Loss of *E2F3a* Function Mitigates the Developmental Defects Associated with *Rb*-deficiency

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Abstract

The Retinoblastoma (*Rb*) gene was the first tumor suppressor discovered. In many, if not all, human tumors *Rb* is functionally inactivated. In a normal cell, the Rb protein is a transcription regulator that controls the cell cycle, differentiation, and cell death. Studies have shown that mice carrying one *Rb* mutant allele ($Rb^{+/-}$) are highly cancer prone and do not live as long as wild type mice. Furthermore, completely Rb deficient embryos ($Rb^{-/-}$) die in mid-gestation around the 14^{th} embryonic day (E14) of development. Analysis of $Rb^{-/-}$ embryos reveals defects in hematopoiesis, neurogenesis, and lens fiber development. At least in part, these defects arise from the inappropriate regulation of the cell cycle. Studies of Rb function have led to the discovery of the E2F transcription family. The E2F family of transcription factors are downstream targets of Rb and work with Rb in controlling the cell. Numerous studies indicate that the E2F family of transcription factors regulates expression of genes that are important for cellular proliferation.

To date, eight E2F family members have been identified. E2F3 is unique in that it has been demonstrated to play a role in the G1/S phase transition. Recently the gene locus for *E2f3* was found to encode two distinct mRNAs, *E2f3a* and *E2f3b*. They share homology throughout the protein sequence from exon 2 to the C-terminus. Both proteins share the same nuclear localization signal, DNA binding, heterodimerization, and pocket protein binding domains; however, E2F3a has an additional cyclin A binding domain. Furthermore, E2F3b is expressed constitutively in quiescent cells, but E2F3a is tightly regulated and present in proliferating cells. Unlike E2F3, mice lacking E2F3a are healthy and show no other signs of reduced viability or livelihood.

E2F3a being a downstream target of Rb suggests that its loss could mitigate the $Rb^{-/-}$ phenotype. To test this hypothesis an in vivo approach was used to generate $E2F3a^{-/-}$; $Rb^{-/-}$ double mutant embryos. The embryos were harvested at E13.5 at which point cells from the placenta, blood, central nervous system, peripheral nervous system, and lens were examined. BrdU incorporation was used to distinguish proliferation and TUNEL assays were used to detect apoptosis. Also, H & E staining was used to examine overall morphology of the different tissue types. As predicted by the hypothesis, the lack of E2F3a completely compensated for the loss of Rb in the placenta and central nervous system. In the peripheral nervous system and the lens the loss of E2F3a partially rescued the $Rb^{-/-}$ phenotype, but the red blood cells showed no rescue in the reduction of nucleated cells. Interestingly, the $E2F3a^{-/-}$; $Rb^{-/-}$ embryos survived up to E17.5. More experiments are being done to assess whether the lack of E2F3a increases the lifespan of $Rb^{+/-}$ mice that are cancer prone.

Elucidating the specific functions of the E2F family members is critical to understanding the intricacies of cell cycle regulation, tumorigenesis, and differentiation. Working to completely comprehend the interaction between E2F3a and Rb during development could lead to new insight into understanding cancer.

Introduction

Over twenty years ago the Retinoblastoma (*Rb*) gene was identified as the first tumor suppressor[1]. Mutations in the Retinoblastoma gene were originally implicated in a childhood eye cancer[2]. Although rare, the retinoblastoma disease is a malignant neoplasm that originally affects the eyes of children usually under the age of three[3]. With proper treatment and baring metastasis, retinoblastoma is usually not lethal[3]. Studying the disease showed the Rb is required for proper lens development[3]. Interestingly, the loss of Rb affected different tissues differently. Lack of Rb in the eye will lead to cancer; whereas, other tissues show no abnormal phenotype in the absence of Rb[4]. Continuous research has greatly expanded the Rb paradigm and emphasized the importance of Rb. In fact, early studies of the gene have unveiled that cancer can result if Rb function is derailed[3]. Most researchers agree that *Rb* is mutated in most, if not all, human cancers[1].

The Rb protein is a large nuclear transcription factor. It is 928 amino acids long and has multiple binding sites for different targets such as HDAC1 and the E2Fs[5,6]. Earlier studies have unearthed Rb as a cornerstone of the cell cycle[3]. It functions as a regulator of transcription that controls the cell cycle, proliferation, and apoptosis[7]. Rb is regulated through the cell cycle by phosphorylation at multiple serine and threonine sites[8]. G_1 and the initiation of replication are controlled by this phosphorylation mechanism. If Rb remains hypophosphorylated then target proteins remain bound and the cell is arrested in G_1[8]. Continued phosphorylation of Rb by G_1 cyclin-dependent kinases (Cdks), primarily D-type cyclins and Cdk 4, results in cell cycle progression[8].

Because of the crucial role of Rb in the cell cycle, numerous studies have been conducted to further elucidate the role of Rb. These studies have shown that the loss of

Rb has deleterious effects on cells. Mice that lack one copy of *Rb* ($Rb^{+/-}$) are highly cancer prone[9]. Cancer primarily arises in the pituitary gland with high levels of metastasis and reduces the viability of these mice to around 10.5 months[9]. These results prove the important role of Rb in cancer. Furthermore, the appearance of pituitary tumors instead of ocular tumors indicates different phenotypes of Rb-induced tumors. The complete inactivation of Rb results in unscheduled cell proliferation, apoptosis and developmental defects[10]. Furthermore, mice that completely lack *Rb* die in midgestation around embryonic day fourteen (E14)[11]. Analysis of $Rb^{-/-}$ embryos reveals defects in hematopoiesis, neurogenesis, and lens development[7]. At least in part, these defects arise from ectopic S-phase entry and apoptosis caused by the loss of Rb. At E13.5 the phenotypic consequences of Rb-deficiency are most apparent[12]. At this time, ectopic proliferation and high levels of apoptosis are not only found in the aforementioned tissues, but also in the placenta[13]. The irregular cell cycle causes dysplasia of trophoblast cells in the labyrinth of the placenta[13]. Oxygen and nutrient exchange between the embryo and mother occurs at the labyrinth, so irregularities in this tissue are detrimental[13].

Two models have been suggested for the lethality of embryos at midgestation. First, some scientists claim that Rb-deficient embryos die of anemia due to abnormal red blood cells (RBCs)[7]. In Rb-deficient mice blood cells remain nucleated, which deprives the cell of the ability to carry oxygen. Through normal embryonic development RBCs lose their nucleus during differentiation. The second model, proposed by Wu et al., suggests that Rb-deficient mice die during midgestation because of defects in the placenta[13]. In their experiment they replaced the placenta of an Rb-deficient embryo with

a wild-type placenta. This placenta replacement rescued the placenta phenotype and other phenotypes affected by Rb-deficiency except the lens tissue. Also, RBCs remained nucleated[13]. Surprisingly, the Rb-deficient embryos with wild-type placentas had extended viabilities instead of dying at E14[13]. A few embryos survived to full-term, though died shortly after birth. Their experiments showed that the viability of an embryo depends on the normal function of the placenta.

Through various experiments, the role of Rb in embryonic development is being elucidated. Apparently Rb is needed at two separate times during embryonic development[7]. First, Rb functions during midgestation to restrict transcription activators such as E2F7. This regulates the cell cycle and ensures normal proliferation. Later in development Rb directs the development of certain tissues. In Wu's study the *Rb*-deficient embryos with wild-type placentas had extended viabilities, but had other developmental defects in tissues due to the lack of Rb[13].

The ever increasing knowledge of Rb has led to the discovery of new proteins and new pathways related to the newly discovered proteins. The discovery of the E2F transcription family are a result of studying Rb[14]. The E2F family of transcription factors is downstream targets of the Rb tumor suppressor and work with Rb in controlling the cell cycle. Currently, eight E2F genes have been discovered as well as two DRTF proteins (DPs). Although the E2Fs heterodimerize to a DP to form a functional complex, the E2F protein dictates the function of the dimerized protein[15]. Based on transcription activity the E2Fs can be broken down into two groups[16]. E2F1, E2F2, and E2F3 are considered to be transcription activators and they bind exclusively to Rb. The activator E2Fs are transcription factors for genes that are required for DNA replication and cell

cycle regulation[1]. They bind at the promoters for genes such as *Orc1*, *cdc6*, *cyclin E*, and *Mcm*[1]. The activator E2Fs are tightly regulated during the cell cycle[1]. Their activities spike at the end of G_1 while they are not expressed nor have low activity in quiescent cells. On the other hand, E2F4, E2F5, E2F6, E2F7 and E2F8 are transcription repressors. E2F4 has the ability to bind to Rb as well as to Rb-related proteins, p107 and p13038. E2F5 can only bind to p13017. The E2F6 protein does not bind to any Rb family member at all; it only associates with the mammalian polycomb complex[17]. E2F4 and E2F5 derive their repressor ability by occupying the promoters of E2F-responsive genes after complexing with an Rb family member and histone deacetylase[17]. Not much is known about the mechanism of E2f6, but recent evidence suggests that it is a repressor[17]. E2f7 and E2f8 were only recently discovered and there is not much information regarding their activity as of yet except that they are repressors.

