The Role of E2F4 in the Growth Suppressive Properties of the Retinoblastoma Protein

by

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ABSTRACT

The growth suppressive functions of the retinoblastoma protein (pRB), the first identified tumor suppressor, are considerably mediated through the repression of the E2F transcription factors. Functional inactivation of pRB, and subsequent deregulation of E2F activity, is a critical event in the formation of most human cancers. pRB is a member of the pocket protein family, which includes p107 and p130. The pocket proteins have some functional redundancy; however, they have differential binding properties to the E2Fs and make very different contributions to the suppression of tumors. The E2F proteins that associate with the pocket proteins can be divided into two groups based on structural and functional similarities. The activating E2Fs, E2F1, E2F2 and E2F3a, are exclusively regulated by pRB and are primarily involved in activating the transcription of E2F-responsive genes that are required for cell cycle progression. The repressive E2Fs, E2F3b, E2F4 and E2F5, are regulated by the entire pocket protein family and are important for transcriptional repression. Mechanistically, the inappropriate proliferation promoted by the absence of pRB is, in large part, attributed to the activating E2Fs. This study investigates the contribution of a repressive E2F, E2F4, to the growth inhibitory properties of pRB during normal development and tumorigenesis. The characterization of mutant mice demonstrated that E2F4 loss significantly suppresses tumor formation in the $Rb^{+/-}$ animals. Molecular analyses suggest a novel mechanism in which p107 and p130 compensate for the loss of pRB by re-establishing the proper regulation of the activating E2Fs. The function of E2F4 in the developmental phenotypes arising from homozygous mutation of *Rb* was also assessed. In contrast to the tumor setting, E2F4 loss aggravated the placental defect in the $Rb^{-/-}$ mice, resulting in earlier lethality. Furthermore, there was no cooperation between E2F4 and pRB within the developing embryo. These results indicate that E2F4 acts in a manner differing from the activating E2Fs in mediating the effects of pRB deficiency in development. Thus, this study has established that E2F4 is a key downstream effector of pRB and has reshaped our understanding of the roles of the activating and repressive E2Fs with respect to the pocket proteins.

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Chapter One

Introduction

Part I: The Retinoblastoma Tumor Suppressor Protein

Genetic factors have long been linked to the development of cancer. This was substantiated by the discovery of oncogenes and tumor suppressor genes, which were primarily identified by their mutated states in human cancers. The retinoblastoma gene (*RB-1*) was the first tumor suppressor to be isolated and has been the prototypical model for the study of this class of genes (Friend et al. 1986; Fung et al. 1987; Lee et al. 1987a). Individuals with one mutated *RB-1* allele develop tumors associated with the loss of the remaining wild-type allele (reviewed in Lohmann and Gallie 2004). The importance of the retinoblastoma protein, pRB, in cell cycle control is underscored by the fact that *RB-1* is mutated in one-third of all human cancers and the gene product is functionally inactivated in most (Weinberg 1992; Sherr 1996). Molecular and cellular studies, as well as analyses of mutant mouse models, have revealed that pRB has critical functions in cell cycle regulation, terminal differentiation and cell survival. Furthermore, studies have demonstrated that pRB shares many functional and structural properties with two related proteins, p107 and p130. Since p107 and p130 have not been identified as tumor suppressor proteins, it will be essential to determine the underlying mechanism responsible for this difference.

A. Discovery and characterization of the retinoblastoma protein

Retinoblastoma is a pediatric tumor of the eye that occurs in familial and sporadic forms (reviewed in Lohmann and Gallie 2004). The sporadic form is usually characterized by a unilateral, unifocal tumor in the retina. Familial retinoblastoma often manifests as bilateral, multifocal retinal tumors and is frequently accompanied by other tumor types, including osteosarcomas. Based on statistical analysis of the inheritance patterns and occurrences of these

two forms of retinoblastoma, A. G. Knudson proposed the two-hit hypothesis, which postulates that two mutations are required for tumor formation (Knudson 1971). Thus, non-hereditary retinoblastoma would result from acquisition of two somatic mutations whereas individuals with one inherited germline mutation would require only one somatic mutation for tumorigenesis.

Retinoblastoma was indeed found to arise from homozygous loss of one genetic locus. Retinoblastoma susceptibility was mapped to chromosome 13q14 based on cytogenetic studies of non-tumorigenic cells, such as lymphocytes and fibroblasts, from retinoblastoma patients (Sparkes et al. 1980; Benedict et al. 1983; Dryja et al. 1986). *RB-1* was subsequently cloned by chromosomal walking techniques and genetic lesions at this locus were verified to occur in retinoblastomas, as well as osteosarcomas (Friend et al. 1986; Fung et al. 1987; Lee et al. 1987a). Mutations in *RB-1* have also been characterized in small cell lung carcinoma (Harbour et al. 1988; Yokota et al. 1988), breast cancer (Lee et al. 1988; T'Ang et al. 1988), and prostate cancer (Bookstein et al. 1990a). Loss of pRB was first demonstrated to be essential for tumorigenesis when the oncogenic transformation properties of *RB-1*-deficient cancer cell lines, including retinoblastoma, osteosarcoma and prostate, were suppressed upon reintroduction of functional pRB (Huang et al. 1988; Bookstein et al. 1990a).

The *RB-1* locus spans 183 kb and encodes a 928 amino acid nuclear protein (reviewed in Lohmann and Gallie 2004). Structurally, the 110 kD protein has three distinct protein binding domains (Figure 1). The A and B domains compose the pocket structure, which is a frequent site for naturally occurring mutations (Hu et al. 1990; Kaelin et al. 1990). In addition, many pRB-interacting proteins bind to the A/B pocket (reviewed in Morris and Dyson 2001). The pocket domain is not sufficient to suppress cellular growth alone (Huang et al. 1992; Qin et al. 1992; Hiebert 1993). A portion of the C-terminal domain is also required for growth suppression,

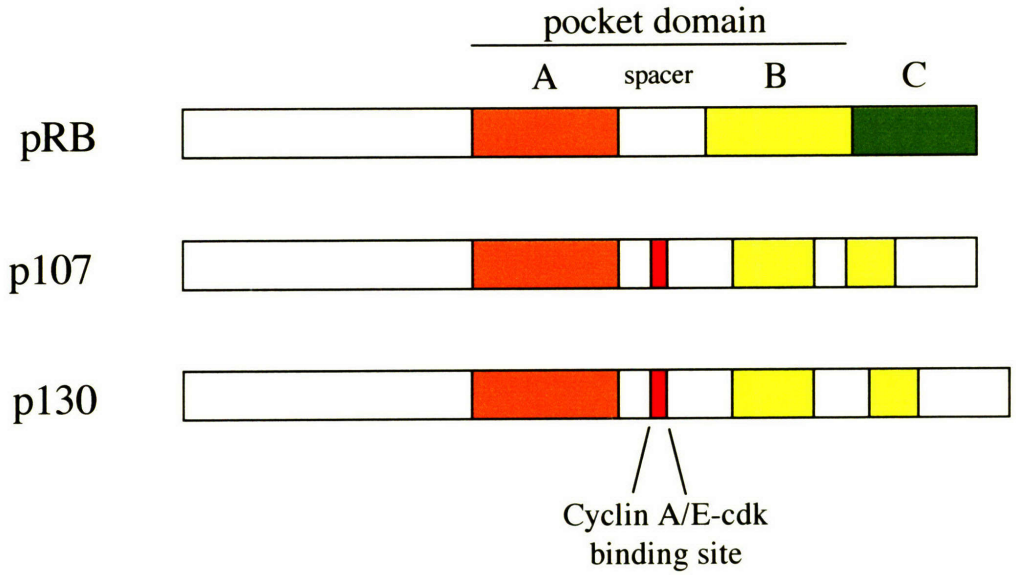

Figure 1. The pocket protein family.

The homologous domains of the pocket proteins, pRB, p107 and p130, are indicated in the schematic diagram. The A and B domains comprise the pocket domain, which is conserved in all three proteins. In contrast, the C domain in pRB is not conserved in the other pocket proteins. p107 and p130 are more closely related to each other than to pRB and they have a larger spacer region which contains a cyclin binding domain.

which together with the A/B pocket are referred to as the large pocket. The C domain is important for binding to a subset of pRB binding partners, such as c-Abl (Welch and Wang 1993), MDM2 (Xiao et al. 1995) and PP1α (Durfee et al. 1993; Ludlow et al. 1993). The A/B pocket and C domains are functionally independent in that they can interact with separate proteins simultaneously (Welch and Wang 1993).

Viral oncoproteins, such as adenovirus early region 1A protein (E1A), simian virus 40 (SV40) large T antigen and human papillomavirus (HPV) type 16 E7 protein, interact with pRB to mediate cellular transformation (DeCaprio et al. 1988; Whyte et al. 1988; Dyson et al. 1989; Dyson et al. 1990). The conserved LxCxE motif in the viral oncoproteins binds to pRB and is required for transformation (DeCaprio et al. 1988; Dyson et al. 1989; Ewen et al. 1989; Dyson et al. 1992). Moreover, the viral oncoproteins bind to the A/B pocket domain of pRB (Hu et al. 1990; Huang et al. 1990; Kaelin et al. 1990). The crystal structure reveals that the LxCxE motif of an HPV E7 peptide directly associates with a conserved groove on the B domain of pocket while the A domain is needed for stable folding of the B domain (Lee et al. 1998). Thus, the viral oncoproteins efficiently inactivate pRB by interfering with interactions between the pocket domain of pRB and numerous cellular proteins.

pRB expression is relatively constant throughout the cell cycle and its activity is regulated by phosphorylation (Lee et al. 1987b). The hypophosphorylated, or active, form of pRB exclusively binds to the SV40 large T oncoprotein (Ludlow et al. 1989; Shew et al. 1989). Once phosphorylated, pRB is inactivated (Cobrinik et al. 1992). The phosphorylation status of pRB varies during the cell cycle (Buchkovich et al. 1989; Chen et al. 1989; DeCaprio et al. 1989; Mihara et al. 1989). In G0/G1-arrested cells, as well as in differentiated cells, pRB is underphosphorylated. In late G1 and S phases, pRB is increasingly phosphorylated by cyclin

D/CDK4 and cyclin D/CDK6 followed by cyclin E/CDK2 (Hinds et al. 1992; Ewen et al. 1993; Kato et al. 1993; Hatakeyama et al. 1994; Lundberg and Weinberg 1998). pRB is then dephosphorylated late in mitosis by protein phosphatase 1 (Ludlow et al. 1993). Thus, the hypophosphorylated form of pRB acts to promote growth suppression.

B. The pocket protein family

The pocket domain of pRB is the hallmark for pRB-related proteins, also referred to as the pocket proteins. The pocket protein family consists of three members, pRB, p107 and p130. p107 and p130 were initially identified as targets bound by small DNA tumor viruses (Harlow et al. 1986; Whyte et al. 1989). Subsequent isolation of their cDNAs revealed significant homology to pRB in the A/B pocket domain (Figure 1) (Ewen et al. 1991; Hannon et al. 1993; Li et al. 1993). Consistent with properties observed for pRB, these proteins are phosphorylated in a cell cycle dependent manner, bind to the E2F transcription factors (discussed in Part II) and can induce G1 growth inhibition when ectopically expressed in a variety of cell lines (reviewed in Classon and Dyson 2001).

Despite the similarities, there are clear differences between the pocket proteins. Structurally, p107 and p130 are most alike with approximately 50% amino acid identity to each other versus 30-35% to pRB (Figure 1) (Ewen et al. 1991; Hannon et al. 1993; Li et al. 1993). p107 and p130 have a larger spacer region between the A and B domains compared to that found in pRB. This region is important for cyclin E/CDK2 and cyclin A/CDK2 binding to p107 and p130 (Ewen et al. 1992; Faha et al. 1992; Hannon et al. 1993; Li et al. 1993). Although the biological significance of cyclin dependent kinase (CDK) binding is unclear, the spacer region has been demonstrated to be necessary for growth suppression by p107. Moreover, p107 and

p130 are not homologous to pRB at the carboxyl terminal regions. Finally, the pocket proteins have distinct expression patterns during the cell cycle. While p130 is induced in quiescent cells, p107 is highly expressed in proliferating cells and pRB is moderately expressed throughout the cell cycle (Chen et al. 1989; Baldi et al. 1995; Beijersbergen et al. 1995; Mayol et al. 1995; Xiao et al. 1996). These properties may reflect functional differences among the pocket proteins.

The most striking physiological difference between the pocket proteins is that only pRB is classified as a tumor suppressor, since homozygous inactivation of *RB-1* has been detected in numerous human cancers. There is emerging evidence for *p130* involvement in cancer; however, it is unclear whether loss of this locus acts in a manner analogous to *RB-1*. In addition to decreased p130 expression correlating with increased tumor malignancy (Baldi et al. 1997; Susini et al. 1998; Tanaka et al. 1999), mutations in the *p130* gene have been identified in nasopharyngeal (Claudio et al. 2000a), and small cell lung and non-small cell lung carcinomas (Baldi et al. 1997; Helin et al. 1997; Claudio et al. 2000b). Accompanying loss of the *RB-1* locus, however, was detected in some cases (Helin et al. 1997), suggesting that loss of pRB function may be the basis for tumorigenesis. Systematic analyses of *RB-1* status will be necessary to determine whether loss of p130 is sufficient for tumor growth in these cases. Mouse models have been used at length to investigate the growth properties associated with the pocket proteins and, consistent with *in vitro* experiments with human cells, the pocket proteins have significant functional redundancy as well as distinct roles. (This will be discussed in detail below.) At any rate, pRB is the most extensively studied pocket protein due to the clinical implications of *RB-1* loss.

C. Mechanisms of transcriptional repression

The growth inhibitory functions of pRB are largely dependent upon its ability to bind to the cellular transcription factor E2F (also identified as DRTF1), the most well characterized downstream effector of the pocket proteins (reviewed in Trimarchi and Lees 2002). The identification of E2F-responsive genes established that E2F activity was essential for control of the G1 to S transition of the cell cycle. This generated a straightforward model for pRB regulation of the cell cycle. In the G1 phase, hypophosphorylated pRB inhibits E2F transcriptional activity. When pRB is inactivated, either by phosphorylation or viral oncoprotein binding, disruption of the pRB-E2F complex and concomitant release of E2F results in the transcriptional activation of E2F target genes that are necessary for S phase entry. Therefore, pRB is a pivotal regulator of E2F activity. Studies into the mechanism of pRB-mediated repression have shown that the repressive action of pRB can be achieved in more than one way. (E2F will be discussed in Part II.)

One mechanism by which pRB inhibits E2F activity is by physically obstructing transcriptional activation. E2F does not have an LxCxE motif, which the viral oncoproteins use to bind to pRB. Instead, E2F interacts with pRB through an 18 amino acid sequence found within the transactivation domain (Helin et al. 1992). Thus, upon stable complex formation, pRB conceals the E2F transactivation domain and prevents transcription of target genes (Hiebert et al. 1992; Flemington et al. 1993; Helin et al. 1993a). This is illustrated by the crystal structure of the pRB pocket domain bound to the pRB binding domain of an E2F transcription factor (Lee et al. 2002). Several highly conserved residues in the transactivation domain of E2F are inaccessible upon interaction with pRB. Therefore, there is a structural basis for this type of 'passive' repression.

Pocket protein-E2F complexes have been found in association with chromatin remodeling enzymes, suggesting a mechanism for the 'active' repression of E2F transcriptional activity. Histone deacetylases (HDACs), whose activities are widely associated with transcriptional repression, interact with pRB to repress E2F target gene expression (Brehm et al. 1998; Luo et al. 1998; Magnaghi-Jaulin et al. 1998). Transcriptional co-repressors that cooperate with HDACs, such as RBP1, also bind to pRB, suggesting that pRB may participate in larger co-repressor complexes to mediate transcriptional repression (Lai et al. 1999). In addition, p107 and p130 interact with HDAC1 at E2F-responsive promoters in G0 and early G1 phases of the cell cycle (Ferreira et al. 1998; Rayman et al. 2002).

pRB may also affect transcription through nucleosome remodeling. pRB can associate with BRG1 and hBRM, human homologs to components of the yeast SWI/SNF complex (Dunaief et al. 1994; Strober et al. 1996; Trouche et al. 1997). Though initial studies demonstrated the requirement for BRG1 and pRB to effectively arrest cells, there is growing evidence that pRB can affect transcription in a BRG1-independent manner. For example, pRB-mediated growth arrest can occur in the presence of BRG1 lacking the ability to bind to pRB (Kang et al. 2004). These differences may be a consequence of over-expression or due to cell type specificity. Further analyses will be necessary to determine the exact nature of the interaction between pRB and members of the human SWI/SNF complex.

Another mode of pRB-mediated 'active' repression may be facilitated by histone methyltransferases. pRB can bind to the histone methyltransferase SUV39H1 at the cyclin E promoter, an E2F target gene (Nielsen et al. 2001). SUV39H1 methylates lysine9 on histone H3, a modification corresponding to transcriptionally silent chromatin, and leads to the recruitment of a heterochromatin protein, HP-1 (Bannister et al. 2001; Lachner et al. 2001). pRB association

with HP-1 at E2F-responsive promoters has also been detected in senescent cells (Narita et al. 2003). Furthermore, interaction between p107 (or p130) and SUV39H1 has been reported at the *dihydrofolate reductase (DHFR)* promoter (Nicolas et al. 2003). The physiological significance of this last observation remains to be determined. In general, much of the data on pocket protein recruitment of chromatin remodeling enzymes is based on over-expression studies. Thus, it is not clear whether the pocket proteins have differential binding properties to the chromatin modifying factors as well as to specific promoters *in vivo*.

The pocket proteins can also mediate transcriptional repression through direct interaction with components of the basal transcriptional machinery. The entire pocket protein family can negatively regulate RNA polymerase III (PolIII) in transfection and *in vitro* transcription assays (White et al. 1995; Sutcliffe et al. 1999). In fact, primary fibroblasts lacking either pRB or p107 and p130 have elevated levels of PolIII transcription. PolIII repression may be achieved through direct interaction with TFIIIB, a multi-subunit complex that recruits PolIII to promoters (Chu et al. 1997; Larminie et al. 1997; Sutcliffe et al. 1999).

D. Regulation of cellular proliferation

To date, there are over 100 proteins reported to interact with pRB (reviewed in Morris and Dyson 2001). In addition to factors that regulate the cell cycle, pRB interacts with cellular proteins involved in additional processes, including differentiation and apoptosis (discussed in the next section). In order to understand how pRB may relate cell cycle control with other biological processes, it is important to determine the mechanisms through which pRB, as well as the pocket proteins, function in each process.

The pocket proteins are key regulators of the G1 phase of the cell cycle. Studies employing microinjection, infection and transfection techniques showed that over-expression of a pocket protein can lead to growth arrest in a variety of cell types (Huang et al. 1988; Bookstein et al. 1990b; Vairo et al. 1995; Zhu et al. 1995). The physiological significance of the pRB family members has been extensively studied through the analysis of the mouse embryonic fibroblasts (MEFs) resulting from mutant mouse models. The role of pRB in the G1 to S transition is exemplified by the fact that *Rb*$^{-/-}$ MEFs have a defect in G1 control. Cell cycle profiling has demonstrated that *Rb*$^{-/-}$ cells have a shortened G1 phase and a proportionally lengthened S phase (Herrera et al. 1996). Although the proliferation rate in these cells is unchanged relative to wild-type cells, there is a multitude of evidence to suggest that this is largely due to functional compensation by the other pocket proteins. First, *Rb*$^{-/-}$ and *p130*$^{-/-}$ cells have elevated p107 mRNA and protein levels (Herrera et al. 1996; Mulligan et al. 1998). In quiescent *p130*$^{-/-}$ T lymphocytes, the increased p107 expression correlates with increased p107/E2F binding (Mulligan et al., 1998). Second, the simultaneous absence of two pocket proteins, p107 and p130 or p107 and pRB, exhibit a further decrease in the proportion of cells in G1 phase relative to *Rb*$^{-/-}$ MEFs (Hurford et al. 1997; Classon et al. 2000; Dannenberg et al. 2000). *Rb*$^{-/-}$;*p107*$^{-/-}$ cells also have a slightly increased proliferative capacity compared to *Rb*$^{-/-}$ cells (Dannenberg et al. 2000). Third, cells deficient for all three pocket proteins are unresponsive to a variety of growth arrest signals, including contact inhibition, serum deprivation, DNA damage, and senescence inducers (Dannenberg et al. 2000; Sage et al. 2000). Finally, acute loss of *Rb* in quiescent primary fibroblasts stimulates cell cycle re-entry due to the inability to induce p107 in a timely manner (Sage et al. 2003). Thus, the pocket proteins have overlapping functions in blocking progression through the cell cycle.

Since only pRB is a bona fide tumor suppressor, there must be functional differences between the pocket proteins. The growth arrest achieved by the pocket proteins is due, in part, to the regulation of different subsets of genes. While loss of either p107 or p130 does not result in E2F target gene deregulation or alterations in cell cycle kinetics, the absence of both induces the expression of a number of E2F target genes (i.e., *cyclin A2*, *E2f1*, *B-myb*, *thymidylate synthase*, *ribonucleotide reductase subunit M2* and *cdc2*) (Hurford et al. 1997). On the other hand, *p107* and *cyclin E* are induced in $Rb^{-/-}$ MEFs (Herrera et al. 1996). Thus, the derepressed E2F-responsive genes in $p107^{-/-};p130^{-/-}$ fibroblasts differ from those seen in $Rb^{-/-}$ MEFs. This may be due to the differential binding properties of the pocket proteins to the E2F transcription factors (see Part II).

There is growing evidence that pRB has roles in the cell cycle outside of the G1 to S transition. pRB mediates repression of *cyclin A*, which subsequently affects cyclin B degradation, suggesting a role in G2/M control (Lukas et al. 1999). More recent work has shown that the gene encoding Mad2, a mitosis spindle checkpoint component, is repressed by pRb-E2F complexes in mouse and human cells (Hernando et al. 2004). In fact, *RB-1* ablation in cells induces *MAD2* expression and results in aneuploidy, implicating pRB in spindle checkpoint regulation. pRB also has a role in S phase checkpoint control in response to DNA damaging agents (Knudsen et al. 1998). Moreover, there is evidence that pRB has a functions in maintaining G0 arrest and senescence. Acute inactivation of pRB in murine senescent cells induces cells to re-enter the cell cycle (Sage et al., 2003). In addition, pRB has been detected at E2F-responsive promoters during senescence (Narita et al., 2003) and growth arrest in response to $p16^{INK4a}$ (Dahiya et al. 2001; Young and Longmore 2004). Furthermore, cyclin C/cdk3 phosphorylation of pRB facilitates transition from G0 to G1 (Ren and Rollins 2004). Thus, these

studies suggest that pRB has functions throughout the cell cycle. Further studies are required to determine the mechanisms through which pRB is functioning in these processes, as well as how it coordinates multiple tasks.

E. Roles of the pocket proteins in development and differentiation

The analyses of genetically modified mice for members of the pocket protein family have underscored the roles of these proteins in normal development and differentiation. It has become apparent that while pRB, p107 and p130 share functional properties, they also possess distinct and separate functions *in vivo*.

i. The retinoblastoma protein

The analyses of mutant mouse models have shown that pRB regulates cellular proliferation, apoptosis and terminal differentiation. $Rb^{-/-}$ mice die *in utero* by embryonic day (E) 15.5 with defects in the nervous system, ocular lens, erythropoeisis and extra-embryonic tissues (Clarke et al. 1992; Jacks et al. 1992; Lee et al. 1992; Wu et al. 2003). The cause of death in these animals is a primary placental defect that results in reduced nutrient and gas delivery to the embryos (Wu et al. 2003). In order to circumvent the early lethality of $Rb^{-/-}$ mice and to analyze the effects of pRB loss on later stages of development and in specific tissues, numerous groups have employed transgenic, chimeric and conditional gene targeting strategies (Robanus-Maandag et al. 1994; Williams et al. 1994b; Zacksenhaus et al. 1996; Lipinski et al. 2001; Ferguson et al. 2002; de Bruin et al. 2003b; MacPherson et al. 2003; Wu et al. 2003; Zhang et al. 2004). These studies have implicated pRB in additional processes, such as muscle differentiation. Furthermore, they have revealed that some of the initially characterized developmental defects in

the *Rb*$^{-/-}$ embryos are cell non-autonomous. Other studies have assessed the involvement of pRB downstream effectors in the developmental functions of pRB through the generation of compound mutant animals (discussed in Part II).

Rb$^{-/-}$ animals exhibit inappropriate cellular proliferation and widespread apoptosis in a number of tissues, including the lens of the eye, the central nervous system (CNS), and the peripheral nervous system (PNS) (Clarke et al. 1992; Jacks et al. 1992; Lee et al. 1992; Lee et al. 1994; Morgenbesser et al. 1994). The status of p53, a critical regulator of apoptosis and growth arrest, was assessed in various tissues of the *Rb*$^{-/-}$ animals (Morgenbesser et al. 1994; Macleod et al. 1996). In the developing lens and the CNS, apoptosis was dependent upon p53. As such, combined loss of p53 and pRB suppressed the apoptosis in these structures. In contrast, p19^{ARF}, an upstream regulator of p53, was dispensable for the programmed cell death in the *Rb*$^{-/-}$ animals since there was no amelioration of the apoptotic defects in *Rb;p19*^{ARF} double mutants (Tsai et al. 2002). With respect to the inappropriate proliferation arising from pRB loss, the absence of p53 or p19^{ARF} increased the level of ectopic DNA synthesis, corresponding with the role of the p53 pathway in growth arrest (Macleod et al. 1996; Tsai et al. 2002). Upon investigation of the apoptosis in the PNS, it was found to be p53-independent (Macleod et al., 1996) and caspase 3-dependent (Simpson et al. 2001). Thus, pRB is critical for cell cycle control and cell survival *in vivo*.

The first indication that the apoptosis originally characterized in the nervous system was a cell non-autonomous effect came from the analysis of chimeric mice (Robanus-Maandag et al. 1994; Williams et al. 1994b; Lipinski et al. 2001). Apoptosis in the CNS is also suppressed in mice with specific homozygous deletion of *Rb* in either the CNS (MacPherson et al. 2003) or the telencephalon (Ferguson et al. 2002). Conditional and tetraploid experiments that resulted in *Rb*

$^{-/-}$ embryos provided with normal functioning placentas further support that apoptosis in the nervous system is not due to the loss of pRB (de Bruin et al. 2003b; Wu et al. 2003). Instead, the programmed cell death was a consequence of defects in the extra-embryonic tissues, which led to hypoxic conditions for the developing embryo. In most of these studies, apoptosis in the ocular lens persisted as well as the increased levels of inappropriate proliferation in the various tissues, indicating cell autonomous functions of pRB. Although the involvement of pRB in cellular survival is not as widespread as initially thought, it does have an important role in the lens.

Analyses of chimeric animals also suggested that the erythroid defect in *Rb*$^{-/-}$ mice was cell non-autonomous (Robanus-Maandag et al. 1994; Williams et al. 1994b). *Rb*$^{-/-}$ embryos die in mid-gestation from E13.5 to E15.5, a time period when the fetal liver is the major site of definitive erythropoiesis (Clarke et al. 1992; Jacks et al. 1992; Lee et al. 1992). *Rb*$^{-/-}$ mice are pale in appearance, have reduced cellularity in the fetal livers and have an increased level of immature nucleated erythrocytes. Chimeric mice, on the other hand, have apparently normal levels of mature enucleated red blood cells. Moreover, *Rb* mutant mice provided with normal functioning placentas also have a significant amelioration in erythropoiesis, similar to the apoptosis in the CNS (de Bruin et al. 2003b). *Rb*$^{-/-}$ fetal liver cells are also able to reconstitute lethally irradiated wild-type recipient mice in adoptive transfer experiments (Hu et al. 1997; Spike et al. 2004). More recently, it was suggested that the insufficient microenvironment provided by the macrophages in the *Rb*$^{-/-}$ fetal livers was responsible for the defects in erythropoiesis (Iavarone et al. 2004). All these lines of evidence point to a cell non-autonomous effect of pRB loss in developing erythrocytes.

While there is no doubt that the placental defect significantly contributes to the erythropoietic defect in *Rb*$^{-/-}$ mice, there are some indications that pRB does have a cell intrinsic

role in red blood cell maturation. First, a significant fraction of nucleated erythrocytes persists in chimeric and conditional *Rb* mutant mice (Hu et al. 1997; de Bruin et al. 2003b). Second, $Rb^{-/-}$ erythroid progenitors fail to differentiate in co-culture experiments with wild-type progenitor cells (Clark et al. 2004). Third, acute deletion of *Rb* in cultured erythroblasts leads to the same defects observed in $Rb^{-/-}$ cells (Spike et al. 2004). Fourth, adoptive transfer experiments with $Rb^{-/-};Rb^{+/+}$ chimeric bone marrow cells have demonstrated that, despite the presence of wild-type cells, the mutant cells failed to exit the cell cycle and did not properly regulate markers required for terminal differentiation (Spike et al. 2004). Finally, it was suggested that the cell intrinsic role of pRB is in stress erythropoiesis since the erythroid defect, which was found to be absent in young $Rb^{-/-};Rb^{+/+}$ chimeras, was evident in aging chimeras developing tumors and in mice with hemolytic anemia (Spike et al., 2004). Thus, there is growing evidence that pRB does, in fact, have a role in erythroblast differentiation although this currently remains controversial.

In rescue experiments where $Rb^{-/-}$ mice survive beyond E15.5, the animals exhibit additional differentiation defects, most notably in the skeletal muscle (Zacksenhaus et al. 1996; de Bruin et al. 2003b). pRB loss results in inappropriate DNA synthesis within myotubes, myoblast apoptosis, abnormal myotubes, and reduced muscle fibers. Marker expression analysis in transgenic *Rb* mutants and *in vitro* differentiation assays of MEFs, C2C12 and Saos-2 cells shows that pRB loss does not disrupt early marker expression (myogenin and MEF2), but does inhibit the expression of late markers (MCK and MRF4) (Novitch et al. 1996; Zacksenhaus et al. 1996; Li et al. 2000). This suggests that while pRB is not involved in cell fate determination, pRB does participate in end-stage differentiation. There seems to be some level of functional compensation by the other pocket proteins during myogenesis since teratocarcinomas from

embryonic stem cells lacking all three pocket proteins have a dramatic lack of muscle cells compared to teratocarcinomas from wild-type cells (Dannenberg et al. 2000).

The molecular mechanism underlying pRB function during muscle differentiation has been extensively investigated *in vitro*. The role of pRB in muscle differentiation depends on pRB association with tissue-specific transcriptional regulators that promote differentiation. It has been proposed that pRB enhances MyoD transcriptional activity to promote MEF2 transcription (Gu et al. 1993). To date, this interaction has not been detected *in vivo*, suggesting that pRB may function by an alternate mechanism to regulate MyoD (Halevy et al. 1995; Zhang et al. 1999b; Li et al. 2000). Recently, loss of N-Ras, a proto-oncogene, in the *Rb*-deficient background resulted in an amelioration of the skeletal muscle defects attributed to pRB loss (Takahashi et al. 2003). In addition to restored muscle fiber density and myotube length, MCK expression (a late marker) was normal and transcriptional activity of MyoD and MEF2 were enhanced *in vitro*. Thus, pRB influences MyoD activity, but the precise mechanism of action remains to be determined. Other pRB interacting proteins, such as HBP1, MRF4 and myogenin, have been suggested to have a role in this process, but further studies are necessary to determine the nature of the interactions (Gu et al. 1993; Shih et al. 1998).

In addition to myogenesis, pRB has been significantly studied in the development and differentiation of other tissues. In neurogenesis and adipogenesis, early marker expression is not affected by pRB loss, though the expression of late differentiation markers is disrupted (Lee et al. 1994; Chen et al. 1996). This is highly reminiscent of pRB function in muscle differentiation, suggesting that pRB may function in an analogous manner to promote differentiation in different cell types. Most evidence for pRB involvement in differentiation, including osteogenesis and adipogenesis, comes from human tumor cell lines and primary murine cells since the available

mouse models do not exhibit defects in these tissues. With the increasing number of tissue-specific *Cre* mouse strains, conditional knockout technology will be important to investigate pRB function in a variety of tissues.

ii. p107 and p130

The analyses of germline and conditional mutant mice have contributed immensely to our understanding of the functional similarities and differences among the pocket proteins. In contrast to $Rb^{-/-}$ mice, $p107^{-/-}$ and $p130^{-/-}$ mice (on a C57Bl/6 background) are viable and have no overt abnormalities (Cobrinik et al. 1996; Lee et al. 1996). On a Balb/c background, however, $p130^{-/-}$ animals die *in utero* from E11 to E13 with neural and muscle defects (LeCouter et al. 1998). In addition, $p107^{-/-}$ animals on a Balb/c background are viable, but are growth retarded and display myeloid hyperproliferation (LeCouter et al. 1998). Recent evidence has shown that the disrupted *p130* allele in the C57Bl/6 background is a hypomorphic allele (Rayman et al. 2002). Thus, the residual p130 activity may be modulating the phenotypes associated with p130 loss, resulting in the observed differences in the different genetic backgrounds. Yet, strain-specific effects can clearly influence the developmental consequences associated with p107 loss and may also be affecting the phenotypes caused by p130 loss.

Compound mutant mouse models have confirmed the redundant nature of the pocket proteins. In general, any combination of homozygous mutations results in decreased survival and accelerated, or novel, defects. $p107^{-/-};p130^{-/-}$ mice (on a 129/Sv x C57Bl/6 genetic background) die perinatally and exhibit endochondral bone defects and defects in chondrocyte cell cycle exit (Cobrinik et al. 1996). Furthermore, the loss of either p107 or p130 in the $Rb^{-/-}$ background causes earlier lethality and leads to increased cell death in the central nervous system

(Lee et al. 1996). In light of the *Rb*-related placental defect, the earlier lethality may be due to an exacerbation of this phenotype. Further studies will be necessary to determine whether p107 and p130 have a role in the extra-embryonic tissues. Finally, the complete ablation of all the pocket proteins results in greatly reduced survival, as evidenced by the inability to generate primary mouse fibroblasts from germline mutant mice (Dannenberg et al. 2000; Sage et al. 2000). Thus, the pocket proteins have substantial overlap in their functions during normal development.

F. The tumor suppressive properties of the retinoblastoma protein

Of the pocket proteins, only pRB is classified as a tumor suppressor. Humans and mice with one mutant *Rb* allele are predisposed to develop tumors. Targeted disruption of the *Rb* gene in mice, however, does not lead to retinoblastoma, as observed in humans. $Rb^{+/-}$ mice have a reduced lifespan of approximately 1 year and are predisposed to develop pituitary adenocarcinomas (90-100% penetrance) and medullary thyroid tumors (approximately 70% penetrance) (Jacks et al. 1992; Hu et al. 1994; Williams et al. 1994a). Similarly, retinoblastoma does not form in chimeric animals generated from injection of $Rb^{-/-}$ embryonic stem cells into wild-type blastocysts (Robanus-Maandag et al. 1994; Williams et al. 1994b). Instead, they develop the same tumors observed in $Rb^{+/-}$ mice at an accelerated rate. It will be important to determine the cause for the tissue specificity of tumor formation between species, as well as within a given organism.

Initially, it was thought that the balance between apoptosis and proliferation was essential for retinal tumor growth in mice. In $Rb^{-/-}$ chimeric retinas, there are considerable levels of ectopic proliferation and cell death (Robanus-Maandag et al. 1994). Transgenic animals expressing SV40 large T antigen or HPV E7 and E6 oncoproteins in the retina develop

retinoblastoma (Windle et al. 1990; Howes et al. 1994; Saenz Robles et al. 1994). These models result in the inactivation of all the pocket proteins as well as p53. On the other hand, mere expression of HPV E7, which inhibits the pocket proteins, in the retina does not lead to tumor formation (Howes et al. 1994). Thus, murine retinoblastoma formation was thought to rely upon loss of p53 to evade programmed cell death.

The first line of evidence that functional redundancy may account for the lack of retinoblastoma formation in *Rb* mutant mice came from compound mutant mouse studies. $Rb^{+/-};p107^{-/-}$ mice develop pituitary and thyroid tumors, but also display retinal dysplasia (Lee et al. 1996). In chimeric and conditional mice with specific loss of pRB and p107 or pRB and p130 in the retina, retinoblastomas develop at high frequencies (Robanus-Maandag et al. 1998; Chen et al. 2004; Dannenberg et al. 2004; MacPherson et al. 2004). Identification of the cell type of origin, amacrine cells, indicated that there is no requirement to override the apoptotic program for retinal tumor development, since the aberrantly proliferating cells originated from a region devoid of apoptosis. Thus, in contrast to humans, the levels of the pocket proteins seem to differ in the murine retinas in which pRB deficiency is insufficient to promote tumor formation.

Functional compensation by p107 and p130 in the absence of pRB has also been observed in other tissues since $Rb^{+/-};p107^{-/-}$ and $Rb^{+/-};p130^{-/-}$ germline mutant mice and $Rb^{-/-};p107^{-/-}$ and $Rb^{-/-};p130^{-/-}$ chimeric animals develop an array of novel tumor types not observed in single mutant chimeras (Robanus-Maandag et al. 1998; Dannenberg et al. 2004). Further studies will be helpful to determine the molecular mechanisms underlying the tissue-specific nature of tumor development in the absence of pRB. It may simply be a matter of the expression levels of the various pocket proteins. Yet, elucidating the regulatory pathways in which the pocket proteins function will be critical since these proteins could merely be acting in the same pathway

or have non-overlapping functions with additive effects. Addressing these issues will provide further insight into the functions of the pocket proteins during oncogenic transformation and normal development, which will inevitably broaden our understanding of how functional inactivation of pRB promotes human cancers.

Part II: The E2F Family of Transcription Factors

A. Discovery and characterization of the E2F transcription factor

The E2F transcription factor was first identified as a cellular component that cooperates with E1A to activate the transcription of the early adenoviral *E2* gene (Kovesdi et al. 1986). Transcriptional activation, and consequently cellular proliferation, by E1A were dependent on E2F DNA recognition sites, 5'-TTTCGCGC-3', in the *E2* promoter and the disruption of cellular E2F-pRB complexes (Kovesdi et al. 1987; Yee et al. 1987). The properties of E2F paralleled those of another cellular activity, DRTF1 (differentiation regulated transcription factor 1), which decreased during F9 embryonal carcinoma stem cell differentiation (La Thangue and Rigby 1987). DRTF1 bound to the same DNA binding motif required for E2F activity and DRTF1 interacted with pRB (La Thangue and Rigby 1987; Bandara and La Thangue 1991). Subsequent cloning of the cDNAs demonstrated that these were in fact the same factor.

The E2F transcription factor plays a pivotal role in the regulation of the G1 to S transition of the cell cycle. E2F recognition sites have been identified in genes involved in cell cycle regulation (e.g., *B-myb*, *cdc2*, *Cyclin E*, *E2f1*, *Rb*), nucleotide metabolism (e.g., *DHFR*, *ribonucleotide reductase*, *thymidine kinase*) and DNA replication (e.g., *PCNA*, *MCMs*, *cdc6*) (reviewed in Trimarchi and Lees 2002). At least one intact E2F DNA binding site is important for the temporal expression of most of these genes, supporting a principal role for E2F transcriptional activity in regulating cell division (Blake and Azizkhan 1989; Means et al. 1992).

B. Members of the E2F transcription factor family

E2F transcriptional activity is carried out by a heterodimer of one E2F protein and one DP protein (Bandara et al. 1993; Helin et al. 1993b; Krek et al. 1993). To date, there are eight

mammalian E2F genes (*E2f1* through *E2f8*) and two DP genes (*DP1* and *DP2*). The DP proteins can bind to E2F1 through E2F6 interchangeably with no differences between DP1- or DP2-containing E2F complexes. The DP moiety enhances DNA binding activity and the E2F subunit confers functional specificity. Structural and functional differences have led to the division of the E2Fs into three subgroups: the activating E2Fs (E2F1, E2F2, E2F3a), the repressive E2Fs (E2F3b, E2F4, E2F5), and the E2Fs that act as transcriptional repressors independently of pocket protein regulation (E2F6, E2F7, E2F8).

i. E2F1

The founding member of the E2F family of transcription factors is now termed E2F1. E2F1 was cloned as a result of its ability to interact with pRB (Helin et al. 1992; Kaelin et al. 1992; Shan et al. 1992). Structural analysis identified distinct domains for DNA binding, DP dimerization and transactivation (Figure 2). The DP dimerization domain consists of a hydrophobic heptad repeat (a putative leucine zipper) and a marked box motif. Both regions are important for DP dimerization and the marked box is also involved in DNA bending (Cress and Nevins 1996). Furthermore, the pRB binding domain lies within the acidic transactivation domain (Helin et al. 1992; Kaelin et al. 1992). Notably, the pRB binding region does not contain the conserved LxCxE motif that is found in other pRB-interacting proteins, such as the viral oncoproteins E1A, SV40 large T antigen and HPV E7. Functional studies revealed that E2F1 is a strong transcriptional activator and is solely regulated by one pocket protein, pRB. In addition, E2F1 expression is cell cycle regulated with induced expression during G1/S.

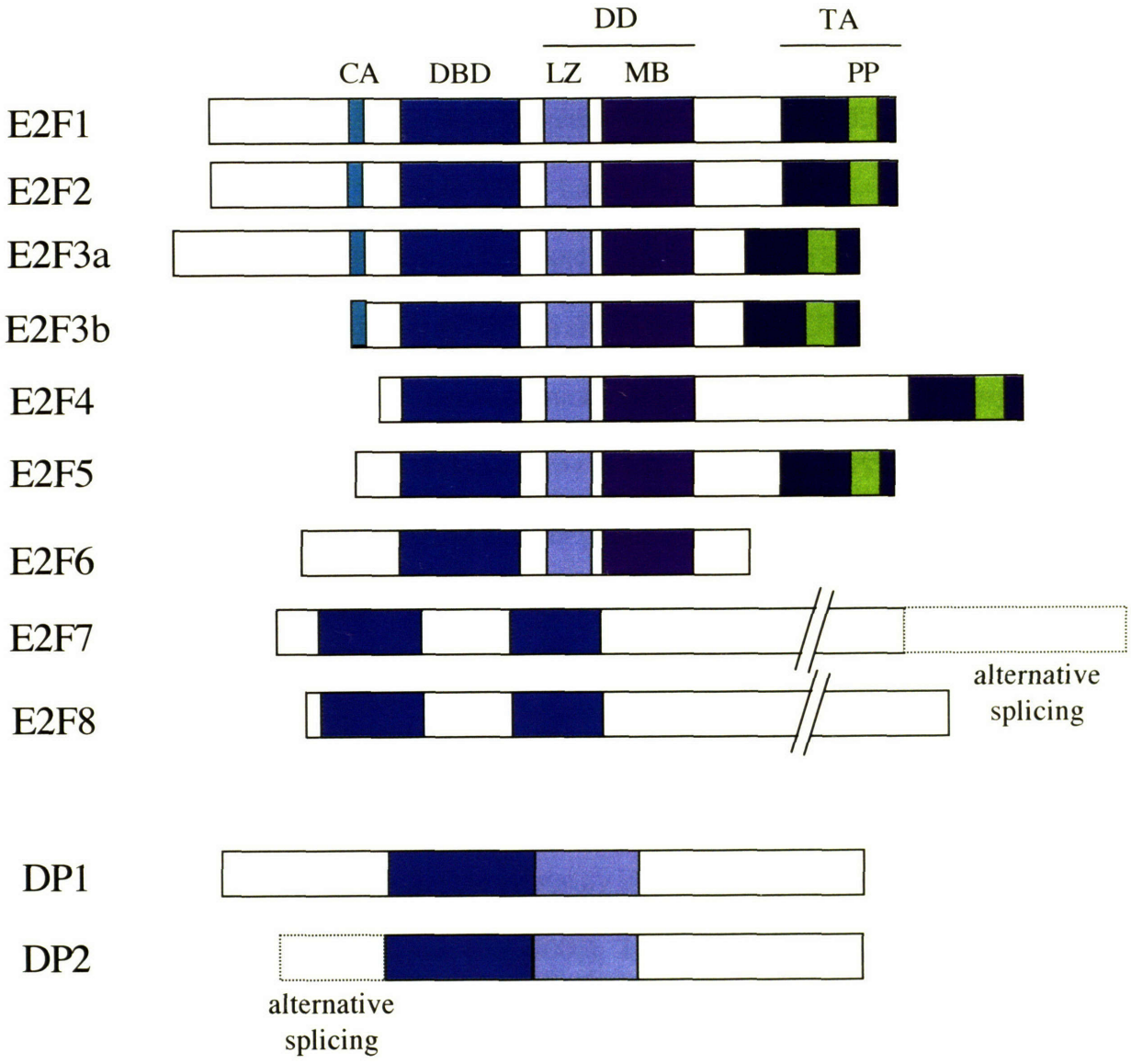

Figure 2. The E2F family of transcription factors.

The conserved domains of the E2F and DP proteins are indicated in the schematic diagram. The DNA binding domain (DBD) is conserved in all of the E2F and DP proteins. Proteins that function as heterodimers composed of an E2F protein (confers functional specificity) and a DP protein (enhances DNA binding) contain a dimerization domain (DD). DDs of E2F1 through E2F6 consist of a putative leucine zipper (LZ) and a marked box (MB) region. The transactivation domain (TA) and sequences for pocket protein binding (PP) are conserved in E2F1 through E2F5. The cyclin A/cdk binding domain (CA) is only present in E2F1, E2F2, E2F3a and E2F3b. Based on the homologous domains in the E2F proteins, they can be divided into three distinct groups: the activating E2Fs (E2F1, E2F2, E2F3a), the repressive E2Fs (E2F3b, E2F4, E2F5), and the E2Fs that are not regulated by the pocket proteins (E2F6, E2F7, E2F8).

ii. E2F2 and E2F3

Electrophoretic mobility shift assays suggested that E2F DNA binding activity in cellular extracts could not be entirely attributed to E2F1. Low stringency library screening with an E2F1 probe yielded two additional E2F species, E2F2 and E2F3 (Ivey-Hoyle et al. 1993; Lees et al. 1993). These proteins are very similar to E2F1 in structure (Figure 2), function and regulation. First, the DNA binding, dimerization, transactivation and pRB binding domains are highly conserved in E2F2 and E2F3 (45%-100% similarity to E2F1) (Lees et al. 1993). Second, E2F2 and E2F3 exclusively bind to pRB and not p107 or p130. Third, E2F2 and E2F3 are expressed in an equivalent cell cycle regulated pattern as E2F1. Fourth, E2F2 and E2F3 are strong transcriptional activators and act analogously to E2F1 in over-expression, reporter and DNA binding assays (Ivey-Hoyle et al. 1993; Lees et al. 1993; Lukas et al. 1996; DeGregori et al. 1997).

