Pocket Protein Family Function in Mesenchymal Tissue Development and Tumorigenesis

By

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ABSTRACT

pRB is a member of the pocket protein family, which includes the closely related proteins p107 and p130. The pocket proteins are critical regulators of the cell cycle and function to restrain proliferation by controlling the activity of the E2F family of transcription factors.

The pocket proteins also play an important role in the development of many tissues. Due to the frequency of mutation of pRB in Osteosarcoma, and its role in the development of tissues of mesenchymal origin, we sought to understand the consequence of the loss of pRb, and its family member p107, in murine mesenchymal cells.

The early lethality of *Rb*$^{-/-}$ mice hampers the study of many mesenchymal tissues, thus we conditionally deleted *Rb* in the mesenchymal progenitors of *p107*$^{-/-}$ mice. These mice develop embryonic skeletal abnormalities characterized by wider and shorter long bones and malformed sternums. Analysis of the defects revealed that inappropriate proliferation of chondrocytes in the growth plate contributed to the phenotype. Mutant adult mice displayed an exacerbated cartilage and growth plate phenotypes, which corresponded to ectopically proliferating growth plate chondrocytes and altered chondrocyte differentiation. Notably, these cartilage defects were consistently associated with the development of enchondromas, a cartilage neoplasm.

We also examined the role of pRb, and its cooperation with the tumor suppressor p53, in the development of murine osteosarcoma. We examined the effect of mutation of *Rb* and *p53* in bone marrow-derived mesenchymal cells, which contains the putative cell of origin of osteosarcoma. We show that *Rb* and *p53* are required for the proper differentiation of mesenchymal cells and mesenchymal cells deficient in these proteins are tumorigenic.

These studies demonstrate that loss of pocket protein function in the mesenchymal lineages can be disastrous for an organism, resulting in tissue deformities and a predisposition to cancer. Thus, pocket proteins play a critical role in regulating skeletogenesis as well as in adult mesenchymal tissue homeostasis and tumor suppression.

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Chapter One

Introduction

Multicellular organisms begin as one cell, and through billions of cellular divisions, develop into an adult animal containing as many as trillions of cells. The process of division is exquisitely regulated to ensure fidelity, standardized embryonic and adult development, and to modulate the total number and type of cells that make up each tissue and organ. When this process becomes deregulated, due to environmental causes or disease states, a number of pathologies can arise, including developmental abnormalities and cancer. Thus, understanding the molecular machinery that controls the timing, length, and frequency of division is critical to our knowledge of the etiology and progression of disease, as well as to our quest for disease cures. The pocket protein family is essential to control cell proliferation during normal development of tissues of mesenchymal origin, and the consequence of its loss is often tumorigenesis.

Part I: Pocket Protein Function in Cellular Proliferation and Cancer

A. Discovery of the Retinoblastoma Protein Family

Retinoblastoma, a malignant tumor of the eye, can be caused by the inheritance of a cancer-associated mutation, but also occurs spontaneously with a similar frequency. Early studies revealed that predisposition to retinoblastoma was linked to mutations in the chromosomal region 13q14, which often appeared as homozygous deletions in retinoblastoma tumor tissue (Dryja et al., 1986; Fung et al., 1987; Sparkes et al., 1980). The cDNA of the gene located in this region, *RB-1*, was subsequently cloned and shown to encode a nuclear phosphoprotein of approximately 110 kilodaltons that likely bound DNA (Friend et al., 1986; Lee et al., 1987a; Lee et al., 1987b).

The product of the *RB-1* gene is called the retinoblastoma protein, known as pRB in humans and pRb in mice. The basic structure and function of pRB were defined through studies of how small DNA tumor viruses cause cellular transformation. Binding of the viral oncoproteins SV40 large T-antigen, Adenovirus E1A, and human papilloma virus (HPV)-16 E7 to pRB was shown to be a necessary step for transformation of cells infected with these viruses, suggesting a role for pRB in growth suppression (DeCaprio et al., 1988; Dyson et al., 1989b; Whyte et al., 1988). Mutational analysis revealed that a short amino acid

sequence, LXCXE, within large T-antigen, E1A, and E7 was required for association with pRB; this region is conserved among these and other viral oncoproteins (Dyson et al., 1990; Dyson et al., 1989b; Munger et al., 1989; Stabel et al., 1985; Vousden and Jat, 1989). The LXCXE motif is necessary to induce cellular transformation, and transferable among different oncoproteins (reviewed in (DeCaprio, 2009). By virtue of their ability to bind these viral oncoproteins, two additional proteins structurally related to pRB were identified. E1A and large T-antigen, through the LXCXE motif, bind a protein with a molecular weight close to that of pRB, called p107 (*RBL1*) and a third protein, p130 (*RBL2*) was found in association with E1A (Dyson et al., 1989a; Li et al., 1993; Whyte et al., 1989). That these proteins were also targeted by viral oncoproteins suggested that they too played an inhibitory role in cell proliferation.

Analysis of mutations in *RB-1* led to the identification of two regions critical for viral oncoprotein binding (Hu et al., 1990; Huang et al., 1990; Kaelin et al., 1990). Interestingly, these non-contiguous C-terminal domains, called the A and B pockets individually, or the pocket domain overall, overlap with regions that contain tumor-associated mutations of *RB-1* consistent with the idea that these regions contribute to growth inhibition (Hu et al., 1990). p107 and p130 also contain these domains, leading this protein family to be called the "pocket protein" family. This region is the most highly conserved among these three proteins. The pocket domain is the critical mediator of pocket protein binding to a large number of cellular proteins, including the E2Fs, the primary targets of pocket-protein regulation (discussed below). p107 and p130 contain additional, unique domains not present in pRB. These are the cyclin A/E binding site, which is situated between the A and B pockets, and a unique N-terminal region that may be important for CDK inhibition (Ewen et al., 1992; Faha et al., 1992; Lees et al., 1992; Zhu et al., 1995)(Figure 1). The biological significance of the cyclin/CDK interaction domains is still unclear, but they may allow p107 and p130 to inhibit G1/S progression in an E2F-independent manner by changing or inhibiting the activity of the S-phase CDKs (Hauser et al., 1997; Zhu et al., 1995).

The activity of the pocket proteins is modulated during the cell cycle by phosphorylation events. During the G0 phase of the cell cycle, p130 expression exceeds that of pRB and p107 and it is uniquely phosphorylated at this stage, in a CDK-independent manner (Canhoto et al., 2000). During G1, pRB and p107 expression increases, as does

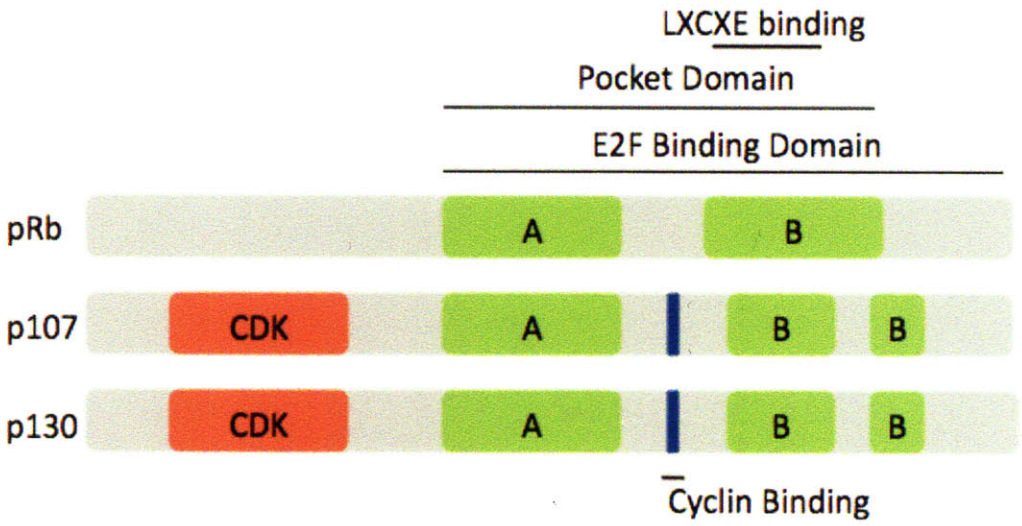

Figure 1: Schematic of the Pocket Proteins
(See text for details.) The pocket proteins contain one large region of homology called the "pocket." The pocket is required for E2F binding and is important for the binding of LXCXE-containing proteins, including viral oncoproteins. p107 and p130 contain two domains that are not present in pRb: a CDK binding domain in the N-terminus, and a cyclin binding domain in the C-terminus.

phosphorylation of all pocket proteins by Cyclin D/CDK4 and Cyclin D/CDK6 complexes (reviewed in (Classon and Dyson, 2001). Most mitogenic signaling pathways converge to activate Cyclin D/CDK complexes, making the phosphorylation of the pocket proteins by this complex the critical node in promoting cell proliferation (Lukas et al., 1996a). By arresting cells in G1 using serum starvation or in S-phase using drugs, it was determined that pRB is underphsophorylated in G0/G1, but promptly becomes phosphorylated as cells begin to proliferate, and reaches its maximum phosphorylated state during S-phase (Chen et al., 1989; Mihara et al., 1989). When cells are induced to differentiate, pRB is rapidly dephosphorylated prior to total growth arrest (Chen et al., 1989). p107 and p130 are phosphorylated in a similar manner to pRB in G1, and are subsequently phosphorylated in S-phase by Cyclin E/CDK2 and Cyclin A/CDK2 (Beijersbergen et al., 1995; Mayol et al., 1995; Xiao et al., 1996).

Viral oncoproteins preferentially bind underphsophorylated pRB in G0/G1, and phosphorylation of pRB in G1/S interferes with this interaction (Ludlow et al., 1989; Ludlow et al., 1990). This suggests that the growth inhibitory, active form of pRB is underphsophorylated. Since phosphorylation of pRB is cell cycle mediated, this, in combination with evidence that viral oncoprotein-pRB binding results in proliferation, led to the realization that pocket proteins likely modulated the cell cycle.

B. Cell Cycle Regulation by Pocket Proteins

Consistent with the idea that they inhibit cell cycle progression, overexpression of pRb, p107, or p130 leads to cell cycle arrest (Claudio et al., 1994; Dick et al., 2000; Zhu et al., 1993). Combined loss of all the pocket proteins in ES cells and mouse embryonic fibroblasts (MEFs) underscores their inhibitory role. *Rb*$^{-/-}$*;p107*$^{-/-}$*;p130*$^{-/-}$ (triple knock-out; TKO) ES cells were unable to differentiate, although all other mutant combinations were phenotypically normal in this regard (Dannenberg et al., 2000). Furthermore, MEFs generated from TKO embryos displayed a variety of cell division defects, including a shortened cell cycle; resistance to G1 arrest after DNA damage, contact inhibition, and serum starvation; and resistance to Ras-induced senescence (Dannenberg et al., 2000; Sage et al., 2000). None of these defects were observed in any of the control genotypes including

the single and double knockouts. These results demonstrated that the pocket proteins have overlapping functions and together are critical regulators of the G1/S transition downstream of several cell cycle control pathways.

One caveat of these experiments, however, is the possibility that single and double knockout MEFs behaved normally in culture due to changes that enable compensation by the remaining pocket protein(s). To address this possibility, MEFs were generated that carried a conditional, *loxP* flanked allele of *Rb*, allowing acute ablation in cultured cells after infection with Adenovirus containing Cre-recombinase (Sage et al., 2003). In this system, acute loss of *Rb* in quiescent MEFs was sufficient for cell cycle reentry, in contrast to earlier results that required loss of all pocket proteins. Furthermore, loss of *Rb* alone was enough to reverse a senescence phenotype. This study revealed that there is developmental compensation among members of the pocket protein family, and moreover, pRb function alone is sufficient to enforce both temporary and permanent G1 arrest in vitro. This study did not address, however, the potential ability of the other pocket proteins to function in this manner; this remains an open question.

C. E2F is a Major Target of the Pocket Proteins

Although the pocket proteins, and particularly pRb, potentially interact with hundreds of different cellular proteins (reviewed by (Morris and Dyson, 2001), their role in restraining the cell cycle seems to be mainly exerted via their interaction with and regulation of the activity of the E2F family of transcription factors. Cell cycle phasing and the specificity of the pocket proteins for different E2Fs determine the composition of the pocket protein-E2F moiety during the cell cycle (Moberg et al., 1996). Furthermore, the composition of the moiety determines the activity of these complexes (Rayman et al., 2002; Takahashi et al., 2000).

E2F was first identified as a cellular factor that could bind to the enhancer sequence of the Adenoviral *E2* gene as well as the *E1A* enhancer and promote transcription (Kovesdi et al., 1987; Yee et al., 1987). Simultaneously, another group identified a cellular activity that could promote transcription from the *E2* promoter, DRTF1 (differentiation-regulated transcription factor-1), which turned out to be identical to E2F (Bandara and La Thangue,

1991; La Thangue and Rigby, 1987). Purification of DTRF1 and sequencing of associated factors led to the identification of an additional component of DTRF1/E2F, called DTRF1-polypeptide 1, or DP1 (Girling et al., 1993). DP1, and another family member DP2, heterodimerize with E2F and are required for its DNA binding and transactivating abilities (Bandara et al., 1993; Girling et al., 1993; Huber et al., 1993; Ormondroyd et al., 1995; Rogers et al., 1996; Wu et al., 1995; Zhang and Chellappan, 1995). E2F binds the *E2* promoter DNA at two sites with the sequence TTTCGCGC (Kovesdi et al., 1987; Yee et al., 1987). It was later demonstrated that E2F binds this same consensus sequence within cellular genes to promote their expression during the G1/S phase of the cell cycle (reviewed by (Trimarchi and Lees, 2002).

There are generally two classes of E2F proteins, the activating E2Fs: E2F1, 2, and 3, and the repressive E2Fs: E2F4 and 5. The transcriptional activity of the activating E2Fs is inhibited by binding of pocket proteins whereas the repressive E2Fs work in concert with the pocket proteins to repress target gene transcription. Thus, distinct classes of E2F activities can direct the cell to either progress through the cell cycle or arrest, making the pocket protein-E2F complexes major regulators of the cell cycle (Figure 2).

i. The Activating E2Fs: E2F1, 2, and 3, and Cell Cycle Entry

E2f1 (E2 promoter binding factor-1) was cloned from cDNA expression libraries on the basis of its interaction with recombinant pRb and its ability to compete pRb away from viral oncoproteins like E1A (Helin et al., 1992; Kaelin et al., 1992; Shan et al., 1992). Soon after, two additional, highly related members of the E2F family, *E2f2* and *E2f3*, were cloned by probing cDNA expression libraries with the DNA sequence of the *E2f1* DNA binding domain (Ivey-Hoyle et al., 1993; Lees et al., 1993). Both new members bound DP and pRb, and they could potently transactivate expression from the *E2* promoter. Recently, it was found that the *E2f3* locus encodes two transcripts from alternate promoters containing different first exons, termed *E2f3a* and *E2f3b* (He et al., 2000; Leone et al., 2000). The individual activities of each isoform remain unresolved, but it is clear that they are at least partially redundant in mice (Chong et al., 2009; Danielian et al., 2008).

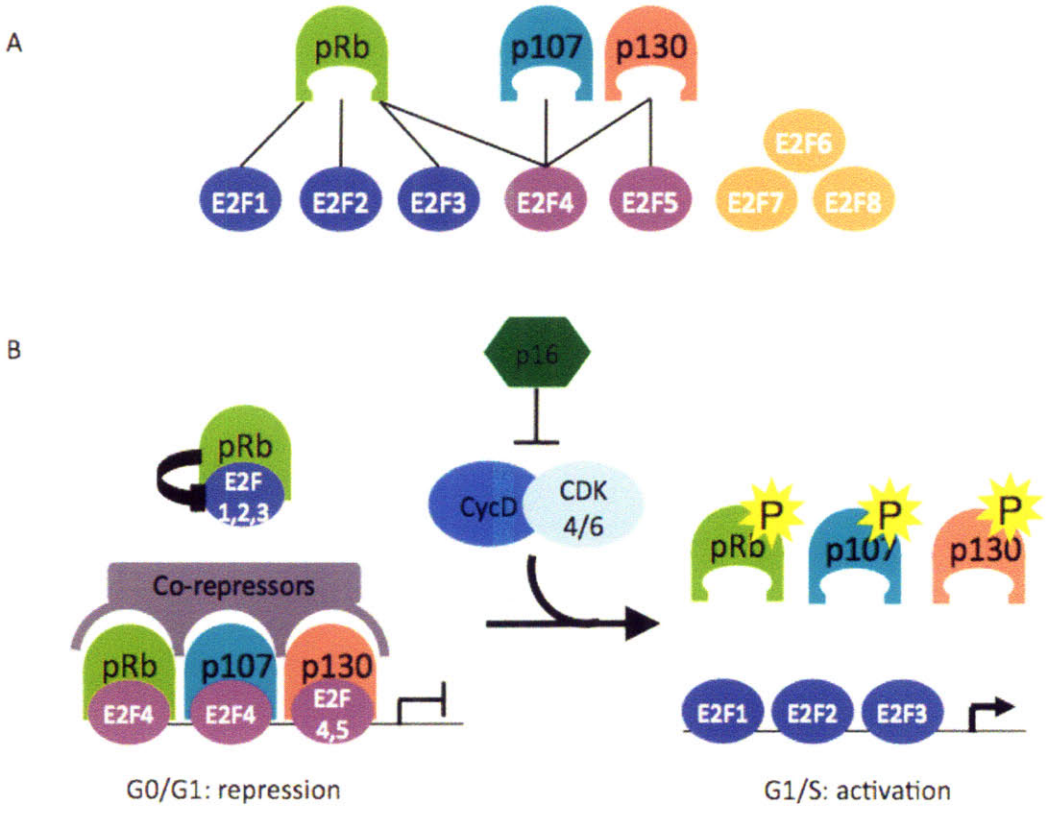

Figure 2: The Pocket Proteins Regulate The E2Fs during the Cell Cycle
(See text for details.) (A) The pocket proteins have E2F binding specificity. pRb preferentially binds E2F1, 2, and 3, while p130 preferentially binds E2F4 and E2F5. p107 only binds E2F4. E2F6, 7, and 8 function independently of the pocket proteins. (B) During the G0/G1 phase, pocket proteins generally act to repress transcription from cell cycle promoters. Upon mitogenic stimulation in G1/S, Cyclin D/CDK complexes phosphorylate the pocket proteins, releasing E2Fs to bind and activate transcription of cell cycle promoters.

The activating E2Fs are bound exclusively by pRb in the G0/G1 phase of the cell cycle, which results in inhibition of E2F activity (Helin et al., 1992; Hiebert et al., 1992; Kaelin et al., 1992). pRb mediates inhibition of E2F transactivation via direct physical occlusion of the transactivation domain of E2F (Flemington et al., 1993; Lee et al., 2002). Crystal structures of pocket protein-E2F complexes established that phosphorylation of the pocket proteins directly prevents E2F from binding (Xiao et al., 2003). Importantly, despite their structural and functional similarities, p107 and p130 do not generally bind the activating E2Fs (Dyson et al., 1993; Lees et al., 1993). This suggests that p107 and p130 do not regulate cell cycle progression in the same manner as pRb. It also was the first indication that the activating E2Fs did not account for all of the E2F activity in the cell.

The activating E2Fs are potent transcriptional activators in transient reporter assays (Helin et al., 1993; Lees et al., 1993). Most E2F target genes encode proteins critical for the G1/S transition and DNA replication (reviewed in (Trimarchi and Lees, 2002). Consistent with their role in promoting S-phase progression, E2F1-3 bind their target promoters during late G1/S, coincident with their transcriptional activation (Rayman et al., 2002; Takahashi et al., 2000). More recently, a role for E2F-mediated expression of genes involved in mitosis, G2/M, and DNA repair has been demonstrated (Hernando et al., 2004; Ishida et al., 2001; Muller et al., 2001; Ren et al., 2002). These data show that the activating E2Fs drive cell cycle progression through their transactivation of genes required for S-phase and beyond.

The activating E2Fs are required for cell cycle entry. Overexpression of *E2f1* drives cell cycle entry from a number of quiescent states including a G1 arrest induced by DNA damage, overexpression of the CDK inhibitor $p16^{INK4A}$, and growth inhibitory TGFβ signaling (DeGregori et al., 1995; DeGregori et al., 1997; Johnson et al., 1993; Kowalik et al., 1995; Lukas et al., 1996b; Mann and Jones, 1996; Qin et al., 1994; Schwarz et al., 1995). *E2f2* and *E2f3* were also shown to be capable of inducing S-phase entry when overexpressed (DeGregori et al., 1997; Lukas et al., 1996b). Further evidence of the requirement of E2F activity to promote cell cycle entry comes from studies of $E2f1^{-/-};E2f2^{-/-};E2f3^{-/-}$ MEFs, which are completely unable to proliferate (Wu et al., 2001). The E2Fs do have some distinct roles, however. For example, $E2f3^{-/-}$ MEFs proliferate more slowly than

WT MEFs and are impaired in cell cycle re-entry from quiescence, while *E2f1*$^{-/-}$ MEFs show no obvious defects (Humbert et al., 2000b). These studies demonstrate the importance of the activating E2Fs in cellular proliferation.

ii. The Repressive E2Fs: E2F4 and 5

The repressive E2Fs, *E2f4* and *E2f5*, were identified and cloned based on their ability to bind either p107 or p130 and their homology to previously identified E2Fs (Beijersbergen et al., 1994; Ginsberg et al., 1994; Hijmans et al., 1995; Sardet et al., 1995). The repressive E2Fs are structurally and functionally similar to the activating E2Fs; they require an association with DP for DNA binding and can activate transcription from an E2F reporter construct when over-expressed (Beijersbergen et al., 1994; Ginsberg et al., 1994; Hijmans et al., 1995; Sardet et al., 1995), although their transactivation ability is not sufficient to promote cell cycle entry from quiescence (Lukas et al., 1996b; Mann and Jones, 1996). Importantly, E2F4 can bind pRb in addition to p107 and p130 (Moberg et al., 1996), whereas E2F5 exclusively binds p130 in the G0/G1 phase of the cell cycle (Hijmans et al., 1995).

Like the activating E2Fs, E2F4 and 5 exhibit cell cycle-dependent associations with pocket proteins and DNA binding activity. E2F4 and E2F5 are both expressed consistently throughout the cell cycle, although E2F5 is less abundant (Sardet et al., 1995). p130/E2F5 complexes are seen in G0 and G1, and then are replaced by pRb-and p107-containing complexes in G1/S and S phase (Chittenden et al., 1993; Cobrinik et al., 1993; Moberg et al., 1996). In G1/S and S phase, E2F4 is the major E2F component of these pocket protein complexes, but also comprises a significant portion of the free E2F activity (Moberg et al., 1996). Despite its high level of expression, examination of the cell cycle in primary cells generated from *E2f4*$^{-/-}$ mice revealed its loss had no effect on proliferation or expression of a variety of E2F target genes such as *E2f1* or *p107* (Humbert et al., 2000a). Proliferation and other cell cycle attributes in *E2f5*$^{-/-}$ MEFs were similarly normal (Lindeman et al., 1998). Together these observations suggest that E2F4 and E2F5 possess redundant functions. Analysis of *E2f4*$^{-/-}$;*E2f5*$^{-/-}$ DKO MEFs revealed, however, that these proteins are dispensable for cell cycle progression, but presence of one or the other is required to exert

a G1 arrest in response to *p16^{INK4A}* (Gaubatz et al., 2000) indicating that their function is required for cell cycle arrest, but not during asynchronous growth.

Pocket protein-repressive E2F complexes are found at transcriptionally silent promoters, including those of genes required for cell cycle progression as well as genes involved in differentiation. Studies of transcriptional control of cell cycle regulated genes demonstrated that E2F recruits pRb to E2F binding sites in promoters to actively repress gene expression (Trimarchi and Lees, 2002). For example, expression of pRb, as well as the presence of E2F sites in the *cdc2* promoter, is required for repression of this locus (Dalton, 1992). Furthermore, *b-myb* expression is directly repressed by E2F binding in G0/G1 when it's associated with pRb; mutation of the E2F consensus sequence leads to constitutive transcription of this locus (Lam and Watson, 1993). In both of the aforementioned cases, loss of direct repression by pRb-E2F at the G1/S transition is sufficient to result in expression from these loci. Other E2F target genes, however, do require additional transactivation by free E2F, for example, the *cyclin A* and *cdc6* promoters (Takahashi et al., 2000). The idea that E2F1-3 are necessary to induce expression of target genes upon S-phase entry is supported by the fact that E2F1/2/3 TKO MEFs are unable to enter S-phase or express many essential E2F target genes (Wu et al., 2001). These studies demonstrate that there are two modes of induction of E2F target genes at the G1/S transition: simple loss of repression and direct activation by free E2F.

Active repression of E2F targets during G0 and G1 involves pocket protein recruitment of a number of co-repressor molecules to enforce transcriptional repression (reviewed in (Frolov and Dyson, 2004). For example, the histone deacetylase HDAC1 is recruited by pRb to endogenous E2F promoters such as *cyclin E* (Brehm et al., 1998; Luo et al., 1998; Rayman et al., 2002). These complexes promote repression of E2F target genes as well as modify chromatin structure during quiescence and dissociate from promoters coincident with gene activation (Brehm et al., 1998; Rayman et al., 2002). HDAC1 was also shown to bind p107 and p130 and requires an E2F consensus site to promote transcriptional repression (Ferreira et al., 1998; Ferreira et al., 2001; Rayman et al., 2002). All class I HDAC molecules associate with pRb-E2F complexes, and both HDAC1 and 2 may do so through LXCXE motifs that bind the pocket domain (Dahiya et al., 2000). Mutations in the pocket of pRb that disrupt binding of HDACs and other LXCXE containing proteins but

not E2F binding lead to impaired repression of *cyclin A* and *cyclin E* during quiescence (Dahiya et al., 2000). Histone H3 Lysine 9 (H3K9) methylation has also been shown to be associated with gene silencing by pRb. The histone methyl transferase (HMT) associated with this methyl mark, SUV39H1, is recruited to E2F promoters by pRb, p107, and p130 and leads to increased HP1 binding and a change in the heterochromatin state of the promoter (Grewal and Elgin, 2002; Nicolas et al., 2003; Nielsen et al., 2001; Vandel et al., 2001).

DNA methlylation also contributes to silencing of E2F target genes. Co-immunoprecipitation experiments demonstrated that the DNA methyltransferase DNMT1 cooperates with pRb-E2F complexes to repress transcription (Robertson et al., 2000). Often, activating E2Fs are required to reverse the effects of these repressive histone marks (Frolov et al., 2003). For example, Adenoviral E1A was shown to promote proliferation in part by binding to E2F promoters and replacing H3K9 methyl marks with histone acetylation (Ghosh and Harter, 2003). The ability of the pocket proteins to promote both histone modification as well as heterochromatic changes suggests that they may play a role in long-term gene silencing, which may be crucial for maintaining cell cycle exit and for tumor suppression.

iii. The Pocket Protein-Independent E2Fs

Three additional E2Fs exist but function independent of pocket protein binding; these are E2F6, E2F7, and E2F8. E2F6 does not contain a pocket protein binding domain or transactivation domain and thus was hypothesized to act as a transcriptional repressor (Trimarchi et al., 1998). Indeed, E2F6 does associate with components of the Polycomb Group Complex (PcG) including Bmi1, Ring1, HP1γ, EZH2, and PHC3, among others (Attwooll et al., 2005; Deshpande et al., 2007; Ogawa et al., 2002; Trimarchi et al., 2001). Consistent with a role in repressing polycomb targets, *E2f6*$^{-/-}$ mice display patterning defects in the skeleton, and cooperates with *Bmi1*-loss in promoting further skeletal defects and deregulation of Hox genes (Courel et al., 2008).

E2F7 and E2F8 are structurally like E2F6, but they do not contain a DP-dimerization domain, instead homodimerizing to gain DNA binding activity without DP (Christensen et al., 2005; de Bruin et al., 2003; Di Stefano et al., 2003; Logan et al., 2004; Logan et al., 2005; Maiti et al., 2005). Overexpression studies demonstrated that these two newest E2Fs are also transcriptional repressors (Christensen et al., 2005; Di Stefano et al., 2003).

D. Tumor Suppressive Properties of the Pocket Protein Family

As discussed above, *Rb* is a key tumor suppressor in humans. Inherited *Rb* mutation, and subsequent somatic loss of the wild-type (WT) copy, is sufficient to induce retinoblastoma development, and increases the risk of other cancers, particularly osteosarcoma as well as small-cell lung carcinoma (SCLC) (reviewed in Burkhart and Sage, 2008). Sporadic *Rb* loss is implicated in enhancing tumor progression when lost at later stages of cancer development, including in prostate, breast, and bladder carcinomas, among others (reviewed in Burkhart and Sage, 2008). HPV-induced cervical carcinoma and squamous cell cancer provides further support for the importance of *Rb*-inactivation in promoting tumor growth (Doorbar, 2006; Perez-Ordonez et al., 2006).

Rb also acts as a murine tumor suppressor, making the mouse an excellent model organism in which to study *Rb*-deficient human cancers. $Rb^{+/-}$ mice develop pituitary and thyroid tumors, although notably not retinoblastoma, starting at 1 year of age (Clarke et al., 1992; Jacks et al., 1992). When combined with mutations in another potent tumor suppressor, *Trp53*, *Rb* mutation can lead to osteosarcoma and SCLC in the mouse (Berman et al., 2008a; Meuwissen et al., 2003; Walkley et al., 2008). Although this is strong evidence for a role for *Rb* in tumor suppression, it is confounding that *Rb* mutation results in different tumor spectrums in mice and humans. One potential reason for this difference is that the other pocket proteins p107 and p130 are able to act as tumor suppressors in disparate tissues in these two organisms. Another possibility, which is not mutually exclusive, is that the requirement for *Rb* function itself varies among different tissues and in different organisms. Mutations in the other pocket proteins are rarely (p130), or never (p107), seen in human tumors (Claudio et al., 2000a; Claudio et al., 2000b; Helin et al., 1997). Furthermore, loss of either p107 or p130, or both does not lead to tumor formation

in the mouse (Cobrinik et al., 1996; Lee et al., 1996). Mouse models have revealed, however, that p107 and p130 can act as tumor suppressors in combination with *Rb* loss in some tissues, leading to novel tumor phenotypes.

Loss of *Rb* alone does not lead to retinoblastoma in the mouse, yet chimeric mice generated with $Rb^{-/-}$;$p107^{-/-}$ or $Rb^{-/-}$;$p130^{-/-}$ ES cells develop retinoblastoma, while $Rb^{+/-}$;$p107^{-/-}$ chimeras develop OS, among other tumors (Dannenberg et al., 2004; Robanus-Maandag et al., 1998). Additionally, using Cre to drive recombination of a floxed allele of *Rb* in retinal cells in a $p107^{-/-}$ or $p130^{-/-}$ background leads to retinoblastoma development (MacPherson et al., 2007; MacPherson et al., 2004). These studies indicate that both p107 and p130, to different degrees, can compensate for the tumor suppressive functions of *Rb* in mouse retinoblasts in a way that they do not in human cells, perhaps due to lack of adequate expression in human cells, or the requirement for *Rb*-specific functions in human but not mouse cells.

Additional mouse models of cancer dependent on combined loss of pocket proteins provide evidence for tumor suppressive functions of p107 and p130 in other epithelial cell types. In a model of K-Ras induced non-small cell lung carcinoma (NSCLC), both *Rb* and *p130* were shown to be suppressors of adenocarcinoma formation (Ho et al., 2009). *Rb* deficiency does not enhance chemically-induced skin cancer, due to p53-induced apoptosis, but combined loss with p107 leads to tumorigenesis both spontaneously and after carcinogen treatment, indicating that p107 can function as a tumor suppressor in *Rb*-deficient epidermis, in part due to its upregulation in response to *Rb* loss (Lara et al., 2008; Santos et al., 2008). Finally, expression of T_{121}, a truncated version of large T-antigen that binds all three pocket proteins but not p53, leads to tumor formation in tissues that are not susceptible to *Rb* loss alone, including in brain choroid epithelium (Lu et al., 2001), astrocytes (Xiao et al., 2002), and mammary epithelium (Simin et al., 2004). These data illustrate that overlapping functions of the pocket protein family members can lead to compensation for one another's tumor suppressive functions, depending on the tissue and context. It is also supports that idea that particular pocket proteins may be essential for the development and differentiation of some tissues and not others, leading to differences in the tumor spectrums in these mice (Classon and Harlow, 2002).

E2F activity is traditionally thought of as oncogenic, since it promotes cell proliferation. To a large extent, the tumor suppressor functions of *Rb* can be attributed to its inhibition of E2F activity (Burkhart and Sage, 2008). This is made clear by a number of mouse models in which E2F loss rescues some tumor phenotypes caused by pocket protein loss. Deletion of *E2f1* suppresses apoptosis and ectopic S-phase entry, and extends the lifespan of $Rb^{-/-}$ embryos (Tsai et al., 1998). *E2f1* loss also extends the lifespan of $Rb^{+/-}$ mice and suppresses the formation of pituitary and thyroid tumors (Yamasaki et al., 1998). Tumor growth in a T_{121}-induced model of tumors in the brain choroid plexus is also suppressed by loss of *E2f1* (Pan et al., 1998). Similarly, *E2f3* may also act as an oncogene downstream of *Rb*, depending on tissue type. $Rb^{-/-};E2f3^{+/-}$ embryos display none of the inappropriate proliferation or apoptosis of $Rb^{-/-}$ embryos (Ziebold et al., 2001). Moreover, *E2f3* loss in $Rb^{+/-}$ mice extends the lifespan and suppresses the formation of pituitary tumors (Ziebold et al., 2003).

This picture is made more complicated, however, by studies that show that E2F1 and E2F3 can also act as tumor suppressors. *E2f1*-loss is oncogenic, resulting in the formation of lymphomas and lung adenocarcinomas among other tumors (Field et al., 1996; Yamasaki et al., 1996). Likewise, suppression of pituitary tumors in $Rb^{+/-};E2f3^{-/-}$ mice is accompanied by the induction of malignant thyroid tumors (Ziebold et al., 2001). Finally, overexpression of *E2f1* can result in tumor suppression due to its ability to elicit p53-dependent apoptosis (Classon and Harlow, 2002; Elliott et al., 2001; Pan et al., 1998). Thus *E2f1* and *E2f3* are responsible for tumor suppression and induction downstream of *Rb*-loss.

Part II: Pocket Protein Regulation of Differentiation and Development

A. General Differentiation Functions of the Pocket Proteins

The pocket proteins act to promote and maintain terminal differentiation. Much of the evidence to support this comes from analysis of mice carrying knockout alleles of *Rb*, *p107*, or *p130*, singularly and in combination. These studies have implicated the pocket proteins in regulating the differentiation of cells in a wide range of tissues, including skin, muscle, intestine, blood, lens, CNS, fat, bone, and cartilage. Below is a description of how the pocket proteins work to induce differentiation, with a focus on their function in tissues of mesenchymal origin.

i. Pocket Protein Function is Required for Proper Development In Vivo

The first indication that pRb was essential during development came from the study of germline $Rb^{-/-}$ mice. These mice die during embryogenesis between e13 and e15 with severe erythrocyte, lens, and neuronal defects (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). These defects seem to have their origin in improper completion of the differentiation program. $Rb^{-/-}$ erythrocytes form but do not fully enucleate, blocking them in an immature state. Moreover, neuronal and lens cells are present, but have reduced expression of late differentiation markers, and exhibit ectopic cell cycle entry and apoptosis (Lee et al., 1994; Morgenbesser et al., 1994).

Some of these developmental defects can, at least in part, be attributed to non-cell autonomous functions of *Rb*. *Rb* is implicated in regulating the development of the extra-embryonic tissues, and techniques used to conditionally delete *Rb* in the embryo proper, thus maintaining a WT placenta, virtually abolish erythroid and neuronal defects (Wu et al., 2003). The initial analysis of $Rb^{-/-}$ chimeras also suggested that the defects in erythropoiesis were attributable to non-cell autonomous effects, since the mice had no obvious erythroid defect and *Rb*-deficient cells contributed significantly to most tissues (Maandag et al., 1994; Williams et al., 1994b). Neuronal and lens defects were still

manifest in these chimeras demonstrating that *Rb* also has cell autonomous functions (Lipinski et al., 2001; Williams et al., 1994b). Later studies, however, definitively established a cell intrinsic role for *Rb* in erythropoiesis. During in vitro erythroid differentiation, after stress-induced anemia, and during erythropoietic reconstitution of irradiated host mice, Rb is required cell autonomously for erythroblasts to terminally differentiate into erythrocytes (Clarke et al., 1992; Spike et al., 2004). Furthermore, although young $Rb^{-/-}$ chimeras with high chimerism are normal, these mice develop age-dependent defects in erythroid maturation, and the erythrocyte compartment is eventually depleted of *Rb*-null cells (Spike et al., 2004). These results are consistent with a cell intrinsic requirement for *Rb* in promoting erythroid differentiation.

Use of an *Rb* mini-gene that expresses *Rb* at low level extends the lifespan of $Rb^{-/-}$ embryos to reveal skeletal muscle defects marked by increased cell death and failure of myoblasts to terminally differentiate (Zacksenhaus et al., 1996). This defect is cell-autonomous, but is not suppressed by E2f1-loss, suggesting that other E2Fs may be responsible for the apoptosis and cell cycle exit failure in this tissue (Tsai et al., 1998; Zacksenhaus et al., 1996). In vitro experiments showed that pRb is required for initiation of skeletal muscle differentiation, but not maintenance of the differentiated state (Huh et al., 2004). pRb likely acts both to inhibit cell proliferation as well as indirectly activate MyoD and other MRFs to promote differentiation in this tissue (Novitch et al., 1996; Novitch et al., 1999).

The fact that $Rb^{-/-}$ mice survive until mid-gestation suggested that *p107* and *p130* might compensate for the loss of pRb function in some tissues. Germline deletion of *p107* or *p130* in the mouse does not lead to any significant phenotypes in mixed background strains (Cobrinik et al., 1996; Lee et al., 1996) although in a pure Balb/c background $p130^{-/-}$ and $p107^{-/-}$ mice die in utero or soon after birth, due to defects in neuronal and muscle development or blood development, respectively (LeCouter et al., 1998a; LeCouter et al., 1998b). This suggests that the requirement for the pocket proteins during development is strain-dependent and may be influenced by the presence of genetic modifiers.

Combined loss of *p107* or *p130* with *Rb* in the mouse confirmed the hypothesis that these family members can substitute for *Rb* function in certain contexts. Germline $Rb^{+/-}$;$p107^{-/-}$ mice have decreased viability and develop retinal dysplasia, a phenotype not

present in *Rb*$^{-/-}$ mice, indicating that *p107* function is sufficient for development of the *Rb*-deficient retina (Lee et al., 1996). Furthermore, germline *Rb*$^{-/-}$;*p107*$^{-/-}$ and *Rb*$^{-/-}$;*p130*$^{-/-}$ mice die earlier during gestation than germline *Rb*$^{-/-}$ mice, with more severe defects in the same tissues (Lee et al., 1996). These studies underscore the ability of *p107* or *p130* to functionally substitute for other pocket protein family members in many developing tissues. Finally, a unique role for *p107* and *p130* was found in developing cartilage; when both were lost, increased proliferation of growth plate chondrocytes was observed, which led to morphological defects that resulted in perinatal lethality (Cobrinik et al., 1996). The tissue-specific redundancy of the pocket proteins may be attributed to the differences in expression levels of the pocket proteins in different tissues, or may hint at specific roles for each protein in particular tissues. Importantly, germline *Rb*$^{-/-}$;*p107*$^{-/-}$;*p130*$^{-/-}$ mice are inviable, demonstrating that pocket protein function overall is essential for survival.

ii. Molecular Mechanisms of Pocket Protein-Mediated Differentiation

Cellular differentiation proceeds through distinct stages and the pocket proteins can control this process through one or more of the following mechanisms: initiation of irreversible cell cycle exit, protection from apoptosis, maintenance of the post-mitotic state, and overall coordination of the timing and procession of differentiation (reviewed in (Lipinski and Jacks, 1999). Notably, the pocket proteins can also participate directly in activation of cell type-specific gene expression. The following sections will focus on the major contributing functions of pocket proteins on differentiation: the regulation of cell cycle exit, and the direct role of these proteins in promoting differentiation.

a. Cell Cycle Exit Functions

One of the important ways in which the pocket proteins control differentiation is through coordination of cell cycle exit and initiation of the differentiation program. During the process of terminal differentiation, the expression and activity of the pocket proteins is regulated in a manner distinct from that during normal cell cycle. Differentiation requires that cells irreversibly exit the cell cycle before initiating tissue-specific gene expression and

undergoing terminal morphological changes. As cells enter G0, p130 expression surpasses that of p107, and pocket protein-E2F complexes are predominantly p130-E2F4. In contrast, pRb expression levels remain relatively constant during cell cycle exit, but the majority is hypophosphorylated, and therefore active. Coincident with cell cycle exit in a variety of tissues, there is an increase in pRb-E2F complexes, which makes a key contribution towards inducing cell cycle exit during differentiation.

Although pRb is the chief player in regulating E2F activity during cell cycle exit, in vitro neuronal differentiation studies provide evidence for important contributions by p107 and p130 in this process. p107 expression is upregulated in $Rb^{-/-}$ neural precursors, and it can form complexes with E2F3, which is normally exclusively bound to pRb (Callaghan et al., 1999). Expression of the E1A oncoprotein in $Rb^{-/-}$ neural precursors leads to apoptosis after induction of differentiation, further implicating a role for p107 and p130 in promoting neuronal differentiation (Callaghan et al., 1999).

In the skin, the pocket proteins play a critical role in ensuring that proliferation and differentiation occur in a stepwise fashion with the proper timing and location. This was demonstrated in $p107^{-/-};p130^{-/-}$ mice in which the basal cells of the epidermis ectopically proliferate, and the spatially restricted differentiation markers K5 and K6 are expanded in their expression (Ruiz et al., 2003). Moreover, K14-Cre mediated deletion of *Rb* in the skin of $p107^{-/-}$ mice also leads to ectopic proliferation, as well as co-expression of the early and late differentiation markers K10 and K5, and inappropriate expression of hair follicle marker K6 in the suprabasal layer of skin (Ruiz et al., 2004) showing that the absence of pocket protein function can result in the uncoupling of proliferation and differentiation. Notably, deletion of *p107* and *Rb* in the skin also leads to carcinogenesis, suggesting that by allowing differentiation to proceed without cell cycle exit pocket protein loss may predispose tissues to tumor formation.

