



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

Eunice Y. Lee<sup>1</sup>, Tina L. Yuan<sup>2</sup>, Paul S. Danielian, Julie C. West, Jacqueline A. Lees<sup>\*</sup>

David H. Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

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### ABSTRACT

The retinoblastoma gene, *RB-1*, was the first identified tumor suppressor. *Rb*<sup>-/-</sup> 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*<sup>-/-</sup> embryos is unknown. Here we establish that *E2f4* deficiency reduced the lifespan of *Rb*<sup>-/-</sup> embryos by exacerbating the *Rb* mutant placental defect. We further show that this reflects the accumulation of trophoblast-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*<sup>-/-</sup>;*E2f4*<sup>-/-</sup> embryos to develop in the presence of *Rb* wild-type placentas. Under these conditions, *Rb*<sup>-/-</sup>;*E2f4*<sup>-/-</sup> mutants survived to birth. These *Rb*<sup>-/-</sup>;*E2f4*<sup>-/-</sup> 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.

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### 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 G<sub>0</sub>/G<sub>1</sub> 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., 2009; 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 G<sub>0</sub>/G<sub>1</sub> 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 (Humbert 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.

\* Corresponding author. Fax: +1 617 253 9863.

E-mail address: [jalees@mit.edu](mailto:jalees@mit.edu) (J.A. Lees).

<sup>1</sup> Current address: Department of Developmental Biology, Stanford University School of Medicine, Stanford, CA 94305, USA.

<sup>2</sup> Current address: Department of Systems Biology, Harvard Medical School, Boston, MA 02115, USA.

Mutant mouse models have also been key in defining the roles of pRB in tumorigenesis and normal development (Burkhardt 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 $\times$ 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 4–6  $\mu$ m. Sections were stained with hematoxylin and eosin for histological analysis. The placental defect was bypassed by breeding  $E2f4^{+/-}$ ,  $Rb^{2lox/2lox}$ , and  $Meox2^{cre/+}$  strains (Humbert et al., 2000a; Sage et al., 2003; Tallquist and Soriano, 2000). Timed  $Rb^{2lox/2lox};E2f4^{+/-}\times Rb^{1lox/+};E2f4^{+/-};Meox2^{cre/+}$  crosses were set up to obtain  $Rb^{1lox/1lox};E2f4^{-/-};Meox2^{cre/+}$  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 4–6  $\mu$ m. 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 h 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% H<sub>2</sub>O<sub>2</sub>, 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 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 2 $\times$  SSC, dehydrated and air dried. Prehybridization was carried out for 1 h followed by overnight hybridization with digoxigenin-labeled riboprobes for *Pl-1* (Colosi et al., 1987) and *4311/Tpbb* (Lescisin et al., 1988). Washes were done in 50% formamide/1 $\times$  SSC/0.1% Tween-20 followed by washes in MABT (100 mM Maleic acid, 150 mM 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  $^{\circ}$ C overnight. Sections were washed in MABT/levamisole (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<sup>®</sup> Mouse Genome 430 2.0 arrays (Affymetrix) were performed according to the manufacturer's instructions. The microarrays were scanned with GeneChip<sup>®</sup> 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  $\geq 2.0$ . The microarray data were deposited in the Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo>) under the accession number GSE13753.

For real-time quantitative PCR analysis, cDNA synthesis was performed using 2  $\mu$ g 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  $\mu$ M primers, and SYBR Green PCR Master Mix in 20  $\mu$ l 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  $G_0$  and early  $G_1$  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  $\times$  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>-/-</sup>; *E2f4*<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>-/-</sup>; *E2f4*<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 (E13.5–E15.5).

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 (Fig. 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.