Recent findings have shown that the originally identified *E2f3* locus encodes two distinct gene products, E2F3a and E2F3b (Leone et al. 2000). E2F3a is the initially characterized E2F3 protein. E2F3b is transcribed from an intronic promoter that results in an alternative exon 1, termed exon 1b. At the protein level, the first 122 residues at the amino terminus of E2F3a are absent in E2F3b and replaced with six distinct amino acids. Yet, the remainder of the sequences, which include domains for cyclin A binding, DNA binding, dimerization, transactivation and pRB binding, are identical in the two proteins. Thus, like E2F3a, E2F3b is solely regulated by pRB. In contrast to E2F3a, E2F3b is expressed throughout the cell cycle. Moreover, there is evidence that instead of transcriptional activation E2F3b participates in transcriptional repression (Aslanian et al. 2004).

iii. E2F4 and E2F5

E2F1, E2F2 and E2F3, which only bind to pRB, did not account for all of the E2F DNA binding activity associated with the three pocket proteins (Shirodkar et al. 1992; Cobrinik et al. 1993). In the pursuit of isolating binding partners to p107 and p130 and/or additional E2F members, E2F4 and E2F5 emerged (Beijersbergen et al. 1994; Ginsberg et al. 1994; Hijmans et al. 1995; Sardet et al. 1995). In general, E2F4 and E2F5 had greater homology to each other (approximately 80% similarity) than to the other identified E2Fs (30-60%) (Figure 2). In comparison to E2F1, there was significant homology in the DNA binding and pRB binding domains. Crystallographic analysis of an E2F4-DP2-DNA complex reveals that the DNA binding domains for both E2F and DP proteins have a winged helix fold and use residues that are strictly conserved among all family members to contact DNA (Zheng et al. 1999). In contrast to E2F1, E2F4 and E2F5 are constitutively expressed throughout the cell cycle and are regulated by various pocket proteins. E2F4 binds to all of the pocket proteins and E2F5 primarily interacts with p130 *in vivo* (Beijersbergen et al. 1994; Ginsberg et al. 1994; Hijmans et al. 1995; Moberg et al. 1996). Although initial studies demonstrated that E2F4 is capable of activating transcription *in vitro*, ectopic expression of E2F4 or E2F5 did not effectively induce cell cycle progression in quiescent cells (Lukas et al. 1996; DeGregori et al. 1997). Thus, E2F4 and E2F5 have overlapping and distinct properties with the previously described E2Fs.

iv. E2F6

The conserved DNA binding and dimerization domains led to the isolation of E2F6 from a yeast two hybrid assay and EST database searching (Figure 2) (Cartwright et al. 1998; Gaubatz et al. 1998; Trimarchi et al. 1998). Like the other E2Fs, E2F6 requires DP dimerization for

efficient DNA binding activity. Consistent with the lack of a pRB binding domain, E2F6 is regulated independently of pocket protein binding. Yet, E2F6 has a role in E2F-dependent repression during the cell cycle. Ectopic expression of E2F6 results in an accumulation of S phase cells and E2F6 has been detected at E2F-responsive promoters during G0 and S phases (Cartwright et al. 1998; Ogawa et al. 2002; Giangrande et al. 2004). Despite such evidence for E2F-related function, E2F6 loss does not have significant effects on murine development or on cell cycle properties of MEFs (Storre et al., 2002; M. Courel and J. A. Lees, unpublished data). Instead, the *E2f6* mutant animals have a mild axial skeletal transformation, consistent with a role for E2F6 in the Polycomb complex (Storre et al. 2002). In further support of this, E2F6 associates with Polycomb Group proteins, which are primarily involved in transcriptional repression of the Hox genes during development (Trimarchi et al. 2001; Ogawa et al. 2002). In addition, E2F6 has been implicated in gene silencing at G0 in complex with histone methyltransferases and other sequence-specific DNA binding transcription factors (Ogawa et al. 2002). Thus, the precise nature of E2F6 function remains unclear and further studies are needed.

v. E2F7 and E2F8

The most divergent E2F members, E2F7 and E2F8, were identified most recently (de Bruin et al. 2003a; Di Stefano et al. 2003; Logan et al. 2004; Maiti et al. 2005). These two proteins are homologous in structure, expression and function (Figure 2). They contain two distinct conserved DNA binding domains, resembling the Arabidopsis E2F-like proteins. Furthermore, DP dimerization is not required for DNA binding and they function as transcriptional repressors independently of the pocket proteins. Their expression is cell cycle regulated and ectopic expression leads to diminished cellular proliferation, as well as repression

of some E2F-responsive genes. Further studies are necessary to determine the physiological role of these novel E2F family members.

vi. DP1 and DP2

DP1 (dimerization partner 1 or DRTF-P1) was isolated as a sequence-specific DNA binding protein in association with DRTF1 (or E2F1) in murine cells (Girling et al. 1993). Subsequently, the human DP1 ortholog was cloned and had 95% identity at the amino acid level to the mouse protein (Helin et al. 1993b). DP1 has significant structural similarities to E2F1 (70% similar in the DNA binding domains), but does not have a pRB binding domain (Figure 2) (Girling et al. 1993). Yet, DP1 is required for stable complex formation between pRB and E2F1 (Helin et al. 1993b). Although it was initially thought that E2F1 proteins homodimerized to bind to DNA (Huber et al. 1993), heterodimerization of E2F1 and DP1 leads to enhanced DNA binding *in vivo* (Helin et al. 1993b; Krek et al. 1993). DP2 was cloned by a variety of methods, such as yeast two hybrid, library screening and degenerate oligonucleotide PCR (Ormondroyd et al. 1995; Wu et al. 1995; Zhang and Chellappan 1995; Rogers et al. 1996). *Dp2* gene has 68% identity to *Dp1*. Although DP2 is expressed in most tissues, there is differential expression of the different isoforms of DP2, which result from alternative splicing. To date, there are no reported functional differences between DP1 and DP2 and they can bind to E2F1 to E2F6 indiscriminately.

It has been over a decade since the first E2F was cloned (Helin et al. 1992; Kaelin et al. 1992; Shan et al. 1992) and new family members are continuing to be discovered 13 years later (Maiti et al. 2005). It is clear that E2F activity is not restricted to the G1/S transition of the cell cycle, and E2F is involved in a number of cellular processes (discussed below). Thus, it is

important to determine the individual physiological contributions of each E2F protein in order to understand the intricacy of E2F function. Our understanding, however, may be incomplete in that there may remain yet unidentified E2Fs.

C. The activating E2Fs versus the repressive E2Fs

The structural differences in the E2F transcription factors are partially reflective of their differential activities in regulating cell division. As indicated by the presence or absence of a pRB binding domain, the pocket proteins regulate E2F1, E2F2, E2F3a, E2F3b, E2F4 and E2F5, but not E2F6, E2F7 or E2F8. The pocket protein regulated E2Fs can be further subdivided into two classes: the activating E2Fs and the repressive E2Fs. For the most part, these two groups have differential functions during the cell cycle. The remainder of Part II will focus on the properties of these two distinct groups in cell cycle control, development and tumorigenesis.

i. Structural and functional properties

E2F1, E2F2, and E2F3a comprise the activating E2Fs and are most similar to each other than to the other E2Fs in structure and function. These proteins have a high degree of structural similarity, as previously described. They are strong activators of transcription (Helin et al. 1992; Kaelin et al. 1992; Shan et al. 1992; Ivey-Hoyle et al. 1993; Lees et al. 1993) and induce quiescent cells to enter the cell cycle when individually over-expressed (Lukas et al. 1996; DeGregori et al. 1997). Accordingly, the absence of E2F3 in murine primary fibroblasts results in delayed proliferation (Humbert et al. 2000b). MEFs lacking E2F1, E2F2 and E2F3 are further impaired in cell division (Wu et al. 2001), supporting that the activating E2Fs have overlapping roles in promoting cellular proliferation.

The role of the activating E2Fs in the G1/S transition of the cell cycle depends upon their ability to activate the transcription of E2F-responsive genes that are necessary for S phase progression. Promoter occupancy experiments have confirmed that the activating E2Fs are associated with E2F-responsive genes coincident with their activation in late G1 and S phases (Takahashi et al. 2000; Rayman et al. 2002). Transcription of many of these genes is dependent on an intact E2F DNA consensus site within the promoter and a functional E2F transactivation domain (Lam and Watson 1993; Hsiao et al. 1994). Moreover, the reduced proliferative capacity of murine primary fibroblasts lacking E2F3 or all of the activating E2Fs was associated with deregulated expression of a number of classical E2F-responsive genes (Humbert et al. 2000b; Wu et al. 2001). Thus, the activating E2Fs are critical for the transcriptional activation of target genes that are necessary for S phase entry.

In contrast, E2F4 and E2F5, the repressive E2Fs, are important for transcriptional repression during G0 and early G1 phases of the cell cycle. Although these proteins can activate transcription in reporter assays, over-expression in Rat1 cells does not efficiently drive progression through the cell cycle (Lukas et al. 1996; DeGregori et al. 1997). In addition, *E2f4*$^{-/-}$ MEFs display normal cell cycle kinetics (Humbert et al. 2000a; Rempel et al. 2000). These results indicate that the repressive E2Fs do not necessarily function in an analogous manner to the activating E2Fs. Moreover, MEFs lacking both E2F4 and E2F5 are not able to arrest in G1 in response to $p16^{INK4a}$ expression (Gaubatz et al. 2000). Similar results are observed for *p107*$^{-/-}$*;p130*$^{-/-}$ cells, suggesting a role for the repressive E2Fs and their associated pocket proteins in maintaining growth arrest through negatively regulating E2F target genes (Hurford et al. 1997; Classon et al. 2000). This model has been reinforced by chromatin immunoprecipitation experiments demonstrating that E2F4-p130 and E2F4-p107 complexes recruit HDAC1 to E2F-

responsive promoters in G0/early G1 and are replaced by the activating E2Fs in late G1/S (Takahashi et al. 2000; Rayman et al. 2002). E2F3b remains relatively uncharacterized; however, initial studies suggest that it also acts as a transcriptional repressor, as demonstrated for the $p19^{ARF}$ gene (Aslanian et al. 2004). Further studies with targeted disruption of *E2f3b* will be helpful to determine the precise nature of E2F3b function *in vivo*.

Although there is strong evidence to support that the activating and repressive E2Fs have opposing functions in gene regulation, it is unclear whether both classes of E2Fs cooperate in the regulation of each E2F target gene. For example, mutation of E2F binding sites in the *B-myb* promoter results in increased transcription (Lam and Watson 1993). Promoter analysis by *in vivo* footprinting found that E2F was bound to this promoter in G0 and early G1, but not in late G1 (Zwicker et al. 1996). Even though a subsequent study detected the activating E2Fs at this promoter during the G1 to S transition (Takahashi et al. 2000), transcriptional activation of the *B-myb* gene does not necessarily require E2F binding (Lam and Watson 1993). This suggests that loss of E2F-pocket protein repressive complexes is sufficient to activate transcription. Extending this concept, it is then unclear if the proliferative defects in the absence of the activating E2Fs are due to the loss of activation or the gain of repression (by the repressive E2Fs) at E2F target genes. In support of the latter, expression of a transactivation-deficient E2F1 mutant, which can bind DNA, was equivalent to the expression of wild-type E2F1 in that both resulted in the derepression of target genes and cell cycle progression (Zhang et al. 1999a; Rowland et al. 2002). On the other hand, activating E2F activity is important for the transcription of certain E2F-responsive genes (e.g., *DHFR*) (Means et al. 1992) and this correlates with entry into S phase (Johnson et al. 1993; Shan and Lee 1994; Qin et al. 1995). Analysis of target gene expression in $E2f1^{-/-};E2F2^{-/-};E2f3^{-/-}$ MEFs indicated that some genes were

induced (e.g., *PCNA* and *Cyclin E1*) and others were repressed (e.g., *cdc6*, *DHFR* and *Mcm3*), suggesting that both activation and repression by the activating E2Fs are important (Wu et al. 2001). Therefore, regulation of E2F-responsive promoters by the activating and/or repressive E2Fs appears to be gene-specific and the overall contribution of the activating and repressive E2Fs to target gene expression may be important in regulating cell growth and division.

One way that the various E2F proteins achieve promoter specificity is through interactions with other transcription factors. TFE3, an E-box binding factor, specifically binds to E2F3a independently of DP to activate transcription of the DNA polymerase α p68 gene (Giangrande et al. 2003). Moreover, E2F2 and E2F3 can bind to RYBP (Ring1 YY1 binding protein), which allows for combinatorial control of promoters that contain E2F and YY1 binding sites (e.g., *cdc6*) (Schlisio et al. 2002). In addition, the activating E2Fs cooperate with NF-Y (nuclear factor Y) transcription factor to trigger transcription of *cdc2* while repression of *cdc2* by E2F4 depends on different promoter elements (Zhu et al. 2004). Thus, these are alternative mechanisms by which E2Fs can regulate transcriptional activity. Other factors that could affect target gene specificity of the E2Fs are pocket protein binding, cell type and species-specific differences.

ii. Regulation of E2F activity

The activity of the E2F transcription factors is tightly regulated by the coordinated action of a number of factors, including pocket protein binding, subcellular localization, post-translational modifications and ubiquitin-mediated proteolysis. The activating and repressive E2Fs have distinct properties with respect to these types of regulation that contribute to their differential functions *in vivo*.

a. Regulation by pocket protein binding

Since mechanisms of repression by the pocket proteins have been described in Part I, important differences between the activating and repressive E2Fs will be highlighted. The activating E2Fs are exclusively regulated by pRB *in vivo* while the repressive E2Fs interact with the entire pocket protein family. Specifically, E2F3b associates with pRB (Leone et al. 2000), E2F4 binds to all three pocket proteins (Beijersbergen et al. 1994; Ginsberg et al. 1994; Moberg et al. 1996) and E2F5 is primarily regulated by p130 (Hijmans et al. 1995; Sardet et al. 1995; Moberg et al. 1996). As previously mentioned, pRB can repress E2F activity via two distinct mechanisms, 'passive' and 'active' repression. Briefly, passive repression occurs when the transactivation domain of E2F is physically masked upon pRB binding, thereby preventing transcriptional activation of target genes. In contrast, the pocket proteins can recruit chromatin remodeling enzymes to promoters in complex with E2F, resulting in active repression. While it is believed that the activating E2Fs are primarily regulated by passive repression, this has not been formally demonstrated *in vivo* since pRB is rarely detected at promoters (Takahashi et al. 2000; Wells et al. 2000). Active repression is widely thought to be a property of the repressive E2Fs, since E2F4 and E2F5 are solely found in the nucleus in association with a pocket protein (discussed below) (Muller et al. 1997; Verona et al. 1997). Furthermore, promoter occupancy experiments have detected E2F4 with p107, p130 and HDAC1 at E2F-responsive promoters coincident with transcriptional repression of the corresponding genes (Rayman et al. 2002). Thus, the current models suggest that the pocket proteins differentially regulate the activating E2Fs and the repressive E2Fs.

b. Regulation by subcellular localization

The activating and repressive E2Fs can be distinguished based on their subcellular localization patterns. E2F1, E2F2 and E2F3a, the activating E2Fs, contain a conserved nuclear localization signal (NLS) in the amino terminal regions and are, therefore, exclusively found in the nucleus (Muller et al. 1997; Verona et al. 1997). On the other hand, E2F4 and E2F5, the repressive E2Fs, lack the amino terminal sequences that encode a NLS (Beijersbergen et al. 1994; Ginsberg et al. 1994; Hijmans et al. 1995; Sardet et al. 1995; Leone et al. 2000). Most of the work detailing the subcellular localization of the repressive E2Fs has been done with E2F4, the predominant E2F *in vivo* (Moberg et al. 1996). In G0 and early G1 phases, E2F4 is primarily found in the nucleus in association with p130 at E2F-responsive promoters (Muller et al. 1997; Verona et al. 1997; Takahashi et al. 2000; Wells et al. 2000; Rayman et al. 2002). Upon entry into S phase, E2F4-p107 complexes replace the nuclear E2F4-p130 complexes and the amount of free E2F4 increases in the cytoplasm as cells reach S phase (Muller et al. 1997; Verona et al. 1997). Consistent with the absence of an NLS, E2F4 is found in the nucleus only when bound to a pocket protein. Upon phosphorylation of the pocket protein, E2F4, which contains two leucine/isoleucine-rich nuclear export signals, is subsequently exported from the nucleus through a mechanism dependent on the CRM1 nuclear export receptor (Gaubatz et al. 2001).

The subcellular localization of the activating and repressive E2Fs largely supports their distinct functions during the cell cycle. The expression pattern and nuclear localization of the activating E2Fs parallel their transcriptional activity at the G1 to S transition of the cell cycle (Magae et al. 1996; Muller et al. 1997; Verona et al. 1997). In contrast, E2F4 is found in the nucleus in association with the pocket proteins during G0/G1, which are times when classic E2F-responsive genes are repressed. Although the subcellular expression patterns of the other

repressive E2Fs remain to be determined, it is likely that E2F5 will be analogous to E2F4 since they share many structural and functional similarities.

c. Regulation by phosphorylation

Phosphorylation of the E2F proteins regulates E2F activity during the cell cycle. The activating E2Fs and E2F3b contain a conserved cyclin A binding domain in the amino terminal region (Figure 2) (Mudryj et al. 1991; Devoto et al. 1992; Pagano et al. 1992; Krek et al. 1994; Xu et al. 1994; Leone et al. 2000). This region specifically binds to cyclin A/CDK2 and cyclin A/CDC2 complexes. Stable complex formation results in phosphorylation of the DP subunit and loss of E2F DNA binding and transactivation activities in S phase (Dynlacht et al. 1994; Xu et al. 1994; Krek et al. 1995). Mutation of the cyclin A binding domain or introduction of a non-phosphorylatable DP leads to the accumulation of cells in S phase and subsequent apoptosis (Krek et al. 1995), suggesting that phosphorylation of E2F-DP is required for cell cycle progression through S phase. Yet, the exact role of DP phosphorylation remains unclear, since a mutant DP lacking potential phosphorylation sites was only able to arrest cells in the presence of ectopic E2F1 expression. Along the same lines, DP phosphorylation in serum deprived cells corresponded with increased E2F binding (Bandara et al. 1994). There are additional studies which demonstrated that E2F1 can be phosphorylated by cyclin A/CDK complexes *in vitro* (Peeper et al. 1995; Adams and Kaelin 1996). Thus, phosphorylation seems to be an important mode of E2F regulation and further work is necessary to elucidate the precise physiological role of E2F and DP phosphorylation.

Although E2F4 and E2F5 lack the cyclin A binding domain, they associate with cyclin/CDK complexes via p107 and p130 and are phosphorylated (Devoto et al. 1992; Faha et

al. 1992; Lees et al. 1992; Shirodkar et al. 1992; Ginsberg et al. 1994). E2F-p107 complexes are found in association with cyclin E/CDK2 in G1 while E2F-p107-cyclin A/CDK2 complexes are predominantly found in S phase (Lees et al. 1992). The functional significance of these complexes is currently unknown. A few studies indicate that phosphorylation is important in transcriptional activation by the repressive E2Fs. For example, phosphorylation of E2F5 by cyclin E/CDK2 in late G1 leads to transcriptional activation and progression through S phase (Morris et al. 2000). Furthermore, dephosphorylation of E2F4/DP1 complexes in human B lymphocytes correlates with reduced DNA binding activity in S phase (van der Sman et al. 1999). While these data suggest that the repressive E2Fs function as transcriptional activators, much of the work entailed over-expression and *in vitro* techniques. Thus, additional studies are necessary to determine the biological significance of phosphorylation of the repressive E2Fs.

d. Regulation by acetylation

Transcriptional activation by the E2Fs has been associated with histone acetyl transferase (HAT) activity. Acetylation of histone tails is widely thought to facilitate transcriptional activation by weakening the interaction between histones and chromatin and, thus, enabling transcription factor binding to DNA. p300 and CREB-binding protein (CBP) form a complex with HAT activity (reviewed in Vo and Goodman 2001). CBP and p300, as well as an associated factor P/CAF, can bind to the activating E2Fs to stimulate their transcriptional activity (Trouche et al. 1996; Martinez-Balbas et al. 2000; Marzio et al. 2000). P/CAF acteylates E2F1 near the DNA binding domain on three lysine residues, which are conserved in E2F2 and E2F3 (Martinez-Balbas et al. 2000; Pediconi et al. 2003). Another HAT, Tip60, associates with ectopically expressed E2F1 at E2F-responsive promoters (Taubert et al. 2004). Thus, acetylation

may be an important mechanism for controlling the transcriptional activity of the activating E2Fs.

It is unclear if acetylation also regulates the activity of the repressive E2Fs. In reporter assays, E2F4 and E2F1 can promote transcriptional activation upon binding to GCN5, a HAT (Lang et al. 2001). Furthermore, E2F5 has been found in association with p300 (Morris et al., 2000). Although these studies demonstrate direct binding, the physiological relevance of these interactions remains to be understood. In contrast, endogenous association of E2F4 and HDAC1 correlates with transcriptional repression in G0 and early G1 (Takahashi et al. 2000; Rayman et al. 2002). Perhaps E2F4 participates in transcriptional activation and repression during different phases of the cell cycle. Further work is necessary to determine whether the repressive E2Fs cooperate with HATs *in vivo* and at which promoters this interaction occurs.

iii. E2F target genes not involved in G1/S regulation

Classic E2F-responsive genes were initially identified by searching for E2F binding sites in promoters of genes with known roles in the G1 to S transition. This type of analysis, while fruitful in understanding the mechanism of E2F activity to promote cell cycle progression, is incomplete. More recently, expression profiling by microarray analysis has been used to identify additional E2F targets genes (Muller et al. 2001). E2F1, E2F2 and E2F3 expression was found to induce the expression of previously identified E2F-responsive genes as well as previously unidentified genes involved in additional pathways, including apoptosis and mitosis. In order to determine direct targets, chromatin immunoprecipitation coupled with microarray analysis has been used (Ren et al. 2002; Weinmann et al. 2002). These studies identified additional novel targets involved in numerous biological processes, such as DNA damage checkpoint and DNA

repair, mitotic spindle checkpoint and chromatin assembly. Thus, E2F functions at many stages of the cell cycle and may coordinate proper cell cycle regulation with other cellular processes.

a. Apoptosis

E2F has a well-established role in apoptosis; however, it is unclear whether this is a property of all the E2Fs or particular ones. Ectopic expression of E2F1 induces apoptosis, in addition to cellular proliferation (Qin et al. 1994; Shan and Lee 1994; Wu and Levine 1994; Kowalik et al. 1995; Vigo et al. 1999). Some studies have demonstrated that this is a specific property of E2F1 (DeGregori et al. 1997; Kowalik et al. 1998; Lissy et al. 2000). In addition, the loss of E2F1 in murine thymocytes results in defects in apoptosis (Field et al. 1996). Furthermore, E2F1 deficiency prevents apoptosis induced by c-Myc expression in MEFs (Leone et al. 2001). Other studies have argued that all of the activating E2Fs promote apoptosis (Vigo et al. 1999; Moroni et al. 2001). Ectopic expression of E2F1, E2F2 or E2F3 can induce apoptosis, though E2F2 and E2F3 were relatively less efficient.

There is also *in vivo* data to support that E2F1 and E2F3 contribute to programmed cell death. As discussed in Part I, *Rb* loss in mice leads to increased levels of apoptosis in the nervous system and the ocular lens (Clarke et al. 1992; Jacks et al. 1992; Lee et al. 1992; Morgenbesser et al. 1994). Despite findings that the apoptosis in the nervous system is a cell non-autonomous effect of *Rb* loss, apoptosis in the lens is a cell intrinsic property (de Bruin et al. 2003b). Loss of either E2F1 or E2F3 in $Rb^{-/-}$ animals results in suppression of the apoptosis in the lens, suggesting that both E2F1 and E2F3 can regulate cell survival *in vivo* (Tsai et al. 1998; Ziebold et al. 2001).

The mechanisms underlying E2F-induced apoptosis are incompletely understood. Most studies focus on E2F1 action in apoptosis since it has an irrefutable role in this process. Ectopic expression of E2F1 can induce p53-dependent (Qin et al. 1994; Wu and Levine 1994; Hiebert et al. 1995) and p53-indendendent apoptosis (Hsieh et al. 1997; Phillips et al. 1997). $p14^{ARF}$, an upstream regulator of p53, was suggested to be an E2F1 target gene and provided a potential link for the p53-dependent apoptosis (Bates et al. 1998). The disruption of $p19^{ARF}$ (murine homolog of human $p14^{ARF}$), however, does not modulate the levels of p53-dependent apoptosis in the absence of pRB (Tsai et al. 2002) or in the presence of increased E2F1 activity (Tolbert et al. 2002). Furthermore, recent work suggests that $p19^{ARF}$ is primarily regulated by E2F3b, not E2F1 (Aslanian et al. 2004). Other apoptotic genes directly regulated by E2F1 are *p73* (Lissy et al. 2000), *Apaf-1* (Moroni et al. 2001; Muller et al. 2001), *Caspase 3* (Muller et al. 2001; Nahle et al. 2002), as well as *Caspase7* (Muller et al. 2001). Although transcriptional activation of these apoptotic E2F target genes has been shown to be important for E2F1 function in apoptosis, the transactivation domain of E2F1 is dispensable for apoptosis induced in Saos-2 cells (Hsieh et al. 1997; Phillips et al. 1997) and WI-38 cells (Moroni et al. 2001). Therefore, the apoptotic response induced by E2F1 is complex and E2F1 may not function through a common mechanism in different settings, such as cell type.

b. DNA damage and repair

There is an emerging role of the E2Fs in DNA damage checkpoint control and DNA repair pathways. In response to DNA damage, E2F1 levels are stabilized (Blattner et al. 1999; Hofferer et al. 1999; Meng et al. 1999) upon phosphorylation by ATM, a kinase activated by DNA damage (Lin et al. 2001). It has been proposed that phosphorylation prevents E2F1

degradation by $p45^{SKP2}$ since the ATM phosphorylation site on E2F1 overlaps with the $p45^{SKP2}$ binding site (Marti et al. 1999; Lin et al. 2001). Furthermore, E2F1 interacts with Nbs1 at origins of DNA replication, which is thought to recruit the Mre11 complex, another ATM substrate (Maser et al. 2001). Expression profiling of human cells by microarray analyses have identified several direct E2F target genes involved in DNA damage checkpoint (*CHK1* and *TP53*) and DNA repair (*MSH2*, *MLH1*, *RAD51*, *RAD54*, *BARD1*) (Ren et al. 2002; Weinmann et al. 2002). A number of these targets were specific for E2F4 (Ren et al. 2002), suggesting that involvement in DNA damage checkpoint control and DNA repair may not be specific to E2F1. There is evidence demonstrating that DNA checkpoint and repair genes also have functions during the normal cell cycle. For example, CHK1 accumulates in S phase to guarantee the completion of DNA replication prior to mitosis (Bartek and Lukas 2003). Thus, E2F regulation of these genes may be a result of their roles in DNA synthesis rather than particular involvement in the DNA damage response.

iv. Roles in development and differentiation

The physiological roles of the individual E2Fs have been probed by analyzing the consequences of targeted gene disruption in mice. These studies have addressed whether the different E2Fs have overlapping functions or separate tissue-specific roles. Consistent with *in vitro* data, the activating and repressive E2Fs have distinct roles during development and differentiation.

a. The activating E2Fs

E2f1$^{-/-}$ (Field et al. 1996; Yamasaki et al. 1996) and *E2f2*$^{-/-}$ (Murga et al. 2001) mice survive to adulthood and display tissue-specific defects. *E2f2*$^{-/-}$ animals often develop

autoimmunity-related defects in adulthood, including splenomegaly and glomerulonephritis (Murga et al. 2001). *E2f1*$^{-/-}$ mice exhibit exocrine gland dysplasia and have an increased predisposition to develop tumors (Field et al. 1996; Yamasaki et al. 1996). Tumor development in these animals was unexpected since loss of an activator was predicted to result in decreased proliferation. The growth inhibitory properties of E2F1 result from its function in apoptosis. In addition, *E2f1*$^{-/-}$ thymocytes exhibit increased proliferation due to apoptotic defects during negative selection (Field et al. 1996; Zhu et al. 1999; Garcia et al. 2000). Furthermore, transient expression of E2F1 in the testes results in apoptosis in transgenic mice (Agger et al. 2005). These data support that E2F1 and E2F2 are not essential for development and that E2F1 has dual roles in cell division and apoptosis *in vivo*.

In contrast, E2F3 (E2F3a and E2F3b) is essential for embryonic development on a pure 129/Sv or C57Bl/6 background (Humbert et al. 2000b; Cloud et al. 2002; Saavedra et al. 2002). These animals die between E13.5 and postnatal day 1 and have reduced cardiomyocyte proliferation (Cloud et al., 2002; J. Cloud and J. A. Lees, unpublished data). Yet, a small proportion of *E2f3*$^{-/-}$ animals can survive to adulthood in a mixed genetic (129/Sv x C57Bl/6) background. The mice that endure have hypoplastic hearts and/or septal defects leading to congestive heart failure in many cases (J. Cloud and J. A. Lees, unpublished data). Furthermore, the surviving animals are not prone to develop tumors, as observed for E2F1 loss. Thus, the activating E2Fs have tissue-specific functions during murine development.

The mild phenotypes associated with loss of an activating E2F are largely due to functional compensation by the remaining activators. *E2f1*$^{-/-}$;*E2f3*$^{-/-}$ and *E2f2*$^{-/-}$;*E2f3*$^{-/-}$ animals die between E9.5 and E10.5 with exacerbated effects in some of the phenotypes described for the single homozygous mutant animals (Wu et al. 2001; Cloud et al. 2002). In addition, *E2f1*$^{-/-}$;*E2f2*

$^{-/-}$ T cells have enhanced proliferation relative to the *E2f2*$^{-/-}$ cells, which hyperproliferate in response to T cell receptor activation (Zhu et al. 2001). Therefore, during murine development, the activating E2Fs have redundant functions in some tissues and unique biological functions in other tissues.

Dp1 mutant mice also indicate that E2F activity is essential for murine development. Since DP binding is required for E2F activity, loss of DP1 would effectively diminish the activity of E2F1 through E2F6. *Dp1*$^{-/-}$ mice die *in utero* by E12.5 due to defects in the extra-embryonic tissues (Kohn et al. 2003). Placental insufficiency in the absence of DP1 was due to impaired proliferation of trophoblast precursors in the ectoplacental cone and defective endoreduplication in the trophoblast giant cells, consistent with the critical role of E2F activity in regulating DNA synthesis. Analysis of *Dp1*$^{+/+}$;*Dp1*$^{-/-}$ chimeras demonstrated that DP1 is not required for the development of embryonic tissues from E10 to E17.5 (Kohn et al. 2004). Based on the tissue-specific defects arising in the adult *E2f* mutant animals, it will be important to establish whether these chimeric animals survive beyond birth and whether they have any developmental defects at later times. Furthermore, in order to correlate DP1 loss with a reduction in E2F activity, the relative levels of E2F DNA binding activity should be determined as well as whether there is functional compensation by DP2.

b. The repressive E2Fs

Consistent with the *in vitro* data, the repressive E2Fs are dispensable for cellular proliferation, but have important roles in differentiation during murine development. *E2f4*$^{-/-}$ mice are viable, but display decreased survival potential due to craniofacial defects that increase susceptibility to bacterial infections (Humbert et al. 2000a). In addition, these animals have

differentiation defects during intestinal development and hematopoiesis (Humbert et al. 2000a; Rempel et al. 2000). More specifically, absence of E2F4 resulted in a failure in crypt formation in the intestinal epithelium (Rempel et al. 2000) and cell autonomous defects during the end stages of red blood cell maturation (Humbert et al. 2000a; Rempel et al. 2000). E2F4 has also been implicated to have roles in adipogenesis (Fajas et al. 2002; Landsberg et al. 2003) and neurogenesis *in vitro* (Persengiev et al. 1999). Ectopic expression of E2F4 enhanced neuronal differentiation in PC12 cells (a pheochromocytoma cell line) and was important for maintaining the differentiated state (Persengiev et al. 1999). In contrast, E2F4 loss predisposed MEFs to differentiate into adipocytes spontaneously as well as in response to hormone induction (Fajas et al. 2002; Landsberg et al. 2003). Adipogenesis entails a period of clonal expansion immediately preceding terminal differentiation, which does not occur during neuronal development. Thus, this difference may reflect the contrasting effects of E2F4 function in the two culture systems.

E2f5$^{-/-}$ mice also have a differentiation, not proliferation, defect (Lindeman et al. 1998). These animals are born at the expected frequency and, on average, died by 6 weeks of age due to hydrocephalus, which resulted from excessive cerebrospinal fluid production in the choroid plexus. The tissue-specific defects associated with either E2F4- or E2F5 loss correspond with their disparate expression patterns in mouse development (Dagnino et al. 1997). Yet, there is some functional compensation between E2F4 and E2F5. The characterization of the double nullizygous animals has not been reported; however, viable mice are not detected at birth (Gaubatz et al. 2000). These mouse model studies confirm that the activating E2Fs and repressive E2Fs regulate different processes during murine development.

v. Roles in tumorigenesis

The activating E2F transcription factors have a well-established role in the regulation of cellular proliferation. As a result, understanding the contribution of the activating E2Fs to tumorigenesis has been a major focus in the field.

Deregulation of E2F1 activity can have oncogenic consequences. Ectopic expression of E2F1 can induce cellular transformation in cooperation with activated *Ras* expression, as assessed by the ability for form tumors in immunocompromised mice (Johnson et al. 1994). Expression of *E2f1* under the *keratin 5* promoter leads to skin tumors in mice (Pierce et al. 1999). In skin tumors induced by chemical carcinogenesis, however, E2F1 expression resulted in an increase in apoptosis and consequently a reduction in tumor size (Pierce et al., 1999). Thus, the net effect of E2F1 functions in proliferation and apoptosis largely dictates its oncogenic properties.

Since the E2F transcription factors are important downstream effectors of the pocket proteins, the requirement for E2F1 activity in tumor promotion has been assessed in tumor mouse models resulting from pocket protein inactivation. SV40 large T antigen inactivates the pocket proteins and p53. A truncation mutant containing the first 121 amino acids of the amino terminus of SV40 large T antigen (T_{121}) specifically targets the pocket proteins, and not p53 (DeCaprio et al. 1989; Dyson et al. 1989; Ewen et al. 1989). Tumor development results from expression of T_{121} in a variety of murine tissues, including the choroid plexus (Saenz Robles et al. 1994), astrocytes (Xiao et al. 2002) and the mammary epithelium (Simin et al. 2004). Although loss of E2F1 did not affect the lifespan of the transgenic mice expressing T_{121} in the choroid plexus, the balance of proliferation and apoptosis was altered in the resulting tumors (Pan et al. 1998). In particular, there was a significant decrease in the levels of apoptosis

accompanied by a decrease in the levels of proliferating cells in the *E2f1*$^{-/-}$;T_{121} tumors compared to those from the T_{121} animals. Thus, these opposing effects did not drastically alter the timing of tumor development. Regardless of the final outcome, it is clear that deregulated E2F1 activity in the absence of the pocket proteins contributes to tumor development through its apoptotic and proliferative roles.

The contribution of the activating E2Fs (E2F1 and E2F3) has also been investigated in tumors arising in *Rb*$^{+/-}$ mice. *Rb*$^{+/-}$ mice die around one year of age due to pituitary tumor development (Jacks et al. 1992; Hu et al. 1994; Williams et al. 1994b). In addition, the animals form thyroid tumors at approximately 70% penetrance. This model is analogous to the T_{121} mouse model since they both result in deregulated E2F activity. Loss of an activating E2F, E2F1 (Yamasaki et al. 1998) or E2F3 (see Appendix C), in the *Rb*$^{+/-}$background results in an extension of lifespan due to a delay in pituitary tumor formation. With regard to thyroid tumor development, loss of E2F1 results in significant suppression while loss of E2F3 increases thyroid tumorigenicity and frequently leads to metastasis. The differential effects in the thyroid may be due to tissue-specific functions of E2F1 and E2F3. Another possibility is that the opposing effects in the pituitary and thyroid tissues in *Rb*$^{+/-}$;*E2f3*$^{-/-}$ animals are reflective of the differential functions of E2F3a and E2F3b,which are both inactivated in *E2f3*$^{-/-}$ mice. In any case, the tumor suppressive properties of pRB are largely dependent upon its ability to regulate the activating E2Fs.

The roles of the activating E2Fs in the phenotypes caused by homozygous loss of *Rb* have also been determined. *Rb*$^{-/-}$ mice die in mid-gestation by E15.5 and exhibit defective erythropoiesis in the fetal liver and widespread inappropriate proliferation and apoptosis in the ocular lens and the nervous system (Clarke et al. 1992; Jacks et al. 1992; Lee et al. 1992). Loss

of an activating E2F, E2F1 or E2F3, led to prolonged survival of the $Rb^{-/-}$ mice to E17 (Tsai et al. 1998; Ziebold et al. 2001). Moreover, the ectopic proliferation and apoptosis were significantly suppressed in the *Rb*-deficient animals with concomitant absence of E2F1, E2F2, or E2F3, though ablation of E2F2 did not rescue as efficiently as the other activating E2Fs (Tsai et al. 1998; Ziebold et al. 2001; Saavedra et al. 2002). Recently, it has been demonstrated that embryonic lethality in the $Rb^{-/-}$ animals is due to a primary placental defect (Wu et al. 2003). In fact, the defect in the extra-embryonic tissues is responsible for the apoptosis in the nervous system and for a significant portion of the erythropoietic defect in the *Rb* mutants (de Bruin et al. 2003b). Notably, the ectopic proliferation is a cell autonomous effect of pRB, as is the apoptosis in the lens. In light of these results, the roles of the activating E2Fs in the placenta need to be examined. It is entirely possible that loss of E2F1 or E2F3 suppressed the placental defect in the $Rb^{-/-}$ mice and, thus, resulted in the observed extension in lifespan. In spite of this, it is clear that E2F1 and E2F3 participate in apoptosis in the lens and in the inappropriate proliferation that arise from pRB loss (Tsai et al. 1998; Ziebold et al. 2001).

Mutant mouse models have significantly extended our understanding of the molecular signals involved in tumorigenesis. With respect to the mechanism through which pRB loss promotes inappropriate proliferation, E2F1 and E2F3, for the most part, have overlapping functions in promoting the phenotypes caused by pRB deficiency. The repressive E2Fs are also downstream effectors of pRB function. Yet, the biological significance of these interactions remains unclear. Therefore, this study is aimed at determining the contribution of E2F4, the major repressive E2F *in vivo*, to the growth suppressive functions of pRB in the contexts of tumor formation (Chapter 2) and normal development (Chapter 3).

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Chapter Two

E2F4 loss suppresses tumorigenesis in *Rb* mutant mice

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The author contributed Table 1 and Figures 1, 6A, B, D. Hieu Cam and Joseph B. Rayman, from Brian D. Dynlacht's laboratory, performed the molecular analyses in Figure 2-5 and 6C with all samples provided by the author. Ulrike Ziebold performed the initial mouse crosses. The author and Jacqueline A. Lees contributed Figure 7. All mouse work and cell culture experiments were done in the laboratory of Jacqueline A. Lees.

ABSTRACT

The E2F transcription factors mediate the activation or repression of key cell cycle regulatory genes under the control of the retinoblastoma protein (pRB) tumor suppressor and its relatives, p107 and p130. Here we investigate how E2F4, the major 'repressive' E2F, contributes to pRB's tumor suppressive properties. Remarkably, E2F4 loss suppresses the development of both pituitary and thyroid tumors in $Rb^{+/-}$ mice. Importantly, E2F4 loss also suppresses the inappropriate gene expression and proliferation of pRB-deficient cells. Biochemical analyses suggest that this tumor suppression occurs via a novel mechanism: E2F4 loss allows p107 and p130 to regulate the pRB-specific, activator E2Fs. We also detect these novel E2F complexes in pRB-deficient cells suggesting that they play a significant role in the regulation of tumorigenesis *in vivo*.

SIGNIFICANCE

Understanding how the E2F and pRB family members contribute to the regulation of tumorigenesis is a key goal. Our finding of tumor suppression in the $Rb^{+/-};E2f4^{-/-}$ mice through the formation of novel E2F complexes in $Rb^{+/-};E2f4^{-/-}$, $Rb^{-/-};E2f4^{-/-}$, and $Rb^{-/-}$ cells strongly suggests tumor formation is critically and exclusively dependent upon the inactivation of pRB, rather than p107 or p130, because it triggers the release of the normally pRB-specific, activator E2Fs. However, p107 and p130 assume significant tumor suppressive properties in pRB-deficient cells because they can substitute for pRB in the regulation of these activator E2Fs. This model suggests a novel strategy for the generation of chemotherapeutics that would act by increasing the available pools of p107 and p130.

INTRODUCTION

The retinoblastoma protein (pRB) was the first identified tumor suppressor, and it is mutated in approximately one third of all human tumors. pRB blocks cells in G1 by inhibiting the activity of a cellular transcription factor, E2F, that controls the expression of key components of the cell cycle and DNA replication machinery (reviewed in Dyson 1998; Trimarchi and Lees 2002). pRB regulates E2F through two distinct mechanisms. First, its association is sufficient to block E2F transcriptional activity. Second, the pRB-E2F complex can recruit histone deacetylases (HDACs) to the promoters of E2F-responsive genes and thereby actively repress their transcription. Cell cycle entry requires the phosphorylation of pRB and its subsequent dissociation from E2F. This phosphorylation is mediated by cell cycle dependent kinase complexes, cyclin D-CDK4/6 and cyclin E-CDK2. Importantly, tumors that retain wild-type pRB almost always carry activating mutations in *cyclin D1* or *CDK4* or inactivating mutations in the cdk4-inhibitor, *p16* (reviewed by Bartek et al. 1996; Sherr 1996). This suggests that the functional inactivation of pRB, and the resulting de-regulation of E2F, is an essential step in tumorigenesis.

pRB belongs to a family of proteins, called the pocket proteins, that also includes p107 and p130 (reviewed by Dyson 1998; Trimarchi and Lees 2002). p107 and p130 share many properties with pRB: they bind to E2F *in vivo*, inhibit E2F transcriptional activity and recruit HDACs to mediate the active repression of E2F-responsive genes. However, there are dramatic differences in the tumor suppressive properties of the individual pocket proteins (reviewed by Mulligan and Jacks 1998). Inheritance of a single *Rb* mutant allele predisposes both mice and humans to tumors with 100% penetrance. The tumors consistently lose the wild-type *Rb* allele,

confirming that pRB behaves as a classical tumor suppressor. In contrast, the loss of p107 and/or p130 does not appear to promote tumorigenicity in mice or cells (Cobrinik et al. 1996; Lee et al. 1996). Yet there is growing evidence that mutation of *p107* and/or *p130* promotes tumor formation when pRB is also inactivated. This is exemplified by $Rb^{-/-};p107^{-/-}$ chimeric mice, which develop an additional tumor type, retinoblastoma, compared to $Rb^{-/-}$ chimeras (Robanus-Maandag et al. 1998), and $Rb^{-/-};p107^{-/-};p130^{-/-}$ mouse embryonic fibroblasts (MEFs), which are more tumorigenic than $Rb^{-/-}$ controls (Dannenberg et al. 2000; Sage et al. 2000). Biochemical and mechanistic studies in cells deficient for different pocket protein family members should help to identify the critical, tumor suppressive function(s) of pRB.

To date, eight genes have been identified as components of the E2F transcriptional activity (reviewed by Dyson 1998; Helin 1998). These genes have been divided into two distinct groups: the *E2fs* (*E2f1* through *E2f6*); and the *DPs* (*DP1* and *DP2*). The protein products from these two groups heterodimerize to give rise to functional E2F activity (Bandara et al. 1993; Helin et al. 1993; Krek et al. 1993). The functional specificity of the E2F-DP complex is primarily determined by the identity of the E2F subunit. The pocket protein-binding E2Fs can be divided into two subgroups that appear to have opposing roles *in vivo* (reviewed by Trimarchi and Lees 2002).

The first E2F subgroup includes E2F1, 2 and 3. These E2Fs play a key role in promoting the activation of E2F-responsive genes, and thereby cell cycle entry. Chromatin immunoprecipitation (ChIP) experiments confirm that these E2Fs associate with the promoters of known target genes coincident with their activation in late G_1 (Takahashi et al. 2000; Rayman et al. 2002). MEFs lacking E2F3 or E2F1, E2F2, and E2F3 exhibit reduced E2F target gene

expression and significant proliferative defects (Humbert et al. 2000b; Wu et al. 2001). Furthermore, the ectopic expression of E2F1, 2 or 3 is sufficient to induce quiescent cells to initiate E2F-responsive gene expression and cell cycle re-entry (Lukas et al. 1996; DeGregori et al. 1997). Importantly, these so-called 'activator' E2Fs are specifically regulated by pRB but not by p107 or p130 *in vivo* (Moberg et al. 1996).

