pRb's Role in Cell Fate, Lineage Commitment, and Tumorigenesis

by

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ABSTRACT

The product of the retinoblastoma gene, pRB, was the first known and cloned tumor suppressor gene and it is functionally inactivated in most human cancers. pRB is thought to suppresses tumorigenesis by restraining cellular proliferation. pRB binds to the E2F family of transcription factors and prevents them from activating genes require for cell cycle progression. In addition, pRB modulates cellular differentiation by binding to master differentiation inducers to either enhance or repress their transcriptional activity. While most of pRB's tumor suppressive functions have been studied in the context of cell cycle control, little is known as to whether pRB's role in differentiation also influences tumorigenesis. We have addressed this issue in the context of bone sarcomas, a tumor type in which pRB is frequently inactivated. To model osteosarcoma in the mouse we used a targeted conditional approach in which *Rb* and/or *p53* were deleted in pre-osteoblasts or mesenchymal stem cells. In osteoblasts we found that *Rb* loss synergized strongly with *p53*-inactivation: it greatly accelerated tumor development and it expanded the tumor spectrum from osteosarcoma in the *p53* single mutants to multiple soft tissue sarcomas in the *Rb;p53* DKO. In mesenchymal stem cells we found that *Rb* acted in a dose dependent manner to modulate the spectrum of tumours arising from p53-deficient, mesenchymal stem cells: osteosarcomas predominated in the presence of *Rb*, while *Rb* loss strongly favoured brown fat tumors. Thus, to directly address the influence of *Rb* status in mesenchymal tumorigenesis we used inducible systems to control pRB's expression. Our data showed that toggling between *Rb* loss or *Rb* re-activation was sufficient to switch the fate commitment of osteosarcoma tumor cells *in vitro* through direct regulation of transcription factors that control mesenchymal differentiation. Consistently, we found that reactivation of *Rb* in tumors generated from *Rb;p53* DKO cells was sufficient to halt tumor progression by promoting differentiation of the tumor cells *in vivo*. Taken together, our data have uncovered three novel roles for pRb. First, *Rb* loss promotes tumorigenesis by deregulating the differentiation potential of committed pre-osteoblasts. Second, pRb regulates fate choice and lineage commitment between the bone and the fat lineages *in vivo*. Third, pRb suppresses tumorigenesis by enforcing cell cycle exit and terminal differentiation.

Thesis Supervisor: Jacqueline A. Lees
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Chapter 1

Introduction

Part I

The Retinoblastoma Tumor Suppressor Gene: Discovery and Cellular Functions

A. Discovery of the Retinoblastoma Gene

The *RB-1* gene (*RB-1* in humans and *Rb* in mice) was the first known and cloned tumor suppressor gene (Weinberg, 1992). The name of this gene derived from retinoblastoma, a malignant tumor of the eye that, if sporadic, manifests in children as a focal lesion in one eye (unilateral). However, retinoblastoma has been observed in pedigree of families, indicating that there is a common genetic predisposition to this disease. In this context, retinoblastoma develops with early onset as multiple focal tumors in one or both eyes (bilateral). This familial predisposition strongly indicated that there was a common inherited factor associated with this disease. Consistent with Knudson two hit hypothesis, this factor was predicted to be a tumor suppressor gene. Susceptibility to retinoblastoma was mapped to chromosome 13q14 through cytogenetic studies of retinoblastoma patients (Benedict *et al.*, 1983; Dryja *et al.*, 1986; Sparkes *et al.*, 1980). A cDNA corresponding to the retinoblastoma gene *(RB-1)* was cloned by chromosome walking techniques, and the homologous mRNA was found to be absent in retinoblastoma-derived cell lines (Friend *et al.* 1986; Fung *et al.* 1987; Lee *et al.* 1987). Molecular analysis found that the protein encoded by *RB-1* is a nuclear phosphoprotein of approximately 110 kilodaltons (Lee *et al.* 1987). Since its discovery, genetic mutations of *RB-1* have been observed in many

other tumor types, particularly osteosarcoma and small cell lung carcinoma. Overall, the *RB-1* locus is mutated in approximately one-third of all human tumors (Weinberg, 1992). Moreover, as I will discuss below, many other human tumors contain mutations in the upstream regulators of the retinoblastoma protein, pRB. Thus, inactivation of the pRB pathway is considered a hallmark of cancer.

B. Structure and Regulation of the Retinoblastoma Tumor Suppressor Protein

pRB's protein domains and function were defined through studies using small DNA tumor viruses that caused cellular transformation. pRB is a common target of viral oncoproteins, including adenovirus (Ad) E1A, human papillomavirus (HPV) E7, and simian virus 40 (SV40) large T antigen (LTa). These small DNA viruses usurp the DNA replication machinery of host cells to achieve viral genome replication by prematurely stimulating cell cycle entry (Liu and Marmorstein 2006). Binding of pRB to these viral oncoproteins was shown to be necessary for transformation of infected cells, suggesting a role for pRB in cell cycle control (DeCaprio *et al.*, 1988; Dyson *et al.*, 1989b; Whyte *et al.*, 1988). In accordance, overexpression of pRB induced cell cycle arrest in cultured cells, while pRB loss results in accelerated cell cycle progression (Herrera *et al.*, 1996; Huang *et al.*, 1988).

Mutational analysis revealed that oncoproteins from DNA tumor viruses contain a conserved LXCXE amino acid sequence, which they use to bind pRB

and inhibit its function (Dyson *et al.*, 1990; Dyson *et al.*, 1989; Munger *et al.*, 1989; Stabel *et al.*, 1985; Vousden and Jat, 1989). Notably, cellular proteins such as histone deacetylases 1 and 2 (HDAC1 and -2) also contain an LXCXE-like sequence, which they use to interact with pRB (discussed below). Mutational analysis in *RB-1* led to the identification of two regions that are critical for the binding of viral oncoprotein (Hu *et al.*, 1990; Huang *et al.*, 1990; Kaelin *et al.*, 1990). These regions were located in the C-terminal portion of pRB, and are called the A and B pockets individually, or the small pocket domain collectively (Figure 1). Importantly these regions overlap with sites that contain tumor-associated mutations of *RB-1* consistent with the idea that these regions contribute to its tumor suppressive functions (Hu *et al.*, 1990). The large pocket domain, which also includes the C-terminal region, is the critical mediator of pRB binding to a large number of cellular proteins, including pRB's main targets, the E2Fs family of transcription factors (discussed in part II).

More-detailed biochemical analysis of pRB revealed that the functions of the *RB-1* protein are governed by multiple posttranslational modifications (Figure 1). pRB has 928 amino acids and does not contain any commonly recognized DNA binding domain. Most of the pRB interacting partners bind to the pocket region, however, some proteins have been shown to bind to other parts of pRB. pRB is phosphorylated in more than ten serine or threonine residues, but is not phosphorylated on tyrosine residues. The binding of many pRB's interacting protein is disrupted by phosphorylation of pRB. Lysine residues 873 and 874 of pRB are modified by acetylation, however the specific molecular mechanism

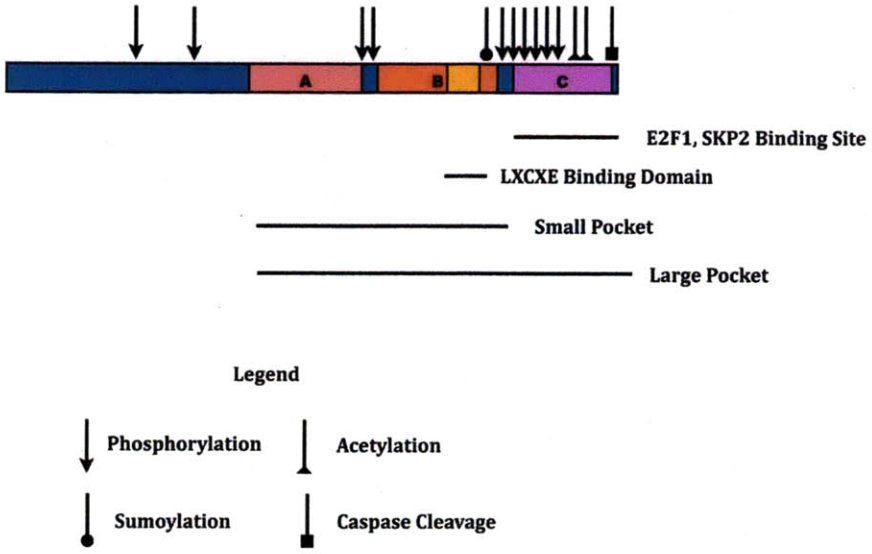

Figure 1: Structure of the retinoblastoma tumor suppressor protein.

mediated by acetylated-pRB needs to be clarified (Chang *et al.*, 2001). pRB also been shown to be sumoylated at lysine 720, which is located in the small pocket near the LXCXE motif, but the function of this modification is unknown (Ledl *et al.*, 2005). During apoptosis, pRB can be cleaved by caspase 8 at the C-terminus and it has been shown that mice lacking pRB recognition sequences are resistant to TNF-alpha-induced apoptosis (Borges *et al.*, 2005). Overall, pRB's posttranslational modifications seem to govern the activity and function of the protein.

C. The Retinoblastoma Tumor Suppressor Gene and Cell Cycle Regulation

There is overwhelming evidence that phosphorylation events govern the activity of pRB during the cell cycle. In G0 and G1, pRB is found in a hypophosphorylated state. In this hypophosphorylated state pRB binds and inhibits, the E2F family of transcription factors to repress their transcriptional activity (Figure 2). Collectively, the E2Fs are a group of transcription factors responsible for the induction of genes required for the G1 to S transition (Trimarchi and Lees, 2002). Extracellular mitogenic signals cause the sequential formation and activation of cyclin-dependent kinases (CDK) where pRB is targeted for phosphorylation initially by cyclin D-CDK4/6 in G1, followed by cyclin E-CDK2 at the G1/S transition (reviewed in Mittnacht 1998). Hyoperphosphorylation of pRB impairs pRB-E2F's interaction, thus enabling the transcription of genes required for cell cycle entry (Buchkovich *et al.*, 1989; Cooper and Shayman, 2001). pRB remains in the hyperphosphorylated state

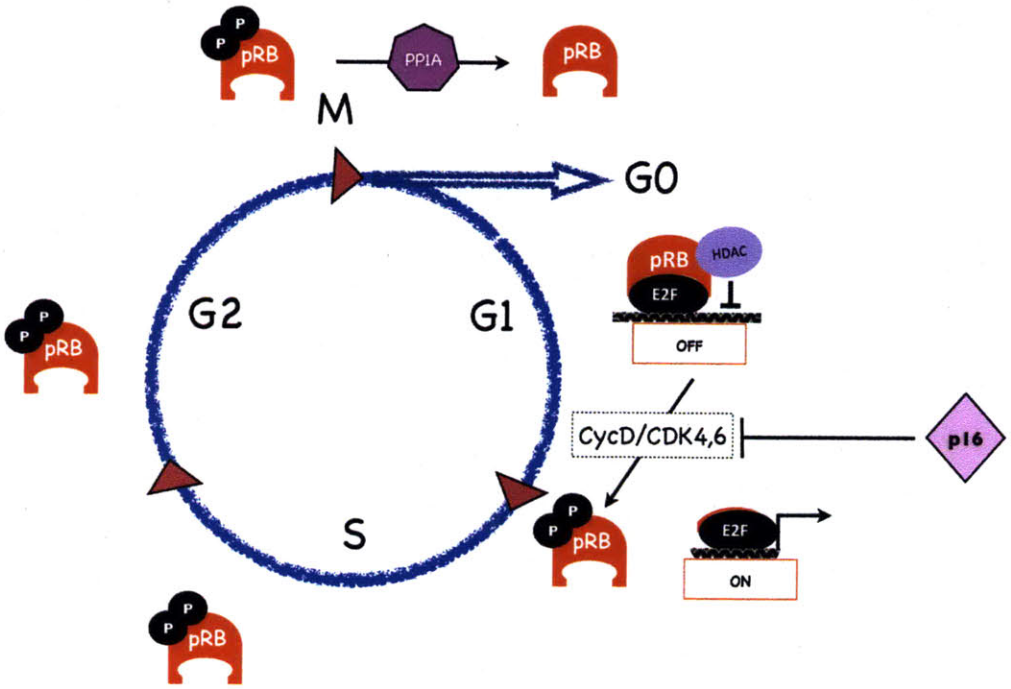

Figure 2: Regulation of cell cycle control by the retinoblastoma protein.

until mitosis (Figure 2), at which point it is dephosphorylated by protein phosphatases 1 and/or 2A (Ludlow *et al.*, 1993; Avni *et al.*, 2003; Cicchillitti *et al.*, 2003). This controlled phosphorylation of pRB by the cyclin-Cdk complexes during different phases of the cell cycle is considered the canonical pathway of cell cycle regulation by pRB (Trimarchi and Lees, 2002). In addition to the Cyclin-CDK complexes, pRB can be phosphorylated by ERK and p38MAPK (Garnovskaya *et al.*, 2004; Nath *et al.*, 2003; Wang *et al.*, 1999), however, the specific mechanisms for these phosphorylation events may represent more specialized functions of pRB, which remain to be elucidated.

pRB-mediated repression of cell cycle genes not only require physical interaction with the E2F family of transcription factors, but also the recruitment/interaction of chromatin remodeling proteins. The pRB-E2F complexes recruit histone deacetylases (HDACs), resulting in an epigenetic remodeling signature that represses transcription of E2F target genes (Brehm *et al.*, 1998; Luo *et al.*,1998; Magnaghi-Jaulin *et al.*, 1998). These complexes typically associate during G0 and G1 phases of the cell cycle and dissociate upon pRB phosphorylation before S phase entry (Rayman *et al.*, 2002; Takahashi *et al.*, 2000; Taubert *et al.*, 2004; Figure 2). These observations underscore the mechanisms by which pRB regulates cell cycle and repress transcription to maintain the G0 or G1 state.

pRB also mediates transcriptional repression by binding to another class of DNA biding proteins, better known as RB binding proteins (RBP). The physical association of pRB with these proteins suggests a role for pRB in mediating

repression in an E2F-independent manner. For example, RBP1 binds to pRB and recruits HDACs to mediate transcriptional repression, resulting in growth arrest (Fattaey *et al.*,1993; Lai *et al.*, 1999a,b; Lai *et al.*, 2001). In a similar manner, RBP2, binds to pRB and mediates repression via histone demethylation (Christensen *et al.*, 2007; Fattaey *et al.*, 1993; Kim *et al.*, 1994; Klose *et al.*, 2007). Through this mechanism, pRB also binds the methyltransferase SUV39H1 at the cyclin E promoter to repress its transcription (Nielsen *et al.*, 2001). Jumonji, which has structural homology to RBP2, interacts with pRB and potentiates pRb-E2F regulation of the cell cycle through a yet unknown mechanism (Jung *et al.*, 2005; Takeuchi *et al.*, 1995). JMJD2A, another protein similar to Jumonji and RBP2, interacts with both pRB and HDACs to mediate repression of cell cycle genes (Gray *et al.*, 2005). Overall, more than 100 proteins have been reported to interact with pRB, many of which are associated with control of transcriptional regulation (Frolov and Dyson, 2004; Morris and Dyson, 2001). pRB also regulates transcription via nucleosome sliding and assembly via its association with components of the SWI/SNF complex (Dunaief *et al.*, 1994; Strober *et al.*, 1996). Through these interactions, pRB mediates repression in an E2F-independent manner. However, the mechanisms by which pRB regulates this process remains to be elucidated.

Finally pRB has also been implicated in the stable repression of E2F target genes during irreversible cell cycle arrest, through a process called cellular senescence. In this scenario, pRB is required for the formation of heterchomatic domains in senescent cells, and interacts with repressed E2F target genes, both

of which are enriched in H3K9 methylation (Narita *et al.* 2003). Additionally, pRB interacts with the DNA methyltransferase DNMT1, which enhances pRB transcriptional repression (Robertson *et al.* 2000).

D. The Retinoblastoma Tumor Suppressor Gene in Tumorigenesis

As mentioned, *RB-1* was identified through a pedigree of families whose children developed retinoblastoma. These patients are also highly predisposed to the development of other malignancies including osteosarcomas. In accordance with a role for pRB in suppressing osteosarcoma development, *RB-1* loss is also observed in about 70% of sporadic osteosarcomas. In addition, papillomavirus-induced cervical carcinoma and squamous cell carcinoma of the head and neck is in part initiated by oncoproteins-mediated inactivation of pRB (Doorbar, 2006; Perez-Ordonez *et al.*, 2006). Moreover, *RB-1* is inactivated in 90% of small cell lung carcinoma and this has been validated using mouse models (Meuwissen *et al.*, 2003). Also, up to 20% of patients with prostate cancer exhibit loss of heterozygosity (LOH) at the *RB-1* locus. Interestingly, in cancers retaining intact pRB, upstream regulators of the pRB pathway are often disrupted. For example, mutations or epigenetic silencing of the cyclin dependent kinase inhibitor CDKN2A/p16 is commonly found in cancers retaining wildtype pRB. In addition, hyper-activation of Cyclin D/CDK4 complexes has also been reported in *RB-1* wildtype tumors (Figure 2). Thus, we can conclude that inactivation of the pRB pathway is an obligatory event for the progression of the majority of human

tumors. However, it is not clear how and in what cell types cancer initiation occurs due to loss of *RB-1* function.

Genetically engineered mouse models carrying mutations in *Rb* have been extensible used to dissect the molecular mechanisms by which pRb suppresses tumorigenesis. Unlike humans, heterozygous mutant mice for *Rb* do not develop retinoblastoma, osteosarcomas, or small cell lung carcinoma. Even conditional inactivation of *Rb* in the retina does not result in the development of retinoblastoma (MacPherson *et al.*, 2004; Robanus-Maandag *et al.*, 1998; Williams *et al.*, 1994a). Instead, heterozygous mutant mice for *Rb* are predisposed to pituitary adenocarcinomas and C-cell-derived medullary thyroid tumors with nearly 100% and 70% penetrance, respectively. However like human cancers, loss of heterozygozity for the remaining wildtype *Rb* allele is observed in murine tumors. This indicates that even in the mouse complete inactivation of the *Rb* gene is required for tumor development. Similar results were observed in *Rb* chimeras. (Hu *et al.*, 1994; Jacks *et al.*, 1992; Robanus-Maandag *et al.*, 1998; Williams *et al.*, 1994a,b).Due to the high penetrance and lethality of these tumors, in the mouse, it is difficult to study whether the loss of *Rb* would lead to the development of other tumor types commonly observed in humans.

This issue was partially overcome by the generation of a mouse with conditional floxable alleles for *Rb* ($Rb^{fl/fl}$). To analyze the effect of *Rb* loss in mice, Maddison *et al.* conditionally deleted *Rb* in the prostate epithelium. By 52 weeks of age, the mice present a multifocal phenotype closely resembling the clinical human disease. On the other hand, the use of Villin-Cre mediated recombination

of pRb in the intestinal tissue suggests that the loss of *Rb* alone is not sufficient to induce tumorigenesis in the gastrointestinal tract (Kucherlapati *et al.*, 2006). This indicates that for, but not others, tissues mutations in pRb can initiate tumorigenesis in the mouse. However, tumor penetrance and progression is influenced by other factors and genetic events that may cooperate with the loss of pRb. For example, several studies have analyzed the effects of *Rb* loss in combination with deletions of the other pocket proteins (discussed below). Others have analyzed the tumorigenic effects of the combined loss of *Rb* and *p53*. For example, conditional deletion of *Rb* and *p53* in the ovaries of mice (Flesken-Nikitin *et al.*, 2003) mimics the human malignancy. Similarly, conditional ablation of *Rb* and *p53* in the lungs of mice promote lung tumorigenesis with characteristics that are similar to human small cell lung cancer (Meuwissen *et al.*, 2003). Thus it seems clear that the use of *Rb* conditional floxable alleles alone or in combination with other tissue specific mutations can provide insight into the development and progression of the related human diseases.