The Rb/E2F pathway (fig. 1) is the cornerstone for the cell cycle by being responsible for initiating the G_1/S-phase transition. Rb blocks transcription by two different mechanisms. Rb is able to bind the E2F activating transcription factors, which inactivates their ability to turn on downstream genes responsible for proliferation[8]. Rb also associates with the repressor E2Fs and histone deacetylases at the E2F promoter target sites; this complex represses transcription via negative regulation. As mentioned earlier, Rb is regulated by phosphorylation. Upon mitogen stimulation, G_1 Cdks, primarily D-type cyclins and Cdk 4, phosphorylate specific sites on the Rb protein. If Rb is hypophosphorylated then the E2Fs are able to bind to the protein. Rb must be phosphorylated at different sites, mainly in the C-terminus region, to cause a conformational change which causes the bound protein to dissociate. One model claims

that the phosphorylation of Ser-567 is the ultimate phosphorylation event to cause the E2F to dissociate and not be able to bind anymore[18]. This phosphorylation disrupts the A-B pocket of Rb and changes the protein shape, which will not bind E2F anymore[18]. Once activator E2Fs are released they are able to activate transcription by binding to the promoters of genes that are required for S-phase.

Many studies have been conducted by altering this pathway by knocking out certain proteins or over expressing others. Through these studies Rb and the activator E2Fs have been found to have opposing roles. Activator E2Fs serve to promote transcription; whereas, Rb binds to E2Fs to repress transcription. In these experiments many different defects have been observed. Mice that lack E2F3 have severe developmental defects and die early of congestive heart failure and $Rb^{+/-}$ mice are highly cancer prone[16,2]. By combining these genotypes ($Rb^{+/-}$; $E2f3^{-/-}$) the phenotypes were ameliorated. The mice lacked the developmental defects associated with loss of E2F3 and exhibited tumor suppression[14]. MEFs that have all three activator E2Fs knocked out ($E2f1^{-/-}$; $E2f2^{-/-}$; $E2f3^{-/-}$) are arrested in G_1[7]. However, if all activator E2Fs are over expressed then the MEFs undergo ectopic S-phase entry[7]. Tumor prone $Rb^{+/-}$ mice that lack E2F1 have extended viability; however, there is not a complete rescue to the wild type phenotype[14]. Studying the numerous E2Fs have exposed the incredible complexity of the family. Some scientists believe that the activator E2Fs have overlapping or redundant functions[16]. For instance, in MEFs that lack E2F3 the over expression of E2F1 rescues the delay in proliferation found in $E2f3^{-/-}$ MEFs[19]. Contrary to this belief, there is evidence that each E2F serves a specific function. Such as E2F3 serves as a transcription factor at the p19ARF promoter[20]. In mice that lack E2F3 p19ARF is over expressed.

There is no redundancy between the E2Fs to regulate the expression of p19ARF[20]. The duality of redundancy and unique properties of the E2Fs further enhance the complexity of the Rb-E2F pathway.

Other complex mechanisms of the Rb/E2F pathway have been discovered to regulate apoptosis. The loss of Rb triggers p53-dependent apoptosis8. Other scientists have found that cells limit growth potential by regulating phosphorylation at Ser-567. Cells that are phosphorylated at the Ser-567 residue are more susceptible to apoptosis58. E2Fs can induce apoptosis too. Free E2Fs can activate both p53-dependent and p53-independent apoptosis[18]. Whether apoptosis in *Rb*-deficient cells is caused by the loss of Rb or free E2Fs is not currently understood. Given the facts that activating E2Fs can promote proliferation and also promote apoptosis makes them interesting. Activator E2Fs act as both an oncogene and a tumor suppressor. At low concentrations they act as an oncogene, but at a high concentration they promote apoptosis and act as a tumor suppressor[14].

Probably the most interesting member of the E2F family is E2F3. E2F3 plays a key role in the activation of most E2F responsive genes[19]. Depending on modifiers in strain backgrounds, E2F3 is critical for full neonatal viability[7]. $E2f3^{-/-}$ that survive birth die early of congestive heart failure resulting from atrial thrombus formation and pulmonary edema[16]. If combined with the loss of E2F1 also, the mice always die before birth[16]. As mentioned earlier the loss of E2F3 in $Rb^{+/-}$ mice suppresses pituitary gland tumors, its loss increases the frequency of tumors in the thyroid gland[14]. This suggests that E2F3 may have tissue-specific functions. Without E2F3, cells do not proliferate sufficiently; MEFs take twice as long to proliferate[19]. However, no apoptosis defect is

noticeable[19]. Surprisingly, an embryo deficient in E2F3 and Rb (*E2F3*$^{-/-}$; *Rb*$^{-/-}$) develops relatively normally even though they die before birth[7]. This suggests that the functions of Rb and E2F3 balance each other. In an *E2F3*$^{-/-}$; *Rb*$^{-/-}$ double mutant embryo at E13.5 (when *Rb*$^{-/-}$ phenotype is most dramatic) the lack of E2F3 completely compensates for the lack of Rb in the central nervous system (CNS) and lens. There were partial rescues in the enucleation of red blood cells (RBCs) and Peripheral Nervous System (PNS)[7]. Based on these observations, E2F3 and Rb play critical roles in embryonic development.

Recently, Dr Leone's lab has discovered that the *E2F3* gene encodes two distinct mRNAs that lead to the synthesis of two E2F3 proteins, E2F3a and E2F3b[21]. This is very interesting because previously researchers assumed that only one gene product was made at the *E2F3* locus. They share homology throughout the protein sequence from exon 2 to the C-terminus (fig. 2). The only difference between the two isoforms is in exon 1. The exon for *E2f3a* is termed exon 1a and is 122 amino acids long. Exon 1b encodes the first exon for *E2f3b* and is only six amino acids in length and are different from exon 1a[21]. Both proteins share the same nuclear localization signal, DNA binding, heterodimerization, and pocket protein binding domains; however, E2F3a has a cyclin A binding domain in exon 1a[21]. The transcription of the two isoforms is regulated by distinct promoters[21]. In the promoter region of E2f3a there are several E2F binding sites as well as a Sp1 sequence and potential Myc binding sites. On the other hand, the promoter region for *E2F3b* does not contain an E2F or Myc binding site[21]. Both gene products bind exclusively with Rb[21]. In fact, E2F3b is believed to be the principle E2F that associates with Rb[21]. Furthermore, E2F3b is constitutively expressed throughout the cell cycle as well as in quiescent cells. On the other hand, the expression pattern of

E2f3a spikes at the G_1/S-phase transition[21]. E2F3a is considered to be grouped with E2F1 and E2F2 as transcription activators; whereas, E2F3b is considered a transcription repressor with E2F4, E2F5, and E2F6.

Materials and Methods

Establishment of *E2f3a* knockout

Cre-loxP gene-targeting techniques were used to generate E2F3a-deficient mice. LoxP sites were placed upstream and downstream of exon1a without affecting E2F3b. The cell lines were elctroporated and selected for by G418. Desired ES cell lines were verified by Southern blot analysis. The ES cell lines were then used to create chimeric mice and then the chimeric mice were further crossed to Cre transgenic mice for deletion of exon1a to generate $E2f3a^{+/-}$ mice. Effective deletion of the *E2F3a* gene was verified using Southern and Western blots. The $E2f3a^{+/-}$ animals were bred in a mixed background (129/sv X FVB) or backcrossed to a FVB background. These mice were further intercrossed to generate $E2f3a^{-/-}$ mice to study E2f3a function.

Manifestation of embryonic mice

$Rb^{+/-}$; $E2f3a^{+/-}$ mice were intercrossed to produce genotypes of interest. Coitus was determined via vaginal plug; the morning after coitus was considered 0.5 days postcoitum. Pregnant females were harvested at 13.5 days postcoitum for histological analysis; while 15.5, 16.5, and 17.5 days postcoitum females were harvested to establish viability. Tissue samples were immediately fixed in formalin. I analyzed three embryos from each genotype (four for $Rb^{-/-}$; $E2f3a^{-/-}$) that were obtained from different litters. Hematoxylin and Eosin (H & E) staining was used to analyze cell morphology. Genotypes of mice and embryos were determined by PCR analysis in which three primers (primer 1: GCTAGCAGTGCCCTTTTGTC; primer 2: TCCAGTGCACTACTCCCTCC; primer 3: CTCCAGACCCCCGATTATTT) were used.

BrdU Incorporation and TUNEL Assay

Pregnant females (13.5 days postcoitum) were injected intraperitoneally with 5'-bromodeoxyuridine (BrdU) (100 ug/grams of body weight) 2 hours prior to harvesting. Desired tissues were fixed in formalin upon harvesting. Sections (5 um) embedded in paraffin were deparffinized, hydrated, treated with Triton X, digested in Proteinase K, and denatured in HCl. Anti-BrdU antibody (DAKO Co. MO-0744) was used to detect BrdU incorporation. Also, an anti-mouse IgG secondary antibody (Vector, MOM Kit PK-2200) detected the primary antibody. For lens tissue, Alexa 594 (rhodamine) was used to tag the secondary antibody followed by a 4',6-Diamidino-2-phenylindole (DAPI) counter stain. In the fourth ventricle, placenta, and dorsal ganglia Vectastain Elite ABC reagent (Vector) followed by DAB peroxidase (Vector, SC-4100) to develop positive signals. DAB stained slides were counterstained with hematoxylin. Apoptotic cells were determined using a TdT-mediated dUTP nick end-labeling (TUNEL) assay. The assay was performed using the manufacturer's protocol (Chemican S7101) except that the TdT enzyme was incubated at room temperature for 1.5 hours and the Anti-Digoxigenin conjugate was incubated at room temperature for 1 hour. All slides were counterstained with hematoxylin.

Determination of Hematopoietic Nucleation

Blood samples were (4 uL) were spread on slides. Giemsa stain (Sigma, diluted 1:20) was used per manufacturer's protocol to determined nucleation of red blood cells. The same embryos were used both for hematopoietic analysis and histochemical analysis.

