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E2F4 cooperates with pRB in the development of extra-embryonic tissues

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Abstract

The retinoblastoma gene, RB-1, was the first identified tumor suppressor. Rb−/− mice die in mid-gestation with defects in proliferation, differentiation and apoptosis. The activating E2F transcription factors, E2F1-3, contribute to these embryonic defects, indicating that they are key downstream targets of the retinoblastoma protein, pRB. E2F4 is the major pRB-associated E2F in vivo, yet its role in Rb−/− embryos is unknown. Here we establish that E2f4 deficiency reduced the lifespan of Rb−/− embryos by exacerbating the Rb-mutant placental defect. We further show that this reflects the accumulation of trophoderm-like cells in both Rb and Rb;E2f4 mutant placentas. Thus, Rb and E2f4 play cooperative roles in placental development. We used a conditional mouse model to allow Rb−/−;E2f4−/− embryos to develop in the presence of Rb wildtype placentas. Under these conditions, Rb−/−;E2f4−/− mutants survived to birth. These Rb−/−;E2f4−/− embryos exhibited all of the defects characteristic of the Rb and E2f4 single mutants and had no novel defects. Taken together, our data show that pRB and E2F4 cooperate in placental development, but play largely non-overlapping roles in the development of many embryonic tissues.

INTRODUCTION

The retinoblastoma protein, pRB, is a member of the family of pocket proteins that includes p107 and p130. The most extensively studied downstream effectors of the pocket proteins are the E2F family of transcription factors (Attwooll et al., 2004; Dimova and Dyson, 2005; Trimarchi and Lees, 2002). Both the pocket proteins and the E2Fs have well defined roles in regulating proliferation and differentiation during development (McClellan and Slack, 2007; Wikenheiser-Brokamp, 2006). In particular, the interplay between the pocket proteins and the E2Fs controls cell cycle exit and entry via the transcriptional regulation of core components of the cell cycle machinery.

The E2F family is comprised of nine members, only six of which are regulated by pocket protein binding. These six proteins play preferential roles in either the activation (E2F1, E2F2, E2F3A and B) or repression (E2F4 and E2F5) of E2F target genes (Attwooll et al., 2004; Dimova and Dyson, 2005; Trimarchi and Lees, 2002). The activating E2Fs are specifically...
regulated by pRB in normal cells. In G0/G1 cells, pRB binds to these E2Fs and suppresses their transcriptional activity. Mitogenic signaling induces pRB phosphorylation by Cyclin/CDK complexes, which causes the release of activating E2Fs, allowing them to bind to and stimulate transcription of E2F-responsive genes. The activating E2Fs drive S-phase progression when they are over-expressed (Lukas et al., 1996) (Muller et al., 1997) and cells that lack these proteins have impaired proliferative capacity (Chong et al., 2008; Danielian et al., 2008; Humbert et al., 2000b; Tsai et al., 2008; Wu et al., 2001). The repressive E2Fs have distinct pocket protein binding properties; E2F5 binds specifically to p130 in vivo, while E2F4 associates with pRB, p107 and p130 (Attwooll et al., 2004; Dimova and Dyson, 2005; Trimarchi and Lees, 2002). E2F4 is the most abundant E2F family member in vivo and it typically accounts for the majority of the endogenous E2F-pocket protein complexes. In G0/G1 cells, E2F4 and E2F5 contribute to the active repression of E2F-responsive promoters by recruiting large complexes that include the pocket proteins and associated histone modifying enzymes (Blais and Dynlacht, 2007). Mitogenic signaling causes dissociation of these repressive complexes and released E2F4 and E2F5 translocate to the cytoplasm. Consistent with their repressive roles, mouse embryo fibroblasts (MEFs) deficient for E2F4 and E2F5 have no proliferation defects, but they are unable to arrest in response to some growth inhibitory signals (Gaubatz et al., 2000). Mouse models have shown that E2f4 mutation causes a wide variety of developmental defects including transient embryonic anemia and defective erythropoiesis (Humert et al., 2000a; Kinross et al., 2006; Rempel et al., 2000), impaired commitment to the lymphoid lineage (Enos et al., 2008), abnormal patterning of the ventral telencephalon (Ruzhynsky et al., 2007), disrupted ossification in some bones (E.S.Miller and J.A.L unpublished observations), poor mammary gland development (Andrechek et al., 2008), and loss of ciliated cells from the airway epithelium (Danielian et al., 2007). In these cases E2F4 appears to have functions both dependent and independent of cell cycle regulation.

Mutant mouse models have also been key in defining the roles of pRB in tumorigenesis and normal development (Burkhart and Sage, 2008; Wikenheiser-Brokamp, 2006). Rb+/− mice are predisposed to develop tumors and this is dependent upon loss of the wild-type Rb allele. Rb−/− embryos die in mid-gestation by E15.5 and exhibit defective erythropoiesis in the fetal liver and widespread inappropriate proliferation and apoptosis in the central nervous system (CNS) peripheral nervous system (PNS) and ocular lens (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992; Macleod et al., 1996; Morgenbesser et al., 1994). Germline and conditional mouse models have also revealed a critical role for Rb in several developmental processes including hematopoiesis and erythroid differentiation (Walkley et al., 2008), skeletal muscle formation (Huh et al., 2004; Zacksenhaus et al., 1996), and bone (Berman et al., 2008), skin (Ruiz et al., 2004; Wikenheiser-Brokamp, 2004) and intestinal epithelium (Haigis et al., 2006; Yang and Hinds, 2007) development.

Studies with chimeric and conditional animals have demonstrated that the apoptosis in the CNS and a portion of the erythroid defects arise as secondary consequences of a placental defect in Rb mutant embryos (Wenzel et al., 2007; Wu et al., 2003). Specifically, inappropriate proliferation of trophoblast stem cells in the labyrinth layer of pRB-deficient placentas results in a compaction of this layer and decreased nutrient and gas exchange between the mother and developing fetus. Consistent with this observation, the mid-gestational lethality of the Rb−/− mice is circumvented by the presence of a wild-type placenta. Notably, analyses of both conditional and chimeric Rb mutant mice show that ectopic proliferation in the lens, CNS and PNS as well as abnormalities in erythrocyte maturation and myogenesis are all cell-autonomous defects (de Bruin et al., 2003; Ferguson et al., 2002; Lipinski et al., 2001; MacPherson et al., 2003; Zacksenhaus et al., 1996).