E. The Retinoblastoma Tumor Suppressor Gene in Differentiation and Development

In addition to cell cycle control, pRB is also required to promote and maintain terminal differentiation. pRB modulates differentiation, not only by promoting cell cycle arrest upon terminal differentiation, but also by binding to tissue specific transcription factors to either enhance or repress their transcriptional activity (Figure 3). Most of the evidence that support a direct role

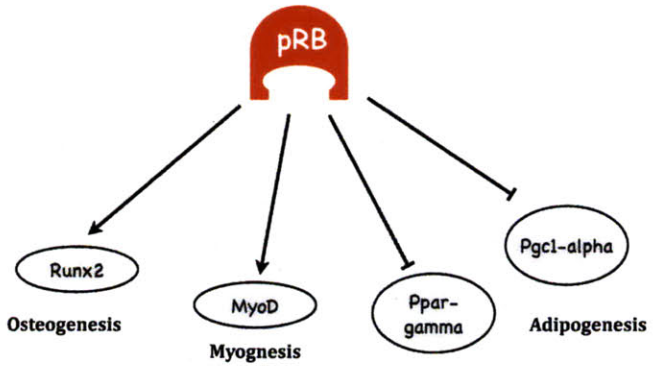

Figure 3: pRb regulates master differentiation inducers of different lineages.

for pRB during differentiation comes from the analysis of mice carrying knockout alleles of *Rb*. *Rb*$^{-/-}$ mutant mice die during midgestation and displayed severe erythrocyte, lens, and neuronal defects (Clarke *et al.*, 1992; Jacks *et al.*, 1992; Lee *et al.*, 1992). These defects arise presumably because of improper completion of the differentiation program. For example, *Rb*$^{-/-}$ erythrocytes develop, but fail to enucleate, neuronal and lens cells are present, but have reduced expression of late differentiation markers, and exhibit continued proliferation and apoptosis (Lee *et al.*, 1994; Morgenbesser *et al.*, 1994). It was revealed that the mid-gestational lethality was due to improper placental development as the presence of a wild-type placenta enables the *Rb*-deficient embryo to survive until birth (de Bruin *et al.*, 2003; Wu *et al.*, 2003). This was also confirmed by analyses of *Rb* mutant chimeras, as these mice have no obvious erythroid defects at birth. However, the neuronal and lens phenotypes manifested in these chimeras show that *Rb* does has cell autonomous functions (Maandag *et al.*, 1994; Williams *et al.*, 1994b). Notably, the observed ectopic proliferation in the aforementioned *Rb*-deficient cell types is a cell autonomous defect. In contrast, many of the apoptotic defects seen in germline Rb$^{-/-}$ embryos have been determined to be cell non-autonomous (Lipinski *et al.*, 2001; Maandag *et al.*, 1994; MacPherson *et al.*, 2003; Williams *et al.*, 1994b).

pRb also plays an important role in modulating the differentiation of several epithelial tissues. In the intestinal epithelium and epidermis, conditional loss of pRb causes increased proliferation and aberrant expression of differentiation markers (Haigis *et al.*, 2006; Yang and Hinds, 2007; Ruiz *et al.*, 2004). In the

lung epithelium, *Rb* loss promotes increased proliferation of neuroendocrine cells (Wikenheiser-Brokamp, 2004). Although it appears that the cell cycle regulatory role of pRb is responsible for the majority of pRb differentiation phenotypes more experiments are required to determine the mechanistic origin these defects. Additionally, pRb has been demonstrated to directly interact with general differentiation-associated transcription factors Mhox, ChxlO, and Pax3, as well as with the neuronal-specific nuclear protein NRP/B during differentiation (Kim *et al.*, 1998). Together, these finding suggest a role for pRB in controlling stem cell differentiation and function and presumable tissue homeostasis.

i. pRB and Stem Cell Biology

pRB also function in the maintenance of stem cell populations. In adult tissues, stem cells are often quiescent, but have strong regenerative potential and the ability to self-renew. Under appropriate signals stem cells give rise to transient amplifying progenitors, which have limited replicative capacity and undergo a maturation process that results in differentiated non-cycling cells. The clearest evidence that pRB is required for stem cell function comes from studies performed in *Arabidopsis* roots. In this biological model, loss of the *RB-1* homolog *RBR* caused reduced ectopic formation of stem cells layers without affecting other mitotically dividing cells and does not alter the differentiation program (Wildwater *et al.*, 2005). Importantly, when RBR is overexpressed the number of stem cells layers is dramatically reduced. Notably, alterations of other members of the canonical pRb-E2F pathway have similar effect on the stem cells

compartment of *Arabidopsis.* More recently, it has been shown that loss of *RB-1* functions in the *Drosophila* retina cause photoreceptor dedifferentiation towards the multipotent state, indicating that pRB may be also required to maintain lineage commitment (reviewed by Conklin and Sage, 2010). However, to date there is no proof in mammals that pRB is required to maintain adult stem cells in the quiescent state. These studies are limited, in part, because *Rb*-null embryos have defective placentas that affect the survival of the embryo (de Bruin *et al.*, 2003; Wu *et al.*, 2003). However, *Rb* deletion in the trophoblast stem cells of the placenta causes an overexpansion of trophoblastic stem cells, and an increase in the distribution of trophoblast markers, which lead to disruption of placental architecture (Wenzel *et al.*, 2007; Wu *et al.*, 2003). These studies strongly suggest that pRb plays a critical role in the maintenance of a mammalian embryonic stem cell population. In accordance with these findings, pRb is differentially activated in differentiating embryonic stem cells (White *et al.*, 2005).

Despite the lack of definitive evidence, pRB is a strong candidate to regulate stem cell self-renewal and differentiation. In tissue culture, pRB has been shown to promote cellular differentiation of multiple lineages by binding and regulating tissues specific transcription factors (Morris and Dyson, 2006; Skapek *et al.*, 2005). In addition, pRb binds to inhibitors of differentiation such as ID2 and EID1 (Ivarone *et al.*, 1995; Lassorella *et al.*, 2000; Maclellan *et al.*, 2000; Miyake *et al.*, 2000). This is without taking into consideration the role of pRB in chromatin remodeling. Thus, it seems plausible to hypothesize that the interactions between pRB, tissue specific transcription factors and chromatin modifiers,

combined with pRB's role in cell cycle control, influence stem cell functions in adult tissues. This thesis provides evidence to support this hypothesis in the context of the role of pRB during mesenchymal stem cells differentiation, which are the cells of origin for the myogenic, adipogenic, and osteogenic lineages (Figure 4).

ii. pRB and Myogenic Differentiation

Transgenic mice expressing low levels of *Rb* reveal a role for pRb in skeletal muscle differentiation (Zacksenhaus *et al.*, 1996).These embryos die at birth with specific skeletal muscle defects. Similarly, muscle defects have been reported in $Rb^{-/-}$ embryos whose lifespan was extended by suppression of placental defects (de Bruin *et al.*, 2003; Wu *et al.*, 2003; Ziebold *et al.*, 2001). Accordingly, *in vitro* studies shows that pRb loss causes decreased expression of late myogenic markers (Huh *et al.*, 2004; Li *et al.*, 2000; Novitch *et al.*, 1996). The inability of *Rb* mutant myocytes to properly differentiate reflects, both a physical interaction between pRb and the muscle-specific transcription factor MyoD (Gu *et al.*, 1993) and pRb's role in cell cycle control (Huh *et al.*, 2004; Li *et al.*, 2000; Zhang *et al.*, 1999). Notably, pRb expression enhances MyoD transcriptional activity by inducing the expression of late muscle differentiation genes such as MHC and MCK, although whether this is via a direct interaction between pRb and MyoD remains to be clarified.

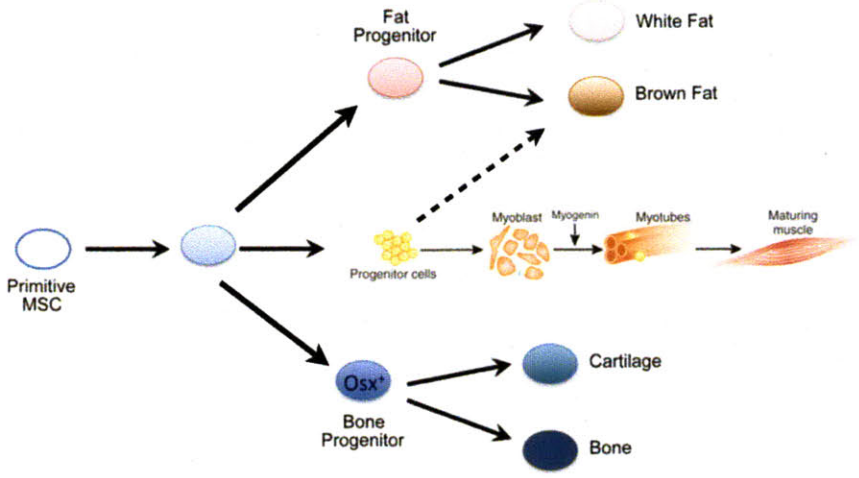

Figure 4: The mesenchymal lineage. Multipotent mesenchymal stem cells have been shown to be the cell of origin for the bone, fat and myogenic lineage.

iii. pRB and Adipogenic Differentiation

The pRB-E2f pathway has been shown to play an important role during adipogenesis. There are two types of fat tissues: white adipose tissue (WAT) and brown adipose tissue (BAT). These tissues are thought to originate from different precursors and their differentiation is triggered by mutually exclusive pathways (Seale *et al.*, 2009). The primary function of WAT is energy storage, while the purpose of BAT is thermogenesis and energy expenditure. Adipogenesis is triggered by the action of two transcription factors: Peroxisome Proliferator-Activated Receptor-gamma (PPAR-gamma; Tontonoz *et al.*, 1994), and the CCAAT/ Enhancer Binding Protein (C/EBP), which has three isoforms, alpha, beta and epsilon (Seale *et al.*, 2009). Importantly, the activity (Figure 3) and/or expression of these transcription factors are regulated by pRB. pRB acts as a co-transcriptional activator for C/EBPs to promote adipogenesis (Chen *et al.*, 1996), while pRB binding to PPAR-gamma attenuates its transcriptional activity by recruiting HDAC3 to silence genes required for adipogenesis (Fajas *et al.*, 2002).

In vivo experiments have shown that pRb acts as a molecular switch between the WAT and BAT lineages (Hansen *et al.*, 2004). This is also consistent with in vitro experiments showing that the promoter of *Pgc1a*, the master determinant of the BAT phenotype is bound and repressed by pRb (Scime *et al.*, 2005; Figure 3). Consistently, adipogenic induction of $Rb^{-/-}$ mouse embryonic fibroblast exhibits brown fat characteristics, including high levels of mitochondria and expression of *Ucp1* and *Pgc1-alpha* (Hansen *et al.*, 2004).

Moreover, PPAR-gamma expression is cell cycle regulated and it is clearly controlled by the pRB-E2F pathway (Fajas *et al.*, 2002).

iv. pRB and Osteogenic Differentiation

The role of pRB in the bone tissue has been previously explored *in vivo* and *in vitro*. Bone tissue forms from mesenchymal progenitor cells via two different differentiation pathways: Through direct differentiation of mesenchymal stem cells into osteoblasts (intramembraneous ossification) or through a cartilage intermediate (endochondral ossification; Nakashima and de Crombrugghe, 2003). The master regulator of the bone lineage is the transcription factor Runx2 (Nakashima and de Crombrugghe, 2003), as *Runx2*-null mice do not develop osteoblasts and therefore lack ossification in all skeletal structures (Komori *et al.*, 1997; Otto *et al.*, 1997)

The first indication that *Rb* plays a role in bone differentiation came from studies of viral oncoprotein-mediated effects on osteoblast differentiation (Beck *et al.*, 1998; Feuerbach *et al.*, 1997). More direct evidence for the involvement of *Rb* osteoblast differentiation came from the inability of $Rb^{-/-}$ mouse embryonic fibroblasts to differentiate into the bone lineage (Thomas *et al.*, 2001; Figure 3). These studies lead to the finding that pRb promotes bone differentiation by enhancing Runx2 transcriptional activity (Thomas *et al.*, 2001). These results have been confirmed *in vivo* as *Rb* null embryos have defects in both intramembraneous and endochondral ossification and showed reduced expression of early osteoblast specific markers (Berman *et al.*, 2008; Gutierrez *et*

al., 2008). The fact that *E2f1* loss partially suppresses the bone defect strongly indicates that the role of *Rb* in bone differentiation is, at least in part, proliferation dependent (Berman *et al.*, 2008). Therefore, pRb regulates bone differentiation by promoting Runx2 transcriptional activity, and indirectly via its impact on cell cycle exit.

F. The Retinoblastoma Family: pRB, p107, and p130 and their related functions.

RB-1 belongs to a family of proteins better known as the pocket proteins. This family consists of three structurally related proteins: pRB, p107, and p130 (Figure 5A). All three members were originally discovered as targets bound to viral oncoproteins and are collectively called the pocket proteins (Harlow *et al.*, 1986; Whyte *et al.*, 1989). The three family members bind a specific subset of E2F transcription factors and their inactivation is controlled by phosphorylation as described above for pRB. Although both p107 and p130 can compensate for certain pRB's function, *RB-1* is the only member of this family of proteins that is commonly mutated in cancer.

The expression pattern of p107 or p130 is different than that of pRB. While pRB is expressed throughout the cell cycle, p130 is most highly expressed in G0 and in quiescent cells, and p107 expression is observed during G1/S (Baldi *et al.*, 1995; Beijersbergen *et al.*, 1995; Chen *et al.*, 1989). Cell cycle wise, p107 and p130 possess similar functions to pRB. Like pRB, overexpression of p107 or p130 induces G1 arrest (Classon and Harlow, 2002) and these proteins are also

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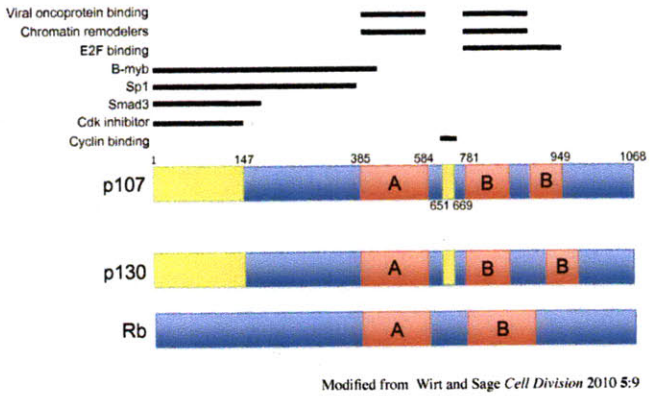

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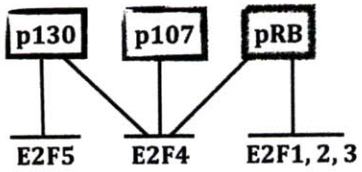

Figure 5: Structure of the pockets proteins and their interaction with the E2F family of transcription factors.

phosphorylated by cyclin-dependent kinases. However, there are also significant differences between these proteins. pRB is the primary binding partner for the activating E2Fs, E2F1, E2F2, and E2F3 (discussed in Part II), while p107 and p130 prefer to bind the repressive E2Fs, E2F4 and E2F5 (Figure 5B). In accordance with this, p107 and p130 associate with HDAC1 at E2F-responsive promoters (Ferreira *et al.*, 1998; Rayman *et al.*, 2002), and the loss of both p107 and p130 induces the expression of genes not observed upon the loss of pRB alone (Herrera *et al.*, 1996; Hurford *et al.*, 1997). Unlike pRB, p107 and p130 can inhibit CDK function (Dynlacht *et al.*, 1997; Zhu *et al.*, 1995).

In mouse embryonic fibroblasts loss of pRb function shorten the G1 phase of the cell cycle. This phenotype is exacerbated by the loss of p107 or p130 (Classon *et al.*, 2000; Dannenberg *et al.*, 2000). Consistently mouse embryonic fibroblasts devoid of all three pocket proteins do not respond to growth arrest signals (Dannenberg *et al.*, 2000; Sage *et al.*, 2000) and when cultured in spheres they reprogram to acquire an aberrant cancer stem cell like phenotype (Liu *et al.*, 2009). The appearance of these stronger phenotypes in the compound mutants suggest that p107 and p130 can compensate, at least partially, for pRb loss (Hurford *et al.*, 1997; Mulligan *et al.*, 1998; Sage *et al.*, 2003). Importantly, this functional compensation has also been implicated *in vivo.* For example, the combinatorial deletion of *Rb* and *p107* causes embryonic lethality at midgestation and accelerated apoptosis in different tissues, including those of the central nervous system (Lee *et al.*, 1996).

This compensatory role for p107 and p130 also extends to tumorigenesis. For example, specific deletion of pRb in the retina combined with loss of *p107* or *p130* results in retinoblastoma tumor formation (MacPherson *et al.*, 2004). Chimeric animals for *Rb* and either *p107* or *p130* also develop retinoblastoma (Dannenberg *et al.*, 2004; Robanus- Maandag *et al.*, 1998). In addition to retinoblastoma, these chimeric animals also develop other tumor types not observed in the *Rb* mutant animals, including osteosarcoma, ovarian cancer, and lung tumors (Dannenberg *et al.*, 2004). These data suggest that the pocket proteins act in a compensatory manner to suppress tumorigenesis in the mouse. Further analysis of combinatorial loss of pocket protein in mice will enable a better understanding of the overlapping and compensatory nature of *Rb, p107,* and *p130* in tumorigenesis, which may also provide insights into the differencial regulation of these proteins in mouse and humans.

Part II

The E2F Family of Transcription Factors

A. Discovery and Structure

E2F were first characterized as cellular proteins that could bind to the adenovirus E2 promoter (E2F stands for E2 Factor), and whose activity increased upon adenoviral infection (Kovesdi *et al.* 1986; La Thangue and Rigby 1987). Upon Adenoviral infection, the early viral protein, E1A, facilitates the induction of several other viral transcripts, including E2. The transcriptional activation of E2 is achieved through recruitment of E1A and a cellular factor called the E2 promoter-binding protein, or E2F (Kovesdi *et al.*, 1986). This factor was known to recognize the 5'-TTTCGCGC-3' DNA sequence in the promoter region of E2 (Kovesdi *et al.*, 1987; Yee *et al.*, 1987). E1A was also shown to induce the transcription of host genes that are required for DNA synthesis and replication, and these promoters also contain the E2F binding site. Subsequently it was discovered that in normal cells, E2F exists in a complex with a second cellular factor, which is dissociated upon E1A expression and this factor was eventually identified as the retinoblastoma protein, pRB (Bagchi *et al.* 1991; Chellappan *et al.* 1991). Based on the ability of the encoded protein to interact with pRB, a cDNA encoding a subunit of E2F was cloned, and this protein was termed E2F-1 (Helin *et al.* 1992; Kaelin *et al.* 1992; Shan *et al.* 1992). Since then, E2F promoter binding sites have been identified in many genes related to cell cycle regulation, nucleotide metabolism, apoptosis, differentiation and DNA replication, indicating an important role for E2F-mediated transcription in cell

cycle progression.

Around the same time that the first E2Fs was characterized, a cellular activity named DRTF (for Differentiation-Regulated Transcription Tactor) was identified. This factor interacted with the E2 promoter and its activity decreased with differentiation of murine embryonal carcinoma stem cells (La Thangue and Rigby 1987). Since DRTF binds to the same DNA sequence as E2F (La Thangue and Rigby 1987), and interacted with pRB (Bandara and La Thangue 1991), it was thought that DRTF and E2F were the same protein or were part of the same transcriptional complex. Later a cDNA encoding an additional component of DRTF/E2F was isolated and named DP1.

To date there are nine E2F genes E2F1 to 8, including a distinct E2F3 gene product, E2F3b (Figure 6). Differences in their transcriptional activity have led to the division of the E2F proteins into three groups. E2F1 through E2F3 comprise the activating E2Fs; E2F4, E2F5, and E2F3b comprise the repressive E2Fs. The other three, E2F6 through E2F8, although similar in structure to the other E2Fs, they act independently of pocket protein regulation (Figure 6).

B. The activating E2Fs

As mentioned, the first cloned E2F, E2F1, was isolated based on its ability to bind pRB (Helin *et al.*, 1992; Kaelin *et al.*, 1992; Shan *et al.*, 1992). E2F1 binds DNA in association with DP1 or DP2 proteins. The resulting complex is a strong transcriptional activator of E2F-responsive promoters (Bandara *et al.*, 1993; Helin *et al.*, 1993; Krek *et al.*, 1993). The other E2F were identified by low-stringency

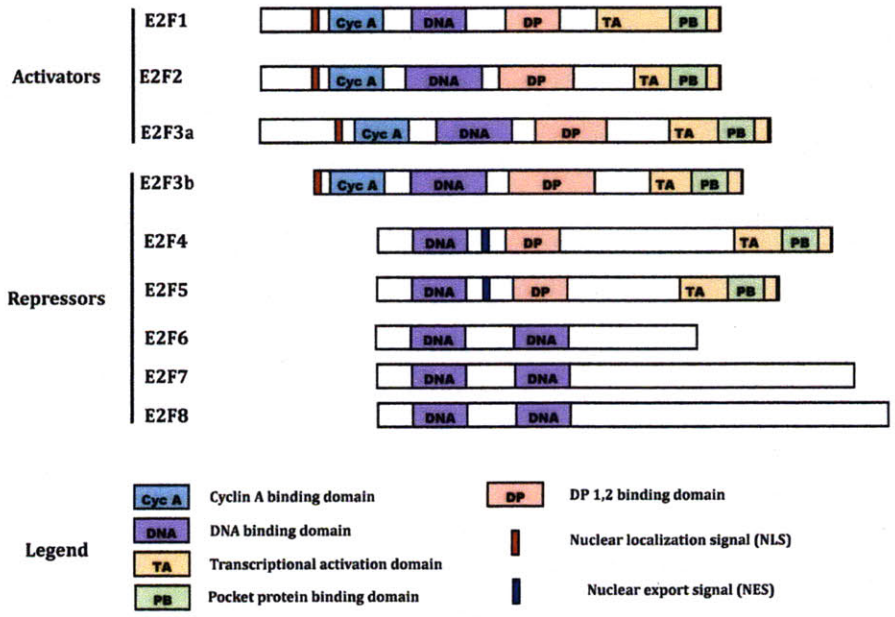

Figure 6: The E2F family of transcription factors.

library screening with an E2F1 probe. This led to the discovery of two additional E2F proteins that were structurally and functionally similar to E2F1; E2F2 and E2F3 (Ivey-Hoyle *et al.*, 1993; Lees *et al.*, 1993; DeGregori *et al.*, 1997; Ivey-Hoyle *et al.*, 1993).

E2F1, 2 and 3 are structurally similar and together they are called the activating E2Fs. They possess distinct domains for DNA binding, DP dimerization, and transactivation (Figure 6). The pocket protein-binding region is located within the transactivation domain near the C-terminus. These domains are highly conserved, as the domain regions in E2F2 and E2F3 are between 45%-100% similar to the equivalent regions in E2F1 (Lees *et al.*, 1993). In addition, all three activating E2Fs possess a conserved nuclear localization signal near the amino-terminus (Muller *et al.*, 1997; Verona *et al.*, 1997).

The activating E2Fs were first characterized for their roles in promoting cell cycle progression. Overexpression of any activating E2F will strongly activate transcription of E2F-responsive genes and will be sufficient to induce quiescent cells to re-enter the cell cycle (Johnson *et al.*, 1993; Lukas *et al.*, 1996; Qin *et al.*, 1994). Consistently, mouse embryonic fibroblasts lacking all three activating E2Fs are blocked in cellular progression (Wu *et al.*, 2001), which clearly suggests that the activating E2Fs function to promote cell cycle progression. This has been confirmed by promoter occupancy experiments in which activating E2Fs are localized and bound to E2F-responsive elements during the G1/S transition. Notably, these associations correspond with the transcriptional activation of these genes (Rayman *et al.*, 2002; Takahaski *et al.*, 2000).

Regulation of the activating E2Fs also corresponds to their function in G1/S progression. All activating E2Fs are regulated predominantly via interaction with the pocket proteins. Notably, the activating E2Fs interact exclusively with pRB during normal physiological conditions (Lees *et al.*, 1993). The binding of pRB masks the E2F transactivation domain and is thought to be the principal method of transcriptional inhibition of these E2Fs (Flemington *et al.*, 1993; Helin *et al.*, 1993; Hiebert *et al.*, 1992; Lee *et al.*, 2002). Upon mitogenic signals, phosphorylation of pRB triggers the release of the E2Fs, indicating that pRB is the major regulator of E2F activity.