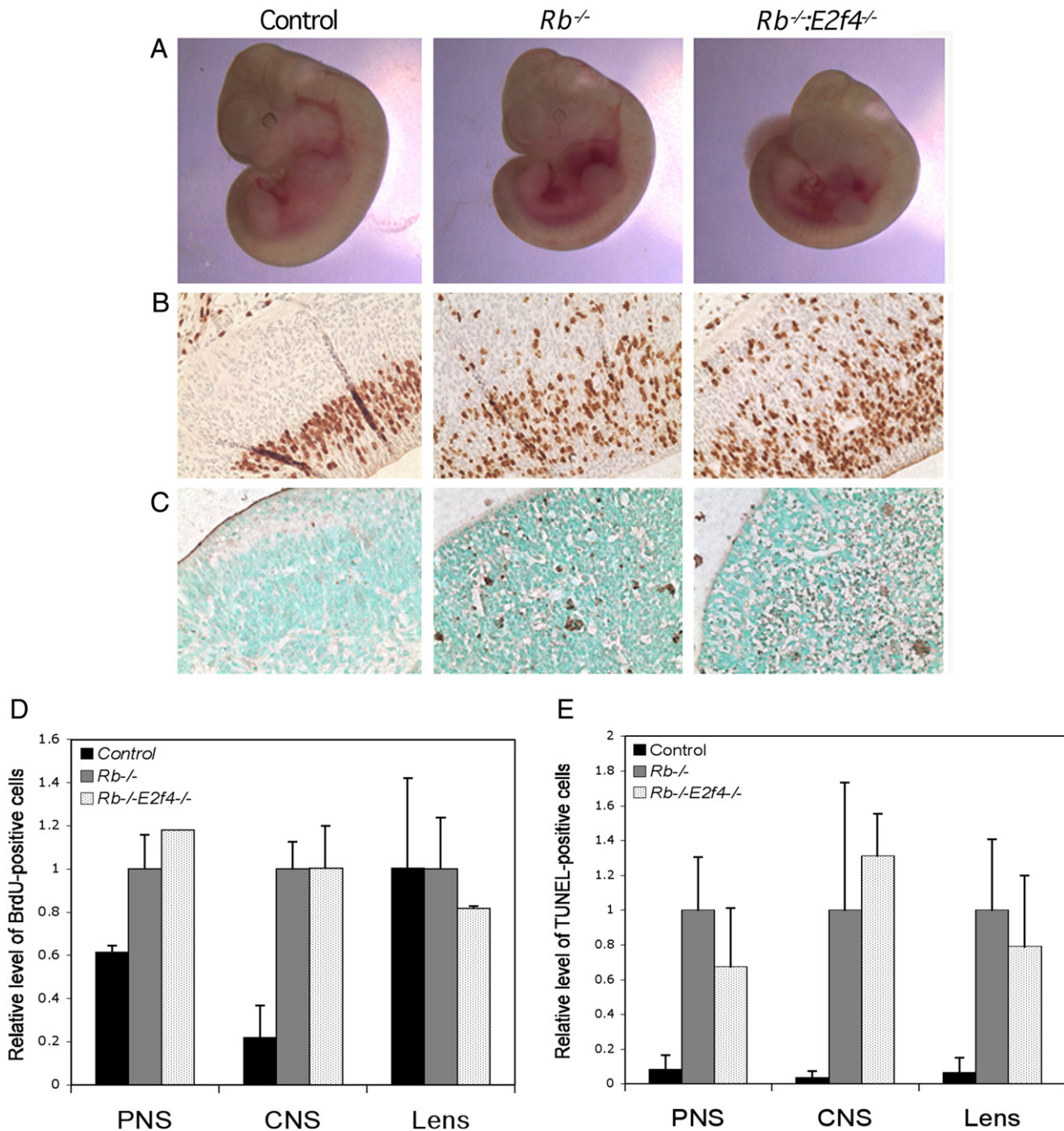
It is well established that the E13.5 *Rb*<sup>-/-</sup> embryos exhibit striking levels of inappropriate S-phase entry and apoptosis in the lens, CNS and PNS (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992; Macleod et al., 1996; Morgenbesser et al., 1994). Therefore we used BrdU incorporation and TUNEL assays to determine whether the levels of ectopic cellular proliferation and apoptosis, respectively, were exacerbated in DKO embryos (Fig. 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 (Fig. 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 (Fig. 1B and D; data not shown). This elevated proliferation occurred at a similar level in *Rb*<sup>-/-</sup> and DKO embryos (Fig. 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>-/-</sup>; *E2f4*<sup>-/-</sup> embryos relative to control littermates (data not shown). This apoptosis defect was more pronounced at E13.5 (Fig. 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>-/-</sup>; *E2f4*<sup>-/-</sup> embryos (Fig. 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.

**Table 1**  
Mutation of *Rb* and *E2f4* leads to embryonic lethality between E10.5 and E13.5.

Age	Total # Embryos	<i>Rb</i> <sup>-/-</sup> ; <i>E2f4</i> <sup>+/+</sup>		<i>Rb</i> <sup>-/-</sup> ; <i>E2f4</i> <sup>+/-</sup>		<i>Rb</i> <sup>-/-</sup> ; <i>E2f4</i> <sup>-/-</sup>	
		obs (lv)	exp	obs (lv)	exp	obs (lv)	exp
E13.5	201	14 (11)	13	26 (24)	25	4 (3)	13
E11.5	366	18 (15)	23	49 (46)	46	26 (14)	23
E10.5	386	27 (24)	24	53 (43)	48	19 (13)	24
E9.5	103	2 (2)	6	15 (13)	13	9 (7)	6

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.