The analyses of compound Rb;E2f mutant mice have shown that the activating E2Fs contribute to many of the Rb mutant phenotypes. First, mutation of either E2f1 or E2f3 modulates the...
tumor phenotype of the Rb+/− mice (Parisi et al., 2007; Yamasaki et al., 1998; Ziebold et al., 2003). Second, germline mutation of E2f1, E2f2 or E2f3 is sufficient to extend the lifespan of Rb−/− embryos (Dirlam et al., 2007; Saavedra et al., 2002; Tsai et al., 1998; Wenzel et al., 2007; Ziebold et al., 2001). In this case, the individual E2Fs play somewhat different roles. Mutation of E2f2 ameliorates many of the erythrocyte defects (Dirlam et al., 2007). Loss of E2f1 or E2f3 has little effect on the abnormalities in erythrocyte maturation or myogenesis, but greatly suppresses both the apoptosis and proliferation defects in the Rb−/− embryos, including the apoptosis that is attributed to the placental defect. Furthermore, mutation of E2f3 in trophoblast stem cell lineage suppresses the extra-embryonic defect of Rb mutants (Wenzel et al., 2007). Taken together, these observations show that pRB-loss allows E2F1 and E2F3 to induce inappropriate proliferation in extra-embryonic tissues causing poor placental function, which leads to apoptosis in the embryo proper.

We have focused our attention on the functional relationship of pRB and E2F4, the major repressive E2F. We have previously shown that the absence of E2F4 suppresses tumors in both Rb+/− mice and Rb chimeras (Lee et al., 2002; Parisi et al., 2008). Although this seems counter to the notion that E2F4 is a repressive E2F, there is some evidence that E2F4 suppresses tumorigenesis through an indirect mechanism (Lee et al., 2002). Given these observations, we wished to determine how E2F4 contributes to the developmental defects arising in Rb−/− embryos.

**MATERIALS AND METHODS**

**Mouse strains**

Mixed (C57BL/6 × 129/Sv) background E2f4+/− and Rb+/− mouse strains (Humbert et al., 2000a; Jacks et al., 1992) were used to obtain germline Rb;E2f4 compound mutant embryos. The detection of a vaginal plug was considered to be E0.5. Embryos were harvested at the indicated times and viability was determined by the presence of a heartbeat under a stereomicroscope. Tissues were fixed in formalin or 4% paraformaldehyde in phosphate buffered saline, embedded in paraffin and sectioned at 4–6µm. Sections were stained with hematoxylin and eosin for histological analysis. The placental defect was bypassed by breeding E2f4+/−, Rb2lox/2lox, and Meox2cre/+ strains (Humbert et al., 2000a; Sage et al., 2003; Tallquist and Soriano, 2000). Timed Rb2lox/2lox;E2f4+/− × Rb+/++;E2f4+/−;Meox2cre/+ crosses were set up to obtain Rb2lox/2lox;E2f4−/−;Meox2cre/+ mice at the indicated developmental ages. At E18.5, the ability to breathe was assessed upon removal of the embryos from the yolk sac. Mice were fixed in Bouin’s fixative or formalin, embedded in paraffin and sectioned at 4–6µm. Sections were stained with hematoxylin and eosin for histological analysis.

**Immunohistochemistry and skeletal staining**

For 5-bromo-2’-deoxyuridine (BrdU) incorporation analysis, pregnant mice were injected intraperitoneally with a BrdU and fluorodeoxyuridine mixture 1 hour prior to sacrifice. Staining for BrdU was performed as previously described (Tsai et al., 2002) and hematoxylin was used for counterstain. Terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) assays were performed to assess levels of apoptosis. Sections were deparaffinized, rehydrated, blocked in 3% H2O2, treated with proteinase K, and incubated with biotin-16-dUTP (Roche) and recombinant terminal deoxynucleotidyltransferase (Invitrogen). Biotin-dUTP was detected with ABC peroxidase detection kit and DAB (Vector Labs). Methyl green was used for counterstain. The number of BrdU- and TUNEL-positive nuclei over the total number of nuclei in a given area was determined for 2–4 independent samples per genotype. Average values were then adjusted relative to the percentages observed in Rb mutants, which were set to 1. Quantification of ectopically proliferating cells in the CNS was determined by counting

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cells in the intermediate zone in the hindbrains. Skeletal stainings were performed as previously described (Humbert et al., 2000a).

**In situ hybridization**

*In situ* hybridization was performed on 4% paraformaldehyde (PFA) fixed, paraffin embedded sections. Sections were deparaffinized, rehydrated, treated with proteinase K, refixed with 4% PFA, incubated in 2x SSC, dehydrated and air dried. Prehybridization was carried out for 1 hour followed by overnight hybridization with digoxigenin-labeled riboprobes for *Pl-1* (Colosi et al., 1987) and *4311/Tpbp* (Lescisin et al., 1988). Washes were done in 50% formamide/1x SSC/0.1% Tween-20 followed by washes in MABT (100mM Maleic acid, 150mM NaCl, pH7.5, 0.5% Tween-20). Sections were blocked in 10% sheep serum/2% blocking reagent (Roche) and then incubated with an anti-digoxigenin antibody conjugated to alkaline phosphatase (Roche) at 4°C overnight. Sections were washed in MABT/levimasole (Sigma) and BM purple (Roche) was used for detection.

