The Role of *E2f4* in Cell Cycle Exit and Bone Development

By

Emily S. Miller

B.S., Biology
University of Massachusetts Amherst, 2003

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ABSTRACT

Members of the E2F family of transcription factors are critical downstream effectors of the pocket protein family and mediate the regulation of genes required for cellular proliferation. The repressive E2Fs act in association with the pocket proteins to promote a G0/G1 phase of the cell cycle. These complexes recruit histone deacetylases to target gene promoters to prevent the transcription of genes required for cell cycle progression. As cell cycle exit is often concomitant with differentiation, it is not surprising that mutation of the E2Fs and pocket proteins results in defective development and differentiation. Mutation of the most abundant E2F, E2F4, is known to disrupt the proper differentiation of several cell types, including erythrocytes and respiratory epithelium cells. Here, I analyzed a novel role for *E2f4* in bone development. I found that mutation of *E2f4* causes defects in intramembranous and endochondral bone development. The calvarial bones of the skull exhibit the most severe defect in development, which is caused by a significant delay in differentiation of osteoblasts. I showed that *E2f4* loss does not alter the differentiation potential of osteoblast progenitors. Instead, loss of *E2f4* impairs the ability of these cells to exit the cell cycle and increases the pool of undifferentiated progenitor cells, delaying bone formation. To further elucidate the role of *E2f4* in cell cycle exit and differentiation, I have generated conditional *E2f4* knockout mice. Analysis of these mice will address the cell autonomous roles *E2f4* plays during differentiation and development, in addition to establish compensatory roles *E2f4* may share with other E2F family members. Taken together, this work has established the in vivo role of *E2f4* in osteoblast differentiation and bone development. Furthermore, this work opens new fields of study regarding *E2f4* function during mouse development.

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Chapter One

Introduction

Historically, members of the E2F family of transcription factors are best known as critical downstream effectors for the retinoblastoma family of pocket proteins. E2F/pocket protein complexes function to regulate cell cycle progression. Early studies of E2F only hinted at the involvement of these proteins in cellular processes beyond cell cycle progression; however, a number of recent studies have revealed a diverse catalog of context-dependent functions for E2Fs *in vivo*. Although many of these functions rely on the cell cycle regulatory properties of associated pocket protein family members, there is emerging evidence that the E2Fs are capable of several cell cycle-independent roles. This chapter describes the functions that pocket protein and E2F family members are responsible for in the cell, focusing on development and differentiation. The first part of this chapter introduces each family member and discusses the roles each protein plays to control cell cycle progression. The second part of this chapter reviews the evidence implicating pocket protein or E2F involvement in development and differentiation. As the majority of the work in this thesis aims to gain a better understanding of bone development, the final part of this chapter describes the cell types and their functions that contribute to skeleton formation.

Part I: Overview of the pocket protein/E2F growth control pathway

A. Discovery and characterization of the retinoblastoma gene family

Retinoblastoma is a malignant cancer of the eye, most commonly occurring in children. Typically, patients with retinoblastoma exhibit either a single tumor in one eye (unilateral retinoblastoma) or multiple focal tumors in both eyes (bilateral retinoblastoma).

The uncommon genetics of the disease led Alfred Knudson to propose that two genetic mutations, or "hits," are required for retinoblastoma formation (Knudson, 1971). Thus, sporadic retinoblastoma results when individuals acquire two somatic mutations in the retinoblastoma susceptibility gene and familial retinoblastoma occurs when an individual carrying a germline mutation in one allele of this gene acquires an additional somatic mutation in the second allele.

The susceptibility to retinoblastoma was mapped to a region of chromosome 13, 13q14, by cytogenetic studies of non-tumorigenic samples from human retinoblastoma patients (Dryja et al, 1986; Sparkes et al, 1980). Chromosomal walking techniques led to the identification of *RB-1*, which encodes a nuclear phosphoprotein that is 110 kilodaltons (Lee et al, 1987b). Indeed, mutations of this locus were verified to occur in retinoblastomas (Friend et al, 1986; Fung et al, 1987; Lee et al, 1987a). The finding that *RB-1* mutations occur in retinoblastoma suggested that the protein product, pRB, plays a role in cell cycle progression. Indeed, studies of DNA tumor viruses and their viral oncoproteins, such as adenovirus E1A, SV40 large T antigen, and the human papillomavirus E7, demonstrated that oncoprotein binding to pRB leads to increased cell proliferation and is required for cellular transformation (DeCaprio et al, 1988; Dyson et al, 1989b; Whyte et al, 1988). Additional targets bound by proteins encoded by small DNA tumor viruses were also found. p107 and p130, which are homologous to pRB, were identified by their ability to associate with adenovirus E1A (Dyson et al, 1989a). Although p107 and p130 share structural similarities to pRB, p107 and p130 are more homologous with each other (50% similarity) than either one is with pRb (30-35% similarity) (Ewen et al, 1991; Hannon et al,

1993; Li et al, 1993). Much of this homology is found within the hallmark pocket domain found in all three proteins; thus, pRB, p107, and p130 are referred to as the pocket proteins. In addition to sharing structural homology to pRB, p107 and p130 also play a role in cell cycle progression, most likely as a consequence of their ability to bind to and regulate the E2F family of transcription factors, like pRB (E2Fs are discussed in Part B).

Although all pocket protein family members play a role in regulating the cell cycle, pRB is the only one that acts as a bona fide tumor suppressor. Somatic mutations in *Rb* have been identified in various cancers, indicating that its tumor suppressor role is not restricted to the retina (Weinberg, 1992). Indeed, it is estimated that *RB-1* is either mutated or altered in approximately one-third of all human tumors (Weinberg, 1992). There is evidence that *p130* mutations have been found in a subset of human tumors (Claudio et al, 2000a; Claudio et al, 2000b; Helin et al, 1997), suggesting that p130 may act as a tumor suppressor in certain contexts. Compound mutant mouse models have also suggested potential tumor suppressive roles for p107 or p130; however, this is limited to cells that lack pRB. For example, retinoblastoma only occurs in mice if both *Rb* and *p107* or *p130* are altered in retinal cells (MacPherson et al, 2007; MacPherson et al, 2004). Furthermore, mice chimeric for *Rb* and *p107* or *Rb* and *p130* develop novel tumor types, such as retinoblastoma, not seen in *Rb* mutants alone (Dannenberg et al, 2004; Robanus-Maandag et al, 1998).

B. The E2F family of transcription factors

i. Discovery of E2F

The E2F transcription factor was first identified as a cellular component required for the transcription of the early adenoviral *E2* gene. Upon infection with adenovirus, the first viral transcript to be produced is *E1A*, which induces the expression of several other early viral transcripts, such as *E2*. Specifically, E1A recruits a cellular activity termed the E2 promoter-binding factor, or E2F, at two DNA recognition sites (5'-TTTCGCGC-3') in the E2 promoter (Kovesdi et al, 1987; Yee et al, 1987). E2F is critical for the activation of cellular genes required for the G1/S transition of the cell cycle, nucleotide biosynthesis, and DNA replication (Trimarchi & Lees, 2002). Studies revealed that E1A causes a cellular protein to dissociate from E2F, which led to the finding that a known E1A interacting protein, pRB, inhibits E2F activity (Trimarchi & Lees, 2002). Based on the ability of the encoded protein to interact with pRB, a subunit of E2F was cloned and named E2F-1 (Helin et al, 1992; Kaelin et al, 1992; Shan et al, 1992). Concomitant with the identification of E2F, studies performed in murine embryonic carcinoma stem cells identified DRTF1, differentiation regulated transcription factor 1, which is down-regulated upon differentiation (La Thangue & Rigby, 1987). Interestingly, DRTF1 binds the same consensus DNA sequence as E2F, and also interacts with pRB (Bandara & La Thangue, 1991; La Thangue & Rigby, 1987). Subsequently, studies proved DRTF1 and E2F were the same factor.

Further studies with DRTF1 led to the identification of DP1, which stands for DRTF-polypeptide 1 (Girling et al, 1993). The related DP2 protein was identified later (Ormondroyd et al, 1995; Rogers et al, 1996; Wu et al, 1995; Zhang & Chellappan, 1995). Both DP proteins can form heterodimers with E2F1-6 *in vivo*, with no differences between

DP1- or DP2- containing E2F complexes (Bandara et al, 1993; Helin et al, 1993; Krek et al, 1993; Trimarchi et al, 1998; Wu et al, 1995). These complexes bind E2F consensus sites and stimulate transcription of target genes. Furthermore, the amino acids that contact E2F binding sites are conserved in both DP and E2F proteins. The DP moiety is required for DNA binding *in vivo* (Bandara et al, 1993; Huber et al, 1993), while the E2F subunit confers different transcriptional and cellular responses depending on which E2F is in the complex (discussed below).

ii. Classification of the E2Fs

Although there are eight *E2f* genes to date, two loci, *E2f3* and *E2f7*, encode two isoforms each: *E2f3a* and *E2f3b*, and *E2f7a* and *E2f7b*, constituting a family of ten distinct gene products. Based on differences in sequence and function, the E2Fs can be classified into three groups. E2F1, 2, and 3 are referred to as the "activating E2Fs," E2F4 and E2F5 comprise the "repressive E2Fs," and E2F6, 7, and 8 are also transcriptional repressors, but function independently of the pocket proteins.

a. The activating E2Fs

The first member of the E2F family of transcription factors, E2F1, was cloned based on its ability to interact with pRB (Helin et al, 1992; Kaelin et al, 1992; Shan et al, 1992). Using the minimal DNA binding domain of E2F1 as a probe, low stringency screening of a cDNA library led to the identification of two related proteins, *E2f2* and *E2f3* (Ivey-Hoyle et al, 1993; Lees et al, 1993). All of the activating E2Fs are structurally similar and possess distinct domains for DNA binding, DP dimerization, and transactivation (Figure 1). The

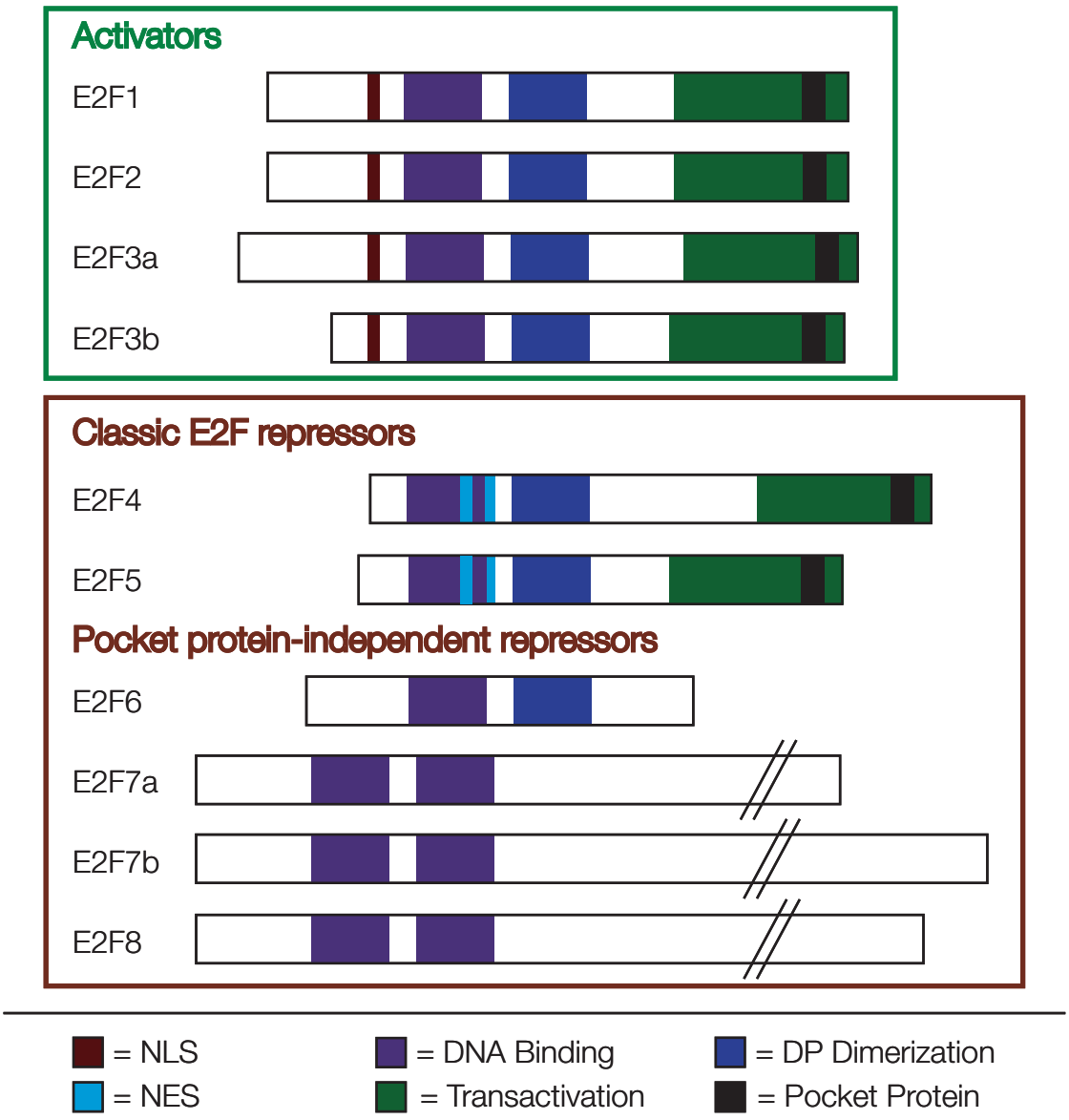

Figure 1. The E2F family of transcription factors

The E2F family is classified into three groups based on their structure and function. All the E2Fs possess homologous DNA binding domains. The activating E2Fs, E2F1, E2F2, E2F3a, and E2F3b each contain a nuclear localization signal (NLS), a DP dimerization domain, and overlapping transactivation and pocket protein binding domains. The classic repressors, E2F4 and E2F5, lack the N-terminal domain found in the activating E2Fs and possess a nuclear export signal (NES), rather than a NLS. E2F6-8 lack sequences required for the transactivation and pocket protein binding domains; therefore, these proteins function as pocket protein-independent repressors of transcription. E2F7a, E2F7b, and E2F8 also lack a DP dimerization domain. Instead, these proteins contain a duplication of the DNA binding domain, allowing them to bind DNA as heterodimers or homodimers with each other, without the aid of a DP protein.

pocket protein-binding domain lies within the transactivation domain near the C-terminus. These domains display a high degree of conservation, ranging from 45-100% identity when compared to equivalent regions in E2F1 (Lees et al, 1993). In addition, E2F1, 2, and 3 possess a nuclear localization signal found in the amino terminus of the proteins (Muller et al, 1997) (Verona et al, 1997).

The activating E2Fs have well-established roles in promoting cell cycle entry. In transient reporter assays, E2F1-3 all act as strong transcriptional activators (Helin et al, 1993; Lees et al, 1993). Indeed, overexpression of any one of these proteins will induce the expression of genes involved in DNA synthesis and cell cycle progression, and is also sufficient to promote cell cycle re-entry in quiescent cells (DeGregori et al, 1997; Johnson et al, 1993; Kowalik et al, 1995; Lukas et al, 1996; Qin et al, 1994). Furthermore,in some cases, overexpression of E2F1, 2, or 3 can override growth arrest signals (DeGregori et al, 1995; Mann & Jones, 1996; Schwarz et al, 1995).

Expression data for the activating E2Fs also provides evidence for their role in promoting proliferation. In quiescent cells, E2F1, 2, and 3 proteins are present at low or barely detectable levels, but their ability to bind DNA of target genes peaks as cells enter the cell cycle (Leone et al, 1998; Leone et al, 2000; Moberg et al, 1996). The localization of these proteins is constitutively nuclear, which is most likely due to their nuclear localization signal (Muller et al, 1997; Verona et al, 1997). Additionally, E2F1-3 are bound to E2F-target gene promoters during G1/S, and these interactions are coincident with the transcriptional activation of these genes (Rayman et al, 2002; Takahashi et al, 2000). Finally, the induction of E2F-responsive genes is dependent on an unaltered E2F DNA consensus sequence in

the target gene promoter as well as a functional transactivation domain in E2F itself (Hsiao et al, 1994; Lam & Watson, 1993).

Genetic studies have provided further evidence that activating E2F activity is required for cell cycle progression. All three activator E2Fs are required for entry into the cell cycle as MEFs lacking E2F1-3 are unable to proliferate (Wu et al, 2001). However, some studies demonstrate the importance of individual E2F activity. The absence of E2F3 in MEFs causes a delay in proliferation due to decreased DNA synthesis and an impaired ability to re-enter the cell cycle from a quiescent state (Humbert et al, 2000b). Although *E2f1*$^{-/-}$ MEFs do not display any cell cycle defects (Humbert et al, 2000b), the acute ablation of *E2f1* or *E2f3* expression with shRNAs prevents quiescent cells from entering the cell cycle upon serum addition (Kong et al, 2007).

The genetic locus for *E2f3* encodes two isoforms, *E2f3a* and *E2f3b*, which are transcribed from two distinct promoters (He et al, 2000; Leone et al, 2000). E2F3a is found predominantly in cycling cells with enrichment in S phase, while E2F3b is expressed throughout the cell cycle and found complexed with pRb in quiescent cells (He et al, 2000; Leone et al, 2000). Based on this expression data, E2F3b was originally proposed to function as a transcriptional repressor. However, recently developed mouse models that conditionally inactivate both *E2f3a* and *E2f3b* argue against this model. Chromatin immunoprecipitation and gene expression analyses have demonstrated that both *E2f3a* and *E2f3b* contribute to G1/S-specific gene expression and cell proliferation (Chong et al, 2009). Moreover, expression of either isoform is sufficient to activate E2F target gene expression and cell proliferation in the absence of *E2f1* and *E2f2*, suggesting redundant

roles between the activating E2Fs (Chong et al, 2009). Despite these overlapping functions, *E2f3a* and *E2f3b* contribute different functions for cell cycle progression. *E2f3a*$^{-/-}$ MEFs have a low penetrance proliferation defect, while MEFS deficient for *E2f3b* do not (Danielian et al, 2008). In addition, *in vitro* studies indicate that *E2f1*$^{-/-}$;*E2f3a*$^{-/-}$ MEFs display significant proliferation defects, but these defects are not seen when *E2f1* loss is combined with the loss of *E2f3b* (Danielian et al, 2008).

b. The repressive E2Fs

The repressive E2Fs, E2F4 and E2F5, were discovered based on their ability to interact with the pocket proteins p107 and p130 (Beijersbergen et al, 1994; Ginsberg et al, 1994; Hijmans et al, 1995; Sardet et al, 1995). Structurally, these proteins are distinct from the activating E2Fs, exhibiting 80% similarity between each other and only 30-60% similarity with the activating E2Fs. E2F4 and 5 possess domains that are required for DNA binding, DP dimerization, and transactivation/pocket protein binding, similar to the activators. However, the repressive E2Fs lack a similar amino terminus present in the activating E2Fs, which includes the nuclear localization signal (Figure 1). Instead, E2F4 and E2F5 contain hydrophobic nuclear export signals, which are responsible for the primarily cytoplasmic localization of E2F4 (Gaubatz et al, 2001). In order for E2F4 to enter the nucleus in G0/G1, it must participate in a pocket protein complex (Lindeman et al, 1997; Magae et al, 1996; Rayman et al, 2002; Verona et al, 1997). Since E2F4 is the most abundant E2F *in vitro* (Moberg et al, 1996), most experiments have focused on ascertaining the role of E2F4 in cell cycle regulation. E2F5 is thought to function in a manner similar to E2F4 due, largely in part, to their high degree of similarity.

The roles of E2F4 and E2F5 differ dramatically from the roles that the activating E2Fs play in cell cycle regulation. Although early studies demonstrated that E2F4 is able to activate transcription (Ginsberg et al, 1994; Lukas et al, 1996), it was subsequently found that overexpression of E2F4 or E2F5 was not sufficient to drive progression through the cell cycle and was also unable to cause arrested cells to re-enter the cell cycle (DeGregori et al, 1997; Lukas et al, 1996; Mann & Jones, 1996). Furthermore, *E2f4*$^{-/-}$ and *E2F5*$^{-/-}$ MEFs display a normal cell cycle profile (Humbert et al, 2000a; Lindeman et al, 1998; Rempel et al, 2000). These studies suggest that E2F4 and E2F5 function, in part, by regulating and maintaining a cell cycle arrest. Consistent with this role, *E2f4*$^{-/-}$*;E2F5*$^{-/-}$ MEFs are unable to properly arrest despite an overexpression of the growth-inhibitory signal p16^{INK4A} (Gaubatz et al, 2000). Similarly, MEFs that lack the pocket protein binding partners exclusive to the repressive E2Fs, p107 and p130, are unable to arrest (Classon et al, 2000b; Hurford et al, 1997).

The expression and subcellular localization of E2F4 and E2F5 also support their role in target gene repression and cell cycle arrest. The activating E2Fs are primarily expressed in cycling cells; however, the repressive E2Fs are present in all phases of the cell cycle, including G0 (Muller et al, 1997; Sardet et al, 1995; Takahashi et al, 2000; Vairo et al, 1995; Verona et al, 1997; Wells et al, 2000). In contrast to the nuclear localization of E2F1-3, E2F4 and E2F5 contain two nuclear export signals (Figure 1) and their predominantly cytoplasmic localization is dependent upon the CRM1 nuclear export factor (Gaubatz et al, 2001). In G0 and early G1, E2F4 and E2F5 are found associated with pocket proteins in the nucleus, bound to target gene promoters when these genes are not expressed (Muller

et al, 1997; Sardet et al, 1995; Takahashi et al, 2000; Vairo et al, 1995; Verona et al, 1997; Wells et al, 2000). As the pocket protein binding domain overlaps with the transactivational domain of E2F, association with pocket proteins eliminates the transactivation potential of E2F4. Finally, E2F4 is able to form complexes with the three pocket proteins, while E2F5 binds pRB and p130 (Hijmans et al, 1995; Moberg et al, 1996).

c. The pocket protein-independent E2Fs

E2F6-8 are structurally distinct from the other members of the E2F family due to the absence of both the transactivation and pocket protein binding domains (Figure 1). E2F6 was originally hypothesized to act as either a transcriptional repressor or a dominant-negative inhibitor of the other E2Fs due to its lack of transactivational activity (Trimarchi et al, 1998). Indeed, E2F6 can cause transcriptional inhibition upon recruitment to a reporter gene promoter using a heterologous DNA binding domain and can block the transcriptional activity of other E2Fs (Cartwright et al, 1998; Gaubatz et al, 1998; Morkel et al, 1997; Trimarchi et al, 1998). E2F6 associates with members of the Polycomb group, which are repressors of homeobox genes that control the anterior-posterior patterning of the developing embryo. E2F6 interacts with Bmi1, Ring1, HP1γ, EZH2, and PHC3 and is likely able to mediate transcriptional repression through these interactions (Attwooll et al, 2005; Deshpande et al, 2007; Ogawa et al, 2002; Trimarchi et al, 1998).

While E2F4 and E2F5 typically regulate genes during G0 and early G1, E2F6 represses the induction of E2F target genes during G1/S. However, a recent study has demonstrated that the transcriptional inhibition of these genes as cells progress through the cell cycle is only partially mediated by E2F6 (Giangrande et al, 2004). If E2F6

expression is lost, E2F4 can play a compensatory role, which may explain the lack of a cell cycle defect in both *E2f6*-null mice and cells (Courel et al, 2008; Giangrande et al, 2004; Pohlers et al, 2005; Storre et al, 2002). If both E2F4 and E2F6 are depleted, the G1/S targets become de-repressed, but no significant change in the cell cycle occurs (Giangrande et al, 2004). E2F6 also exerts tissue-specific repression in gonad-specific isoforms of structural proteins, such as *Stag3*, *SMC1β, TUBA3*, and *TUBA7*. Loss of E2F6 results in the expression of these genes in other tissues, rather than being restricted to the testis only (Pohlers et al, 2005; Storre et al, 2002). Thus, E2F6 is limited to binding only a subset of E2F target genes while simultaneously having a broadened number of unique targets. The mechanism behind this, however, is unclear.

Similar to E2F6, E2F7 and E2F8 do not possess domains required for transcriptional activation and pocket protein binding, but they differ from E2F6 because they lack the DP dimerization domain. Rather, E2F7 and E2F8 contain a duplication of the DNA binding domain (Figure 1), which allows them to bind DNA as homodimers or heterodimers with each other without the aid of a DP protein (Christensen et al, 2005; de Bruin et al, 2003a; Di Stefano et al, 2003; Logan et al, 2004; Logan et al, 2005; Maiti et al, 2005). The *E2f7* locus encodes two isoforms: E2F7a is the shorter protein of 728 amino acids, while E2F7b, the more abundant protein, is 911 amino acids (Di Stefano et al, 2003). The two proteins differ only in their C-terminal tails starting from amino acid 713 (Di Stefano et al, 2003). Overexpression studies with E2F7 and E2F8 demonstrate that they are capable of blocking E2F transcriptional activation, suggesting that E2F7 and E2F8 play repressive roles in transcription. Furthermore, during S phase, *E2f7* and *E2f8* become

induced and E2F7 binds to the promoters of some E2F-responsive genes (Christensen et al, 2005; Di Stefano et al, 2003). Thus, the proposed function of E2F7 and E2F8 is to repress E2F-target genes once they have become activated in S phase, in a role analogous to that of E2F6.

C. Regulation of the cell cycle by pocket protein and E2F family members

Members from both the E2F and pocket protein families form various complexes that are regulated throughout the cell cycle. In G0/G1, E2F is in a transcriptionally inactive complex with a hypophosphorylated pocket protein. The domain responsible for pocket protein binding overlaps with the transcriptional activation domain (Figure 1) (Helin et al, 1992) (Kaelin et al, 1992), thus E2F binding to a pocket protein renders this complex unable to induce transcriptional activity (Flemington et al, 1993; Hiebert et al, 1992). Indeed, when pRB is bound to E2F, several residues required for transactivation become concealed (Lee et al, 2002a). In response to mitogenic signaling, the pocket proteins become hyperphosphorylated by cyclin D-CDK4/6 in G1 and subsequently by cyclin E-CDK2 and cyclin A-CDK2 in G1/S (Mittnacht, 1998). Pocket protein phosphorylation modifies key residues at the interface of E2F binding (Xiao et al, 2003), causing the release of an E2F subunit capable of transcriptional activity.

The activity of the pocket protein/E2F complex in the cell cycle is dictated by the function of the E2F moiety, leading to the following model of cell cycle regulation (Figure 2). The activating E2Fs promote cell cycle progression, while the repressive E2Fs function to maintain a G0/G1 state. In G0/G1, hypophosphorylated pRB binds to the activating E2Fs,

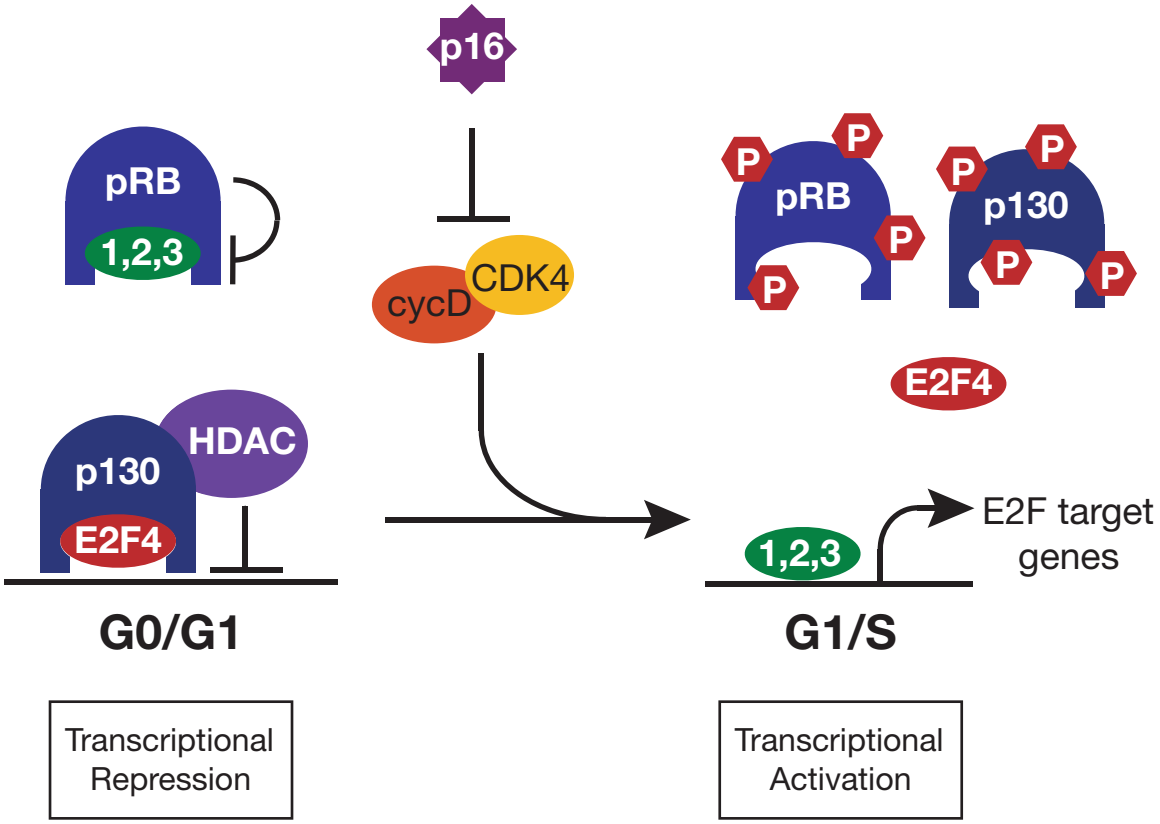

Figure 2. Cell cycle dependent transcription by E2F and pocket protein family members

In G0/G1, repressive complexes consisting primarily of p130 and E2F4 occupy promoters of E2F target genes. p130/E2F4 complexes are able to recruit chromatin remodeling enzymes such as histone deacetylases (HDAC), further contributing to transcriptional repression of E2F-responsive genes. The activating E2Fs are bound to pRB preventing their transcriptional activity. Upon mitogenic signaling, cyclin-CDK complexes are able to overcome inhibition by CDK inhibitors such as p16, and phosphorylate pRB and p130. Phosphorylation disrupts pocket protein-E2F binding, causing E2F4 to be exported from the nucleus. Concomitantly, the activating E2Fs are free to bind cell cycle-regulated target gene promoters and activate their transcription.

preventing their ability to transactivate genes required for cell cycle progression. To further prevent entry into the cell cycle, the repressive E2Fs, E2F4 and E2F5, associate with p107, p130, and possibly pRB at target gene promoters to prevent entry into S-phase. As cells are stimulated to enter the cell cycle, the pocket proteins become hyperphosphorylated by cyclin/CDK complexes, causing the release of free E2F. E2F4 and E2F5 contain nuclear export signals and are exported from the nucleus upon release from members of the pocket protein family, thereby relieving transcriptional repression. Concomitantly, the activating E2Fs are now free of their pRB-mediated inhibition and activate the transcription of genes required for cell division.

i. Transcriptional repression

There is a growing amount of evidence that the repressive E2Fs actively repress the transcriptional activation of target genes when bound to their promoters in addition to merely preventing their transcription. Alteration of the E2F-binding sites in the *B-myb*, *cdc2*, and *E2f1* promoters leads to an increase in activity during G0/G1, when all activating E2Fs are still transcriptionally inhibited by pRB (Dalton, 1992; Hsiao et al, 1994; Lam & Watson, 1993). Thus, the E2F complex that binds these promoters must actively repress transcriptional activity during G0/G1. Since DNA footprinting analysis revealed that the promoters of *B-myb*, *cdc2* and *cyclin A2* are bound by E2F only in quiescent cells, it was presumed that active repression was the only form of gene regulation (Huet et al, 1996; Tommasi & Pfeifer, 1995; Zwicker et al, 1996). However, the more sensitive chromatin immunoprecipitation technique revealed that activating E2Fs were found to associate with

the promoter of *B-myb* during G1/S (Takahashi et al, 2000), but *B-myb* activation may not require E2F binding (Lam & Watson, 1993). In addition, overexpression of an E2F1 expression construct capable of binding DNA and displacing endogenous E2F complexes, but lacking a transactivation domain, was able to induce the transcription of E2F target genes (Zhang et al, 1999a). These data clearly underscore the importance of repressive pocket protein/E2F complexes in preventing the activation of E2F target genes.

Pocket protein/E2F complexes are also able to recruit various factors that alter chromatin structure leading to further repression of E2F target genes. All three pocket proteins are able to recruit histone deacetylases (HDACs) (Brehm et al, 1998; Ferreira et al, 1998; Luo et al, 1998) and, furthermore, HDAC1 and HDAC2 associate with E2F4 and p107 or p130 at target gene promoters in G0/G1 (Rayman et al, 2002). In experiments where the interaction between Rb and HDACs has been weakened, the ability of these mutants to inhibit transcriptional activation was not altered, but they were unable to actively repress the transcription of some E2F target genes (Chen & Wang, 2000; Dahiya et al, 2000). Chromatin structure is also regulated by nucleosome sliding complexes, such as BRG/BRM. pRb can interact with both these proteins (Dunaief et al, 1994; Singh et al, 1995) and it has also been shown that pRb can simultaneously bind to both BRM and E2F, suggesting that a SWI/SNF/Rb/E2F complex can form at promoters with E2F binding sites (Trouche et al, 1997).

Another mechanism for active transcriptional repression is via histone methyltransferases, such as SUV39H1. pRB interacts with SUV39H1 and recruits it to the cyclin E promoter, causing the methylation of lysine 9 of histone H3 (H3K9) and HP1

binding to repress the cyclin E promoter (Nielsen et al, 2001). In quiescent cells, the binding of E2F4 and p130 is accompanied by H3K9 methylation at the promoters of *cyclin A* and *cdc6* (Ghosh & Harter, 2003). Finally, pRb, E2F1, and HDAC1 were identified in complex with DNMT1 during its purification. This study revealed that the effect of pRb repression was enhanced by DNMT1 (Robertson et al, 2000).

Recent studies suggest that E2F4 plays a key role in the transcriptional repression that is mediated via TGF-β signaling. Cycling HaCaT cells treated with TGF-β induces the formation of repressive E2F4-pRb and E2F4-p107 complexes at the promoters of *E2F1*, *B-myb*, and *HsORC1* gene (Li et al, 1997). Mutation of the E2F binding site results in increased expression from these promoters during TGF-β treatment (Li et al, 1997). Similarly, the cell cycle arrest program activated by TGF-β in human keratinocytes causes the generation of E2F4-p130 complexes that associate HDAC1, to inhibit the activity of the *cdc25* promoter (Iavarone & Massague, 1999). Further study revealed that E2F4-p107 and E2F5-p107 form complexes with Smad3 in the cytoplasm. Upon treatment with TGF-β, these complexes translocate to the nucleus, associate with Smad4, and bind to the TGF-β inhibitory element in the *c-myc* promoter to repress is transcription (Chen et al, 2002). Finally, TGF-β signaling has been implicated in repressing the expression of survivin in prostate epithelial cells. Ligand-bound TGF-β receptors activate Smad 2 and 3, which hypophosphorylate pRb and allow E2F4 binding at the survivin promoter, inhibiting transcriptional activity (Yang et al, 2008).

ii. Transcriptional activation

As the repressive E2Fs play an active role in transcriptional repression, it is possible that the activating E2Fs play an analogous role in transcriptional activation. Indeed, E2F transcriptional activation has been associated with histone acetyl transferase (HAT) activity. Together, p300 and Creb-binding protein (CBP) form a complex capable of HAT activity (Vo & Goodman, 2001) and interact with the activating E2Fs, stimulating their transcriptional activity (Martinez-Balbas et al, 2000; Marzio et al, 2000; Trouche et al, 1996). Three lysine residues located near the DNA-binding domain are acetylated by P/CAF (Martinez-Balbas et al, 2000; Pediconi et al, 2003). Other HAT-containing complexes such as GCN5/TRRAP and Tip60 also interact with the activating E2Fs (Dyson, 1998). Further evidence supporting the role of acetylation in transcriptional activation is the finding that promoter binding at the G1/S transition occurs concomitantly with HAT binding and before histone acetylation occurs (Caretti et al, 2003; Taubert et al, 2004). E2F/HAT activity is likely to facilitate transcriptional activation by weakening the interaction between the histone and chromatin, allowing transcription factors to have more accessibility to the DNA.

iii. E2F target genes

Once the E2F-binding sequence was found in the *E2* promoter, similar sequences were found in promoter regions of several genes, suggesting that these genes are potential E2F targets. Several studies have identified the presence of E2F-binding sites and confirmed that mutation of this site reveals a critical role for E2F in cell cycle-regulated transcription. For example, alteration of the *dihydrofolate reducatase* (*DHFR*) promoter

demonstrated that E2F binding is essential for its activation during G1/S (Means et al, 1992; Wade et al, 1992). Indeed, early studies identified E2F as a regulator of many cell cycle-regulated genes responsible for S-phase entry, such as *cyclin E*, *c-Myb*, and *CDK2*. Other targets are involved in the assembly of pre-replication complexes at origins of replication: Cdc6, ORC proteins, and MCMs, and genes directly involved in DNA synthesis have also been identified as E2F targets: *ribonucleotide reductase*, *thymidine kinase*, and *DNA polymerase* α (Stevaux & Dyson, 2002). The E2F pathway is also able to regulate itself positively and negatively via E2F binding and activation of *E2f1*, *E2f2, E2f3, RB-1*, *p107*, and *E2F7* promoters.

In addition to regulating genes directly involved in cell cycle entry, other E2F targets were found that are involved in mitosis and DNA repair. E2F regulation has been confirmed for genes with mitotic functions such as *cyclin B1* and *B2*, *cdc2*, *cdc20*, *cdc25a*, and *smc2* and *4* (Ishida et al, 2001; Muller et al, 2001; Ren et al, 2002). Studies have demonstrated that several classes of DNA repair enzymes are also regulated by E2F. The mismatch repair genes *msh2* and *mlh1*, excision repair genes such as *Fanconi anemia* and *rpa3*, and the recombination repair genes *rad51* and *rad54* have all been identified in microarray analyses as novel classes of E2F target genes (Ren et al, 2002; Weinmann et al, 2002). The physiological relevance of these target genes is not clear, nor is the mechanism as to how E2F regulates these promoters *in vivo*, but it does suggest that E2F regulation may extend past its well-defined role in G1/S regulation. Indeed, a recent study using genome-wide chromatin immunoprecipitation revealed that cycling cells exhibit layers of regulation from both activator and repressive E2F-pocket protein complexes

(Balciunaite et al, 2005). In addition, this study also revealed unique target gene regulation by individual E2F and pocket protein species, such as E2F4, p107, and p130 (Balciunaite et al, 2005).

Part II: Roles of the pocket proteins and E2Fs in development and differentiation

Analyses of both single and compound mutant mice have revealed not only roles for the pocket proteins and E2F family members in cell cycle regulation, but also their roles in the normal development and the differentiation of several cell lineages. Not surprisingly, many of these novel roles in development depend on proper cell cycle regulation. However, members of both families are capable of exerting cell cycle-independent roles in influencing the differentiation of various cell types. Although it has become increasingly clear that the pocket proteins and the E2F family of transcription factors exhibit overlapping, compensatory roles, they also possess remarkable specificity of function *in vivo*.

A. The pocket proteins

i. The tumor suppressor, pRb

Analyses of *Rb*-mutant mice demonstrated its importance in development and differentiation. *Rb*-null mice are embryonic lethal, dying between embryonic day (e) 13 and 15 with defective development of the nervous system, lens, erythroid, muscle, and extra-embryonic tissue (Clarke et al, 1992; de Bruin et al, 2003b; Jacks et al, 1992; Lee et al, 1992; Wu et al, 2003). These embryos exhibit abnormal erythropoiesis, apoptosis, and ectopic proliferation. Later studies demonstrated that a placental defect prevents efficient nutrient and gas exchange to the embryo, and a wildtype placenta allows $Rb^{-/-}$ embryos to survive until birth (de Bruin et al, 2003b; Wu et al, 2003). In addition, a wildtype placenta also ameliorates some of the apoptotic defects, suggesting a non-cell autonomous

phenotype for *Rb* loss (Lipinski et al, 2001; Maandag et al, 1994; MacPherson et al, 2003; Williams et al, 1994), but does not affect the ectopic proliferation defect.

Further elucidation of the function of pRb in several cell types has established its role in regulating differentiation. Early studies reported that neither $Rb^{-/-}$ adult chimeric mice nor *Rb*-null embryos with wildtype placentas exhibited significant defects in erythropoiesis. However, *Rb* chimeric embryos exhibited a slight increase in the number of nucleated red blood cells, and the presence of a wildtype placenta in $Rb^{-/-}$ embryos was not able to completely rescue abnormal erythrocyte development (de Bruin et al, 2003b; Maandag et al, 1994; Williams et al, 1994; Wu et al, 2003). Moreover, acute ablation of *Rb* in erythroid progenitor cells causes defects in the ability of these cells to properly exit the cell cycle and terminally differentiate *in vitro* (Clark et al, 2004; Spike et al, 2004). Finally, *Rb* is required to regulate the expansion of erythrocytes and to promote the enucleation of red blood cells (Spike et al, 2004).

Another tissue that is affected in $Rb^{-/-}$ animals is the lens, which undergoes ectopic cell cycles and apoptosis. When these animals are supplied with a wildtype placenta, the defects in the lens are not suppressed, suggesting that these defects are cell autonomous (de Bruin et al, 2003b; Wu et al, 2003). In addition, *Rb*-null animals exhibit reduced expression of the late markers for lens differentiation, *filensin* and *γ-crystallin*, when compared to wildtype levels of these genes (Liu & Zacksenhaus, 2000; Morgenbesser et al, 1994). This defect appears to be independent of cell cycle exit defects, suggesting that pRb is playing a more direct role in the differentiation of this tissue. Indeed, it was found

that pRb forms a functional complex with Pax6, which is required for lens development (Cvekl et al, 1999).

Skeletal muscle differentiation occurs coincident with an increase in mRNA and protein levels of *Rb* (Coppola et al, 1990). Consistent with this, myocytes lacking *Rb* are unable to properly differentiate due to the loss of a physical interaction between the muscle-specific transcription factor, MyoD, and pRb, which augments the activity of MyoD (Gu et al, 1993). Moreover, animals deficient for *Rb* die at birth with severe defects in skeletal muscle differentiation, such as increased apoptosis, increased numbers of elongated nuclei that actively synthesize DNA within myotubes, reduced muscle fibers, and reduced expression of late markers of muscle differentiation (Zacksenhaus et al, 1996). These defects are unable to be rescued by a wildtype placenta (de Bruin et al, 2003b; Wu et al, 2003). A direct role for pRb in myogenic differentiation is still under investigation as the physical interaction between pRb and MyoD has not been demonstrated *in vivo* (Li et al, 2000). However, it is clear that defects in $Rb^{-/-}$ skeletal muscle differentiation are due to deregulation of the cell cycle (Huh et al, 2004; Li et al, 2000; Zhang et al, 1999b). Furthermore, conditional ablation of *Rb* in skeletal muscle cells provides compelling evidence that pRb is essential for cell cycle exit and the initiation of differentiation in this tissue (Huh et al, 2004).

Recent studies have also revealed an important role for pRb in the differentiation of several types of epithelial cells. A lung-specific knockout of *Rb* causes increased proliferation in the epithelia of the lung. Interestingly, only neuroendocrine cells are affected, while other lung epithelial cells, such as Clara cells and ciliated cells, are normal

(Wikenheiser-Brokamp, 2004). It is likely that the role of *Rb* as a cell cycle regulator is the underlying cause of the neuroendocrine defect. Conditional deletion of *Rb* in the epidermis of the skin causes an increase in proliferation and altered expression of the differentiation markers *K5*, *K6*, and *K10* (Ruiz et al, 2004). Similarly, *Rb* ablation in the intestine results in ectopic entry into S-phase and mitosis (Haigis et al, 2006; Yang & Hinds, 2007). These effects were accompanied by up-regulation of the differentiation markers *Cdx1* and *Cdx2* (Haigis et al, 2006).

pRb has also been implicated in the maintenance of certain stem cell populations. Proper proliferation and differentiation of stem cells is not only reliant on intrinsic signals, but also on extrinsic cues that come from a stem cell's microenvironment. *Rb*-null mice display defects in hematopoiesis (Spike et al, 2004), and one group found that *Rb* loss causes a myeloproliferative disorder only when *Rb* is ablated from both hematopoietic cells and the bone marrow niche (Walkley et al, 2007). This study suggests that *Rb* plays an important role in maintaining the proper niche that is conducive to proper hematopoiesis. The role that *Rb* plays in directly regulating hematopoietic stem cells is controversial, and more research is required to elucidate the role of pRb. Loss of pRb in both germline and conditional knockout mice has demonstrated the importance of pRb function in the placenta and for embryonic survival (Clarke et al, 1992; de Bruin et al, 2003b; Jacks et al, 1992; Lee et al, 1992; Wu et al, 2003). A recent study has shown an *Rb*-null placenta is sufficient to cause embryonic lethality even if the embryo is wildtype (Wenzel et al, 2007). Embryos lacking *Rb* in trophoblast stem cells exhibit hyperproliferation of these cells, broader expression of trophoblast markers, and a global disruption of placental

architecture, suggesting a crucial role for *Rb* in stem cell population maintenance (Wenzel et al, 2007)}(Wu et al, 2003). Moreover, differentiation of embryonic stem cells occurs concomitantly with the activation of pRb (White et al, 2005). Another group demonstrated that pRb reduction in Arabidopsis increases the amount of stem cells without affecting the duration of mitosis, while overexpression of pRb decreases the number of stem cells (Wildwater et al, 2005).

The neuronal compartment also undergoes massive apoptosis upon loss of Rb (Clarke et al, 1992; Jacks et al, 1992; Lee et al, 1992). However, this defect in neuronal lineages is non-cell autonomous (Ferguson et al, 2002; Lipinski et al, 2001; MacPherson et al, 2003; Wu et al, 2003). Loss of *Rb* correlated with ectopic proliferation and the decreased expression of several neuronal markers, including neurotrophin receptors TrkA, TrkB, and p75 (Lee et al, 1994). The use of a neuronal specific promoter that drives the LacZ reporter gene demonstrated that loss of *Rb* causes defects in several parts of the developing nervous system, including the olfactory epithelium, the retina, and the neocortex (Slack et al, 1998). These studies underscore the importance of pRb in neuronal fate commitment. More recently, loss of *Rb* in the telencephalon demonstrated that the aberrant migration of a specific subpopulation of interneurons in the brain occurs independent of any cell cycle defects (Ferguson et al, 2005; Ferguson et al, 2002).

Although most evidence linking pRb and differentiation is dependent on the role of pRb as a cell cycle regulator, there is mounting evidence that implicates the direct involvement of pRb in the differentiation of several cell types. One example is the role of pRb in osteoblast differentiation. pRb physically interacts with Runx2/CBFA1, an essential

transcription factor required for osteogenesis, and, together, they bind osteoblast-specific promoters *in vivo* (Thomas et al, 2001). Moreover, *in vitro* data demonstrate that pRb acts as a transcriptional activator for Runx2 (Thomas et al, 2001). Furthermore, while wildtype MEFs are able to secrete a calcified matrix upon BMP-induced differentiation, no mineralization is observed in *Rb*-null MEFs (Thomas et al, 2001). Embryos that have been conditionally deleted for *Rb*, both in the embryo proper and in the bone, exhibit impaired bone formation (Berman et al, 2008; Gutierrez et al, 2008). These defects are accompanied by altered expression of bone differentiation markers, such as *alkaline phosphatase* and *collagen1a1* (Berman et al, 2008; Gutierrez et al, 2008). One caveat is that these studies suggest that *Rb* plays a major role in promoting cell cycle arrest in order to facilitate terminal osteoblast differentiation. However, these data do not discount the possibility that pRb plays a direct role in osteoblast differentiation. Indeed, it was found that Rb loss in osteoblasts enabled adipogenic differentiation, suggesting a higher multipotency inherent in these cells compared to wildtype (Gutierrez et al, 2008). Further study is needed in order to determine what cell cycle-independent role pRb is playing in osteoblast differentiation and bone development.

Perhaps the most convincing studies implicating a cell cycle-independent role for Rb is in promoting adipogenesis. pRb physically interacts with CCAAT/enhancer-binding protein (C/EBP) transcription factors, which are essential for inducing adipocyte differentiation (Chen et al, 1996). In addition, pRB enhances both C/EBP binding to DNA and the transactivation by C/EBPs (Chen et al, 1996). Furthermore, *Rb*-deficient MEFs lack the ability to differentiate into adipocytes, either spontaneously or in response to hormone

treatment (Chen et al, 1996; Classon et al, 2000a). This block in differentiation can be circumvented by the addition of a PPARγ ligand (Classon et al, 2000a; Hansen et al, 1999), which is a transcription factor critical for adipocyte differentiation that is induced by upstream factors such as C/EBP. This data suggests that the role of *Rb* in adipogenesis is not linked to its ability to repress E2F-responsive promoters.

Since E2F1 induces *PPARγ* transcription during clonal expansion, it is possible that the loss of pRb results in unrestricted activation of *PPARγ* (Fajas et al, 2002b). However, one group reported that PPARγ promotes adipogenesis more efficiently in the absence of pRb (Fajas et al, 2002a). Furthermore, pRb interacts with PPARγ and HDAC3 (Fajas et al, 2002a). It is possible that *Rb* plays a dual role in adipogenesis, first by promoting cell cycle exit and transactivation by C/EBPs, but then regulates the extent of adipogenesis by antagonizing downstream PPARγ signaling. Although further studies are required to fully understand the role of pRb in adipogenesis, it is clear that, *in vitro*, the function of pRb is to regulate adipocyte differentiation in a cell cycle-independent manner.

pRb also plays a role in regulating the decision to become either brown or white fat. In embryonic and neonatal mice, white fat precursors express pRb, while the lack of nuclear pRb characterizes brown fat adipocytes (Hansen et al, 2004). Another difference between these tissues is that pRb becomes hyperphosphorylated in brown fat after adult mice are exposed to cold temperatures whereas white fat remains unchanged (Hansen et al, 2004). Similarly, *in vitro* data further suggest that pRb plays an inhibitory role in brown fat conversion. In the presence of PPARγ ligand, genes that are differentially expressed in white fat and brown fat show markedly different expression pattern profiles during the

differentiation of wildtype and *Rb*$^{-/-}$ MEFs. In MEFs lacking *Rb*, the brown fat-specific markers *UCP-1* and *PGC-1α* were expressed at levels comparable to normal brown adipose tissue (Hansen et al, 2004). Conversely, Cre-mediated deletion of *Rb* in adult pre-adipocytes resulted in an almost complete block in white adipose tissue differentiation (Scime et al, 2005). These data suggest that the presence of pRb drives adipocytes to become white fat. Consistent with this, chromatin immunoprecipitation assays demonstrate that pRb binds to the *PGC-1α* promoter and represses transcription (Scime et al, 2005), thereby regulating the switch between white and brown fat differentiation from adult progenitors in adipose tissue.

ii. p107 and p130

Genetic analyses of *p107*$^{-/-}$ and *p130*$^{-/-}$ mice also uncover roles in promoting differentiation. Mice deficient for *p107* or *p130* reveal that there are strain-specific differences in the resulting phenotypes. There are no obvious phenotypic consequences for *p107* or *p130* loss in either a C57Bl/6 or 129/Sv background (Cobrinik et al, 1996; Lee et al, 1996); however, *p130*$^{-/-}$ embryos do not survive past e13.5 and display defective neural, muscle, and heart development in a Balb/c background (LeCouter et al, 1998b). Similarly, Balb/c mice deficient for *p107* experience impaired growth, exhibit a myeloproliferative disorder, have defective white fat development, and 70% die before weaning (LeCouter et al, 1998a; Scime et al, 2005). Upon a single backcross to C57Bl/6, the phenotypes of both *p107*$^{-/-}$ and *p130*$^{-/-}$ mice are reverted to wildtype, highlighting the importance of both genes in development, but in a strain-specific manner (LeCouter et al, 1998a; LeCouter et al, 1998b).

iii. Overlapping roles of the pocket proteins

Compound mutant mice reveal overlapping and distinct contributions made by each pocket protein member during development. Not surprisingly, creating double mutant animals frequently exacerbates pre-existing phenotypes. For example, losing one copy of *Rb* in addition to lacking *p107* results in mice that are born at lower than expected Mendelian frequencies and are growth retarded (Lee et al, 1996). Complete loss of *Rb* and *p107* causes lethality two days earlier than embryos lacking *Rb* alone (Lee et al, 1996). In addition, *Rb* loss in the intestinal epithelium, combined with the deletion of either *p107* or *p130*, resulted in epithelial hyperplasia not observed in $Rb^{-/-}$ mice (Haigis et al, 2006). Similarly, when skin-specific deletions of *Rb* are combined with loss of *p107*, the severity of hyperplasia, hyperkeratosis, and abnormal epidermal differentiation increases (Ruiz et al, 2004). Conditional ablation of all three pocket proteins in the lung causes the number of neuroendocrine cells to increase, blocks differentiation of Clara and ciliated cells, and results in lethality within the first three weeks of age (Wikenheiser-Brokamp, 2004).