E2F4 and E2F5 represent the second E2F subgroup. The transcriptional properties of these E2Fs are largely determined by their subcellular localization (Magae et al. 1996; Muller et al. 1997; Verona et al. 1997; Gaubatz et al. 2001). The endogenous E2F4-DP and E2F5-DP complexes are localized in the cytoplasm and are therefore unable to contribute to the activation of E2F-responsive genes. However, pocket protein binding enables the nuclear localization of E2F4 and E2F5. As a result, E2F4 and E2F5 appear to be primarily involved in the active repression of E2F-responsive genes. E2F4 associates with pRB, p107 and p130 *in vivo* and accounts for the majority of the repressive-pocket protein complexes (Moberg et al. 1996). E2F5 is expressed in G_0 cells and is primarily regulated by p130 (Hijmans et al. 1995; Sardet et al. 1995). ChIP assays confirm that E2F4, p107, p130, and HDAC specifically associate with E2F-responsive promoters in G_0/G_1 cells under physiological conditions (Takahashi et al. 2000; Rayman et al. 2002). Importantly, MEFs deficient for E2F4 and E2F5 are unable to arrest in G1 in response to a variety of growth arrest signals, suggesting that the repressive E2Fs promote cell cycle arrest (Gaubatz et al. 2000).

Considerable attention has focused on understanding how the growth suppressive properties of pRB relate to its role in the inhibition of the activating E2Fs versus its participation in repressive E2F-pRB complexes. The analysis of *Rb;E2f1* and *Rb;E2f3* compound mutant mice

has shown that the absence of E2F1 or E2F3 is sufficient to suppress both the ectopic S-phase entry and p53-dependent apoptosis arising in pRB-deficient embryos (Tsai et al. 1998; Ziebold et al. 2001). Moreover, E2F1 deficiency significantly diminishes the development of tumors in $Rb^{+/-}$ mice (Yamasaki et al. 1998). These data suggest that the inappropriate release of the activator E2Fs makes a significant contribution to the phenotypic consequences of pRB deficiency. However, these experiments do not rule out a role for the repressive pRB-E2F complexes in tumor suppression. Indeed, numerous over-expression studies have led to the conclusion that regulation of E2F-responsive genes, and therefore cell cycle entry, is largely controlled by the repressive, and not activating, E2Fs (Zhang et al. 1999; Zhang et al. 2000; Dahiya et al. 2001). In this study, we use *Rb;E2f4* compound mutant mice to investigate whether repressive E2F complexes contribute to tumor suppression. This analysis shows that the absence of E2F4 suppresses the formation of pRB-deficient tumors by promoting the formation of novel complexes between the activating E2Fs and p107 and p130 as well as correcting inappropriate target gene expression and cell growth. Most significantly, these data provide support for a model in which pocket proteins function as tumor suppressors by controlling activator E2Fs rather than by forming repressive E2F complexes.

RESULTS

Loss of E2F4 extends lifespan and alters tumorigenesis in *Rb* mutant mice

Over-expression studies strongly suggest that the repressive E2F-pocket protein complexes play a critical role in controlling the expression of E2F-responsive genes. Given this finding, we wished to establish whether these repressive E2F-pocket protein complexes contribute to tumor suppression. E2F4 is the major repressive E2F *in vivo*, accounting for the majority of the endogenous pRB-, p107- and p130-associated E2F activity. Thus, if the repressive E2F complexes are important, E2F4 loss should exacerbate the formation of pRB-deficient tumors. To test this hypothesis, we intercrossed *Rb* and *E2f4* mutant mouse strains with the same C57BL/6 X 129/Sv mixed background. We then compared the lifespan and tumor phenotype of $Rb^{+/-}$, $Rb^{+/-}$;$E2f4^{+/-}$ and $Rb^{+/-}$;$E2f4^{-/-}$ littermates.

The phenotype of the $Rb^{+/-}$ mice was entirely consistent with previous studies (reviewed by Mulligan and Jacks, 1998). All mice died between 8.5 and 13.9 months of age (Figure 1A). Histological examination confirmed that the cause of death was intermediate lobe pituitary tumors and that the vast majority of the $Rb^{+/-}$ animals (23/27) also displayed c-cell thyroid tumors (Figure 1A and B; data not shown). Mutation of a single *E2f4* allele did not significantly alter the lifespan of $Rb^{+/-}$ animals (Figure 1A). Moreover, the $Rb^{+/-}$;$E2f4^{+/-}$ mice developed pituitary (55/57) and thyroid (47/57) tumors that were comparable to those arising in the $Rb^{+/-}$ controls with respect to both incidence and size (Figure 1B; data not shown). Thus, a reduction in the levels of E2F4 had no notable effect on tumorigenicity in the *Rb* mutant mice.

Remarkably, the phenotype of $Rb^{+/-}$;$E2f4^{-/-}$ animals diverged considerably from those of their littermate controls. First, there was a significant difference ($p=0.0033$) in lifespan of

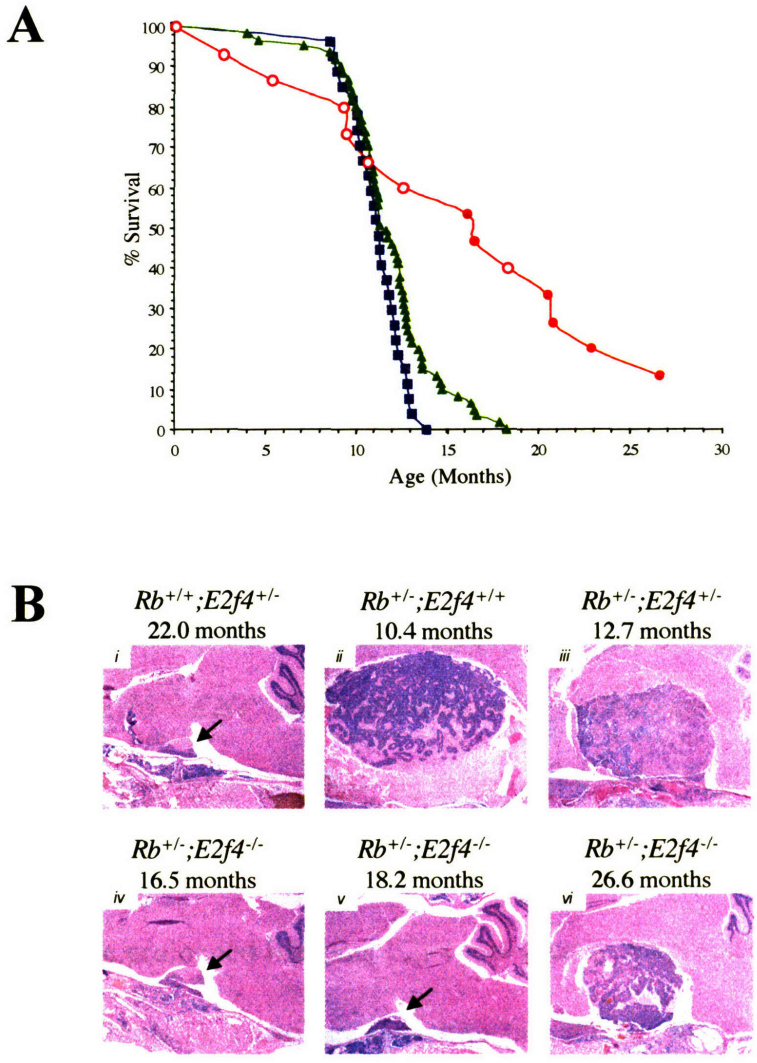

Figure 1. Loss of E2F4 extends the lifespan of $Rb^{+/-}$ adults by reducing the tumor incidence.

A, Survival curves for $Rb^{+/-};E2f4^{+/+}$ (blue, n=27), $Rb^{+/-};E2f4^{+/-}$ (green, n=61), and $Rb^{+/-};E2f4^{-/-}$ (red, n=15) mice. $Rb^{+/-};E2f4^{-/-}$ mice with no evidence of tumor formation (open circles) and those with tumors (closed circles) are shown, but the animals that were prematurely sacrificed are not included. B, Representative H&E stained median sections of adult heads including the pituitary (marked with an arrow) from (i) control $Rb^{+/+};E2f4^{+/-}$ animal, (ii) $Rb^{+/-};E2f4^{+/+}$, (iii) $Rb^{+/-};E2f4^{+/-}$, and (iv-vi) $Rb^{+/-};E2f4^{-/-}$ mice. The older $Rb^{+/-};E2f4^{-/-}$ mice have a variety of pituitary phenotypes including (iv) small, early pituitary tumors, (v) normal pituitaries, and (vi) medium intermediate lobe pituitary tumors. Magnification, 20x.

the $Rb^{+/-}$;$E2f4^{-/-}$ versus the $Rb^{+/-}$ animals (Figure 1A; Table 1). Two of the $Rb^{+/-}$;$E2f4^{-/-}$ mice died at early ages (2.7 and 5.4 mo.) as a result of an increased susceptibility to infections. This is a characteristic phenotype of the $E2f4^{-/-}$ mice and was therefore an anticipated outcome for a fraction of the $Rb^{+/-}$;$E2f4^{-/-}$ mice. However, we unexpectedly found that neither of these animals had any evidence of tumorigenic lesions (data not shown), even though such lesions are routinely observed in the pituitaries of $Rb^{+/-}$ mice by 3 months of age (Nikitin and Lee 1996). Most importantly, the majority of the $Rb^{+/-}$;$E2f4^{-/-}$ mice (17/19) survived at least until the window of lethality of the $Rb^{+/-}$ littermate controls (8.5-13.9 mo.). Moreover, 4 months after the death of the oldest surviving $Rb^{+/-}$ animal, half of the $Rb^{+/-}$;$E2f4^{-/-}$ mice remained alive and healthy. Indeed, a significant fraction of the $Rb^{+/-}$;$E2f4^{-/-}$ animals lived to an age (20-27 months) comparable to wild-type controls (Figure 1A; Table 1). Thus, the absence of E2F4 actually extended the lifespan of the $Rb^{+/-}$ mice.

Consistent with the prolonged lifespan, E2F4 loss greatly suppressed the formation of tumors in the $Rb^{+/-}$ mice (Table 1). Histological examination showed that the vast majority of the $Rb^{+/-}$;$E2f4^{-/-}$ animals died as a result of defects typical of the $E2f4^{-/-}$ mice. Indeed, prior to 16 months of age none of the $Rb^{+/-}$;$E2f4^{-/-}$ mice displayed any evidence of pituitary tumors, although they were detected in some of the older $Rb^{+/-}$;$E2f4^{-/-}$ mice (Table 1). However, the incidence of pituitary tumors was significantly lower than in the $Rb^{+/-}$ controls ($p=0.000092$), and there was a considerable range in size in the tumors that did arise in the $Rb^{+/-}$;$E2f4^{-/-}$ animals (Table 1; Figure 1B; data not shown). Three of the older $Rb^{+/-}$;$E2f4^{-/-}$ animals (16.2, 20.5, and 23 mo.) eventually developed tumors comparable to those seen in the $Rb^{+/-}$ mice (8.5-13.9 mo.), but others

Table 1. Histological Analysis of $Rb^{+/-};E2f4^{-/-}$ Mice

Age (Months)	Pituitary Tumor	Thyroid Tumor	Cause of Death
2.7	-	-	Sinusitis
5.4	-	-	Pylonephritis
8.7	-	-	Sacrificed early
9.3	-	-	Unknown
9.4	-	-	Severe dermatitis
10.7	-	-	Dermatitis, Aspiration
12.6	-	-	Severe dermatitis
15.6	-	-	Sacrificed early
16.2	++++	-	Pituitary tumor
16.5	+	++++	Thyroid tumor and metastasis
18.2	-	-	Histiocytic sarcoma
18.5	-	-	Sacrificed early
18.6	++	-	Sacrificed early
20.5	++++	-	Pituitary tumor
20.8	++	-	Hemangiosarcoma
23.0	++++	-	Pituitary tumor and pheochromocytoma
26.0	nd	nd	Alive
26.6	+++	-	Infection of reproductive organs
27.0	nd	nd	Alive

nd = not determined

Tumor size is indicated as follows: "+" indicates very early tumor growth and "++++" indicates tumors comparable to those of $Rb^{+/-}$ mice.

developed very early lesions or mid-sized tumors (16.5 and 26.6 mo.), and two animals had completely normal pituitaries (18.2 and 18.5 mo.).

E2F4 loss had an even greater effect on the development of thyroid tumors in $Rb^{+/-}$ mice (p=0.00000034). Despite the extremely high incidence of c-cell thyroid tumors in the $Rb^{+/-}$ (23/27) and $Rb^{+/-};E2f4^{+/-}$ (47/57) animals, only 1/17 of the $Rb^{+/-};E2f4^{-/-}$ mice developed a thyroid tumor (Table 1; data not shown). Indeed, there was no evidence of thyroid hyperplasia in the remaining 16/17 $Rb^{+/-};E2f4^{-/-}$ animals. Thus, we conclude that the absence of E2F4 dramatically suppresses the development of both pituitary and thyroid tumors in the $Rb^{+/-}$ mice and thereby greatly extends their lifespan.

Loss of E2F4 induces profound rearrangement of E2F-pocket protein complexes

We initiated the tumor studies with the expectation that E2F4 loss would either have no effect on, or would exacerbate, the formation of pRB-deficient tumors depending on whether or not the repressive E2F-pocket protein complexes were important for tumor suppression. Instead, our data clearly show that E2F4 loss inhibits the formation of tumors. To establish the underlying mechanism, we characterized the effect that E2F4 loss had on the remaining E2F-pocket protein complexes. Initially, we compared the E2F complexes present in extracts from wild-type, $Rb^{+/-}$, and $Rb^{+/-};E2f4^{-/-}$ MEFs by immunoprecipitating specific E2Fs and then western blotting to identify the associated pocket proteins. Consistent with previous studies, E2F1 and E2F3 bound specifically to pRB in wild-type and $Rb^{+/-}$ cells (Figure 2 and data not shown). In contrast, in $Rb^{+/-};E2f4^{-/-}$ MEFs, activating E2Fs participated in novel pocket protein complexes in addition to binding to pRB. Specifically, E2F1 bound to p130, and E2F3 associated with p107.

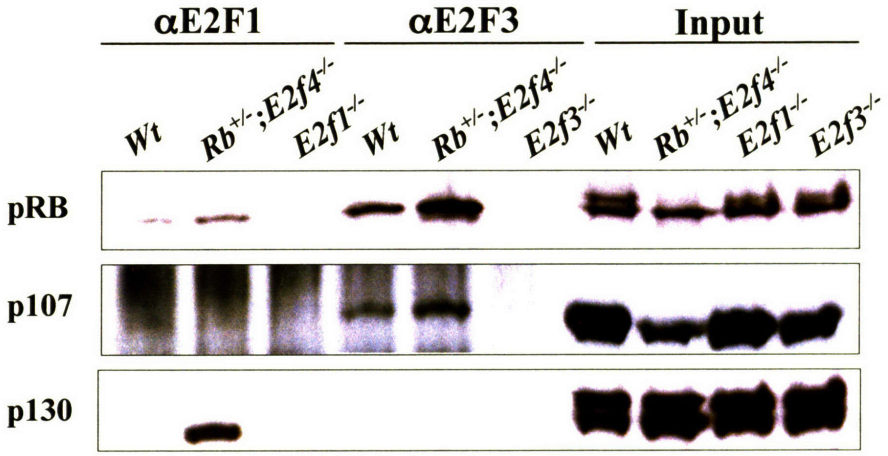

Figure 2. E2F complex rearrangement in *Rb*$^{+/-}$; *E2f4*$^{-/-}$ MEFs.

Western blot detection of pRB, p107, and p130 after immunoprecipitation of lysates from wild-type and *Rb*$^{+/-}$; *E2f4*$^{-/-}$ MEFs with anti-E2F1 and E2F3 antibodies. Input lysate (10% of total) is shown at right.

This was not due to an alteration of E2F1 or E2F3 levels, since steady state amounts of these proteins were not affected in cells deficient for either pRB or pRB and E2F4 relative to wild-type MEFs (Figure 3 and data not shown). Thus, E2F4 loss allows p107 and p130 to substitute for pRB by binding E2F1 and E2F3.

Since the activating E2Fs are known to be important downstream targets of the pRB tumor suppressor, the formation of novel complexes between activating E2Fs and p107 and p130 could account for the suppression of tumors in the $Rb^{+/-}$;$E2f4^{-/-}$ mice. To address this issue, we used electrophoretic mobility shift assays to establish whether these novel complexes were present in $Rb^{+/-}$;$E2f4^{-/-}$ tissues. For these experiments, we immunoprecipitated p107 from extracts derived from several tissues, including the pituitary, which is prone to tumors in $Rb^{+/-}$ animals. The associated E2F species were released by the addition of the detergent deoxycholate (DOC) and then identified in electrophoretic mobility shift assays (Figure 4). Regardless of the tissue examined, p107 associated specifically with E2F4 in the wild-type and $Rb^{+/-}$ mutant mice (Figure 4A, B, D). In contrast, p107 bound at least three distinct E2F complexes in the tissues derived from the $Rb^{+/-}$;$E2f4^{-/-}$ mice (Figure 4C, D). An anti-E2F5 antibody recognized one of these species, and the other E2F complexes were completely retarded by a combination of antibodies against E2F1 and E2F3. E2F1 and E2F5 were also observed when the immunoprecipitations were conducted with anti-p130 antibodies (data not shown). Thus, the absence of E2F4 enables p107 and p130 to bind activator E2Fs in a variety of tissues, including the tumor-prone pituitary.

It is well documented that the formation of tumors in $Rb^{+/-}$ mice is dependent upon the inactivation of the wild-type *Rb* allele. Therefore, we also determined the nature of the E2F

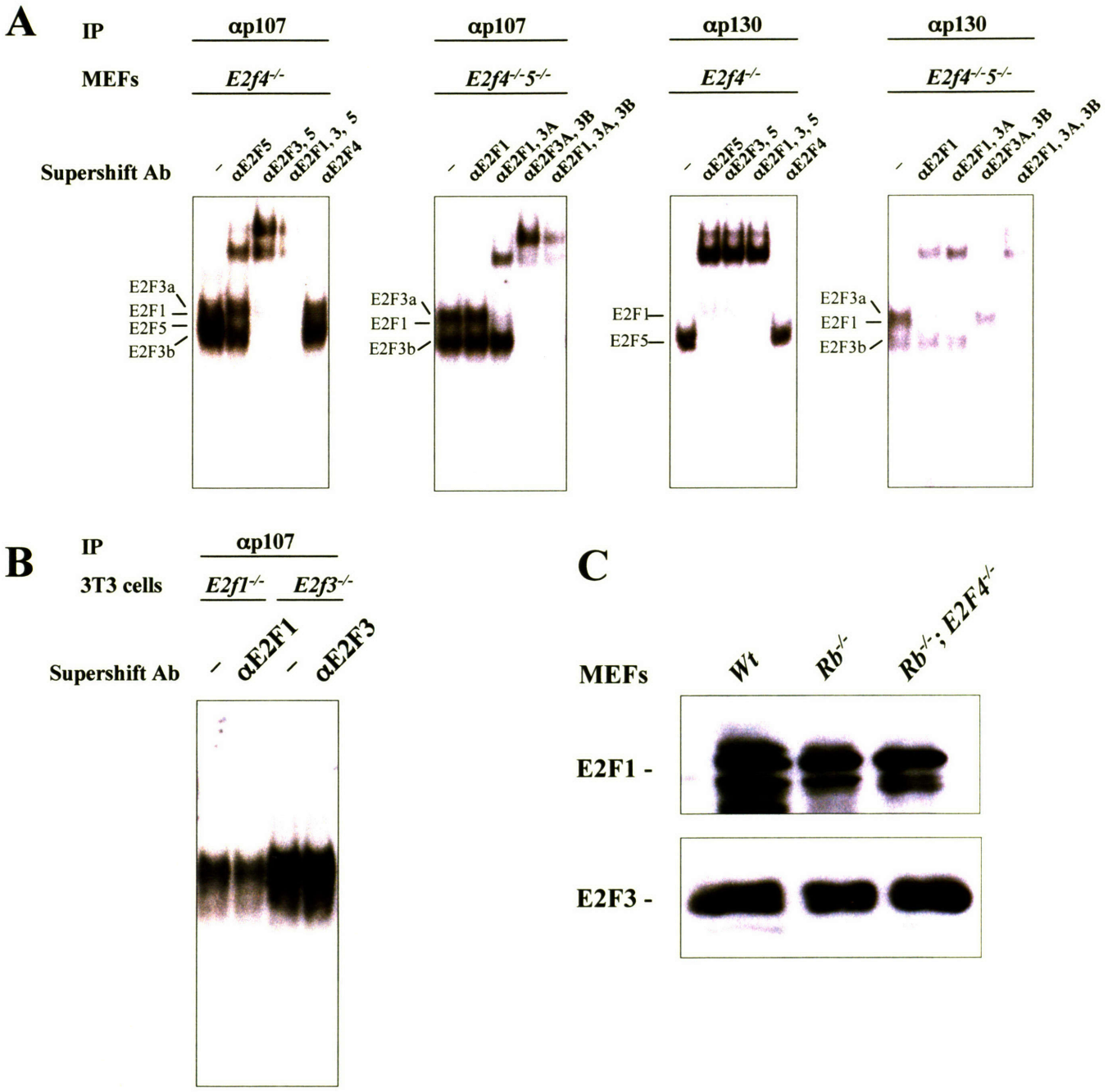

Figure 3. p107/p130 association with activator E2Fs in *E2f4*$^{-/-}$ MEFs is further enhanced in *E2f4*$^{-/-}$;*E2f5*$^{-/-}$ MEFs.

A, *E2f4*$^{-/-}$ and *E2f4*$^{-/-}$;*E2f5*$^{-/-}$ MEFs were lysed with E2F extraction buffer and were subjected to immunoprecipitation with anti-p107 and/or p130 antibodies, DOC release, and EMSA. B, Coupled Immunoprecipitation-DOC release with anti-p107 antibodies in *E2f1*$^{-/-}$ and *E2F3*$^{-/-}$ 3T3s to test the specificity of the antibodies. C, Western blot analysis of E2F1 and E2F3 proteins in *Rb*$^{-/-}$ and *Rb-/-;E2f4*$^{-/-}$ MEFs.

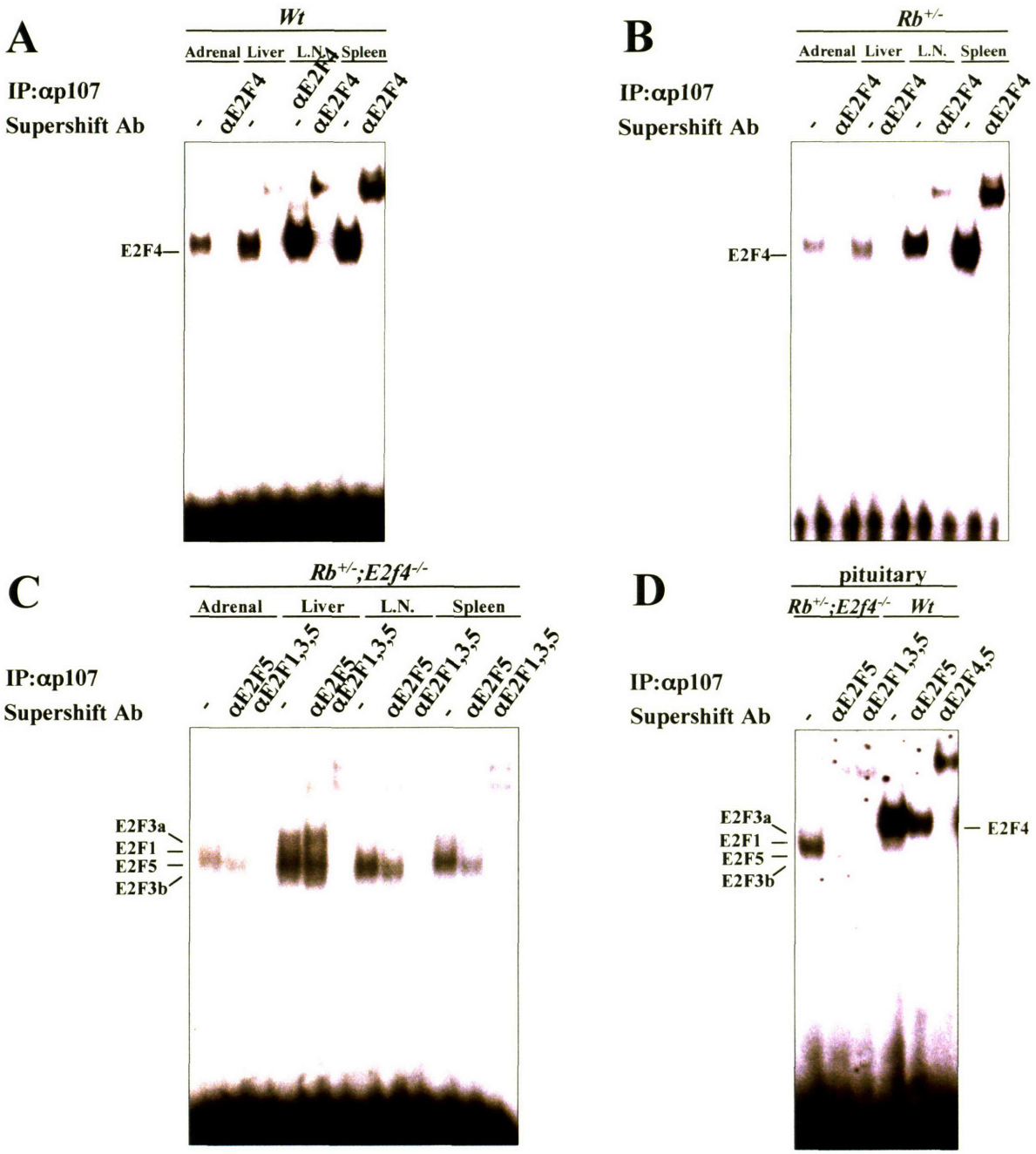

Figure 4. p107 associates exclusively with E2F4 in organs of wild type and *Rb*$^{+/-}$ mice but associates with E2F1, 3, and 5 in *Rb*$^{+/-}$;*E2f4*$^{-/-}$ mice.

A-C, Immunoprecipitation, DOC release, and EMSA were performed with anti-p107 antibody, using homogenates of adrenal glands (A, C, 500 μg; B, 300 μg), liver (A-C, 2 mg), lymph nodes (L.N.)(A, C, 500 μg; B, 250 μg), and spleen (A, C, 200 μg; B, 100 μg) of wild-type, *Rb*$^{+/-}$, and *Rb*$^{+/-}$;*E2f4*$^{-/-}$ mice. D, Coupled immunoprecipitation-DOC release of E2F proteins in pituitary homogenates of wild-type (130 μg) and *Rb*$^{+/-}$;*E2f4*$^{-/-}$ (200 μg) mice with anti-p107 antibody. Specific E2F-DNA complexes in the absence of antibody retardation are indicated. The identities of each of the distinct p107/E2F complexes in *Rb*$^{+/-}$;*E2F4*$^{-/-}$ tissues were deduced by performing EMSA assays on MEFs deficient for individual E2F family members as well as compound E2F mutant cells.

complexes in *Rb*$^{-/-}$ versus *Rb*$^{-/-}$;*E2f4*$^{-/-}$ cells. Since the *Rb*$^{-/-}$ and *Rb*$^{-/-}$;*E2f4*$^{-/-}$ animals both die *in utero* (reviewed by Mulligan and Jacks, 1998; E.Y.L. and J.A.L., see chapter 3), these experiments were conducted using MEFs (Figure 5A). DOC release and electrophoretic mobility shift assays confirmed that E2F4 and E2F5 accounted for all of the p107- and p130-associated E2F activity in wild-type cells (Figure 5A). In agreement with our analysis of *Rb*$^{+/-}$;*E2f4*$^{-/-}$ samples, some of the p107- and p130-associated E2F species in *Rb*$^{-/-}$;*E2f4*$^{-/-}$ MEFs were unaffected by anti-E2F4 and anti-E2F5 antibodies. The remaining complexes corresponded to E2F1, E2F3a and E2F3b (data not shown). Importantly, we found that the spectrum of E2F complexes in the *Rb*$^{-/-}$ MEFs was a composite of those of the wild-type and *Rb*$^{-/-}$;*E2f4*$^{-/-}$ MEFs. Specifically, whereas E2F4 accounted for either all or a large fraction of the p107-associated E2F activity in wild-type and *Rb*$^{-/-}$ cells, respectively, p107 bound significant quantities of activator E2Fs in *Rb*$^{-/-}$ and *Rb*$^{-/-}$;*E2f4*$^{-/-}$ MEFs. Further, p130 associated almost exclusively with E2F4 in *Rb*$^{-/-}$ cells, but in the doubly deficient cells, it associated to a large extent with E2F1 and E2F5 (Figure 5A and see below).

This analysis raised the possibility that p107 and p130 might act in pRB-deficient cells to bind to E2F1 and E2F3 even in the presence of physiological levels of E2F4. To further test this hypothesis, we examined the pocket protein binding properties of E2F1 and E2F3 in *Rb*$^{-/-}$ and *Rb*$^{-/-}$;*E2f4*$^{-/-}$ MEFs by immunoprecipitating the activator E2Fs and western blotting for associated pocket proteins (Figure 5B). These experiments confirmed that there was a robust association between the activating E2Fs and p107 and p130 in *Rb*$^{-/-}$ cells. Furthermore, the absence of both E2F4 and pRB strikingly increased the level of E2F1-associated p130 when compared to *Rb*$^{-/-}$ cells. Taken together, these data yield two important conclusions. First, in

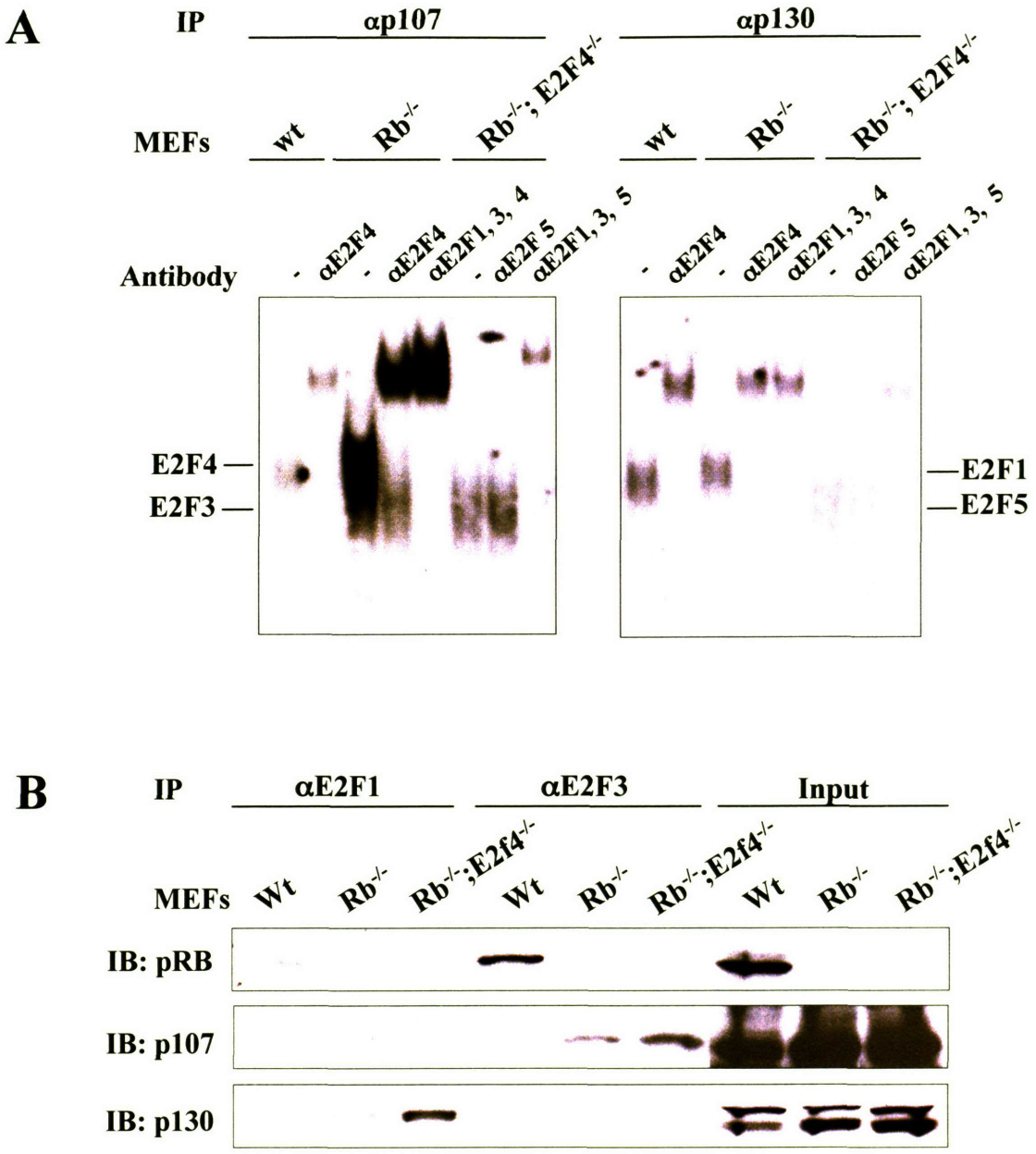

Figure 5. E2F complex rearrangement in *Rb*$^{-/-}$ MEFs and enhanced by further loss of E2F4.

A-B, MEFs were lysed with ELB buffer and were subjected to immunoprecipitation, DOC release, and EMSA with p107 or p130 antibodies (A) or were immunoprecipitated with anti-E2F1 or anti-E2F3 antibodies and Western blotted with pocket protein antibodies (B). Specific E2F-DNA complexes in the absence of antibody retardation are indicated.

pRB-deficient cells, p107 and p130 appear to substitute for pRB in the regulation of the activating E2Fs. Second, E2F4 loss enhances the formation of these novel complexes, presumably by increasing the levels of the free pools of p107 and p130.

Loss of E2F4 suppresses inappropriate E2F target gene expression and cell proliferation in pRB-deficient cells

Our experiments indicated that tumor suppression in animals lacking both pRB and E2F4 resulted from the reassortment of complexes such that p107/p130 associated with activating E2Fs. Given these findings, we investigated whether loss of E2F4 had an impact on the proliferative capacity of *Rb*-deficient MEFs. To address this issue, we compared the levels of proliferation in wild-type, $Rb^{-/-}$, and $Rb^{-/-};E2f4^{-/-}$ MEFs grown to confluence. Wild-type cells incorporated BrdU at low levels, as expected for a quiescent population (Figure 6A and B). In contrast, $Rb^{-/-}$ cells largely failed to arrest in response to confluent growth, and approximately 40% of the cells entered S-phase. Remarkably, loss of *E2f4* completely suppressed this inappropriate proliferation and restored the low levels of BrdU incorporation observed in wild-type cells.

The abnormal proliferation observed in confluent $Rb^{-/-}$ MEFs has been shown to correlate with the inappropriate expression of known E2F-responsive genes, cyclin E and p107 (Herrera et al. 1996; Hurford et al. 1997). Given the apparent rescue of the $Rb^{-/-}$ proliferation defect in $Rb^{-/-};E2f4^{-/-}$ MEFs, we hypothesized that the loss of E2F4 might also modulate the expression of E2F-responsive genes. We investigated this possibility by examining expression of the *cyclin E* gene in wild-type, $Rb^{-/-}$ and $Rb^{-/-};E2f4^{-/-}$ MEFs grown to confluence. As expected from previous

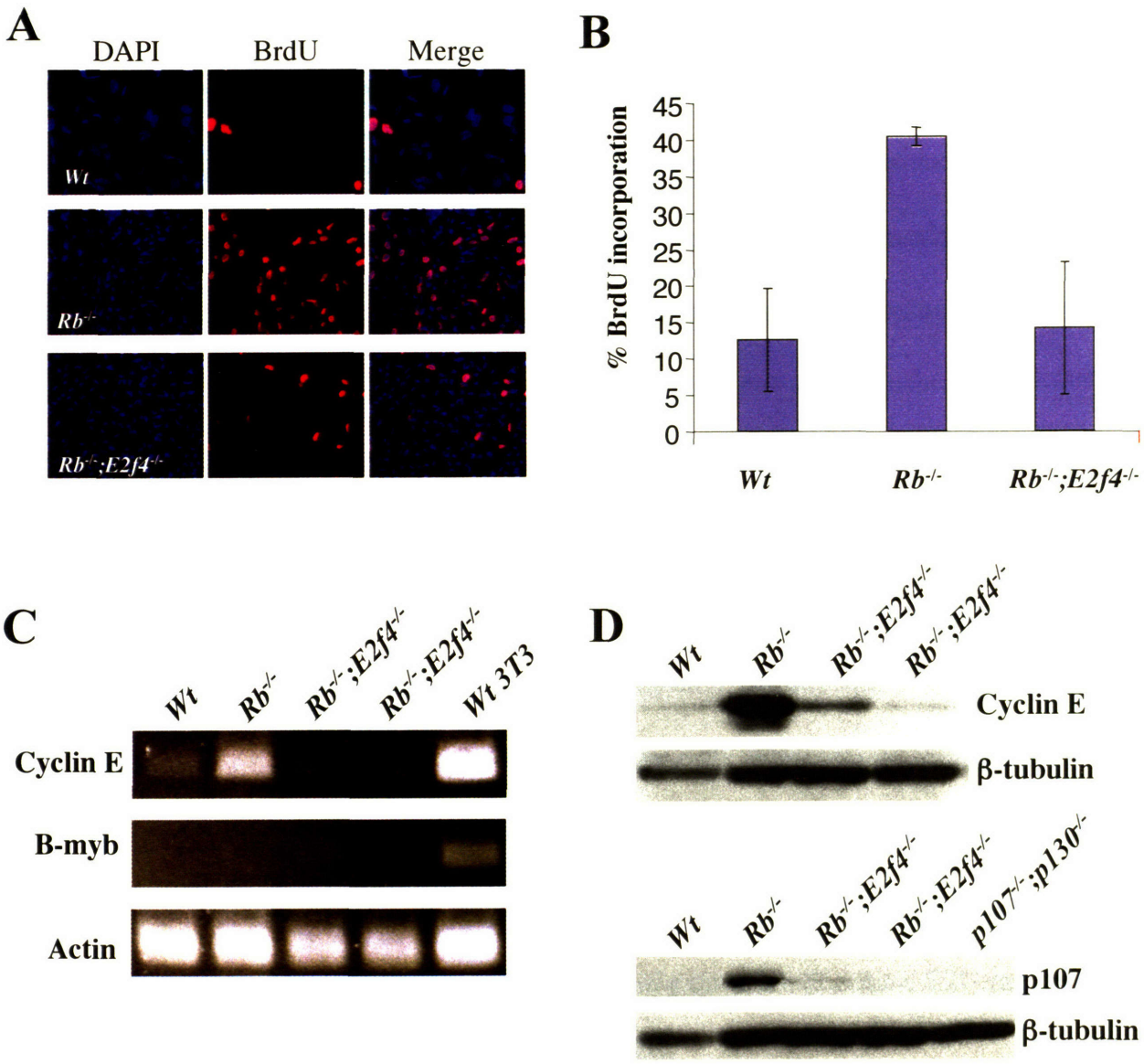

Figure 6. Loss of E2F4 restores confluence arrest and regulation of target genes in *Rb*$^{-/-}$ MEFs.

A, Immunofluorescence for BrdU (red) and DAPI (blue) on wild-type, *Rb*$^{-/-}$ and *Rb*$^{-/-}$*;E2f4*$^{-/-}$ MEFs treated with BrdU 2 days after reaching confluence. B, Quantification of BrdU incorporation. For each genotype, the percentage of BrdU positive nuclei was calculated. The graph depicts the average of two experiments with standard deviation. C, RT-PCR analysis of E2F target genes, *cyclin E* and *B-myb* (not deregulated in *Rb*$^{-/-}$ MEFs), and actin (loading control) on day 2 confluent cells. Wild-type 3T3 cells were used as a positive control. D, Western blot analysis of E2F target genes, cyclin E and p107, and β-tubulin (loading control) on day 2 confluent cells. Asynchronously growing *p107*$^{-/-}$*;p130*$^{-/-}$ MEFs were used as a negative control for the p107 blot.

studies (Hererra et al. 1996; Hurford et al. 1997), cyclin E was expressed at very low levels in confluent, wild-type cells but was markedly elevated in cells deficient for *Rb* (Figure 6C). In striking contrast, cyclin E RNA levels were dramatically and consistently reduced in cells deficient for both *Rb* and *E2f4* to levels that approximated those observed in wild-type cells. We demonstrated that each of these effects was specific, since expression of a second E2F target gene, *B-myb*, known to be under the control of p107/p130 but not pRB (Hurford et al., 1997; Rayman et al., 2002), was not affected by mutation of *Rb* or *Rb* and *E2f4* (data not shown).

To extend these findings, we performed western blotting on extracts derived from wild-type and mutant MEFs and examined expression of several E2F target genes. These experiments confirmed our RT-PCR studies and showed that expression of cyclin E and a second established pRB target, p107, was markedly elevated in *Rb*-deficient cells. Furthermore, simultaneous loss of *E2f4* largely reversed this de-regulation in two independent preparations of doubly null MEFs (Figure 6D). These findings strongly suggest that loss of *E2f4* suppresses tumorigenic growth of *Rb* deficient cells by restoring both appropriate levels of expression of critical E2F target genes and a normal response to cues that limit cell proliferation.

DISCUSSION

The goal of these studies was to establish whether the formation of repressive E2F complexes contributes to the tumor suppressive properties of pRB. Since E2F4 cooperates with the pocket proteins in gene repression, we anticipated that E2F4 loss would either exacerbate or have no effect on the tumor phenotype of the $Rb^{+/-}$ mice depending upon whether or not repression was important. Instead, we found that the absence of E2F4 greatly inhibited the formation of both pituitary and thyroid tumors, enabling a significant fraction of the $Rb^{+/-};E2f4^{-/-}$ mice to live as long as wild-type controls. Indeed, the degree of tumor suppression significantly exceeded that resulting from the loss of the activating E2Fs, E2F1 or E2F3, in an $Rb^{+/-}$ background (Yamasaki et al. 1998). Furthermore, we demonstrated that loss of *E2f4* in *Rb*-deficient cells restored the control of E2F-responsive genes and the inhibition of DNA synthesis characteristic of wild-type, confluence-arrested cells. Since the loss of contact inhibition is one of the hallmarks of a cancer cell, we suggest that this finding could explain the tumor suppression we observe in pituitaries and thyroids of $Rb^{+/-}$; $E2f4^{-/-}$ mice. Thus, this study provides the first evidence for a critical role of E2F4 in pRB function.

E2F4 loss could be exerting its tumor suppressive effects via several possible mechanisms. The simplest model is that E2F4 contributes to the activation of E2F-responsive genes and is therefore a key downstream target of pRB in a similar manner to E2F1 and E2F3. This conclusion is supported by early studies that showed that E2F4 has significant transcriptional activity in over-expression experiments (Beijersbergen et al. 1994; Ginsberg et al. 1994). However, analysis of the endogenous E2F4 protein does not support this conclusion. First, the predominant cytoplasmic localization of the free E2F4-DP complexes is inconsistent

with their role in transcriptional activation (Magae et al. 1996; Muller et al. 1997; Verona et al. 1997; Gaubatz et al. 2001). Second, ChIP assays strongly suggest that E2F4 specifically occupies E2F-responsive promoters in association with p107 and p130 during the G0/G1 stages of the cell cycle when these targets are transcriptionally repressed (Takahashi et al. 2000; Rayman et al. 2002). Finally, primary cells that are deficient for E2F4 and E2F5 are defective in cell cycle arrest but not proliferative functions (Gaubatz et al. 2000). Clearly, these data do not rule out the possibility that E2F4 could contribute to the activation of E2F-responsive genes in pRB-deficient tumor cells, and experiments that investigate both expression profiles and promoter occupancy of target genes will be needed to address this issue further. Moreover, it is important to note that although it is widely assumed that E2F1 and E2F3 contribute to the formation of tumors through this mechanism, this has not yet been demonstrated. Therefore, experiments with *Rb;E2f* compound mutant cells will be critical in testing this hypothesis as well.

An alternative model arising from our data suggests that E2F4 loss could increase the apoptotic potential of pRB-deficient cells. Under these conditions, cells in the $Rb^{+/-};E2f4^{-/-}$ mice that lose the wild-type *Rb* allele might be eliminated by apoptosis rather than become tumorigenic. This a reasonable concern because there is considerable evidence supporting a role for the E2F proteins in the regulation of many apoptosis genes (reviewed by Trimarchi and Lees 2002). We have not observed any obvious difference in the apoptotic potential of $Rb^{-/-};E2f4^{-/-}$ versus $Rb^{-/-}$ MEFs (our unpublished observations). However, since this does not address the consequences of E2F4 loss in the adult pituitary and thyroid, we are attempting to establish $Rb^{-/-};E2f4^{-/-}$ ES cell lines that can be used to generate chimeric mutant mice. Such mutant animals will be invaluable because they will allow us to establish whether $Rb^{-/-};E2f4^{-/-}$ cells can contribute to

adult tissues. Since it is well established that the formation of tumors in $Rb^{+/-}$ mice depends upon the inactivation of the wild-type *Rb* allele, it is also possible that the rearrangement in pocket protein complexes in the $Rb^{+/-};E2f4^{-/-}$ tissues somehow diminishes the selective pressure for loss of heterozygosity. In addition, our data do not rule out that the observed tumor suppressive effect of E2F4 loss is cell non-autonomous. Thus, the generation of both conditional and chimeric mice will also be essential in allowing us to address these two issues.

A final model suggests that E2F4 loss suppresses tumors by simply altering the spectrum of the remaining E2F complexes. We currently favor this hypothesis, based on our biochemical analysis. Specifically, our data show that E2F4 loss promotes the formation of novel E2F complexes in which p107 and p130 associate with the normally pRB-specific E2Fs, E2F1 and 3 (Figure 7). Previous studies have shown that inappropriate release of the activating E2Fs makes a major contribution to the phenotypic consequences of pRB loss (Tsai et al. 1998; Yamasaki et al. 1998; Ziebold et al. 2001). We therefore propose that E2F4 loss suppresses tumorigenesis by increasing the free pools of p107 and p130 and thereby enabling them to substitute for pRB in the inhibition of the activating E2Fs (Figure 7). This could also account for the observed suppression of inappropriate E2F-responsive gene expression and cell cycle entry of confluence-arrested $Rb^{-/-}$ MEFs (Figure 6B and C). Additional tumor studies will be required to distinguish between these models. However, regardless of the precise mechanism by which E2F4 loss is operating, our studies do not provide any support for a role of repressive E2F-pocket protein complexes in tumor suppression. Instead, they strongly suggest that the critical tumor suppressive role of pRB is to inhibit E2F family members that mediate the activation of E2F-responsive genes.