The notion that the pocket proteins are required to coordinate cell cycle exit and differentiation is further supported by data from hair cells in the ear. Tissue-specific deletion of *Rb* in these cells leads to increased numbers and proliferation of hair cells (Sage et al., 2005). Although the hair cells were ectopically proliferating, they were terminally differentiated based on morphology and expression of differentiation markers (Sage et al., 2005). These data demonstrated that in this cell type, *Rb* is required for switching off

proliferation but not for execution of the differentiation program. Together, these data demonstrate that in the absence of pocket proteins, cell cycle exit and differentiation may become uncoupled, leading to proliferation of differentiated cell types and tissue dysplasia.

b. Direct Control of Differentiation

In addition to its role in cell cycle regulation, the pocket proteins can directly influence differentiation through interactions with and modulation of the activity of many differentiation-specific transcription factors. For the most part, pRb has been demonstrated to augment the activity of tissue-specific transcription factors in tissues of the mesenchymal lineage, discussed in greater detail below. In general, there is evidence for pRb promoting tissue-specific gene expression by binding to a variety of transcription factors involved in differentiation (Figure 3).

pRb expression can enhance MyoD activity and induce expression of late muscle differentiation markers like MHC and MCK, although whether this is via a direct interaction between pRb and MyoD remains controversial (Guo et al., 1995). Additionally, pRb has been demonstrated to directly interact with general differentiation-associated transcription factors Mhox, Chx10, and Pax3 (Arnold and Winter, 1998), as well as the neuronal-specific nuclear protein NRP/B during differentiation (Kim et al., 1998). Finally, pRb interacts directly with C/EBPβ and PPARγ to modulate adipocyte differentiation, and with Runx2 to induce osteoblast differentiation; the importance of these interactions will be discussed below. Importantly, both MyoD and C/EBPα, another adipose-specific transcription factor, have been shown to induce the expression of the CDK inhibitor p21, which causes cell cycle arrest and retains pRb in the active, hypophosphorylated form (Guo et al., 1995; Halevy et al., 1995; Timchenko et al., 1996). This suggests that regulation of tissue-specific factors by pRb may result in a feed-forward mechanism to promote differentiation in which pRb promotes cell cycle exit and induces differentiation-specific gene expression.

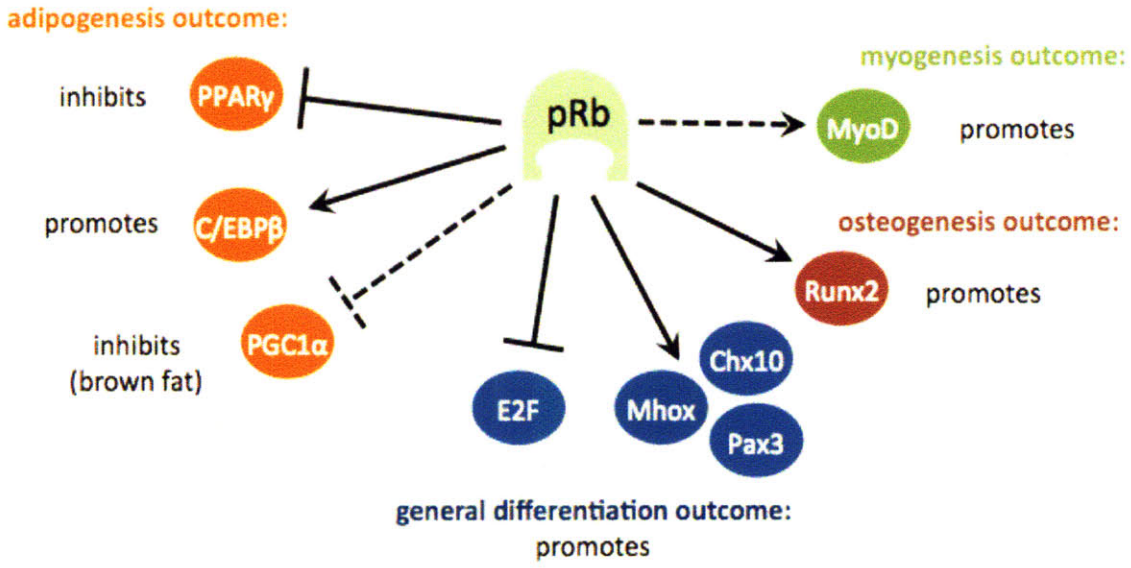

Figure 3: pRb Promotes Differentiation Through Regulation of Tissue-Specific Differentiation Factors

(See text for details.) pRb can influence the outcome of terminal differentiation by directly (solid lines) or indirectly (dotted lines) interacting with a number of factors involved in adipogenesis, myogenesis, and osteogenesis, as well as general differentiation functions. The stated outcomes indicate pRb's contribution to the differentiation program and do not necessarily indicate the function of the protein with which it interacts. For example, pRb inhibits PPARγ and thus adipogenesis; PPARγ promotes adipogenesis.

B. Regulation of Mesenchymal Tissue Development and Differentiation

i. Embryonic Origins of Mesenchymal Tissues

Osteoblasts (bone), adipocytes (fat), chondrocytes (cartilage), myoblasts (muscle), and fibroblasts (connective tissue) are the cell types considered to make up the mesenchymal tissues. These cells arise from both the ectodermal and mesodermal germ layers during embryogenesis. Importantly, despite the strong similarities among the skeletal elements, they arise from three different tissue types within these different germ layers. The cartilage, bone, and connective tissues of the face and head develop from the craniofacial mesenchyme, which originates as cranial neural crest tissue, of ectodermal origin. The remaining skeletal, muscle, and connective elements come from one of two regions of the mesoderm. Somites derived from the paraxial mesoderm further specialize into either the sclerotome, which gives rise to the bone and cartilage of the vertebrae and ribs (the axial skeleton), or the dermamyotome, from which brown adipose tissue and the muscles of the back and around the limbs arises. Finally, the bone, cartilage and connective tissue of the limbs (the appendicular skeleton) develop from the lateral plate mesoderm. The origin of white adipose tissue is unclear, but it may arise from mesodermal derived pericytes that reside on the outside of blood vessels (Tang et al., 2008). The distinct fate choices of cells within these different tissue regions depend on the location of these cells within the mesodermal compartment and the signals that they receive from the surrounding tissue (Gilbert, 2006) and references therein).

The pocket proteins have been found to play a significant role in regulating the differentiation of osteoblasts, chondrocytes, myocytes, and adipocytes. Our studies have further explored the role of the pocket proteins in cartilage, bone, and fat development. The rest of this section will focus in more detail on their function in these mesenchymal tissue types.

ii. Fat Development

Fat tissue is located in a number of anatomical sites throughout the body and each depot is comprised of one either white adipose tissue (WAT) or brown adipose tissue (BAT). These originate from different cellular precursors, differentiate through mutually exclusive pathways, and perform distinct functions (reviewed in (Seale et al., 2009). The primary function of WAT is energy storage, which it achieves through de novo synthesis of triglycerides from glucose and import of fatty acids from the blood. In contrast, the purpose of BAT is thermogenesis and energy expenditure. The expression of the BAT-specific protein uncoupling protein 1 (UCP1) is responsible for the ability of brown adipocytes to generate heat and expend chemical energy. UCP1 is a mitochondrial membrane protein that catalyzes proton release and thus disruption of the membrane potential, preventing ATP production and instead allowing energy dissipation. Unsurprisingly, BAT contains the greatest amount of mitochondria of any mammalian tissue except cardiomyocytes (Seale et al., 2009).

Two key transcription factors control the process of adipocyte differentiation in both WAT and BAT. The first, PPARγ (peroxisome proliferator-activated receptor-γ), a nuclear hormone receptor, is induced early during differentiation and stimulates adipogenesis in response to lipids (Tontonoz et al., 1994). The second factor, C/EBP (CCAAT/enhancer binding protein), has three isoforms, α, β, and δ, which induce PPARγ expression, among other adipogenesis genes (Seale et al., 2009). C/EBPα and PPARγ work cooperatively to coordinate a differentiation program that results in a stably differentiated adipocyte (Wu et al., 1999). Interestingly, pRb can function both to promote and inhibit adipogenesis through interactions with these proteins. pRb directly binds C/EBPs and co-activates C/EBPβ transcriptional activity to promote adipogenesis (Chen et al., 1996). However, it has also been demonstrated that pRb can bind PPARγ and attenuate its transactivation capacity by recruiting HDACs to silence genes required for adipogenesis (Fajas et al., 2002).

BAT, unlike WAT, requires an additional set of transcription factors for its unique developmental program. These proteins, and thus BAT differentiation, is also highly regulated by the pocket proteins. For example, PGC1α (PPARγ coactivator-1α), a critical regulator of mitochondrial biogenesis, promotes, but is not necessary for, BAT

development and function (Uldry et al., 2006). When ectopically expressed in WAT, PGC1α causes UCP1 upregulation, among other BAT-specific genes (Puigserver et al., 1998; Tiraby et al., 2003; Uldry et al., 2006). The *PGC1α* promoter is bound and repressed by pRb (Scime et al., 2005), suggesting that pRb can modulate brown fat differentiation specifically. Indeed, $Rb^{-/-}$ MEFs induced to differentiate into adipocytes exhibit brown fat characteristics, including high levels of mitochondria and expression of UCP1 and PGC1α (Hansen et al., 2004). Furthermore, WAT in $p107^{-/-}$ mice contains abundant brown fat-like cells, containing numerous mitochondria and expressing BAT-specific genes (Scime et al., 2005). These results support the idea that pocket proteins negatively regulate PGC1α expression, and thereby inhibit adoption of the brown fat fate.

iii. Bone Development

Bones serve both mechanical/locomotive and protective functions in vertebrate organisms, as well as house the hematopoietic niche within the marrow. All bones begin as condensations of mesenchymal cells, but depending upon anatomical location, can develop through two distinct pathways called intramembraneous and endochondral ossification (Nakashima and de Crombrugghe, 2003). Intramembraneous ossification occurs in most of the skull and craniofacial bones, as well as part of the clavicles, and proceeds through direct differentiation of osteoblasts from the mesenchymal condensations. In contrast, endochondral ossification takes place in the axial (spine and ribs) and appendicular (limb) skeletons. In these bones, mesenchymal condensations differentiate first into chondrocytes, which proliferate and further differentiate, providing a cartilage scaffold. Osteoblasts then invade this cartilage mold, where they terminally differentiate and produce the characteristic calcified matrix of bone (Kronenberg, 2003). In both modes of bone development, osteoclasts, which derive from the monocytic lineage, are responsible for bone resorption, an important activity involved in the bone remodeling process that occurs throughout the life of a vertebrate organism (Karsenty and Wagner, 2002). This section will deal with specific mechanisms of osteoblast differentiation through both

developmental modalities; cartilage development and endochondral ossification are discussed in the next section.

The primary function of osteoblasts is to synthesize the extra-cellular matrix characteristic of bone. Several proteins identified in vitro have been shown to be critical for this process, including alkaline phosphatase (AP), type I collagens, osteopontin (OP), osteocalcin (OC), and bone sialoprotein (Bsp) (Karsenty and Wagner, 2002). Although not exclusively expressed by osteoblasts, the combinatorial expression of these molecules provides a signature of osteoblast differentiation. The final event in bone formation is mineralization of the matrix into hydroxyapatite, which is composed predominantly of calcium phosphate and hydroxyl ions, and also contains potassium, fluoride, and magnesium.

The transcription factor Runx2 (also called Cbfa1) is a considered the master regulator of osteoblast differentiation from mesenchymal precursors (reviewed in (Nakashima and de Crombrugghe, 2003). *Runx2* was identified as the gene mutated in human cleidocranial dysplasia, an inherited syndrome characterized by a multitude of skeletal patterning and growth defects (Mundlos et al., 1997). In the mouse, expression of Runx2 in the mesoderm begins at e9.5, and is present at high levels in e13.5 mesenchymal condensations. The importance of *Runx2* in bone development is exemplified by the striking phenotype of *Runx2*$^{-/-}$ mice, which have no osteoblasts and completely lack ossification in any skeletal elements (Komori et al., 1997; Otto et al., 1997). Osterix (Osx) is another critical osteoblast-specific transcription factor expressed early during differentiation and required for bone formation. Like *Runx2*$^{-/-}$ mice, *Osx*$^{-/-}$ mice lack ossified skeletal elements, and osteoblasts are found arrested within the mesenchymal condensations, regardless of the mode of ossification (Nakashima et al., 2002). *Osx*-null osteoblasts show decreased expression of the osteoblast-specific collagen Col1a1, and undetectable levels of other proteins required for proper bone differentiation including Bsp, OP and OC. Notably, *Runx2* expression is normal in *Osx*$^{-/-}$ mice, although *Osx* is not expressed in *Runx2*$^{-/-}$ mice, suggesting that Osx acts downstream of Runx2 (Nakashima et al., 2002).

Rb critically regulates bone differentiation, and does so directly by augmentation of Runx2 transcriptional activity, and indirectly via its impact on cell cycle exit. The first

indication that *Rb* played a role in bone differentiation came from studies of viral oncoprotein-mediated effects on osteoblast differentiation from stromal cells. Stromal cells immortalized by SV40 T-antigen are unable to differentiate into osteoblasts (Feuerbach et al., 1997). Moreover, E1A expression in immortalized osteoblast precursors represses osteoblast differentiation, and this requires the pocket protein-binding domain (Beck et al., 1998). Direct evidence of *Rb* involvement in osteoblast differentiation came from the inability of $Rb^{-/-}$ MEFs to differentiate into bone after treatment with BMP2, a molecule known to induce differentiation (Thomas et al., 2001). pRb was also shown to interact with and enhance the transcriptional activity of Runx2, in association with another Runx2 binding protein, Hes1 (Lee et al., 2006; Luan et al., 2007; Thomas et al., 2001).

More recent studies have shown that *Rb* is critical for proper bone development in vivo. Mice lacking *Rb* have defects in both intramembraneous and endochondral bone development, concurrent with decreased ossification and reduced expression of the osteoblast markers alkaline phosphatase and collagen I (Berman et al., 2008b; Gutierrez et al., 2008). These in vivo defects are suppressed by concomitant *E2f1* loss, indicating that the role of *Rb* in bone differentiation is *E2f1* dependent, and is, at least in part, cell cycle exit mediated (Berman et al., 2008b). Interestingly, colony-forming unit (Cfu) assays to detect osteoprogenitors revealed increased numbers of these cells in the $Rb^{-/-}$ skulls, suggesting that *Rb*-deficiency also leads to inappropriate maintenance of a progenitor pool, perhaps through failure of progenitors to exit the cell cycle and/or due to a direct role of *Rb* in promoting osteoblast differentiation (Gutierrez et al., 2008).

iv. Cartilage Development

Cartilage is comprised of chondrocytes, which secrete a specialized extra-cellular matrix rich in type II collagen. Cartilage exists in several forms throughout the body but most studies have focused on the development of the temporary cartilage of the growth plate required to prefigure the bones during endochondral ossification. During this process, chondrocytes differentiate directly from mesenchymal condensations, then undergo a precisely defined program of proliferation, hypertrophic differentiation, and

apoptosis, thereby generating the cartilage scaffold upon which bone later forms, illustrated in Figure 4. In this model, chondrocytes first proliferate and line up in stacks exhibiting a characteristic coin-like appearance. Upon receiving the appropriate signals, these chondrocytes terminally differentiate into hypertrophic chondrocytes and secrete a specialized collagen, Collagen X. Finally, hypertrophic chondrocytes promote the differentiation and invasion of osteoblasts from the surrounding undifferentiated mesenchyme to form the bone collar, and then die. Successive iterations of this process result in the longitudinal growth of the long bones. This differentiation program is controlled extrinsically by diffusible signaling molecules, and by an intrinsic genetic network of differentiation-specific transcription factors (Kronenberg, 2003) (Figure 4). Below, the most relevant molecules will be discussed.

SOX9 is the master regulator of chondrocyte differentiation from mesenchymal condensations, and cells lacking *Sox9* expression within chimeric mice are excluded from mesenchymal condensations (Bi et al., 1999). SOX9 is responsible for inducing the expression of a number of critical differentiation genes, including *Col1a1*, *Col2a1*, and *aggrecan*, in addition to downstream transcriptional regulators of chondrocyte differentiation, *Sox5* and *Sox6*. SOX9 is required at both early and late time points of chondrocyte differentiation as demonstrated by tissue-specific mouse knockouts. When *Sox9* is deleted early in limb bud mesenchyme, cartilage condensations do not form, *Sox5* and *Sox6* are not expressed, and mesenchymal condensations are susceptible to Bmp-induced apoptosis (Akiyama et al., 2002). Furthermore, conditional loss of *Sox9* driven by Col2-Cre results in decreased chondrocyte proliferation, premature hypertrophic differentiation, and muted Ihh/PTHrP signaling (discussed below). *Sox5*$^{-/-}$;*Sox6*$^{-/-}$ mice phenocopy *Sox9*-deficiency, further demonstrating that these transcription factors are essential to mediate proper chondrocyte differentiation growth plate development (Akiyama et al., 2002; Smits et al., 2001).

The most well characterized morphogenic signaling pathway that controls growth plate development is the Indian Hedgehog (Ihh)/Parathyroid Hormone Related-protein (PTHrP) signaling loop. Mouse knockouts of *Ihh*, *PTHrP*, and the *PTHrP receptor* (*PPR*) demonstrate that they coordinate the timing and location of chondrocyte differentiation by promoting proliferation and inhibiting differentiation of chondrocytes in a location-

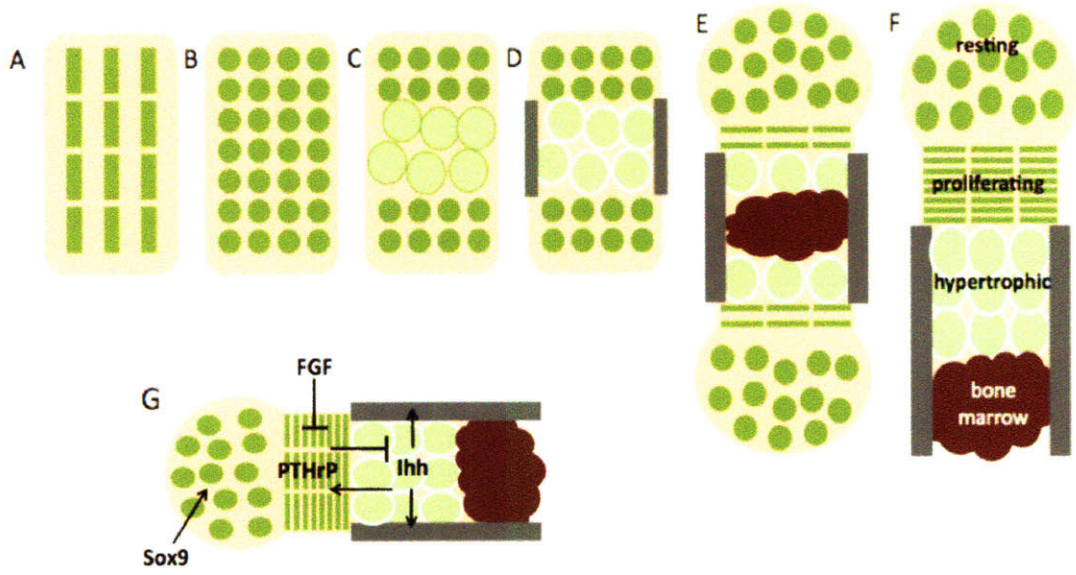

Figure 4: Endochondral Ossification of the Long Bones
(A) Endochondral ossification begins from the condensation of mesenchymal cells in the site of the future bone. (B) Mesenchymal cells differentiate into chondrocytes through the actions of the master regulator Sox 9 (see G). (C) Chondrocytes proliferate and begin to terminally differentiate into hypertrophic chondrocytes. (D) Hypertrophic chondrocytes secrete the specialized collagen, Collagen X and express Ihh, which promotes bone collar formation (grey bars; see G). (E) Hypertrophic chondrocytes recruit hematopoietic cells to generate the bone marrow and osteoblasts, which will form the bone matrix upon the cartilage scaffold, then die. (F) The embryonic growth plate eventually contains chondrocytes in all stages of differentiation: resting, proliferative, and hypertrophic. Waves of proliferation and differentiation lead to longitudinal growth of the long bone. (G) Signaling molecules can inhibit (FGF) or promote (Ihh/PTHrP) chondrocyte proliferation in the growth plate.

dependent manner. *Ihh*$^{-/-}$, *PTHrP*$^{-/-}$, and *PPR*$^{-/-}$ mice all exhibit decreased proliferation and increased and ectopic hypertrophy of chondrocytes, leading to shorter and malformed long bones (Karaplis et al., 1994; Lanske et al., 1996; St-Jacques et al., 1999). Overexpression of PTHrP or PPR has the opposite effect, resulting in delayed chondrocyte differentiation and malformed bones (Schipani et al., 1997; Weir et al., 1996). Analysis of Ihh and PTHrP chimeric mice demonstrated that local feedback between Ihh and PTHrP is responsible for determining the length of the proliferative region in the growth plate by ensuring that proximity to cells secreting the growth signal controls the decision to proliferate or differentiate (Chung et al., 1998; Chung et al., 2001). Ihh is also responsible for inducing osteoblast differentiation from the mesenchymal cells adjacent to hypertrophic chondrocytes, as *Ihh*$^{-/-}$ mice lack osteoblasts in the bone collar (St-Jacques et al., 1999).

FGF signaling also plays a critical role in maintaining the appropriate balance of proliferation and differentiation in the growth plate. Three of the four FGF receptors (FGFR1, 2, and 3) have confirmed expression at various time points during endochondral ossification, and several FGF genes have been implicated as critical ligands (Kronenberg, 2003). Mouse studies have revealed that the major function of FGFs in growth plate chondrocytes is inhibition of proliferation and acceleration of differentiation, which is achieved both directly, through downstream JAK/STAT signaling (Sahni et al., 1999), and indirectly via of Ihh expression (Minina et al., 2002). Both *Fgfr3*$^{-/-}$ and *Fgf18*$^{-/-}$ mice display similar phenotypes, namely, increased chondrocyte proliferation, expansion in the length of the growth plate, delayed ossification, and increased Ihh expression (Colvin et al., 1996; Deng et al., 1996; Liu et al., 2002; Minina et al., 2002; Ohbayashi et al., 2002) supporting the role of FGF in limiting chondrocyte proliferation.

Both *p107* and *p130* have been shown to specifically regulate proliferation and differentiation of the growth plate and endochondral ossification. Combined loss of *p107* and *p130* in the germline leads to shorter long bones and reduced endochondral ossification (Cobrinik et al., 1996). These defects are attributed to inappropriate proliferation of chondrocytes in the long bone epiphyses. The requirement for *p107* and *p130* in the growth plate is in part due to their participation in the FGF signaling pathway (Dailey et al., 2003). FGF signaling leads to G1 arrest and dephosphorylation of all pocket proteins in rat chondrosarcoma cells as well as micromass cultures and bone rudiments

(Laplantine et al., 2002). FGF directly induces the rapid dephosphorylation of p107 through activation of PP2A, while dephosphorylation of pRb and p130 is delayed and likely due to inhibition of cyclin/CDK complexes (Kolupaeva et al., 2008). FGF-induced growth arrest is ablated in the absence of *p107* and *p130* (Laplantine et al., 2002). Moreover, *p107* and *p130* are required for the coordination of proliferation and differentiation in the developing growth plate by indirectly promoting the expression of Runx2, Ihh and PPR after cell cycle exit (Rossi et al., 2002). In a manner similar to *p130*, *p27*-loss in combination with *p107*-loss leads to shorter long bones with increased chondrocyte proliferation and decreased differentiation (Yeh et al., 2007). Consistent with the importance of the Rb pathway in regulating cartilage development, Cyclin D has been shown to be required for chondrocyte proliferation downstream of TGFβ and PTHrP signaling in both primary chondrocytes and chondrodysplasia syndromes (Beier et al., 2001; Beier and LuValle, 2002).

Part III: The p53 Tumor Suppressor

The p53 tumor suppressor is considered the "guardian of the genome" due to its role in responding to intrinsic cellular stress (Lane, 1992). p53 is activated under conditions that jeopardize genomic integrity, and in response can induces the expression of a host of target genes resulting in cell cycle arrest or apoptosis depending on the severity of the damage. The importance of these functions in tumor suppression is evidenced by the frequency of p53 mutation in human cancers. p53 mutations are often the causative factor in Li-Fraumeni syndrome, a familial cancer syndrome typified by multiple malignancies, including osteosarcoma (Varley, 2003). p53 mutations also play a critical role in the progression of spontaneous osteosarcoma (Kansara and Thomas, 2007). Most of the cancer-associated mutations identified affect the ability of p53 to bind DNA, underscoring the importance of p53 transcriptional activity in tumor suppression (Olivier et al., 2004; Pfeifer, 2000).

A. Regulation of p53

p53 is predominantly regulated post-translationally. The major negative regulator of p53 is Mdm2, an E3 ubiquitin-ligase that ubiquitinates p53 and targets it for degradation. Through binding, Mdm2 also physically inhibits p53 by blocking the transactivation domain. The significance of this interaction is demonstrated by the fact that *Mdm2*$^{-/-}$ mice are inviable, but concomitant loss of *p53* rescues this phenotype (Jones et al., 1995; Montes de Oca Luna et al., 1995). The Mdm2 homologue, MdmX, although not a ubiquitin-ligase, also inhibits p53 activity (Marine and Jochemsen, 2005). Like *Mdm2*$^{-/-}$ mice, *MdmX*$^{-/-}$ mice are embryonic lethal, and this is similarly rescued by *p53*-loss, suggesting that this interaction is biologically significant (Parant et al., 2001).

Mdm2-mediated ubiquitination of p53 is antagonized by p19^{Arf}, making p19^{Arf} an indirect positive regulator of p53 (Honda and Yasuda, 1999; Kamijo et al., 1998; Pomerantz et al., 1998; Stott et al., 1998; Zhang et al., 1998). *Arf* is expressed as an alternative product from the *Cdkn2a/Ink4a* promoter, which also encodes the CDK inhibitor p16^{INK4A}, but their protein products are quite distinct due to the different reading frames (Quelle et al., 1995).

$p19^{Arf}$ is thought to sequester and inhibit Mdm2 in the nucleolus, where it resides, thereby activating p53 (Tao and Levine, 1999; Weber et al., 1999). Interestingly, *Arf* is both positively and negatively regulated through binding of the E2Fs to the promoter, thereby implicating the Rb pathway in p53 regulation (Aslanian et al., 2004).

E2F has been implicated in influencing p53 activity in other ways. E2F regulates the expression of *ATM* and *CHK2*, indirectly regulating phosphorylation of p53 by these kinases (Berkovich and Ginsberg, 2003; Powers et al., 2004; Rogoff et al., 2002; Rogoff et al., 2004). E2F also activates the expression of many p53 co-factors like ASPP1, ASPP2, JMY, and TP53INP1, which modulate p53 transcriptional activity on different targets (Hershko et al., 2005). It has been hypothesized that the crosstalk between the Rb and p53 pathways allow the cell to monitor proliferation levels, but this remains to be examined in mouse models.

p53 is also regulated by post-translational modifications, predominantly phosphorylation, after DNA damage (Bode and Dong, 2004). These modifications often lead to increased DNA binding activity (Lane et al., 1994). Phosphorylation of particular p53 residues also disrupts Mdm2 binding, which leads to accumulation of p53 in the absence of ubiquitin-mediated degradation (Shieh et al., 1997). The major kinases associated with p53 phosphorylation are the ATM (ataxia telangiectasia mutated) and ATR (ATM-related) kinases, which are activated in response to UV- and γ-irradiation-induced DNA damage. ATM and ATR, and their downstream kinases Chk1 and Chk2, phosphorylate the N-terminus of p53 in response to DNA damage (Zhou and Elledge, 2000). These phosphorylation events are critical for tumor suppression. Patients who carry inherited mutations in *ATM* or *CHK2* are predisposed to a variety of cancers, including sarcomas (Bell et al., 1999; Lee et al., 2001; Reed et al., 1966).

B. p53 Target Genes

The transcriptional targets of p53 fall into two categories, those that promote cell cycle arrest, and those involved in apoptosis. p53 can activate both classes of genes in response to stress, but how specificity of the p53 response is achieved is unclear and is an active area of study. One possible mechanism of specificity is mediated through the ASPP

p53-binding proteins, which enhance p53 binding to pro-apoptotic targets (Samuels-Lev et al., 2001). However, additional mechanisms are still to be discovered.

Of the p53 targets that regulate cell cycle arrest, p21 is the best understood, and probably the most critical. The importance of p21 in the p53 response has been demonstrated in *p21*$^{-/-}$ MEFs, which are defective in the p53-dependent arrest in response to DNA damage (Brugarolas et al., 1995; Deng et al., 1995). p21 induces G1 arrest by inhibiting the G1/S kinases Cyclin D/CDK4 and 6 and Cyclin E/CDK2 (Sherr and Roberts, 1995). p53 also regulates G2/M arrest by inducing the Cyclin B/CDK1-inhibitors *GADD45* and *14-3-3*σ (Hermeking et al., 1997; Hollander et al., 1993).

The apoptosis-promoting class of target genes induced by p53 includes mediators of both the intrinsic (mitochondrial-mediated) and extrinsic (Fas-ligand activated) pathways of cell death. p53 induces the Bcl2-family member Bax, which is involved in cytochrome c release from the mitochondria (Scorrano and Korsmeyer, 2003). The BH3-only proteins Bid, Noxa, and Puma are also induced by p53, and function to increase the pro-apoptotic activity of Bax or decrease the protective functions of Bcl2. Finally, p53 can induce *Apaf1*, an apoptotic effector that cooperates with Caspase9 to initiate caspase activation (Moroni et al., 2001).

C. Role of p53 in Cancer and Development

Mouse models of cancer dependent on *p53* mutations have provided valuable tools to elucidate the tumor suppressive functions of *p53*. Analysis of *p53*$^{-/-}$ mice demonstrated that *p53* is virtually dispensable for embryonic development, but adult mice develop a variety of spontaneous tumors by 6 months of age (Donehower et al., 1992). Further investigation revealed that mice heterozygous for *p53* deletion were also tumor prone, and that in most cases, loss of the WT allele of *p53* occurred (Jacks et al., 1994). Importantly, these two mouse strains developed distinct tumor spectrums, indicating that *p53* status can influence tumor latency and tissue distribution of tumors (Jacks et al., 1994).

p53 enhances tumorigenesis in a variety of mouse models of cancer (Attardi and Jacks, 1999). Although there is abundant evidence for *p53*-loss interacting with a number

of tumor suppressors and oncogenes to promote tumorigenesis in the literature, I will focus on the interactions between *p53* and *Rb* in mouse models, since this is most relevant to the work described in this thesis. *p53* and *Rb* are found mutated together in many human cancers, but the first evidence that they cooperate in tumorigenesis in the mouse came from a study by Williams, et al. *Rb* mutation reduces the viability of $p53^{+/-}$ and $p53^{-/-}$ mice, while *p53*-loss accelerates pituitary and thyroid tumorigenesis (Williams et al., 1994a). These mice also develop novel tumors like pinealoblastomas, which are not seen in $Rb^{+/-}$ or *p53* mutant mice (Williams et al., 1994a). Furthermore, we and others have shown that deleting *p53* in the osteoblast lineage using *Osx-Cre* leads to fully penetrant osteosarcoma, and tumorigenesis is accelerated and exacerbated by *Rb*-loss (Berman et al., 2008a; Walkley et al., 2008). Deletion of both *p53* and *Rb* in this lineage also leads to a broader spectrum of tumor types, including hibernomas and neuroendocrine tumors (Berman et al., 2008a).

p53 was not considered to be a developmental regulator, given that $p53^{-/-}$ mice survive to adulthood with no developmental abnormalities (Donehower et al., 1992). Recent studies, however, have demonstrated a role for *p53* in negatively regulating osteoblast differentiation. $p53^{-/-}$ mice display an increased bone mass phenotype, and $p53^{-/-}$ osteoblasts differentiate into bone faster than WT osteoblasts, with increased expression of the osteogenic marker *Osx* (Wang et al., 2006). This study also showed that *p53* could directly repress the *Osx* promoter in vitro. In addition, deletion of *Mdm2* in osteoblast precursors leads to skeletal deformities, and osteoblasts from these mice show increased p53 activity, reduced proliferation, and increased activity of Runx2, a master regulator of osteoblast differentiation (Lengner et al., 2006). It still remains to be seen whether *p53* plays a direct role in differentiation of this lineage in vivo, however, these studies clearly demonstrate a role for *p53* in this process.

REFERENCES

Akiyama, H., Chaboissier, M.C., Martin, J.F., Schedl, A., and de Crombrugghe, B. (2002). The transcription factor Sox9 has essential roles in successive steps of the chondrocyte differentiation pathway and is required for expression of Sox5 and Sox6. Genes Dev *16*, 2813-2828.

Arnold, H.H., and Winter, B. (1998). Muscle differentiation: more complexity to the network of myogenic regulators. Curr Opin Genet Dev *8*, 539-544.

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

Attardi, L.D., and Jacks, T. (1999). The role of p53 in tumour suppression: lessons from mouse models. Cell Mol Life Sci *55*, 48-63.

Attwooll, C., Oddi, S., Cartwright, P., Prosperini, E., Agger, K., Steensgaard, P., Wagener, C., Sardet, C., Moroni, M.C., and 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*, 1199-1208.

Bandara, L.R., Buck, V.M., Zamanian, M., Johnston, L.H., and La Thangue, N.B. (1993). Functional synergy between DP-1 and E2F-1 in the cell cycle-regulating transcription factor DRTF1/E2F. EMBO J *12*, 4317-4324.

Bandara, L.R., and La Thangue, N.B. (1991). Adenovirus E1a prevents the retinoblastoma gene product from complexing with a cellular transcription factor. Nature *351*, 494 -497.

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

Beier, F., Ali, Z., Mok, D., Taylor, A.C., Leask, T., Albanese, C., Pestell, R.G., and LuValle, P. (2001). TGFbeta and PTHrP control chondrocyte proliferation by activating cyclin D1 expression. Mol Biol Cell *12*, 3852-3863.

Beier, F., and LuValle, P. (2002). The cyclin D1 and cyclin A genes are targets of activated PTH/PTHrP receptors in Jansen's metaphyseal chondrodysplasia. Mol Endocrinol *16*, 2163-2173.

Beijersbergen, R.L., Carlee, L., Kerkhoven, R.M., and Bernards, R. (1995). Regulation of the retinoblastoma protein-related p107 by G1 cyclin complexes. Genes Dev *9*, 1340 -1353.

Beijersbergen, R.L., Kerkhoven, R.M., Zhu, L., Carlee, L., Voorhoeve, P.M., and 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*, 2680-2690.

Bell, S.M., Schreiner, C.M., Schultheis, P.J., Miller, M.L., Evans, R.L., Vorhees, C.V., Shull, G.E., and Scott, W.J. (1999). Targeted disruption of the murine Nhe1 locus induces ataxia, growth retardation, and seizures. Am J Physiol *276*, C788-795.

Berkovich, E., and Ginsberg, D. (2003). ATM is a target for positive regulation by E2F-1. Oncogene *22*, 161-167.

Berman, S.D., Calo, E., Landman, A.S., Danielian, P.S., Miller, E.S., West, J.C., Fonhoue, B.D., Caron, A., Bronson, R., Bouxsein, M.L., *et al.* (2008a). Metastatic osteosarcoma induced by inactivation of Rb and p53 in the osteoblast lineage. Proc Natl Acad Sci U S A *105*, 11851-11856.

Berman, S.D., Yuan, T.L., Miller, E.S., Lee, E.Y., Caron, A., and Lees, J.A. (2008b). The retinoblastoma protein tumor suppressor is important for appropriate osteoblast differentiation and bone development. Mol Cancer Res *6*, 1440-1451.

Bi, W., Deng, J.M., Zhang, Z., Behringer, R.R., and de Crombrugghe, B. (1999). Sox9 is required for cartilage formation. Nat Genet *22*, 85-89.

Bode, A.M., and Dong, Z. (2004). Post-translational modification of p53 in tumorigenesis. Nat Rev Cancer *4*, 793-805.

Brehm, A., Miska, E.A., McCance, D.J., Reid, J.L., Bannister, A.J., and Kouzarides, T. (1998). Retinoblastoma protein recruits histone deacetylase to repress transcription. Nature *391*, 597-601.

Brugarolas, J., Chandrasekaran, C., Gordon, J.I., Beach, D., Jacks, T., and Hannon, G.J. (1995). Radiation-induced cell cycle arrest compromised by p21 deficiency. Nature *377*, 552-557.

Burkhart, D.L., and Sage, J. (2008). Cellular mechanisms of tumour suppression by the retinoblastoma gene. Nat Rev Cancer *8*, 671-682.

Callaghan, D.A., Dong, L., Callaghan, S.M., Hou, Y.X., Dagnino, L., and Slack, R.S. (1999). Neural precursor cells differentiating in the absence of Rb exhibit delayed terminal mitosis and deregulated E2F 1 and 3 activity. Dev Biol *207*, 257-270.

Canhoto, A.J., Chestukhin, A., Litovchick, L., and DeCaprio, J.A. (2000). Phosphorylation of the retinoblastoma-related protein p130 in growth-arrested cells. Oncogene *19*, 5116-5122.

Chen, P.L., Riley, D.J., Chen, Y., and Lee, W.H. (1996). Retinoblastoma protein positively regulates terminal adipocyte differentiation through direct interaction with C/EBPs. Genes Dev *10*, 2794-2804.

Chen, P.L., Scully, P., Shew, J.Y., Wang, J.Y., and Lee, W.H. (1989). Phosphorylation of the retinoblastoma gene product is modulated during the cell cycle and cellular differentiation. Cell *58*, 1193-1198.

Chittenden, T., Livingston, D.M., and DeCaprio, J.A. (1993). Cell cycle analysis of E2F in primary human T cells reveals novel E2F complexes and biochemically distinct forms of free E2F. Mol Cell Biol *13*, 3975-3983.

Chong, J.L., Tsai, S.Y., Sharma, N., Opavsky, R., Price, R., Wu, L., Fernandez, S.A., and Leone, G. (2009). E2f3a and E2f3b contribute to the control of cell proliferation and mouse development. Mol Cell Biol *29*, 414-424.

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

Chung, U.I., Lanske, B., Lee, K., Li, E., and Kronenberg, H. (1998). The parathyroid hormone/parathyroid hormone-related peptide receptor coordinates endochondral bone development by directly controlling chondrocyte differentiation. Proc Natl Acad Sci U S A *95*, 13030-13035.

Chung, U.I., Schipani, E., McMahon, A.P., and Kronenberg, H.M. (2001). Indian hedgehog couples chondrogenesis to osteogenesis in endochondral bone development. J Clin Invest *107*, 295-304.

Clarke, A.R., Maandag, E.R., van Roon, M., van der Lugt, N.M., van der Valk, M., Hooper, M.L., Berns, A., and te Riele, H. (1992). Requirement for a functional Rb-1 gene in murine development. Nature *359*, 328-330.

Classon, M., and Dyson, N. (2001). p107 and p130: versatile proteins with interesting pockets. Exp Cell Res *264*, 135-147.

Classon, M., and Harlow, E. (2002). The retinoblastoma tumour suppressor in development and cancer. Nat Rev Cancer *2*, 910-917.

Claudio, P.P., Howard, C.M., Baldi, A., De Luca, A., Fu, Y., Condorelli, G., Sun, Y., Colburn, N., Calabretta, B., and Giordano, A. (1994). p130/pRb2 has growth suppressive properties similar to yet distinctive from those of retinoblastoma family members pRb and p107. Cancer Res *54*, 5556-5560.

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

Claudio, P.P., Howard, C.M., Pacilio, C., Cinti, C., Romano, G., Minimo, C., Maraldi, N.M., Minna, J.D., Gelbert, L., Leoncini, L., *et al.* (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*, 372-382.

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

Cobrinik, D., Whyte, P., Peeper, D.S., Jacks, T., and Weinberg, R.A. (1993). Cell cycle-specific association of E2F with the p130 E1A-binding protein. Genes Dev *7*, 2392-2404.

Colvin, J.S., Bohne, B.A., Harding, G.W., McEwen, D.G., and Ornitz, D.M. (1996). Skeletal overgrowth and deafness in mice lacking fibroblast growth factor receptor 3. Nat Genet *12*, 390-397.

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

Dahiya, A., Gavin, M.R., Luo, R.X., and Dean, D.C. (2000). Role of the LXCXE binding site in Rb function. Mol Cell Biol *20*, 6799-6805.

Dailey, L., Laplantine, E., Priore, R., and Basilico, C. (2003). A network of transcriptional and signaling events is activated by FGF to induce chondrocyte growth arrest and differentiation. J Cell Biol *161*, 1053-1066.

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

Danielian, P.S., Friesenhahn, L.B., Faust, A.M., West, J.C., Caron, A.M., Bronson, R.T., and Lees, J.A. (2008). E2f3a and E2f3b make overlapping but different contributions to total E2f3 activity. Oncogene *27*, 6561-6570.

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

Dannenberg, J.H., van Rossum, A., Schuijff, L., and te Riele, H. (2000). Ablation of the retinoblastoma gene family deregulates G(1) control causing immortalization and increased cell turnover under growth-restricting conditions. Genes Dev *14*, 3051 -3064.

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

DeCaprio, J.A. (2009). How the Rb tumor suppressor structure and function was revealed by the study of Adenovirus and SV40. Virology *384*, 274-284.

DeCaprio, J.A., Ludlow, J.W., Figge, J., Shew, J.Y., Huang, C.M., Lee, W.H., Marsilio, E., Paucha, E., and Livingston, D.M. (1988). SV40 large tumor antigen forms a specific complex with the product of the retinoblastoma susceptibility gene. Cell *54*, 275-283.

DeGregori, J., Kowalik, T., and Nevins, J.R. (1995). Cellular targets for activation by the E2F1 transcription factor include DNA synthesis- and G1/S-regulatory genes. Mol Cell Biol *15*, 4215-4224.

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

Deng, C., Wynshaw-Boris, A., Zhou, F., Kuo, A., and Leder, P. (1996). Fibroblast growth factor receptor 3 is a negative regulator of bone growth. Cell *84*, 911-921.

Deng, C., Zhang, P., Harper, J.W., Elledge, S.J., and Leder, P. (1995). Mice lacking p21CIP1/WAF1 undergo normal development, but are defective in G1 checkpoint control. Cell *82*, 675-684.

Deshpande, A.M., Akunowicz, J.D., Reveles, X.T., Patel, B.B., Saria, E.A., Gorlick, R.G., Naylor, S.L., Leach, R.J., and Hansen, M.F. (2007). PHC3, a component of the hPRC-H complex, associates with E2F6 during G0 and is lost in osteosarcoma tumors. Oncogene *26*, 1714-1722.