Analyses of mice lacking both *p107* and *p130* revealed compensatory functions in the regulation of chondrocyte growth and differentiation in long bone development (Cobrinik et al, 1996). These defects are caused by inappropriate cell cycle progression and loss of *Runx2* induction, preventing these cells from differentiating (Cobrinik et al, 1996; Laplantine et al, 2002; Rossi et al, 2002). $p107^{-/-}$;$p130^{-/-}$ mice exhibit an altered epidermis with defective keratinocyte differentiation and the development of abnormal hair follicles (Ruiz et al, 2003). In addition, double knockout MEFs exhibit an increased ability to differentiate into adipocytes *in vitro* (Landsberg et al, 2003), which is similar to the

differentiation seen when either pocket protein is absent in 3T3-L1 pre-adipocyte cells (Classon et al, 2000a). However, anti-sense oligonucleotides against *p107* in 3T3-L1 cells block adipocyte differentiation (May et al, 2001), which is consistent with the defective adipogenesis described in *p107*$^{-/-}$ mice (Scime et al, 2005).

B. The activating E2Fs

i. Single knockout mice

Studies of E2F knockout mice have not only revealed roles in regulating the cell cycle, but also identified diverse functions that are context dependent and, in some cases, cell cycle-independent. Mice deficient for *E2f1* are fully viable, but exhibit testicular atrophy, exocrine abnormalities, increased T cell proliferation due to defective negative selection, and a range of tissue-specific tumors (Field et al, 1996; Garcia et al, 2000; Yamasaki et al, 1996; Zhu et al, 1999). Tumor types include lymphomas, reproductive tract sarcomas, uterine tumors, and lung adenocarcinomas (Field et al, 1996; Yamasaki et al, 1996). *E2f1*$^{-/-}$ mice are also resistant to obesity when fed a high-fat diet, likely due to the inability to stimulate adipogenesis through activation of *PPARγ* (Fajas et al, 2002b). Another study implicated a role of *E2f1* in bone development. When *E2f1* is overexpressed, chondrocytes are unable to properly exit the cell cycle, preventing chondrocyte differentiation and delaying endochondral bone development (Scheijen et al, 2003). Similarly, *E2f1* overexpression in keratinocytes *in vitro* also prevents proper differentiation (Wong et al, 2003). Recently, results from a sensitive subtractive screen identified E2F1 as an activator of several atypical cell cycle-independent targets (Iwanaga et al, 2006). Several of the genes identified may be involved in development or differentiation, such as Neogenin,

WASF1, and SGEF (Iwanaga et al, 2006). Additionally, the neuronal migration gene, *NRP-1*, contains an E2F binding sequence in its promoter and is activated upon E2F1 binding (Jiang et al, 2007). E2F1 is able to block the effects of G-CSF, which acts as a survival factor and promotes granulocyte differentiation (Strom et al, 1998; Wells et al, 2002).

E2f2$^{-/-}$ mice are born at the expected frequency and are viable (Murga et al, 2001). However, these animals die prematurely due to autoimmunity-related defects caused by excessive T cell proliferation, such as splenomegaly and glomerulonephritis (Murga et al, 2001). In addition, mice deficient for *E2f2* exhibit erythroid maturation defects (Li et al, 2003) and a high incidence of tumors, including hematopoietic malignancies, a histiocytic sarcoma, and a lung adenoma (Zhu et al, 2001).

In contrast to both *E2f1*$^{-/-}$ and *E2f2*$^{-/-}$ mice, *E2f3*$^{-/-}$ mice in a pure genetic background are embryonic lethal, while only a small proportion of mixed background *E2f3*-null mice are able to survive (Cloud et al, 2002; Humbert et al, 2000b). Loss of *E2f3* impairs myocardium proliferation, resulting in hypoplastic ventricular walls, septal defects, and embryonic lethality (King et al, 2008). Interestingly, surviving *E2f3*$^{-/-}$ mice do not develop tumors like *E2F1*-null mice; rather, these mice die prematurely due to congestive heart failure (Cloud et al, 2002; King et al, 2008). *In vitro*, *E2f3*$^{-/-}$ MEFs exhibit a slightly reduced capacity to differentiate into adipocytes, suggesting that E2F3 may promote adipogenesis through the activation of *PPARγ* (Fajas et al, 2002b).

Conditional mouse models have further elucidated the extent to which each *E2f3* isoform contributes to development. Both *E2f3a* and *E2f3b* single knockouts are viable and develop normally (Danielian et al, 2008; Tsai et al, 2008), suggesting that these

proteins have redundant functions throughout development. However, further examination has revealed unique context-specific roles that each isoform is required for in development. For example, miRNA-mediated ablation of *E2f3b* revealed an essential role in promoting myogenic differentiation (Asp et al, 2009). In addition, chromatin immunoprecipitation-on-chip analysis has suggested divergent roles for each isoform of *E2f3*. While genes targeted by E2F3a are predominantly proliferation-associated, the majority of E2F3b targets are involved in differentiation and development (Asp et al, 2009). Indeed, despite the ability of E2F3a to bind E2F3b-specific gene promoters when overexpressed, E2F3a is unable to stimulate the transcription of these genes (Asp et al, 2009).

ii. Compound mutant mice

Analysis of double and triple knockout mice have elucidated redundant functions among the activating E2Fs, explaining the relatively mild single mutant phenotypes, while also revealing individual E2F-specific roles during development. *E2f1*$^{-/-}$;*E2f2*$^{-/-}$ mice are viable, but develop diabetes and die prematurely (Iglesias et al, 2004). They also exhibit exacerbation of defects characteristic of single knockout animals, such as autoimmunity, T cell defects, and tumor development (DeRyckere & DeGregori, 2005; Zhu et al, 2001). In addition, hematopoietic defects arise from impaired S-phase progression in hematopoietic progenitors, and B-cells exhibit defective maturation, presumably due to a failed cell cycle exit (Li et al, 2003; Zhu et al, 2001). Moreover, the loss of *E2f3* in addition to *E2f1* or *E2f2* exacerbated the developmental and age-related phenotypes present in *E2f1* and *E2f2* single knockouts (Cloud et al, 2002; Wu et al, 2001). Although mice doubly-deficient for

E2f1 and *E2f3b* are viable and do not exhibit any tissue-specific defects, *E2f1*$^{-/-}$;*E2f3a*$^{-/-}$ mice die within the first few weeks of life, suggesting that *E2f3a* plays a more critical role in development *in vivo* than *E2f3b* (Danielian et al, 2008). Finally, these mice exhibit defective cartilage development, likely due to an exacerbation of the existing chondrocyte differentiation defect present in single *E2f1*$^{-/-}$ and *E2f3*$^{-/-}$ mice (Danielian et al, 2008).

C. The repressive E2Fs

i. Classical repressors

Although E2F4 and E2F5 exhibit overlapping roles in promoting cell cycle exit, *in vivo* studies have provided evidence for distinct roles during development. Loss of E2F4 results in developmental defects and bacterial infections that cause poor postnatal survival (Humbert et al, 2000a). The susceptibility to bacterial infections results from the replacement of ciliated cells in the respiratory epithelium with mucin-secreting columnar secretary cells; this defect occurs independently from any alterations in cell cycle progression (Danielian et al, 2007). In addition, *E2f4*$^{-/-}$ mice exhibit craniofacial defects that may contribute to bacterial infections (Humbert et al, 2000a). *E2f4* has also been implicated in development of other types of epithelial cells. In the proximal lung epithelium, loss of E2F4 results in the aberrant expression of the Clara cell marker CC10 (Danielian et al, 2007). Similarly, abnormal crypt formation occurs in the intestinal epithelium of *E2f4*-deficient mice (Rempel et al, 2000).

During development, *E2f4*$^{-/-}$ mice are anemic and display defects in red blood cell maturation (Humbert et al, 2000a), likely due to an impaired cell cycle progression (Kinross et al, 2006). *E2f4*-deficient mice exhibit severe early eye patterning defects, such as altered

optic cup formation, coloboma, and abnormal eye pigmentation (Ruzhynsky et al, 2009). Additionally, these embryos also display patterning defects in the optic vesicle and altered marker gene expression of the optic stalk and the ventral cup (Ruzhynsky et al, 2009). Loss of E2F4 also has an effect on T lymphocytes. Acute ablation of *E2f4* during CD8+ T cell priming results in increased primary proliferation and has a negative effect in secondary stimulation, demonstrating the importance of E2F4 in the proper formation of functional memory T cells (Bancos et al, 2009). The absence of E2F4 results in the reduction of the ventral telencephalon as well as the complete loss of Sonic Hedgehog expression (Ruzhynsky et al, 2007). While there are no proliferation defects in neural progenitor cells, these cells experience a reduced ability to self-renew (Ruzhynsky et al, 2007). The self-renewal defect can be rescued by restoring Sonic Hedgehog signaling, which is specifically reduced in the absence of E2F4 (Ruzhynsky et al, 2007). Together, these *in vivo* data suggest that E2F4 contributes to development and differentiation of several lineages through both cell cycle-dependent and -independent mechanisms.

In vitro studies have further implicated *E2f4* in differentiation. Overexpression of E2F4 enhances NGF-induced neuronal differentiation as well as the maintenance of a differentiated state upon loss of NGF induction (Persengiev et al, 1999). E2F4 forms repressive complexes with p107 or p130 at the FGFR1 promoter in proliferating myoblasts and myotubes, thereby regulating skeletal muscle differentiation (Parakati & DiMario, 2005; Parakati & Dimario, 2005). Finally, E2F4 is required to repress adipocyte differentiation. In association with p107 or p130, E2F4 can bind to and repress *PPARγ* expression during terminal adipocyte differentiation (Fajas et al, 2002b; Landsberg et al, 2003). In chimeric

mice, *E2f4*$^{-/-}$ cells contribute to a greater proportion of white adipose tissue compared to control chimeric cells (Fajas et al, 2002b).

The generation of *E2f5*$^{-/-}$ mice demonstrated that *E2f4* and *E2f5* make differential contributions during development. *E2f5*-null mice are born at expected frequencies, but soon develop ataxia, ruffled coats, and dehydration (Lindeman et al, 1998). These mice die prematurely due to intracerebral hemorrhage and hydrocephalus, implicating *E2f5* in the regulation of cerebral spinal fluid secretion (Lindeman et al, 1998). Combined loss of both transcriptional repressors, E2F4 and E2F5, results in embryonic lethality early in development (Gaubatz et al, 2000), demonstrating the functional redundancy between these two proteins.

ii. Pocket protein-independent repressors

E2F6 represses transcription through its association with Polycomb group proteins, which are repressors of homeobox genes that control the anterior-posterior patterning of the developing embryo. Indeed, mutation of E2F6 results in mice that exhibit posterior transformations of the axial skeleton (Courel et al, 2008; Storre et al, 2002). These mice also exhibit defective spermatocyte development, but this defect does not affect fertility (Storre et al, 2002).

E2F7 and E2F8 are the newest members to the E2F family, and conditional mouse models are beginning to elucidate the roles these proteins play in development and differentiation. Both *E2f7*$^{-/-}$ and *E2f8*$^{-/-}$ mice are viable and do not exhibit any abnormalities; however, *E2f7*$^{-/-}$;*E2f8*$^{-/-}$ embryos are unable to survive past e11.5 (Li et al, 2008). Moreover, *E2f7*$^{+/-}$;*E2f8*$^{-/-}$ mice develop normally, but *E2f7*$^{-/-}$;*E2f8*$^{+/-}$ mice die within 3 months,

suggesting a greater contribution of E2F7 versus E2F8 during development (Li et al, 2008). Mice lacking both *E2f7* and *E2f8* exhibit vascular defects, multifocal hemorrhages, and massive apoptosis (Li et al, 2008). Interestingly, both E2F7 and E2F8 occupy the *E2f1* promoter and repress its transcription. Upon loss of both proteins, E2F1 becomes overexpressed and results in the activation of an apoptotic response (Li et al, 2008). Finally, a recent *in vitro* study has found that E2F7 was able to inhibit proliferation and initiate differentiation of keratinocytes (Endo-Munoz et al, 2009).

D. E2F and pocket protein double knockout mice

The analysis of compound mutant mice that are deficient for both an E2F and a pocket protein can reveal novel phenotypes, which may not have been discovered in crosses with a member of its own family. For example, analysis of *Rb*-null mice revealed an up-regulation E2F2 (Dirlam et al, 2007). Consistent with its expression pattern, deletion of *E2f2* restored the ability of erythroid cells to terminally differentiate, revealing a tissue specific role of E2F2 in promoting erythropoiesis (Dirlam et al, 2007). Moreover, pRb mediates placental differentiation and nervous system development by inhibiting the activation of E2F target genes by E2F3a (Chong et al, 2009). Furthermore, neuronal differentiation in the retina is regulated via pRb suppression of E2F3a, but in a manner independent of cell cycle regulation (Chen et al, 2007). A similar result was found for *Rb* and *E2f3a* in regulating neuronal differentiation and migration in the brain by a cell cycle-independent mechanism (Chen et al, 2007; Chong et al, 2009; McClellan et al, 2007).

Combining *E2f* and *Rb* mutations can lead to unexpected effects on tumorigenesis. For example, *E2f3*, in combination with loss of *Rb*, has been shown to both suppress and

promote tumorigenesis depending on the tissue (Parisi et al, 2007; Ziebold et al, 2003). One of the more surprising results was that loss of *E2f4* suppresses tumorigenesis in $Rb^{+/-}$ mice. Since loss of *E2f1* significantly diminishes the development of pituitary and thyroid tumors in $Rb^{+/-}$ animals, it was originally hypothesized that loss of *E2f4*, which accounts for the majority of endogenous pocket protein activity, would exacerbate tumorigenesis. Unexpectedly, deletion of *E2f4* significantly extends the lifespan of $Rb^{+/-}$ mice (Lee et al, 2002b). Biochemical analyses suggest that loss of E2F4 allows p107 and p130 to bind to and inhibit the activator E2Fs in a process the authors refer to as “pocket protein reshuffling” (Lee et al, 2002b). Indeed, loss of E2F4 rescues inappropriate proliferation and E2F target gene expression (Lee et al, 2002b).

Part III: Bone development and osteoblast differentiation

Pocket protein and E2F family members are essential for the terminal differentiation of numerous cell types. E2F4 comprises the majority of E2F/pocket protein complexes *in vivo* and is able to associate with all of the pocket proteins. Interestingly, all pocket protein family members have been shown to contribute to proper bone development in mice. Therefore, we sought to determine if E2F4 could also contribute to osteoblast differentiation and bone formation. The following is an overview of bone development.

A. Overview and anatomy of the bone

Bone is a specialized connective tissue and comprises the skeleton, which provides support that gives the body shape. In combination with associated muscles, the bones of the skeleton can also provide a means of locomotion and related movement. The skeleton also encases vital organs of the body, protecting them from damage. Finally, bones act as a reservoir for inorganic ions, such as calcium and phosphate.

The skeleton is made of two types of bone, cortical and trabecular bone. Cortical bone (also referred to as compact bone) is found along the shafts of long bones, such as the femur, tibia, radius, and ulna. It is also the primary component of flat bones like the skull and ribs. Since cortical bone is quite strong, it is responsible for supporting body weight and protecting internal organs. Cortical bone makes up approximately 80% of skeletal mass while the second type of bone, trabecular, comprises the remaining 20%. Trabecular bone is also called cancellous or spongy bone due to its less dense and more porous appearance. This type of bone is found in the vertebrae of the spinal cord and in the epiphyses of the long bones.

Despite the differences in appearance and strength, compact and trabecular bone have identical chemical compositions. Both are composites of inorganic and organic material, with organic making up about 75% of bone. The initial event in bone formation is the deposition of type 1 collagen, termed osteoid, which is made of two Collagen1a1 proteins chains and one Collagen1a2 protein chain. In addition to collagen, bone-forming cells called osteoblasts also secrete Osteocalcin and Osteopontin into the collagen matrix. Bone formation is incomplete until minerals are incorporated into the osteoid. Calcium, phosphate, and hydroxyl ions constitute the majority of hydroxyapatite, which is the inorganic component that creates a mineralized bone matrix. Hydroxyapatite also contains magnesium, fluoride, and potassium, providing an accessible source of ions for the body.

B. The coordination of players involved in bone formation

The relatively static appearance of bone belies the complexities of patterning and cellular differentiation required during development. The skeleton is made of two tissues (cartilage and bone) and three cell types (chondrocytes, osteoblasts, and osteoclasts) that need to be exquisitely coordinated in order to form more than 200 different skeletal elements throughout the body. The first step in bone formation is skeletal morphogenesis, which is the migration of mesenchymal cells to their ultimate location and their subsequent condensation into precursors of cartilage and bone. Mesenchymal precursors forming the craniofacial skeleton are derived from cranial neural crest cells, the axial skeleton is derived from the paraxial mesoderm or somites, and the limb skeleton is formed from lateral plate mesoderm cells. Once these cell lineages migrate to a location where skeletal elements will

develop, they form characteristic mesenchymal condensations and differentiate into either chondrocytes, the cell type specific to cartilage, or osteoblasts (Hall & Miyake, 1992).

Endochondral ossification is responsible for the formation of most bones in the body, including long bones, ribs, and vertebrae. During this process, mesenchymal condensations differentiate into chondrocytes. These cells proliferate and secrete cartilage matrix, forming a cartilaginous template that becomes replaced by bone. Both proliferation and cartilage matrix deposition cause the template to elongate and expand. Chondrocytes in the center of the template differentiate into hypertrophic chondrocytes, which stop proliferating and enlarge. Hypertrophic chondrocytes secrete a unique extracellular matrix, which includes Collagen X, and also induce osteoblast differentiation in cells residing in the perichondrium, forming the bone collar. In addition, hypertrophic chondrocytes cause blood vessels to grow out from the perichondrium by releasing angiogenic factors. These blood vessels deliver osteoblasts, hematopoietic cells, and also bone-resorbing osteoclasts, creating a primary ossification center. At these centers, the Collagen X-containing matrix is degraded by osteoclasts. Simultaneously, hypertrophic chondrocytes undergo apoptosis, which clears the way for invading osteoblast cells to secrete an osteoid matrix and hematopoietic cells to create bone marrow.

Flat bones such as the calvarial bones of the skull and part of the clavicle form via the second process, intramembranous ossification. Bones formed through intramembranous ossification do not form via a cartilage intermediate; rather, mesenchymal condensations differentiate directly into osteoblasts to produce bone. Once both types of bones are formed, bone mass is constantly regulated by a process called

bone remodeling. During bone remodeling, the bone undergoes periods of bone resorption and bone formation.

i. Chondrocytes

During the development of endochondral bones, mesenchymal condensations must first differentiate into chondrocytes. Although further investigation is required to fully understand the complex signaling involved in cartilage development, it is clear that Sox9 is an early transcription factor required for chondrogenesis. Chimeric mouse studies revealed that *Sox9*-null cells do not express any chondrocyte-specific markers and are excluded from chondrogenic condensations, indicating that *Sox9* is required for chondrocyte differentiation and cartilage formation (Bell et al, 1997; Bi et al, 1999). Sox9 is essential for the expression of cartilage-specific components of the extracellular matrix, such as type II collagen (Bi et al, 1999; Lefebvre & de Crombrugghe, 1998). Sox9 belongs to a family of transcription factors that include Sox5 and Sox6, which are also involved in the expression of the type II collagen gene, *Col2a1*. Since Sox5, 6, and 9 form a complex with other nuclear proteins in chondrocytes, and Sox5 and Sox6 are co-expressed with Sox9 in sites of chondrogenesis, it is believed that all three *Sox* genes cooperatively activate *Col2a1* (Zhou et al, 1998). Finally, $Sox5^{-/-}$;$Sox6^{-/-}$ mice die at e16.5, and growth plate chondrocytes exhibit a failure to differentiate into hypertrophic chondrocytes (Lefebvre et al, 1998).

Mutant mouse models have elucidated some of the transcriptional regulators that cause chondrocytes to exit the cell cycle and become hypertrophic, such as Runx2 and HDAC4. Mice deficient for the transcription factor *Runx2* exhibit skeletal elements that lack hypertrophic chondrocytes (Kim et al, 1999). Moreover, *Runx2* is transiently expressed in

pre-hypertrophic chondrocytes, the subpopulation of proliferating chondrocytes that will differentiate into hypertrophic chondrocytes (Takeda et al, 2001). Furthermore, constitutive expression of *Runx2* enhances hypertrophy and causes tracheal cartilage to convert into bone (Takeda et al, 2001; Ueta et al, 2001). HDAC4 is also expressed in pre-hypertrophic chondrocytes, and associates with and inhibits Runx2 (Vega et al, 2004). Indeed, overexpression of HDAC4 disrupts hypertrophy and results in a phenotype similar to that of *Runx2*-null mice.

Other factors implicated in coordinating chondrocyte proliferation and hypertrophy are Indian hedgehog (Ihh) and PTHrP. Ihh, a member of the Hedgehog family of signaling molecules, stimulates the proliferation of growth plate chondrocytes and prevents differentiation into hypertrophic chondrocytes. *Ihh*-null mice exhibit severe dwarfism in axial and appendicular skeletal elements and do not form endochondral bones (St-Jacques et al, 1999). Ihh activates the expression of *PTHrP*, a secreted molecule, in chondrocytes, and PTHrP signals through its receptor to inhibit chondrocyte hypertrophy and to suppress *Ihh* expression by keeping chondrocytes in a proliferating state (Lanske et al, 1996; St-Jacques et al, 1999). Null mutations of *PTHrP* or its receptor decreases the number of proliferating growth plate chondrocytes and increases the hypertrophic zone (Karaplis et al, 1994; Lanske et al, 1996). Interestingly, both null mutations and overexpression of *PTHrP* resulted in dwarfism, likely due to the importance of chondrocyte hypertrophy in longitudinal bone growth.

ii. Osteoblasts

Osteoblasts are responsible for synthesizing the components of the bone matrix in both intramembranous and endochondral bones. *In vitro* studies have identified several proteins that are specific to or highly expressed in differentiating osteoblasts (Figure 3). Individually, the up-regulation of these genes may not be unique to bone development, but together they collectively define a characteristic bone signature during osteoblast differentiation. The enzyme alkaline phosphatase, which plays a role in matrix mineralization, and type I collagen are expressed early during the commitment to an osteoblastic fate. Early- to mid-stage markers of osteoblast differentiation are *Osteopontin* and *Osteonectin*, while *Osteocalcin* is specifically expressed by mature osteoblasts. Finally, the production of calcium-containing bone nodules is characteristic of terminally differentiated osteoblasts. Although *in vitro* studies of bone marker induction has facilitated the elucidation of osteoblast differentiation, they do not address how osteoblast markers become induced.

Osteoblasts originate from mesenchymal cells, and many proteins contribute to the regulation of this differentiation process. One such protein is Runx2, which is the earliest master regulator of osteoblast differentiation. The transcription factor, Runx2, was identified based on its ability to regulate the expression of several bone proteins, including Osteocalcin, alkaline phosphatase, and type I collagen (Ducy et al, 1997; Lee et al, 2007). Runx2 is expressed in cells that will eventually become the skeleton as early as e10.5 and regulates many genes required for osteoblast differentiation (Ducy, 2000). Consistent with its pattern of expression and function *in vitro*, mice deficient for *Runx2* exhibit a complete

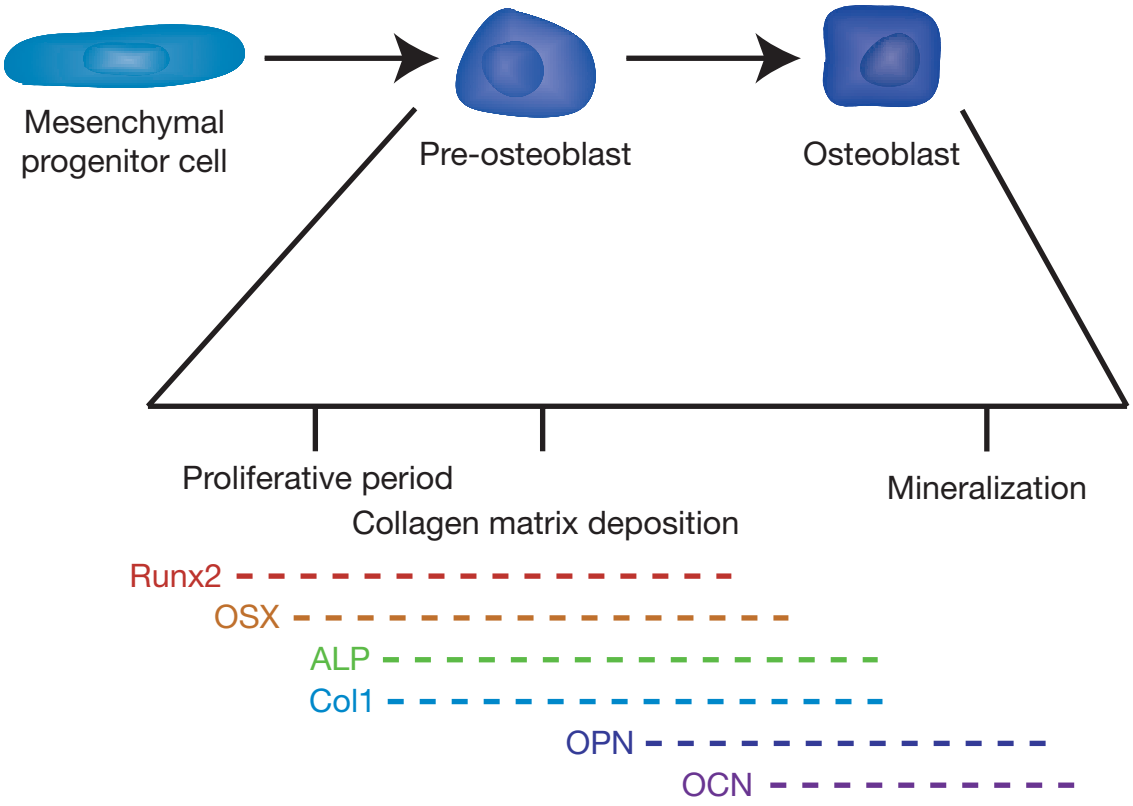

Figure 3. *In vitro* osteoblast differentiation

Schematic representation of the expression of bone differentiation markers during *in vitro* osteoblast differentiation. Mesenchymal progenitor cells destined to differentiate into pre-osteoblasts are characterized by the expression of the master regulators of bone differentiation, Runx2 and Osterix, OSX. Other bone markers that are expressed early during *in vitro* osteoblast differentiation are alkaline phosphatase (ALP) and type 1 collagen (Col1). *Osteopontin* (OPN) is a bone differentiation marker that is expressed in early to mid-stage osteoblasts, while *Osteocalcin* (OCN) is specifically expressed by terminally differentiated, mineralized matrix-secreting osteoblasts.

absence of bone tissue despite a relatively normal cartilage skeleton (Komori et al, 1997; Otto et al, 1997). Indeed, no early or late osteoblast markers are expressed in these embryos, demonstrating that *Runx2* activity is required for bone matrix deposition by mature osteoblasts (Ducy et al, 1997). Conversely, overexpression of *Runx2* in fibroblasts or myoblasts *in vitro* induces the expression of osteoblast specific markers in these cell types (Ducy et al, 1997).

Another transcription factor that is essential for osteoblast differentiation in mice is Osterix (Osx). Osx is expressed in and highly specific to the osteoblast lineage, although some studies have observed expression in chondrocytes *in vivo* and other tissues *in vitro* (Milona et al, 2003; Nakashima et al, 2002; Rodda & McMahon, 2006; Yagi et al, 2003). Mice lacking *Osx* expression are perinatal lethal due to the absence of bone formation (Nakashima et al, 2002), a phenotype similar to that of the *Runx2* mutants. Additionally, there is no detectable expression of bone-specific markers in *Osx*-null mice (Nakashima et al, 2002). *In situ* hybridization analysis revealed that *Osx* is not expressed in *Runx2*-null animals, but *Osx*-deficient animals express *Runx2* suggesting that *Osx* acts downstream of *Runx2* in osteoblast differentiation (Nakashima et al, 2002). Indeed, *in vitro* overexpression of *Osx* is sufficient to induce differentiation and expression of osteoblast markers (Nakashima et al, 2002; Tai et al, 2004; Wang et al, 2006).

Several other proteins are involved in the proper regulation of osteoblast differentiation and function. A recent study suggested that the transcription factor ATF4 is essential for terminal differentiation of osteoblasts and their function. ATF4 can bind to an osteoblast-specific element in the *Osteocalcin* promoter and directly activate its

transcription (Ducy & Karsenty, 1995; Schinke & Karsenty, 1999; Yang et al, 2004). In addition, ATF4 and its associated kinase, RSK2, post-transcriptionally regulate the expression of type I collagen (Yang et al, 2004). Mutation of either *Atf4* or *Rsk2* cause reduced bone mass in mice due to impaired bone formation (Yang et al, 2004). The nuclear protein SATB2 regulates osteoblast differentiation and function by physically interacting with both Runx2 and ATF4 (Dobreva et al, 2006). Moreover, SATB2 binds to and regulates the *Osteopontin* and *Osteocalcin* promoters (Dobreva et al, 2006). *In vivo* bone development is also negatively impacted when mutations are introduced into *Ihh* (Long et al, 2004; Rodda & McMahon, 2006), *β-catenin* (Day et al, 2005; Hill et al, 2005; Hu et al, 2005; Rodda & McMahon, 2006), *Msx2* (Satokata et al, 2000), or *Dlx5* (Acampora et al, 1999). Finally, pRb can associate with Runx2 and bind osteoblast-specific promoters, such as *Osteopontin* (Thomas et al, 2001). Moreover, this association enhances the transcriptional activity of Runx2; however, these findings have only been demonstrated *in vitro* (Thomas et al, 2001).

iii. Osteoclasts

Unlike osteoblasts and chondrocytes, which originate from mesenchymal stem cells, the osteoclast is derived from the monocyte/macrophage hematopoietic lineage. Osteoclasts are multinucleated cells that release acid and lytic enzymes that degrade the bone to which they are attached. Proper regulation of osteoclast activity is critical for maintaining the density of bone. Most adult skeletal diseases are the result of excessive osteoclast activity (Rodan & Martin, 2000), such as osteoporosis, rheumatoid arthritis, and

periodontal disease. Conversely, too little osteoclast activity causes osteopetrosis, which is characterized by increased bone mass that fills the bone marrow cavity.

Co-culture studies using bone marrow or spleen cultures with stromal cells yielded osteoclasts, creating a breakthrough in the understanding of osteoclastogenesis (Takahashi et al, 1988). Osteoblasts secrete three main factors that regulate osteoclast differentiation: CSF-1, RANKL, and Osteoprotegrin (OPG). Both CSF-1 and RANKL are required for the differentiation of osteoclasts and the induction of osteoclast-specific genes, including tartrate-resistant acid phosphatase. Indeed, mice with inactivating mutations of CSF-1 lack osteoclasts (Yoshida et al, 1990). Similarly, mice lacking the ligand for the TNF-related RANK receptor, RANKL, do not produce osteoclasts and also exhibit osteopetrosis (Kong et al, 1999; Yasuda et al, 1998b). The last factor secreted by osteoblasts, OPG, acts as a decoy receptor for RANKL (Simonet et al, 1997; Yasuda et al, 1998a). Since OPG can sequester RANKL, OPG strongly inhibits osteoclastogenesis. Accordingly, mice deficient for *OPG* exhibit osteopetrosis. Although osteoblasts do not play a direct role in bone resorption, their ability to secrete factors that influence osteoclast differentiation further underscores the importance of osteoblasts in bone remodeling.

Mutant mouse models have significantly contributed to our understanding of E2F function. In addition to the vast number of studies that link E2Fs to pocket protein-mediated regulation of the cell cycle, there is a growing body of knowledge that highlights the functions of the E2Fs in development and differentiation. Indeed, many of these roles are intimately linked to cell cycle exit. Therefore, this study is aimed at determining the role

of E2F4, the major repressive E2F *in vivo*, during bone development (Chapter 2). The highly homologous repressive E2F, E2F5, may compensate and preclude the complete understanding of E2F4 function in single knockout animals. Therefore, we have generated a conditional *E2f4* mouse that can be bred with *E2f5*$^{+/-}$ mice to address the synergistic roles that E2F4 and E2F5 may play during cell cycle exit and bone development (Chapter 3).

REFERENCES

Acampora D, Merlo GR, Paleari L, Zerega B, Postiglione MP, Mantero S, Bober E, Barbieri O, Simeone A, Levi G (1999) Craniofacial, vestibular and bone defects in mice lacking the Distal-less-related gene Dlx5. Development 126(17): 3795-3809

Asp P, Acosta-Alvear D, Tsikitis M, van Oevelen C, Dynlacht BD (2009) E2f3b plays an essential role in myogenic differentiation through isoform-specific gene regulation. Genes Dev 23(1): 37-53

Attwooll C, Oddi S, Cartwright P, Prosperini E, Agger K, Steensgaard P, Wagener C, Sardet C, Moroni MC, Helin K (2005) A novel repressive E2F6 complex containing the polycomb group protein, EPC1, that interacts with EZH2 in a proliferation-specific manner. J Biol Chem 280(2): 1199-1208

Balciunaite E, Spektor A, Lents NH, Cam H, Te Riele H, Scime A, Rudnicki MA, Young R, Dynlacht BD (2005) Pocket protein complexes are recruited to distinct targets in quiescent and proliferating cells. Mol Cell Biol 25(18): 8166-8178

Bancos S, Cao Q, Bowers WJ, Crispe IN (2009) Dysfunctional memory CD8+ T cells after priming in the absence of the cell cycle regulator E2F4. Cell Immunol

Bandara LR, Buck VM, Zamanian M, Johnston LH, La Thangue NB (1993) Functional synergy between DP-1 and E2F-1 in the cell cycle-regulating transcription factor DRTF1/E2F. EMBO J 12(11): 4317-4324

Bandara LR, La Thangue NB (1991) Adenovirus E1a prevents the retinoblastoma gene product from complexing with a cellular transcription factor. Nature 351(6326): 494-497

Beijersbergen RL, Kerkhoven RM, Zhu L, Carlee L, Voorhoeve PM, Bernards R (1994) E2F-4, a new member of the E2F gene family, has oncogenic activity and associates with p107 in vivo. Genes Dev 8(22): 2680-2690

Bell DM, Leung KK, Wheatley SC, Ng LJ, Zhou S, Ling KW, Sham MH, Koopman P, Tam PP, Cheah KS (1997) SOX9 directly regulates the type-II collagen gene. Nat Genet 16(2): 174-178

Berman SD, Yuan TL, Miller ES, Lee EY, Caron A, Lees JA (2008) The retinoblastoma protein tumor suppressor is important for appropriate osteoblast differentiation and bone development. Mol Cancer Res 6(9): 1440-1451

Bi W, Deng JM, Zhang Z, Behringer RR, de Crombrugghe B (1999) Sox9 is required for cartilage formation. Nat Genet 22(1): 85-89

Brehm A, Miska EA, McCance DJ, Reid JL, Bannister AJ, Kouzarides T (1998) Retinoblastoma protein recruits histone deacetylase to repress transcription. Nature 391(6667): 597-601

Caretti G, Salsi V, Vecchi C, Imbriano C, Mantovani R (2003) Dynamic recruitment of NF-Y and histone acetyltransferases on cell-cycle promoters. J Biol Chem 278(33): 30435-30440

Cartwright P, Muller H, Wagener C, Holm K, Helin K (1998) E2F-6: a novel member of the E2F family is an inhibitor of E2F-dependent transcription. Oncogene 17(5): 611-623

Chen CR, Kang Y, Siegel PM, Massague J (2002) E2F4/5 and p107 as Smad cofactors linking the TGFbeta receptor to c-myc repression. Cell 110(1): 19-32

Chen D, Opavsky R, Pacal M, Tanimoto N, Wenzel P, Seeliger MW, Leone G, Bremner R (2007) Rb-Mediated Neuronal Differentiation through Cell-Cycle-Independent Regulation of E2f3a. PLoS Biol 5(7): e179

Chen PL, Riley DJ, Chen Y, Lee WH (1996) Retinoblastoma protein positively regulates terminal adipocyte differentiation through direct interaction with C/EBPs. Genes Dev 10(21): 2794-2804

Chen TT, Wang JY (2000) Establishment of irreversible growth arrest in myogenic differentiation requires the RB LXCXE-binding function. Mol Cell Biol 20(15): 5571-5580

Chong JL, Tsai SY, Sharma N, Opavsky R, Price R, Wu L, Fernandez SA, Leone G (2009) E2f3a and E2f3b contribute to the control of cell proliferation and mouse development. Mol Cell Biol 29(2): 414-424

Christensen J, Cloos P, Toftegaard U, Klinkenberg D, Bracken AP, Trinh E, Heeran M, Di Stefano L, Helin K (2005) Characterization of E2F8, a novel E2F-like cell-cycle regulated repressor of E2F-activated transcription. Nucleic Acids Res 33(17): 5458-5470

Clark AJ, Doyle KM, Humbert PO (2004) Cell-intrinsic requirement for pRb in erythropoiesis. Blood 104(5): 1324-1326

Clarke AR, Maandag ER, van Roon M, van der Lugt NM, van der Valk M, Hooper ML, Berns A, te Riele H (1992) Requirement for a functional Rb-1 gene in murine development. Nature 359(6393): 328-330

Classon M, Kennedy BK, Mulloy R, Harlow E (2000a) Opposing roles of pRB and p107 in adipocyte differentiation. Proc Natl Acad Sci U S A 97(20): 10826-10831

Classon M, Salama S, Gorka C, Mulloy R, Braun P, Harlow E (2000b) Combinatorial roles for pRB, p107, and p130 in E2F-mediated cell cycle control. Proc Natl Acad Sci U S A 97(20): 10820-10825

Claudio PP, Howard CM, Fu Y, Cinti C, Califano L, Micheli P, Mercer EW, Caputi M, Giordano A (2000a) Mutations in the retinoblastoma-related gene RB2/p130 in primary nasopharyngeal carcinoma. Cancer Res 60(1): 8-12

Claudio PP, Howard CM, Pacilio C, Cinti C, Romano G, Minimo C, Maraldi NM, Minna JD, Gelbert L, Leoncini L, Tosi GM, Hicheli P, Caputi M, Giordano GG, Giordano A (2000b) Mutations in the retinoblastoma-related gene RB2/p130 in lung tumors and suppression of tumor growth in vivo by retrovirus-mediated gene transfer. Cancer Res 60(2): 372-382

Cloud JE, Rogers C, Reza TL, Ziebold U, Stone JR, Picard MH, Caron AM, Bronson RT, Lees JA (2002) Mutant mouse models reveal the relative roles of E2F1 and E2F3 in vivo. Mol Cell Biol 22(8): 2663-2672

Cobrinik D, Lee MH, Hannon G, Mulligan G, Bronson RT, Dyson N, Harlow E, Beach D, Weinberg RA, Jacks T (1996) Shared role of the pRB-related p130 and p107 proteins in limb development. Genes Dev 10(13): 1633-1644

Coppola JA, Lewis BA, Cole MD (1990) Increased retinoblastoma gene expression is associated with late stages of differentiation in many different cell types. Oncogene 5(11): 1731-1733

Courel M, Friesenhahn L, Lees JA (2008) E2f6 and Bmi1 cooperate in axial skeletal development. Dev Dyn 237(5): 1232-1242

Cvekl A, Kashanchi F, Brady JN, Piatigorsky J (1999) Pax-6 interactions with TATA-box-binding protein and retinoblastoma protein. Invest Ophthalmol Vis Sci 40(7): 1343-1350

Dahiya A, Gavin MR, Luo RX, Dean DC (2000) Role of the LXCXE binding site in Rb function. Mol Cell Biol 20(18): 6799-6805

Dalton S (1992) Cell cycle regulation of the human cdc2 gene. EMBO J 11(5): 1797-1804

Danielian PS, Bender Kim CF, Caron AM, Vasile E, Bronson RT, Lees JA (2007) E2f4 is required for normal development of the airway epithelium. Dev Biol 305(2): 564-576

Danielian PS, Friesenhahn LB, Faust AM, West JC, Caron AM, Bronson RT, Lees JA (2008) E2f3a and E2f3b make overlapping but different contributions to total E2f3 activity. Oncogene 27(51): 6561-6570

Dannenberg JH, Schuijff L, Dekker M, van der Valk M, te Riele H (2004) Tissue-specific tumor suppressor activity of retinoblastoma gene homologs p107 and p130. Genes Dev 18(23): 2952-2962

Day TF, Guo X, Garrett-Beal L, Yang Y (2005) Wnt/beta-catenin signaling in mesenchymal progenitors controls osteoblast and chondrocyte differentiation during vertebrate skeletogenesis. Dev Cell 8(5): 739-750

de Bruin A, Maiti B, Jakoi L, Timmers C, Buerki R, Leone G (2003a) Identification and characterization of E2F7, a novel mammalian E2F family member capable of blocking cellular proliferation. J Biol Chem 278(43): 42041-42049

de Bruin A, Wu L, Saavedra HI, Wilson P, Yang Y, Rosol TJ, Weinstein M, Robinson ML, Leone G (2003b) Rb function in extraembryonic lineages suppresses apoptosis in the CNS of Rb-deficient mice. Proc Natl Acad Sci U S A 100(11): 6546-6551

DeCaprio JA, Ludlow JW, Figge J, Shew JY, Huang CM, Lee WH, Marsilio E, Paucha E, Livingston DM (1988) SV40 large tumor antigen forms a specific complex with the product of the retinoblastoma susceptibility gene. Cell 54(2): 275-283

DeGregori J, Leone G, Miron A, Jakoi L, Nevins JR (1997) Distinct roles for E2F proteins in cell growth control and apoptosis. Proc Natl Acad Sci U S A 94(14): 7245-7250

DeGregori J, Leone G, Ohtani K, Miron A, Nevins JR (1995) E2F-1 accumulation bypasses a G1 arrest resulting from the inhibition of G1 cyclin-dependent kinase activity. Genes Dev 9(23): 2873-2887

DeRyckere D, DeGregori J (2005) E2F1 and E2F2 are differentially required for homeostasis-driven and antigen-induced T cell proliferation in vivo. J Immunol 175(2): 647-655

Deshpande AM, Akunowicz JD, Reveles XT, Patel BB, Saria EA, Gorlick RG, Naylor SL, Leach RJ, Hansen MF (2007) PHC3, a component of the hPRC-H complex, associates with E2F6 during G0 and is lost in osteosarcoma tumors. Oncogene 26(12): 1714-1722

Di Stefano L, Jensen MR, Helin K (2003) E2F7, a novel E2F featuring DP-independent repression of a subset of E2F-regulated genes. EMBO J 22(23): 6289-6298

Dirlam A, Spike BT, Macleod KF (2007) Deregulated E2f-2 underlies cell cycle and maturation defects in retinoblastoma null erythroblasts. Mol Cell Biol 27(24): 8713-8728

Dobreva G, Chahrour M, Dautzenberg M, Chirivella L, Kanzler B, Farinas I, Karsenty G, Grosschedl R (2006) SATB2 is a multifunctional determinant of craniofacial patterning and osteoblast differentiation. Cell 125(5): 971-986

Dryja TP, Rapaport JM, Joyce JM, Petersen RA (1986) Molecular detection of deletions involving band q14 of chromosome 13 in retinoblastomas. Proc Natl Acad Sci U S A 83(19): 7391-7394

Ducy P (2000) Cbfa1: a molecular switch in osteoblast biology. Dev Dyn 219(4): 461-471

Ducy P, Karsenty G (1995) Two distinct osteoblast-specific cis-acting elements control expression of a mouse osteocalcin gene. Mol Cell Biol 15(4): 1858-1869

Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G (1997) Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. Cell 89(5): 747-754

Dunaief JL, Strober BE, Guha S, Khavari PA, Alin K, Luban J, Begemann M, Crabtree GR, Goff SP (1994) The retinoblastoma protein and BRG1 form a complex and cooperate to induce cell cycle arrest. Cell 79(1): 119-130

Dyson N (1998) The regulation of E2F by pRB-family proteins. Genes Dev 12(15): 2245-2262

Dyson N, Buchkovich K, Whyte P, Harlow E (1989a) The cellular 107K protein that binds to adenovirus E1A also associates with the large T antigens of SV40 and JC virus. Cell 58(2): 249-255

Dyson N, Howley PM, Munger K, Harlow E (1989b) The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. Science 243(4893): 934-937

Endo-Munoz L, Dahler A, Teakle N, Rickwood D, Hazar-Rethinam M, Abdul-Jabbar I, Sommerville S, Dickinson I, Kaur P, Paquet-Fifield S, Saunders N (2009) E2F7 can regulate proliferation, differentiation, and apoptotic responses in human keratinocytes: implications for cutaneous squamous cell carcinoma formation. Cancer Res 69(5): 1800-1808

Ewen ME, Xing YG, Lawrence JB, Livingston DM (1991) Molecular cloning, chromosomal mapping, and expression of the cDNA for p107, a retinoblastoma gene product-related protein. Cell 66(6): 1155-1164

Fajas L, Egler V, Reiter R, Hansen J, Kristiansen K, Debril MB, Miard S, Auwerx J (2002a) The retinoblastoma-histone deacetylase 3 complex inhibits PPARgamma and adipocyte differentiation. Dev Cell 3(6): 903-910

Fajas L, Landsberg RL, Huss-Garcia Y, Sardet C, Lees JA, Auwerx J (2002b) E2Fs regulate adipocyte differentiation. Dev Cell 3(1): 39-49

Ferguson KL, McClellan KA, Vanderluit JL, McIntosh WC, Schuurmans C, Polleux F, Slack RS (2005) A cell-autonomous requirement for the cell cycle regulatory protein, Rb, in neuronal migration. EMBO J 24(24): 4381-4391

Ferguson KL, Vanderluit JL, Hebert JM, McIntosh WC, Tibbo E, MacLaurin JG, Park DS, Wallace VA, Vooijs M, McConnell SK, Slack RS (2002) Telencephalon-specific Rb knockouts reveal enhanced neurogenesis, survival and abnormal cortical development. EMBO J 21(13): 3337-3346

Ferreira R, Magnaghi-Jaulin L, Robin P, Harel-Bellan A, Trouche D (1998) The three members of the pocket proteins family share the ability to repress E2F activity through recruitment of a histone deacetylase. Proc Natl Acad Sci U S A 95(18): 10493-10498

Field SJ, Tsai FY, Kuo F, Zubiaga AM, Kaelin WG, Jr., Livingston DM, Orkin SH, Greenberg ME (1996) E2F-1 functions in mice to promote apoptosis and suppress proliferation. Cell 85(4): 549-561

Flemington EK, Speck SH, Kaelin WG, Jr. (1993) E2F-1-mediated transactivation is inhibited by complex formation with the retinoblastoma susceptibility gene product. Proc Natl Acad Sci U S A 90(15): 6914-6918

Friend SH, Bernards R, Rogelj S, Weinberg RA, Rapaport JM, Albert DM, Dryja TP (1986) A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. Nature 323(6089): 643-646

Fung YK, Murphree AL, T'Ang A, Qian J, Hinrichs SH, Benedict WF (1987) Structural evidence for the authenticity of the human retinoblastoma gene. Science 236(4809): 1657-1661

Garcia I, Murga M, Vicario A, Field SJ, Zubiaga AM (2000) A role for E2F1 in the induction of apoptosis during thymic negative selection. Cell Growth Differ 11(2): 91-98

Gaubatz S, Lees JA, Lindeman GJ, Livingston DM (2001) E2F4 is exported from the nucleus in a CRM1-dependent manner. Mol Cell Biol 21(4): 1384-1392

Gaubatz S, Lindeman GJ, Ishida S, Jakoi L, Nevins JR, Livingston DM, Rempel RE (2000) E2F4 and E2F5 play an essential role in pocket protein-mediated G1 control. Mol Cell 6(3): 729-735

Gaubatz S, Wood JG, Livingston DM (1998) Unusual proliferation arrest and transcriptional control properties of a newly discovered E2F family member, E2F-6. Proc Natl Acad Sci U S A 95(16): 9190-9195

Ghosh MK, Harter ML (2003) A viral mechanism for remodeling chromatin structure in G0 cells. Mol Cell 12(1): 255-260

Giangrande PH, Zhu W, Schlisio S, Sun X, Mori S, Gaubatz S, Nevins JR (2004) A role for E2F6 in distinguishing G1/S- and G2/M-specific transcription. Genes Dev 18(23): 2941-2951

Ginsberg D, Vairo G, Chittenden T, Xiao ZX, Xu G, Wydner KL, DeCaprio JA, Lawrence JB, Livingston DM (1994) E2F-4, a new member of the E2F transcription factor family, interacts with p107. Genes Dev 8(22): 2665-2679

Girling R, Partridge JF, Bandara LR, Burden N, Totty NF, Hsuan JJ, La Thangue NB (1993) A new component of the transcription factor DRTF1/E2F. Nature 365(6445): 468

Gu W, Schneider JW, Condorelli G, Kaushal S, Mahdavi V, Nadal-Ginard B (1993) Interaction of myogenic factors and the retinoblastoma protein mediates muscle cell commitment and differentiation. Cell 72(3): 309-324

Gutierrez GM, Kong E, Sabbagh Y, Brown NE, Lee JS, Demay MB, Thomas DM, Hinds PW (2008) Impaired bone development and increased mesenchymal progenitor cells in calvaria of RB1-/- mice. Proc Natl Acad Sci U S A 105(47): 18402-18407

Haigis K, Sage J, Glickman J, Shafer S, Jacks T (2006) The related retinoblastoma (pRb) and p130 proteins cooperate to regulate homeostasis in the intestinal epithelium. J Biol Chem 281(1): 638-647

Hall BK, Miyake T (1992) The membranous skeleton: the role of cell condensations in vertebrate skeletogenesis. Anat Embryol (Berl) 186(2): 107-124

Hannon GJ, Demetrick D, Beach D (1993) Isolation of the Rb-related p130 through its interaction with CDK2 and cyclins. Genes Dev 7(12A): 2378-2391

Hansen JB, Jorgensen C, Petersen RK, Hallenborg P, De Matteis R, Boye HA, Petrovic N, Enerback S, Nedergaard J, Cinti S, te Riele H, Kristiansen K (2004) Retinoblastoma protein functions as a molecular switch determining white versus brown adipocyte differentiation. Proc Natl Acad Sci U S A 101(12): 4112-4117

Hansen JB, Petersen RK, Larsen BM, Bartkova J, Alsner J, Kristiansen K (1999) Activation of peroxisome proliferator-activated receptor gamma bypasses the function of the retinoblastoma protein in adipocyte differentiation. J Biol Chem 274(4): 2386-2393