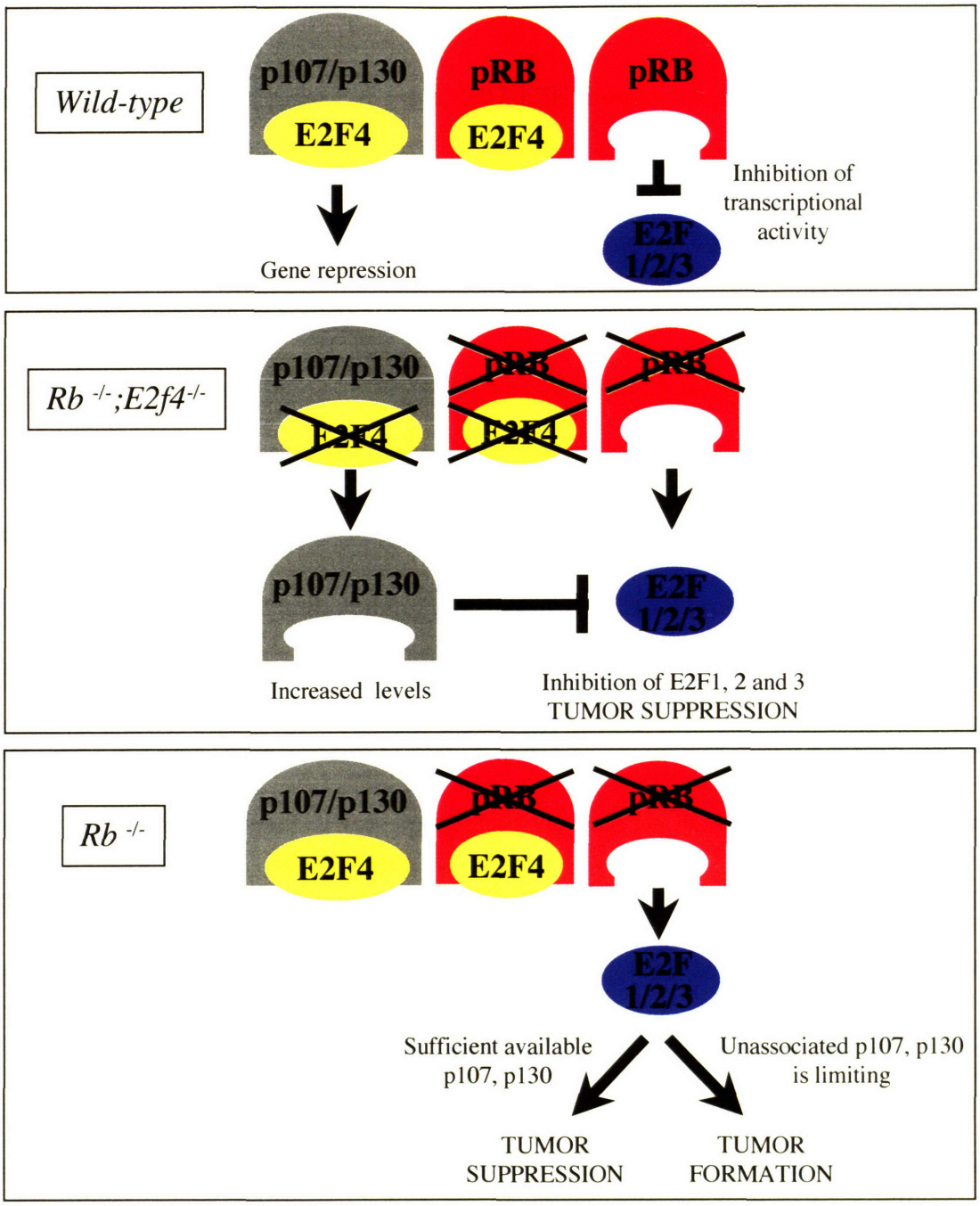

Figure 7. Model for tumor suppression resulting from simultaneous deficiency of *E2f4* and *Rb*.

In wild-type cells, the activator E2Fs are specifically regulated by pRB while E2F4 associates with pRB, p107 and p130. In the absence of *Rb,* E2F1 and E2F3 activators are released, activating inappropriate S-phase target gene expression and thereby promoting uncontrolled proliferation and tumor formation. The simultaneous deficiency of *Rb* and *E2f4* in $Rb^{+/-}$;$E2f4^{-/-}$ mice results in the association of p107 and p130 with the activator E2Fs, conferring tumor suppressive functions on p107 and p130. Loss of pRB alone also promotes some binding of p107 and p130 to E2F1 and E2F3. Thus, the levels of available p107 and p130 in individual tissues may account for the tissue specificity of $Rb^{-/-}$ tumor formation.

Importantly, we also detected p130-E2F1 and p107-E2F3 complexes in cells that had physiological levels of E2F4, but lacked the pRB tumor suppressor. Since the generation of $Rb^{-/-}$ cells is a key step in the development of many naturally occurring tumors, we believe that the formation of novel E2F-pocket protein complexes has significant *in vivo* relevance. There is extensive evidence from both human tumors and mutant mouse models that the pocket proteins play non-overlapping roles in the suppression of tumors (Cobrinik et al. 1996; Lee et al. 1996; Robanus-Maandag et al. 1998; Dannenberg et al. 2000; Sage et al. 2000). Specifically, pRB is a classical tumor suppressor, but mutation of *p107* and/or *p130* promotes tumor formation only when pRB is also inactivated. We believe that our observations can account for these differential properties. First, we propose that tumor formation is dependent upon the inappropriate release of the activating E2Fs. Since these E2Fs are specifically regulated by pRB in normal cells, their release can only be triggered by the loss of pRB and not p107 and/or p130, explaining why pRB is the key tumor suppressor *in vivo*. Second, our data suggest that pRB loss causes p107 and p130 to substitute for pRB in the regulation of the activating E2Fs. In this manner, p107 and p130 become significant tumor suppressors in pRB-deficient cells. Consistent with this hypothesis, $Rb^{-/-};p107^{-/-}$ chimeric mice develop an additional tumor type, retinoblastoma, compared to $Rb^{-/-}$ chimeras (Robanus-Maandag et al. 1998), and the combined mutation of pRB, p107 and p130 has been shown to be highly tumorigenic (Dannenberg et al. 2000; Sage et al. 2000). Moreover, *p107* and/or *p130* mutations have been detected at a low frequency in certain pRB-deficient human tumors (Helin et al. 1997; Claudio et al. 2000a; Claudio et al. 2000b).

It is important to note that mutation of *p107* and/or *p130* is not required for the formation of most pRB-deficient tumors. We must therefore conclude that p107 and p130 are unable to

compensate for the loss of pRB in tumor-prone tissues. Inheritance of germline *Rb* mutations results in a highly tissue-specific tumor spectrum in both humans (retinoblastoma) and mice (pituitary and thyroid tumors). Since pRB is believed to play a key role in all tissue types, the underlying basis for this tissue-specific spectrum is not understood. We believe that our observations could also explain this phenomenon. Our data show that the absence of E2F4 increases the levels of p107 and p130 that associate with the activating E2Fs in $Rb^{-/-}$ cells, suppressing the formation of both pituitary and thyroid tumors in $Rb^{-/-}$ mice. This raises the possibility that the tumor-prone tissues may simply be those where the levels of available p107 and p130 are insufficient to substitute fully for pRB in the inhibition of the activating E2Fs (Figure 5). By extension of this logic, a relatively modest increase in the free pools of p107 and p130 may be sufficient to prevent the formation of tumors. This suggests a novel strategy for the generation of chemotherapeutic agents that would either release free pools of p107 and p130 by depleting cells of E2F4 (as in this study) or increasing intracellular p107/p130 levels.

EXPERIMENTAL PROCEDURES

Generation, genotyping, and analysis of mice and MEFs

The *Rb*$^{+/-}$;*E2f4*$^{+/-}$ strain was generated by intercrossing *Rb*$^{+/-}$ and *E2f4*$^{+/-}$ 129/Sv x C57BL/6 mice carrying germline mutations in *Rb* or *E2f4*. Genotyping was conducted as previously described (Jacks et al. 1992; Humbert et al. 2000a). Soft tissues were fixed in 10% formalin, stained with hematoxylin and eosin, and scored for tumors histologically and/or macroscopically. For comparison, the tumors were measured at the widest diameter. MEFs were generated using embryos between 10.5 and 13.5 days post coitum from *Rb*$^{+/-}$;*E2f4*$^{+/-}$ intercrosses as previously described (Humbert et al. 2000b). MEFs were used before passage 7. The statistical significance of the differential lifespan and tumor incidence of the *Rb*$^{+/-}$ versus the *Rb*$^{+/-}$;*E2f4*$^{-/-}$ animals was determined by the log-rank test and 2-tailed Fisher's exact test (as previously described by Yamasaki et al. 1998), respectively.

Immunoprecipitation, deoxycholate release, and electrophoretic mobility shift assays.

Asynchronously growing MEFs were lysed in ELB buffer (50 mM Hepes pH 7.0, 250 mM NaCl, 5 mM EDTA, 0.1% NP-40, and 10 % glycerol containing a protease inhibitor cocktail). Immunoprecipitation using ELB buffer and western blotting were conducted using anti-E2F1 (sc-193, Santa Cruz Biotech), anti-E2F3 (AB) (sc-878), anti-pRB (G3-245, PharMingen), anti-p107 (sc-318) and anti-p130 (sc-317) antibodies. Whole organs were lysed in E2F extraction buffer (20 mM Hepes pH 7.8, 450 mM NaCl, 0.2 mM EDTA pH 8.0, 0.1% NP-40, 25% glycerol) by three rounds of freeze-thaw and then homogenized in 1.5 ml Eppendorf tubes with a tight-fitting pestle. Deoxycholate (DOC) release and electrophoretic mobility shift assays were

conducted as described (Woo et al. 1997) using anti-E2F1 (KH129, Neomarkers), anti-E2F3 (sc-878), anti-E2F4 (sc-1082), and anti-E2F5 (sc-1083) antibodies. The specificity of these antibodies was confirmed by performing electrophoretic mobility shift assays with extracts from various E2F-deficient MEFs.

Confluence arrest, BrdU incorporation, and target gene expression assays

MEFs were grown to confluence, and two days later, cells were labeled with 10 μM 5-bromo-2'-deoxyuridine (BrdU; Sigma) for 8 hours. Incorporation was quantified by indirect immunofluorescence with anti-BrdU (347580, Becton Dickinson) antibodies and DAPI. The percentage of BrdU positive cells was determined by counting more than 875 cells per genotype. Whole cell extracts from day 2 confluent MEFs were prepared as previously described (Moberg et al., 1996), and western blotting was performed using anti-cyclin E (sc-481), anti-p107 (sc-318)(each from Santa Cruz Biotech), and anti-β-tubulin (T-4026, Sigma) antibodies. RT-PCR assays were carried out as described in Ren et al., 2002 using an Invitrogen RT-PCR Superscript One Step kit.

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Chapter Three

E2F4 cooperates with pRB in the development of extra-embryonic but not embryonic tissues

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The author contributed all the text, Tables 1-4 and Figures 1-6. Jing Zhang, from Harvey F. Lodish's laboratory, performed the erythroid cell analyses represented in Tables 5 and 6 and Figure 8 through a collaborative effort with the author. Tina Yuan and the author contributed Figure 7. Most of the work was performed in the laboratory of Jacqueline A. Lees.

ABSTRACT

Homozygous mutation of the murine retinoblastoma tumor suppressor gene, *Rb*, causes embryonic lethality between E13.5 and E15.5 with defects in cellular proliferation, differentiation and apoptosis. Many of these defects are suppressed by mutation of the activating E2Fs, *E2f1* or *E2f3*, indicating that they are key downstream targets of the retinoblastoma protein, pRB. We have previously shown that loss of E2F4, a repressive E2F, suppresses tumor formation in the $Rb^{+/-}$ background. In this study, we have assessed how E2F4 contributes to the developmental consequences of pRB loss. In stark contrast to the activating *E2fs*, homozygous mutation of *E2f4* shortened the lifespan of $Rb^{-/-}$ embryos. This resulted from an exacerbation of the extra-embryonic tissue defect of the $Rb^{-/-}$ mice, indicating that E2F4 and pRB cooperate in the development of this tissue. To determine the relative roles of E2F4 and pRB in the embryo, we provided $Rb^{-/-}$;$E2f4^{-/-}$ embryos with a normal functioning placenta. Under these conditions, the $Rb^{-/-}$;$E2f4^{-/-}$ mice survived to birth and exhibited all of the defects that were observed in the *E2f4* and *Rb* single mutant embryos. This was most striking in the hematopoietic compartment, which displayed both the stress erythropoiesis and Howell-Jolly body phenotypes of the $E2f4^{-/-}$ embryos and the excessive levels of nucleated erythrocytes characteristic of $Rb^{-/-}$ embryos. Thus, we conclude that pRB and E2F4 play non-overlapping roles in the development of many embryonic tissues.

INTRODUCTION

The retinoblastoma gene, *RB-1*, was the first tumor suppressor to be identified. The E2F transcription factors are the most extensively studied downstream effectors of the retinoblastoma protein, pRB, and they make a key contribution to its growth inhibitory activities (reviewed in Trimarchi and Lees 2002). In the G1 phase, hypophosphorylated pRB binds to E2F and consequently inhibits the activation of E2F-responsive genes, which are required for cell cycle progression. Upon mitogenic stimulation, pRB is phosphorylated by cyclin D/CDK4 and cyclin E/CDK2 complexes. This leads to the release of transcriptionally active E2F and entry into S phase. The importance of this pathway is highlighted by the fact that tumors with wild-type pRB typically have mutations in *Cyclin D1*, *CDK4* or $p16^{INK4a}$, a cyclin dependent kinase inhibitor (reviewed in Sherr 1996). Therefore, functional inactivation of pRB, which leads to deregulated E2F activity, is a critical event in tumorigenesis.

E2F transcriptional activity is conferred by a heterodimer of one E2F protein and one DP protein. To date, there are eight mammalian E2F genes (*E2f1* through *E2f8*) and two DP genes (*DP1* and *DP2*) (reviewed in Attwooll et al. 2004; Maiti et al. 2005). The DP moiety enhances DNA binding and the E2F subunit confers functional specificity. Based on structural and functional differences, the E2Fs that are regulated by pRB and its relatives, p107 and p130, (collectively known as the pocket proteins) can be divided into two distinct subgroups: the activating E2Fs (E2F1, E2F2, E2F3a) and the repressive E2Fs (E2F3b, E2F4, E2F5). The activating E2Fs are primarily involved in the transcriptional activation of E2F target genes and when over-expressed each of these E2Fs induces quiescent cells to enter S phase (Lukas et al. 1996; DeGregori et al. 1997). Coincident with the transcription of E2F-responsive genes and initiation of DNA synthesis, the activating E2Fs are bound to E2F-responsive promoters

(Takahashi et al. 2000; Rayman et al. 2002). In addition, the activating E2Fs are solely regulated by pRB *in vivo* and can induce apoptosis when ectopically expressed (Lees et al. 1993; Moberg et al. 1996; Vigo et al. 1999).

In contrast, the repressive E2Fs have pivotal roles in transcriptional repression of E2F target genes. They are constitutively expressed throughout the cell cycle and their subcellular localization largely influences their transcriptional activity (Magae et al. 1996; Muller et al. 1997; Verona et al. 1997; Gaubatz et al. 2001). E2F4 and E2F5 are found in the nucleus when bound to the pocket proteins. E2F4, the major E2F species *in vivo*, interacts with all of the pocket proteins and E2F5 predominantly binds to p130 (Beijersbergen et al. 1994; Ginsberg et al. 1994; Ikeda et al. 1996; Moberg et al. 1996). Promoter occupancy experiments show that E2F4 and E2F5 are found at classic E2F target genes in association with pocket proteins and chromatin remodeling factors during G0/G1 (Takahashi et al. 2000; Rayman et al. 2002). Consistent with this observation, cells lacking both E2F4 and E2F5 are unable to arrest in G1 in response to $p16^{INK4a}$ expression (Degregori et al., 1997; Gaubatz et al., 2000; Lukas et al., 1996). E2F3b is less well understood, but it also associates with classic E2F-responsive promoters during G0/G1 and it is required to maintain the appropriate repression of the $p19^{ARF}$ tumor suppressor in primary mouse cells (Aslanian et al. 2004). Taken together, these findings indicate a role for the repressive E2Fs, and their associated pocket proteins, in enforcing the repression of critical growth regulatory genes.

Mutant mouse models have been an important tool in defining the role of pRB during tumorigenesis and development. Consistent with human genetics, $Rb^{+/-}$ mice develop tumors associated with loss of the remaining wild-type allele (Hu et al. 1994; Williams et al. 1994). pRB is also essential for murine development. $Rb^{-/-}$ mice 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). Studies with chimeric and conditional animals have demonstrated that the programmed cell death in the CNS and a considerable fraction of the erythroid defects are cell non-autonomous effects of pRB loss (Maandag et al. 1994; Williams et al. 1994; Lipinski et al. 2001; Ferguson et al. 2002; de Bruin et al. 2003; Wu et al. 2003). In fact, they are secondary consequences of a primary placental defect that is responsible for the embryonic death of the $Rb^{-/-}$ mice (Wu et al. 2003). The loss of pRB leads to uncontrolled proliferation of trophoblast stem cells in the labyrinth layer of the placenta, which results in decreased nutrient and gas exchange between the mother and developing fetus. When the early lethality of the $Rb^{-/-}$ animals is circumvented by providing a wildtype placenta, the rescued animals continue to exhibit ectopic proliferation in the lens, CNS and PNS and also display abnormalities in erythrocyte maturation and myogenesis (de Bruin et al. 2003; Wu et al. 2003). Taken together, these data show that pRB plays critical roles in cell cycle regulation, terminal differentiation and cell survival.

Studies with compound *Rb;E2f* mutant mice have illustrated that the activating E2Fs have significant roles in mediating the developmental phenotypes associated with pRB deficiency. Homozygous mutation of either *E2f1* or *E2f3* leads to an extension in lifespan in the $Rb^{-/-}$ mice. It also suppresses both the apoptosis and the unscheduled proliferation that occurs in the lens, CNS and PNS of the $Rb^{-/-}$ embryos, although the abnormalities in erythrocyte maturation or myogenesis persist (Tsai et al. 1998; Ziebold et al. 2001). These studies do not assess the roles of *E2f1* and *E2f3* in the extra-embryonic relative to the embryonic tissues of the $Rb^{-/-}$ animals. The phenotypes of the compound mutant mice, however, are widely interpreted to indicate that the activating E2Fs

act in a cell autonomous manner to promote the inappropriate proliferation of both the placental trophoblast stem cells and the embryonic lens, CNS and PNS.

We have investigated the functional relationship of E2F4, the major repressive E2F, and pRB. We previously reported that the absence of E2F4 in the $Rb^{+/-}$ mice results in a dramatic suppression of tumor formation (Lee et al. 2002). Our analysis reveals that, in contrast to the tumor setting, the absence of E2F4 decreases the lifespan of the *Rb*-deficient mice due to an exacerbation of the placental defect. Furthermore, E2F4 does not cooperate with pRB within the developing embryo. These findings clearly indicate that E2F4 functions in a distinct manner from the activating E2Fs in mediating the effects of pRB loss during normal development.

RESULTS

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 (Lukas et al. 1996; DeGregori et al. 1997; Takahashi et al. 2000; Rayman et al. 2002). Our recent data demonstrated that E2F4, which accounts for the majority of the endogenous pRB-, p107- and p130-associated E2F activity, significantly contributes to tumor formation in $Rb^{+/-}$ mice (Lee et al. 2002). We propose that the oncogenic activity of E2F4 reflects its ability to restrict the levels of p107 and p130 that are available to bind, and suppress, the activating E2Fs in place of pRB. It is formally possible, however, that E2F4 can act as a transcriptional activator in a manner analogous to the activating E2Fs. In order to better define the role of E2F4 in the growth suppressive properties of pRB, we have assessed the developmental consequences of E2F4 loss in $Rb^{-/-}$ mice.

$Rb^{-/-}$ animals die in mid-gestation from E13.5 to E15.5 (Clarke et al. 1992; Jacks et al. 1992; Lee et al. 1992). To analyze the effects of E2F4 deficiency on the *Rb* mutant mice, we set up double heterozygous crosses on a mixed C57Bl/6 x 129/Sv background. Since $E2f4^{-/-}$ mice are viable to adulthood, we began to assess embryo viability at E13.5, the beginning of the window of lethality for the $Rb^{-/-}$ mice. Consistent with previous findings, the $Rb^{-/-}$ mice were present at the expected frequency with most of the animals found alive (Table 1). We also detected the $Rb^{-/-}$;$E2f4^{+/-}$ embryos at the expected Mendelian ratio, indicating that the loss of one wild-type allele of *E2f4* has no effect on the survival of $Rb^{-/-}$ animals at this developmental stage. In contrast, the $Rb^{-/-}$;$E2f4^{-/-}$ embryos were clearly underrepresented at E13.5; arising at approximately one-third of the expected frequency. The high mortality rate of the double knockout animals is specifically

attributed to the additional loss of E2F4 since the vast majority of the *Rb* homozygous mutants are alive at this age. Thus, the combined loss of pRB and E2F4 results in early embryonic death prior to E13.5.

To ascertain the timing of lethality for the *Rb*$^{-/-}$;*E2f4*$^{-/-}$ mice we looked at progressively earlier times in development. At E11.5, the *Rb*$^{-/-}$;*E2f4*$^{-/-}$ embryos arose at roughly the expected frequency, but slightly more than half of the embryos were found dead, as assessed by the absence of a heartbeat (Table 1). All other genotypes were present at the expected frequency, confirming that loss of E2F4 reduces the lifespan of the *Rb*$^{-/-}$ embryos. At E10.5, a larger proportion (~70%) of the double knockout animals had a detectable heartbeat and at E9.5 viable *Rb*$^{-/-}$;*E2f4*$^{-/-}$ embryos were present at the expected frequency. Therefore, the *Rb*$^{-/-}$;*E2f4*$^{-/-}$ animals die between E10 and E14, significantly earlier than the *Rb*$^{-/-}$ mice (E13.5-E15.5).

Although homozygous loss of E2F4 dramatically shifted the window of lethality of the *Rb* homozygous mutant animals, the phenotypic consequences on embryonic development were negligible. First, gross morphological and histological examination detected the same spectrum of defects in the *Rb*$^{-/-}$;*E2f4*$^{-/-}$ animals that had previously been reported for each of the single homozygous mutants. The *Rb*$^{-/-}$ and *E2f4*$^{-/-}$ embryos appear paler than their wild-type littermates at E13.5 as a result of erythrocyte differentiation defects (Jacks et al. 1992; Humbert et al. 2000; Rempel et al. 2000). The *Rb*$^{-/-}$;*E2f4*$^{-/-}$ animals showed a similar pallid coloration. Furthermore, *Rb*$^{-/-}$;*E2f4*$^{-/-}$ embryos were smaller than littermate controls as previously reported for the *E2f4* mutants (Figure 1A) (Humbert et al. 2000; Rempel et al. 2000). Apart from these characteristic *Rb*$^{-/-}$ and *E2f4*$^{-/-}$ phenotypes, the compound mutant mice did not display any novel morphological defects.

Table 1. Offspring viability from $Rb^{+/-}E2f4^{+/-}$ intercrosses

Age	Total # Embryos	$Rb^{-/-};E2f4^{+/+}$		$Rb^{-/-};E2f4^{+/-}$		$Rb^{-/-};E2f4^{-/-}$	
		# alive/obs	# expected	# alive/obs	# expected	# alive/obs	# expected
E13.5	201	11/14	13	24/26	25	3/4	13
E11.5*	366	15/18	23	46/49	46	14/26	23
E10.5*	386	24/27	24	43/53	48	13/19	24
E9.5*	103	2/2	6	13/15	13	7/9	6

* Offspring viability was assessed by the presence of a heartbeat under a stereomicroscope.

We also examined the *Rb*$^{-/-}$;*E2f4*$^{-/-}$ embryos for evidence of the inappropriate S phase entry and apoptosis, which are well established features of the E13.5 *Rb*$^{-/-}$ embryos (Lee et al. 1994; Morgenbesser et al. 1994; Macleod et al. 1996). For these experiments, we assayed the level of BrdU incorporation and TUNEL staining in wildtype, *E2f4*$^{-/-}$, *Rb*$^{-/-}$ and *Rb*$^{-/-}$;*E2f4*$^{-/-}$ embryos (Figure 1B, C). At E11.5, a time when a significant proportion of the double knockouts are viable, the *Rb*$^{-/-}$ embryos have elevated levels of BrdU-positive cells in the intermediate zone of the hindbrain (CNS) and the dorsal root ganglia (PNS) compared to control and *E2f4*$^{-/-}$ animals (Figure 1D and data not shown). There was no alteration in the levels of BrdU-positive cells between the *Rb*$^{-/-}$ and *Rb*$^{-/-}$;*E2f4*$^{-/-}$ mice. We also quantified the number of proliferating cells in the lens vesicle. There was no change among control, *Rb*$^{-/-}$ and *Rb*$^{-/-}$;*E2f4*$^{-/-}$ animals, which is most likely indicative of the fact that the lens is actively developing and cells are just beginning to withdraw from the cell cycle around E11.5 (Kaufman 1995). Taken together, these findings suggest that E2F4 does not contribute to the inappropriate proliferation observed in the absence of pRB.

We also assessed the levels of apoptosis in the animals. At E11.5, there were increased numbers of apoptotic cells in the hindbrain in the *Rb* mutant embryos relative to control littermates. This elevated level of apoptosis persisted to the same extent in the *Rb*$^{-/-}$;*E2f4*$^{-/-}$ mice (data not shown). These findings were confirmed at E13.5, a developmental stage when this phenotype is considerably enhanced in the *Rb*$^{-/-}$ mice. Consistent with previous studies, there were significant amounts of programmed cell death in the hindbrain near the fourth ventricle, trigeminal ganglia (PNS) and ocular lens of the *Rb*$^{-/-}$ mice, compared to control and *E2f4*$^{-/-}$ littermates (Figure1C and data not shown). We found that the degree of apoptosis was relatively unchanged in the *Rb*$^{-/-}$;*E2f4*$^{-/-}$ mice from the *Rb* homozygous mutants. Thus, increased

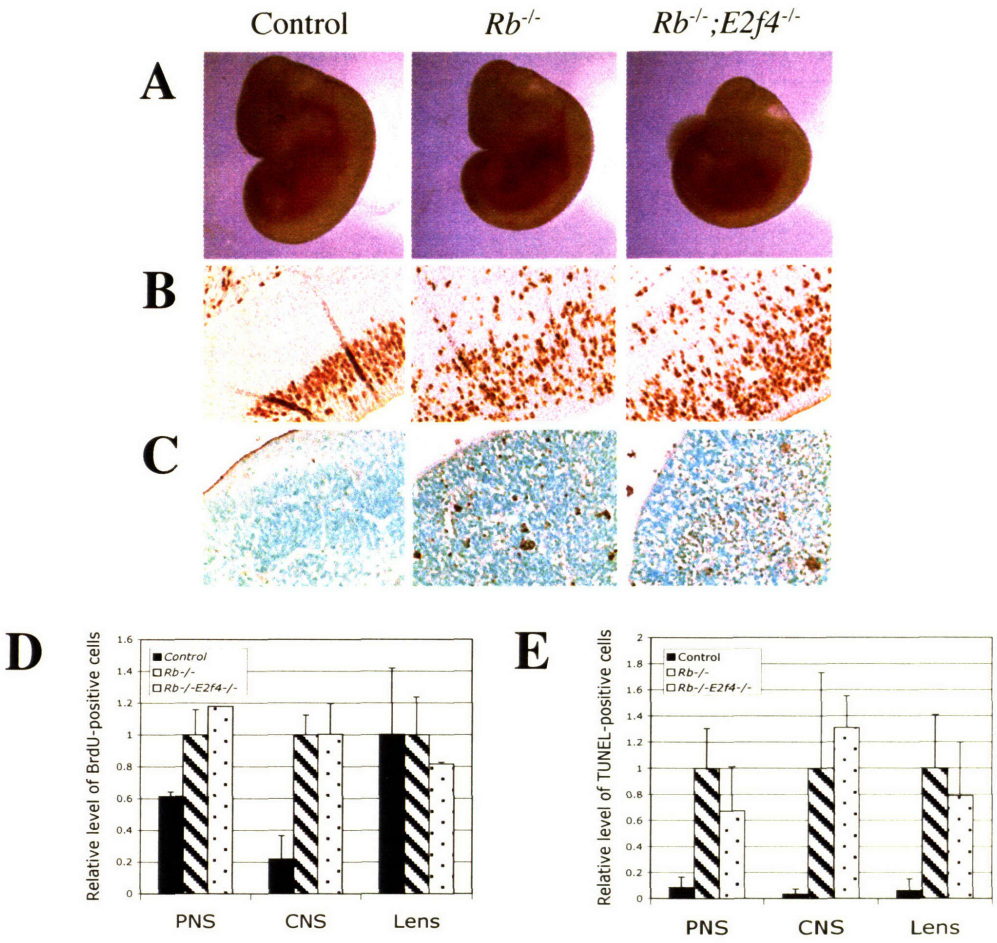

Figure 1. Loss of E2F4 does not affect inappropriate proliferation and apoptosis in the *Rb*$^{-/-}$ embryos.

A) Control, *Rb*$^{-/-}$ and *Rb*$^{-/-}$;*E2f4*$^{-/-}$ mice at E11.5. B) Sagittal sections of the hindbrain near the fourth ventricle in control and mutant embryos at E11.5 are stained for BrdU incorporation to identify S phase cells. In the control, BrdU-positive cells are restricted to the ventricular zone while in the *Rb*$^{-/-}$ and *Rb*$^{-/-}$;*E2f4*$^{-/-}$ sections BrdU-positive cells are present in the intermediate zone, indicating ectopic proliferation. Magnification, 40x. C) Transverse sections of the hindbrain near the fourth ventricle in control and mutant embryos at E13.5 are stained for apoptotic cells. There are increased levels of TUNEL-positive cells in the *Rb*$^{-/-}$ and *Rb*$^{-/-}$;*E2f4*$^{-/-}$ sections. Magnification, 40x. D) Quantitation of BrdU-positive cells in control and mutant animals at E11.5. The elevated levels of proliferating cells in the PNS (trigeminal ganglia) and CNS (hindbrain) in the *Rb* mutants are unchanged with the additional loss of E2F4. In contrast, the amounts of S phase cells in the developing lens are similar in control and mutant animals. For the PNS and lens, the overall percentage of BrdU-positive cells was quantified. For the CNS, BrdU-positive cells in the intermediate zone were counted. E) Quantitation of TUNEL-positive cells in control and mutant animals at E13.5. The elevated levels of apoptotic cells in the PNS (dorsal root ganglia), CNS (hindbrain), and ocular lens in the *Rb* mutants are unchanged with the additional loss of E2F4. All data are represented relative to the percentages observed in *Rb* mutants (+ standard deviation), which were set to 1.

programmed cell death does not contribute to the reduced survival potential of the double knockout animals.

Extra-embryonic tissue defect in *Rb*$^{-/-}$;*E2f4*$^{-/-}$ embryos

Since there were no clear indications as to the cause for early lethality of the *Rb*$^{-/-}$;*E2f4*$^{-/-}$ animals upon examination of the embryos, we analyzed the extra-embryonic tissues. pRB has been found to have a critical role in placental development (Wu et al. 2003). Loss of pRB results in hyperproliferation of trophoblast stem cells in the labyrinth layer of the placenta, leading to reduced nutrient and gas delivery to the embryo. Consistent with this finding, the *Rb*-related defect was highly penetrant at E13.5 in our crosses (Figure 2A, B). At earlier times, E10.5 and E11.5, the defect in the labyrinth was variable and subtle in many instances (Figure 3B). Histological examination at E11.5 revealed that loss of E2F4 enhanced the labyrinth layer defect in the *Rb*-deficient background and had no discernable effect on the other placental layers (Figure 3B). In contrast to the *Rb*$^{-/-}$ extra-embryonic tissues, a considerable proportion (about 60%) of *Rb*$^{-/-}$;*E2f4*$^{-/-}$ placentas had a decreased villous appearance of the labyrinth layer. In addition, the yolk sacs of the compound mutants were not well vascularized (Figure 3A). At E13.5, the placental defect was more apparent and was detectable in every *Rb*$^{-/-}$;*E2f4*$^{-/-}$ placenta examined (Figure 2A, B). There was a profound reduction in size and number of fetal and maternal blood cavities, which was associated with a smaller and more compacted labyrinth layer in the double mutant placentas. Since the *Rb*$^{-/-}$;*E2f4*$^{-/-}$ embryos are as small as *E2f4*$^{-/-}$ mice at E13.5, we compared the sizes of their placentas and found that the labyrinth layer of the double mutants was smaller than those of *E2f4*$^{-/-}$ littermates (data not shown). Therefore, the decrease in placental size is not just a consequence of growth retardation, but also reflects the defective nature of the placenta. Taken together, these

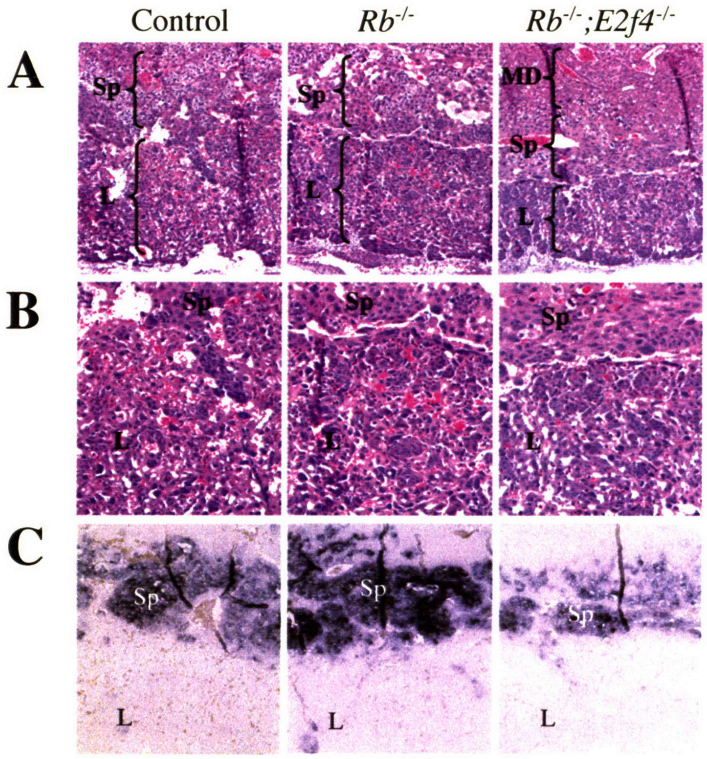

Figure 2. The defect in the *Rb*$^{-/-}$ extra-embryonic tissues is exacerbated in *Rb*$^{-/-}$;*E2f4*$^{-/-}$ mice at E13.5.

A and B) Hematoxylin and eosin stained placental sections of control and mutant littermates at a magnification of 10x and 20x, respectively. The labyrinth layer (L) is highly villous in control sections while the loss of pRB results in a compact appearance. In the *Rb*$^{-/-}$;*E2f4*$^{-/-}$ placentas, this layer is denser and has a further reduction in blood spaces when compared to the *Rb*$^{-/-}$ sections. C) *In situ* hybridization for 4311 (Tpbp) indicates that the spongiotrophoblast layer (Sp) is relatively normal in the mutant placentas. Magnification, 10x. MD, maternal decidua.

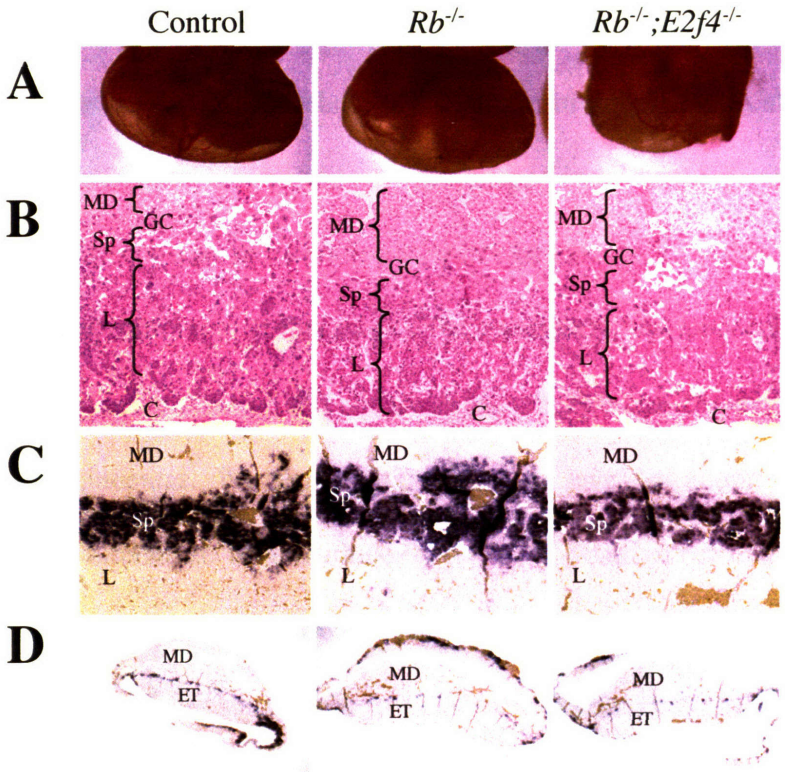

Figure 3. Loss of E2F4 aggravates the placental defect in the *Rb* mice at E11.5.

A) Control yolk sacs are highly vascularized. In contrast, there is a decrease in the number of blood vessels in the *Rb* mutant yolk sacs and 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 labyrinth layer (L) defect in the $Rb^{-/-}$ placenta is subtle. In the $Rb^{-/-};E2f4^{-/-}$ placentas, this layer is slightly smaller and has a noticeably compact appearance. C) *In situ* hybridization for 4311 (Tpbp) indicates that the differentiation of the cells in the spongiotrophoblast layer (Sp) is relatively unaffected in the mutant placentas. Magnification, 10x. D) *In situ* hybridization for placental lactogen-1 indicates that trophoblast giant cell (GC) differentiation is comparable in the control and mutant placentas. Magnification, 2x. MD, maternal deciduas; C, chorionic plate.

data reveal that the early lethality of the *Rb*$^{-/-}$;*E2f4*$^{-/-}$ embryos is due to placental insufficiency since E2F4 loss aggravates the labyrinth layer defect in the *Rb*$^{-/-}$ placentas.

In order to determine whether the compact appearance of the labyrinth layer is a result of excessive cell division, we assessed the levels of proliferation in the placentas at E11.5. The percentage of cells undergoing DNA synthesis was unchanged in all of the genotypes examined (data not shown). Yet, inappropriate proliferation may not be detectable at this time due to the active formation of the labyrinth layer at this stage. The extremely low frequency with which viable *Rb*$^{-/-}$;*E2f4*$^{-/-}$ embryos arise at E13.5 did not allow us to assess the levels of proliferating cells in the compound mutant placentas. We further determined the levels of apoptosis in the E11.5 placentas. There were no alterations in the number of TUNEL-positive cells between wild-type, *Rb*$^{-/-}$, and *Rb*$^{-/-}$;*E2f4*$^{-/-}$ placentas (data not shown). These results indicate that the placental defect in the *Rb*$^{-/-}$;*E2f4*$^{-/-}$ animals at E11.5 does not result from an overall change in the levels of proliferation or apoptosis within the labyrinth.

To further dissect the placental defect, we compared the expression of several placental layer specific differentiation markers in the various genotypes. Due to the small number of *Rb*$^{-/-}$;*E2f4*$^{-/-}$ embryos at E13.5, our analysis of these samples was limited to *in situ* hybridization. In contrast, the E13.5 *Rb*$^{-/-}$ and control littermates, and all pertinent E11.5 genotypes were examined by both quantitative real-time PCR and *in situ* hybridization. There was no significant difference in the expression levels of two trophoblast giant cell markers, placental lactogen-1 (Pl-1) and proliferin-1 (Plf-1), in *Rb*$^{-/-}$ and *Rb*$^{-/-}$;*E2f4*$^{-/-}$ placentas versus the wildtype or *E2f4*$^{-/-}$ control littermates by real-time PCR (Figure 4A, B and data not shown). This finding was confirmed by *in situ* hybridization, which further showed that the spatial expression of Pl-1 was equivalent in all of the genotypes examined (Figure 3D). Similarly, there was no difference in either the expression

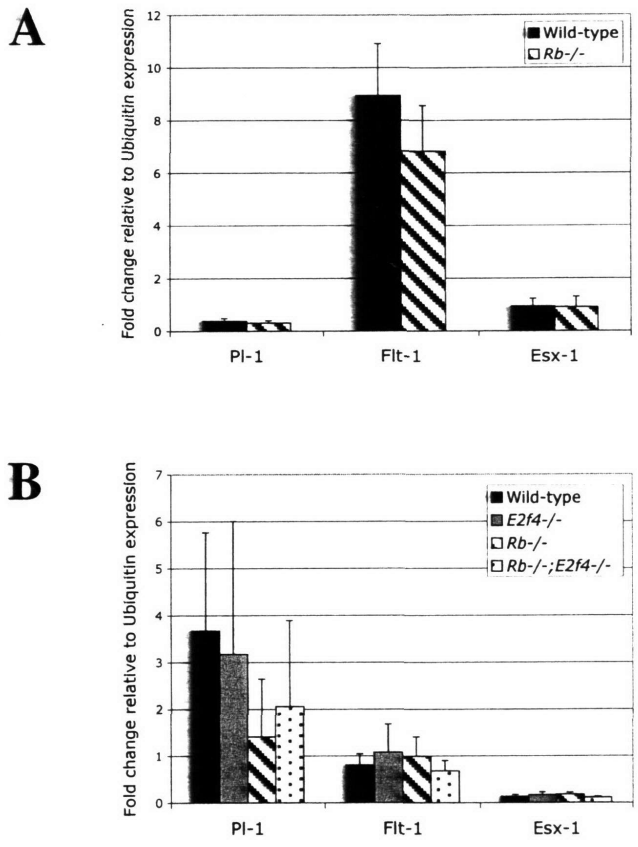

Figure 4. mRNA expression levels for placental layer-specific markers are unchanged in mutant placentas.

A and B) At E13.5 and E11.5, respectively, 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(labyrinthine 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.

levels of two spongiotrophoblast markers, 4311 (Tpbp) and Flt-1 (Figure 4A, B and data not shown), or the *in situ* hybridization pattern of 4311 (Figure 2C and Figure 3C), between the various genotypes . We were unable to detect a good *in situ* hybridization signal for a labyrinth marker. Yet, quantitative real-time PCR showed that Esx-1, a labyrinth trophoblast marker, was expressed at similar levels in wildtype, *E2f4*$^{-/-}$, *Rb*$^{-/-}$, and *Rb*$^{-/-}$;*E2f4*$^{-/-}$ embryos (Figure 4A, B). In combination with the hematoxylin and eosin staining of the placentas, these results confirm that the trophoblast giant cell and spoingiotrophoblast layers are not adversely affected by the loss of E2F4 and/or pRB. Moreover, the defect in the *Rb*$^{-/-}$;*E2f4*$^{-/-}$ placentas is not associated with a defect in trophoblast differentiation in the labyrinth layer.

Expression profile of mutant placentas

To determine the molecular basis of the placental defect, we analyzed the transcriptional expression profiles of the *Rb*$^{-/-}$ and *Rb*$^{-/-}$;*E2f4*$^{-/-}$ extra-embryonic tissues. We began by comparing the expression profiles of wild-type and *Rb*$^{-/-}$ placentas at E13.5, the time when the placental defect is clearly evident in most of *Rb*$^{-/-}$ samples. Using the Affymetrix whole mouse genome (430 2.0) microarray, we identified 135 differentially expressed genes with a $\geq$ 2-fold difference and an adjusted p-value < 0.05 in the *Rb*$^{-/-}$ tissues. Of these genes, 71 were induced while 64 were repressed (Table 2A and 2B). As an internal control, *Rb* was detected as a significantly repressed gene.

Consistent with the classical view that pRB loss promotes E2F transcriptional activity, a number of classic E2F-responsive genes were significantly upregulated in the *Rb* mutant placentas. These included MCM2, MCM3, MCM6, MCM7, cyclin E1, cyclin E2 and E2F1. Importantly, we were able to confirm the induction of all of these E2F-target genes using real-time PCR (Figure 5A

and data not shown). These results provide direct support for the previous conclusion that loss of pRB results in increased proliferation at E13.5 (Wu et al., 2003). Due to the small amount of E13.5 *Rb*$^{-/-}$;*E2f4*$^{-/-}$ animals, we compared the transcript levels of the same cell cycle genes in wild-type, *E2f4*$^{-/-}$, *Rb*$^{-/-}$ and *Rb*$^{-/-}$;*E2f4*$^{-/-}$ placentas at E11.5. At this earlier time point, we did not detect any significant differences (Figure 5B). This finding is entirely consistent with our BrdU immunohistochemistry data, which did not detect any difference on the level of proliferating cells between wild-type and mutant placentas at E11.5.