Mouse Embryonic Fibroblasts

Mouse embryonic fibroblasts (MEFs) of E13.5 embryos were prepared using standard methods. When cells were almost confluent, they were frozen with liquid nitrogen in a freezing medium (Dulbecco Minimal Essential Medium with 10% DMSO and 25% FBS). The MEFs were unfrozen using standard protocols for analysis.

Quantification of data

To quantify the proliferation and apoptosis data I counted positive-labeled cells and then divided the total number of cells. The quotient was a standardized percentage that was easy to compare across genotypes. About ten areas from each embryo's labyrinth layer of the placenta were counted for comparison. The post-mitotic zone of the fourth ventricle was used to quantify the CNS. In the PNS several dorsal ganglia in different vertebra were counted. All the cells of the lens fiber, which is dorsal of the actual lens, were counted for several sections from each embryo's eyes.

Results

Earlier studies have shown that the ablation of *E2f3* leads to reduced viability and health problems in mice[19]. With the discovery of two distinct gene products from the *E2f3* locus, understanding the contribution of each protein is of interest. *E2f3a* knockout mice were generated by using cre-loxP gene-targeting techniques (Fig. 3a). Afterwards, the successful knockout was confirmed by Southern and Western blot analyses with no difference of expression of E2F3b (Fig. 3b & c). We found that the E2f3a-deficient mice were completely viable and appeared with proper Mendelian proportions. They show no defects and have the same livelihood as their wild type counterparts. They breed normally and have the same lifespan as $E2f3a^{+/+}$ mice. From these results, E2F3a is not needed for neonatal viability. Furthermore, the *E2f3a*-deficient mice show no signs of congestive heart failure which is a trademark of $E2f3^{-/-}$ mice. Largely, there are no apparent anatomical or histological differences between adult $E2f3a^{-/-}$ mice and $E2f3a^{+/+}$ littermates.

MEFs were used to analyze the result of E2f3a-deficiency at the cellular level without affecting *E2f3b* expression. As expected, $E2f3a^{-/-}$ cells were delayed in entering S-phase as compared to wild type MEFs cultured from the same litter (courtesy of Shih-Yin Tsai). Because E2F3a is a transcription activator, the loss of it should reduce initiation of transcription. Other experiments have determined the E2F-responsive genes that E2F3a induces. Some of these are *cdc6*, *cyclinE*, *mcm3*, *polα*, *DHFR*, and *PCNA*. After serum stimulation the gene expression of these downstream targets in $E2f3a^{-/-}$ MEFs is significantly lower than $E2f3^{+/+}$ MEFs. The reduction in expression of these

genes that are required for transcription could be responsible for the delay of S-phase entry.

Since neonatal viability of E2f3a-deficient mice was established, I next focused on embryonic development. *E2f3a*$^{+/-}$ mice were intercrossed and mothers were harvested at E13.5 to analyze the embryos. Visually there was no difference between *E2f3a*$^{-/-}$ mice and *E2f3a*$^{+/+}$ littermates (Fig. 4). Further internal analysis reinforced the initial observation. To examine morphology, slide sections of embryos were stained with Hematoxylin and Eosin. The embryo sections showed no difference in cell morphology or anatomical proximity (Fig. 4).

E2f3a and Rb have opposing roles in the Rb/E2f pathway; Rb regulates the activities of E2Fs by binding to them. Loss of Rb results in unregulated E2F activity which leads to ectopic S-phase entry and inappropriate apoptosis. The loss of Rb regulation is lethal and results in embryonic death around E14. Previous studies indicate that the loss of certain E2Fs suppresses the developmental defects caused by Rb-deficiency and even extends embryonic viability up to E17.5. I was interested in determining if E2F3a was responsible for contributing to the phenotype caused by Rb-deficiency. Crosses of either *Rb*$^{+/-}$*; E2f3a*$^{+/-}$ *or Rb*$^{+/-}$*; E2f3a*$^{-/-}$ mice with *Rb*$^{+/-}$*; E2f3a*$^{+/-}$ were used to achieve *Rb*$^{-/-}$*; E2f3a*$^{-/-}$ embryos. Notably, these double knockout embryos had extended viability up to E17.5 (Table 1). This shows that E2F3a significantly impairs the proper development of Rb-deficient embryos. No double knockout embryos survived to full term. Possibly, the role of Rb in late embryonic development of certain tissues causes the lethality of embryos that lack Rb.

The increased viability of the embryos suggests that the loss of E2f3a must suppress the phenotype of Rb-deficient embryos. Because E2F3a and Rb oppose each other in the respective pathway the loss of E2F3a could rescue ectopic proliferation and inappropriate apoptosis arising from Rb-deficiency. To determine this I examined proliferation, apoptosis, and cell morphology of tissues that are affected by Rb-deficiency. Examining the development of tissues that express Rb at E13.5, a critical time for Rb expression, will give insight into what is occurring in the embryo. The placenta, fourth ventricle of the brain, dorsal ganglia, lens fiber, and red blood cells were all analyzed for the rescue capability of the double knockout.

$Rb^{-/-}$ embryos show a strong phenotype in the placenta. Particularly, dysplasia of trophoblast cells occurs and they invade the labyrinth layer of the placenta where oxygen exchange occurs between the mother and the embryo. With high levels of proliferation and apoptosis, the morphology of the labyrinth layer of the placenta is distorted and vascularization is decreased which reduces oxygen exchange. In *$Rb^{-/-}$* embryos, phenotypes arising from Rb-deficiency were rescued by the incorporation of a wild type placenta for the embryo. Because E2F3a opposes Rb in cell cycle, I was interested in discovering if E2f3a contributed to the phenotype in Rb-deficient placentas. To accomplish this I examined placentas from placentas from *$Rb^{+/+}$; $E2f3a^{+/+}$*, *$Rb^{-/-}$; $E2f3a^{+/+}$*, *$Rb^{-/-}$; $E2f3a^{-/-}$*, and *$Rb^{+/+}$; $E2f3a^{-/-}$* embryos. The embryo sections were H & E stained in order to show cell morphology. Embryos were dissected at E13.5 because that is when Rb is critical for development and *$Rb^{-/-}$; $E2f3a^{+/+}$* embryos usually die after E14. As expected the E13.5 *$Rb^{-/-}$* embryo's placentas phenotypes matched previously published results[7]. Furthermore, *$E2F3a^{-/-}$* placentas showed no variation from the wild

type placentas (Fig. 5a). In *Rb*$^{-/-}$; *E2f3a*$^{-/-}$ embryos the morphology was restored to the wild type phenotype; there were no clusters of trophoblast cells in the labyrinth. Furthermore, vascularization appears to be improved over the Rb-deficient embryos which should lead to better oxygen exchange.

In light of morphology being rescued, other cellular processes might exhibit wild type activity also. Because the loss of Rb results in ectopic S-phase entry and inappropriate apoptosis, I tested the same placenta samples for proliferation and apoptosis. To test for proliferation I used BrdU incorporation. BrdU was injected into the pregnant mother two hours before harvesting. In the wild type placenta an average of 22.01% of the cells in the labyrinth were in S-phase (Fig. 5b). In *Rb*$^{-/-}$; *E2f3a*$^{+/+}$ placentas nearly 43% of the cells were in S-phase. The loss of E2F3a, *Rb*$^{-/-}$; *E2f3a*$^{-/-}$, rescued the proliferation in the placenta, only 19.26% of the cells are in S-phase. As expected, there is no difference between *Rb*$^{+/+}$; *E2f3a*$^{-/-}$ and wild type embryos. Next, inappropriate apoptosis was quantified using TUNEL assays. The loss of E2F3a significantly reduced inappropriate apoptosis in the Rb-deficient embryo; however, a complete rescue was not observed (Fig. 5c).

In previous experiments the loss of E2F3 has shown to completely suppress the neurological defects of Rb-deficiency in murine embryos[7]. I was interested whether the loss of E2F3a would have the same effect. Using the same embryos from previous experiments, I examined both the fourth ventricle of the CNS and the dorsal ganglia from the PNS in *Rb*$^{+/+}$; *E2f3a*$^{+/+}$, *Rb*$^{-/-}$; *E2f3a*$^{+/+}$, *Rb*$^{-/-}$; *E2f3a*$^{-/-}$, and *Rb*$^{+/+}$ *E2f3a*$^{-/-}$ embryos at E13.5. Morphologically there was a small detectable difference in the CNS between genotypes (Fig. 6a). However, quantification of the proliferating cells in the post-mitotic

region of the fourth ventricle showed an Rb-deficient phenotype. As expected the $Rb^{+/+}$; $E2f3a^{+/+}$ and $Rb^{+/+}$; $E2f3a^{-/-}$ embryos had very low levels of proliferation in the post-mitotic region. The $Rb^{-/-}$; $E2f3a^{+/+}$ embryos had cells proliferating in the post-mitotic region (about 7%) as previously characterized (Fig. 6b). Like in the placenta, the $Rb^{-/-}$; $E2f3a^{-/-}$ embryos showed a complete rescue with virtually no proliferating cells in the post-mitotic region. The dorsal ganglia cells were quantified to compare proliferation and apoptosis in the PNS (Fig. 6c). Like in the CNS, the dorsal ganglia demonstrated ectopic proliferation in the $Rb^{-/-}$; $E2f3a^{+/+}$ embryo (Fig.7). Consistent with other tissues, the $Rb^{+/+}$; $E2f3a^{+/+}$ and $Rb^{+/+}$; $E2f3a^{-/-}$ embryos resembled each other in regards to S-phase entry of the cells. The loss of E2f3a only partially rescues ectopic proliferation by about 70% (Fig. 7b). Apoptosis in the nervous system was also measured by TUNEL assays. The fourth ventricle showed a complete rescue. On the other hand, the dorsal ganglia only showed a reduction in cells that stained positive for apoptosis (Fig. 7c).

