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pRB and E2F4 play distinct cell-intrinsic roles in fetal erythropoiesis

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Abstract

The retinoblastoma tumor suppressor protein pRB functions, at least in part, by directly binding to and modulating the activity of the E2F transcription factors. Previous studies have shown that both E2F4 and pRB play important roles in fetal erythropoiesis. Given that these two proteins interact directly we investigated the overlap of E2F4 and pRB function in this process by analyzing *E2f4*$^{-/-}$, conditional *Rb* knockout (*Rb*$^{1lox/1lox}$), and compound *E2f4*$^{-/-}$;*Rb*$^{1lox/1lox}$ embryos. At E15.5 *E2f4*$^{-/-}$ and *Rb*$^{1lox/1lox}$ fetal erythroid cells display distinct abnormalities in their differentiation profiles. When cultured *in vitro*, both *E2f4*$^{-/-}$ and *Rb*$^{1lox/1lox}$ erythroid cells show defects in cell cycle progression. Surprisingly, analysis of cell cycle profiling suggests that E2F4 and pRB control cell cycle exit through different mechanisms. Moreover, only pRB, but not E2F4, promotes cell survival in erythroid cells. We observed an additive rather than a synergistic impact upon the erythroid defects in the compound *E2f4*$^{-/-}$;*Rb*$^{1lox/1lox}$ embryos. We further found that fetal liver macrophage development is largely normal regardless of genotype. Taken together, our results show that E2F4 and pRB play independent cell-intrinsic roles in fetal erythropoiesis.

Keywords

retinoblastoma; pRB; E2F4; erythroid differentiation; cell cycle

Introduction

The retinoblastoma protein (pRB) plays cell-intrinsic and non cell-intrinsic roles in hematopoiesis.[1] Germline *Rb*$^{-/-}$ embryos die *in utero* by embryonic day 15.5 (E15.5) with a pale appearance indicative of anemia, reduced fetal liver cellularity and an increase in immature nucleated erythroid cells in their peripheral blood. These severe erythropoietic defects are partially rescued in conditional *Rb* mutant mice and chimeric mice comprised of *Rb*$^{-/-}$ and wild-type cells, indicating that loss of pRB in non-erythroid cells, in particular mutation of *Rb* within the placenta, contributes to the erythropoietic defects in pRB-deficient embryos.[2–7] Despite the partial rescue in chimeric mice, abnormalities in erythroid

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differentiation persist, including impaired cell cycle exit, defective enucleation in late erythroblasts and mild anemia.[8–10] These data indicate a cell-intrinsic role of pRB in promoting cell cycle exit and terminal differentiation.

E2F4, the major repressive member of the E2F transcription factor family, also plays an important role in erythropoiesis. *E2f4*$^{-/-}$ embryos exhibit severe, but transient, fetal anemia having a pale appearance and reduced fetal liver cellularity.[11–13] Analysis of peripheral blood smears reveals a significant increase in abnormal erythrocytes, including erythrocytes of enlarged cell size (macrocytosis), erythrocytes containing Howell-Jolly bodies (fragments of DNA remaining from incomplete enucleation), and occasionally nucleated erythrocytes. Furthermore, transplantation experiments using *E2f4*$^{-/-}$ fetal liver cells (FLCs) suggest that E2F4 acts cell-autonomously in erythroid cells.[13] Unlike pRB, E2F4 is largely dispensable for cellular proliferation and cell survival *in vivo* and in mouse embryo fibroblasts even though a subset of E2F4 target genes are involved in cell cycle progression.[14]

Mounting evidence suggests that pRB and E2F4 act cooperatively in regulating many cellular processes. First, E2F4 forms a complex with pRB in the nucleus.[15, 16] Second, E2F4 binds many E2F-target gene promoters that are likely targets of pRB function[17–19], and *Rb* itself is an E2F4 target gene.[20] Third, mutation of *E2f4* in *Rb*$^{+/-}$ mice results in a dramatic suppression of tumor formation.[2122] Fourth, the absence of E2F4 decreases the lifespan of the germline pRB-deficient mice due to an exacerbation of the placental defect.[23] Finally, mutation of *Rb* in *E2f4*$^{+/-}$ embryos results in an absence of ciliated epithelial cells in the airway, a phenotype normally only observed in *E2f4*$^{-/-}$ embryos.[24]

Since both *E2f4*$^{-/-}$ and *Rb*$^{-/-}$ embryos display erythropoietic defects, we hypothesized that, as in other cellular processes, E2F4 and pRB functionally interact in regulating erythropoiesis. To avoid the cell non-autonomous disruption of erythropoiesis resulting from placental defects in *Rb*$^{-/-}$embryos, we used conditional *Rb* knockout animals (*Rb*$^{1lox/1lox}$) in which *Rb* is mutated throughout the embryo but not in the extra-embryonic placenta. To determine whether E2F4 and pRb have overlapping roles in erythropoiesis we generated compound mutant mice (*E2f4*$^{-/-}$*;Rb*$^{1lox/1lox}$) and compared the fetal erythropoietic defects in these embryos to those in wild-type, *E2f4*$^{-/-}$ and *Rb*$^{1lox/1lox}$ embryos.