The most induced gene in *Rb*$^{-/-}$ placentas, compared to wild-type tissues, was not a classic E2F-responsive gene. Instead, it was *developmental pluripotency associated 5* (*Dppa5*), which is also known as *embryonal stem cell specific gene 1* (*Esg-1*). This gene is specifically expressed in embryonic stem (ES) cells and in the trophoectoderm, which gives rise to most cell lineages in the placenta (Astigiano et al. 1991; Bierbaum et al. 1994; Tanaka et al. 2002; Bortvin et al. 2003; Ahn et al. 2004). In contrast, Dppa5 is not expressed in lineage-committed stem cells (i.e., neural stem cells and trophoblast stem cells) or somatic cells. Thus, the expression pattern of Dppa5 denotes it as a marker for pluripotency, but its function is currently unknown. Quantitative real-time PCR corroborated our microarray data, as there was approximately 10 times more Dppa5 mRNA in *Rb*$^{-/-}$ placentas than in wild-type tissues (Figure 5C). Furthermore, Dppa5 was barely expressed in wild-type trophoblast stem cells, in accordance with previous reports (data not shown; Tanaka et al., 2002). Importantly, the levels of Dppa5 were also increased (approximately 4 fold) in extra-embryonic tissues derived from *Rb*$^{-/-}$ and *Rb*$^{-/-}$;*E2f4*$^{-/-}$ versus wild-type and *E2f4*$^{-/-}$ animals at E11.5 (Figure 5D). Thus, the upregulation of Dppa5 is detected at, or before, the placental defect can be consistently observed in the *Rb*$^{-/-}$ embryos. Based on these observations, we conclude that there is

Table 2A. Significantly induced genes in E13.5 *Rb*$^{-/-}$ placentas identified by microarray analysis

Probe set	Gene Title	Fold change	Adj p-value
1418199_at	hemogen	4.733	1.71E-19
1447360_at	transforming growth factor beta 1 induced transcript 4	4.636	1.71E-19
1416552_at	developmental pluripotency associated 5	4.197	1.71E-19
1420773_at	deubiquitinating enzyme 1	3.859	1.71E-19
1420720_at	neuronal pentraxin 2	3.451	1.71E-19
1438239_at	midline 1	3.061	1.71E-19
1450555_at	testis expressed gene 13	2.786	1.95E-11
1460570_at	RIKEN cDNA 2900019M05 gene	2.547	6.58E-08
1433623_at	zinc finger protein 367	2.473	6.48E-09
1419014_at	Rhesus blood group-associated A glycoprotein	2.289	1.30E-04
1444454_at	Transcribed sequences	2.237	5.23E-07
1442334_at	Transcribed seq, similarity to sp:O95522 (H.sapiens) YA02_HYPOTHETICAL PROTEIN DJ1198H6.2	2.179	4.06E-07
1427603_at	cDNA sequence BC018510	2.158	3.88E-05
1425753_a_at	uracil-DNA glycosylase	2.133	3.53E-05
1435998_at	Similar to chromosome 14 open reading frame 18 isoform a; enhancer of invasion 10 (LOC239083)	2.104	1.29E-05
1418283_at	claudin 4	2.087	1.05E-11
1419348_at	parotid secretory protein	2.048	3.18E-10
1438852_x_at	minichromosome maintenance deficient 6 (MIS5 homolog, S. pombe) (S. cerevisiae)	1.980	4.65E-08
1443961_at	expressed sequence AU017962	1.956	1.31E-04
1458659_at	Transcribed sequences	1.935	1.60E-08
1452590_a_at	hypothetical LOC211623	1.932	6.12E-07
1419571_at	solute carrier family 28 (sodium-coupled nucleoside transporter), member 3	1.819	9.30E-04
1456005_a_at	BCL2-like 11 (apoptosis facilitator)	1.770	2.85E-03
1422628_at	RIKEN cDNA 4632417K18 gene	1.727	3.87E-05
1419570_at	solute carrier family 28 (sodium-coupled nucleoside transporter), member 3	1.725	3.88E-05
1435275_at	cytochrome c oxidase subunit VIb, testes-specific	1.713	7.22E-03
1449061_a_at	DNA primase, p49 subunit	1.706	3.82E-07
1420719_at	testis expressed gene 15	1.658	7.82E-03
1431554_a_at	annexin A9	1.641	5.09E-03
1452458_s_at	peptidylprolyl isomerase (cyclophilin) like 5	1.629	1.60E-03
1418203_at	phorbol-12-myristate-13-acetate-induced protein 1	1.608	1.11E-06
1449960_at	neuronal pentraxin 2	1.574	2.46E-04
1423809_at	transcription factor 19	1.561	2.63E-03

Probe set	Gene Title	Fold change	Adj p-value
1436708_x_at	minichromosome maintenance deficient 4 homolog (S. cerevisiae)	1.542	1.74E-04
1424638_at	cyclin-dependent kinase inhibitor 1A (P21)	1.530	1.27E-02
1419172_at	dihydrofolate reductase	1.515	5.06E-05
1416326_at	cysteine-rich protein 1 (intestinal)	1.504	6.71E-04
1434734_at	RIKEN cDNA E130016E03 gene	1.495	4.41E-02
1449052_a_at	DNA methyltransferase 3B	1.488	2.59E-03
1417938_at	RAD51 associated protein 1	1.487	4.73E-03
1420028_s_at	minichromosome maintenance deficient 3 (S. cerevisiae)	1.486	3.10E-05
1418600_at	Kruppel-like factor 1 (erythroid)	1.483	1.67E-02
1416251_at	minichromosome maintenance deficient 6 (MIS5 homolog, S. pombe) (S. cerevisiae)	1.479	3.55E-05
1416641_at	ligase I, DNA, ATP-dependent	1.475	1.66E-04
1449708_s_at	checkpoint kinase 1 homolog (S. pombe)	1.464	6.78E-03
1418369_at	DNA primase, p49 subunit	1.439	2.53E-05
1423877_at	chromatin assembly factor 1, subunit B (p60)	1.434	1.82E-02
1418719_at	RIKEN cDNA 2410004L22 gene	1.432	2.62E-02
1426652_at	minichromosome maintenance deficient 3 (S. cerevisiae)	1.425	2.87E-04
1422944_a_at	diaphanous homolog 3 (Drosophila)	1.416	5.98E-03
1456280_at	RIKEN cDNA E130314M08 gene	1.414	2.98E-03
1428304_at	RIKEN cDNA 2410004I17 gene	1.410	2.58E-02
1416492_at	cyclin E1	1.406	2.12E-04
1434695_at	RIKEN cDNA 2810047L02 gene	1.373	6.65E-03
1448899_s_at	RAD51 associated protein 1	1.373	2.22E-02
1452598_at	RIKEN cDNA 2810418N01 gene	1.369	1.06E-02
1415945_at	minichromosome maintenance deficient 5, cell division cycle 46 (S. cerevisiae)	1.368	3.46E-04
1449705_x_at	minichromosome maintenance deficient 3 (S. cerevisiae)	1.347	5.03E-04
1425837_a_at	CCR4 carbon catabolite repression 4-like (S. cerevisiae)	1.346	6.90E-04
1417541_at	helicase, lymphoid specific	1.345	4.35E-04
1428738_a_at	DNA segment, Chr 14, ERATO Doi 449, expressed	1.339	1.15E-04
1422535_at	cyclin E2	1.338	1.75E-04
1439269_x_at	MCM7 (S. cerevisiae)	1.335	5.79E-03
1435114_at	RIKEN cDNA D630024B06 gene	1.331	4.81E-03
1435217_at	up-regulated in Myc liver	1.327	4.62E-02
1452912_at	RIKEN cDNA 2600005O03 gene	1.322	2.37E-02
1424608_a_at	xanthine dehydrogenase	1.321	5.09E-03

Probe set	Gene Title	Fold change	Adj p-value
1436808_x_at	minichromosome maintenance deficient 5, cell division cycle 46 (S. cerevisiae)	1.302	3.48E-05
1432566_at	RIKEN cDNA 1700129I15 gene	1.301	1.50E-03
1452715_at	RIKEN cDNA 2310022K01 gene	1.299	4.58E-02
1423653_at	ATPase, Na+/K+ transporting, alpha 1 polypeptide	1.297	1.46E-02
1434079_s_at	minichromosome maintenance deficient 2 mitotin (S. cerevisiae)	1.296	4.17E-03
1422430_at	fidgetin-like 1	1.294	2.37E-03
1421278_s_at	spectrin alpha 1	1.266	1.78E-02
1417926_at	RIKEN cDNA 5830426I05 gene	1.258	2.50E-02
1418281_at	RAD51 homolog (S. cerevisiae)	1.256	1.13E-03
1450677_at	checkpoint kinase 1 homolog (S. pombe)	1.238	4.09E-02
1418264_at	SoxLZ/Sox6 leucine zipper binding protein in testis	1.229	1.33E-02
1416915_at	mutS homolog 6 (E. coli)	1.226	1.18E-02
1439562_at	RIKEN cDNA F730047E07 gene	1.224	3.29E-02
1441910_x_at	cyclin E1	1.212	1.99E-03
1448777_at	minichromosome maintenance deficient 2 mitotin (S. cerevisiae)	1.210	3.33E-03
1424953_at	cDNA sequence BC021614	1.207	3.76E-02
1435710_at	expressed sequence AI661384	1.203	1.16E-02
1437309_a_at	replication protein A1	1.201	8.86E-04
1416802_a_at	RIKEN cDNA 2610036L13 gene	1.185	1.36E-02
1445849_at	RIKEN cDNA A430089I19 gene	1.170	6.25E-03
1424607_a_at	xanthine dehydrogenase	1.168	4.11E-05
1426687_at	mitogen activated protein kinase kinase kinase 3	1.156	2.66E-02
1426242_at	polymerase (RNA) II (DNA directed) polypeptide A	1.153	4.51E-06
1455990_at	kinesin family member 23	1.149	8.76E-03
1426473_at	DnaJ (Hsp40) homolog, subfamily C, member 9	1.141	5.75E-03
1421731_a_at	flap structure specific endonuclease 1	1.138	1.93E-03
1426817_at	antigen identified by monoclonal antibody Ki 67	1.130	5.91E-03
1438320_s_at	minichromosome maintenance deficient 7 (S. cerevisiae)	1.126	6.36E-03
1439012_a_at	deoxycytidine kinase	1.121	2.30E-02
1422016_a_at	centromere autoantigen H	1.117	2.39E-02
1424143_a_at	retroviral integration site 2	1.101	6.78E-03
1424609_a_at	xanthine dehydrogenase	1.095	2.27E-05
1436390_a_at	Mid-1-related chloride channel 1	1.094	2.34E-02
1416118_at	RIKEN cDNA 2310035M22 gene	1.065	1.82E-02
1439436_x_at	inner centromere protein	1.056	4.60E-02

Probe set	Gene Title	Fold change	Adj p-value
1416031_s_at	minichromosome maintenance deficient 7 (S. cerevisiae)	1.055	2.31E-02
1452543_a_at	secretoglobin, family 1A, member 1 (uteroglobin)	1.054	1.78E-02
1426731_at	desmin	1.050	1.45E-03
1423293_at	replication protein A1	1.046	2.64E-02
1424144_at	retroviral integration site 2	1.030	2.43E-02
1436459_at	G protein-coupled receptor 161	1.024	1.77E-02
1416030_a_at	minichromosome maintenance deficient 7 (S. cerevisiae)	1.014	1.00E-02
1436454_x_at	flap structure specific endonuclease 1	1.006	7.36E-03
1449033_at	tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	1.004	2.66E-02
1448393_at	claudin 7	1.001	3.34E-02

Table 2B. Significantly repressed genes in E13.5 *Rb*$^{-/-}$ placentas identified by microarray analysis

Probe set	Gene Title	Fold change	Adj p-value
1416646_at	alpha fetoprotein	-5.226	1.71E-19
1416645_a_at	alpha fetoprotein	-4.071	1.71E-19
1459737_s_at	transthyretin	-4.002	1.71E-19
1454608_x_at	transthyretin	-3.917	1.71E-19
1438840_x_at	apolipoprotein A-I	-3.787	1.71E-19
1436879_x_at	alpha fetoprotein	-3.784	1.71E-19
1419232_a_at	apolipoprotein A-I	-3.709	1.71E-19
1455201_x_at	apolipoprotein A-I	-3.588	1.71E-19
1435648_at	RIKEN cDNA B430119L13 gene	-3.191	1.84E-10
1419233_x_at	apolipoprotein A-I	-3.089	6.00E-17
1455913_x_at	transthyretin	-3.077	1.13E-14
1419725_at	solute carrier family 26, member 4	-2.909	1.71E-19
1451580_a_at	transthyretin	-2.726	3.13E-10
1417761_at	apolipoprotein A-IV	-2.466	1.65E-09
1418282_x_at	serine (or cysteine) proteinase inhibitor, clade A, member 1a	-2.462	1.71E-19
1458070_at	cDNA sequence BC032925	-2.445	7.90E-12
1417950_a_at	apolipoprotein A-II	-2.441	3.19E-06
1426990_at	cubilin (intrinsic factor-cobalamin receptor)	-2.367	1.00E-17
1452270_s_at	cubilin (intrinsic factor-cobalamin receptor)	-2.361	8.44E-12
1418689_at	cathepsin 6	-2.346	1.28E-09
1418310_a_at	retinaldehyde binding protein 1	-2.333	6.90E-08
1424265_at	N-acetylneuraminate pyruvate lyase	-2.277	1.61E-05
1436504_x_at	apolipoprotein A-IV	-2.273	8.89E-12
1426082_a_at	solute carrier family 16 (monocarboxylic acid transporters), member 4	-2.203	5.44E-12
1456418_at	Transcribed seq, sim to protein pir:A55119 (H.sapiens) A55119 protein romk-1	-2.200	1.54E-06
1416025_at	fibrinogen, gamma polypeptide	-2.182	4.90E-16
1434354_at	monoamine oxidase B	-2.174	1.00E-17
1415938_at	serine protease inhibitor, Kazal type 3	-2.107	3.00E-17
1418916_a_at	secreted phosphoprotein 2, 24kDa	-2.065	7.03E-13
1455593_at	apolipoprotein B	-2.053	1.03E-13
1428079_at	fibrinogen, B beta polypeptide	-2.003	1.81E-10
1424279_at	fibrinogen, alpha polypeptide	-1.993	1.60E-16
1432198_at	RIKEN cDNA 6330414G02 gene	-1.993	1.37E-08
1431701_a_at	PDZ domain containing 1	-1.990	1.47E-05
1448680_at	serine (or cysteine) proteinase inhibitor, clade A, member 1a	-1.980	1.71E-19
1421063_s_at	SNRPN upstream reading frame	-1.968	5.80E-03

Probe set	Gene Title	Fold change	Adj p-value
1424338_at	solute carrier family 6 (neurotransmitter transporter, GABA), member 13	-1.941	2.31E-03
1420553_x_at	serine (or cysteine) proteinase inhibitor, clade A, member 1a	-1.924	3.33E-11
1451513_x_at	serine (or cysteine) proteinase inhibitor, clade A, member 1a	-1.889	5.39E-14
1423436_at	glutathione S-transferase, alpha 3	-1.863	2.47E-09
1425260_at	albumin 1	-1.813	3.63E-10
1417850_at	retinoblastoma 1	-1.813	7.15E-06
1436101_at	RIKEN cDNA 2810473M14 gene	-1.792	2.78E-03
1429379_at	extra cellular link domain-containing 1	-1.788	2.38E-05
1431226_a_at	fibronectin type III domain containing 4	-1.783	6.90E-04
1418724_at	complement component factor i	-1.770	3.10E-11
1418370_at	troponin C, cardiac/slow skeletal	-1.747	2.45E-03
1419095_a_at	apolipoprotein M	-1.714	1.69E-06
1422837_at	sciellin	-1.631	3.55E-05
1426547_at	group specific component	-1.600	3.18E-10
1435578_s_at	RIKEN cDNA C630028C02 gene	-1.576	9.78E-03
1439111_at	16 days neonate cerebellum cDNA, RIKEN full-length enriched library, clone:9630032E14 product:unknown EST, full insert sequence	-1.570	1.28E-02
1452424_at	G protein-coupled receptor 23	-1.568	6.26E-07
1425841_at	solute carrier family 26, member 7	-1.566	1.78E-02
1425546_a_at	transferrin	-1.552	7.69E-05
1426260_a_at	UDP-glucuronosyltransferase 1 family, member 2	-1.536	9.83E-07
1460541_at	expressed sequence AI643885	-1.536	6.23E-04
1418880_at	glial cell line derived neurotrophic factor family receptor alpha 3	-1.447	2.76E-04
1458401_at	RIKEN cDNA 4932438A13 gene	-1.445	2.63E-05
1431142_s_at	apoptosis-inducing factor (AIF)-like mitochondrion-associated inducer of death	-1.442	1.73E-02
1436279_at	solute carrier family 26, member 7	-1.427	1.79E-02
1440409_at	RIKEN cDNA 2210401J11 gene	-1.425	7.76E-07
1424808_at	laminin, alpha 4	-1.407	4.80E-02
1424901_at	glucosaminyl (N-acetyl) transferase 3, mucin type	-1.376	2.38E-05
1416776_at	crystallin, mu	-1.376	8.35E-04
1437259_at	solute carrier family 9 (sodium/hydrogen exchanger), member 2	-1.365	6.10E-03
1455193_at	zinc finger and BTB domain containing 8	-1.356	4.94E-02
1451297_at	gulonolactone (L-) oxidase	-1.345	3.65E-02
1432418_a_at	creatine kinase, mitochondrial 1, ubiquitous	-1.327	8.61E-04

Probe set	Gene Title	Fold change	Adj p-value
	Transcribed sequence with moderate similarity to protein pir:T00261 (H.sapiens) T00261		
1435292_at	hypothetical protein KIAA0603 - human	-1.298	3.65E-02
1425875_a_at	leptin receptor	-1.295	5.34E-03
1417079_s_at	lectin, galactose-binding, soluble 2	-1.289	1.00E-02
1450770_at	RIKEN cDNA 3632451O06 gene	-1.286	7.17E-03
1451426_at	DNA segment, Chr 11, Lothar Hennighausen 2, expressed	-1.275	2.71E-03
1421641_at	solute carrier family 6 (neurotransmitter transporter, noradrenalin), member 2	-1.268	1.34E-02
1424649_a_at	transmembrane 4 superfamily member 3	-1.259	4.50E-03
1451738_at	O-linked N-acetylglucosamine (GlcNAc) transferase (UDP-N-acetylglucosamine:polypeptide-N-acetylglucosaminyl transferase)	-1.252	4.81E-02
1428223_at	RIKEN cDNA 1700018O18 gene	-1.248	1.25E-02
1425644_at	leptin receptor	-1.221	4.41E-02
1449077_at	erythroid associated factor	-1.220	6.78E-03
1451657_a_at	cytosolic ovarian carcinoma antigen 1	-1.214	3.21E-02
1439163_at	Transcribed sequence with weak similarity to protein ref:NP_079268.1 (H.sapiens) hypothetical protein FLJ12547 [Homo sapiens]	-1.196	4.01E-03
1427975_at	RIKEN cDNA 2210403B10 gene	-1.190	1.76E-02
1442025_a_at	expressed sequence AI467657	-1.187	3.62E-02
1429523_a_at	solute carrier family 39 (metal ion transporter), member 5	-1.183	1.83E-03
1426697_a_at	low density lipoprotein receptor-related protein associated protein 1	-1.176	4.44E-02
1416842_at	glutathione S-transferase, mu 5	-1.169	3.66E-02
1433514_at	ethanolamine kinase 1	-1.149	7.04E-03
1422852_at	calcium and integrin binding family member 2	-1.139	1.45E-02
1418352_at	hydroxysteroid (17-beta) dehydrogenase 2	-1.113	2.30E-02
1437213_at	Transcribed sequence with moderate similarity to protein ref:NP_008937.1 (H.sapiens) cleavage and polyadenylation specific factor 5, 25 kD subunit; pre-mRNA cleavage factor Im	-1.093	2.00E-02
1418069_at	apolipoprotein C-II	-1.089	6.61E-03
1449043_at	N-acetyl galactosaminidase, alpha	-1.084	1.78E-02
1429901_at	RIKEN cDNA 6330571D19 gene	-1.052	1.96E-02
1419403_at	cDNA sequence BC017612	-1.038	1.76E-03
1439995_at	expressed sequence C80638	-1.037	9.64E-03
1422723_at	stimulated by retinoic acid gene 6	-1.031	1.03E-02

Probe set	Gene Title	Fold change	Adj p-value
1459034_at	RIKEN cDNA D330023L08 gene	-1.026	2.50E-03
1421261_at	lipase, endothelial	-1.019	9.32E-03
1455093_a_at	alpha-2-HS-glycoprotein	-1.018	4.25E-02
1457551_at	Transcribed sequences	-1.016	3.23E-05
1455966_s_at	Transcribed sequence with moderate similarity to protein ref:NP_008937.1 (H.sapiens) cleavage and polyadenylation specific factor 5, 25 kD subunit; pre-mRNA cleavage factor Im	-1.009	2.40E-02

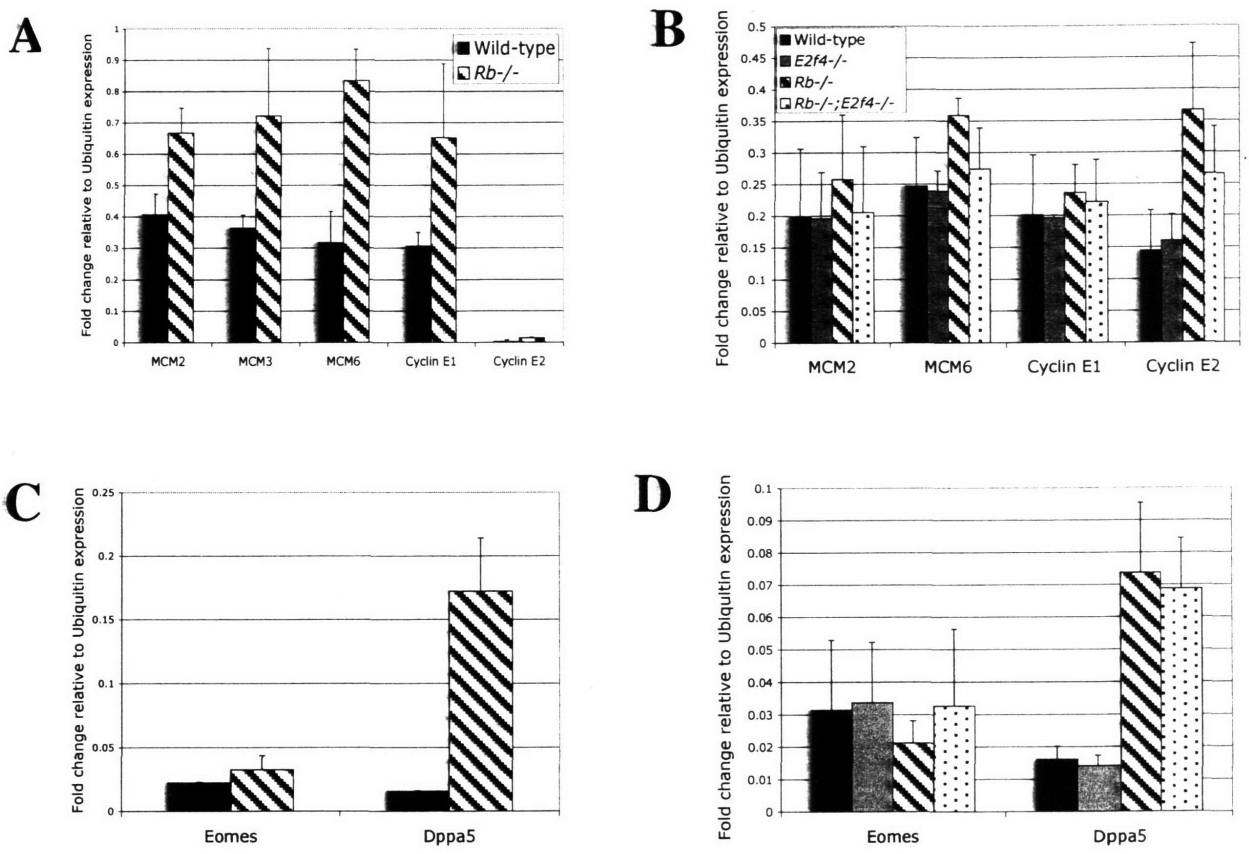

Figure 5. Confirmation of microarray data by quantitative real-time PCR analysis.

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, indicating that loss of pRB and/or E2F4 does not affect proliferation in this tissue at this time. C and D) Expression of Eomes (a trophoblast stem cell marker) and Dppa5 (a pluripotency marker) in wild-type and mutant placentas at E13.5 and E11.5, respectively. Eomes is expressed at similar levels among the various genotypes while Dppa5 expression is significantly elevated in $Rb^{-/-}$ and $Rb^{-/-};E2f4^{-/-}$ placentas relative to wild-type and $E2f4^{-/-}$ tissues. Gene expression levels are normalized to ubiquitin expression levels and error bars represent standard deviation.

a population of pluripotent cells in the *Rb* mutant placentas, which is absent or very minimally present in the wild-type placentas.

Wu et al. (2003) previously concluded that placental insufficiency in the *Rb* mutants results from the uncontrolled proliferation of trophoblast stem cells within the labyrinth layer. This was based on their ability to detect increased expression of Eomes, a trophoblast stem cell marker. In contrast with this finding, we did not detect any significant difference in Eomes expression between $Rb^{-/-}$ (either single or double mutant) and control placentas at either E11.5 and E13.5, as assessed by real-time PCR (Figure 5C, D). Moreover, Eomes was not identified as a differentially expressed gene in our microarray studies. Although these results indicate that Eomes is equivalently expressed in wild-type, $Rb^{-/-}$, $E2f4^{-/-}$, and $Rb^{-/-};E2f4^{-/-}$ placentas we cannot rule out the possibility that the localization of Eomes expressing cells is altered in the mutant tissues. Our data, however, suggest that the labyrinth layer defect of $Rb^{-/-}$ and $Rb^{-/-};E2f4^{-/-}$ placentas results from the presence of a misplaced pool of pluripotent cells rather than an increased population of trophoblast stem cells.

For a number of the differentially expressed genes that were analyzed, the mRNA expression levels in the double knockout placentas paralleled those in the *Rb* mutants while the wild-type and $E2f4^{-/-}$ placentas exhibited comparable levels (Figure 4, 5 and data not shown). These findings suggest that these genes are influenced by pRB and not E2F4. Presumably, different genes are affected by the simultaneous loss of pRB and E2F4 and cause the intensified placental defect. In order to identify these genes, we performed microarray analyses on wild-type and $Rb^{-/-};E2f4^{-/-}$ placentas at E11.5. We chose this time point because a significant proportion of viable double mutant animals can be recovered at this stage. Using the same arrays and thresholds as before, we identified 14 significantly induced genes and 30 repressed genes in the $Rb^{-/-};E2f4^{-/-}$

placentas compared to wild-type tissues. Of the 14 induced genes, 3 were also induced in expression profiles from the E13.5 $Rb^{-/-}$ placentas. Furthermore, 4 of the 30 repressed genes were in the previous array. The complete table of differentially expressed genes is listed in Table 3A and 3B. None of the E2F-responsive cell cycle genes were differentially expressed in the E11.5 $Rb^{-/-};E2f4^{-/-}$, placentas, supporting our real-time PCR and proliferation index results. Dppa5 was still one of the most induced genes in the compound mutant placentas, which is also consistent with our real-time PCR analysis (Figure 5D). Further analysis is necessary to validate the candidates that arose from these microarrays, as well as to determine whether differential expression is a consequence of losing pRB and/or E2F4.

Early lethality is rescued in conditionally mutant animals

In order to determine whether the placental defect is the primary cause of death in the $Rb^{-/-};E2f4^{-/-}$ embryos, we generated conditional mice resulting in double mutant embryos provided with normal functioning placentas. Using a similar strategy employed by (Wu et al. 2003), we bred $Rb^{2lox/2lox};E2f4^{+/-}$ mice to an epiblast-specific *Cre* line, MORE (Mox2Cre) (Tallquist and Soriano 2000; MacPherson et al. 2003). Although *Cre* expression from the Mox2 promoter is detectable in the embryo proper by E6, recombination efficiency is variable (Tallquist and Soriano 2000; Hayashi et al. 2002). Despite the possibility for embryo-specific genetic mosaics, the majority of extra-embryonic tissues do not exhibit *Cre* activity, allowing us to establish whether early lethality associated with simultaneous loss of pRB and E2F4 results from an aggravated placental defect.

$Rb^{1lox/1lox};Mox2^{cre/+}$ mice are found dead at birth (Wu et al. 2003). We also found that all $Rb^{1lox/1lox};Mox2^{cre/+}$ mice, irrespective of the *E2f4* genotype, were underrepresented at birth. We believe this is partially due to episodes of cannibalism, since the *Rb* mutant animals have a

Table 3A. Significantly induced genes in E11.5 *Rb*$^{-/-}$;*E2f4*$^{-/-}$ placentas identified by microarray analysis

Probe set	Gene Title	Fold change	Adj p-value
1429287_a_at	prolactin	3.247	0.0E+00
1443961_at	Similar to THO complex subunit 4 (Tho4) (RNA and export factor binding protein 1) (REF1-I) (Ally of AML-1 and LEF-1) (Aly/REF)	2.679	0.0E+00
1416552_at	developmental pluripotency associated 5	2.441	0.0E+00
1460613_x_at	growth hormone	2.208	0.0E+00
1427346_at	ovary testis transcribed	2.207	0.0E+00
1437522_x_at	growth hormone	2.119	0.0E+00
1456595_x_at	growth hormone	2.019	0.0E+00
1420773_at	deubiquitinating enzyme 1	1.954	0.0E+00
1436936_s_at	inactive X specific transcripts	1.871	0.0E+00
1415897_a_at	microsomal glutathione S-transferase 1	1.618	5.9E-04
1427262_at	inactive X specific transcripts	1.613	0.0E+00
1423859_a_at	prostaglandin D2 synthase (brain)	1.578	0.0E+00
1418766_s_at	T-cell immunoglobulin and mucin domain containing 2	1.565	1.5E-04
1425220_x_at	cDNA sequence AF067061	1.498	3.6E-02
1456655_at	Exostoses (multiple) 1	1.437	0.0E+00
1427263_at	inactive X specific transcripts	1.387	0.0E+00
1438512_at	epididymal protein Av381126	1.313	1.9E-03
1453287_at	RIKEN cDNA 5730557B15 gene	1.285	0.0E+00
1420720_at	neuronal pentraxin 2	1.128	5.0E-02
1421041_s_at	glutathione S-transferase, alpha 2 (Yc2)	1.096	1.6E-03
1435217_at	Hypothetical LOC236262 /// Hypothetical LOC385178	1.068	3.2E-02
1429982_at	RIKEN cDNA 4933426K21 gene	1.048	0.0E+00
1416676_at	kininogen 1	1.025	2.0E-05

Table 3B. Significantly repressed genes in E11.5 *Rb*$^{-/-}$;*E2f4*$^{-/-}$ placentas identified by microarray analysis

Probe set	Gene Title	Fold Change	Adj p-value
1417850_at	retinoblastoma 1	-2.849	0.00E+00
1426438_at	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, Y-linked	-2.417	0.00E+00
1425182_x_at	kallikrein 9 /// kallikrein 22	-2.314	0.00E+00
1452077_at	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, Y-linked	-2.272	0.00E+00
1426439_at	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, Y-linked	-2.243	0.00E+00
1424749_at	WD repeat and FYVE domain containing 1	-2.063	1.00E-10
1419725_at	solute carrier family 26, member 4	-2.026	0.00E+00
1417210_at	eukaryotic translation initiation factor 2, subunit 3, structural gene Y-linked	-1.931	0.00E+00
1424903_at	jumonji, AT rich interactive domain 1D (Rbp2 like)	-1.923	0.00E+00
1418310_a_at	retinaldehyde binding protein 1	-1.754	2.72E-04
1435648_at	RIKEN cDNA B430119L13 gene	-1.695	2.61E-04
1426225_at	retinol binding protein 4, plasma	-1.573	3.28E-03
1425841_at	solute carrier family 26, member 7	-1.530	4.07E-02
1438325_at	ecotropic viral integration site 1	-1.511	1.46E-04
1427308_at	RIKEN cDNA C630028C02 gene	-1.507	1.41E-02
1448291_at	matrix metalloproteinase 9	-1.496	1.20E-09
1450188_s_at	lipase, endothelial	-1.489	1.02E-03
1426598_at	ubiquitously transcribed tetratricopeptide repeat gene, Y chromosome	-1.459	0.00E+00
1423135_at	thymus cell antigen 1, theta	-1.435	2.34E-07
1437093_at	dynein, axonemal, intermediate chain 1	-1.419	1.00E-10
1452656_at	zinc finger, DHHC domain containing 2	-1.400	8.18E-03
1421262_at	lipase, endothelial	-1.399	1.75E-03
1451933_a_at	cathepsin 7	-1.390	5.00E-10
1426263_at	immunoglobulin superfamily, member 4C	-1.338	4.61E-02
1453485_s_at	RIKEN cDNA 1110005A03 gene	-1.320	7.93E-04
1435577_at	RIKEN cDNA C630028C02 gene	-1.291	0.00E+00
1451862_a_at	perforin 1 (pore forming protein)	-1.276	1.15E-08
1424477_at	cDNA sequence BC019731	-1.272	3.59E-02
1436544_at	ATPase, Class V, type 10D	-1.232	2.31E-02
1452655_at	zinc finger, DHHC domain containing 2	-1.202	2.75E-02
1452417_x_at	immunoglobulin kappa chain, constant region	-1.195	3.73E-05
1455735_at	adaptor-related protein complex AP-1, sigma 3	-1.151	4.16E-02
1452424_at	G protein-coupled receptor 23	-1.114	1.30E-02
1448729_a_at	septin 4	-1.093	7.91E-04
1455422_x_at	septin 4	-1.084	5.95E-04

Probe set	Gene Title	Fold Change	Adj p-value
1422643_at	monooxygenase, DBH-like 1	-1.044	4.11E-02
1429566_a_at	homeodomain interacting protein kinase 2	-1.039	7.66E-03
1423669_at	procollagen, type I, alpha 1	-1.037	3.40E-02
1450792_at	TYRO protein tyrosine kinase binding protein	-1.013	3.19E-02

reduced survival potential once born. To confirm that the $Rb^{1lox/1lox};Mox2^{cre/+}$ animals and the $Rb^{1lox/1lox};E2f4^{-/-};Mox2^{cre/+}$ mice developed to term, we assessed the frequency with which they arose at E18.5, one day prior to birth, as well as at earlier time points. Consistent with previous findings, the $Rb^{1lox/1lox};Mox2^{cre/+}$ mice were present at the expected Mendelian ratio at E13.5, E15.5 and E18.5 (Table 4). In contrast to the germline mutants, loss of *E2f4* did not reduce the viability of the $Rb^{1lox/1lox};Mox2^{cre/+}$ animals since the $Rb^{1lox/1lox};E2f4^{+/-};Mox2^{cre/+}$ and the $Rb^{1lox/1lox};E2f4^{-/-};Mox2^{cre/+}$ mice were recovered at the expected numbers. All of the $Rb^{1lox/1lox};Mox2^{cre/+}$ mice, regardless of *E2f4* status, were not able to survive upon removal from the uterus, because they could not begin or sustain breathing. Thus, the $Rb^{1lox/1lox};E2f4^{-/-};Mox2^{cre/+}$ mice had the same timing of lethality as the $Rb^{1lox/1lox};Mox2^{cre/+}$ mice. Moreover, these results support our findings that the reduced viability of the $Rb^{-/-};E2f4^{-/-}$ embryos is indeed due to an acute placental defect.

Since the conditional double mutant mice survive beyond E13.5, we were able to assess the consequences of pRB- and E2F4 loss on later stages of embryonic development. We assessed the efficiency of recombination in the mutant animals by western blot analysis, which confirmed that pRB expression was undetectable in $Rb^{1lox/1lox};Mox2^{cre/+}$ and $Rb^{1lox/1lox};E2f4^{-/-};Mox2^{cre/+}$ mouse embryonic fibroblasts (data not shown). Consistent with our analysis of the germline mutant embryos, gross morphological examination revealed that the rescued double knockout mice exhibited defects reminiscent of both the $E2f4^{-/-}$ and the $Rb^{1lox/1lox};Mox2^{cre/+}$ littermates. In general, the phenotypes were detectable in most animals by E13.5 and continued to birth. The $Rb^{1lox/1lox};E2f4^{-/-};Mox2^{cre/+}$ animals were similar in coloration, size and weight to the $E2f4^{-/-}$ littermates, which were paler and smaller than the control and $Rb^{1lox/1lox};Mox2^{cre/+}$ littermates (Figure 6A; data not shown). The $Rb^{1lox/1lox};E2f4^{-/-};Mox2^{cre/+}$ animals also had a hunched appearance as seen in the $Rb^{1lox/1lox};Mox2^{cre/+}$ mice, which was reflective of the altered spinal

Table 4. Offspring viability from $Rb^{2lox/2lox};E2f4^{+/-}$ x $Rb^{1lox/+};E2f4^{+/-};Mox2^{cre/+}$ crosses*

Age	Total # Embryos	$Rb^{1lox/1lox};E2f4^{+/+}$		$Rb^{1lox/1lox};E2f4^{+/-}$		$Rb^{1lox/1lox};E2f4^{-/-}$	
		# observed	# expected	# observed	# expected	# observed	# expected
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

* All animals represented in this table are $Mox2^{cre/+}$.

curvature of the skeletons (Figure 6B). Furthermore, all of the rescued *Rb*-deficient animals had misshapen ribs and absence of a muscle attachment site on the humerus, both consequences of abnormal musculature. The $Rb^{1lox/1lox};Mox2^{cre/+}$ animals had decreased muscle density associated with myoblast apoptosis (assessed by TUNEL staining), shortened myotubes and reduced muscle fibers in the skeletal muscle (Figure 6C). The extent of the defect was unchanged in the compound mutants. Finally, as discussed below, we analyzed the peripheral blood from the E18.5 animals since both E2F4 and pRB have been implicated to have roles in erythropoiesis (Figure 6E). Taken together, our data demonstrates that loss of E2F4 does not modulate the phenotypes of the $Rb^{1lox/1lox};Mox2^{cre/+}$ embryos and does not lead to additional phenotypes in the *Rb*-deficient background.

Although unscheduled proliferation is a cell autonomous effect of pRB loss, it has been demonstrated that the apoptosis observed in the nervous system, but not in the lens, is secondary 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 conditional double mutant animals. As determined from BrdU incorporation assays at E18.5, the levels of ectopic proliferation in the ocular lens and in the hindbrain (CNS) were unchanged in the $Rb^{1lox/1lox};E2f4^{-/-};Mox2^{cre/+}$ animals compared to the $Rb^{1lox/1lox};Mox2^{cre/+}$ mice (Figure 6D and data not shown). Furthermore, the degree of apoptosis in the lens paralleled that observed in the *Rb* single mutant embryos at E18.5 (data not shown). There were also significant levels of TUNEL-positive cells in the retina of $Rb^{1lox/1lox};Mox2^{cre/+}$ and the concomitant loss of E2F4 had no effect (data not shown). Consistent with our analyses of the $Rb^{-/-};E2f4^{-/-}$ embryos, E2F4 loss had no influence on the levels of ectopic proliferation and apoptosis in the $Rb^{1lox/1lox};Mox2^{cre/+}$ mice, suggesting that E2F4 does not participate in these processes regulated by pRB.

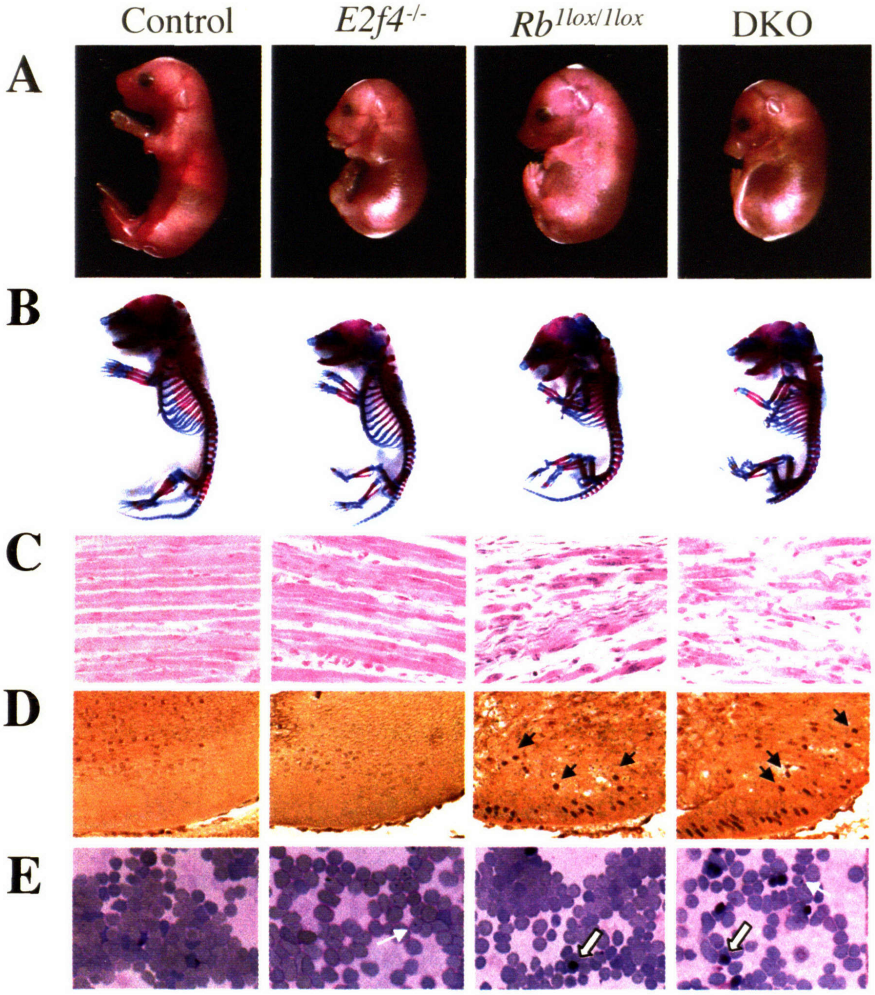

Figure 6. Conditional *Rb*$^{-/-}$;*E2f4*$^{-/-}$ mice exhibit a combination of *Rb* and *E2f4* mutant phenotypes at E18.5.

A) Control, *E2f4*$^{-/-}$, *Rb*$^{1lox/1lox}$;*Mox*$^{cre/+}$ (*Rb*$^{1lox/1lox}$) and *Rb*$^{1lox/1lox}$;*E2f4*$^{-/-}$;*Mox*$^{cre/+}$ (DKO) embryos. DKO embryos are growth retarded, as seen in the *E2f4*$^{-/-}$ mice, and have a hunched appearance similar to the *Rb*$^{1lox/1lox}$ animals. B) Skeletal staining with Alcian blue (cartilage) and Alizarin red (bone) of control and mutant embryos. The *Rb*$^{1lox/1lox}$ and DKO embryos have altered curvatures of the spine and ribcage. C) Hematoxylin and eosin stained skeletal muscle sections at a magnification of 40x. There is a decrease in muscle density and organization in the *Rb* mutants, which is unchanged with the additional loss of E2F4. D) Transverse sections of the ocular lens in control and mutant embryos are stained for BrdU incorporation to identify S phase cells. Darkly stained BrdU-positive cells were detected in the lens fiber cell compartment (indicated by the black arrows) in the *Rb*$^{1lox/1lox}$ and DKO lenses, but not in the control and *E2f4*$^{-/-}$ lenses. Magnification, 40x. E) Giemsa stained peripheral blood smears indicates the presence of abnormal erythrocytes in the absence of E2F4 and/or pRB. There is a considerable amount of erythroblasts containing Howell-Jolly bodies (white arrows) with the loss of E2F4 and the loss of pRB leads to a number of immature nucleated erythroblasts (outlined arrows).

E2F4 and pRB in erythropoiesis

E2F4 and pRB have demonstrated roles in erythroid differentiation. *E2f4*$^{-/-}$ mice exhibit severe fetal anemia from E13.5 to E16.5 (Humbert et al. 2000; Rempel et al. 2000). Analysis of the peripheral blood smears reveals a significant proportion of abnormal erythrocytes, including nucleated erythrocytes and cells containing Howell-Jolly bodies, which are remnants of DNA that have not been completely extruded during enucleation. *Rb*$^{-/-}$ mice also have an erythroid defect. They 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 mice and chimeras composed of *Rb*-deficient and wild-type cells have shown that a considerable amount of the red blood cell defect in *Rb*$^{-/-}$ animals is cell non-autonomous (Maandag et al. 1994; Williams et al. 1994; de Bruin et al. 2003). Despite the significant rescue, abnormalities in erythrocyte maturation persist in the conditional and chimeric animals, indicative of a cell autonomous role for pRB in erythropoiesis (de Bruin et al. 2003; Spike et al. 2004). Thus, the nature of pRB function in this process is not entirely clear. In order to determine whether pRB acts cell autonomously or non-autonomously in red blood cell development, we have investigated the effects of pRB deficiency in our conditional system. Furthermore, we have analyzed the effects of simultaneous loss of E2F4 and pRB with the aim of understanding whether these two proteins cooperate during erythroid differentiation.