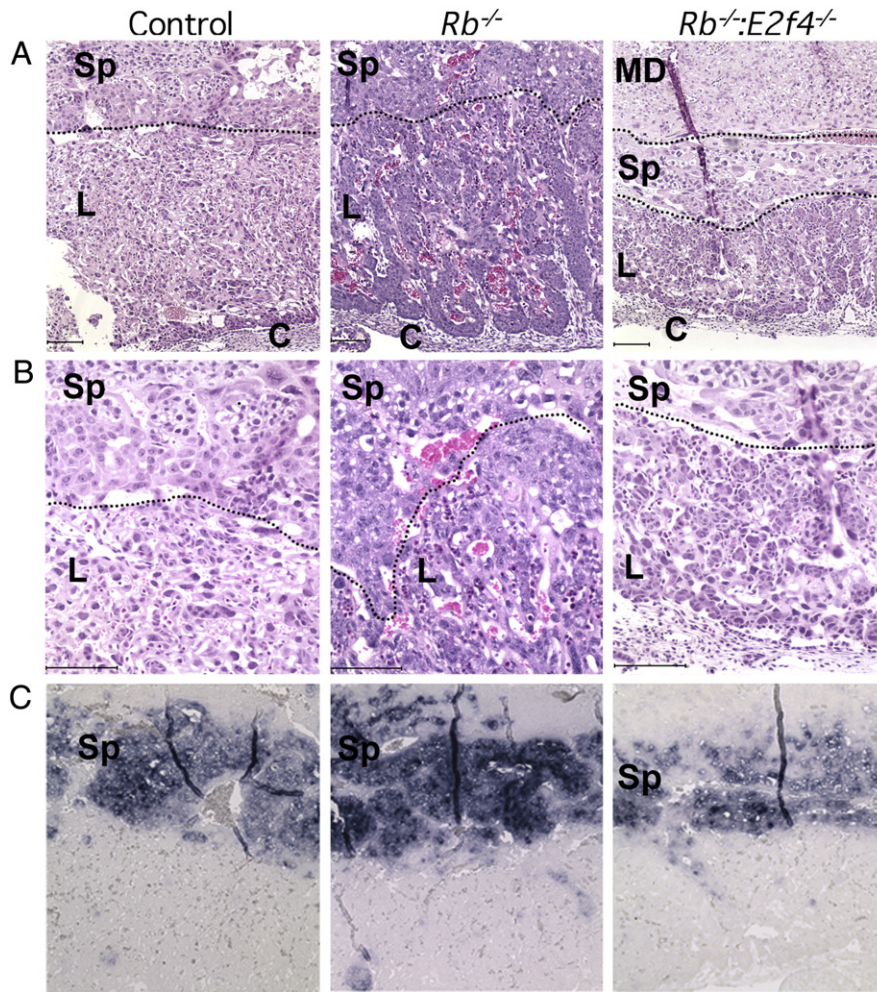


**Fig. 1.** E2F4-loss does not affect inappropriate proliferation and apoptosis in the *Rb*<sup>-/-</sup> embryos. (A) Control, *Rb*<sup>-/-</sup> and *Rb*<sup>-/-</sup>;*E2f4*<sup>-/-</sup> embryos at E11.5. (B) BrdU incorporation shows that S-phase cells are restricted to the ventricular zone of the 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 E11.5 (40× 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.

#### 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 (Fig. 2A and 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 Fig. 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 placental layers (Fig. 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 wild-type control extra-embryonic tissues (Fig. 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 wild-type control (Supplementary Fig. 1). At this same timepoint, the *Rb*<sup>-/-</sup> placentas displayed a smaller reduction (9.3%) that was not statistically significant



**Fig. 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 10 $\times$  (A) and 20 $\times$  (B); the bar in each panel corresponds to 100  $\mu$ m. 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* (*Tpbp*) indicates that the spongiotrophoblast layer (Sp) is relatively normal in the mutant placentas. Magnification, 10 $\times$ . MD, maternal decidua; C, chorionic plate.

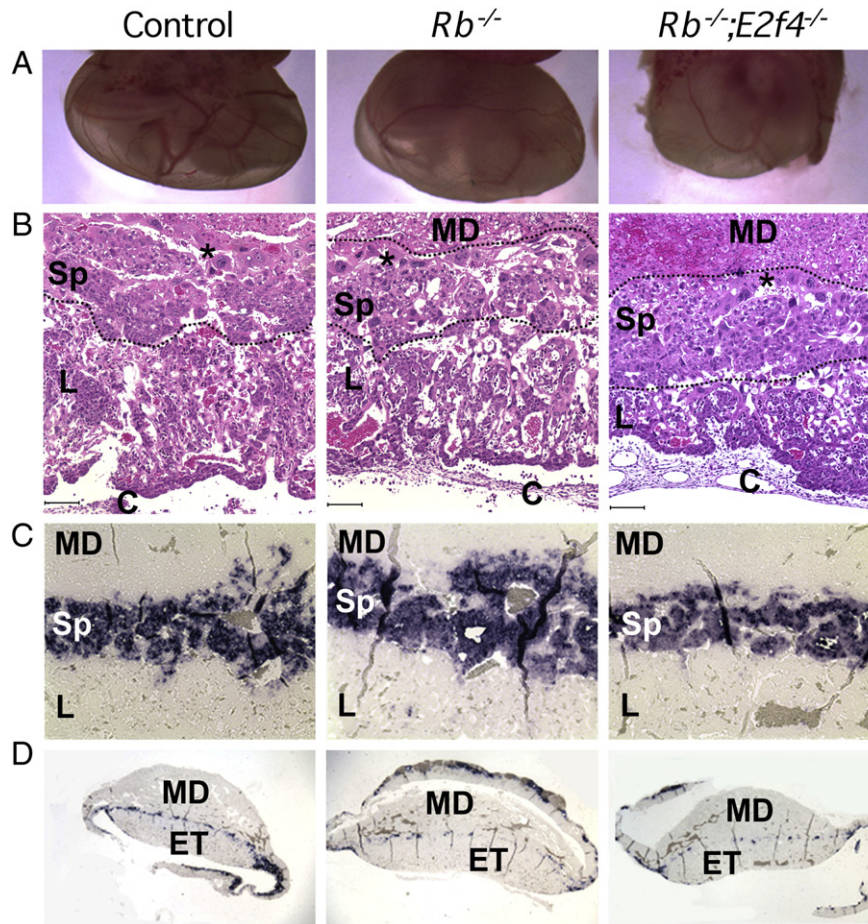
(Supplementary Fig. 1). Additionally the yolk sacs of the compound mutants were typically not well vascularized (Fig. 3A) at this stage.