We found that E2F4 and pRB have distinct and cell-intrinsic roles in fetal erythropoiesis. In all the phenotypes we analyzed, the compound *E2f4*$^{-/-}$*;Rb*$^{1lox/1lox}$ mutant embryos displayed additive but not synergistic effects of E2F4 loss and pRB loss, showing that pRB and E2F4 regulate fetal erythropoiesis through independent mechanisms.

Results

E2f4$^{-/-}$ and *Rb*$^{1lox/1lox}$ embryos have distinct defects in fetal erythropoiesis *in vivo*

To study the relationship of E2F4 and pRB in fetal erythropoiesis, we analyzed *E2f4*$^{-/-}$, conditional *Rb* knockout (*Rb*$^{1lox/1lox}$), and *E2f4* and *Rb* double knockout (*E2f4*$^{-/-}$*;* *Rb*$^{1lox/1lox}$) embryos. As previously reported, we found that *E2f4*$^{-/-}$ embryos at E15.5 were smaller in size with reduced fetal liver cellularity and pale body color compared to wild-type (WT) embryos[13]. *Rb*$^{1lox/1lox}$ embryos appeared pale in body color, hunched in posture, and translucent along the spinal cord (Figure 1, left panel). *E2f4*$^{-/-}$*;Rb*$^{1lox/1lox}$ embryos showed all the morphological phenotypes displayed by *E2f4*$^{-/-}$ and *Rb*$^{1lox/1lox}$ embryos[23]. Interestingly, the morphological defects in these embryos were not exacerbated; the body size and cellularity of the fetal livers was comparable to the *E2f4*$^{-/-}$ embryos (Figure 1, left panel; data not shown). These findings are consistent with our previous work showing that anemia in E18.5 single and double mutants is correlated with decreased hematocrit levels and red blood cell defects [23].

We then examined definitive erythropoiesis in vivo in *E2f4*$^{-/-}$, *Rb*$^{1lox/1lox}$, and *E2f4*$^{-/-}$;*Rb*$^{1lox/1lox}$ FLCs using the flow cytometric analysis we developed [25]. Briefly, FLCs were double-labeled for erythroid-specific TER119 and non erythroid-specific CD71 (transferrin receptor). Five distinct populations of cells (R1– R5), corresponding to erythroblasts at different differentiation stages, were defined by multiple criteria, from R1 through R5 representing the least to most differentiated, respectively (Figure 1, right panel). In general, R1–R5 cells mainly contained CFU-E (colony-forming unit-erythroid) progenitors and proerythroblasts; proerythroblasts and early basophilic erythroblasts; early and late basophilic erythroblasts; chromatophilic and orthochromatophilic erythroblasts; and late orthochromatophilic erythroblasts and reticulocytes, respectively.

We quantified R1–R5 cells isolated from embryos of different genotypes (Table 1). At E15.5, WT FLCs contained 2.7% of erythroid progenitor-enriched R1 cells. Compared to WT controls, *E2f4*$^{-/-}$ FLCs showed a modest elevation in the R1 cells (4.1%, p-value < 0.05). This result is consistent with our erythroid colony assay, which also showed a consistent increase of erythroid progenitors in *E2f4*$^{-/-}$ embryos (data not shown). In addition, the proportion of *E2f4*$^{-/-}$ R5 cells decreased significantly compared to WT controls (0.6% versus 1.2%, p-value < 0.05). In contrast to *E2f4*$^{-/-}$ FLCs, *Rb*$^{1lox/1lox}$ FLCs showed similar percentages of R1 cells (2.6%) and R5 cells (1.2%) to those of WT controls (2.7% and 1.2%, respectively). However, *Rb*$^{1lox/1lox}$ FLCs displayed a significant decrease in the proportion of basophilic erythroblasts (R3 cells, 60.3% versus 70.3% in wild-type controls p-value < 0.01) concomitant with a considerable increase in the level of orthochromatophilic erythroblasts (R4 cells, 28.3% versus 17.8% in wild-type controls, p-value < 0.01). This indicates that terminal erythroid differentiation is disrupted in the absence of pRB. In the double mutant *E2f4*$^{-/-}$;*Rb*$^{1lox/1lox}$ FLCs, we observed the distinct characteristics of both *E2f4*$^{-/-}$ and *Rb*$^{1lox/1lox}$ FLCs: an increase in the proportions of R1 (4.8%) and R4 cells (23.0%) and a decrease in the percentages of R3 (60.3%) and R5 cells (0.5%). Due to the small sample size all of the changes were not statistically significant; however, these embryos clearly exhibited additive rather than exacerbated or ameliorated erythroid defects. The distinct erythroid abnormalities in *E2f4*$^{-/-}$ and *Rb*$^{1lox/1lox}$ FLCs and their additive effects in *E2f4*$^{-/-}$;*Rb*$^{1lox/1lox}$ FLCs suggest that E2F4 and pRB play distinct roles in fetal erythropoiesis *in vivo*.