To confirm that the erythroid defects in the *E2f4* and *Rb* mutants, we analyzed the peripheral blood of E18.5 embryos. Consistent with previous findings, the *E2f4*$^{-/-}$ animals were anemic at E18.5, as evidenced by a 30% reduction in hematocrit levels compared to those of wild-type littermates (p-value $< 3 \times 10^{-6}$; Figure 7A). The *Rb*$^{1lox/1lox}$;*Mox2*$^{cre/+}$ mice had a similar decrease in hematocrits (p-value < 0.02), supporting that pRB loss has a significant effect on erythrocyte

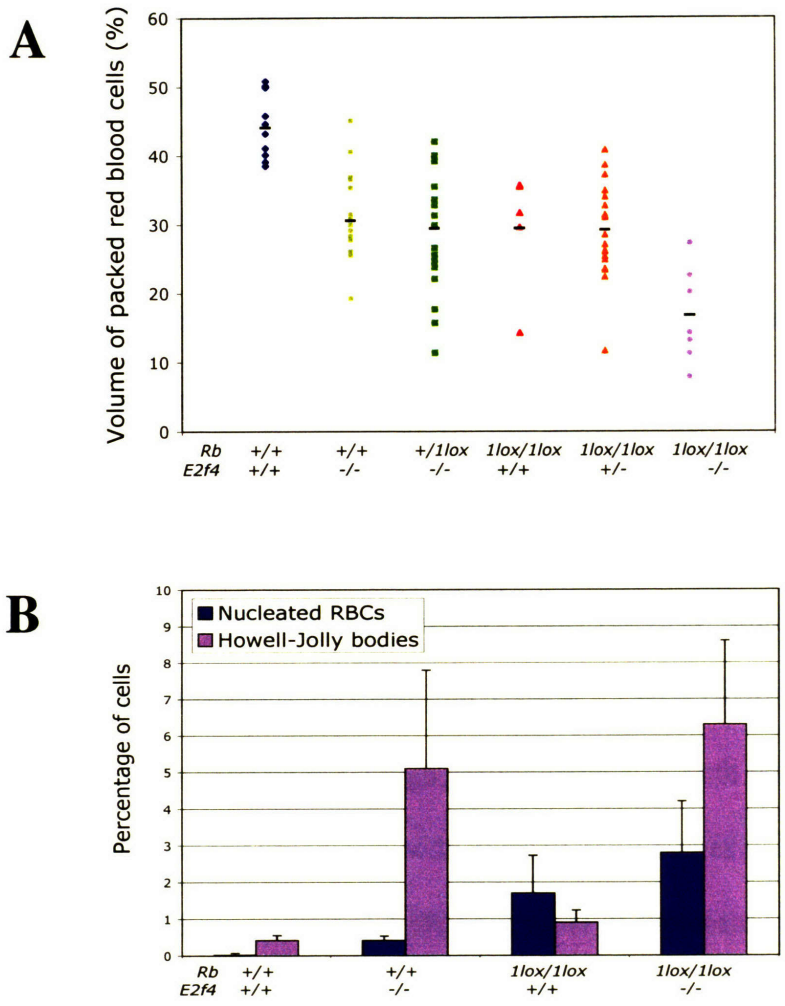

Figure 7. Loss of E2F4 and pRB results in anemia in E18.5 mice.

A) Hematocrits of peripheral blood from control and mutant mice. Loss of E2F4 or pRB leads to similar decreases in the volume of packed red blood cells and the conditional double mutants have a further reduction compared to wild-type embryos. Each data point represents the hematocrit value from a single animal and black bars (-) reflect the average hematocrit levels. B) Quantitation of abnormal erythrocytes from control and mutant Giemsa stained peripheral blood smears. The conditional double knockout animals have increased levels of nucleated red blood cells and erythrocytes containing Howell-Jolly bodies, which are additive effects of E2F4- and pRB-loss.

development. There was a further decrease (approximately 60%) in hematocrits of the *Rb*$^{1lox/1lox}$;*E2f4*$^{-/-}$;*Mox2*$^{cre/+}$ mice relative to wild-type animals (p-value < $5x10^{-6}$).

Cytological investigation of the peripheral blood cells at E18.5 revealed that the *Rb*$^{1lox/1lox}$;*E2f4*$^{-/-}$;*Mox2*$^{cre/+}$ animals had additive phenotypes of the *Rb*$^{1lox/1lox}$;*Mox2*$^{cre/+}$ and *E2f4*$^{-/-}$ mice (Figure 6E; Figure 7B). The vast majority of red blood cells in the wild-type animals are mature enucleated erythrocytes with a very low amount of immature nucleated cells (0.02% $\pm$ 0.05) and mature erythrocytes containing Howell-Jolly bodies (0.42% $\pm$ 0.13). In contrast, there was a modest increase in the level of erythrocytes containing Howell-Jolly bodies in the *Rb*$^{1lox/1lox}$;*Mox2*$^{cre/+}$ mice (0.9% $\pm$ 0.33) while there was a greater elevation in the number of nucleated red blood cells (1.7% $\pm$ 1.03). On the other hand, the *E2f4*$^{-/-}$ animals exhibited the converse (0.41% $\pm$ 0.12 nucleated erythrocytes and 5.1% $\pm$ 2.7 with Howell-Jolly bodies). We found that the double mutant animals had a high preponderance of both nucleated immature erythrocytes (2.8% $\pm$ 1.4) and red blood cells with Howell-Jolly bodies (6.3% $\pm$ 2.3), suggesting that there may be synergy between E2F4 and pRB during erythropoiesis.

Since erythrocyte defects are first evident during embryonic development in the *E2f4* and *Rb* single mutant mice, we analyzed erythropoiesis in the fetal livers, the site of the first wave of definitive erythropoiesis from E12 to late gestation (Car and Eng 2001). Colony formation assays revealed that the *E2f4*$^{-/-}$ and *Rb*$^{1lox/1lox}$;*E2f4*$^{-/-}$;*Mox2*$^{cre/+}$ fetal livers had a significant, and comparable, increase in erythroid progenitor cells, colony forming units (CFU-Es) and burst forming units (BFU-Es), compared to the wild-type fetal livers at E13.5 and E15.5 (data not shown). Yet, there was no difference in the number of progenitor cells in the *Rb*$^{1lox/1lox}$;*Mox2*$^{cre/+}$ fetal livers compared to that in wild-type littermates. This is in contrast to the initial characterization of the erythropoietic defect in the *Rb*$^{-/-}$ embryos, which demonstrated that there

was an increase in erythroid progenitors in $Rb^{-/-}$ fetal livers (Jacks et al. 1992). Although the increased erythropoiesis in the *Rb* germline mutants is secondary to the placental defect, the cytological analysis of E18.5 peripheral blood smears indicated that an erythrocyte maturation defect is present in the conditional *Rb* mutant animals.

In order to determine the precise stage at which red blood cell development is affected by E2F4 and/or pRB loss, we used a flow cytometry-based analysis to quantify the erythroblasts at the various differentiation states in E15.5 fetal livers (Zhang et al. 2003). As committed progenitor cells differentiate into erythrocytes, expression of the non-erythroid specific transferrin receptor (CD71) is induced initially and repressed thereafter, while the expression of the erythroid specific marker TER119 is very low at first and induced upon differentiation (Ikuta et al. 1990; Kina et al. 2000). This allows the stages of erythroid differentiation to be monitored by double labeling freshly isolated fetal liver cells for CD71 and TER119.

Consistent with our *in vitro* progenitor assays, the $E2f4^{-/-}$ and $Rb^{1lox/1lox};E2f4^{-/-};Mox2^{cre/+}$ fetal livers had a modest elevation in the $CD71^{med}TER119^{low}$ population, which is highly enriched for CFU-E progenitor cells, compared to wild-type controls (Table 5). $Rb^{1lox/1lox};Mox2^{cre/+}$ fetal livers had normal levels of progenitor cells, but there was a significant decrease in the proportion of basophilic erythroblasts ($CD71^{hi}TER119^{hi}$ population) and a considerable increase in the level of orthochromatophilic erythroblasts ($CD71^{med}TER119^{hi}$ population). This indicates that there is a block in the end stage of erythroid differentiation in the absence of pRB. Surprisingly, the erythroid differentiation profile of the $Rb^{1lox/+}$ fetal livers was similar to that observed for the $Rb^{1lox/1lox};Mox2^{cre/+}$ fetal livers, which may reveal that pRB functions in a haploinsufficient manner during erythrocyte development (data not shown). We observed the same trend, though to a lesser

Table 5. Flow cytometric analysis of erythrocyte development in E15.5 fetal livers

Genotype	$CD71^{med}TER119^{lo}$ (%)	$CD71^{hi}TER119^{lo}$ (%)	$CD71^{hi}TER119^{hi}$ (%)	$CD71^{med}TER119^{hi}$ (%)	$CD71^{lo}TER119^{hi}$ (%)
Differentiation Stage+	Unipotent stem cell	Proerythrocyte	Basophilic erythroblast	Orthochromatic erythroblast	Reticulocyte
Wild-type n=19	2.6 ± 0.4	2.9 ± 1.8	72.1 ± 8.7	16.4 ± 8.4	1.1 ± 0.6
E2f4$^{-/-}$ n=8	3.6 ± 1.1*	4.6 ± 2.5	72.3 ± 3.3	11.3 ± 3.0	0.8 ± 0.5
Rb$^{flox/flox}$ n=20	2.5 ± 0.5	2.4 ± 1.0	60.9 ± 13.0 **	28.0 ± 13.1 **	1.3 ± 0.9
Rb$^{flox/flox}$;*E2f4*$^{-/-}$ n=3	3.7 ± 0.5	2.8 ± 1.3	64.0 ± 12.0	22.3 ± 12.0	0.6 ± 0.2

+ The majority of cells represented by each population are in the indicated stage of erythrocyte development.

* denotes statistical significance, p-value = 0.03, compared to wild-type.

** denotes statistical significance, p-value < 0.005, compared to wild-type.

extent, in the $Rb^{1lox/1lox}$;$E2f4^{-/-}$;$Mox2^{cre/+}$ fetal livers. In general, the double mutants exhibited characteristics of both *E2f4* and *Rb* single mutant mice.

We next used an *in vitro* culture system that closely resembles erythrocyte development *in vivo* to determine whether erythroblast proliferation defects are associated with the differentiation abnormalities (Zhang et al., 2003). $TER119^-$ cells were purified from individual E15.5 fetal livers and cultured on fibronectin-coated plates in the presence of erythropoietin, which specifically supports the survival of erythroid cells, for one day. By two days in culture, most of the wild-type cells are in the end stages of erythropoiesis. Accordingly, cell cycle analysis demonstrated that the vast majority of wild-type cells had exited the cell cycle after two days, and there were low levels of cells in S and G2/M phases (Table 6). As previously demonstrated, there was a considerable increase in the $Rb^{1lox/1lox}$;$Mox2^{cre/+}$ cells in S phase and a mild, though significant, increase in the G2/M population. In conjunction with the erythroid differentiation profile of the $Rb^{1lox/1lox}$;$Mox2^{cre/+}$ fetal livers, our data is consistent with the notion that the significant accumulation of late stage erythroblasts results from a defect in terminal cell cycle exit. The failure to exit the cell cycle in the absence of pRB is concomitant with their failure to enucleate properly (data not shown).

Unexpectedly, we found that loss of E2F4 also resulted in a significant decrease in the proportion of cells in G0/G1 phases of the cell cycle relative to the wild-type erythroblasts (Table 6). In contrast to the $Rb^{1lox/1lox}$;$Mox2^{cre/+}$ cells, there was more than twice the number of $E2f4^{-/-}$ erythroblasts in both S and G2/M phases. Cytological analysis of the cultured erythroid cells showed that enucleated red blood cells formed in the absence of E2F4, but with increased cell size, consistent with the macrocytosis identified *in vivo* (data not shown; Humbert et al. 2000). Our data suggest that E2F4 is not necessarily acting in an analogous manner to pRB.

Table 6. Cell cycle analysis of erythroid cells isolated from E15.5 fetal livers

Genotype	G0/G1 (%)	S (%)	G2/M (%)
Wild-type, n=17	87.4 ± 3.1	7.5 ± 1.9	5.1 ± 1.7
E2f4$^{-/-}$, n=9	72.2 ± 6.0 **	15.5 ± 4.9 **	12.4 ± 3.1 **
Rb$^{1lox/1lox}$, n=16	76.4 ± 5.1 **	16.8 ± 3.8 *	6.9 ± 2.2 **
Rb$^{1lox/1lox}$*E2f4*$^{-/-}$, n=2	46.5 ± 1.4 **	37.6 ± 0.1 **	16.1 ± 1.5 **

* denotes statistical significance, p-value = 0.019, compared to wild-type.

** denotes statistical significance, p-value < 0.001, compared to wild-type.

This is further supported by the relatively normal differentiation profiles of the *E2f4*-/- fetal livers (Table 5). Taken together, these findings indicate that the absence of E2F4 does not result in an erythrocyte defect at a particular differentiation stage, but rather may be in cell cycle progression.

Currently, we have only been able to analyze erythroblasts from two $Rb^{1lox/1lox}$;$E2f4^{-/-}$;$Mox2^{cre/+}$ embryo. Undoubtedly, further samples will be needed, but our initial findings indicate that there are additive effects of E2F4- and pRB loss in the erythroid compartment. Therefore, these results suggest that E2F4 and pRB have distinct functions in erythropoiesis.

Recently, it has been proposed that the *Rb*-null defects in erythropoiesis are secondary to abnormalities in fetal liver macrophages (Iavarone et al. 2004). In order to determine if the erythrocyte abnormalities in the $Rb^{1lox/1lox}$;$Mox2^{cre/+}$ embryos are indeed a cell non-autonomous effect of improper differentiation of the macrophages, we quantitatively assessed the population of macrophages in E15.5 fetal livers by flow cytometric analysis. Among all of the genotypes examined, there was no difference in the distribution of macrophage progenitor cells, as determined by expression of CD31, an antigen on endothelial cells and macrophage progenitors (Figure 8A). We also found that there were no differences in the granulocyte and macrophage progenitors by colony formation assays with $Rb^{1lox/1lox}$;$Mox2^{cre/+}$ and wild-type fetal livers at E13.5 and E15.5 (data not shown). Upon analysis of F4/80 expression, a mature macrophage marker, there was a slight decrease in the proportion of F4/80-positive cells in the $Rb^{1lox/1lox}$;$Mox2^{cre/+}$ fetal livers compared to wild-type cells (Figure 8B). Although the mild reduction was statistically significant compared to wild-type littermates (p-value = 0.006), it is not nearly as dramatic as previously described in the germline *Rb* mutant fetal livers (Iavorone et al., 2004). Thus, the macrophage differentiation defect in the $Rb^{-/-}$ embryos appears to be largely due to the primary placental defect. Moreover, it does not seem likely that a minimal reduction in the amount of fully

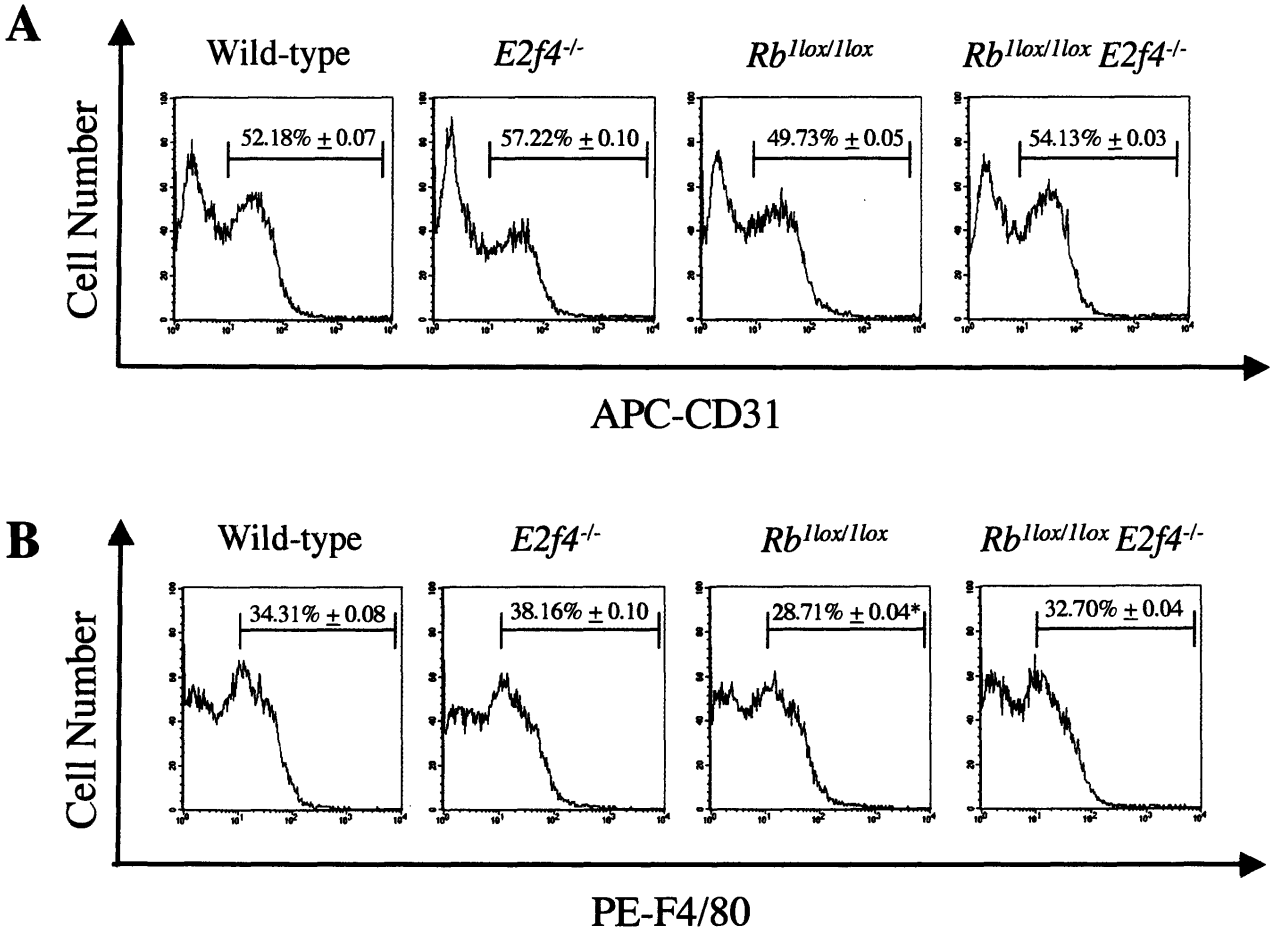

Figure 8. Macrophage differentiation is minimally affected in conditional *Rb* mutant animals.

A and B) Histogram representations of flow cytometry analysis of TER119$^-$ cells immunostained with Allophycocyanin (APC)-conjugated anti-CD31and phycoerythrin(PE)-conjugated anti-F4/80 antibodies, respectively. Among the various genotypes, there was no alteration in the proportion of macrophage progenitor cells, as determined by expression of CD31 (an antigen on endothelial cells and macrophage progenitors). There was a slight decrease in the proportion of F4/80-positive cells (a mature macrophage marker) in the $Rb^{1lox/1lox}$;$Mox2^{cre/+}$ fetal livers compared to wild-type littermates (*p-value = 0.006). All data represented as average values ± standard deviation.

differentiated macrophages is entirely responsible for the observed erythrocyte abnormalities in the conditional *Rb* mutant animals, since our erythroblast differentiation culture system is performed in the absence of macrophages. These findings support that pRB has a cell autonomous function in red blood cell development.

We performed the same analyses on *E2f4*$^{-/-}$ fetal livers and found that the microenvironment of the erythroblasts in these fetal livers was normal. Despite previous findings that there is an increase in monocyte and granulocyte populations in *E2f4*$^{-/-}$ bone marrow (Rempel et al. 2000), we did not detect any changes within the fetal livers, as judged by the expression of Mac1 (a monocyte and granulocyte differentiation marker) and Gr-1 (primarily a granulocyte differentiation marker, but also expressed on monocytes) (data not shown). When we examined the *Rb*$^{1lox/1lox}$;*E2f4*$^{-/-}$;*Mox2*$^{cre/+}$ fetal livers, they recapitulated the results for the *Rb*$^{1lox/1lox}$;*Mox2*$^{cre/+}$ embryos. Thus, all of the erythroid defects associated with *E2f4* and conditional *Rb* single mutant animals manifest in the conditional double homozygous mutants in a purely additive manner, illustrating that E2F4 and pRB do not cooperate during erythrocyte differentiation.

DISCUSSION

The E2F transcription factors are the most well studied downstream effectors of pRB. Compound mutant mouse models have been used to determine the specific roles of the individual E2Fs to the growth suppressive properties of pRB. In general, the phenotypic effects of pRB deficiency are largely dependent on the activating E2Fs (Tsai et al. 1998; Ziebold et al. 2001), consistent with their role in promoting cell cycle progression. In this study, we have assessed the contribution of E2F4, a repressive E2F, to the developmental consequences of pRB loss. We found that E2F4 is critical during development of the $Rb^{-/-}$ mice and the functional significance of the interaction between pRB and E2F4 was tissue-specific. Our analysis clearly supports that E2F4 has distinct properties from the activating E2Fs in mediating the effects of pRB loss during normal development.

pRB and E2F4 do not cooperate in the developing embryo

We previously demonstrated that E2F4 considerably contributes to tumor formation in $Rb^{+/-}$ mice (Lee et al. 2002). We proposed that E2F4 indirectly influences tumor progression by limiting the levels of p107 and p130 that can compensate for the absence of pRB. It is formally possible, however, that E2F4 could promote cellular proliferation by activating the transcription of E2F-responsive genes in a manner analogous to the activating E2Fs. E2F4 can act as a strong transcriptional activator when ectopically expressed and E2F4 has been found at some murine promoters during the G1 to S transition of the cell cycle (Beijersbergen et al. 1994; Ginsberg et al. 1994; Wells et al. 2000). Thus, if E2F4 functions to activate gene transcription, then E2F4 loss should suppress the developmental defects arising in the $Rb^{-/-}$ mice and result in a significant extension in lifespan, as observed in mice with simultaneous loss of an activating E2F and pRB

(Tsai et al. 1998; Ziebold et al. 2001). Instead, we found that the absence of E2F4 accelerated embryonic lethality in the $Rb^{-/-}$ mice. Furthermore, there was minimal, if any, cooperation between E2F4 and pRB in the developing embryo. Loss of E2F4 had no effect on the levels of inappropriate proliferation and apoptosis in the ocular lens, CNS and PNS in the *Rb*-deficient animals. Moreover, using a conditional deletion strategy, we found that loss of E2F4 did not modulate any defects associated with the absence of pRB in later stages of development, including inappropriate proliferation and apoptosis in the lens, as well as differentiation defects in the skeletal muscle and in erythroid cells. Thus, E2F4 does not have overlapping properties with the activating E2Fs in promoting the phenotypes in the embryo proper caused by pRB loss.

There is currently serious debate as to the nature of the role of pRB in erythrocyte development. Early studies demonstrated that *Rb*-null mice had defective definitive erythropoiesis in the fetal livers (Clarke et al. 1992; Jacks et al. 1992; Lee et al. 1992). Subsequent analyses of $Rb^{-/-};Rb^{+/+}$ chimeras showed that the presence of wild-type cells alleviated the erythroid abnormalities and, thus, the erythroid defect in the absence of pRB is a cell non-autonomous effect (Maandag et al. 1994; Williams et al. 1994). This was confirmed by the recent finding that the placental defect is largely responsible for the incomplete erythropoiesis in the *Rb* mutant mice (de Bruin et al. 2003; Wu et al. 2003). Although these results indicate that pRB loss in the erythroid compartment does not have as great an influence as initially believed, there are several lines of evidence to suggest that there is indeed a cell intrinsic function of pRB during red blood cell maturation. First, $Rb^{-/-}$ cells fail to differentiate properly in irradiated wild-type mice reconstituted with $Rb^{-/-}$ or $Rb^{-/-};Rb^{+/+}$ chimeric fetal liver cells (Hu et al. 1997; Spike et al. 2004). Second, the end stages of differentiation are perturbed in $Rb^{-/-}$ erythroid progenitors in the presence of wild-type cells, as well as upon acute deletion of *Rb in vitro* (Clark et al. 2004; Spike et al. 2004).

Third, *Rb* mutant mice provided with a normal placenta have a persistence of nucleated immature erythrocytes in the peripheral blood (de Bruin et al. 2003; Wu et al. 2003).

In the process of determining the interplay between pRB and E2F4 in erythrocyte development, we characterized the defects arising in the single and compound mutants. Although there was no data to suggest that pRB and E2F4 cooperate during this process, our analysis led to some new insights into pRB and E2F4 function. Our characterization of the erythroid defect in the conditional *Rb* mutant animals support the view that pRB has a cell autonomous function in erythrocyte development. In accordance with previous studies, we found that the increase in immature erythrocytes (at the normoblast stage) in the absence of pRB is linked to a cell cycle exit defect. These defects appear to be a cell intrinsic effect of pRB in the erythroblasts, and not entirely a consequence of abnormal macrophage differentiation as recently suggested by Iavarone et al. (2004). There was a substantial decrease in the proportion of mature macrophages in the conditional *Rb* mutant cells compared to wild-type cells. This indicates that the previously described reduction in macrophages in $Rb^{-/-}$ fetal livers is largely influenced by the placental defect and the $Rb^{-/-}$ macrophages that were previously analyzed had already acquired a defect from the hypoxic environment *in utero*. These data support that there is a cell autonomous effect of pRB in the erythroid cells. Future studies using macrophage-specific conditional deletion strategies will be helpful to clarify the role of pRB in this cell lineage and to determine the extent to which erythroid cell development is affected.

Unexpectedly, the $Rb^{1lox/+}$ fetal livers had erythroid differentiation defects similar to those observed in the conditional *Rb* mutants, suggesting that pRB can act in a haploinsufficient manner. Along the same lines, $Rb^{+/-}$ fetal liver cells had a diminished reconstitution potential compared to wild-type cells in adoptive transfer experiments (Spike et al. 2004). Yet, when we cultured the *Rb*

heterozygous cells, they became indistinguishable from wild-type cells. Since the erythroid differentiation profile is obtained from freshly isolated fetal liver cells and the cell cycle profile relies on erythroblasts that differentiate in culture, the properties may be affected by the varying differentiation environments. On the other hand, the cells completely lacking pRB have defects in both differentiation and cell cycle control, suggesting that these defects can be uncoupled in the $Rb^{+/-}$ fetal liver cells.

In contrast to pRB, E2F4 loss does not result in an erythrocyte defect at a particular differentiation stage, but rather seems to affect cellular proliferation. A significantly greater proportion of *E2f4*$^{-/-}$ erythroblasts were in the cell cycle at a time when most wild-type cells had undergone terminal cell cycle exit. This was not anticipated since the loss of E2F4 in MEFs does not lead to defects in cellular proliferation (Humbert et al. 2000; Rempel et al. 2000). It will be important to determine whether loss of E2F4 promotes cellular proliferation of the erythroblasts or causes a delay in cell cycle progression. Coupled with the increase in erythrocytes containing Howell-Jolly bodies, DNA fragments that have not been properly extruded from mature erythrocytes, lack of cell cycle control in the absence of E2F4 could be responsible for these abnormal red blood cells. Promoter occupancy experiments have shown that *Mad2* and *Bub3*, mitotic spindle checkpoint components, are specific targets of E2F4 (Ren et al. 2002). Thus, E2F4 deficiency may lead to the derepression of these genes and result in abnormal chromosome separation at the mitotic spindle, giving rise to red blood cells containing Howell-Jolly bodies. Alternatively, Howell-Jolly bodies can arise from a defect in enucleation, a process that has not been well characterized. Further work will be needed to explore these possibilities.

pRB and E2F4 are essential for extra-embryonic development

Recently it has been demonstrated that the *Rb* homozygous mutant mice die from placental insufficiency caused by a defect in the labyrinthine layer of the extra-embryonic tissues (Wu et al. 2003). We found that additional loss of E2F4 aggravated this phenotype as the labyrinth had a more compact appearance in the $Rb^{-/-};E2f4^{-/-}$ placentas compared to $Rb^{-/-}$ littermate placentas. Based on placental layer specific marker analysis, trophoblast differentiation in the three layers of the $Rb^{-/-};E2f4^{-/-}$ placentas was not affected, consistent with findings for $Rb^{-/-}$ placentas. We also determined the expression levels of Eomes, a trophoblast stem cell marker, since loss of pRB leads to its increased spatial expression and consequent hyperproliferation of trophoblast stem cells (Wu et al. 2003). We were unable to detect any significant differences in Eomes expression in wild-type, $E2f4^{-/-}$, $Rb^{-/-}$ and $Rb^{-/-};E2f4^{-/-}$ placentas by microarray and real-time PCR analyses. Since the previous report of increased Eomes expression was assessed by *in situ* hybridization, it is possible that the pattern of Eomes expression is merely altered in the mutant placentas while the overall expression levels are equivalent.

Although we found no evidence for an expanded trophoblast stem (TS) cell population, our data suggest that there is an atypical population of pluripotent cells in the *Rb* mutant placentas. We found that Dppa5 (Esg-1), a marker for pluripotency, was one of the most differentially expressed genes in the *Rb* mutant placentas compared to wild-type tissues. The function of Dppa5 is currently unknown, but its expression is limited to ES and trophoectoderm cells, but repressed in lineage-restricted stem cells and terminally differentiated cells (Astigiano et al. 1991; Bierbaum et al. 1994; Tanaka et al. 2002; Bortvin et al. 2003; Ahn et al. 2004). Since we did not detect any alterations in the expression of other markers that are unique to pluripotent ES cells, such as Oct3/4 and FGF4, the cell population expressing Dppa5 is not necessarily equivalent to ES cells.

In fact, the trophoectoderm, which only contributes to the placental tissues, has been reported to express equivalent levels of Dppa5 as observed in the inner cell mass (ICM), which gives rise to the embryo proper (Tanaka et al., 2002). Yet, in contrast to the ICM, Dppa5 induction in the trophoectoderm was not accompanied by expression of Oct3/4 and FGF4, indicating that the trophoectoderm is not analogous to the ICM. Thus, placental insufficiency due to loss of pRB may result from increased proliferation or atypical persistence of cells resembling the trophoectoderm within the labyrinth layer. Further support that loss of pRB leads to an increased pool of stem cell-like cells comes from observations with Id2, a transcriptional repressor that interacts with pRB. In the placenta, the human ortholog of *Id2* is highly expressed in the cytotrophoblasts, the equivalent of murine trophoblast stem cells, and is repressed as the cells differentiate (Jen et al. 1997; Janatpour et al. 2000). Loss of Id2 results in a significant extension in lifespan and a general suppression of defects in the $Rb^{-/-}$ mice (Lasorella et al. 2000). The requirement for Id2 was not assessed in the $Rb^{-/-}$ placentas; however, it is very likely that Id2 has an opposing effect to pRB in the undifferentiated trophoblasts, and, thus, the loss of Id2 results in developmental rescue of the *Rb* mutant embryos. Further work will be needed to determine the role of Id2 in the murine placenta, as well as to establish the functional consequences of increased expression of Dppa5 in *Rb* mutant placentas.

We expected that the genes differentially expressed in the *Rb* mutants would be further affected by the simultaneous loss of E2F4, if E2F4 and pRB are cooperating during placental development. E2f4 deficiency does not influence the expression levels of Dppa5 in the *Rb*-deficient background. In general, we found that mRNA expression levels of genes with enhanced or diminished expression in the *Rb* mutants were unchanged in the double knockout placentas, as assessed by real-time PCR. In order to assess the gene expression changes in the $Rb^{-/-};E2f4^{-/-}$ extra-

embyronic tissues more systematically, we used microarray analysis to determine the differential expression profiles from the double mutant placentas relative to the wild-type placentas. There were a number of potentially interesting candidates with demonstrated roles in placental development, such as Growth Hormone, Prolactin, and Matrix Metalloproteinase 9. Further work is required to determine whether their expression is regulated by pRB and/or E2F4 and how their deregulation contributes to placental insufficiency.

Our data is consistent with the notion that E2F4 and pRB may have independent roles in the placenta. In this case, accelerated placental failure due to concomitant loss of E2F4 and pRB would result from a combination of distinct defects. This is supported by our findings in the compound mutant embryos. First, in many tissues in the embryo proper, E2F4 was dispensable for the phenotypes caused by pRB loss. Furthermore, during erythropoiesis, a process where both E2F4 and pRB are documented to have functions, we found no evidence for crosstalk and the double mutant animals simply displayed additive defects. Second, expression analysis suggests that the pRB-related defect in the placenta is associated with an increase in a stem cell-like population which is unaffected by the additional loss of E2F4. Third, *E2f4* homozygous mutant embryos are growth retarded and exhibit fetal anemia with an increase in erythroid progenitor cells, which are characteristics often associated with inefficient placental function.

While it is formally possible that loss of E2F4 disturbs extra-embryonic tissue development, it remains unclear whether E2F4 in fact functions in the placenta. There were no obvious histological abnormalities in the *E2f4*$^{-/-}$ placentas. In addition, the erythropoietic defect in the *E2f4* mutant mice is a cell intrinsic effect (Humbert et al. 2000). Ultimately, these data do not rule out the possibility that E2F4 has a significant impact on placental development. Conditional deletion experiments with E2F4 would determine whether the reduced embryo size is a

consequence of *E2f4* loss in the placenta. Moreover, further analysis of our microarray results may yield E2F4-regulated target genes, which may also be helpful to establish a requirement for E2F4 in the placenta.

Alternatively, E2F4 and pRB may function in the same pathway during the development of the extra-embryonic tissues. The phenotype of the *Rb*$^{-/-}$;*E2f4*$^{-/-}$ animals is highly reminiscent of that observed for the *Rb*$^{-/-}$;*p107*$^{-/-}$ embryos. For example, the window of lethality for the *Rb*$^{-/-}$;*p107*$^{-/-}$ embryos is between E10.5 and E13 (Lee et al. 1996), which is comparable to that for the *Rb*$^{-/-}$;*E2f4*$^{-/-}$ mice. Although the cause of death in the *Rb*$^{-/-}$;*p107*$^{-/-}$ animals was not determined, the elevated levels of CNS apoptosis, the lack of defects in the embryo and the yolk sac abnormalities suggest a more severe defect in the extra-embryonic tissues compared to the *Rb*$^{-/-}$ embryos. The placentas from the *Rb*$^{-/-}$;*p107*$^{-/-}$ mice need to be examined; however, loss of E2F4 appears to have analogous effects as p107 deficiency in the *Rb*$^{-/-}$ embryos, suggesting that there is a critical requirement for repressive pocket protein-E2F complexes in the extra-embryonic tissues.

Despite the mechanism by which E2F4 and pRB promote embryonic development, it is clear that the pRB pathway plays a major role in the extra-embryonic tissues. In addition to our study, others have identified upstream and downstream regulators of pRB activity that have profound effects in the placenta upon targeted inactivation. $p57^{KIP2}$ and $p27^{KIP1}$, cyclin dependent kinase inhibitors, regulate the proliferation and differentiation of trophoblasts (Zhang et al. 1998; Takahashi et al. 2000). Furthermore, mice mutant for cyclin E1 and cyclin E2 exhibit endoreduplication defects in the trophoblast giant cells (Geng et al. 2003; Parisi et al. 2003). Mice mutant for DP1, the dimerization partner for E2F proteins, die in mid-gestation and have a significant reduction in trophoblast precursors (Kohn et al. 2003). The absence of DP1 would be expected to indirectly inactivate the E2Fs that require DP binding for E2F activity. Thus, by

extension, the E2Fs would be expected to function in the placenta as well. While we have demonstrated that E2F4 does indeed function in the placenta in association with pRB, it is very likely that the activating E2Fs also contribute to the development of the extra-embryonic tissues. Loss of an activating E2F may suppress, to some extent, the placental defect in the $Rb^{-/-}$ embryos, since the double mutant animals survive longer than the *Rb*-null mice and have an amelioration of the cell non-autonomous apoptosis in the CNS (Tsai et al. 1998; Ziebold et al. 2001). Studies addressing the role of the activating E2Fs in the placentas will be needed.

Many pathways regulating the development of the embryo proper are conserved in extra-embryonic development (reviewed in Cross et al. 2003). The pRB pathway is no exception and, thus, it will be important to understand whether pRB and its regulators have retained their established functions in cell cycle control and differentiation or whether they have novel functions in the placenta.

EXPERIMENTAL PROCEDURES

Mouse strains

E2f4$^{+/-}$ and *Rb*$^{+/-}$ mice were crossed to generate *Rb*$^{+/-}$;*E2f4*$^{+/-}$ mice on a mixed C57BL/6 x 129/Sv background (Jacks et al. 1992; Humbert et al. 2000). *Rb*$^{+/-}$;*E2f4*$^{+/-}$ mice were interbred to obtain *Rb*$^{-/-}$;*E2f4*$^{-/-}$ animals. The detection of a vaginal plug was considered E0.5. Embryos were harvested at the indicated times and viability was determined by the presence of a heartbeat under a stereomicroscope. Embryos and placentas were fixed in 10% formalin or 4% paraformaldehyde, embedded in paraffin and sectioned at 4-6μm. Sections were stained with hematoxylin and eosin for histological analysis.

Genetic rescue experiments were carried out by breeding *E2f4*$^{+/-}$, *Rb*$^{2lox/2lox}$, and *Mox2*$^{cre/+}$ mice (Humbert et al. 2000; Tallquist and Soriano 2000; Sage et al. 2003). *Mox2*$^{cre/+}$ transgenic mice were obtained from Jackson Laboratories. Timed *Rb*$^{2lox/2lox}$;*E2f4*$^{+/-}$ x *Rb*$^{1lox/+}$;*E2f4*$^{+/-}$;*Mox2*$^{cre/+}$ crosses were set up to obtain *Rb*$^{1lox/1lox}$;*E2f4*$^{-/-}$;*Mox2*$^{cre/+}$ mice at the indicated developmental ages. At E18.5, the ability to breathe was assessed upon removal from the yolk sac. Mice were fixed in Bouin's fixative or 10% formalin, embedded in paraffin and sectioned at 4-6μm. Sections were stained with hematoxylin and eosin for histological analysis.

Immunohistochemistry and skeletal staining

For bromodeoxyuridine (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 (Vector Labs) followed by 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. 2000).

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 digoxygenin-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. Sections were blocked in10% sheep serum/2% blocking reagent (Roche) and then incubated with an anti-digoxigenin antibody conjugated to alkaline phosphatase 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 3 independently prepared samples per genotype were used 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 normalized using GCRMA normalization method (Wu and Irizarry 2004). LPE test was applied for variance stabilization between the biological replicates (Jain et al. 2003) and statistical significance was determined using Benjamini-Hochberg False Discovery Rate rule (Benjamini et al. 2001). Differential expression of a gene was considered significant if the adjusted p-value < 0.05 and the fold change ≥ 2.0.

For real-time 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. Reactions were carried out with 50 ng cDNA, 16 μM primers, and SYBR Green PCR Master Mix in 20 μl total volume (Applied Biosystems). Reactions were carried out 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.

Purification and *in vitro* culture of erythroid progenitors

Fetal liver cells (FLC) were isolated from individual E15.5 embryos. FLC were mechanically dissociated by pipetting in PBS containing 2% fetal bovine serum (FBS). Single cell suspensions from each individual embryo were prepared by passing the dissociated cells through a 25 μm cell strainer. Alive cells were counted using 0.4% Trypan Blue (Cellgro). TER119$^-$ cells were purified and cultured as previously described (Zhang et al., 2003).

Immunostaining, cell cycle and flow cytometry analyses of erythroid differentiation

Freshly isolated FLC and cultured Day 2 cells were stained for CD71 and TER119 simultaneously as previously described (Zhang et al., 2003). For macrophage analysis, TER119$^-$ cells purified from individual embryos were immunostained with phycoerythrin(PE)-conjugated anti-F4/80 (1:100; Caltag Laboratories) and Allophycocyanin (APC)-conjugated anti-CD31 (1:100; BD Pharmingen) antibodies simultaneously. For cell cycle analysis, cultured erythroid cells were harvested on Day 2 and stained in hypotonic Propidium Iodide (PI) solution (0.1% sodium citrate and 50 mg/ml PI). Flow cytometry was carried out on a Becton Dickinson FACSCalibur (Franklin Lakes, NJ) and collected data was analyzed by ModFit software (Verity Software House, Inc., Topsham, ME).

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Chapter Four

Conclusions

Our understanding of the extent to which the E2F family of transcription factors contributes to the growth suppressive properties of pRB is limited to the activating E2Fs. This study is the first demonstration of the biological significance of the interplay between pRB and E2F4, a repressive E2F, in cell cycle regulation during normal development and tumorigenesis. In chapter two, we established that E2F4 loss has profound effects on tumor formation in the $Rb^{+/-}$ mice. Our molecular and biochemical analyses suggest a novel mechanism through which E2F4 may influence tumor progression. In chapter three, we found that E2F4 is critical during development of the $Rb^{-/-}$ mice and the functional impact of the interaction between pRB and E2F4 is tissue-specific. Our findings further indicate that E2F4 does not act in an equivalent manner to the activating E2Fs in mediating the effects of pRB loss. Thus, E2F4 is an important downstream effector of pRB function and this study has provided insights into the requirement for the various E2F proteins in the cellular processes regulated by pRB.

pRB and E2F4 in tumorigenesis

We assessed the consequences of E2F4 loss on the tumor phenotype in $Rb^{+/-}$ mice. Homozygous mutation of *E2f4* in the $Rb^{+/-}$ animals considerably suppressed tumor formation in the pituitary and thyroid glands and subsequently extended their lifespan. We also found that loss of E2F4 suppressed inappropriate proliferation in *Rb*-deficient MEFs. Molecular analyses detected novel complexes of p107 and p130 with the activating E2Fs in the absence of pRB and/or E2F4. These findings support a model whereby p107 and p130 compensate for pRB deficiency by re-establishing the proper regulation of the activating E2Fs. Therefore, E2F4 indirectly promotes tumor progression in the $Rb^{+/-}$ mice by restricting the levels of free p107 and p130. These data suggest that tumor formation is dependent on the relative levels of activating

E2F activity, instead of the widely held view that increased tumorgenicity is due to the loss of repressive pocket protein-E2F complexes.

Despite the fact that our proposed mechanism can account for the differential tumor suppressive properties of the pocket proteins, the functional relevance of the rearranged pocket protein-E2F complexes remains to be determined. It has not been technically feasible to assess the levels of the free activating E2Fs in the wild-type, $Rb^{+/-}$ and $Rb^{+/-};E2f4^{-/-}$ tissues due to the relatively low cellular levels of these proteins. Another way to substantiate our model would be to directly alter the levels of the compensatory pocket proteins in the $Rb^{+/-};E2f4^{-/-}$ background. If diminishing the levels of p107 and/or p130 did not affect tumor formation in the $Rb^{+/-};E2f4^{-/-}$ mice, then this would indicate that an alternative mechanism is at work. We have combined the *Rb*, *E2f4* and *p107* mutations with this aim; however, the results have been inconclusive thus far (Appendix A). Tumor development was not possible to analyze in the $Rb^{+/-};E2f4^{-/-};p107^{-/-}$ animals, because mice with homozygous mutations in the *E2f4* and *p107* genes had a severely reduced survival potential. In order to circumvent the early lethality, conditional knockout mice could be generated. Additionally, transgenic mice expressing the truncated SV40 large T antigen, T_{121}, in a tissue-specific manner could be used (Saenz Robles et al. 1994; Xiao et al. 2002; Simin et al. 2004). Since T_{121} results in the inactivation of all the pocket proteins, loss of E2F4 would be predicted to have no effect on tumor formation. Along the same lines, analogous experiments could be performed in MEFs. For example, acute loss of *Rb* in quiescent primary fibroblasts stimulates cell cycle re-entry due to an inability to induce *p107* in a timely manner (Sage et al. 2003). Based on our model, acute loss of *Rb* in quiescent *E2f4*-deficient MEFs should resemble $Rb^{-/-}$ cells and remain arrested, since the absence of E2F4 would liberate sufficient amounts of p107 and p130 to compensate for the abrupt loss of pRB.

A complementary approach to validate the complex rearrangement model would be to modulate the levels of the activating E2Fs. Loss of an activating E2F, E2F1 or E2F3, in $Rb^{+/-}$ mice also leads to suppression of the pituitary tumors (Yamasaki et al. 1998; Appendix C). Thus, decreasing the amounts of the activating E2Fs should have the same effect as the loss of E2F4 in *Rb*-deficient MEFs. Our preliminary analyses of the cell cycle properties of $Rb^{-/-};E2f1^{-/-}$, $Rb^{-/-};E2f3^{-/-}$ and $Rb^{1lox/1lox};E2f4^{-/-}$ primary fibroblasts have not been consistent (Appendix B), which may reflect the differing functions or expression levels of the E2Fs. Furthermore, we cannot rule out the possibility that another mechanism is responsible. Curiously, $Rb^{-/-};E2f1^{-/-}$, $Rb^{-/-};E2f3^{-/-}$ and $Rb^{1lox/1lox};E2f4^{-/-}$ cells transformed with activated Ras and Myc act in the same manner and are less tumorigenic than transformed $Rb^{-/-}$ cells. This may suggest that there is a differential requirement for the E2Fs and pRB in tumorigenesis than in normal cell cycle control. Further investigation is necessary to make any definitive conclusions, but these initial results indicate that loss of E2F4 acts in a cell autonomous manner to suppress the oncogenic capacity of $Rb^{-/-}$ cells. Since it has been difficult to process the results from the cell cycle characterizations, an additional method to test our model would be to knock down expression of p107 or p130 in the various compound mutant cells using RNAi technology. According to our model, this should lead to a reversion of the *Rb* mutant phenotype in the $Rb^{1lox/1lox};E2f4^{-/-}$ MEFs while there should not be a significant effect in the $Rb^{-/-};E2f3^{-/-}$ cells.

Although our data are consistent with the complex rearrangement model, this does not exclude other possibilities. One potential mechanism is that loss of E2F4 may sensitize $Rb^{+/-}$ cells to undergo apoptosis upon loss of the remaining wild-type allele, an event that normally promotes tumorigenesis in the $Rb^{+/-}$ mice. This model is supported by our observation that there

was increased apoptosis in cultured $Rb^{1lox/1lox};E2f4^{-/-}$ erythroblasts (data not shown). This effect, however, may be cell type-specific since $Rb^{1lox/1lox};E2f4^{-/-}$ MEFs did not display a decreased survival potential when expanded in culture or in response to DNA damaging agents (Appendix B; data not shown). Furthermore, E2F4 is generally not thought to be involved in apoptosis (DeGregori et al. 1997; Wang et al. 2000), and in the studies that do link E2F4 to this process, overexpression induces programmed cell death (Dirks et al. 1998; Chang et al. 2000), which is in opposition to this model. A straightforward way to address the apoptotic potential of the $Rb^{-/-};E2f4^{-/-}$ cells in the pituitary and thryroid tissues would be through the analysis of chimeric animals generated from double mutant ES cells injected into wild-type blastocysts. If the double knockout cells were present in the tumor-prone tissues at adulthood, the apoptosis model would not be supported. On the other hand, if the double knockout cells initially contribute to the tissues during development and then are not detectable by adulthood, this would strongly support the notion of decreased viability of $Rb^{-/-};E2f4^{-/-}$ cells. This outcome is also consistent with a cell non-autonomous effect; however, our preliminary characterization of transformed $Rb^{1lox/1lox};E2f4^{-/-}$ cells supports that the reduced oncogenic capacity is a cell intrinsic function.