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

Dick, F.A., Sailhamer, E., and Dyson, N.J. (2000). Mutagenesis of the pRB pocket reveals that cell cycle arrest functions are separable from binding to viral oncoproteins. Mol Cell Biol *20*, 3715-3727.

Donehower, L.A., Harvey, M., Slagle, B.L., McArthur, M.J., Montgomery, C.A., Jr., Butel, J.S., and Bradley, A. (1992). Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. Nature *356*, 215-221.

Doorbar, J. (2006). Molecular biology of human papillomavirus infection and cervical cancer. Clin Sci (Lond) *110*, 525-541.

Dryja, T.P., Rapaport, J.M., Joyce, J.M., and Petersen, R.A. (1986). Molecular detection of deletions involving band q14 of chromosome 13 in retinoblastomas. Proc Natl Acad Sci U S A *83*, 7391-7394.

Dyson, N., Bernards, R., Friend, S.H., Gooding, L.R., Hassell, J.A., Major, E.O., Pipas, J.M., Vandyke, T., and Harlow, E. (1990). Large T antigens of many polyomaviruses are able to form complexes with the retinoblastoma protein. J Virol *64*, 1353-1356.

Dyson, N., Buchkovich, K., Whyte, P., and 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*, 249-255.

Dyson, N., Dembski, M., Fattaey, A., Ngwu, C., Ewen, M., and Helin, K. (1993). Analysis of p107-associated proteins: p107 associates with a form of E2F that differs from pRB -associated E2F-1. J Virol *67*, 7641-7647.

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

Elliott, M.J., Dong, Y.B., Yang, H., and McMasters, K.M. (2001). E2F-1 up-regulates c-Myc and p14(ARF) and induces apoptosis in colon cancer cells. Clin Cancer Res *7*, 3590 -3597.

Ewen, M.E., Faha, B., Harlow, E., and Livingston, D.M. (1992). Interaction of p107 with cyclin A independent of complex formation with viral oncoproteins. Science *255*, 85-87.

Faha, B., Ewen, M.E., Tsai, L.H., Livingston, D.M., and Harlow, E. (1992). Interaction between human cyclin A and adenovirus E1A-associated p107 protein. Science *255*, 87-90.

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

Ferreira, R., Magnaghi-Jaulin, L., Robin, P., Harel-Bellan, A., and 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*, 10493-10498.

Ferreira, R., Naguibneva, I., Mathieu, M., Ait-Si-Ali, S., Robin, P., Pritchard, L.L., and Harel -Bellan, A. (2001). Cell cycle-dependent recruitment of HDAC-1 correlates with deacetylation of histone H4 on an Rb-E2F target promoter. EMBO Rep *2*, 794-799.

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

Field, S.J., Tsai, F.Y., Kuo, F., Zubiaga, A.M., Kaelin, W.G., Jr., Livingston, D.M., Orkin, S.H., and Greenberg, M.E. (1996). E2F-1 functions in mice to promote apoptosis and suppress proliferation. Cell *85*, 549-561.

Flemington, E.K., Speck, S.H., and Kaelin, W.G., 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*, 6914-6918.

Friend, S.H., Bernards, R., Rogelj, S., Weinberg, R.A., Rapaport, J.M., Albert, D.M., and Dryja, T.P. (1986). A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. Nature *323*, 643-646.

Frolov, M.V., and Dyson, N.J. (2004). Molecular mechanisms of E2F-dependent activation and pRB-mediated repression. J Cell Sci *117*, 2173-2181.

Frolov, M.V., Stevaux, O., Moon, N.S., Dimova, D., Kwon, E.J., Morris, E.J., and Dyson, N.J. (2003). G1 cyclin-dependent kinases are insufficient to reverse dE2F2-mediated repression. Genes Dev *17*, 723-728.

Fung, Y.K., Murphree, A.L., T'Ang, A., Qian, J., Hinrichs, S.H., and Benedict, W.F. (1987). Structural evidence for the authenticity of the human retinoblastoma gene. Science *236*, 1657-1661.

Gaubatz, S., Lindeman, G.J., Ishida, S., Jakoi, L., Nevins, J.R., Livingston, D.M., and Rempel, R.E. (2000). E2F4 and E2F5 play an essential role in pocket protein-mediated G1 control. Mol Cell *6*, 729-735.

Ghosh, M.K., and Harter, M.L. (2003). A viral mechanism for remodeling chromatin structure in G0 cells. Mol Cell *12*, 255-260.

Gilbert, S.F. (2006). Developmental biology, 8th edn (Sunderland, Mass., Sinauer Associates Publishers).

Ginsberg, D., Vairo, G., Chittenden, T., Xiao, Z.X., Xu, G., Wydner, K.L., DeCaprio, J.A., Lawrence, J.B., and Livingston, D.M. (1994). E2F-4, a new member of the E2F transcription factor family, interacts with p107. Genes Dev *8*, 2665-2679.

Girling, R., Partridge, J.F., Bandara, L.R., Burden, N., Totty, N.F., Hsuan, J.J., and La Thangue, N.B. (1993). A new component of the transcription factor DRTF1/E2F. Nature *362*, 83-87.

Grewal, S.I., and Elgin, S.C. (2002). Heterochromatin: new possibilities for the inheritance of structure. Curr Opin Genet Dev *12*, 178-187.

Guo, K., Wang, J., Andres, V., Smith, R.C., and Walsh, K. (1995). MyoD-induced expression of p21 inhibits cyclin-dependent kinase activity upon myocyte terminal differentiation. Mol Cell Biol *15*, 3823-3829.

Gutierrez, G.M., Kong, E., Sabbagh, Y., Brown, N.E., Lee, J.S., Demay, M.B., Thomas, D.M., and Hinds, P.W. (2008). Impaired bone development and increased mesenchymal progenitor cells in calvaria of RB1-/- mice. Proc Natl Acad Sci U S A *105*, 18402 -18407.

Halevy, O., Novitch, B.G., Spicer, D.B., Skapek, S.X., Rhee, J., Hannon, G.J., Beach, D., and Lassar, A.B. (1995). Correlation of terminal cell cycle arrest of skeletal muscle with induction of p21 by MyoD. Science *267*, 1018-1021.

Hansen, J.B., Jorgensen, C., Petersen, R.K., Hallenborg, P., De Matteis, R., Boye, H.A., Petrovic, N., Enerback, S., Nedergaard, J., Cinti, S., *et al.* (2004). Retinoblastoma protein functions as a molecular switch determining white versus brown adipocyte differentiation. Proc Natl Acad Sci U S A *101*, 4112-4117.

Hauser, P.J., Agrawal, D., Chu, B., and Pledger, W.J. (1997). p107 and p130 associated cyclin A has altered substrate specificity. J Biol Chem *272*, 22954-22959.

He, Y., Armanious, M.K., Thomas, M.J., and Cress, W.D. (2000). Identification of E2F-3B, an alternative form of E2F-3 lacking a conserved N-terminal region. Oncogene *19*, 3422-3433.

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

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

Helin, K., Wu, C.L., Fattaey, A.R., Lees, J.A., Dynlacht, B.D., Ngwu, C., and Harlow, E. (1993). Heterodimerization of the transcription factors E2F-1 and DP-1 leads to cooperative trans-activation. Genes Dev *7*, 1850-1861.

Hermeking, H., Lengauer, C., Polyak, K., He, T.C., Zhang, L., Thiagalingam, S., Kinzler, K.W., and Vogelstein, B. (1997). 14-3-3 sigma is a p53-regulated inhibitor of G2/M progression. Mol Cell *1*, 3-11.

Hernando, E., Nahle, Z., Juan, G., Diaz-Rodriguez, E., Alaminos, M., Hemann, M., Michel, L., Mittal, V., Gerald, W., Benezra, R., *et al.* (2004). Rb inactivation promotes genomic instability by uncoupling cell cycle progression from mitotic control. Nature *430*, 797-802.

Hershko, T., Chaussepied, M., Oren, M., and Ginsberg, D. (2005). Novel link between E2F and p53: proapoptotic cofactors of p53 are transcriptionally upregulated by E2F. Cell Death Differ *12*, 377-383.

Hiebert, S.W., Chellappan, S.P., Horowitz, J.M., and Nevins, J.R. (1992). The interaction of RB with E2F coincides with an inhibition of the transcriptional activity of E2F. Genes Dev *6*, 177-185.

Hijmans, E.M., Voorhoeve, P.M., Beijersbergen, R.L., van 't Veer, L.J., and Bernards, R. (1995). E2F-5, a new E2F family member that interacts with p130 in vivo. Mol Cell Biol *15*, 3082-3089.

Ho, V.M., Schaffer, B.E., Karnezis, A.N., Park, K.S., and Sage, J. (2009). The retinoblastoma gene Rb and its family member p130 suppress lung adenocarcinoma induced by oncogenic K-Ras. Oncogene *28*, 1393-1399.

Hollander, M.C., Alamo, I., Jackman, J., Wang, M.G., McBride, O.W., and Fornace, A.J., Jr. (1993). Analysis of the mammalian gadd45 gene and its response to DNA damage. J Biol Chem *268*, 24385-24393.

Honda, R., and Yasuda, H. (1999). Association of p19(ARF) with Mdm2 inhibits ubiquitin ligase activity of Mdm2 for tumor suppressor p53. EMBO J *18*, 22-27.

Hu, Q.J., Dyson, N., and Harlow, E. (1990). The regions of the retinoblastoma protein needed for binding to adenovirus E1A or SV40 large T antigen are common sites for mutations. EMBO J *9*, 1147-1155.

Huang, S., Wang, N.P., Tseng, B.Y., Lee, W.H., and Lee, E.H. (1990). Two distinct and frequently mutated regions of retinoblastoma protein are required for binding to SV40 T antigen. EMBO J *9*, 1815-1822.

Huber, H.E., Edwards, G., Goodhart, P.J., Patrick, D.R., Huang, P.S., Ivey-Hoyle, M., Barnett, S.F., Oliff, A., and Heimbrook, D.C. (1993). Transcription factor E2F binds DNA as a heterodimer. Proc Natl Acad Sci U S A *90*, 3525-3529.

Huh, M.S., Parker, M.H., Scime, A., Parks, R., and Rudnicki, M.A. (2004). Rb is required for progression through myogenic differentiation but not maintenance of terminal differentiation. J Cell Biol *166*, 865-876.

Humbert, P.O., Rogers, C., Ganiatsas, S., Landsberg, R.L., Trimarchi, J.M., Dandapani, S., Brugnara, C., Erdman, S., Schrenzel, M., Bronson, R.T., *et al.* (2000a). E2F4 is essential for normal erythrocyte maturation and neonatal viability. Mol Cell *6*, 281-291.

Humbert, P.O., Verona, R., Trimarchi, J.M., Rogers, C., Dandapani, S., and Lees, J.A. (2000b). E2f3 is critical for normal cellular proliferation. Genes Dev *14*, 690-703.

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

Ivey-Hoyle, M., Conroy, R., Huber, H.E., Goodhart, P.J., Oliff, A., and Heimbrook, D.C. (1993). Cloning and characterization of E2F-2, a novel protein with the biochemical properties of transcription factor E2F. Mol Cell Biol *13*, 7802-7812.

Jacks, T., Fazeli, A., Schmitt, E.M., Bronson, R.T., Goodell, M.A., and Weinberg, R.A. (1992). Effects of an Rb mutation in the mouse. Nature *359*, 295-300.

Jacks, T., Remington, L., Williams, B.O., Schmitt, E.M., Halachmi, S., Bronson, R.T., and Weinberg, R.A. (1994). Tumor spectrum analysis in p53-mutant mice. Curr Biol *4*, 1 -7.

Johnson, D.G., Schwarz, J.K., Cress, W.D., and Nevins, J.R. (1993). Expression of transcription factor E2F1 induces quiescent cells to enter S phase. Nature *365*, 349-352.

Jones, S.N., Roe, A.E., Donehower, L.A., and Bradley, A. (1995). Rescue of embryonic lethality in Mdm2-deficient mice by absence of p53. Nature *378*, 206-208.

Kaelin, W.G., Jr., Ewen, M.E., and Livingston, D.M. (1990). Definition of the minimal simian virus 40 large T antigen- and adenovirus E1A-binding domain in the retinoblastoma gene product. Mol Cell Biol *10*, 3761-3769.

Kaelin, W.G., Jr., Krek, W., Sellers, W.R., DeCaprio, J.A., Ajchenbaum, F., Fuchs, C.S., Chittenden, T., Li, Y., Farnham, P.J., Blanar, M.A., *et al.* (1992). Expression cloning of a cDNA encoding a retinoblastoma-binding protein with E2F-like properties. Cell *70*, 351-364.

Kamijo, T., Weber, J.D., Zambetti, G., Zindy, F., Roussel, M.F., and Sherr, C.J. (1998). Functional and physical interactions of the ARF tumor suppressor with p53 and Mdm2. Proc Natl Acad Sci U S A *95*, 8292-8297.

Kansara, M., and Thomas, D.M. (2007). Molecular pathogenesis of osteosarcoma. DNA Cell Biol *26*, 1-18.

Karaplis, A.C., Luz, A., Glowacki, J., Bronson, R.T., Tybulewicz, V.L., Kronenberg, H.M., and Mulligan, R.C. (1994). Lethal skeletal dysplasia from targeted disruption of the parathyroid hormone-related peptide gene. Genes Dev *8*, 277-289.

Karsenty, G., and Wagner, E.F. (2002). Reaching a genetic and molecular understanding of skeletal development. Dev Cell *2*, 389-406.

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

Kolupaeva, V., Laplantine, E., and Basilico, C. (2008). PP2A-mediated dephosphorylation of p107 plays a critical role in chondrocyte cell cycle arrest by FGF. PLoS ONE *3*, e3447.

Komori, T., Yagi, H., Nomura, S., Yamaguchi, A., Sasaki, K., Deguchi, K., Shimizu, Y., Bronson, R.T., Gao, Y.H., Inada, M., *et al.* (1997). Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. Cell *89*, 755-764.

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

Kowalik, T.F., DeGregori, J., Schwarz, J.K., and Nevins, J.R. (1995). E2F1 overexpression in quiescent fibroblasts leads to induction of cellular DNA synthesis and apoptosis. J Virol *69*, 2491-2500.

Kronenberg, H.M. (2003). Developmental regulation of the growth plate. Nature *423*, 332 -336.

La Thangue, N.B., and Rigby, P.W. (1987). An adenovirus E1A-like transcription factor is regulated during the differentiation of murine embryonal carcinoma stem cells. Cell *49*, 507-513.

Lam, E.W., and Watson, R.J. (1993). An E2F-binding site mediates cell-cycle regulated repression of mouse B-myb transcription. EMBO J *12*, 2705-2713.

Lane, D.P. (1992). Cancer. p53, guardian of the genome. Nature *358*, 15-16.

Lane, D.P., Lu, X., Hupp, T., and Hall, P.A. (1994). The role of the p53 protein in the apoptotic response. Philos Trans R Soc Lond B Biol Sci *345*, 277-280.

Lanske, B., Karaplis, A.C., Lee, K., Luz, A., Vortkamp, A., Pirro, A., Karperien, M., Defize, L.H., Ho, C., Mulligan, R.C., *et al.* (1996). PTH/PTHrP receptor in early development and Indian hedgehog-regulated bone growth. Science *273*, 663-666.

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

Lara, M.F., Santos, M., Ruiz, S., Segrelles, C., Moral, M., Martinez-Cruz, A.B., Hernandez, P., Martinez-Palacio, J., Lorz, C., Garcia-Escudero, R., *et al.* (2008). p107 acts as a tumor suppressor in pRb-deficient epidermis. Mol Carcinog *47*, 105-113.

LeCouter, J.E., Kablar, B., Hardy, W.R., Ying, C., Megeney, L.A., May, L.L., and Rudnicki, M.A. (1998a). Strain-dependent myeloid hyperplasia, growth deficiency, and accelerated cell cycle in mice lacking the Rb-related p107 gene. Mol Cell Biol *18*, 7455-7465.

LeCouter, J.E., Kablar, B., Whyte, P.F., Ying, C., and Rudnicki, M.A. (1998b). Strain-dependent embryonic lethality in mice lacking the retinoblastoma-related p130 gene. Development *125*, 4669-4679.

Lee, C., Chang, J.H., Lee, H.S., and Cho, Y. (2002). Structural basis for the recognition of the E2F transactivation domain by the retinoblastoma tumor suppressor. Genes Dev *16*, 3199-3212.

Lee, E.Y., Chang, C.Y., Hu, N., Wang, Y.C., Lai, C.C., Herrup, K., Lee, W.H., and Bradley, A. (1992). Mice deficient for Rb are nonviable and show defects in neurogenesis and haematopoiesis. Nature *359*, 288-294.

Lee, E.Y., Hu, N., Yuan, S.S., Cox, L.A., Bradley, A., Lee, W.H., and Herrup, K. (1994). Dual roles of the retinoblastoma protein in cell cycle regulation and neuron differentiation. Genes Dev *8*, 2008-2021.

Lee, J.S., Thomas, D.M., Gutierrez, G., Carty, S.A., Yanagawa, S., and Hinds, P.W. (2006). HES1 cooperates with pRb to activate RUNX2-dependent transcription. J Bone Miner Res *21*, 921-933.

Lee, M.H., Williams, B.O., Mulligan, G., Mukai, S., Bronson, R.T., Dyson, N., Harlow, E., and Jacks, T. (1996). Targeted disruption of p107: functional overlap between p107 and Rb. Genes Dev *10*, 1621-1632.

Lee, S.B., Kim, S.H., Bell, D.W., Wahrer, D.C., Schiripo, T.A., Jorczak, M.M., Sgroi, D.C., Garber, J.E., Li, F.P., Nichols, K.E., *et al.* (2001). Destabilization of CHK2 by a missense mutation associated with Li-Fraumeni Syndrome. Cancer Res *61*, 8062-8067.

Lee, W.H., Bookstein, R., Hong, F., Young, L.J., Shew, J.Y., and Lee, E.Y. (1987a). Human retinoblastoma susceptibility gene: cloning, identification, and sequence. Science *235*, 1394-1399.

Lee, W.H., Shew, J.Y., Hong, F.D., Sery, T.W., Donoso, L.A., Young, L.J., Bookstein, R., and Lee, E.Y. (1987b). The retinoblastoma susceptibility gene encodes a nuclear phosphoprotein associated with DNA binding activity. Nature *329*, 642-645.

Lees, E., Faha, B., Dulic, V., Reed, S.I., and Harlow, E. (1992). Cyclin E/cdk2 and cyclin A/cdk2 kinases associate with p107 and E2F in a temporally distinct manner. Genes Dev *6*, 1874-1885.

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

Lengner, C.J., Steinman, H.A., Gagnon, J., Smith, T.W., Henderson, J.E., Kream, B.E., Stein, G.S., Lian, J.B., and Jones, S.N. (2006). Osteoblast differentiation and skeletal development are regulated by Mdm2-p53 signaling. J Cell Biol *172*, 909-921.

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

Li, Y., Graham, C., Lacy, S., Duncan, A.M., and 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*, 2366-2377.

Lindeman, G.J., Dagnino, L., Gaubatz, S., Xu, Y., Bronson, R.T., Warren, H.B., and Livingston, D.M. (1998). A specific, nonproliferative role for E2F-5 in choroid plexus function revealed by gene targeting. Genes Dev *12*, 1092-1098.

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

Lipinski, M.M., Macleod, K.F., Williams, B.O., Mullaney, T.L., Crowley, D., and Jacks, T. (2001). Cell-autonomous and non-cell-autonomous functions of the Rb tumor suppressor in developing central nervous system. EMBO J *20*, 3402-3413.

Liu, Z., Xu, J., Colvin, J.S., and Ornitz, D.M. (2002). Coordination of chondrogenesis and osteogenesis by fibroblast growth factor 18. Genes Dev *16*, 859-869.

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

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

Lu, X., Magrane, G., Yin, C., Louis, D.N., Gray, J., and Van Dyke, T. (2001). Selective inactivation of p53 facilitates mouse epithelial tumor progression without chromosomal instability. Mol Cell Biol *21*, 6017-6030.

Luan, Y., Yu, X.P., Xu, K., Ding, B., Yu, J., Huang, Y., Yang, N., Lengyel, P., Di Cesare, P.E., and Liu, C.J. (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*, 16860-16870.

Ludlow, J.W., DeCaprio, J.A., Huang, C.M., Lee, W.H., Paucha, E., and Livingston, D.M. (1989). SV40 large T antigen binds preferentially to an underphosphorylated member of the retinoblastoma susceptibility gene product family. Cell *56*, 57-65.

Ludlow, J.W., Shon, J., Pipas, J.M., Livingston, D.M., and DeCaprio, J.A. (1990). The retinoblastoma susceptibility gene product undergoes cell cycle-dependent dephosphorylation and binding to and release from SV40 large T. Cell *60*, 387-396.

Lukas, J., Bartkova, J., and Bartek, J. (1996a). Convergence of mitogenic signalling cascades from diverse classes of receptors at the cyclin D-cyclin-dependent kinase-pRb -controlled G1 checkpoint. Mol Cell Biol *16*, 6917-6925.

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

Luo, R.X., Postigo, A.A., and Dean, D.C. (1998). Rb interacts with histone deacetylase to repress transcription. Cell *92*, 463-473.

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

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

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

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

Mann, D.J., and Jones, N.C. (1996). E2F-1 but not E2F-4 can overcome p16-induced G1 cell -cycle arrest. Curr Biol *6*, 474-483.

Marine, J.C., and Jochemsen, A.G. (2005). Mdmx as an essential regulator of p53 activity. Biochem Biophys Res Commun *331*, 750-760.

Mayol, X., Garriga, J., and Grana, X. (1995). Cell cycle-dependent phosphorylation of the retinoblastoma-related protein p130. Oncogene *11*, 801-808.

Meuwissen, R., Linn, S.C., Linnoila, R.I., Zevenhoven, J., Mooi, W.J., and Berns, A. (2003). Induction of small cell lung cancer by somatic inactivation of both Trp53 and Rb1 in a conditional mouse model. Cancer Cell *4*, 181-189.

Mihara, K., Cao, X.R., Yen, A., Chandler, S., Driscoll, B., Murphree, A.L., T'Ang, A., and Fung, Y.K. (1989). Cell cycle-dependent regulation of phosphorylation of the human retinoblastoma gene product. Science *246*, 1300-1303.

Minina, E., Kreschel, C., Naski, M.C., Ornitz, D.M., and Vortkamp, A. (2002). Interaction of FGF, Ihh/Pthlh, and BMP signaling integrates chondrocyte proliferation and hypertrophic differentiation. Dev Cell *3*, 439-449.

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

Montes de Oca Luna, R., Wagner, D.S., and Lozano, G. (1995). Rescue of early embryonic lethality in mdm2-deficient mice by deletion of p53. Nature *378*, 203-206.

Morgenbesser, S.D., Williams, B.O., Jacks, T., and DePinho, R.A. (1994). p53-dependent apoptosis produced by Rb-deficiency in the developing mouse lens. Nature *371*, 72 -74.

Moroni, M.C., Hickman, E.S., Lazzerini Denchi, E., Caprara, G., Colli, E., Cecconi, F., Muller, H., and Helin, K. (2001). Apaf-1 is a transcriptional target for E2F and p53. Nat Cell Biol *3*, 552-558.

Morris, E.J., and Dyson, N.J. (2001). Retinoblastoma protein partners. Adv Cancer Res *82*, 1 -54.

Muller, H., Bracken, A.P., Vernell, R., Moroni, M.C., Christians, F., Grassilli, E., Prosperini, E., Vigo, E., Oliner, J.D., and Helin, K. (2001). E2Fs regulate the expression of genes involved in differentiation, development, proliferation, and apoptosis. Genes Dev *15*, 267-285.

Mundlos, S., Otto, F., Mundlos, C., Mulliken, J.B., Aylsworth, A.S., Albright, S., Lindhout, D., Cole, W.G., Henn, W., Knoll, J.H., *et al.* (1997). Mutations involving the transcription factor CBFA1 cause cleidocranial dysplasia. Cell *89*, 773-779.

Munger, K., Werness, B.A., Dyson, N., Phelps, W.C., Harlow, E., and Howley, P.M. (1989). Complex formation of human papillomavirus E7 proteins with the retinoblastoma tumor suppressor gene product. EMBO J *8*, 4099-4105.

Nakashima, K., and de Crombrugghe, B. (2003). Transcriptional mechanisms in osteoblast differentiation and bone formation. Trends Genet *19*, 458-466.

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

Nicolas, E., Roumillac, C., and Trouche, D. (2003). Balance between acetylation and methylation of histone H3 lysine 9 on the E2F-responsive dihydrofolate reductase promoter. Mol Cell Biol *23*, 1614-1622.

Nielsen, S.J., Schneider, R., Bauer, U.M., Bannister, A.J., Morrison, A., O'Carroll, D., Firestein, R., Cleary, M., Jenuwein, T., Herrera, R.E., *et al.* (2001). Rb targets histone H3 methylation and HP1 to promoters. Nature *412*, 561-565.

Novitch, B.G., Mulligan, G.J., Jacks, T., and Lassar, A.B. (1996). Skeletal muscle cells lacking the retinoblastoma protein display defects in muscle gene expression and accumulate in S and G2 phases of the cell cycle. J Cell Biol *135*, 441-456.

Novitch, B.G., Spicer, D.B., Kim, P.S., Cheung, W.L., and Lassar, A.B. (1999). pRb is required for MEF2-dependent gene expression as well as cell-cycle arrest during skeletal muscle differentiation. Curr Biol *9*, 449-459.

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

Ohbayashi, N., Shibayama, M., Kurotaki, Y., Imanishi, M., Fujimori, T., Itoh, N., and Takada, S. (2002). FGF18 is required for normal cell proliferation and differentiation during osteogenesis and chondrogenesis. Genes Dev *16*, 870-879.

Olivier, M., Hussain, S.P., Caron de Fromentel, C., Hainaut, P., and Harris, C.C. (2004). TP53 mutation spectra and load: a tool for generating hypotheses on the etiology of cancer. IARC Sci Publ, 247-270.

Ormondroyd, E., de la Luna, S., and La Thangue, N.B. (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*, 1437-1446.

Otto, F., Thornell, A.P., Crompton, T., Denzel, A., Gilmour, K.C., Rosewell, I.R., Stamp, G.W., Beddington, R.S., Mundlos, S., Olsen, B.R., *et al.* (1997). Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. Cell *89*, 765-771.

Pan, H., Yin, C., Dyson, N.J., Harlow, E., Yamasaki, L., and Van Dyke, T. (1998). Key roles for E2F1 in signaling p53-dependent apoptosis and in cell division within developing tumors. Mol Cell *2*, 283-292.

Parant, J., Chavez-Reyes, A., Little, N.A., Yan, W., Reinke, V., Jochemsen, A.G., and Lozano, G. (2001). Rescue of embryonic lethality in Mdm4-null mice by loss of Trp53 suggests a nonoverlapping pathway with MDM2 to regulate p53. Nat Genet *29*, 92-95.

Perez-Ordonez, B., Beauchemin, M., and Jordan, R.C. (2006). Molecular biology of squamous cell carcinoma of the head and neck. J Clin Pathol *59*, 445-453.

Pfeifer, G.P. (2000). p53 mutational spectra and the role of methylated CpG sequences. Mutat Res *450*, 155-166.

Pomerantz, J., Schreiber-Agus, N., Liegeois, N.J., Silverman, A., Alland, L., Chin, L., Potes, J., Chen, K., Orlow, I., Lee, H.W., *et al.* (1998). The Ink4a tumor suppressor gene product, p19Arf, interacts with MDM2 and neutralizes MDM2's inhibition of p53. Cell *92*, 713-723.

Powers, J.T., Hong, S., Mayhew, C.N., Rogers, P.M., Knudsen, E.S., and Johnson, D.G. (2004). E2F1 uses the ATM signaling pathway to induce p53 and Chk2 phosphorylation and apoptosis. Mol Cancer Res *2*, 203-214.

Puigserver, P., Ribot, J., Serra, F., Gianotti, M., Bonet, M.L., Nadal-Ginard, B., and Palou, A. (1998). Involvement of the retinoblastoma protein in brown and white adipocyte cell differentiation: functional and physical association with the adipogenic transcription factor C/EBPalpha. Eur J Cell Biol *77*, 117-123.

Qin, X.Q., Livingston, D.M., Kaelin, W.G., Jr., and Adams, P.D. (1994). Deregulated transcription factor E2F-1 expression leads to S-phase entry and p53-mediated apoptosis. Proc Natl Acad Sci U S A *91*, 10918-10922.

Quelle, D.E., Zindy, F., Ashmun, R.A., and Sherr, C.J. (1995). Alternative reading frames of the INK4a tumor suppressor gene encode two unrelated proteins capable of inducing cell cycle arrest. Cell *83*, 993-1000.

Rayman, J.B., Takahashi, Y., Indjeian, V.B., Dannenberg, J.H., Catchpole, S., Watson, R.J., te Riele, H., and Dynlacht, B.D. (2002). E2F mediates cell cycle-dependent transcriptional repression in vivo by recruitment of an HDAC1/mSin3B corepressor complex. Genes Dev *16*, 933-947.

Reed, W.B., Epstein, W.L., Boder, E., and Sedgwick, R. (1966). Cutaneous manifestations of ataxia-telangiectasia. JAMA *195*, 746-753.

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

Robanus-Maandag, E., Dekker, M., van der Valk, M., Carrozza, M.L., Jeanny, J.C., Dannenberg, J.H., Berns, A., and te Riele, H. (1998). p107 is a suppressor of retinoblastoma development in pRb-deficient mice. Genes Dev *12*, 1599-1609.

Robertson, K.D., Ait-Si-Ali, S., Yokochi, T., Wade, P.A., Jones, P.L., and Wolffe, A.P. (2000). DNMT1 forms a complex with Rb, E2F1 and HDAC1 and represses transcription from E2F-responsive promoters. Nat Genet *25*, 338-342.

Rogers, K.T., Higgins, P.D., Milla, M.M., Phillips, R.S., and Horowitz, J.M. (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*, 7594-7599.

Rogoff, H.A., Pickering, M.T., Debatis, M.E., Jones, S., and Kowalik, T.F. (2002). E2F1 induces phosphorylation of p53 that is coincident with p53 accumulation and apoptosis. Mol Cell Biol *22*, 5308-5318.

Rogoff, H.A., Pickering, M.T., Frame, F.M., Debatis, M.E., Sanchez, Y., Jones, S., and Kowalik, T.F. (2004). Apoptosis associated with deregulated E2F activity is dependent on E2F1 and Atm/Nbs1/Chk2. Mol Cell Biol *24*, 2968-2977.

Rossi, F., MacLean, H.E., Yuan, W., Francis, R.O., Semenova, E., Lin, C.S., Kronenberg, H.M., and Cobrinik, D. (2002). p107 and p130 Coordinately regulate proliferation, Cbfa1 expression, and hypertrophic differentiation during endochondral bone development. Dev Biol *247*, 271-285.

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

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

Sage, C., Huang, M., Karimi, K., Gutierrez, G., Vollrath, M.A., Zhang, D.S., Garcia-Anoveros, J., Hinds, P.W., Corwin, J.T., Corey, D.P., *et al.* (2005). Proliferation of functional hair cells in vivo in the absence of the retinoblastoma protein. Science *307*, 1114-1118.

Sage, J., Miller, A.L., Perez-Mancera, P.A., Wysocki, J.M., and Jacks, T. (2003). Acute mutation of retinoblastoma gene function is sufficient for cell cycle re-entry. Nature *424*, 223 -228.

Sage, J., Mulligan, G.J., Attardi, L.D., Miller, A., Chen, S., Williams, B., Theodorou, E., and Jacks, T. (2000). Targeted disruption of the three Rb-related genes leads to loss of G(1) control and immortalization. Genes Dev *14*, 3037-3050.

Sahni, M., Ambrosetti, D.C., Mansukhani, A., Gertner, R., Levy, D., and Basilico, C. (1999). FGF signaling inhibits chondrocyte proliferation and regulates bone development through the STAT-1 pathway. Genes Dev *13*, 1361-1366.

Samuels-Lev, Y., O'Connor, D.J., Bergamaschi, D., Trigiante, G., Hsieh, J.K., Zhong, S., Campargue, I., Naumovski, L., Crook, T., and Lu, X. (2001). ASPP proteins specifically stimulate the apoptotic function of p53. Mol Cell *8*, 781-794.

Santos, M., Ruiz, S., Lara, M.F., Segrelles, C., Moral, M., Martinez-Cruz, A.B., Ballestin, C., Lorz, C., Garcia-Escudero, R., and Paramio, J.M. (2008). Susceptibility of pRb-deficient epidermis to chemical skin carcinogenesis is dependent on the p107 allele dosage. Mol Carcinog *47*, 815-821.

Sardet, C., Vidal, M., Cobrinik, D., Geng, Y., Onufryk, C., Chen, A., and Weinberg, R.A. (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*, 2403-2407.

Schipani, E., Lanske, B., Hunzelman, J., Luz, A., Kovacs, C.S., Lee, K., Pirro, A., Kronenberg, H.M., and Juppner, H. (1997). Targeted expression of constitutively active receptors for parathyroid hormone and parathyroid hormone-related peptide delays endochondral bone formation and rescues mice that lack parathyroid hormone -related peptide. Proc Natl Acad Sci U S A *94*, 13689-13694.

Schwarz, J.K., Bassing, C.H., Kovesdi, I., Datto, M.B., Blazing, M., George, S., Wang, X.F., and Nevins, J.R. (1995). Expression of the E2F1 transcription factor overcomes type beta transforming growth factor-mediated growth suppression. Proc Natl Acad Sci U S A *92*, 483-487.

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

Scorrano, L., and Korsmeyer, S.J. (2003). Mechanisms of cytochrome c release by proapoptotic BCL-2 family members. Biochem Biophys Res Commun *304*, 437-444.

Seale, P., Kajimura, S., and Spiegelman, B.M. (2009). Transcriptional control of brown adipocyte development and physiological function--of mice and men. Genes Dev *23*, 788-797.

Shan, B., Zhu, X., Chen, P.L., Durfee, T., Yang, Y., Sharp, D., and Lee, W.H. (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*, 5620-5631.

Sherr, C.J., and Roberts, J.M. (1995). Inhibitors of mammalian G1 cyclin-dependent kinases. Genes Dev *9*, 1149-1163.

Shieh, S.Y., Ikeda, M., Taya, Y., and Prives, C. (1997). DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2. Cell *91*, 325-334.

Simin, K., Wu, H., Lu, L., Pinkel, D., Albertson, D., Cardiff, R.D., and Van Dyke, T. (2004). pRb inactivation in mammary cells reveals common mechanisms for tumor initiation and progression in divergent epithelia. PLoS Biol *2*, E22.

Smits, P., Li, P., Mandel, J., Zhang, Z., Deng, J.M., Behringer, R.R., de Crombrugghe, B., and Lefebvre, V. (2001). The transcription factors L-Sox5 and Sox6 are essential for cartilage formation. Dev Cell *1*, 277-290.

Sparkes, R.S., Sparkes, M.C., Wilson, M.G., Towner, J.W., Benedict, W., Murphree, A.L., and Yunis, J.J. (1980). Regional assignment of genes for human esterase D and retinoblastoma to chromosome band 13q14. Science *208*, 1042-1044.

Spike, B.T., Dirlam, A., Dibling, B.C., Marvin, J., Williams, B.O., Jacks, T., and Macleod, K.F. (2004). The Rb tumor suppressor is required for stress erythropoiesis. EMBO J *23*, 4319-4329.

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

Stabel, S., Argos, P., and Philipson, L. (1985). The release of growth arrest by microinjection of adenovirus E1A DNA. EMBO J *4*, 2329-2336.

Stott, F.J., Bates, S., James, M.C., McConnell, B.B., Starborg, M., Brookes, S., Palmero, I., Ryan, K., Hara, E., Vousden, K.H., *et al.* (1998). The alternative product from the human CDKN2A locus, p14(ARF), participates in a regulatory feedback loop with p53 and MDM2. EMBO J *17*, 5001-5014.

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

Tang, W., Zeve, D., Suh, J.M., Bosnakovski, D., Kyba, M., Hammer, R.E., Tallquist, M.D., and Graff, J.M. (2008). White fat progenitor cells reside in the adipose vasculature. Science *322*, 583-586.

Tao, W., and Levine, A.J. (1999). P19(ARF) stabilizes p53 by blocking nucleo-cytoplasmic shuttling of Mdm2. Proc Natl Acad Sci U S A *96*, 6937-6941.

Thomas, D.M., Carty, S.A., Piscopo, D.M., Lee, J.S., Wang, W.F., Forrester, W.C., and Hinds, P.W. (2001). The retinoblastoma protein acts as a transcriptional coactivator required for osteogenic differentiation. Mol Cell *8*, 303-316.

Timchenko, N.A., Wilde, M., Nakanishi, M., Smith, J.R., and Darlington, G.J. (1996). CCAAT/enhancer-binding protein alpha (C/EBP alpha) inhibits cell proliferation through the p21 (WAF-1/CIP-1/SDI-1) protein. Genes Dev *10*, 804-815.

Tiraby, C., Tavernier, G., Lefort, C., Larrouy, D., Bouillaud, F., Ricquier, D., and Langin, D. (2003). Acquirement of brown fat cell features by human white adipocytes. J Biol Chem *278*, 33370-33376.

Tontonoz, P., Hu, E., and Spiegelman, B.M. (1994). Stimulation of adipogenesis in fibroblasts by PPAR gamma 2, a lipid-activated transcription factor. Cell *79*, 1147-1156.

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

Trimarchi, J.M., Fairchild, B., Wen, J., and Lees, J.A. (2001). The E2F6 transcription factor is a component of the mammalian Bmi1-containing polycomb complex. Proc Natl Acad Sci U S A *98*, 1519-1524.

Trimarchi, J.M., and Lees, J.A. (2002). Sibling rivalry in the E2F family. Nat Rev Mol Cell Biol *3*, 11-20.

Tsai, K.Y., Hu, Y., Macleod, K.F., Crowley, D., Yamasaki, L., and 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*, 293-304.

Uldry, M., Yang, W., St-Pierre, J., Lin, J., Seale, P., and Spiegelman, B.M. (2006). Complementary action of the PGC-1 coactivators in mitochondrial biogenesis and brown fat differentiation. Cell Metab *3*, 333-341.

Vandel, L., Nicolas, E., Vaute, O., Ferreira, R., Ait-Si-Ali, S., and Trouche, D. (2001). Transcriptional repression by the retinoblastoma protein through the recruitment of a histone methyltransferase. Mol Cell Biol *21*, 6484-6494.

Varley, J.M. (2003). Germline TP53 mutations and Li-Fraumeni syndrome. Hum Mutat *21*, 313-320.

Vousden, K.H., and Jat, P.S. (1989). Functional similarity between HPV16E7, SV40 large T and adenovirus E1a proteins. Oncogene *4*, 153-158.

Walkley, C.R., Qudsi, R., Sankaran, V.G., Perry, J.A., Gostissa, M., Roth, S.I., Rodda, S.J., Snay, E., Dunning, P., Fahey, F.H., *et al.* (2008). Conditional mouse osteosarcoma, dependent on p53 loss and potentiated by loss of Rb, mimics the human disease. Genes Dev *22*, 1662-1676.

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

Weber, J.D., Taylor, L.J., Roussel, M.F., Sherr, C.J., and Bar-Sagi, D. (1999). Nucleolar Arf sequesters Mdm2 and activates p53. Nat Cell Biol *1*, 20-26.

Weir, E.C., Philbrick, W.M., Amling, M., Neff, L.A., Baron, R., and Broadus, A.E. (1996). Targeted overexpression of parathyroid hormone-related peptide in chondrocytes causes chondrodysplasia and delayed endochondral bone formation. Proc Natl Acad Sci U S A *93*, 10240-10245.

Whyte, P., Buchkovich, K.J., Horowitz, J.M., Friend, S.H., Raybuck, M., Weinberg, R.A., and Harlow, E. (1988). Association between an oncogene and an anti-oncogene: the adenovirus E1A proteins bind to the retinoblastoma gene product. Nature *334*, 124 -129.

Whyte, P., Williamson, N.M., and Harlow, E. (1989). Cellular targets for transformation by the adenovirus E1A proteins. Cell *56*, 67-75.

Williams, B.O., Remington, L., Albert, D.M., Mukai, S., Bronson, R.T., and Jacks, T. (1994a). Cooperative tumorigenic effects of germline mutations in Rb and p53. Nat Genet *7*, 480-484.

Williams, B.O., Schmitt, E.M., Remington, L., Bronson, R.T., Albert, D.M., Weinberg, R.A., and Jacks, T. (1994b). Extensive contribution of Rb-deficient cells to adult chimeric mice with limited histopathological consequences. EMBO J *13*, 4251-4259.

Wu, C.L., Zukerberg, L.R., Ngwu, C., Harlow, E., and Lees, J.A. (1995). In vivo association of E2F and DP family proteins. Mol Cell Biol *15*, 2536-2546.

Wu, L., de Bruin, A., Saavedra, H.I., Starovic, M., Trimboli, A., Yang, Y., Opavska, J., Wilson, P., Thompson, J.C., Ostrowski, M.C., *et al.* (2003). Extra-embryonic function of Rb is essential for embryonic development and viability. Nature *421*, 942-947.

Wu, L., Timmers, C., Maiti, B., Saavedra, H.I., Sang, L., Chong, G.T., Nuckolls, F., Giangrande, P., Wright, F.A., Field, S.J., *et al.* (2001). The E2F1-3 transcription factors are essential for cellular proliferation. Nature *414*, 457-462.

Wu, Z., Rosen, E.D., Brun, R., Hauser, S., Adelmant, G., Troy, A.E., McKeon, C., Darlington, G.J., and Spiegelman, B.M. (1999). Cross-regulation of C/EBP alpha and PPAR gamma controls the transcriptional pathway of adipogenesis and insulin sensitivity. Mol Cell *3*, 151-158.

Xiao, A., Wu, H., Pandolfi, P.P., Louis, D.N., and Van Dyke, T. (2002). Astrocyte inactivation of the pRb pathway predisposes mice to malignant astrocytoma development that is accelerated by PTEN mutation. Cancer Cell *1*, 157-168.

Xiao, B., Spencer, J., Clements, A., Ali-Khan, N., Mittnacht, S., Broceno, C., Burghammer, M., Perrakis, A., Marmorstein, R., and Gamblin, S.J. (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*, 2363-2368.

Xiao, Z.X., Ginsberg, D., Ewen, M., and Livingston, D.M. (1996). Regulation of the retinoblastoma protein-related protein p107 by G1 cyclin-associated kinases. Proc Natl Acad Sci U S A *93*, 4633-4637.