**Oligonucleotide microarray analysis and real time PCR**

Extra-embryonic tissues were separated from the maternal deciduas under a stereomicroscope. Total RNA was prepared with Trizol reagent (Invitrogen). RNA quality and concentration were determined using an Agilent 2100 Bioanalyzer. For microarray analysis, 2–3 biological replicates per genotype were used at E13.5 and at E11.5. Target preparation and hybridization to GeneChip® Mouse Genome 430 2.0 arrays (Affymetrix) were performed according to the manufacturer’s instructions. The microarrays were scanned with GeneChip® Scanner 3000 and images were analyzed with GeneChip® Operating Software. Data was processed and normalized using the Affymetrix (1.20.0) and gcRMA 2.14.0; bioconductor packages (Gentleman et al., 2004; Wu and Irizarry, 2004). The LPE test with Benjamini-Hochberg False Discovery Rate correction was used for differential expression analysis (Benjamini et al., 2001, Jain et al., 2003). Differential expression of a gene was considered significant if the adjusted p-value < 0.05 and the fold change ≥ 2.0.

For real-time quantitative PCR analysis, cDNA synthesis was performed using 2 μg total RNA and SuperScriptIII (Invitrogen). At E13.5, 2–4 independently prepared samples were used per genotype while at E11.5 3–4 biological replicates were tested per genotype. qPCR reactions were carried out with 50 ng cDNA, 16 μM primers, and SYBR Green PCR Master Mix in 20 μl total volume (Applied Biosystems) in the ABI Prism 7000 Sequence Detection System. Gene expression was normalized against ubiquitin expression. Primer sequences are available upon request. For each gene, reactions were performed 2–6 times per sample.

**RESULTS AND DISCUSSION**

**Loss of E2F4 causes earlier lethality in Rb-deficient mice**

Over-expression and promoter occupancy studies strongly suggest that the repressive E2F-pocket protein complexes play a critical role in regulating the expression of E2F-responsive genes in G0 and early G1 phases of the cell cycle (Blais and Dynlacht, 2007). E2F4 typically accounts for the majority of the endogenous pRB-, p107- and p130-associated E2F activity in *vivo* and E2F4-loss has a profound effect on the formation of pRB-deficient tumors (Lee et al., 2002; Parisi et al., 2008). To explore the interplay between E2F4 and pRB in normal development, we intercrossed mixed C57BL/6 × 129/Sv background *Rb*<sup>+/−</sup>;*E2f4*<sup>+/−</sup> mice to generate wild-type, *Rb* and *E2f4* single and double mutant embryos. We have previously shown that *E2f4*<sup>−/−</sup> embryos are present at the expected frequency at all gestational stages (Humbert et al., 2000a). Thus, we first assessed embryo viability at E13.5, the beginning of the window of lethality for *Rb*<sup>−/−</sup> mice (Table 1). Consistent with previous observations, *Rb*<sup>−/−</sup> mice were present at the expected frequency and most, but not all, were alive as judged by the presence...
of a heartbeat. We also detected Rb<sup>-/-;E2f4<sup>+/-</sup></sup> embryos at the expected Mendelian ratio, indicating that the loss of one E2f4 allele has no effect on the survival of Rb<sup>-/-</sup> animals at this developmental stage. In contrast, the Rb<sup>-/-;E2f4<sup>-/-</sup></sup> embryos (herein referred to as DKO embryos) were clearly underrepresented at E13.5, arising at approximately one-third of the expected frequency. Therefore, the combined loss of pRB and E2F4 results in embryonic death prior to E13.5.

To ascertain the timing of lethality for DKO embryos, we screened for their presence at progressively earlier developmental stages (Table 1). At E11.5, DKO animals were detected at the expected frequency, but only 54% (14 out of 26) were viable, as assessed by the presence of a beating heart (Table 1). All of the other genotypes were present and alive at the expected frequency, reinforcing our conclusion that E2F4-loss reduces the lifespan of Rb<sup>-/-</sup> embryos.

At E10.5, an increased proportion (68%, 13 out of 19) of DKO embryos had a detectable heartbeat and at E9.5 viable DKOs were present at the expected frequency. Embryos at E12.5 were not examined as already at E11.5 only one half of the DKOs were viable (approximately 1 in 30 embryos). Therefore, the DKO embryos die between E10-E14, which is significantly earlier than Rb<sup>-/-</sup> embryos.

Given the clear shift in the window of lethality of DKO versus Rb<sup>-/-</sup> embryos we conducted careful analyses of these embryos. By gross morphological and histological examination, DKO embryos displayed the same spectrum, and similar severity of defects that had been previously reported for the single homozygous mutants. E13.5 Rb<sup>-/-</sup> and E2f4<sup>-/-</sup> embryos both appear paler than their wild-type littermate controls as a result of their respective erythrocyte differentiation defects (Humbert et al., 2000a; Jacks et al., 1992) and DKO embryos had a similar pallid coloration. Furthermore, DKO embryos were smaller than littermate controls as previously reported for the E2f4 mutants (Figure 1A) (Humbert et al., 2000a; Rempel et al., 2000). Therefore, apart from the characterized Rb<sup>-/-</sup> and E2f4<sup>-/-</sup> phenotypes, the DKOs did not display any additional morphological defects.

It is well established that the E13.5 Rb<sup>-/-</sup> embryos exhibit striking levels of inappropriate S-phase entry and apoptosis in the lens, CNS and PNS (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992; Macleod et al., 1996; Morgenbesser et al., 1994). Therefore we used BrdU incorporation and TUNEL assays to determine whether the levels of ectopic cellular proliferation and apoptosis, respectively, were exacerbated in DKO embryos (Figure 1B–E; data not shown). Since a significant fraction of DKO embryos are dead by E13.5, we assayed S-phase entry in E11.5 embryos. In the lens vesicles, we detected a similar high level of BrdU-positive cells in all genotypes (Figure 1D). This is consistent with the fact that the lens is actively developing at E11.5 and cells are only just beginning to exit the cell cycle (Kaufman et al., 1995). In contrast, Rb<sup>-/-</sup> embryos had increased levels of BrdU-positive cells in the intermediate zone of the hindbrain (CNS) and the dorsal root ganglia (PNS) compared to control and E2f4<sup>-/-</sup> embryos (Figure 1B and D; data not shown). This elevated proliferation occurred at a similar level in Rb<sup>-/-</sup> and DKO embryos (Figure 1B and D) indicating that E2F4 does not modulate the inappropriate proliferation that arises in pRB-deficient embryos.