He Y, Armanious MK, Thomas MJ, Cress WD (2000) Identification of E2F-3B, an alternative form of E2F-3 lacking a conserved N-terminal region. Oncogene 19(30): 3422-3433

Helin K, Holm K, Niebuhr A, Eiberg H, Tommerup N, Hougaard S, Poulsen HS, Spang-Thomsen M, Norgaard P (1997) Loss of the retinoblastoma protein-related p130 protein in small cell lung carcinoma. Proc Natl Acad Sci U S A 94(13): 6933-6938

Helin K, Lees JA, Vidal M, Dyson N, Harlow E, Fattaey A (1992) A cDNA encoding a pRB-binding protein with properties of the transcription factor E2F. Cell 70(2): 337-350

Helin K, Wu CL, Fattaey AR, Lees JA, Dynlacht BD, Ngwu C, Harlow E (1993) Heterodimerization of the transcription factors E2F-1 and DP-1 leads to cooperative trans-activation. Genes Dev 7(10): 1850-1861

Hiebert SW, Chellappan SP, Horowitz JM, Nevins JR (1992) The interaction of RB with E2F coincides with an inhibition of the transcriptional activity of E2F. Genes Dev 6(2): 177-185

Hijmans EM, Voorhoeve PM, Beijersbergen RL, van 't Veer LJ, Bernards R (1995) E2F-5, a new E2F family member that interacts with p130 in vivo. Mol Cell Biol 15(6): 3082-3089

Hill TP, Spater D, Taketo MM, Birchmeier W, Hartmann C (2005) Canonical Wnt/beta-catenin signaling prevents osteoblasts from differentiating into chondrocytes. Dev Cell 8(5): 727-738

Hsiao KM, McMahon SL, Farnham PJ (1994) Multiple DNA elements are required for the growth regulation of the mouse E2F1 promoter. Genes Dev 8(13): 1526-1537

Hu H, Hilton MJ, Tu X, Yu K, Ornitz DM, Long F (2005) Sequential roles of Hedgehog and Wnt signaling in osteoblast development. Development 132(1): 49-60

Huber HE, Edwards G, Goodhart PJ, Patrick DR, Huang PS, Ivey-Hoyle M, Barnett SF, Oliff A, Heimbrook DC (1993) Transcription factor E2F binds DNA as a heterodimer. Proc Natl Acad Sci U S A 90(8): 3525-3529

Huet X, Rech J, Plet A, Vie A, Blanchard JM (1996) Cyclin A expression is under negative transcriptional control during the cell cycle. Mol Cell Biol 16(7): 3789-3798

Huh MS, Parker MH, Scime A, Parks R, Rudnicki MA (2004) Rb is required for progression through myogenic differentiation but not maintenance of terminal differentiation. J Cell Biol 166(6): 865-876

Humbert PO, Rogers C, Ganiatsas S, Landsberg RL, Trimarchi JM, Dandapani S, Brugnara C, Erdman S, Schrenzel M, Bronson RT, Lees JA (2000a) E2F4 is essential for normal erythrocyte maturation and neonatal viability. Mol Cell 6(2): 281-291

Humbert PO, Verona R, Trimarchi JM, Rogers C, Dandapani S, Lees JA (2000b) E2f3 is critical for normal cellular proliferation. Genes Dev 14(6): 690-703

Hurford RK, Jr., Cobrinik D, Lee MH, Dyson N (1997) pRB and p107/p130 are required for the regulated expression of different sets of E2F responsive genes. Genes Dev 11(11): 1447-1463

Iavarone A, Massague J (1999) E2F and histone deacetylase mediate transforming growth factor beta repression of cdc25A during keratinocyte cell cycle arrest. Mol Cell Biol 19(1): 916-922

Iglesias A, Murga M, Laresgoiti U, Skoudy A, Bernales I, Fullaondo A, Moreno B, Lloreta J, Field SJ, Real FX, Zubiaga AM (2004) Diabetes and exocrine pancreatic insufficiency in E2F1/E2F2 double-mutant mice. J Clin Invest 113(10): 1398-1407

Ishida S, Huang E, Zuzan H, Spang R, Leone G, West M, Nevins JR (2001) Role for E2F in control of both DNA replication and mitotic functions as revealed from DNA microarray analysis. Mol Cell Biol 21(14): 4684-4699

Ivey-Hoyle M, Conroy R, Huber HE, Goodhart PJ, Oliff A, Heimbrook DC (1993) Cloning and characterization of E2F-2, a novel protein with the biochemical properties of transcription factor E2F. Mol Cell Biol 13(12): 7802-7812

Iwanaga R, Komori H, Ishida S, Okamura N, Nakayama K, Nakayama KI, Ohtani K (2006) Identification of novel E2F1 target genes regulated in cell cycle-dependent and independent manners. Oncogene 25(12): 1786-1798

Jacks T, Fazeli A, Schmitt EM, Bronson RT, Goodell MA, Weinberg RA (1992) Effects of an Rb mutation in the mouse. Nature 359(6393): 295-300

Jiang SX, Sheldrick M, Desbois A, Slinn J, Hou ST (2007) Neuropilin-1 is a direct target of the transcription factor E2F1 during cerebral ischemia-induced neuronal death in vivo. Mol Cell Biol 27(5): 1696-1705

Johnson DG, Schwarz JK, Cress WD, Nevins JR (1993) Expression of transcription factor E2F1 induces quiescent cells to enter S phase. Nature 365(6444): 349-352

Kaelin WG, Jr., Krek W, Sellers WR, DeCaprio JA, Ajchenbaum F, Fuchs CS, Chittenden T, Li Y, Farnham PJ, Blanar MA, et al. (1992) Expression cloning of a cDNA encoding a retinoblastoma-binding protein with E2F-like properties. Cell 70(2): 351-364

Karaplis AC, Luz A, Glowacki J, Bronson RT, Tybulewicz VL, Kronenberg HM, Mulligan RC (1994) Lethal skeletal dysplasia from targeted disruption of the parathyroid hormone-related peptide gene. Genes Dev 8(3): 277-289

Kim IS, Otto F, Zabel B, Mundlos S (1999) Regulation of chondrocyte differentiation by Cbfa1. Mech Dev 80(2): 159-170

King JC, Moskowitz IP, Burgon PG, Ahmad F, Stone JR, Seidman JG, Lees JA (2008) E2F3 plays an essential role in cardiac development and function. Cell Cycle 7(23): 3775-3780

Kinross KM, Clark AJ, Iazzolino RM, Humbert PO (2006) E2f4 regulates fetal erythropoiesis through the promotion of cellular proliferation. Blood 108(3): 886-895

Knudson AG, Jr. (1971) Mutation and cancer: statistical study of retinoblastoma. Proc Natl Acad Sci U S A 68(4): 820-823

Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi K, Shimizu Y, Bronson RT, Gao YH, Inada M, Sato M, Okamoto R, Kitamura Y, Yoshiki S, Kishimoto T (1997) Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. Cell 89(5): 755-764

Kong LJ, Chang JT, Bild AH, Nevins JR (2007) Compensation and specificity of function within the E2F family. Oncogene 26(3): 321-327

Kong YY, Yoshida H, Sarosi I, Tan HL, Timms E, Capparelli C, Morony S, Oliveira-dos-Santos AJ, Van G, Itie A, Khoo W, Wakeham A, Dunstan CR, Lacey DL, Mak TW, Boyle WJ, Penninger JM (1999) OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. Nature 397(6717): 315-323

Kovesdi I, Reichel R, Nevins JR (1987) Role of an adenovirus E2 promoter binding factor in E1A-mediated coordinate gene control. Proc Natl Acad Sci U S A 84(8): 2180-2184

Kowalik TF, DeGregori J, Schwarz JK, Nevins JR (1995) E2F1 overexpression in quiescent fibroblasts leads to induction of cellular DNA synthesis and apoptosis. J Virol 69(4): 2491-2500

Krek W, Livingston DM, Shirodkar S (1993) Binding to DNA and the retinoblastoma gene product promoted by complex formation of different E2F family members. Science 262(5139): 1557-1560

La Thangue NB, Rigby PW (1987) An adenovirus E1A-like transcription factor is regulated during the differentiation of murine embryonal carcinoma stem cells. Cell 49(4): 507-513

Lam EW, Watson RJ (1993) An E2F-binding site mediates cell-cycle regulated repression of mouse B-myb transcription. EMBO J 12(7): 2705-2713

Landsberg RL, Sero JE, Danielian PS, Yuan TL, Lee EY, Lees JA (2003) The role of E2F4 in adipogenesis is independent of its cell cycle regulatory activity. Proc Natl Acad Sci U S A 100(5): 2456-2461

Lanske B, Karaplis AC, Lee K, Luz A, Vortkamp A, Pirro A, Karperien M, Defize LH, Ho C, Mulligan RC, Abou-Samra AB, Juppner H, Segre GV, Kronenberg HM (1996) PTH/PTHrP receptor in early development and Indian hedgehog-regulated bone growth. Science 273(5275): 663-666

Laplantine E, Rossi F, Sahni M, Basilico C, Cobrinik D (2002) FGF signaling targets the pRb-related p107 and p130 proteins to induce chondrocyte growth arrest. J Cell Biol 158(4): 741-750

LeCouter JE, Kablar B, Hardy WR, Ying C, Megeney LA, May LL, Rudnicki MA (1998a) Strain-dependent myeloid hyperplasia, growth deficiency, and accelerated cell cycle in mice lacking the Rb-related p107 gene. Mol Cell Biol 18(12): 7455-7465

LeCouter JE, Kablar B, Whyte PF, Ying C, Rudnicki MA (1998b) Strain-dependent embryonic lethality in mice lacking the retinoblastoma-related p130 gene. Development 125(23): 4669-4679

Lee C, Chang JH, Lee HS, Cho Y (2002a) Structural basis for the recognition of the E2F transactivation domain by the retinoblastoma tumor suppressor. Genes Dev 16(24): 3199-3212

Lee EY, Cam H, Ziebold U, Rayman JB, Lees JA, Dynlacht BD (2002b) E2F4 loss suppresses tumorigenesis in Rb mutant mice. Cancer Cell 2(6): 463-472

Lee EY, Chang CY, Hu N, Wang YC, Lai CC, Herrup K, Lee WH, Bradley A (1992) Mice deficient for Rb are nonviable and show defects in neurogenesis and haematopoiesis. Nature 359(6393): 288-294

Lee EY, Hu N, Yuan SS, Cox LA, Bradley A, Lee WH, Herrup K (1994) Dual roles of the retinoblastoma protein in cell cycle regulation and neuron differentiation. Genes Dev 8(17): 2008-2021

Lee MH, Williams BO, Mulligan G, Mukai S, Bronson RT, Dyson N, Harlow E, Jacks T (1996) Targeted disruption of p107: functional overlap between p107 and Rb. Genes Dev 10(13): 1621-1632

Lee NK, Sowa H, Hinoi E, Ferron M, Ahn JD, Confavreux C, Dacquin R, Mee PJ, McKee MD, Jung DY, Zhang Z, Kim JK, Mauvais-Jarvis F, Ducy P, Karsenty G (2007) Endocrine regulation of energy metabolism by the skeleton. Cell 130(3): 456-469

Lee WH, Bookstein R, Hong F, Young LJ, Shew JY, Lee EY (1987a) Human retinoblastoma susceptibility gene: cloning, identification, and sequence. Science 235(4794): 1394-1399

Lee WH, Shew JY, Hong FD, Sery TW, Donoso LA, Young LJ, Bookstein R, Lee EY (1987b) The retinoblastoma susceptibility gene encodes a nuclear phosphoprotein associated with DNA binding activity. Nature 329(6140): 642-645

Lees JA, Saito M, Vidal M, Valentine M, Look T, Harlow E, Dyson N, Helin K (1993) The retinoblastoma protein binds to a family of E2F transcription factors. Mol Cell Biol 13(12): 7813-7825

Lefebvre V, de Crombrugghe B (1998) Toward understanding SOX9 function in chondrocyte differentiation. Matrix Biol 16(9): 529-540

Lefebvre V, Li P, de Crombrugghe B (1998) A new long form of Sox5 (L-Sox5), Sox6 and Sox9 are coexpressed in chondrogenesis and cooperatively activate the type II collagen gene. EMBO J 17(19): 5718-5733

Leone G, DeGregori J, Yan Z, Jakoi L, Ishida S, Williams RS, Nevins JR (1998) E2F3 activity is regulated during the cell cycle and is required for the induction of S phase. Genes Dev 12(14): 2120-2130

Leone G, Nuckolls F, Ishida S, Adams M, Sears R, Jakoi L, Miron A, Nevins JR (2000) Identification of a novel E2F3 product suggests a mechanism for determining specificity of repression by Rb proteins. Mol Cell Biol 20(10): 3626-3632

Li FQ, Coonrod A, Horwitz M (2000) Selection of a dominant negative retinoblastoma protein (RB) inhibiting satellite myoblast differentiation implies an indirect interaction between MyoD and RB. Mol Cell Biol 20(14): 5129-5139

Li FX, Zhu JW, Hogan CJ, DeGregori J (2003) Defective gene expression, S phase progression, and maturation during hematopoiesis in E2F1/E2F2 mutant mice. Mol Cell Biol 23(10): 3607-3622

Li J, Ran C, Li E, Gordon F, Comstock G, Siddiqui H, Cleghorn W, Chen HZ, Kornacker K, Liu CG, Pandit SK, Khanizadeh M, Weinstein M, Leone G, de Bruin A (2008) Synergistic function of E2F7 and E2F8 is essential for cell survival and embryonic development. Dev Cell 14(1): 62-75

Li JM, Hu PP, Shen X, Yu Y, Wang XF (1997) E2F4-RB and E2F4-p107 complexes suppress gene expression by transforming growth factor beta through E2F binding sites. Proc Natl Acad Sci U S A 94(10): 4948-4953

Li Y, Graham C, Lacy S, Duncan AM, Whyte P (1993) The adenovirus E1A-associated 130-kD protein is encoded by a member of the retinoblastoma gene family and physically interacts with cyclins A and E. Genes Dev 7(12A): 2366-2377

Lindeman GJ, Dagnino L, Gaubatz S, Xu Y, Bronson RT, Warren HB, Livingston DM (1998) A specific, nonproliferative role for E2F-5 in choroid plexus function revealed by gene targeting. Genes Dev 12(8): 1092-1098

Lindeman GJ, Gaubatz S, Livingston DM, Ginsberg D (1997) The subcellular localization of E2F-4 is cell-cycle dependent. Proc Natl Acad Sci U S A 94(10): 5095-5100

Lipinski MM, Macleod KF, Williams BO, Mullaney TL, Crowley D, Jacks T (2001) Cell-autonomous and non-cell-autonomous functions of the Rb tumor suppressor in developing central nervous system. EMBO J 20(13): 3402-3413

Liu Y, Zacksenhaus E (2000) E2F1 mediates ectopic proliferation and stage-specific p53-dependent apoptosis but not aberrant differentiation in the ocular lens of Rb deficient fetuses. Oncogene 19(52): 6065-6073

Logan N, Delavaine L, Graham A, Reilly C, Wilson J, Brummelkamp TR, Hijmans EM, Bernards R, La Thangue NB (2004) E2F-7: a distinctive E2F family member with an unusual organization of DNA-binding domains. Oncogene 23(30): 5138-5150

Logan N, Graham A, Zhao X, Fisher R, Maiti B, Leone G, La Thangue NB (2005) E2F-8: an E2F family member with a similar organization of DNA-binding domains to E2F-7. Oncogene 24(31): 5000-5004

Long F, Chung UI, Ohba S, McMahon J, Kronenberg HM, McMahon AP (2004) Ihh signaling is directly required for the osteoblast lineage in the endochondral skeleton. Development 131(6): 1309-1318

Lukas J, Petersen BO, Holm K, Bartek J, Helin K (1996) Deregulated expression of E2F family members induces S-phase entry and overcomes p16INK4A-mediated growth suppression. Mol Cell Biol 16(3): 1047-1057

Luo RX, Postigo AA, Dean DC (1998) Rb interacts with histone deacetylase to repress transcription. Cell 92(4): 463-473

Maandag EC, van der Valk M, Vlaar M, Feltkamp C, O'Brien J, van Roon M, van der Lugt N, Berns A, te Riele H (1994) Developmental rescue of an embryonic-lethal mutation in the retinoblastoma gene in chimeric mice. EMBO J 13(18): 4260-4268

MacPherson D, Conkrite K, Tam M, Mukai S, Mu D, Jacks T (2007) Murine bilateral retinoblastoma exhibiting rapid-onset, metastatic progression and N-myc gene amplification. EMBO J 26(3): 784-794

MacPherson D, Sage J, Crowley D, Trumpp A, Bronson RT, Jacks T (2003) Conditional mutation of Rb causes cell cycle defects without apoptosis in the central nervous system. Mol Cell Biol 23(3): 1044-1053

MacPherson D, Sage J, Kim T, Ho D, McLaughlin ME, Jacks T (2004) Cell type-specific effects of Rb deletion in the murine retina. Genes Dev 18(14): 1681-1694

Magae J, Wu CL, Illenye S, Harlow E, Heintz NH (1996) Nuclear localization of DP and E2F transcription factors by heterodimeric partners and retinoblastoma protein family members. J Cell Sci 109 (Pt 7): 1717-1726

Maiti B, Li J, de Bruin A, Gordon F, Timmers C, Opavsky R, Patil K, Tuttle J, Cleghorn W, Leone G (2005) Cloning and characterization of mouse E2F8, a novel mammalian E2F family member capable of blocking cellular proliferation. J Biol Chem 280(18): 18211-18220

Mann DJ, Jones NC (1996) E2F-1 but not E2F-4 can overcome p16-induced G1 cell-cycle arrest. Curr Biol 6(4): 474-483

Martinez-Balbas MA, Bauer UM, Nielsen SJ, Brehm A, Kouzarides T (2000) Regulation of E2F1 activity by acetylation. EMBO J 19(4): 662-671

Marzio G, Wagener C, Gutierrez MI, Cartwright P, Helin K, Giacca M (2000) E2F family members are differentially regulated by reversible acetylation. J Biol Chem 275(15): 10887-10892

May JS, Prince AM, Lyle RE, McGehee RE, Jr. (2001) Antisense suppression of p107 inhibits 3T3-L1 adipocyte differentiation. Biochem Biophys Res Commun 283(4): 837-842

McClellan KA, Ruzhynsky VA, Douda DN, Vanderluit JL, Ferguson KL, Chen D, Bremner R, Park DS, Leone G, Slack RS (2007) Unique requirement for Rb/E2F3 in neuronal migration: evidence for cell cycle-independent functions. Mol Cell Biol 27(13): 4825-4843

Means AL, Slansky JE, McMahon SL, Knuth MW, Farnham PJ (1992) The HIP1 binding site is required for growth regulation of the dihydrofolate reductase gene promoter. Mol Cell Biol 12(3): 1054-1063

Milona MA, Gough JE, Edgar AJ (2003) Expression of alternatively spliced isoforms of human Sp7 in osteoblast-like cells. BMC Genomics 4(1): 43

Mittnacht S (1998) Control of pRB phosphorylation. Curr Opin Genet Dev 8(1): 21-27

Moberg K, Starz MA, Lees JA (1996) E2F-4 switches from p130 to p107 and pRB in response to cell cycle reentry. Mol Cell Biol 16(4): 1436-1449

Morgenbesser SD, Williams BO, Jacks T, DePinho RA (1994) p53-dependent apoptosis produced by Rb-deficiency in the developing mouse lens. Nature 371(6492): 72-74

Morkel M, Wenkel J, Bannister AJ, Kouzarides T, Hagemeier C (1997) An E2F-like repressor of transcription. Nature 390(6660): 567-568

Muller H, Bracken AP, Vernell R, Moroni MC, Christians F, Grassilli E, Prosperini E, Vigo E, Oliner JD, Helin K (2001) E2Fs regulate the expression of genes involved in differentiation, development, proliferation, and apoptosis. Genes Dev 15(3): 267-285

Muller H, Moroni MC, Vigo E, Petersen BO, Bartek J, Helin K (1997) Induction of S-phase entry by E2F transcription factors depends on their nuclear localization. Mol Cell Biol 17(9): 5508-5520

Murga M, Fernandez-Capetillo O, Field SJ, Moreno B, Borlado LR, Fujiwara Y, Balomenos D, Vicario A, Carrera AC, Orkin SH, Greenberg ME, Zubiaga AM (2001) Mutation of E2F2 in mice causes enhanced T lymphocyte proliferation, leading to the development of autoimmunity. Immunity 15(6): 959-970

Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR, de Crombrugghe B (2002) The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. Cell 108(1): 17-29

Nielsen SJ, Schneider R, Bauer UM, Bannister AJ, Morrison A, O'Carroll D, Firestein R, Cleary M, Jenuwein T, Herrera RE, Kouzarides T (2001) Rb targets histone H3 methylation and HP1 to promoters. Nature 412(6846): 561-565

Ogawa H, Ishiguro K, Gaubatz S, Livingston DM, Nakatani Y (2002) A complex with chromatin modifiers that occupies E2F- and Myc-responsive genes in G0 cells. Science 296(5570): 1132-1136

Ormondroyd E, de la Luna S, La Thangue NB (1995) A new member of the DP family, DP-3, with distinct protein products suggests a regulatory role for alternative splicing in the cell cycle transcription factor DRTF1/E2F. Oncogene 11(8): 1437-1446

Otto F, Thornell AP, Crompton T, Denzel A, Gilmour KC, Rosewell IR, Stamp GW, Beddington RS, Mundlos S, Olsen BR, Selby PB, Owen MJ (1997) Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. Cell 89(5): 765-771

Parakati R, DiMario JX (2005) Dynamic transcriptional regulatory complexes, including E2F4, p107, p130, and Sp1, control fibroblast growth factor receptor 1 gene expression during myogenesis. J Biol Chem 280(22): 21284-21294

Parakati R, Dimario JX (2005) Repression of fibroblast growth factor receptor 1 gene expression by E2F4 in skeletal muscle cells. Dev Dyn 232(1): 119-130

Parisi T, Yuan TL, Faust AM, Caron AM, Bronson R, Lees JA (2007) Selective requirements for E2f3 in the development and tumorigenicity of Rb-deficient chimeric tissues. Mol Cell Biol 27(6): 2283-2293

Pediconi N, Ianari A, Costanzo A, Belloni L, Gallo R, Cimino L, Porcellini A, Screpanti I, Balsano C, Alesse E, Gulino A, Levrero M (2003) Differential regulation of E2F1 apoptotic target genes in response to DNA damage. Nat Cell Biol 5(6): 552-558

Persengiev SP, Kondova, II, Kilpatrick DL (1999) E2F4 actively promotes the initiation and maintenance of nerve growth factor-induced cell differentiation. Mol Cell Biol 19(9): 6048-6056

Pohlers M, Truss M, Frede U, Scholz A, Strehle M, Kuban RJ, Hoffmann B, Morkel M, Birchmeier C, Hagemeier C (2005) A role for E2F6 in the restriction of male-germ-cell-specific gene expression. Curr Biol 15(11): 1051-1057

Qin XQ, Livingston DM, Kaelin WG, Jr., Adams PD (1994) Deregulated transcription factor E2F-1 expression leads to S-phase entry and p53-mediated apoptosis. Proc Natl Acad Sci U S A 91(23): 10918-10922

Rayman JB, Takahashi Y, Indjeian VB, Dannenberg JH, Catchpole S, Watson RJ, te Riele H, Dynlacht BD (2002) E2F mediates cell cycle-dependent transcriptional repression in vivo by recruitment of an HDAC1/mSin3B corepressor complex. Genes Dev 16(8): 933-947

Rempel RE, Saenz-Robles MT, Storms R, Morham S, Ishida S, Engel A, Jakoi L, Melhem MF, Pipas JM, Smith C, Nevins JR (2000) Loss of E2F4 activity leads to abnormal development of multiple cellular lineages. Mol Cell 6(2): 293-306

Ren B, Cam H, Takahashi Y, Volkert T, Terragni J, Young RA, Dynlacht BD (2002) E2F integrates cell cycle progression with DNA repair, replication, and G(2)/M checkpoints. Genes Dev 16(2): 245-256

Robanus-Maandag E, Dekker M, van der Valk M, Carrozza ML, Jeanny JC, Dannenberg JH, Berns A, te Riele H (1998) p107 is a suppressor of retinoblastoma development in pRb-deficient mice. Genes Dev 12(11): 1599-1609

Robertson KD, Ait-Si-Ali S, Yokochi T, Wade PA, Jones PL, Wolffe AP (2000) DNMT1 forms a complex with Rb, E2F1 and HDAC1 and represses transcription from E2F-responsive promoters. Nat Genet 25(3): 338-342

Rodan GA, Martin TJ (2000) Therapeutic approaches to bone diseases. Science 289(5484): 1508-1514

Rodda SJ, McMahon AP (2006) Distinct roles for Hedgehog and canonical Wnt signaling in specification, differentiation and maintenance of osteoblast progenitors. Development 133(16): 3231-3244

Rogers KT, Higgins PD, Milla MM, Phillips RS, Horowitz JM (1996) DP-2, a heterodimeric partner of E2F: identification and characterization of DP-2 proteins expressed in vivo. Proc Natl Acad Sci U S A 93(15): 7594-7599

Rossi F, MacLean HE, Yuan W, Francis RO, Semenova E, Lin CS, Kronenberg HM, Cobrinik D (2002) p107 and p130 Coordinately regulate proliferation, Cbfa1 expression, and hypertrophic differentiation during endochondral bone development. Dev Biol 247(2): 271-285

Ruiz S, Santos M, Segrelles C, Leis H, Jorcano JL, Berns A, Paramio JM, Vooijs M (2004) Unique and overlapping functions of pRb and p107 in the control of proliferation and differentiation in epidermis. Development 131(11): 2737-2748

Ruiz S, Segrelles C, Bravo A, Santos M, Perez P, Leis H, Jorcano JL, Paramio JM (2003) Abnormal epidermal differentiation and impaired epithelial-mesenchymal tissue interactions in mice lacking the retinoblastoma relatives p107 and p130. Development 130(11): 2341-2353

Ruzhynsky VA, Furimsky M, Park DS, Wallace VA, Slack RS (2009) E2F4 Is Required for Early Eye Patterning. Dev Neurosci

Ruzhynsky VA, McClellan KA, Vanderluit JL, Jeong Y, Furimsky M, Park DS, Epstein DJ, Wallace VA, Slack RS (2007) Cell cycle regulator E2F4 is essential for the development of the ventral telencephalon. J Neurosci 27(22): 5926-5935

Sardet C, Vidal M, Cobrinik D, Geng Y, Onufryk C, Chen A, Weinberg RA (1995) E2F-4 and E2F-5, two members of the E2F family, are expressed in the early phases of the cell cycle. Proc Natl Acad Sci U S A 92(6): 2403-2407

Satokata I, Ma L, Ohshima H, Bei M, Woo I, Nishizawa K, Maeda T, Takano Y, Uchiyama M, Heaney S, Peters H, Tang Z, Maxson R, Maas R (2000) Msx2 deficiency in mice causes pleiotropic defects in bone growth and ectodermal organ formation. Nat Genet 24(4): 391-395

Scheijen B, Bronk M, van der Meer T, Bernards R (2003) Constitutive E2F1 overexpression delays endochondral bone formation by inhibiting chondrocyte differentiation. Mol Cell Biol 23(10): 3656-3668

Schinke T, Karsenty G (1999) Characterization of Osf1, an osteoblast-specific transcription factor binding to a critical cis-acting element in the mouse Osteocalcin promoters. J Biol Chem 274(42): 30182-30189

Schwarz JK, Bassing CH, Kovesdi I, Datto MB, Blazing M, George S, Wang XF, Nevins JR (1995) Expression of the E2F1 transcription factor overcomes type beta transforming growth factor-mediated growth suppression. Proc Natl Acad Sci U S A 92(2): 483-487

Scime A, Grenier G, Huh MS, Gillespie MA, Bevilacqua L, Harper ME, Rudnicki MA (2005) Rb and p107 regulate preadipocyte differentiation into white versus brown fat through repression of PGC-1alpha. Cell Metab 2(5): 283-295

Shan B, Zhu X, Chen PL, Durfee T, Yang Y, Sharp D, Lee WH (1992) Molecular cloning of cellular genes encoding retinoblastoma-associated proteins: identification of a gene with properties of the transcription factor E2F. Mol Cell Biol 12(12): 5620-5631

Simonet WS, Lacey DL, Dunstan CR, Kelley M, Chang MS, Luthy R, Nguyen HQ, Wooden S, Bennett L, Boone T, Shimamoto G, DeRose M, Elliott R, Colombero A, Tan HL, Trail G, Sullivan J, Davy E, Bucay N, Renshaw-Gegg L, Hughes TM, Hill D, Pattison W, Campbell P, Sander S, Van G, Tarpley J, Derby P, Lee R, Boyle WJ (1997) Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. Cell 89(2): 309-319

Singh P, Coe J, Hong W (1995) A role for retinoblastoma protein in potentiating transcriptional activation by the glucocorticoid receptor. Nature 374(6522): 562-565

Slack RS, El-Bizri H, Wong J, Belliveau DJ, Miller FD (1998) A critical temporal requirement for the retinoblastoma protein family during neuronal determination. J Cell Biol 140(6): 1497-1509

Sparkes RS, Sparkes MC, Wilson MG, Towner JW, Benedict W, Murphree AL, Yunis JJ (1980) Regional assignment of genes for human esterase D and retinoblastoma to chromosome band 13q14. Science 208(4447): 1042-1044

Spike BT, Dirlam A, Dibling BC, Marvin J, Williams BO, Jacks T, Macleod KF (2004) The Rb tumor suppressor is required for stress erythropoiesis. EMBO J 23(21): 4319-4329

St-Jacques B, Hammerschmidt M, McMahon AP (1999) Indian hedgehog signaling regulates proliferation and differentiation of chondrocytes and is essential for bone formation. Genes Dev 13(16): 2072-2086

Stevaux O, Dyson NJ (2002) A revised picture of the E2F transcriptional network and RB function. Curr Opin Cell Biol 14(6): 684-691

Storre J, Elsasser HP, Fuchs M, Ullmann D, Livingston DM, Gaubatz S (2002) Homeotic transformations of the axial skeleton that accompany a targeted deletion of E2f6. EMBO Rep 3(7): 695-700

Strom DK, Cleveland JL, Chellappan S, Nip J, Hiebert SW (1998) E2F-1 and E2F-3 are functionally distinct in their ability to promote myeloid cell cycle progression and block granulocyte differentiation. Cell Growth Differ 9(1): 59-69

Tai G, Polak JM, Bishop AE, Christodoulou I, Buttery LD (2004) Differentiation of osteoblasts from murine embryonic stem cells by overexpression of the transcriptional factor osterix. Tissue Eng 10(9-10): 1456-1466

Takahashi N, Yamana H, Yoshiki S, Roodman GD, Mundy GR, Jones SJ, Boyde A, Suda T (1988) Osteoclast-like cell formation and its regulation by osteotropic hormones in mouse bone marrow cultures. Endocrinology 122(4): 1373-1382

Takahashi Y, Rayman JB, Dynlacht BD (2000) Analysis of promoter binding by the E2F and pRB families in vivo: distinct E2F proteins mediate activation and repression. Genes Dev 14(7): 804-816

Takeda S, Bonnamy JP, Owen MJ, Ducy P, Karsenty G (2001) Continuous expression of Cbfa1 in nonhypertrophic chondrocytes uncovers its ability to induce hypertrophic chondrocyte differentiation and partially rescues Cbfa1-deficient mice. Genes Dev 15(4): 467-481

Taubert S, Gorrini C, Frank SR, Parisi T, Fuchs M, Chan HM, Livingston DM, Amati B (2004) E2F-dependent histone acetylation and recruitment of the Tip60 acetyltransferase complex to chromatin in late G1. Mol Cell Biol 24(10): 4546-4556

Thomas DM, Carty SA, Piscopo DM, Lee JS, Wang WF, Forrester WC, Hinds PW (2001) The retinoblastoma protein acts as a transcriptional coactivator required for osteogenic differentiation. Mol Cell 8(2): 303-316

Tommasi S, Pfeifer GP (1995) In vivo structure of the human cdc2 promoter: release of a p130-E2F-4 complex from sequences immediately upstream of the transcription initiation site coincides with induction of cdc2 expression. Mol Cell Biol 15(12): 6901-6913

Trimarchi JM, Fairchild B, Verona R, Moberg K, Andon N, Lees JA (1998) E2F-6, a member of the E2F family that can behave as a transcriptional repressor. Proc Natl Acad Sci U S A 95(6): 2850-2855

Trimarchi JM, Lees JA (2002) Sibling rivalry in the E2F family. Nat Rev Mol Cell Biol 3(1): 11-20

Trouche D, Cook A, Kouzarides T (1996) The CBP co-activator stimulates E2F1/DP1 activity. Nucleic Acids Res 24(21): 4139-4145

Trouche D, Le Chalony C, Muchardt C, Yaniv M, Kouzarides T (1997) RB and hbrm cooperate to repress the activation functions of E2F1. Proc Natl Acad Sci U S A 94(21): 11268-11273

Tsai SY, Opavsky R, Sharma N, Wu L, Naidu S, Nolan E, Feria-Arias E, Timmers C, Opavska J, de Bruin A, Chong JL, Trikha P, Fernandez SA, Stromberg P, Rosol TJ, Leone G (2008) Mouse development with a single E2F activator. Nature 454(7208): 1137-1141

Ueta C, Iwamoto M, Kanatani N, Yoshida C, Liu Y, Enomoto-Iwamoto M, Ohmori T, Enomoto H, Nakata K, Takada K, Kurisu K, Komori T (2001) Skeletal malformations caused by overexpression of Cbfa1 or its dominant negative form in chondrocytes. J Cell Biol 153(1): 87-100

Vairo G, Livingston DM, Ginsberg D (1995) Functional interaction between E2F-4 and p130: evidence for distinct mechanisms underlying growth suppression by different retinoblastoma protein family members. Genes Dev 9(7): 869-881

Vega RB, Matsuda K, Oh J, Barbosa AC, Yang X, Meadows E, McAnally J, Pomajzl C, Shelton JM, Richardson JA, Karsenty G, Olson EN (2004) Histone deacetylase 4 controls chondrocyte hypertrophy during skeletogenesis. Cell 119(4): 555-566

Verona R, Moberg K, Estes S, Starz M, Vernon JP, Lees JA (1997) E2F activity is regulated by cell cycle-dependent changes in subcellular localization. Mol Cell Biol 17(12): 7268-7282

Vo N, Goodman RH (2001) CREB-binding protein and p300 in transcriptional regulation. J Biol Chem 276(17): 13505-13508

Wade M, Kowalik TF, Mudryj M, Huang ES, Azizkhan JC (1992) E2F mediates dihydrofolate reductase promoter activation and multiprotein complex formation in human cytomegalovirus infection. Mol Cell Biol 12(10): 4364-4374

Walkley CR, Shea JM, Sims NA, Purton LE, Orkin SH (2007) Rb regulates interactions between hematopoietic stem cells and their bone marrow microenvironment. Cell 129(6): 1081-1095

Wang X, Kua HY, Hu Y, Guo K, Zeng Q, Wu Q, Ng HH, Karsenty G, de Crombrugghe B, Yeh J, Li B (2006) p53 functions as a negative regulator of osteoblastogenesis, osteoblast-dependent osteoclastogenesis, and bone remodeling. J Cell Biol 172(1): 115-125

Weinberg RA (1992) The retinoblastoma gene and gene product. Cancer Surv 12: 43-57

Weinmann AS, Yan PS, Oberley MJ, Huang TH, Farnham PJ (2002) Isolating human transcription factor targets by coupling chromatin immunoprecipitation and CpG island microarray analysis. Genes Dev 16(2): 235-244

Wells J, Boyd KE, Fry CJ, Bartley SM, Farnham PJ (2000) Target gene specificity of E2F and pocket protein family members in living cells. Mol Cell Biol 20(16): 5797-5807

Wells J, Graveel CR, Bartley SM, Madore SJ, Farnham PJ (2002) The identification of E2F1-specific target genes. Proc Natl Acad Sci U S A 99(6): 3890-3895

Wenzel PL, Wu L, de Bruin A, Chong JL, Chen WY, Dureska G, Sites E, Pan T, Sharma A, Huang K, Ridgway R, Mosaliganti K, Sharp R, Machiraju R, Saltz J, Yamamoto H, Cross JC, Robinson ML, Leone G (2007) Rb is critical in a mammalian tissue stem cell population. Genes Dev 21(1): 85-97

White J, Stead E, Faast R, Conn S, Cartwright P, Dalton S (2005) Developmental activation of the Rb-E2F pathway and establishment of cell cycle-regulated cyclin-dependent kinase activity during embryonic stem cell differentiation. Mol Biol Cell 16(4): 2018-2027

Whyte P, Ruley HE, Harlow E (1988) Two regions of the adenovirus early region 1A proteins are required for transformation. J Virol 62(1): 257-265

Wikenheiser-Brokamp KA (2004) Rb family proteins differentially regulate distinct cell lineages during epithelial development. Development 131(17): 4299-4310

Wildwater M, Campilho A, Perez-Perez JM, Heidstra R, Blilou I, Korthout H, Chatterjee J, Mariconti L, Gruissem W, Scheres B (2005) The RETINOBLASTOMA-RELATED gene regulates stem cell maintenance in Arabidopsis roots. Cell 123(7): 1337-1349

Williams BO, Schmitt EM, Remington L, Bronson RT, Albert DM, Weinberg RA, Jacks T (1994) Extensive contribution of Rb-deficient cells to adult chimeric mice with limited histopathological consequences. EMBO J 13(18): 4251-4259

Wong CF, Barnes LM, Dahler AL, Smith L, Serewko-Auret MM, Popa C, Abdul-Jabbar I, Saunders NA (2003) E2F modulates keratinocyte squamous differentiation: implications for E2F inhibition in squamous cell carcinoma. J Biol Chem 278(31): 28516-28522

Wu CL, Zukerberg LR, Ngwu C, Harlow E, Lees JA (1995) In vivo association of E2F and DP family proteins. Mol Cell Biol 15(5): 2536-2546

Wu L, de Bruin A, Saavedra HI, Starovic M, Trimboli A, Yang Y, Opavska J, Wilson P, Thompson JC, Ostrowski MC, Rosol TJ, Woollett LA, Weinstein M, Cross JC, Robinson ML, Leone G (2003) Extra-embryonic function of Rb is essential for embryonic development and viability. Nature 421(6926): 942-947

Wu L, Timmers C, Maiti B, Saavedra HI, Sang L, Chong GT, Nuckolls F, Giangrande P, Wright FA, Field SJ, Greenberg ME, Orkin S, Nevins JR, Robinson ML, Leone G (2001) The E2F1-3 transcription factors are essential for cellular proliferation. Nature 414(6862): 457-462

Xiao B, Spencer J, Clements A, Ali-Khan N, Mittnacht S, Broceno C, Burghammer M, Perrakis A, Marmorstein R, Gamblin SJ (2003) Crystal structure of the retinoblastoma tumor suppressor protein bound to E2F and the molecular basis of its regulation. Proc Natl Acad Sci U S A 100(5): 2363-2368

Yagi K, Tsuji K, Nifuji A, Shinomiya K, Nakashima K, DeCrombrugghe B, Noda M (2003) Bone morphogenetic protein-2 enhances osterix gene expression in chondrocytes. J Cell Biochem 88(6): 1077-1083

Yamasaki L, Jacks T, Bronson R, Goillot E, Harlow E, Dyson NJ (1996) Tumor induction and tissue atrophy in mice lacking E2F-1. Cell 85(4): 537-548

Yang HS, Hinds PW (2007) pRb-mediated control of epithelial cell proliferation and Indian hedgehog expression in mouse intestinal development. BMC Dev Biol 7: 6

Yang J, Song K, Krebs TL, Jackson MW, Danielpour D (2008) Rb/E2F4 and Smad2/3 link survivin to TGF-beta-induced apoptosis and tumor progression. Oncogene 27(40): 5326-5338

Yang X, Matsuda K, Bialek P, Jacquot S, Masuoka HC, Schinke T, Li L, Brancorsini S, Sassone-Corsi P, Townes TM, Hanauer A, Karsenty G (2004) ATF4 is a substrate of RSK2 and an essential regulator of osteoblast biology; implication for Coffin-Lowry Syndrome. Cell 117(3): 387-398

Yasuda H, Shima N, Nakagawa N, Mochizuki SI, Yano K, Fujise N, Sato Y, Goto M, Yamaguchi K, Kuriyama M, Kanno T, Murakami A, Tsuda E, Morinaga T, Higashio K (1998a) Identity of osteoclastogenesis inhibitory factor (OCIF) and osteoprotegerin (OPG): a mechanism by which OPG/OCIF inhibits osteoclastogenesis in vitro. Endocrinology 139(3): 1329-1337

Yasuda H, Shima N, Nakagawa N, Yamaguchi K, Kinosaki M, Mochizuki S, Tomoyasu A, Yano K, Goto M, Murakami A, Tsuda E, Morinaga T, Higashio K, Udagawa N, Takahashi N, Suda T (1998b) Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. Proc Natl Acad Sci U S A 95(7): 3597-3602

Yee AS, Reichel R, Kovesdi I, Nevins JR (1987) Promoter interaction of the E1A-inducible factor E2F and its potential role in the formation of a multi-component complex. EMBO J 6(7): 2061-2068

Yoshida H, Hayashi S, Kunisada T, Ogawa M, Nishikawa S, Okamura H, Sudo T, Shultz LD (1990) The murine mutation osteopetrosis is in the coding region of the macrophage colony stimulating factor gene. Nature 345(6274): 442-444

Zacksenhaus E, Jiang Z, Chung D, Marth JD, Phillips RA, Gallie BL (1996) pRb controls proliferation, differentiation, and death of skeletal muscle cells and other lineages during embryogenesis. Genes Dev 10(23): 3051-3064

Zhang HS, Postigo AA, Dean DC (1999a) Active transcriptional repression by the Rb-E2F complex mediates G1 arrest triggered by p16INK4a, TGFbeta, and contact inhibition. Cell 97(1): 53-61

Zhang JM, Zhao X, Wei Q, Paterson BM (1999b) Direct inhibition of G(1) cdk kinase activity by MyoD promotes myoblast cell cycle withdrawal and terminal differentiation. EMBO J 18(24): 6983-6993

Zhang Y, Chellappan SP (1995) Cloning and characterization of human DP2, a novel dimerization partner of E2F. Oncogene 10(11): 2085-2093

Zhou G, Lefebvre V, Zhang Z, Eberspaecher H, de Crombrugghe B (1998) Three high mobility group-like sequences within a 48-base pair enhancer of the Col2a1 gene are required for cartilage-specific expression in vivo. J Biol Chem 273(24): 14989-14997

Zhu JW, DeRyckere D, Li FX, Wan YY, DeGregori J (1999) A role for E2F1 in the induction of ARF, p53, and apoptosis during thymic negative selection. Cell Growth Differ 10(12): 829-838

Zhu JW, Field SJ, Gore L, Thompson M, Yang H, Fujiwara Y, Cardiff RD, Greenberg M, Orkin SH, DeGregori J (2001) E2F1 and E2F2 determine thresholds for antigen-induced T-cell proliferation and suppress tumorigenesis. Mol Cell Biol 21(24): 8547-8564

Ziebold U, Lee EY, Bronson RT, Lees JA (2003) E2F3 loss has opposing effects on different pRB-deficient tumors, resulting in suppression of pituitary tumors but metastasis of medullary thyroid carcinomas. Mol Cell Biol 23(18): 6542-6552

Zwicker J, Liu N, Engeland K, Lucibello FC, Muller R (1996) Cell cycle regulation of E2F site occupation in vivo. Science 271(5255): 1595-1597

Chapter Two

E2F4 is required for proper bone development and normal cell cycle exit of osteoblast progenitor cells *in vivo*

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Author's contribution: Figure 3, 4D, 5, and 6

ABSTRACT

The E2F family of transcription factors, in association with the pocket protein family members, function to regulate genes required for cellular proliferation. The most abundant E2F, E2F4, maintains a G0/G1 cell cycle state through the transcriptional repression of genes that encode key proliferation regulators. E2F4's deletion in mice disrupts the development of specific tissues such as the airway epithelium. Here, we investigate E2F4's role in bone development. We found that E2F4 loss impairs the formation of several bones that arise through intramembranous or endochondral ossification. The most severe defect occurred in the calvarial bones of the skull where we observed a striking delay in the differentiation of calvarial osteoblasts. Through both *in vivo* and *in vitro* analyses, we show that E2F4 loss did not abolish the differentiation potential of osteoblastic progenitors, but it impaired their ability to exit the cell cycle and increased the endogenous pool of undifferentiated progenitor cells. We conclude that E2F4 plays an important role in enabling osteoblastic progenitors to exit the cell cycle and therby contributes to the commitment of these cells to the osteoblast lineage and to bone formation.

INTRODUCTION

The E2F family of transcription factors is best known for its role in regulating genes required for cellular proliferation (Attwooll et al, 2004; Dimova & Dyson, 2005). To date, ten E2F family members have been identified. These can be subdivided into two distinct groups based on their predisposition to either activate or repress transcription of E2F-responsive genes. E2F4 functions primarily as a transcriptional repressor and its nuclear localization is largely dependent on its association with pocket protein family members pRb, p107, and p130 (Gaubatz et al, 2000; Rayman et al, 2002; Verona et al, 1997). These complexes recruit histone deacetylases to E2F-responsive gene promoters to directly repress transcription (Blais & Dynlacht, 2007; Rayman et al, 2002; Takahashi et al, 2000; Wells et al, 2000). Upon mitogen-induced phosphorylation of the pocket proteins by cyclin dependent kinases, the pocket protein/E2F4 complexes dissociate and E2F4 is exported to the cytoplasm because it contains nuclear export signals (Attwooll et al, 2004; Dimova & Dyson, 2005; Trimarchi & Lees, 2002). This removes E2F4 from promoters, thereby relieving the active repression of the target genes.

While the role of the E2F family of transcription factors in cell cycle progression has been well characterized, we are only beginning to understand the role of E2Fs in development (McClellan & Slack, 2007). E2F4 affects the differentiation and development of many cell lineages through cell cycle-dependent and cell cycle-independent mechanisms. For example, *E2f4* knockout embryos are transiently anemic and exhibit cell autonomous defects in red blood cell maturation (Humbert et al, 2000; Kinross et al, 2006; Rempel et al, 2000). These animals show incomplete enucleation and an increased

population of progenitor and immature erythrocytes (Humbert et al, 2000; Rempel et al, 2000). Mutation of *E2f4* also leads to neonatal lethality caused by chronic rhinitis and associated opportunistic bacterial infections (Humbert et al, 2000). The susceptibility to infection seems to result from a defect in the differentiation of the nasal epithelium that causes mucin-secreting cells to replace ciliated cells leading to impairment of mucus clearance (Danielian et al, 2007). Although the mechanism underlying the cilial cell defect is unknown, it appears to be independent of disregulated cellular proliferation. E2F4 also contributes to adipocyte differentiation and this seems to occur through both cell cycle-dependent and -independent mechanisms (Fajas et al, 2002; Landsberg et al, 2003).

A role for E2F4 in chondrocyte or osteoblast differentiation has not been investigated. However, its pocket protein binding partners have been implicated in the differentiation of these cell types. Specifically, mutation of *p107* and *p130 in vivo* prevents chondrocytes from properly exiting the cell cycle (Cobrinik et al, 1996), while loss of pRb inhibits osteoblast differentiation *in vitro* (Berman et al, 2008; Thomas et al, 2001). Therefore, it is plausible that E2F4 may also function in the differentiation of these cell types and, consequently, bone development. Bone development occurs via two distinct processes (Wagner & Karsenty, 2001). In the first process, intramembranous ossification, mesenchymal progenitor cells differentiate directly into osteoblasts, which secrete the calcified extracellular matrix that constitutes bone. This occurs primarily in the flat bones of the cranium and the medial clavicles. Most bones in the skeleton form via the second process, endochondral ossification, where condensations of mesenchymal progenitor cells differentiate first into chondrocytes. These chondrocytes proliferate and secrete a cartilage

matrix, forming the template for ossified bone formation. Upon terminal differentiation, chondrocytes become hypertrophic and undergo apoptosis, which provides space for osteoblasts to invade and generate the bone matrix. In this study, we show that *E2f4*$^{-/-}$ embryos exhibit a defect in the normal development of bones formed through either endochondral or intramembranous ossification and establish that E2F4 is required for appropriate differentiation of osteoblasts.

RESULTS

E2f4$^{-/-}$ embryos exhibit defects in bone development

To determine the effect of E2F4 loss on skeletal development, we examined the skeletons of wildtype and *E2f4*$^{-/-}$ embryos at e18.5 by Alizarin Red staining for bone and Alcian Blue staining of cartilage. At this stage of development, several bones in the *E2f4*$^{-/-}$ embryos were less ossified compared to the bones of wildtype littermate controls (Figure 1). The sternebrae, xiphoid process, and presphenoid bone, which form through endochondral ossification, had reduced Alizarin Red staining in *E2f4*-null embryos, indicating reduced ossification (Figure 1A, B). A similar defect is seen in the bones that form through intramembranous ossification; in *E2f4*$^{-/-}$ embryos, the frontal and parietal bones of the skull demonstrate dramatically reduced ossification compared to wildtype embryos (Figure 1C).

We analyzed embryos at earlier time points to determine if there were any skeletal defects in the initiation of the ossification process. At e13.5, the first and only bone to become ossified is the clavicle. In *E2f4*$^{-/-}$ embryos, the clavicle is not ossified as judged by the absence of Alizarin Red staining of this bone in comparison with wildtype littermates (Figure 2). At e15.5, there is also a reduction in the amount of Alizarin Red staining in the skulls of *E2f4*-null embryos compared to wildtype (Figure 2). In addition, we observed a decrease in the amount of Alcian Blue staining in the basisphenoid and the presphenoid bones at this stage (Figure 2). Interestingly, this finding suggests that the presphenoid bone ossification defect in e18.5 embryos may, at least in part, be a result of defective or delayed cartilage differentiation, which forms the template for this bone. The data from

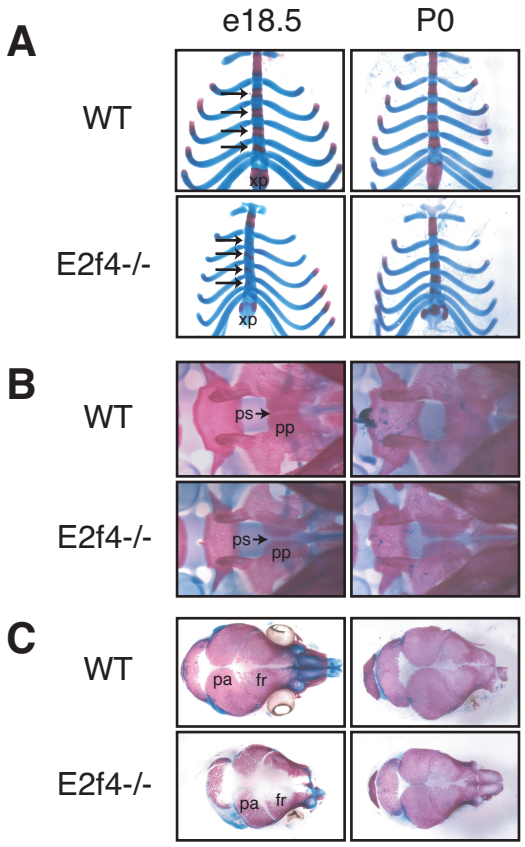

Figure 1. Deletion of *E2f4* causes defects in embryonic bone development.

(A-C) Alizarin Red (bone) and Alcian Blue (cartilage) staining of e18.5 and P0 littermate embryos. e18.5 *E2f4*$^{-/-}$ embryos exhibit less ossification in the sternebrae (arrows) and xiphoid (A), presphenoid bone (B), and cranium (C). Abbreviations: xp, xiphoid process; pp, palatine process; ps (with arrow), presphenoid; fr, frontal bone; pa, parietal bone.