It is also formally possible that E2F4 contributes to tumor development in the same manner that the activating E2Fs function. In support of this, there are a number of studies showing that E2F4 induces proliferation and activation of E2F-responsive genes when ectopically expressed (Ginsberg et al. 1994; Lukas et al. 1996; DeGregori et al. 1997; Wang et al. 2000). These effects are most likely a consequence of overexpression since loss of E2f4 has no physiological effect on proliferation (Humbert et al. 2000; Rempel et al. 2000). Furthermore, E2F4 loss has opposing effects to the absence of an activating E2F in the $Rb^{-/-}$ background (Tsai et al. 1998; Ziebold et al. 2001), which is inconsistent with this model. This model could be

addressed by generating a knock-in mouse which expresses E2F4 with a mutated transactivation domain, and an intact pocket protein binding domain, and then crossing this strain with the $Rb^{+/-}$ mice. If there is significant tumor suppression in the resulting animals, similar to the $Rb^{+/-};E2f4^{-/-}$ mice, then the ability of E2F4 to activate transcription is important for tumorigenesis. On the other hand, if tumor formation is comparable to that observed in the $Rb^{+/-}$ mice, then it would imply that the function of E2F4 in tumor development relies upon pocket protein binding, supporting our complex rearrangement model. Thus, these experiments are aimed at exploring the mechanism by which the absence of E2F4 suppresses tumors in the $Rb^{+/-}$ mice.

We currently favor the complex rearrangement model, but further work is needed to confirm this. Determining the precise mechanism through which loss of E2F4 suppresses tumor formation in the $Rb^{+/-}$ mice will be important for better understanding the role of the E2Fs in the tumor suppressive properties of pRB. Ultimately, this may provide new strategies for developing chemotherapeutic treatments.

pRB and E2F4 in development

We also determined the effects of concomitant loss of E2F4 and pRB on murine development. E2F4 deficiency resulted in earlier lethality of the $Rb^{-/-}$ mice due to placental insufficiency. When compound mutant mice were provided with a normal functioning placenta, the animals were present at birth, confirming that the absence of E2F4 resulted in aggravation of the extra-embryonic tissue defect in the *Rb* mutant animals. With respect to the embryonic phenotypes, loss of E2F4 did not modulate any defects associated with the absence of pRB, including inappropriate proliferation, apoptosis and differentiation defects. These results are in stark contrast to the consequences of ablating an activating E2F in the $Rb^{-/-}$ background. Thus,

E2F4 does not have overlapping functions with the activating E2Fs in mediating the effects of pRB loss during normal development.

Although we were not able to determine the precise nature of the defect in the *Rb*$^{-/-}$;*E2f4*$^{-/-}$ placentas, we provide new insight into the placental defect arising from the loss of pRB. It has previously been reported that pRB loss results in overproliferation of trophoblast stem (TS) cells in the labyrinth layer (Wu et al. 2003). Our data, however, indicate that the proportion of TS cells in the *Rb*$^{-/-}$ placentas is similar to that in the wild-type placentas. Instead, we suggest that a stem cell population distinct from TS cells is overrepresented in the *Rb* mutant placentas compared to wild-type tissues. We found that Dppa5 (Esg-1), a pluripotent ES cell marker, was strikingly elevated in the *Rb* mutant placentas. Since the expression levels of other ES cell-specific markers were unchanged and this expression pattern is only observed in trophoectoderm cells (Tanaka et al. 2002), we propose that loss of pRB results in an increase in cells resembling the trophoectoderm, which are precursors of TS cells and give rise to many cell lineages in the extra-embryonic tissues (reviewed in Rossant and Cross 2001).

Further work will be needed to verify that a trophoectoderm-like population persists beyond the blastocyst stage in the absence of pRB. First, in order to correlate increased Dppa5 expression with the histological defect in the *Rb*$^{-/-}$ placentas, it will be important to determine the spatial localization of the Dppa5-expressing cells in the *Rb*$^{-/-}$ placentas by *in situ* hybridization. Second, if Dppa5 expression is indicative of a persistent cell population analogous to the trophectoderm, a systematic analysis of the timing of Dppa5 expression from E3.0 (blastocyst stage) to E13.5 (time of obvious placental dysfunction) will be useful. We detected increased Dppa5 transcript levels as early as E11.5, a time when the *Rb* mutant defect is not obvious, and it will be necessary to look at earlier time points. This experiment would be most informative if

done by *in situ* hybridization since it may indicate whether the formation of trophoectoderm-derived tissues is affected. For example, the trophoectoderm gives rise to the extra-embryonic ectoderm and the ectoplacental cone by E6.0, and if Dppa5 is confined to one of these regions, then it may suggest that there is a defect in cell fate determination to that compartment. On the other hand, if *Dppa5* expression is repressed in $Rb^{-/-}$ placentas after E3.5, paralleling that in wild-type placentas, and then reappears, this could indicate de-differentiation of a subset of cells. This is a possibility since we did not detect any differences in differentiation markers for the various placental layers. In this case, investigating the differentiation properties of *Rb* mutant TS cells in culture would be helpful. These experiments would also be beneficial for determining whether there is simply a failure to repress *Dppa5* in TS cells. Third, promoter analysis of the *Dppa5* gene may yield insight into its upstream regulation by pRB. Our data indicate that E2F4 does not participate in regulating *Dppa5* (chapter 3) and preliminary analysis of the *Dppa5* promoter did not reveal any E2F binding sites (data not shown). Thus, a more in-depth analysis for other pRB-binding transcription factor consensus sites, as well as for Id2 interactors, should be performed. Finally, the physiological function of Dppa5 remains to be determined and understanding its role *in vivo* may provide insight into its relationship with pRB.

It is clear that loss of E2F4 leads to a more severe placental defect in the $Rb^{-/-}$ mice. As such, it will be important to pinpoint the defect in the compound mutant placentas. Our microarray analysis of $Rb^{-/-}$;$E2f4^{-/-}$ and wild-type placentas identified 44 differentially expressed genes in the $Rb^{-/-}$;$E2f4^{-/-}$ tissues. Four of the genes (i.e., *prolactin*, *growth hormone*, *matrix metalloproteinase 9* and *X inactive specific transcript*) are potential candidates for further investigation since they have previously been linked to placental development. To begin analyzing the involvement of these factors in the $Rb^{-/-}$;$E2f4^{-/-}$ placentas, the differential expression

of these genes needs to be verified by an alternative method, such as quantitative real-time PCR or *in situ* hybridization. Then, performing the same analysis on $Rb^{-/-}$ and $E2f4^{-/-}$ placentas will clarify whether deregulation is a result of pRB and/or E2F4 loss. Promoter analysis followed by chromatin immunoprecipitation assays could establish if these potential targets are directly regulated by E2F4 in cooperation with the pocket proteins. Thus, further investigation of these promising targets may aid in defining the cause of placental dysfunction in the $Rb^{-/-};E2f4^{-/-}$ placentas.

Since the *Rb*-related defect has not been fully characterized, it has been difficult to establish whether E2F4 cooperates with pRB in the extra-embryonic tissues or whether they have separate functions. There are many similarities between the $Rb^{-/-};E2f4^{-/-}$ mice and the $Rb^{-/-};p107^{-/-}$ animals (Lee et al. 1996), suggesting that the loss of repressive E2F-pocket protein complexes is important for placental development. If E2F4 has a redundant function to p107, then it would be predicted that $Rb^{-/-};E2f4^{-/-};p107^{-/-}$ mice would resemble $Rb^{-/-};E2f4^{-/-}$ embryos, since both genotypes result in the same repressive complexes. On the other hand, if E2F4 loss alone affects placental development, then determining the defect in the $E2f4^{-/-}$ placentas may help to define the defect in the $Rb^{-/-};E2f4^{-/-}$ placentas. The generation of conditional $E2f4^{-/-}$ animals provided with a wild-type placenta could address this. If the resulting $E2f4^{-/-}$ animals were no longer growth retarded, then this would support that E2F4 may have a distinct role in the placenta and further investigation would be required. Thus, the functional relevance of pRB-E2F4 complexes in regulating placental development remains incomplete.

The role of E2F4 in pRB-mediated growth suppresion

It is curious that E2F4 loss significantly extends the lifespan of the $Rb^{+/-}$ mice, but decreases the viability of the $Rb^{-/-}$ embryos. These differing outcomes may indicate differential mechanisms through which E2F4 functions in these two settings. We currently favor the idea that inappropriate proliferation in the developing embryo proper and tumorigenesis in the absence of pRB are critically dependent upon the activating E2Fs, while the repressive E2F-pocket protein complexes are important for regulating placental development. Therefore, the overall contribution of the activating and repressive E2Fs to target gene expression may be dictating cell growth and division. We found that loss of E2F4 suppressed inappropriate cell division in the $Rb^{-/-}$ MEFs, but had no effect on proliferation levels in the $Rb^{-/-}$ embryos. In contrast, loss of E2F3, an activating E2F, suppresses ectopic proliferation in the $Rb^{-/-}$ mice (Ziebold et al. 2001), yet did not alter levels of inappropriate proliferation in $Rb^{-/-}$ MEFs cultured in low serum (data not shown). Further investigation will be needed to determine the levels of the various E2Fs and the pocket proteins in these mutant cells and whether the relative proportions change in different cell types. It will also be important to determine whether the activating E2Fs rescue the $Rb^{-/-}$ embryos through suppression of the placental defect. Understanding the role of E2F4 and the activating E2Fs in the extra-embryonic tissues may help to clarify their functions.

Alternatively, E2F4 may be acting through one mechanism to mediate the effects in the $Rb^{+/-}$ and $Rb^{-/-}$ mice. For example, if cells lacking E2F4 and pRB are inclined to undergo apoptosis (as discussed above), then this could result in the suppression of tumors as well as the earlier embryonic lethality. Furthermore, this may be linked to the inappropriate expression of pituitary-specific hormones, prolactin and growth hormone, detected in the double mutant

placentas. If they are also deregulated in the pituitary, then it may result in cellular abnormalities, such as apoptosis. Additional work will be needed to determine whether these factors are also deregulated in the tumor-prone tissues of the *Rb*$^{+/-}$;*E2f4*$^{-/-}$ mice, and the consequences of the inappropriate expression.

This has been the first study to investigate the physiological role of a repressive E2F in the growth suppressive functions of pRB. Although our analyses have led to a number of unresolved issues, we have clearly demonstrated that E2F4 loss has significant effects in the *Rb*-deficient mice. Therefore, these findings have broadened our understanding of the molecular signals contributing to tumorigenesis and development in the absence of pRB.

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Appendix A

Partial reduction of p107 levels does not alter the tumor phenotype in *Rb*$^{+/-}$;*E2f4*$^{-/-}$ mice

Eunice Y. Lee and Jacqueline A. Lees

In chapter two, we found that E2F4 loss dramatically suppressed tumorigenesis in the $Rb^{+/-}$ mice and promoted the formation of novel E2F-pocket protein complexes *in vivo*. These results led to a model whereby E2F4 loss indirectly inhibited tumor progression in the $Rb^{+/-}$ mice by increasing the levels of free p107 and p130 that can compensate for pRB deficiency. In order to determine whether this is the underlying mechanism for the tumor suppression, we have introduced the *p107* mutation in the $Rb^{+/-};E2f4^{-/-}$ background. Directly reducing the levels of a compensatory pocket protein should lead to a reversion of the $Rb^{+/-}$ tumor phenotype. If there were no effect, however, then this would indicate that an alternative mechanism is at work.

Since a fraction of the expected number of $Rb^{+/-};p107^{-/-}$ animals survive to adulthood (Lee et al., 1996), we thought that additional loss of E2F4 may not alter the recovery rate. In order to generate $Rb^{+/-};E2f4^{-/-};p107^{-/-}$ animals, three crosses were set up on a mixed C57Bl/6 x 129/Sv background: 1) $Rb^{+/-};E2f4^{+/-};p107^{-/-}$ x $Rb^{+/-};E2f4^{+/-};p107^{+/-}$, 2) $Rb^{+/-};E2f4^{+/-};p107^{+/-}$ x $Rb^{+/-};E2f4^{+/-};p107^{+/-}$, 3) $Rb^{+/-};E2f4^{+/-};p107^{+/-}$ x $Rb^{+/+};E2f4^{+/-};p107^{+/-}$. We did not detect any $Rb^{+/-};E2f4^{-/-};p107^{-/-}$ animals from our crosses at 3 weeks of age (Table 1). In fact, none of the $E2f4^{-/-};p107^{-/-}$ animals were present at this time, suggesting that these animals have severely reduced viability. Further work is needed to determine the precise timing and cause of lethality in the $E2f4^{-/-};p107^{-/-}$ animals.

Given that it was not possible to analyze tumor formation in the $Rb^{+/-};E2f4^{-/-};p107^{-/-}$ animals, we assessed the $Rb^{+/-};E2f4^{-/-};p107^{+/-}$ animals. The frequency at which these mice arose was near the expected value (Table 1). Furthermore, these animals survived to adulthood, allowing for scoring of tumor development. The majority of the $Rb^{+/-};E2f4^{-/-};p107^{+/-}$ animals (4/6) lived longer than the $Rb^{+/-}$ littermates, which died between 8 and 14 months of age (Table

Table 1. Offspring viability from *Rb;E2f4;p107* intercrosses at 3 weeks of age

Genotype	# Expected	# Observed
$Rb^{+/-};E2f4^{-/-};p107^{+/-}$	8.4	6
$Rb^{+/-};E2f4^{-/-};p107^{-/-}$	5.8	0
$Rb^{+/+};E2f4^{-/-};p107^{-/-}$	5.6	0
$E2f4^{-/-}$ *	33.75	14
$p107^{-/-}$ *	46.75	21
Total number of mice		135

* Includes all possible genotypes at other two loci.

Table 2. Histological Analysis of $Rb^{+/-};E2f4^{-/-};p107^{+/-}$ Mice

Age (Months)	Pituitary Tumor*	Thyroid Tumor*	Cause of Death
9	++++	-	Pituitary tumor
13	-	-	Unknown
14.9	++++	-	Pituitary tumor
14.9	nd	nd	Unknown
15.3	+++	-	Pituitary tumor
20.7	-	-	Unknown

nd = not determined

* Tumor size is indicated as follows: "-" indicates no tumor growth and "++++" indicates tumors comparable to those of $Rb^{+/-}$ mice.

2). Upon inspection of tumor development, 3/5 $Rb^{+/-};E2f4^{-/-};p107^{+/-}$ animals had pituitary tumors and 0/5 had thyroid lesions (Table 2). Therefore, the loss of one wild-type allele of *p107* has no effect on the lifespan and tumorigenesis in the $Rb^{+/-};E2f4^{-/-}$ animals (chapter 2).

These data are inconclusive with respect to defining the mechanistic basis of tumor suppression in the $Rb^{+/-};E2f4^{-/-}$ animals. While we can clearly state that partial reduction in p107 is insufficient to overcome the tumor suppression, it is entirely possible that the remaining pool of free p107 and p130 is more than enough to compensate for diminished pRB function. Thus, alternative approaches are required to test the complex rearrangement model.

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Appendix B

Cell cycle characterization of *Rb;E2f* mutant primary fibroblasts

Eunice Y. Lee, Tiziana Parisi, Alison Taylor and Jacqueline A. Lees

The author contributed all of the text and Figures 1A, 1B, 1D, 1E and Figure 2A. The author and Tiziana Parisi contributed to Figure 2B. Alison Taylor contributed Figure 1C and 1F. All work was done in the laboratory of Jacqueline A. Lees.

Based on mutant mouse studies in the $Rb^{+/-}$ background, loss of E2F4 (chapter 2) or an activating E2F results in significant tumor suppression in the pituitary gland (Yamasaki et al. 1998; Ziebold et al. 2003). In order to determine if these E2Fs are working through a common mechanism to regulate cell cycle progression, we characterized various *Rb;E2f* mutant MEFs. We hypothesized that the compound mutant cells should have equivalent properties, according to the complex rearrangement model proposed in chapter 2. For the most part, our results are preliminary and somewhat inconclusive.

We analyzed $Rb^{-/-}$;*E2f1*$^{-/-}$, $Rb^{-/-}$;*E2f3*$^{-/-}$, and $Rb^{1lox/1lox}$;*E2f4*$^{-/-}$ cells in proliferation, cell cycle re-entry, apoptosis and transformation assays. All MEFs were generated from E13.5 embryos. Since $Rb^{-/-}$;*E2f4*$^{-/-}$ mice were severely underrepresented at this time, we generated *Rb;E2f4* mutant cells from the conditional double knockout animals provided with a functional placenta, as described in chapter 3. It has been reported that asynchronous $Rb^{-/-}$ primary fibroblasts display a normal proliferation rate (Herrera et al. 1996). In our assays, most MEF lines generated from *Rb* mutant embryos had growth rates similar to the control MEFs (Figure 1A and 1B), though some proliferated slightly faster (Figure 1C). We also noticed that some lines reached a higher saturation density than controls (Figure 1A), which is most likely indicative of the defective confluence arrest response in the absence of pRB. For the most part, we found that simultaneous loss of pRB and E2F4 (Figure 1A) or E2F1 (Figure 1C) had equivalent proliferation rates as the control fibroblasts. Consistent with previous findings (Humbert et al. 2000), the E2F3-deficient cells had a proliferation defect (Figure 1B). Furthermore, the $Rb^{-/-}$;*E2f3*$^{-/-}$ cells had slightly reduced proliferation rates intermediate to the *E2f3*$^{-/-}$ and wild-type cells, suggesting that in the absence of pRB the deregulation of the remaining activating E2Fs is responsible for the increased proliferation over the *E2f3*$^{-/-}$ cells.

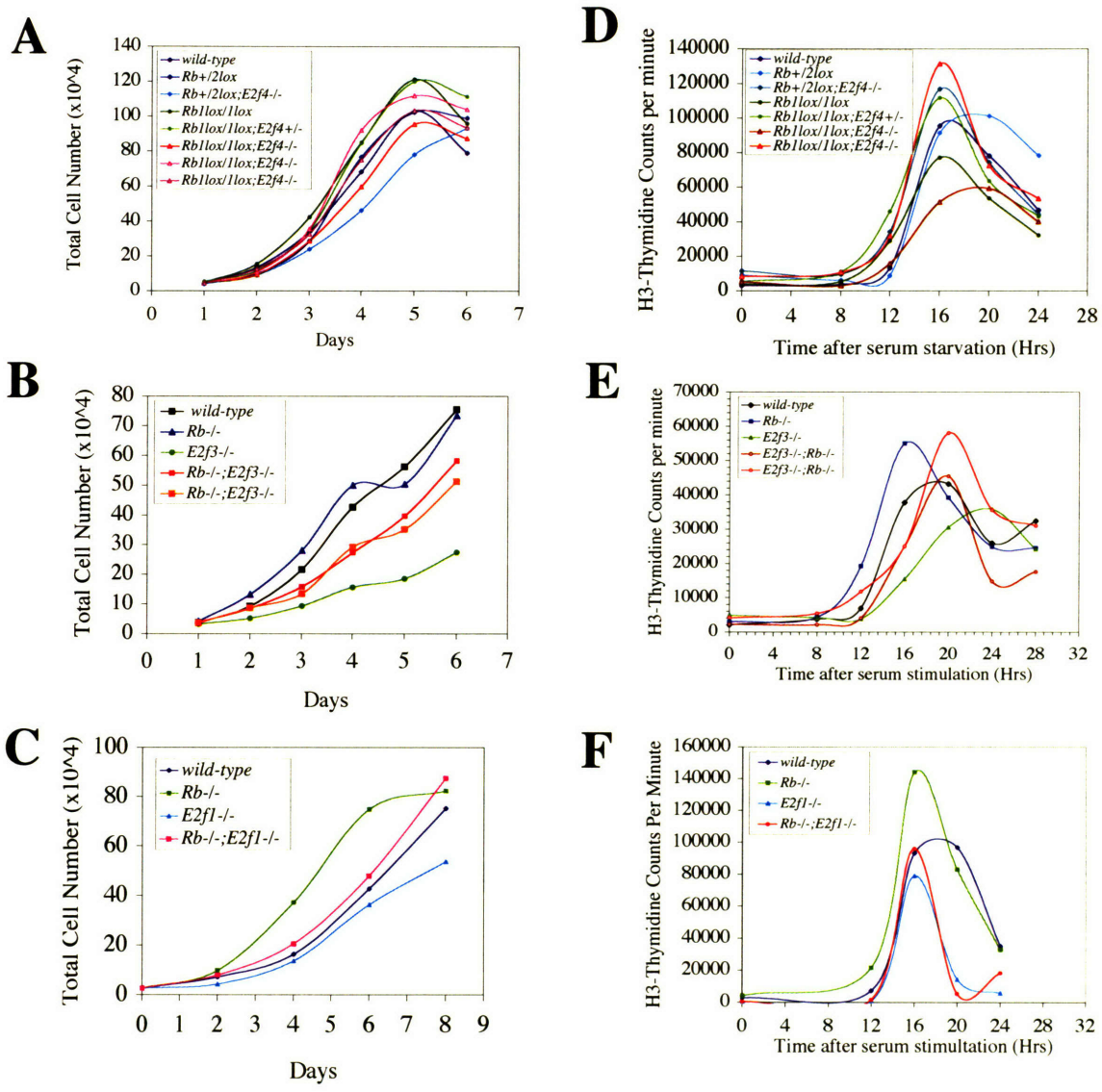

Figure 1. Cell cycle properties of *Rb;E2F* mutant MEFs.

A-C) The proliferation rates of asynchronously dividing wild-type, *Rb* mutant, $Rb^{1lox/1lox}$;$E2f4^{-/-}$ (A), $Rb^{-/-}$;$E2f3^{-/-}$ (B) and $Rb^{-/-}$;$E2f1^{-/-}$ (C) fibroblasts were determined by plating $2x10^4$ cells in a 12-well plate well on day 0 and then counting the cells at the indicated times. D-F) Serum starved wild-type, *Rb* mutant, $Rb^{1lox/1lox}$;$E2f4^{-/-}$ (D), $Rb^{-/-}$;$E2f3^{-/-}$ (E) and $Rb^{-/-}$;$E2f1^{-/-}$ (F) MEFs were induced to re-enter the cell cycle upon readdition of serum. Cell cycle progression was monitored by H3-Thymidine incorporation.

We also performed cell cycle re-entry assays to determine the cell cycle kinetics. MEFs were synchronized in G0/G1 by serum starvation and then stimulated enter S phase upon addition of serum. As previously reported, *Rb* mutant MEFs progressed through G1 more rapidly than control cells. In general, the $Rb^{-/-};E2f1^{-/-}$ (Figure 1F) and $Rb^{1lox/1lox};E2f4^{-/-}$ (Figure 1D) MEFs either resembled the control cells or the $Rb^{-/-}$ fibroblasts. We examined the protein levels of p107 and cyclin E, which are derepressed in *Rb* mutant cells, and found that their expression mimicked their cell cycle kinetics (data not shown). For example, in the compound mutant cells that entered S phase more quickly, p107 and cyclin E were elevated to equivalent levels as observed for the *Rb* mutant cells. In contrast, we consistently found that the $Rb^{-/-};E2f3^{-/-}$ cells had normal cell cycle kinetics, which was in-between the $Rb^{-/-}$ cells and the $E2f3^{-/-}$ cells (Figure 1E). It may be worthwhile to investigate the differences in the two classes of $Rb^{-/-};E2f1^{-/-}$ cells, as well as of $Rb^{1lox/1lox};E2f4^{-/-}$ cells. If the cause for the differences within a given genotype can be determined, this may aid in understanding the functional relevance of the various E2F-pRB complexes.

We also determined whether the $Rb^{-/-};E2f1^{-/-}$ and the $Rb^{1lox/1lox};E2f4^{-/-}$ cells were sensitive to apoptosis in response to DNA damaging agents. Asynchronously dividing MEFs were treated with cisplatin (8μM or 16μM) or γ-irradiation (5G or 10G) and then stained for annexin V and propidium iodide followed by flow cytometry analysis. In response to γ-irradiation, there was no alteration in apoptosis levels for all of the genotypes examined (data not shown). In contrast, the $Rb^{-/-};E2f1^{-/-}$ MEFs were highly susceptible to undergo programmed cell death after cisplatin treatment (40.6% versus 19.6% in control cells and 14.7% in $E2f1^{-/-}$ cells). E2F1 has been demonstrated to have roles in apoptosis and the DNA damage checkpoint (discussed in Introduction), which may reflect the observed differences.

Finally, we transformed the various *Rb;E2f* mutant cells with activated Ras and Myc in order to analyze the oncogenic capacity of these cells. We confirmed that Ras and Myc were expressed in all the transformed cells by Western blot analysis (data not shown). In contrast to the cell cycle results which were variable, transformed $Rb^{-/-};E2f1^{-/-}$, $Rb^{-/-};E2f3^{-/-}$, and $Rb^{1lox/1lox};E2f4^{-/-}$ MEFs all had diminished transformation potential to form tumors in immunocompromised mice relative to transformed $Rb^{-/-}$ cells (Figure 2). Most notably, the tumors induced by transformed $Rb^{1lox/1lox};E2f4^{-/-}$ cells at 25 days post injection (dpi) were comparable in volume to those arising from $Rb^{1lox/1lox}$ cells at 16 dpi (Figure 2A). This has only been done with one MEF line per genotype and needs to be confirmed. Yet, these results thus far indicate that loss of E2F4 acts in a cell autonomous manner to suppress the oncogenic potential of $Rb^{-/-}$ cells.

Our preliminary analyses of the cell cycle properties of $Rb^{-/-};E2f1^{-/-}$ and $Rb^{1lox/1lox};E2f4^{-/-}$ primary fibroblasts have not been consistent. In contrast, the $Rb^{-/-};E2f3^{-/-}$ cells exhibit reduced cell proliferation and cell cycle kinetics in all the assays performed. These differences may reflect the differing functions of the E2Fs during cell cycle regulation or the differing levels of the available activating E2Fs. Since it is not possible to determine the relative amounts of the free activating E2Fs, analyses of target gene expression and promoter occupancy may be useful. With respect to oncogenic potential, the various *Rb;E2f* mutant cells functioned in the same manner, which may suggest a differential requirement for the E2Fs and pRB in tumorigenesis than in normal cell cycle control. Further investigation is necessary to make any definitive conclusions.

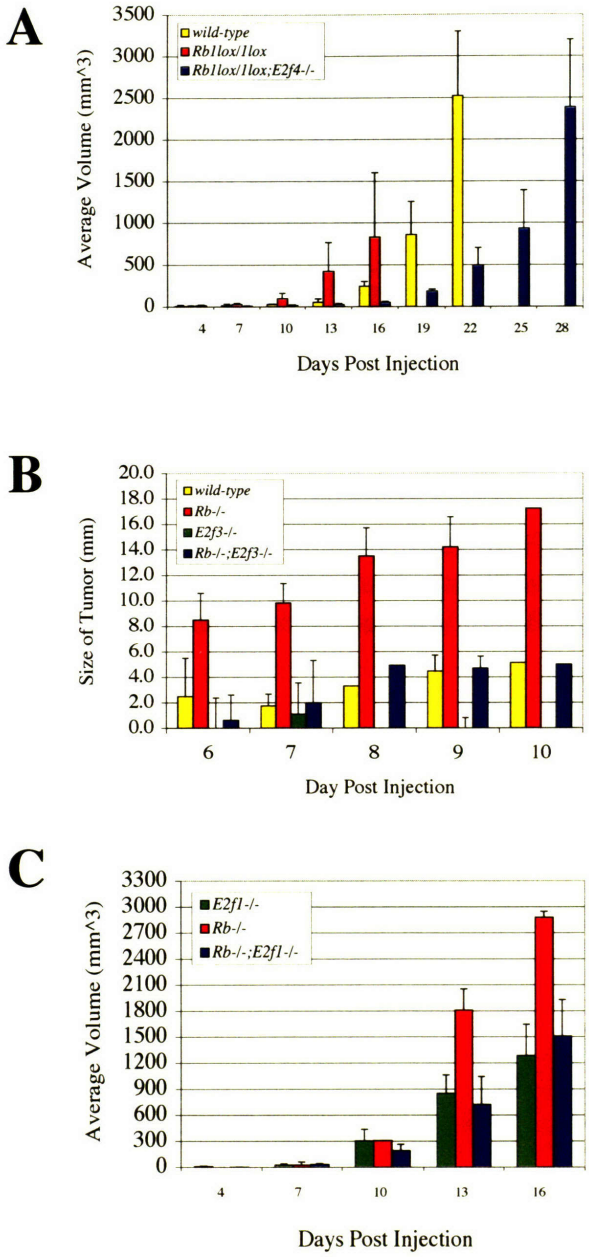

Figure 2. Oncogenic capacity of *Rb;E2F* mutant MEFs.

Wild-type, *Rb* mutant, $Rb^{1lox/1lox}$;$E2f4^{-/-}$ (A), $Rb^{-/-}$;$E2f3^{-/-}$ (B) and $Rb^{-/-}$;$E2f1^{-/-}$ (C) MEFs were transformed with activated Ras and Myc. The abilities of the transformed cells to form tumors in nude mice were assessed. Briefly, 10^6 cells were injected per injection site and tumor growth was monitored at the indicated times post injection.

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Appendix C

E2F3 loss has opposing effects on different pRB-deficient tumors resulting in suppression of pituitary tumors but metastasis of medullary thyroid carcinomas

Ulrike Ziebold, Eunice Y. Lee, Roderick T. Bronson and Jacqueline A. Lees

Mol Cell Biol (2003) **23**: 6542-6552

Ulrike Ziebold contributed all of the text, tables and figures, except Figure 5A which was contributed by the author. Roderick T. Bronson consulted on murine tumor pathology. All work was done in the laboratory of Jacqueline A. Lees.

ABSTRACT

The E2F transcription factors are key downstream targets of the retinoblastoma protein (pRB) tumor suppressor. We have previously shown that E2F3 plays a critical role in mediating the mitogen-induced activation of E2F-responsive genes and contributes to both the inappropriate proliferation and p53-dependent apoptosis that arises in pRB-deficient embryos. Here we show that E2F3 also has a significant effect on the phenotype of the tumor prone $Rb^{+/-}$ mice. The absence of E2F3 results in a significant expansion in the lifespan of these animals that correlates with a dramatic alteration in the tumor spectrum. E2F3 loss suppresses the development of the pituitary tumors that normally account for the death of $Rb^{+/-}$ mice. However, it also promotes the development of the medullary thyroid carcinomas yielding metastases at high frequency. This increased aggressiveness does not seem to result from any change in p53 levels or activity in these tumors. Instead, we show that E2F3 loss leads to an increase in the rate of tumor initiation. Finally, analysis of $Rb^{+/-};E2f3^{+/-}$ mice shows that this tumor suppressive function of E2F3 is dose dependent.

INTRODUCTION

The retinoblastoma gene (*RB-1*) was identified by virtue of its absence in early childhood retinoblastoma (Friend et al. 1986; Fung et al. 1987; Lee et al. 1987). Subsequent studies revealed that *RB-1* is mutated in approximately one third of all human tumors (reviewed by Weinberg 1992). To elucidate the precise role of the retinoblastoma protein (pRB) in both tumorigenesis and development, a number of mutant mouse strains have been established (reviewed by Mulligan and Jacks 1998). Their analyses underscore the importance of pRB as both a tumor suppressor and as a key regulator of cellular growth in normal development. Consistent with the familial cancer syndromes, mice carrying a single *Rb* mutant allele are highly cancer prone (Hu et al. 1994; Williams et al. 1994). These animals develop pituitary tumors with almost complete penetrance and a significant number also display medullary thyroid tumors (MTCs). In addition, pRB is essential for embryogenesis (Clarke et al. 1992; Jacks et al. 1992; Lee et al. 1992). The mid-gestation lethality of pRB-deficient embryos is accompanied by the defective development of the fetal liver, erythrocytes, neurons and lens that result from a combination of ectopic S phase entry and inappropriate programmed cell death. Subsequent studies showed that the apoptosis can be either p53-dependent or p53-independent depending on the particular tissue (Morgenbesser et al. 1994; Macleod et al. 1996).

The growth suppressive properties of pRB are thought to be largely dependent upon its ability to regulate the E2F transcription factors (reviewed by Dyson 1998; Trimarchi and Lees 2002). The E2Fs control the expression of genes essential for cell proliferation, including key components of both the DNA replication and cell cycle machinery. pRB binds to E2F during the G_1 phase of the cell cycle and inhibits the activation of E2F dependent target genes. In response to mitogenic signals, pRB is phosphorylated by the cell cycle dependent kinases cyclin D/cdk4,6

and cyclinE/cdk2 and transcriptionally active E2F is released. Notably, almost all pRB-positive tumors contain activating mutations in *cycD1* or *cdk4* or inactivating mutations in the cdk inhibitor *p16* (reviewed by Sherr 1996). This suggests that functional inactivation of pRB, and thereby inappropriate release of E2F activity, is an essential step in tumorigenesis.

To date, six genes that encode for members of the E2F family have been cloned (reviewed by Dyson 1998; Trimarchi and Lees 2002). Although most of these genes encode a single protein product, *E2f3* has two alternative promoters that yield two distinct proteins, called E2F3a and E2F3b, which differ in their N-terminal sequences. E2F3b has only recently been identified and its biological properties are not well understood. The remaining E2F proteins have been divided into three distinct subgroups based on significant differences in their structure and function.

E2F1, 2 and 3a represent one subgroup and they are believed to be the key downstream targets of pRB. These three E2Fs are specifically regulated by pRB, and not the pRB-related proteins p107 or p130 (Lees et al. 1993). When over-expressed, E2F1, 2 and 3a are potent transcriptional activators and are each sufficient to induce quiescent cells to enter S phase (Lukas et al. 1996; DeGregori et al. 1997). The endogenous E2F1, 2 and 3a proteins are released from pRB during late G_1 and then associate with E2F-responsive promoters just prior to the activation of E2F-responsive genes (Takahashi et al. 2000; Rayman et al. 2002). Taken together, these data suggest that E2F1, 2 and 3a play a key role in the induction of cellular proliferation. Consistent with this hypothesis, mouse embryonic fibroblasts (MEFs) that lack E2F3a and E2F3b (for simplicity, herein referred to as E2F3) have a defect in the mitogen-induced activation of almost all known E2F-responsive genes that impairs the proliferation of both primary and transformed cells (Humbert et al. 2000b). Moreover, the combined loss of E2F1, E2F2 and E2F3 completely

blocks cellular proliferation suggesting that these proteins have overlapping roles *in vivo* (Wu et al. 2001). Finally, these "activator E2Fs" can trigger cells to undergo apoptosis through both p53-dependent (Qin et al. 1994; Shan and Lee 1994; Wu and Levine 1994) and p53-independent mechanisms (Hsieh et al. 1997; Phillips et al. 1997).

The analysis of *E2f* mutant mouse strains suggests that the activating E2Fs play both overlapping and unique roles in normal development. E2F1 and E2F2 are not required for embryonic development, but adult *E2f1*$^{-/-}$ and *E2f2*$^{-/-}$ mice each develop a unique spectrum of tissue-specific abnormalities including defined, but distinct, defects in T lymphocyte development (Yamasaki et al. 1996; Zhu et al. 1999; Garcia et al. 2000; Murga et al. 2001; Zhu et al. 2001). *E2f1* mutant mice are also susceptible to hematopoietic malignancies and the tumor incidence appears to be increased by *E2f2* mutation (Yamasaki et al. 1996; Zhu et al. 2001). Tumorigenesis is not dependent upon loss of the remaining wild-type *E2f1* and/or *E2f2* allele and there is still considerable debate about the underlying basis for these tumor suppressive properties (reviewed by Trimarchi and Lees 2002). Initially, this was thought to be due to E2F1's role in the active repression of E2F-responsive genes via recruitment of pRB and associated histone deacetylases. However, others have suggested that E2F1 acts as a tumor suppressor through its ability to induce apoptosis and/or its participation in a DNA damage response (Meng et al. 1999; Lin et al. 2001; Maser et al. 2001).

In contrast to the other activating E2Fs, the loss of E2F3 causes a high frequency of neonatal lethality (Humbert et al. 2000b; Cloud et al. 2002). In certain mixed strain backgrounds, a small proportion of the *E2f3*$^{-/-}$ mice survive to adulthood, but most die prematurely of congestive heart failure (Cloud et al. 2002). The analysis of *E2f1;E2f3* compound mutant mice indicates that the developmental defects arising in the individual *E2f1* or

E2f3 mutant mice are exacerbated by their combined mutation (Cloud et al. 2002). This strongly suggests that these genes have critical, overlapping functions in development. However, *E2f3* mutation has no detectable effect on the tumor incidence of *E2f1* mutant mice raising the possibility that tumor suppression is a specific property of *E2f1* and *E2f2*, but not *E2f3* (Cloud et al. 2002).

Compound *Rb;E2f* mutant mouse models have been used to determine how pRB's growth suppressive properties relate to its role in the inhibition of the activating E2Fs. These studies show that the absence of either E2F1 or E2F3 greatly suppresses the ectopic S-phase entry, p53-dependent and p53-independent apoptosis arising in pRB-deficient embryos and thereby significantly extends their lifespan (Tsai et al. 1998; Ziebold et al. 2001). This suggests that E2F1 and E2F3 both make significant contributions to the phenotypic consequences of pRB-deficiency. Consistent with this hypothesis, E2F1 loss has been shown to significantly reduce the development of pRB-deficient tumors (Yamasaki et al. 1998). However, the lack of a complete rescue raised the possibility that one or more additional E2Fs might also contribute to the tumor phenotype. This notion is supported by the finding that E2F4 loss greatly suppresses the formation of tumors in Rb+/- mice by enabling p107 and p130 to bind, and presumably inhibit, both E2F1 and E2F3 (Lee et al. 2002). In this study, we have generated *Rb;E2f3* compound mutant mice to assess how E2F3 contributes to the *Rb* mutant phenotype. Our analysis reveals an unexpected role for E2F3 in suppressing metastasis of pRB-deficient medullary thyroid tumors.

RESULTS

Rb mutation increases the viability of the *E2f3*$^{-/-}$ neonates

We have previously shown E2F3 is critical for cellular proliferation and contributes to the inappropriate proliferation and apoptosis that arise in pRB-deficient embryos (Humbert et al. 2000b; Ziebold et al. 2001). Given these observations, we hypothesized that E2F3 might contribute to the development of pRB-deficient tumors. Since the viability of the *E2f3*$^{-/-}$ mice and the severity of the tumor phenotype of the *Rb*$^{+/-}$ mice are both influenced by strain specific modifiers (Cloud et al. 2002), we conducted crosses between *Rb* (Jacks et al. 1992) and *E2f3* (Humbert et al. 2000b) mutant mouse strains in both the 129/Sv and mixed (129/Sv x C57BL/6) backgrounds.

Initially, we examined the ability of the compound *Rb;E2f3* mutant genotypes to survive until weaning. In the pure 129/Sv background (data not shown), we observed complete embryonic lethality of the *E2f3*$^{-/-}$ mice as previously described (Cloud et al. 2002). Unfortunately, the presence of a single *Rb* mutant allele did not suppress the lethality of the *E2f3*$^{-/-}$ animals. However, *Rb*$^{+/-}$ and *Rb*$^{+/-}$;*E2f3*$^{+/-}$ mice were both generated at the expected Mendelian ratio and were aged for analysis of adult phenotypes. In the mixed, 129/Sv x C57BL/6, strain background, a small fraction of the *E2f3*$^{-/-}$ mice survived to weaning (Cloud et al. 2002). Significantly, matings of mixed background *Rb*$^{+/-}$;*E2f3*$^{+/-}$ females with either *Rb*$^{+/-}$;*E2f3*$^{+/-}$ or *Rb*$^{+/-}$;*E2f3*$^{-/-}$ males yielded surviving *E2f3*$^{-/-}$ animals at a significantly higher frequency in the presence (25.75 ± 4.05% of expected) versus the absence (11.4 ± 0.3% of expected frequency) of one *Rb* mutant allele (Table I). This strongly suggests that a reduction in pRB levels is sufficient to overcome the requirement for E2F3 in one or more developmental processes that are essential for neonatal viability.

Table I. *Rb* mutation increases the viability of the *E2f3*$^{-/-}$ neonates

Cross	Genotype	Number of animals	% expected frequency
Rb$^{+/-}$;*E2f3*$^{+/-}$ x *Rb*$^{+/-}$;*E2f3*$^{+/-}$	WT	60	100%
	E2f3$^{+/-}$	110	104.5%
	Rb$^{+/-}$	85	91.7%
	Rb$^{+/-}$; *E2f3*$^{+/-}$	191	70.8%
	E2f3$^{-/-}$	7	11.7%
	Rb$^{+/-}$; *E2f3*$^{-/-}$	26	21.7%
		(Σ=479 animals)	
Rb$^{+/-}$;*E2f3*$^{+/-}$ x *Rb*$^{+/-}$;*E2f3*$^{-/-}$	*E2f3*$^{+/-}$	99	100%
	Rb$^{+/-}$; *E2f3*$^{+/-}$	206	104%
	E2f3$^{-/-}$	11	11.1%
	Rb$^{+/-}$; *E2f3*$^{-/-}$	59	29.8%
		(Σ=375 animals)	

Genotypic analysis of the progeny was performed at weaning or 21 days of age.

E2f3 mutation increases the lifespan of the tumor-prone $Rb^{+/-}$ mice

To determine whether E2F3 loss alters the viability of $Rb^{+/-}$ adults, we aged a large cohort of surviving neonatal *E2f3;Rb* mutant littermates and compared their lifespan (Figure 1, Table II). In the inbred 129/Sv mice, we observed a significant increase (log rank test, p=0.0008) in the mean survival of the $Rb^{+/-};E2f3^{+/-}$ (9.8 ± 1.3 months) versus the $Rb^{+/-}$ (8.6 ± 1.2 months) littermates. Similarly, in the mixed (129/Sv x C57BL/6) strain, the lifespan of the $Rb^{+/-}$ mice (10.2 ± 1.6 months) was progressively increased by the presence of either one (10.8 ± 1.8 months) or two (13 ± 2.6 months) *E2f3* mutant alleles. Both of these changes are statistically significant (log rank test, p=0.0128 and <0.00001, respectively). Thus, *E2f3* mutation increases the viability of the $Rb^{+/-}$ mice in a dose dependent manner. Importantly, the $Rb^{+/-};E2f3^{-/-}$ mice could also be clearly distinguished from their $E2f3^{-/-}$ littermate controls; they died earlier and had no evidence of the congestive heart failure that causes the death of most $E2f3^{-/-}$ adults (Cloud et al. 2002). Thus, the *Rb* mutation is influencing the viability of these $Rb^{+/-};E2f3^{-/-}$ adults.

E2f3 mutation suppresses development of pituitary tumors in $Rb^{+/-}$ mice

Since *E2f3* mutation extends the lifespan of the $Rb^{+/-}$ animals, we first examined whether it had any effect on either the incidence or size of the intermediate lobe pituitary tumors that is the documented cause of death of *Rb* heterozygotes (Hu et al. 1994; Riley et al. 1994; Williams et al. 1994; Harrison et al. 1995; Nikitin and Lee 1996). For these studies, we focused primarily on the mixed 129/Sv x C57BL/6 background because all genotypes of interest were

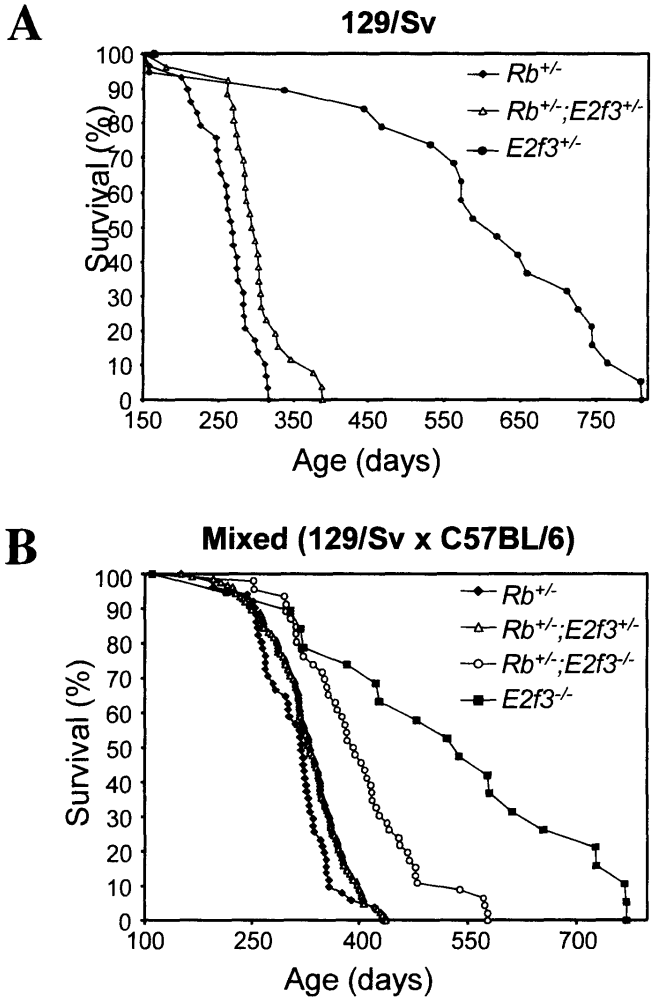


Figure 1. Effect of *E2f3* status on the lifespan of *Rb* heterozygous mice.

Progeny arising from $Rb^{+/-};E2f3^{+/-}$ intercrosses were aged together. Log-rank survival curves depict the survival (%) over the age of the animals (days). A) In the pure 129/Sv background, the lifespan of $Rb^{+/-};E2f3^{+/-}$ animals (n=25) was significantly increased as compared to $Rb^{+/-}$ animals (n=28). Additionally, $E2f3^{+/-}$ animals (n=19) were aged as a control. B) In the mixed 129/Sv x C57BL/6 background, the lifespan of the $Rb^{+/-};E2f3^{+/-}$ (n=125) and $Rb^{+/-};E2f3^{-/-}$ animals (n=40) increased progressively relative to that of the $Rb^{+/-}$ (n=50) animals, but was decreased as compared to $E2f3^{-/-}$ animals (n=18).