E2F4 and pRB regulate distinct stages of erythroid differentiation

To determine the requirements for E2F4 and pRB in erythroid differentiation, we used an *in vitro* culture system that closely resembles erythroid proliferation and differentiation *in vivo*[25]. Briefly, TER119-negative cells were purified from individual E15.5 fetal livers and cultured on fibronectin-coated plates. By two days in culture, 87.4% of the WT cells exited the cell cycle and were in G0/G1 phase indicating that most cells had reached the end stages of erythroid differentiation (Table 2). Consistent with previous reports [4, 9, 10], we observed that in the absence of pRB, a significantly decreased proportion of erythroid cells exited the cell cycle (76.4% in G0/G1 phase, p-value < 0.001). The failure to exit the cell cycle in pRB-deficient erythroid cells was concomitant with their failure to enucleate and become reticulocytes (data not shown). Surprisingly, *E2f4*$^{-/-}$ erythroid cells also showed a defect in achieving G0/G1 (only 72.2% in G0/G1 phase, p-value < 0.001). In addition, we found that *E2f4*$^{-/-}$ erythroid cells had a considerable increase in both G2/M and S phase (12.4% and 15.5%, respectively), whereas pRB-deficient erythroid cells had only a modest increase in G2/M phase (6.8%) but a significant increase in S phase (16.8%, Table 2). However, the *E2f4*$^{-/-}$ erythroid cells did not display the enucleation defect seen in pRB-deficient erythroid cells and the proportion of reticulocytes was comparable to that of WT controls (data not shown). As observed in *E2f4*$^{-/-}$ peripheral blood smears[11–13], the *E2f4*$^{-/-}$ reticulocytes that formed *in vitro* also showed enlarged cell size (macrocytosis) and a

significant proportion of them contained Howell Jolly bodies (data not shown). The difference in the cell cycle profiles indicates that E2F4 and pRB regulate terminal erythroid proliferation through distinct mechanisms. In further support of this notion, cultured *E2f4*$^{-/-}$;*Rb*$^{1lox/1lox}$ cells showed additive defects in cell cycle profile including a high percentage of cells in S-phase, 37.5% and a low percentage of cells in the G0/G1 stage, 48.0% (Table 2).

Since loss of pRb has been implicated in apoptosis[1] we then analyzed the apoptotic effects of E2F4- and pRB-loss, individually and in combination (Table 3). By two days in culture, only 12.6% of WT cells were apoptotic. Compared to WT controls, *E2f4*$^{-/-}$ erythroid cells showed a similar apoptotic rate (13.3%, p-value = 0.82) whereas, loss of pRB resulted in a significant increase of apoptotic cells (20.7%, p-value = 9.7×10^{-10}). Removal of E2F4 from pRB-deficient erythroid cells (*E2f4*$^{-/-}$;*Rb*$^{1lox/1lox}$ cells) did not increase the apoptotic rate significantly (28.5%; p-value = 0.2, relative to *Rb*$^{1lox/1lox}$ cells). These results indicate that pRB, but not E2F4, has a cell-intrinsic role in regulating cell survival in erythroid cells, further supporting the conclusion that E2F4 and pRB have independent roles during erythroid differentiation.