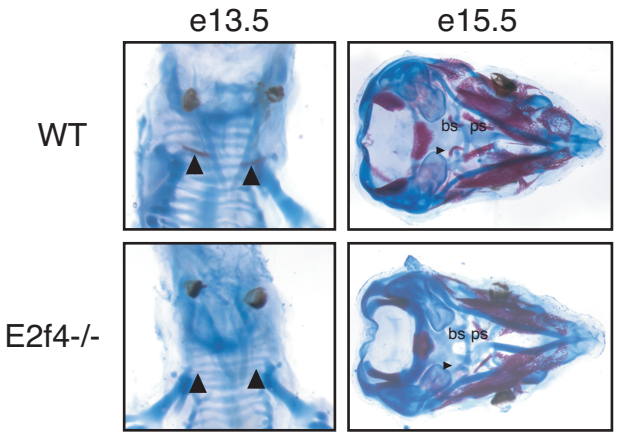

Figure 2. Deletion of *E2f4* causes defects in early embryonic cartilage and bone development.

Alizarin Red (bone) and Alcian Blue (cartilage) staining of e13.5 (A) and e15.5 (B) embryos. (Left column) e13.5 *E2f4*$^{-/-}$ embryos exhibit less ossification in the clavicles (arrowheads). (Right column) e15.5 *E2f4*$^{-/-}$ embryos display less ossification of bone (arrowhead) and less deposition of cartilage matrix in the presphenoid and basisphenoid elements. Abbreviations: bs, basisphenoid; ps, presphenoid.

these embryos indicate that loss of E2F4 affects the normal embryonic development of several bones of the skeleton.

We also examined the skeletons of mice at birth (P0). At P0, the skeletal defects were less apparent (Figure 1). Indeed, many of the affected bones appeared to be ossified appropriately at this timepoint, although the presphenoid bone still showed decreased ossification relative to the controls (Figure 1B). Taken together, these data show that E2F4 disrupts the normal timing of bone development but its loss does not prevent ossification of the murine skeleton.

Loss of E2f4 affects osteoblast differentiation in vivo

Bone levels are influenced by three different cell types: osteoclasts, chondrocytes and osteoblasts. Osteoclasts resorb bone matrix; thus, we considered the possibility that E2F4 loss delayed bone ossification by increasing osteoclast activity. However, by performing a tartrate resistant acid phosphatase (TRAP) assay, a well-established test for osteoclast activity, we showed that there was no difference in osteoclast activity between *E2f4*-null and wildtype frontal bones (data not shown). Chondrocytes and osteoblasts both play a positive role in bone formation. As we described above, a delay in cartilage development may contribute to the impaired endrochondral differentiation in the *E2f4* mutants, raising the possible involvement of a chondrocyte defect. However, this could not explain the defect in intramembranous ossification, such as the frontal bone of the skull, because this does not involve a cartilage intermediate. Thus, we focused our attention on osteoblasts because these are required for both types of bone differentiation. To eliminate

any possible influence of chondrocytes, we used the frontal bones to assess osteoblast differentiation. First, we examined the expression of alkaline phosphatase (ALP), an early marker of osteoblast differentiation, at e17.5 (Figure 3A). *E2f4*$^{-/-}$ frontal bones display significantly less ALP activity than the wildtype control and this correlated with a significant difference in the extent of Alizarin Red staining between the two genotypes (Figure 3A). Thus, these data indicate that E2F4 loss disrupts osteoblast differentiation at an early step.

Given that regulation of cell cycle progression and exit are important processes during the initial steps of osteoblast differentiation and that E2F4 acts to prevent cells from entering the cell cycle, we hypothesized that osteoblast defects in the *E2f4* mutants may be associated with a cell cycle defect. Therefore, we examined cell cycle progression in the frontal bones by assessing incorporation of the nucleotide analogue 5-Bromo-2-deoxyuridine (BrdU) and also expression of Ki67, a proliferation marker. At both e16.5 and e17.5, we observed a higher level of BrdU-positive osteoblast progenitor cells in the frontal bones of *E2f4*$^{-/-}$ embryos than the wildtype littermate controls that was statistically significant (data not shown, Figure 3B). Consistent with this finding, a greater percentage of e17.5 *E2f4*$^{-/-}$ osteoblasts in the frontal bone stained positively for Ki67 compared to wildtype osteoblasts (Figure 3B). The increased number of cells cycling in the e17.5 *E2f4*$^{-/-}$ frontal bone was not associated with an apoptotic response, as determined by TUNEL staining (data not shown). Importantly, by e18.5, there was no significant difference in the level of osteoblast proliferation between the two genotypes (data not shown). Taken together, these data suggest that E2F4 inactivation impairs the ability of osteoblasts to exit

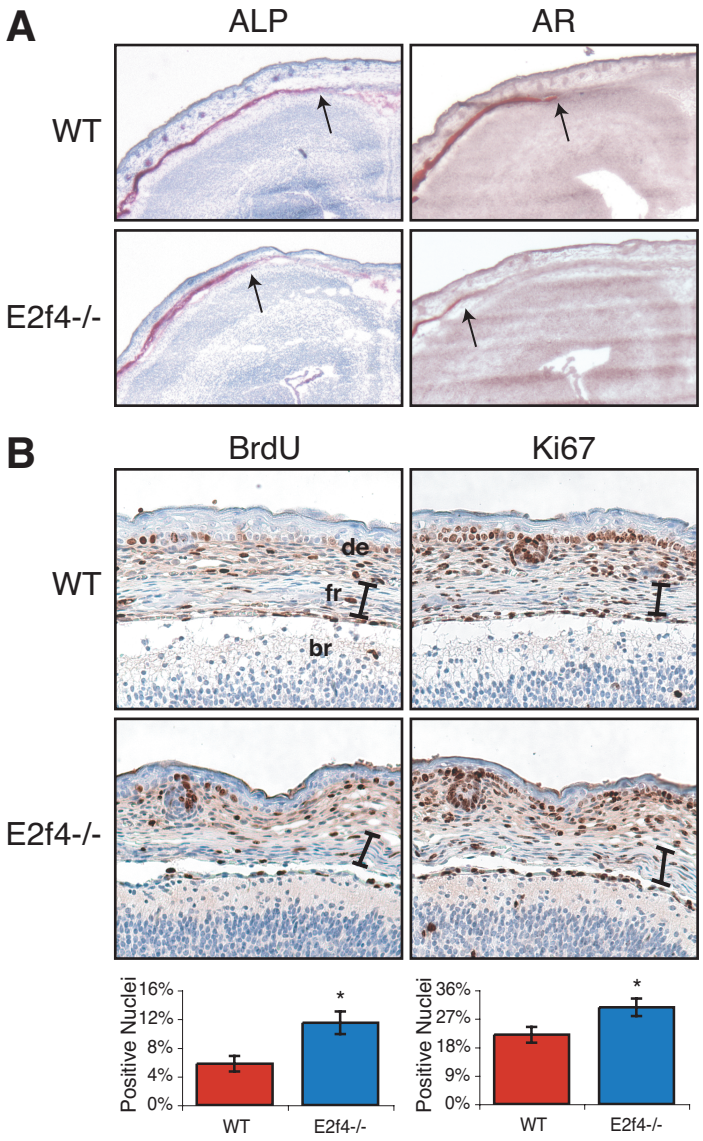

Figure 3. *E2f4*-deficient frontal bones display decreased levels of alkaline phosphatase while *E2f4*-deficient progenitor osteoblasts do not properly exit the cell cycle *in vivo*.

(A) Coronal sections of frontal bones from e17.5 embryos were assessed by histochemical analysis of alkaline phosphatase activity (left column, ALP) and Alizarin Red staining of bone (right column, AR). *E2f4*$^{-/-}$ frontal bone sections (bottom row) exhibit decreased levels of both markers compared to wildtype (top row). 2X magnification shown. Arrows indicate the front of activity or staining, respectively. (B) Immunohistochemical analysis of BrdU incorporation (left column) or Ki67 protein expression (right column) in coronal sections of frontal bones from e17.5 embryos. Pregnant females were labeled with BrdU for two hours. *E2f4*$^{-/-}$ frontal bones (bottom) exhibit a greater percentage of nuclei positively staining for BrdU or Ki67 than wildtype frontal bones (top). 20X magnification shown. Frontal bones (bar). Results of five separate experiments are quantified below the images; bars, 1 SD; *, $P<0.05$. Abbreviations: de, dermis; fr, frontal bone; br, brain.

the cell cycle at the appropriate developmental stage *in vivo* and thereby significantly delays the bone differentiation program.

E2f4$^{-/-}$ *calvarial preparations differentiate to a greater extent in osteoblasts than wildtype calvarial preparations in vitro*

We used an *in vitro* osteoblast differentiation assay to better understand how loss of E2F4 may affect osteoblast differentiation. Specifically, we generated calvarial preparations from e18.5 *E2f4*-null and wildtype embryos and then assessed their ability to differentiate into osteoblasts. Unexpectedly, we found that the *E2f4*-deficient osteoblasts consistently produced a greater amount of calcified bone matrix than their wildtype counterparts as determined by Alizarin Red staining (Figure 4A). Notably, we first observed calcified bone matrix the same number of days after the initiation of differentiation in both genotypes (data not shown), suggesting that the rate of matrix formation was comparable in the wildtype and *E2f4* mutant cells. To further explore this, we used quantitative RT-PCR to determine the levels of several osteoblast markers during the differentiation time course. For these studies, we examined *Runx2* and *Osterix* (*Osx*), two transcription factors that are master regulators of osteoblast differentiation (Ducy et al, 1997; Nakashima et al, 2002; Otto et al, 1997), and also the early markers, alkaline phosphatase (*ALP*) and *Collagen1* (*Col1*), the early/mid-marker, *Osteopontin* (*OPN*) and the late-marker, *Osteocalcin* (*OC*), indicative of osteoblast differentiation. This showed that there was no obvious disruption in the relative timing expression of these genes in the *E2f4*-deficient cells (Figure 4B), indicating that E2F4 loss does not alter the differentiation process. However, these cells showed a significant up-regulation of *OSX*, *OPN*, and at times, *ALP* compared to wildtype

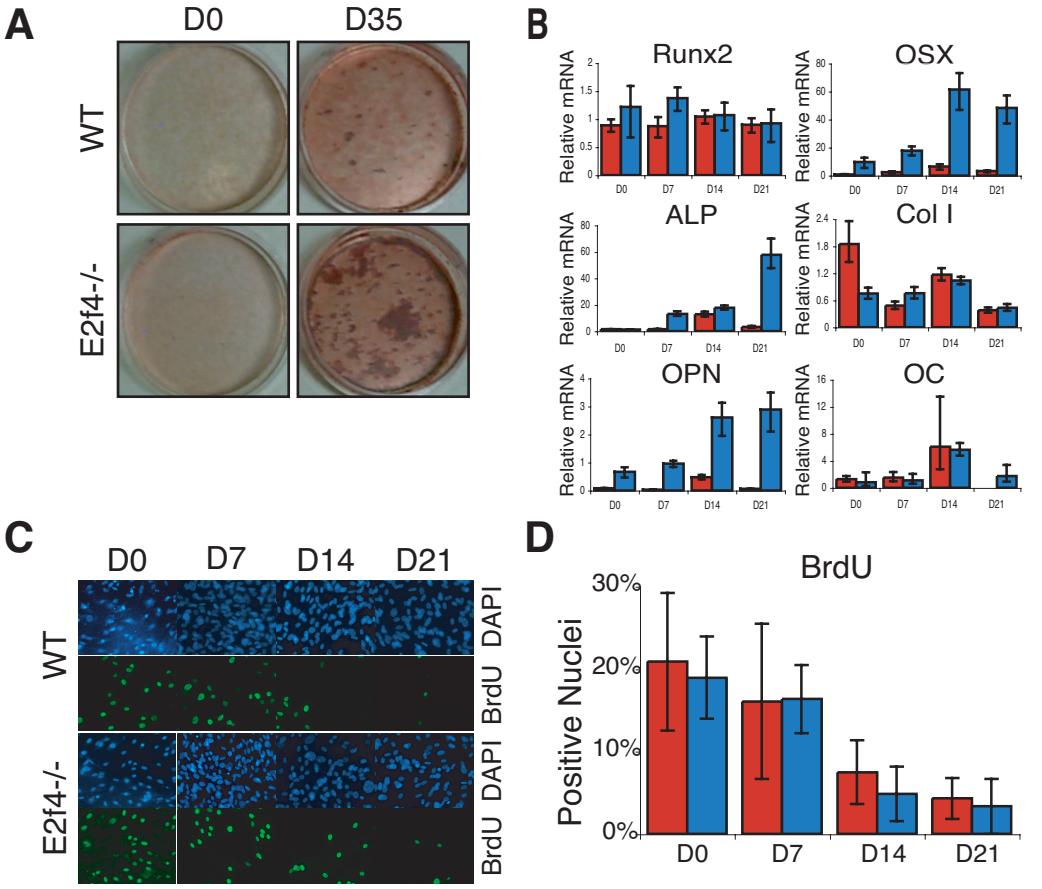

Figure 4. *E2f4*$^{-/-}$ calvarial cells differentiate to a greater extent than wildtype, but do not display altered cell cycle arrest.

(A) Terminal differentiation of primary calvarial cells was determined by Alizarin Red staining of secreted calcium deposits after 0 and 35 days. *E2f4*$^{-/-}$ calvarial cells (bottom row) secrete a greater number of calcium deposits than wildtype osteoblasts (top row). (B) Quantitative RT-PCR measurements of bone marker mRNA levels from wildtype (red bars) and *E2f4*$^{-/-}$ (blue bars) calvarial cells during differentiation. *E2f4*$^{-/-}$ calvarial cells express greater levels of *Osterix, Osteopontin*, and, at times, *Alkaline Phosphatase* mRNAs compared to wildtype cells. Ubiquitin was used as an internal control to normalize for RNA levels within each sample. Each time point is an average of three samples. Columns, results from a representative littermate pair; bars, 1 SD. (C) Indirect immunofluorescence analysis of cell cycle proliferation in confluence arrested cells. Wildtype (top two rows) and *E2f4*$^{-/-}$ (bottom two rows) calvarial cells were cultured with BrdU for 24 hours at the indicated time points during differentiation *in vitro*. Nuclei were stained for BrdU incorporation (green) and DAPI (blue). 20X magnification shown. (D) Quantitation of the immunofluorescence in (C). A minimum of 250 cells was analyzed per sample from three separate experiments; bars, 1 SD. Wildtype (red bars) and *E2f4*$^{-/-}$ (blue bars) calvarial cells display similar confluence arrest profiles.

preparations (Figure 4B), consistent with the increased matrix production (Figure 4A). These results demonstrate that *E2f4*$^{-/-}$ calvarial preparations secrete a greater amount of calcified extracellular matrix and possess increased mRNA levels of several osteoblast differentiation markers upon induction of differentiation compared to wildtype cells.

It is well established that the density of osteoblasts correlates positively with their ability to differentiate *in vitro* (Gerber et al 2001, Purpura et al 2004). Thus, we hypothesized that the enhanced differentiation properties of the *E2f4*$^{-/-}$ calvarial preparations might reflect a defect in their ability to exit the cell cycle as the cells reach confluence. To address this possibility, we compared the cell cycle kinetics of the wildtype and *E2f4*$^{-/-}$ calvarial cells during differentiation by assessing their ability to incorporate BrdU during a 24-hour pulse. Contrary to our test hypothesis, the two genotypes had the same percentage of BrdU-positive cells at every stage of the differentiation time-course (Figure 4C, D). Moreover, in cycling populations, there was also no detectable difference in the percentage of *E2f4*$^{-/-}$ and wildtype calvarial cells that incorporated BrdU during a two-hour pulse (data not shown). Taken together, these experiments show that *E2f4*$^{-/-}$ calvarial osteoblasts have an increased ability to differentiate *in vitro* and this occurs without any change in their proliferative capacity during either asynchronous proliferation or confluence arrest and terminal differentiation.

E2f4 deficiency increases the pools of osteoblastic progenitors in vivo

Given that E2F4 loss *in vivo* resulted in less ossification of various bones, along with a cell cycle exit defect, we were surprised to find that *E2f4*$^{-/-}$ osteoblasts have increased

potential to differentiate *in vitro* and display no cell cycle defect. We considered two possible hypotheses to reconcile the *in vitro* and *in vivo* phenotypes. E2F4 could play a direct role in bone differentiation that is apparent *in vitro* but obscured *in vivo* because E2F4 is also required for cell cycle exit. Alternatively, the increased differentiation *in vitro* could reflect an increase in the level of osteoblastic progenitors in the calvarium due to the cell cycle exit and differentiation defects *in vivo*. Thus, we tested both of these hypotheses.

We used two complementary approaches to determine whether E2F4 plays a direct role in osteoblast differentiation. Initially, we tested the effect of restoring E2F4 in the deficient osteoblasts by isolating *E2f4*-deficient calvarial cells and infected them with either a control retrovirus or one expressing the human E2F4 protein and then compared their ability to differentiate. After staining with Alizarin Red, we found that, although cells infected with human *E2f4* robustly expressed E2F4 (Figure 5B), there was no difference in the amount of calcium deposits secreted by these cells and those infected with empty virus (Figure 5A). Accordingly, there was no significant difference in the expression levels of osteoblast markers between these two populations (Figure 5C). It seemed plausible that the *E2f4* mutant cells had passed a critical point in osteoblast differentiation such that the re-introduction of E2F4 was unable to reverse the defect. Therefore, we performed the converse experiment in which we isolated wildtype calvarial cells, infected them with retroviruses carrying either a hairpin against *E2f4* or a control hairpin against luciferase and induced them to differentiate. Consistent with the add-back experiment, we saw no difference in the extent of mineralization between either cell population despite a strong

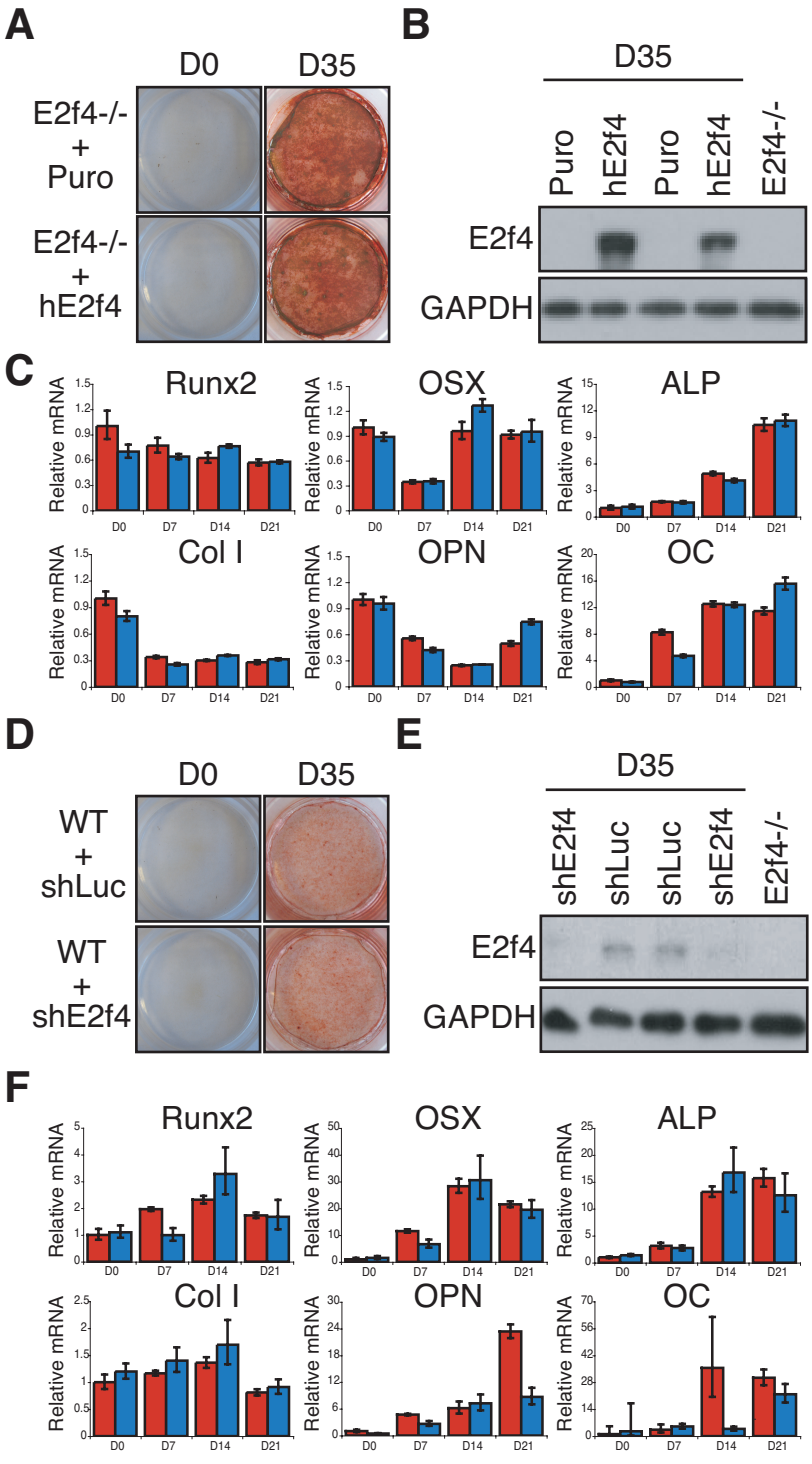
A
D0
D35
E2f4-/- + Puro
E2f4-/- + hE2f4
B
D35
Puro
hE2f4
Puro
hE2f4
E2f4-/-
E2f4
GAPDH
C
Runx2
OSX
ALP
Col I
OPN
OC
Relative mRNA
D0
D7
D14
D21
D
D0
D35
WT + shLuc
WT + shE2f4
E
D35
shE2f4
shLuc
shLuc
shE2f4
E2f4-/-
E2f4
GAPDH
F
Runx2
OSX
ALP
Col I
OPN
OC
Relative mRNA
D0
D7
D14
D21

Figure 5. E2F4 does not function to modulate osteoblast differentiation *in vitro*

(A) Alizarin Red staining of secreted calcium deposits after 0 and 35 days. *E2f4*$^{-/-}$ calvarial cells were stably infected with *hE2f4* or an empty virus. No difference is observed between *E2f4*$^{-/-}$ cells over-expressing hE2F4 or *E2f4*$^{-/-}$ control cells. (B) Stable infection of the *hE2f4* cDNA construct constitutively over-expresses hE2F4 protein through the 35 day duration of a differentiation time course experiment. GAPDH is shown as a loading control. (C) Quantitative RT-PCR results of (A) display no differences in the expression of osteoblast markers between control cells (red bars) and cells over-expressing E2F4 (blue bars). Quantitative RT-PCR was performed as described in Figure 3B. (D) Alizarin Red staining of secreted calcium deposits after 0 and 35 days. Wildtype calvarial cells were stably infected with either a control hairpin against luciferase or a hairpin against *E2f4*. No difference is observed between wildtype cells with E2F4 knockdown or with the luciferase control hairpin. (E) Stable infection of the *E2f4* hairpin constitutively knocks down the E2F4 protein through the 35 day duration of a differentiation time course experiment. GAPDH is shown as a loading control. (F) Quantitative RT-PCR results of (D) display no differences in the expression of osteoblast markers between control cells (red bars) and cells with knock down of E2F4 (blue bars). Quantitative RT-PCR was performed as described in Figure 4B.

knockdown of E2F4 expression (Figure 5D, E). There was also no change in the expression levels of bone differentiation markers (Figure 5F). Together, these experiments suggest that E2F4 loss does not modulate osteoblast differentiation *in vitro*.

Our alternate hypothesis is that the increased *in vitro* differentiation potential of the *E2f4*$^{-/-}$ calvarial preparations, relative to the wildtype controls, reflects the presence of a greater number of osteoblastic progenitors in the *E2f4* mutant calvarium due to the impaired osteoblast differentiation *in vivo*. To test this hypothesis, we performed an osteoblast progenitor assay in which wildtype and *E2f4*$^{-/-}$ calvarial cells were plated at very low density directly into differentiation media. This allows for the growth of individual colonies capable of differentiating into osteoblasts, which stain with alkaline phosphatase. Notably, starting at day 7, the *E2f4* mutants had a higher number of alkaline phosphatase-expressing colonies than the wildtype controls (Figure 6A). To quantify this difference, we performed a limiting dilution assay and determined the number of alkaline phosphatase-positive colonies one week later. This showed that the frequency of osteoblastic progenitor cells is 1 in 21 for *E2f4*$^{-/-}$ cells versus 1 in 50 for wildtype controls (Figure 6B). Taken together, our data strongly suggest that E2F4 loss impairs bone formation *in vivo* by disrupting the ability of osteoblastic progenitors to exit the cell cycle at the appropriate point in development, thereby increasing the levels of osteoblastic progenitors. These cells are able to differentiate *in vitro*, accounting for the higher differentiation potential of E2F4 mutant versus wildtype calvarial cells.

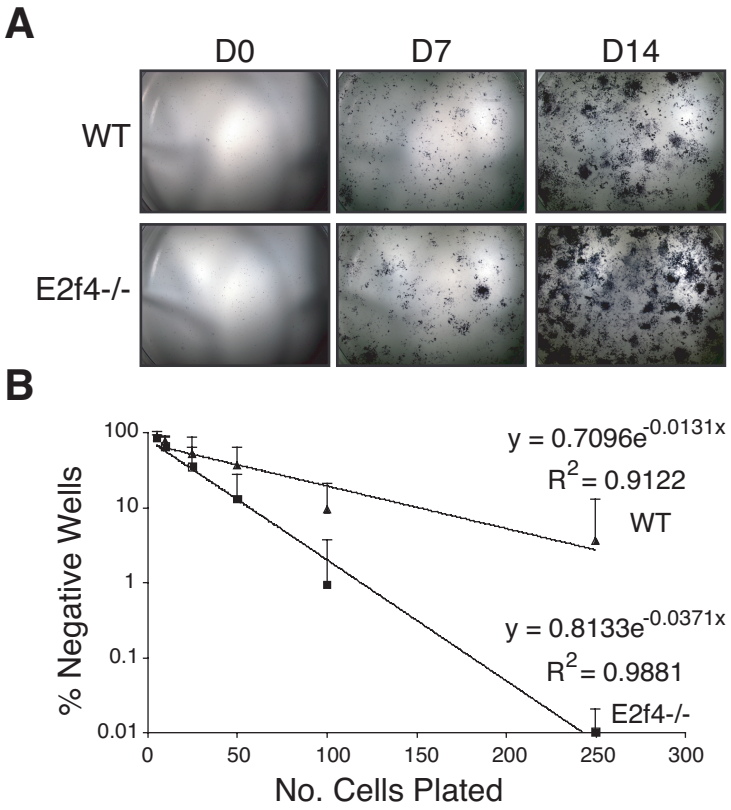

Figure 6. *E2f4*$^{-/-}$ calvarial preparations contain more progenitor osteoblasts than wildtype preparations

(A) Alkaline phosphatase staining of osteoblast progenitor cells at the indicated time points. Calvarial preparations were isolated and sparsely plated directly into differentiation media. *E2f4*$^{-/-}$ progenitor osteoblasts (bottom row) produce more alkaline phosphatase-positive colonies than wildtype cells (top row). (B) Limiting dilution assay of wildtype and *E2f4*$^{-/-}$ osteoblast progenitor cells. Cells were plated directly into induction media and alkaline-phosphatase-positive colonies were assessed 7 days later. *E2f4*$^{-/-}$ progenitor osteoblasts exhibited a higher clonal frequency of alkaline phosphatase-positive colonies compared to wildtype cells.

DISCUSSION

E2F4 is primarily considered to be a repressive E2F that functions in G0/G1 to maintain growth arrest. E2F4 can bind to all three pocket proteins, pRb, p107, and p130 *in vivo* (Moberg et al, 1996). Interestingly, all of these pocket proteins have been implicated in bone development. Deletion of *p107* and p*130* in the mouse causes an increase in chondrocyte proliferation *in vivo*, affecting the development of long bones (Cobrinik et al, 1996), whereas *pRb* loss perturbs osteoblast differentiation *in vitro* and *in vivo* (Berman et al; Thomas et al, 2001). Therefore, we hypothesized that E2F4 may have a role in bone development. Our results suggest that E2F4 plays a key role in establishing the appropriate timing of osteoblast differentiation that reflects its role in enabling cell cycle exit of osteoblast progenitor cells, as opposed to a more direct role in the differentiation process. Specifically, we find that E2F4 loss delays osteoblast differentiation *in vivo* at an early stage, as judged by analysis of ALP activity, and this correlates with an increase in the level of proliferating cells. Accordingly, *in vitro* differentiation assays confirm that the *E2f4*-deficient calvaria retain a higher level of osteoblastic progenitors than the wildtype controls. Moreover, both add-back and knockdown experiments argue against a requirement for E2F4 in the actual osteoblast differentiation process.

It is formally possible that defective cell cycle exit could reflect a non-cell autonomous role of E2F4. For example, *E2f4* knockout mice are anemic during embryogenesis (Humbert et al, 2000), and thus the anemic state of the embryo could prevent adequate nutrients and gases from reaching cells of all types, including osteoblasts. In this situation, many cellular processes could be adversely affected, causing

a delay in the development of various embryonic tissues. We are now generating an *E2f4* conditional model knockout mouse, which will allow us to directly address whether E2F4's role in osteoblast differentiation is cell autonomous. Despite this uncertainty, we favor the notion that E2F4 acts in a cell autonomous manner to promote cell cycle exit in osteoblasts through its role as a transcriptional repressor of E2F-responsive genes.

In order to maintain cell cycle arrest, E2F4 associates with pocket protein family member pRb and recruits histone deacetylases to E2F-responsive gene promoters to actively repress transcription. We have previously reported that *Rb*-deficient embryos also display defects in bone development. Specifically, *Rb* inactivation leads to defects in the formation of the cranium, the hyoid bone, the palatine process, and the sternum. In addition, both *in vivo* and *in vitro* studies demonstrate that $Rb^{-/-}$ calvarial osteoblasts fail to properly exit the cell cycle (Berman et al, 2008). Although both *E2f4*- and *Rb*-deficient embryos have defective bone formation in the cranium and sternum, they display unique defects in the formation of many other bones. These phenotypic differences suggest that E2F4 and pRb play overlapping, but distinct roles in bone development.

We believe that there are two non-mutually exclusive possibilities that could account for the difference in phenotype between *E2f4*- and *Rb*- deficient embryos. First, it is possible that this reflects the varying abilities of the other E2F and pocket protein members to compensate for the loss of E2F4 versus pRb in individual tissues. We have previously shown that E2F4 loss alters the interaction between the pocket proteins and the remaining E2Fs, presumably altering their transcriptional properties (Lee et al, 2002). Moreover, it is well established that p107 and p130 can compensate for the loss of pRb to

varying degrees in different settings (Sage et al, 2003; Wikenheiser-Brokamp, 2004). Presumably, the relative expression levels of E2F4 and pRb, in the normal tissues, and the remaining E2F and pocket proteins, in the mutant tissues, would account for the specific spectrum of bone defects that are observed in *E2f4* versus *Rb* mutants. For example, E2F4 might be the predominant E2F protein in the presphenoid bone, but play a lesser role, compared to the other E2Fs, in the hyoid bone and palatine process. The alternative possibility is that while both E2F4 and pRb play indirect roles in bone development by promoting cell cycle exit, pRb plays an additional, more direct role in bone development by regulating Runx2, one of the master regulators of bone development (Luan et al, 2007; Thomas et al, 2001).

Our data indicate that E2F4 loss disrupts both intramembranous and endochondral ossification of bones. As endochondral bones ossify via a cartilage intermediate, it is possible that some of the defects in bone development could be attributed to disrupted chondrocyte differentiation. If true, we speculate that this defect would also reflect the inability of these cells to exit the cell cycle. It is interesting to note that both osteoblasts and chondrocytes arise from a common mesenchymal precursor cell. If E2F4 loss prevents osteoblast progenitor cells from exiting the cell cycle, thereby preventing terminal differentiation into mature osteoblasts, it is also possible that chondrocyte progenitor cells would have the same problem. Although our *in vitro* data suggest that E2F4 does not directly influence terminal differentiation of osteoblasts, it is entirely possible that E2F4 could play a direct role in the differentiation of earlier progenitor cells, such as osteochondro progenitors or even mesenchymal progenitor cells.

EXPERIMENTAL PROCEDURES

Animal maintenance and histological preparations

The generation of *E2f4*$^{-/-}$ mice has been described previously (Humbert et al, 2000). Gestation was dated by detection of a vaginal plug. Pregnant mice were injected with 10 μl/gm body weight of 5 mg/ml 5-Bromo-2'-deoxyuridine (BrdU) in phosphate buffered solution (PBS) two hours prior to tissue collection. Collected embryonic tissue was immediately embedded in OCT or fixed in 4% paraformaldehyde (PFA) and embedded in paraffin. Histological sections were cut at 6-8 microns.

Histological analyses

Enzymatic alkaline phosphatase assays were performed on unfixed frozen sections. Briefly, 0.06g sodium nitrite was dissolved into 1.5 ml of water and added to 600 μl of 50 mg/ml of new fuchsin (Sigma) in 2M HCl. This solution was added to 210 ml Tris buffer (pH 9.0). Finally, 1.8 ml of 83.3 mg/ml Naphthol AS-Bi-Phosphate (Sigma) in DMF (Sigma) was added. Sections were incubated with this solution for 15 minutes, washed in PBS and counterstained with hematoxylin. Alizarin red staining was performed by incubating unfixed frozen sections for 5 minutes in 20 mg/ml alizarin red (Sigma), pH 4.2. Immunohistochemical analyses were performed using antibodies against BrdU (1:50 347580, BD Biosciences) and Ki67 (1:50 550609, BD Biosciences) as described (Danielian et al, 2007). Statistical significance was determined using the two sample Student's T Test with two-tailed distribution and unequal variance.

Skeletal staining

Embryos were sacrificed, skinned, and eviscerated. The remaining tissue was fixed in 95% ethanol for 4 days, transferred to acetone for 3 days, and subsequently transferred to staining solution (final volume of 0.015% alcian blue 8GX (Sigma), 0.005% alizarin red S (Sigma) and 5% glacial acetic acid in ethanol) at 37°C for two days and room temperature for a third day. Tissue was cleared in 1% potassium hydroxide for several days and ultimately stored in glycerol.

Calvarial preparations and culture

Calvaria from e17.5 or e18.5 embryos were removed, treated with several rounds of collagenase/trypsin digests at 37°C, and plated onto 6-well plates. Cells were grown and expanded in αMEM with 10% fetal bovine serum and Pen/Strep. For differentiation, 250,000 cells were plated onto 3 cm tissue culture plates. Upon reaching confluence, calvarial osteoblasts were treated with media supplemented with 50 μg/ml of ascorbic acid and 10 mM β-glycerol-phosphate. To assay for calcium deposits, plates were stained with 1% alizarin red S solution (pH 5.0) for 15 minutes. For osteoblast progenitor assays, 450 cells/cm^2 were plated directly into induction media. Osteoblast differentiation was detected by staining with BCIP/NBT Liquid Substrate System (Sigma) by manufacturer's instructions. In limiting dilution assays, 5, 10, 25, 100, and 250 cells were plated into 96-well plates containing differentiation media and stained with BCIP/NBT Liquid Substrate System (Sigma) 7 days later. Statistical significance was determined using L-Calc Software (StemCell Technologies).

E2f4 and luciferase hairpins were cloned into the MSCV-LMP retroviral vector (EAV4679 Open Biosystems). The luciferase hairpin was excised from pPRIME-CMV-GFP-FF3 (Stegmeier et al., 2005) with EcoRI and XhoI and subcloned into MSCV-LMP. The *E2f4* hairpin, 5' CAGAGATTTAGAAAGATTT 3', was cloned into MSCV-LMP as described in the manufacturer's instructions (Open Biosystems). Phoenix cells at 60% confluence were transfected with 2 μg/ml MSCV-LMP. The media was replaced 8 hours later and supernatants were collected at 24 hours and filtered. Supernatants containing 10% FBS and 8 μg/ml polybrene were added to calvarial cells. Infected cells were selected with 2.5 μg/ml of puromycin for 2 days. Knockdown of E2F4 protein was confirmed by quantitative RT-PCR and by Western, using a monoclonal antibody against human E2F4 (1:10 LLF4.2 (Moberg et al, 1996)) and GAPDH (1:5000, AM4300, Ambion) as a loading control.

pBabe-*E2f4* (Landsberg et al, 2003) was used to overexpress *E2f4*. Calvarial cells were infected as described above. Infected cells were selected with 2.5 μg/ml puromycin for 2 days. Overexpression of hE2F4 was confirmed by Western analysis using the same antibodies described for the knockdown experiment.

Immunofluorescence

For *in vitro* BrdU incorporation, osteoblasts were plated onto coverslips prior to achieving confluence. BrdU was added to the media (final concentration of 10 μM) and incubated for 24 hours prior to 4% PFA fixation. Antigen was detected using an antibody against BrdU (1:50 347580, BD Biosciences) and Texas Red-X goat anti-mouse secondary (1:1000, Invitrogen). Statistical significance was determined using the Student's T Test.

Quantitative real-time PCR

RNA was isolated from differentiation plates using the Qiagen RNeasy kit. First-strand cDNA was transcribed from 1 μg of RNA using Superscript III Reverse Transcriptase (Invitrogen) following manufacturer's instructions. Quantitative RT-PCR with 100 ng cDNA was performed using SYBR Green (Applied Biosystems). Reactions were run on the ABI Prism 7000 Sequence Detection System and analyzed using the 7000 SDS software. Primers are listed in Table 1.

Table 1. Quantitative RT-PCR Primer Pairs

Gene	Primer Sequence
Alkaline Phosphatase	For: TCT CCA GAC CCT GCA ACC TC Rev: CAT CCT GAG CAG ACC TGG TC
Collagen1a1	For: CGA GTC ACA CCG GAA CTT GG Rev: GCA GGC AGG GCC AAT GTC TA
Cyclin A	For: AGT TTG ATA GAT GCT GAC CC Rev: TAG GTC TGG TGA AGG TCC
Cyclin E	For: TGT TTT TGC AAG ACC CAG ATG A Rev: GGC TGA CTG CTA TCC TCG CT
Osteocalcin	For: CTC TGT CTC TCT GAC CTC ACA G Rev: CAG GTC CTA AAT AGT GAT ACC G
Osteopontin	For: TGC TTT TGC CTG TTT GGC AT Rev: TTC TGT GGC GCA AGG AGA TT
Osterix	For: GCA AGG CTT CGC ATC TGA AA Rev: AAC TTC TTC TCC CGG GTG TGA
Runx2	For: TGA GAT TTG TGG GCC GGA Rev: TCT GTG CCT TCT TGG TTC CC
Ubiquitin	For: TGG CTA TTA ATT ATT CGG TCT GCA T Rev: GCA AGT GGC TAG AGT GCA GAG TAA

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REFERENCES

Attwooll C, Lazzerini Denchi E, Helin K (2004) The E2F family: specific functions and overlapping interests. *EMBO J* **23**(24): 4709-4716

Berman SD, Yuan TL, Miller ES, Lee EY, Caron A, Lees JA (2008) The retinoblastoma protein tumor suppressor is important for appropriate osteoblast differentiation and bone development. *Mol Cancer Res* **6**(9): 1440-1451

Blais A, Dynlacht BD (2007) E2F-associated chromatin modifiers and cell cycle control. *Curr Opin Cell Biol* **19**(6): 658-662

Cobrinik D, Lee MH, Hannon G, Mulligan G, Bronson RT, Dyson N, Harlow E, Beach D, Weinberg RA, Jacks T (1996) Shared role of the pRB-related p130 and p107 proteins in limb development. *Genes Dev* **10**(13): 1633-1644

Danielian PS, Bender Kim CF, Caron AM, Vasile E, Bronson RT, Lees JA (2007) E2f4 is required for normal development of the airway epithelium. *Dev Biol* **305**(2): 564-576

Dimova DK, Dyson NJ (2005) The E2F transcriptional network: old acquaintances with new faces. *Oncogene* **24**(17): 2810-2826

Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G (1997) Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. *Cell* **89**(5): 747-754

Fajas L, Landsberg RL, Huss-Garcia Y, Sardet C, Lees JA, Auwerx J (2002) E2Fs regulate adipocyte differentiation. *Dev Cell* **3**(1): 39-49

Gaubatz S, Lindeman GJ, Ishida S, Jakoi L, Nevins JR, Livingston DM, Rempel RE (2000) E2F4 and E2F5 play an essential role in pocket protein-mediated G1 control. *Mol Cell* **6**(3): 729-735

Humbert PO, Rogers C, Ganiatsas S, Landsberg RL, Trimarchi JM, Dandapani S, Brugnara C, Erdman S, Schrenzel M, Bronson RT, Lees JA (2000) E2F4 is essential for normal erythrocyte maturation and neonatal viability. *Mol Cell* **6**(2): 281-291

Kinross KM, Clark AJ, Iazzolino RM, Humbert PO (2006) E2f4 regulates fetal erythropoiesis through the promotion of cellular proliferation. *Blood* **108**(3): 886-895

Landsberg RL, Sero JE, Danielian PS, Yuan TL, Lee EY, Lees JA (2003) The role of E2F4 in adipogenesis is independent of its cell cycle regulatory activity. *Proc Natl Acad Sci U S A* **100**(5): 2456-2461

Lee EY, Cam H, Ziebold U, Rayman JB, Lees JA, Dynlacht BD (2002) E2F4 loss suppresses tumorigenesis in Rb mutant mice. *Cancer Cell* **2**(6): 463-472

Luan Y, Yu XP, Xu K, Ding B, Yu J, Huang Y, Yang N, Lengyel P, Di Cesare PE, Liu CJ (2007) The retinoblastoma protein is an essential mediator of osteogenesis that links the p204 protein to the Cbfa1 transcription factor thereby increasing its activity. *J Biol Chem* **282**(23): 16860-16870

McClellan KA, Slack RS (2007) Specific in vivo roles for E2Fs in differentiation and development. *Cell Cycle* **6**(23): 2917-2927

Moberg K, Starz MA, Lees JA (1996) E2F-4 switches from p130 to p107 and pRB in response to cell cycle reentry. *Mol Cell Biol* **16**(4): 1436-1449

Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR, de Crombrugghe B (2002) The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. *Cell* **108**(1): 17-29

Otto F, Thornell AP, Crompton T, Denzel A, Gilmour KC, Rosewell IR, Stamp GW, Beddington RS, Mundlos S, Olsen BR, Selby PB, Owen MJ (1997) Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. *Cell* **89**(5): 765-771

Rayman JB, Takahashi Y, Indjeian VB, Dannenberg JH, Catchpole S, Watson RJ, te Riele H, Dynlacht BD (2002) E2F mediates cell cycle-dependent transcriptional repression in vivo by recruitment of an HDAC1/mSin3B corepressor complex. *Genes Dev* **16**(8): 933-947

Rempel RE, Saenz-Robles MT, Storms R, Morham S, Ishida S, Engel A, Jakoi L, Melhem MF, Pipas JM, Smith C, Nevins JR (2000) Loss of E2F4 activity leads to abnormal development of multiple cellular lineages. *Mol Cell* **6**(2): 293-306

Sage J, Miller AL, Perez-Mancera PA, Wysocki JM, Jacks T (2003) Acute mutation of retinoblastoma gene function is sufficient for cell cycle re-entry. *Nature* **424**(6945): 223-228

Takahashi Y, Rayman JB, Dynlacht BD (2000) Analysis of promoter binding by the E2F and pRB families in vivo: distinct E2F proteins mediate activation and repression. *Genes Dev* **14**(7): 804-816

Thomas DM, Carty SA, Piscopo DM, Lee JS, Wang WF, Forrester WC, Hinds PW (2001) The retinoblastoma protein acts as a transcriptional coactivator required for osteogenic differentiation. *Mol Cell* **8**(2): 303-316

Trimarchi JM, Lees JA (2002) Sibling rivalry in the E2F family. *Nat Rev Mol Cell Biol* **3**(1): 11-20

Verona R, Moberg K, Estes S, Starz M, Vernon JP, Lees JA (1997) E2F activity is regulated by cell cycle-dependent changes in subcellular localization. *Mol Cell Biol* **17**(12): 7268-7282

Wagner EF, Karsenty G (2001) Genetic control of skeletal development. *Curr Opin Genet Dev* **11**(5): 527-532

Wells J, Boyd KE, Fry CJ, Bartley SM, Farnham PJ (2000) Target gene specificity of E2F and pocket protein family members in living cells. *Mol Cell Biol* **20**(16): 5797-5807

Wikenheiser-Brokamp KA (2004) Rb family proteins differentially regulate distinct cell lineages during epithelial development. *Development* **131**(17): 4299-4310

Chapter Three

The synergistic roles of E2F4 and E2F5 in cell cycle exit and terminal differentiation

Emily Miller and Jacqueline A. Lees

ABSTRACT

The E2F transcription factors are critical downstream effectors of pocket protein family members that function to regulate genes required for cell cycle progression. The repressor E2Fs, E2F4 and E2F5, function primarily to maintain a G0/G1 cell cycle state and actively repress transcription of E2F target genes. The deletion of either protein disrupts the development of specific tissues, such as the calvarium and the choroid plexus. Moreover, loss of both E2F4 and E2F5 causes lethality as early as embryonic day 8.5. Here, we describe the generation of conditional *E2f4* knockout mice with the aim of addressing the overlapping roles that E2F4 and E2F5 play during development. We also show that acute ablation of *E2f4* in wildtype and *E2f5*$^{-/-}$ MEFs impairs proliferation, suggesting that the combined loss of both repressor proteins will have profound effects on cell cycle progression and the differentiation of several cell lineages.

INTRODUCTION

The E2F family regulates the transcription of genes required for DNA synthesis and cell cycle progression (Attwooll et al, 2004; Dimova & Dyson, 2005). To date, eight *E2F* genes (*E2f1-8*) have been identified, which can be divided into two distinct groups based on differences in their predominant function. The activator E2Fs, E2F1-3, play a key role in activating genes required to enter the cell cycle, while E2F4-8 function to repress the transcription of E2F target genes (Attwooll et al, 2004; DeGregori & Johnson, 2006; Dimova & Dyson, 2005; Trimarchi & Lees, 2002). E2F4 and E2F5 were identified based on their ability to associate with the pocket proteins p107 and p130 (Beijersbergen et al, 1994; Ginsberg et al, 1994; Hijmans et al, 1995; Sardet et al, 1995). E2F4 functions primarily as a transcriptional repressor, and its nuclear localization largely depends on association with members of the pocket protein family, pRb, p107, and p130 (Gaubatz et al, 2001; Rayman et al, 2002; Verona et al, 1997). The resulting complexes recruit histone deacetylases to E2F target gene promoters, reducing transcription (Blais & Dynlacht, 2007; Rayman et al, 2002; Takahashi et al, 2000; Wells et al, 2000). Upon mitogenic signaling, cyclin-dependent kinases phosphorylate the pocket proteins and cause the release of E2F4, which can be exported from the nucleus due to the presence of nuclear export signals (Gaubatz et al, 2001). E2F5 is thought to function in a manner analogous to that of E2F4 based, in large part, on their high degree of structural similarity.

Early studies found that E2F4 could activate transcription (Ginsberg et al, 1994; Lukas et al, 1996); however, in contrast to the activating E2Fs, subsequent studies demonstrated that over-expression of either E2F4 or E2F5 was not sufficient to drive

progression through the cell cycle and was also unable to stimulate serum-arrested cells to re-enter the cell cycle (DeGregori et al, 1997; Lukas et al, 1996; Mann & Jones, 1996). Given that chromatin immunoprecipitation assays suggest that E2F4/5-p130/p107 complexes are predominantly found at the promoters of key cell cycle-regulated genes during G0/G1 (Conboy et al, 2007; Takahashi et al, 2000) and that *p107*$^{-/-}$;*p130*$^{-/-}$ MEFs exhibit a deregulated cell cycle and altered E2F target gene expression (Hurford et al, 1997), it was expected that the loss of either E2F4 or E2F5 would cause ectopic S-phase entry. However, MEFs lacking *E2f4* or *E2f5* exhibit a normal cell cycle profile (Humbert et al, 2000; Lindeman et al, 1998; Rempel et al, 2000). Due to the structural similarity between E2F4 and E2F5, it is possible that these proteins function in a redundant manner. Indeed, *E2f4*$^{-/-}$;*E2f5*$^{-/-}$ MEFs continue to enter S-phase despite growth arrest signals from p16^{INK4A}, while MEFs deficient for *E2f4* or *E2f5* alone properly growth arrest (Gaubatz et al, 2000).

There is an increasing amount of evidence implicating the E2F family of transcription factors in the differentiation of several cell types. Although many of these novel roles in development are dependent upon proper regulation of the cell cycle, many studies have found cell cycle-independent roles for E2F in the differentiation of specific cell types. Analyses of *E2f5* knockout mice revealed that embryonic and neonatal development in these animals is normal. However, around 3-4 weeks old, these mice develop ruffled coats, ataxia, and dehydration (Lindeman et al, 1998). These *E2f5*-null mice die prematurely due to intracerebral hemorrhage and hydrocephalus. Further studies indicated that the hydrocephalus was a consequence of increased cerebral spinal fluid secretion in

E2f5$^{-/-}$mice (Lindeman et al, 1998). *E2f4* mutants have completely non-overlapping defects. Embryos lacking *E2f4* are transiently anemic and exhibit cell autonomous defects in red blood cell maturation, resulting in increased numbers of progenitor and immature erythrocytes and incomplete enucleation (Humbert et al, 2000; Kinross et al, 2006; Rempel et al, 2000). *E2f4*$^{-/-}$ mice are neonatal lethal due to chronic rhinitis and associated opportunistic bacterial infections (Humbert et al, 2000). The susceptibility to infections results from defective differentiation of the nasal epithelium, resulting in mucin-secreting cells in place of ciliated cells within the airway, which causes chronic rhinitis (Danielian et al, 2007). It is unclear what the underlying mechanism is that causes the cilial cell defect, but it appears to be independent of an altered cell cycle progression. E2F4 also plays a role in adipocyte differentiation, through both cell cycle-dependent and -independent functions (Fajas et al, 2002; Landsberg et al, 2003). Recently, we have found that E2F4 contributes to the commitment of osteoblast progenitor cells to the osteoblast lineage and to bone formation by enabling these cells to properly exit the cell cycle (Chapter 2).

Although the phenotypes of *E2f4* and *E2f5* single knockout mice suggest that they make differential contributions during development, mice deficient for both *E2f4* and *E2f5* are embryonic lethal (Gaubatz et al, 2000; unpublished observations, J. Sero, T. Yuan, and J.A.L), suggesting that E2F4 and E2F5 perform largely overlapping functions. Given that both E2F4 and E2F5 are involved in promoting and maintaining a cell cycle arrest, and that the ability to properly exit the cell cycle is often concomitant with terminal differentiation of several cell types, we have generated an *E2f4* conditional knockout mouse to elucidate the synergistic roles that E2F4 and E2F5 play during development.

RESULTS

Generation of *E2f4* conditional knockout mice

To create conditional *E2f4* mutants, we used recombineering to introduce loxP sites surrounding exons 2, 3, and 4 (Figure 1). Upon Cre-mediated recombination, the majority of the gene encoding the DNA binding domain and the DP dimerization domain is deleted from the genome, thereby constituting a null allele. Figure 1 shows the targeting vector construct, which contains the 5' LoxP site and a neomycin cassette flanked by Frt sites in intron 1 and the 3' LoxP site in intron 4. The vector also includes the diptheria toxin (DTA) gene for negative selection of random integration events. After electroporation of the targeting construct into 129/B6 F1 hybrid embryonic stem (ES) cells, recombinant ES cells were selected based on their resistance to G418. Out of 223 clones picked, 8 correctly targeted clones were verified by Southern blot analysis (Figure 2a) using the 5' and 3' probes shown in Figure 1. Clone 4C6 was injected into blastocysts, resulting in 7 male chimeric pups with all but one displaying 99%, or more, chimerism based on coat color. Using the PCR genotyping strategy in Figure 2b, we have confirmed germline transmission of the targeted allele (Figure 2c). We are now breeding these mice to *E2f5* mutants in the presence of various Cre recombinases to assess the overlap of E2F4 and E2F5 function in key tissues.