In the lens proliferation and apoptosis were also measured. I was interested in quantifying the post-mitotic region known as the lens fiber which is on the dorsal side of the actual lens. The morphology of the lens in the double knockout embryo is severely distorted (Fig. 8). The morphology of the $Rb^{-/-}$; $E2f3a^{-/-}$ embryos was between the wild type and $Rb^{+/+}$; $E2f3a^{-/-}$ embryos on the basis of cells present in the lens fiber tissue samples. The $Rb^{+/+}$; $E2f3a^{+/+}$ and $Rb^{+/+}$; $E2f3a^{-/-}$ embryos contained a little less than 100 cells per tissue section of the lens fiber and the $Rb^{+/+}$; $E2f3a^{-/-}$ embryos contained around 265 cells per tissue section. Interestingly, there were about 200 cells per tissue sample in the $Rb^{-/-}$; $E2f3a^{-/-}$ embryos. Quantifying S-phase entry gave insight into this observation.

In the lens fiber tissue, the loss of E2F3a reduces ectopic S-phase entry of *Rb*$^{+/+}$*; E2f3a*$^{-/-}$ embryos by 45% (Fig. 9a). Furthermore, apoptosis was only reduced slightly (Fig. 9b).

Defects in erythroid development have been implicated in causing the *Rb*$^{-/-}$ embryonic lethality around E14. Our lab showed that replacing an Rb-deficient placenta in with a wild type placenta in an *Rb*$^{-/-}$ embryo will extend viability, but will not overcome the erythropoiesis defect[13]. In *Rb*$^{-/-}$ embryos red blood cells (RBCs) do not enucleate properly and are ineffective at carrying oxygen[13]. Previous experiments showed that the loss of E2F3 only partially rescued the enucleation defect in Rb-deficient mice[7]. I wanted to discern if the loss of E2F3a could also reduce the lack of enucleation. By Giemsa staining blood samples of harvested *Rb*$^{+/+}$*; E2f3a*$^{+/+}$, *Rb*$^{-/-}$*; E2f3a*$^{+/+}$, *Rb*$^{-/-}$*; E2f3a*$^{-/-}$, and *Rb*$^{+/+}$*; E2f3a*$^{-/-}$ embryos I could quantify if the loss of E2F3a could cause more RBCs in Rb-deficient embryos to properly enucleate (Fig 10a). I discovered that there was no difference in the percentage of enucleated cells in *Rb*$^{+/+}$*; E2f3a*$^{-/-}$ and *Rb*$^{-/-}$*; E2f3a*$^{-/-}$ embryos (T-test: $p>0.1$). As seen throughout these experiments, there was no detectable difference between enucleated RBCs in *Rb*$^{+/+}$*; E2f3a*$^{+/+}$ and *Rb*$^{+/+}$*; E2f3a*$^{-/-}$ embryos (Fig. 10b). Extended viability did not result from rescued erythroid processes.

Discussion

The explosion of recent knowledge regarding the Rb/E2F pathway has expanded the roles of the proteins involved and has increased the understanding how cells are complexly regulated. Previous experiments have shown that Rb and E2F3 are both necessary for viability. E2F3 has shown to be a major contributor to the pathway with unique functions. Without E2F3 MEFs do not divide as quickly as wild type cells. Also, loss of E2F3 up regulates p19ARF expression and E2F3 is required for proper centrosome division[22]. The discovery of to *E2F3* gene products, E2F3a and E2F3b, has prompted many questions about the functional specificity of each protein. Fully understanding the pathway relies on elucidating the individual functions of each protein and determining redundancy effects.

Unlike E2F3-deficient mice, E2F3a-deficient mice are fully viable and exhibit normal livelihoods and breeding capabilities. Whether E2F3b is required for viability or if only a component of E2F3 (be it E2F3a or E2F3b) is not known at this time. Experiments with E2F3b-deficient mice will help answer this question. Determining what E2F3 activities E2F3a is responsible for is of interest of cell biology and cancer research. Loss of E2F3 has shown to rescue most of the phenotypes caused by Rb-deficiency as well as extend the life of the embryos from E14 up to E17. Importantly, my study determined that losing just E2F3a has the most of the same rescue capability of losing all of the E2F3 protein. $E2F3a^{-/-}; Rb^{-/-}$ mice show a complete rescued phenotype in the placenta and CNS. Furthermore, there is a partial rescue in the PNS and the lens fiber. However, there is no rescue of the erythropoietic defects caused by Rb-deficiency. These findings compared to earlier studies that focused on losing all of E2F3 suggest that

E2f3a and E2F3b act differently in different tissues. They may be expressed in different amounts in specific tissues or may also have the ability to mimic each others function. Further study of E2F3b will fully answer this question. These studies show that E2F3a is the major contributor of the *E2F3* gene products for proliferation and apoptosis regulation. This is not to say that E2F3b does not have any of this ability also, although E2F3b may act in different tissues than E2F3a.

The complete rescue of the placenta is an interesting development. Our lab previously showed that replacing an Rb-deficient placenta with a wild type placenta will extend the viability of $Rb^{-/-}$ embryos[13]. Interestingly the loss of E2F3a extended the lives of these embryos also without rescuing any of the erythropoietic defects. This strengthens the argument that Rb-/- embryos die at E14 because of placenta defects instead of erythropoietic defects. Based on prior experiments, the rescue of the placenta caused by the loss of E2f3a in Rb-deficient embryos could be the reason that the embryos had extended viabilities up to E17.5.

One of the most interesting characteristics of the Rb/E2F pathway is the regulation of apoptosis. Two different models have been proposed for the inappropriate apoptosis resulting from Rb-deficiency. First, the loss of Rb triggers p53-dependent apoptosis. The second model claims that a high concentration of free E2F can trigger apoptosis also through both p53-dependent and p53-independent mechanisms. This may be a method to safeguard against unwanted proliferation and ultimately cancer. My data concerning apoptosis was striking in that the rescue of apoptosis varied from a complete rescue in some tissues and barely any rescue in other tissues. $Rb^{-/-}; E2F3a^{+/+}$ embryos are expected to have a high level of apoptosis because of the loss of Rb and the ability to

control apoptosis. However, the variation in $Rb^{-/-}$; $E2F3a^{-/-}$ embryonic apoptosis in tissues is shocking. My data supports that high levels of free E2F can cause apoptosis. By losing E2F3a in the Rb-deficient embryo the levels of free E2F are reduced, especially if E2F3a is expressed highly in these tissues in wild type embryos. The difference in apoptosis frequency in other tissues may be a result of high E2F3b expression or low expression of E2F3a in that tissue in wild type embryos. One problem with this model must be worked out. Experiments need to determine if the loss of E2F3a in specific tissue up regulates expression of other E2Fs in that tissue.

E2F3a is characterized as a transcription activator. MEFs that lack E2F3a are slow in initiated replication after serum starvation. My results show that losing E2F3a in the Rb-deficient embryo reduces proliferation. These combined results suggest that E2F3a has oncogenic ability. E2F3a promotes transcription; without regulation from Rb ectopic proliferation happens because of the unregulation of the activator E2Fs. Further experiments need to be done to determine to what extent E2F3a has oncogenic ability; to study this, E2F3a could be over expressed in mice to see whether tumor formation results.

Rb inactivation occurs in most if not all cancers. Loss of Rb in cancer leads to deleterious phenotypes; there is no longer any regulation of proliferation, apoptosis, and the cell cycle. By losing Rb the Rb/E2F pathway loses regulation and the activator E2Fs are able to make cells proliferate ectopically. Study of this pathway in cancer has produced many valuable results. Simultaneously losing an antagonist of Rb can lessen the phenotypical consequences of Rb-deficiency and can lead to improved viability by suppressing tumor formation. For instance $Rb^{+/-}$; $E2F3^{-/-}$ mice have extended viability

and suppress pituitary tumors in comparison to $Rb^{+/-}$ cancer-prone mice. My results show that the loss of E2F3a mitigates the effects of Rb-deficiency in embryos. Judging by these results, the loss of E2F3a may also suppress tumor formation in $Rb^{+/-}$ mice. Currently, I am conducting a tumor study to test for this hypothesis.

The E2Fs play an interesting role in cancer. There purpose is to initiate the G_1/S-phase transition. Deregulation of the activator E2Fs will lead to ectopic proliferation which is a trademark of cancer. Previous experiments have shown both redundant and unique functions among the E2F family. Some of the E2Fs have a wide array of functions. For instance, E2F3 has proven to be the most unique functions. There are binding sites for the Myc oncogene on the promoter for E2F3. During a cancerous state, E2F3 will be up regulated by increased Myc expression. An increase in E2F3 expression will lead to ectopic proliferation which will promote tumor formation. Also, E2F3 has been shown to regulate centrosome formation in dividing cells. Cells that lack E2F3 have abnormal numbers of centrosomes and exhibit aneuploidy. The resultant abnormal cells are irregular and have been shown to lead to increased metastasis. Completely understanding the role of E2F3a will give further insight into the genetics behind cancer. Knocking out E2F3a may be a prime choice to reduce cancer phenotypes associated with E2F3. E*2f3a* has a Myc binding site at the promoter while *E2f3b* does not. Ablation of the *E2F3a* gene may not increase proliferation in the presence of Myc up regulation, because Myc cannot promote transcription of the protein. Furthermore, the presence of E2F3b may be enough to sustain proper centrosome formation.