In addition to pocket protein regulation, the activating E2Fs are also regulated by phosphorylation and acetylation. The activating E2Fs possess a cyclin A binding domain in the amino terminus (Devoto *et al.*, 1992; Krek *et al.*, 1994; Leone *et al.*, 2000; Mudryj *et al.*, 1991; Pagano *et al.*, 1992; Figure 6). Through this region, cyclin A/CDK complexes can phosphorylate E2F1 (Adams and Kaelin, 1996; Peeper *et al.*, 1995), as well as DP, resulting in loss of E2F DNA binding and consequently transcriptional activity (Dynlacht *et al.*, 1994; Krek *et al.*, 1995; Xu *et al.*, 1994). E2Fs acetylation is mediated by P/CAF, likely with p300 and CBP (Martinez-Balbas *et al.*, 2000; Pediconi *et al.*, 2003). The association of E2Fs with P/CAF, p300, and CBP on chromatin is thought to stimulate transcription (Martinez-Balbas *et al.*, 2000; Marzio *et al.*, 2000; Trouche *et al.*,1996). E2F1 is also acetylated and stabilized in response to DNA damage (Galbiati *et al.*, 2005; Ianari *et al.*, 2004). However, the physiological relevance and function of E2F phosphorylation and acetylation with respect to cell cycle

regulation and DNA damage are unclear and remain to be elucidated.

In addition to its role in cell cycle, the activating E2Fs also play a role in regulating apoptosis. E2F1 overexpression induces both proliferation and apoptosis (Kowalik *et al.*, 1995; Qin *et al.*, 1994; Shan and Lee, 1994; Wu and Levine, 1994; Vigo *et al.*, 1999). In accordance, E2F1 loss *in vivo* suppresses the apoptosis observed in the Rb null embryos (Tsai *et al.*, 1998). Similar results were obtained with E2F3 loss (Ziebold *et al.*, 2001). On the other hand, microarray analysis of $E2F1^{-/-};E2F3^{-/-}$ cells showed enrichment of an apoptotic signature (Black *et al.*, 2005), which indicates that E2F1, and possibly other activating E2Fs are potent inducers of apoptosis. This apoptotic effects have been shown to be mediated in a p53-dependent and independent manner (Aslanian *et al.*, 2004; Iaquinta *et al.*, 2005; Hsieh *et al.*, 1997; Phillips *et al.*, 1997) as activating E2Fs can directly regulate transcription of pro-apoptotic target genes (Zhao *et al.*, 2005).

C. The repressive E2Fs

The repressive E2Fs, E2F4 and E2F5, were identified and cloned based on their ability to bind p107 or p130 and their homology to previously identified E2Fs. These E2Fs are structurally and functionally similar to the activating E2Fs (Figure 6). They associate with DP proteins to bind DNA 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 in

quiescence cells (Lukas *et al.*, 1996; Mann and Jones, 1996). This suggests that the function of E2F4 and E2F5 is different from that ofthe activating E2Fs. 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; Figure 6).

Like the activating E2Fs, E2F4 and E2F5 exhibit cell cycle-dependent associations with pocket proteins and DNA binding activity. p130/E2F5 complexes are seen in G0 and G1, and these 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 their cell cycle dependent regulation, mouse embryonic fibroblasts lacking E2F4 or E2F5 have no proliferation or cell cycle defects (Humbert *et al.*, 2000a; Lindeman *et al.*, 1998). Analysis of *E2f4*$^{-/-}$;*E2f5*$^{-/-}$ mouse embryonic fibroblasts revealed, that these proteins are dispensable for cell cycle progression, but individually required to promote G1 arrest in response to *INK4a* (Gaubatz *et al.*, 2000). Together these observations suggest that E2F4 and E2F5 possess redundant functions. Moreover, their function is solely required for cell cycle arrest, but not for asynchronous growth.

Unlike the activating E2Fs, E2F4 and E2F5 are expressed in all phases of the cell cycle (Muller *et al.*, 1997; Sardet *et al.*, 1995; Takahashi *et al.*, 2000; Vairo *et al.*, 1995; Verona *et al.*, 1997; Wells *et al.*, 2000). Also, their cellular

localization is predominantly cytoplasmic, which is consistent with the lack of a nuclear localization signal in these proteins (Figure 6). On the contrary, E2F4 possesses two nuclear export signals, and its cytoplasmic localization has been shown to be dependent on the CRM1 nuclear export factor (Gaubatz *et al.*, 2001). However, upon association to pocket protein the repressive E2Fs localize to the nucleus and bind to E2F-responsive promoters (Muller *et al.*, 1997; Sardet *et al.*, 1995; Takahashi *et al.*, 2000; Vairo *et al.*, *1995;* Verona *et al.*, 1997; Wells *et al.*, 2000). In accordance, in G0/G1 E2F4 and E2F5 account for most of the nuclear, E2F-pocket protein complexes. This pocket protein dependent nuclear localization is thought to account for the predominant role of E2F4 and 5 in the repression and not activation of E2F target genes.

The role of the repressive E2Fs during development has been explored *in vivo* and *in vitro*. *E2f4-null* mice display several developmental defects and die prematurely due to bacterial infections (Humbert *et al.*, 2000a) caused by the lack of ciliated cells in the airway epithelium (Danielian *et al.*, 2007). In addition, the *E2f4*-null mice are anemic and have cell autonomous defects in red blood cell maturation (Humbert *et al.*, 2000a; Rempel *et al.*, 2000). The *E2f5*$^{-/-}$ animals have been generated and are born at expected frequencies and show no detectable developmental defects. However, these animals die by 6 weeks due to hydrocephalus (Lindeman *et al.*, 1998).

In vitro, overexpression of E2F4 enhances neuronal differentiation and plays an important role in maintenance of the differentiation state (Persengiev *et al.*, 1999). In myoblasts, E2F4 complexes with p130 or p107 to repress FGFR1,

which is an important regulator of skeletal muscle differentiation (Parakati and DiMario, 2005a,b). During adipogenesis, E2F4 plays an important role in terminal differentiation *in vitro,* at least in part, by repressing of PPAR-gamma (Fajas *et al.*, 2002b; Landsberg *et al.*, 2003). Notably, *E2f4*-null embryonic stem cells in chimeric mice significantly contribute to white adipose tissue (Fajas *et al.*, 2002).

During tumorigenesis the axis between cell cycle exit and terminal differentiation is clearly disrupted. However, the specific mechanisms and the pathways that deregulate the differentiation potential of normal cells to make them tumorigenic remain to be elucidated. This is of particular interest because reduced expression of master differentiation inducers is observed in high-grade tumors. Given the role of the pRB-E2F pathway in cell cycle control and differentiation, it is seems plausible that inactivation of this pathway affects not only cell cycle control, but also cellular differentiation. Thus, in this thesis we explored this hypothesis by studying the role of the pRb pathway in mesenchymal differentiation and tumorigenesis. This offered us a unique experimental system to disect the relationship between cell cycle control, cellular differentiation, and tumorigenesis.

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Chapter 2

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

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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 play 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 a 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 *Osxl- GFP::Cre*transgene (Rodda & McMahon, 2006). In this Cre transgene (herein called Osx1-*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 *Osx1-Cre;*$Rb^{+/fl}$, *Osx1-Cre;*$p53^{+/fl}$ or *Osx1-Cre;*$Rb^{+/fl}$;$p53^{+/fl}$ males with $Rb^{fl/fl}$, $p53^{fl/fl}$, or $Rb^{fl/fl}$;$p53^{fl/fl}$ females, we generated animals carrying every possible combination of *Rb* and *p53* alleles, with or without *Osx1-Cre.* All genotypes arose at approximately the expected frequency (Table S1**).** Mice carrying *Osx1-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 *Osx1-Cre* was expressed specifically in osteoblasts and not other mesenchymal lineages using reporter mice (A.S.L. and J.A.L., unpublished data; Chapter 3 Figure 1). We also showed that the *Osx1-Cre* transgene catalyzed efficient recombination of the conditional *Rb* and *p53* alleles in the bone, by using PCR-based genotyping assays (Fig. S1).

Table 1. Incidence of osteosarcoma (OS), neuroendocrine (NE), hibernoma (HIB) and other tumor types in *Rb;p53;Osx1-GFP::Cre* genotypes

Genotype	*Mice w. tumors*	*Tumor type*			*Mice with mets (%)*	*Av. age of euth.* (days ± s.d.)*
		OS	*NE*	*HIB*		
		Other				
$Rb^{fl/fl}$	2		2(pit)			397±47
$p53^{fl/fl}$	25	25			32	281±55
$Rb^{+/fl};p53^{+/fl}$	16	16			19	299±84
$Rb^{fl/fl};p53^{+/fl}$	18	17	4(pit)		22	251±87
$Rb^{+/fl};p53^{fl/fl}$	21	21	1(pit)		43	207±33
$Rb^{fl/fl};p53^{fl/fl}$ (DKO)	43	28 rhabdo	24	19	37	147±31

*Age of euth comparison t-test: DKO vs. p53 $p<0.0001$, $Rb^{+/fl};p53^{+/fl}$ vs. p53 $p=0.13$, $Rb^{+/fl};p53^{fl/fl}$ vs. p53 $p<0.0001$ and $Rb^{fl/fl};p53^{+/fl}$ vs. p53 $p=0.17$.

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 *Osx1-Cre;Rb*$^{fl/fl}$ 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 *Osx1-Cre* transgene is expressed at low levels in neuroendocrine tissues/precursors. Because the *Osx1-Cre* transgene is known to act in osteoblast precursors and histological analysis did not reveal tumorigenic lesions in the bones of adult *Osx1-Cre;Rb*$^{fl/fl}$ animals (data not shown), we conclude that *Rb* loss is not sufficient to promote the transformation of murine osteoblast precursors.

Consistent with the presence of osteosarcoma in humans and mice with germ-line *p53* mutations (Kansara &Thomas, 2007), a large fraction of the *Osx1-Cre;p53*$^{fl/fl}$ 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 *Osx1-Cre;Rb*$^{+/fl}$*;p53*$^{fl/fl}$ and *Osx1-Cre;Rb*$^{fl/fl}$*;p53*$^{+/fl}$ genotypes were highly predisposed to develop osteosarcoma, and their mean survival time was considerably shorter than that of the *Osx1-Cre;p53*$^{fl/fl}$ animals (Fig. 1A and Table 1). In addition, osteosarcomas

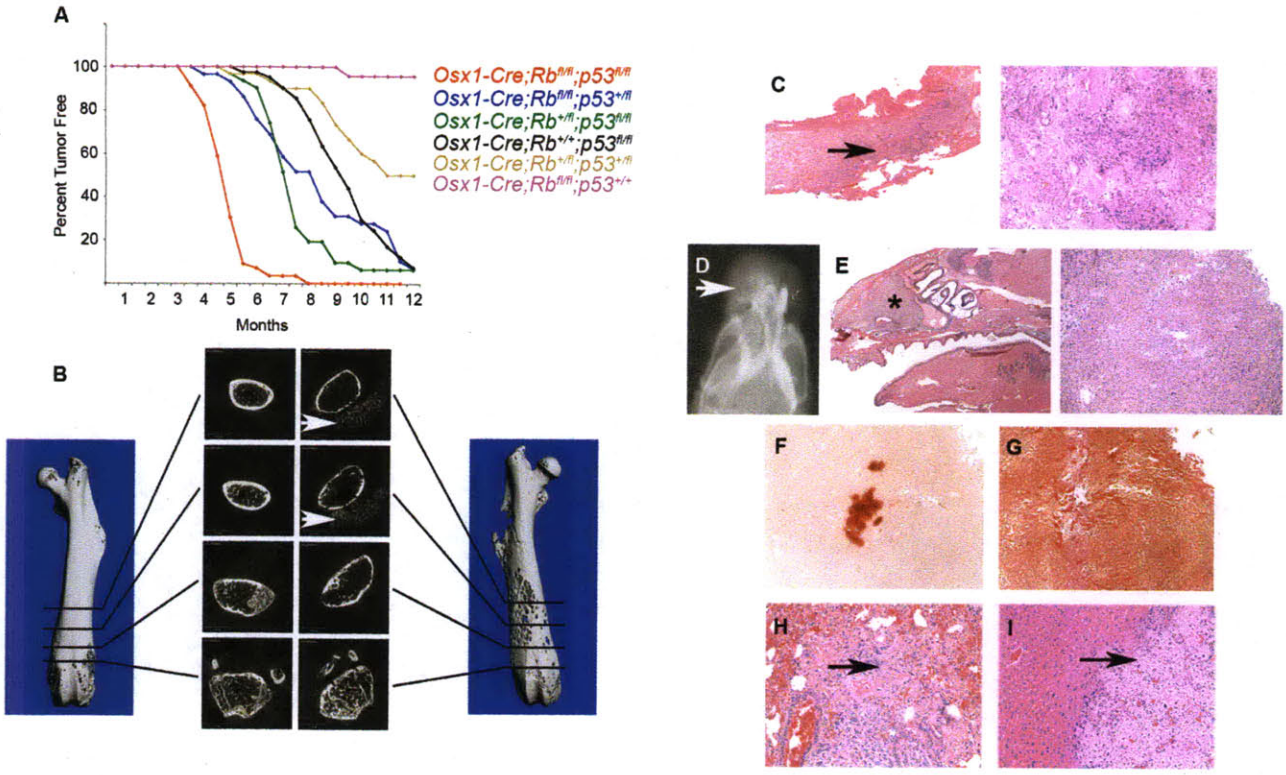


Figure 1. A mouse model of metastatic osteosarcoma.

(**A**) Kaplan-Mayer plot of the indicated genotypes carrying the *Osx1-GFP::Cre* up to 12 months of age. Histological and radiological analyses of osteosarcomas, and associated metastases, arising from *DKO* mice: (**B**) 3D reconstructed images from micro-Computerised Tomography are shown for control femur (left) versus a femur containing an osteosarcoma (right). Central panels show individual images at the indicated positions. Note the loss of bone cortex and the presence of bone specules located in the tumor that has grown beyond the periosteum (arrows). (**C**) Histological analyses of an osteosarcoma located in a femur show areas of bone cortex erosion (arrow on left) and the presence of little mineralized bone within the tumor (right). (**D-G**) Analysis of a representative snout tumor by (**D**) soft X-ray image to show the typical sunburst pattern (arrow), (**E**) H&E staining and staining of adjacent sections of undecalcified tumor with (**F**) Alizarin Red to detect calcified bone matrix or (**G**) Sirius Red to detect collagen. (**H-I**) Representative examples of osteosarcoma metastasis (arrow), in (**H**) lung and (**I**) liver containing detectable bone matrix. Histological sections were stained with H&E. Panels A, C, E shown at 2X, and remaining panels at 40X, magnification.

arose in a significant fraction of the *Osx1-Cre;Rb*$^{+/fl}$*;p53*$^{+/fl}$ animals, but rarely *(Osx1-Cre;p53*$^{+/fl}$*)* or never *(Osx1-Cre;Rb*$^{+/fl}$*)* 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 *Osx1-Cre;Rb*$^{+/fl}$*;p53*$^{fl/fl}$, *Osx1-Cre;Rb*$^{fl/fl}$*;p53*$^{+/fl}$, *Osx1-Cre;p53*$^{fl/fl}$ and *Osx1-Cre;Rb*$^{+/fl}$*;p53*$^{+/fl}$. 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 *Osx1-Cre;Rb*$^{fl/fl}$*;p53*$^{fl/fl}$ (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. 2). Many *DKO*s 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 *DKO*s and their associated tumor types (data not shown). Lack of correlation suggests that the shortened lifespan of the *DKO*s, versus 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.

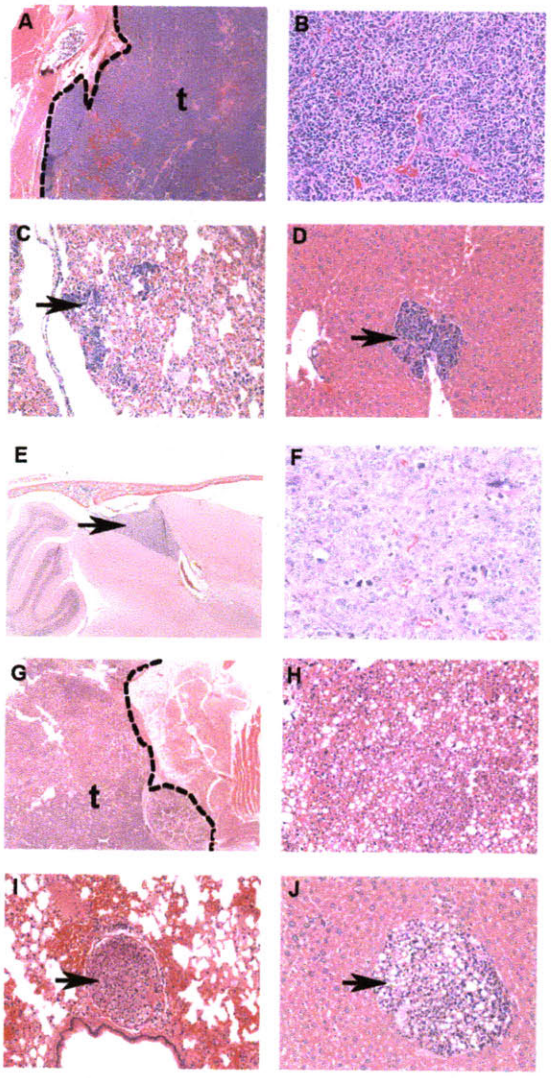


Figure 2. Histological analyses of neuroendocrine tumors and hibernomas arising in *DKO* mice.

(**A,B**) Typical neuroendocrine tumor located near the neck shown at (**A**) 2X magnification and (**B**) 40X magnification, showing densely packed cells with little cytoplasm, oval nuclei, and granular nucleoplasm surrounded by fibrovascular stroma. Neuroendocrine tumor cells metastasize to both the (**C**) lung and (**D**) liver. (**E-F**) Neuroendocrine tumor located in the brain (arrow), likely a pinealoma, shown at (**E**) 2X magnification and (**F**) 40X magnification. (**G**) Example of a hibernoma (2X magnification) 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 (40X magnification located in the (**I**) lung and (**J**) liver. All sections were stained with HNE and t indicates tumor.

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. 3). These tumors contained mRNAs associated with early to mid stages of bone differentiation, such as *Runx2, Osx, Alkaline Phosphatase* (Alp), and *Collagen1* (Coll), 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. 3). Finally, as noted above, a significant fraction of the osteosarcomas metastasized to lung

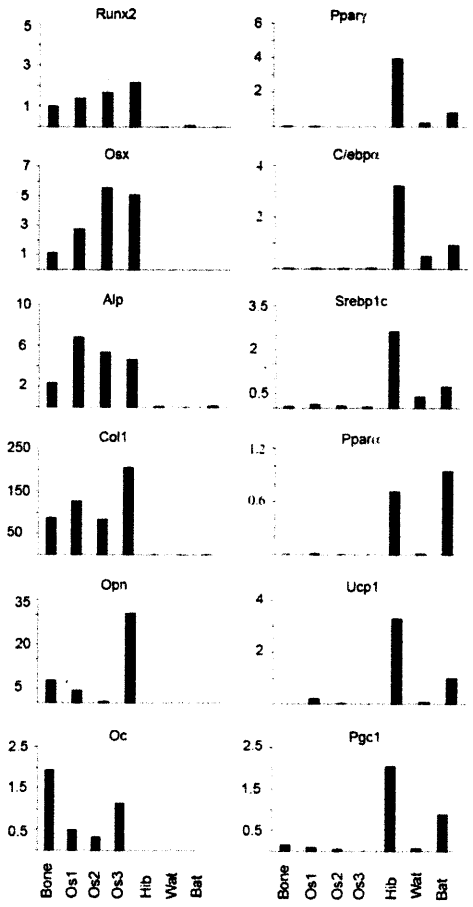


Figure 3. Analyses of gene expression in osteosarcomas derived from *Osx1-Cre;Rb$^{fl/fl}$;p53$^{fl/fl}$* mice.

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 hand column: 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), *collagen a1* (Col1); and late stages of osteoblast differentiation *osteopontin* (Opn) and *osteocalcin* (Oc). Osteosarcomas expressed genes associated with the osteoblast lineage although in general slightly lower levels of late differentiation markers. Right hand mRNA profiles of genes associated with adipogenesis (*Pparγ*, *C/ebpα*, and *Srebp1c*) and specifically brown adipose tissue (*Pparα*, *Ucp1* and *Pgc1*).

and liver (Fig. 1 H-I, Table 1, and Table S2). Thus, mutation of *Rb* and *p53* using this Cre transgene induces formation of metastatic osteosarcomas that resemble the human disease.

Cell lines derived from osteosarcomas are immortal and form osteogenic tumors when transplanted in nude mice.

To further characterize these tumors, we dissected primary osteosarcomas from three different *DKO* mice, mechanically disaggregated the cells, and placed them in culture. The tumors used for this experiment span the range of osteosarcoma phenotypes 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. 4 A and B). All three tumors yielded rapidly growing cell populations, and PCR verified that the $Rb^{fl/fl}$ and $p53^{fl/fl}$ 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. 4 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. 4 C-H). However, they were poorly differentiated or

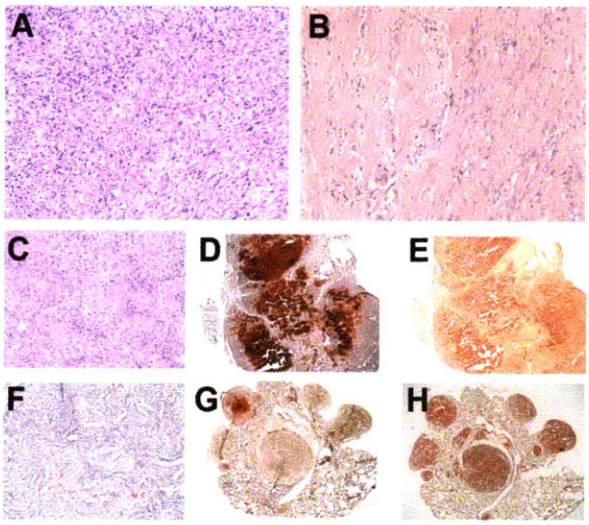


Figure 4. *DKO* osteosarcoma cell lines can form bone tumors in immunocompromised mice.