Macrophage differentiation is normal in the absence of E2F4 and pRB

It has been proposed that erythropoietic defects in germline *Rb*$^{-/-}$ embryos are secondary to abnormalities in fetal liver macrophages.[26] To determine whether the distinct erythropoietic defects in *E2f4*$^{-/-}$ and *Rb*$^{1lox/1lox}$ FLCs are indeed a cell non-autonomous effect of improper differentiation of macrophages, we quantitatively assessed fetal liver macrophage development in E15.5 embryos by flow cytometric analysis (Figure 2). TER119-negative cells were purified from individual E15.5 FLCs and antibodies against CD31 and F4/80 were used to label immature macrophage progenitors and mature macrophages, respectively. Among all of the genotypes examined, there was no significant difference in the distribution of immature macrophage progenitors (Figure 2 top panel). Upon analysis of F4/80 expression, there was a slight decrease in the proportion of F4/80-positive cells in *Rb*$^{1lox/1lox}$ FLCs (29.0%) and a further reduction in *E2f4*$^{-/-}$; *Rb*$^{1lox/1lox}$ FLCs (26.5%) compared to WT cells (33.1%) (Figure 2, bottom panel). However, these mild reductions were not statistically significant. These data indicate normal macrophage development and support the conclusion that E2F4 and pRB play cell-intrinsic roles in erythroid differentiation.

Discussion

Here we provide several insights into the cell-intrinsic functions of E2F4 and pRB in fetal erythropoiesis. First, we demonstrated that pRB promotes cell survival in late erythroblasts in a cell-autonomous manner. Second, we identified a role for E2F4 in regulating cell cycle exit, which seems to act through a different mechanism from pRB. Third, E2F4 and pRB act distinctly in regulating fetal erythropoiesis. Finally, our results indicate that the effects of pRB-loss on macrophage development are largely influenced by placental dysfunction in *Rb*$^{-/-}$ germline mutants.

pRB in fetal erythropoiesis and macrophage development

pRB promotes cell cycle exit, terminal differentiation and cell survival during fetal erythropoiesis *in vivo*.[27–29] The cell-intrinsic role of pRB in these processes was convincingly established by further experiments using *in vitro* cultures, pRB-deficient hematopoietic stem cells or mice with an erythroid lineage-specific mutation of *Rb*.[4, 9, 10] Our results are consistent with these reports and the cell-intrinsic functions of pRB observed *in vitro* are consistent with the phenotypes identified *in vivo*.

Fetal liver macrophages are indispensable for fetal erythropoiesis.[30] In germline $Rb^{-/-}$ embryos, macrophage development is severely impaired.[26] When co-cultured with wild-type macrophages, $Rb^{-/-}$ erythroid cells enucleate properly. Moreover, a novel form of pRB, which is truncated at the N-terminus, is expressed specifically in a subset of mature macrophages and granulocytes in fetal livers.[31] These results suggest a cell-autonomous role for pRB in macrophage differentiation. We investigated this issue in conditional *Rb* mutant mice using flow cytometric analyses. The proportion of F4/80-positive cells (mature macrophages) in $Rb^{1lox/1lox}$ embryos is slightly reduced in comparison with their wild-type littermates but this was not statistically significant. This, along with other studies in conditional *Rb* mutant embryos[7] or in *Rb* mutant macrophages[10], suggests that macrophage differentiation is largely unaffected in $Rb^{1lox/1lox}$ embryos and thus the severe defect observed in germline $Rb^{-/-}$ embryos is likely to be a secondary consequence of the placental defect.

E2F4 in fetal erythropiesis

Although a subset of E2F4 target genes are involved in cell cycle regulation, E2F4 is largely dispensable for cellular proliferation both *in vitro* and *in vivo*.[14] In our study, however, we found a role for E2F4 in regulating cell cycle progression in erythroid cells. Although both *E2f4* and *Rb* mutant erythroid cells displayed a reduction in the G1 population, indicating failure to achieve cell cycle arrest, unlike pRB-deficient erythroid cells, which displayed an S-phase increase relative to wild-type erythroid cells, $E2f4^{-/-}$ erythroid cells had increases in both the G2/M and S-phases of the cell cycle. This result is in agreement with previous studies.[12] The significant increase in the G2/M population suggests that E2F4 works through a distinct mechanism from pRB in regulating cell cycle progression.

We speculate that loss of E2F4 could lead to uniform increases in the G2/M and S-phases by reducing the time spent in G1, by reducing the rate at which cells progress through S-phase and/or by activating the G2/M checkpoint. The latter possibility could occur if E2F4-loss led to impaired DNA replication and/or DNA damage during S-phase. This is supported by two observations. Firstly, $E2f4^{-/-}$ erythrocytes are larger than wild-type erythrocytes cells and often contain Howell Jolly bodies an indicator of damaged chromosomal DNA.[11–13] In mice treated with DNA damaging agents, the formation of Howell Jolly bodies is substantially elevated due to damaged chromosomal DNA that fails to be extruded in the enucleation process.[32] Secondly, a remarkable cadre of E2F4 target genes encode proteins involved in DNA damage checkpoint and repair pathways, chromosome assembly/condensation, chromosome segregation, and mitotic spindle checkpoints.[18–20]