We also assessed the levels of apoptosis in the developing embryos. At E11.5, we detected a similar, subtle increase in apoptotic cells in the hindbrains of both Rb<sup>-/-</sup> and Rb<sup>-/-;E2f4<sup>-/-</sup></sup> embryos relative to control littermates (data not shown). This apoptosis defect was more pronounced at E13.5 (Figure 1C and E). As previously reported, Rb<sup>-/-</sup> embryos displayed high levels of programmed cell death in the hindbrain near the fourth ventricle (CNS), the trigeminal ganglia (PNS) and the ocular lens. We observed a similar level of apoptosis in each of these tissues in the Rb<sup>-/-;E2f4<sup>-/-</sup></sup> embryos (Figure 1E). Taken together, these data suggest that E2f4-loss does not exacerbate either the inappropriate proliferation or apoptosis arising in the
Rb<sup>−/−</sup> embryos. Thus, increased cell death in these embryonic tissues does not contribute to the reduced survival of the DKO versus the Rb<sup>−/−</sup> embryos.

**Extra-embryonic tissue defects in DKO embryos**

Since our embryonic analyses did not provide any cause for the early lethality of the DKO animals, we next examined the extra-embryonic tissues. pRB-loss causes hyperproliferation of trophoblast cells in the labyrinth layer of the placenta, leading to reduced nutrient and gas delivery to the embryo (Wenzel et al., 2007; Wu et al., 2003). Consistent with these reports, the labyrinth layer defect was highly penetrant in our E13.5 Rb<sup>−/−</sup> embryos (Figure 2A, B). At earlier timepoints, E10.5 and E11.5, this defect was only apparent in a small subset of Rb<sup>−/−</sup> embryos and was extremely subtle (data not shown). At E11.5 no significant morphological defect was observed in E2f4<sup>−/−</sup> placentas in comparison with wild-type placentas (Supplementary Figure 1, data not shown). Histological examination at E11.5 showed that E2F4-loss enhanced the labyrinth layer defect in the Rb-deficient background but had no discernable effect on the other plental layers (Figure 3B). Specifically, the size of the labyrinth layer relative to the entire placenta was reduced in the DKO placentas when compared with that of the wildtype control extra-embryonic tissues (Figure 3B). Quantification of the labyrinth layer reduction at E11.5 indicated a statistically significant reduction (16%, p = 0.015) in the DKO embryos relative to the wildtype control (Supplementary Figure 1). At this same timepoint, the Rb<sup>−/−</sup> placentas displayed a smaller reduction (9.3%) that was not statistically significant (Supplementary Figure 1).Additionally the yolk sacs of the compound mutants were typically not well vascularized (Figure 3A) at this stage.

The synergy between pRB- and E2F4-loss was even more pronounced at E13.5 (Figure 2A and B). Every Rb<sup>−/−</sup>;E2f4<sup>−/−</sup> placenta examined had a smaller and more compacted labyrinth layer than the Rb<sup>−/−</sup> placentas (Figure 2A and B; data not shown). This clearly reflects synergy between E2f4 and Rb in plental function as the E2f4 single mutants had no detectable morphological differences and the height of the labyrinth layer, relative to the total placenta (average ± S.D. = 50 ± 15%) was not significantly different from wild-type controls at E13.5 (54.2 ± 5.6%, p = 0.577; data not shown) or E11.5 (Supplementary Figure 1). Taken together, these data show that E2F4-loss aggravates the labyrinth layer defect in Rb<sup>−/−</sup> placentas, strongly suggesting that the early lethality of DKO versus Rb<sup>−/−</sup> embryos results from an exacerbation of the placental insufficiency.

To determine whether the reduction or compaction of the labyrinth layer was a result of changes in cell division, we used BrdU incorporation to compare the levels of proliferation in the mutant placentas. This analysis was conducted at E11.5, in large part, because viable DKO embryos are well represented at this stage. Although the labyrinth defect was clearly apparent in E11.5 DKO placentas, we observed a similar, high percentage of proliferating cells in DKO, Rb<sup>−/−</sup> and control labyrinth layers (data not shown). This high proliferative index likely reflects the active development of the labyrinth at E11.5. Unfortunately, the rarity of viable DKO embryos at later developmental stages precluded comparison of BrdU incorporation in Rb<sup>−/−</sup> versus DKO placentas at E13.5. We screened E11.5 placentas for apoptosis and found no difference in the levels of TUNEL-positive cells between wild-type, Rb<sup>−/−</sup>, and DKO placentas (data not shown). Thus, the enhanced labyrinth defect observed in DKO versus Rb<sup>−/−</sup> animals at E11.5 does not result from any detectable change in the levels of proliferation or apoptosis in this tissue.

We also examined the expression levels of representative plental layer-specific differentiation markers at E11.5 and E13.5 using a combination of in situ hybridization and quantitative real-time PCR (qPCR). There was no significant difference in the expression levels/pattern of the spongiotrophoblast markers 4311/Tpbp (Figure 2C and Figure 3C) and/or flt1 (Figure 4A and B) between control, Rb<sup>−/−</sup> and DKO placentas at either timepoint.
Similarly, the trophoblast giant cell markers placental lactogen-1 (Figure 3D, Figure 4A and B) and proliferin-1 (data not shown) were expressed at comparable levels in DKO, \( Rb^{-/-} \) and control littermates. Together with the hematoxylin and eosin staining of the placentas, these data show that the trophoblast giant cell and spongiotrophoblast layers are not appreciably affected by the loss of E2F4 and/or pRB. Finally, we examined the expression of \( Esx-1 \), a marker of differentiated labyrinth trophoblasts, at E11.5 and E13.5 (Figure 4A and B). Remarkably, despite the aberrant morphology of the labyrinth layer in \( Rb^{-/-} \) and DKO placentas, we did not detect any difference in the expression of \( Esx-1 \) relative to littermate controls. Thus, the compaction of the labyrinth layer in \( Rb^{-/-} \) or DKO placentas is not associated with increased expression of differentiated labyrinth trophoblast markers.

