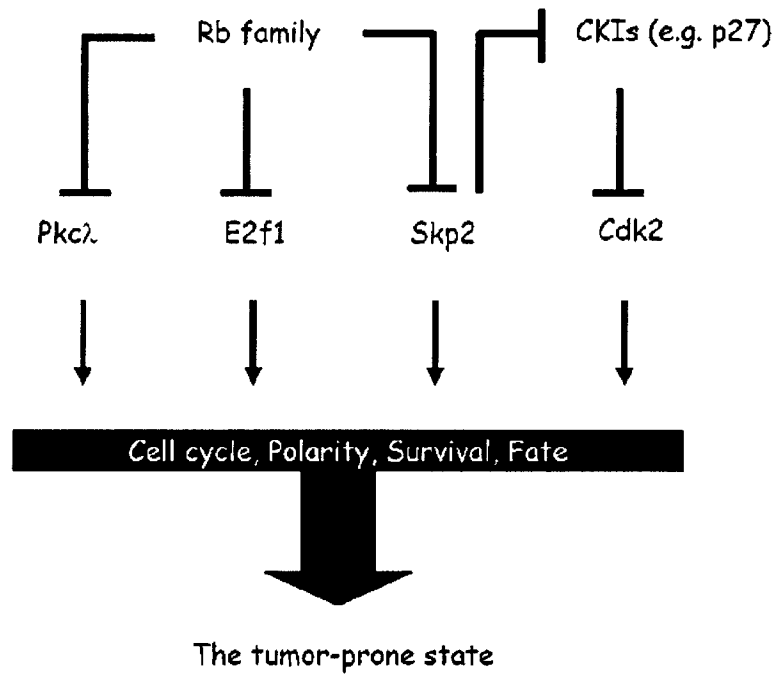


Figure 24

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2012/001058

A. CLASSIFICATION OF SUBJECT MATTER IPC: A61K 31/194 (2006.01) , A61K 31/4709 (2006.01) , A61K 31/506 (2006.01) , A61K 31/52 (2006.01) , A61K 31/713 (2006.01) , A61K 33/00 (2006.01) (more IPCs on the last page) According to International Patent Classification (IPC) or to both national classification and IPC														
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) A61K 31 ; C12Q 1/25 ; C12Q 1/48 , G01N 33/48 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used) STN, Epoque (Epodoc, TXTEN, Medline) (retinoblastoma, Rb, Cdk, CKI, Skp2, aPKC, E2f, p27, p107, , HLM006474, lithium, CYC202, R547, aurothiomalate, ICA-1, cancer, malignancy, neoplasia, tumourgenesis and related terms)														
C. DOCUMENTS CONSIDERED TO BE RELEVANT <table border="1"> <thead> <tr> <th>Category*</th> <th>Citation of document, with indication, where appropriate, of the relevant passages</th> <th>Relevant to claim No.</th> </tr> </thead> <tbody> <tr> <td>X</td> <td>WO 2009/117484 (University of South Florida) 24 September 2009 (24-09-2009) see entire document</td> <td>1-5, 8-10, 15-17</td> </tr> <tr> <td>X</td> <td>Ma, Y. et al., "A small-molecule E2f inhibitor blocs growth in a melanoma culture model", CANCER RES, 1 August 2008, 68: 6292-6299. see entire document</td> <td>1-5, 8-10, 15-17</td> </tr> <tr> <td>X</td> <td>Barrera, G. et al., "4-hydroxynoneal and regulation of cell cycle: effects on the pRb/E2F pathway", FREE RADICAL BIOLOGY & MEDICINE, 1 September 2004, 37(5): 597-606. see entire document</td> <td>1-5, 8-10, 15-17</td> </tr> </tbody> </table>			Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.	X	WO 2009/117484 (University of South Florida) 24 September 2009 (24-09-2009) see entire document	1-5, 8-10, 15-17	X	Ma, Y. et al., "A small-molecule E2f inhibitor blocs growth in a melanoma culture model", CANCER RES, 1 August 2008, 68: 6292-6299. see entire document	1-5, 8-10, 15-17	X	Barrera, G. et al., "4-hydroxynoneal and regulation of cell cycle: effects on the pRb/E2F pathway", FREE RADICAL BIOLOGY & MEDICINE, 1 September 2004, 37(5): 597-606. see entire document	1-5, 8-10, 15-17
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[X] Further documents are listed in the continuation of Box C. [X] See patent family annex. <table border="1"> <tbody> <tr> <td>* Special categories of cited documents :</td> <td>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</td> </tr> <tr> <td>"A" document defining the general state of the art which is not considered to be of particular relevance</td> <td>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</td> </tr> <tr> <td>"E" earlier application or patent but published on or after the international filing date</td> <td>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</td> </tr> <tr> <td>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</td> <td>"&" document member of the same patent family</td> </tr> <tr> <td>"O" document referring to an oral disclosure, use, exhibition or other means</td> <td></td> </tr> <tr> <td>"P" document published prior to the international filing date but later than the priority date claimed</td> <td></td> </tr> </tbody> </table>			* Special categories of cited documents :	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention	"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone	"E" earlier application or patent but published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art	"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family	"O" document referring to an oral disclosure, use, exhibition or other means		"P" document published prior to the international filing date but later than the priority date claimed	
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"P" document published prior to the international filing date but later than the priority date claimed														
Date of the actual completion of the international search 24 January 2012 (24-01-2013)		Date of mailing of the international search report 14 February 2013 (14-02-2013)												
Name and mailing address of the ISA/CA Canadian Intellectual Property Office Place du Portage I, C114 - 1st Floor, Box PCT 50 Victoria Street Gatineau, Quebec K1A 0C9 Facsimile No.: 001-819-953-2476		Authorized officer Tania Nish (819) 934-3592												