E2F4 and pRB act distinctly in fetal erythropoiesis

Despite accumulating evidence that E2F4 and pRB function together in many cellular processes, we found that they play different roles in fetal erythropoiesis. Our conclusion is supported by the following observations. First, although both E2F4-loss and pRB-loss affect the cell cycle the consequences upon cell cycle profiles are different. Second, in all the analyses we performed, $E2f4^{-/-}$ erythroid cells and $Rb^{-/-}$ erythroid cells display distinct phenotypes. This correlates with previous studies describing distinct morphological defects in $E2f4^{-/-}$ erythroid cells and $Rb^{1lox/1lox}$ erythroid cells.[9, 10, 12, 13] Third the possibility that E2F4 and pRB function independently in erythropoiesis is confirmed by our analyses of compound $E2f4^{-/-}; Rb^{1lox/1lox}$ mutants where we only observed additive erythroid cell defects relative to the single mutants. In addition, we previously found quantitatively additive defects in hematocrit values, the percentage of red blood cells that contained either nuclei or Howell-Jolly bodies *in vivo* in E18.5 $E2f4^{-/-}; Rb^{1lox/1lox}$ embryos in comparison with $Rb^{1lox/1lox}$ and $E2f4^{-/-}$ embryos[23]. Fourth in the study by Dirlam and co-workers[33], the binding of pRB and E2F4 to E2F-target genes was found to be largely mutually exclusive

indicating that E2F4 and pRB regulate different sets of target genes in fetal liver cells further supporting the conclusion that during erythropoiesis E2F4 and pRB have distinct cell-intrinsic roles. Fifth, the mutation of *E2f2* suppresses many of the cell cycle and maturation defects in pRB-deficient erythroblasts, indicating that, during erythropoiesis, E2F2, rather than E2F4 is the major downstream target of pRB function.[33]

Mutation of *E2f4* does not significantly impact the consequences of *pRb* mutation in erythropoiesis (this study and[23]), muscle or nervous system development.[23] In contrast loss of E2F4 suppresses tumor formation in *Rb* mutant mice[2122] but exacerbates the placental phenotype seen in $Rb^{-/-}$ embryos.[23] Furthermore pRB and E2F4 can cooperate in airway development.[24] Thus the functional interaction between E2F4 and pRB is highly context dependent and likely depends upon which E2Fs and E2F-pocket protein complexes are able to regulate particular target genes.

Materials and Methods

Mouse strains and breeding

Mox2cre/+ transgenic mice[34] were obtained from Jackson Laboratories, Bar Harbor, ME (#003755). Intercrosses of $E2f4^{+/-}$ mice[13] (MMHCC# 01XK7) to obtain $E2f4^{-/-}$ embryos, crosses between $Rb^{2lox/2lox}$[35] and $Rb^{1lox/+}$;*Mox2cre/+* mice to obtain the conditional *Rb* knockout $Rb^{1lox/1lox}$;*Mox2cre/+* ($Rb^{1lox/1lox}$) embryos, and crosses between $Rb^{2lox/2lox}$;$E2f4^{+/-}$ and $Rb^{1lox/+}$;$E2f4^{+/-}$;*Mox2cre/+* animals to obtain $Rb^{1lox/1lox}$;$E2f4^{-/-}$;*Mox2cre/+* ($Rb^{1lox/1lox}$;$E2f4^{-/-}$) mice. The detection of a vaginal plug was considered E0.5. Fetal livers were isolated from individual embryos at E15.5. Genotyping was conducted on tail tissue as described.[13, 35] All mice were on a mixed 129/Sv × C57/BL6 genetic background and results obtained from different crosses were combined for the same genotype in all the analyses.

Purification and *in vitro* culture of TER119-negative cells

Single cell suspensions of individual E15.5 fetal livers were prepared by passing the dissociated cells through a 25 μm cell strainer. TER119-negative fetal liver cells (FLCs) were purified by magnetic beads following the manufacturer's protocol (StemCell Technologies, Vancouver, BC, Canada). Purified cells were then cultured for two days as previously described.[25]

Immunostaining and flow cytometric analysis

To obtain erythroid differentiation profiles, freshly isolated FLC and cultured cells were simultaneously stained for CD71 and TER119 as previously described.[25] To analyze macrophage differentiation, purified TER119-negative cells were simultaneously labeled with phycoerythrin (PE)-conjugated anti-F4/80 (1:100; Caltag Laboratories, Burlingame, CA) and allophycocyanin (APC)-conjugated anti-CD31 (1:100; BD Pharmingen, Franklin Lakes, NJ) antibodies. To perform apoptosis analysis, cultured cells were stained for Annexin V and 7- AAD according to the manufacturer's instructions (BD Pharmingen). Flow cytometry was carried out on a Becton Dickinson FACSCalibur machine (BD Biosciences, Franklin Lakes, NJ).