Table II. E2f3 loss increases lifespan of $Rb^{+/-}$ mice in a mixed (129/Sv x C57BL/6) and in a pure (129/Sv) genetic background

Genetic background	Genotype of animals	Number (months)	Mean survival (days)	Survival range
Mixed (129/Sv x C57BL/6)	$Rb^{+/-}$	50	10.2 $^{+}/_{-}$ 1.6	194 to 431
	$Rb^{+/-}$; $E2f3^{+/-}$	125	10.8 $^{+}/_{-}$ 1.8	166 to 437
	$Rb^{+/-}$; $E2f3^{-/-}$	50	13.0 $^{+}/_{-}$ 2.6	252 to 582
	$E2f3^{-/-}$	18	17.1 $^{+}/_{-}$ 5.5	213 to 770
Pure 129/Sv	$Rb^{+/-}$	28	8.6 $^{+}/_{-}$ 1.2	157 to 316
	$Rb^{+/-}$; $E2f3^{+/-}$	25	9.8 $^{+}/_{-}$ 1.3	262 to 388
	$E2f3^{+/-}$	19	19.5 $^{+}/_{-}$ 5.3	157 to 810

available for analysis. The phenotypes of the $Rb^{+/-}$ littermate controls were entirely consistent with previous reports. We observed melanotroph carcinomas that had arisen from the intermediate lobe of the pituitary (Figure 2A and data not shown). In almost every case, the tumors had expanded to compress adjacent brain structures, causing the death of these animals. In contrast, microscopic histological examination showed that the pituitaries of *E2f3* mutant littermates were completely normal (Figure 2A and data not shown).

At the time of death, the pituitary tumors of the $Rb^{+/-};E2f3^{+/-}$ mice were indistinguishable from those of the $Rb^{+/-}$ controls as judged by incidence, size and pathological criteria (Figure 2A, C and data not shown). Since there is a short increase in lifespan of the $Rb^{+/-};E2f3^{+/-}$ animals relative to the $Rb^{+/-}$ controls, it seems likely that these tumors develop slightly slower. Consistent with this hypothesis, the complete absence of E2F3 had a major effect on pituitary tumor development. Although all of the $Rb^{+/-};E2f3^{-/-}$ mice displayed pituitary carcinomas, these were significantly smaller than those detected in the $Rb^{+/-}$ controls (Figure 2B and C). Indeed, in several cases, the tumor was still primarily contained within the intermediate layer of the pituitary causing little disruption of the other layers (Figure 2B). The suppression of tumor formation is particularly striking given that the $Rb^{+/-};E2f3^{-/-}$ animals live longer than their $Rb^{+/-}$ controls. Importantly, in about 30% of the $Rb^{+/-};E2f3^{-/-}$ animals the pituitary tumor was ruled out as the cause of death. We therefore conclude that E2F3 acts to promote the development of pituitary tumors in *Rb* mutant mice.

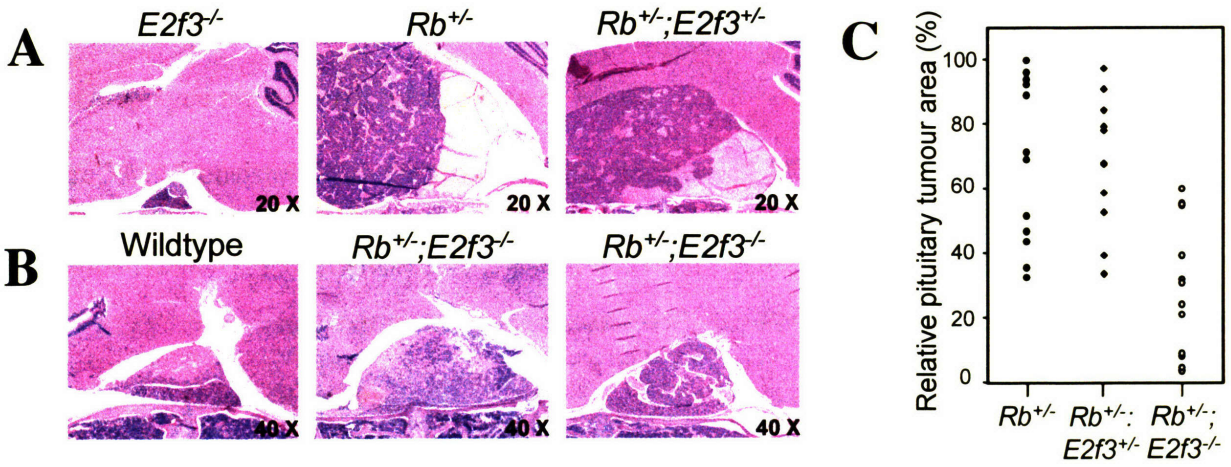


Figure 2. Loss of *E3f3* suppresses tumor formation in the pituitary.

A) Hematoxylin and Eosin stained sections of pituitaries from a healthy $E2f3^{-/-}$ animal who died at 14 months due to congestive heart failure, and $Rb^{+/-}$ and $Rb^{+/-};E2f3^{+/-}$ mice that died at 10.5 months of age due to the pituitary tumors (20X). B) Hematoxylin and Eosin stained sections of pituitaries from a healthy wildtype animal sacrificed at 12 months of age and two $Rb^{+/-};E2f3^{-/-}$ mice that died at 10.5 and 12 months (40X). C) Comparison of the pituitary tumor size (as judged by area of median section) at the time of death of $Rb^{+/-}$ (n=12), $Rb^{+/-};E2f3^{+/-}$ (n=11) and $Rb^{+/-};E2f3^{-/-}$ (n=15) mice.

_E2f3_ mutation promotes the development of pRB-deficient medullary thyroid tumors

Since the pituitary tumors could not account for the death of all $Rb^{+/-};E2f3^{-/-}$ animals, we next examined whether *E2f3* status altered the known MTC tumor phenotype of the *Rb* heterozygotes. $Rb^{+/-}$ mice are predisposed to develop c-cell hyperplasia that subsequently progress into medullary thyroid carcinomas (Hu et al. 1994; Williams et al. 1994; Harrison et al. 1995; Park et al. 1999). Depending on the specific recombinant mouse mutant used and genetic background, the incidence of these tumors varies from 50-90%. In our study, 56% of $Rb^{+/-}$ cohort displayed either c-cell hyperplasia or MTCs
(Figure 3A). Consistent with previous reports, the ensuing MTCs were mostly small, predominantly unilateral, non-aggressive and showed sings of necrosis. In contrast, ten $E2f3^{-/-}$ and 15 $E2f3^{+/-}$ littermates that were aged together with our $Rb^{+/-}$ cohort showed no signs of c-cell abnormalities (data not shown).

Significantly, the mutation of *E2f3* dramatically increased the development of these tumors. In the $Rb^{+/-};E2f3^{+/-}$ animals, MTCs were detected with almost complete penetrance (98%). Moreover, almost all of these compound mutants had bilateral, rather than unilateral, tumors and these were much larger than those observed in the $Rb^{+/-}$ controls (Figure 3A). In many cases, these tumors had invaded adjacent structures and were palpable in the living mice (Figure 3A). In a subset of the $Rb^{+/-};E2f3^{+/-}$ animals, this tumor had begun to compress the trachea and impede breathing. In these mice, the pituitary and c-cell tumors were both sufficiently severe to be considered as the cause of death. The relatively small difference in the time of death of $Rb^{+/-}$ (10.2 ± 1.6 mo) and $Rb^{+/-};E2f3^{+/-}$ (10.8 ± 1.8 mo) mice, strongly

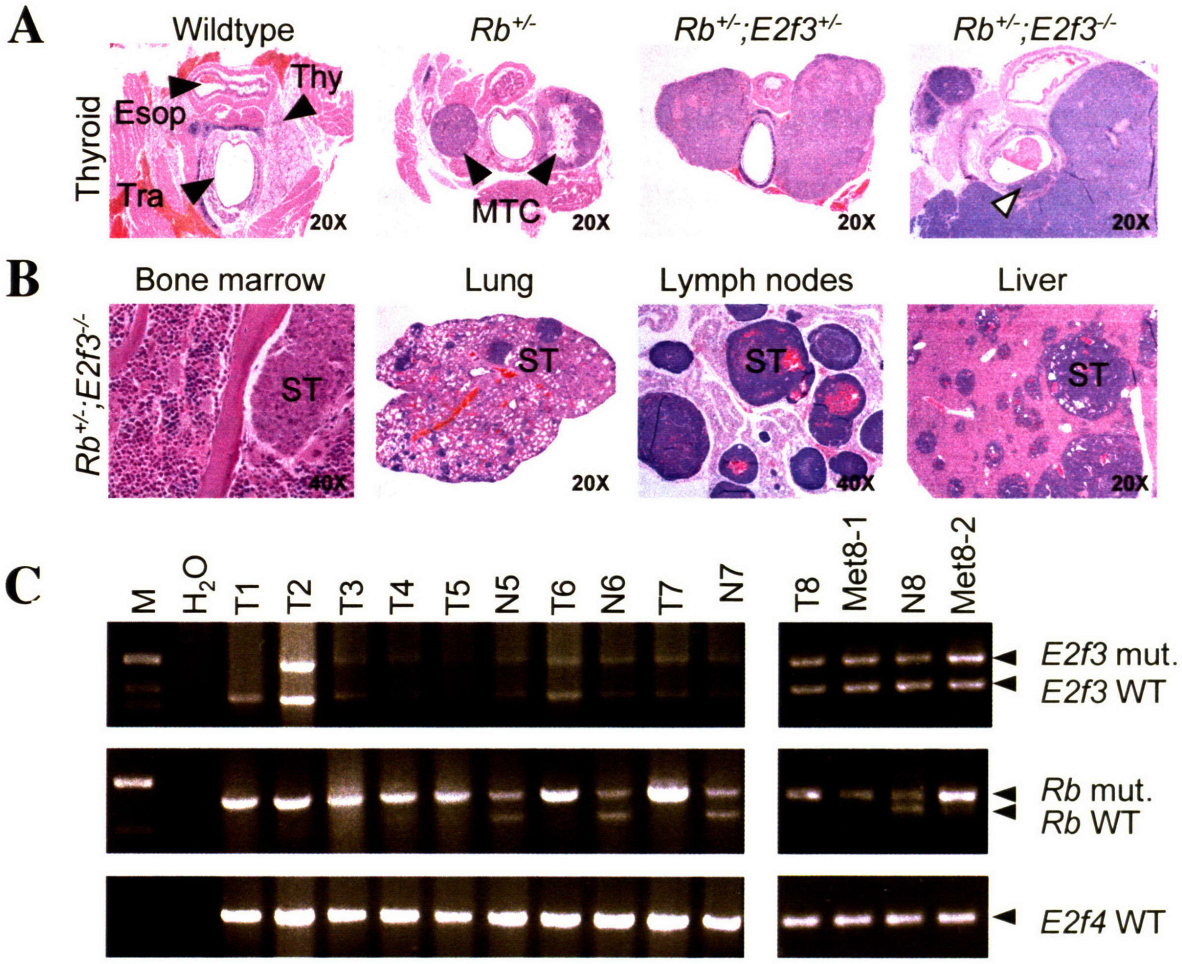


Figure 3. *E3f3* mutation promotes thyroid tumor development.

A) Hematoxylin and Eosin stained sections of thyroids from a healthy control wild-type animal sacrificed at 10.5 months of age versus MTCs derived from $Rb^{+/-}$, $Rb^{+/-}$;$E2f3^{+/-}$ and $Rb^{+/-}$;$E2f3^{-/-}$ mice that died at 10.5, 11 and 12 months of age, respectively. Arrowheads indicate the location of thyroid (Thy), trachea (Tra) and esophagus (Esop) in the wild-type controls. The open arrowhead highlights the infiltration of the tumor into the intra-tracheal space in an $Rb^{+/-}$;$E2f3^{-/-}$ animal. B) Cross-section of metastatic tumor growth in the bone marrow, lung, lymph nodes and liver of four different $Rb^{+/-}$;$E2f3^{-/-}$ animals. Selected sites of secondary tumor growth (ST) are indicated. C) PCR based LOH analysis of DNA extracted from freshly isolated biopsies from either MTCs (T), mestastases (Met) or matching normal tissues (N) derived from either $Rb^{+/-}$ (T1) or $Rb^{+/-}$;$E2f3^{+/-}$ (T2-8, Met8-1 and Met8-2) mice alongside a water only control reaction (H_2O). All of the tumors showed LOH of *Rb*, but not *E2f3*. An *E2f4*-specific PCR was used to confirm equal input of DNA.

suggests that the increase in MTC development is induced by the change in *E2f3* dosage rather than the change in lifespan.

The aggressiveness of this particular tumor located in the thyroid was further increased in the $Rb^{+/-};E2f3^{-/-}$ mice. MTCs were detected at high frequency (92%) and the vast majority were bilateral and showed extensive infiltration into adjacent structures (Figure 3A). In several animals (3/38), the tumors were found to have traversed the muscle and cartilage layers and invaded into tracheal space (open arrowhead in Figure 3A). Histologically, the MTCs of the $Rb^{+/-};E2f3^{-/-}$ mice also appeared much more aggressive than those of the $Rb^{+/-}$ and $Rb^{+/-};E2f3^{+/-}$ littermates as judged by a significant increase in the nuclear/cytoplasmatic ratio (data not shown). Importantly, these highly aggressive MTCs and/or the resulting metastases (see below) could account for the death of the subset of $Rb^{+/-};E2f3^{-/-}$ mice that had insignificant pituitary tumors. Thus, in the same animal, *E2f3* mutation is suppressing tumor development in the pituitary while promoting the development of c-cell tumors.

_E2f3_ mutation promotes metastasis of pRB-deficient medullary thyroid tumors in a dose dependent manner

In a significant fraction of the $Rb^{+/-};E2f3^{-/-}$ mice (37.5%), we observed metastasis of the MTC (Figure 3B, Table II). These secondary tumors were easily observed in the liver and lung upon macroscopic inspection. Further histo-pathological analyses revealed the presence of metastases in numerous other organs including the kidneys, intestines, lymph nodes and adrenals and more importantly into the marrow of various bones. Importantly, metastases were detected in $Rb^{+/-};E2f3^{-/-}$ mice that died at the earliest (e.g., 252 days) as well as the latest time points (e.g., 582 days) indicating that onset of metastasis is not merely as a result of the extension of lifespan.

In comparison, only a small fraction of the $Rb^{+/-}$ controls (9%) developed metastases and these were primarily located in the lung (Table III). Interestingly, there was also an increase in the frequency of metastasis in the $Rb^{+/-};E2f3^{+/-}$ mice (23%) although these were also mostly present in the lung and liver and merely a few animals developed secondary tumors in numerous organs comparable to the $Rb^{+/-};E2f3^{-/-}$ mice (Table III). Additionally, onset of metastasis was detected despite similar age of these animals as compared to $Rb^{+/-}$ mice (Table II). Taken together, these data show that E2F3 acts to suppress the development of the pRB-deficient thyroid tumor and to inhibit its metastasis.

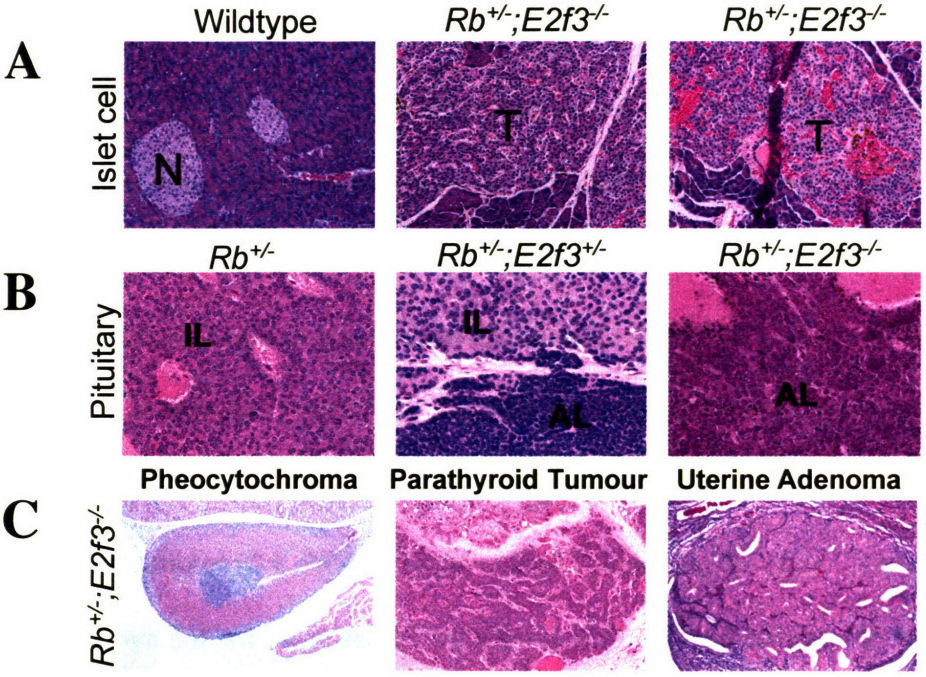
We wished to establish whether the increased aggressiveness of MTCs and ensuing metastasis in the $Rb^{+/-};E2f3^{+/-}$ mice required inactivation of the wild-type *E2f3* allele. To address this issue, we micro-dissected late-stage MTCs from the various *Rb;E2f3* genotypes and performed semi-quantitative PCR to screen for loss of heterozygosity (LOH) of both the *Rb* and *E2f3* genes. Consistent with previous analysis (Nikitin and Lee 1996), we observed LOH of *Rb* in all of the tumors derived from the $Rb^{+/-}$, $Rb^{+/-};E2f3^{+/-}$ and $Rb^{+/-};E2f3^{-/-}$ animals (Figure 3C; data not shown) confirming that our tumor samples are of sufficient purity to detect LOH. In contrast, we consistently detected the wild-type *E2f3* allele in tumors derived from the eight $Rb^{+/-}$ and 15 $Rb^{+/-};E2f3^{+/-}$ mice (Figure 3C; data not shown). Importantly, there was no evidence of LOH of *E2f3* in either the primary thyroid tumors or any of the ten metastatic lesions that we examined. We therefore conclude that E2F3 is acting in a dose dependent manner to suppress the development and the metastatic growth of MTCs in mice.

Rb;E2f3 mutant animals display several novel tumorigenic lesions

In addition to the change in the MTC phenotype, our necropsy studies showed that the *Rb;E2f3* compound mutant mice also developed novel tumorigenic lesions at low frequency (Figure 4). We detected tumors that had clearly initiated in the anterior lobe of the pituitary in both an $Rb^{+/-};E2f3^{-/-}$ and an $Rb^{+/-};E2f3^{+/-}$ animal. In the latter case, the anterior lobe tumor had developed alongside, but clearly independently from, the typical intermediate lobe pituitary tumor. The remaining, novel tumors were all specifically detected in the $Rb^{+/-};E2f3^{-/-}$ and not the $Rb^{+/-};E2f3^{+/-}$ mice. First, two animals developed islet cell tumors that were large, highly vascularized and non-necrotic. Although this incidence is low, histological examination revealed a significant incidence of islet cell hyperplasia in the majority of $Rb^{+/-};E2f3^{-/-}$ animals that is clearly distinct from the background level of occasional hyperplastic islet cells that arise in many ageing animals of this specific genetic background. Finally, we also observed one parathyroid tumor and one pineoblastoma in two independent $Rb^{+/-};E2f3^{-/-}$ animals. Interestingly, islet cell tumors, parathyroid tumors and pineoblastomas are not observed in the $Rb^{+/-}$ controls (this study; (Hu et al. 1994; Riley et al. 1994; Williams et al. 1994; Harrison et al. 1995; Nikitin and Lee 1996) but were previously detected in $Rb^{+/-};p53^{+/-}$ and $Rb^{+/-};p53^{-/-}$ mutant mice (Williams et al. 1994; Vooijs et al. 2002). The presence of these novel tumor types in the $Rb^{+/-};E2f3^{-/-}$ mice reinforces our conclusion that E2F3 can act to suppress tumor formation in the mouse.

E2f3 mutation does not alter p53 levels or activity in the pRB-deficient tumors

Inhibition of apoptosis is often a critical event in tumor development and *p53*, or its upstream regulators, are frequent targets for mutation (reviewed by Hanahan and Weinberg



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Observed lesion	*Rb*$^{+/-}$	*Rb*$^{+/-}$; *E2f3*$^{+/-}$	*Rb*$^{+/-}$; *E2f3*$^{-/-}$	*E2f3*$^{+/-}$
Islet cell tumour	0/36	0/46	2/31	0/16
Anterior pituitary tumour	0/36	1/46	2/31	0/16
Pheocytochroma	1/36	1/22	1/28	0/16
Parathyroid tumour	0/36	0/46	1/31	0/16
Pineoblastoma	0/36	0/46	1/31	0/16
Uterine endometrial adenoma	0/18	0/23	1/15	0/8

Figure 4. Additional, novel tumor types arise in the *Rb*$^{+/-}$;*E2f3*$^{-/-}$ mice.

A) Comparison of normal islets (N) in a wild-type mouse versus the islet-cell tumors (T) from two *Rb*$^{+/-}$;*E2f3*$^{-/-}$ mice (all 20x magnification). B) Histological appearance of a typical *Rb* mutant intermediate lobe (IL) pituitary tumor versus pituitary tumors from two different *Rb*$^{+/-}$;*E2f3*$^{-/-}$ mice that had initiated in both the intermediate (IL) and anterior lobe (AL) or solely the anterior lobe (AL) of a pituitary (all 40x magnification). C) A pheocytochroma, parathyroid tumor and an uterine endometrial adenoma detected in different *Rb*$^{+/-}$;*E2f3*$^{-/-}$ mice. D) Incidence of various lesions in *Rb*$^{+/-}$, *Rb*$^{+/-}$;*E2f3*$^{+/-}$, *Rb*$^{+/-}$;*E2f3*$^{-/-}$ and *E2f3*$^{+/-}$ mice (all 40x magnification).

2000). We have previously shown that *E2f3* contributes to the induction of p53-dependent apoptosis arising in pRB-deficient embryos (Ziebold et al. 2001) and now find that the $Rb^{+/-}$;$E2f3^{-/-}$ mice develop a similar spectrum (although not the same incidence) of novel tumors as the $Rb^{+/-}$;$p53^{+/-}$ and $Rb^{+/-}$;$p53^{+/-}$ animals. These observations suggested that *E2f3* mutation might promote the development of pRB-deficient tumors by reducing the activation of p53 and therefore reducing the need to functionally inactivate this protein. To test this hypothesis, we first compared p53 protein levels in size matched, late-stage MTCs derived from eight $Rb^{+/-}$, twelve $Rb^{+/-}$;$E2f3^{+/-}$ and nine $Rb^{+/-}$;$E2f3^{-/-}$ animals (Figure 5A and data not shown). Regardless of genotype, p53 was expressed at very low levels in most of the tumor samples. Although a small fraction of the tumor samples (2/8 $Rb^{+/-}$, 3/12 $Rb^{+/-}$;$E2f3^{+/-}$ and 1/9 $Rb^{+/-}$;$E2f3^{-/-}$) expressed slightly higher levels of p53, we did not observe any significant differences between the various genotypes (Figure 5A and data not shown).

To determine whether the p53 protein was functionally active, we used electrophoretic mobility shift assays (EMSAs) to screen for p53 DNA binding activity. For the majority of tumor samples, including those with the highest levels of p53, there was a direct correlation between p53 protein levels and DNA binding activity (Figure 5A and data not shown). This was also true of the metastases that were assayed (data not shown). Although we cannot rule out the possibility that a low level of mutant p53 is present in a subset of these tumors, our data suggest that the majority of the MTCs and their metastases still maintain a low level of functional wild-type p53. These data suggest that the functional inactivation of p53 is not a pre-requisite for the development or metastasis of the MTCs in the $Rb^{+/-}$ mice. Thus, there is little reason to believe that E2F3 loss promotes the development of these tumors through the regulation of p53.

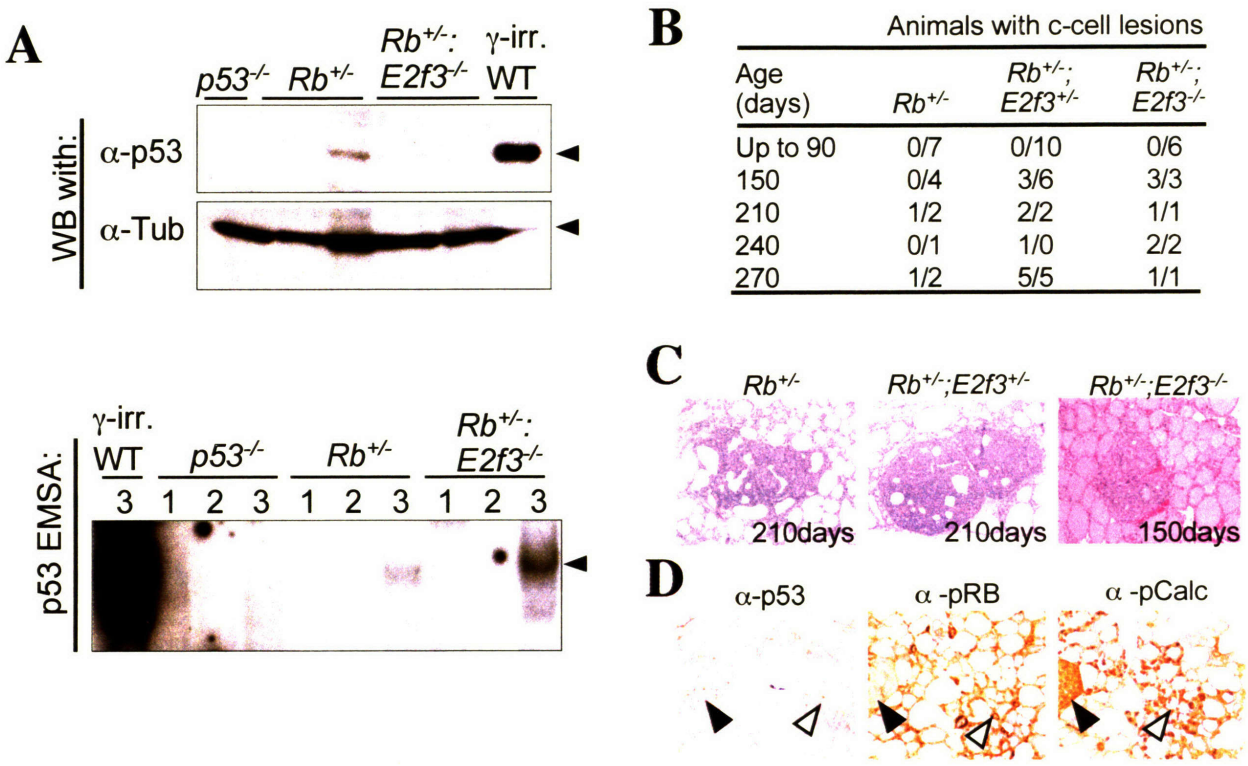

Figure 5. Further characterization of tumors derived from *Rb;E2f3* mice.

A) The levels p53 protein in MTCs derived from $Rb^{+/-}$ versus $Rb^{+/-};E2f3^{-/-}$ mice was assayed by western blotting (WB) whole cellular extracts derived from various tumors (upper panel). Whole cell lysates from wild-type MEFs that had been subjected to 1 Grey of γ-irradiation (γ-irr. WT) or a lymphoma derived from a $p53^{-/-}$ mouse ($p53^{-/-}$) were used as positive and negative controls, respectively. β-tubulin (β-Tub) was used as a loading control. The same cell extracts were also screened for the presence of p53 activity using EMSAs (lower panel). The samples were incubated with mutant competitor (1), wild-type competitor (2) and/or mutant competitor that included p53-specific antibody (3). The p53/DNA complex is indicated with an arrow. B) Number of $Rb^{+/-}$, $Rb^{+/-};E2f3^{+/-}$ and $Rb^{+/-};E2f3^{-/-}$ animals that had c-cell lesions or developed MTCs at the indicated time after histological analysis of serial sections of thyroids. C) There was a considerable difference in the size of the tumors in the various *Rb;E2f3* genotypes. For example, the tumor in a 210 day old $Rb^{+/-}$ animal was smaller than that present in an age-matched $Rb^{+/-};E2f3^{+/-}$ mouse and closely resembled that of a 150 day old $Rb^{+/-};E2f3^{-/-}$ animal. D) Analysis of p53, pRB and pCalc (Calcitonin gene-product) expression in early c-cell adenomas of $Rb^{+/-};E2f3^{-/-}$ mice using immunohistochemistry. Closed arrowheads highlight presence of low level p53 and high level pCalc, but absence of pRB expression in early adenomas. Open arrowheads depict expression of p53, pRB and pCalc in c-cells of the thyroid. All lesions in C) and D) were photographed with the same magnification (40x).

E2f3 mutation promotes the initiation of the MTC

We next tested whether *E2f3* status affected the development of pRB-deficient MTC by influencing the time of onset. To address this question, $Rb^{+/-}$, $Rb^{+/-}$;$E2f3^{+/-}$ and $Rb^{+/-}$;$E2f3^{-/-}$ littermates were sacrificed at 90, 150, 210, 240 and 270 days of age and their thyroids were screened for the presence of hyperplastic c-cell lesions or early tumors by serial sectioning (Figure 5C and data not shown). Up to 90 days of age, there was no evidence of lesions in any of the animals examined. However, by 150 days we observed a significant difference in both the frequency and size of tumors in the three genotypes. While tumors were not detected in the $Rb^{+/-}$ controls (0/4), they were present in half of the $Rb^{+/-}$;$E2f3^{+/-}$ (3/6) and all of the $Rb^{+/-}$;$E2f3^{-/-}$ (3/3) animals that we examined. However, two of the lesions in the $Rb^{+/-}$;$E2f3^{-/-}$ mice were larger than those arising in the $Rb^{+/-}$;$E2f3^{+/-}$ littermates (data not shown). C-cell tumors were first detected in the $Rb^{+/-}$ mice at 210 days of age. Importantly, these were much smaller than those arising in age matched, $Rb^{+/-}$;$E2f3^{+/-}$ mice and were of a similar size to the tumors of $Rb^{+/-}$;$E2f3^{-/-}$ mice at just 150 days of age. Immunohistochemistry confirmed that early tumorigenic lesions of the $Rb^{+/-}$;$E2f3^{-/-}$ mice had all of the hallmarks of pRB-deficient MTCs (Figure 5D). The tumor stained positive for calcitonin, a specific marker of c-cells (open arrowhead). Moreover, pRB was clearly expressed in the c-cells, but not the MTC, confirming that its loss is required for tumorigenesis. Finally, in agreement with our analysis of late-stage tumors, we detected low level p53-staining in the early MTCs derived from $Rb^{+/-}$;$E2f3^{-/-}$ and also $Rb^{+/-}$ and $Rb^{+/-}$;$E2f3^{+/-}$ mice (Figure 5D and data not shown). Taken together, these data indicate that *E2f3* acts in a dose dependent manner to suppress the initiation of MTCs.

DISCUSSION

We have previously described an *E2f3* mutant mouse strain in which expression of E2F3a and E2F3b is abrogated and shown that E2F3 contributes to the induction of cellular proliferation in both wild-type and pRB-deficient cells (Humbert et al. 2000b; Ziebold et al. 2001). In this study, we have used *Rb;E2f3* compound mutant mice to determine how E2F3 contributes to the development of pRB-deficient tumors. This analysis reveals considerable interplay between E2F3 and pRB in both normal development and tumorigenicity (Figure 6).

E2F3 and pRB act in opposition to one another in normal development

With regard to development, all of our findings support a simple model in which pRB and E2F3 act in direct opposition to one another (Figure 6). Our data clearly show that mutation of a single *Rb* allele is sufficient to increase the fraction of surviving $E2f3^{-/-}$ neonates by two- to threefold. Moreover, it also completely suppresses the congestive heart failure that is responsible for the death of most $E2f3^{-/-}$ adults (this study (Cloud et al. 2002). This directly complements the previous finding that *E2f3* mutation suppresses the inappropriate proliferation and apoptosis in pRB-deficient embryos and thereby greatly extends their lifespan (Ziebold et al. 2001; Saavedra et al. 2002). Significantly, many of the properties of E2F3 in normal development are shared by E2F1. The phenotypes of $Rb^{-/-};E2f3^{-/-}$ and $Rb^{-/-};E2f1^{-/-}$ embryos are similar to one another (Tsai et al. 1998; Ziebold et al. 2001). Moreover, the analysis of *E2f1;E2f3* compound mutant mice shows that the developmental and age-related defects of the individual *E2f1* or *E2f3* mice are exacerbated by the mutation of the other *E2f* (Cloud et al. 2002). Together, these data indicate that E2F1 and E2F3 play critical, overlapping roles in the

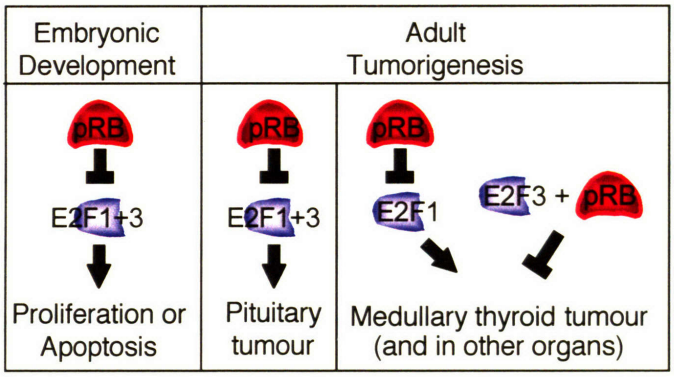

Figure 6. Relative roles of pRB and E2F3 in both development and tumorigenesis.

development and maintenance of a variety of tissues and pRB opposes the action of these factors. Importantly, this regulation appears to be critically dependent upon the appropriate balance of pRB and E2F proteins since a reduction in *Rb* or *E2f* gene dosage can either disrupt or restore development.

E2f3 acts as either an oncogene or tumor suppressor in different *Rb* mutant tumors

In contrast to the developmental regulation, the relative roles of pRB and E2F3 in tumorigenesis appear highly complex. It is well documented that the retinoblastoma gene behaves as a classic tumor suppressor. Humans or mice carrying a germline *Rb* mutation develop tumors with complete penetrance and tumor development is accompanied by LOH (reviewed by Mulligan and Jacks 1998). One significant difference is the spectrum of tumors, retinoblastoma in humans versus pituitary and c-cell carcinomas in mice. In this study, we show that E2F3 loss in mice has a differential effect on the development of these specific tumor types. In the pituitary, the absence of E2F3 significantly suppressed tumor formation resulting in an extension of lifespan. Thus, E2F3 acts as an oncogene in this tissue. In contrast, in the c-cells, E2F3 loss clearly increases tumorigenicity leading to the formation of highly aggressive carcinomas that metastasize to form secondary tumors within a wide variety of other tissues. Indeed, these MTCs and/or their metastases, and not pituitary tumors, are responsible for the death of a significant fraction of the $Rb^{+/-};E2f3^{-/-}$ animals. This represents the first evidence that E2F3 can collaborate with pRB to act as a tumor suppressor *in vivo*. This conclusion is supported by the finding that $Rb^{+/-};E2f3^{-/-}$ mice develop additional tumor types that are not observed in the $Rb^{+/-}$ controls. Thus, in different tissues of a single animal, E2F3 acts as either an oncogene or a tumor suppressor (Figure 6). This underscores the importance of tissue-specific

roles for E2F3 and raises questions about the underlying mechanism of these opposing activities. Clearly, since the *E2f3* mutation affects expression of both E2F3a and E2F3b it is entirely possible that this results from differential activities of the two E2F isoforms.

Before further discussion of potential mechanisms, it is important to consider what we now know about the tumorigenic properties of the various E2F family members. The concept that *E2f*s could function as tumor suppressors was originally deduced from the analysis of *E2f1* mutant mice (Yamasaki et al. 1996; Yamasaki et al. 1998). This early study showed that both $E2f1^{+/-}$ and $E2f1^{-/-}$ mice had an increased propensity to develop tumors with late onset and low penetrance (Yamasaki et al. 1996). It was subsequently shown that *E2f2* mutant mice are tumor prone and that the combined mutation of *E2f1* and *E2f2* increases both the incidence and time of onset of tumorigenesis (Zhu et al. 2001). Thus, at least in certain tissues, E2F1 and E2F2 have similar, additive roles in the suppression of tumors. In contrast, we have found that the mutation of *E2f3*, either alone or in combination with *E2f1*, or *E2f4* has no detectable effect on tumorigenesis (Humbert et al. 2000a; Cloud et al. 2002). This initially suggested that tumor suppression might be a specific property of *E2f1* and *E2f2*, but not *E2f3* or *E2f4*.

Significantly, the tumorigenic properties of the individual *E2fs* appear quite different when analyzed in the context of the $Rb^{+/-}$ mutant background. In this setting, *E2f1* clearly displays oncogenic not tumor suppressive properties. E2F1 loss suppresses the development of pRB-deficient brain tumors in a large T-antigen transgenic mouse model (Pan et al. 1998) and the formation of both pituitary and thyroid tumors in $Rb^{+/-}$ mice yielding a dramatic extension of lifespan (Yamasaki et al. 1998). In contrast, E2F3 loss has opposing effects on the pituitary tumors (suppressing) and the thyroid tumors (promoting) indicating that it is behaving as both oncogene and tumor suppressor. The phenotype of the $Rb^{+/-};E2f4^{-/-}$ mice adds to this complexity.

E2F4 loss suppresses the pituitary and thyroid tumors arising the $Rb^{+/-}$ mice more effectively that the loss of any other E2F tested to date (Lee et al. 2002). However, molecular analyses suggest that this occurs via an indirect mechanism in which the absence of E2F4 allows it associated pocket proteins, p107 and p130, to bind, and presumably suppress the activity of, E2F1 and E2F3 (Lee et al. 2002). Thus, it appears that the complete loss of E2F3 has a different effect on the tumor spectrum of $Rb^{+/-}$ mice than restoration of the normal pocket protein regulation of the endogenous E2F1 and E2F3. We cannot rule out the possibility that the consequences of E2F3 loss result from changes in the regulation or activity of the remaining E2F proteins. However, together these observations show that the activating E2Fs (E2F1, 2 and 3) all have the ability to either promote or suppress tumorigenicity depending on the setting.

It is widely believe that the activating E2Fs' oncogenic activity results from the known, shared (cell autonomous) role of these proteins in the transcriptional activation of E2F-responsive genes and the induction of cellular proliferation (Humbert et al. 2000b; Wu et al. 2001). In contrast, there is still considerable debate about the underlying basis for the E2F's tumor suppressive activity. Several models have been proposed to account for the E2Fs' tumor suppressive activity, all of which are cell autonomous. One of the most popular is that this results in the known ability of the E2Fs to activate p53-dependent apoptosis (Qin et al. 1994; Shan and Lee 1994; Wu and Levine 1994). Since, apoptosis requires a higher threshold level of E2F activity than the induction of cellular proliferation, this offers a simple explanation for the E2Fs ability to act as an oncogene (low level induces proliferation) or a tumor suppressor (high level induces apoptosis). Our analysis of *Rb;E2f3* mutant tumors provides some insight into this model. Initially, our observation that $Rb^{+/-};E2f3^{-/-}$ animals develop the same novel tumor types as $Rb^{+/-};p53^{+/-}$ and $Rb^{+/-};p53^{-/-}$ mutant mice seemed to support it. Perhaps E2F3 loss promoted

tumor development by reducing the requirement to inactivate p53-dependent apoptosis. However, our additional analysis did not support this conclusion. Regardless of whether the MTCs and their metastases were isolated from $Rb^{+/-}$, $Rb^{+/-}$;$E2f3^{+/-}$ or $Rb^{+/-}$;$E2f3^{-/-}$ mice, the majority retained a low level of functional p53 protein. Thus, p53-inactivation is not a pre-requisite for the development of MTCs or subsequent metastases and *E2f3* status does not appear to alter its frequency. This suggests that, at least in this specific tumor type, *E2f3* does not exert its tumor suppressor effect by altering the need to inactivate p53. Obviously, this does not rule out the possibility that the apoptosis mechanism is operating in other tumor types including the additional, rare tumors arising in the $Rb^{+/-}$;$E2f3^{-/-}$ mice that phenocopy those arising in the $Rb^{+/-}$;*p53* mutant animals.

Importantly, our analysis of staged tumors strongly suggests that *E2f3* status affects the rate of tumor onset. Specifically, tumorigenic lesions arise at a progressively earlier time in the thyroids of $Rb^{+/-}$;$E2f3^{-/-}$, $Rb^{+/-}$;$E2f3^{+/-}$ and $Rb^{+/-}$ mice. This observed acceleration of tumor onset is consistent with three other models of E2Fs' tumor suppressor function. First, since the activating E2Fs bind pRB family members and their associated histone deacetylases it is widely believed that they can participate in the repression of E2F-responsive genes [see (Rayman et al. 2002) for example]. Second, E2F1 has been also implicated as a component of the ATM/ATR-Nbs1/Mre11 damage response pathway (Meng et al. 1999; Lin et al. 2001; Maser et al. 2001). Finally, it has recently been reported that E2F3 loss can lead to centrosome amplification, mitotic spindle defects and aneuploidy (Saavedra et al. 2003). Significantly, these latter two mechanisms have been primarily linked to E2F1 and E2F3 respectively. This raises the possibility that the activating E2Fs might suppress tumor formation through distinct mechanisms.

The tumor suppressive properties of *E2f1* and *E2f3* do have at least one, unusual characteristic in common. The analysis of tumors derived from $E2f1^{+/-}$ mice showed that there is no evidence of LOH of *E2f1* (Yamasaki et al. 1996; Zhu et al. 2001; Cloud et al. 2002). This is entirely consistent with the finding that $E2f1^{+/-}$ and $E2f1^{-/-}$ mice have a similar spectrum, incidence and kinetics of tumor formation and it strongly suggests that a reduction in E2F1 levels is sufficient to negate its tumor suppressive properties. Here we now show that the development of the pRB-deficient thyroid tumors is promoted by the heterozygous mutation of *E2f3* and the primary and secondary tumors retain the wild-type *E2f3* allele (in a mixed background). This is further supported by the fact that heterozygous mutation of *E2f3* also increases both the incidence of MTCs in the pure 129/Sv background $Rb^{+/-}$ mutant mice (data not shown). Thus, in an analogous manner to *E2f1*, a mere reduction in *E2f3* dosage is sufficient to impair tumor suppression. This is not a unique phenomenon, but it has been ascribed to only a small number of tumor suppressors (reviewed by Cook and McCaw 2000; Quon and Berns 2001). Thus, the finding that this is a shared property of *E2f1* and *E2f3* supports the notion that they may suppress tumor formation via a similar mechanism.

Interestingly, the tumorigenicity of $Rb^{+/-}$;$E2f3^{+/-}$ and $Rb^{+/-}$;$E2f3^{-/-}$ animals is not equivalent. Mutation of the second *E2f3* allele increases the aggressiveness of the MTC, broadens the tissue spectrum of the resulting metastases and allows the formation of novel tumor types. There are two possible explanations for these different tumor phenotypes. *E2f3* could simply act to suppress tumors in a dose dependent manner. Alternatively, the heterozygous and homozygous mutation of *E2f3* could promote tumorigenesis via different mechanisms. Indeed, it seems possible that *E2f3* haploinsufficiency could promote tumor formation through a mechanism that is shared with E2F1 while complete loss of E2F3 might act through a unique

mechanism, for example centrosome amplification. Obviously, additional experiments will be required to address all potential models.

Regardless of the underlying mechanism, it now seems clear that the activating E2Fs (E2F1, 2 and 3) all display similar abilities to promote or suppress tumorigenicity. However, the observed behavior appears to be highly context dependent, being greatly influenced by the absence or presence of pRB and the levels/activity of the remaining E2F species. The simplest explanation for this finding is that the individual E2Fs have both positive and negative functions that are integrated to determine the rate of cell proliferation and/or survival. In this manner, the changes in the individual E2Fs could either promote or suppress tumor formation depending on the balance of positive and negative activities in individual tissues.

EXPERIMENTAL PROCEDURES

Animal maintenance and histological analysis

The *Rb* and *E2f3* mutant mouse strains were genotyped using previously described PCR protocols (Jacks et al. 1992; Humbert et al. 2000b). Animals were sacrificed just prior to death or as warranted by the size of the tumors. In some cases, animals were examined after they were found dead. Full necropsy was performed on all animals according to standard procedures. For histology all tissues were fixed in 10% phosphate buffered formalin or Bouin's solution, embedded in paraffin blocks and 4-6 μm representative sections were stained with hematoxylin and eosin. For more detailed analysis of the pathology of thyroids and c-cell lesions as well as ensuing metastasis in lungs sections from four to six different levels of these organs were produced. Tumor genotypes were determined by conducting semi-quantitative PCR in tissues that had been isolated using a dissection microscope.

Statistical analysis and establishment of relative tumor areas

Survival and longevity statistical analyses were calculated with Excel (Microsoft) and Stata 6.0 (Stata-Corporation) programs. To establish relative pituitary tumor areas, low power microscopic views of *Rb* mutant pituitary tumors were photographed and a normalized grid was electronically merged with the entire tumor area. The largest pituitary carcinoma was set to 100% and tumors of all other genotypes were measured accordingly.

Immunohistochemistry, western blotting and gel retardation assays.

Immunohistochemical detection of p53 (CM5; Novocastra), pRB (144011A; BD Bioscience) and Calcitonin (CMC-101; Cell Marque) was performed on formalin fixed, paraffin embedded 4 μm sections. Antigen retrieval was performed by boiling the specimen in Trilogy

(Cell Marque). Detection of primary antibodies was performed using biotinylated secondary antiserum facilitated by ABC, avidin-peroxidase-biotin complex, as suggested by manufacturer (Vector Lab.). Specificity of α-pRB primary antibodies was verified using CNS sections of WT and viable $Rb^{-/-}$ E13.5 embryos. All stainings had a control reaction that did not contain any primary antibody. Western blotting and gel retardation assays were performed on whole cell extracts of dissected late stage MTCs as previously described (Tsai et al. 2002).

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