(**A-B**) H&E stained section of the primary osteosarcomas #985 and #2380 respectively. Tumors derived from (**C-E**) subcutaneous or (**F-H**) intravenous injection of OS-*DKO*-985. (**C**, **F**) H&E staining at 40X magnification. Adjacent sections were stained with either (**D**,**G**) Alizarin Red or (**E**,**H**) Sirius Red to stain calcified bone matrix and collagen respectively (2X magnification).

undifferentiated, as only small regions of the tumor produced calcified bone (Fig. 4 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 *Osx1-Cre* transgene, characteristics of the primary osteosarcomas, and osteoblastic properties of the transplanted tumor cell lines all suggest that the tumors result from transformation of cells committed to the bone lineage. Thus, we asked whether the cultured tumor cells retained their ability to differentiate into bone *in vitro.* For these experiments, we allowed the tumor cells to reach confluence and then cultured them in osteogenic induction media. *DKO*-OS-985 (Fig. 5), *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. 5 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

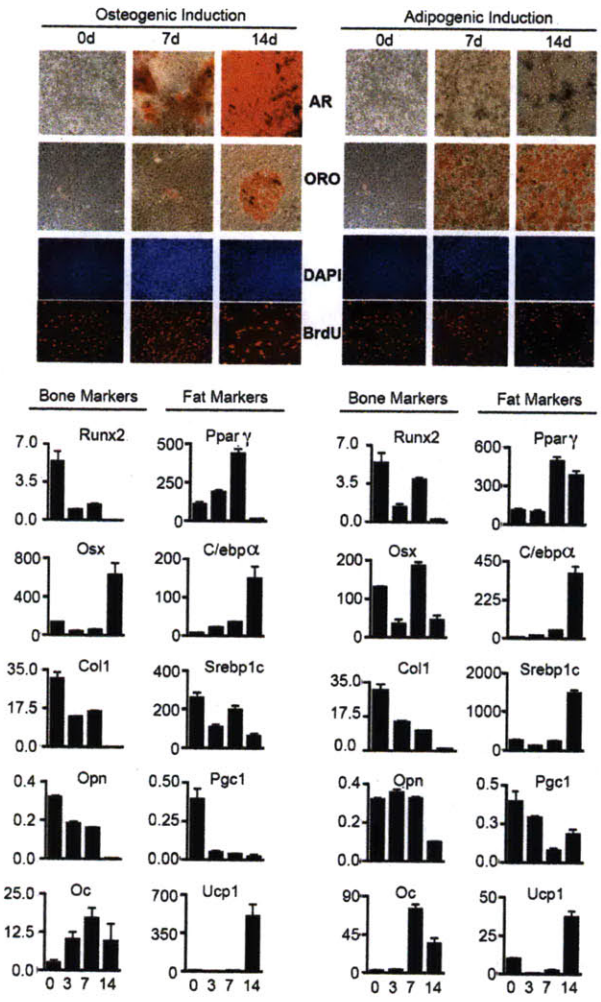


Figure 5. Osteosarcoma cells lines are multi-potent in *vitro*.

Osteosarcoma cell lines were induce to differentiate into the bone (left panel) and fat (right panel) lineages. At the indicated timepoints (days), cells were assayed. Mineral deposits were stained with Alizarin Red (AR) as a marker for osteogenic differentiation. The lipophylic dye Oil- Red O (ORO) was use to stain lipid droplet accumulation during adipogenic induction. Cells were pulse with BrdU to determine cells proliferative status during differentiation. Expression of differentiation markers for bone and fat was determined by qRT-PCR.

differentiation media adopted the adipogenic fate, as judged by Oil Red-O staining for lipid droplets (Fig. 5). Consistent with this finding, adipocyte differentiation markers were induced in these cells (Fig. 5). To explore adipocyte differentiation further, we cultured the tumor cells in adipogenic differentiation media (Fig. 5). 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. 5). 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).

DKO osteosarcoma cell lines express Sca-1, a marker of early mesenchymal progenitors, and this correlates with their tumorigenic potential.

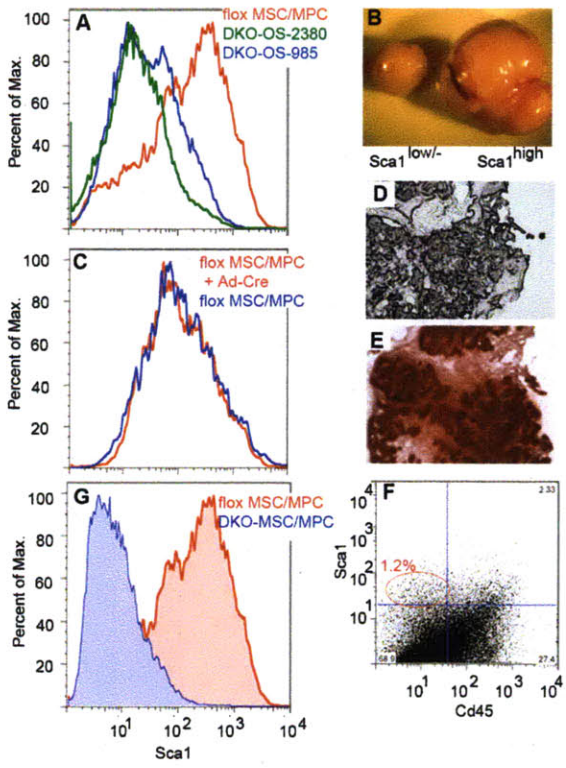


Figure 6: Sca1 expression and *Rb*- and *p53*-loss are both required for efficient tumorigenesis *in vivo.*

Sca1 expression in (**A**) *DKO*-OS-985 and *DKO*-OS-2380 cell lines versus flox MSC/MPCs or (**C**) flox MSC/MPCs before and after Adeno-cre infection and inactivation of *Rb* and *p53*. (**B**) Tumors arising in immuno-compromised mice injected subcutaneously with 105 *DKO*-OS-985 cells sorted for either $Sca1^{+}$ (left) or $Sca\text{-}1^{low/-}$ (right). (D and E) Tumors arising in immuno-compromised mice injected subcutaneously with 10^6 flox MSC/MPCs + Ad-cre stained for (**D**) Alkalinephosphate expression or (**E**) Alizarin Red. Sca-1 expression in (**F**) *DKO* MSC/MPCs versus flox MSC/MPCs and (**G**) primary *DKO* osteosarcomas.

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. 6A 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-1high population (Table S3). In another experiment, the Sca-1^{high} cells produced a much larger tumor than the Sca-$1^{low/-}$ cells (Fig. 6B). 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-1^{high} MSC/MPC preparations is sufficient to confer tumorigenicity. For this experiment, we isolated stromal cells from the bone marrow of $Rb^{fl/fl};p53^{fl/fl}$ 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-1^{high}/$CD45^-$ cells (Fig. 6C 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. 6 D and E and Table S3). Thus, we conclude that the loss of *Rb* and *p53* in Sca-1^{high} MSC/MPCs is sufficient to create osteosarcoma-initiating cells. Long-term passaging of the flox MSC/MPC;+Ad-Cre cultures confirmed that these cells are fully immortalized *in vitro.* Furthermore, the composition of the cell population shifted over time to give a mixture of Sca-1^{high} and Sca-1^{-} cells (data not shown), indicating that division of the Sca-1^{+} tumor-initiating cells can yield Sca-1^{-} progeny.

The presence of Sca-1^{+} cells within the OS cell lines was somewhat unexpected because Cre expression, and therefore *p53* and *Rb* inactivation, occurs in committed osteoblast precursors (i.e., cells that are presumed to be Sca-1^{-}). To determine whether these Sca-1^{+} cells exist in the endogenous tumors, we dissociated primary osteosarcomas from *DKO*s 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. 6F). 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. 6G). Notably, this cellular composition represents a clear departure from the properties of wild-type flox MSC/MPCs (which are predominantly Sca-1^{high}) (Fig. 6G), 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-I$^{low/-}$ osteoprogenitors *in vivo.* First, they could result from the accumulation and expansion of Sca-I$^{low/-}$ 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,

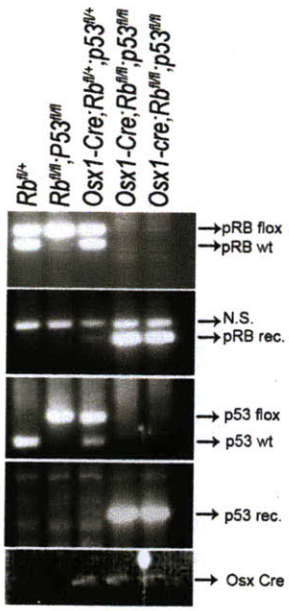
and a high incidence of metastases.

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^{-}/$Osx1^{+}$ 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^{-}/$Osx1^{+}$ 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.



Supplementary Figure 1. 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 $Rb^{fl/fl}$, $Rb^{fl/fl}$;$p53^{fl/fl}$, and *Osx-Cre*;$Rb^{fl/fl}$;$p53^{fl/fl}$ 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.

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										
$Rb^{c/c};p53^{-/c}$	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

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

Experimental Procedures

Animal Maintenance and Histological Analyses.

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

Analysis of Tumor Study Mice.

The criteria for euthanasia by CO_2 inhalation were a total tumor burden of $2cm^3$, tumor ulceration/bleeding, signs of infection, respiratory distress, impaired mobility, 20% reduction in body weight, or general cachexia. All tissues were collected and hipbones, 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 80^0C and cut at 10 tm for frozen sections. All paraffin embedded sections were cut, 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 1h, 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.

PrimersforMouse Genotyping.

To identify the *Rb* conditional allele we usedn the following primers:

5 lox: 5'-CTCTAGATCCTCTCATTCTTC-3'

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 Osxl-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- pm filter, and plated in normal growth medium (10% FBS in DMEM, 1% P/S, 1-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 FACS-Calibur HTS (Becton-Dickinson) using Sca-I and Cd45 antibodies (BD Pharmigen). For transplant assays, 10^5-10^6 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.

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Chapter 3

Rb regulates fate choice and lineage commitment *in vivo*

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The author contributed to all text, graphs, tables and figures.

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Abstract

Mutation of the *RB-1* tumour suppressor occurs in one third of all human tumours and is particularly associated with retinoblastoma and osteosarcoma (Burkhart and Sage, 2008). Numerous functions have been ascribed to the product of the human *RB-1* gene, pRB. The best known is pRB's ability to promote cell cycle exit through inhibition of the E2F transcription factors and the transcriptional repression of genes encoding cell cycle regulators (Burkhart and Sage, 2008). In addition, pRB has been shown *in vitro* to regulate several transcription factors that are master differentiation inducers (Korenjak and Brehm, 2005). Depending on the differentiation factor and cellular context, pRB can either suppress or promote their transcriptional activity. For example, pRB binds to Runx2 and potentiates its ability to promote osteogenic differentiation program *in vitro* (Thomas *et al.*, 2001). In contrast, pRB acts together with E2F to suppress *Pparγ* , the master activator of adipogenesis (Fajas *et al.*, 2002a; Fajas *et al.*, 2002b). Since osteoblasts and adipocytes can both arise from mesenchymal stem cells, these observations suggest that pRB might play a role in the choice between these two fates. However, to date, there is no evidence for this *in vivo.* Here we use mouse models to address this hypothesis in the context of mesenchymal tissue development and tumorigenesis. Our data show that *Rb* status plays a key role in establishing fate choice between bone and brown adipose tissue *in vivo*.

Mutations in *RB-1* (70-90% of cases) and *TP53* (50-70% of cases) are strongly associated with human osteosarcoma (Kansara and Thomas, 2007; Clark *et al.*, 2008). To model osteosarcoma in the mouse, we crossed $Rb^{fl/fl}$ (Sage *et al.*, 2003) and $p53^{fl/fl}$ (Jonkers *et al.*, 2001) conditional mutant mice with a transgenic line, *Prx1-Cre* (Logan *et al.*, 2002), which expresses Cre recombinase in uncommitted mesenchymal cells that contribute to bone, muscle, and both white and brown adipose tissue (Figure 1a-c). The homozygous deletion of *Rb* and/or *p53* by *Prx1-Cre* yielded viable neonates with no detectable developmental defects (data not shown), allowing us to determine the affect of *Rb* and/or *p53* loss on sarcomagenesis (Figure 2a). The *Prx1-Cre;*$p53^{fl/fl}$ animals developed osteosarcoma (62%), rhabdomyosarcomas (15%) and/or undifferentiated sarcomas (12%). In contrast, deletion of *Rb* alone did not yield sarcomas. However, *Rb* mutation had a profound effect on the tumour spectrum of *Prx1-Cre;*$p53^{fl/fl}$ mice (Figure 2a,b): deletion of one *Rb* allele increased the frequency of osteosarcomas (to 92%), while mutation of both *Rb* alleles shifted the tumour spectrum away from osteosarcoma (now 18%) and towards hibernomas (91%; Supplementary figure 2). This propensity for brown fat, as opposed to white fat, tumors fits with prior studies showing that *Rb* loss promotes brown fat over white fat differentiation (Hansen *et al.*, 2004; Scime *et al.*, 2005; Dali-Youcef *et al.*, 2007). Genotyping confirmed that the tumour cells had undergone Cre-mediated recombination of *Rb* and/or *p53* (Figure 2c, data not shown). Moreover, it showed that the *Prx1-Cre;*$Rb^{+/fl}$*;*$p53^{fl/fl}$ tumours consistently retained the wildtype *Rb* allele (Figure 2c, data not shown). Thus *Rb* acts in a

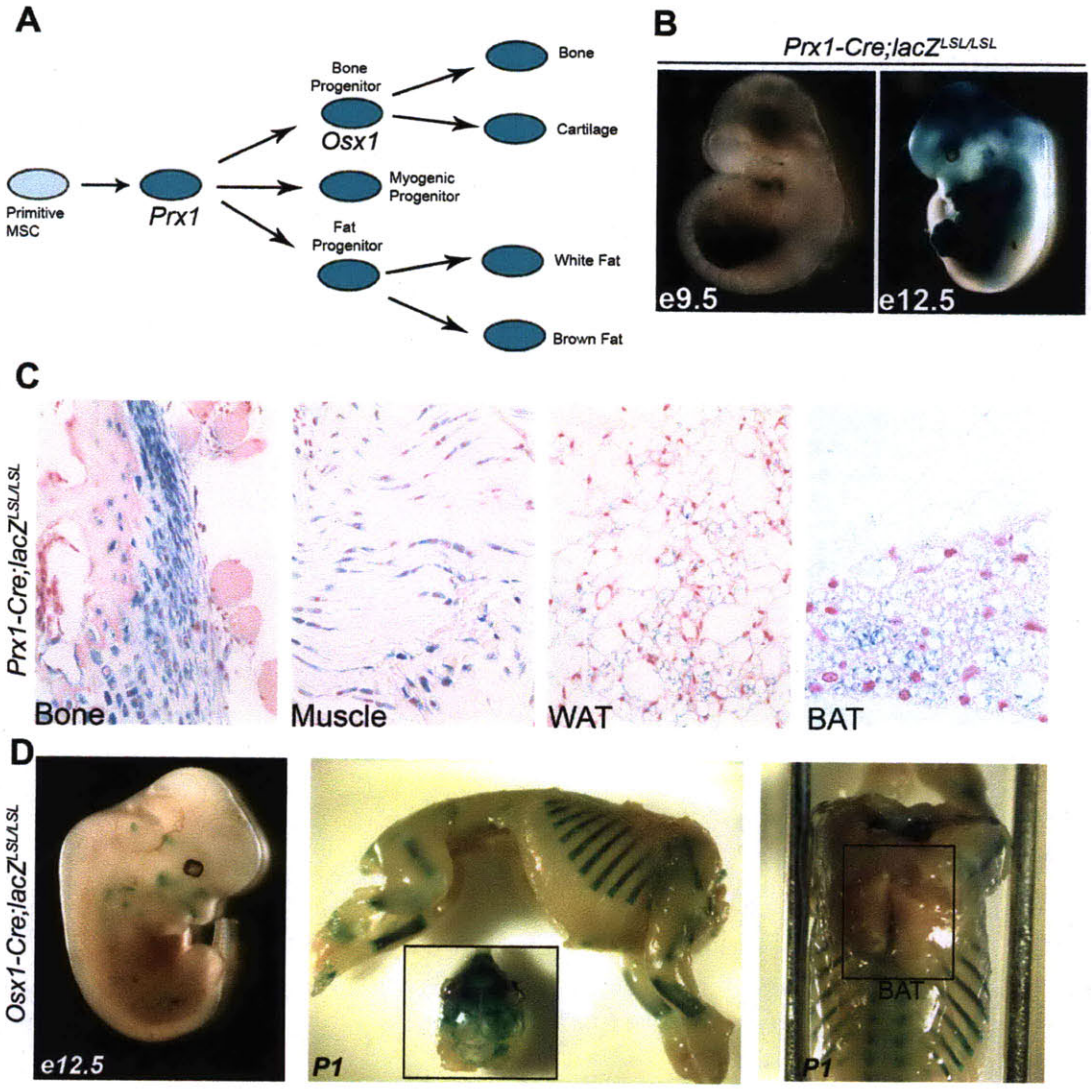


Figure 1: Prx1-Cre is expressed in an uncommitted mesenchymal compartment that gives rise to bone, fat, and myogenic lineages in the adult.

a, Diagram shows the onset of expression of *Prx1* versus *Osx1* in uncommitted mesenchymal stem cells versus committed osteoblast progenitors respectively. **b-c,** Intercrossing of *Prx1-Cre* with the *lox-stop-lox-LacZ* reporter mice and LacZ staining shows **(b)** that Prx-cre is expressed in the developing limb buds as early as e9.5 and is more evident at e12.5 as previously reported[10] and **(c)** it establishes extensive contribution of Cre-expressing cells to the bone, muscle, white and brown adipose tissues in 4-6 weeks old animals. **d**, Intercrossing of *Osx1-Cre* with the *lox-stop-lox-LacZ* reporter mice and staining for LacZ shows that unlike *Prx1-Cre*, *Osx1-Cre* is barely expressed at e12.5. In addition *Osx1-Cre* expression at postnatal day 1 is restricted to the bone tissue, and undetectable in the muscle and the brown fat compartments.

a.

Genotype			Tumor Distribution (%)				Mice	Average Latency
Prx1	*Rb*	*p53*	RMS	SAR	OS	HIB	#	(days±SD)
+	fl/fl	+/+	0	0	0	0	7	428±130
+	+/+	fl/fl	15	12	**62**	0	19	342±101
+	+/fl	fl/fl	9	18	**92**	4	33	273±77
+	fl/fl	fl/fl	12	3	18	**91**	40	163±34

RMS=Rhabdomyosarcoma; SAR=Undifferentiated Sarcoma; OS=Osteosarcoma
HIB=Hibernoma.

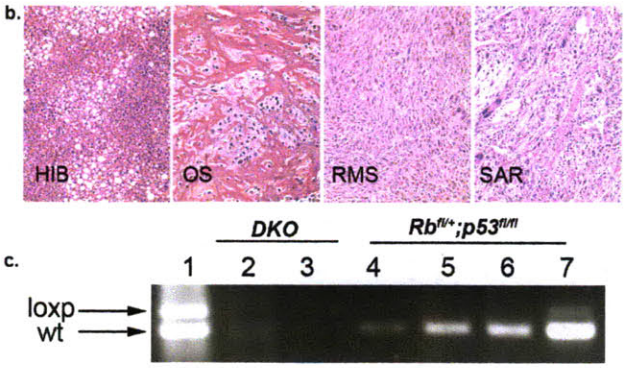


Figure 2: *Rb* cooperates with *p53* and modulates mesenchymal tumor fate in a dosage-dependent manner.

a, Mesenchymal tumor distribution (percentage of animals analyzed up to 24 months of age) for *Prx1-Cre;Rb* and/or *p53* compound mutant animals. **b**, H+E staining of representative sarcomas (20x magnification). **c**, PCR genotyping to detect *Rb* wildtype (wt) and recombined conditional mutant (loxp) alleles in control $Rb^{fl/+};p53^{fl/+}$ tissues (lane 1) or cell lines derived from $Prx1\text{-}Cre;Rb^{fl/fl};p53^{fl/fl}$ (DKO) or $Prx1\text{-}Cre;Rb^{fl/+};p53^{fl/fl}$ osteosarcomas. Cell lines were cultured for ≥20 passages prior to genotyping to eliminate stromal cell contribution.

dose dependent manner to modulate the spectrum of tumours arising from *p53*-deficient, uncommitted mesenchymal stem cells: osteosarcomas predominant in the presence of *Rb*, while *Rb* loss strongly favours hibernoma formation.