INTERNATIONAL SEARCH REPORTInternational application No.
PCT/CA2012/001058**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of the first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons :

1. ☒ Claim Nos. : 1-14

because they relate to subject matter not required to be searched by this Authority, namely :

Claims 1-14 are directed to a method for treatment of the human or animal body by surgery or therapy which the International Search Authority is not required to search (Rule 39.1, PCT). However, this Authority has carried out a search based on the alleged effects or purposes/uses of the product defined in claims RB pathway inhibitors.

2. ☐ Claim Nos. :

because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically :

3. ☐ Claim Nos. :

because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows :

See Extra Sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☒ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claim Nos. :

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim Nos. :

Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2012/001058

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Silva A.K., et al., " <i>Lithium chloride regulated the proliferation of stem-like cells in retinoblastoma cell lines: a potential role for the canonical Wnt signalling pathway</i> ", MOLECULAR VISION, 2010, 16:36-45. see entire document	1-5, 8-10, 15-17
X	EP 2 065 039 (Max-Planck-Gesellschaft) 3 June 2009 (03-06-2009) see entire document	1-5, 8-10, 15-17
X	WO 2010/056931 (Inteligent Oncotherapeutics Inc.) 20 May 2010 (20-05-2010) see entire document	1-5, 8-10, 15-17
X	DePinto, W. et al., " <i>In vitro and in vivo activity of R547: a potent and selective cyclin-dependent kinase inhibitor currently in phase I clinical trials</i> ", MOL CANCER THER., 2006, 5: 2644-2658. see entire document	1-7, 11, 15-17
X	WO2007/059111 (Entremed Inc.) 24 May 2007 (24-05-2007) see entire document	1-7, 11, 15-17
X	WO 2010/104923 (Research Development Foundation) 16 September 2010 (16-09-2010) see entire document	1-7, 11, 15-17
X	EP 1 726 953 (F. Hoffmann-La Roche) 29 November 2006 (29-11-2006) see entire document	1-7, 11, 15-17
X	Liu & Yamauchi, " <i>p27-associated G1 arrest induced by hinokitiol in human malignant melanoma cells is mediated via down-regulation of pRb, Skp2 ubiquitin ligase and impairment of Cdk2 function</i> ", CANCER LETTERS, 2009, 286: 240-249. see entire document	1-7, 11, 15-17
X	CA 2 597 193 (University of Montfort) 13 February 2009 (13-02-2009) see entire document	1-7, 11, 15-17
X	WO 2009/061345 (Cornell Research Foundation) 14 May 2009 (14-05-2009) see entire document	1-7, 11, 15-17
X	Senderowicz, A.M., " <i>Small-molecule cyclin-dependent kinase modulators</i> ", ONCOGENE, 2003, 22:6609-6620. see entire document	1-7, 11, 15-17

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2012/001058

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Stallings-Mann, M. et al., " <i>A novel small-molecule inhibitor of protein kinase C₁ blocks transformed growth of non-small cell lung cancer cells</i> ", CANCER RES, 2006, 66: 1767-1774. see entire document	1-4, 12-13, 15-17
X	WO 2009/036414 (University of South Florida) 19 March 2009 (19-03-2009) see entire document	1-4, 12, 14, 15-17
X	Pillai, P. et al., "A novel PKC _γ inhibitor abrogates cell proliferation and induces apoptosis in neuroblastoma", THE INTERNATIONAL JOURNAL OF BIOCHEMISTRY & CELL BIOLOGY, 16 February 2011, 43: 784-794. see entire document	1-4, 12-17
X	US 5 650 287 (Nevins et al.) 22 July 1997 (22-07-1997) see entire document	21-22
P,X	Sangwan, M et al., " <i>Established and new mouse models reveal E2F1 and Cdk2 dependency of retinoblastoma, and expose effective strategies to block tumour initiation</i> ", ONCOGENE, 30 January 2012, 31: 5019-5028. see entire document	1-22