Expression profile of mutant placentas

We hypothesized that the placental defect would be associated with gene expression changes, either as a direct result of the loss of the transcriptional properties of E2F4 and/or pRB or as a downstream consequence. To better understand the molecular basis of the defect, we used Affymetrix whole mouse genome (430 2.0) microarrays to compare the gene expression profiles of control and mutant extra-embryonic tissues. We began by comparing wild-type and \( Rb^{-/-} \) placentas at E13.5, the time when the placental defect is evident in the majority of \( Rb^{-/-} \) samples. After excluding sex-linked genes, this screen identified 236 genes that were differentially expressed between wild-type and \( Rb^{-/-} \) replicates with a ≥ 2-fold difference in expression level and an adjusted p-value < 0.05. Of these genes, 107 were down-regulated and 129 were up-regulated by the inactivation of \( Rb \) (Figure 5; Supplemental Table 1 and Supplemental Table 2). \( Rb \) was identified as a repressed gene, providing validation for the array analysis.

Consistent with the prevailing view that pRB-loss promotes E2F transcriptional activity, a subset of the induced genes are known E2F target genes. To further validate these findings, we performed qPCR on representative E2F–responsive genes, \( mcm2, mcm3, mcm6, mcm7, cyclin E1, cyclin E2 \) and \( E2f1 \). All seven of these targets were significantly up-regulated in \( Rb^{-/-} \) placentas at E13.5 (Figure 6A and data not shown). These results are consistent with the previous report of inappropriate proliferation in pRB-deficient placentas at this timepoint (Wu et al., 2003). Given the success of the \( Rb \) mutant analysis, we extended our studies to compare the gene expression profiles of DKO placentas and wild-type controls. For this analysis, we used E11.5 samples due to the difficulty in recovering viable DKO embryos at E13.5. At this earlier stage 35 genes (17 down-regulated and 18 up-regulated) were significantly de-regulated in the E11.5 DKO placentas (Figure 5; Supplemental Table 3 and Supplemental Table 4). As an internal control for this array experiment, \( Rb \) was identified as a down-regulated gene. \( E2f4 \) expression was also down-regulated although this did not reach statistical significance.

By comparing our array results from the DKO placentas with those from the \( Rb^{-/-} \) placentas we identified 2/17 of the down-regulated genes (\( Rb \) and B430119L13Rik) and 3/18 of the up-regulated genes (\( Dppa5, Tsc22d, Bzw2 \)) in common between the two sets of experiments. Deregulation of these genes is likely to be important for the placental defect.

We presume that the relatively small number of differentially expressed genes identified in DKO (n=35) versus \( Rb^{-/-} \) (n=236) placentas reflects the different timing of the analyses (E11.5 and E13.5, respectively) and that the 35 genes constitute the earliest detectable expression changes that coincide with the emerging placental defect. Notably, while E2F–responsive genes were induced genes in E13.5 \( Rb^{-/-} \) placentas, there was no significant de-regulation (either up- or down-regulation) of E2F–responsive genes in DKO placentas at E11.5 (Figure 6B; Supplemental Table 3 and Supplemental Table 4). To further explore this finding, we used qPCR to screen E11.5 placentas from wildtype, \( E2f4^{-/-}, Rb^{-/-} \) and DKO embryos for four classic E2F–responsive genes (\( mcm2, mcm6, cyclinE1 \) and \( cyclinE2 \)) that were clearly up-
Early lethality is rescued in conditionally mutant animals

To determine whether the placental defect is the primary cause of death of the DKO embryos, we generated a conditional mouse model in which DKO embryos were provided with \( Rb^{+/−} \) placentas. Using the strategy employed by Wu et al., (2003), we interbred the \( E2f4^{+/−} \) mice with a conditional \( Rb \) mutant strain, \( Rh^{2lox/2lox} \) (Sage et al., 2003), and an epiblast-specific \( Cre \) line, MORE (\( Meox2-Cre \)) (Hayashi et al., 2002; Tallquist and Soriano, 2000). Cre recombinase expression from the \( Meox2 \) promoter is detected in the embryo proper by E6, but the majority of extra-embryonic tissues do not express Cre (Hayashi et al., 2002; Tallquist and Soriano, 2000). Thus, through comparative analysis of \( Rh^{lox/lox,Meox2^{cre/+}} \) and \( Rh^{lox/lox,E2f4^{−/−};Meox2^{cre/+}} \) embryos we can determine whether the early lethality associated with the loss of both pRB and E2F4 is due to the aggravated placental defect. For simplicity, we will refer to these conditional mutant embryos as \( cRb^{−/−} \) and \( cRb^{−/−};E2f4^{−/−} \).

As previously reported (de Bruin et al., 2003; Wu et al., 2003), \( cRb^{−/−} \) mice were dead at birth. We also recovered inviable \( cRb^{−/−};E2f4^{−/−} \) animals at this timepoint (data not shown).
Irrespective of their E2f4 genotype, the cRb−/− embryos were present at less than the expected frequency. Since this under-representation could be due to the cannibalism of dead or unhealthy newborns, we used timed pregnancies to compare the frequency of cRb−/− and cRb−/−;E2f4−/− embryos in utero. cRb−/− embryos, regardless of their E2f4 status, were present and alive at the expected frequency at E13.5, E15.5 and immediately prior to birth (E18.5), as judged by the presence of a heartbeat (Table 2). At E18.5 the cRb−/− and cRb−/−;E2f4−/− embryos died once they were removed from the uterus, because they could not begin or sustain breathing (data not shown). Thus, the presence of an Rb wild-type placenta allows cRb−/− and cRb−/−;E2f4−/− embryos to survive up to, but not beyond, birth. This is definitive proof that the reduced lifespan of the germline DKO embryos (E10-E14) is directly related to the increased severity of the placental defect.