Yamasaki, L., Bronson, R., Williams, B.O., Dyson, N.J., Harlow, E., and Jacks, T. (1998). Loss of E2F-1 reduces tumorigenesis and extends the lifespan of Rb1(+/-)mice. Nat Genet *18*, 360-364.

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

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

Yeh, N., Miller, J.P., Gaur, T., Capellini, T.D., Nikolich-Zugich, J., de la Hoz, C., Selleri, L., Bromage, T.G., van Wijnen, A.J., Stein, G.S., *et al.* (2007). Cooperation between p27 and p107 during endochondral ossification suggests a genetic pathway controlled by p27 and p130. Mol Cell Biol *27*, 5161-5171.

Zacksenhaus, E., Jiang, Z., Chung, D., Marth, J.D., Phillips, R.A., and Gallie, B.L. (1996). pRb controls proliferation, differentiation, and death of skeletal muscle cells and other lineages during embryogenesis. Genes Dev *10*, 3051-3064.

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

Zhang, Y., Xiong, Y., and Yarbrough, W.G. (1998). ARF promotes MDM2 degradation and stabilizes p53: ARF-INK4a locus deletion impairs both the Rb and p53 tumor suppression pathways. Cell *92*, 725-734.

Zhou, B.B., and Elledge, S.J. (2000). The DNA damage response: putting checkpoints in perspective. Nature *408*, 433-439.

Zhu, L., Enders, G., Lees, J.A., Beijersbergen, R.L., Bernards, R., and Harlow, E. (1995). The pRB-related protein p107 contains two growth suppression domains: independent interactions with E2F and cyclin/cdk complexes. EMBO J *14*, 1904-1913.

Zhu, L., van den Heuvel, S., Helin, K., Fattaey, A., Ewen, M., Livingston, D., Dyson, N., and Harlow, E. (1993). Inhibition of cell proliferation by p107, a relative of the retinoblastoma protein. Genes Dev *7*, 1111-1125.

Ziebold, U., Lee, E.Y., Bronson, R.T., and Lees, J.A. (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*, 6542-6552.

Ziebold, U., Reza, T., Caron, A., and Lees, J.A. (2001). E2F3 contributes both to the inappropriate proliferation and to the apoptosis arising in Rb mutant embryos. Genes Dev *15*, 386-391.

Chapter Two

Rb and *p107* Regulate Cartilage Development and Tumorigenesis

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The author designed and performed all experiments with the exception of acquisition of mice and colony generation, done by S.D.B., and a portion of the embryo and adult immunohistochemistry performed by J.C.W. Contributions to experimental design and data interpretation were made by S.D.B. and J.A.L.

ABSTRACT

The pocket proteins pRb, p107, and p130 have established roles in regulating the cell cycle through the control of E2F activity. These proteins have also been shown to regulate differentiation in cell cycle-dependent and –independent manners in a variety of tissues. Concomitant deletion of *p107* and *p130* in the mouse leads to defects in cartilage development during endochondral ossification, the process by which long bones form. Despite evidence of a role for pRb in osteoblast differentiation, it is unknown whether it functions during endochondral ossification. Here, we show that deletion of *Rb* in the mesenchymal progenitors of *p107*-null mice results in severe cartilage defects in the growth plates of long bones. In the embryo, the malformation of long bone cartilage is attributable to inappropriate chondrocyte proliferation in the growth plate. In the adult animals, inappropriate and persistent proliferation in the growth plate as well as altered expression of chondrocyte differentiation markers leads to enchondroma formation as early as 8 weeks of age. These results reveal a novel role for pRb in cartilage development and endochondral ossification. We have developed the first mouse model that defines a role for the pocket proteins in enchondroma formation. Furthermore, we show that pRb and p107 are required to coordinate proliferation and differentiation to prevent tumorigenesis in this tissue.

INTRODUCTION

The retinoblastoma protein, pRB, is a key tumor suppressor. It is mutated in 30% of human tumors and its function is disrupted by mutations in upstream regulators in most others (Weinberg, 1995). Inheritance of a mutant copy of *RB* leads to early onset retinoblastoma and can increase the risk of osteosarcoma (OS) 500-fold (Gurney et al., 1995). *Rb* also functions as a tumor suppressor in the mouse, and a variety of mouse models have implicated *Rb* mutation or loss in the development of pituitary and thyroid tumors although notably not retinoblastoma (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992).

pRb belongs to a family of structurally and functionally related proteins called the pocket protein family. This family of proteins, which includes p107 and p130, acts to govern progression through the cell cycle by binding and regulating the activity of the E2F family of transcription factors, which primarily control the expression of genes required at the G1/S transition (reviewed in Trimarchi and Lees, 2002). Upon mitogenic signaling, or oncogenic stress, CyclinD-CDK4/6 complexes, and subsequently CyclinE-CDK2 complexes, phosphorylate the pocket proteins, causing the release of free E2F. The activating E2Fs then bind and transactivate target gene promoters leading to the expression of cell cycle genes, thus promoting cellular proliferation.

pRb is the only pocket protein demonstrated to be a *bona fide* tumor suppressor in humans although mouse models have revealed that p107 and p130 can act as tumor suppressors in some *Rb*-deficient tissues. Notably, mouse model experiments suggest that the tumor spectrum differences in human vs. mouse may be driven by differences in compensatory mechanisms of pocket proteins in these two organisms. Specifically, loss of *Rb* does not lead to retinoblastoma in the mouse, but chimeric mice generated with $Rb^{-/-}$;$p107^{-/-}$ or $Rb^{-/-}$;$p130^{-/-}$ ES cells develop retinoblastoma, while $Rb^{+/-}$;$p107^{-/-}$:WT chimeras develop OS, among other tumors (Dannenberg et al., 2004; Robanus-Maandag et al., 1998). Furthermore, *Rb* mutation in the embryo proper leads to bone differentiation defects, but does not promote tumorigenesis (Berman et al., 2008b). However, *Rb* mutation can give rise to osteosarcoma when combined with mutations in *Trp53* (Berman et al., 2008a; Walkley et al., 2008). These data illustrate that pocket protein family members can

compensate for one another's tumor suppressive functions, depending on the tissue and context, highlighting the need for multiple mutations to drive tumorigenesis.

Rb$^{-/-}$ mice display a developmental defect in erythropoiesis, neuronal and lens development (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992), and there is abundant evidence that the pocket protein family plays a crucial role in regulating differentiation and development in a number of tissues. It is not completely understood when and how the pocket proteins may compensate for one another in this process. One way pocket proteins can promote differentiation is by promoting cell cycle exit. For example, conditional deletion of *Rb* in the skin results in differentiation defects, which are exacerbated upon concurrent loss of *p107* (Ruiz et al., 2005; Ruiz et al., 2004). Loss of these pocket proteins is associated with downregulation of epidermal differentiation genes, as well as upregulation of E2F activity, consistent with increasing proliferation. These data argue that loss of pocket proteins in a stem cell/progenitor cell may increase proliferation, thereby preventing exit, and thus differentiation. Cells may then continue to cycle while incompletely undergoing a differentiation program (Burkhart and Sage, 2008).

There is also evidence for pocket proteins directly impacting differentiation by binding and activating differentiation-specific transcription factors. For example, pRb can promote adipogenesis through its interaction with the fat-specific transcription factor family, CCAAT/enhancer-binding proteins (C/EBPs) and subsequent enhancement of its transcriptional activity (Chen et al., 1996). pRb can also bind and inhibit PPARγ, a key transcription factor in promoting adipogenesis (Fajas et al., 2002). Furthermore, both pRb and p107 have been implicated in regulating the decision of adipocytes to become white or brown fat (Hansen et al., 2004; Scime et al., 2005). Additional support for the role of *Rb* in direct initiation of differentiation comes from the bone. Loss of *Rb* leads to a delay or defect in bone formation in vivo and in vitro (Berman et al., 2008b; Gutierrez et al., 2008; Thomas et al., 2001). Consistent with its role in differentiation in this tissue, pRb augments the transcriptional activity of Runx2 (Cbfa1), a transcription factor required for osteoblast differentiation (Thomas et al., 2001).

Both *p107* and *p130* have been shown to specifically regulate proliferation and differentiation of the growth plate during endochondral ossification. Combined loss of

p107 and *p130* in the germline leads to shorter long bones and reduced endochondral ossification (Cobrinik et al., 1996). These defects are attributed to inappropriate proliferation of chondrocytes in the long bone epiphyses. The requirement for p107 and p130 in the growth plate is in part due to their participation in the FGF signaling pathway, which acts to restrict chondrocyte proliferation through the upregulation of genes involved in cell cycle arrest and differentiation (Dailey et al., 2003).

It is clear that regulation of pocket protein function is critical for maintaining the balance between proliferation and differentiation in the growth plate. Although much is known about p107 and p130 function in the growth plate in vitro and in vivo, the role of pRb in the growth plate has not been explored. We sought to determine if pRb, like p107 and p130, is required for proper cartilage development, and if concomitant loss of *Rb* and *p107* in the mesenchymal progenitor cell (MPC) would lead to novel developmental and/or neoplastic phenotypes. To do this, we crossed $p107^{-/-}$ mice to mice containing a floxed allele of *Rb* whose recombination was dependent on expression of *Prx1-Cre* transgene in the MPC. Our study revealed a novel role for pRb in growth plate development. We found that combined loss of *Rb* and *p107* in the MPC does lead to a significant cartilage defect that is, at least in part, dependent on increased expression of E2F target genes. Furthermore, we found that loss of these two genes results in the development of enchondromas and severe long bone abnormalities in the adult mouse.

RESULTS

DKO mice have decreased viability

The pocket proteins p107 and p130 have established roles in regulating the growth plate. On the other hand, it is unclear what, if any, role pRb has in this tissue. To explore if and how pRb and p107 function together in the growing skeleton, we used mice carrying a null allele of *p107*, a floxed allele of *Rb*, and the *Prx1-Cre* transgene, which is expressed in mesenchymal progenitor cells (Logan et al., 2002). To confirm the expression of the *Prx1-Cre* transgene in mesenchymally derived tissues we crossed mice bearing this transgene with the Rosa26-LSL-LacZ reporter mouse (Soriano, 1999) and stained adult tissues with X-gal to assess the expression of β-galactosidase. As expected, staining was restricted to tissues of mesenchymal origin, including chondrocytes, osteoblasts, connective tissue, smooth and skeletal muscle, brown adipose tissue and white adipose tissue (Fig. 1).

We generated mice of the appropriate experimental genotypes by intercrossing $Rb^{+/c}$;$p107^{+/-}$;*Prx1-Cre* and $Rb^{c/c}$;$p107^{-/-}$ animals. $Rb^{+/c}$;$p107^{+/-}$;*Prx1-Cre* (HH or control), $Rb^{+/c}$;$p107^{-/-}$;*Prx1-Cre* (p107 KO), $Rb^{c/c}$;$p107^{+/-}$;*Prx1-Cre* (Rb KO), and $Rb^{c/c}$;$p107^{-/-}$;*Prx1-Cre* (DKO) animals comprised an aging cohort of 189 mice that was monitored for the development of tumors and other pathologies. Since $p107^{-/-}$;$p130^{-/-}$, and $Rb^{c/c}$;*Mox-Cre* animals have reduced viability or perinatal lethality, we were interested if any of the above genotypes were underrepresented after birth. In fact, DKOs were present at less than the expected Mendelian frequency at 3 weeks of age; approximately 2.4% compared to the expected 12.5% for this cross (Table 1). $p107^{-/-}$ mice with and without the *Prx1-Cre* transgene were also underrepresented, although not significantly, at 3 weeks of age. This is likely due to their smaller size when born (Lee et al., 1996). To ascertain when the DKO animals were dying, we genotyped animals at embryonic day (e) 15.5, e17.5, e18.5, and 0, 1, 2, and 3 days after birth. DKOs were found at the expected frequency before birth (Table 2; $\chi^2 = 0.96$), but no DKOs were found in any of 4 litters right after birth, suggesting that they were dying perinatally.

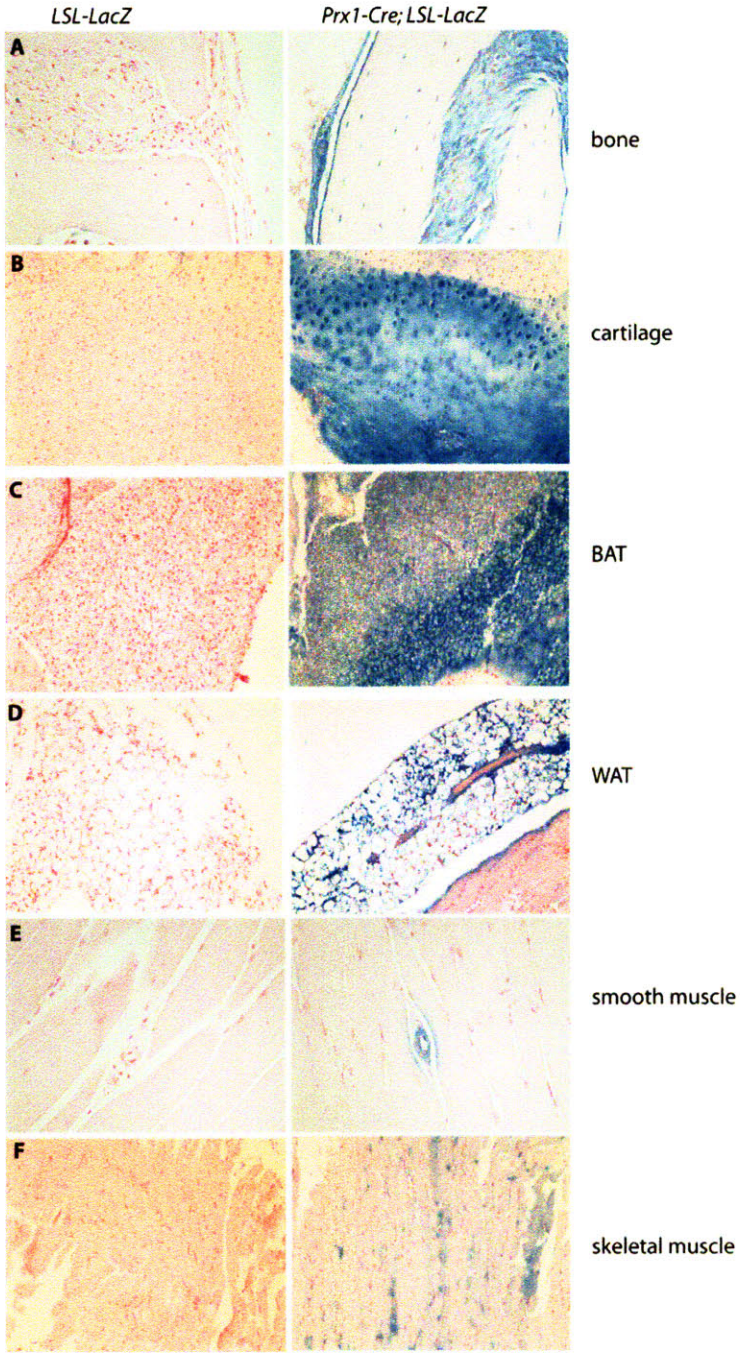

Figure 1: X-Gal Staining of Mesenchymal Tissues
Tissues were dissected from 3 WT and 3 *Prx1-Cre* mice carrying the *Rosa26-LSL-LacZ* reporter allele and stained with X-Gal to mark cells expressing β-galactosidase. Staining was seen only in tissues carrying *Prx1-Cre*, and never in tissues from mice lacking the *Prx1-Cre* transgene.

Table 1: Viability at weaning of progeny from *Rb*$^{+/c}$;*p107*$^{+/-}$;*Prx1-Cre* x *Rb*$^{c/c}$;*p107*$^{-/-}$ Cross

Genotype	Observed	Percentage†
Rb$^{+/c}$ *p107*$^{+/-}$ Cre+	75	17.5
Rb$^{+/c}$ *p107*$^{-/-}$ Cre+	34	8.3
Rb$^{c/c}$ *p107*$^{+/-}$ Cre+	70	16.6
Rb$^{c/c}$ *p107*$^{-/-}$ Cre+	10	2.4
Rb$^{+/c}$ *p107*$^{+/-}$	77	18.2
Rb$^{+/c}$ *p107*$^{-/-}$	38	9.0
Rb$^{c/c}$ *p107*$^{+/-}$	77	18.2
Rb$^{c/c}$ *p107*$^{-/-}$	41	9.7

†53 mice expected for each genotype (12.5%); 422 progeny counted.

Table 2: Viability at e18.5 of progeny from *Rb*$^{+/c}$;*p107*$^{+/-}$;*Prx1-Cre* x *Rb*$^{c/c}$;*p107*$^{-/-}$ Cross

Genotype	Observed	Percentage†
Rb$^{+/c}$ *p107*$^{+/-}$ Cre+	15	13.3
Rb$^{+/c}$ *p107*$^{-/-}$ Cre+	12	10.6
Rb$^{c/c}$ *p107*$^{+/-}$ Cre+	15	13.3
Rb$^{c/c}$ *p107*$^{-/-}$ Cre+	13	11.5
Rb$^{+/c}$ *p107*$^{+/-}$	12	10.6
Rb$^{+/c}$ *p107*$^{-/-}$	13	11.5
Rb$^{c/c}$ *p107*$^{+/-}$	20	17.7
Rb$^{c/c}$ *p107*$^{-/-}$	13	11.5

†14 mice expected for each genotype (12.5%); 113 progeny counted from 15 litters

DKO embryos have growth plate abnormalities

To determine the reason for early lethality of the DKOs, we examined e17.5 embryos for defects, paying particular attention to tissues of mesenchymal origin. Given the established roles of the pocket proteins in bone development, we looked at this tissue first. Whole skeletons dissected from e17.5 embryos were stained with Alizarin Red and Alcian Blue, to mark calcified bone and cartilage, respectively. The cranial and other skull bones, which develop through intramembraneous ossification, manifested no morphological abnormalities (data not shown). However, we found striking defects in the DKO limbs that were either not present (HH and Rb KO) or much more subtle (p107 KO) in the control animals. Overall limb length, as well as the length of the regions of ossification, was much

shorter in DKOs than in the controls (Fig. 2A). Furthermore, the cartilaginous regions in the DKOs were wider than in controls (Fig. 2A). DKO sternebrae and xiphoid processes were also under-ossified and abnormally wide (Fig. 2B). The long bones and sternum form through endochondral ossification, where the generation of a cartilage mold precedes bone formation and mineralization. Thus, the presence of skeletal defects in the long bones and sternum, but not in bones of the skull, indicates that only endochondral, and not intramembraneous, ossification was affected in these mice. These results suggest that the shortened long bones are due to a primary cartilage defect.

To understand the DKO skeletal defect in more detail, we examined H&E stained histological sections of limbs from e17.5 embryos. At this stage of development, limbs contain three distinct zones of chondrocytes in different stages of differentiation, illustrated in Figure 2C. At the epiphysis or "top" of the bone, chondrocytes are in a resting phase. Just below is a zone of highly proliferative chondrocytes that form stack-like structures. Finally, adjacent to the nascent bone marrow are terminally differentiated hypertrophic chondrocytes. In a 2D section, on either side of the hypertrophic chondrocytes is the nascent bone collar. The bone collar is formed by osteoblasts that differentiate directly from the perichondrium, a region of undifferentiated mesenchymal cells surrounding the growing bone.

At e17.5 both the proliferative and hypertrophic zones of chondrocytes were expanded in the DKO limbs compared to controls (Fig. 2D-G) and the hypertrophic, terminally differentiated chondrocytes were disorganized, with more compact nuclei, compared to the other genotypes. Despite the abnormal shape and organization of the hypertrophic zone, the bone collar had formed and ossified properly, indicating that osteoblast differentiation was occurring normally in the DKO limbs (Fig. 3). This was consistent with what we observed in the skeletal staining, and suggests that pRb and p107 are playing a specific role in chondrocytes, but not osteoblasts, in these mice.

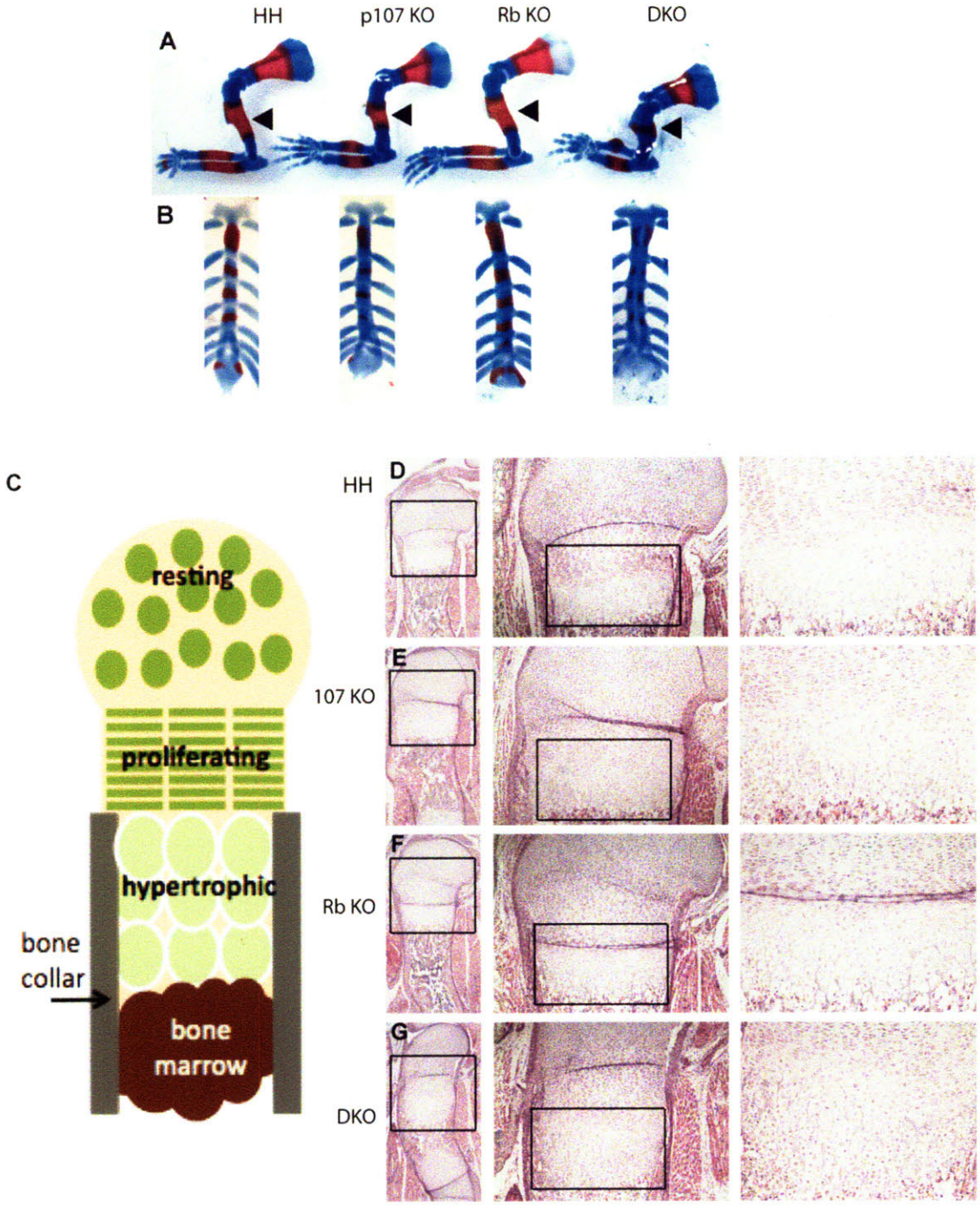

Figure 2: DKO embryos display long bone and growth plate defects.
e17.5 skeletons from 5 litters were stained with Alizarin Red and Alcian Blue to mark the bone and cartilage, respectively. (A) The arms and (B) sternums were analyzed for morphological and ossification defects. (C) Embryonic growth plate development is illustrated here and described in the text. H&E stained sections of proximal humoral epiphyses from 4 litters were examined in control (D), p107 KO (E), Rb KO (F) and DKO (G) embryo littermates. Original magnification: panels on left, 4X; middle, 10X; right, 20X.

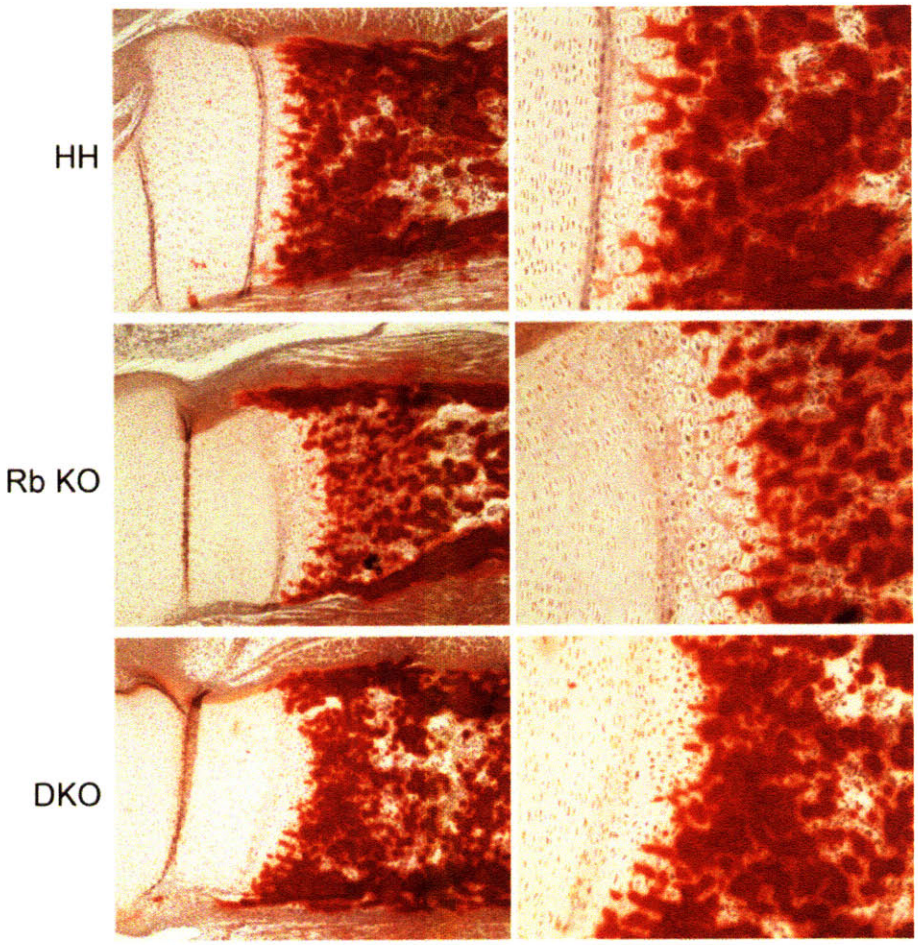

Figure 3: DKO Embryonic Limbs Exhibit Normal Ossification at e18.5
Embryonic limbs from 2 e18.5 litters were stained with Alizarin Red to indicate calcified matrix. DKO limbs stained normally compared to control limbs. Original magnification: left panel, 10X; right panel, 20X.

Embryonic long bone defects are due to inappropriate chondrocyte proliferation

Based on the well-known roles of pRb and p107 in cell cycle control, we were interested in whether the cartilage defect was due to inappropriate proliferation in the growth plate. Pregnant females were injected with BrdU and e17.5 embryos were harvested 2 hours later. BrdU-positive nuclei in the proliferative and hypertrophic zones in sections of humoral growth plates were quantified. Compared to all other genotypes, DKO animals had a significantly greater percentage of BrdU positive chondrocytes in the proliferative zone (Fig. 4A). This observation indicates that the abnormal morphology of the cartilage anlagen is at least partly due to inappropriate proliferation of chondrocytes in the embryonic growth plate. Notably, in many DKOs, a sizable percentage of BrdU-positive cells were observed in the terminally differentiated region of hypertrophic chondrocytes, but this was never observed in other genotypes (Fig. 4B). The presence of cycling cells in the terminally differentiated zone of chondrocytes suggests that proliferation and differentiation have become uncoupled upon loss of pRb and p107.

To determine whether the differentiation process was also perturbed in the embryonic cartilage, we stained embryonic limb sections for expression of Collagen X, a collagen species expressed exclusively in terminally differentiated hypertrophic chondrocytes. We found that Collagen X expression was restricted to cells with hypertrophic chondrocyte morphology in control and DKO animals, even though this region was expanded in the DKOs (Fig. 5A and B). Thus, the increased area of hypertrophic chondrocytes in the DKO is likely a consequence of the greater total number of proliferative zone chondrocytes undergoing differentiation, and not due to inappropriate levels or location of differentiation. Taken together, these data suggest that increased chondrocyte proliferation, and thus a defect in cell cycle exit, in the DKO embryonic growth plates results in abnormally shaped, shortened long bones.

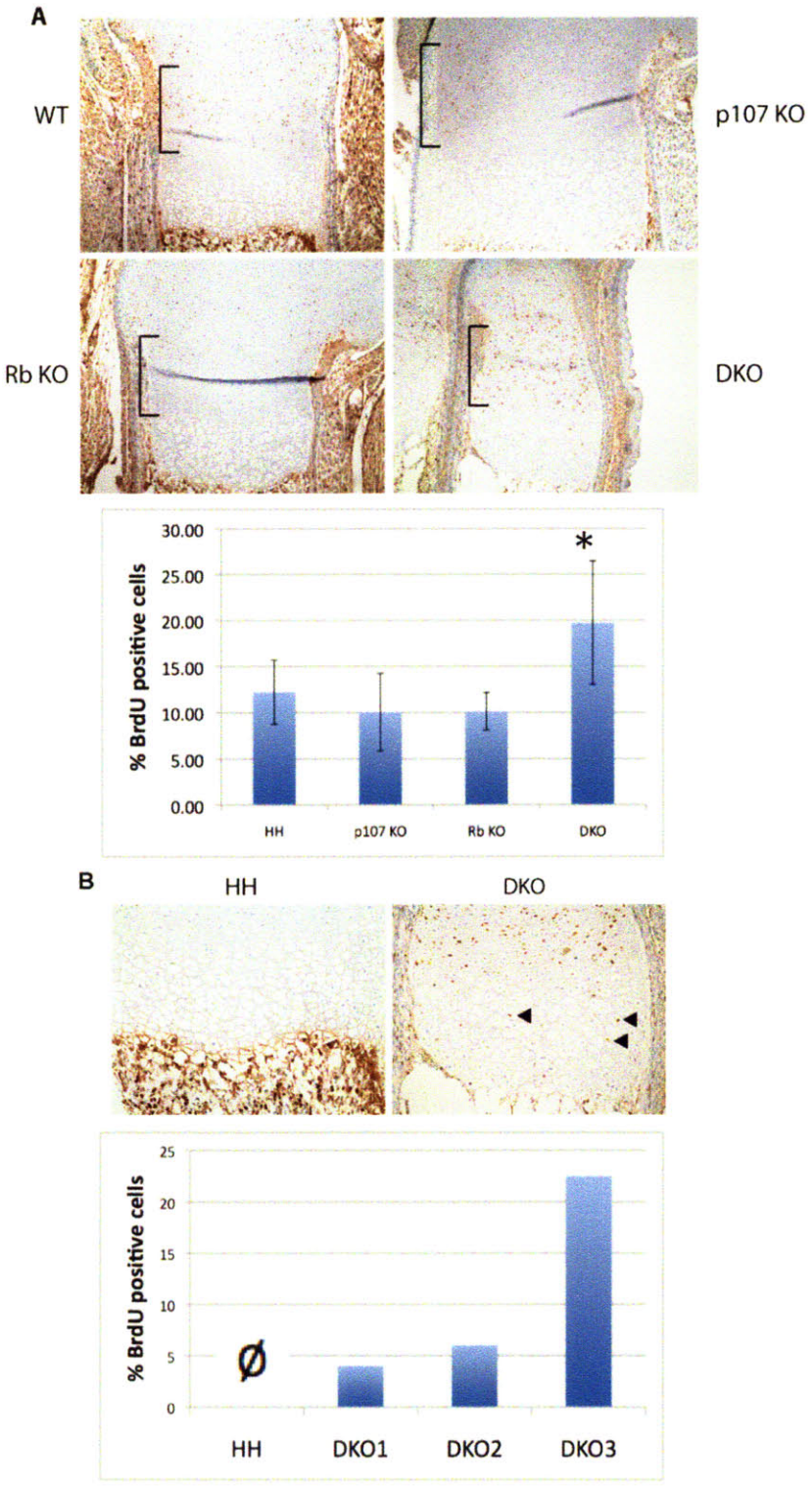

Figure 4: Chondrocyte proliferation is significantly increased in DKO embryonic growth plates. e17.5 pregnant females were injected with BrdU and the embryos harvested two hours later. Sections of the humerus were stained and BrdU incorporation was quantified in the proliferative zone chondrocytes (A, zone marked by extent of line) and the hypertrophic terminally differentiated zone chondrocytes (B, positive cells marked by arrowhead) in at least 2 mice/genotype in 4 litters. *Student's t-test: $p<0.003$

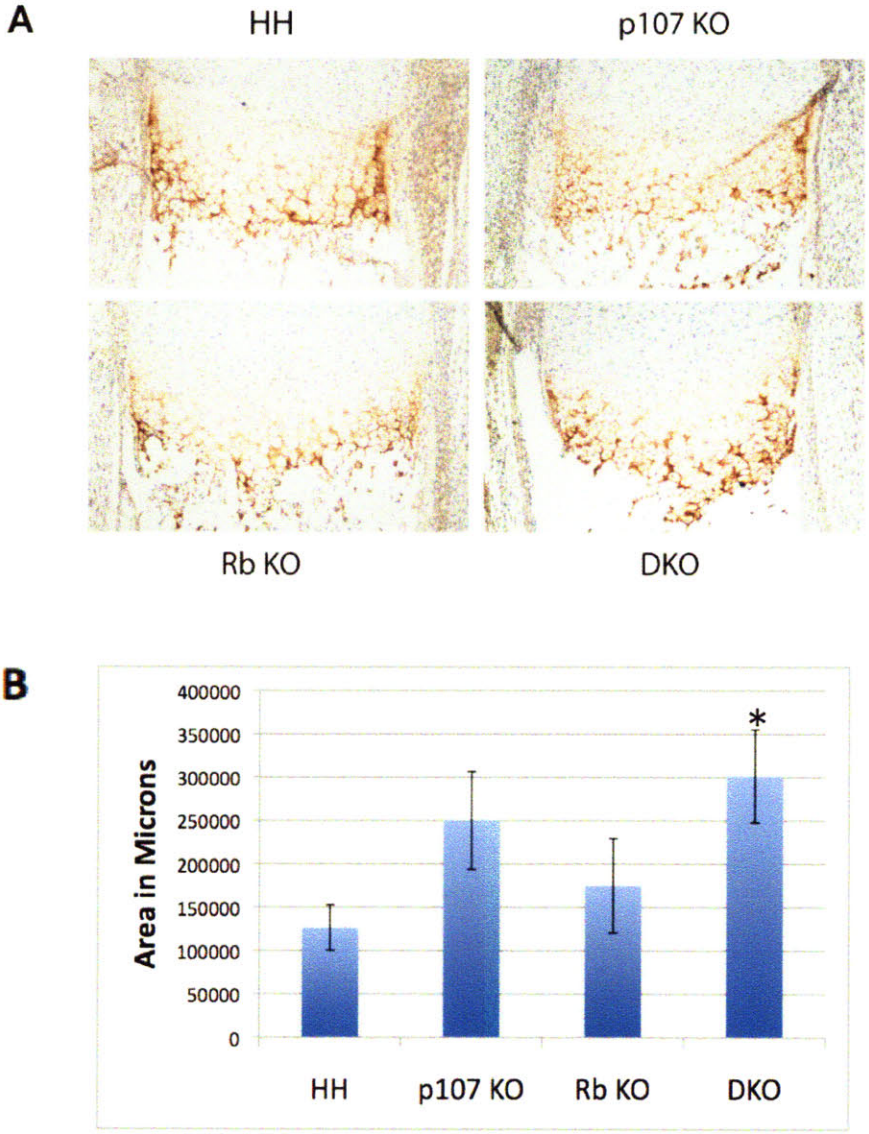

Figure 5: The terminal differentiation marker Collagen X is appropriate expressed in DKO embryos. Immunohistochemistry was performed for Collagen X on e17.5 humoral sections (A). The area of the region containing hypertrophic chondrocytes by morphology was quantified in (B). In both cases, at least 2 mice/genotype in 3 litters were analyzed. *Student's t-test: $p<0.003$

Surviving DKO animals develop growth plate enchondromas by 8 weeks of age

Out of over 400 mice generated from our crosses (described above), only 10 DKO animals survived to adulthood, and they had a significantly reduced life span (Table 3), which was predominantly due to the development of abdominal masses that were either preputial adenomas or leiomyosarcomas. Since most DKOs were dying between e18.5 and P1, we speculate that the few surviving DKOs represented those with a weaker phenotype due to incomplete penetrance of the *Rb* and *p107* mutations. Upon dissection of DKO animals, we observed dramatic defects in the long bones. The bones of the limbs were thicker and shorter than those of wild-type animals and had a characteristic abnormal shape. We confirmed this severe morphological defect at high resolution by micro-computerized tomography (μCT) of HH and DKO femurs at 6 months (Fig. 6A). This analysis permitted a precise determination of femur length, revealing significantly shortened long bones in the DKOs (Fig. 6B).

Table 3: Disease Phenotypes of Aging Cohort

Genotype (all Cre+):	$Rb^{+/c}$ $p107^{+/-}$	$Rb^{+/c}$ $p107^{-/-}$	$Rb^{c/c}$ $p107^{+/-}$	$Rb^{c/c}$ $p107^{-/-}$
Avg. Age at Euth. (days)[a]	531	543	508	265
# animals (histology)	10	9	17	10
Chondrones/Enchondromas[b]	0	0	7	10
Cystic Uterus	2	3	4	0
Leiomyosarcoma	0	0	1[c]	2
Mammary Fibroadenoma	0	0	2	0
Hibernoma	0	0	1	0
Preputial Adenoma	0	0	3	4
C-cell (metastatic)	0	0	2(2)	3(2)
Histiocytic Sarcoma	4	2	1	0
B-cell lymphoma	2	0	4	0

a: Student's T-test demonstrates $p < 0.0001$ for age of euthanasia between DKO and Double Het.
b: $Rb^{c/c};p107^{+/-};$*Prx1-Cre* mice only developed chondrones, not enchondromas.
c: This was a leiomyoma in the $Rb^{c/c};p107^{+/-};$*Prx1-Cre* animal.

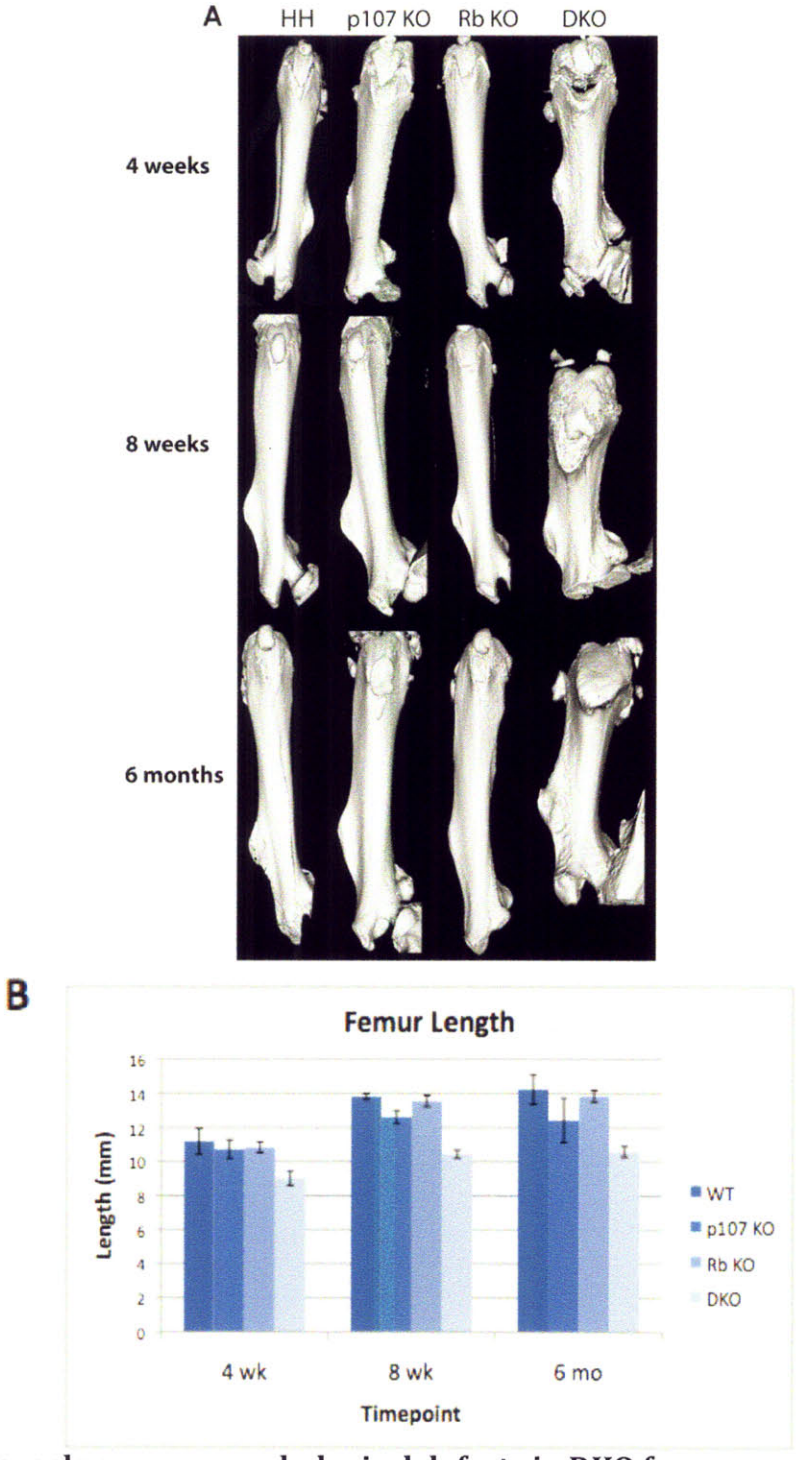

Figure 6: μCT reveals severe morphological defects in DKO femurs.
(A) Femurs were analyzed by μCT at 4 and 8 weeks and 6 months. DKO femurs were shorter, wider, and abnormally shaped compared to controls. (B) Femur length was precisely quantified by μCT. DKO femurs are significantly shorter than controls at all timepoints. Students t-test for significance comparing DKO to HH at each timepoint: $p<0.003$

Histological analysis of adult long bones revealed that all *Rb* KO and DKO mice possessed chondrones, an osteoarthritic-like cartilage abnormality, but only DKOs developed enchondromas, a benign cartilage tumor containing abundant atypical chondrocytes and an abnormal matrix similar to human enchondromas (Table 2; Fig. 7A). These lesions most often occurred in the growth plates of long bones, although one was found in the spine, and arose as early as 6 months of age in this cohort. The enchondromas were remarkably similar to human enchondromas both by histology and location in the growth plate. The enchondromas expressed Collagen X (Fig. 7C), indicating that they contained terminally differentiated chondrocytes. Notably, the enchondromas were not positive for the proliferative marker Ki67 (data not shown), or BrdU positive after injection of BrdU and euthanasia after 2 hours (Fig. 7B). This raises the possibility that that the proliferative event that led to these lesions had occurred earlier during the lifespan of the animal.