Given that *Rb* loss in *p53* mutant uncommitted mesenchymal cells disfavours osteosarcoma formation, we also investigated the affect of *Rb* loss in a bone-committed progenitor. For this, we deleted *Rb* and/or *p53* using the *Osx-Cre* transgenic (Rodda and McMahon, 2006) which uses *Osterix* promoter sequences to express Cre in the pre-oestoblast (Figure 1d). In this model(Berman *et al.*, 2008a), *Osx-Cre;p53*$^{fl/fl}$ mice specifically develop osteosarcoma (100%) while *Osx-Cre;Rb*$^{fl/fl}$*;p53*$^{fl/fl}$ develop osteosarcoma (53%), hibernomas (46%) and sarcomas (2%). We established cell lines from multiple (≥3) independent *Osx-Cre;p53*$^{fl/fl}$ and *Osx-Cre;Rb*$^{fl/fl}$*;p53*$^{fl/fl}$ osteosarcomas and discovered that the two genotypes have distinct differentiation properties (Figure 3, data not shown). The *Rb;p53* (*DKO*) osteosarcoma (OS) cell lines expressed mRNAs that are characteristic of bone and fat differentiation (Figure 3a). Indeed, their expression pattern more closely resembled that of mesenchymal stem cells (MSCs) than primary osteoblasts (Supplementary Figure 2). Accordingly, culture in the appropriate differentiation media induced these *DKO* cells to adopt either the adipogenic or osteoblastic fate (Figure 3a). In contrast, the *p53KO* OS cell lines closely resembled pre-osteoblasts based on their gene expression patterns, but these cells were unable to differentiate into either bone or fat (Figure 3a and Supplementary Figure 2). Since this differentiation block occurs in the p53-null OS cell lines, but not p53-deficient primary osteoblasts (Lengner *et al.*, 2006;

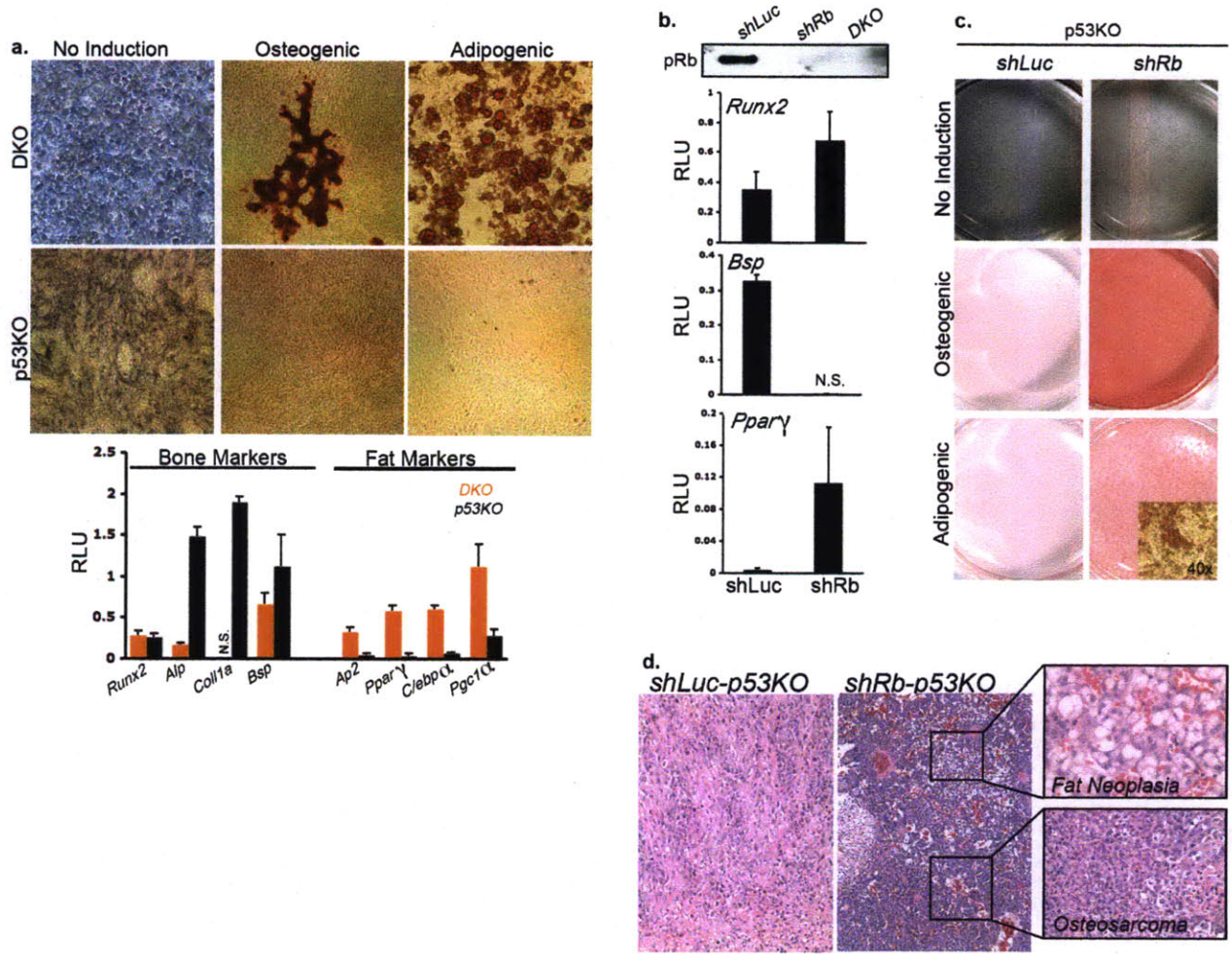


Figure 3: *Rb* regulates osteosarcoma-cell lineage plasticity *in vitro* and *in vivo*.

a, The differentiation potential of 3 different *DKO* and *p53KO* OS cell lines was assessed by addition of osteogenic or adipogenic differentiation media. Expression of bone and fat markers was assessed by qPCR of un-induced *DKO* (orange) and *p53KO* (black) OS cells. **b**, *Rb* or control (Luc) shRNAs were expressed in the *p53KO* cell lines. *Rb* knockdown was confirmed by immunoprecipitation and qPCR showed that this caused downregulation of the bone marker *Bsp* and upregulation of the fat marker *Pparγ* without culture in differentiating media. **c**, The osteogenic and adipogenic potential of *shLuc*- and *shRb-p53KO* cell lines was assessed. **d**, H+E staining of representative tumors derived from *shLuc*- and *shRb-p53KO* cell lines injected subcutaneously into immunocompromised mice. Moreover, the *shRb-p53KO* OS derived tumors were frequently mixed lineage (top inset shows fat neoplasm; bottom inset bone/undifferentiated sarcoma), while the control *shLuc-p53KO* tumors were uniformly. Bars represent the mean of three independent experiments (+/- SD). NS = not significantly expressed.

Figure 6a), it likely reflects their transformed state. We used these *p53KO* OS cells to determine whether *Rb* loss was sufficient to alter the differentiation potential of blocked pre-oestoblasts by introducing control (*shLuc*) or *Rb*-specific (*shRb*) shRNAs. pRb was readily detectable in *shLuc-p53KO*, but not *shRb-p53KO*, OS cells (Figure 3b). Strikingly, without addition of differentiation media, pRb knockdown downregulated the bone-specific mRNA *Bsp* and upregulated the fat regulator *Pparγ* (Figure 3b). Accordingly, these *shRb-p53KO* OS cells were now able to differentiate into either bone or fat *in vitro* (Figure 3c). Moreover, when transplanted into nude mice, the *shRb-p53KO* OS cells formed more aggressive tumors than the parental *p53KO* OS cells, and these were of mixed lineage (fat, bone and undifferentiated sarcomas), in stark contrast to the undifferentiated osteoblastic tumours arising from either control *shLuc-p53KO* or parental *p53KO* OS cell lines (Figure 3d, Supplementary Figure 3, and data not shown). Thus, pRB loss is sufficient to over-ride the differentiation block of these *p53*-deficient, tumor cell lines and also expand their fate commitment to include the adipogenic state.

We also examined the consequences of reintroducing *Rb* into the *DKO* OS cells. For this, we induced pRb in confluence-arrested DKO cells using a doxocycline-inducible expression system (*DKO-Rb*$^{Dox\text{-}ON}$; Figure 4). Remarkably, pRb restoration caused the *DKO* OS cells to adopt the differentiation state of the *p53KO* OS cell lines within two days: it induced down-regulation of adipogenic markers and up-regulation of osteogenic markers, and the cells were unable to

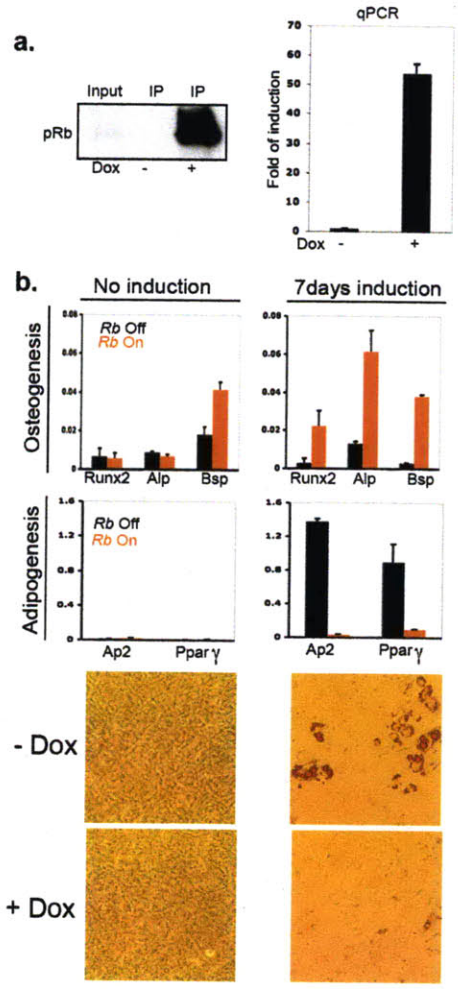


Figure 4: Restoration of *Rb* function promotes lineage commitment in osteosarcoma cell lines.

Immunoprecipitates of *DKO-Rb*$^{Dox\text{-}ON}$ OS cells showed pRb expression 48h after doxocycline (Dox) treatment (left, upper panel). *Rb* expression was also confirmed by qPCR (left, lower panel). OS cells were treated either with Dox (*Rb* On) or PBS (*Rb* Off) for 48h and then were induced to differentiate into the adipogenic and osteogenic lineages by addition of differentiation media. qPCR for osteogenic and adipogenic markers was used to analyzed the differentiation potential of these cells either prior to (-Diff. media) or 7 days after [+Diff. media (7d)] addition of differentiation media in the *Rb* On (orange) versus *Rb* Off (black) populations.

differentiate into fat (Figure 4). Thus removal or re-introduction of *Rb* appears sufficient to switch lineage specification between osteoblastic commitment and multipotency.

In vitro studies have shown that pRB can act with E2F to enforce transcriptional repression of *Pparγ* (Fajas *et al.*, 2002a; Fajas *et al.*, 2002b), and also bind, and potentiate the transcriptional activity of, the osteogenic regulator RUNX2 (Thomas *et al.*, 2001). We hypothesized that pRb's role in these processes might underlie *Rb*'s affect on adipogenesis versus osteogenesis. Thus, we used our *DKO-Rb*$^{Dox\text{-}ON}$ cells to determine whether the presence or absence of pRb modulated these transcriptional regulators (Figure 5). First, we used chromatin-immunoprecipitation assays to investigate promoter regulation of *Pparγ* and representative Runx2-responsive genes *Coll1a* (Figure 5a) and osteocalcin (*Oc*; data not shown). pRb-induction caused both pRb and E2f4, the predominant repressive E2f, to be recruited to the *Pparγ* promoter (Figure 5a) and this correlated with *Pparγ* mRNA downregulation (Figure 5a). Contemporaneously, pRb bound to *Coll1a* and *Oc* and this was accompanied by increased promoter occupancy of Runx2 and upregulation of *Coll1a* and *Oc* mRNAs (Figure 5a, data not shown). Importantly, these changes in *Pparγ* , *Coll1a* and *Oc* regulation were all detected within 2 days of pRb induction and without addition of differentiation-inducing media. In addition, we found that Runx2 associated with pRb in the *p53KO*, but not the *DKO*, OS cells and its transcriptional activity was 8 fold higher in the former, versus the latter, population (Figure 5b). Thus the presence or absence of pRb directly modulates

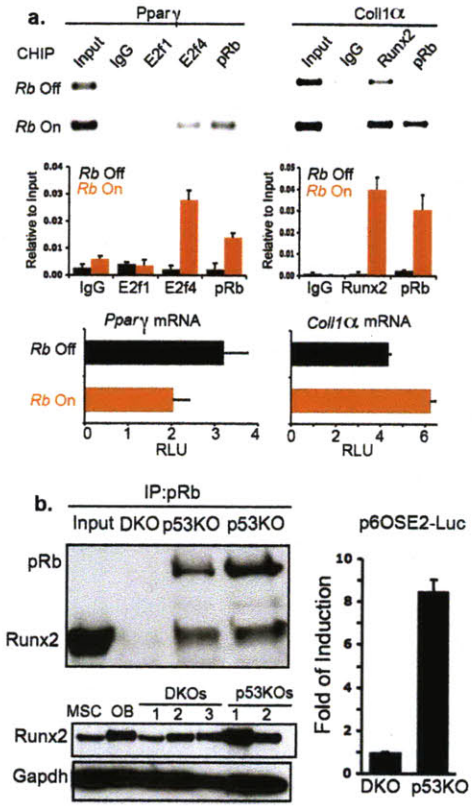


Figure 5: pRb modulates the activity and the expression of the master lineage regulators Runx2 and Pparγ.

a, *DKO-Rb*$^{Dox\text{-}ON}$ cells were cultured for two days in the absence (*Rb Off*) or presence (*Rb On*) of doxocycline and then analyzed. Results are representative of three independent experiments. Promoter occupancy was assessed by chromatin immunoprecipitation. Sequence analysis identified two potential E2f binding sites (-278 and –160) within the *Pparγ* promoter. pRb induction caused a dramatic upregulation of both pRb and E2F4 binding to the proximal site. (No binding was observed at the distal element.) Similarly, pRb induction allowed pRb to bind to the known Runx2 response element of Col1a[16] and also increased the binding of Runx2. These changes correlated with the downregulation of *Pparγ* mRNA and upregulation of Col1a mRNA as judged by qPCR. **b**, Western blotting detected Runx2 in pRb-immunoprecipitates from *p53KO*-OS cell lines (left, top panel). Western blotting of whole cell extracts confirmed that Runx2 was expressed in both DKO and *p53KO* OS cell lines (left, bottom panel). MSCs and osteoblasts were used as a positive control. Right panel: Runx2 transcriptional activity was shown to be higher in the *p53KO*- versus the DKO OS cell lines as judged by activation of the artificial Runx2-responsive reporter p6OSE2-Luc. Results are the average of six independent samples.

the levels and activity of *Pparγ* and Runx2 in accordance with the preferential commitment of our OS cell lines to the osteogenic versus the adipogenic lineage.

The preceding experiments establish a clear role for pRb in fate commitment bias *in vivo* and *in vitro*. However, since this analysis was conducted in *p53*-deficient cells, it is unclear whether *Rb* alone is sufficient to determine this plasticity or whether transformation is also required. To address this, we isolated primary osteoblasts from the calvaria of $Rb^{fl/fl}$, $p53^{fl/fl}$ or $Rb^{fl/fl};p53^{fl/fl}$ e18.5 embryos. We brought these cells to confluence, to minimize the influence of altered proliferation, infected them with adenoviruses expressing Cre or a GFP control and then assayed differentiation. As expected, the control-infected osteoblasts were able to undergo osteogenesis but not adipogenesis (Figure 6a; data not shown). Similarly, p53 loss had no effect on this fate commitment (Lengner *et al.*, 2006; Figure 6a). In stark contrast, deletion of *Rb*, either alone or together with *p53*, allowed these cells to adopt either the bone or fat lineage (Figure 6a; data not shown). This switch to multipotency correlated with the significant upregulation of adipogenic markers prior to the induction of differentiation (Figure 6b). Thus, pRb-loss is sufficient to alter the fate commitment in otherwise wildtype calvarial osteoblasts.

In vitro culture can modulate the plasticity of cells. Thus, we wished to examine *Rb*'s role in fate choice *in vivo*. For this, we employed a third transgenic strain, *Meox2-Cre*, which expresses Cre in the embryo proper from e6.5 (Tallquist and Soriano, 2000). *Meox2-Cre;*$Rb^{fl/fl}$ embryos survive to birth

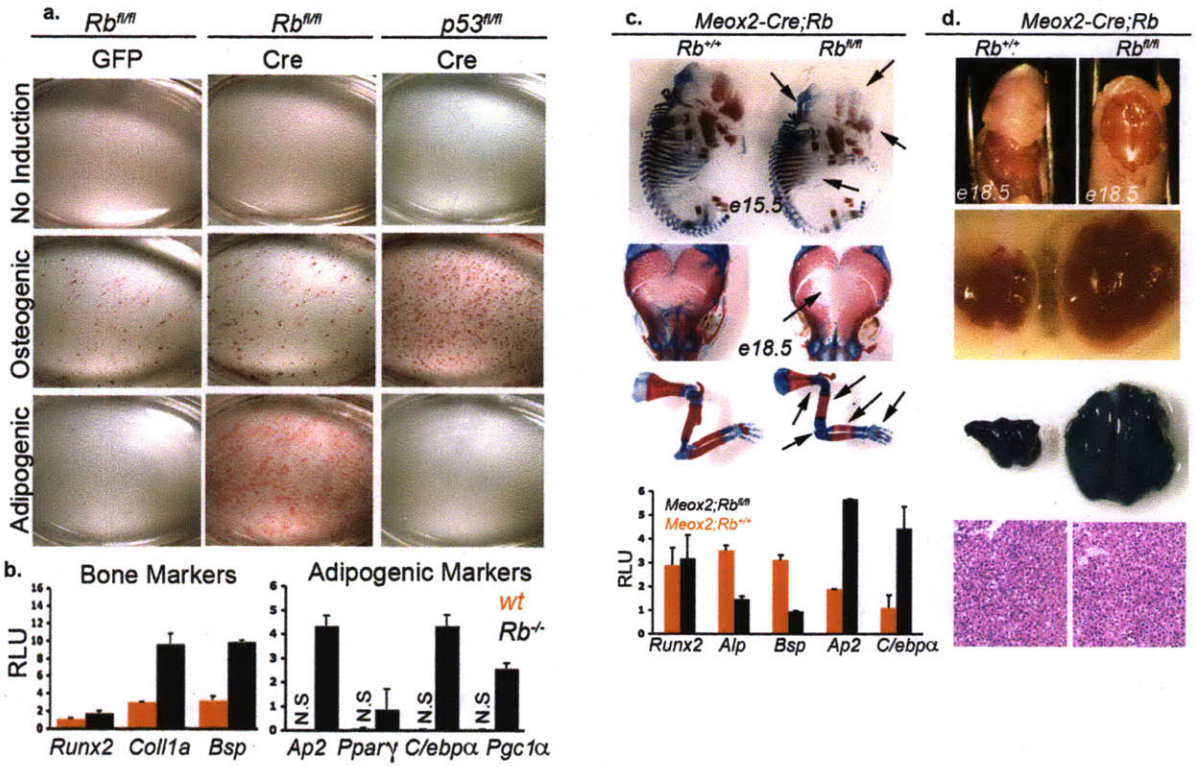


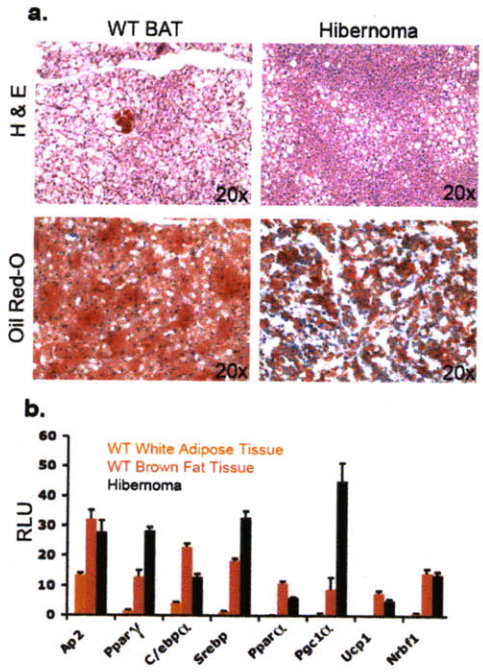
Figure 6: *Rb* maintains the osteoblastic fate commitment in normal osteoblasts and regulates fate choice during normal development *in vivo*.

a, Calvarial osteoblasts were prepared from e18.5 $Rb^{fl/fl}$ or $p53^{fl/fl}$ embryos and infected with Ad-GFP or Ad-Cre at P1. Five days later, the cells were induced with differentiation media and then assayed for osteogenesis and adipogenesis at 0, 14 and 25 days by staining with Alizarin Red and Oil-Red-O. A representative timepoint (25 days) is shown. **b,** qPCR was also used to assess osteogenic and adipogenic markers in the un-induced $Rb^{fl/fl}$ (wt) versus $Rb^{fl/fl}$+Ad-Cre ($Rb^{-/-}$) osteoblasts. Bars represent the mean of three independent experiments (+/- SD). **c**, Alizarian Red (bone mineralization) and Alcian Blue (cartilage) staining of e15.5 skeletons (top panel), e18.5 calvaria (middle panel) and e18.5 limbs (botton panel) from *Meox2-Cre;*$Rb^{+/+}$ and *Meox2-Cre;*$Rb^{fl/fl}$ littermate embryos. Arrows mark visible skeletal defects. qPCR was used to assess osteogenic (*Runx2*, *Alp*, and *Bsp*) and adipogenic (*Ap2* and *C/ebpα)* markers in mRNA extracted from the calvarial bones of e18.5 *Meox2-Cre;*$Rb^{+/+}$ and *Meox2-Cre;*$Rb^{fl/fl}$ embryos. Bars show the mean of three embryos arising in two independent crosses (+/- SD). **d**, Brown adipose tissue (BAT) was dissected from the backs of *Meox2-Cre;*$Rb^{fl/fl}$ embryos (n=10) and their *Meox2-Cre;*$Rb^{+/+}$ littermate controls. All 10 showed a dramatic expansion of the brown fat compartment. A representative example is shown (upper two panels). Introduction of the LSL-LacZ reporter into this model, and LacZ staining confirmed equal, widespread expression of Cre in the control and *Rb* mutant BAT (third panel). H+E staining of BAT (bottom panel).

(Wu *et al.*, 2003). We isolated wildtype (*Meox2-Cre;Rb*$^{+/+}$) and *Rb* mutant (*Meox2-Cre;Rb*$^{fl/fl}$) littermates at e15.5 and e18.5 and examined both bone and brown fat development. First, there was a significant reduction in the level of calcified bone matrix in both the calvaria and long bones of *Rb* mutant versus wildtype embryos (Berman *et al.*, 2008b; Figure 6c). Moreover, qPCR analysis established that *Runx2* mRNA was present at appropriate levels in the *Rb* mutant e18.5 calvarial osteoblasts, but there was a downregulation of other bone markers and a clear upregulation of fat-associated mRNAs (Figure 6c). In parallel, we found that the level of brown fat was dramatically increased in the e18.5 *Rb* mutant versus the wildtype controls (Figure 6d and Supplementary Figure 4). Thus, *Rb* loss in an, otherwise wildtype, embryo impairs bone differentiation and expands the fat compartment.