Controlling E2F function will be paramount for cancer therapeutics in the future. If ectopic proliferation is reduced then tumor growth and ultimately metastasis can be

delayed. The various E2Fs all have shared and unique functions. They can exhibit both tumor suppressor and oncogene properties. Understanding the key relationships and activities of E2F proteins is vital in understanding and harnessing cancer. Ablation of an E2F may have either positive or negative effects in cancer suppression. Furthermore, the actual function of an E2F may vary between tissues. Considerable research into understanding E2F functions very well may lead to devising cancer therapies in the future.

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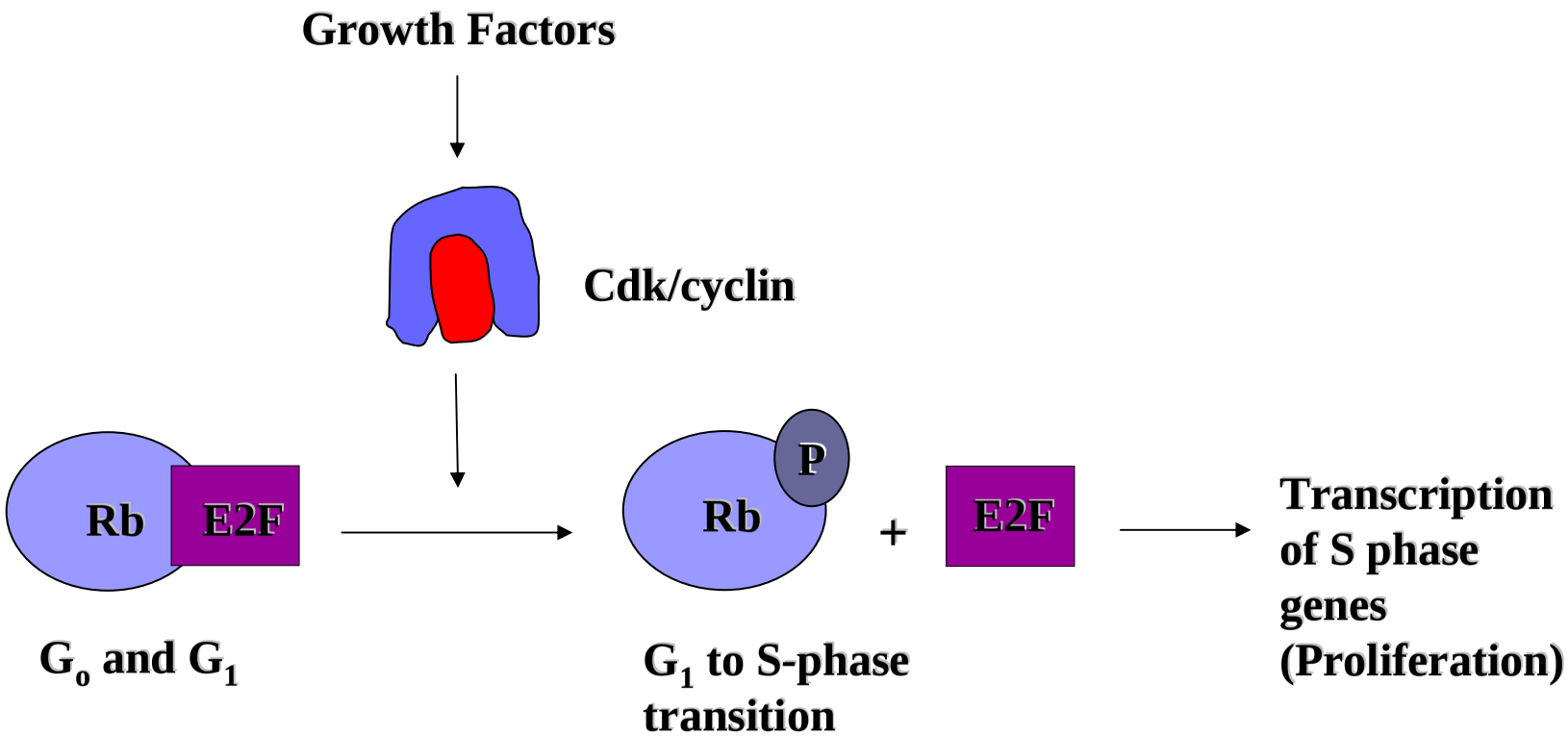
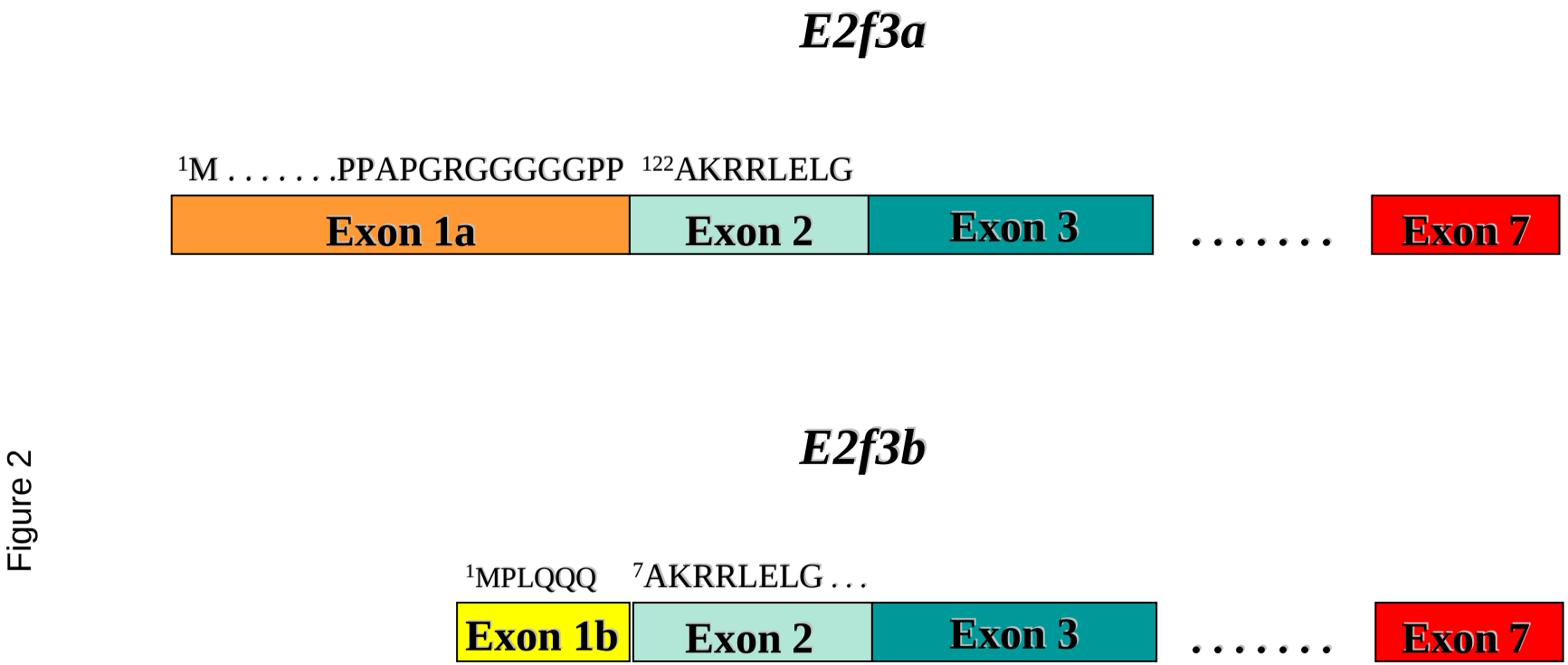
Figure 1



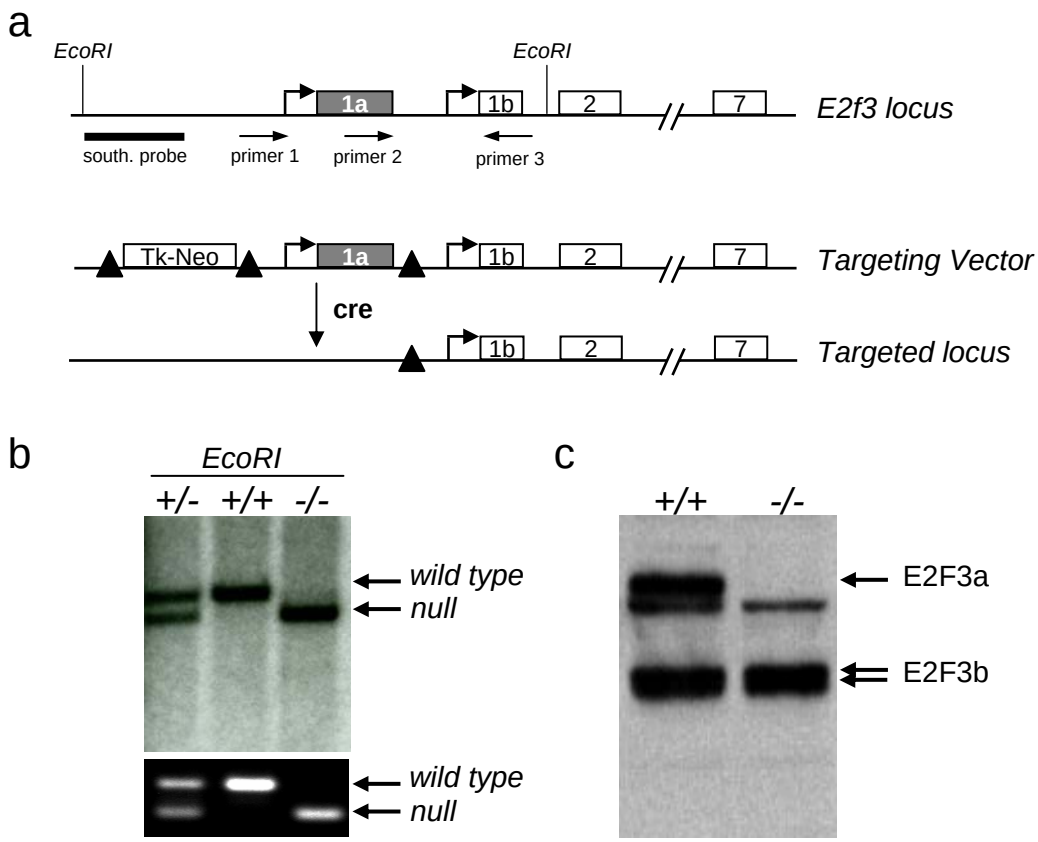
Overview of the Rb/E2F Pathway

Figure 2



Protein sequences for E2F3a and E2F3b. Exon 1a is 122 amino acids long while exon 1b is 6 amino acids long. Exons 2-7 are the same.