The enchondroma phenotype in older DKO mice led us to assess long bone abnormalities in younger mice to determine the timing of enchondroma formation. To do this, we generated cohorts of mice that were euthanized at 4 weeks and 8 weeks and 6 months of age (described in Fig. 7). We chose these timepoints based on the growth features of the growth plate, which is still active at 4 weeks, but enters quiescence around 8 weeks, when the mice become sexually mature. We included the 6 month timepoint to ensure that we would see enchondroma formation. μCT analysis of femurs at 4 and 8 weeks also revealed severe morphological defects, and confirmed the shortened length of these bones compared to controls, even at this timepoint (Fig. 6A and B).

H&E sections of long bones at 4 weeks of age revealed that DKO animals had greatly expanded cartilage regions in the proximal epiphysis of the humerus (humoral head) compared to the control (Fig. 8A), and in the distal epiphysis of the femur (data not shown). Whereas the growth plate in the control humerus was organized and consisted of neat stacks of proliferating and differentiating chondrocytes, the DKO growth plates were disorganized and contained chondrocytes with a range of shapes and sizes. Similarly, articular cartilage regions were expanded with disorganized chondrocytes in the DKOs

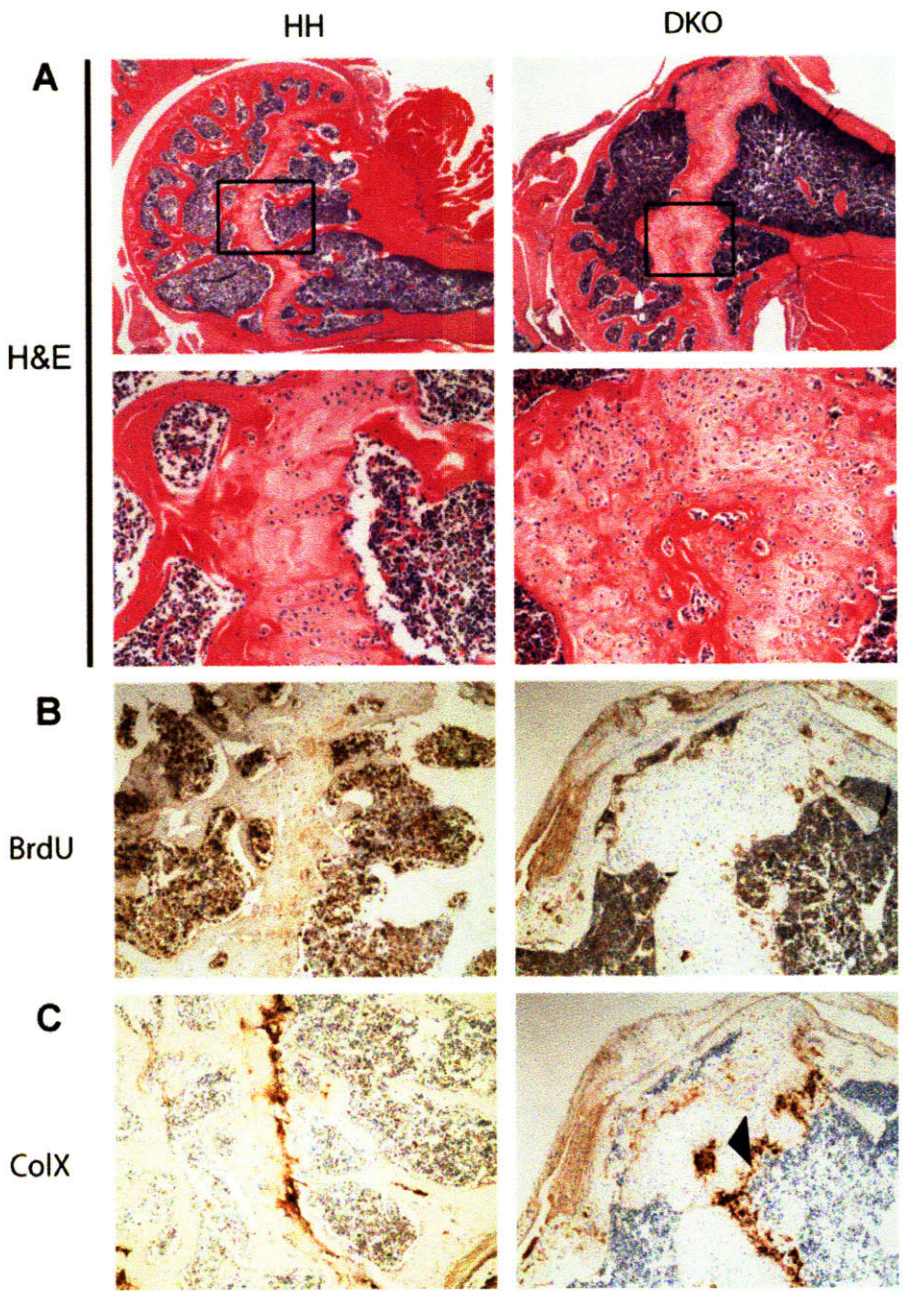

Figure 7: Enchondroma Formation in 6 mo. old DKO mice.
H&E stained humoral sections from 6 mo. old mice revealed the presence of enchondromas in the growth plate of DKOs but not control animals (A; magnification: top panels 4X, bottom panels 20X). Enchondromas and the corresponding growth plate regions of control animals were stained for (B) BrdU incorporation and (C) ColX expression (all at 10X magnification).

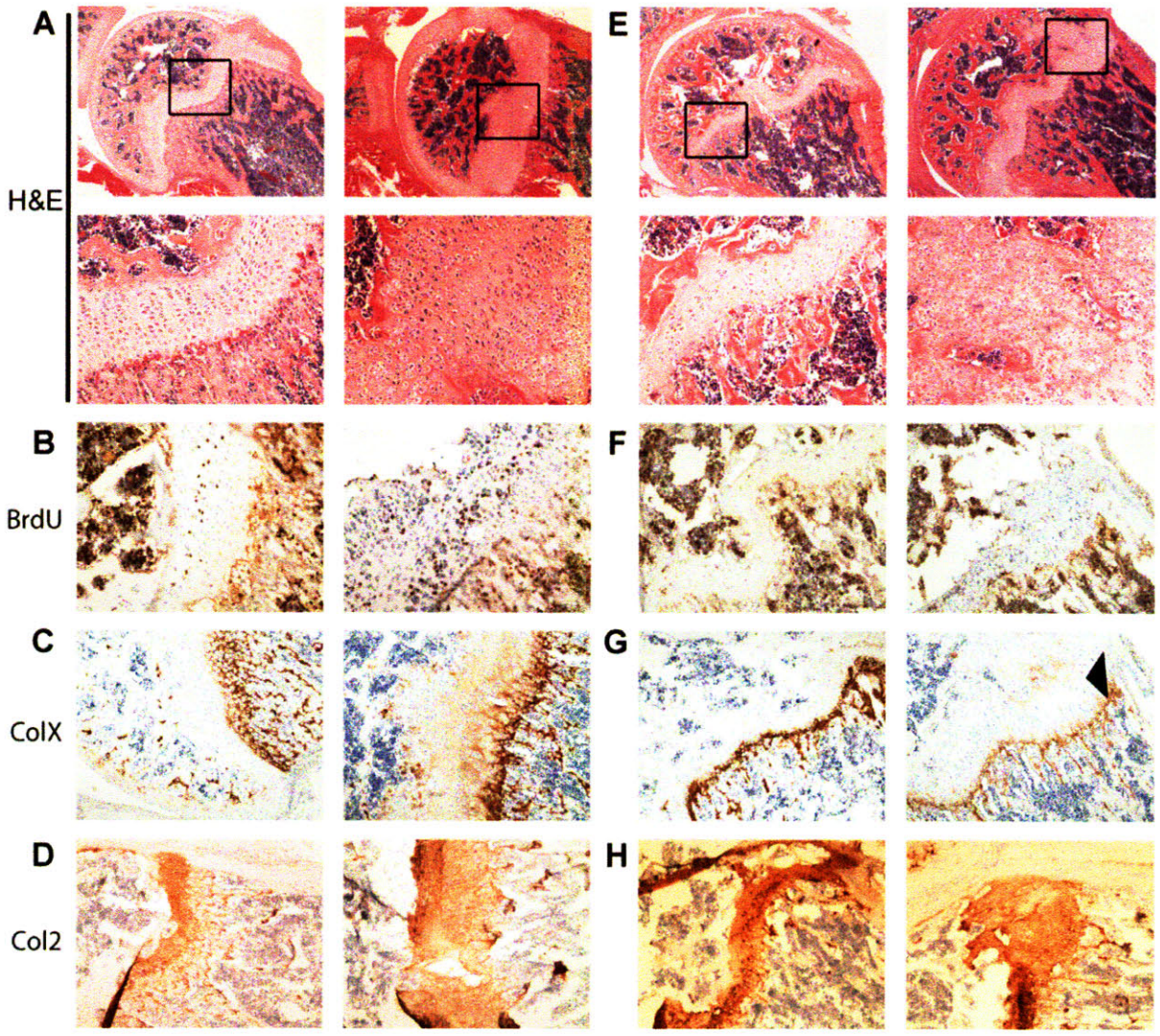

Figure 8: Growth Plate Abnormalities and Enchondroma formation in 4 wk. and 8 wk. old DKO mice. Sections from 4wk old and 8wk old humerii were stained with H&E (A and E, repectively) to reveal growth plate abnormalities and the presence of enchondromas in the 8 week old DKOS but not control animals (magnification: top panels 4X, bottom panels 20X). Growth plates were stained for BrdU incorporation (B, F), ColX expression (C, G), and Col2 expression (D, H) (all 10X magnification).

(Fig. 8A). These growth plate defects were exacerbated in the adult long bones compared to the embryos.

The growth plates of the DKO animals were also more highly proliferative, as demonstrated by staining for BrdU positive chondrocytes (Fig. 8B). Interestingly, the disorganized region of the DKO growth plate was positive for expression of Collagen X, although its expression was restricted to chondrocytes with hypertrophic morphology in the control (Fig. 8C). Since the region of chondrocytes that is proliferative is equivalent to the region expressing Collagen X, it appears that the expanded region of chondrocytes inappropriately expresses a marker of terminal differentiation without having entered the differentiation program.

To examine the differentiation status of these cells further, we performed immunohistochemistry for Collagen II, an early differentiation marker for resting and proliferative chondrocytes. Interestingly, the expanded region of chondrocytes in the DKO growth plate also stained positive for this marker (Fig. 8D), consistent with the idea that these chondrocytes were mis-expressing differentiation markers and are in a hybrid differentiation state. These data indicate that the effect of *Rb*- and *p107* loss on proliferation and differentiation is exacerbated in the 4-week old DKO mice compared to the DKO embryos.

At the 8-week timepoint, the growth plate and articular cartilage regions were still expanded compared to controls (Fig. 8E) and importantly, it was at this age that we first detected developing enchondromas. These enchondromas were present in each DKO mouse we observed at this age and all enchondromas originated from the growth plate (Fig. 8E, arrowheads). Interestingly, these enchondromas were not very proliferative, as shown by lack of BrdU positive cells (Fig. 8F). This suggests that the enchondromas are slow-growing lesions, and/or much of the proliferation required to form this cartilage mass occurred between 4 and 8 weeks. The enchondromas were positive for Collagen X staining, although the morphology of the chondrocytes was not consistent with normal terminally differentiated, hypertrophic cells (Fig. 8G). Additionally, the entire region of the enchondroma stained positive for Collagen II expression (Fig. 8H). Together, these data suggest that enchondromas arise from inappropriately proliferative growth plate chondrocytes that are expressing a mix of early and late differentiation markers. These

results demonstrate that combined loss of *Rb* and *p107* in the mesenchymal progenitor leads to fully penetrant development of enchondromas in the growth plate that are similar to human enchondromas, non-proliferative, and comprised of chondrocytes expressing both early and late differentiation markers.

DKO embryonic chondrocytes express elevated levels of proliferation and early differentiation markers

We wished to understand the consequence of *Rb*- and *p107*-loss in chondrocytes at the molecular level. To do so, we generated cultures of primary chondrocytes from the sternums of e18.5 embryos derived from the crosses described above, which gave rise to all genotypes of interest. Sternums were chosen because growth plate chondrocytes in this structure are more abundant and easier to isolate than in the long bones, and because sternum cartilage at this timepoint was found to be abnormal in the DKO animals (Fig. 2B). Primary chondrocytes were maintained in culture no longer than 2 weeks and passaged fewer than 4 times before analysis to ensure maintenance of the chondrocyte phenotype. We analyzed the expression of several differentiation and proliferation genes by qRT-PCR in asynchronously growing cultures. The expression of the E2F target genes *E2f1*, *Cyclin A*, and *Cyclin E* was increased at least 2-fold in the DKO chondrocytes and the increase in expression correlated with decreasing dosage of pocket proteins (Fig. 9A-C). In addition, an early marker of chondrocyte development, *Sox9*, was upregulated in both the p107 KO and DKO chondrocytes, consistent with the expression of early markers of differentiation in the adult DKO growth plates (Fig. 9D). Finally, *Fibrillin-2* an ECM protein component of the cartilage matrix was also upregulated in the p107 KO and DKO chondrocytes, consistent with evidence that it is an E2F target gene (Fig. 9E) (Yanagino et al., 2009).

Thus, we can attribute the chondrocyte and cartilage defects in part to increased expression of proliferation-associated proteins such as E2F1, and inappropriate expression of early chondrocyte differentiation markers in the growth plate. This kind of analysis reveals that pRb and p107 are involved in both processes that govern growth plate development: proliferation and differentiation. Furthermore, although embryos exhibit only a proliferation phenotype, gene expression analysis demonstrates that differentiation

genes are deregulated in embryonic growth plate chondrocytes, presaging the adult growth plate defects.

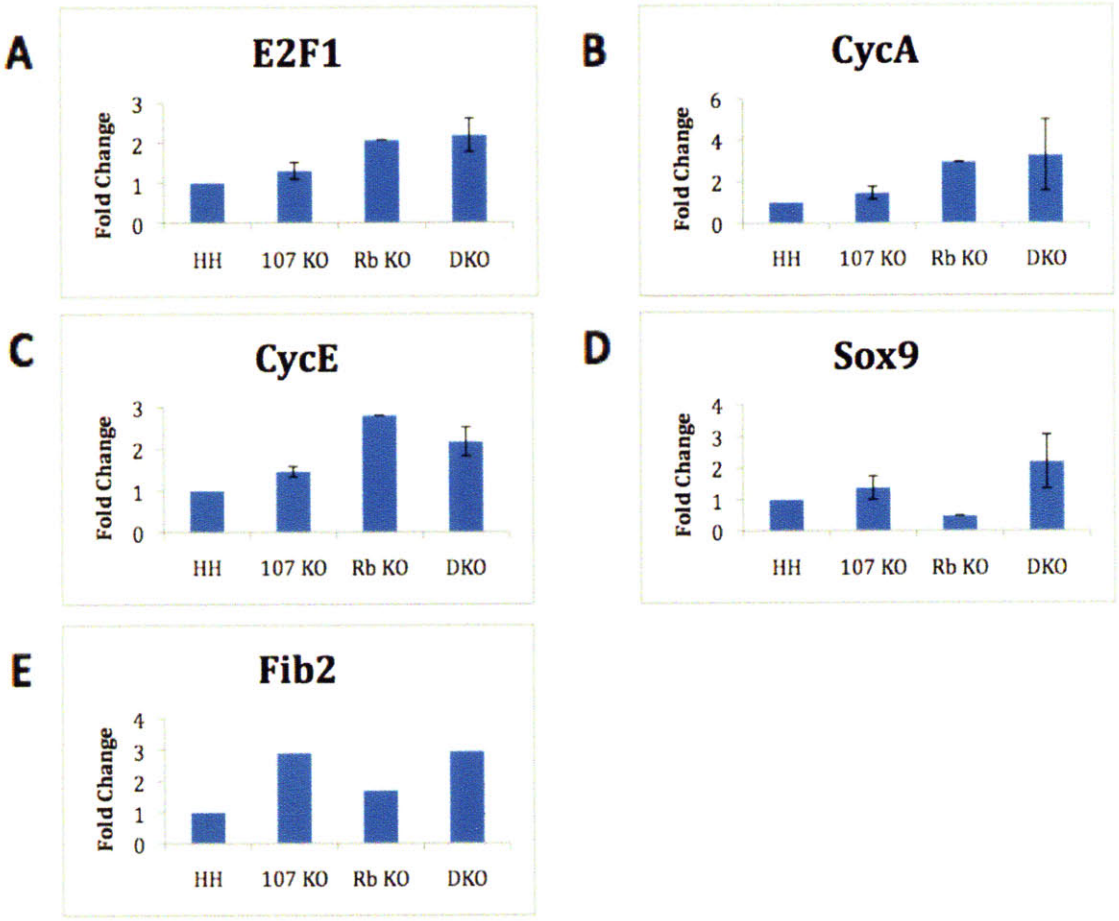

Figure 9: DKO Chondrocytes Express Elevated Levels of Proliferation and Early Differentiation Markers. RNA was harvested from e18.5 sternum chondrocytes, transcribed into cDNA, and gene expression was analyzed by quantitative RT-PCR. These genes represent E2F target genes involved in proliferation (A-C) or genes involved in chondrocyte differentiation (D and E). Primers are listed in Experimental Procedures.

DISCUSSION

It is well accepted that the pocket proteins function in the development of different tissues, but the details of how they do this are still emerging (Classon and Harlow, 2002; Lipinski and Jacks, 1999). pRb, p107, and p130 participate in differentiation through initiating cell cycle exit as well as by promoting the expression or activity of differentiation-specific proteins. Although pRb functions in bone development (Berman et al., 2008b; Gutierrez et al., 2008; Thomas et al., 2001) and bone tumorigenesis (Berman et al., 2008a; Walkley et al., 2008), no role for it has yet been demonstrated in cartilage, the precursor to bone formation during endochondral ossification. The other pocket protein family members p107 and p130 play a crucial role in regulating chondrocyte proliferation downstream of FGF signaling (Laplantine et al., 2002; Rossi et al., 2002). Intriguingly, pRb function is also affected downstream of FGF signaling in chondrocytes, but its role in this tissue has not yet been explored.

Using a tissue-specific Cre-expressing transgene to drive *Rb* loss in MPCs in *p107*$^{-/-}$ mice, we have uncovered a novel role for pRb in chondrocytes. We found that pRb and p107 function together during embryogenesis to regulate proliferation in growth plate chondrocytes, which is important for appropriate bone shape and growth in the adult. Loss of *Rb* and *p107* in the MPC is partially lethal, suggesting that improper development of the growth plate during embryogenesis can have consequences on overall viability. pRb and p107 also play a role in maintaining the adult growth plate by restraining proliferation and enforcing terminal differentiation to prevent tumorigenesis. Loss of both pRb and p107, but not either one alone, leads to increased expression of proliferation and early differentiation markers in chondrocytes. Either of these latter abnormalities could lead to initiation and progression of the enchondromas that develop in DKO animals by 8 weeks. Alterations in the expression of p16, pRb, and CDK4, including loss of the chromosomal region containing *RB*, have been identified in human chondrosarcomas, highlighting the importance of this pathway in tumor suppression in this tissue (Asp et al., 2001; Asp et al., 2000; Ropke et al., 2006; Yamaguchi et al., 1996). Our study represents the first mouse model that defines a role for the pocket proteins in enchondroma formation.

Since decreased viability has been reported for p107 mutations in some backgrounds (LeCouter et al., 1998), and *Rb*$^{-/-}$;*p107*$^{-/-}$ mice die around embryonic day 11.5 (Lee et al., 1996), we were not surprised to find viability issues in our DKO animals. Although it is often difficult to determine the cause of perinatal lethality, we speculate that most of the DKO animals died due to sternum abnormalities that are apparent in the Alizarin Red/Alcian Blue stained skeletons (Fig. 2B). *p107*$^{-/-}$;*p130*$^{-/-}$ embryos have a similar rib cage defect and perinatal lethality (Cobrinik et al., 1996), and the authors speculate that the rib defect leads to an inability to breath after birth. It is not unreasonable that the Rb/p107 DKOs might be succumbing to the same defect. Interestingly, a small number of DKO animals survive to adulthood. We suspect that the presence of survivors is due to incomplete penetrance of the Rb/p107 phenotype, and that these animals represent the animals with the weakest phenotypes.

Our data show that loss of pRb and p107 leads to increased chondrocyte proliferation during embryogenesis, but only mild, if any, changes to differentiation (Fig. 4 and 5). Nonetheless, DKO chondrocytes with hypertrophic morphology incorporate BrdU, suggesting that proliferation and at least some steps of differentiation have been uncoupled. Although proliferation is increased in *p107*$^{-/-}$;*p130*$^{-/-}$ growth plates, proliferation of hypertrophic chondrocytes is not observed, and the overall phenotype is less severe compared to the *Rb*$^{c/c}$;*p107*$^{-/-}$;*Prx1-Cre* animals in this study (Cobrinik et al., 1996).

Downstream of the pocket proteins, the activating E2Fs have been shown to play a role in growth plate development in the mouse. *E2f1* overexpression inhibits hypertrophic chondrocyte differentiation resulting in shorter long bones (Scheijen et al., 2003). Additionally, in *E2f1*$^{-/-}$;*E2f3a*$^{-/-}$ mice, the growth plate is disorganized and the chondrocytes are much larger than normal (Danielian et al., 2008). If the chondrocyte defect in pRb/p107 DKO animals were due to deregulated E2F activity downstream of Rb and p107-loss, we would expect to see increased proliferation as well as shortened long bones, which is indeed what we observe. Consistent with this result, our qRT-PCR data from e18.5 sternum chondrocytes show that E2F target genes are indeed upregulated in the DKOs. Thus, the cartilage defects can be in part attributed to increased E2F activity.

The cartilage phenotype is intensified in the adult DKO animals that we examined. In growth plates at 4wks and 8 wks, proliferation was increased in the DKO growth plates compared to controls, similar to the situation in the embryos. However, enchondromas observed at 8wks and 6 months are not proliferative, but they do express a mixture of differentiation markers from both early (Col2) and late stage (ColX) chondrocyte differentiation. This first suggests that the proliferation event required to form the enchondromas occurs between 4 and 8 weeks, but that cells eventually enter quiescence. Secondly, after entering quiescence, enchondroma cells are still unable to properly execute the differentiation program and continue to express a mix of early and late differentiation markers. Differentiation marker expression was observed to be abnormal as early as e18.5 (Fig. 9), prior to any obvious differentiation defect, suggesting that even at this timepoint, pRb and p107 loss was eliciting a change in differentiation gene expression.

Uncoupling of proliferation and differentiation in *Rb* and *p107* double-deficient cells has been reported before in the skin (Ruiz et al., 2004). In this study, DKO cells actively proliferate while expressing both early (K5) and late (K10) epidermal differentiation markers. This could be an indirect result of cells continuing to follow a differentiation program but lacking the ability to exit the cell cycle, that is, a failure of *Rb* and *p107*-deficient cells to properly coordinate differentiation with cell cycle exit. Alternatively, *Rb* and *p107* could be directly responsible for enforcing the differentiation program of skin cells, and this program cannot be executed properly in their absence.

Our study does not distinguish between a direct role of pRb and p107 in promoting the differentiation of chondrocytes versus their role in coordinating cell cycle exit with the initiation of differentiation. In our system, most DKO chondrocytes within enchondromas must eventually exit the cell cycle since enchondromas are not proliferative, nor do they advance to malignancy. This is could be due to redundant pathways of cell cycle arrest compensating for the lack of pocket protein function. The compensatory mechanism may depend on *p130* expression. A mouse model that combines loss of all three pocket proteins in chondrocytes will be necessary to address this possibility, although our preliminary qRT-PCR data do not show an upregulation of *p130* mRNA in DKO chondrocytes (A. Landman and J. Lees, unpublished observations). Additionally, secondary mutations in the Rb or other pathways may be required for chondrosarcoma development. To test the

requirement for additional oncogenic events to drive tumor formation, the Rb/p107 mice could be crossed to other tumor suppressor knockouts, perhaps *p53*, which drives bone tumorigenesis in the presence of an *Rb* mutation (Berman et al., 2008a; Walkley et al., 2008).

Another reason why the enchondromas do not develop into chondrosarcomas can be gleaned from other mouse models of enchondroma. Enchondroma formation in the proximal humerus and distal femur can be induced to occur in mice by 8.5 weeks of age through expression of a mutant form of the parathyroid hormone-related protein receptor (PPR) that constitutively activates Indian Hedgehog signaling (Hopyan et al., 2002). The authors speculate that enchondromas in this model form from proliferating growth plate chondrocytes that are aberrantly maintained in the adult. These chondrocytes improperly express differentiation markers and/or are unable to receive the appropriate differentiation signals from the surrounding cells. *Pten$^{c/c}$;Col2a1-Cre* mice also develop enchondromas due to increased *Hif1α* expression and subsequent "stalling" of the growth plate chondrocyte differentiation program (Yang et al., 2008). This study also suggests that lingering undifferentiated chondrocytes in the growth plate can give rise to enchondromas. Based on the results from these studies, we can speculate that cycling Rb/p107-null chondrocytes are aberrantly maintained in the adult growth plate. These chondrocytes attempt to differentiate, but due to their improper location in the growth plate and the potential lack of appropriate extrinsic signaling molecules, cannot. These cells are then primed to generate improperly differentiated cartilage lesions.

In conclusion, pRb and p107 are required for normal chondrocyte growth. It also seems that pRb and p107 are required for cell cycle exit coincident with initiation of differentiation, but that in some cases, the absence of cell cycle exit does not prohibit differentiation. pRb and p107 are also responsible for ensuring, either directly or indirectly, the appropriate timing of expression of chondrocyte differentiation markers. Cells that express a mixture of early and late differentiation markers will not be able to either function appropriately for their cell type nor differentiate properly. When these cells sit in the growth plate, left behind after normal cells would normally have

differentiated or been removed, they can contribute to benign lesions, which have the potential for malignant transformation.

EXPERIMENTAL PROCEDURES

Animal Maintenance and Histology

The generation of $Rb^{c/c}$ mice, $p107^{-/-}$ mice, *Prx1-Cre* mice, and *Rosa26-LSL-LacZ* mice has been described previously (Lee et al., 1996; Logan et al., 2002; Sage et al., 2003; Soriano, 1999). All mice were maintained on a mixed background. Gestation was dated by detection of a vaginal plug. Mice were injected with 10μg/gm body weight of 5mg/ml 5-Bromo-2'-deoxyuridine (BrdU) in phosphate buffered saline (PBS) two hours prior to tissue collection. Collected tissues were fixed in 10% formalin (adult) or 4% paraformaldehyde (embryonic) and embedded in paraffin. Adult tissues containing bone were decalcified in 0.46M EDTA, 2.5% Ammonium Hydroxide, pH7.2, for two weeks prior to paraffin embedding. Histological sections were cut at 5μm.

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 room temperature for a third day. Tissue was cleared in 1% potassium hydroxide for several days and ultimately stored in glycerol.

Histological Analyses

Adult tissues were stained for β-galactosidase activity as described in Parisi et al., 2007. Briefly, dissected tissues were fixed, rinsed, and then stained overnight at 37°C in X-Gal staining solution. Samples were then rinsed and fixed again before being processed for paraffin embedding. Sections were cut at 5μm and counterstained with Nuclear Fast Red. Paraffin sections were stained with Alizarin Red S staining by deparaffinizing and dehydrating in ethanol, rinsing briefly in distilled H_2O, and staining in freshly made 2% Alizarin Red S (Sigma) in H_2O (pH 4.2) for 5 minutes. Slides were dipped 20 times in acetone, 20 times in acetone:xylene, 1:1, cleared in xylene and mounted.

Immunohistochemistry for BrdU, Collagen X, and Collagen 2 were performed by standard procedures on paraffin slides with the following exception: slides prepared for Collagen X staining were incubated in 0.1% pepsin in 0.5M glacial acetic acid (in H_2O) at 37°C for 2 hours in lieu of the sodium citrate antigen retrieval method. Antibodies used were mouse anti-BrdU (1:50, BD Biosciences), goat anti-Collagen 2 (1:500, Santa Cruz sc-7764), and mouse anti-Collagen X (1:20, Quartett clone ColX53). HRP-conjugated secondary antibodies were provided in the Vectastain ABC Kit Elite (Vector Labs), and antibody staining was visualized with DAB (Vector Labs).

Micro-computerized Tomography (μCT)

Analysis of 3D bone structure was performed as described in Glatt et al., 2007. 3D image data was collected from euthanized animals using a GE Healthcare Micro-CT machine and analyzed using the Microview 2.2 software from GE Healthcare.

Isolation of Primary Chondrocytes

Chondrocytes were isolated from e18.5 sternums following a procedure modified from Lefebvre et al., 1994. e18.5 embryos were sacrificed and sternums removed and rinsed in 1% FBS/PBS. Sternums were subjected to a series of digestions at 37°C with vigorous rocking: first 2mg/ml pronase (Roche) in PBS for 30 min., then 3mg/ml Collagenase D (Roche) in 10% FBS in DME for 1hr. Sternums were rinsed in 1% FBS in PBS, then fresh 3mg/ml Collagenase D was added, and sternums were incubated for an additional 4-6 hours with vigorous rocking until the cartilage regions were released from the ossified nodules. The preparation was filtered through a 70μm filter, washed, and plated in 10% FBS in DME at $1X10^5$ cells/cm^2. Cells were maintained at this density in 10% FBS in DME for 2 weeks or less.

Quantitative real-time PCR

RNA was isolated from subconfluent embryonic sternum chondrocytes using the Qiagen RNeasy kit. First-strand cDNA synthesis was performed using 0.5-1μg RNA using Superscript III Reverse Transcriptase (Invitrogen). Quantitative RT-PCR with 50-100ng

cDNA was performed using SYBR Green (Applied Biosystems) using Ubiquitin as an internal control. Reactions were run on the ABI Prism 7000 Sequence Detection System and analyzed using the 7000 SDS software. Primers are as follows:

Gene	Real-Time Primer Sequence
E2f1	Forward: 5'-TGCTGAGGCCCAAGCAGTTTAT-3'
	Reverse: 5'-ATCCATGGCTGTCAGTCTGTCT-3'
Cyclin A	Forward: 5'-AGTTTGATAGATGCTGACCC-3'
	Reverse: 5'-TAGGTCTGGTGAAGGTCC-3'
Cyclin E	Forward: 5'-GGCTAATGGAGGTGTGCGAA-3'
	Reverse: 5'-AGTAGAACGTCTCTCTGTGGAGCTTA-3'
SOX9	Forward: 5'-GAGGCCACGGAAGAGACTCA-3'
	Reverse: 5'-CAGCGCCTTGAAGATAGCATT-3'
Fibrillin-2	Forward: 5'-GGCCGAATGGCAAAAGCTC-3'
	Reverse: 5'-CCCCCAACGCCAGGAGAAAAG-3'
Ubiquitin	Forward: 5'-TGGCTATTAATTATTCGGTCTGCAT-3'
	Reverse: 5'-GCAAGTGGCTAGAGTGCAGAGTAA-3'

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REFERENCES

Asp, J., Inerot, S., Block, J.A., and Lindahl, A. (2001). Alterations in the regulatory pathway involving p16, pRb and cdk4 in human chondrosarcoma. J Orthop Res *19*, 149-154.

Asp, J., Sangiorgi, L., Inerot, S.E., Lindahl, A., Molendini, L., Benassi, M.S., and Picci, P. (2000). Changes of the p16 gene but not the p53 gene in human chondrosarcoma tissues. Int J Cancer *85*, 782-786.

Berman, S.D., Calo, E., Landman, A.S., Danielian, P.S., Miller, E.S., West, J.C., Fonhoue, B.D., Caron, A., Bronson, R., Bouxsein, M.L., *et al.* (2008a). Metastatic osteosarcoma induced by inactivation of Rb and p53 in the osteoblast lineage. Proc Natl Acad Sci U S A *105*, 11851-11856.

Berman, S.D., Yuan, T.L., Miller, E.S., Lee, E.Y., Caron, A., and Lees, J.A. (2008b). The retinoblastoma protein tumor suppressor is important for appropriate osteoblast differentiation and bone development. Mol Cancer Res *6*, 1440-1451.

Burkhart, D.L., and Sage, J. (2008). Cellular mechanisms of tumour suppression by the retinoblastoma gene. Nat Rev Cancer *8*, 671-682.

Chen, P.L., Riley, D.J., Chen, Y., and Lee, W.H. (1996). Retinoblastoma protein positively regulates terminal adipocyte differentiation through direct interaction with C/EBPs. Genes Dev *10*, 2794-2804.

Clarke, A.R., Maandag, E.R., van Roon, M., van der Lugt, N.M., van der Valk, M., Hooper, M.L., Berns, A., and te Riele, H. (1992). Requirement for a functional Rb-1 gene in murine development. Nature *359*, 328-330.

Classon, M., and Harlow, E. (2002). The retinoblastoma tumour suppressor in development and cancer. Nat Rev Cancer *2*, 910-917.

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

Dailey, L., Laplantine, E., Priore, R., and Basilico, C. (2003). A network of transcriptional and signaling events is activated by FGF to induce chondrocyte growth arrest and differentiation. J Cell Biol *161*, 1053-1066.

Danielian, P.S., Friesenhahn, L.B., Faust, A.M., West, J.C., Caron, A.M., Bronson, R.T., and Lees, J.A. (2008). E2f3a and E2f3b make overlapping but different contributions to total E2f3 activity. Oncogene *27*, 6561-6570.

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

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

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

Gurney, J.G., Severson, R.K., Davis, S., and Robison, L.L. (1995). Incidence of cancer in children in the United States. Sex-, race-, and 1-year age-specific rates by histologic type. Cancer *75*, 2186-2195.

Gutierrez, G.M., Kong, E., Sabbagh, Y., Brown, N.E., Lee, J.S., Demay, M.B., Thomas, D.M., and Hinds, P.W. (2008). Impaired bone development and increased mesenchymal progenitor cells in calvaria of RB1-/- mice. Proc Natl Acad Sci U S A *105*, 18402 -18407.

Hansen, J.B., Jorgensen, C., Petersen, R.K., Hallenborg, P., De Matteis, R., Boye, H.A., Petrovic, N., Enerback, S., Nedergaard, J., Cinti, S., *et al.* (2004). Retinoblastoma protein functions as a molecular switch determining white versus brown adipocyte differentiation. Proc Natl Acad Sci U S A *101*, 4112-4117.

Hopyan, S., Gokgoz, N., Poon, R., Gensure, R.C., Yu, C., Cole, W.G., Bell, R.S., Juppner, H., Andrulis, I.L., Wunder, J.S., *et al.* (2002). A mutant PTH/PTHrP type I receptor in enchondromatosis. Nat Genet *30*, 306-310.

Jacks, T., Fazeli, A., Schmitt, E.M., Bronson, R.T., Goodell, M.A., and Weinberg, R.A. (1992). Effects of an Rb mutation in the mouse. Nature *359*, 295-300.

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

LeCouter, J.E., Kablar, B., Hardy, W.R., Ying, C., Megeney, L.A., May, L.L., and Rudnicki, M.A. (1998). Strain-dependent myeloid hyperplasia, growth deficiency, and accelerated cell cycle in mice lacking the Rb-related p107 gene. Mol Cell Biol *18*, 7455-7465.

Lee, E.Y., Chang, C.Y., Hu, N., Wang, Y.C., Lai, C.C., Herrup, K., Lee, W.H., and Bradley, A. (1992). Mice deficient for Rb are nonviable and show defects in neurogenesis and haematopoiesis. Nature *359*, 288-294.

Lee, M.H., Williams, B.O., Mulligan, G., Mukai, S., Bronson, R.T., Dyson, N., Harlow, E., and Jacks, T. (1996). Targeted disruption of p107: functional overlap between p107 and Rb. Genes Dev *10*, 1621-1632.

Lefebvre, V., Garofalo, S., Zhou, G., Metsaranta, M., Vuorio, E., and De Crombrugghe, B. (1994). Characterization of primary cultures of chondrocytes from type II collagen/beta-galactosidase transgenic mice. Matrix Biol *14*, 329-335.

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

Logan, M., Martin, J.F., Nagy, A., Lobe, C., Olson, E.N., and Tabin, C.J. (2002). Expression of Cre Recombinase in the developing mouse limb bud driven by a Prxl enhancer. Genesis *33*, 77-80.

Parisi, T., Yuan, T.L., Faust, A.M., Caron, A.M., Bronson, R., and Lees, J.A. (2007). Selective requirements for E2f3 in the development and tumorigenicity of Rb-deficient chimeric tissues. Mol Cell Biol *27*, 2283-2293.

Robanus-Maandag, E., Dekker, M., van der Valk, M., Carrozza, M.L., Jeanny, J.C., Dannenberg, J.H., Berns, A., and te Riele, H. (1998). p107 is a suppressor of retinoblastoma development in pRb-deficient mice. Genes Dev *12*, 1599-1609.

Ropke, M., Boltze, C., Meyer, B., Neumann, H.W., Roessner, A., and Schneider-Stock, R. (2006). Rb-loss is associated with high malignancy in chondrosarcoma. Oncol Rep *15*, 89-95.

Rossi, F., MacLean, H.E., Yuan, W., Francis, R.O., Semenova, E., Lin, C.S., Kronenberg, H.M., and Cobrinik, D. (2002). p107 and p130 Coordinately regulate proliferation, Cbfa1 expression, and hypertrophic differentiation during endochondral bone development. Dev Biol *247*, 271-285.

Ruiz, S., Santos, M., Lara, M.F., Segrelles, C., Ballestin, C., and Paramio, J.M. (2005). Unexpected roles for pRb in mouse skin carcinogenesis. Cancer Res *65*, 9678-9686.

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

Sage, J., Miller, A.L., Perez-Mancera, P.A., Wysocki, J.M., and Jacks, T. (2003). Acute mutation of retinoblastoma gene function is sufficient for cell cycle re-entry. Nature *424*, 223 -228.

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

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

Soriano, P. (1999). Generalized lacZ expression with the ROSA26 Cre reporter strain. Nat Genet *21*, 70-71.

Thomas, D.M., Carty, S.A., Piscopo, D.M., Lee, J.S., Wang, W.F., Forrester, W.C., and Hinds, P.W. (2001). The retinoblastoma protein acts as a transcriptional coactivator required for osteogenic differentiation. Mol Cell *8*, 303-316.

Trimarchi, J.M., and Lees, J.A. (2002). Sibling rivalry in the E2F family. Nat Rev Mol Cell Biol *3*, 11-20.

Walkley, C.R., Qudsi, R., Sankaran, V.G., Perry, J.A., Gostissa, M., Roth, S.I., Rodda, S.J., Snay, E., Dunning, P., Fahey, F.H., *et al.* (2008). Conditional mouse osteosarcoma, dependent on p53 loss and potentiated by loss of Rb, mimics the human disease. Genes Dev *22*, 1662-1676.

Weinberg, R.A. (1995). The retinoblastoma protein and cell cycle control. Cell *81*, 323-330.

Yamaguchi, T., Toguchida, J., Wadayama, B., Kanoe, H., Nakayama, T., Ishizaki, K., Ikenaga, M., Kotoura, Y., and Sasaki, M.S. (1996). Loss of heterozygosity and tumor suppressor gene mutations in chondrosarcomas. Anticancer Res *16*, 2009-2015.

Yanagino, T., Yuasa, K., Nagahama, M., Matsuda, Y., and Tsuji, A. (2009). Transcriptional regulation of fibrillin-2 gene by E2F family members in chondrocyte differentiation. J Cell Biochem *106*, 580-588.

Yang, G., Sun, Q., Teng, Y., Li, F., Weng, T., and Yang, X. (2008). PTEN deficiency causes dyschondroplasia in mice by enhanced hypoxia-inducible factor 1alpha signaling and endoplasmic reticulum stress. Development *135*, 3587-3597.

Chapter Three

Rb and *p53*-Deficient Bone Marrow-Derived Mesenchymal Cells Exhibit Altered Differentiation and are Tumorigenic

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INTRODUCTION

Tumors of mesenchymal origin are called sarcomas, and osteosarcoma is the most common tumor of bone. About 900 cases are diagnosed each year in the US and the majority of these occur in adolescents around the time of the growth spurt (Gurney et al., 1995). Mutation of the tumor suppressor *Rb* is found in all cases of inherited and most cases of sporadic osteosarcoma (Kansara and Thomas, 2007). The tumor suppressor gene *p53* is also found mutated in a large percentage of these tumors, and inheritance of *p53* mutations predisposes patients to osteosarcoma as well as other cancers (Kansara and Thomas, 2007; Malkin, 1993; Wunder et al., 2005).

Recently, we and another group developed mouse models of osteosarcoma that depend on the loss of *Rb* and/or *p53* in the osteoblast lineage (Berman et al., 2008a; Walkley et al., 2008, see also Appendix A). In both models, Cre-recombinase mediated deletion of *loxP*-flanked exons within *Rb* and *p53* initiated tumorigenesis. Since the *Osx-Cre* transgene is used to drive recombinase expression, the cells in which recombination occurs are likely osteoblast precursors (Rodda and McMahon, 2006). However, several of our results suggest that the osteoblastic tumor cells in this model acquire characteristics of a mesenchymal progenitor cell (MPC), which are osteoblast progenitors. Firstly, cell cultures generated from $Rb^{c/c}$; $p53^{c/c}$; *Osx-Cre* (DKO) osteosarcomas are capable of multipotent differentiation in vitro, expressed bone and fat differentiation markers, and expressed the murine stem-cell surface marker Sca1 (Berman et al., 2008a). Secondly, the osteosarcoma cell lines were competent to form tumors in immuno-compromised mice, but did so more efficiently if they were sorted first for Sca1-expressing cells. Finally, in addition to osteosarcomas, DKO mice unexpectedly developed hibernomas, a tumor of brown adipose tissue (BAT). Since the BAT is also descended from the mesenchyme, we hypothesized that the cellular origin of hibernomas could be the same as the cellular origin of the osteosarcomas.