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/CA2012/001058

Patent Document Cited in Search Report	Publication Date	Patent Family Member(s)	Publication Date
WO2012037553A2	22 March 2012 (22-03-2012)	WO2012037553A2 WO2012037553A9	22 March 2012 (22-03-2012) 05 July 2012 (05-07-2012)
WO2009117484A2	24 September 2009 (24-09-2009)	US2011053977A1 US8202886B2 WO2009117484A3	03 March 2011 (03-03-2011) 19 June 2012 (19-06-2012) 04 March 2010 (04-03-2010)
EP2065039A1	03 June 2009 (03-06-2009)	EP2065039A1 WO2009068322A1	03 June 2009 (03-06-2009) 04 June 2009 (04-06-2009)
WO2010056931A1	20 May 2010 (20-05-2010)	CA2742489A1 US2012028907A1	20 May 2010 (20-05-2010) 02 February 2012 (02-02-2012)
WO2007059111A2	24 May 2007 (24-05-2007)	US2007185069A1 WO2007059111A3	09 August 2007 (09-08-2007) 14 May 2009 (14-05-2009)
WO2010104923A1	16 September 2010 (16-09-2010)	CA2754917A1 US2012114636A1	16 September 2010 (16-09-2010) 10 May 2012 (10-05-2012)
EP1726953A1	29 November 2006 (29-11-2006)	AT405828T CA2545426A1 CN101078716A DE602006002311D1 EP1726953B1 ES2313556T3 JP2006325593A SG127843A1 US2006269482A1	15 September 2008 (15-09-2008) 25 November 2006 (25-11-2006) 28 November 2007 (28-11-2007) 02 October 2008 (02-10-2008) 20 August 2008 (20-08-2008) 01 March 2009 (01-03-2009) 07 December 2006 (07-12-2006) 29 December 2006 (29-12-2006) 30 November 2006 (30-11-2006)
CA2597193A1	13 February 2009 (13-02-2009)	None	
WO2009061345A2	14 May 2009 (14-05-2009)	US2011009353A1 WO2009061345A3	13 January 2011 (13-01-2011) 03 December 2009 (03-12-2009)
WO2009036414A1	19 March 2009 (19-03-2009)	US2009075932A1	19 March 2009 (19-03-2009)
US5650287A	22 July 1997 (22-07-1997)	AU3609693A WO9315227A1	01 September 1993 (01-09-1993) 05 August 1993 (05-08-1993)

The claims are directed to a plurality of inventive concepts as follows:

Group A - Claims 1-5 (in part), 6-7, 11, 15-17 (in part) are directed to methods and uses of Cdk inhibitors to prevent retinoblastoma via inhibiting the RB pathway.

Group B - Claims 1-5 (in part), 8-10, 15-17 (in part) are directed to methods and uses of E2f inhibitors to prevent retinoblastoma via inhibiting the RB pathway.

Group C - Claims 1-4 (in part), 12 (in part), 13-14, 15-17 (in part) are directed to methods and uses of atypical protein kinase C inhibitors to prevent retinoblastoma via inhibiting the RB pathway.

Group D - Claims 1-4 (in part), 12 (in part), 15-17 (in part) are directed to methods and uses of Skp2 inhibitors to prevent retinoblastoma via inhibiting the RB pathway.

Group E - Claims 18-20 are directed to method of determining the likelihood of a population of cells to be cancer-prone comprising determining the level of E2f and Cdk activity of said cells.

Group F - Claim 21 is directed to method of determining the likelihood of a population of cells to be cancer-prone comprising determining the level of atypical protein kinase C activity of said cells.

Group G - Claim 22 is directed to method of determining the likelihood of a population of cells to be cancer-prone comprising determining the level of Skp2 activity of said cells.

The claims must be limited to one inventive concept as set out in Rule 13 of the PCT.

The therapeutic effects of Groups A-D are directed to the specific cancer retinoblastoma and the diagnostic methods of Groups E-G are directed to cancer in general. The link between these groups relies on the substrates of the RB pathway for preventative effects or diagnostics. The RB pathway is the universal pathway in cell cycle and cancer development and is both well known and well studied for new treatments or diagnosis of cancer (D1-D18). As such, it cannot be the special technical feature to link Groups A-D and Groups E-G. Likewise, the use of inhibitors of the RB pathway to treat cancers such as retinoblastoma is known (D1-D17) and thus there is no linking feature between the inhibitors of each of Groups A-D. There also lacks an inventive feature as a link between the diagnostic methods of Groups E-G as the methods implemented are commonly used in the art and the substrates being measured for cancer identification are known (D1-D18). Therefore, there lacks unity of invention between Groups A-G.

INTERNATIONAL SEARCH REPORT

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A61P 35/00 (2006.01) , *C12Q 1/25* (2006.01) , *C12Q 1/48* (2006.01) , *G01N 33/48* (2006.01)