Cell cycle analysis

Purified TER119-negative cells were cultured for two days and stained in Hypotonic Propidium Iodide (PI) solution as previously described.[36] Flow cytometry was carried out on a BD FACSCalibur machine and collected data was analyzed by ModFit software (Verity Software House, Inc., Topsham, ME).

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Abbreviations

FLCs	fetal liver cells
CFU-E	colony forming unit erythroid
WT	wild type

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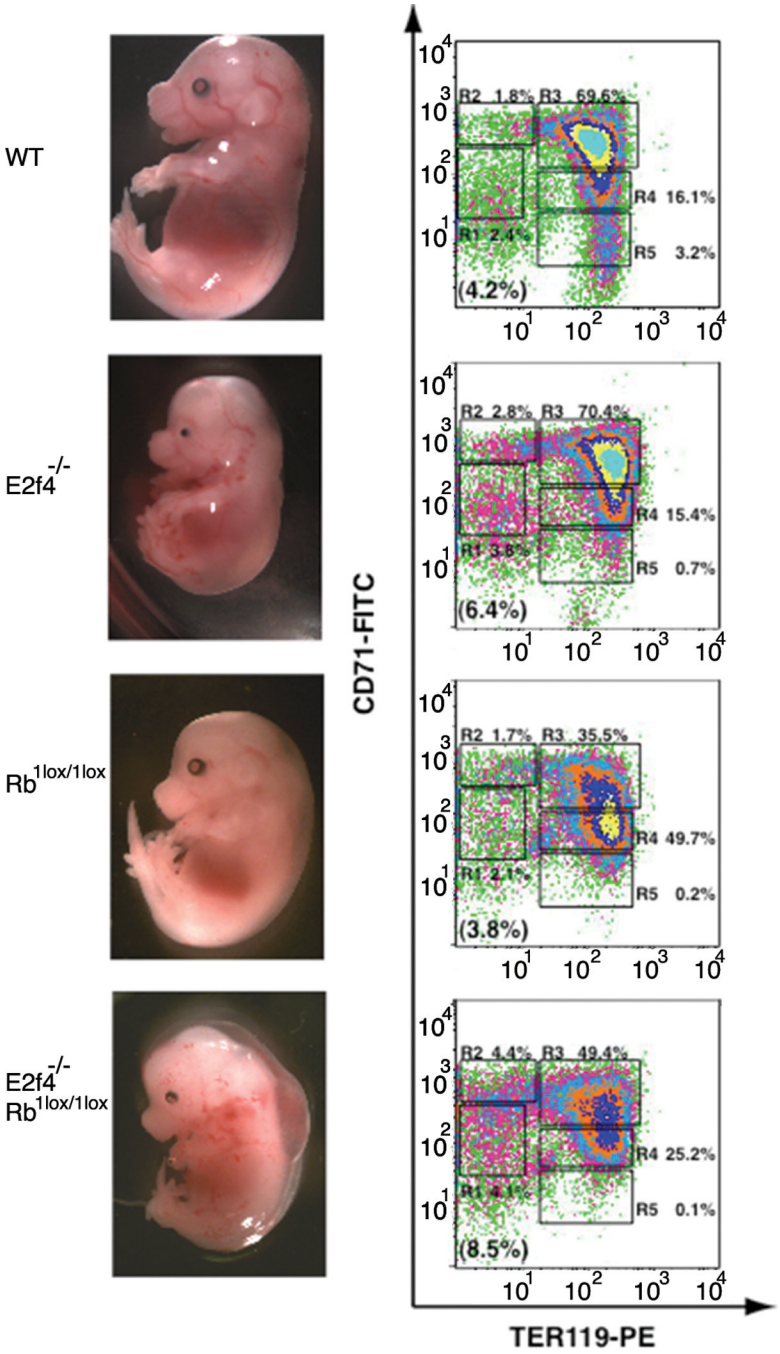

Figure 1. *E2f4*$^{-/-}$, *Rb*$^{1lox/1lox}$, and *E2f4*$^{-/-}$; *Rb*$^{1lox/1lox}$ embryos have abnormal erythroid differentiation profiles