**E2F4 and pRB play largely independently roles in embryonic development**

Since cRb−/− and cRb−/−;E2f4−/− mutant mice survive beyond E13.5, we were able to determine the consequences of E2F4- and pRB-deficiency on later stages of embryonic development. Initially we verified the efficiency of Cre recombination by showing that pRB was no longer detectable in mouse embryonic fibroblasts generated from cRb−/− or cRb−/−;E2f4−/− embryos (data not shown). Then we conducted a careful comparison of the single and double Rb/E2f4 mutants. In a similar manner to the germline mutant embryos, we found that cRb−/−;E2f4−/− embryos exhibited a pattern of defects that phenocopied those present in E2f4−/− and cRb−/− littermate controls. These phenotypes were detected at E13.5 and continued to birth. Figure 7 shows defects that were present at E18.5. At a gross morphological level, E18.5 cRb−/−;E2f4−/− animals displayed the pallid coloration and reduced size and weight that was characteristic of their E2f4−/− littermates (Figure 7A; data not shown). In addition, cRb−/−;E2f4−/− mutants had the same hunched appearance as cRb−/− single mutants that results from an alteration in the curvature of the spine (Figure 7A and B). The cRb−/− and cRb−/−;E2f4−/− mutants also had misshapen ribs and absence of a muscle attachment site on the humerus, both known consequences of abnormal musculature (Figure 7B; data not shown). Histological inspection confirmed that cRb−/− and cRb−/−;E2f4−/− embryos had a reduced muscle density that was associated with myoblast apoptosis (as assessed by TUNEL staining), shortened myotubes, and reduced muscle fibers in the skeletal muscle, including the intercostal, forelimb and hindlimb muscles (Figure 7C; data not shown).

Germline inactivation of Rb leads to ectopic S-phase entry and increased programmed cell death in the lens of the eye and the nervous system (Lee et al., 1992; Macleod et al., 1996; Morgenbesser et al., 1994). The unscheduled proliferation associated with pRB-loss is cell autonomous; however, the apoptosis observed in the nervous system, but not in the lens, is largely due to the placental defect in Rb−/− embryos (de Bruin et al., 2003). To determine if E2F4 participates in cell cycle control and cell survival associated with pRB, we analyzed the levels of proliferation and apoptosis in the rescued double mutant animals. Through analysis of incorporated BrdU, we showed that there was a considerable degree of ectopic proliferation in both the ocular lens and hindbrain of E18.5 cRb−/−;E2f4−/− embryos which closely paralleled that observed in the cRb−/− controls (Figure 7D; data not shown). We also detected a comparable level of apoptosis in the lenses of cRb−/−;E2f4−/− and cRb single mutant embryos at E18.5 (data not shown). Finally, we observed significant levels of TUNEL-positive cells in the retina of cRb−/− mutants and the concomitant loss of E2F4 had no effect on this phenotype (data not shown). Thus, in concordance with our analyses of the germline DKO embryos, E2F4-loss had no influence on the levels of ectopic proliferation and apoptosis in cRb−/− embryos, showing that E2F4-loss does not impact these pRB-regulated processes. This also suggests that E2F4-p107 and E2F4-p130 complexes are unable to compensate for the loss of pRB in these tissues during development.
E2F4 and pRB each have demonstrated roles in erythroid differentiation. E2f4−/− embryos exhibit severe fetal anemia from E13.5 to E16.5, resulting in pallid coloration (Humbert et al., 2000a; Rempel et al., 2000). Analysis of peripheral blood smears reveals a significant proportion of abnormal erythrocytes, including nucleated erythrocytes and cells containing Howell-Jolly bodies, which are remnants of chromatin that have not been completely extruded during enucleation (Humbert et al., 2000a; Kinross et al., 2006). Rb−/− embryos also have a pale appearance, reduced cellularity in the fetal livers and an increase in immature nucleated erythrocytes (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). Analyses of conditional and chimeric mutant mice show that the red blood cell (RBC) defect in Rb−/− animals results from both non-cell-autonomous and cell autonomous effects (Clark et al., 2004; de Bruin et al., 2003; Dirlam et al., 2007; Maandag et al., 1994; Sankaran et al., 2008; Spike et al., 2004; Williams et al., 1994).