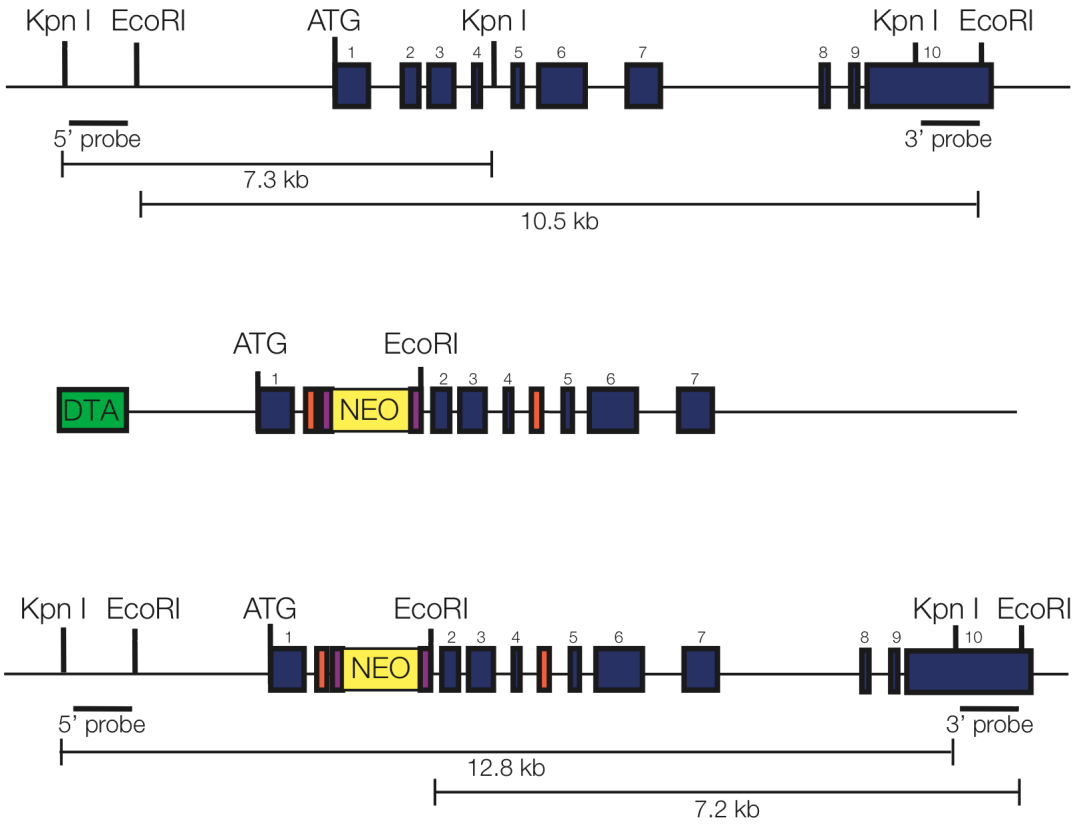

Figure 1. Schematic of the targeting construct and the endogenous *E2f4* locus

The endogenous *E2f4* locus is pictured on top, the targeting vector is in the middle, and the targeted allele is pictured on the bottom. The *E2f4* exons are represented by dark blue boxes. DTA represents the diptheria toxin negative selection cassette, the purple boxes are *Frt* sites flanking the neomycin positive selection cassette, and the orange boxes are *LoxP* sites. The bracketed lines represent the predicted sizes of genomic fragments identified by the 5' and 3' probe sequences used for Southern blot analysis after homologous recombination.

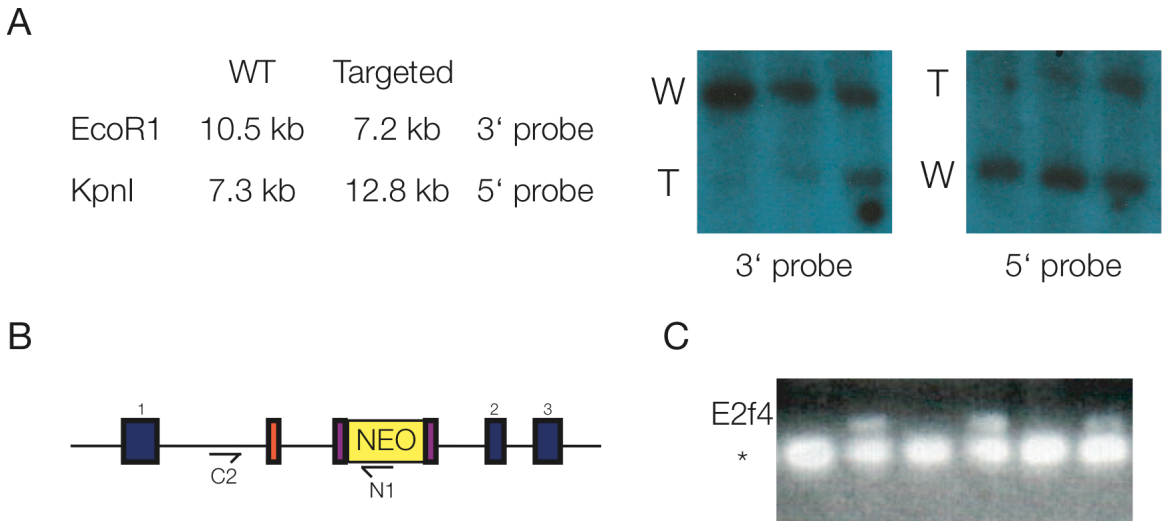

Figure 2. Germline transmission of the conditional *E2f4* allele

(A) The predicted sizes of the genomic fragments after digestion with either KpnI or EcoRI and hybridization with the 5’ probe or 3’ probe, respectively, are shown along with Southern analysis of representative targeted clones. In each case, one non-targeted clone and two targeted clones are shown. Abbreviations: W, wildtype band; T, targeted band(B) PCR screening strategy to identify mice carrying the targeted allele. Primers C2 and N1 amplify a 195 bp fragment of DNA. (C) PCR from ear clips confirming germline transmission of the conditional *E2f4* allele (upper band). Asterisk denotes a non-specific band.

Acute ablation of *E2f4* disrupts normal asynchronous proliferation in MEFs

It has been previously reported that MEFs lacking either *E2f4* or *E2f5* display a normal cell cycle profile (Humbert et al, 2000; Lindeman et al, 1998; Rempel et al, 2000). Only when MEFs are deficient for both *E2f4* and *E2f5* do they exhibit the inability to exit the cell cycle while in the presence of the growth arrest signal, $p16^{INK4A}$ (Gaubatz et al, 2000). In addition, $E2f4^{-/-};E2f5^{-/-}$ MEFs generated from e13.5-14.5 embryos exhibit normal growth arrest in response to serum-starvation, normal proliferation kinetics following serum-stimulation, and normal E2F target gene regulation (Gaubatz et al, 2000). In our laboratory, however, $E2f4^{-/-};E2f5^{-/-}$ embryos were very rarely detected at e13.5, indicating that genetic background has an impact on the penetrance of the phenotype (unpublished observations, J. Sero, T. Yuan, and J.A.L). These data suggest that other E2Fs, or other factors, may compensate for the loss of *E2f4* and *E2f5* in germline deficient embryos. Indeed, a compensatory role for E2F4 has been found in cells that are deficient for E2F6 (Giangrande et al, 2004). Given these findings, we were interested in the effect of combining germline loss of *E2f5* with the acute ablation of *E2f4 in vitro*. Other studies have indicated that acute ablation of pocket proteins or E2Fs have more severe effects *in vitro* than germline mutations (Kong et al, 2007; Sage et al, 2003)

Wildtype and $E2f5^{-/-}$ MEFs previously generated in the lab were infected with a retrovirus carrying either a hairpin against *E2f4* or a control hairpin against luciferase, and the properties of the resulting cell pools were compared in standard proliferation assays.

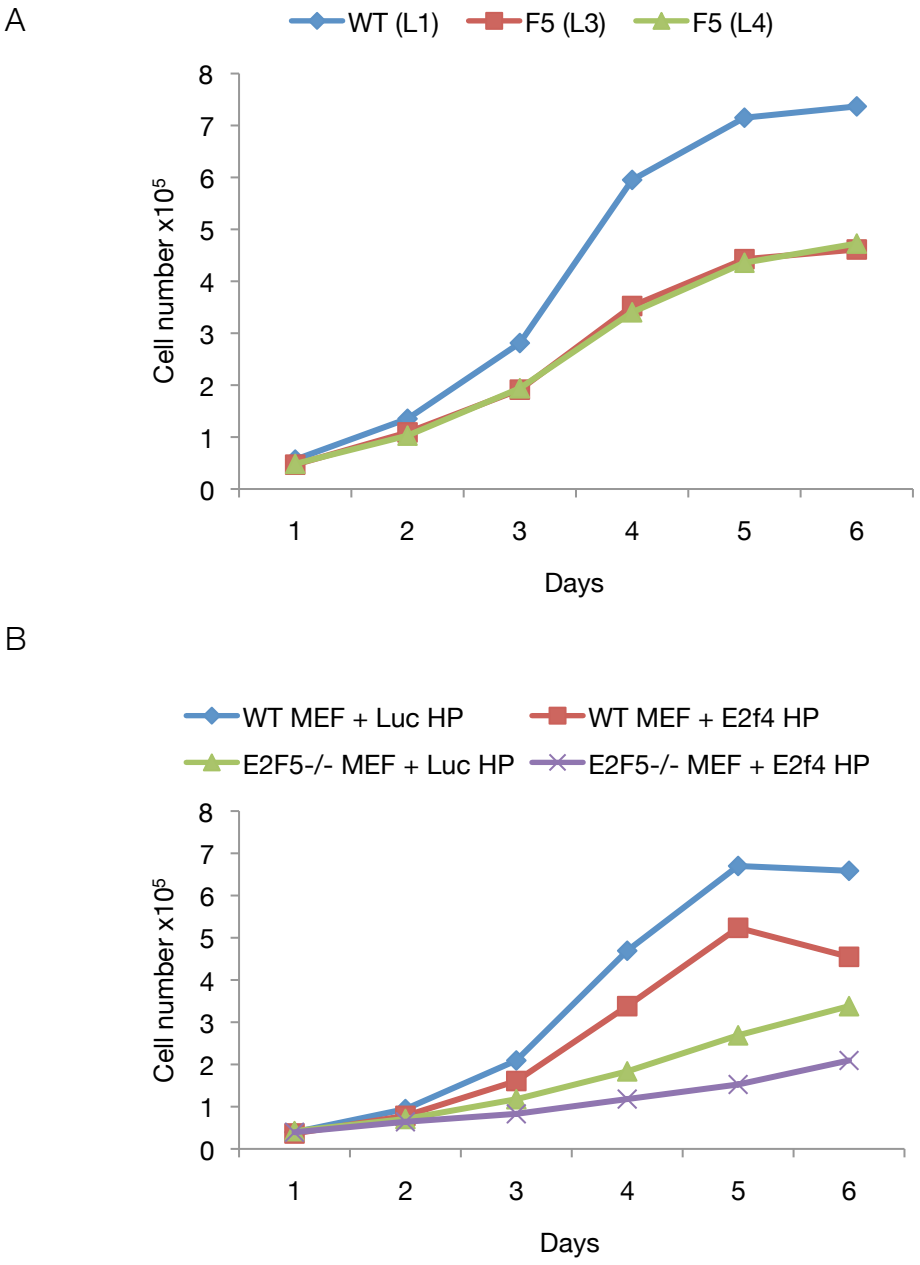

Figure 3. Acute ablation of *E2f4* alters asynchronous proliferation in MEFs

Asynchronous proliferation assays of wildtype and *E2f5*$^{-/-}$ MEFs (A) or wildtype and *E2f5*$^{-/-}$ MEFs infected with either a hairpin against *E2f4* or a luciferase control (B). A total of 4 x 104 cells were plated in duplicate in a 3 cm dish and their growth was monitored by daily counting for 6 days.

E2f5$^{-/-}$ MEFs expressing the *E2f4* hairpin exhibited a marked decrease in their ability to asynchronously proliferate compared to *E2f5*$^{-/-}$ MEFs expressing the luciferase control hairpin (Figure 3b). Surprisingly, wildtype MEFs expressing the *E2f4* hairpin also displayed a reduced proliferative capacity compared to the luciferase control infected wildtype MEFs (Figure 3b). It should be noted that in this preliminary experiment, the *E2f5*$^{-/-}$ MEFs began with a reduced ability to proliferate before being infected with the retrovirus (Figure 3a). These results suggest that acute *E2f4* knockdown causes alterations in asynchronous proliferation in both wildtype and *E2f5*$^{-/-}$ MEFs.

DISCUSSION

Members of the E2F family of transcription factors are key regulators of the cell cycle. E2F4 and E2F5 primarily function as transcriptional repressors that promote and maintain a cell cycle arrest (Attwooll et al, 2004; Dimova & Dyson, 2005; Trimarchi & Lees, 2002). Previous studies of *E2f4* and *E2f5* knockout mice have revealed critical roles in promoting cell cycle exit and enabling the terminal differentiation of several cell types, such as erythrocytes, osteoblasts, and neuronal epithelial cells in the choroid plexus (Humbert et al, 2000; Kinross et al, 2006; Lindeman et al, 1998). Since mice doubly deficient for *E2f4* and *E2f5* are embryonic lethal beginning as early as embryonic day 8.5 (Gaubatz et al, 2000; unpunlished observations, J. Sero, T. Yuan, and J.A.L), it has not been possible to fully explore the overlapping contributions that E2F4 and E2F5 make during embryonic development. In order to investigate the loss of both E2F4 and E2F5 during embryonic and adult development, we have generated conditional *E2f4* knockout mice.

To begin elucidating the roles that the repressor E2Fs share in regulating the cell cycle and promoting terminal differentiation, we acutely ablated *E2f4* in wildtype and *E2F5*$^{-/-}$ MEFs. We found that the sudden loss of *E2f4* in an *E2f5*$^{-/-}$ null background decreases the ability of these cells to proliferate. As E2F4 and E2F5 are transcriptional repressors, we were expecting the proliferative capacity of these cells to increase, not decrease. In addition, decreased proliferation was also observed in wildtype cells that expressed the hairpin against *E2f4*. These results could suggest a pro-proliferative role for these proteins. Alternatively, these data could support the pocket protein reshuffling model that our lab has previously proposed to account for the tumor suppression in *Rb*$^{+/-}$;*E2f4*$^{-/-}$ mice in

comparison to *Rb*$^{+/-}$ mice (Lee et al, 2002). Since E2F4 accounts for the majority of endogenous pocket protein binding activity, the loss of E2F4 in this case caused the released pocket proteins, p107 and p130, to form novel complexes with the normally pRb-specific activating E2Fs, E2F1-3, *in vitro* and *in vivo*, thereby reducing their activity (Lee et al, 2002). It is likely that the acute loss of *E2f4* is also increasing the free pools of pocket proteins in wildtype and *E2F5*$^{-/-}$ MEFs, which could bind and inhibit the transcriptional activity of activator E2Fs. To confirm this hypothesis, co-immunoprecipitation or gel shift assays could be performed using extracts from wildtype and *E2f5*-null MEFs expressing either the luciferase or *E2f4* hairpin.

In this experiment, the *E2f5*-null MEFs appear to have a proliferation defect, which is in contrast to what has been previously published. Only two distinct MEF lines have been characterized in this assay, and we feel that analysis of additional lines is necessary to conclude whether or not germline loss of *E2f5* causes proliferation defects. Although these are preliminary data, we are encouraged by the observation that acute ablation of *E2f4* decreases the current proliferative state of the *E2f5*-deficient cells to a similar degree to the effect on wildtype cells.

We recently found that germline loss of *E2f4* prevents calvarial osteoblast precursor cells from properly exiting the cell cycle and interferes with the ability of these cells to commit to the osteoblast lineage, resulting in defective bone development (Chapter 2). During mouse development, E2F4 is widely expressed, while E2F5 expression is restricted to terminally differentiated cells (Dagnino et al, 1997a; Dagnino et al, 1997b). It is possible that both E2F4 and E2F5 work in concert to ensure exit from the cell cycle and promote

differentiation. E2F4 could be largely responsible for promoting cell cycle exit and initiating differentiation, while E2F5 maintains both cell cycle arrest and, thus, terminal differentiation. Osteoblasts deficient for *E2f4* display a transient inability to exit the cell cycle, and the transient nature of the phenotype could be a consequence of compensation by other E2F members, such as E2F5. We are interested in determining what effect the combined loss of E2F4 and E2F5 has on osteoblast differentiation and bone development; thus, we are generating homozygous conditional *E2f4* mice (*E2f4*$^{c/c}$) to cross with Osterix1-GFP::Cre transgene (Osx-Cre) carrying mice (Rodda & McMahon, 2006) and *E2f5*$^{+/-}$;Osx-Cre mice to obtain control, *E2f4*$^{c/c}$;*Osx-Cre* mice, and *E2f4*$^{c/c}$;*E2f5*$^{-/-}$;*Osx-Cre* mice. The Osterix transcription factor is one of the master regulators of bone development expressed in pre-osteoblasts and is required for their commitment to their osteoblastic fate (Nakashima et al, 2002). Using these lines it will be possible to assess if the additional loss of *E2f5* exacerbates the bone defects that are predicted to arise from the loss of E2F4 and/or reveals novel bone phenotypes.

EXPERIMENTAL PROCEDURES

Cloning of the targeting construct and confirmation of germline transmission of the conditional *E2f4* allele

The BAC clones 33, 104, and 119 from the RPCI-22 library were verified to contain the *E2f4* genomic locus by Southern blot analysis using a probe made by amplifying a region from exon 10 with the primers 3' exon 10 and 5' exon 10 (all primer sequences are listed in Table 1). Glycerol stocks were made from the recombinogenic bacterial strains DY380 that were transformed with BACs 104 and 119 (as described in http://recombineering.ncifcrf.gov/). Two sets of primers (RecombineeringPrimer 1F and pL451 5' neo, RecombineeringPrimer 2R and pL451 3' neo) were used to amplify overlapping halves of the Frt-Neo-Frt cassette using Hi-Fidelity Taq (55° C anneal, 1.5 min extension, 30 cycles). The two PCR fragments were digested with BssHII and EagI, ligated, purified, and recombineered into DY380 cells containing BAC 104. pBR322 was PCR amplified with Hi-Fidelity Taq using primers pBR322-1554,12600 F and pBR322-4330,10100 R to subclone the *E2f4* locus genomic DNA using gap repair (http://recombineering.ncifcrf.gov/), creating a pBR322+104+Neo plasmid. The 3' LoxP site was annealed by combining 9 μl of 10 μM NewKpnILoxP F, 9 μl of 10 μM NewKpnILoxP R, and 2 μl 1x NTE (10mM Tris, 10mM EDTA pH 7.4, 100mM NaCl), heating to 80° C, and allowed to cool to below 30° C. pBR322+104+Neo was cut using EcoRV, ligated with the 3' LoxP site, and transformed into DH5α cells (pBR322+104+N+L). The NewLoxP R primer was used to sequence the plasmid to verify clones with the correct LoxP orientation. The targeting construct from pBR322+104+N+L

was sequentially digested with AflII then XhoI. pBR322 was digested with EcoRV then SalI, ligated with the insert from pBR322+104+N+L, and transformed into DH5α cells.

To ensure no mutations were introduced into the exons found in the targeting construct, DNA sequencing was performed with the following primers: upstream 5' loxP F, downstream exon 2 R, mid-exon 3, 4, 5 F, exon 6 and 7 F, and exon 8 F. The targeting vector was linearized and sent to the Rippel Transgenic Facility for electroporation into 129/B6 F1 hybrid ES cells and selected with G418. *E2f4*$^{c/+}$ clones were detected by Southern blot analysis using external 5' and 3' probes in addition to a neomycin probe. Clone 4C6 was used to generate chimeras and all pups resulting from matings were genotyped by PCR analysis of ear clip DNA using the common primer, Conditional E2F4 C2, and the neomycin-specific primer, Conditional E2F4 N1.

E2f4 and luciferase knockdown and MEF analyses

The cloning of the *E2f4* and luciferase hairpins and the stable integration into primary cells has been previously described (Chapter 2). The proliferation assay was performed essentially as described (Aslanian et al, 2004).

Table 2. Primers used for the generation of conditional *E2f4* mice

Primer Name	Sequence
3' exon 10	TCCTCCGACTTTCTCCACCTCC
5' exon 10	CTCTGCAATGGCTCTAAATGAGGG
3' probe F	TGAGGTTCCTAATGGGCCTGTGTG
3' probe R	GAGCTGGAGCCATTCTTAGGTACC
5' probe F	CAGTGCTGATTGGTGGTTGAAGGC
5' probe R	AGGTAAGTGTGGCTCAGTGGCTGA
RecombineeringPrimer 1F	tgcaggccctcgtggcccttggcgactaggacagggagccgagcatcaga aATAACTTCGTATAGCATACATTAT
RecombineeringPrimer 2R	ccttccaatcactccgcgagtgtgtcgggaagagtcttggcttagctgtg GAATTCCGAAGTTCCTATTCTCTAGAAAGTAT
pL451 3' neo	CCTCGCTAGCTGATCACTCAGAAGAACTCGTCAAGAAGGCG
pL451 5' neo	GGTCGCTAGCCGGATCGGCCATTGAACAAGATGG
pBR322-1554,12600 F	tggggatggagagtgatgcatgtgcattcctgcccccaagatgttccagg CCGATACGCGAGCGAACGTGAAGC
pBR322-4330,10100 R	cctggagcgggtccactttgcatcgcctgtgcttccggagccaggggctc GACGAAAGGGCCTCGTGATACGCC
NewKpnILoxP F	ATAACTTCGTATAGCATACATTATACGAAGTTATGTAC
NewKpnILoxP R	ATAACTTCGTATAATGTATGCTATACGAAGTTATGTAC
NewLoxP R	CTGGAACTTGCAATGTAGACAAGG
Conditional E2F4 C2	GACTAGGACAGGGAGCCGAGCATC
Conditional E2F4 N1	ATGCTGGGGATGCGGTGGGCTCTA
upstream 5' loxP F	TAGCAAGGAAGAGTCGGGTGGTTC
downstream exon 2 R	TATGCCTTCCACACAGCCCTCAGA
mid-exon 3, 4, 5 F	ACCAGCACAAGGTGTGGGTGCAGC
exon 6 and 7 F	GTACCCAGTGCTCTAGGGAGGTAG
exon 8 F	CACACTGGTTGTCCTGGCCATAGG

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REFERENCES

Aslanian A, Iaquinta PJ, Verona R, Lees JA (2004) Repression of the Arf tumor suppressor by E2F3 is required for normal cell cycle kinetics. *Genes Dev* **18**(12): 1413-1422

Attwooll C, Lazzerini Denchi E, Helin K (2004) The E2F family: specific functions and overlapping interests. *EMBO J* **23**(24): 4709-4716

Beijersbergen RL, Kerkhoven RM, Zhu L, Carlee L, Voorhoeve PM, Bernards R (1994) E2F-4, a new member of the E2F gene family, has oncogenic activity and associates with p107 in vivo. *Genes Dev* **8**(22): 2680-2690

Blais A, Dynlacht BD (2007) E2F-associated chromatin modifiers and cell cycle control. *Curr Opin Cell Biol* **19**(6): 658-662

Conboy CM, Spyrou C, Thorne NP, Wade EJ, Barbosa-Morais NL, Wilson MD, Bhattacharjee A, Young RA, Tavare S, Lees JA, Odom DT (2007) Cell cycle genes are the evolutionarily conserved targets of the E2F4 transcription factor. *PLoS ONE* **2**(10): e1061

Dagnino L, Fry CJ, Bartley SM, Farnham P, Gallie BL, Phillips RA (1997a) Expression patterns of the E2F family of transcription factors during mouse nervous system development. *Mech Dev* **66**(1-2): 13-25

Dagnino L, Fry CJ, Bartley SM, Farnham P, Gallie BL, Phillips RA (1997b) Expression patterns of the E2F family of transcription factors during murine epithelial development. *Cell Growth Differ* **8**(5): 553-563

Danielian PS, Bender Kim CF, Caron AM, Vasile E, Bronson RT, Lees JA (2007) E2f4 is required for normal development of the airway epithelium. *Dev Biol* **305**(2): 564-576

DeGregori J, Johnson DG (2006) Distinct and Overlapping Roles for E2F Family Members in Transcription, Proliferation and Apoptosis. *Curr Mol Med* **6**(7): 739-748

DeGregori J, Leone G, Miron A, Jakoi L, Nevins JR (1997) Distinct roles for E2F proteins in cell growth control and apoptosis. *Proc Natl Acad Sci U S A* **94**(14): 7245-7250

Dimova DK, Dyson NJ (2005) The E2F transcriptional network: old acquaintances with new faces. *Oncogene* **24**(17): 2810-2826

Fajas L, Landsberg RL, Huss-Garcia Y, Sardet C, Lees JA, Auwerx J (2002) E2Fs regulate adipocyte differentiation. *Dev Cell* **3**(1): 39-49

Gaubatz S, Lees JA, Lindeman GJ, Livingston DM (2001) E2F4 is exported from the nucleus in a CRM1-dependent manner. *Mol Cell Biol* **21**(4): 1384-1392

Gaubatz S, Lindeman GJ, Ishida S, Jakoi L, Nevins JR, Livingston DM, Rempel RE (2000) E2F4 and E2F5 play an essential role in pocket protein-mediated G1 control. *Mol Cell* **6**(3): 729-735

Giangrande PH, Zhu W, Schlisio S, Sun X, Mori S, Gaubatz S, Nevins JR (2004) A role for E2F6 in distinguishing G1/S- and G2/M-specific transcription. *Genes Dev* **18**(23): 2941-2951

Ginsberg D, Vairo G, Chittenden T, Xiao ZX, Xu G, Wydner KL, DeCaprio JA, Lawrence JB, Livingston DM (1994) E2F-4, a new member of the E2F transcription factor family, interacts with p107. *Genes Dev* **8**(22): 2665-2679

Hijmans EM, Voorhoeve PM, Beijersbergen RL, van 't Veer LJ, Bernards R (1995) E2F-5, a new E2F family member that interacts with p130 in vivo. *Mol Cell Biol* **15**(6): 3082-3089

Humbert PO, Rogers C, Ganiatsas S, Landsberg RL, Trimarchi JM, Dandapani S, Brugnara C, Erdman S, Schrenzel M, Bronson RT, Lees JA (2000) E2F4 is essential for normal erythrocyte maturation and neonatal viability. *Mol Cell* **6**(2): 281-291

Hurford RK, Jr., Cobrinik D, Lee MH, Dyson N (1997) pRB and p107/p130 are required for the regulated expression of different sets of E2F responsive genes. *Genes Dev* **11**(11): 1447-1463

Kinross KM, Clark AJ, Iazzolino RM, Humbert PO (2006) E2f4 regulates fetal erythropoiesis through the promotion of cellular proliferation. *Blood* **108**(3): 886-895

Kong LJ, Chang JT, Bild AH, Nevins JR (2007) Compensation and specificity of function within the E2F family. *Oncogene* **26**(3): 321-327

Landsberg RL, Sero JE, Danielian PS, Yuan TL, Lee EY, Lees JA (2003) The role of E2F4 in adipogenesis is independent of its cell cycle regulatory activity. *Proc Natl Acad Sci U S A* **100**(5): 2456-2461

Lee EY, Cam H, Ziebold U, Rayman JB, Lees JA, Dynlacht BD (2002) E2F4 loss suppresses tumorigenesis in Rb mutant mice. *Cancer Cell* **2**(6): 463-472

Lindeman GJ, Dagnino L, Gaubatz S, Xu Y, Bronson RT, Warren HB, Livingston DM (1998) A specific, nonproliferative role for E2F-5 in choroid plexus function revealed by gene targeting. *Genes Dev* **12**(8): 1092-1098

Lukas J, Petersen BO, Holm K, Bartek J, Helin K (1996) Deregulated expression of E2F family members induces S-phase entry and overcomes p16INK4A-mediated growth suppression. *Mol Cell Biol* **16**(3): 1047-1057

Mann DJ, Jones NC (1996) E2F-1 but not E2F-4 can overcome p16-induced G1 cell-cycle arrest. *Curr Biol* **6**(4): 474-483

Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR, de Crombrugghe B (2002) The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. *Cell* **108**(1): 17-29

Rayman JB, Takahashi Y, Indjeian VB, Dannenberg JH, Catchpole S, Watson RJ, te Riele H, Dynlacht BD (2002) E2F mediates cell cycle-dependent transcriptional repression in vivo by recruitment of an HDAC1/mSin3B corepressor complex. *Genes Dev* **16**(8): 933-947

Rempel RE, Saenz-Robles MT, Storms R, Morham S, Ishida S, Engel A, Jakoi L, Melhem MF, Pipas JM, Smith C, Nevins JR (2000) Loss of E2F4 activity leads to abnormal development of multiple cellular lineages. *Mol Cell* **6**(2): 293-306

Rodda SJ, McMahon AP (2006) Distinct roles for Hedgehog and canonical Wnt signaling in specification, differentiation and maintenance of osteoblast progenitors. *Development* **133**(16): 3231-3244

Sage J, Miller AL, Perez-Mancera PA, Wysocki JM, Jacks T (2003) Acute mutation of retinoblastoma gene function is sufficient for cell cycle re-entry. *Nature* **424**(6945): 223-228

Sardet C, Vidal M, Cobrinik D, Geng Y, Onufryk C, Chen A, Weinberg RA (1995) E2F-4 and E2F-5, two members of the E2F family, are expressed in the early phases of the cell cycle. *Proc Natl Acad Sci U S A* **92**(6): 2403-2407

Takahashi Y, Rayman JB, Dynlacht BD (2000) Analysis of promoter binding by the E2F and pRB families in vivo: distinct E2F proteins mediate activation and repression. *Genes Dev* **14**(7): 804-816

Trimarchi JM, Lees JA (2002) Sibling rivalry in the E2F family. *Nat Rev Mol Cell Biol* **3**(1): 11-20

Verona R, Moberg K, Estes S, Starz M, Vernon JP, Lees JA (1997) E2F activity is regulated by cell cycle-dependent changes in subcellular localization. *Mol Cell Biol* **17**(12): 7268-7282

Wells J, Boyd KE, Fry CJ, Bartley SM, Farnham PJ (2000) Target gene specificity of E2F and pocket protein family members in living cells. *Mol Cell Biol* **20**(16): 5797-5807

Chapter Four

Discussion

The E2F family of transcription factors, in association with pRb and the related pocket protein family members, p107 and p130, control the regulation of the cell cycle. As a result, these proteins play an integral role in other cellular processes, such as differentiation. Here, I analyzed the *in vivo* role of E2F4 in murine embryonic development and differentiation. I found that E2F4 is essential for calvarial cells to exit the cell cycle and begin their subsequent commitment to become osteoblasts, thereby contributing to bone development (Chapter 2). To further explore the effects that loss of E2F4 causes *in vivo*, I have generated conditional *E2f4* knockout mice. By investigating the phenotypes that arise in mice lacking either E2F4 alone or E2F4 and E2F5, we will gain new insight about the synergistic roles each protein plays during development.

E2F4 in bone development and osteoblast differentiation

E2F4 functions primarily as a transcriptional repressor and can bind to all of the pocket protein family members (Moberg et al, 1996). Interestingly, all three binding partners have been implicated in the differentiation of cell types that contribute to bone formation. Both p107 and p130 exert overlapping roles to ensure proper differentiation of long bone chondrocytes (Cobrinik et al, 1996), while pRb is required to promote osteoblast differentiation (Berman et al, 2008; Thomas et al, 2001). Therefore, we hypothesized that E2F4 may play a similar role in bone development. Examination of embryonic skeletons revealed that bone development is disrupted from an early stage, as *E2f4*-deficient mice exhibit decreased expression of the early bone differentiation marker, alkaline phosphatase. Moreover, loss of E2F4 increases the number of osteoblast progenitor cells

that continue to enter the cell cycle compared to wildtype cells. Therefore, we believe that abrogating E2F4 function prevents pre-osteoblasts from exiting the cell cycle, a step that is coincident with differentiation in many cell types. My *in vitro* data demonstrate that, while E2F4 does not play a cell cycle-independent role in osteoblast differentiation, E2F4 loss increases the number of osteoblast progenitor cells due to the cell cycle exit defect.

Although these data suggest that loss of E2F4 affects osteoblast differentiation *in vivo*, it is unclear if E2F4 is influencing bone development in a cell autonomous manner. As *E2f4*$^{-/-}$ embryos are transiently anemic, it is possible that the anemic state of the embryo prevents nutrients and gases from being efficiently delivered to cells of all types, such as osteoblasts. Therefore, many cellular processes could be disrupted, causing a delay in the development of multiple embryonic tissues. I have generated conditional *E2f4* mice with the goal of addressing the cell autonomy of E2F4 function. To study the effect of E2F4 loss specifically in osteoblasts, one could cross *E2f4*$^{c/c}$ mice with *Osx-Cre* transgenic mice. If E2F4 plays a cell autonomous role in promoting osteoblast differentiation, then conditional deletion of *E2f4* in *Osx*-expressing cells should cause similar bone defects as those observed in embryos with germline loss of E2F4.

However, it is also possible that *E2f4*$^{c/c}$;*Osx-Cre* mice will appear normal, suggesting that E2F4 does not play a cell intrinsic role in promoting cell cycle exit in osteoblast precursor cells. However, this outcome would not rule out the possibility that E2F4 is required for bone development. Although my data indicate that loss of E2F4 affects an early step in osteoblast differentiation, it did not address in which cell type E2F4 function is necessary for proper bone development. Since the original skeletal analysis was

performed in mice with germline loss of E2F4, it is possible that E2F4 is required in osteoblasts and/or the cells from which they arise, the mesenchymal stem cell (MSC). To better define the period in which E2F4 is required for bone development, one could determine the *in vitro* and *in vivo* requirement for E2F4 in MSCs. MSCs are able to differentiate into osteoblasts *in vitro* without the requirement of a confluence arrest. One could isolate *E2f4*$^{c/c}$ MSCs and infect them with either a control adenovirus containing GFP (Adeno-GFP) or an adenovirus expressing the Cre recombinase gene (Adeno-Cre). By performing a similar progenitor assay as described in Chapter 2, one could determine if E2F4 is essential for cell cycle exit in MSCs by comparing the number of alkaline phosphatase-positive colonies in the acutely ablated MSCs compared to the control infected *E2f4*$^{c/c}$ MSCs. Assuming cell cycle exit is necessary for MSCs to initiate osteoblast differentiation, one should observe a delay in the secretion of calcium deposits in *E2f4*-null MSCs compared to *E2f4*$^{c/c}$ MSCs after osteogenic induction. One caveat is that we do not know if MSCs continue to proliferate while they differentiate *in vitro*. Therefore, it will be important to assess the cell cycle profile of Adeno-Cre infected cells and Adeno-GFP infected cells during differentiation. If it is found that the control-infected MSCs continue to proliferate as they differentiate, it will be difficult to determine what role, if any, E2F4 plays in promoting cell cycle exit and, consequently, osteoblast differentiation in MSCs.

Conditional *E2f4* mice will elucidate the *in vivo* role E2F4 plays in MSC differentiation. By crossing *E2f4*$^{c/c}$ mice with mice carrying the *Prx-Cre* transgene, *E2f4* will be deleted from mesenchymal progenitor cells (Logan et al, 2002). One could examine embryonic skeletons stained with Alizarin Red to determine if *E2f4*$^{c/c}$;*Prx-Cre* embryos

display disrupted bone ossification compared to *E2f4*$^{c/c}$ controls. Subsequently, one could analyze cell cycle progression of osteoblasts found in affected bones by comparing the number of nuclei that have incorporated BrdU and express Ki67 to the number observed in control mice.

The original skeletal analysis of *E2f4*$^{-/-}$ embryos revealed defects in both intramembranous and endochondral bone development. I demonstrated that E2F4 loss specifically affects osteoblasts in intramembranous bones; thus, it is possible that loss of E2F4 alters endochondral bone development by disrupting chondrocyte differentiation. To investigate this possibility, one could study cartilage formation in the presphenoid and basisphenoid bones, which exhibit reduced cartilage matrix secretion at e15.5. Immunohistochemistry analyses of these bones could be used to determine if loss of E2F4 affects chondrocyte differentiation. By analyzing BrdU incorporation or Ki67-positive nuclei, we could determine if E2F4 promotes cell cycle exit in chondrocytes. In addition, one could analyze the expression of chondrocyte differentiation markers, including *Sox9* and *collagen type II*, to determine if a particular step in chondrocyte differentiation is affected in *E2f4*$^{-/-}$ embryos.

Using my conditional *E2f4* mouse, one could delete *E2f4* in chondrocyte progenitor cells using a cartilage-specific Cre recombinase transgene, such as *Sox9-Cre* (Akiyama et al, 2005). This will enable one to determine if E2F4 is essential for an early step in cartilage development. Alcian Blue staining of *E2f4*$^{c/c}$;*Sox9-Cre* embryonic skeletons can reveal defective endochondral bone formation. In addition, BrdU incorporation and Ki67 staining analyses will reveal any chondrocyte progenitor proliferation defects. Similarly, one could

examine a later step in cartilage development by depleting *E2f4* in cells that express *collagen II* using the *Col2a1-Cre* transgene (Ovchinnikov et al, 2000), elucidating the requirement for E2F4 in chondrocytes to maintain a terminally differentiated state. Since chondrocytes form a cartilage template for subsequent osteoid matrix deposition, bone defects can also be analyzed in both mouse models using the assays described in Chapter 2.

Finally, one can characterize the effect E2F4 loss has on *in vitro* chondrocyte differentiation. One could isolate embryonic chondrocytes and their progenitor cells from *E2f4*-null (germline and conditionally ablated) and control limbs to study possible proliferation and differentiation defects (Pfander et al, 2003; Shakibaei, 1995). An alternative approach is to differentiate ES cells into chondrocytes and investigate any alterations in proliferation, differentiation, and chondrocyte gene expression caused by the loss of E2F4.

Synergistic roles of E2F4 and E2F5 in cell cycle exit and terminal differentiation

E2F4 and E2F5 are thought to act primarily as transcriptional repressors and are present during all phases of the cell cycle (Muller et al, 1997; Sardet et al, 1995; Takahashi et al, 2000; Vairo et al, 1995; Verona et al, 1997; Wells et al, 2000). Mice deficient for both *E2f4* and *E2f5* die in the early stages of embryogenesis (Gaubatz et al, 2000), suggesting that they perform overlapping functions during development. My data show that loss of E2F4 causes an increase in the number of osteoblast progenitors due to continued S-phase entry. However, this phenotype is transient, and we hypothesize that other E2Fs

may compensate for the loss of E2F4, such as E2F5. My conditional *E2f4* mice will also help to address the interplay between *E2f4* and *E2f5* in various tissues. The clavicle, which is the first bone to develop, becomes ossified at e13.5. In our laboratory, it is rare to find a double knockout mouse at e13.5. Therefore, the use of conditional *E2f4* mice will enable us to perform this analysis.

We are in the process of generating *E2f4*$^{c/c}$;*Osx-Cre*, *E2f4*$^{c/c}$;*E2f5*$^{-/-}$;*Osx-Cre*, *E2f4*$^{c/c}$; *Prx-Cre*, and *E2f4*$^{c/c}$;*E2f5*$^{-/-}$;*Prx-Cre* animals that lack E2F4 expression in osteoblasts and mesenchymal stem cells, respectively. Alizarin Red and Alcian Blue staining of embryonic skeletons will reveal possible exacerbations of the *E2f4*$^{-/-}$ phenotype, as well as any novel phenotypes caused by the additional loss of *E2f5*. Additional alterations in cell cycle progression and bone differentiation marker expression can be determined by using analyses described in Chapter 2.

To complement the *in vivo* analysis, one could also determine the synergistic roles E2F4 and E2F5 play in osteoblast and chondrocyte differentiation *in vitro*. One could isolate *E2f4*$^{c/c}$ and *E2f4*$^{c/c}$*;E2f5*$^{-/-}$ MSCs, infect parallel populations with Adeno-GFP or Adeno-Cre, and induce either osteogenic or chondrogenic differentiation. By comparing the amount of secreted cartilage or osteoid matrix between *E2f4*$^{c/c}$ and *E2f4*$^{c/c}$*;E2f5*$^{-/-}$ cells, one could determine the impact that *E2f5* loss has on the differentiation of these cell lineages. BrdU incorporation, Ki67 staining, and osteoblast and chondrocyte differentiation marker analysis can also be performed as described in Chapter 2 and in the above section.

Another cell type of interest to study with the conditional *E2f4* mouse is the adipocyte, which, like the osteoblast and the chondrocyte, arises from mesenchymal stem cells. Previously, our lab reported that E2F4 loss enhances the ability of MEFs to differentiate into adipocytes. This can be largely attributed to that fact that E2F4, in association with p107 and p130, can bind to and repress PPARγ expression during terminal adipocyte differentiation (Fajas et al, 2002; Landsberg et al, 2003). However, these experiments do not take into account the possibility that more adipocyte progenitors may be present in the population of *E2f4*$^{-/-}$ MEFs compared to wildtype MEFs, a situation similar to that seen in *E2f4*$^{-/-}$ osteoblast progenitors. One could test this hypothesis by comparing the number of BrdU-positive nuclei in *E2f4*$^{c/c}$ MSCs that have been infected with either Adeno-Cre or Adeno-GFP. To further elucidate how E2F4 affects adipogenesis, one could differentiate *E2f4*-deleted MSCs or control MSCs into adipocytes *in vitro*. By comparing the number of lipid vacuoles that stain with Oil Red O in both *E2f4*-deleted and control cells, one could confirm that loss of E2F4 causes increased adipogenic differentiation. In addition, one could analyze the expression of general adipocyte genes, including PPARγ, C/EBPα, and SREBP-1c, to corroborate the differentiation results. One could further examine adipocyte differentiation by looking at possible changes in the expression of genes specific for brown fat, such as UCP-1, NRBF1, and PGC-1. This analysis will allow one to determine if loss of E2f4 affects the commitment of adipocyte progenitors to either lineage. By adding *E2f4*$^{c/c}$*;E2f5*$^{-/-}$ MSCs into the above analysis, one could determine if E2F4 and E2F5 have cooperative roles in adipogenesis.

The conditional *E2f4* mouse also allows investigation of white fat development *in vivo*. While brown fat begins to develop during gestation, white fat does not visibly accumulate until after birth (Rangwala & Lazar, 2000). This has been hard to study in *E2f4*$^{-/-}$ mice because of their neonatal lethality. By comparing *E2f4*$^{c/c}$ and *E2f4*$^{c/c}$;Prx-Cre mice, one will be able to investigate the effect that deletion of *E2f4* in adipocytes will have on white and brown fat development. As loss of E2F4 in MEFs causes spontaneous adipocyte differentiation, it will be interesting to determine if *E2f4*$^{c/c}$;Prx-Cre mice will have more white fat than control mice. Additional deletion of *E2f5* can reveal the extent that E2F4 and E2F5 are able to compensate for each other during *in vivo* adipogenesis.

During development, E2F4 is widely expressed, while E2F5 expression is restricted to terminally differentiated cells. It is possible that these proteins predominantly act during separate stages of cellular differentiation. E2F4 could primarily be responsible for promoting cell cycle exit and initiating differentiation. E2F5 could function later in the differentiation process by maintaining both cell cycle arrest and a terminally differentiated state. One could test this hypothesis by inducing *E2f4*-null osteoblast progenitors, chondrocyte progenitors, or MSCs to differentiate into bone, cartilage, or fat *in vitro*. One could infect these cells with a lentivirus expressing either a hairpin against *E2f5* or luciferase and subsequently examine these cells for alterations in cell cycle profile and differentiation status.

Synergy of E2F4 and the pocket proteins in mesenchymal stem cell differentiation

Given that E2F4 associates with the pocket proteins to repress transcription and to promote cell cycle arrest, it is not surprising that many of the same cell lineages become disrupted upon the loss of one of these repressive complex components. Some of these cell types include osteoblasts, chondrocytes, and adipocytes. In our laboratory, we have conditional mouse models of both *E2f4* and *Rb*, enabling us to determine the overlapping roles these proteins play during development and differentiation. Recently, it was found that loss of *Rb* affects osteoblast differentiation (Berman et al, 2008; Gutierrez et al, 2008). It would be interesting to combine *E2f4*$^{-/-}$;*Rb*$^{c/c}$ animals with either *Osx-Cre* or *Prx-Cre* to study bone development. Although some bones are similarly affected in *Rb* and *E2f4* single mutant mice (frontal and parietal bones, sternebrae), some bone defects are unique to each mutant (hyoid and palatine process in *Rb*$^{-/-}$ mice and presphenoid bone in *E2f4*$^{-/-}$ mice) (Berman et al, 2008). This data suggests that the overall contribution to cell cycle exit and differentiation that each protein is involved in during bone development is different for certain bones. By analyzing double mutant embryos using assays described in Chapter 2, one could determine the functional overlap between E2F4 and pRb in osteoblast progenitor cell cycle exit and differentiation. One could perform similar studies as described above for cartilage differentiation by deleting *E2f4* and *Rb* using *Sox9-Cre*, *Col2a1-Cre*, and/or *Prx-Cre*. Our lab has shown that *E2f4*$^{-/-}$;*p107*$^{-/-}$;*p130*$^{-/-}$ animals display similar defects in long bone development (Landsberg, 2003); thus it would be interesting to see if the specific loss of *E2f4* in *E2f4*$^{c/c}$;*p107*$^{-/-}$;*p130*$^{-/-}$;*Prx-Cre* animals would exacerbate the defects characteristic of *p107*$^{-/-}$;*p130*$^{-/-}$ animals. Finally, like E2F4, pocket proteins have

been implicated in the differentiation of adipocytes. Although our lab has already characterized the adipogenic phenotypes of MEFs lacking *E2f4*, *p107*, and *p130* in addition to *E2f4*$^{-/-}$;*Rb*$^{-/-}$ MEFs (unpublished data, R. Landsberg and J.A.L), it would be interesting to explore the roles of each of these proteins in adipogenesis in the more physiologically relevant mesenchymal stem cell.

Acute ablation of E2F4 in vitro causes reduced proliferation in MEFs

I have presented preliminary data suggesting that acute ablation of *E2f4* causes reduced proliferation in MEFs (Chapter 3). Since E2F4 comprises the majority of endogenous E2F/pocket protein complexes, we believe that loss of E2F4 increases the number of free pocket proteins available in the cell. As a result, the pocket proteins can form novel complexes with the activator E2Fs, preventing both their association with target gene promoters and subsequent transcriptional activation. Indeed, this has been observed in the *Rb*$^{+/-}$;*E2f4*$^{-/-}$ and *Rb*$^{+/-}$;*E2f4*$^{+/-}$ MEFs (Lee et al, 2002). By performing co-immunoprecipitation experiments and gel shift assays, one could verify if pocket protein reshuffling is indeed occurring in E2F4 knockdown MEFs. One could also compare the cell cycle profiles of *E2f4*-depleted MEFs and MEFs expressing the control hairpin to determine if the reduced proliferation is the result of a block in the cell cycle. Another possibility as to why the sudden loss of E2F4 causes decreased proliferation is that it activates a novel apoptotic response. One could test this by comparing the number of TUNEL-positive cells found in the population of cells expressing the *E2f4* hairpin and cells expressing the luciferase control. At this point, it is unclear what the physiological relevance of pocket

protein reshuffling will have in the development and differentiation *in vivo* of cell types that require E2F4 and/or E2F5.

Conclusion

The ability of cells to form specialized tissues relies on the exquisite coordination of the proteins that regulate cell cycle division and subsequent terminal differentiation. Indeed, defects in tissue development are often accompanied by the continued proliferation of constituent cells, preventing the initiation of differentiation. Here, I describe a novel role for the transcriptional repressor protein, E2F4, in bone development. I show loss of E2F4 causes defects in intramembranous bone formation. These defects arise due to the inability of osteoblast progenitor cells to exit the cell cycle *in vivo*, thus preventing terminal differentiation of osteoblasts and proper bone formation.

E2f4$^{-/-}$ mice are neonatal lethal, limiting research on this mouse model to embryonic studies. As a result, many studies of cellular differentiation must be done in an *in vitro* setting, which may inadequately address the importance of E2F4 function *in vivo*. Furthermore, the synergistic roles that E2F4 shares with other E2F and pocket protein family members often result in earlier embryonic lethality, making many studies impossible. Thus, I have generated a conditional *E2f4* mouse that will elucidate the biological functions of E2F4 in postnatal development and allow potential compensatory roles by related proteins to be revealed.