The synergy between pRB- and E2F4-loss was even more pronounced at E13.5 (Fig. 2A and B). Every  $Rb^{-/-};E2f4^{-/-}$  placenta examined had a smaller and more compacted labyrinth layer than the  $Rb^{-/-}$  placentas (Fig. 2A and B; data not shown). This clearly reflects synergy between *E2f4* and *Rb* in placental function as the *E2f4* single mutants had no detectable morphological differences and the height of the labyrinth layer, relative to the total placenta (average  $\pm$  S.D. =  $50 \pm 15\%$ ) was not significantly different from wild-type controls at E13.5 ( $54.2 \pm 5.6\%$ ,  $p = 0.577$ ; data not shown) or E11.5 (Supplementary Fig. 1). Taken together, these data show that E2F4-loss aggravates the labyrinth layer defect in  $Rb^{-/-}$  placentas, strongly suggesting that the early lethality of DKO versus  $Rb^{-/-}$  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,  $Rb^{-/-}$  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  $Rb^{-/-}$  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,  $Rb^{-/-}$ , and DKO placentas (data not shown). Thus, the enhanced labyrinth defect observed in DKO versus  $Rb^{-/-}$  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 placental 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* (Figs. 2C and 3C) and/or *flt1* (Fig. 4A and B) between control,  $Rb^{-/-}$  and DKO placentas at either timepoint. Similarly, the trophoblast giant cell markers *placental lactogen-1* (Figs. 3D, 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 (Fig. 4A and B). Remarkably, despite the aberrant morphology of the



**Fig. 3.** Loss of E2F4 aggravates the placental defect in *Rb*<sup>-/-</sup> 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 10×; the bar in each panel represents 100 μm. In *Rb*<sup>-/-</sup>;*E2f4*<sup>-/-</sup> 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, 10×. (D) Trophoblast giant cell differentiation is largely unaffected in mutant placentas as assessed by *in situ* hybridization for placental lactogen-1. Magnification, 2×. \*Position of trophoblast giant cells; MD, maternal decidua; C, chorionic plate; ET, embryonic tissue.

labyrinth layer in *Rb*<sup>-/-</sup> 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*<sup>-/-</sup> or DKO placentas is not associated with increased expression of differentiated labyrinth trophoblast markers.

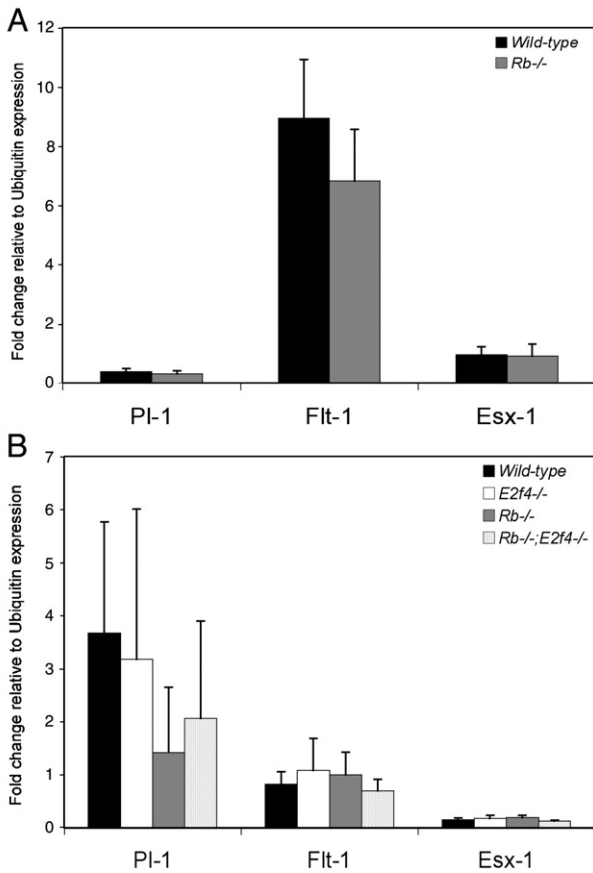
#### Expression profile of mutant placentas

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*<sup>-/-</sup> placentas at E13.5, the time when the placental defect is evident in the majority of *Rb*<sup>-/-</sup> samples. After excluding sex-linked genes, this screen identified 236 genes that were differentially expressed between wild-type and *Rb*<sup>-/-</sup> 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* (Fig. 5; Supplemental Tables 1 and 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*<sup>-/-</sup> placentas at E13.5 (Fig. 6A; 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 deregulated in the E11.5 DKO placentas (Fig. 5; Supplemental Tables 3 and 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*<sup>-/-</sup> 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*<sup>-/-</sup> (*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



**Fig. 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.

*Rb*<sup>-/-</sup> placentas, there was no significant de-regulation (either up- or down-regulation) of E2F-responsive genes in DKO placentas at E11.5 (Fig. 6B; Supplemental Tables 3 and 4). To further explore this finding, we used qPCR to screen E11.5 placentas from wild-type, *E2f4*<sup>-/-</sup>, *Rb*<sup>-/-</sup> and DKO embryos for four classic E2F-responsive genes (*mcm2*, *mcm6*, *cyclinE1* and *cyclinE2*) that were clearly up-regulated in the E13.5 placentas from *Rb*<sup>-/-</sup> embryos (Fig. 6A). We observed a modest induction of *cyclinE2* in the DKO and *Rb*<sup>-/-</sup> placentas (*p*-values of 0.1 and 0.01, respectively). The other three genes were expressed at similar levels in the control versus *Rb*<sup>-/-</sup> or DKO E11.5 placentas. These findings are entirely consistent with our BrdU labeling studies, which detected a difference in the level of proliferating cells in *Rb* mutant versus wild-type placentas at E13.5, but not at E11.5.