We therefore analyzed peripheral blood from the E18.5 conditional embryos to determine whether the RBC defects arising in the pRB mutant embryos are modulated by the loss of E2F4, or vice versa. Consistent with previous findings, E2F4 and pRB are both required for normal RBC development, but their epistasis is unknown. In toto, our analyses show that cRb−/−;E2f4−/− embryos display all of the defects of the cRb−/− and E2f4−/− single mutants without any detectable increase in severity or evidence of new phenotypes. In consideration of this, and our analysis of the germline Rb;E2f4 mutants, we conclude that E2F4 and pRB play distinct roles in the development of embryonic versus extraembryonic tissues. In the former setting, E2F4 and pRB appear to function largely independently from one another. This finding was somewhat surprising, given that E2F4 is the predominant pRB-associated E2F in most cell types, including embryonic fibroblasts. It remains to be established whether the non-overlapping roles of E2F4 and pRB result from their differential regulation of specific E2F-responsive genes, as suggested by chromatin immunoprecipitation studies in fetal liver cells (Dirlam et al., 2007), or whether they reflect broader roles for E2F4 and/or pRB. In support of the latter hypothesis, E2F4 and pRB have both been shown to function as co-factors for other transcriptional regulators (Chen et al., 2002; Hansen et al., 2004; Scime et al., 2005; Thomas et al., 2001). In contrast to the embryonic tissues, E2F4 and pRB clearly act synergistically in extra-embryonic tissues by promoting normal development of the labyrinth layer of the placenta. Our data strongly suggest that the...
placenta defect in both Rb−/− and DKO mutants reflects the inappropriate persistence of trophoectoderm-like cells. Specifically, expression profiling of the mutant placentas shows an increase in levels of Dppa5 mRNA, a marker of early trophoectoderm but not trophoblast stem cells. Dppa5 upregulation is clearly detectable at E11.5, when wildtype, Rb−/− and DKO extraembryonic tissues show a similar, high degree of proliferation. There are no consensus E2F binding sites within the upstream sequences or first intron of the Dppa5 gene (data not shown) suggesting that an indirect mechanism results in increased Dppa5 mRNA levels in pRb−/− and DKO placentas; for example an increase in the trophoectoderm-like cell population within the placenta. High Dppa5 mRNA levels persist at E13.5, and are accompanied by an upregulation of E2F–responsive mRNAs and inappropriate proliferation in both the Rb−/− and DKO placentas. Given these observations, we hypothesize that pRB-loss impairs the development of the labyrinth layer by promoting proliferation, and presumably impeding differentiation, of the trophoectoderm-like cells. Loss of E2F4 exacerbates the placental phenotype, leading to an earlier disruption of placental function and the concomitant failure of embryonic development. It is interesting to note that Rb−/−;p107−/− embryos also die a couple of days earlier than Rb−/− embryos (Lee et al., 1996), similar to the Rb−/−;E2f4−/− embryos. The study of Rb−/−;p107−/− embryos was conducted before pRB’s role in placental development was discovered, and the extra-embryonic tissues were not examined. However, since the extra-embryonic defect accounts for the mid-gestational lethality of the Rb mutants, the shortened lifespan of the Rb−/−;p107−/− embryos may reflect an exacerbation of the Rb mutant placental defect. Furthermore, we can detect E2F4-p107/p130 complexes in E11.5 placental extracts by immunoprecipitation experiments but were unable to detect E2F4-pRB complexes due to the low amounts of pRB and E2F4 in this tissue (data not shown). It is well established that E2F4 and p107 act together to mediate the repression of E2F–responsive genes (Balciunaite et al., 2005). Together with our observation that known E2F–responsive genes are deregulated in Rb−/− and Rb−/−;E2f4−/− placentas this strongly suggests that the synergy between E2F4 and pRB in placental development reflects the shared roles of these proteins, and likely p107, in regulating the transcription of these genes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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REFERENCES


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Parisi T, Bronson RT, Lees JA. Inhibition of pituitary tumors in Rb mutant chimeras through E2f4 loss reveals a key suppressive role for the pRB/E2F pathway in urothelium and ganglionic carcinogenesis. Oncogene. 2008


Figure 1. E2F4-loss does not affect inappropriate proliferation and apoptosis in the Rb<sup>-/-</sup> embryos

A) Control, Rb<sup>-/-</sup> and Rb<sup>-/-</sup>;E2f4<sup>-/-</sup> embryos at E11.5. B) BrdU incorporation shows that S-phase cells are restricted to the ventricular zone of the hindbrain in control embryos, but are also present in the intermediate zone in Rb<sup>-/-</sup> and Rb<sup>-/-</sup>;E2f4<sup>-/-</sup> embryos at 11.5 (40x magnification). C) TUNEL staining shows similar levels of apoptotic cells in the hindbrains of Rb<sup>-/-</sup> and Rb<sup>-/-</sup>;E2f4<sup>-/-</sup> at E13.5. Quantitation of BrdU-positive (D) and TUNEL-positive (E) cells in control and mutant animals at E11.5 and E13.5, respectively, in the PNS (trigeminal ganglia), CNS (hindbrain) and ocular lens. Loss of E2F4 has no effect on the levels of proliferating and apoptotic cells in Rb mutant embryos. All data are represented relative to the percentages observed in Rb mutants (+ standard deviation), which were set to 1.

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Figure 2. The placental defect in Rb−/− embryos is exacerbated in Rb−/−;E2f4−/− embryos at E13.5. Representative hematoxylin and eosin stained placental sections of control and mutant littermates at a magnification of 10x (A) and 20x (B), the bar in each panel corresponds to 100µm. The loss of Rb results in a denser labyrinth layer (L) compared to the control. In the Rb−/−;E2f4−/− placentas, this layer is further compacted and reduced in size. C) In situ hybridization for 4311 (Tphp) indicates that the spongiotrophoblast layer (Sp) is relatively normal in the mutant placentas. Magnification, 10x. MD, maternal decidua; C, chorionic plate.
Figure 3. Loss of E2F4 aggravates the placental defect in Rb−/− embryos at E11.5
A) Control yolk sacs are highly vascularized while there is a decrease in the number of blood vessels in the Rb mutant yolk sacs. Loss of E2F4 further impairs vascularization in the yolk sac.
B) Hematoxylin and eosin stained placental sections of control and mutant littermates at a magnification of 10x, the bar in each panel represents 100µm. In Rb−/−;E2f4−/− placentas the height of the labyrinth layer is reduced. C) In situ hybridization for 4311 (Tpbp) indicates that the spongiotrophoblast layer (Sp) is relatively unaffected in the mutant placentas. Magnification, 10x. D) Trophoblast giant cell differentiation is largely unaffected in mutant placentas as assessed by in situ hybridization for placental lactogen-1. Magnification, 2x.
*Position of trophoblast giant cells; MD, maternal decidua; C, chorionic plate; ET, embryonic tissue.
Figure 4. Placental layer-specific markers are unchanged in mutant placentas
At E13.5 (A) and E11.5 (B) wild-type and mutant placentas expressed equivalent levels of placental lactogen-1 (pl-1, trophoblast giant cell layer marker), Flt-1 (spongiotrophoblast layer marker), and Esx-1 (labyrinth layer marker), as assessed by quantitative real-time PCR. Gene expression levels are normalized to ubiquitin expression levels. Standard deviation is represented by the error bars.
Figure 5. Microarray data analysis identifies common genes deregulated in Rb−/− and Rb−/−;E2f4−/− placentas at E13.5 and E11.5
At E13.5, microarray analysis was used to determine which genes are deregulated in Rb−/− placentas in comparison with wild-type placentas. At E11.5 Rb−/−;E2f4−/− placental cDNA was compared with wild-type placental cDNA. Comparison of the deregulated gene sets identified common sets of up- and down-regulated genes including Dppa5 (a marker of pluripotent cell types) and Rb respectively.
Figure 6. E2F-target genes and Dppa5 expression are deregulated in Rb and Rb;E2f4 mutant placentas
A) Cell cycle genes are expressed at increased levels in E13.5 Rb$^{-/-}$ placentas compared to wild-type placentas. B) At E11.5, wild-type and mutant placentas express cell cycle genes at similar levels. Expression of Eomes (a trophoblast stem cell marker) and Dppa5 (a pluripotency marker) in wild-type and mutant placentas at E13.5 (C) and E11.5 (D). Eomes is expressed at similar levels among the various genotypes whilst Dppa5 expression is significantly elevated in Rb$^{-/-}$ and Rb$^{-/-}$;E2f4$^{-/-}$ placentas. Gene expression levels are normalized to ubiquitin expression levels and error bars represent standard deviation.
Figure 7. Conditional Rb<sup>−/−</sup>;E2f4<sup>−/−</sup> embryos exhibit Rb and E2f4 mutant phenotypes at E18.5