REFERENCES

Akiyama H, Kim JE, Nakashima K, Balmes G, Iwai N, Deng JM, Zhang Z, Martin JF, Behringer RR, Nakamura T, de Crombrugghe B (2005) Osteo-chondroprogenitor cells are derived from Sox9 expressing precursors. *Proc Natl Acad Sci U S A* **102**(41): 14665-14670

Berman SD, Yuan TL, Miller ES, Lee EY, Caron A, Lees JA (2008) The retinoblastoma protein tumor suppressor is important for appropriate osteoblast differentiation and bone development. *Mol Cancer Res* **6**(9): 1440-1451

Cobrinik D, Lee MH, Hannon G, Mulligan G, Bronson RT, Dyson N, Harlow E, Beach D, Weinberg RA, Jacks T (1996) Shared role of the pRB-related p130 and p107 proteins in limb development. *Genes Dev* **10**(13): 1633-1644

Fajas L, Landsberg RL, Huss-Garcia Y, Sardet C, Lees JA, Auwerx J (2002) E2Fs regulate adipocyte differentiation. *Dev Cell* **3**(1): 39-49

Gaubatz S, Lindeman GJ, Ishida S, Jakoi L, Nevins JR, Livingston DM, Rempel RE (2000) E2F4 and E2F5 play an essential role in pocket protein-mediated G1 control. *Mol Cell* **6**(3): 729-735

Gutierrez GM, Kong E, Sabbagh Y, Brown NE, Lee JS, Demay MB, Thomas DM, Hinds PW (2008) Impaired bone development and increased mesenchymal progenitor cells in calvaria of RB1-/- mice. *Proc Natl Acad Sci U S A* **105**(47): 18402-18407

Landsberg RL (2003) The Role of E2F Pocket Protein Repressive Complexes in Cell Cycle Control and Differentiation. PhD Thesis, Biology, MIT, Cambridge

Landsberg RL, Sero JE, Danielian PS, Yuan TL, Lee EY, Lees JA (2003) The role of E2F4 in adipogenesis is independent of its cell cycle regulatory activity. *Proc Natl Acad Sci U S A* **100**(5): 2456-2461

Lee EY, Cam H, Ziebold U, Rayman JB, Lees JA, Dynlacht BD (2002) E2F4 loss suppresses tumorigenesis in Rb mutant mice. *Cancer Cell* **2**(6): 463-472

Logan M, Martin JF, Nagy A, Lobe C, Olson EN, Tabin CJ (2002) Expression of Cre Recombinase in the developing mouse limb bud driven by a Prxl enhancer. *Genesis* **33**(2): 77-80

Moberg K, Starz MA, Lees JA (1996) E2F-4 switches from p130 to p107 and pRB in response to cell cycle reentry. *Mol Cell Biol* **16**(4): 1436-1449

Muller H, Moroni MC, Vigo E, Petersen BO, Bartek J, Helin K (1997) Induction of S-phase entry by E2F transcription factors depends on their nuclear localization. *Mol Cell Biol* **17**(9): 5508-5520

Ovchinnikov DA, Deng JM, Ogunrinu G, Behringer RR (2000) Col2a1-directed expression of Cre recombinase in differentiating chondrocytes in transgenic mice. *Genesis* **26**(2): 145-146

Pfander D, Cramer T, Schipani E, Johnson RS (2003) HIF-1alpha controls extracellular matrix synthesis by epiphyseal chondrocytes. *J Cell Sci* **116**(Pt 9): 1819-1826

Rangwala SM, Lazar MA (2000) Transcriptional control of adipogenesis. *Annu Rev Nutr* **20**: 535-559

Sardet C, Vidal M, Cobrinik D, Geng Y, Onufryk C, Chen A, Weinberg RA (1995) E2F-4 and E2F-5, two members of the E2F family, are expressed in the early phases of the cell cycle. *Proc Natl Acad Sci U S A* **92**(6): 2403-2407

Shakibaei M (1995) Integrin expression on epiphyseal mouse chondrocytes in monolayer culture. *Histol Histopathol* **10**(2): 339-349

Takahashi Y, Rayman JB, Dynlacht BD (2000) Analysis of promoter binding by the E2F and pRB families in vivo: distinct E2F proteins mediate activation and repression. *Genes Dev* **14**(7): 804-816

Thomas DM, Carty SA, Piscopo DM, Lee JS, Wang WF, Forrester WC, Hinds PW (2001) The retinoblastoma protein acts as a transcriptional coactivator required for osteogenic differentiation. *Mol Cell* **8**(2): 303-316

Vairo G, Livingston DM, Ginsberg D (1995) Functional interaction between E2F-4 and p130: evidence for distinct mechanisms underlying growth suppression by different retinoblastoma protein family members. *Genes Dev* **9**(7): 869-881

Verona R, Moberg K, Estes S, Starz M, Vernon JP, Lees JA (1997) E2F activity is regulated by cell cycle-dependent changes in subcellular localization. *Mol Cell Biol* **17**(12): 7268-7282

Wells J, Boyd KE, Fry CJ, Bartley SM, Farnham PJ (2000) Target gene specificity of E2F and pocket protein family members in living cells. *Mol Cell Biol* **20**(16): 5797-5807

Appendix A

Metastatic osteosarcoma induced by inactivation of *Rb* and *p53* in the osteoblast lineage

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Author's contributions: Figure 1F and 3

ABSTRACT

Mutation of the *RB-1* and *p53* tumor suppressors is associated with the development of human osteosarcoma. With the goal of generating a mouse model of this disease, we used conditional and transgenic mouse strains to inactivate *Rb* and/or *p53* specifically in osteoblast precursors. The resulting *Rb;p53* double mutant (DKO) animals are viable but develop early onset osteosarcomas with complete penetrance. These tumors display many of the characteristics of human osteosarcomas, including being highly metastatic. We established cell lines from the DKO osteosarcomas to further investigate their properties. These immortalized cell lines are highly proliferative and they retain their tumorigenic potential, as judged by their ability to form metastatic tumors in immunocompromised mice. Moreover, they can be induced to differentiate and, depending on the inductive signal, will adopt either the osteogenic or adipogenic fate. Consistent with this multipotency, a significant portion of these tumor cells express Sca-1, a marker that is typically associated with stem cells/uncommitted progenitors. By assaying sorted cells in transplant assays, we demonstrate that the tumorigenicity of the osteosarcoma cell lines correlates with the presence of the Sca-1 marker. Finally, we show that loss of *Rb* and *p53* in Sca-1-positive mesenchymal stem/progenitor cells is sufficient to yield transformed cells that can initiate osteosarcoma formation *in vivo*.

INTRODUCTION

Osteosarcomas account for ≈30% of malignant bone tumors and 3–4% of all childhood malignancies (Clark et al, 2008; Kansara & Thomas, 2007). They arise primarily around the knee joint, lower femur and upper tibia, which are all regions of active bone growth and repair. These tumors are predominantly osteoblastic in nature, although there is a correlation between loss of differentiation and poor prognosis. The generation of new therapeutic treatments for osteosarcoma has improved the 5-year survival rate of affected individuals. However, like other mesenchymal neoplasms, osteosarcomas are predisposed to metastasize via the hematogenous route, and thus, pulmonary metastasis is a major cause of death. Analyses of both sporadic and hereditary tumors show that inactivation of the *p53* and *RB-1* tumor suppressors plays a key role in the development of this tumor type (Clark et al, 2008; Kansara & Thomas, 2007). Li-Fraumeni patients, who often carry germ-line mutations in *p53*, are predisposed to a variety of tumors, 12% of which are bone sarcomas (Bell et al, 1999; Malkin, 1993). *p53* mutations are also observed in 20–60% of sporadic osteosarcomas (Tsuchiya et al, 2000; Wunder et al, 2005). Similarly, patients carrying germ-line mutations in *RB-1* have an ≈500-fold higher incidence of osteosarcoma than the general population (Gurney et al, 1995). Moreover, *RB-1* mutations are detected in 70% of all adolescent osteosarcomas (Feugeas et al, 1996). Finally, human osteosarcomas can carry mutations in both *p53* and *RB-1* (Toguchida et al, 1988).

Mouse models have provided considerable insight into the role of *p53* in bone development and tumorigenesis. Experiments from three different settings suggest that *p53* plays an important role in bone development by modulating the differentiation of

osteoblasts. First, p53-deficient mice display both accelerated osteoblast differentiation and increased bone density (Wang et al, 2006). Second, hyperactivation of p53, via deletion of the p53-inhibitor Mdm2, suppresses osteoblast differentiation by inhibiting expression of the bone-specific transcription factor Runx2 (Lengner et al, 2006). Finally, *in vitro* studies show that deletion of p53 from mesenchymal stem cells (MSCs) and osteoblast precursors *in vitro* promotes transcriptional changes associated with the early stages of osteogenesis but impairs end-stage differentiation to mature osteocytes (Tataria et al, 2006). Together, these experiments suggest that p53-loss promotes commitment to the osteoblast lineage but blocks the terminal differentiation of these progenitors. Importantly, mice carrying tumor-associated alleles of *p53* develop a variety of tumor types including osteosarcoma (Iwakuma et al, 2005). The status of *Rb* in these tumors has not been investigated. However, sarcomas arising in $Rb^{+/-}$;$p53^{-/-}$ mice do undergo loss of heterozygosity of *Rb* (Williams et al, 1994).

Analyses of cell lines and mouse models also provide intriguing links between *Rb* and osteogenesis. The retinoblastoma protein pRb has been shown to physically interact with Runx2, and the resulting complex transcriptionally activates the late osteoblast marker osteocalcin (Thomas et al, 2001). Loss of pRb, but not the pRb-related pocket proteins p107 and p130, can suppress the terminal osteogenic differentiation of cultured cell lines (Thomas et al, 2001). Moreover, we have recently shown that embryos conditionally deleted for *Rb* display defects in both endochondral and intramembranous ossification that result, at least in part, from a cell cycle exit defect (Berman et al, 2008). Unfortunately, these conditional *Rb* mutant animals die at birth, precluding analysis of adult bone

phenotypes. Heterozygous *Rb* mutant mice and *Rb*$^{-/-}$/*wild type* chimeras are viable, but they develop pituitary and thyroid tumors, never osteosarcomas (Vooijs & Berns, 1999). Thus, to date, there is no mouse model of *Rb* mutant osteosarcoma.

In this study, we have used conditional and transgenic mouse strains to inactivate *Rb* and/or *p53*, specifically in osteoblast precursors. The resulting compound mutant animals developed metastatic osteosarcomas that closely resemble human tumors. Analysis of these tumors shows that their tumorigenic potential correlates with their expression of the Sca-1 stem cell marker and other aspects of the stem cell gene expression program.

RESULTS

Mutation of Rb and p53 in Osteoblast Precursors Results in Osteosarcomas.

To generate a mouse model of osteosarcoma, we used mice carrying three alleles: the conditional alleles of *Rb* (Sage et al, 2003) and *p53* (Jonkers et al, 2001) and the *Osx1-GFP::Cre* transgene (Rodda & McMahon, 2006). In this Cre transgene (herein called *Cre*), expression of Cre recombinase is driven by promoter sequences of *Osterix1* (*Osx1*), a master regulator of bone differentiation, and is therefore restricted to osteogenic precursors derived from skeletal progenitors (Rodda & McMahon, 2006). By crossing $Rb^{+/c}$;Cre^{+}, $p53^{+/c}$;Cre^{+} or $Rb^{+/c}$;$p53^{+/c}$;Cre^{+} males with $Rb^{c/c}$,$p53^{c/c}$, or $Rb^{c/c}$;$p53^{c/c}$ females, we generated animals carrying every possible combination of *Rb* and *p53* alleles, with or without *Cre*. All genotypes arose at approximately the expected frequency [supporting information (SI) Table S1]. Mice carrying *Cre* were slightly smaller than their littermates at birth, but this did not affect their survival. By 2–3 months of age, mice of all genotypes were of similar size (data not shown). Consistent with previous reports (Rodda & McMahon, 2006), we confirmed that *Cre* was expressed specifically in osteoblasts and not other mesenchymal lineages using reporter mice (A.S.L. and J.A.L., unpublished data). We also showed that the *Cre* transgene catalyzed efficient recombination of the conditional *Rb* and *p53* alleles in the bone, by using PCR-based genotyping assays (Fig. S1).

To screen for tumors, we established an aging colony of the various *Rb*;*p53* mutant genotypes and monitored them carefully. Moribund animals were euthanized and all

Table 1.

Incidence of osteosarcoma, neuroendocrine, hibernoma, and other tumor types in *Rb;p53;Osx1-GFP::Cre* genotypes

Genotype (all Cre^+)	Fraction of mice with tumors by 1 year	Mice analyzed by histopathy	Tumor type*				Mice with mets, %	Average age of euthanasia†, days ± SD
			OS	NE	HIB	Other		
$Rb^{c/c}$	1/23	2		2 (2 pit)			0	ND
$p53^{c/c}$	36/41	25	25				32	281 ± 55
$Rb^{+/c};p53^{+/c}$	15/30	16	16				19	299 ± 84
$Rb^{c/c};p53^{+/c}$	26/29	18	17	4 (3 pit)			22	251 ± 87
$Rb^{+/c};p53^{c/c}$	29/31	21	21	1			43	207 ± 33
$Rb^{c/c};p53^{c/c}$	56/56	43	28	24	19	rhabdo	37	147 ± 31

- *Tumor types: OS, osteosarcoma; NE, neuroendocrine tumor; HIB, hibernoma; rhabdo, rhabdomyosarcoma.
- †Age of euthanasia comparison *t* test: DKO vs. p53, $P < 0.0001$; $Rb^{+/c};p53^{+/c}$ vs. p53, $P = 0.13$; $Rb^{+/c};p53^{c/c}$ vs. p53, $P < 0.0001$; and $Rb^{c/c};p53^{+/c}$ vs. p53, $P = 0.17$.

tissues were analyzed for tumor phenotypes by histopathology. Up to 1 year of age (Fig. 1A and Table 1) and beyond (data not shown), the vast majority of $Rb^{c/c}$;Cre^{+} mice remained tumor-free. Two of these animals did develop tumors at 9 and 12 months of age. However, these were pituitary tumors, the typical tumor of $Rb^{+/-}$ germ-line mutant and $Rb^{-/-}$ chimeric mutant animals (Vooijs & Berns, 1999). This result suggests that the *Cre* transgene is expressed at low levels in neuroendocrine tissues/precursors. Because the *Cre* transgene is known to act in osteoblast precursors and histological analysis did not reveal tumorigenic lesions in the bones of adult $Rb^{c/c}$;Cre^{+} animals (data not shown), we conclude that *Rb* loss is not sufficient to promote the transformation of murine osteoblast precursors.

Consistent with the presence of osteosarcoma in humans and mice with germ-line *p53* mutations (Kansara & Thomas, 2007), a large fraction of the $p53^{c/c}$;Cre^{+} mice developed osteosarcoma, but not other tumor types, by 1 year of age (Fig. 1A and Table 1). Although *p53* loss is clearly sufficient to promote tumorigenesis, our data reveal strong synergy between *Rb* and *p53* mutations in osteosarcoma development (Fig. 1A and Table 1). The $Rb^{+/c}$;$p53^{c/c}$;Cre^{+} and $Rb^{c/c}$;$p53^{+/c}$;Cre^{+} genotypes were highly predisposed to develop osteosarcoma, and their mean survival time was considerably shorter than that of the $p53^{c/c}$;Cre^{+} animals (Fig. 1A and Table 1). In addition, osteosarcomas arose in a significant fraction of the $Rb^{+/c}$;$p53^{+/c}$;Cre^{+} animals, but rarely ($p53^{+/c}$;Cre^{+}) or never ($Rb^{+/c}$;Cre^{+}) in the single heterozygous mutants (Table 1 and data not shown). Importantly, with the exception of the occasional neuroendocrine tumor, osteosarcoma was the only tumor type arising in $Rb^{+/c}$;$p53^{c/c}$;Cre^{+}, $Rb^{c/c}$;$p53^{+/c}$;Cre^{+}, $p53^{c/c}$;Cre^{+} and $Rb^{+/c}$;$p53^{+/c}$;Cre^{+}

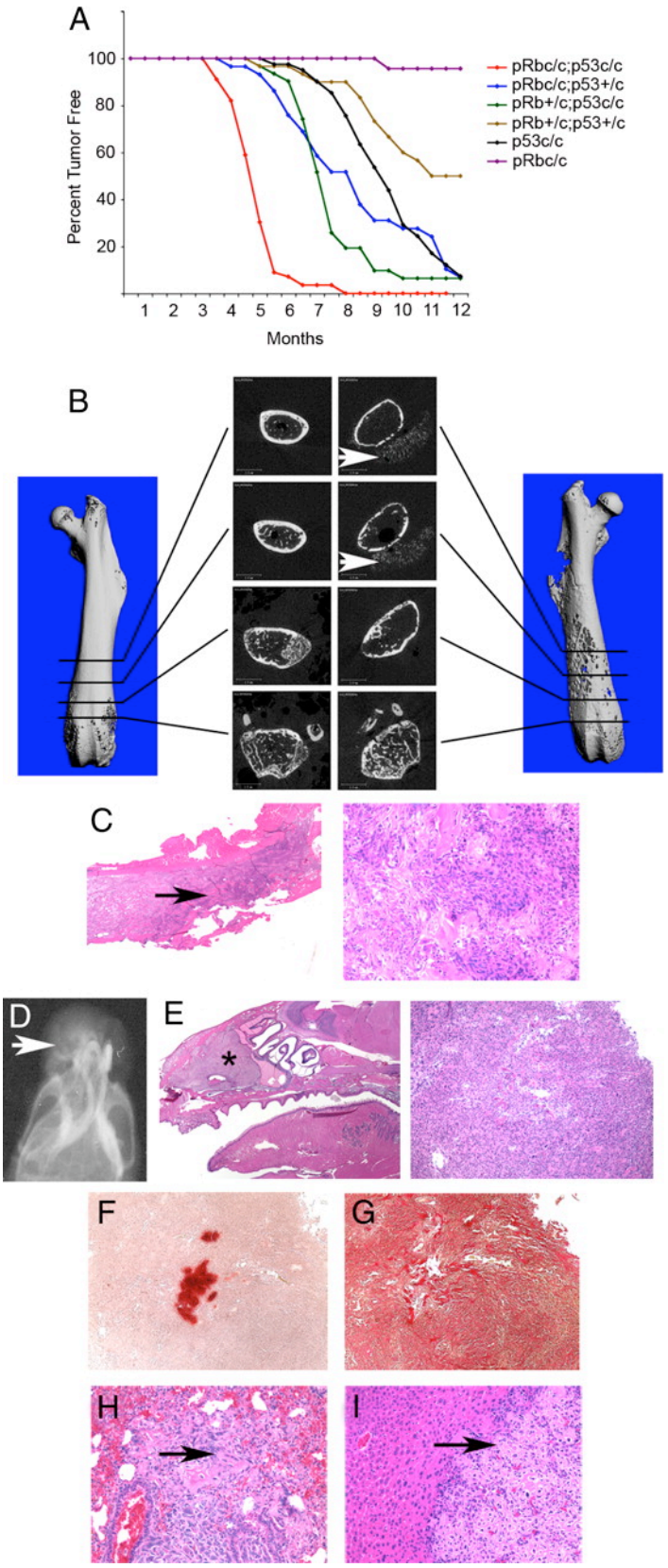
A
Percent Tumor Free
100
80
60
40
20
1 2 3 4 5 6 7 8 9 10 11 12
Months
pRbc/c;p53c/c
pRbc/c;p53+/c
pRb+/c;p53c/c
pRb+/c;p53+/c
p53c/c
pRbc/c
B
C
D
E
*
F
G
H
I

Figure 1. A mouse model of metastatic osteosarcoma.

(A) Kaplan–Meier plot of the indicated genotypes carrying *Osx1-GFP::Cre* up to 12 months of age. (B–I) Analyses of osteosarcomas and associated metastases arising in DKO mice. (B) 3D reconstructed images from microComputerised Tomography are shown for a control femur (Left) versus a femur containing an osteosarcoma (Right). Central panels show 2D images at the indicated positions. Note the loss of bone cortex and the presence of bone spicules located in the tumor that has grown beyond the periosteum (arrows). (C) Histological analyses of an osteosarcoma in a femur show areas of bone cortex erosion (Left, arrow) and the presence of little mineralized bone within the tumor (Right). (D–G) Analysis of a representative snout tumor by soft x-ray image to show the typical sunburst pattern (arrow) (D), H&E staining and analysis of adjacent sections of undecalcified tumor (E) with Alizarin Red to detect calcified bone matrix (F) or Sirius Red to detect collagen (G). (H and I) Representative examples of osteosarcoma metastases (arrow), in lung (H) and liver (I) containing detectable bone matrix. (Magnification: C and E ×2; F–I ×40.)

animals. This observation supports the view that the *Cre* transgene is highly tissue-specific and strongly suggests that these osteosarcomas arise through transformation of osteoblast precursors. Like human osteosarcomas, a significant fraction of these tumors were metastatic (Table 1). The metastases were most commonly seen in the lung and liver, but they also arose in the spleen, kidney, ovary, and adrenal glands (Fig. 1 and Table S2).

The synergy between *Rb* and *p53* is underscored by the phenotype of the $Rb^{c/c}$;$p53^{c/c}$;Cre^{+} (herein called DKO) mice. These animals had a substantially shorter mean lifespan than the intermediate genotypes (Fig. 1A and Table 1) and developed osteosarcomas (75% of animals), neuroendocrine tumors (60% of animals), and hibernomas (44% of animals), tumors derived from brown adipose tissue (Fig. S2). Many DKOs presented with multiple tumor types, and in 40% of cases metastasis of at least one of the primary tumors was observed (Table 1 and Table S2). There was no obvious correlation between the time of death of the DKOs and their associated tumor types (data not shown). Lack of correlation suggests that the shortened lifespan of the DKOs, vs. other genotypes, is not due simply to the presence of additional tumor types but likely reflects the accelerated onset and/or aggressiveness of the tumors.

The osteosarcomas arose in a variety of locations, including the femur, a major site for human osteosarcoma, and the snout (the most common site in our model), spine, and skull. These tumors displayed characteristics typical of human osteosarcomas (Fig. 1 and data not shown). For example, microComputerized Tomography and H&E staining of femoral osteosarcomas showed destruction of the bone cortex and the presence of ossified spicules in the tumor mass located outside of the periosteum (Fig. 1 B and C).

Similarly, x-ray analysis of a typical snout tumor revealed the classic sunburst pattern indicative of osteoid tissue (osseous tissue before calcification: Fig. 1D). Moreover, the osteosarcomas were largely composed of osteoblastic cells, as judged by H&E staining and Sirius Red staining for collagen (Fig. 1 C, E, and G). However, like many human osteosarcomas, these tumors were predominantly poorly differentiated or undifferentiated, as judged by low levels of Alizarin Red staining of calcified bone matrix (Fig. 1F). We also used quantitative real-time PCR (qRT-PCR) to analyze the expression of differentiation markers in primary osteosarcomas derived from DKO mice (Fig. S3). These tumors contained mRNAs associated with early to mid stages of bone differentiation, such as *Runx2*, *Osx*, *Alkaline Phosphatase* (Alp), and *Collagen1* (Col1), at the same or higher levels than control bone tissue. In contrast, *Osteocalcin* (Oc) mRNA, associated with fully differentiated osteoblasts that have secreted bone matrix, was present at lower levels than in the control. Notably, mRNAs associated with adipose tissue were not expressed in the primary osteosarcomas, but were present in hibernomas (Fig. S3). Finally, as noted above, a significant fraction of the osteosarcomas metastasized to lung and liver (Fig. 1 H–I, Table 1, and Table S2). Thus, mutation of *Rb* and *p53* using this Cre transgene induces formation of metastatic osteosarcomas that resemble the human disease.

Cell Lines Derived from Osteosarcomas Are Immortal and Form Osteogenic Tumors When Transplanted in Nude Mice.

To further characterize these tumors, we dissected primary osteosarcomas from three different DKO mice, mechanically disaggregated the cells, and placed them in culture. The tumors used for this experiment span the range of osteosarcoma phenotypes

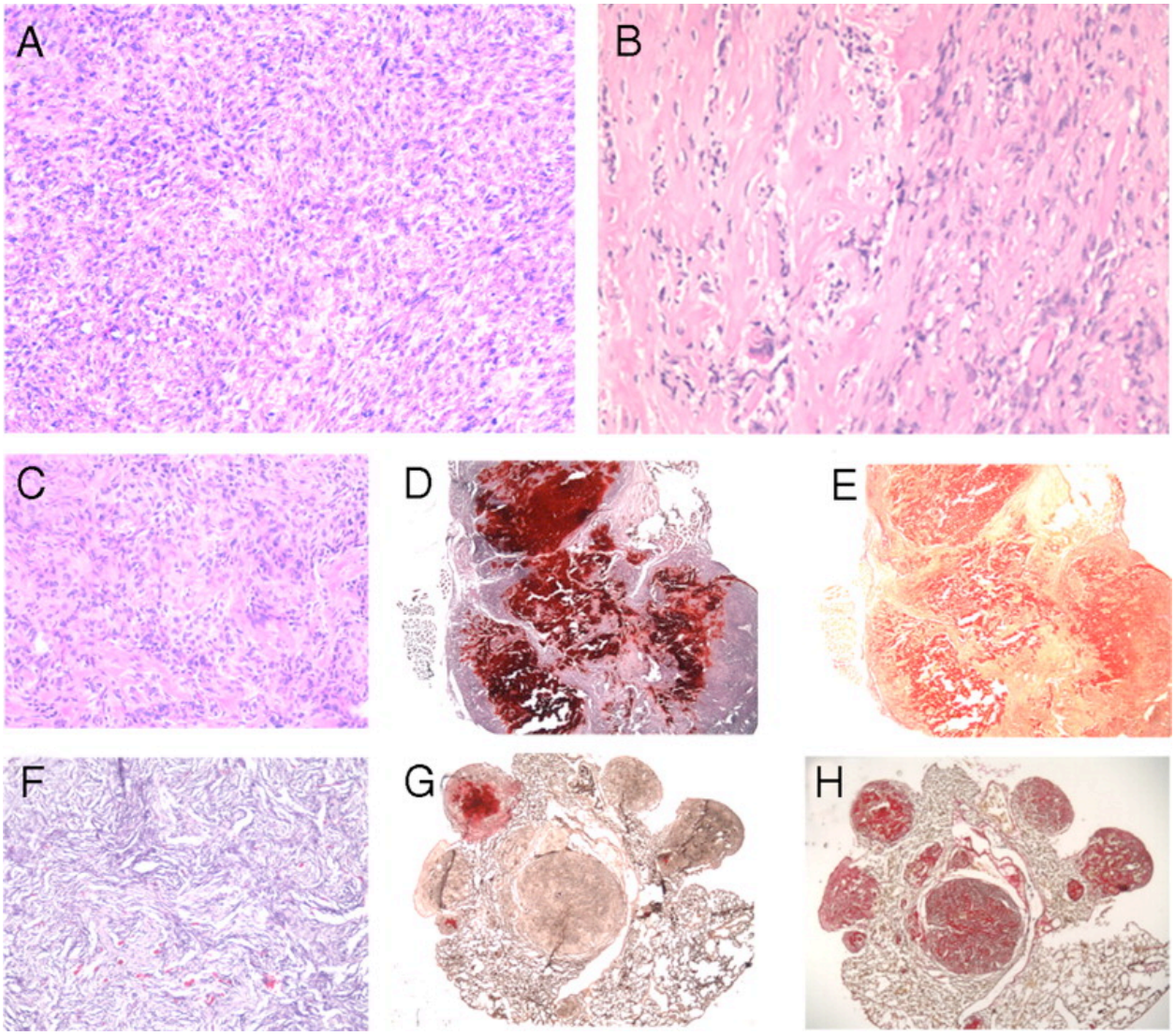

Figure 2. OS cell lines can form bone tumors in immuno-compromised mice.

(A and B) H&E stained section of the primary osteosarcomas 985 and 2380, respectively. Tumors derived from s.c. (C–E) or i.v. (F–H) injection of DKO-OS-985. (C and F) H&E staining. Adjacent sections were stained with either Alizarin Red (D, G) or Sirius Red (E, H) to stain calcified bone matrix and collagen, respectively. (Magnification: A–C and F ×40; D, E, G, and H ×2.)

seen in our mice: two of the tumors (985 and 2674) were largely undifferentiated, whereas the third (2380) had a higher level of osteoid matrix (Fig. 2 A and B). All three tumors yielded rapidly growing cell populations, and PCR verified that the $Rb^{c/c}$ and $p53^{c/c}$ conditional alleles had undergone complete recombination (data not shown). The resulting cell lines (called DKO-OS-985, DKO-OS-2380, and DKO-OS-2674) were fully immortalized.

To investigate their tumorigenic potential, we injected the osteosarcoma (OS) cell lines into immuno-compromised mice, both s.c. and i.v. DKO-OS-985, DKO-OS-2380, and DKO-OS-2674 all yielded ≥1 cm^3 masses (s.c.) or bone nodules in the lungs (i.v.) between 50 and 100 days (Fig. 2 and Table S3). The resulting tumors closely resembled the parental osteosarcomas. They were osteoblastic in nature, as determined by H&E, Sirius Red, and Alizarin red staining (Fig. 2 C–H). However, they were poorly differentiated or undifferentiated, as only small regions of the tumor produced calcified bone (Fig. 2 C–H). Moreover, the s.c. tumors were highly invasive and in some (DKO-OS-2380 and DKO-OS-2674) or all (DKO-OS-985) instances, they metastasized to the liver and other organs (data not shown). Thus, the OS cell lines retained their ability to form metastatic osteosarcomas *in vivo*.

Osteosarcoma Cell Lines Demonstrate Properties of Mesenchymal Stem/Progenitor Cells in Vitro.

The specificity of the Cre transgene, characteristics of the primary osteosarcomas, and osteoblastic properties of the transplanted tumor cell lines all suggest that the tumors result from transformation of cells committed to the bone lineage. Thus, we asked whether

the cultured tumor cells retained their ability to differentiate into bone *in vitro*. For these experiments, we allowed the tumor cells to reach confluence and then cultured them in osteogenic induction media. DKO-OS-985 (Fig. 3), DKO-OS-2380, and DKO-OS-2674 (data not shown) all gave similar results: The bone differentiation program was rapidly activated as judged by the detection of bone matrix by Alizarin Red staining and by the expression of key bone differentiation markers. Notably, the OS cell lines all retained a large number of proliferating cells throughout the differentiation time course, as assessed by BrdU incorporation (Fig. 3 and data not shown). In contrast, wild-type osteoblast and MSC preparations consistently stopped proliferating before they produced bone matrix (data not shown). The OS cells lines displayed one other unexpected phenotype: Some of the cells in bone differentiation media adopted the adipogenic fate, as judged by Oil Red O staining for lipid droplets (Fig. 3). Consistent with this finding, adipocyte differentiation markers were induced in these cells (Fig. 3). To explore adipocyte differentiation further, we cultured the tumor cells in adipogenic differentiation media (Fig. 3). Under these conditions, a significant fraction of the cells differentiated into adipocytes, as confirmed by both Oil Red O staining and gene expression analysis of adipocyte differentiation markers (Fig. 3). Notably, these cells also expressed bone differentiation markers. They did not stain with Alizarin Red, but this is likely because of the absence of inorganic phosphate (a component of osteogenic but not adipogenic differentiation media), which is essential for formation of the mineralized bone matrix. Contrary to normal adipogenesis, proliferating cells persisted throughout the differentiation time course. Thus, for both bone and fat differentiation, the normal link between differentiation stimuli and cell cycle exit is disrupted

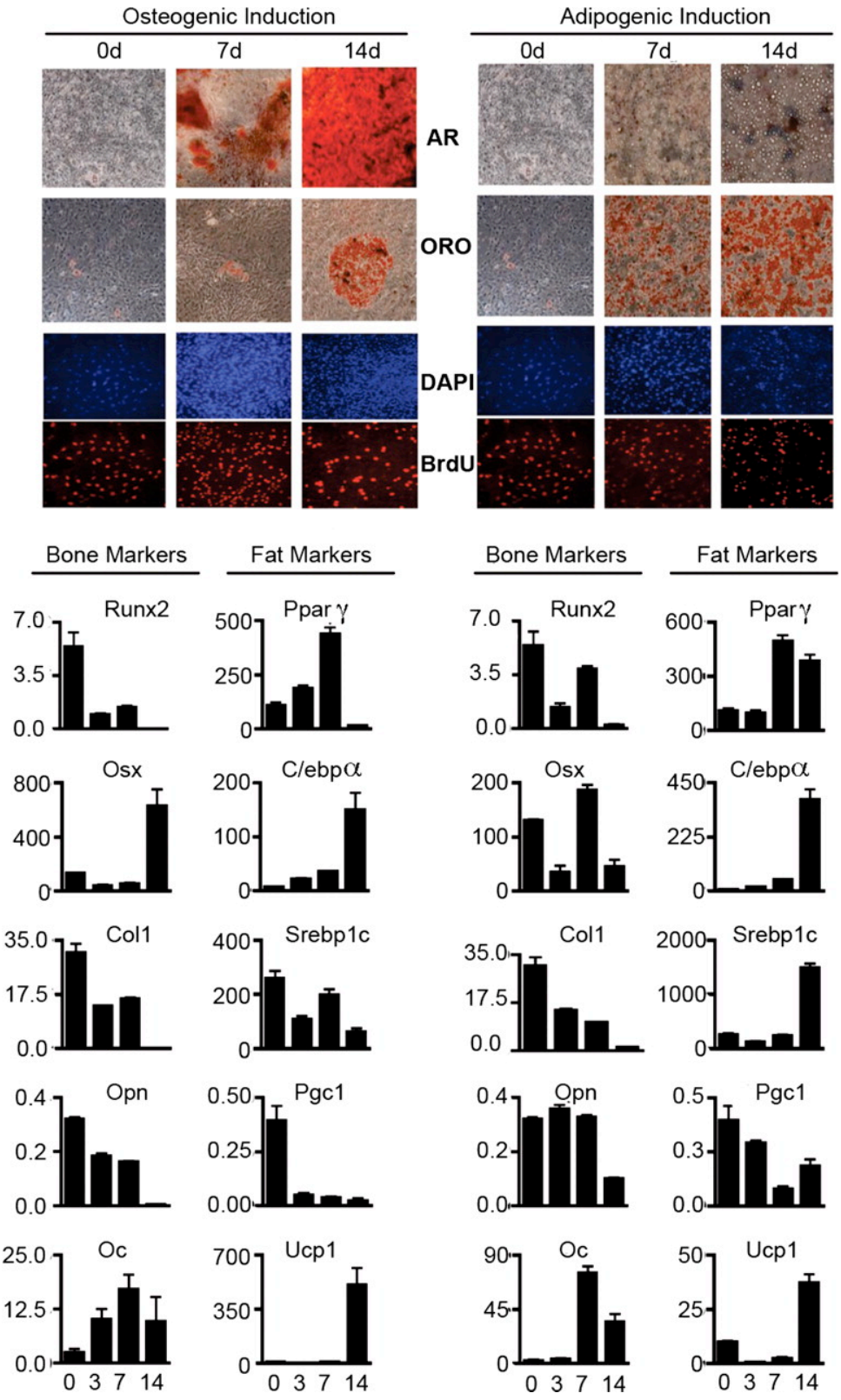
Osteogenic Induction
0d
7d
14d
Adipogenic Induction
0d
7d
14d
AR
ORO
DAPI
BrdU
Bone Markers
Fat Markers
Bone Markers
Fat Markers
Runx2
Pparγ
Runx2
Pparγ
Osx
C/ebpα
Osx
C/ebpα
Col1
Srebp1c
Col1
Srebp1c
Opn
Pgc1
Opn
Pgc1
Oc
Ucp1
Oc
Ucp1
0 3 7 14
0 3 7 14
0 3 7 14
0 3 7 14

Figure 3. Osteosarcoma cells lines are multipotent *in vitro*.

DKO-OS-985 cells were induced to differentiate into the bone (Left) and fat (Right) lineages and assayed at the indicated time points (days). Mineral deposits were stained with Alizarin Red (AR) as a marker for osteogenic differentiation. Oil-Red O (ORO) was used to stain lipid droplet accumulation during adipogenic induction. Cells were pulsed with BrdU to determine the proliferative status during differentiation. Expression of differentiation markers for bone and fat was determined by qRT-PCR.

in these OS cell lines. Finally, preliminary studies suggest that the OS cell lines can also be induced to differentiate into cartilage-producing chondrocytes when cultured in chondrogenic media (data not shown). Taken together, these data suggest that the DKO-OS cell lines possess characteristics reminiscent of MSCs/mesenchymal progenitor cells (MPCs).

Osteosarcoma Cell Lines Express Sca-1, a Marker of Early Mesenchymal Progenitors, and This Correlates with Their Tumorigenic Potential.

Given the multipotency of the OS cell lines, we tested them for the expression of a known MSC/MPC marker, Sca-1. We found that a significant fraction of the DKO-OS-985, DKO-OS-2380, and DKO-OS-2674 cells expressed Sca-1 (Fig. 4A and data not shown). We then asked whether the presence or absence of Sca-1 influenced the tumorigenicity of the OS cell lines. To answer this question, we used FACS to isolate populations of DKO-OS-985 that had either high or low/no Sca-1 expression and were all $CD45^-$ (to eliminate any hematopoietic stem cells) and assayed their tumorigenicity by s.c. injection in immunocompromised mice. In one experiment, tumors arose only from the $Sca\text{-}1^{high}$ population (Table S3). In another experiment, the $Sca\text{-}1^{high}$ cells produced a much larger tumor than the $Sca\text{-}1^{low/-}$ cells (Fig. 4B). Therefore, the tumorigenicity of the OS cell lines correlates with the presence of the Sca-1 marker.

Given this finding, we wished to establish whether the inactivation of *Rb* and *p53* in $Sca\text{-}1^{high}$ MSC/MPC preparations is sufficient to confer tumorigenicity. For this experiment, we isolated stromal cells from the bone marrow of $Rb^{c/c};p53^{c/c}$ mice and placed the cells in culture to establish flox MSC/MPCs. After two passages, the flox MSC/MPCs were

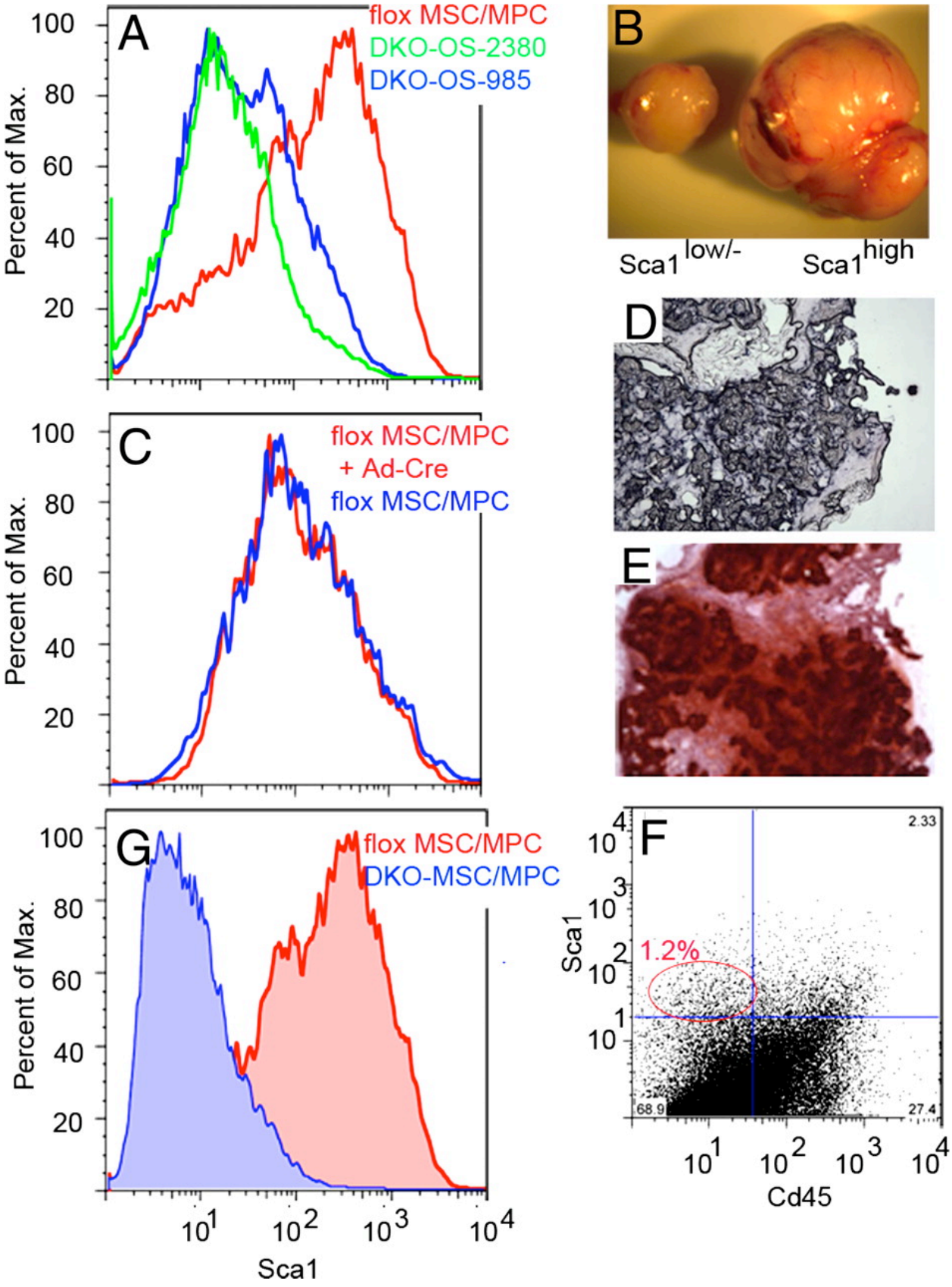
A
flox MSC/MPC
DKO-OS-2380
DKO-OS-985
Percent of Max.
100
80
60
40
20
B
Sca1 low/-
Sca1 high
C
flox MSC/MPC
+ Ad-Cre
flox MSC/MPC
D
E
G
flox MSC/MPC
DKO-MSC/MPC
Sca1
F
2.33
1.2%
68.9
27.4
Cd45

Figure 4. Sca-1 expression and *Rb*- and *p53*-loss are both required for efficient tumorigenesis *in vivo*.

Sca-1 expression in DKO-OS-985 and DKO-OS-2380 cell lines versus flox MSC/MPCs (A) or flox MSC/MPC+Ad-Cre cells, in which *Rb* and *p53* have been inactivated, versus flox MSC/MPCs (C). (B) Tumors arising in immunocompromised mice injected s.c. with 105 DKO-OS-985 cells sorted for either $Scal^{low/-}$ or $Scal^{high}$. (D and E) Tumors arising in immunocompromised mice injected s.c. with 106 flox MSC/MPC+Ad-cre stained for *Alp* expression (D) or Alizarin Red (E). Sca-1 expression in primary DKO osteosarcomas (F) and DKO MSC/MPCs versus flox MSC/MPCs (G).

infected with a Cre-expressing adenovirus and recombination of the conditional alleles was confirmed by PCR genotyping (data not shown). Untreated and recombined (flox MSC/MPC+Ad-Cre) MSC/MPCs were briefly expanded to yield sufficient cells for s.c. injection into immunocompromised mice. At this time point, the two populations were similarly composed of predominantly Sca-1^{high}/CD45^{-} cells (Fig. 4C and data not shown). However, whereas the wild-type flox MSC/MPCs did not form tumors, the flox MSC/MPC+Ad-Cre yielded tumors that stained positive for both the bone marker *Alp* and Alizarin Red (Fig. 4 D and E and Table S3). Thus, we conclude that the loss of *Rb* and *p53* in Sca-1^{high} MSC/MPCs is sufficient to create osteosarcoma-initiating cells. Long-term passaging of the flox MSC/MPC+Ad-Cre cultures confirmed that these cells are fully immortalized *in vitro*. Furthermore, the composition of the cell population shifted over time to give a mixture of Sca-1^{high} and Sca-1^{-} cells (data not shown), indicating that division of the Sca-1^{+} tumor-initiating cells can yield Sca-1^{-} progeny.

The presence of Sca-1^{+} cells within the OS cell lines was somewhat unexpected because Cre expression, and therefore *p53* and *Rb* inactivation, occurs in committed osteoblast precursors (i.e., cells that are presumed to be Sca-1–). To determine whether these Sca-1^{+} cells exist in the endogenous tumors, we dissociated primary osteosarcomas from DKOs and analyzed them directly by FACS. Importantly, Sca-1^{+}/CD45^{-} cells consistently constituted a relatively small percentage (≈1%) of the tumor, with the bulk consisting of Sca-1^{-}/CD45^{-} cells (Fig. 4F). To further explore this finding, we isolated bone marrow stromal cells from 6- to 10-week-old DKO mice before the presence of gross osteosarcomas. We placed these cells in culture and assayed the passage 1 DKO

MSC/MPC population by FACS. Remarkably, the majority of the DKO MSC/MPCs were Sca-1$^{low/-}$ (Fig. 4G). Notably, this cellular composition represents a clear departure from the properties of wild-type flox MSC/MPCs (which are predominantly Sca-1^{high}) (Fig. 4G), and it more closely resembles that of the primary osteosarcoma. Thus, inactivation of *Rb* and *p53* had greatly altered the properties of the bone marrow mesenchymal cells by 6–10 weeks of age. Given the short culture time of the DKO MSC/MPC preparations, we conclude that the Sca-1$^{low/-}$ osteoprogenitors must exist in the DKO bone marrow, and their predominance within the culture suggests that their levels are significantly elevated compared with wild-type bone marrow. Additionally, the absence of *Rb* and *p53* may help enable these cells to be established in culture. We believe there are two potential sources for the Sca-1$^{low/-}$ osteoprogenitors *in vivo*. First, they could result from the accumulation and expansion of Sca-1$^{low/-}$-committed osteoblast precursors that were the target of *Rb* and *p53* loss. Second, they could be the progeny of the DKO Sca-1^{+} osteoprogenitors that arose after the loss of *Rb* and *p53* in the committed osteoblast. Taken together, our findings provide insight into the cell lineages that contribute to osteosarcoma in our model. First, loss of *Rb* and *p53* occurs in committed osteoblast precursors. Second, DKO Sca-1^{+} cells arise at low frequency *in vivo* and Sca-1 expression correlates with tumor-initiating capacity. Finally, the DKO Sca-1^{+} cells can give rise to Sca-1^{-} progeny, and such Sca-1^{-} cells constitute the bulk of the endogenous osteosarcomas.

DISCUSSION

Mutation of *Rb* and *p53* is associated with development of human osteosarcoma. We have used an *Osx1-Cre* transgene (Rodda & McMahon, 2006) to induce inactivation of these tumor suppressors in murine osteoblast precursors. Loss of *Rb* alone is insufficient to establish osteosarcoma in these animals. However, because other *Rb/p53* genotypes are tumor prone, the lack of osteosarcomas is not because of an inability of the *Cre*-expressing precursors to become tumor-initiating cells. Instead, we presume that the tumorigenic consequences of *Rb*-loss are suppressed in these cells. It seems likely that other pocket proteins contribute to this suppression, because chimeras generated with *Rb*;*p107*, but not *Rb*, mutant ES cells develop osteosarcomas at low frequency (Dannenberg et al, 2004). In addition, our data underscore the key role of *p53* in osteosarcoma development. First, *p53*-loss in osteoblast precursors is sufficient to allow osteosarcoma formation. Second, we see robust synergy between *p53* and *Rb* in tumorigenesis. The rapidity with which these mice die from osteosarcoma correlates with the dosage of *p53* and *Rb* mutant alleles. Moreover, the DKO mice show a broadened tumor spectrum that includes hibernomas and neuroendocrine tumors and osteosarcomas. Indeed, these mice can develop multiple tumor types and die as early as 4 months of age. Importantly, irrespective of the starting genotype, the osteosarcomas display many of the characteristics of human osteosarcomas, including a shared predisposition to develop tumors within the femur, a similar cellular composition, and a high incidence of metastases.

Our study also has important implications for questions regarding the osteosarcoma cell-of-origin. To date, much of our understanding of tumor stem cells has come from the study of hematological malignancies. For example, it has been shown that acute myeloid leukemia can arise from a committed progenitor cell (Krivtsov et al, 2006). In these studies, although normal progenitor cells lost the expression of self-renewal pathways, transformed progenitor cells "acquired" the aberrant activation of self-renewal pathways. The resultant tumor-initiating cells thus contained a hybrid gene expression program, with some elements of progenitor cells and some elements of more primitive stem cells. In contrast to hematopoietic tumors, very little is known about tumor-initiating cells in osteosarcomas. The analysis of gene expression programs in Ewing's sarcoma, a tumor of bone and soft tissue, revealed an expression program that resembles MSCs (Tirode et al, 2007). Notably, silencing or inhibiting the EWS/ETS fusion gene product in sarcoma cell lines released them from their undifferentiated state and permitted both adipocytic and osteoblastic differentiation, implying that Ewing's sarcomas retain a population of undifferentiated cells that resembles MSCs. However, whether these MSC-like cells could reinitiate tumors (and thus represent a putative tumor stem-cell population), or conversely, whether differentiated cells lost their tumor initiating potential, was not established.

Here, we show that cell-lines derived from DKO osteosarcomas can differentiate into at least two lineages *in vitro* and retain gene expression programs of multiple lineages even after commitment to one lineage. Thus, although these cells necessarily arise from a cell that expresses Osx1 (and has thus committed to the osteoblast pathway), they display

a capacity for multipotent differentiation. Furthermore, these cell lines are also capable of reinitiating secondary tumors, and this capacity correlates with their expression of Sca-1, an antigen that is widely recognized as a marker of stem cells/uncommitted progenitors. Importantly, we confirm that these Sca-1^{+}/CD45^{-} cells exist in the endogenous osteosarcomas. How do these cells arise? One possibility (Model 1) is that Sca-1 and Osx1 are actually coexpressed in a small fraction of cells *in vivo*, presumably during the transition from uncommitted progenitor to early osteoblast precursor. These Sca-1^{+}/Osx1^{+} cells would represent the key target for transformation by *Rb* and *p53*. Alternatively (Model 2), expression of Sca-1 and Osx1 is mutually exclusive, but loss of *Rb* and *p53* in the Sca-1^{-}/Osx^{+} committed bone precursor changes the property of these cells to allow, at low frequency, reactivation of a stem-cell-like phenotype that includes Sca-1 expression. Notably, by 6–10 weeks of age, the loss of *Rb* and *p53* has altered the properties of the bone marrow mesenchymal cells such that MSC/MPC preparations shift from being predominately Sca-1^{high}/CD45^{-} (wild type) to predominantly Sca-1$^{low/-}$/CD45^{-} (DKO). We speculate that this shift reflects the expansion of the DKO Sca-1^{-}/Osx^{+} osteoblast precursors *in vivo*. Presumably, this population either already contains rare DKO Sca-1^{+}/Osx1^{+} recombinants (Model 1) or is a fertile ground for the rare dedifferentiation event that creates the DKO Sca-1^{+}/Osx1^{+} (Model 2) cells.

Irrespective of the mechanism by which the DKO Sca-1^{+}/Osx1^{+} cells arise, they clearly have hybrid properties. First, they have elements of more primitive stem cells that allow multilineage differentiation, expression of a stem cell antigen, and tumor reinitiating capacity. Second, they have elements of osteoblast precursor cells, as evidenced by their

strong commitment to form osteosarcomas *in vivo*. Further experiments are required to understand the nature of this Sca-1$^+$ cell population and, because Sca-1 is a murine marker, to translate these findings to human tumors. However, we hypothesize that these Sca-1$^+$ cells represent, or at least include, the tumor-initiating cell for the osteosarcomas arising in this mouse model.

EXPERIMENTAL PROCEDURES

Animal Maintenance and Histological Analyses.

All animal procedures followed protocols approved by the Institute's Committee on Animal Care. The *Rb*$^{c/c}$ (Sage et al, 2003), *p53*$^{c/c}$ (Jonkers et al, 2001), and *Osx1-GFP::Cre* (Rodda & McMahon, 2006) mice were maintained on a mixed genetic background. The criteria for euthanizing aging animals and the preparation and staining of sections are described in SI Experimental Procedures. Analysis of 3D bone structure was performed by using high-resolution microtomographic imaging, as described in (Glatt et al, 2007).

Analysis of Tumor Study Mice.

The criteria for euthanasia by CO_2 inhalation were a total tumor burden of 2 cm_3, tumor ulceration/bleeding, signs of infection, respiratory distress, impaired mobility, 20% reduction in body weight, or general cachexia. All tissues were collected and hip bones, femurs and tibias were separated and fixed overnight in PBS with 3.7% formaldehyde. Soft tissues were transferred into 70% ethanol and dehydrated via an ethanol series before embedding in paraffin for sectioning. Tissues containing bone were either decalcified in 0.46M EDTA, 2.5% Ammonium Hydroxide pH 7.2 for 2 weeks, and processed for paraffin sectioning or fixed, transferred directly into OCT Compound (Tissue-Tek) and frozen at 80°C and cut at 10 μm for frozen sections. All paraffin embedded sections were cut at 4 μm, dewaxed, and stained with H&E. Blood smears were fixed in methanol for 10 min and then stained with Wright's (Sigma,WS16) and Giemsa (Sigma GS-500) stains following standard protocols. For Sirius red staining, sections were briefly stained with hematoxylin, then with 0.1% Sirius red in saturated picric acid (Electron Microscopy Sciences) for 1 h,

washed in 5% vol/vol glacial acetic acid, and then dehydrated in ethanol/xylene before mounting. For Alizarin red staining, sections were rinsed in water, placed in 2% Alizarin Red S (pH 4.2) for 5 min, dipped 20 times in acetone followed by acetone:xylene (1:1), and then mounted. For Alkaline Phosphatase staining, sections were incubated with BCIP/NBT solution (Sigma-Aldrich) per the manufacturer's instructions.

Primers for Mouse Genotyping.

To identify the Rb conditional allele we used primer 5 lox: 5 -CTCTAGATCCTCTCATTCTTC- 3 and primer 3 lox: 5 -CCTTGACCATAGCCCAGCAC- 3 . Primer Rbcre3.2 was used in conjunction with primer 5 lox to detect the recombined allele: 5 -GGTTAATGAAGGACTGGG- 3 . To identify the p53 conditional allele we used primer p53A: 5 -CACAAAAACAGGTTAAACCCAG-3 and primer p53B: 5 -AGCACATAGGAGGCAGAGAC-3 . The recombined allele was detected using primer p53A in conjunction with primer p53D: 5 -GAAGACAGAAAAGGGGAGGG-3 . To determine the presence of the Osx1-GFP::Cre transgene we used primers TGCK5 : 5 -GCCAGGCAGGTGCCTGGACAT- 3 and Osx-10(3): 5 -CTCTTCATGAGGAGGACCCT- 3.

Isolation and Analysis of OS Cell Lines and MSC/MPCs.