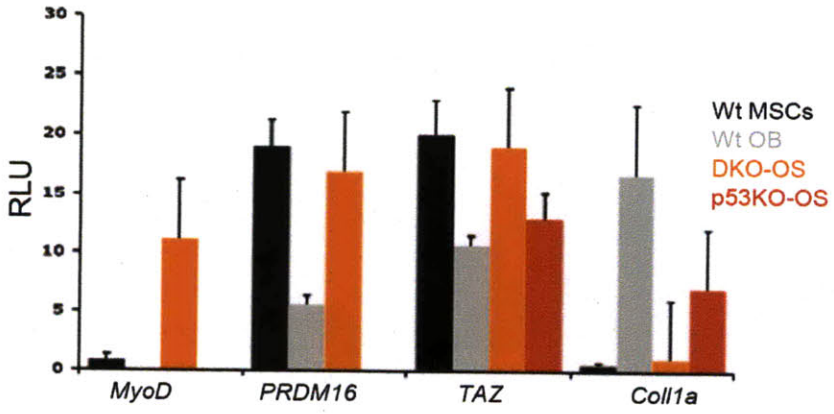
Our data establish a clear role for pRB in determining the fate choice of mesenchymal progenitors and the lineage commitment of pre-osteoblasts. This occurs both *in vitro* and *in vivo* and irrespective of whether these cells are transformed or otherwise wildtype. *In vivo*, *Rb*-loss favours adipogenesis over osteogenesis to the extent that it can reduce the levels of calcified bone and greatly increase the levels of brown fat. Moreover, *Rb*-loss in pre-osteoblasts is sufficient to disfavour commitment to the osteogenic state and restore multipotency. It is possible that *Rb* loss allows expansion of a rare multipotent progenitor population that exist within the pre-osteoblast compartment. Alternatively, *Rb* loss could be actively reprogramming the pre-osteoblasts by driving either trans-differentiation to the adipogenic lineage or true

dedifferentiation to the multipotent progenitor stage. Between the two reprogramming models we favour de-differentiation based on both the expression of multi-lineage differentiation markers in the DKO OS cells (Supplementary Figure 4) and the broadening of the tumor spectrum from solely oesteosarcomas in the *Osx-Cre;p53*$^{fl/fl}$ animals to include not only osteosarcomas and hibernomas but also sarcomas in the *Osx-Cre;Rb*$^{fl/fl}$*;p53*$^{fl/fl}$ mice. Finally, our data offers potential insight into the cell of origin for osteosarcomas. Specifically, given the high frequency of *RB-1* mutations in human osteosarcoma, we were surprised to find that *Rb* mutation predisposes mesenchymal cells away from the osteoblastic state. Given this finding, we speculate that *RB-1* mutant osteosarcomas are likely to arise from more committed osteoblastic lineages than from uncommitted mesenchymal progenitors. In this setting, *RB-1* loss could enable de-differentiation and thereby synergize with other mutations to promote tumorigenesis.

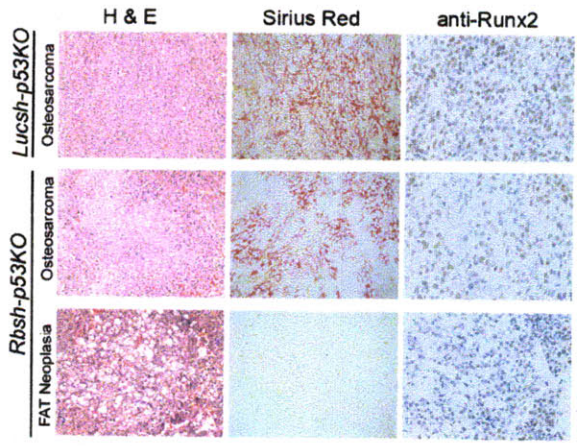


Supplementary Figure 1: Characterization of brown fat adipogenic sarcomas (Hibernomas).

a, H&E staining of brown adipose tissue (WT BAT) and hibernoma (upper panel). To confirm the adipogenic nature of the HIB, frozen sections for both WT BAT and HIB were stained with Oil Red-O, which marks accumulation of lipid droplets. **b**, The expression profile of HIB for different adipogenic marker was compared to that of white and brown adipose tissues. This data clearly shows that the adipogenic sarcomas observed in our mouse model are indeed from the brown fat compartment.

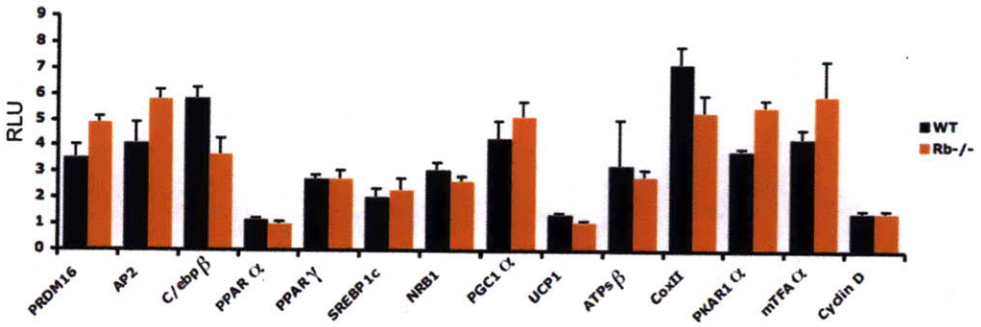


Supplementary Figure 2: Expression of multilineage specific markers in DKO-OS cell lines.
Different lineage specific markers for mesenchymal lineages were analyzed by qPCR in DKO-OS cell lines and compared to wt MSCs, wt osteoblasts and the p53KO OS cells. MyoD is a myogenic specific transcription factor, PRDM16 is a molecular determinant for the brown fat/skeletal muscle lineages, TAZ is a molecular determinant for the bone/fat lineages, and Coll1a is a bone specific factor. This expression analysis showed that the p53KO OS cells closely resemble the committed osteoblasts, consistent with the stage at which the Cre was expressed and therefore *p53* deleted. In contrast, the *DKO OS* cells more closely resembled the multipotent progenitors, consistent with the notion that these cells have undergone de-differentiation.



Supplementary Figure 3: *Rb*-loss promotes tumor-cell plasticity *in vivo*.

Tumors arising from *Lucsh-p53KO* and *Rbsh-p53KO* cell lines were stained with Sirius Red, which marks collagen fibbers and immuno-stained for the bone-specific transcription factor Runx2. Both collagen and Runx2 were clearly detected in all *Lucsh-p53KO* cells, but low levels of collagen and Runx2 were present in *Rbsh-p53KO* cells. In addition, neither Runx2 nor Collagen staining was observed in the fat neoplasia observed in the *Rbsh-p53KO* tumors.



Supplementary Figure 4: Expression profile of *Rb*-mutant and –wt brown adipogenic tissue.

The brown fat compartment of e18.5 *Meox2-Cre;Rb*$^{+/+}$ and *Meox2-Cre;Rb*$^{fl/fl}$ embryos was collected and analysed for the expression of different adipogenic markers using qPCR. This confirms that the adipogenic nature of the tissues analyzed for this study.

Experimental Procedures

Animal maintenance and histological analyses

Animal procedures followed protocols approved by MIT's Committee on Animal Care. The *Rb*$^{fl/fl}$, *p53*$^{fl/fl}$, *Osx1-GFP::Cre, Prx1-Cre, Rosa26-LSL-lacZ* (REF), and *Meox2-Cre* animals were maintained on a mixed genetic background. Skeletal stainings were conducted as described. The transplant assays were conducted in NOD/SCID mice using 10^4 cells. Tissues were fixed in PBS with 3.7% formaldehyde. Prior to paraffin sectioning, soft tissues were dehydrated via an ethanol series, while bone-containing tissue was decalcified in 0.46M EDTA, 2.5% Ammonium Hydroxide pH 7.2.

Mouse genotyping

The *Rb* conditional band was detected using the primers 5'lox: 5'-CTCTAGATCCTCTCATTCTTC-3' and 3'lox: 5'-CCTTGACCATAGCCCAGCAC-3'. Primer Rbcre3.2 (5'-GGTTAATGAAGGACTGGG-3') was used in conjunction with primer 5'lox to detect the recombined band. 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'.

Tumor monitoring and analysis

The criteria for euthanasia (by CO2 inhalation) were a total tumor burden of $2cm^3$, tumor ulceration/bleeding, signs of infection, respiratory distress, impaired mobility, ≥20% reduction in body weight or general cachexia. All tissues were collected and hip bones, femurs and tibias were separated and fixed overnight in PBS with 3.7% formaldehyde. Soft tissues were transferred into 70% ethanol and dehydrated via an ethanol series prior to embedding in paraffin for sectioning. Tissues containing bone was either decalcified in 0.46M EDTA, 2.5% Ammonium Hydroxide pH 7.2 for two weeks then processed for paraffin sectioning. All paraffin embedded sections were cut at 5μm, dewaxed and stained with H&E. Sirius red staining was performed by treating sections briefly stained with hematoxylin with 0.1% Sirius red in saturated picric acid (Electron Microscopy Sciences) for one hour, washing in 5% v/v glacial acetic acid and then dehydration in ethanol/xylene prior to mounting.

Generation of osteosarcoma cell lines

Osteosarcomas were dissected, minced, filtered through a 70μm filter, and plated in normal growth medium (10% FBS in DME, 1% P/S, L-glutamine) to generate the OS cell lines. Cells were passaged as they reached confluence. For RNA purification, cells were rinsed 2x with PBS, and RNA extraction was performed using RNAeasy kit (Quiagen). First-strand cDNA was transcribed from 1 μg of RNA using Superscript III reverse transcriptase (Invitrogen) following the manufacturer's instructions. Quantitative RT-PCR with 20 to 100 ng cDNA was done using SYBR Green (Applied Biosystems). Reactions were run on the ABI Prism 7000 Sequence Detection System and analyzed using the 7000 SDS

software. Primers used for qPCR are shown in Supplementary Table 1. Knock-down of *Rb* in the *p53KO-OS* cells was achieved using the pMLP-miR30-based shorthairpin (*Rb* targeted sequence: CACGGACGTGTGAACTTATATA). Adenoviruses expressing Cre or GFP were provided by the U. of Iowa Gene Transfer Vector Core. For immunoprecipitations and immunoblotting, proteins were extracted with a Triton X-100 based buffer and quantified by the BCA assay reagent (Pierce, Inc). Antibodies were from Santa Cruz Biotechnology [pRb (H-153), E2F1 (C-20), and E2F4 (C-20)], BD Pharmingen (pRb), Ambion (GAPDH) and MBL (Runx2). Dual luciferase assays were performed as described by the manufacturer (Promega). The Runx2 reporter p6OSE2-Luc and control p4Luc were provided by Dr. Gerard Karsenty.

Immunohistochemistry (IHC)

Runx2 IHC was performed using a modified citric acid unmasking protocol. Briefly, slides were deparaffinized in xylene and rehydrated through ethanol. Slides were boiled for 30 min in citrate buffer, pH 6.0, and then cooled in running tap water. Slides were then washed in PBS for 5 min followed by inactivation of endogenous peroxidases by incubation 0.5% H_2O_2 in methanol. Slides were blocked in 10% Goat Serum for 1 h at room temperature. Primary antibody (MBL anti-Runx2 Clone 8G5) was diluted 1:200 in PBS 0.15% Triton and incubated overnight at 4 °C. The following day, slides were washed three times in PBS. Secondary antibodies (Vectastatin ABC kits, Vector laboratories) were diluted 1:500 in PBS containing 0.4% Goat Serum and detected using a DAB substrate (Vector Laboratories). All samples were counterstained with hematoxylin.

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 of 0.015% Alcian blue 8GX (Sigma), 0.005% alizarin red S (Sigma), and 5% glacial acetic acid in ethanol at 37°C for 2 days and at room temperature for a one more day. Tissue was cleared in 1% potassium hydroxide for several days and then stored in glycerol.

Calvarial Osteoblasts Preparation and Culture

Calvaria from embryonic day 18.5 embryos were removed and carefully cleaned in sterile PBS from contaminating tissue. Then treated with several rounds of collagenase/trypsin digestion at 37°C, and plated onto six-well plates for 2 days in αMEM with 10% fetal bovine serum and penicillin/streptomycin. For differentiation, $3.5x10^5$ cells were plated onto a well of a 6-well tissue culture plates. Upon reaching confluence, calvarial osteoblasts were treated with medium supplemented with 50 μg/mL of ascorbic acid and 10 mmol/L of β-glycerol-phosphate. Adenovirus (University of Iowa Gene Transfer Vector Core) was added to the medium at 100 plaque-forming units per cell and washed away 24 h later. To assay for calcium deposits, plates were stained with 1% alizarin red S solution (pH 5.0).

Chromatin Immunoprecipitation assay

Protein complexes were cross-linked to DNA in living nuclei by adding formaldehyde (Sigma, Inc.) to give a final concentration of 1%. After incubation for 10 min at 37 °C, crosslinking was stopped by addition of glycine to a final concentration of 0.125 M for 5 min. Cross-linked cells were washed twice with PBS containing PMSF 1mM (phenylmethylsulfonyl fluoride), scraped and pelleted. Nuclei were extracted with a 20mM Tris pH 8, 3mM MgCl2, 20 mM KCl buffer containing protease inhibitors, pelleted by microcentrifugation and lysed by incubation in SDS lysis buffer (1% sodium dodecyl sulfate, 10 mM EDTA, 50 mM Trischloride pH 8.1), containing protease inhibitors. The resulting chromatin solution was sonicated to generate 500-1000 bp DNA fragments. After microcentrifugation, the supernatant was diluted 1:10 with a dilution buffer (0.01% sodium dodecyl sulfate, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Trischloride pH 8.1, 167 mM NaCl, containing protease inhibitors), precleared with blocked protein A-positive Staph cells (Santa Cruz, Inc), and divided into aliquots. Five micrograms of the indicated antibodies was added to each aliquot and incubated for 12 to 16 hours at 4°C with rotation. Antibody-protein-DNA complexes were isolated by immunoprecipitation with blocked protein A-positive Staph A cells. Following extensive washing, bound DNA fragments were eluted and analyzed by Quantitative RT-PCR using primers shown in Supplementary Table 2.

Supplementary Table 1: Real Time PCR Primers

Gene	Forward Primer	Reverse Primer
Alkaline Phosph.	TCT CCA GAC CCT GCA ACC TC	CAT CCT GAG CAG ACC TGG TC
Collagen 1a1	CGA GTC ACA CCG GAA CTT GG	GCA GGC AGG GCC AAT GTC TA
Osteocalcin	CTC TGT CTC TCT GAC CTC ACA G	CAG GTC CTA AAT AGT GAT ACC G
Osteopontin	TGC TTT TGC CTG TTT GGC AT	TTC TGT GGC GCA AGG AGA TT
Runx2	TGA GAT TTG TGG GCC GGA	TCT GTG CCT TCT TGG TTC CC
Ap2	ATCCCTTTGTGGGAACCTGGAA	ACGCTGATGATCATGTTGGGCT
C/ebp α	CAAGAACAGCAACGAGTACCG	GTCACTGGTCAACTCCAGCAC
Pparγ	GAGCTGACCCAATGGTTGCTG	GCTTCAATCGGATGGTTCTTC
Srebp-1c	GGAGCCATGGATTGCACATT	GCTTCCAGAGAGGAGGCCAG
Ucp-1	AGCCGGCTTAATGACTGGAG	TCTGTAGGCTGCCCAATGAAC
Pgc-1	GTCCTCACAGAGACACTGGA	TGGTTCTGAGTGCTAAGACC
Nbrf-1	CGGCACCTAGCGCCCGG	CGGCACCTAGCGCCCGG
MyoD	CGCCACTCCGGGACATAG	GAAGTCGTCTGCTGTCTCAAAGG
Prdm16	GACCACGGTGAAGCCATTC	GCGTGCATCCGCTTGTG
Taz	GTCACCAACAGTAGCTCAGATC	AGTGATTACAGCCAGGTTAGAAAG
Gapdh	CAAGGTGGCAGAGGCCTTT	TCCAGCTGCTCAATGGACGCATTT

Supplementary Table 2: CHIP Primers

Promoter	Forward Primer	Reverse Primer
Pparγ (E2f Proximal Site)	ACGCGGAAGAAGAGACCT	TCCTGTCAGAGTGTGACTTCTCCT
Pparγ (E2f Distal Site)	TCGCACTCAGAGCGGCAG	AGGTCTCTTCCGCGTCCCT
Coll1a1 (Runx2 site)	TGCTTCCACGTTTACAGCTCTAAAG	GTCAGGAAAGGGTCATCTGTAGTCC
Osteocalcin (Runx2 Site)	GAGAGCACACAGTAGGAGTGGTGGAG	TCCAGCATCCAGTAGCATTTATATCG

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Chapter 4

Deregulation of the pRb-E2f pathway contributes to tumorigenesis by antagonizing terminal differentiation.

Eliezer Calo and Jacqueline A. Lees.

The author contributed to all the text, graphs, and figures.

Abstract

Sarcoma of soft tissues and bone are mesenchymal tumors that are fatal in approximately one-third of the patients. Genetic analyses of sarcomas have linked inactivation of the *RB-1* tumor suppressor gene with the pathogenesis these malignancies. We have previously study the role of *Rb* in sarcomas and found that *Rb*-loss modulate the biology of tumors arising from *p53* mutant animals in a dosage dependent manner not only to enhance tumor progression, but also to alter the tumor spectrum. To further understand the role of the *Rb* pathway in sarcomas we focused on the activating E2fs (E2f1, 2, & 3), which are cell cycle regulators and direct downstream targets of pRb. Specifically, we studied the role of the major activating E2f, E2f1, in tumors initiated from *Rb;p53*-defficient mesenchymal progenitor cells. In this context, complete inactivation of *E2f1* suppresses mesenchymal tumorigenesis. Importantly this phenotype was not due to reduced E2f activity as loss of *E2f3a*, either alone or in combination with *E2f1*, did not alter tumor development. To investigate whether this role for *E2f1* is cell type dependent we inactivated *E2f1* in the osteoblastic lineage, which is a more committed mesenchymal compartment. To do that we crossed the *Osx1-Cre;*$Rb^{fl/fl}$*;*$p53^{fl/fl}$ animals into an *E2f1*-null background. In this context we found that *E2f1*-loss suppresses osteosarcomagenesis in a dosage dependent manner and altered both physiological tumor distribution and tumor spectrum. In addition, we observed that *E2f1*-loss altered the osteogenic differentiation program as judged by the degree of differentiation of the tumors and the enhanced differentiation potential of *E2f1*-null calvarial osteoblasts. Together our data suggest that inactivation of the pRb pathway antagonizes cellular differentiation and therefore its inactivation is more critical in committed progenitor cells than in the stem cell compartment.

Introduction

The retinoblastoma gene, *RB-1*, was the first identified tumor suppressor gene and its functionally inactivated in one third of all human cancers (Weinberg, 1992). In particular, *RB-1*-loss is strongly associated with the development of retinoblastoma, osteosarcoma, and small cell lung carcinoma (Burkhart and Sage, 2008). The protein product of the *RB-1* gene, pRB, is thought to suppress tumorigenesis primarily by restraining cellular proliferation (Burkhart and Sage, 2008). pRB does so by binding to the E2F family of transcription factors and prevents them from activating genes required for cell cycle progression (Trimarchi and Lees, 2002). pRB-mediated repression of cell cycle genes not only requires physical interaction with the E2Fs, but also the recruitment and/or interaction with chromatin remodeling enzymes (Zhu, 2005). This indicates that pRB acts as a molecular scaffold to ensure proper cell cycle exit. The stability of the pRB-E2F complexes is controlled by phosphorylated. pRB can be hyperphosphorylation by the activation of Cyclin/CDK complexes. These phosphorylation events impair pRb's ability to bind the E2Fs, allowing free E2Fs to transcriptionally activate genes require for cell cycle entry (Weinberg, 1995). This is the canonical pathway by which pRB regulates the cell cycle and its thought to be pRB's main barrier against tumor development.

Genetic analyses of *RB-1*-deficient tumors have provided insights into the role of pRB as a tumor suppressor. In patients with familial retinoblastoma, which is characterized by inheritance of a defective copy of the *RB-1* gene, loss of the remaining wild type *RB-1* allele was thought to be the driving force for tumor

development. Although this is generally true, recent studies have shown that these tumors also harbor inactivating mutations in the TP53 pathway (van de Rijn and Fletcher, 2006; Lefevre *et al.*, 2001). This indicates that a second inactivating mutation in the *RB-1* gene might be insufficient to initiate or maintain the tumor phenotype and that additional oncogenic events are required. Unfortunately, cases of familial predisposition syndromes are rare which make difficult the continuity of these studies in human patients. Studies from mouse models for familial predisposition syndromes have not provided sufficient insights into the role of *Rb* in tumorigenesis. This is in part because homozygous mutant animals for *Rb* are not viable and in heterozygozity they develop pituitary tumors, never osteosarcoma nor retinoblastoma (Jacks *et al.*, 1992; Clarke *et al.*, 1992). Combined, $Rb^{+/-};p53^{-/-}$ animals developed tumor fasters than the single mutant animals and loss-of-heterozygosity for the *Rb* gene was observed (Williams *et al.*, 1994). This clearly indicates that there is a selective pressure for inactivating the *Rb* pathway in a *p53* deficient tumorigenic background. Unfortunately, this system is not feasible to study the mechanisms by which *Rb*-loss promotes tumorigenesis because tumor formation is unpredictable and these animals die mostly from hematopoietic tumors and/or neuroendocrine malignancies.

To better understand the role of the *Rb* pathway in a disease-relevant tumor model we have developed a feasible and clinically relevant mouse model for osteosarcoma that is potentiated by loss of *Rb* (Berman *et al.*, 2008a; Walkley *et al.*, 2008). To generate this mouse model we used a targeted conditional approach in which the tumor suppressors genes *Rb* and/or *p53* were inactivated

either in mesenchymal progenitor cells or in committed osteoblasts. We have reported that in osteoblasts deletion of *Rb* alone was not sufficient to initiate osteosarcomas. In contrast, *p53* mutant animals developed osteosarcomas with short latency and complete penetrance. However, *Rb* loss strongly synergized with *p53*-inactivation: it greatly accelerated tumor development and it expanded the tumor spectrum from osteosarcoma in the *p53* single mutants to multiple soft tissue sarcomas in the *Rb;p53 DKO* (Berman *et al.*, 2008b). Interestingly, we observed a similar reprogramming effect in mesenchymal progenitor cells. Specifically, *Rb* acted in a dosage dependent manner to modulate the spectrum of tumors arising from *p53* mutant mesenchymal progenitor cells: osteosarcoma predominated in the presence of *Rb*, while *Rb* loss strongly favored brown fat tumors (Calo *et al.*, 2010).