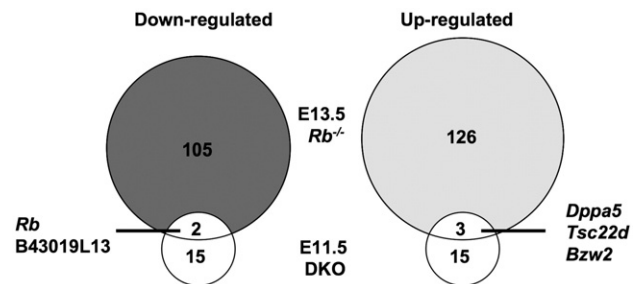
Given these observations, we focused our attention on the list of genes that were deregulated in DKOs. The most striking was a gene named developmental pluripotency associated 5 (*Dppa5*) or embryonal stem cell-specific gene 1 (*Esg-1*). *Dppa5/Esg-1* was one of the three genes induced in both DKO and *Rb*<sup>-/-</sup> placentas (Fig. 5, Supplemental Tables 1 and 3). In both comparisons, *Dppa5* probes report a >8-fold increase in expression in the mutant placentas with adjusted *p*-values of approximately zero. *Dppa5/Esg-1* was originally identified as a gene expressed in embryonal carcinoma cells and embryonal stem (ES) cells, but not in lineage-committed stem cells such as trophoblast stem (TS) cells, or somatic cells (Astigiano et al., 1991; Bierbaum et al., 1994). This led to the initial conclusion that *Dppa5/Esg-1* was an ES cell-specific marker. Subsequent studies, however, have shown that *Dppa5/Esg-1* is also expressed in the

trophoblast, a TS cell precursor, and germ cells (Ahn et al., 2004; Bortvin et al., 2003; Tanaka et al., 2002). Quantitative real-time PCR confirmed our microarray data; *Dppa5* mRNA levels were induced 10 fold in *Rb*<sup>-/-</sup> placentas at E13.5 and approximately 4-fold in both *Rb*<sup>-/-</sup> and DKO placentas at E11.5 relative to age matched controls (Fig. 6C and D). Previous studies concluded that the placental insufficiency of the *Rb* mutants results from the uncontrolled proliferation of trophoblast stem cells within the labyrinth layer (Wenzel et al., 2007; Wu et al., 2003). This was based on detection of increased expression of *Eomes*, a known marker of TS cells. Contrary to this finding, we did not observe any significant difference in *Eomes* expression when comparing *Rb*<sup>-/-</sup> and/or DKO placentas with wild-type controls at E11.5 and E13.5, as judged by microarray and qPCR (Fig. 6C, D Supplemental Tables 1–4). In accordance with a prior report (Tanaka et al., 2002), we find that *Dppa5/Esg-1* is expressed in wild-type trophoblast, but not in TS cells (data not shown). Thus, taken together, our data suggests that the loss of pRB causes an increase in the numbers of trophoblast-like cells at E11.5 and this contributes to the increased cellularity of the labyrinth layer, which ultimately results in placental insufficiency. The further induction of *Dppa5/Esg-1* and the detectable increase of cell cycle gene expression at E13.5 suggests that the trophoblast-like cells are proliferating inappropriately and this may account for the progressive worsening of the placental defect at this later developmental stage.

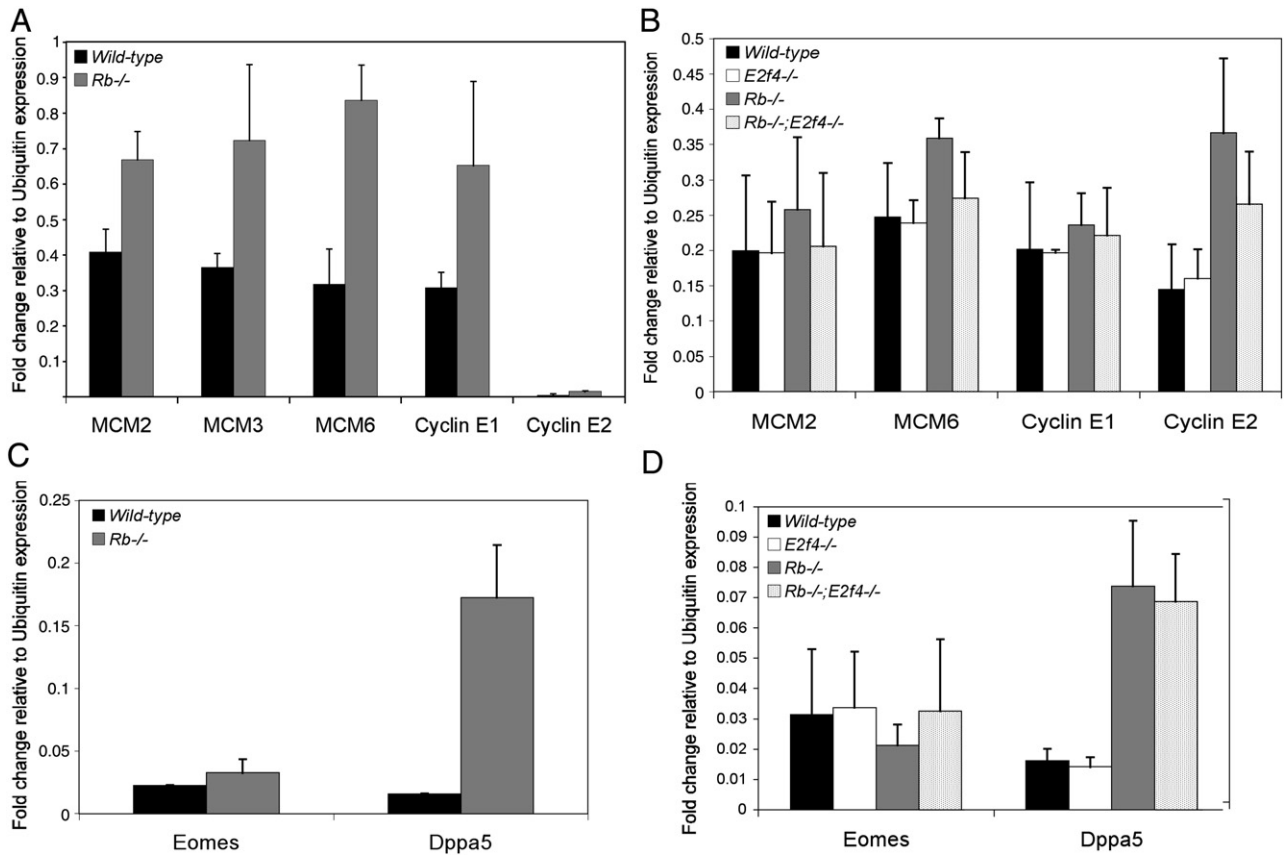
#### Early lethality is rescued in conditionally mutant animals