Figure 3

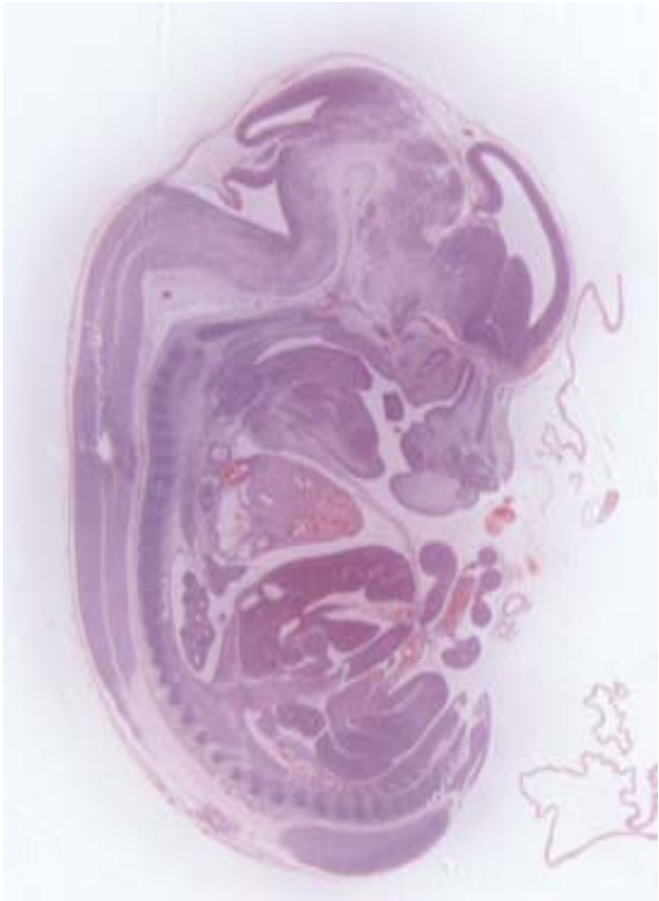


Generation of E2f3a-deficient mice. A, genomic structure of the *E2f3a* locus. Exon of interested is exon 1a. Three loxp sites and Cre-mediated deletion removes the exon. The southern probe is upstream of exon 1a. B, Southern blot analysis of DNA from embryos with the indicated genotypes. C, Western blot analysis showing deletion of E2F3a protein and unaffected expression of E2F3b protein

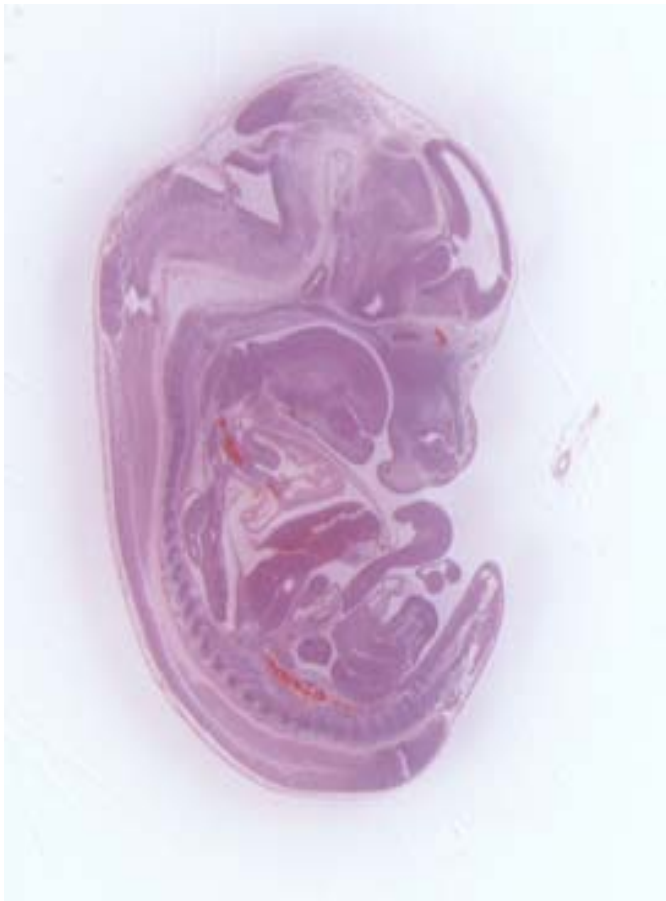
Figures courtesy of Shih-Yen Tsai and Rene Oparvsky

Figure 4

Comparison of H & E Stained Embryos (E13.5)



Wild Type



E2f3a mutant

Genotypic analysis of embryos derived from *Rb*$^{+/-}$;*E2f3a*$^{+/-}$ and *Rb*$^{+/-}$;*E2f3a*$^{-/-}$ intercrosses

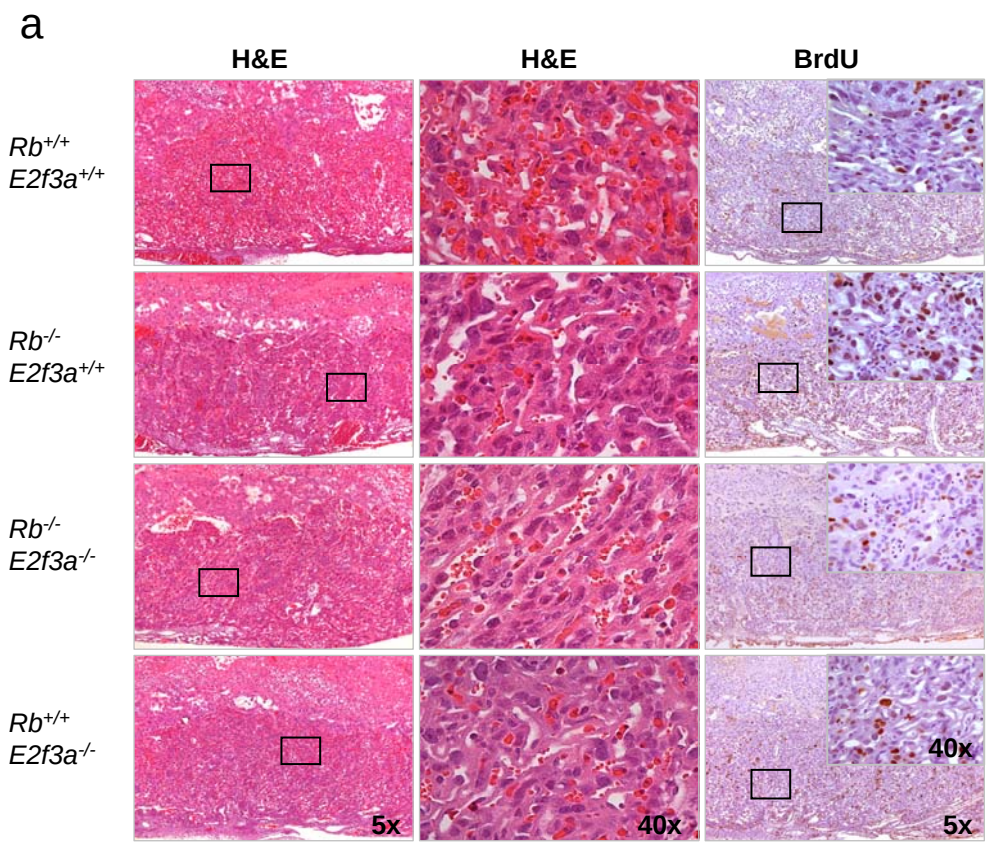
Table 1

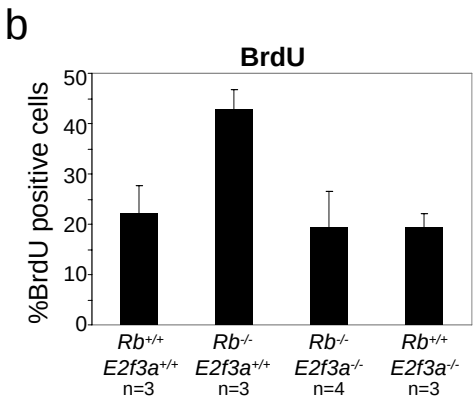
	Rb$^{+/+}$			*Rb*$^{+/-}$			*Rb*$^{-/-}$			total
	E2f3a$^{+/+}$	*E2f3a*$^{+/-}$	*E2f3a*$^{-/-}$	*E2f3a*$^{+/+}$	*E2f3a*$^{+/-}$	*E2f3a*$^{-/-}$	*E2f3a*$^{+/+}$	*E2f3a*$^{+/-}$	*E2f3a*$^{-/-}$	
E13.5	6	20	7[a]	11	33 (1)	16 (1)	4 (2)[a]	14 (5)	6 (2)[b]	128
expected	*9*	*15*	*9*	*13*	*31*	*22*	*7*	*15*	*9*	
E15.5	12	15	9[a]	16 (1)	31 (1)	11	0 (9)[b]	2 (9)	5 (3)[b]	124
expected	*9*	*13*	*9*	*19*	*26*	*18*	*9*	*13*	*9*	
E16.5	-	6	7[a]	-	5	8	-	-	2 (4)[b]	32
expected	*-*	*3*	*5*	*-*	*7*	*8*	*-*	*3*	*5*	
E17.5	2	3	4[a]	5	4	12	0 (1)	0 (2)	1 (7)[c]	41
expected	*1*	*3*	*6*	*3*	*6*	*13*	*1*	*3*	*6*	