Osteosarcomas were dissected, minced, filtered through a 70-μm filter, and plated in normal growth medium (10% FBS in DMEM, 1% P/S, l-glutamine) to generate the OS cell lines. Cells were passaged as they reached confluence. For differentiation into bone and fat, cells were plated, allowed to reach confluence, and induced to differentiate as described in (Mukherjee et al, 2008). For RNA purification, cells were rinsed two times with PBS, and RNA extraction was performed by using the RNeasy kit (Qiagen). Gene

expression was performed by SYBR-Green quantitative RT-PCR, using Ubiquitin mRNA to normalize RNA inputs. Primers used for qRT-PCR and mouse genotyping are shown in SI Experimental Procedures and Table S4.

MSC/MPCs were generated as described in (Mukherjee et al, 2008). Conditional MSC/MPCs were infected with Ad5CMVCre-eGFP at ≈100 pfu per cell (University of Iowa Gene Transfer Vector Core). FACS analysis of OS and MSC/MPCs was performed on a FACSCalibur HTS (Becton-Dickinson) using ScaI and Cd45 antibodies (BD Pharmigen). For transplant assays, 105–106 unsegregated or sorted cells were injected either s.c. or i.v. into NOD/SCID mice. Moribund animals were euthanized, and tumors were collected for further experiments.

SUPPORTING INFORMATION

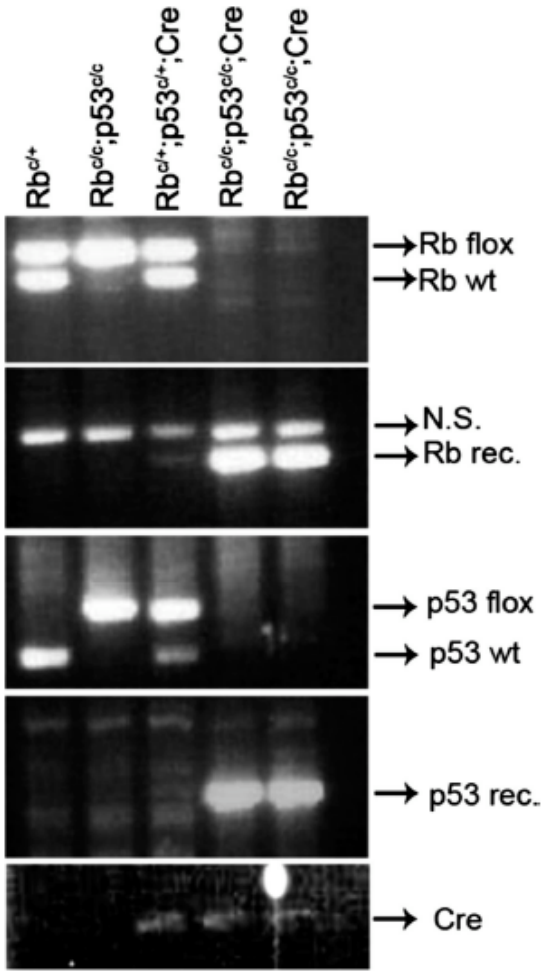

Fig. S1. Analysis of *Rb* and *p53* recombination in DKO osteosarcomas.

PCR genotyping was used to test for the efficiency of Cre-mediated recombination. As controls, DNA from $Rb^{c/c}$, $Rb^{c/c};p53^{c/c}$, and $Rb^{c/c};p53^{c/c}$ *Osx*-cre mice were used. DNA for the left three samples was extracted from ear-clips and from osteosarcoma cell lines for the right two samples. All the bands migrated at the expected sizes. The recombined (rec) bands for *Rb* (second row) and *p53* (fourth row) correlate with the presence of the *Osx-cre* transgene (fifth row). NS, nonspecific band.

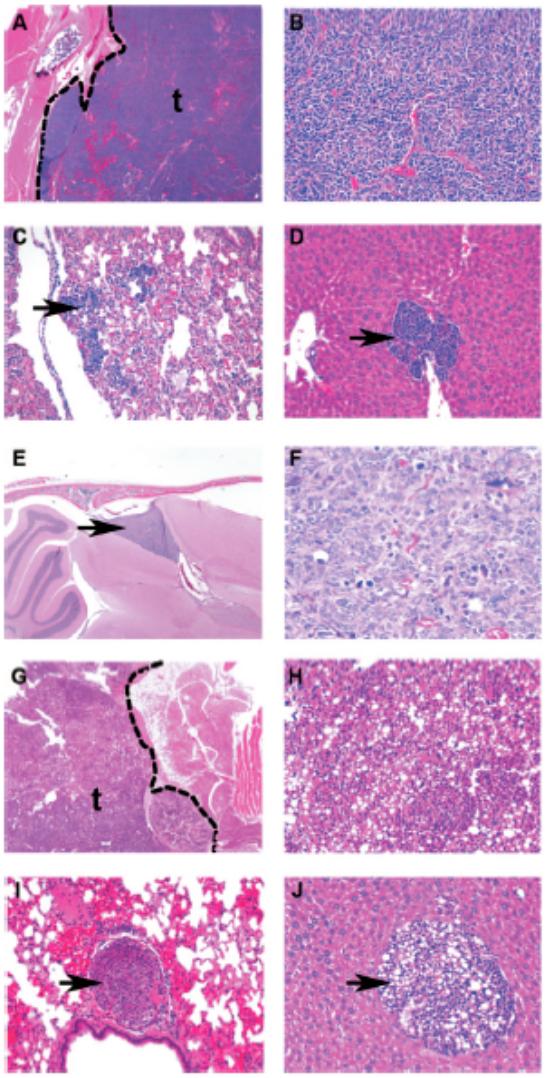

Fig. S2. Histological analyses of neuroendocrine tumors and hibernomas arising in DKO mice.

(A and B) A typical neuroendocrine tumor located near the neck, showing densely packed cells with little cytoplasm, oval nuclei, and granular nucleoplasm surrounded by fibrovascular stroma. Neuroendocrine tumor cells metastasize to both the lung (C) and liver (D). (E and F) A neuroendocrine tumor located in the brain (arrows), likely a pinealoma. (G) Example of a hibernoma located on the back near the forelimbs. Hibernomas were comprised of sheets of malignant vacuolated cells containing polymorphic nuclei and lipid droplets of varying sizes, sometimes arranged in small clusters surrounded by fibrous stroma. In many cases, multinucleate giant cells were also present. (H) Hibernoma metastasis located in the lung (I) and liver (J). All sections were stained with H&E, and t indicates tumor. (Magnification: A, E, and G 2; B–D, F, H–J, 40.)

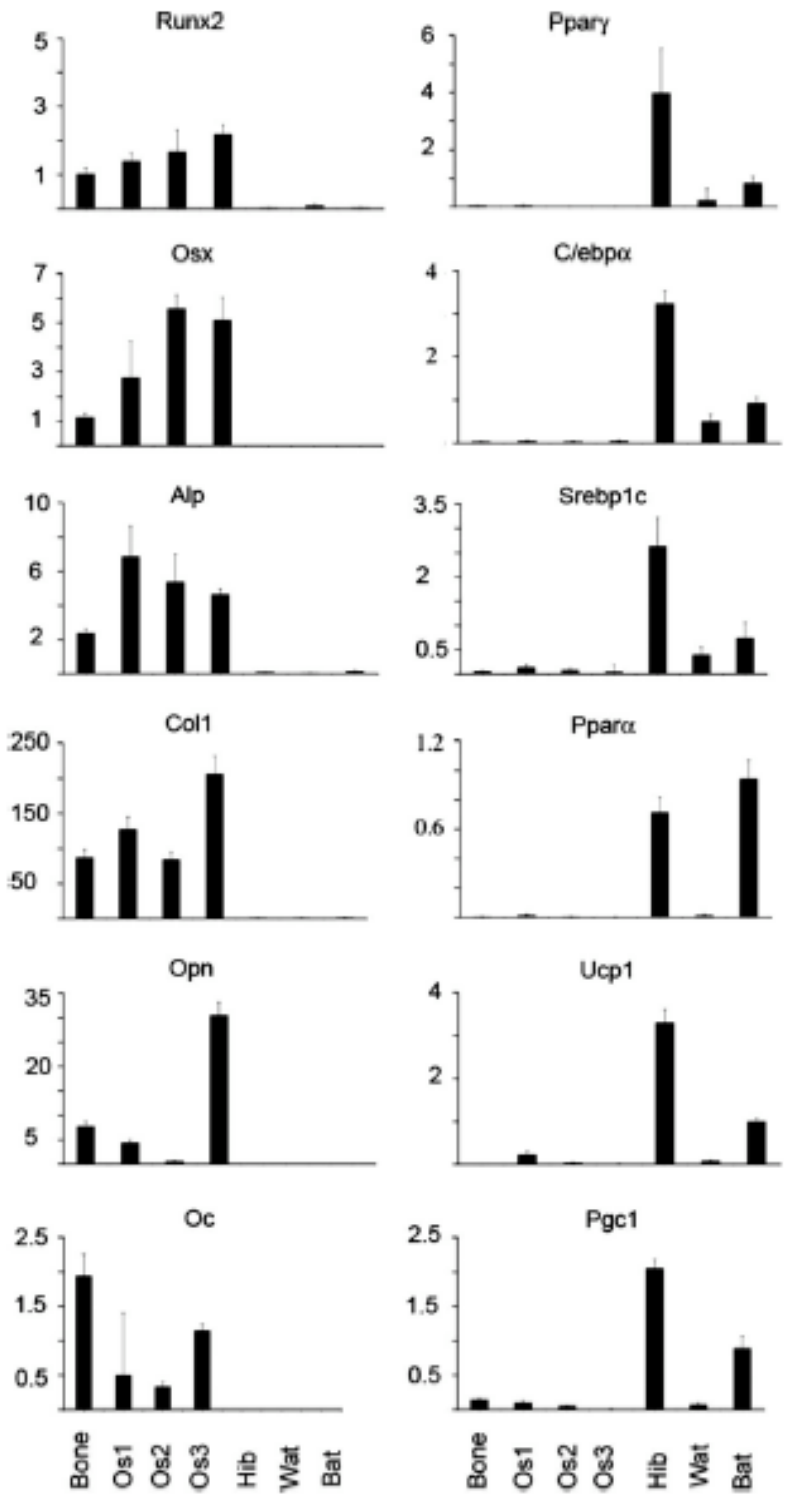
Runx2
Pparγ
Osx
C/ebpα
Alp
Srebp1c
Col1
Pparα
Opn
Ucp1
Oc
Pgc1
Bone
Os1
Os2
Os3
Hib
Wat
Bat

Fig. S3. Analyses of gene expression in osteosarcomas derived from $Rb^{c/c}$;$p53^{c/c}$;Cre^{+} mice.

qRT-PCR was used to assess the expression levels of the indicated genes in normal bone (Bone), three independent osteosarcomas (OS1–3) and, as controls, a hibernoma (Hib), white adipose tissue (Wat), and brown adipose tissue (Bat). (Left) mRNA profiles of genes associated with bone differentiation, including markers of osteoblast progenitors *Runx2* and *Osterix1* (*Osx*); early stages of osteoblast differentiation, *alkaline phosphatase* (*Alp*), *collagena1* (*Col1*); and late stages of osteoblast differentiation *osteopontin* (*Opn*) and *osteocalcin* (*Oc*). Osteosarcomas expressed genes associated with the osteoblast lineage, although, in general, with slightly lower levels of late differentiation markers. (Right) mRNA profiles of genes associated with adipogenesis (Pparγ, C/ebpα, and Srebp1c) and genes specific for brown adipose tissue (Pparγ, Ucp1, and Pgc1).

Table S1. Frequency of key mutant genotypes arising from $pRb^{+/c};p53^{+/c};Cre^{+}$ x $pRb^{c/c};p53^{c/c}$ crosses

Genotype (Cre^{+})	$pRb^{+/c};p53^{+/c}$	$pRb^{c/c};p53^{+/c}$	$pRb^{+/c};p53^{c/c}$	$pRb^{c/c};p53^{c/c}$
Observed	43	50	43	35
Expected %	25	25	25	25
Observed %	25.1	29.2	25.1	20.5

Animals were first genotyped for *Cre*, and positives were then genotyped for *Rb* and *p53* alleles.

Table S2. Location and number of metastases arising from primary osteosarcomas, neuroendocrine tumors and hibernomas

	Osteosarcoma mets						Neuroendocrine mets				Hibernoma mets			
Genotype	Lv	Lu	Ad	Ov	Kd	Spl	Lv	Lu	Spl	BM	Lv	Lu	BM	Kd
$Rb^{o/c}$	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
$p53^{o/c}$	5	2				3								
$Rb^{+/c};p53^{+/c}$	3	2	1	1										
$Rb^{o/c};p53^{+/c}$	1	4			2									
$Rb^{+/c};p53^{c/c}$	8	3			1		1							
$Rb^{o/c};p53^{o/c}$ (DKO)	5	5			1		3	4	1	1	2	4	1	1

Lv, liver; Lu, lung; Ad, adrenal; Ov, ovary; BM, bone marrow; Spl, spleen; Kd, kidney.

Table S3. Tumorigenicity of OS and MSC/MPC cells in immunocompromised mice

Cells injected	Type of Injection	Tumors	Tumor histology	Mice with mets	Avg age of euthanisation, days
DKO-OS-985	s.c.	4/4	Osteosarcoma	4/4	46
DKO-OS-985	i.v.	3/3	Osteosarcoma	N/A	50.7
DKO-OS-2380	s.c.	2/2	Osteosarcoma	1/2	69
DKO-OS-2380	i.v.	2/2	Osteosarcoma	N/A	63
DKO-OS-2674	s.c.	2/2	Osteosarcoma	1/2	71
DKO-OS-Sca1^{high}	s.c.	2/2	Osteosarcoma	0/2	48
DKO-OS-Sca1$^{low/-}$	s.c	1/2	Osteosarcoma	0/2	48
flox MSC/MPC + Ad-CRE	s.c.	2/2	Osteosarcoma	0/2	50
flox MSC/MPC	s.c.	0/1	No Tumor	N/A	>50

Table S4. Primers for qRT-PCR

	Primer sequences	
mRNA	Forward	Reverse
Alk Phos	TCTCCAGACCCTGCAACCTC	CATCCTGAGCAGACCTGGTC
Col-1a	CGAGTCACACCGAACTTGG	GCAGGCAGGGCCAATGTCTA
Osteocalcin	CTCTGTCTCTCTGACCTCACAG	CAGGTCCTAAATAGTGATACCG
Osteopontin	TGCTTTTGCCTGTTTGGCAT	TTCTGTGGCGCAAGGAGATT
Osterix	GCAAGGCTTCGCATCTGAAA	AACTTCTTCTCCCGGGTGTGA
Runx2	TGAGATTTGTGGGCCGGA	TCTGTGCCTTCTTGGTTCCC
Ubiquitin	TGGCTATTAATTATTCGGTCTGCAT	GCAAGTGGCTAGAGTGCAGAGTAA
PPARg	GAGCTGACCCAATGGTTGCTG	GCTTCAATCGGATGGTTCTTC
C/EBPa	CAAGAACAGCAACGAGTACCG	GTCACTGGTCAACTCCAGCAC
SREBP-1c	GGAGCCATGGATTGCACATT	GCTTCCAGAGAGGAGGCCAG
UCP-1	AGCCGGCTTAATGACTGGAG	TCTGTAGGCTGCCCAATGAAC
PGC-1	GTCCTCACAGAGACACTGGA	TGGTTCTGAGTGCTAAGACC

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REFERENCES

Bell DW, Varley JM, Szydlo TE, Kang DH, Wahrer DC, Shannon KE, Lubratovich M, Verselis SJ, Isselbacher KJ, Fraumeni JF, Birch JM, Li FP, Garber JE, Haber DA (1999) Heterozygous germ line hCHK2 mutations in Li-Fraumeni syndrome. Science 286(5449): 2528-2531

Berman SD, Yuan TL, Miller ES, Lee EY, Caron A, Lees JA (2008) The retinoblastoma protein tumor suppressor is important for appropriate osteoblast differentiation and bone development. Mol Cancer Res 6(9): 1440-1451

Clark JC, Dass CR, Choong PF (2008) A review of clinical and molecular prognostic factors in osteosarcoma. J Cancer Res Clin Oncol 134(3): 281-297

Dannenberg JH, Schuijff L, Dekker M, van der Valk M, te Riele H (2004) Tissue-specific tumor suppressor activity of retinoblastoma gene homologs p107 and p130. Genes Dev 18(23): 2952-2962

Feugeas O, Guriec N, Babin-Boilletot A, Marcellin L, Simon P, Babin S, Thyss A, Hofman P, Terrier P, Kalifa C, Brunat-Mentigny M, Patricot LM, Oberling F (1996) Loss of heterozygosity of the RB gene is a poor prognostic factor in patients with osteosarcoma. J Clin Oncol 14(2): 467-472

Glatt V, Canalis E, Stadmeyer L, Bouxsein ML (2007) Age-related changes in trabecular architecture differ in female and male C57BL/6J mice. J Bone Miner Res 22(8): 1197-1207

Gurney JG, Severson RK, Davis S, Robison LL (1995) Incidence of cancer in children in the United States. Sex-, race-, and 1-year age-specific rates by histologic type. Cancer 75(8): 2186-2195

Iwakuma T, Lozano G, Flores ER (2005) Li-Fraumeni syndrome: a p53 family affair. Cell Cycle 4(7): 865-867

Jonkers J, Meuwissen R, van der Gulden H, Peterse H, van der Valk M, Berns A (2001) Synergistic tumor suppressor activity of BRCA2 and p53 in a conditional mouse model for breast cancer. Nat Genet 29(4): 418-425

Kansara M, Thomas DM (2007) Molecular pathogenesis of osteosarcoma. DNA Cell Biol 26(1): 1-18

Krivtsov AV, Twomey D, Feng Z, Stubbs MC, Wang Y, Faber J, Levine JE, Wang J, Hahn WC, Gilliland DG, Golub TR, Armstrong SA (2006) Transformation from committed progenitor to leukaemia stem cell initiated by MLL-AF9. Nature 442(7104): 818-822

Lengner CJ, Steinman HA, Gagnon J, Smith TW, Henderson JE, Kream BE, Stein GS, Lian JB, Jones SN (2006) Osteoblast differentiation and skeletal development are regulated by Mdm2-p53 signaling. J Cell Biol 172(6): 909-921

Malkin D (1993) p53 and the Li-Fraumeni syndrome. Cancer Genet Cytogenet 66(2): 83-92

Mukherjee S, Raje N, Schoonmaker JA, Liu JC, Hideshima T, Wein MN, Jones DC, Vallet S, Bouxsein ML, Pozzi S, Chhetri S, Seo YD, Aronson JP, Patel C, Fulciniti M, Purton LE, Glimcher LH, Lian JB, Stein G, Anderson KC, Scadden DT (2008) Pharmacologic targeting of a stem/progenitor population in vivo is associated with enhanced bone regeneration in mice. J Clin Invest 118(2): 491-504

Rodda SJ, McMahon AP (2006) Distinct roles for Hedgehog and canonical Wnt signaling in specification, differentiation and maintenance of osteoblast progenitors. Development 133(16): 3231-3244

Sage J, Miller AL, Perez-Mancera PA, Wysocki JM, Jacks T (2003) Acute mutation of retinoblastoma gene function is sufficient for cell cycle re-entry. Nature 424(6945): 223-228

Tataria M, Quarto N, Longaker MT, Sylvester KG (2006) Absence of the p53 tumor suppressor gene promotes osteogenesis in mesenchymal stem cells. J Pediatr Surg 41(4): 624-632; discussion 624-632

Thomas DM, Carty SA, Piscopo DM, Lee JS, Wang WF, Forrester WC, Hinds PW (2001) The retinoblastoma protein acts as a transcriptional coactivator required for osteogenic differentiation. Mol Cell 8(2): 303-316

Tirode F, Laud-Duval K, Prieur A, Delorme B, Charbord P, Delattre O (2007) Mesenchymal stem cell features of Ewing tumors. Cancer Cell 11(5): 421-429

Toguchida J, Ishizaki K, Sasaki MS, Ikenaga M, Sugimoto M, Kotoura Y, Yamamuro T (1988) Chromosomal reorganization for the expression of recessive mutation of retinoblastoma susceptibility gene in the development of osteosarcoma. Cancer Res 48(14): 3939-3943

Tsuchiya T, Sekine K, Hinohara S, Namiki T, Nobori T, Kaneko Y (2000) Analysis of the p16INK4, p14ARF, p15, TP53, and MDM2 genes and their prognostic implications in osteosarcoma and Ewing sarcoma. Cancer Genet Cytogenet 120(2): 91-98

Vooijs M, Berns A (1999) Developmental defects and tumor predisposition in Rb mutant mice. Oncogene 18(38): 5293-5303

Wang X, Kua HY, Hu Y, Guo K, Zeng Q, Wu Q, Ng HH, Karsenty G, de Crombrugghe B, Yeh J, Li B (2006) p53 functions as a negative regulator of osteoblastogenesis, osteoblast-dependent osteoclastogenesis, and bone remodeling. J Cell Biol 172(1): 115-125

Williams BO, Remington L, Albert DM, Mukai S, Bronson RT, Jacks T (1994) Cooperative tumorigenic effects of germline mutations in Rb and p53. Nat Genet 7(4): 480-484

Wunder JS, Gokgoz N, Parkes R, Bull SB, Eskandarian S, Davis AM, Beauchamp CP, Conrad EU, Grimer RJ, Healey JH, Malkin D, Mangham DC, Rock MJ, Bell RS, Andrulis IL (2005) TP53 mutations and outcome in osteosarcoma: a prospective, multicenter study. J Clin Oncol 23(7): 1483-1490

Appendix B

The Retinoblastoma Protein Tumor Suppressor Is Important for Appropriate Osteoblast Differentiation and Bone Development

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Mol Cancer Res 6(9): 140-1451, 2008

Author's Contributions: Supplemental Figure 2

ABSTRACT

Mutation of the retinoblastoma (*RB*) tumor suppressor gene is strongly linked to osteosarcoma formation. This observation and the documented interaction between the retinoblastoma protein (pRb) and Runx2 suggests that pRb is important in bone development. To assess this hypothesis, we used a conditional knockout strategy to generate pRb-deficient embryos that survive to birth. Analysis of these embryos shows that *Rb* inactivation causes the abnormal development and impaired ossification of several bones, correlating with an impairment in osteoblast differentiation. We further show that *Rb* inactivation acts to promote osteoblast differentiation *in vitro* and, through conditional analysis, establish that this occurs in a cell-intrinsic manner. Although these *in vivo* and *in vitro* differentiation phenotypes seem paradoxical, we find that Rb-deficient osteoblasts have an impaired ability to exit the cell cycle both in vivo and in vitro that can explain the observed differentiation defects. Consistent with this observation, we show that the cell cycle and the bone defects in *Rb*-deficient embryos can be suppressed by deletion of *E2f1*, a known proliferation inducer that acts downstream of *Rb*. Thus, we conclude that pRb plays a key role in regulating osteoblast differentiation by mediating the inhibition of E2F and consequently promoting cell cycle exit.

INTRODUCTION

The first tumor suppressor to be cloned was the retinoblastoma gene, *RB*. *RB* is mutated in approximately one-third of all sporadic human tumors, but there is strong correlation with certain tumor types. Specifically, *RB* mutations are observed in almost all retinoblastomas (Weinberg, 1992) and also in a large percentage of osteosarcomas and small cell lung carcinomas. For patients who carry germ line *RB* mutations, osteosarcoma is the second most common tumor type after retinoblastoma (Gurney et al, 1995). Overall, >70% of osteosarcomas show a molecular change or mutation at the *RB* locus (Belchis et al, 1996; Feugeas et al, 1996).

The gene product, pRb, belongs to a family of proteins, including p107 and p130, termed the pocket proteins, although only pRb has been shown to possess significant tumorsuppressive properties (Lipinski & Jacks, 1999). The best characterized role of pRb is its regulation of cell cycle progression. Overexpression of pRb causes G1 cell cycle arrest (Huang et al, 1988), whereas acute ablation of pRb induces cell cycle re-entry in quiescent cells (Sage et al, 2003). To execute its cell cycle–inhibitory function, hypophosphorylated pRb binds to and inhibits the E2F family of transcription factors (Trimarchi & Lees, 2002). During G1, pRb becomes hyperphosphorylated by the cyclin D-cdk4/6 complex and subsequently by cyclin E-cdk2. This phosphorylation releases the E2Fs from pRb to induce the transcription of cellular genes essential for S phase entry and cell division.

The analyses of *in vivo* mouse models and *in vitro* experiments show that pRb is required for the differentiation of specific tissues. In erythropoiesis, the loss of *Rb* results in

inefficient enucleation and incomplete terminal differentiation of erythroid cells (Clark et al, 2004; Spike et al, 2004). In skeletal muscle, pRb is required for proper cell cycle exit and differentiation (Huh et al, 2004). Conditional deletion of *Rb* in the intestine causes increased proliferation and abnormal expression of differentiation markers (Haigis et al, 2006; Yang & Hinds, 2007). The loss of pRb affects the normal expression of differentiation genes, such as β- and γ-crystallines, in the lens (Morgenbesser et al, 1994). These deficiencies in differentiation seem to be due, at least partially, to a defect in cell cycle exit, a step believed to be required in most differentiation pathways. However, this does not rule out the possibility that pRb contributes to differentiation in a more distinct and specific manner. Notably, pRb binds to NRP/B, a protein up-regulated during neuronal differentiation and involved in neuronal process formation (Kim et al, 1998). Relevant to this, other markers of neuronal differentiation are decreased in the *Rb*-deficient embryo (Lee et al, 1994). With respect to fat cells, pRb physically interacts with CAAT/enhancer binding protein-β, and the loss of this interaction inhibits adipocyte differentiation (Chen et al, 1996).

Several studies implicate a role for pRb in osteoblast differentiation. SV40-derived large T-antigen, which targets the pocket proteins, prevents the differentiation of stromal cell lines into osteoblasts (Feuerbach et al, 1997). The adenoviral E1A 12S protein also represses osteoblast differentiation, and this is dependent on a functional E1A pocket protein-binding domain (Beck et al, 1998). Most striking is the finding that in immortalized cell lines, pRb physically interacts with Runx2/CBFA1, one of the transcription factors essential for osteoblast differentiation (Luan et al, 2007; Thomas et al, 2001). This latter

observation suggests that pRb may play a role in osteoblast differentiation that is independent of cell cycle regulation.

Determining the role of pRb in osteoblast differentiation *in vivo* may ultimately provide some important insights concerning the high prevalence of *Rb* mutations in osteosarcoma. However, murine embryos deficient for pRb die between embryonic days 13.5 and 15.5 (Clarke et al, 1992; Jacks et al, 1992; Lee et al, 1992). This early lethality has thus far precluded the study of pRb in bone development, which primarily does not occur until embryonic day 15.5 in mice. To circumvent this problem, we generated a conditional *Rb* mouse strain that allows pRb-deficient embryos to survive until birth. This mouse model has enabled us to perform *in vitro* and *in vivo* studies to determine the effects of pRb loss in osteoblast differentiation and bone development.

RESULTS

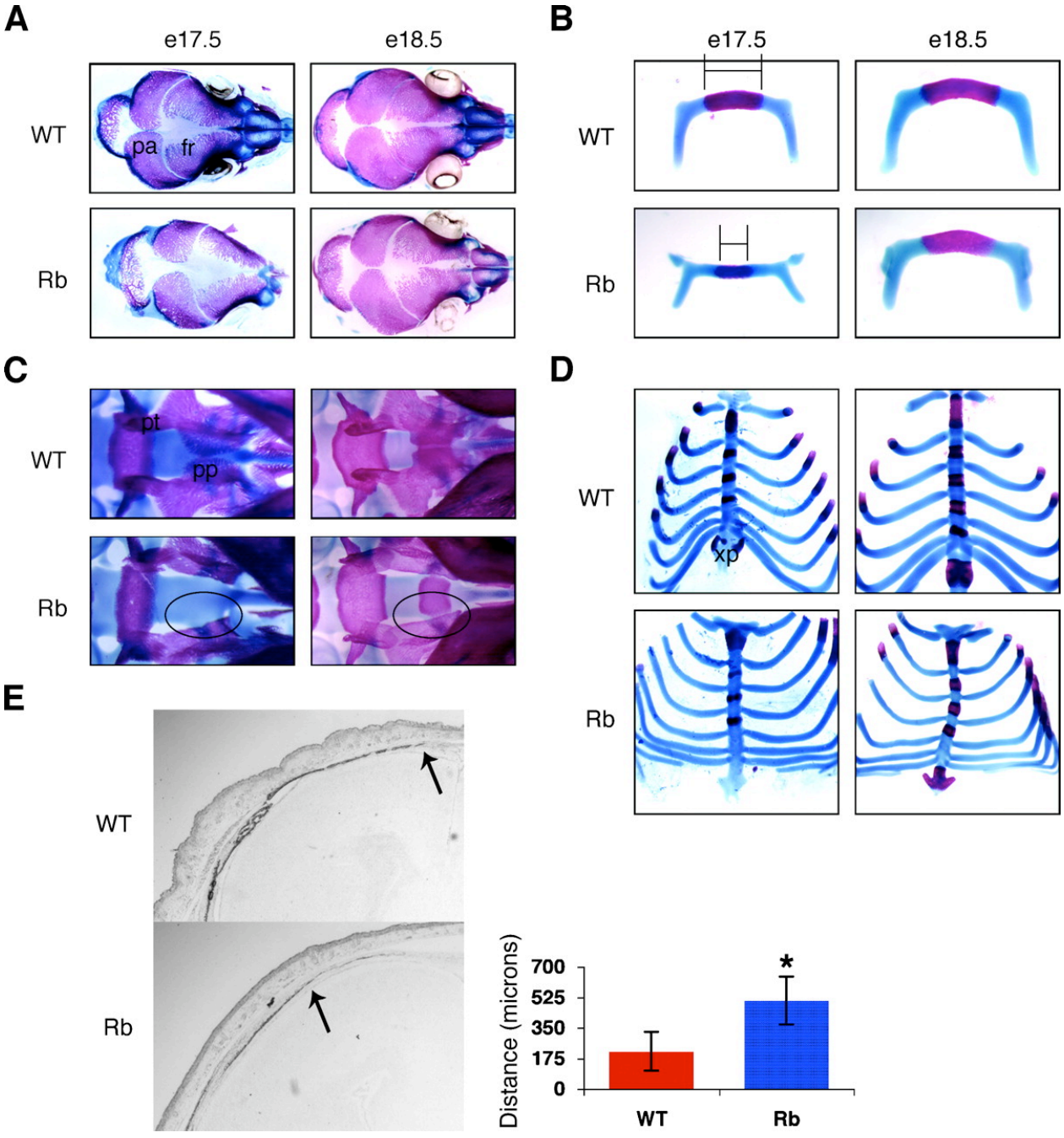
pRb-Deficient Embryos Exhibit Bone Defects during Development

The retinoblastoma gene, *RB*, is mutated in a large proportion of osteosarcomas. I*n vitro* studies suggest that *Rb* may play a direct role in bone development (Beck et al, 1998; Feuerbach et al, 1997; Thomas et al, 2001), but this has not been examined *in vivo*. The germ line $Rb^{-/-}$ mice die in mid-gestation (between embryonic days 13.5 and 15.5), prior to the formation of most bones. However, recent studies show that this mid-gestational lethality results from a placental defect (Wenzel et al, 2007; Wu et al, 2003). Thus, we generated a conditional mouse strain that allows *Rb* mutant embryos to develop in the presence of a wildtype placenta. Specifically, we crossed an *Rb* mutant mouse line with loxP sites flanking the third exon of *Rb* ($Rb^{c/c}$; (Sage et al, 2003)) with a *Mox2-Cre* transgenic line ($Mox2^{+/Cre}$) that expresses the Cre recombinase in the embryo proper, but not in the placenta, beginning approximately at embryonic day 6.5 (Tallquist & Soriano, 2000). The resulting $Mox2^{+/Cre}$ conditionally null *Rb* embryos ($Rb^{c-/-c-}$) survive until birth, allowing us to assess pRb's role in bone development. Importantly, we observed no difference between wild-type embryos or cells ($Rb^{+/c}$; $Mox2^{+/+}$) and heterozygous animals or cells ($Rb^{+/c}$;$Mox2^{+/Cre}$) in any of our *in vivo* or *in vitro* experiments, and therefore have used wild-type animals as controls in our study.

Initially, we examined skeletons of wild-type and $Rb^{c-/c-}$ embryos at embryonic day 17.5 by alizarin red staining of bone and Alcian blue staining of cartilage. Compared with wild-type littermate controls, the embryonic day 17.5 $Rb^{c-/c-}$ embryos displayed less ossification in a variety of bones (Fig. 1). These include the frontal and parietal calvarial

bones of the skull (Fig. 1A) that arise through intramembranous ossification and the hyoid bone (Fig. 1B) that develops by endochondral ossification. These defects were partially penetrant, as 9 of 13 $Rb^{c-/c-}$ embryos exhibited the decreased ossification. Moreover, other bones in the $Rb^{c-/c-}$ embryos, including the pterygoid bone and palatine process in the head and the xiphoid process of the sternum, were appropriately ossified but showed an abnormal structure (Fig. 1C and D). These abnormal structures were observed in all 13 $Rb^{c-/c-}$ embryos examined. Finally, several other bones such as the long bones of the forelimbs and hind limbs did not exhibit any differing phenotypes between the $Rb^{c-/c-}$ and wild-type embryos. It is possible that certain embryonic bones, such as the limbs, are less susceptible to the effects of *Rb* loss than others, perhaps due to the compensation effects of p107 and p130. Alternatively, the *Mox2-Cre* transgene may be less efficient in some settings.

To further explore the defects that were observed in the $Rb^{c-/c-}$ embryos, we examined the skeletons of mutant embryos at other developmental stages. At earlier time points, embryonic days 15.5 and 16.5, the $Rb^{c-/c-}$ embryos displayed all of the bone defects described above (data not shown). At the later time points, embryonic days 18.5 and 19.5/birth, the phenotype was altered somewhat: we still observed aberrantly developed bones, such as the pterygoid, palatine process, and xiphoid process (Fig. 1C and D; data not shown) with nearly complete penetrance (seven of eight embryonic day 18.5 $Rb^{c-/c-}$ embryos). However, we observed a similar alizarin red staining in the calvaria and hyoid bone of $Rb^{c-/c-}$ embryos versus wild-type littermate controls (Fig. 1A and B; data


A
e17.5
e18.5
WT
Rb
pa
fr
B
e17.5
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C
WT
Rb
pt
pp
D
WT
Rb
xp
E
WT
Rb
Distance (microns)
700
525
350
175
0
WT
Rb
*

Figure 1. Deletion of *Rb* causes defects in embryonic bone development.

A to D. Alizarin red (bone) and Alcian blue (cartilage) staining of embryos. Embryonic day 17.5 $Rb^{c-/c-}$ mice exhibit less ossification in the cranium (A) and hyoid bone (B). Bar, the difference in hyoid bone ossification at embryonic day 17.5 (B). $Rb^{c-/c-}$ embryos at embryonic days 17.5 and 18.5 display aberrant formation of bones in the head (ventral view of head in C) and sternum (D). The aberrantly shaped or missing palatine process in the $Rb^{c-/c-}$ embryos is circled in C. E. Pregnant mothers were injected at embryonic day 18 with calcein for 12 h. Coronal sections of the frontal bone of embryonic day 18.5 mice were analyzed for calcein incorporation. $Rb^{c-/c-}$ embryos incorporate less calcein than their wild-type littermates. Original magnification, ×2. The distance from the front of calcein incorporation (arrow) to the midline of the suture was measured in nine $Rb^{c-/c-}$ and nine wild-type embryo sections. Columns, mean; bars, 1 SD; *, $P < 0.001$, statistically significant difference. Abbreviations: fr, frontal bone; pa, parietal bone; pp, palatine process; pt, pterygoid bone; xp, xiphoid process. WT, $Rb^{+/c}$;$Mox2^{+/+}$; Rb, $Rb^{c-/c-}$;$Mox2^{+/Cre}$.

not shown). We considered two explanations for this latter observation. The first possibility was that pRb loss initially impaired or delayed bone differentiation, but this defect was then corrected by acceleration in the rate of bone deposition after embryonic day 17.5. The second possibility was that pRb loss impaired bone differentiation at all developmental stages, but this impairment was not apparent at later time points because the alizarin red detection method is more qualitative than quantitative. In other words, by embryonic day 18.5, there was some ossification in the appropriate regions of the $Rb^{c-/c-}$ calvaria and hyoid bone but the level of deposited bone was still lower than in the wild-type controls. To distinguish between these two possibilities, we directly assessed the rate of new bone formation after embryonic day 18.5 using calcein incorporation. Calcein is a fluorescent compound that can be injected into an animal and is then incorporated into newly forming bones. We analyzed the amount of calcein incorporation into the frontal bone of embryonic day 18.5 embryos 12 hours after the calcein injection of pregnant females. Notably, the $Rb^{c-/c-}$ frontal bones incorporated significantly less calcein compared with wild-type littermates (Fig. 1E). Similar results were obtained when calcein was injected 12 hours prior to birth (data not shown). These data indicate that pRb loss does not cause an acceleration in frontal bone formation in the late stages of gestation. Instead, the rate of ossification remains considerably lower than that observed in wild-type embryos. Taken together, our data indicate that the loss of pRb causes a defect in the rate of ossification and/or proper formation of several bones throughout embryonic skeletal development.

The Loss of pRb Affects an Early Step in the Differentiation of Osteoblasts In vivo

Notably, pRb loss impairs the development of bones that arise through two distinct mechanisms, termed endochondral (e.g., the hyoid) and intramembranous (e.g., the calvaria) ossification. The former is influenced by three cell types: chondrocytes, which form an essential cartilage template; osteoblasts, which differentiate to secrete the bone matrix; and osteoclasts, which oppose bone formation by degrading and reabsorbing bone. In contrast, intramembranous ossification is influenced by osteoblasts and osteoclasts but occurs in a cartilage-independent manner. This fact, along with the apparently normal development of the cartilage skeleton within $Rb^{c-/c-}$ embryos (Fig. 1B-D; data not shown), suggests that a chondrocyte defect cannot fully account for the defective bone development. Therefore, we examined both osteoblast and osteoclast function. To assess osteoclast levels, we screened the frontal bones of embryonic day 17.5 embryos for the presence of tartrate-resistant acid phosphatase activity, an osteoclast specific marker. There were no active osteoclasts present in either the wild-type or the $Rb^{c-/c-}$ frontal bones (Supplementary Fig. S1). Thus, the decreased ossification in $Rb^{c-/c-}$ embryos is likely not due to either cartilage defects or increased osteoclast activity.

Given these findings, we next screened embryonic day 17.5 frontal bones for the presence of osteoblast-specific markers. Two early markers of differentiating osteoblasts are alkaline phosphatase (ALP) activity and Collagen1a1 (*Col1*) mRNA expression. The activity and expression, respectively, of these two markers were significantly decreased in the $Rb^{c-/c-}$ frontal bone compared with those in wild-type sections (Fig. 2). Moreover, the expression levels of osteopontin (*OPN*), an early to mid-differentiation marker, were also

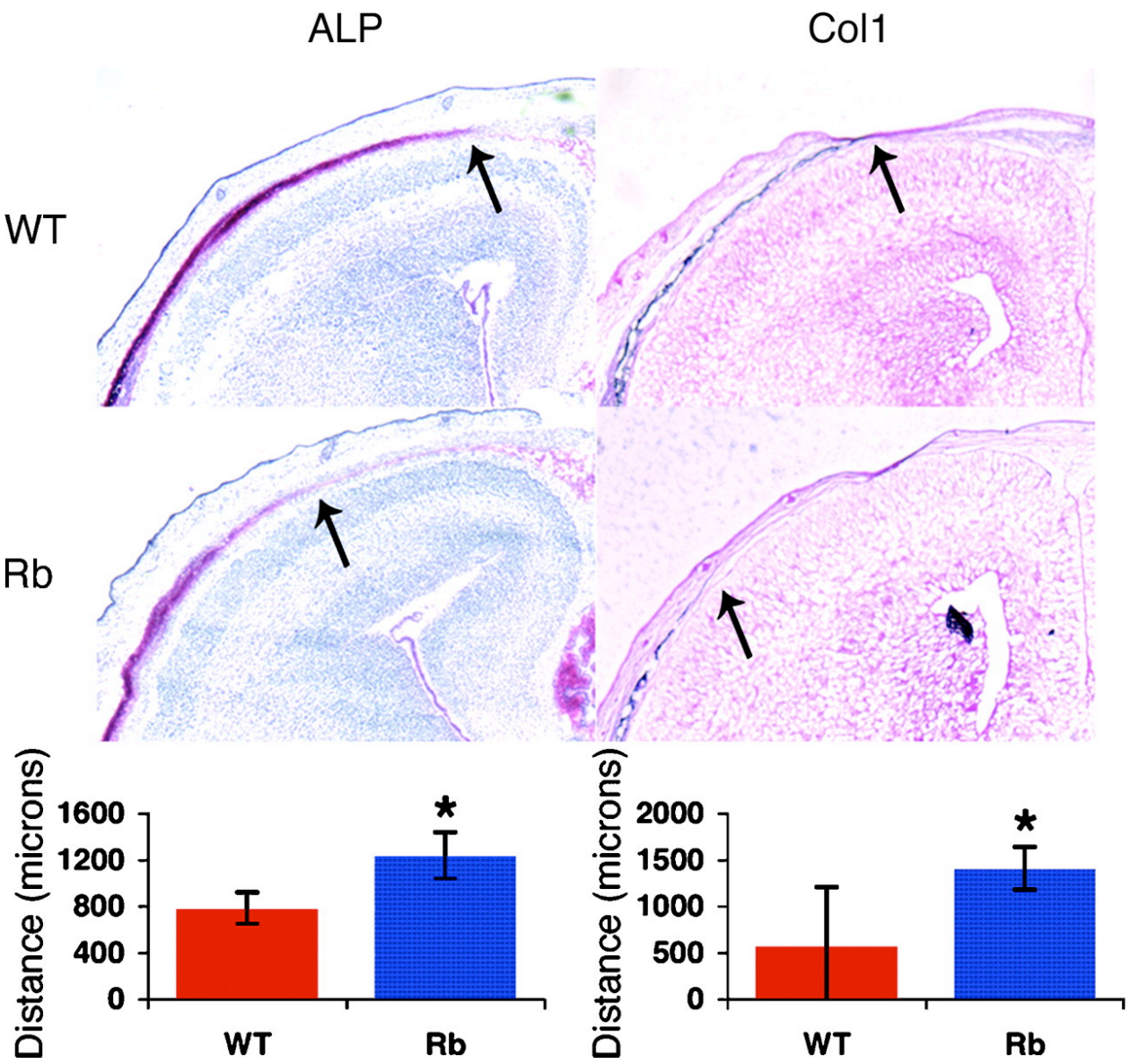


Figure 2. pRb-deficient frontal bones display decreased levels of osteoblast markers.

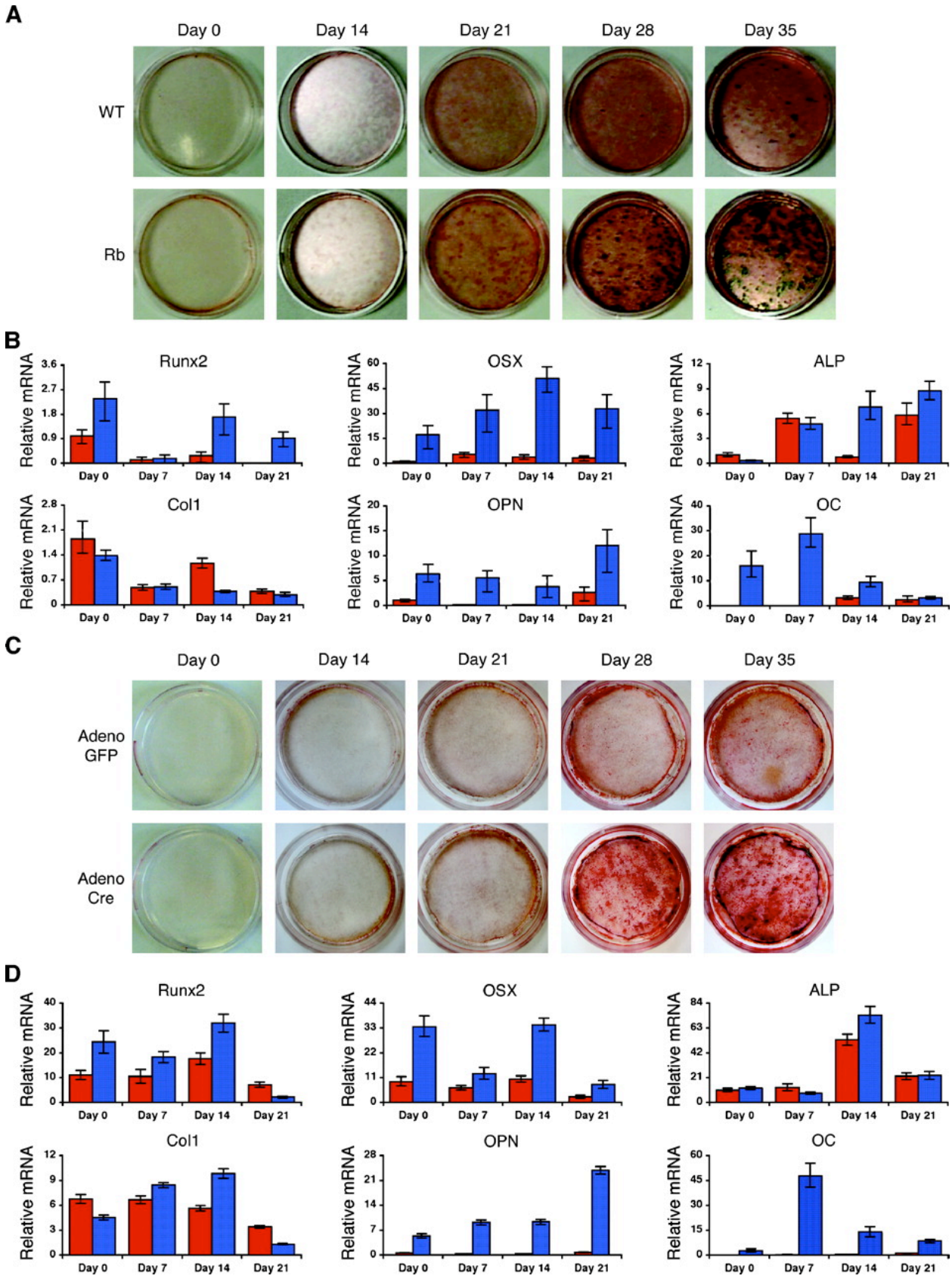
Coronal sections of frontal bones from embryonic day 17.5 embryos were assessed by histochemical analysis of alkaline phosphatase activity (left column) and *in situ* analysis of *Collagen1a1* mRNA (right column). $Rb^{c-/c-}$ frontal bone sections (bottom row) exhibit decreased levels of both markers compared with wild-type (top row). Original magnification, ×2. The distance from the front of activity or expression (arrows) to the midline of the suture was measured in at least 8 embryo pairs for *Col1* and in 12 pairs for ALP. Columns, mean; bars, 1 SD; *, $P < 0.01$, statistically significant difference. WT, $Rb^{+/c}$;$Mox2^{+/+}$; Rb, $Rb^{c-/c-}$;$Mox2^{+/Cre}$.

typically downregulated in the $Rb^{c-/c-}$ embryos relative to wild-type controls (data not shown). These data indicate that osteoblast differentiation is perturbed in $Rb^{c-/c-}$ embryos at the earliest stages of the pathway.

pRb-Deficient Osteoblasts Differentiate to a Greater Extent than Wild-type Cells In vitro

Our *in vivo* data show that an early step in osteoblast differentiation is affected. One possibility is that pRb regulates osteoblast differentiation directly. For example, it has been reported previously that pRb can interact with and co-activate *Runx2/CBFA1*, one of the transcription factors essential for osteoblast differentiation (Luan et al, 2007; Thomas et al, 2001). To further dissect the role of pRb in osteoblast differentiation, we used a well-defined and often used *in vitro* osteoblast differentiation system. Specifically, primary cells were isolated from the calvaria of wild-type and $Rb^{c-/c-}$ embryos and expanded. Two hundred and fifty thousand cells were plated onto 3-cm tissue culture dishes and then induced to differentiate upon confluency. In this system, bone-like calcium deposits are secreted by fully differentiated osteoblasts and can be analyzed by alizarin red staining. Based on our *in vivo* data and previous *in vitro* differentiation studies with fibroblasts (Thomas et al, 2001), we anticipated that $Rb^{c-/c-}$ osteoblasts would differentiate to a lesser extent than wild-type cells. Contrary to this hypothesis, however, the $Rb^{c-/c-}$ osteoblasts secreted a greater number of calcium deposits than wild-type osteoblasts based on the alizarin red staining (Fig. 3A).

We then used quantitative real-time PCR (RT-PCR) to analyze the mRNA levels of several osteoblast markers during the differentiation of these cells. Although the


A
Day 0
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OSX
ALP
Col1
OPN
OC
Relative mRNA
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Day 7
Day 14
Day 21
C
Day 0
Day 14
Day 21
Day 28
Day 35
Adeno GFP
Adeno Cre
D
Runx2
OSX
ALP
Col1
OPN
OC
Relative mRNA
Day 0
Day 7
Day 14
Day 21

Figure 3. *Rb*$^{c-/c-}$ primary osteoblasts differentiate to a greater extent than wild-type.

A. Terminal differentiation of primary calvarial osteoblasts was determined by alizarin red staining of secreted calcium deposits from 0 to 35 d. *Rb*$^{c-/c-}$ osteoblasts (bottom row) secrete a greater number of calcium deposits than wild-type osteoblasts (top row). B. Quantitative RT-PCR results of bone marker expression levels from wild-type (red columns) and *Rb*$^{c-/c-}$ (blue columns) osteoblasts during differentiation. *Rb*$^{c-/c-}$ osteoblasts express greater mRNA levels of *Runx2*, osterix, osteopontin, and osteocalcin but not alkaline phosphatase or Collagen1a1 compared with wild-type osteoblasts. Ubiquitin was used as an internal control to normalize for RNA levels within the samples. Each time point is an average of four reactions. Columns, results from a representative littermate pair; bars, 1 SD. WT, *Rb*$^{+/c}$;*Mox2*$^{+/+}$; Rb, *Rb*$^{c-/c-}$;*Mox2*$^{+/Cre}$. C. *Rb*$^{c/c}$ primary calvarial osteoblasts were infected with adenovirus expressing either the Cre recombinase enzyme or green fluorescent protein 2 d prior to differentiation. Terminal differentiation was assessed by alizarin red staining. Rbc/c osteoblasts acutely ablated for pRb (bottom row) secrete a greater number of calcium deposits than control-infected osteoblasts (top row). D. Quantitative RT-PCR analysis done as described in B. Osteoblasts acutely ablated for pRb (blue columns) express greater mRNA levels of *Runx2*, osterix, osteopontin, and osteocalcin but not alkaline phosphatase or Collagen1a1 compared with control-infected osteoblasts (red columns).

transcriptional levels of *Alp* and *Col1* were unchanged, the $Rb^{c-/c-}$ osteoblasts exhibited significantly greater levels of expression for several other osteoblast genes compared with the wild-type cells (Fig. 3B). Notably, *Runx2* and osterix (*OSX*), two transcription factors that are necessary to induce osteoblast differentiation (Ducy et al, 1997; Nakashima et al, 2002; Otto et al, 1997), were up-regulated in the $Rb^{c-/c-}$ cells from the earliest stages of the differentiation process (Fig. 3B). *Runx2* and *OSX* have been shown to induce the transcription of downstream osteoblast differentiation genes (Ducy et al, 1997; Nakashima et al, 2002; Wang et al, 2006). In accordance with these findings, we observed the increased expression of the early/mid- and late-differentiation markers, osteopontin (*OPN*) and osteocalcin (*OC*), respectively, in the $Rb^{c-/c-}$ osteoblasts. Together, these data suggest that osteoblasts deficient for pRb differentiate to a greater extent than wildtype cells *in vitro*, and this correlates with the increased transcriptional levels of *Runx2*, *OSX*, and their downstream targets.