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*<sup>+/+</sup> placentas. Using the strategy employed by Wu et al., (2003), we interbred the *E2f4*<sup>+/-</sup> mice with a conditional *Rb* mutant strain, *Rb*<sup>2lox/2lox</sup> (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 *Rb*<sup>1lox/1lox</sup>; *Meox2*<sup>cre/+</sup> and *Rb*<sup>1lox/1lox</sup>; *E2f4*<sup>-/-</sup>; *Meox2*<sup>cre/+</sup> 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*<sup>-/-</sup> and *cRb*<sup>-/-</sup>; *E2f4*<sup>-/-</sup>.

As previously reported (de Bruin et al., 2003; Wu et al., 2003), *cRb*<sup>-/-</sup> mice were dead at birth. We also recovered inviable *cRb*<sup>-/-</sup>; *E2f4*<sup>-/-</sup> animals at this timepoint (data not shown). Irrespective of their *E2f4* genotype, the *cRb*<sup>-/-</sup> 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



**Fig. 5.** Microarray data analysis identifies common genes deregulated in *Rb*<sup>-/-</sup> and *Rb*<sup>-/-</sup>; *E2f4*<sup>-/-</sup> placentas at E13.5 and E11.5. At E13.5, microarray analysis was used to determine which genes are deregulated in *Rb*<sup>-/-</sup> placentas in comparison with wild-type placentas. At E11.5, *Rb*<sup>-/-</sup>; *E2f4*<sup>-/-</sup> 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.



**Fig. 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*<sup>-/-</sup> 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*<sup>-/-</sup> and *Rb*<sup>-/-</sup>;*E2f4*<sup>-/-</sup> placentas. Gene expression levels are normalized to ubiquitin expression levels and error bars represent standard deviation.

pregnancies to compare the frequency of *cRb*<sup>-/-</sup> and *cRb*<sup>-/-</sup>;*E2f4*<sup>-/-</sup> embryos *in utero*. *cRb*<sup>-/-</sup> 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*<sup>-/-</sup> and *cRb*<sup>-/-</sup>;*E2f4*<sup>-/-</sup> 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*<sup>-/-</sup> and *cRb*<sup>-/-</sup>;*E2f4*<sup>-/-</sup> 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 independent roles in embryonic development

Since *cRb*<sup>-/-</sup> and *cRb*<sup>-/-</sup>;*E2f4*<sup>-/-</sup> 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*<sup>-/-</sup> or *cRb*<sup>-/-</sup>;*E2f4*<sup>-/-</sup> 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*<sup>-/-</sup>;*E2f4*<sup>-/-</sup> embryos exhibited a pattern of defects that phenocopied those present in *E2f4*<sup>-/-</sup> and *cRb*<sup>-/-</sup> littermate controls. These phenotypes were detected at E13.5 and continued to birth. Fig. 7 shows defects that were present at E18.5. At a gross morphological level, E18.5 *cRb*<sup>-/-</sup>;*E2f4*<sup>-/-</sup> animals displayed the pallid coloration and reduced size and weight that was characteristic

of their *E2f4*<sup>-/-</sup> littermates (Fig. 7A; data not shown). In addition, *cRb*<sup>-/-</sup>;*E2f4*<sup>-/-</sup> mutants had the same hunched appearance as *cRb*<sup>-/-</sup> single mutants that results from an alteration in the curvature of the spine (Fig. 7A and B). The *cRb*<sup>-/-</sup> and *cRb*<sup>-/-</sup>;*E2f4*<sup>-/-</sup> mutants also had misshapen ribs and absence of a muscle attachment site on the humerus, both known consequences of abnormal musculature (Fig. 7B; data not shown). Histological inspection confirmed that *cRb*<sup>-/-</sup> and *cRb*<sup>-/-</sup>;*E2f4*<sup>-/-</sup> 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 (Fig. 7C; data not shown).