Understanding the properties of the MPC is important because progenitor cells have been implicated in tumorigenesis as the tumor initiating or cancer stem cells in other tissues (Burkhart and Sage, 2008). The existence of an adult stem cell that can give rise to all mesenchymal tissues has been hypothesized, but the evidence for this cell in the mouse is limited and controversial (Bianco et al., 2008). This is not necessarily surprising, given

that these different lineages, and even cells within these lineages, arise from vastly different embryonic tissues, as well as end up in anatomically diverse locations (discussed in Chapter 1). Although there is evidence that bone marrow-derived mesenchymal cells can give rise to several lineages in vitro (Grigoriadis et al., 1988), most work has confirmed the existence of only one type of multipotent mesenchymal progenitor cell in vivo in the adult mouse, which can give rise to two lineages; the osteo-chondro progenitor which resides in the bone marrow and can generate both osteoblast and chondrocyte progeny in vivo (Ducy et al., 1997, reviewed in Karsenty and Wagner, 2002). Additionally, a multipotent progenitor that can give rise to either skeletal myoblasts or brown adipocytes has recently been identified, but its embryonic location, and whether it is also present in the adult animal, is still unclear (Seale et al., 2008). Finally, an adult white adipocyte progenitor cell has been identified within the white adipose compartment, but it is, so far, only unipotent (Rodeheffer et al., 2008).

Efforts to identify bone marrow MPCs by cell surface markers has been confounded by the rarity of this cell in vivo and their plasticity in culture; each preparation of non-hematopoietic bone marrow from mouse seems to have distinct immunophenotypes and it has been difficult to correlate the expression of a particular surface marker with a particular cell type or differentiation state (Bianco et al., 2008)(our unpublished observations). Nevertheless, MPCs are considered the progenitor cells for the tissue lineages that comprise the skeletal elements: bone, cartilage, connective tissue, muscle, and brown and white fat (Harada and Rodan, 2003). Despite the complicated and controversial picture of MPCs in mesenchymal tissue biology, we sought to study MPCs and non-hematopoietic bone marrow cells to elucidate the mechanism of tumorigenesis in our osteosarcoma model, and help define the properties of the mesenchymal progenitor.

The osteosarcoma model provides an excellent foundation for this study since pRb and p53 have already been implicated in regulating the differentiation of mesenchymal lineages. *Rb*-deficient mice exhibit a delay in intramembraneous ossification (Berman et al., 2008b) and an increased progenitor number (Gutierrez et al., 2008). pRb influences the differentiation capacity of osteoblasts in vitro, in part by modulating the activity of the osteoblast-specific transcription factor Runx2 (Berman et al., 2008a; Berman et al., 2008b; Thomas et al., 2001). Furthermore, pRb is important for controlling white and brown fat

differentiation (Classon et al., 2000; Hansen et al., 2004; Scime et al., 2005), muscle differentiation (Huh et al., 2004; Novitch et al., 1996; Schneider et al., 1994; Zacksenhaus et al., 1996) and cartilage differentiation (discussed in Chapter 2). There is also evidence of a role for p53 in bone development and differentiation. Studies of *p53*$^{-/-}$ or *Mdm2*$^{-/-}$ mice suggest that p53 blocks the differentiation of osteoblasts (Lengner et al., 2006; Wang et al., 2006) and analysis of differentiating *p53*$^{-/-}$ bone marrow-derived mesenchymal cells indicates that *p53* loss leads to incomplete osteoblast differentiation (Tataria et al., 2006).

Using our *Rb*$^{c/c}$;*p53*$^{c/c}$;*Osx-Cre* transgenic mouse lines, we examined the immunophenotype, differentiation capacity, and tumorigenicity of a variety of WT and DKO bone marrow-derived mesenchymal preparations (BM-MPC preps or just BM-MPCs) and assessed the stem cell-like properties of these cells in comparison with the osteosarcoma-derived cells. Furthermore, we assayed in vivo for the presence of DKO cells in brown fat pads to explore the origin of the hibernomas. We found that *Rb* and *p53* play important roles in controlling the phenotype of cultured BM-MPCs and that combined loss of *Rb* and *p53* in these cells does give rise to a Sca1^{+} tumor-initiating cell as early as 8 weeks of age. These studies provide evidence that the osteosarcoma-initiating cell has properties of a progenitor cell. Moreover, we found that the *Osx-Cre* transgene is expressed in brown adipocytes, and concomitant loss of *Rb* and *p53* permits/enhances survival of this cell type, likely resulting in hibernoma formation.

RESULTS

Osx-Cre induced mutation of Rb and p53 in brown fat cells enhances survival/proliferation

We generated a mouse model of osteosarcoma via mutation of *Rb* and/or *p53* specifically in the osteoblast lineage (Berman et al., 2008a). This was accomplished by using an *Osx-Cre* transgene to express Cre-recombinase, which had been shown to be expressed in committed osteoblast precursors (Rodda and McMahon, 2006). In our model, $Rb^{c/c};p53^{c/c};Osx\text{-}Cre$ (DKO) mice develop osteosarcoma (OS) by 5 months of age. Surprisingly, in addition to osteosarcoma, DKO animals develop hibernomas, a tumor of brown adipose tissue, which, like bone, is of mesenchymal origin. Although the *Osx-Cre* transgene was expected to be expressed exclusively in osteoblasts one possible explanation for hibernoma formation in the DKO mice is unanticipated expression of the transgene in the brown fat. In this scenario, cells in which *Rb* and *p53* are mutated upon *Osx-Cre* expression would survive and over-proliferate, eventually giving rise to tumors. An alternative hypothesis, based on the stem cell-like behavior of the osteosarcoma tumor cell lines, is that osteoblasts would de-differentiate upon loss of *Rb* and *p53*, migrate to brown fat sites, and proliferate to form a tumor. Although there is no direct evidence of this phenomenon in the literature, recently it was observed that loss of *Rb* in some cell types causes the acquisition of a stem cell-like phenotype, including expression of stem cell markers (Liu et al., 2009).

To determine which of the above possibilities could account for hibernoma formation, we developed a reporter system that allowed us to assess the presence of WT and DKO cells that had expressed the *Osx-Cre* transgene, or were the progeny of such a cell, in any tissue. *Osx-Cre* or $Rb^{c/c};p53^{c/c};Osx\text{-}Cre$ (DKO) mice were interbred with *Rosa26-LSL-LacZ* reporter mice and tissues were stained with X-Gal to identify which cells expressed β-galactosidase (β-gal) as a result of Cre-mediated recombination of the LSL cassette. To confirm that the system worked, we examined β-gal expression in osteoblasts, where we expected expression of the transgene in cells of all genotypes. Indeed, β-gal positive cells

were present in osteoblasts carrying the *Osx-Cre* and *LSL-LacZ* transgenes regardless of *Rb* and *p53* status, but they were not present in osteoblasts containing only *LSL-LacZ* or *Osx-Cre* (data not shown). Surprisingly, we also found expression of the *Osx-Cre* transgene in brown fat cells in wild-type mice at e15.5, P1, 4 weeks, and 8 weeks of age (Fig. 1 and data not shown). Importantly, $Rb^{c/c};p53^{c/c};$*Osx-Cre;LSL-LacZ* mice exhibited a far greater population of cell that stained in the brown fat pads compared to WT (compare Fig. 1A and B to Fig. 1D). Mice heterozygous for *Rb* and *p53* that carried the transgene and reporter construct displayed an intermediate level of staining (Fig. 1C). These results demonstrate that the *Osx-Cre* transgene is expressed in brown adipose tissue, and that mutation of *Rb* and *p53* in these cells favors their survival and expansion relative to normal cells. Thus, the hibernomas likely arise in situ due to expression of *Osx-Cre* in brown fat cells and subsequent survival and expansion of Rb/p53 DKO cells.

In vitro ablation of Rb and p53 in BM-MPC preps increases Sca1 expression and multilineage differentiation

Our studies of osteosarcoma-derived cell lines from DKO mice revealed that they had characteristics of mesenchymal progenitor cells. First, they could give rise to bone, fat, and cartilage in vitro, in contrast to cultured primary osteoblasts, which give rise only to bone, demonstrating their multipotency. Second, a percentage of the population expressed the stem cell marker Sca1 (Berman et al., 2008a). Furthermore, when the tumor cell lines were sorted based on Sca1 expression, the $Sca1^{+}$ populations more efficiently gave rise to tumors in immuno-compromised CrTac:NCr-$Foxn1^{nu}$ (nude) mice (Berman et al., 2008a). Since Sca1 expression seemed to correlate with the stem cell-like phenotype and tumorigenicity of the osteosarcoma cell lines, we decided to assay Sca1 expression, differentiation capacity, and tumorigenicity in cultured BM-MPC preps, which are thought to contain mesenchymal progenitor cells. BM-MPCs were isolated from $Rb^{c/c}$, $p53^{c/c}$, and $Rb^{c/c};p53^{c/c}$ mice. After brief culture in vitro, cells were infected with Adenovirus expressing Cre-recombinase to delete the conditional alleles, resulting in $Rb^{c-/c-}$ (Rb KO), $p53^{c-/c-}$ (p53 KO), and $Rb^{c-/c-};p53^{c-/c-}$ (DKO) cell lines. As a control, $Rb^{c/c};p53^{c/c}$ cells were infected with Adenovirus expressing GFP. After three passages, cells were assayed for Sca1

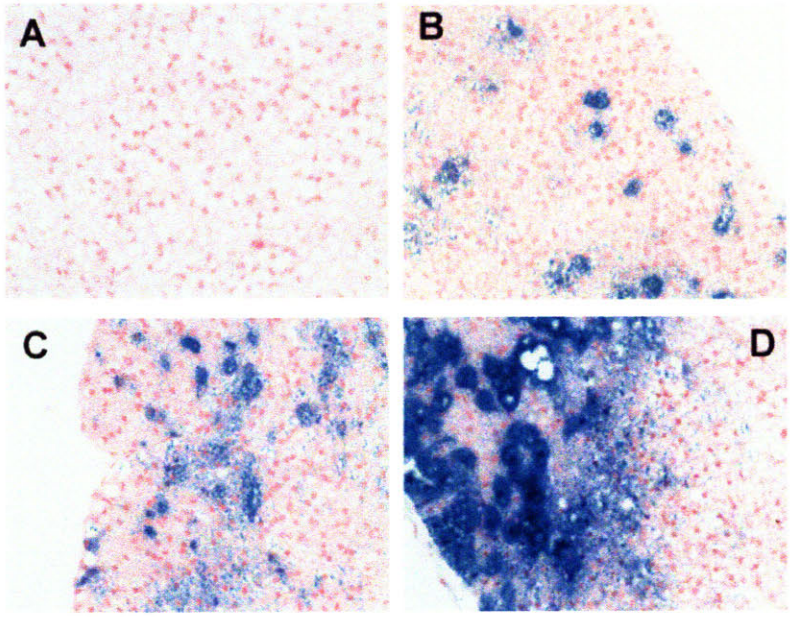

Figure 1: X-Gal Staining of BAT from P1 mice reveals expression of the *Osx-Cre* transgene in this tissue.
BAT dissected from P1 mice was stained with X-Gal to identify β-gal expressing cells. (A) *LSL-LacZ* mice do not exhibit staining, but (B) *Osx-Cre;LSL-LacZ*, (C) $Rb^{c/c};p53^{c/c}$;*Osx-Cre;LSL-LacZ*, and (D) $Rb^{c/c};p53^{c/c}$;*Osx-Cre;LSL-LacZ* exhibit different degrees of staining.

expression by FACS. Expression of CD45, a hematopoietic cell marker, was concomitantly examined, to separate the mesenchymal Sca1$^+$ cells from the hematopoietic Sca1$^+$ cells, which are known to be CD45$^+$. The control culture contained a small percentage of cells (~1%) that were Sca1$^+$ but CD45$^-$ (and therefore non-hematopoietic). The Rb KO, p53 KO, and DKO cultures, however, had expanded Sca1$^+$/CD45$^-$ populations (Fig. 2). Of these cultures, the Rb KO cells contained the smallest Sca1$^+$ population, which was still greatly increased compared to the control population, while loss of *p53* alone, or both tumor suppressors, increased the Sca1$^+$ population to as much as 30% (Fig. 2). This was comparable to the expression of Sca1 in the OS tumor cell lines (Berman et al., 2008a). These data indicate that loss of either tumor suppressor, in vitro, in BM-MPC preps leads to an increase in the Sca1$^+$ population.

We were interested whether loss of these two tumor suppressors and consequent increase in Sca1 expression influenced the differentiation capacity of the BM-MPC preps. To investigate this, we induced the same Adeno-cre and Adeno-GFP infected cells described above to differentiate into osteoblasts or adipocytes, and assessed the degree of differentiation 7, 14, and 21 days later. As expected, WT cells differentiated into both cell types by 21 days, as determined by Alizarin Red staining for calcified bone matrix (Fig. 3A) and Oil Red O staining for fat droplets (Fig. 3B). p53 KO and DKO cells also differentiated down both lineages, and notably, these cells did so earlier and to a greater extent than WT (Fig. 3A and B). In contrast, the Rb KO cells did not differentiate into either lineage by 21 days (Fig. 3A and B). Based on these experiments, we can conclude that loss of *p53* alone or *Rb* and *p53* together in the BM-MPC prep does not reduce the in vitro multipotency of the overall population compared to WT BM-MPCs, and in fact may promote differentiation down both lineages. Loss of *Rb* alone eliminates the ability of the BM-MPCs to differentiate into either lineage consistent with evidence that *Rb* is required for both bone and fat differentiation (Chen et al., 1996; Thomas et al., 2001).

BM-MPC preps that lose *Rb* and *p53* in vitro can generate tumors in nude mice

Given the phenotypic differences among the MPC preps, we wondered if there was a connection between differentiation capacity, Sca1 expression and tumorigenicity. To test

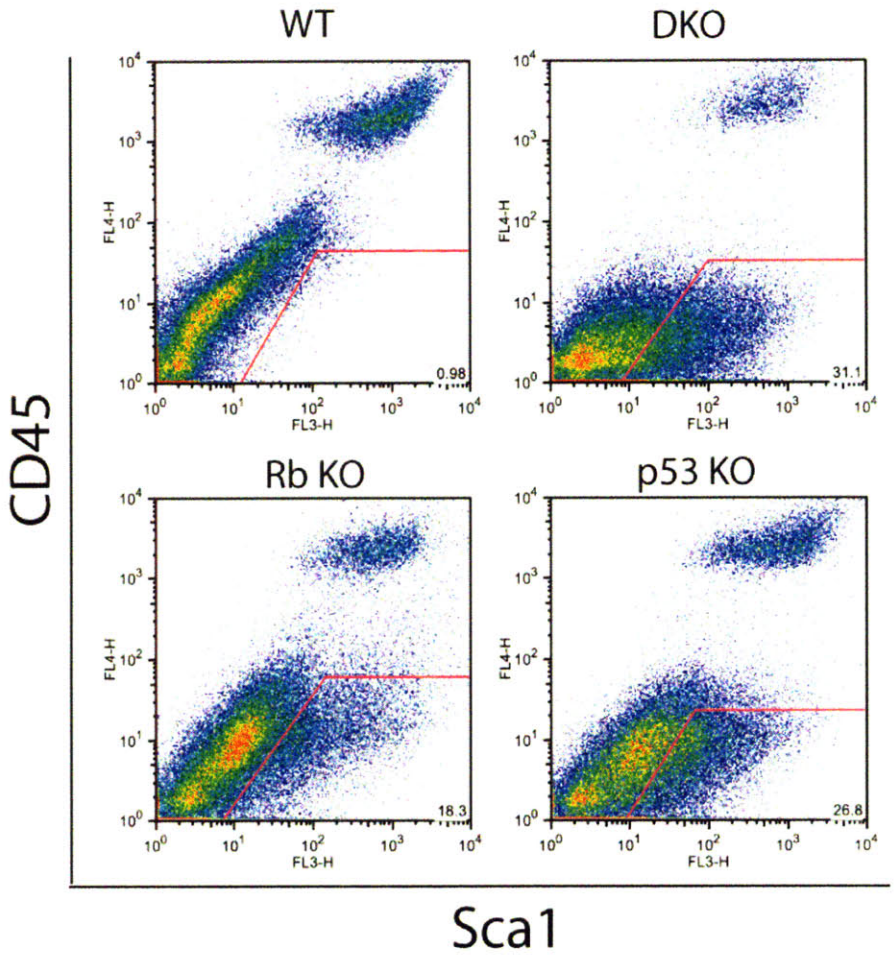

Figure 2: Sca1 expression increases in BM-MPC preps after Ad-Cre infection in vitro
BM-MPC preps were analyzed by FACS to determine the expression of Sca1. Compared to WT ($Rb^{c/c}$;$p53^{c/c}$ BM-MPCs infected with Ad-GFP) BM-MPCs, a greater population of $Rb^{c/c}$, $p53^{c/c}$; and $Rb^{c/c}$;$p53^{c/c}$ BM-MPCs after Ad-cre infection express Sca1.

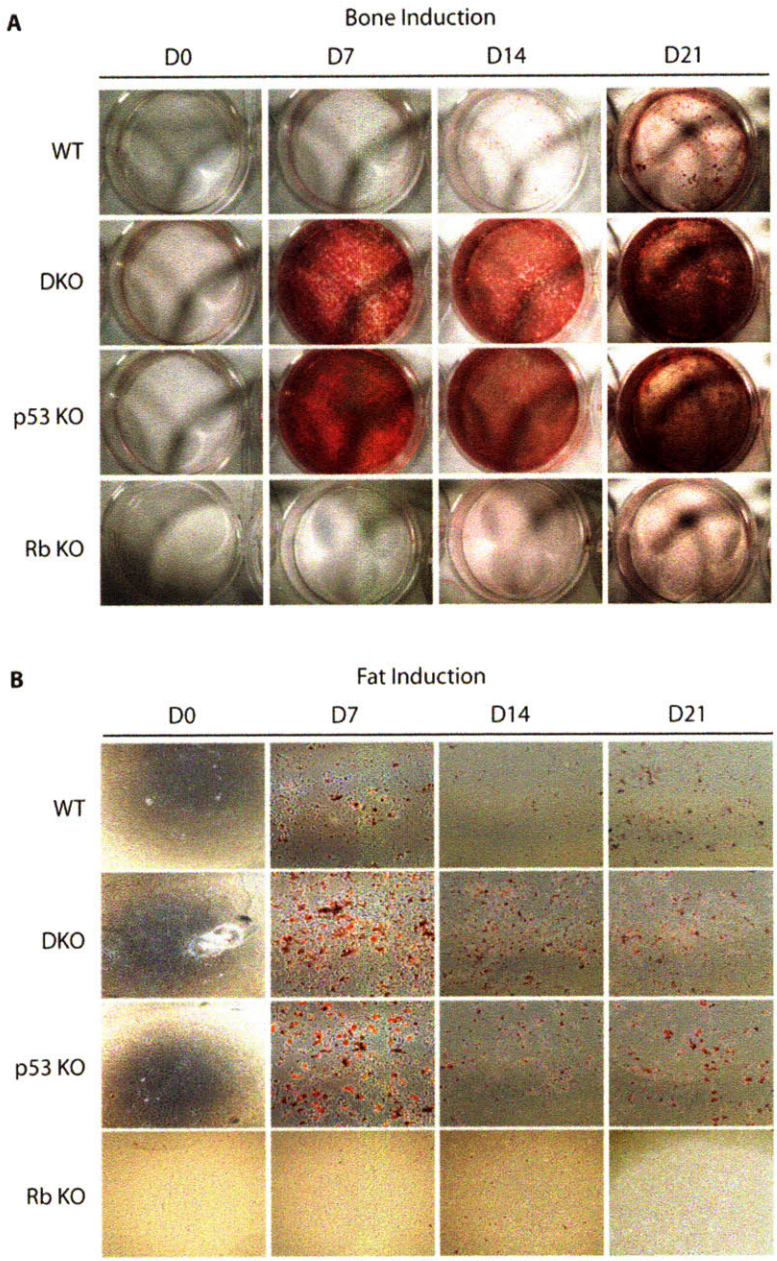

Figure 3: Bone and Fat Differentiation in BM-MPC preps after Ad-Cre infection
Confluent BM-MPC preps after Ad-GFP or Ad-Cre infection were induced to differentiate into the (A) bone, or (B) fat lineage and wells were stained with Alizarin Red S (bone) or Oil Red O (fat) to measure the extent of differentiation into each lineage at different timepoints. Compared to WT cultures, DKO and p53 KO cultures exhibited increased and more rapid differentiation into both lineages. Rb KO cultures did not differentiate into either lineage.

Genotype	Cell #	Surface Markers	# of injections	# of tumors	Metastases	Avg. Time to Tumor	Tumor type
WT MSCs	10^6	unsorted	2	0	N/A	N/A	
Rb/p53 DKO BM-MPCs	10^6	unsorted	4	2	no	2 months	sarcoma
	5×10^5	unsorted	1	0	N/A	N/A	
p53 KO BM-MPCs	10^6	unsorted	2	0	N/A	N/A	
S3012 DKO osx	10^6	unsorted	4	3	no	4 months	sarcoma
	2.5×10^5	unsorted	1	0	N/A	N/A	
SA991 DKO osx	10^6	unsorted	2	2	lungs	6.5 months	
	5×10^5	unsorted	1	1	multiple	5.5 months	sarcoma with bony region
	10^6	Sca1+ CD90-	1	1	no	4 months	sarcoma with bony region
	5×10^5	Sca1+ CD90-	1	1	no	4 months	sarcoma with bony region

Table 1: Tumorigenicity of BM-MPCs in nude mice after in vitro or in vivo ablation of *Rb* and/or *p53*

this, we injected low passage WT, Rb KO, p53 KO, and DKO BM-MPC preps subcutaneously into nude mice and monitored them for tumor development. As expected, WT BM-MPCs did not give rise to any tumors (Table 1). Rb KO BM-MPCs also did not give rise to any tumors (data not shown), consistent with the absence of osteosarcomas in the *Rb$^{c/c}$;Osx-Cre* mice. Surprisingly, the p53 KO BM-MPCs did not give rise to tumors in the nude mice, in contrast to the ability of *p53$^{c/c}$;Osx-Cre* cells to promote primary osteosarcoma in our in vivo model (Table 1; (Berman et al., 2008a). Although these results are contradictory, the lack of tumors in our experiments could be explained by changes that occurred to the p53 KO BM-MPCs during in vitro culture or differences in environmental signals between endogenously arising tumors and the nude mouse transplants. Finally, DKO BM-MPCs were capable of giving rise to tumors in nude mice (Table 1). These tumors arose by 2 months and were undifferentiated sarcomas based on histological analysis (data not shown). Thus, loss of both *Rb* and *p53* in the BM-MPC population was sufficient for transformation.

The Osteosarcoma-initiating cells are present in the bone marrow of DKO mice as early as 8 weeks of age

So far, we have shown that BM-MPC preps that lose *Rb* and *p53* in vitro possess properties similar to the OS cell lines from our osteosarcoma model. Given this, we were interested in identifying cells in vivo with BM-MPC-like properties prior to tumor formation. To answer this question, we generated BM-MPCs from 8-week-old DKO mice, cultured them briefly to expand the population and deplete hematopoietic cells, and then assessed their differentiation capacity, Sca1 expression, and/or injected them into nude mice to assess tumor formation. In a first experiment, BM-MPCs derived from a DKO mouse (line S3012) experienced complete recombination of the *Rb* and *p53* by passage 5, although at earlier time points cells containing unrecombined conditional *Rb* and *p53* alleles were still present by PCR (data not shown). We were able to culture S3012 for more than 23 passages, indicating that they were immortalized. S3012 cells had a moderate level of Sca1 expression after 1 passage in culture (P1). About 15% of S3012 cells expressed Sca1, compared to ~1% in WT cells and ~30% in BM-MPCs that lost *Rb* and *p53* in vitro

(compare Figs. 2 and 4A). The expression of Sca1 increased modestly after 23 passages (P23) (Fig. 4A). Thus, the Sca1-expressing population in the DKO bone marrow had expanded relative to the WT bone marrow and continued to expand during culture.

Early passage (P1) S3012 cultures that retained cells with unrecombined *Rb* and *p53* conditional alleles behaved similarly to both the OS tumor cell lines and the in vitro ablated DKO BM-MPCs when induced to differentiate into osteoblasts or adipocytes. Alizarin Red positive nodules covered the well as early as 7 days after bone induction, and lipid droplets appeared by 14 days after fat induction (Fig. 5A). To determine if there were changes in the differentiation capacity of these cells after extended culturing, we induced P23 S3012 cells to differentiate into osteoblasts and adipocytes. The P23 cells retained differentiated into osteoblasts, although it took longer, and was reduced, compared to the P1 S3012 cells, as shown by the appearance of Alizarin Red positive nodules between 7 and 14 days after induction (Fig. 5B). In contrast, the P23 cells did not differentiate into adipocytes even after 21 days of induction (Fig. 5B). Therefore P23 S3012 cultures had lost differentiation capacity compared to the parental low-passage culture.

We then wanted to determine whether the pre-tumorigenic BM-MPCs could initiate tumorigenesis in transplant. When $1X10^6$ S3012 cells from passage 4 (P4) were injected subcutaneously into nude mice, 3 out of 4 injections resulted in the growth of undifferentiated sarcomas within 4 months, although an injection of $2.5X10^5$ cells did not result in a tumor (Table 1). The ability of these cells to initiate tumors indicates that tumor-initiating cells are present in the bone marrow, although at low frequency, as early as 8 weeks of age. We analyzed these tumors by FACS to determine the level of Sca1 expression. In 2 out of 3 tumors (nude tumor 1 and 2a), the percentage of cells that expressed Sca1 but were $CD45^-$ was between 30 and 40%; greater than the percentage of cells expressing Sca1 in the original injected population (Fig. 4B). In the third tumor (nude tumor 2b), Sca1 expression was about 5%, reduced compared to the injected population (Fig. 4B). Interestingly, this tumor was the most differentiated and contained a significant amount of osteoid matrix (data not shown). These results suggest that Sca1 expression level correlates with the differentiation status of the tumor, and expression of Sca1 may either influence or be a consequence of maintenance of the undifferentiated state of the tumor-initiating cells.

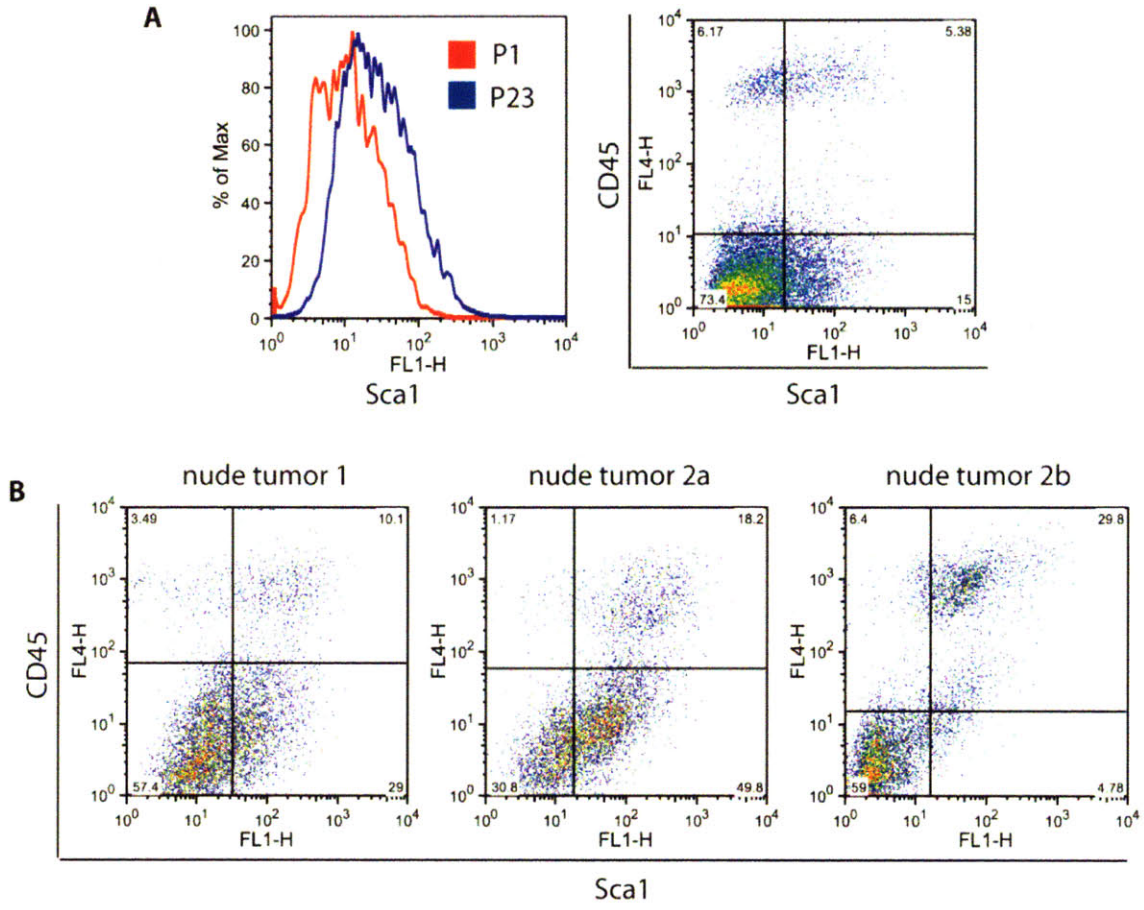

Figure 4: BM-MPCs derived from $Rb^{c/c}$;$p53^{c/c}$;Osx-Cre mice express Sca1 and form tumors in nude mice

(A) BM-MPCs from line S3012 express Sca1 during early passages in vitro. The population expressing Sca1 increases as the culture is passaged over 23 times. (B) BM-MPCs at P4 can give rise to tumors in nude mice. Of three tumors generated, two expressed Sca1 at a greater level than the parental population (see A). The third tumor showed a decrease in Sca1 expression.

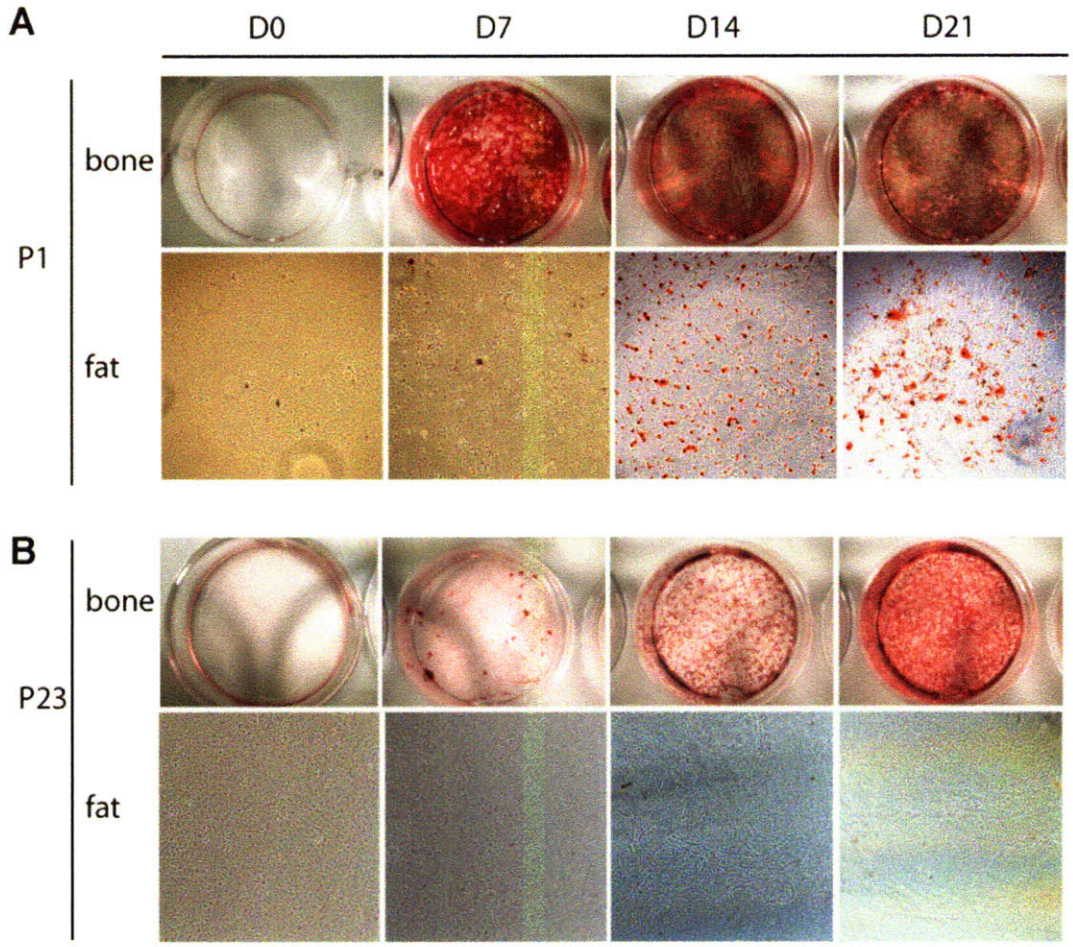

Figure 5: Bone and Fat Differentiation in BM-MPC prep S3012 at P1 and P23
Confluent S3012 cells were induced to differentiate into the bone or fat lineage and wells were stained with Alizarin Red S (bone) or Oil Red O (fat) to measure the extent of differentiation into each lineage at different timepoints. (A) At P1, S3012 cells can differentiate into both lineages. (B) At P23, S3012 cells are only able to differentiate into bone.

In a second experiment, BM-MPCs were again generated from an 8 week old DKO mouse and cultured briefly in vitro (line SA991). We injected either $1X10^6$ (2 injections) or $5X10^5$ (1 injection) cells subcutaneously in nude mice and all three injections gave rise to

tumors by 6.5 months (Table 1). These tumors were classified largely as undifferentiated sarcomas by histology, and contained large regions of bony matrix (Table 1; Fig. 6A). Intriguingly, mice injected with SA991 cells also presented with a variety of metastases, either just in the lungs ($1X10^6$ cells/injection) or in multiple tissues including the lungs, liver, kidneys, and on the spine ($5X10^5$ cells/injection) (Fig. 6B). Since SA991 and S3012 were generated from mice of the same genotype, it is surprising that SA991 cells were capable of metastasizing while S3012 cells weren't. One possible explanation is the greater amount of time SA991 animals survived while bearing tumors (5-6 months instead of 4 months), which may have increased the chances of metastatic spread, although we have no data to support this. Alternatively, there may be unidentified differences between the S3012 and SA991 cell lines. Nonetheless, these studies together support the idea that a tumor-initiating cell is present in the DKO bone marrow that is capable of giving rise to both primary tumors and metastases in transplant, as early as 8 weeks of age.

Sca1$^+$ expression marks the tumor-initiating cell in DKO bone marrow

Our initial study of OS tumor cell lines revealed that the Sca1$^+$ population of cells had enhanced tumorigenicity in nude mice, and tumors resulting from injection of S3012 cells showed that Sca1 expression changes in tumors compared to the original population. Based on these observations, we decided to directly examine the role of Sca1 in tumorigenicity of the DKO BM-MPCs. Based on previously reported expression of the surface marker CD90 on murine BM-MPCs (Nadri et al., 2007), we chose to examine the expression of this marker as well. Almost 50% of the SA991 cells were Sca1$^+$ (Fig. 7A). We also found a small but significant population of CD90$^+$ cells, a proportion of which were also Sca1$^+$ (Fig. 7A). We sorted SA991 MPCs for the Sca1$^+$/CD90$^-$ population and injected either $1X10^6$ or $5X10^5$ cells subcutaneously into nude mice to determine the relative contributions of Sca1 and CD90 to tumorigenesis. These injections gave rise to undifferentiated sarcomas after 4 months, but no metastases (Table 1; Fig. 6C, D). FACS analysis of the tumors revealed the presence of a Sca1$^+$/CD90$^-$ population as well as a Sca1$^+$/CD90$^+$ population in both tumors (Fig. 7B, C). These tumor studies confirm previous observations that Sca1$^+$ cells have enhanced tumorigenicity, since the sorted populations

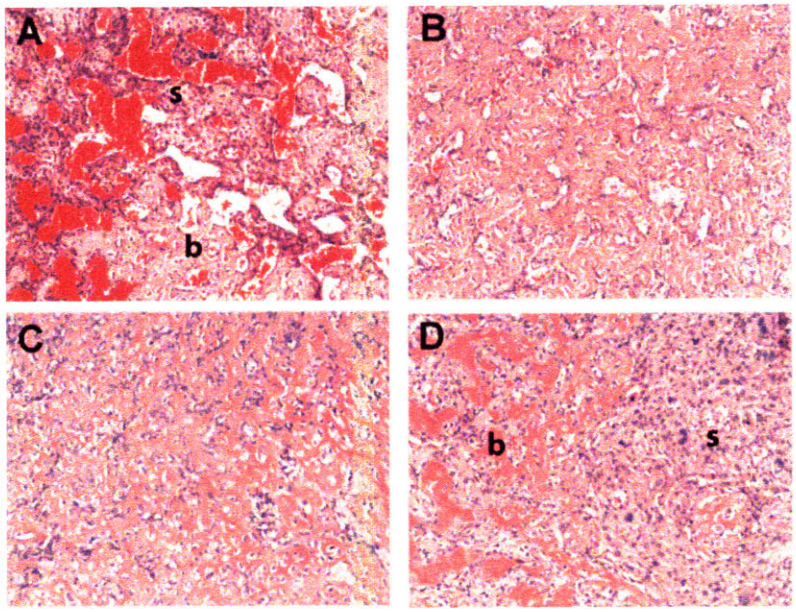

Figure 6: Tumors Generated in Nude Mice from BM-MPC line SA991
(A) Tumor generated from $1X10^6$ unsorted SA991 cells, containing both bony (b) and undifferentiated sarcoma (s) regions. (B) Lung metastasis that arose in a nude mouse injected with $1X10^6$ unsorted SA991 cells. (C) Undifferentiated sarcoma that arose in a nude mouse injected with $1X10^6$ $Sca1^+/CD90^-$ SA991 cells. (D) Tumor generated in a nude mouse injected with $5X10^5$ $Sca1^+/CD90^-$ SA991 cells. This tumor displays bony (b) and undifferentiated sarcoma regions (s).

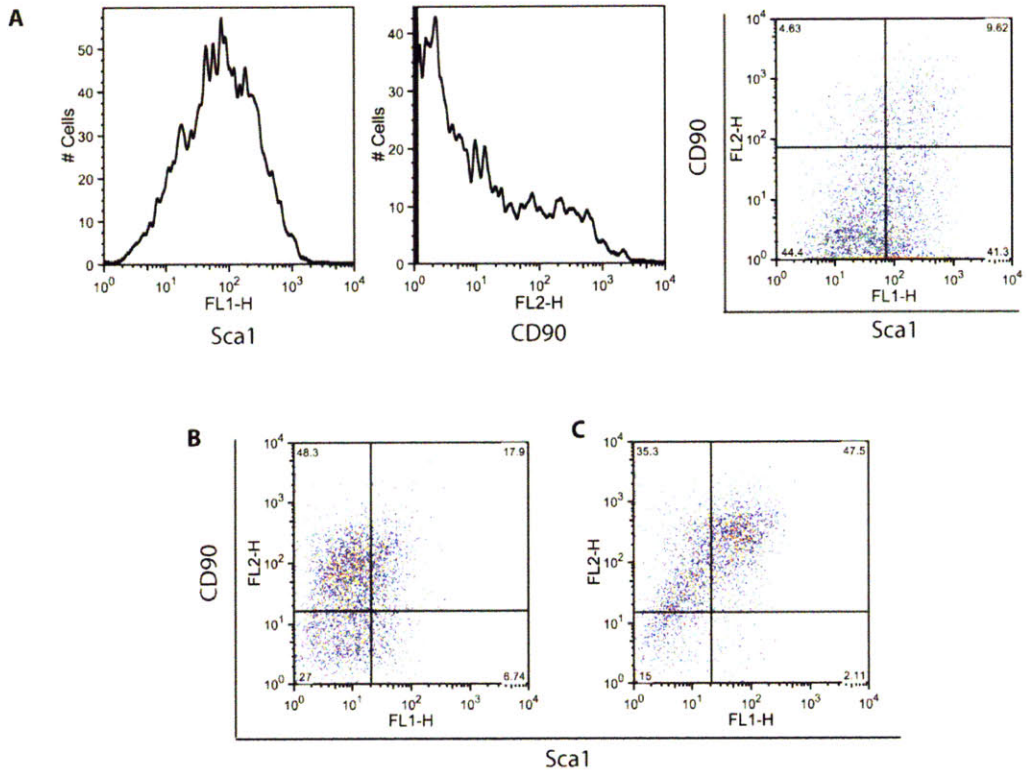

Figure 7: BM-MPC line SA991 expresses Sca1 and CD90
(A) BM-MPCs from line SA991 express Sca1 and CD90 during early passages in vitro. Either $1X10^6$ (B) or $5X10^5$ (C) SA991 cells sorted for the $Sca1^+/CD90^-$ population were injected into nude mice. In both cases a tumor was generated, each displaying a Sca1/CD90 expression profile that is different from the parental population (see A).

gave rise to tumors faster than the unsorted populations. Taken together, the transplant studies show that tumor-initiating cells exists in pre-tumor DKO bone marrow, they are likely Sca1^{+}, and can give rise to populations of cells of varying immunophenotypes within a subcutaneous tumor. Further studies will be required to pinpoint the phenotype of the tumor-initiating cells as well as establish the in vivo characteristics, such as differentiation capacity and stem cell-like properties.

DISCUSSION

In this study, we used several different experimental approaches to examine the properties of bone marrow-derived mesenchymal progenitor cells (BM-MPCs). Our interest in these cells stemmed from the analysis of tumor-derived cell lines from our mouse model of osteosarcoma (Berman et al., 2008a). These cell lines exhibited properties associated with mesenchymal progenitor cells; namely, potential to differentiate down multiple mesenchymal lineages in vitro and expression of the murine stem cell marker Sca1. Moreover, Sca1 expression in these cells correlated with their ability to efficiently give rise to secondary tumors in nude mice, suggesting that the tumor-initiating cell had stem cell-like properties. Thus, we sought to compare the tumor cell lines to BM-MPC cultures that had lost *Rb* and *p53* in vitro, or in vivo prior to obvious tumor formation, to better understand the developmental and tumorigenic properties of mesenchymal cells. We also investigated the origin of the hibernomas in DKO animals.