The increased tumor spectrum observed in the absence of pRb raised the possibility that pRb's tumor suppressive function goes beyond cell cycle control and that control of cellular differentiation might be part of pRb's tumor suppressive network. This is plausible because pRB has been shown to modulate cellular differentiation *in vitro*, not only by promoting cell cycle arrest upon terminal differentiation, but also by binding to tissue specific transcription factors to either enhance or repress their transcriptional activity (Galderisi *et al.*, 2006). The role of pRb in cellular differentiation has also been study *in vivo* using *Rb/E2fs* compound mutant animals. These studies have shown that some of the phenotypes observed in the *Rb*-mutant embryos, including the proliferation defects, can be rescued by inactivation of the activating E2fs (E2f1, 2, and 3).

For example, we have shown that the ossification defects observed in the *Rb*-mutant embryos can be partially rescued by deletion of *E2f1* (Berman *et al.*, 2008b). In this context, *E2f1* loss reduces the ectopic proliferation observed in the *Rb*-mutant osteoblasts. From these observations we concluded that pRb-mediated inhibition of E2f1 is important for osteoblasts differentiation, at least in part by enforcing cell cycle exit. This is consistent with recent reports showing that *in vivo* the activating E2fs are dispensable for cellular proliferation, but together with pRb, they are required for the maintenance of the differentiated state (Chong *et al.*, 2009; Chen *et al.*, 2009). Moreover, pRb can aid differentiation in an E2f-independent manner. For example, during osteogenesis pRb acts as a cotranscriptional activator for Runx2 (Berman *et al.*, 2008b; Thomas *et al.*, 2001), which is the master regulator of the bone lineage. Taken together, these studies clearly indicate that pRb can regulate cellular differentiation in a cell cycle dependent- and independent-manners. Based on these observations, we hypothesized that during tumorigenesis inactivation of the pRB pathway enhances tumor progression by antagonizing cellular differentiation.

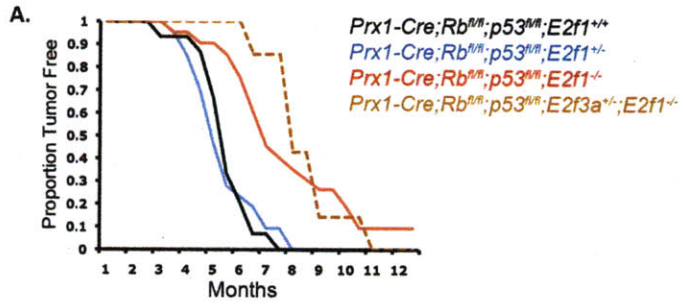
To test this hypothesis *in vivo*, we have analyzed the role of the activating E2fs, which are direct downstream effectors of pRb, in mesenchymal tumorigenesis. Since we have shown that *E2f1*-loss partially rescued the bone defects in the *Rb*-mutant embryos (Berman *et al.*, 2008b), we also wanted to investigate whether inactivation of *E2f1* will affect the development of bone tumors. Here we report that *E2f1* role during sarcomagenesis is cell type

dependent. In mesenchymal stem cells, we found that *E2f1*-loss increases the tumor free survival of the animals, but it does not alter tumor spectrum. However, in committed osteoblasts, *E2f1*-loss increases the tumor free survival of the animals in a dosage-dependent manner and it modulates the differentiation state of the tumors. These observations reflect, at least in part, a novel role for the pRb-E2f pathway in modulating the differentiation during tumorigenesis. Overall, our study supports a model in which inactivation of the pRb pathway alters the differentiation potential of transformed cells in an E2f1-depedent manner and this is more critical in committed progenitor cells than in the stem cell compartment.

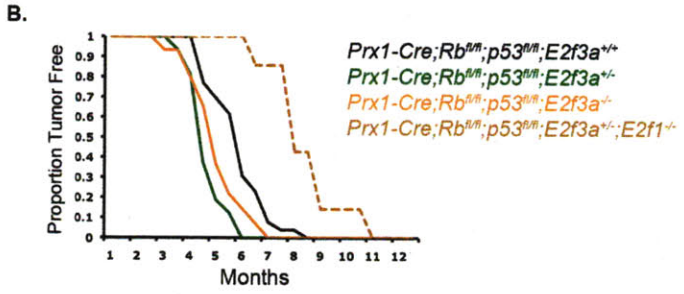
Results

The activating E2fs have non-overlapping roles during to sarcomagenesis

It is known that *Rb* loss synergizes with *p53* mutations during tumor development. We have confirmed these results, in the mouse, by studying the role of *Rb* and *p53* in mesenchymal tumors (Berman *et al.*, 2008a; Calo *et al.*, 2010). We have reported that *Rb*-loss increases both the tumorigenic potential and the tumor spectrum of *p53*-deficeint mesenchymal progenitor cells. Thus, we wanted to explore how different components of the *Rb* pathway contribute to this synergy. In this study, we focused on the E2f-family of transcription factors, which are cell cycle regulators and direct downstream targets of pRb. We asked whether the major activating E2f, E2f1, would impact the kinetics at which sarcomas develop and/or the *Rb*-dependent change in tumor fate. To do this, we used our previously characterized mouse model of sarcoma, which is driven by *Prx1-Cre*-mediated inactivation of *Rb* and *p53* in mesenchymal progenitor cells. Thus, we crossed the *Prx1-Cre;*$Rb^{fl/fl}$*;*$p53^{fl/fl}$ (Prx1-DKO) animals into an *E2f1*-null background to generate the *Prx1-Cre;*$Rb^{fl/fl}$*;*$p53^{fl/fl}$*;*$E2f1^{-/-}$ (*Prx1-f1TKO*) mutant animals (Figure 1A). These animals came at the expected mendelian ratios and showed no detectable developmental defects (data not shown). We compared the tumor free survival of *Prx1-f1TKO* animals to the corresponding *Prx1-DKO* and *Prx1-Cre;*$Rb^{fl/fl}$*;*$p53^{fl/fl}$*;*$E2f1^{-/+}$ genotypes. Kaplan Meier analyses revealed that the complete loss of *E2f1* does impacted the kinetics at which sarcomas developed, but does not altered the spectrum of tumors observed in the *Prx1-DKO* mutant animals (Figure 1A). Specifically, *Prx1-f1TKO* animals showed a



Genotype	Animals #	Osteosarcoma (%)	Hibernoma (%)	Survival Ave.±S.D.
Prx1-Cre;Rb$^{fl/fl}$;p53$^{fl/fl}$;E2f1$^{+/+}$	15	29	71	156±30
Prx1-Cre;Rb$^{fl/fl}$;p53$^{fl/fl}$;E2f1$^{+/-}$	22	13	87	153±37
Prx1-Cre;Rb$^{fl/fl}$;p53$^{fl/fl}$;E2f1$^{-/-}$	21	29	71	195±55



Genotype	Animals #	Osteosarcoma (%)	Hibernoma (%)	Survival Ave.±S.D.
Prx1-Cre;Rb$^{fl/fl}$;p53$^{fl/fl}$;E2f3a$^{+/+}$	13	19	81	167±29
Prx1-Cre;Rb$^{fl/fl}$;p53$^{fl/fl}$;E2f3a$^{+/-}$	16	0	100	133±19
Pre-Cre;Rb$^{fl/fl}$;p53$^{fl/fl}$;E2f3a$^{-/-}$	15	14	86	144±32

Figure 1: *E2f1*, but not *E2f3a*, contributes to sarcoma genesis.

Kaplan Meier plots of the *Prx1-Cre;Rb$^{fl/fl}$;p53$^{fl/fl}$* mutant animals crossed into either an *E2f1* (**A**), an *E2f3a* (**B**) or an *E2f3a$^{+/-}$;E2f1$^{-/-}$* mutant background. Animals were monitores for tumor development for up to 12 months. Tables contain the number of animals analyzed and the tumor distribution associated to each genotype. The dashed curve (brown dashed lines) represent the same cohort of animals in both survival curve (*Prx1-Cre;Rb$^{fl/fl}$;p53$^{fl/fl}$;E2f3a$^{+/-}$;E2f1$^{-/-}$*, n=7 animals).

dramatic increase in the tumor free survival (195±55) when compared to *Prx1-Cre;Rb*$^{fl/fl}$*;p53*$^{fl/fl}$*;E2f1*$^{-/+}$ (153±37) and/or *Prx1-DKO* (156±30) animals. Moreover, regardless of the starting genotype, these animals were predisposed to hibernomas (brown fat tumors) and osteosarcomas (Figure 1A). Thus, we concluded that E2f1 cooperate with mutation in the *Rb* and *p53* pathways to promote sarcomagenesis.

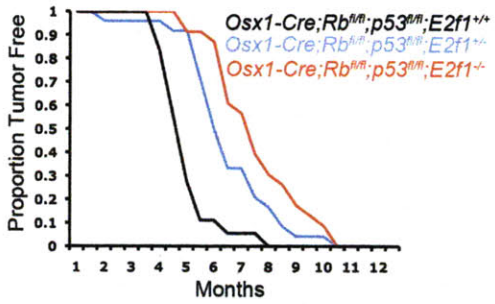
We then explored whether the increased tumor free survival observed in our tumor model was specific to *E2f1*-loss, or whether it was due to a reduction in total E2f activity. The rational behind this experiment is that it has been shown that activating E2fs (E2f1, 2, and 3a) can compensate for each other during normal mouse development (Trimarchi and Lees, 2002). However it is unclear whether this compensation also occurs during tumorigenesis. To address this issue we crossed the *Prx1-DKO* animals into an *E2f3a*-null background. We decided to use *E2f3a*-null animals because both *E2f1* and *E2f3a* are known to have compensatory roles during mesenchymal tissue development (Danielian *et al.*, 2008; Tsai *et al.*, 2008), which is the tissue from were sarcomas originate (Coindre, 2006). To our surprise, *E2f3a*-loss has no effect during sarcomagenesis as *Prx1-Cre;Rb*$^{fl/fl}$*;p53*$^{fl/fl}$*;E2f3a*$^{-/-}$ animals developed tumors at the same rate as the *Prx1-DKO*s (Figure 1B). This data clearly indicates that *E2f1* and *E2f3a* have non-overlapping roles during mesenchymal tumorigenesis. Moreover, it shows that *E2f1* has an oncogenic role and cooperates with mutations in the *Rb* and *p53* pathways to drive mesenchymal tumors. To further validate this hypothesis we have generated the *Prx1-Cre;Rb*$^{fl/fl}$*;p53*$^{fl/fl}$*;E2f3a*$^{+/-}$

;*E2f1*$^{-/-}$ and the *Prx1-Cre;Rb*$^{fl/fl}$;*p53*$^{fl/fl}$;*E2f3a*$^{-/-}$;*E2f1*$^{-/-}$ mutant animals. Consistent with our previous observations, *Prx1-Cre;Rb*$^{fl/fl}$;*p53*$^{fl/fl}$;*E2f3a*$^{+/-}$;*E2f1*$^{-/-}$ animals developed tumors at the same rate as the *Prx1-f1TKO* mutant animals (Figure 1A-B). As expected, Prx1-*Cre;Rb*$^{fl/fl}$;*p53*$^{fl/fl}$;*E2f3a*$^{-/-}$;*E2f1*$^{-/-}$ did not survive past 3 moths of ages due to severe developmental defects (Tsai *et al.*, 2008; Danielian *et al.*, 2008). These results strongly suggest that *E2f1* role during sarcomagenesis is not simply due to reduced E2f activity. Thus, we concluded that, in the context of sarcomas, E2f1 is the major oncogenic E2fs. In addition, we have dissected that E2f1 and E2f3a have overlapping roles during development, but not during tumorigenesis.

E2f1 oncogenic activity is more critical in committed progenitor cells

Given that in mesenchymal progenitor cells *E2f1* promotes tumor development, we wanted to explore whether *E2f1* role during sarcomagenesis is cell type dependent. Since mesenchymal stem cells are the cell of origin for the bone, fat, and myogenic lineages (Minguell *et al.*, 2001), we decided to investigate the role of *E2f1* in a more committed mesenchymal compartment. To address this we explored the consequences of inactivating *E2f1* in *Rb;p53* double mutant osteoblasts *in vivo* (Figure 2). We have chosen bone over other mesenchymal tissue because we have previously studied the role of *E2f1*-loss in *Rb*-mutant osteoblasts and demonstrated that the ossification defects observed in these embryos can be partially rescued by deletion of *E2f1* (Berman *et al.*, 2008b). This provided us with a unique experimental system to dissect the

A.



Genotype	Animals #	Osteosarcoma (%)	Hibernoma (%)	Survival Average±S.D.
Osx1-Cre;Rb$^{fl/fl}$;p53$^{fl/fl}$;E2f1$^{+/+}$	19	65	35	144±28
Osx1-Cre;Rb$^{fl/fl}$;p53$^{fl/fl}$;E2f1$^{+/-}$	18	100	0	190±54
Osx1-Cre;Rb$^{fl/fl}$;p53$^{fl/fl}$;E2f1$^{-/-}$	23	86	14	221±48

B.

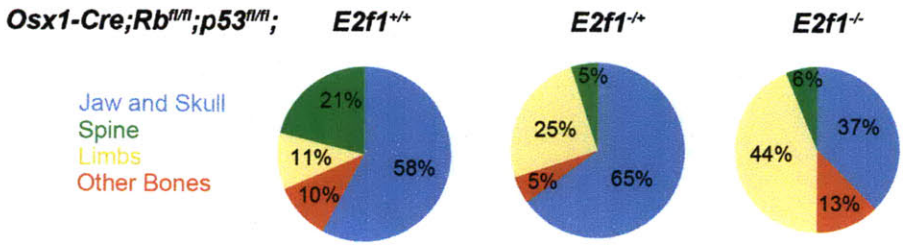


Figure 2: *E2f1* modulates osteosarcoma tumor development in a dosage dependent manner.

(**A**) Kaplan-Meier plot of the indicated genotypes carrying the *Osx1::GFP-Cre* transgene up to 12 months of age. The table contains the genotype, the number of animals analyzed, and tumor spectrum. (**B**) Pie charts represent the distribution of osteosarcomas observed among the different genotypes.

contribution of E2f1 during osteoblast differentiation and transformation. To do this we used the *Osx1-Cre* transgene, which expresses Cre recombinase specifically in committed osteoblasts (Rodda and McMahon, 2006). We and others have shown that *Osx1-Cre;Rb$^{fl/fl}$;p53$^{fl/fl}$* (*Osx1-DKO*) animals are fully viable and develop tumors by six months of age (Berman *et al.*, 2008a; Walkley *et al.*, 2008).

We crossed the *Osx1-DKO*s into an *E2f1*-null background to generate the *Osx1-Cre;Rb$^{fl/fl}$;p53$^{fl/fl}$;E2f1$^{-/-}$* (*Osx1-f1TKO*), as well as the *Osx1-Cre;Rb$^{fl/fl}$;p53$^{fl/fl}$;E2f1$^{-/+}$* and the *Osx1-DKO* compound mutant animals (Figure 2A). Similar to what we observed in the *Prx1-f1TKO* animals we found that even in committed osteoblasts *E2f1* function still oncogenic as *Osx1-f1TKO* animals showed the highest average survival rate (221±48). To our surprise and in contrast to the *Prx1-Cre* model, *E2f1* modulates the tumorigenic potential of the *Osx1-DKO* animals in a dosage dependent manner. Specifically, the average survival rate for the *Osx1-Cre;Rb$^{fl/fl}$;p53$^{fl/fl}$;E2f1$^{-/+}$* animals was higher (190±54) than the one for the *Osx1-DKO*s (144±28 and Figure 2A). These results strongly argue that *E2f1* transforming activity is more critical in committed progenitor cells than in the stem cell compartment.

In addition to its oncogenic activity, we found that *E2f1*-loss has a dramatic effect in both primary tumor formation and physiological tumor location. As expected, osteosarcoma was the predominant tumor type in all the animals analyzed (Figure 1A). Interestingly, the development of osteosarcomas in the *Osx1-DKO* and the *Osx1-Cre;Rb$^{fl/fl}$;p53$^{fl/fl}$;E2f1$^{-/+}$* animals were primarily

observed in the head bones (58% and 65% respectively). In contrasts, a small fraction (37%) of the *Osx1-f1TKO* developed tumors in the head bones. The remaining 63% of tumors observed in the *Osx1-f1TKO*s appeared in the long bones of the animals (13% spine + 44% limbs and Figure 2B). In addition to osteosarcomas, we have reported that the *Osx1-Cre;Rb*$^{fl/fl}$*;p53*$^{fl/fl}$ animals develop hibernomas at small frequencies (Berman *et al.*, 2008a). To our surprise, *E2f1*–loss altered the development of hibernomas. While 35% of the *Osx1-DKO*s animals developed hibernomas, only 14% of the *Osx1-f1TKO*s developed this malignancy, suggesting that *E2f1*-loss suppresses hibernoma tumor formation in this tumor model (Figure 2A). Taken together, inactivation of *E2f1* in committed osteoblasts partially suppresses *Rb*-dependent tumor fate and alters tumor distribution.

E2f1 modulates the differentiation state of the tumors.

We also explored whether *E2f1* status will have an impact in the differentiation state of the osteosarcomas. Histological analyses revealed that *E2f1*-null tumors were consistently more differentiate than tumors retaining wildtype E2f1. This was judged by the presence of calcified bone matrix in histological sections of the *Osx1-f1TKO* tumors when compared to the other genotypes. This was also confirmed by staining the tumors sections with Sirius Red, which marks collagen, and Runx2 immunohistochemistry. Strikingly, *E2f1* null tumors expressed high levels of both collagen and Runx2 when compared to tumors in which *E2f1* is present (Figure 3A). Consistently, cell lines generated

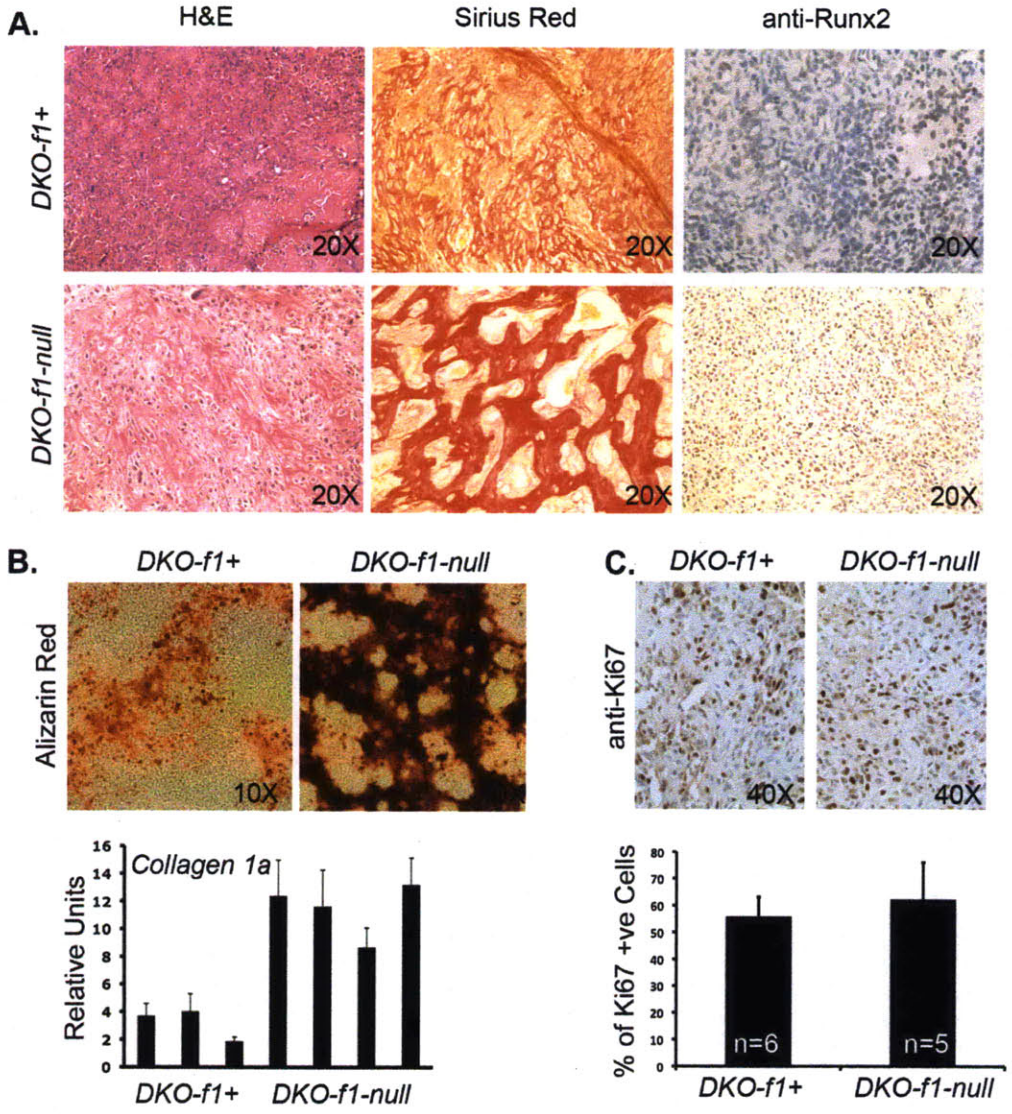


Figure 3: *E2f1* alters osteosarcomas' degree of differentiation.

(**A**) Histological analyses of osteosarcomas from *Osx1-Cre;Rb$^{fl/fl}$;p53$^{fl/fl}$;E2f1$^{+/+}$* and *Osx1-Cre;Rb$^{fl/fl}$;p53$^{fl/fl}$;E2f1$^{-/-}$* animals. Parrafin embeded tumor sections were analyzed for their degree of differentiation by Runx2 immunohistochemistry and by collagen staining using Sirius Red. Pictures are representative of 11 different tumors per genotype. Similar analyses were conducted in *Osx1-Cre;Rb$^{fl/fl}$;p53$^{fl/fl}$;E2f1$^{+/-}$* tumors and the results obtained were indistinguishable from the *Osx1-Cre;Rb$^{fl/fl}$;p53$^{fl/fl}$;E2f1$^{+/+}$*. (B) Alizarin Red staining of osteosarcoma tumor cell lines derived from *Osx1-Cre;Rb$^{fl/fl}$;p53$^{fl/fl}$;E2f1$^{+/+}$* and *Osx1-Cre;Rb$^{fl/fl}$;p53$^{fl/fl}$;E2f1$^{-/-}$* tumors. The differentiation potential of the cells was confirmed by real time PCR experiments for the bone specific marker collagen 1a. Each bar represents an individual cell line. (C) Ki67 immunostaining of paraffin embedded sections to assay for the proliferation potential of the tumors. Bars represent the quantification of the indicated number of independent tissue samples. Error bar equals the standard deviation from at least three independent experiments.

from *Osx1-Cre;Rb*$^{-/-}$*;p53*$^{-/-}$*;E2f1*$^{-/-}$ osteosarcomas expressed high level of the bone specific marker collange1a and when induced to differentiate, these cells secreted calcified matrix at a greater extend than either the *Osx1-DKO* or *Osx1-Cre;Rb*$^{-/-}$*;p53*$^{-/-}$*;E2f1*$^{+/-}$ control cells (Figure 3B) This suggests that E2f1 contribute to tumorigenesis by modulating the differentiation state of the tumors.