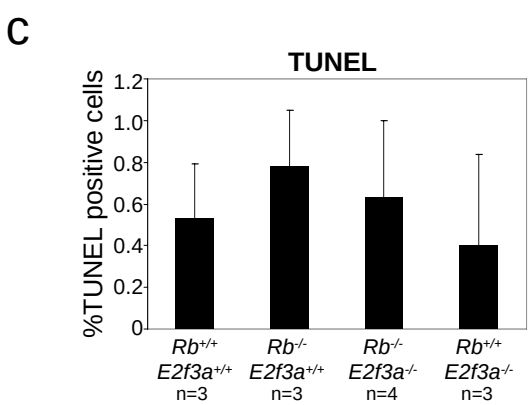
[a] not significant ($p>0.1$); [b] significant ($p<0.05$); [c] highly significant ($p<0.025$).

Viability table for different embryonic ages, parenthesis indicates number of embryos that are unviable at a given age. Significant data is noted under the table.

Figure 5

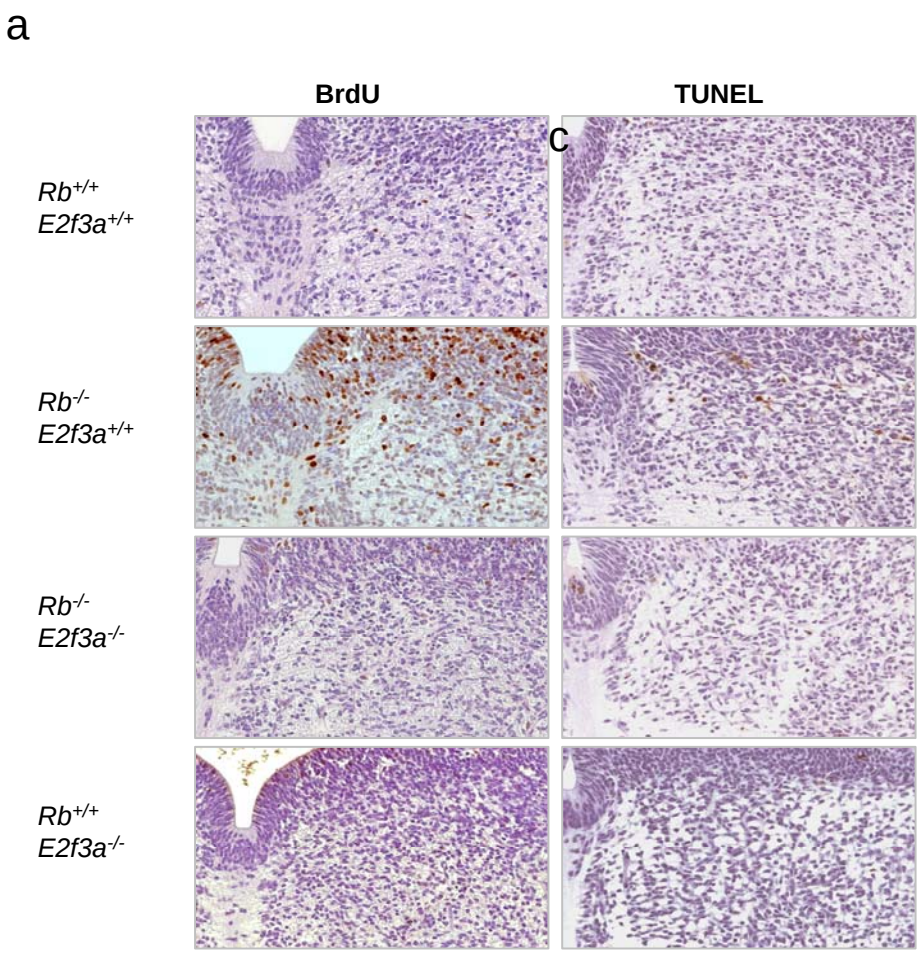


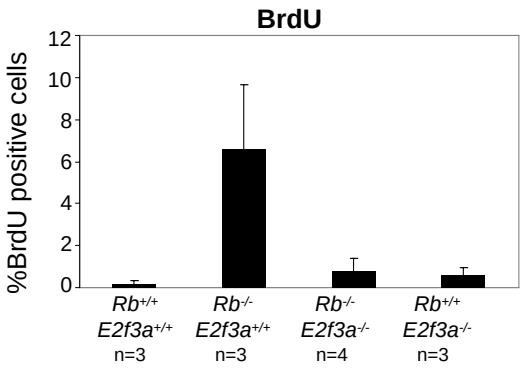


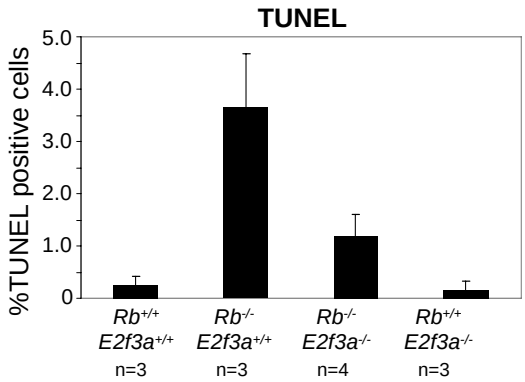


Comparison of the labyrinth of the placenta from different genotypes

Figure 6

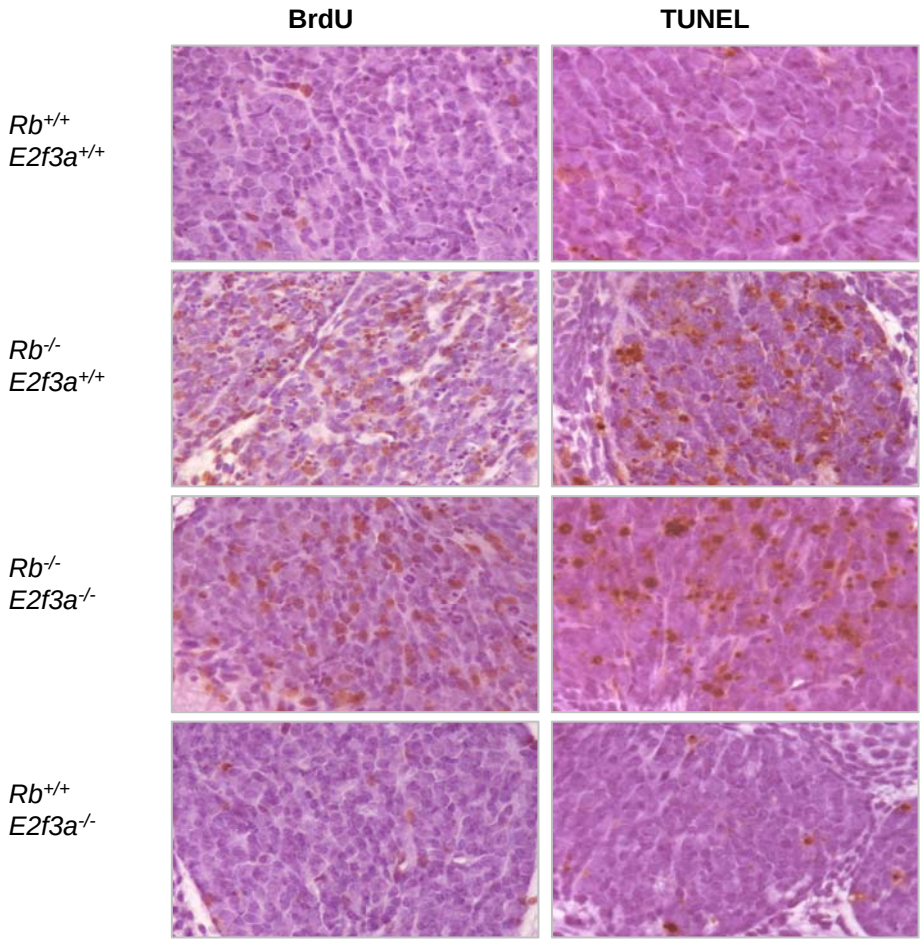