We found that, contrary to previous reports, the *Osx-Cre* transgene is expressed in the BAT. This is consistent with the notion that hibernomas likely arise in situ from rare *Osx-Cre*-expressing brown fat cells and not from DKO osteoblast precursors that became progenitor-like cells and traveled to the site of brown fat to differentiate down that lineage. Interestingly, mutation of both *Rb* and *p53* is required for hibernoma formation, while *p53*-loss alone is sufficient for osteosarcoma formation (Berman et al., 2008a). Our *Rosa26-LSL-LacZ* data show that mice heterozygous for *Rb* and *p53* have a modest expansion of X-gal stained cells in the BAT (Fig. 1). This suggests that loss of just one allele of *Rb* and *p53* is enough to promote over-proliferation, but is not sufficient to drive cells towards tumorigenesis. It would be interesting to determine the minimum requirements for tumorigenesis in this tissue.

Our experiments do not address whether low levels of endogenous *Osx* are expressed normally in the BAT or if this is a unique property of the transgene. This could easily be tested by assaying for *Osx* expression by qRT-PCR from the BAT. Notably, *Osx* is not expressed in DKO hibernomas, suggesting that the transgene may have an expression pattern distinct from the endogenous locus (Berman et al., 2008a). Alternatively,

endogenous *Osx* may be expressed early during BAT development but is eventually silenced, preventing its detection in the adult tissue.

We found that combined loss of *Rb* and *p53* in BM-MPC preps in vitro phenocopied the behavior of the tumor cell lines in differentiation assays, tumorigenesis assays, and in Sca1 expression (Fig. 2 and 3;Table 1). Interestingly, although loss of *Rb* and *p53* enhanced osteogenesis and adipogenesis in vitro, these cells formed undifferentiated tumors in vivo. This is consistent with studies that indicate that *p53* may act to inhibit terminal osteogenesis, but its loss only promotes the early stages of the process, leaving cells in an incompletely differentiated state that may be competent to initiate tumorigenesis (Tataria et al., 2006). These results suggest that even transformed cells can be induced to differentiate, which presents a possible method of treating osteosarcoma: by promoting differentiation of the cancer cells.

Our results show that in vitro ablation of *Rb* alone in BM-MPC cultures inhibits both osteogenic and adipogenic differentiation (Fig. 3). This is consistent with the known roles of pRb in modulating the activity of bone and fat specific transcription factors to impact the differentiation program of osteoblast and adipocyte precursor cells (Chen et al., 1996; Hansen et al., 2004; Thomas et al., 2001). In contrast, loss of *p53* at the same time as *Rb* in vitro not only suppresses this differentiation defect but also enhances differentiation into both lineages. It is unclear what function(s) of *p53* are responsible for this effect in *Rb*-deficient cells but we can postulate that the inhibitory effect of *p53* on differentiation may be stronger in an *Rb*-null background, perhaps due to activation of the p53 pathway downstream of *Rb*-loss (Pan et al., 1998; Tsai et al., 1998; Ziebold et al., 2001).

In contrast to BM-MPC preps that lose *Rb* and *p53* in vitro, analysis of BM-MPCs from DKO mice showed that the cells that lose *Rb* and *p53* in vivo, when expanded in vitro, are not capable of multilineage differentiation, although they do express Sca1 and can give rise to tumors in nude mice (Fig. 5 and 6;Table 1). These data suggest that the cells giving rise to osteosarcoma may express Sca1 but also retain their osteoblast identity. Only after becoming a full-fledged tumor, presumably with additional mutations do osteosarcoma cells exhibit in vitro multipotency.

One major caveat to our experiments is the heterogeneity of our cultured BM-MPCs, which likely contain mesenchymal progenitor cells, committed precursor cells, and

differentiated progeny (Bianco et al., 2008). This generates at least two difficulties. First, during differentiation, multiple populations of cells with distinct lineage commitments cannot be distinguished from a clonal population of cells that is multipotent; that is, both cultures will differentiate down both the bone and fat lineages. Second, in cultures where *Rb* and *p53* are ablated in vitro, we cannot be sure which cells in the culture are affected by Adeno-Cre infection, and therefore likely undergo immortalization. When these cells are injected into nude mice, we have no information about which cells within the population are tumorigenic. One solution to these problems is to develop a system to identify distinct mesenchymal cell populations within the bone marrow. Characterization of cell surface markers that define cells within the bone marrow based on differentiation status is currently underway in our laboratory and others. This is discussed in greater detail in Chapter 4.

The heterogeneity of BM-MPC preps may explain our observation that early passage BM-MPCs from DKO mice are multipotent, but extensively cultured DKO BM-MPCs differentiate only into bone, yet retain expression of the stem cell marker Sca1 (Fig. 4 and 5). We propose that the following is occurring: we assume the BM-MPC prep contains a mixture of WT mesenchymal progenitor cells, *Osx-Cre* expressing, and therefore probably DKO osteoblast precursors, and WT adipocyte precursors with limited replicative potential. At P1, the osteoblast and adipocyte precursors differentiate in response to their respective inductive signals, while the BM-MPCs either differentiate slowly or not at all. After 23 passages, however, the untransformed adipocyte precursors have probably been eliminated from the population. The *Osx-Cre* expressing cells have immortalized and remain committed to the bone lineage, resulting in bone differentiation but not adipocyte differentiation. Furthermore, this immortalized osteoblast has gained the stem cell-like property of Sca1 expression.

Nude mouse experiments have shown that BM-MPCs from young DKO mice prior to tumor development can give rise to secondary tumors suggesting that the osteosarcoma-initiating cell is present in the bone marrow at this stage. These BM-MPCs and the tumors they generate in nude mice contain a large population of cells expressing Sca1. Therefore, Sca1 expression is associated with both the identity of the mesenchymal progenitor cell as well as the tumor-initiating cell. The second BM-MPC culture that was also revealed that

injection of Sca1$^+$/CD90$^-$ cells into mice led to the development of a tumor containing a Sca1$^+$/CD90$^+$ population. Our data, however, do not allow us to determine if the cells within the tumor are host- or donor-derived, therefore, we cannot definitively prove that the Sca1$^+$/CD90$^+$ cells arose within the tumor from the BM-MPCs. To address this, the BM-MPCs could be labeled with a fluorescent marker like GFP prior to injection, which would enable us to distinguish between host- and donor-derived cells. Further experiments using BM-MPCs that have been sorted for populations expressing different combinations of cell surface markers could help us define a surface marker signature for tumorigenic populations. Additionally, serial dilution and serial passaging experiments in nude mice using BM-MPCs could help define the tumor-initiating cell or cells.

EXPERIMENTAL PROCEDURES

Animal Maintenance and Histology

The generation of *Rb*$^{c/c}$ mice, *p53*$^{c/c}$ mice, *Osx-Cre* mice, and *Rosa26-LSL-LacZ* mice has been described previously (Jonkers et al., 2001; Rodda and McMahon, 2006; Sage et al., 2003; Soriano, 1999). All mice were maintained on a mixed background. Gestation was dated by detection of a vaginal plug. CrTac:NCr-Foxn1^{nu} nude mice were purchased from Taconic. Nude mice were injected with BM-MPCs subcutaneously. Moribund animals were euthanized and tumors and other tissues were either fixed in 10% formalin and embedded in paraffin or analyzed by FACS. Histological sections were cut at 5μm.

X-Gal Staining

Adult and embryonic tissues were stained for β-galactosidase activity as described in (Parisi et al., 2007). Briefly, dissected tissues were fixed, rinsed, and then stained overnight at 37°C in X-Gal staining solution. Samples were then rinsed and fixed again before being processed for paraffin embedding. Sections were cut at 5μm and counterstained with Nuclear Fast Red.

Isolation and Analysis of BM-MPCs

BM-MPCs were generated as described in (Mukherjee et al., 2008). Briefly, femurs and tibias were crushed using a mortar and pestle in 1% FBS in PBS, then washed and filtered through a 70μm filter. Red blood cells were lysed using ACK Lysis Buffer (1mM $KHCO_3$, 0.15M NH_4Cl, 0.1mM EDTA, pH 7.2), cells were washed again and plated in T75 flasks in 20% FBS in DME. Cells were passaged upon confluence in 15-20% FBS in DME. Conditional BM-MPCs were infected with Ad5CMVCre-eGFP or Ad5CMVeGFP at ~100 pfu per cell (University of Iowa Gene Transfer Vector Core). For differentiation into bone and fat, BM-MPCs were allowed to become confluent and then induced to differentiate as described in (Mukherjee et al., 2008). To assess the extent of differentiation, cells were stained with Alizarin Red S (calcified bone nodules) or Oil Red O (lipid droplets).

FACS Analysis and Sorting

Cells were prepared for FACS either by isolation directly from the bone marrow (as described above but without plating in vitro), from tumors arising in nude mice by mincing and filtering through a 70μm filter, or from cultures by trypsinization, washed in 1%FBS in PBS, and stained with antibodies in 1% FBS in PBS on ice for 30 minutes (primary antibody) or 15 minutes (secondary antibody if necessary). Primary antibodies were used at 1:100: rat anti-mouse CD45-APC (LCA/Ly-5, BD Pharmingen), rat anti-mouse Sca1-FITC (Ly-6A/E, BD Pharmingen), rat anti-mouse CD90-APC (Thy1.2, Abcam), rat anti-mouse CD90-biotin (BD Pharmingen). The secondary Streptavidin-PE was used at 1:500 (eBioscience). Samples were either run on a FACSCalibur HTS (Becton-Dickinson) and analyzed with FlowJo software or were sorted on a FACSAria (Becton-Dickinson) before injection into nude mice.

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REFERENCES

Berman, S.D., Calo, E., Landman, A.S., Danielian, P.S., Miller, E.S., West, J.C., Fonhoue, B.D., Caron, A., Bronson, R., Bouxsein, M.L., *et al.* (2008a). Metastatic osteosarcoma induced by inactivation of Rb and p53 in the osteoblast lineage. Proc Natl Acad Sci U S A *105*, 11851-11856.

Berman, S.D., Yuan, T.L., Miller, E.S., Lee, E.Y., Caron, A., and Lees, J.A. (2008b). The retinoblastoma protein tumor suppressor is important for appropriate osteoblast differentiation and bone development. Mol Cancer Res *6*, 1440-1451.

Bianco, P., Robey, P.G., and Simmons, P.J. (2008). Mesenchymal stem cells: revisiting history, concepts, and assays. Cell Stem Cell *2*, 313-319.

Burkhart, D.L., and Sage, J. (2008). Cellular mechanisms of tumour suppression by the retinoblastoma gene. Nat Rev Cancer *8*, 671-682.

Chen, P.L., Riley, D.J., Chen, Y., and Lee, W.H. (1996). Retinoblastoma protein positively regulates terminal adipocyte differentiation through direct interaction with C/EBPs. Genes Dev *10*, 2794-2804.

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

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

Grigoriadis, A.E., Heersche, J.N., and Aubin, J.E. (1988). Differentiation of muscle, fat, cartilage, and bone from progenitor cells present in a bone-derived clonal cell population: effect of dexamethasone. J Cell Biol *106*, 2139-2151.

Gurney, J.G., Severson, R.K., Davis, S., and Robison, L.L. (1995). Incidence of cancer in children in the United States. Sex-, race-, and 1-year age-specific rates by histologic type. Cancer *75*, 2186-2195.

Gutierrez, G.M., Kong, E., Sabbagh, Y., Brown, N.E., Lee, J.S., Demay, M.B., Thomas, D.M., and Hinds, P.W. (2008). Impaired bone development and increased mesenchymal progenitor cells in calvaria of RB1-/- mice. Proc Natl Acad Sci U S A *105*, 18402 -18407.

Hansen, J.B., Jorgensen, C., Petersen, R.K., Hallenborg, P., De Matteis, R., Boye, H.A., Petrovic, N., Enerback, S., Nedergaard, J., Cinti, S., *et al.* (2004). Retinoblastoma protein functions as a molecular switch determining white versus brown adipocyte differentiation. Proc Natl Acad Sci U S A *101*, 4112-4117.

Harada, S., and Rodan, G.A. (2003). Control of osteoblast function and regulation of bone mass. Nature *423*, 349-355.

Huh, M.S., Parker, M.H., Scime, A., Parks, R., and Rudnicki, M.A. (2004). Rb is required for progression through myogenic differentiation but not maintenance of terminal differentiation. J Cell Biol *166*, 865-876.

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

Kansara, M., and Thomas, D.M. (2007). Molecular pathogenesis of osteosarcoma. DNA Cell Biol *26*, 1-18.

Karsenty, G., and Wagner, E.F. (2002). Reaching a genetic and molecular understanding of skeletal development. Dev Cell *2*, 389-406.

Lengner, C.J., Steinman, H.A., Gagnon, J., Smith, T.W., Henderson, J.E., Kream, B.E., Stein, G.S., Lian, J.B., and Jones, S.N. (2006). Osteoblast differentiation and skeletal development are regulated by Mdm2-p53 signaling. J Cell Biol *172*, 909-921.

Liu, Y., Clem, B., Zuba-Surma, E.K., El-Naggar, S., Telang, S., Jenson, A.B., Wang, Y., Shao, H., Ratajczak, M.Z., Chesney, J., *et al.* (2009). Mouse fibroblasts lacking RB1 function form spheres and undergo reprogramming to a cancer stem cell phenotype. Cell Stem Cell *4*, 336-347.

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

Mukherjee, S., Raje, N., Schoonmaker, J.A., Liu, J.C., Hideshima, T., Wein, M.N., Jones, D.C., Vallet, S., Bouxsein, M.L., Pozzi, S., *et al.* (2008). Pharmacologic targeting of a stem/progenitor population in vivo is associated with enhanced bone regeneration in mice. J Clin Invest *118*, 491-504.

Nadri, S., Soleimani, M., Hosseni, R.H., Massumi, M., Atashi, A., and Izadpanah, R. (2007). An efficient method for isolation of murine bone marrow mesenchymal stem cells. Int J Dev Biol *51*, 723-729.

Novitch, B.G., Mulligan, G.J., Jacks, T., and Lassar, A.B. (1996). Skeletal muscle cells lacking the retinoblastoma protein display defects in muscle gene expression and accumulate in S and G2 phases of the cell cycle. J Cell Biol *135*, 441-456.

Pan, H., Yin, C., Dyson, N.J., Harlow, E., Yamasaki, L., and Van Dyke, T. (1998). Key roles for E2F1 in signaling p53-dependent apoptosis and in cell division within developing tumors. Mol Cell *2*, 283-292.

Parisi, T., Yuan, T.L., Faust, A.M., Caron, A.M., Bronson, R., and Lees, J.A. (2007). Selective requirements for E2f3 in the development and tumorigenicity of Rb-deficient chimeric tissues. Mol Cell Biol *27*, 2283-2293.

Rodda, S.J., and McMahon, A.P. (2006). Distinct roles for Hedgehog and canonical Wnt signaling in specification, differentiation and maintenance of osteoblast progenitors. Development *133*, 3231-3244.

Rodeheffer, M.S., Birsoy, K., and Friedman, J.M. (2008). Identification of white adipocyte progenitor cells in vivo. Cell *135*, 240-249.

Sage, J., Miller, A.L., Perez-Mancera, P.A., Wysocki, J.M., and Jacks, T. (2003). Acute mutation of retinoblastoma gene function is sufficient for cell cycle re-entry. Nature *424*, 223 -228.

Schneider, J.W., Gu, W., Zhu, L., Mahdavi, V., and Nadal-Ginard, B. (1994). Reversal of terminal differentiation mediated by p107 in Rb-/- muscle cells. Science *264*, 1467 -1471.

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

Seale, P., Bjork, B., Yang, W., Kajimura, S., Chin, S., Kuang, S., Scime, A., Devarakonda, S., Conroe, H.M., Erdjument-Bromage, H., *et al.* (2008). PRDM16 controls a brown fat/skeletal muscle switch. Nature *454*, 961-967.

Soriano, P. (1999). Generalized lacZ expression with the ROSA26 Cre reporter strain. Nat Genet *21*, 70-71.

Tataria, M., Quarto, N., Longaker, M.T., and Sylvester, K.G. (2006). Absence of the p53 tumor suppressor gene promotes osteogenesis in mesenchymal stem cells. J Pediatr Surg *41*, 624-632; discussion 624-632.

Thomas, D.M., Carty, S.A., Piscopo, D.M., Lee, J.S., Wang, W.F., Forrester, W.C., and Hinds, P.W. (2001). The retinoblastoma protein acts as a transcriptional coactivator required for osteogenic differentiation. Mol Cell *8*, 303-316.

Tsai, K.Y., Hu, Y., Macleod, K.F., Crowley, D., Yamasaki, L., and 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*, 293-304.

Walkley, C.R., Qudsi, R., Sankaran, V.G., Perry, J.A., Gostissa, M., Roth, S.I., Rodda, S.J., Snay, E., Dunning, P., Fahey, F.H., *et al.* (2008). Conditional mouse osteosarcoma, dependent on p53 loss and potentiated by loss of Rb, mimics the human disease. Genes Dev *22*, 1662-1676.

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

Wunder, J.S., Gokgoz, N., Parkes, R., Bull, S.B., Eskandarian, S., Davis, A.M., Beauchamp, C.P., Conrad, E.U., Grimer, R.J., Healey, J.H., *et al.* (2005). TP53 mutations and outcome in osteosarcoma: a prospective, multicenter study. J Clin Oncol *23*, 1483-1490.

Zacksenhaus, E., Jiang, Z., Chung, D., Marth, J.D., Phillips, R.A., and Gallie, B.L. (1996). pRb controls proliferation, differentiation, and death of skeletal muscle cells and other lineages during embryogenesis. Genes Dev *10*, 3051-3064.

Ziebold, U., Reza, T., Caron, A., and Lees, J.A. (2001). E2F3 contributes both to the inappropriate proliferation and to the apoptosis arising in Rb mutant embryos. Genes Dev *15*, 386-391.

Chapter Four

Discussion

We have examined the roles of the pocket proteins pRb and p107 in controlling differentiation and tumorigenesis in two mesenchymal tissues: cartilage and bone. By conditionally mutating *Rb* in mesenchymal progenitor cells in $p107^{-/-}$ mice, we showed that these genes are required for proper endochondral ossification of the long bones and sternum (Chapter 2). Loss of *Rb* and *p107* in this lineage results in developmental cartilage defects and eventually leads to the formation of enchondromas in the joints of adult mice. These phenotypes can be explained in part by inappropriate proliferation of embryonic growth plate chondrocytes, and abnormal and altered proliferation and differentiation of adult chondrocytes (Chapter 2).

Using a similar method, we conditionally mutated *Rb* and *p53* in osteoblast precursors to develop a mouse model of osteosarcoma (Appendix A). Further investigation into tumor etiology in this model led to the finding that the likely cell of origin in this tumor retains the osteoblast identity but also acquires a stem cell-like feature of Sca1 expression, which correlates with tumorigenesis (Chapter 3). We also demonstrated that expression of the *Osx-Cre* transgene is not restricted to osteoblasts, and is active in at least one other tissue, brown fat. This finding explains the presence of hibernomas in the $Rb^{c/c};p53^{c/c};$*Osx-Cre* mice (Chapter 3).

These studies add to the growing body of evidence that tumor suppressor function is required not only for cell cycle regulation but also for appropriate differentiation. However, many questions remain unanswered; these are discussed below in the context of the data described in Chapters 2 and 3.

The Role of Rb and p107 in Cartilage

Rb and *p107*-Dependent Development of Chondrosarcomas

An interesting research avenue that emerges from our study is to determine the additional events that would allow the enchondromas in the $Rb^{c/c};p107^{-/-};$*Prx1-Cre* mice to progress to chondrosarcomas. In our model, the Rb/p107 DKO chondrocytes cycle more and continue to proliferate at a time when WT growth plates enter quiescence (Chapter 2). The regions of over-proliferation in the adult growth plates also exhibit abnormal

differentiation status, and express early and late markers of differentiation, indicating that they are unable to terminally differentiate. By 6 months, however, the enchondromas are quiescent, and they never advance to malignancy. What causes this block in tumorigenesis? Is it due to extrinsic inhibitory signals, a lack of growth factors required for complete transformation, the cells having reached a differentiated, quiescent or senescent state from which transformation is improbable and/or was the time span of the experiment insufficient for the additional mutation(s) required for malignancy to occur? We have not examined any of these phenomena in detail.

Although human chondrosarcomas are associated with mutations in the *Rb* pathway (Asp et al., 2001; Asp et al., 2000; Ropke et al., 2006; Yamaguchi et al., 1996), there are few clues in this literature to suggest what mutations might make the distinction between a benign enchondroma and a chondrosarcoma. One way to study this question would be to delete *p53* in the context of *Rb* and *p107* mutations, since *p53* is also implicated in human chondrosarcoma development (Yamaguchi et al., 1996), and *p53* mutation has already been shown to be tumorigenic in the mesenchymal lineage, both on its own and in cooperation with *Rb* mutation (Berman et al., 2008; Jacks et al., 1994; Walkley et al., 2008; Williams et al., 1994). Our preliminary results show loss of *Rb* and *p53* using the *Prx1-Cre* transgene leads to malignant osteosarcomas and hibernomas (A. Landman, E. Calo, P. Danielian, S. Berman, J. Lees, unpublished data). One caveat is that the potential ability of this combination of deletions to generate chondrosarcomas could be masked by the development of more malignant tumors in other tissues.

Another way to try to promote chondrosarcomagenesis would be to irradiate a cohort of young *Rb$^{c/c}$;p107$^{-/-}$;Prx1-Cre* mice and monitor for tumor development. The major caveats in this experiment are the likelihood of promoting additional irrelevant tumors, as well as the infrequent survival of DKO mice that could be used for the study. Finally, another option is to cross *Rb$^{c/c}$;p107$^{-/-}$;Prx1-Cre* mice to another mouse model of enchondroma formation, namely mice carrying a constitutively active allele of *PPR* (discussed in Chapter 1). Would these mutations synergize to promote advanced disease? Before interbreeding these mice, we would perform preliminary experiments to better understand the crosstalk between PPR signaling and the pocket proteins. First, we could determine if PPR signaling is indeed perturbed in the Rb/107 DKO mice by looking at

PTHrP and *PPR* expression in the growth plate by RNA in situ hybridization. Additionally, the *PPR* mutant mice could be examined for the dependence of the enchondroma phenotype on the function of *Rb* and *p107*.

We are also interested in studying the development of uterine leiomyosarcoma, a smooth muscle tumor. In humans, uterine leiomyomata, or fibroids, are common in reproductive-age women, but malignant transformation to leiomyosarcoma is rare. Leiomyosarcoma formation is associated with mutations on Chromosome 1, but the molecular mechanisms that drive malignancy remain unclear (reviewed in Hodge and Morton, 2007). Although there are indications that *RB* is important for tumor suppression in this tissue, as well in leiomysarcomas in other organs like the bladder, its molecular function in this tissue is unknown (Bleoo et al., 2003; Dei Tos et al., 1996). One mouse model of uterine leiomyosarcoma exists; these tumors are generated after ubiquitous Cre-mediated expression of SV40 Large T-antigen, which impairs the activity of the pocket proteins, as well as p53 (Politi et al., 2004). These data suggest that it is worthwhile to generate a more straightforward model of this tumor type by mutating the tumor suppressors we're interested in, specifically in the tissue of interest. For example, to explore the contribution of the pocket proteins specifically towards uterine smooth muscle tumorigenesis, one could cross $Rb^{c/c};p107^{-/-}$ mice to mice carrying a smooth muscle-specific Cre-expressing transgene such as *smMHC-Cre* (Regan et al., 2000) and monitor for tumor development.

The Role of the Activating E2Fs in Cartilage Development

Since the growth plates of Rb/p107 DKO mice exhibit inappropriate chondrocyte proliferation, and E2F target genes are upregulated at the mRNA level in embryonic chondrocytes, an obvious direction to take this work would be to examine the effect of the loss of the activating E2Fs in this model. Based on the canonical functions of the activating E2Fs (Trimarchi and Lees, 2002) we expect that loss of activating E2Fs might suppress the proliferation defect in the Rb/p107 DKO embryonic and adult growth plates. There is evidence in the literature that loss of either *E2f1* or *E2f3* in an $Rb^{-/-}$ background can suppress both differentiation and tumor phenotypes via their effect on proliferation as well

as apoptosis (McClellan et al., 2007; Parisi et al., 2007; Saavedra et al., 2002; Tsai et al., 1998; Yamasaki et al., 1998; Ziebold et al., 2003; Ziebold et al., 2001). Alternatively, *E2f1* and *E2f3* are involved in the differentiation of a number of tissues, as their absence results in a variety of differentiation defects that are often cell cycle-dependent (Cloud et al., 2002; Field et al., 1996; Humbert et al., 2000; King et al., 2008; Yamasaki et al., 1996). Therefore, it seems reasonable that their loss could also contribute to differentiation defects.

We are currently breeding the *Rb$^{c/c}$;p107$^{-/-}$;Prx1-Cre* animals to *E2f1$^{-/-}$*, *E2f3a$^{-/-}$*, and *E2f3b$^{-/-}$* animals to assess the effect of E2F loss on proliferation and differentiation of the growth plate at embryonic and later time points. Since both E2F1 and E2F3a have been shown to directly modulate the differentiation of chondrocytes, there is a chance that deleting them in a *Rb* and *p107*-deficient background could exacerbate the defect (Danielian et al., 2008; Scheijen et al., 2003). In contrast, although *E2f3$^{-/-}$* mice are neonatal lethal, E2F3b is generally redundant with E2F3a function and *E2f3b$^{-/-}$* mice display no phenotype, suggesting that *E2f3b*-loss might not influence chondrocyte proliferation or differentiation (Chong et al., 2009; Cloud et al., 2002; Danielian et al., 2008; Tsai et al., 2008). Consistent with this latter observation, our preliminary results show that concomitant loss of *E2f3b* with *Rb* and *p107* in chondrocytes does not alter the phenotype of DKO embryos; the DKO and TKO animals display almost identical defects in long bone length, width, and chondrocyte proliferation in the growth plate. The question still remains whether this result will hold up at later time points. Although we haven't fully analyzed the *Rb$^{c/c}$;p107$^{-/-}$;E2f3a$^{-/-}$;Prx1-Cre* animals yet, they do seem to arise at the expected frequency at weaning, strongly suggesting that loss of *E2F3a* does suppress the neonatal lethality of the *Rb$^{c/c}$;p107$^{-/-}$;Prx1-Cre* mice.

Potential Roles of *E2f4* and *p130* in *Rb$^{c/c}$;p107$^{-/-}$;Prx1-Cre* Mice

E2F4 is the only E2F known to associate with all pocket proteins (Trimarchi and Lees, 2002). E2F4 binds pRb and p130 at various points during the cell cycle and p130-E2F4 complexes are known to repress transcription of cell cycle genes during G0 (Moberg et al., 1996). Interestingly, p107 associates exclusively with E2F4 and it is thought that the p107-E2F4 complex promotes gene repression during G1 and S phase (Balciunaite et al.,

2005; Blais and Dynlacht, 2004; Rayman et al., 2002). Therefore, it is important to consider the role that loss of pRb- and p107-containing E2F4 complexes might play in the phenotypes we see in *Rb$^{c/c}$;p107$^{-/-}$;Prx1-Cre* mice.

If E2F4 functions in chondrocytes solely through pocket protein-mediated repression of target genes, loss of pRb and p107 would leave p130 as the only pocket protein available to regulate E2F4. Since p130-E2F4 complexes seem to be active during G0, rather than G1 or S like pRb-E2F4 or p107-E2F4 complexes, it is unclear if the former complex could functionally replace the latter complexes. These new complexes may have an altered spectrum of target genes and/or may be non-functional or irrelevant in this tissue. Thus, gene promoters normally bound by Rb-E2F4 or p107-E2F4 complexes might become de-repressed or otherwise deregulated in their absence, potentially leading to increased expression of cell cycle genes, differentiation genes, or both. This, in turn, could lead to increased proliferation and perturbed differentiation. Furthermore, there is some evidence that E2F4 may have pocket protein-independent activity that may be enhanced in the absence of Rb and p107 due to increased amounts of free E2F4 (Landsberg et al., 2003).

The role of pocket protein-E2F4 complexes in regulating cartilage development is currently unknown. However, there is evidence that E2F4 function is important for differentiation of some mesenchymal cell types. For example, E2F4 is critical for regulating osteoblast progenitor cell number, and thus for the proper timing of bone differentiation, in the murine skull (E. Miller, S. Berman, T. Yuan, and J. Lees, submitted). Furthermore, *E2f4$^{-/-}$* MEFs have an increased propensity for adipogenesis in vitro (Fajas et al., 2002; Landsberg et al., 2003). During fat differentiation, E2F4, in cooperation with p107 and/or p130 can bind and repress the *PPARγ1* promoter (Fajas et al., 2002). PPARγ1 is a transcription factor required for adipogenesis, so this is consistent with the idea that E2F4 inhibits adipocyte differentiation. Since loss of p107 and p130 in MEFs also results in increased adipogenesis (Classon et al., 2000), it is possible that p107, p130, and E2F4 act in concert to inhibit adipogenesis, and loss of either pocket protein function or E2F4 activity has the same outcome. Thus, loss of p107 and pRb in chondrocytes may produce an analogous situation where E2F4's role in repressing a gene(s) important for differentiation would be ablated, leading to differentiation abnormalities like those seen in our model.

Although no cartilage defects have been reported in *E2f4*$^{-/-}$ embryos or animals since these mice generally don't survive beyond 3 weeks of age, the role of E2F4 in cartilage could be more rigorously assessed by crossing a mouse strain carrying a conditional knockout allele of *E2f4* recently developed in our lab (E. Miller and J. Lees, unpublished data) with mice carrying a mesenchymal tissue-specific Cre-expressing transgene like *Prx1-Cre*. By comparing the embryonic and adult growth plate phenotypes in these mice with those in our study, we could determine if loss of E2F4 function is responsible for the proliferative or differentiation defects we observed. This would help define which aspects of the Rb/p107 DKO phenotype were due to deregulation of E2F4 vs. E2F4-independent effects. Furthermore, by using the conditional *E2f4* mice to generate *Rb*$^{c/c}$*;p107*$^{-/-}$*E2f4*$^{c/c}$*;Prx1-Cre* compound mutant mice we can determine whether the pocket proteins and E2F4 act synergistically upon different aspects of cartilage differentiation.

To more specifically probe the activity of E2F4 and p130-E2F4 complexes in chondrocytes, p130 and E2F4 expression and complex formation could be assessed in primary chondrocytes in the presence and absence of pRb and p107. These studies could reveal if p130 and/or E2F4 expression or activity is only triggered when pRb and p107 are lost. Preliminary results show that mRNA expression of p130 is the same in WT, Rb KO, p107 KO, and DKO primary chondrocytes, however, p130 may be regulated at the post-transcriptional level. To determine whether p130-E2F4 complexes are functional and which genes they might regulate, ChIP analysis and concomitant qRT-PCR of candidate cell cycle and differentiation genes could be used. Recently, ChIP-chip analyses, whereby genome-wide promoter occupancy of proteins of interest can be assayed in an unbiased manner, was determined for E2F4, p130, and p107 in a synchronized human cell line (Balciunaite et al., 2005). This study revealed novel and distinct classes of targets for these proteins, alone and in combination. Although most of the promoters that were identified were related to cell cycle progression, there is no reason to believe these genes don't also influence differentiation. Studies like these can inform our examination of genes that may be differentially regulated by E2F4 and the pocket proteins during chondrocyte differentiation.

Tissue-Specific Consequences of *Rb*- and *p107*-Loss

Since pRb and p107 loss has such a striking effect on the development of cartilage and the smooth muscle of the uterus (discussed below), it is noteworthy that other tissues in which *Prx1-Cre* expression was confirmed (Chapter 2) we did not detect a phenotype. This could be due to the existence of compensatory mechanisms at work in the unaffected tissues or because these proteins have minor functions in these tissues. The most likely compensatory proteins are p130 and the pocket protein-independent E2Fs since they since also regulates E2F target gene expression. p130 can compensate for p107 loss in cartilage, as demonstrated by the proliferative defect in this tissue only observed when both p130 and p107 are mutated but not one or the other (Cobrinik et al., 1996; Lee et al., 1996). Furthermore, there are abundant examples of pocket protein-dependent compensation in several tissues during development (Lipinski and Jacks, 1999). p130 may compensate for the lack of pRb and p107 through E2F4/5-dependent or –independent mechanisms. For example, $Rb^{-/-};E2f4^{-/-}$ mice display increased tumor latency compared to $Rb^{-/-}$ mice (Lee et al., 2002). The tumor suppressive effects of *E2f4* loss can be attributed to the formation of non-canonical pocket protein-E2F complexes, in which p107 binds E2F3 and p130 binds E2F1 in these tissues (Lee et al., 2002). This likely reduces the oncogenic effect caused by the lack of pRb regulation of activating E2Fs. Therefore, p130 could act in place of p107 and pRb in some tissues to regulate the activating E2Fs in a non-canonical manner.

There is also evidence that E2F6 can substitute for E2F4 activity in terms of promoter occupancy and transcriptional repression during G0, in human cell lines (Ogawa et al., 2002; Xu et al., 2007). Therefore it is possible that in certain tissues, E2F6 or other pocket protein-independent E2Fs can act in place of E2F4, or pocket protein-E2F4 complexes. To test whether p130 or E2F6 are acting in place of pRb and p107 in our model, the expression levels and promoter occupancy of E2F6 and p130-containing compounds in a variety of WT and Rb/p107 DKO tissues could be examined. If compensation is occurring, it is possible that expression and/or activity of the compensating protein should either be greater in WT tissues that are unaffected compared to affected upon *Rb* and *p107* deletion, or that the expression or activity of these proteins would be upregulated in unaffected tissues upon *Rb* and *p107* deletion but not in affected

tissues. Furthermore, to identify a direct genetic interaction, the *Rb$^{c/c}$;p107$^{-/-}$;Prx1-Cre* mice could be interbred with *p130$^{-/-}$* or *E2f6$^{-/-}$* mice. In either case, if p130 or E2F6 could act in place of pRb and p107 in a particular tissue, we would expect to see a novel phenotype in the triple knockout mice.

Regulation of Chondrocyte Proliferation and Differentiation Mediated by Rb and p107

We are interested in the connection between upstream regulators of chondrocyte proliferation and differentiation, and the pocket proteins. During both embryogenesis and adulthood, the growth plate forms, functions, and eventually quiesces under the guidance of several interconnected signaling networks that influence proliferation as well as differentiation (Kronenberg, 2003). Understanding the ways in which the developmental signaling networks influence the activity of pRb and p107 will help us understand the developmental consequences of cell non-autonomous inputs in *Rb/p107*-deficient chondrocytes. Below I describe the potential crosstalk between two critical growth plate signaling pathways, Ihh/PTHrP and FGF, and the pocket proteins and how their disruption might lead to the cartilage developmental abnormalities we see in our mice.

There is abundant evidence implicating p107 and p130 as downstream effectors of FGF signaling in the growth plate (Dailey et al., 2003; Laplantine et al., 2002; Rossi et al., 2002). However, what is less clear is which cells are producing and which are responding to FGF. One FGF molecule known to be involved in inhibition of chondrocyte proliferation in vivo is FGF18 (Ohbayashi et al., 2002). The expression of *Fgf18* in the perichondrium, the undifferentiated mesenchymal cells surrounding the growth plate, is controlled by Runx2, and impinges on chondrocytes within the growth plate by diffusing into this region (Hinoi et al., 2006). One possible mechanism of inappropriate proliferation in our model is based on the reduced ability of this critical extrinsic mediator of cell cycle arrest via the pocket proteins in Rb/p107 null chondrocytes, leading to inappropriate proliferation. Importantly, *Prx1-Cre* is expressed in the mesenchymal cells of the perichondrium (Logan et al., 2002), thus *Rb* and *p107* are also mutated in this cells. Thus, the alternative, but not mutually exclusive possibility is that Runx2-dependent induction of *Fgf18* expression is

attenuated in the absence of its binding partner pRb (Thomas et al., 2001). Therefore, FGF signaling could be both up- and downstream of the pocket proteins in the embryonic growth plate.

One way to examine FGF signaling in our model is using RNA in situ analysis and/or IHC to look for the expression of *Fgf18* in the perichondrium as well as the presence of the molecule on the growth plate. Normal expression of *Fgf18* in Rb/p107 mutant tissues would suggest that the absence of these pocket proteins from the perichondrium did not reduce Runx2-mediated induction of this gene. Furthermore, one could examine the responsiveness of *Rb/p107*-null growth plates to FGF, and other signals, through the culture of embryonic bone rudiments (Yeh et al., 2007). Bone rudiments can be treated with a variety of soluble factors in vitro and the effect can be assessed either by sectioning of the bone and performing immunohistochemistry (IHC) or by dissociation of the rudiment and subsequent analysis by FACS, qRT-PCR, or Western blot for the expression of proliferation and differentiation markers of interest.

There are also intriguing but complicated connections between the Ihh/PTHrP signaling loop and the pocket proteins. As described in Chapter 1, Ihh and PTHrP comprise a feed-forward loop in the growth plate that promotes proliferation and delays differentiation (hypertrophy) of chondrocytes, and coordinates the location of these processes (reviewed in (Kronenberg, 2003). Interestingly, like FGF signaling, Ihh and PTHrP seem to function both up- and downstream of the pocket proteins, as well as in parallel pathways. Ihh promotes proliferation through induction of PTHrP as well as through PTHrP–independent mechanisms (Karp et al., 2000). One such mechanism is direct activation of the *Cyclin E* promoter by the Ihh downstream signaling molecule Gli, as well as indirect induction of *Cyclin D*, both of which act to inhibit the function of pocket proteins and promote cell cycle entry (Duman-Scheel et al., 2002). Furthermore, PTHrP promotes proliferation and inhibits differentiation in part by indirectly inhibiting the CDK inhibitor p57, which itself indirectly inhibits the actions of the pocket proteins (MacLean et al., 2004). Loss of *Rb*, however, has been found to promote the expression of *Ihh*, and its downstream effector *Gli3*, through an unknown mechanism, in the intestine (Yang and Hinds, 2007), thus creating a negative feedback loop in which the pocket proteins and Ihh/PTHrP signaling antagonizes one another.

To determine whether Ihh/PTHrP signaling in the growth plate contributes to the cartilage defect of *Rb/p107*-null mice, we should first determine if the expression of these genes is altered. We have examined the mRNA expression levels of these two genes by qRT-PCR from primary sternum chondrocytes, but both were variable within genotypes and between litters, preventing us from drawing a conclusion. A more robust way to measure the expression of these genes as well as identify which cells are expressing them is by RNA in situ hybridization in the growth plates. Because Ihh/PTHrP and the pocket proteins seem to function in a negative feedback loop, we would expect to see increased levels of one or both of these proteins in *Rb/p107*-deficient growth plates, which would be consistent with increased chondrocyte proliferation. Furthermore, ChIP analysis could be used to test whether pRb, p107, or the activating E2Fs directly regulate the expression of Ihh or PTHrP in chondrocytes. Finally, since the adult growth plate phenotype is partly due to the maintenance of differentiating/cycling chondrocytes past when they should have been taken over by bone, it would be instructive to determine if Ihh/PTHrP signaling was intact or amplified at that stage, based on their role in controlling the location of proliferation vs. differentiation.

The Role of Rb and p53 in Osteosarcoma and BM-MPCs

Rb$^{c/c}$;p53$^{c/c}$;Osx-Cre (Rb/p53 DKO) mice develop bony, malignant osteosarcomas but the tumor cells possess some attributes of stem cells, namely expression of the stem cell marker Sca1, and multipotent differentiation potential in vitro (Berman et al., 2008). Moreover, the Rb/p53 DKO mice develop an additional mesenchymal tumor type, hibernomas, with high frequency (Berman et al., 2008). We wanted to better understand the properties of the osteosarcoma cells, as well as the potential similarities between the tumor cells and mesenchymal progenitor cells. In Chapter 3, we asked whether BM-MPCs that lost *Rb* and/or *p53* in vitro or in vivo would behave similarly to the tumor-derived cell lines from the osteosarcoma model. To do this, we examined the differentiation capacity and tumorigenicity of BM-MPCs after losing Rb, p53, or both, either in vitro or in vivo. We found that loss of Rb and p53 in vitro enhances the differentiation capacity of BM-MPC preps, while in vivo deletion of these genes by *Osx-Cre* expression and subsequent culturing

results in immortalization of a cell that differentiates only into bone. However, regardless of whether the BM-MPCs lose *Rb* and *p53* in vitro or in vivo, they are tumorigenic when injected into nude mice.

Like the tumor cell lines, BM-MPCs that had lost Rb and p53 in vitro were multipotent in vitro, expressed Sca1, and were tumorigenic. Notably, tumor cell lines and cultured BM-MPCs retain an important distinction. While the tumor cell lines were derived originally from a population of cells that had expressed Cre in vivo, and therefore had a specific set of cellular identities, BM-MPCs are probably a more heterogeneous mixture of adherent mesenchymal cells from the bone marrow, as described in the discussion section of Chapter 3 (Bianco et al., 2008). Our studies could not distinguish between the different populations in these cultures, therefore it is unclear, after Adeno-Cre infection, which population(s) was immortalized and responsible for the differentiation and/or tumorigenic phenotypes we observed. Although it is difficult to directly compare these cells to the tumor cells, we did identify several characteristics that were common between the cultures. It is important to note that the culturing of cells in vitro could contribute to this by selecting for particular cells well adapted to grow under in vitro tissue culture conditions.

It is also difficult to compare BM-MPCs derived from Rb/p53 DKO mice with the tumor cell lines. Although both preparations lost Rb and p53 in vivo in the same cell population, DKO BM-MPCs did not possess the same differentiation properties as the tumor cell lines. These differences could be attributed to in vitro culturing effects or changes that occurred in the tumor cells prior to in vitro culture. Nevertheless, because we cannot identify the cell types present in the BM-MPC preps, we are hampered in our efforts to characterize the tumor cell of origin of osteosarcoma. Thus, we, and others, are making efforts to define the populations of mesenchymal cells within the bone marrow through use of gene expression profiling and identification of cell surface markers, which I discuss below.

Using Cell Surface Markers to Identify Mesenchymal Cell Populations in vivo

An important caveat with this work is that BM-MPCs were cultured in vitro before performing any experiments. Ultimately, we are interested in the properties and behavior of the tumor-initiating cell in vivo, but that is currently unfeasible due to the paucity of validated cell surface markers to identify and prospectively isolate distinct cell populations within the bone marrow. Much work has been done on examining surface marker expression in vitro, identifying cell surface proteins, such as CD105, STRO-1, and CD90, as being expressed on bone marrow-derived mesenchymal cells, however, we know that in vitro culture can lead to plasticity in the expression of these markers (Mukherjee et al., 2008; Nadri et al., 2007; Simmons and Torok-Storb, 1991). Although we used Sca1 and CD90 in this study to mark cells derived from the bone marrow, it is important to note that only Sca1 has been implicated to function in mesenchymal progenitors. The best evidence that Sca1 marks osteoprogenitors in the bone marrow comes from a study of $Sca1^{-/-}$ mice, in which osteoprogenitor numbers in the bone marrow, as measured by colony forming unit assays, as well as the proliferative index of these cells, was decreased (Bonyadi et al., 2003).