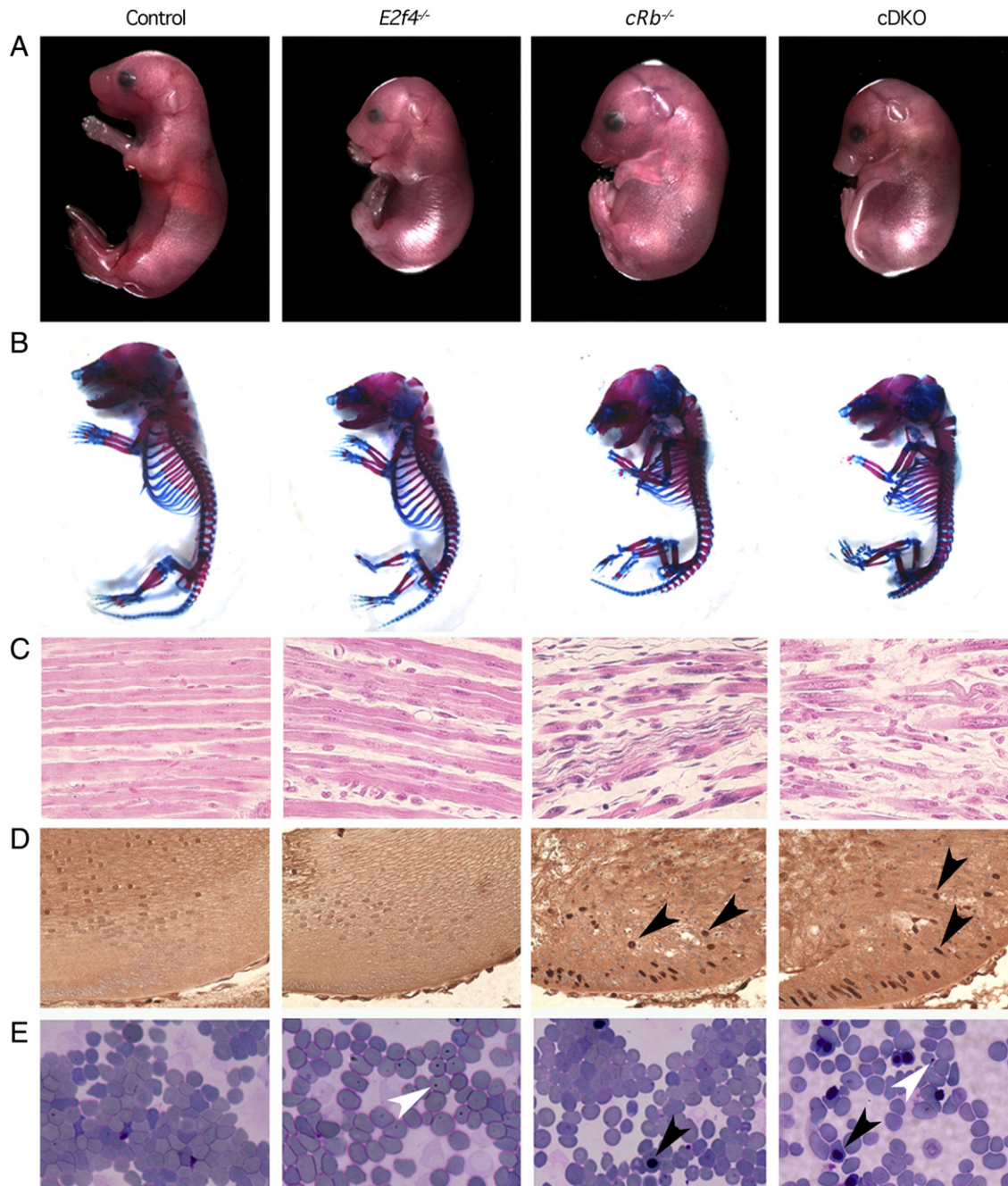
Germline inactivation of *Rb* leads to ectopic S-phase entry and increased programmed cell death in the lens of the eye and the nervous system (Lee et al., 1992; Macleod et al., 1996; Morgenbesser et al., 1994). The unscheduled proliferation associated with pRB-loss is cell-autonomous; however, the apoptosis observed in the nervous system, but not in the lens, is largely due to the placental defect in

**Table 2**  
Conditional mutation of *Rb* allows survival of *cRb*<sup>-/-</sup>;*E2f4*<sup>-/-</sup> embryos up to E18.5.

Age	Total # Embryos	<i>cRb</i> <sup>-/-</sup> ; <i>E2f4</i> <sup>+/+</sup>		<i>cRb</i> <sup>-/-</sup> ; <i>E2f4</i> <sup>+/-</sup>		<i>cRb</i> <sup>-/-</sup> ; <i>E2f4</i> <sup>-/-</sup>	
		obs	exp	obs	exp	obs	exp
E18.5	258	19	16	39	32	12	16
E15.5	100	6	6	15	12	4	6
E13.5	65	6	4	9	8	5	4

Frequency of embryos arising from *Rb*<sup>c/c</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.





**Fig. 7.** Conditional  $Rb^{-/-};E2f4^{-/-}$  embryos exhibit  $Rb$  and  $E2f4$  mutant phenotypes at E18.5. A) Control,  $E2f4^{-/-}$ ,  $Rb^{1lox/1lox};Meox^{cre/+}$  ( $cRb^{-/-}$ ) and  $Rb^{1lox/1lox};E2f4^{-/-};Meox^{cre/+}$  ( $cRb^{-/-};E2f4^{-/-}$ , cDKO) embryos at E18.5. cDKO embryos are growth retarded, like  $E2f4^{-/-}$  mice, and have a hunched appearance similar to  $cRb^{-/-}$  embryos. B) Skeletal staining with Alcian blue (cartilage) and Alizarin red (bone) of control and mutant embryos reveals altered curvatures of the spine and ribcage in the  $cRb^{-/-}$  and cDKO 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, 40 $\times$ . D) Darkly stained BrdU-positive cells were detected in the lens fiber cell compartment (indicated by the black arrows) in the  $cRb^{-/-}$  and cDKO lenses, but not in the control and  $E2f4^{-/-}$  lenses. Magnification, 40 $\times$ . 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 cDKO blood preparations.

$Rb^{-/-}$  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 (Fig. 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.