A) Control, E2f4<sup>−/−</sup>, Rb<sup>1lox/1lox</sup>;Meox<sup>cre/+</sup> (cRb<sup>−/−</sup>) and Rb<sup>1lox/1lox</sup>;E2f4<sup>−/−</sup>;Meox<sup>cre/+</sup> (cRb<sup>−/−</sup>;E2f4<sup>−/−</sup>, DKO) embryos at E18.5. DKO embryos are growth retarded, like E2f4<sup>−/−</sup> mice, and have a hunched appearance similar to cRb<sup>−/−</sup> embryos. B) Skeletal staining with Alcian blue (cartilage) and Alizarin red (bone) of control and mutant embryos reveals altered curvatures of the spine and rib cage in the cRb<sup>−/−</sup> and DKO embryos. C) Decrease in muscle density and fiber organization in the Rb mutants is unchanged with the additional loss of E2F4 as judged by hematoxylin and eosin staining. Magnification, 40x. D) Darkly stained BrdU-positive cells were detected in the lens fiber cell compartment (indicated by the black arrows) in the cRb<sup>−/−</sup> and DKO lenses, but not in the control and E2f4<sup>−/−</sup> lenses. Magnification, 40x.
E) Giemsa stained peripheral blood smears from control and mutant animals. There are a considerable percentage of erythroblasts containing Howell-Jolly bodies (white arrowheads) following the loss of E2F4 while the loss of pRB leads to a number of immature nucleated erythroblasts (black arrowheads). Both of these phenotypes are present in DKO blood preparations.
Figure 8. Anemia and morphological red blood cell defects in c Rb\textsuperscript{−/−} and E2f4\textsuperscript{−/−} embryos are additive in c Rb\textsuperscript{−/−};E2f4\textsuperscript{−/−} embryos

A) Hematocrit analyses of blood from E18.5 embryos of the indicated genotypes. Each dot represents one embryo and the mean is indicated by a horizontal bar. In comparison with wild-type embryos both the c Rb\textsuperscript{−/−} and E2f4\textsuperscript{−/−} embryos display reduced levels of red blood cells and the effect is compounded in the double mutants.

B) Quantification of the percentage of red blood cells displaying failed enucleation or Howell-Jolly bodies, which are characteristic phenotypes of c Rb\textsuperscript{−/−} and E2f4\textsuperscript{−/−} embryonic erythrocytes at E18.5, respectively. There is an additive effect in double mutant embryos. The error bars represent one standard deviation from the mean.
Table 1

Mutation of Rb and E2f4 leads to embryonic lethality between E10.5 and E13.5.

<table>
<thead>
<tr>
<th>Age</th>
<th>Total #</th>
<th>Embryos</th>
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<th>Rb&lt;sup&gt;-/-&lt;/sup&gt;;E2f4&lt;sup&gt;+-/-&lt;/sup&gt;</th>
<th>Rb&lt;sup&gt;-/-&lt;/sup&gt;;E2f4&lt;sup&gt;-/-&lt;/sup&gt;</th>
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<td>26 (24) 25</td>
<td>4 (3) 13</td>
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<tr>
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<td></td>
<td>18 (15) 23</td>
<td>49 (46) 46</td>
<td>26 (14) 23</td>
</tr>
<tr>
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<td></td>
<td>27 (24) 24</td>
<td>53 (43) 48</td>
<td>19 (13) 24</td>
</tr>
<tr>
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<td></td>
<td>2 (2) 6</td>
<td>15 (13) 13</td>
<td>9 (7) 6</td>
</tr>
</tbody>
</table>

Frequency of embryos arising from Rb<sup>+/+</sup>;E2f4<sup>+/+</sup> intercrosses. Only embryos homozygous for the Rb mutation are shown. Obs=observed number, lv=live (as judged by heartbeat or morphology), exp=expected number.
Table 2

Conditional mutation of Rb allows survival of cRb<sup>−/−</sup>;E2f4<sup>−/−</sup> embryos up to E18.5.

<table>
<thead>
<tr>
<th>Age</th>
<th>Total # Embryos</th>
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<th>cRb&lt;sup&gt;−/−&lt;/sup&gt;;E2f4&lt;sup&gt;−/−&lt;/sup&gt; obs</th>
<th>cRb&lt;sup&gt;+/−&lt;/sup&gt;;E2f4&lt;sup&gt;−/−&lt;/sup&gt; obs</th>
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<td>E13.5</td>
<td>65</td>
<td>6 4</td>
<td>9 8</td>
<td>5 4</td>
</tr>
</tbody>
</table>

Frequency of embryos arising from Rb<sup>+/−</sup>;E2f4<sup>+/−</sup> × cRb<sup>+/−</sup>;E2f4<sup>+/−</sup>;Meox2<sup>+/cre</sup> intercrosses. Only Meox2<sup>+/cre</sup> embryos shown. Obs=observed number, exp=expected number.