Therefore, there is a need for functionally relevant cell surface markers that can be used to define different populations of bone marrow mesenchymal cells, their differentiation state and function, in vivo. One way to approach this problem is through validation of proteins currently known to be expressed by BM-MPCs, in a fashion similar to that used to define hematopoietic stem cells (HSCs) and their progeny (Morrison et al., 1995). A definitive, albeit time-consuming, way to assay the relevance of a cell surface marker for defining a particular subset of cells would be to generate a knockout mouse for that protein and examine the affected tissues. Alternatively, cells must be isolated directly from the bone marrow by FACS, using known negative selection markers against hematopoietic and endothelial cells (CD45, CD31), and candidate positive selection markers. These cells must then be examined for self-renewal capability, differentiation status/potential (eg osteoblast progenitor vs. bipotent osteo-chondroprogenitor), and expression of other markers. Self-renewal capability can be tested in vitro by examining colony forming ability of single cells. However, differentiation status and marker expression ideally should be performed without in vitro culture. Moreover, the

differentiation capability of the cells absolutely must be tested in vivo, to ensure that we are not assaying in vitro artifacts.

Finally, the identity of the bona fide mesenchymal stem cell, if it exists, must be subjected to the standard set of stem cell criteria (Bianco et al., 2008), although the ability of a single mesenchymal stem cell to reconstitute in vivo all of its descendent lineages would be an incredible challenge. This is because, unlike HSCs and the hematopoietic system, which can relatively easily be removed from a mouse, no such procedure exists for mesenchymal tissues, which exist throughout the body as independent organs. One possible way to achieve this definition would be to show that genetically, epigenetically, and immunophenotypically identical mesenchymal cell clones derived from the bone marrow could separately reconstitute individual mesenchymal tissues like bone, fat, and muscle.

A more systematic approach to define the populations of mesenchymal cells in the bone marrow would be through high-throughput expression arrays of phenotypically different subsets of WT and transformed mesenchymal cells. This work is currently ongoing in the lab, with a focus on the genome-wide mRNA expression changes in WT BM-MPCs compared to the tumor cell lines. Not only will this reveal gene expression changes that have occurred downstream of *Rb* and *p53* mutation in the tumor cell lines, but may reveal a subset of differentially regulated genes that are correlated with differentiation status and identify novel cell surface markers. To make this data useful, it is imperative that gene expression changes are correlated with the differentiation and cell surface marker phenotypes of sub-populations of bone marrow and tumor cells, derived from studies like those described above. Furthermore, using validated cell surface markers one could compare the overall gene expression of different isolated populations. These studies would allow us to define a molecular signature for bone marrow-derived mesenchymal cells at different stages of differentiation. Finally, combining phenotypic data with gene expression data, as well as cell surface marker expression might make it possible to confirm that an osteoblast transformed by deletion of *Rb* and *p53* gains so-called stem cell properties.

CONCLUSION

The balance between proliferation and differentiation contributes to whether a cell can become a cancer cell, but the mechanisms that regulate this switch are still emerging. The pocket proteins pRb, p107, and p130 are at the center of this decision point due to their critical role in regulating cell cycle entry and exit, which is concomitant with cellular differentiation. These studies highlight an important connection between defects in differentiation and the susceptibility to tumorigenesis in mesenchymal tissues. We have shown that pRb and p107 are necessary for restraining proliferation and instructing normal differentiation of chondrocytes in the growth plate, thus revealing a novel role for these pocket proteins in this tissue. Mice lacking these proteins in mesenchymal tissues are predisposed to develop cartilage defects and enchondromas. Furthermore, we have demonstrated that pRb and p53 influence the differentiation properties of bone marrow-derived mesenchymal cells, and their loss can result in tumor formation in several tissues and may lead to the de-differentiation of committed mesenchymal cells. These studies provide insights into how both cell cycle and differentiation functions of pocket proteins regulate normal mesenchymal tissue differentiation and how these functions are perturbed during mesenchymal tissue tumorigenesis.

REFERENCES

Asp, J., Inerot, S., Block, J.A., and Lindahl, A. (2001). Alterations in the regulatory pathway involving p16, pRb and cdk4 in human chondrosarcoma. J Orthop Res *19*, 149-154.

Asp, J., Sangiorgi, L., Inerot, S.E., Lindahl, A., Molendini, L., Benassi, M.S., and Picci, P. (2000). Changes of the p16 gene but not the p53 gene in human chondrosarcoma tissues. Int J Cancer *85*, 782-786.

Balciunaite, E., Spektor, A., Lents, N.H., Cam, H., Te Riele, H., Scime, A., Rudnicki, M.A., Young, R., and Dynlacht, B.D. (2005). Pocket protein complexes are recruited to distinct targets in quiescent and proliferating cells. Mol Cell Biol *25*, 8166-8178.

Berman, S.D., Calo, E., Landman, A.S., Danielian, P.S., Miller, E.S., West, J.C., Fonhoue, B.D., Caron, A., Bronson, R., Bouxsein, M.L., *et al.* (2008). Metastatic osteosarcoma induced by inactivation of Rb and p53 in the osteoblast lineage. Proc Natl Acad Sci U S A *105*, 11851-11856.

Bianco, P., Robey, P.G., and Simmons, P.J. (2008). Mesenchymal stem cells: revisiting history, concepts, and assays. Cell Stem Cell *2*, 313-319.

Blais, A., and Dynlacht, B.D. (2004). Hitting their targets: an emerging picture of E2F and cell cycle control. Curr Opin Genet Dev *14*, 527-532.

Bleoo, S.L., Godbout, R., Rayner, D., Tamimi, Y., and Moore, R.B. (2003). Leiomyosarcoma of the bladder in a retinoblastoma patient. Urol Int *71*, 118-121.

Bonyadi, M., Waldman, S.D., Liu, D., Aubin, J.E., Grynpas, M.D., and Stanford, W.L. (2003). Mesenchymal progenitor self-renewal deficiency leads to age-dependent osteoporosis in Sca-1/Ly-6A null mice. Proc Natl Acad Sci U S A *100*, 5840-5845.

Chong, J.L., Tsai, S.Y., Sharma, N., Opavsky, R., Price, R., Wu, L., Fernandez, S.A., and Leone, G. (2009). E2f3a and E2f3b contribute to the control of cell proliferation and mouse development. Mol Cell Biol *29*, 414-424.

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

Cloud, J.E., Rogers, C., Reza, T.L., Ziebold, U., Stone, J.R., Picard, M.H., Caron, A.M., Bronson, R.T., and Lees, J.A. (2002). Mutant mouse models reveal the relative roles of E2F1 and E2F3 in vivo. Mol Cell Biol *22*, 2663-2672.

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

Dailey, L., Laplantine, E., Priore, R., and Basilico, C. (2003). A network of transcriptional and signaling events is activated by FGF to induce chondrocyte growth arrest and differentiation. J Cell Biol *161*, 1053-1066.

Danielian, P.S., Friesenhahn, L.B., Faust, A.M., West, J.C., Caron, A.M., Bronson, R.T., and Lees, J.A. (2008). E2f3a and E2f3b make overlapping but different contributions to total E2f3 activity. Oncogene *27*, 6561-6570.

Dei Tos, A.P., Maestro, R., Doglioni, C., Piccinin, S., Libera, D.D., Boiocchi, M., and Fletcher, C.D. (1996). Tumor suppressor genes and related molecules in leiomyosarcoma. Am J Pathol *148*, 1037-1045.

Duman-Scheel, M., Weng, L., Xin, S., and Du, W. (2002). Hedgehog regulates cell growth and proliferation by inducing Cyclin D and Cyclin E. Nature *417*, 299-304.

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

Field, S.J., Tsai, F.Y., Kuo, F., Zubiaga, A.M., Kaelin, W.G., Jr., Livingston, D.M., Orkin, S.H., and Greenberg, M.E. (1996). E2F-1 functions in mice to promote apoptosis and suppress proliferation. Cell *85*, 549-561.

Hinoi, E., Bialek, P., Chen, Y.T., Rached, M.T., Groner, Y., Behringer, R.R., Ornitz, D.M., and Karsenty, G. (2006). Runx2 inhibits chondrocyte proliferation and hypertrophy through its expression in the perichondrium. Genes Dev *20*, 2937-2942.

Hodge, J.C., and Morton, C.C. (2007). Genetic heterogeneity among unterine leiomyomata: insights into malignant progression. Hum Mol Genet *16, R7-13.*

Humbert, P.O., Verona, R., Trimarchi, J.M., Rogers, C., Dandapani, S., and Lees, J.A. (2000). E2f3 is critical for normal cellular proliferation. Genes Dev *14*, 690-703.

Jacks, T., Remington, L., Williams, B.O., Schmitt, E.M., Halachmi, S., Bronson, R.T., and Weinberg, R.A. (1994). Tumor spectrum analysis in p53-mutant mice. Curr Biol *4*, 1 -7.

Karp, S.J., Schipani, E., St-Jacques, B., Hunzelman, J., Kronenberg, H., and McMahon, A.P. (2000). Indian hedgehog coordinates endochondral bone growth and morphogenesis via parathyroid hormone related-protein-dependent and -independent pathways. Development *127*, 543-548.

King, J.C., Moskowitz, I.P., Burgon, P.G., Ahmad, F., Stone, J.R., Seidman, J.G., and Lees, J.A. (2008). E2F3 plays an essential role in cardiac development and function. Cell Cycle *7*, 3775-3780.

Kronenberg, H.M. (2003). Developmental regulation of the growth plate. Nature *423*, 332 -336.

Landsberg, R.L., Sero, J.E., Danielian, P.S., Yuan, T.L., Lee, E.Y., and Lees, J.A. (2003). The role of E2F4 in adipogenesis is independent of its cell cycle regulatory activity. Proc Natl Acad Sci U S A *100*, 2456-2461.

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

Lee, E.Y., Cam, H., Ziebold, U., Rayman, J.B., Lees, J.A., and Dynlacht, B.D. (2002). E2F4 loss suppresses tumorigenesis in Rb mutant mice. Cancer Cell *2*, 463-472.

Lee, M.H., Williams, B.O., Mulligan, G., Mukai, S., Bronson, R.T., Dyson, N., Harlow, E., and Jacks, T. (1996). Targeted disruption of p107: functional overlap between p107 and Rb. Genes Dev *10*, 1621-1632.

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

Logan, M., Martin, J.F., Nagy, A., Lobe, C., Olson, E.N., and Tabin, C.J. (2002). Expression of Cre Recombinase in the developing mouse limb bud driven by a Prxl enhancer. Genesis *33*, 77-80.

MacLean, H.E., Guo, J., Knight, M.C., Zhang, P., Cobrinik, D., and Kronenberg, H.M. (2004). The cyclin-dependent kinase inhibitor p57(Kip2) mediates proliferative actions of PTHrP in chondrocytes. J Clin Invest *113*, 1334-1343.

McClellan, K.A., Ruzhynsky, V.A., Douda, D.N., Vanderluit, J.L., Ferguson, K.L., Chen, D., Bremner, R., Park, D.S., Leone, G., and Slack, R.S. (2007). Unique requirement for Rb/E2F3 in neuronal migration: evidence for cell cycle-independent functions. Mol Cell Biol *27*, 4825-4843.

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

Morrison, S.J., Uchida, N., and Weissman, I.L. (1995). The biology of hematopoietic stem cells. Annu Rev Cell Dev Biol *11*, 35-71.

Mukherjee, S., Raje, N., Schoonmaker, J.A., Liu, J.C., Hideshima, T., Wein, M.N., Jones, D.C., Vallet, S., Bouxsein, M.L., Pozzi, S., *et al.* (2008). Pharmacologic targeting of a stem/progenitor population in vivo is associated with enhanced bone regeneration in mice. J Clin Invest *118*, 491-504.

Nadri, S., Soleimani, M., Hosseni, R.H., Massumi, M., Atashi, A., and Izadpanah, R. (2007). An efficient method for isolation of murine bone marrow mesenchymal stem cells. Int J Dev Biol *51*, 723-729.

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

Ohbayashi, N., Shibayama, M., Kurotaki, Y., Imanishi, M., Fujimori, T., Itoh, N., and Takada, S. (2002). FGF18 is required for normal cell proliferation and differentiation during osteogenesis and chondrogenesis. Genes Dev *16*, 870-879.

Parisi, T., Yuan, T.L., Faust, A.M., Caron, A.M., Bronson, R., and Lees, J.A. (2007). Selective requirements for E2f3 in the development and tumorigenicity of Rb-deficient chimeric tissues. Mol Cell Biol *27*, 2283-2293.

Politi, K., Szabolcs, M., Fisher, P., Kljuic, A., Ludwig, T., and Efstratiadis, A. (2004). A mouse model of uterine leiomyosarcoma. Am J Pathol *164*, 325-336.

Rayman, J.B., Takahashi, Y., Indjeian, V.B., Dannenberg, J.H., Catchpole, S., Watson, R.J., te Riele, H., and Dynlacht, B.D. (2002). E2F mediates cell cycle-dependent transcriptional repression in vivo by recruitment of an HDAC1/mSin3B corepressor complex. Genes Dev *16*, 933-947.

Regan, C.P., Manabe, I., and Owens, G.K. (2000). Development of a smooth muscle-targeted cre recombinase mouse reveals novel insights regarding smooth muscle myosin heavy chain promoter regulation. Circ Res *87*, 363-369.

Ropke, M., Boltze, C., Meyer, B., Neumann, H.W., Roessner, A., and Schneider-Stock, R. (2006). Rb-loss is associated with high malignancy in chondrosarcoma. Oncol Rep *15*, 89-95.

Rossi, F., MacLean, H.E., Yuan, W., Francis, R.O., Semenova, E., Lin, C.S., Kronenberg, H.M., and Cobrinik, D. (2002). p107 and p130 Coordinately regulate proliferation, Cbfa1 expression, and hypertrophic differentiation during endochondral bone development. Dev Biol *247*, 271-285.

Saavedra, H.I., Wu, L., de Bruin, A., Timmers, C., Rosol, T.J., Weinstein, M., Robinson, M.L., and Leone, G. (2002). Specificity of E2F1, E2F2, and E2F3 in mediating phenotypes induced by loss of Rb. Cell Growth Differ *13*, 215-225.

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

Simmons, P.J., and Torok-Storb, B. (1991). Identification of stromal cell precursors in human bone marrow by a novel monoclonal antibody, STRO-1. Blood *78*, 55-62.

Thomas, D.M., Carty, S.A., Piscopo, D.M., Lee, J.S., Wang, W.F., Forrester, W.C., and Hinds, P.W. (2001). The retinoblastoma protein acts as a transcriptional coactivator required for osteogenic differentiation. Mol Cell *8*, 303-316.

Trimarchi, J.M., and Lees, J.A. (2002). Sibling rivalry in the E2F family. Nat Rev Mol Cell Biol *3*, 11-20.

Tsai, K.Y., Hu, Y., Macleod, K.F., Crowley, D., Yamasaki, L., and 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*, 293-304.

Tsai, S.Y., Opavsky, R., Sharma, N., Wu, L., Naidu, S., Nolan, E., Feria-Arias, E., Timmers, C., Opavska, J., de Bruin, A., *et al.* (2008). Mouse development with a single E2F activator. Nature *454*, 1137-1141.

Walkley, C.R., Qudsi, R., Sankaran, V.G., Perry, J.A., Gostissa, M., Roth, S.I., Rodda, S.J., Snay, E., Dunning, P., Fahey, F.H., *et al.* (2008). Conditional mouse osteosarcoma, dependent on p53 loss and potentiated by loss of Rb, mimics the human disease. Genes Dev *22*, 1662-1676.

Williams, B.O., Remington, L., Albert, D.M., Mukai, S., Bronson, R.T., and Jacks, T. (1994). Cooperative tumorigenic effects of germline mutations in Rb and p53. Nat Genet *7*, 480-484.

Xu, X., Bieda, M., Jin, V.X., Rabinovich, A., Oberley, M.J., Green, R., and Farnham, P.J. (2007). A comprehensive ChIP-chip analysis of E2F1, E2F4, and E2F6 in normal and tumor cells reveals interchangeable roles of E2F family members. Genome Res *17*, 1550 -1561.

Yamaguchi, T., Toguchida, J., Wadayama, B., Kanoe, H., Nakayama, T., Ishizaki, K., Ikenaga, M., Kotoura, Y., and Sasaki, M.S. (1996). Loss of heterozygosity and tumor suppressor gene mutations in chondrosarcomas. Anticancer Res *16*, 2009-2015.

Yamasaki, L., Bronson, R., Williams, B.O., Dyson, N.J., Harlow, E., and Jacks, T. (1998). Loss of E2F-1 reduces tumorigenesis and extends the lifespan of Rb1(+/-)mice. Nat Genet *18*, 360-364.

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

Yang, H.S., and Hinds, P.W. (2007). pRb-mediated control of epithelial cell proliferation and Indian hedgehog expression in mouse intestinal development. BMC Dev Biol *7*, 6.

Yeh, N., Miller, J.P., Gaur, T., Capellini, T.D., Nikolich-Zugich, J., de la Hoz, C., Selleri, L., Bromage, T.G., van Wijnen, A.J., Stein, G.S., *et al.* (2007). Cooperation between p27 and p107 during endochondral ossification suggests a genetic pathway controlled by p27 and p130. Mol Cell Biol *27*, 5161-5171.

Ziebold, U., Lee, E.Y., Bronson, R.T., and Lees, J.A. (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*, 6542-6552.

Ziebold, U., Reza, T., Caron, A., and Lees, J.A. (2001). E2F3 contributes both to the inappropriate proliferation and to the apoptosis arising in Rb mutant embryos. Genes Dev *15*, 386-391.

Appendix A

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

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Author's contributions: Figure 4A, C-E, G, and Table S3

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 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.

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, $P = 0.17$.

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^{+} 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.

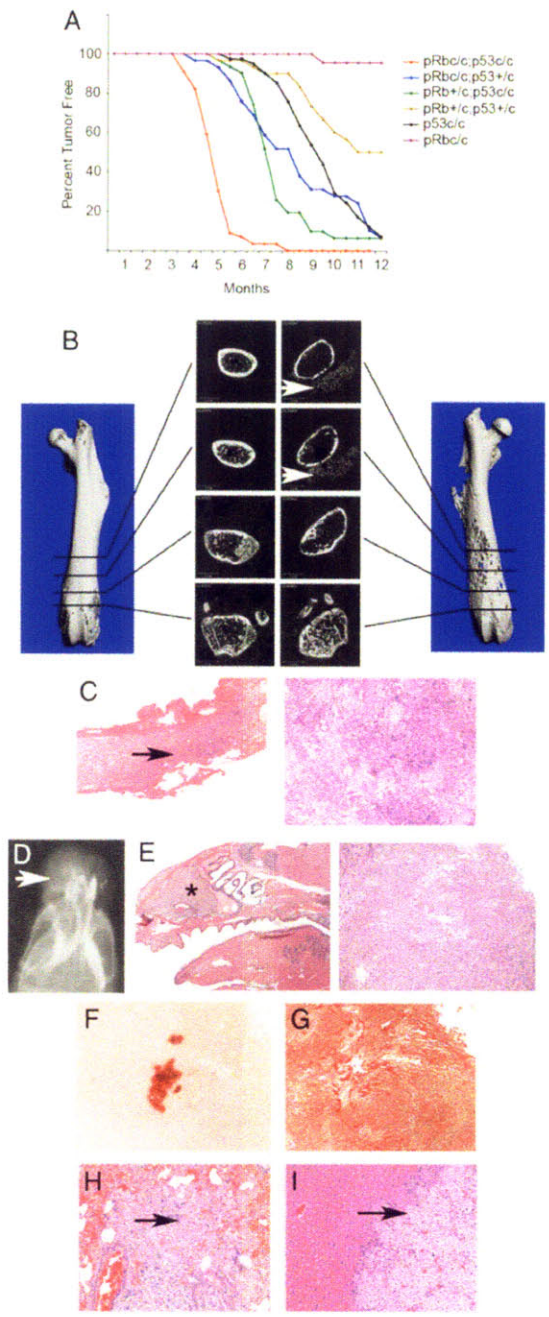

Figure 1. A mouse model of metastatic osteosarcoma.
(A) Kaplan–Meier plot of the indicated genotypes carrying *Osx1-GFP::Cre* up to 12 months of age. (B–I) Analyses of osteosarcomas and associated metastases arising in DKO mice. (B) 3D reconstructed images from microComputerised Tomography are shown 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.

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 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

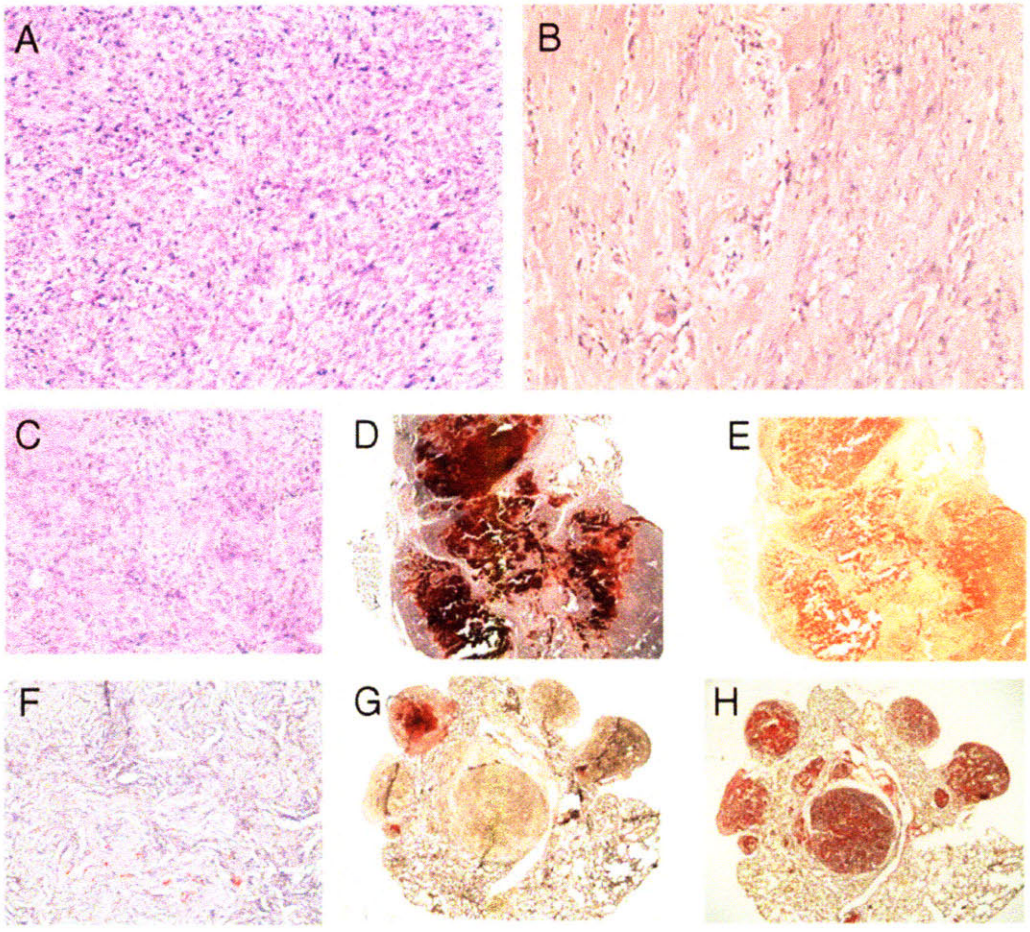

Figure 2. OS cell lines can form bone tumors in immuno-compromised mice.
(A and B) H&E stained section of the primary osteosarcomas 985 and 2380, respectively. Tumors derived from s.c. (C–E) or i.v. (F–H) injection of DKO-OS-985. (C and F) H&E staining. Adjacent sections were stained with either Alizarin 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.)

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 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).

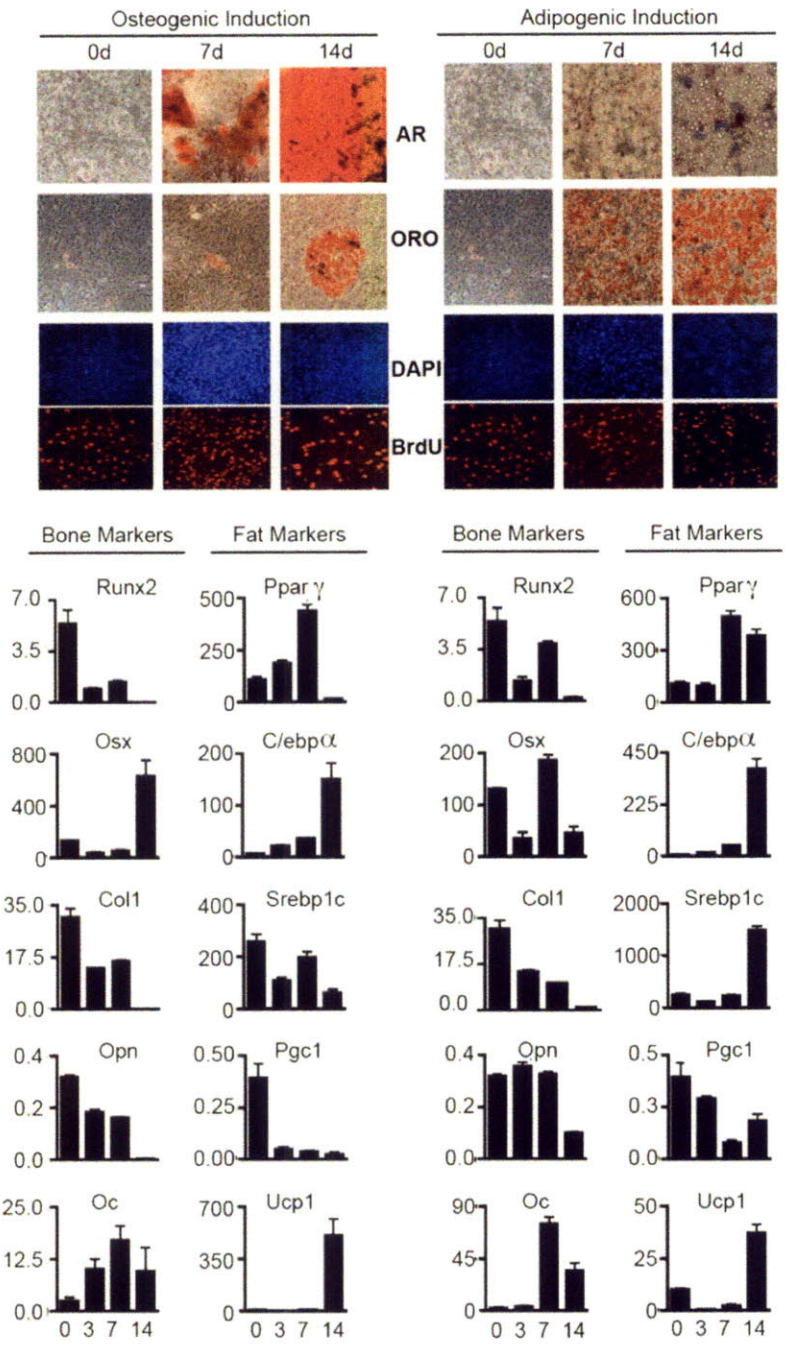

Figure 3. Osteosarcoma cells lines are multipotent *in vitro*.
DKO-OS-985 cells were induced to differentiate into the bone (Left) and fat (Right) lineages and assayed at the indicated time points (days). Mineral deposits were stained with Alizarin Red (AR) as a marker for osteogenic differentiation. 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.

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 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 $Sca\text{-}1^{high}/CD45^-$ 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 $Sca\text{-}1^{high}$ 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 $Sca\text{-}1^{high}$ and $Sca\text{-}1^-$ cells (data not shown), indicating that division of the $Sca\text{-}1^+$ tumor-initiating cells can yield $Sca\text{-}1^-$ progeny.

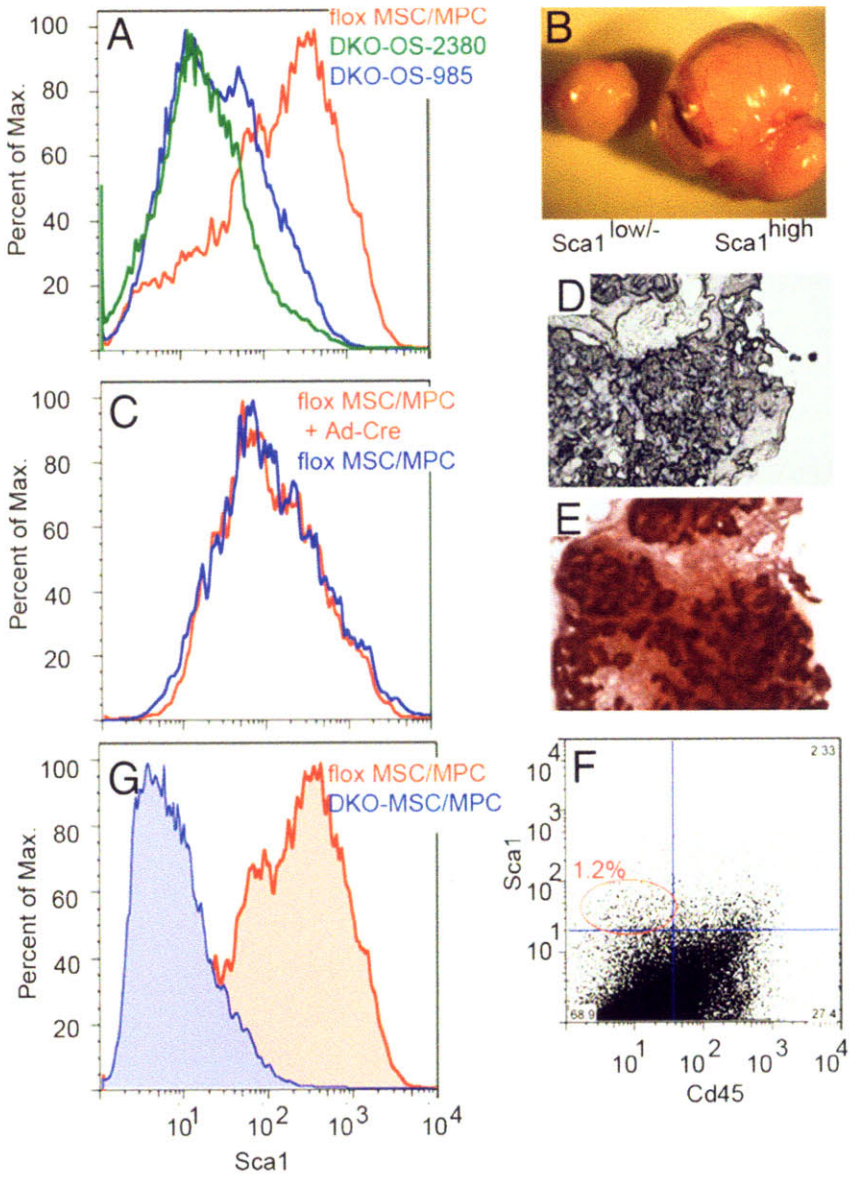

Figure 4. Sca-1 expression and *Rb*- and *p53*-loss are both required for efficient tumorigenesis *in vivo*.

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 Sca1$^{low/-}$ or Sca1^{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).

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 CO2 inhalation were a total tumor burden of 2 cm3, 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

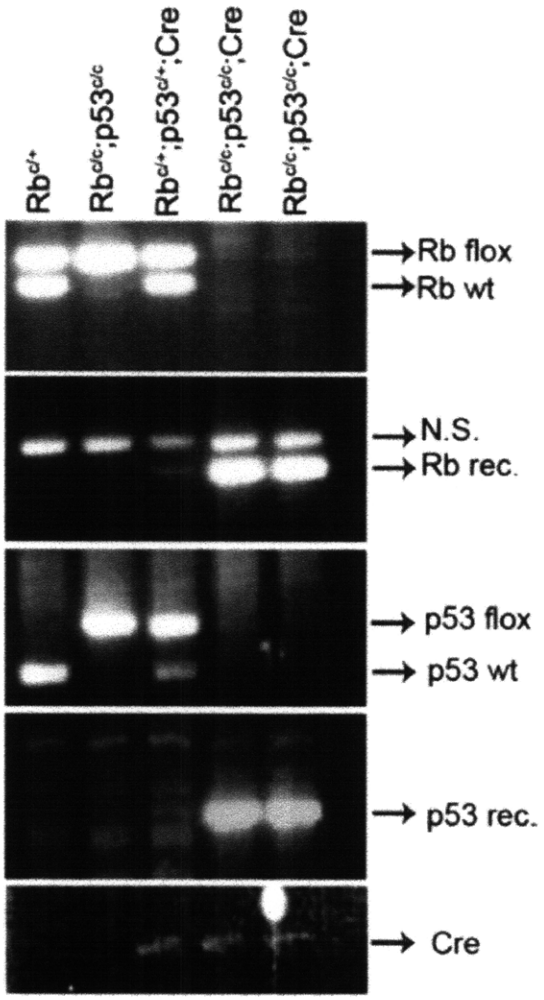

Fig. S1. Analysis of *Rb* and *p53* recombination in DKO osteosarcomas.
PCR genotyping was used to test for the efficiency of Cre-mediated recombination. As controls, DNA from $Rb^{c/c}$, $Rb^{c/c};p53^{c/c}$, and $Rb^{c/c};p53^{c/c}$ *Osx*-cre mice were used. DNA for the left three samples was extracted 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.

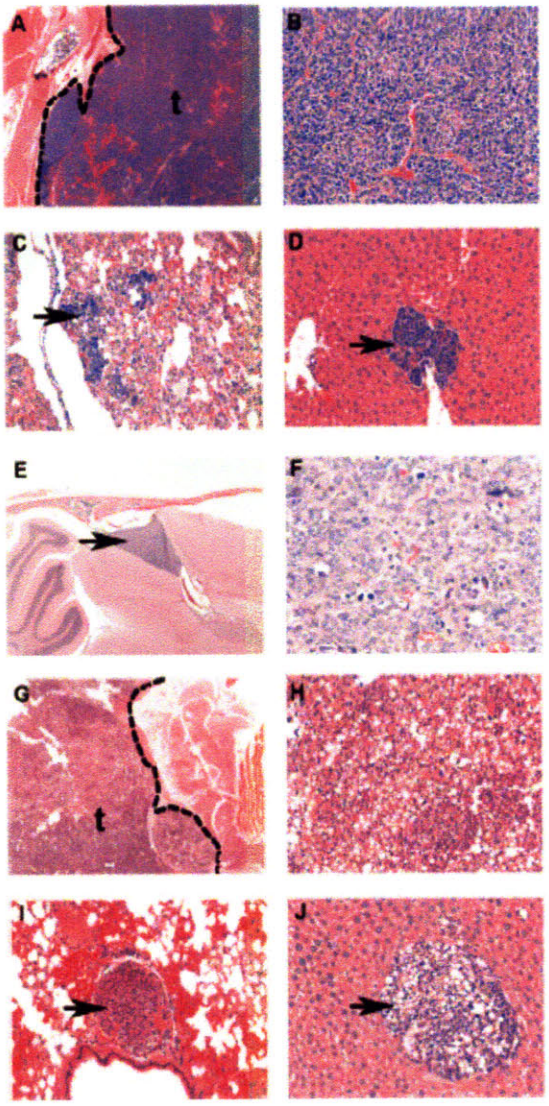

Fig. S2. Histological analyses of neuroendocrine tumors and hibernomas arising in DKO mice.

(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.)

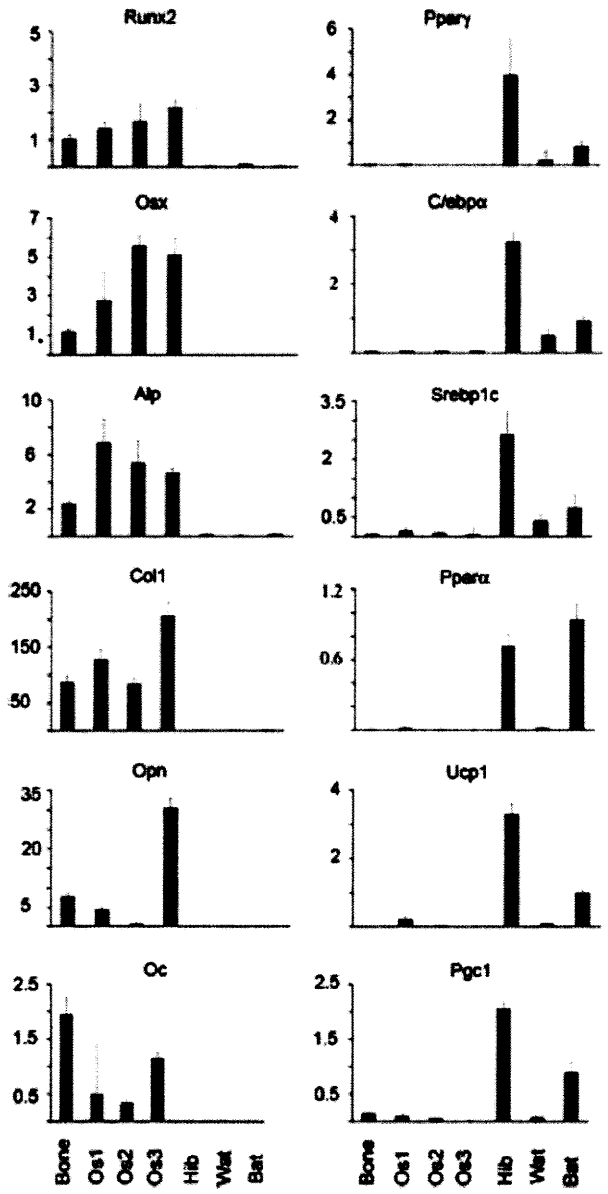

Fig. S3. Analyses of gene expression in osteosarcomas derived from *Rb*$^{c/c}$;*p53*$^{c/c}$;*Cre*$^{+}$ mice.

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
$Rb^{c/c}$	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
$p53^{c/c}$	5	2				3								
$Rb^{+/c};p53^{+/c}$	3	2	1	1										
$Rb^{c/c};p53^{+/+}$	1	4			2									
$Rb^{+/c};p53^{c/c}$	8	3			1		1							
$Rb^{c/c};p53^{c/c}$ (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

mRNA	Primer sequences	
	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, et al. (1999) Heterozygous germ line hCHK2 mutations in Li-Fraumeni syndrome. Science 286, 2528-31.

Berman SB, et al. The retinoblastoma protein tumor suppressor is important for appropriate osteoblast differentiation and bone development. Submitted

Clark JC, Dass CR, Choong PF (2008) A review of clinical and molecular prognostic factors in osteosarcoma. J Cancer Res Clin Oncol 134, 281-97.

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, 2952-62.

Feugeas O, et al. (1996) Loss of heterozygosity of the RB gene is a poor prognostic factor in patients with osteosarcoma. J Clin Oncol 14, 467-72.

Gokgoz N, et al. (2001) Comparison of p53 mutations in patients with localized osteosarcoma and metastatic osteosarcoma. Cancer 92, 2181-9.

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, 2186-95.

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

Jonkers J, et al. (2001) Synergistic tumor suppressor activity of BRCA2 and p53 in a conditional mouse model for breast cancer. Nat Genet 29, 418-25.

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

Krivtsov AV, et al. (2006) Transformation from committed progenitor to leukaemia stem cell initiated by MLL-AF9. Nature 442, 818-22.

Lengner CJ, et al. (2006) Osteoblast differentiation and skeletal development are regulated by Mdm2-p53 signaling. J Cell Biol 172, 909-21.

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

Mukherjee S, et al. (2008) Pharmacologic targeting of a stem/progenitor population in vivo is associated with enhanced bone regeneration in mice. J Clin Invest 118, 491-504.

Pierroz DD, Bouxsein ML, Rizzoli R, Ferrari SL (2006) Combined treatment with a beta -blocker and intermittent PTH improves bone mass and microarchitecture in ovariectomized mice. Bone 39, 260-7.

Rodda SJ, McMahon AP (2006) Distinct roles for Hedgehog and canonical Wnt signaling in specification, differentiation and maintenance of osteoblast progenitors. Development 133, 3231-44.

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, 223-8.

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, 624-32.

Thomas DM, et al. (2001) The retinoblastoma protein acts as a transcriptional coactivator required for osteogenic differentiation. Mol Cell 8, 303-16.

Tirode F, et al. (2007) Mesenchymal stem cell features of Ewing tumors. Cancer Cell 11, 421-9.

Toguchida J, et al. (1988) Chromosomal reorganization for the expression of recessive mutation of retinoblastoma susceptibility gene in the development of osteosarcoma. Cancer Res 48, 3939-43.

Tsuchiya T, et al. (2000) Analysis of the p16INK4, p14ARF, p15, TP53, and MDM2 genes and their prognostic implications in osteosarcoma and Ewing sarcoma. Cancer Genet Cytogenet 120, 91-8.

Vooijs M, Berns A (1999) Developmental defects and tumor predisposition in Rb mutant mice. Oncogene 18, 5293-303.

Wang X, et al. (2006) p53 functions as a negative regulator of osteoblastogenesis, osteoblast-dependent osteoclastogenesis, and bone remodeling. J Cell Biol 172, 115 -25.

Wunder JS, et al. (2005) TP53 mutations and outcome in osteosarcoma: a prospective, multicenter study. J Clin Oncol 23, 1483-90.

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Publications

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osteosarcoma induced by inactivation of Rb and p53 in the osteoblast lineage. *Proc. Natl. Acad. Sci., 105*(33): 11851-11856.

Coïc, E., Feldman, T., **Landman A.S.**, Haber, J.E. (2008) Mechanisms of Rad52-independent spontaneous and UV-induced mitotic recombination in Saccharomyces cerevisiae. *Genetics*, 179(1): 199-211.

Wijnen, H., **Landman, A.**, Futcher, B. (2002) The G(1) cyclin Cln3 promotes cell cycle entry via the transcription factor Swi6. *Mol. Cell. Biol., 22*(12): 4402-18.

Meetings and Abstracts

2008 Cold Spring Harbor Meeting on Mechanisms and Models of Cancer
Poster: pRb and p107 Loss in Mesenchymal Progenitors Leads to Cartilage Neoplasia

2008 Keystone Symposium on Cell Death and Senescence
Poster: *Rb* and *p53* in Mesenchymal Lineage Development and Tumorigenesis

2007 Colrain Meeting
Talk: A New Mouse Model of Osteosarcoma

Awards and Honors

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