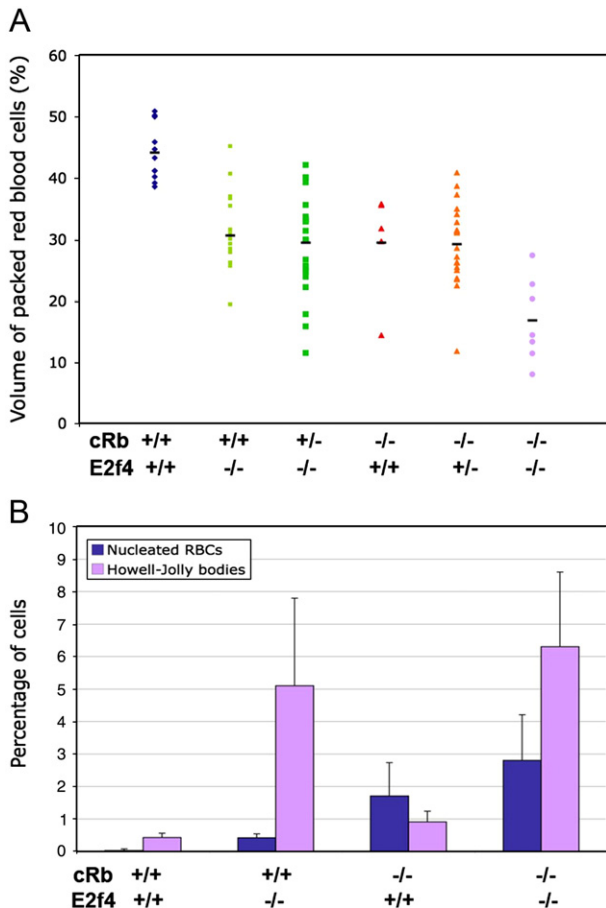
$E2f4$  and pRB each have demonstrated roles in erythroid differentiation.  $E2f4^{-/-}$  embryos exhibit severe fetal anemia from E13.5 to

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).

E2F4 and pRB are both required for normal RBC development, but their epistasis is unknown. 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,  $E2f4^{-/-}$  embryos were anemic at E18.5, as evidenced by a 30% reduction in hematocrit levels compared to those of wild-type littermates ( $p$ -value  $< 3 \times 10^{-6}$ ; Fig. 8A). The  $cRb^{-/-}$  embryos had a similar decrease in hematocrits ( $p$ -value  $< 0.02$ ), supporting the observation that pRB-loss has a significant effect on erythrocyte development. We found

that there was a further decrease ( $\sim 60\%$ ) in hematocrits of  $cRb^{-/-}; E2f4^{-/-}$  embryos relative to wild-type embryos ( $p$ -value  $< 5 \times 10^{-6}$ ). Cytological investigation of the peripheral blood cells at E18.5 revealed that  $cRb^{-/-}; E2f4^{-/-}$  embryos displayed the additive phenotypes of the  $cRb^{-/-}$  and  $E2f4^{-/-}$  mice (Figs. 7E and 8B). Consistent with previous studies, most of the RBCs in E18.5 wild-type embryos were mature enucleated erythrocytes and we rarely observed immature nucleated RBCs ( $0.02\% \pm 0.05$ ) or mature erythrocytes containing Howell–Jolly bodies ( $0.42\% \pm 0.13$ ). In  $cRb^{-/-}$  embryos, we observed a more than 80-fold higher level of immature nucleated RBCs ( $1.7\% \pm 1.03$ ) and there was also a small increase in the proportion of RBCs with Howell–Jolly bodies ( $0.9\% \pm 0.33$ ). In contrast,  $E2f4^{-/-}$  embryos showed a less dramatic increase in the level of immature nucleated RBCs ( $0.41\% \pm 0.12$ ), but there was a large elevation in the fraction of RBCs with Howell–Jolly bodies ( $5.1\% \pm 2.7$ ). Remarkably,  $cRb^{-/-}; E2f4^{-/-}$  embryos had a high proportion of both immature nucleated erythrocytes ( $2.8\% \pm 1.4$ ) and RBCs with Howell–Jolly bodies ( $6.3\% \pm 2.3$ ) that was essentially additive of the  $cRb^{-/-}$  and  $E2f4^{-/-}$  mutant phenotypes. In line with this finding, E2F4 and pRB show mutually exclusive occupation of E2F-responsive promoters in E15.5 fetal liver cells (Dirlam et al., 2007). These observations show that pRB and E2F4 play important and independent roles in embryonic erythroid development.

*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 extra-embryonic 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 wild-type,  $Rb^{-/-}$  and DKO extra-embryonic 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



**Fig. 8.** Anemia and morphological red blood cell defects in  $cRb^{-/-}$  and  $E2f4^{-/-}$  embryos are additive in  $cRb^{-/-}; E2f4^{-/-}$  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  $cRb^{-/-}$  and  $E2f4^{-/-}$  embryos display reduced levels of red blood cells and the effect is compounded in the double mutant embryos. (B) Quantification of the percentage of red blood cells displaying failed enucleation or Howell–Jolly bodies, which are characteristic phenotypes of  $cRb^{-/-}$  and  $E2f4^{-/-}$  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.

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*<sup>-/-</sup>; *p107*<sup>-/-</sup> 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 in this tissue (data not shown). It is well established that E2F4 and p107 act together to mediate the repression of E2F-responsive genes (Balcianaite et al., 2005). Together with our observation that known E2F-responsive genes are deregulated in *Rb*<sup>-/-</sup> and *Rb*<sup>-/-</sup>; *E2f4*<sup>-/-</sup> 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.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2009.05.541.

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