Acute Ablation of pRb Promotes the Differentiation of Osteoblasts In vitro

The wildtype and $Rb^{c-/c-}$ osteoblasts were prepared on embryonic day 17.5, when there was a significant difference in the degree of calvarial differentiation (Fig. 1A). This raised the possibility that the increased *in vitro* differentiation of the $Rb^{c-/c-}$ versus wildtype cells simply reflected the presence of a larger pool of progenitor osteoblasts in the $Rb^{c-/c-}$ versus wildtype calvaria. To address this hypothesis, we isolated conditional $Rb^{c/c}$ osteoblasts. These cells were brought to confluence and then infected with either a control adenovirus containing green fluorescent protein (Adeno-GFP) or one expressing the Cre

recombinase gene (Adeno-Cre). This strategy yielded parallel populations of control and $Rb^{c-/c-}$ osteoblasts that had identical starting numbers of progenitors. Consistent with previous studies (Sage et al, 2003), we found that the Adeno-Cre was sufficient to acutely ablate pRb within 2 days of infection (data not shown). Therefore, 2 days post-infection (denoted day 0 in Figs. 3C and D and Figs. 4D-F) we placed the confluent wildtype and $Rb^{c-/c-}$ cells in differentiation media. The acutely ablated $Rb^{c-/c-}$ osteoblasts differentiated to a greater extent than the control-infected $Rb^{c/c}$ cells, just as we had observed with the germ line $Rb^{c-/c-}$ osteoblasts (compare Fig. 3C and A). Moreover, the acutely ablated $Rb^{c-/c-}$ cells expressed increased levels of *Runx2*, *OSX*, *OPN*, and *OC* relative to the Adeno-GFP–infected cells in a comparable manner to that observed in the germ line $Rb^{c-/c-}$ osteoblasts (compare Figs. 3D and B). These data show that loss of pRb acts in an intrinsic manner to increase the differentiation of primary osteoblast cultures *in vitro*.

Depletion of pRb in Progenitor Osteoblasts Causes Cell Cycle Exit Defects In vitro

We aimed to understand the molecular changes that accompanied this increased differentiation. One possibility is that pRb possesses a cell cycle–independent repressive function in osteoblast differentiation. In this manner, loss of pRb would allow for the deregulated increase in osteoblast genes such as *Runx2* and *OSX*. We have attempted several experiments to test the potential contribution of this interaction, including conducting chromatin immunoprecipitations of Runx2 at osteoblast-specific promoters in wildtype, $Rb^{c-/c-}$, and $Rb^{c/c}$; $E2f1^{-/-}$ calvarial preparations (data not shown). These studies did not yield any evidence that *Rb* loss altered Runx2 promoter -binding activity. Moreover,

we did not detect any pRb binding to the *Runx2* and *OSX* promoters. This latter, negative chromatin immunoprecipitation result is not particularly informative because pRb chromatin immunoprecipitation works poorly in murine cells. However, the *Runx2* and *OSX* promoter both lack conventional E2F binding sites. Thus, although these observations do not rule out a direct, repressive role for pRb in osteoblast differentiation *in vitro*, we have no data to support this model.

A second potential cause of the observed increase in osteoblast differentiation *in vitro* upon pRb loss may be related to cell cycle defects. Notably, the increased density of osteoblast cultures is known to enhance their differentiation (Gerber & ap Gwynn, 2001; Purpura et al, 2004). We hypothesized that loss of pRb may affect the normal confluence arrest of the calvarial cells, leading to an increase in proliferation and consequently, an increase in cell density. Thus, we compared the proliferation of wildtype versus germ line $Rb^{c-/c-}$ cells throughout the differentiation process. At all time points, we found that a higher proportion of the $Rb^{c-/c-}$ osteoblast nuclei incorporated 5-bromo-2- deoxyuridine (BrdU) compared with the wildtype controls (Fig. 4A and B). In agreement with these findings, the $Rb^{c-/c-}$ osteoblasts showed elevated levels of cyclin A and cyclin E mRNAs (Fig. 4C). Finally, total cell counts during the initiation of differentiation showed an increase in the total number of cells present in $Rb^{c-/c-}$ confluent cultures compared with wildtypes (Table 1). Similar results in all of these assays were observed in the analyses of osteoblasts acutely ablated for pRb (Fig. 4D-F; Table 1). Thus, we conclude that pRb loss increases the proliferation, and consequently, the density of confluent osteoblast cultures, thereby leading to an increase in primary calvarial osteoblast differentiation *in vitro*. Notably, the

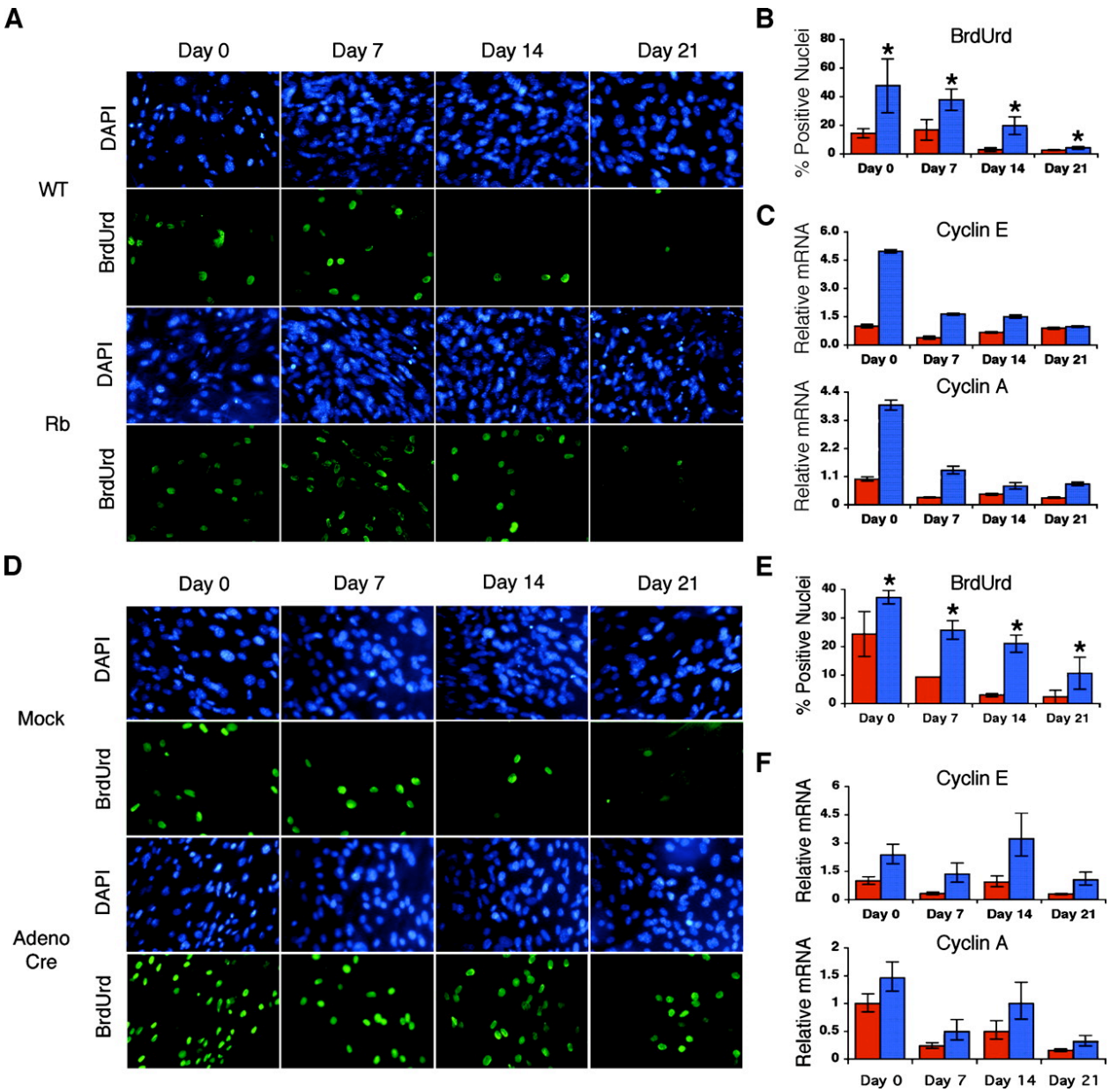

A
Day 0
Day 7
Day 14
Day 21
DAPI
BrdUrd
WT
Rb
B
BrdUrd
% Positive Nuclei
C
Cyclin E
Cyclin A
Relative mRNA
D
Mock
Adeno Cre
E
F

Figure 4. Confluent osteoblasts *in vitro* exhibit excess proliferation upon loss of pRb.

A. Immunofluorescence analysis of BrdU incorporation in differentiating osteoblasts. Wild-type (top two rows) and $Rb^{c-/c-}$ (bottom two rows) osteoblasts were treated with BrdU (green) for 24 h at the indicated time points during differentiation *in vitro*. Nuclei are stained with 4′,6-diamidino-2-phenylindole (blue). Original magnification, ×20. B. Quantitation of the immunofluorescence analysis in A. A minimum of 250 cells was counted from each of three or more separate images for each sample. A greater percentage of $Rb^{c-/c}$ osteoblasts incorporate BrdU compared with wild-type cells at all time points. C. Quantitative RT-PCR analysis was done as described in Fig. 3. $Rb^{c-/c}$ osteoblasts (blue columns) express greater mRNA levels of *cyclin E* and *cyclin A* relative to wild-type osteoblasts (red columns) during in vitro differentiation. D. Nuclei of mock-infected (top two rows) and acutely ablated (bottom two rows) $Rb^{c/c}$ osteoblasts were stained for BrdU (green) and 4′,6-diamidino-2-phenylindole (blue). Original magnification, ×20. E. Quantitation of the immunofluorescence analysis in D. A greater percentage of $Rb^{c/c}$ osteoblast nuclei acutely ablated for pRb (blue columns) stain positively for BrdUrd incorporation than control nuclei (red columns). F. Quantitative RT-PCR shows that acutely ablated $Rb^{c/c}$ osteoblasts (blue columns) express greater mRNA levels of *cyclin E* and *cyclin A* compared with mock-infected osteoblasts (red columns). Bars, 1 SD. *, $P < 0.05$, a statistically significant difference. WT, $Rb^{+/c}$;$Mox2^{+/+}$; Rb, $Rb^{c-/c-}$;$Mox2^{+/Cre}$.

Table 1.

Cell Numbers at Day 0 of Differentiation

Genotype	Germ line			Conditional	
		Wild-type	$Rb^{c-/c-}$	Adeno-GFP	Adeno-Cre
Cell count (×1,000)	481 ±16.5	656 ± 14.1	483 ± 24.5	579 ± 17.6	

- NOTE: Two hundred and fifty thousand cells were plated onto a 3-cm tissue culture dish and allowed to reach confluency (typically 4 days later). For "germ line" cells, this confluency arrest constituted day 0 of differentiation, and the number of cells was ascertained. For "conditional" cells ($Rb^{c/c}$) at confluence, adenovirus containing either green fluorescent protein or Cre recombinase was added to the medium. Two days after adenovirus addition (designated as day 0 of differentiation) cells were counted. Average cell counts from at least three separate experiments ± SD are shown.

increased proliferation in $Rb^{c/c}$ cultures is not perpetual, as the percentage of proliferating cells does decrease to almost zero by day 35 (Fig. 4; data not shown). This suggests that compensatory mechanisms, perhaps through the pocket proteins p107 and p130, exist to eventually enable cell cycle exit in the osteoblasts.

The Loss of Rb Prevents Osteoblasts from Properly Exiting the Cell Cycle In vivo

Having established a likely basis for the increased differentiation of pRb-deficient osteoblasts *in vitro*, we wished to determine whether a similar mechanism could explain the impaired bone development *in vivo*. Specifically, because appropriate cell cycle exit is important for the early stages of osteoblast differentiation *in vivo*, we hypothesized that pRb loss might impair cell cycle exit *in vivo* and cause a negative effect on bone formation. Thus, to assess cell cycle progression *in vivo*, we analyzed coronal sections of embryonic day 17.5 frontal bones for BrdU, which incorporates into newly synthesized DNA during S phase. Embryos deficient for pRb exhibited a significantly greater percentage of osteoblast nuclei that incorporated BrdU compared with the wildtype embryos (Fig. 5A). We also tested frontal bone sections for protein expression of proliferating cell nuclear antigen (PCNA), a known proliferation marker. Consistent with our BrdU data, we observed a greater number of $Rb^{c-/c-}$ osteoblast nuclei that stained positively for PCNA compared with wildtype nuclei (Fig. 5B). Interestingly, at the apex of the frontal bone (the midline of the skill) where most of the osteoprogenitors were still proliferating, we did not observe a difference in BrdU or PCNA staining between the wildtype and $Rb^{c-/c-}$ embryos (data not shown). This would indicate that the loss of pRb does not affect the proliferation rate of

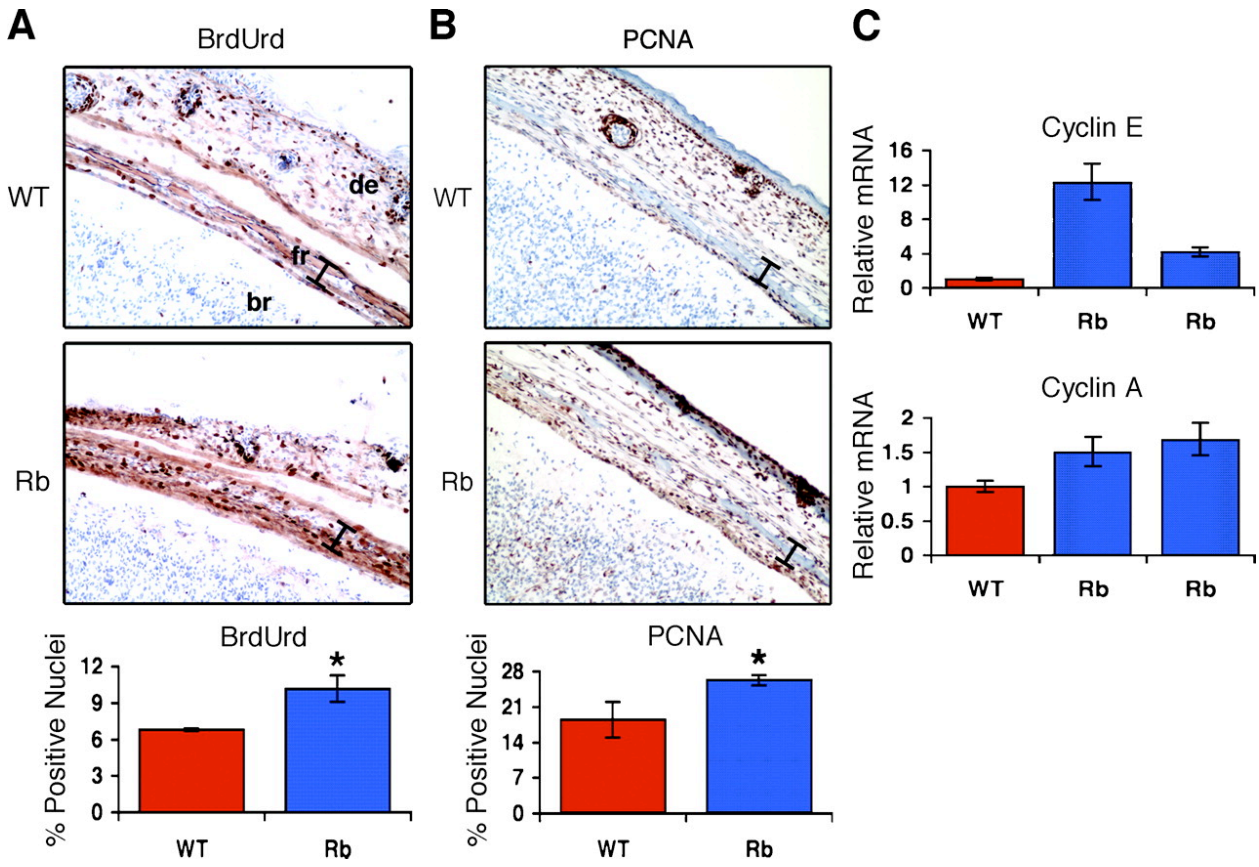


Figure 5. pRb-deficient osteoblasts do not properly exit the cell cycle *in vivo*.

A and B. Immunohistochemical analysis of BrdU incorporation (A) or PCNA protein expression (B) in coronal sections of frontal bones from embryonic day 17.5 embryos. Pregnant females were injected with BrdU for 2 h. $Rb^{c-/c-}$ frontal bones (bottom) exhibit a greater number of nuclei positively staining for BrdU or PCNA than wild-type littermates (top). Original magnification, ×20. Frontal bones (bar). Columns, quantified results from four pairs of $Rb^{c-/c-}$ and wild-type frontal bone sections; bars, 1 SD; *, $P < 0.05$, statistically significant difference. C. Quantitative RT-PCR analysis of *cyclin E* (top) and *cyclin A* (bottom) mRNA levels from $Rb^{c-/c-}$ and control littermates. mRNA was isolated from the calvaria of embryonic day 16.5 embryos. Analysis done as described in Fig. 3. $Rb^{c-/c-}$ calvaria (blue columns) express increased levels of *cyclin A* and *cyclin E* relative to wild-type littermates (red columns). Bars, 1 SD. Abbreviations: br, brain; de, dermis; fr, frontal bone. WT, $Rb^{+/c}$;$Mox2^{+/+}$; Rb, $Rb^{c-/c-}$;$Mox2^{+/Cre}$.

osteoprogenitors but does affect their ability to properly exit the cell cycle and to remain outside of the cell cycle. We did not observe any proliferative differences between $Rb^{c-/c-}$ and wildtype forelimbs (data not shown), corresponding with our finding that there was no difference in the forelimbs based on alizarin red staining.

We also extracted RNA from the calvaria of $Rb^{c-/c-}$ and wildtype embryos to examine the transcript levels of cyclin A and cyclin E. Like PCNA, these transcripts are specifically induced in proliferating cells. $Rb^{c-/c-}$ calvaria typically expressed greater mRNA levels of cyclin A and cyclin E than wildtype skulls (Fig. 5C). Importantly, the unrestricted cell cycle progression in $Rb^{c-/c-}$ frontal bones was not associated with an apoptotic response, as determined by terminal nucleotidyl transferase–mediated nick end labeling staining (data not shown). These data suggest that pRb deficiency impairs osteoblasts from exiting the cell cycle *in vivo* at the appropriate developmental stage.

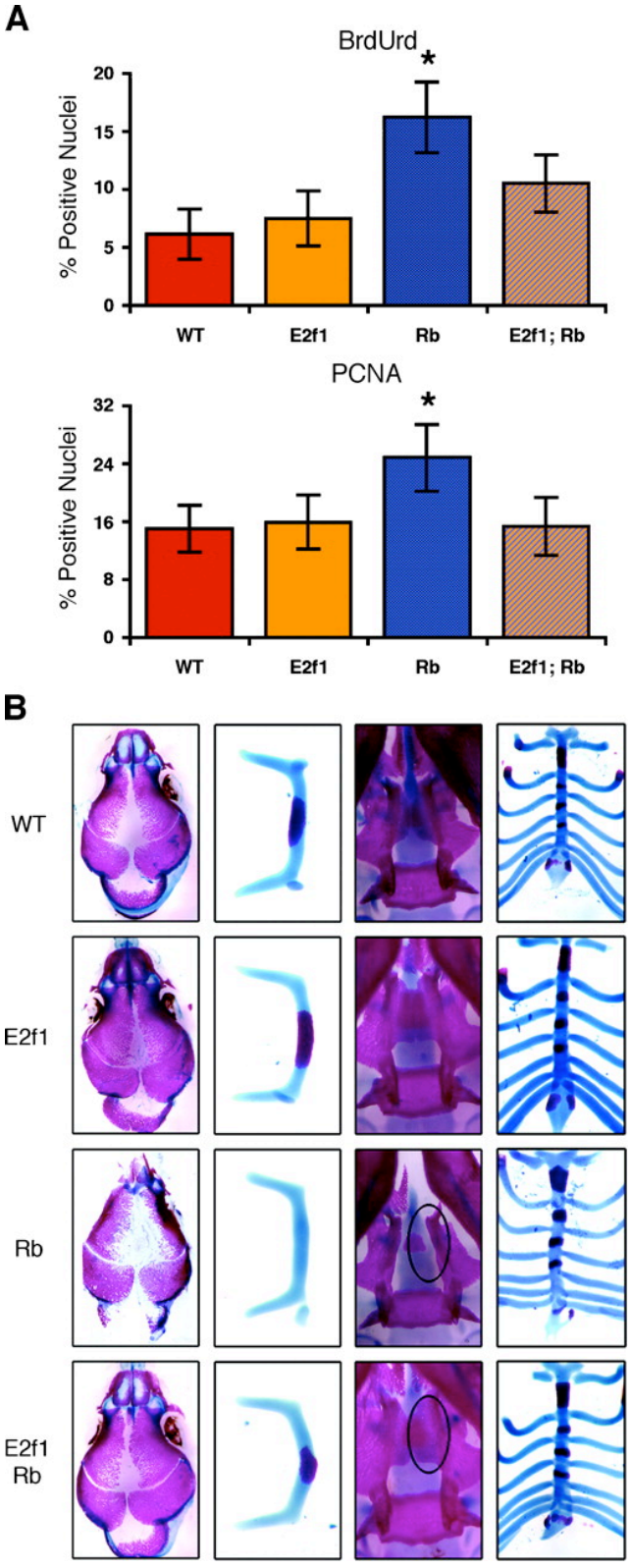
Deletion of E2f1 Suppresses the Cell Cycle and Ossification Defects in $Rb^{c-/c-}$ Embryos

The cell cycle regulatory activity of pRb is known to be at least partially dependent on its ability to suppress the E2F transcription factors and prevent the activation of genes such as *PCNA*, *cyclin A* and *cyclin E* that control cell cycle progression. E2F1 is an archetypal member of the E2F family. It is bound to and inhibited by pRb in arrested cells, and it contributes to the activation of target genes once pRb is inactivated by either mitogenic signaling in wildtype cells or genetic lesions in tumor cells. Previous work has shown that the loss of E2F1 can suppress the ectopic cell cycles arising from the loss of *Rb* in other tissues (Tsai et al, 1998). We found that *Rb* and *E2f1* are both expressed in the

calvaria (Supplemental Fig. S2). Thus, we crossed a mouse possessing a deletion of E2f1 into our conditional Rb model, and we then examined the compound mutant embryos to determine if E2F activity contributes to the excess proliferation and ossification defects arising in the $Rb^{c-/c-}$ embryos.

First, we assessed the level of cellular proliferation in the embryonic osteoblasts through analysis of both BrdU incorporation and PCNA expression in frontal bone sections from embryonic day 17.5 embryos (Fig. 6A). These two assay methods yielded highly concordant results. First, there was no significant difference in the levels of either BrdU- or PCNA positive nuclei in the wildtype versus the $E2f1^{-/-}$ osteoblasts. Thus, loss of *E2f1* alone seems insufficient to perturb osteoblast proliferation. Second, consistent with our prior analysis, proliferating osteoblasts were present at significantly higher levels in the $Rb^{c-/c-}$ frontal bone compared with the wildtype and $E2f1^{-/-}$ controls. Finally, the deletion of *E2f1* was sufficient to almost fully suppress the excess proliferation arising in the $Rb^{c-/c-}$ embryos. The loss of *E2f1* on its own or in the $Rb^{c-/c-}$ background did not affect proliferation at the apex of the frontal bone (data not shown), suggesting that the rate of progenitor proliferation remained unaffected. Therefore, we conclude that inappropriate activation of E2F1 contributes to the inability of pRb-deficient osteoprogenitors to properly exit the cell cycle *in vivo*.

We then assessed whether *E2f1* inactivation modulated the $Rb^{c-/c-}$ embryonic skeletal defects observed at embryonic day 17.5 (Fig. 6B). Consistent with the absence of any proliferation defects, the deletion of *E2f1* alone did not cause any detectable defects in skeletal development. As observed previously, *Rb* deficiency caused decreased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PCNA
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32
24
16
8
0
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WT
E2f1
Rb
E2f1; Rb
B
WT
E2f1
Rb
E2f1
Rb

Figure 6. Deletion of *E2f1* suppresses the bone defects due to the loss of pRb.

A. Immunohistochemical analysis of BrdU incorporation (top) or PCNA protein expression (bottom) in coronal sections of frontal bones from embryonic day 17.5 embryos, done with four to six samples of each genotype. Deletion of *E2f1* suppresses the increased BrdU incorporation and PCNA expression observed in $Rb^{c-/c-}$ frontal bone osteoblasts. B. Skeletal staining of embryonic day 17.5 embryos as described in Fig. 1. Deletion of *E2f1* suppresses the decreased ossification found in the $Rb^{c-/c-}$ calvaria (first column) and hyoid bone (second column). Deletion of *E2f1* also suppresses the aberrant formation of the palatine process and pterygoid bone (third column) and xiphoid process (fourth column) observed in $Rb^{c-/c-}$ skeletons. An aberrant palatine process in the $Rb^{c-/c-}$ and a suppressed palatine process in the double mutant are circled (third column). Bars, 1 SD; *, $P < 0.05$, statistically significant difference between $Rb^{c-/c-}$ and wild-type, $E2f1^{-/-}$, or $Rb^{c-/c-}$;$E2f1^{-/-}$. WT, $Rb^{+/c}$; $Mox2^{+/+}$; $E2f1^{+/+}$; E2f1, $Rb^{+/c}$; $Mox2^{+/+}$; $E2f1^{-/-}$; Rb, $Rb^{c-/c-}$; $Mox2^{+/Cre}$; $E2f1^{+/+}$; RbE2f1, $Rb^{c-/c-}$; $Mox2^{+/Cre}$;$E2f1^{-/-}$.

ossification in the skull and hyoid, and aberrant formation of the xiphoid process, palatine process, and pterygoid bone. Notably, in almost all *E2f1*$^{-/-}$;*Rb*$^{c-/c-}$ double mutant embryos (12of 13), the reduced ossification was partially or completely ameliorated (Fig. 6B, first two columns). Moreover, f40% (5 of 13) of the double mutants exhibited normal formation of the palatine process, pterygoid bone, and the xiphoid process was completely normal (Fig. 6B, latter two columns). Taken together, these data show that deletion of *Rb* causes defects in embryonic skeletal development that are due, at least in part, to the inappropriate release of E2F1.

DISCUSSION

The *RB* locus is mutated or altered in >70% of all osteosarcomas (Belchis et al, 1996; Feugeas et al, 1996). Moreover, several *in vitro* studies implicate pRb and the pocket proteins in osteoblast differentiation (Beck et al, 1998; Feuerbach et al, 1997; Luan et al, 2007; Thomas et al, 2001). Given these observations, we used the $Mox2^{+/Cre}$ transgene to conditionally inactivate *Rb* in the $Rb^{c/c}$ embryo proper, but not in the placenta, and thereby generate pRb deficient embryos that survive until birth. This conditional strategy allows us to assess pRb's role in bone development *in vivo* and primary osteoblast differentiation *in vitro*. Our analyses reveal a role for pRb in the promotion of osteogenesis via the regulation of proper cell cycle exit.

In the developing embryo, the loss of pRb impaired bone formation in a manner that caused two types of defects. Some bones, such as the pterygoid bone, palatine process, and xiphoid process, developed abnormally and were misshapen, whereas the skull and hyoid bone exhibited decreased bone formation. The decreased ossification in the $Rb^{c-/c}$ frontal bone was accompanied by reduced alkaline phosphatase activity and decreased levels of *Col1* and *OPN* mRNA. Previous studies have shown that deletion of the pRb-related proteins, p107 and p130, or overexpression of E2F1 affect chondrocyte differentiation and development (Cobrinik et al, 1996; Rossi et al, 2002; Scheijen et al, 2003). Although our data do not rule out a role for pRb in cartilage development, they clearly show that pRb plays a role in bone development that is independent of chondrocytes. Specifically, $Rb^{c-/c-}$ skeletons did not show any apparent defects in cartilage formation, and several of the affected bones formed via intramembranous ossification, a

process that does not involve chondrocytes. Moreover, the bone defects in the $Rb^{c-/c-}$ frontal bone, and presumably in other affected bones, were not the result of increased osteoclast activity or apoptosis. Therefore, our data suggest that the loss of *Rb* impairs osteoblast differentiation *in vivo* at the earliest stages of the pathway.

One caveat of the *in vivo* studies is that they do not prove that pRb's requirement for osteoblast differentiation is cell autonomous. To address this issue, we determined how the loss of pRb affects the differentiation of primary osteoblasts *in vitro*. Given our *in vivo* defects and the prior observation that pRb-deficient MEFs were impaired in their ability to undergo osteogenesis (Thomas et al, 2001), we anticipated that primary osteoblasts isolated from $Rb^{c-/c}$ embryos would display an impaired differentiation phenotype in vitro. However, the exact opposite was observed: the $Rb^{c-/c-}$ osteoblasts differentiated to a greater extent than the wildtype controls. Importantly, we found that the acute ablation of *Rb* in confluent osteoblasts was sufficient to trigger increased differentiation. These data show that loss of pRb acts in a cell autonomous manner to promote osteoblast differentiation *in vitro*.

Our study shows that two distinct molecular changes accompany the improved *in vitro* differentiation upon loss of pRb. First, we observe a dramatic up-regulation of osteoblast genes, such as *Runx2* and *OSX* in differentiating pRb-deficient osteoblasts to levels that are sometimes not reached by wildtype cells. At this time, we do not know if the extreme up-regulation in $Rb^{c-/c-}$ cultures is due to an increased ability of individual cells to induce osteoblast genes, an increased percentage of terminally differentiated cells in the culture, or both. Interestingly, in these *in vitro* assays, pRb loss clearly induces some (e.g.,

Runx2 and *OSX*) but not all (*ALP* and *Col1*) osteoblast genes. The reason for this differential response is unclear. However, we note that even prior to the induction of differentiation, the *ALP* and *Col1* mRNAs are present at much higher levels in the cultured osteoblasts than in the endogenous calveria. This suggests that the in vitro culture somehow induces *ALP* and *Col1* expression or that it selects for a subpopulation of the calverial cells that are committed to the osteoblast lineage and therefore have high *ALP* and *Col1* expression.

The second molecular change that accompanies the improved *in vitro* differentiation of pRb-deficient osteoblasts is an increase in the fraction of cells that are proliferating and the sustained presence of proliferating cells at later time points in the differentiation process. Because the density of osteoblasts has been reported to correlate positively with their ability to differentiate *in vitro* (Gerber & ap Gwynn, 2001; Purpura et al, 2004), we believe that the increased proliferation of the pRb-deficient osteoblasts contributes to their improved differentiation by increasing the density of the confluent cells. We tried two distinct approaches to directly test this model. First, we attempted to maintain the $Rb^{c/c}$ osteoblasts in the presence of anti-proliferative drugs prior to the ablation of pRb. However, the experiment requires several days of drug treatment to which the cells faired poorly. Second, because our *in vivo* data indicate that deletion of *E2f1* suppresses excess proliferation due to the loss of *Rb*, we analyzed the consequence of *E2f1* deficiency in acutely ablated and germ line–deleted $Rb^{c-/c}$ osteoblasts. Unfortunately, the loss of *E2f1* did not suppress the cell cycle defects of osteoblasts in this *in vitro* setting. Thus, we have been unable to prove that a cell cycle exit defect can account for the increased

differentiation of *Rb*-depleted osteoblasts *in vitro*. Despite this limitation, our *in vivo* studies provide strong support for this model. Specifically, we find that osteoblasts of the $Rb^{c-/c-}$ frontal bone fail to exit the cell cycle at the appropriate stage of development, and we can completely suppress both the proliferation defect and the decreased ossification of the skull and hyoid bones through inactivation of *E2f1*, a known pRb target and proliferation inducer.

If a cell cycle exit defect is the major underlying cause of both the *in vitro* and *in vivo* defects, how does this account for the apparently opposing effects on bone differentiation seen in the two settings? One possibility is that this is an aberrant consequence of the i*n vitro* culture that somehow enables the *Rb*-deficient cells to overcome their differentiation defect. The alternative possibility, which we favor, is that pRb loss affects cells at early and late stages of osteoblast differentiation in a differential manner, and the *in vivo* and the *in vitro* studies highlight the defects in these distinct populations. Specifically, we hypothesize that pRb loss leads to ectopic proliferation that prevents early progenitors from entering osteoblast differentiation but concomitantly enhances the differentiation of late stage osteoblasts. In this model, the *in vitro* cultures could favor analysis of the late stage osteoblasts, thereby showing that pRb loss promotes osteoblast differentiation. In contrast, the *in vivo* phenotype would be more complex. Specifically, our data clearly show ectopic proliferation of $Rb^{c-/c-}$ cells in the developing frontal bone, but we cannot know whether these represent uncommitted early progenitor cells or differentiating osteoblasts that are proliferating inappropriately. In fact, we believe that both populations coexist. In this event, at early time points in the bone differentiation process, the shortage of

committed osteoprogenitors would initially impair bone formation—exactly as we observe in the late stage embryos. However, as the committed osteoblasts accumulate, their increased proliferation would eventually allow, and perhaps ultimately enhance, bone differentiation—as we observe in the *in vitro* assays. Unfortunately, because the $Rb^{c-/c-}$ animals die at birth, we cannot determine whether their osteoblast density and bone deposition ultimately exceeds that seen in wildtype animals.

There is considerable evidence to suggest that pRb plays a direct role in regulating the transcriptional programs that control osteoblast differentiation. Most compelling is the finding that pRb can positively regulate Runx2 *in vitro* (Luan et al, 2007; Thomas et al, 2001). Our findings do not discount the possibility that pRb plays a direct role in bone differentiation through Runx2, or some other mechanism, or that this might contribute to the bone defects we observe *in vivo*. However, they argue that the primary role of pRb in bone differentiation is to inhibit E2F1 and thereby facilitate cell cycle exit. Given that *Rb* inactivation is observed in a large proportion of osteosarcomas, it will be important to develop additional models that allow a comparison of the mechanisms by which loss of *Rb* affects bone development versus osteosarcoma formation.

EXPERIMENTAL PROCEDURES

Animal Maintenance and Histologic Preparations

The generation of *Rb*$^{c/c}$ and *Mox2-Cre* mice has been described previously (Sage et al, 2003; Tallquist & Soriano, 2000). *Rb*$^{c/c}$ and *E2f1*$^{-/-}$ mice were provided by Tyler Jacks. *Mox2-Cre* mice were purchased from The Jackson Laboratory. Gestation was dated by detection of a vaginal plug. Pregnant mice were injected with 10 μL/g body weight of 5 mg/mL BrdU in PBS 2hours prior to tissue collection. For calcein incorporation, pregnant mice were injected with 10 μL/g body weight of 2.5 mg/mL calcein 12 or 24 hours prior to tissue collection. Collected embryonic tissue was fixed in 4% paraformaldehyde and embedded in optimal cutting temperature. Frozen sections were cut at 6 to 8 μm except for those for in situ analysis, which were cut at 10 to 12 μm. The morphology of the brain and presphenoid bone were used to ensure that equivalent planes of the frontal bone were analyzed in all samples.

Histologic Analyses

Enzymatic ALP assays were done on unfixed frozen sections. Briefly, 0.06 g of sodium nitrite was dissolved into 1.5 mL of water and added to 600 μL of 50 mg/mL of new fuchsin (Sigma) in 2mol/L of HCl. This solution was added to 210 mL of Tris buffer (pH 9.0). Finally, 1.8 mL of 83.3 mg/mL naphthol AS-Bi-phosphate (Sigma) in DMF (Sigma) was added. Sections were incubated with this overall solution for 15 min, washed in PBS and counterstained with hematoxylin. Immunohistochemical analyses were done using antibodies against BrdU (1:50 347580; BD Biosciences) and PCNA (1:2,000 sc56; Santa Cruz) as previously described (Danielian et al, 2007). For Collagen1a1 *in situ*, digoxigenin-

11-UTP–labeled single-strand riboprobe was prepared (probe was a gift from B. Olsen), and hybridization was carried out overnight in 50% formamide at 55° C. Washing, detection, staining, and mounting of slides were carried out as described previously (Bohme et al, 1995). Statistical significance was determined using the two-sample Student's *t* test with two-tailed distribution and unequal variance.

Skeletal Staining

Embryos were sacrificed, skinned, and eviscerated. The remaining tissue was fixed in 95% ethanol for 4 days, transferred to acetone for 3 days, and subsequently transferred to staining solution [final volume of 0.015% Alcian blue 8GX (Sigma), 0.005% alizarin red S (Sigma), and 5% glacial acetic acid in ethanol] at 37° C for 2 days and at room temperature for a 3rd day. Tissue was cleared in 1% potassium hydroxide for several days and ultimately stored in glycerol.

Calvarial Preparations and Culture

Calvaria from embryonic day 17.5 embryos were removed, treated with several rounds of collagenase/trypsin digests at 37° C, and plated onto six-well plates. Cells were grown and expanded in αMEM with 10% fetal bovine serum and penicillin/streptomycin. For differentiation, 250,000 cells were plated onto 3-cm tissue culture plates. Upon reaching confluence, calvarial osteoblasts were treated with medium supplemented with 50 μg/mL of ascorbic acid and 10 mmol/L of h-glycerol-phosphate. Adenovirus (University of Iowa Gene Transfer Vector Core) was added to the medium at 100 plaque-forming units per cell and washed away 24 h later. To assay for calcium deposits, plates were stained with 1% alizarin red S solution (pH 5.0).

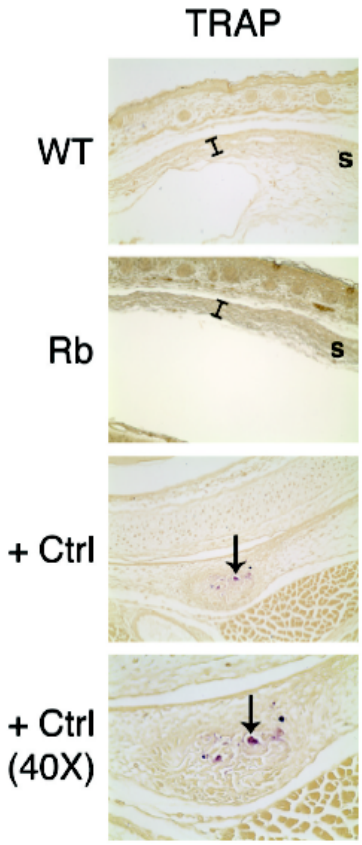
Immunofluorescence

For BrdU incorporation, osteoblasts were plated onto coverslips prior to achieving confluence. BrdU was added to the medium (final concentration of 10 μmol/L) and incubated for 24 h prior to 4% paraformaldehyde fixation. Antigen was detected using antibody against BrdU (1:50 347580; BD Biosciences) with Texas red-X goat anti-mouse secondary (1:1,000; Invitrogen). Statistical significance was determined using Student's *t* test.

Quantitative RT-PCR

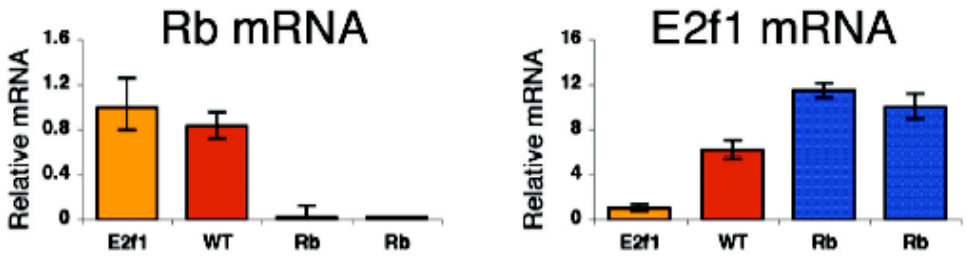
RNA was isolated from differentiation plates using the Qiagen RNeasy kit. First-strand cDNA was transcribed from 1 μg of RNA using Superscript III reverse transcriptase (Invitrogen) following the instructions of the manufacturer. Quantitative RT-PCR with 20 to 100 ng cDNA was done using SYBR Green (Applied Biosystems). Reactions were run on the ABI Prism 7000 Sequence Detection System and analyzed using the 7000 SDS software. Primers are listed in Table 2.

SUPPLEMENTARY FIGURES



Supplemental Figure 1. Deletion of *Rb in vivo* does not affect osteoclast activity.

TRAP analysis of coronal sections from wild-type and $Rb^{c-/c-}$ frontal bones of e17.5 embryos. Neither wild-type nor $Rb^{c-/c-}$ frontal bones exhibit osteoclasts as assessed by TRAP staining. A positively stained osteoclast (arrow) within a facial bone from the same section as the wild-type frontal bone is shown ("+ Ctrl" and "+ Ctrl 40X"). 20X magnification shown except for "+ Ctrl 40X," which is 40X magnification. Frontal bones are marked with the bar. Abbreviations: S, suture. WT = $Rb^{+/c};Mox2^{+/+}$, Rb = $Rb^{c-/c-};Mox2^{+/Cre}$.



Supplemental Figure 2. *Rb* calvaria display increased mRNA levels of *E2f1*.

Quantitative RT-PCR analysis of *Rb* (left) and *E2f1* (right) mRNA levels from $Rb^{c-/c-}$ and control littermates. mRNA was isolated from the calvaria of e16.5 embryos. Analysis performed as described in Figure 3. (Left) *Rb* mRNA is expressed in the calvaria of $E2f1^{-/-}$, *Rb* heterozygous, and wild-type embryos. (Right) $Rb^{c-/c-}$ calvaria express increased levels of *E2f1* relative to control littermates. Error bars signify one standard deviation. WT = $Rb^{+/c}$;$Mox2^{+/+}$;$E2f1^{+/+}$, E2f1 = $Rb^{+/c}$;$Mox2^{+/+}$;$E2f1^{-/-}$, Rb = $Rb^{c-/c-}$;$Mox2^{+/Cre}$;$E2f1^{+/+}$.

Table 2. Cell numbers at Day 0 of differentiation*

	Germline		Conditional	
Genotype	Wild-type	$Rb^{c-/c-}$	Adeno-GFP	Adeno-Cre
Cell Count (X1000)	481 ±16.5	656 ± 14.1	483 ± 24.5	579 ± 17.6

*250,000 cells were plated onto a three-cm tissue culture dish and allowed to reach confluency (typically four days later). For "Germline" cells, this confluency arrest constituted Day 0 of differentiation, and the number of cells was ascertained. For "Conditional" cells ($Rb^{c/c}$) at confluence, adenovirus containing either GFP or Cre recombinase was added to the media. Two days after adenovirus addition (designated as Day 0 of differentiation) cells were counted. Average cell counts from at least 3 separate experiments ± standard deviation are shown.

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REFERENCES

Beck GR, Jr., Sullivan EC, Moran E, Zerler B (1998) Relationship between alkaline phosphatase levels, osteopontin expression, and mineralization in differentiating MC3T3-E1 osteoblasts. *J Cell Biochem* **68**(2): 269-280

Belchis DA, Meece CA, Benko FA, Rogan PK, Williams RA, Gocke CD (1996) Loss of heterozygosity and microsatellite instability at the retinoblastoma locus in osteosarcomas. *Diagn Mol Pathol* **5**(3): 214-219

Bohme K, Li Y, Oh PS, Olsen BR (1995) Primary structure of the long and short splice variants of mouse collagen XII and their tissue-specific expression during embryonic development. *Dev Dyn* **204**(4): 432-445

Chen PL, Riley DJ, Chen Y, Lee WH (1996) Retinoblastoma protein positively regulates terminal adipocyte differentiation through direct interaction with C/EBPs. *Genes Dev* **10**(21): 2794-2804

Clark AJ, Doyle KM, Humbert PO (2004) Cell-intrinsic requirement for pRb in erythropoiesis. *Blood* **104**(5): 1324-1326

Clarke AR, Maandag ER, van Roon M, van der Lugt NM, van der Valk M, Hooper ML, Berns A, te Riele H (1992) Requirement for a functional Rb-1 gene in murine development. *Nature* **359**(6393): 328-330

Cobrinik D, Lee MH, Hannon G, Mulligan G, Bronson RT, Dyson N, Harlow E, Beach D, Weinberg RA, Jacks T (1996) Shared role of the pRB-related p130 and p107 proteins in limb development. *Genes Dev* **10**(13): 1633-1644

Danielian PS, Bender Kim CF, Caron AM, Vasile E, Bronson RT, Lees JA (2007) E2f4 is required for normal development of the airway epithelium. *Dev Biol* **305**(2): 564-576

Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G (1997) Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. *Cell* **89**(5): 747-754

Feuerbach D, Loetscher E, Buerki K, Sampath TK, Feyen JH (1997) Establishment and characterization of conditionally immortalized stromal cell lines from a temperature-sensitive T-Ag transgenic mouse. *J Bone Miner Res* **12**(2): 179-190

Feugeas O, Guriec N, Babin-Boilletot A, Marcellin L, Simon P, Babin S, Thyss A, Hofman P, Terrier P, Kalifa C, Brunat-Mentigny M, Patricot LM, Oberling F (1996) Loss of heterozygosity of the RB gene is a poor prognostic factor in patients with osteosarcoma. *J Clin Oncol* **14**(2): 467-472

Gerber I, ap Gwynn I (2001) Influence of cell isolation, cell culture density, and cell nutrition on differentiation of rat calvarial osteoblast-like cells in vitro. *Eur Cell Mater* **2**: 10-20

Gurney JG, Severson RK, Davis S, Robison LL (1995) Incidence of cancer in children in the United States. Sex-, race-, and 1-year age-specific rates by histologic type. *Cancer* **75**(8): 2186-2195

Haigis K, Sage J, Glickman J, Shafer S, Jacks T (2006) The related retinoblastoma (pRb) and p130 proteins cooperate to regulate homeostasis in the intestinal epithelium. *J Biol Chem* **281**(1): 638-647

Huang HJ, Yee JK, Shew JY, Chen PL, Bookstein R, Friedmann T, Lee EY, Lee WH (1988) Suppression of the neoplastic phenotype by replacement of the RB gene in human cancer cells. *Science* **242**(4885): 1563-1566

Huh MS, Parker MH, Scime A, Parks R, Rudnicki MA (2004) Rb is required for progression through myogenic differentiation but not maintenance of terminal differentiation. *J Cell Biol* **166**(6): 865-876

Jacks T, Fazeli A, Schmitt EM, Bronson RT, Goodell MA, Weinberg RA (1992) Effects of an Rb mutation in the mouse. *Nature* **359**(6393): 295-300

Kim TA, Lim J, Ota S, Raja S, Rogers R, Rivnay B, Avraham H, Avraham S (1998) NRP/B, a novel nuclear matrix protein, associates with p110(RB) and is involved in neuronal differentiation. *J Cell Biol* **141**(3): 553-566

Lee EY, Chang CY, Hu N, Wang YC, Lai CC, Herrup K, Lee WH, Bradley A (1992) Mice deficient for Rb are nonviable and show defects in neurogenesis and haematopoiesis. *Nature* **359**(6393): 288-294

Lee EY, Hu N, Yuan SS, Cox LA, Bradley A, Lee WH, Herrup K (1994) Dual roles of the retinoblastoma protein in cell cycle regulation and neuron differentiation. *Genes Dev* **8**(17): 2008-2021

Lipinski MM, Jacks T (1999) The retinoblastoma gene family in differentiation and development. *Oncogene* **18**(55): 7873-7882

Luan Y, Yu XP, Xu K, Ding B, Yu J, Huang Y, Yang N, Lengyel P, Di Cesare PE, Liu CJ (2007) The retinoblastoma protein is an essential mediator of osteogenesis that links the p204 protein to the Cbfa1 transcription factor thereby increasing its activity. *J Biol Chem* **282**(23): 16860-16870

Morgenbesser SD, Williams BO, Jacks T, DePinho RA (1994) p53-dependent apoptosis produced by Rb-deficiency in the developing mouse lens. *Nature* **371**(6492): 72-74

Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR, de Crombrugghe B (2002) The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. *Cell* **108**(1): 17-29

Otto F, Thornell AP, Crompton T, Denzel A, Gilmour KC, Rosewell IR, Stamp GW, Beddington RS, Mundlos S, Olsen BR, Selby PB, Owen MJ (1997) Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. *Cell* **89**(5): 765-771

Purpura KA, Aubin JE, Zandstra PW (2004) Sustained in vitro expansion of bone progenitors is cell density dependent. *Stem Cells* **22**(1): 39-50

Rossi F, MacLean HE, Yuan W, Francis RO, Semenova E, Lin CS, Kronenberg HM, Cobrinik D (2002) p107 and p130 Coordinately regulate proliferation, Cbfa1 expression, and hypertrophic differentiation during endochondral bone development. *Dev Biol* **247**(2): 271-285

Sage J, Miller AL, Perez-Mancera PA, Wysocki JM, Jacks T (2003) Acute mutation of retinoblastoma gene function is sufficient for cell cycle re-entry. *Nature* **424**(6945): 223-228

Scheijen B, Bronk M, van der Meer T, Bernards R (2003) Constitutive E2F1 overexpression delays endochondral bone formation by inhibiting chondrocyte differentiation. *Mol Cell Biol* **23**(10): 3656-3668

Spike BT, Dirlam A, Dibling BC, Marvin J, Williams BO, Jacks T, Macleod KF (2004) The Rb tumor suppressor is required for stress erythropoiesis. *EMBO J* **23**(21): 4319-4329

Tallquist MD, Soriano P (2000) Epiblast-restricted Cre expression in MORE mice: a tool to distinguish embryonic vs. extra-embryonic gene function. *Genesis* **26**(2): 113-115

Thomas DM, Carty SA, Piscopo DM, Lee JS, Wang WF, Forrester WC, Hinds PW (2001) The retinoblastoma protein acts as a transcriptional coactivator required for osteogenic differentiation. *Mol Cell* **8**(2): 303-316

Trimarchi JM, Lees JA (2002) Sibling rivalry in the E2F family. *Nat Rev Mol Cell Biol* **3**(1): 11-20

Tsai KY, Hu Y, Macleod KF, Crowley D, Yamasaki L, Jacks T (1998) Mutation of E2f-1 suppresses apoptosis and inappropriate S phase entry and extends survival of Rb-deficient mouse embryos. *Mol Cell* **2**(3): 293-304

Wang X, Kua HY, Hu Y, Guo K, Zeng Q, Wu Q, Ng HH, Karsenty G, de Crombrugghe B, Yeh J, Li B (2006) p53 functions as a negative regulator of osteoblastogenesis, osteoblast-dependent osteoclastogenesis, and bone remodeling. *J Cell Biol* **172**(1): 115-125

Weinberg RA (1992) The retinoblastoma gene and gene product. *Cancer Surv* **12**: 43-57

Wenzel PL, Wu L, de Bruin A, Chong JL, Chen WY, Dureska G, Sites E, Pan T, Sharma A, Huang K, Ridgway R, Mosaliganti K, Sharp R, Machiraju R, Saltz J, Yamamoto H, Cross JC, Robinson ML, Leone G (2007) Rb is critical in a mammalian tissue stem cell population. *Genes Dev* **21**(1): 85-97

Wu L, de Bruin A, Saavedra HI, Starovic M, Trimboli A, Yang Y, Opavska J, Wilson P, Thompson JC, Ostrowski MC, Rosol TJ, Woollett LA, Weinstein M, Cross JC, Robinson ML, Leone G (2003) Extra-embryonic function of Rb is essential for embryonic development and viability. *Nature* **421**(6926): 942-947

Yang HS, Hinds PW (2007) pRb-mediated control of epithelial cell proliferation and Indian hedgehog expression in mouse intestinal development. *BMC Dev Biol* **7**: 6