The left panels show pictures of wild-type (WT), *E2f4*$^{-/-}$, conditional *Rb* knockout (*Rb1lox/1lox*), and double knockout (*E2f4*$^{-/-}$;*Rb*$^{1lox/1lox}$) embryos at E15.5. The right panels are representative flow cytometry density plots of fetal liver cells (FLCs) from each corresponding genotype. FLCs were freshly isolated and double labeled with a FITC-conjugated anti-CD71 monoclonal antibody (mAb) and a PE-conjugated anti-TER119 mAb. Dead cells (propidium iodide positive) and debris (low forward scatter) were excluded from analysis. Axes indicate relative logarithmic fluorescence units for PE (X-axis) and FITC (Y-axis). Regions R1–R5 are defined by characteristic staining pattern of cells. The percentage

of TER119-negative cells (R1 + R2) is in parentheses at the bottom of each density plot. The quantification of R1–R5 cells for each genotype is summarized in Table 1.

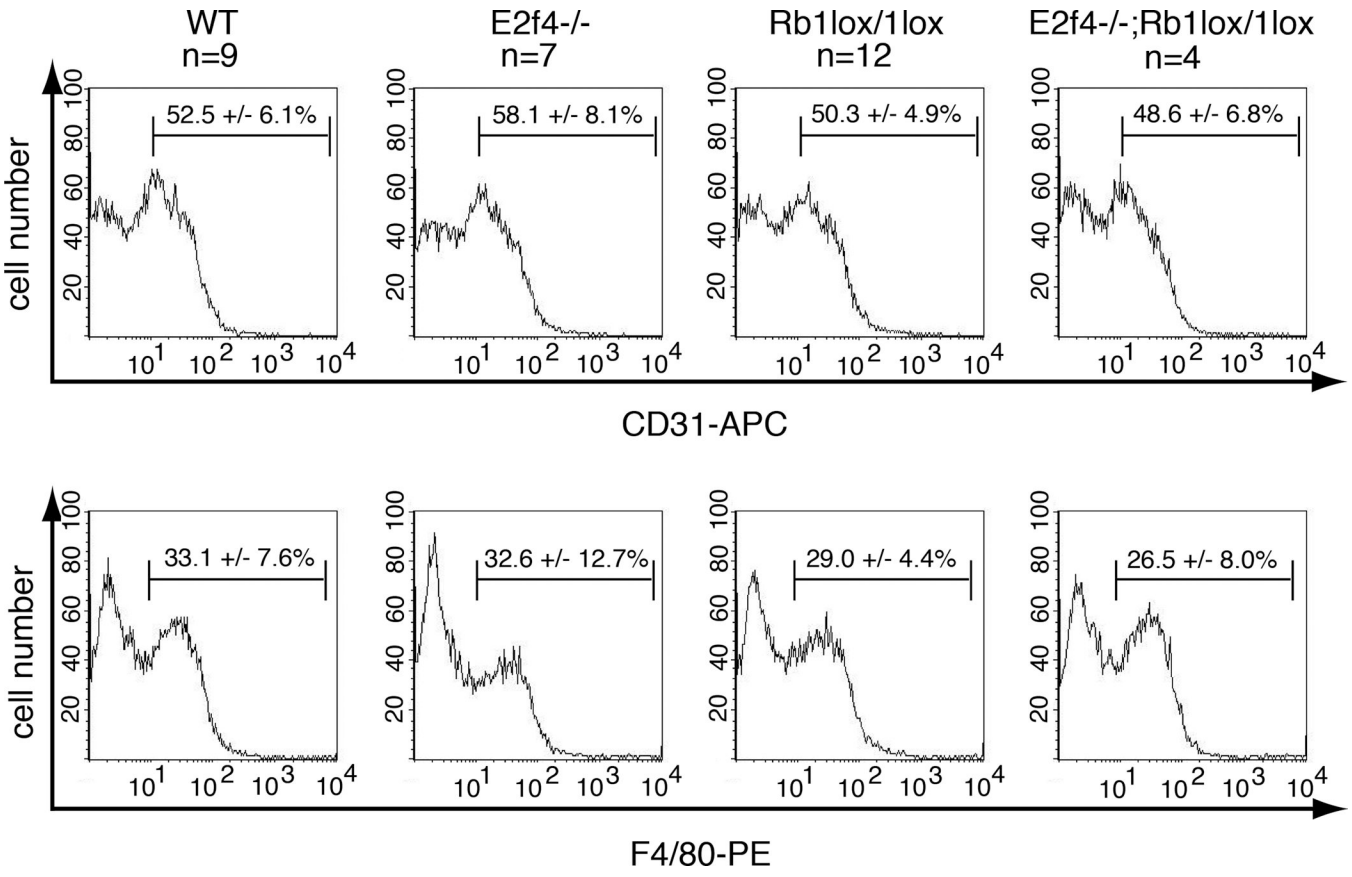

Figure 2. Macrophage differentiation in *E2f4*$^{-/-}$, *Rb*$^{1lox/1lox}$, and *E2f4*$^{-/-}$; *Rb*$^{1lox/1lox}$ embryos is normal