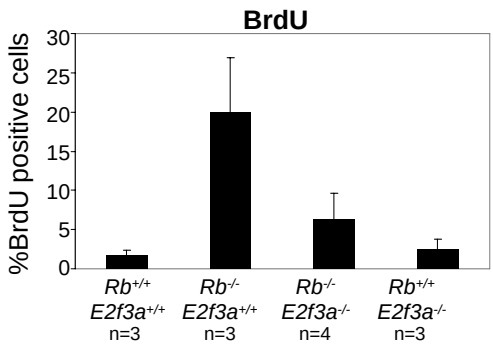


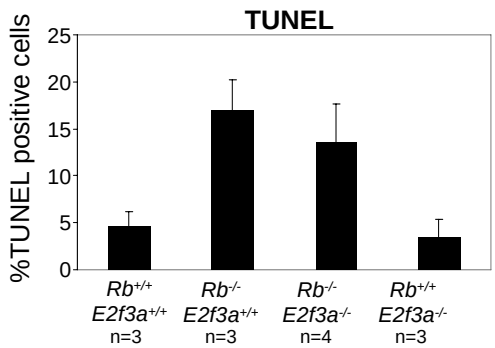


Fourth Ventricle

Figure 7







Dorsal Ganglia

Figure 8

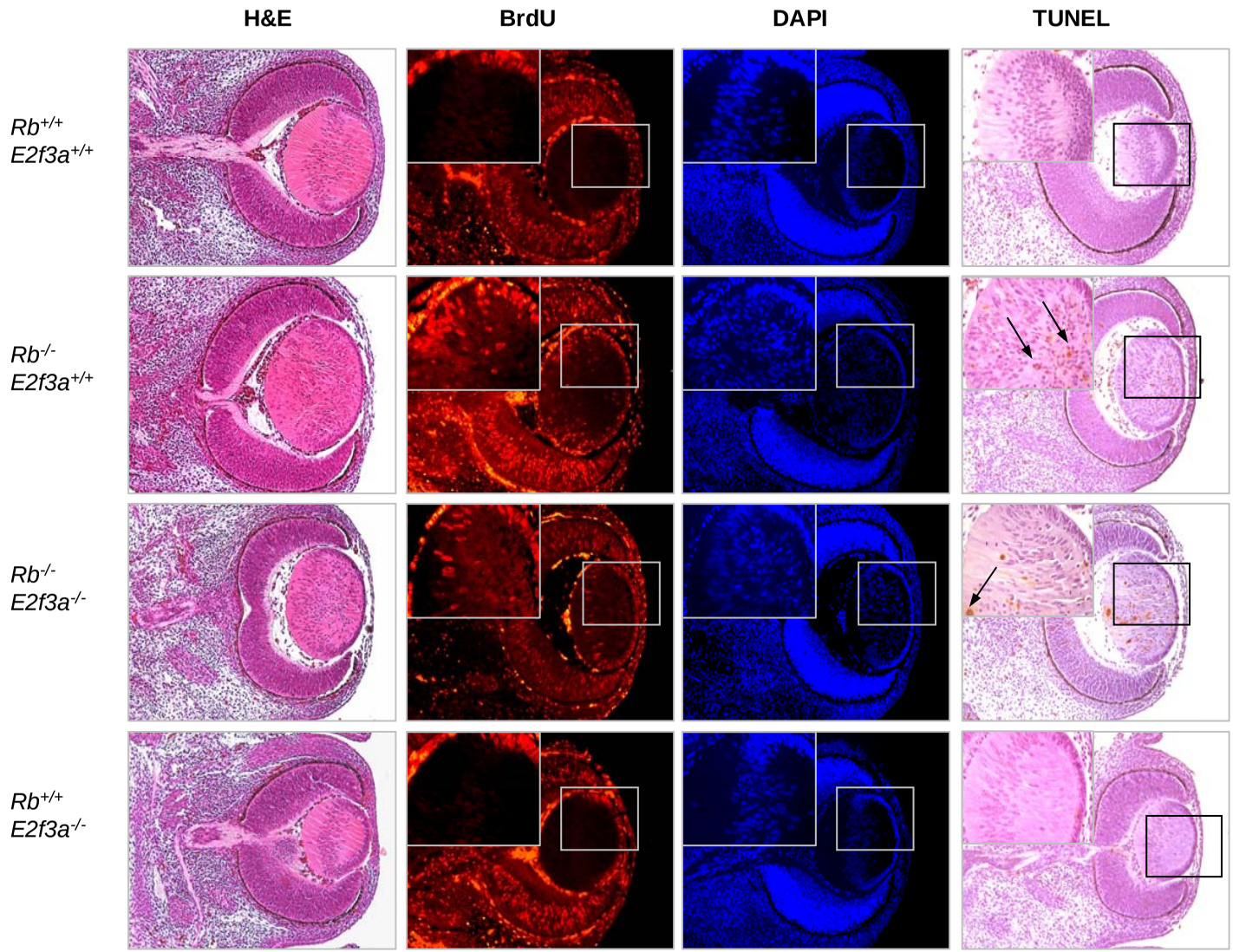
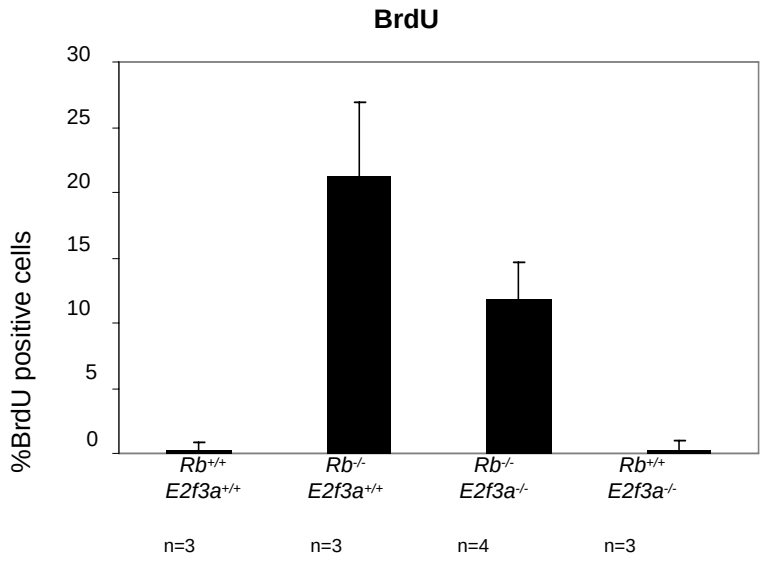

H&E
BrdU
DAPI
TUNEL
Rb+/+
E2f3a+/+
Rb-/-
E2f3a+/+
Rb-/-
E2f3a-/-
Rb+/+
E2f3a-/-
Lens Fiber

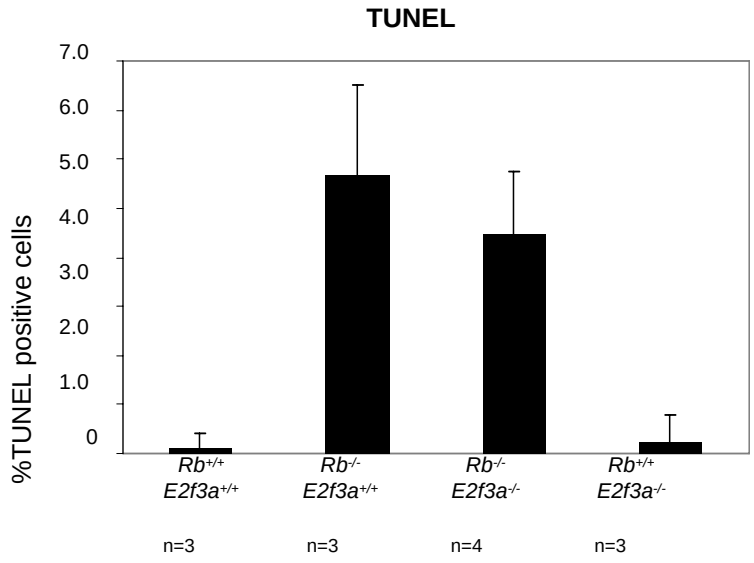
Figure 9

a



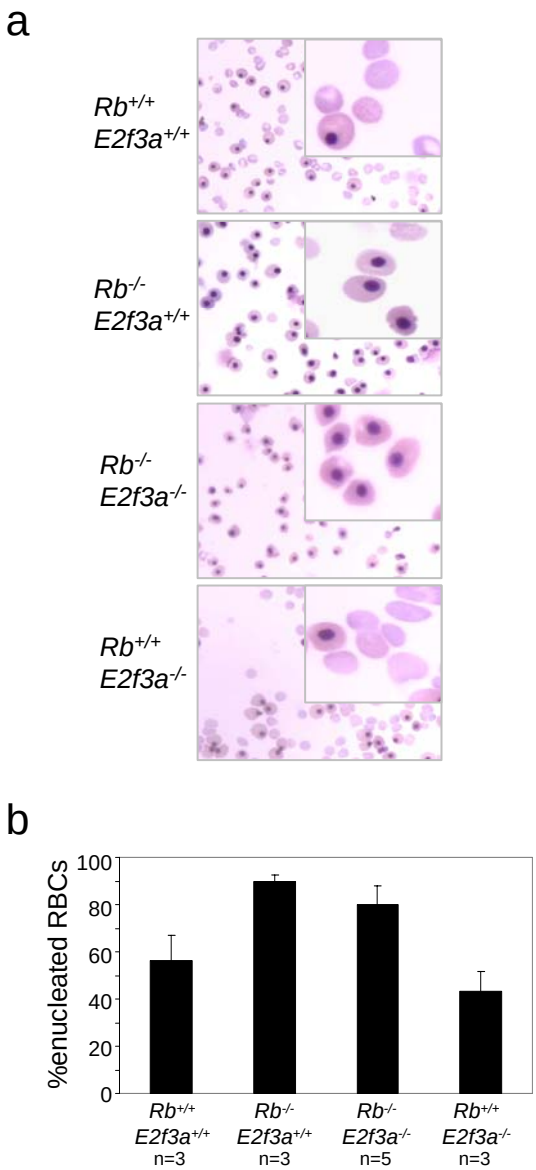
BrdU positive cell graph for lens fiber

b



TUNEL positive cells graph for lens fiber

Figure 10



A, Giemsa stain of RBCs. B, Nucleated RBC data