TER119-negative cells were purified from individual embryos at E15.5. Purified cells were double labeled with an APC-conjugated anti-CD31 monoclonal antibody (mAb) and a PE-conjugated anti-F4/80 mAb. Dead cells (propidium iodide positive) and debris (low forward scatter) were excluded from analysis as described in Figure 1. Graphs illustrate flow cytometry histograms of cell numbers (Y axis) versus relative logarithmic fluorescence units for APC (top panels) or PE (bottom panels) (X-axis). The CD31- or F4/80-positive cells are defined as indicated. Results are presented as average percentages ± standard derivation of total cells analyzed. None of the values is statistically significant compared to the corresponding wild-type (WT) value.

Table 1

Flow cytometry analysis of erythroid development in E15.5 fetal liver cells

Genotype	R1 (%)	R2 (%)	R3 (%)	R4 (%)	R5 (%)
Wild-type n=18	2.7 ± 0.4	2.5 ± 1.2	70.3 ± 9.5	17.8 ± 9.1	1.2 ± 0.6
E2f4$^{-/-}$ n=8	4.1 ± 1.6 **	3.1 ± 1.4	64.2 ± 15.6	18.9 ± 14.7	0.6 ± 0.5 **
Rb$^{1lox/1lox}$ n=22	2.6 ± 0.6	2.6 ± 1.2	60.3 ± 13.8 *	28.3 ± 13.7 *	1.2 ± 0.9
Rb$^{1lox/1lox}$;*E2f4*$^{-/-}$ n=4	4.8 ± 2.3	4.2 ± 3.2	60.3 ± 12.2	23.0 ± 9.9	0.5 ± 0.3 *

Fetal liver cells were freshly isolated from individual E15.5 embryos. The cells were stained and analyzed as described in Figure 1. Results are presented as average percentages ± standard derivation of total cells analyzed.

* denotes statistical significance relative to the corresponding wild-type value, p-value < 0.01.

** denotes statistical significance relative to the corresponding wild-type value, p-value < 0.05.

Table 2

Cell cycle analysis of erythroid cells cultured for two days *in vitro*

Genotype	G0/G1 (%)	G2/M (%)	S (%)
Wild-type, n=17	87.4 ± 3.1	5.1 ± 1.7	7.5 ± 1.9
E2f4$^{-/-}$, n=9	72.2 ± 6.0 **	12.4 ± 3.1 **	15.5 ± 4.9 **
Rb$^{1lox/1lox}$, n=17	76.4 ± 5.1 **	6.8 ± 2.2 *	16.8 ± 3.8 **
Rb$^{1lox/1lox}$*E2f4*$^{-/-}$, n=3	48.0 ± 2.8 **	14.5 ± 2.8 *	37.5 ± 0.1 **

Fetal liver cells were freshly isolated from individual E15.5 embryos. TER119-negative cells were purified and cultured *in vitro* for two days. Cultured cells were harvested, incubated in Hypotonic PI solution, and analyzed by flow cytometry. The data was analyzed by ModFit software for cell cycle distribution. Results are presented as average percentages ± standard derivation of total cells analyzed.

* denotes statistical significance relative to the corresponding wild-type value, p-value = 0.02.

** denotes statistical significance relative to the corresponding wild-type value, p-value < 0.001.

Table 3

Apoptosis analysis of erythroid cells cultured for two days *in vitro*

Genotype	Apoptotic cells (%)	p-value (relative to wt)
Wild-type, n=18	12.6 ± 3.1	n/a
E2f4$^{-/-}$, n=3	13.3 ± 4.4	0.82
Rb$^{1lox/1lox}$, n=7	20.7 ± 0.8	9.7×10^{-10}
Rb$^{1lox/1lox}$*E2f4*$^{-/-}$, n=3	28.5 ± 8.2	0.08

Fetal liver cells were freshly isolated from individual E15.5 embryos. TER119-negative cells were purified and cultured *in vitro* for two days. Cultured cells were harvested, labeled with Annexin V-APC and 7-AAD, and analyzed by flow cytometry. The Annexin V-positive and 7-AAD-negative cells were considered apoptotic cells. Results are presented as average percentages ± standard derivation of total cells analyzed.