One possible explanation for this enhanced differentiation is that *E2f1*-loss decreases the proliferative capacity of the cells. To address this we performed immunostaining for the proliferation maker Ki67 in osteosarcoma tumor sections taken from *Osx1-DKO*, *Osx1-Cre;Rb*$^{-/-}$*;p53*$^{-/-}$*;E2f1*$^{+/-}$, and *Osx1-f1TKOs*. We observed no differences in the proliferation status of the tumors (Figure 3C). These results are in contrasts to what we observed in our developmental studies. In that context, *E2f1*-loss rescue the proliferation defects observed in *Rb*-mutant calvarial osteoblasts (Berman *et al.*, 2008b). Thus, E2f1 role during sarcomagenesis seems to be proliferation independent.

E2f1 negatively regulates osteoblast differentiation during normal development.

The unexpected changes observed in tumor outcome, in the *Osx1-Cre* mouse model and the altered differentiation state of the tumors suggested to us that E2f1 might be modulating osteoblasts differentiation. Based on the fact that cellular differentiation opposed transformation, we hypothesized that the increased survival rate of *Osx1-f1TKOs* may be, in part, attributed to a novel role for E2f1 in antagonizing terminal differentiation. To explore this possibility, we

have isolated calvarial osteoblasts from e18.5 wild type and *E2f1*-null embryos. This system is suitable to study the role of E2f1 in bone differentiation because we have shown that loss of *E2f1* does not affect the proliferative capacity of calvarial osteoblasts *in vitro* (Berman *et al.*, 2008b). Thus, we cultured these primary osteoblasts to confluence and assayed them for their ability to secrete mineralized matrix, which is indicative of terminal differentiation. Consistent with our tumor data, *E2f1*-null osteoblasts were able to secrete more calcified matrix than wild type osteoblast as judged by Alizarin Red staining (Figure 4A). This enhanced differentiation potential was confirmed by qPCR analyses for early and late bone markers before and after osteogenic induction (Figure 4A).

Next, we decided to explore whether this role for E2f1 during osteogenesis is pRb-dependent. To do that we generated calvarial osteoblasts from $Rb^{fl/fl};E2f1^{-/-}$ and $Rb^{fl/fl};E2F1^{+/+}$ embryos and assayed them for their ability to differentiate into the bone lineage. Similar to the previous experiment, we cultured these osteoblasts to confluence and infected them with Adenoviruses expressing either Cre (Ad-Cre), to inactivate *Rb*, or control GFP (Ad-GF). At two days post infection we induced these osteoblasts to differentiate into the bone lineage. To our surprise we found that even in the absence of *Rb*, *E2f1*-loss enhances osteoblasts differentiation. We observed that $AdCre^{+};Rb^{fl/fl};E2f1^{-/-}$ osteoblasts were able to differentiate at a greater extend than $AdCre^{+};Rb^{fl/fl}$ cells (Figure 4A). These results were confirmed by qPCR experiments for different bone specific genes (Figure 4A). Thus we concluded that *in vitro* E2f1 modulates osteogenic differentiation in an *Rb*-independent manner. To explore this in an *in vivo* setting

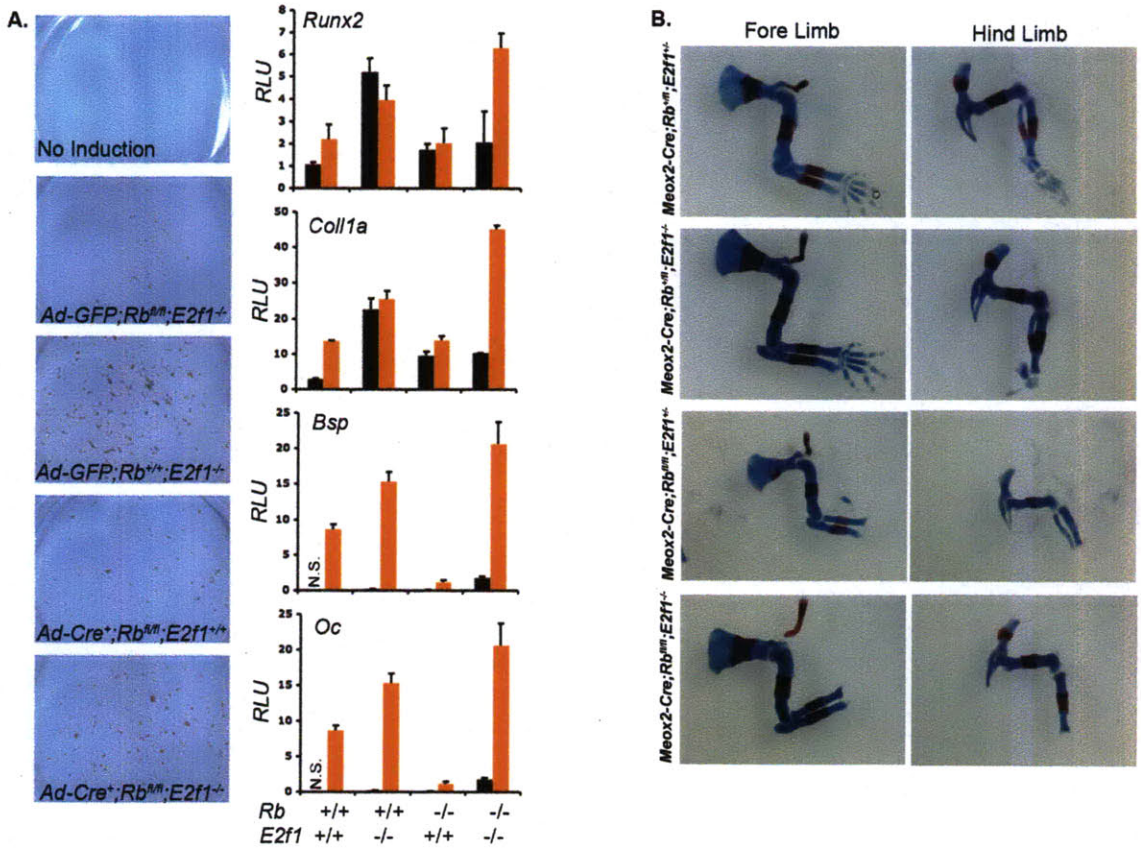


Figure 4: E2f1 modulate osteoblasts differentiation *in vitro* and *in vivo*.

(**A**) Calvarial osteoblasts, from the indicate genotypes were isolated from e18.5 embryos and assayed for their ability to terminally differentiate into the bone lineage *in vitro*. Calcium deposits indicative of terminal differentiation were analyzed by Alizarin Red staining. The differentiation potential of these osteoblasts was confirmed by qPCR before (**black bars**) and after (orange bars) differentiation for early and late bone specific genes. (**B**) The role of E2f1 in osteogenesis was further explored *in vivo* by investigating whether loss of E2f1 will rescued the lack of ossification phenotype observe in the *Meox2-Cre;Rb*$^{fl/fl}$ animals. Pictures are representative of the limbs from e15.5 embryos stained with dyes that mark collagen (blue staining) and calcified bone matrix (red staining). More than 10 embryos were analyzed for this experiments. Representative pictures are shown here.

we took advantage of the *Meox2-Cre;Rb*$^{fl/fl}$ mutant embryos, which survive until birth and displayed defects in multiple mesenchymal tissues, including lack of skeletal ossification (Berman *et al.*, 2008b). We asked whether *E2f1* loss would rescue the ossification defect observed in the *Rb* mutant embryos. To do this we analyzed the skeletons of e15.5 *Meox2-Cre;Rb*$^{fl/fl}$*;E2f1*$^{-/-}$ and *Meox2-Cre;Rb*$^{fl/fl}$*;E2f1*$^{+/+}$ and compared them to the skeleton of their wild type littermates. Consistent with our *in vitro* results and our previous published data, *E2f1* loss rescued the ossification defects observed in the limbs of the *Rb* mutant embryos (Figure ; (Berman *et al.*, 2008b). Together, these results clearly show that E2f1 negatively regulates terminal differentiation in an *Rb*-independent manner and strongly suggests that E2f1 contributes to tumorigenesis by antagonizing terminal differentiation.

_E2f1_-loss affects _Rb_-dependent plasticity, but has no effect on _Rb_-dependent fate choice.

Our data shows that *E2f1*-loss promotes differentiaton, which may be attributed to the delayed osteosarcoma formation observed in *Osx1-f1TKO* animals. Thus, if our hypothesis holds true, we would expect *E2f1* activity to be require for *Rb*-dependent cellular plasticity. We have previously shown that *Rb*-loss renders calvarial osteoblasts multipotent as judged by the ability of *Rb* mutant cells to undergo adipogenic and osteogenic differentiation *in vitro (Calo et al., 2010)*. Based on these findings we wanted to explore whether *E2f1* would influence this reprogramming event *in vitro*. To do this, we assayed the

adipogenic differentiation potential of $Rb^{fl/fl};E2f1^{+/+}$, and $Rb^{fl/fl};E2f1^{-/-}$ e18.5 calvarial osteoblasts. As described, we culture these osteoblasts to confluence, infect them with Ad-GFP or Ad-Cre and assayed them for their ability to differentiate into the adipogenic lineage. Consistent with out previous results, Ad-$Cre^{+};Rb^{fl/fl};E2f1^{+/+}$ were able to undergo adipogenesis, while Ad-GFP;$Rb^{fl/fl};E2f1^{-/-}$ and Ad-GFP;$Rb^{fl/fl};E2f1^{+/+}$ failed to do so. To our surprise, Ad-$Cre^{+};Rb^{fl/fl};E2f1^{-/-}$ ostoeblasts showed a reduced, but not abolished, adipogenic differentiation potential when compared to the Ad-$Cre^{+};Rb^{fl/fl};E2f1^{+/+}$ cells, which underwent adipogenesis even without the addition of induction media (Figure 5A). These results were confirmed by real time PCR analyses for adipogenic specific markers. Taken together, our data indicates that *E2f1* status partially modulates *Rb*-dependent cellular plasticity. However, *E2f1*-loss has no effect on *Rb*-depedent cell fate as loss of *E2f1* does not affect the expanded brown fat compartment observed in the *Meox2-Cre;*$Rb^{fl/fl};E2f1^{+/+}$ mutant embryos (Figure 5B). Thus, we concluded that the role of pRb in modulating fate choice is independent of E2f1 function. Collectively, our data suggest that E2f1 contributes to tumorigenesis by antagonizing terminal differentiation.

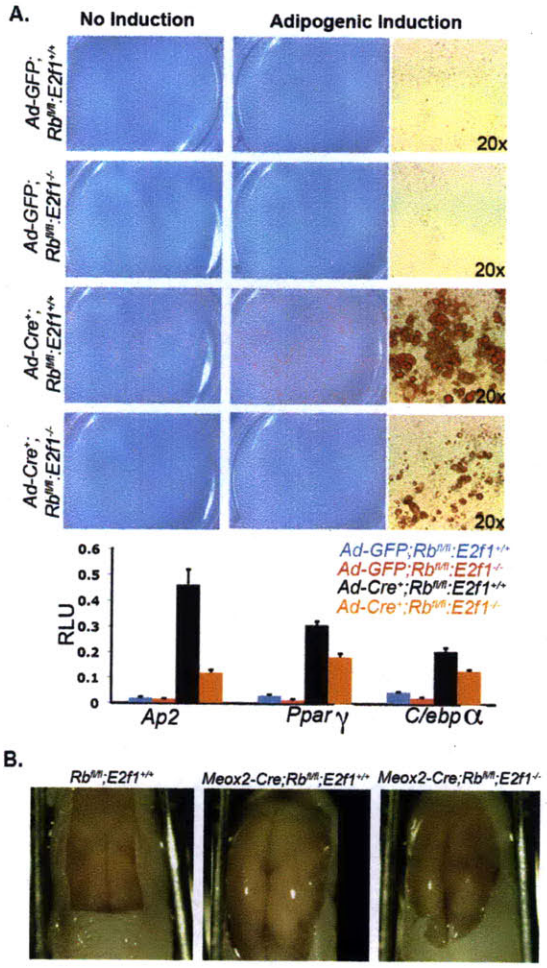


Figure 5: *E2f1*-loss partially affects *Rb*-dependent plasticity but has no effect on *Rb*'s-dependent fate choice.

(**A**) The ability of osteoblast to reprogram towards the adipogenic state was analyzed by isolating e18.5 calvarial osteoblasts of the indicated genotypes. These osteoblasts were induced to differentiated into adipocytes for 21 and stained with Oil Red-O. The differentiation state was confirmed by qPCR analyses after induction. (**B**) Representative sample of the brown fat compartment from e18.5 embryos for the indicated genotype.

Discussion

Inactivation of the *RB-1* and *TP53* pathways are hallmark of cancer. However the specific mechanisms by which these two tumor suppressor genes contribute to transformation are not fully understood. Here we provided insights into the role of the pRB-E2F pathway in mesenchymal tumorigenesis. We took advantage of our previously published mouse models for sarcoma to dissect the contribution of the activating E2fs, E2f1 and E2f3a, during sarcomagenesis. Due to their role in promoting cell cycle progression, these E2fs were thought to have oncogenic properties and therefore contribute to tumor development by stimulating cellular proliferation. However, E2f1 and E2f3a have also been shown to have pro-apoptotic roles, which suggest that they can behave as tumor suppressors (Iaquinta and Lees, 2007). In this context, these E2f have been shown to transcriptionally activate genes involved in cellular processes related to cell death. Most of the functional evidence on E2fs and cancer came from *in vitro* studies. Thus, to date it is controversial whether these genes will behave as tumor suppressor genes or oncogenes *in vivo*.

Our data clearly shows that in sarcomas, E2f1, but E2f3a, has oncogenic properties *in vivo*. We observed that E2f1-loss suppresses the tumorigenic potential of *Rb;p53*-deficeient mesenchymal progenitor cells. This is of particular interest because E2f1 and E2f3a compensate for each other during development. However, we found that this is not the case during tumorigenesis. This is supported by the fact that *Prx1-Cre;Rb*$^{fl/fl}$*;p53*$^{fl/fl}$*;E2f1*$^{-/-}$*;E2f3a*$^{+/-}$ developed tumors with similar kinetics than the *Prx1-f1TKO*. Taken together these results

show that *E2f1* and E2f3a have non-overlapping roles during tumorigenesis and that E2f1 is oncogenic and cooperate with mutations in the *Rb* and *p53* pathway to promote sarcomagenesis.

We also explored the role of E2f1 in a more committed mesenchymal compartment. In this context E2f1 function still oncogenic, but in contrast to the results obtained using the *Prx1-Cre* transgene, *E2f1* act in a dosage dependent manner not only to expand the life span of the *Prx1-f1TKO* mutant animals, but also to reduce the incidence of brown fat tumors. This indicates that E2f1 transforming activity is more critical in committed progenitor cells than in the stem cell compartment. In addition, we observed that E2f1-loss modulates physiological tumor location and this correlates with a novel role for E2f1 in antagonizing osteoblasts differentiation. These conclusions were derived from three independent observations. First, histological analyses from *E2f1* null tumors showed that these osteosarcomas were more differentiated than tumors retaining wildtype E2f1. Second, cell lines derived from *E2f1*-null tumors showed an increase osteogenic differentiation potential as judged by high expression of the bone specific marker collagen1a and the ability of these cells to undergo terminal differentiation upon induction. Third, *E2f1*-null calvarial osteoblasts differentiate at a greater extend than control osteoblasts. Thus, E2f1 role in preventing differentiation is independent of transformation.

Given that cellular proliferation opposes transformation, it is plausible to hypothesize that this phenotype is proliferation-dependent. However we do not think that this is the case. First, we have shown that E2f1 status does not alter

the proliferation potential of committed osteoblasts (Berman *et al.*, 2008b). Similar results were obtained in E2f1-null mouse embryonic fibroblasts (Humbert *et al.*, 2000; Wu *et al.*, 2001). Also, the osteosarcoma derived cell lines use in this experiment are *Rb;p53*-deficient and regardless of E2f1 status, they proliferate in culture with similar kinetics. This was confirmed by staining of tumors sections with the proliferation marker Ki67. Therefore, we concluded that E2f1 role during osteogenic differentiation is independent of proliferation.

In accordance with our previous observations, this role for *E2f1* in modulating bone differentiation was also independent of *Rb* status. The fact that *E2f1*-loss does not increases the incidence of osteosarcomas in the *Prx1-Cre;Rb*$^{fl/fl}$*;p53*$^{fl/fl}$ animals clearly indicates that E2f1 role in osteoblasts differentiation and tumorigenesis is specific to committed osteoblast and not to the stem cell compartment. Based on this we proposed a model to explain the role of E2f1 during mesenchymal differentiation and tumorigenesis. During differentiation, pRb-Runx2 complexes are activated, in the stem cell compartment, to initiate the osteogenic program. This interaction has been shown to be critical for promoting commitment towards the osteoblastic state (Thomas *et al.*, 2001). After commitment is made, pRb is required to binds E2f1 in order to properly form chromatin bound repressive complexes that facilitate permanent cell cycle exit and therefore allow terminal differentiation. However during tumorigenesis, *Rb* loss causes deregulation of the differentiation program because these repressive complexes fail to form, allowing free E2f1 to inhibit the ability of cells to terminally differentiate and/or maintain terminal differentiation. If

our model proves to be correct, this will underscore why pRb, and not the other pocket proteins (p107 and p130), is commonly mutated in cancer. In fact, our model is consistent with recent reports showing that, during normal mouse development, activating E2fs are dispensable for cellular proliferation *in vivo*, but are instead required for the survival of the differentiated state by forming chromatin-bound repressive complexes with their negative regulators, the pocket proteins: pRb, p107 and p130 (Chong *et al.*, 2009; Chen *et al.*, 2009). Thus, we hypothesized that, in cancer, inactivation of the pRb alters the differentiation state of committed cells and therefore *Rb* loss is more critical in committed progenitor cells than in the stem cell compartment.

Experimental Procedures

Animal maintenance and histological analyses

Animal procedures followed protocols approved by MIT's Committee on Animal Care. The *Rb*$^{fl/fl}$, *p53*$^{fl/fl}$, *Osx1-GFP::Cre, Prx1-Cre, E2f1*$^{-/-}$, *E2f3a*$^{-/-}$, and *Meox2-Cre* animals were maintained on a mixed genetic background. Tissues were fixed in PBS with 3.7% formaldehyde. Prior to paraffin sectioning, soft tissues were dehydrated via an ethanol series, while bone-containing tissue was decalcified in 0.46M EDTA, 2.5% Ammonium Hydroxide pH 7.2.

Mouse genotyping

The *Rb* conditional band was detected using the primers 5'lox: 5'-CTCTAGATCCTCTCATTCTTC-3' and 3'lox: 5'-CCTTGACCATAGCCCAGCAC-3'. Primer Rbcre3.2 (5'-GGTTAATGAAGGACTGGG-3') was used in conjunction with primer 5'lox to detect the recombined band. 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'. The E2f3a null and wild type allele was detected using the primers A12: 5'-GGT CAG GGC GCC ACT TTG GAG G-3' and A16: 5'-AGC AAT ACG TTA ATA TAT CGT AAC AC-3'. The E2f1 null and wild type allele was detected using the primers: 5'-GCTGGAATGGTGT CAGCACAGCG-3', 5'-TCCAAGAATCATATCCAGTGGCT-3' and 5'-CTACCCGGTAGAATTGACCTGCA -3'.

Tumor monitoring and analysis

The criteria for euthanasia (by CO2 inhalation) were a total tumor burden of $2cm^3$, tumor ulceration/bleeding, signs of infection, respiratory distress, impaired mobility, ≥20% reduction in body weight or general cachexia. All tissues were collected and hip bones, femurs and tibias were separated and fixed overnight in PBS with 3.7% formaldehyde. Soft tissues were transferred into 70% ethanol and dehydrated via an ethanol series prior to embedding in paraffin for sectioning. Tissues containing bone was either decalcified in 0.46M EDTA, 2.5% Ammonium Hydroxide pH 7.2 for two weeks then processed for paraffin sectioning. All paraffin embedded sections were cut at 5µm, dewaxed and stained with H&E. Sirius red staining was performed by treating sections briefly stained with hematoxylin with 0.1% Sirius red in saturated picric acid (Electron Microscopy Sciences) for one hour, washing in 5% v/v glacial acetic acid and then dehydration in ethanol/xylene prior to mounting.

Immunohistochemistry (IHC)

Runx2 IHC was performed using a modified citric acid unmasking protocol. Briefly, slides were deparaffinized in xylene and rehydrated through ethanol. Slides were boiled for 30 min in citrate buffer, pH 6.0, and then cooled in running tap water. Slides were then washed in PBS for 5 min followed by inactivation of endogenous peroxidases by incubation 0.5% H_2O_2 in methanol. Slides were blocked in 10% Goat Serum for 1 h at room temperature. Primary antibody (MBL anti-Runx2 Clone 8G5) was diluted 1:200 in PBS 0.15% Triton and incubated

overnight at 4 °C. The following day, slides were washed three times in PBS. Secondary antibodies (Vectastatin ABC kits, Vector laboratories) were diluted 1:500 in PBS containing 0.4% Goat Serum and detected using a DAB substrate (Vector Laboratories). All samples were counterstained with hematoxylin.

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 of 0.015% Alcian blue 8GX (Sigma), 0.005% alizarin red S (Sigma), and 5% glacial acetic acid in ethanol at 37°C for 2 days and at room temperature for a one more day. Tissue was cleared in 1% potassium hydroxide for several days and then stored in glycerol.

Calvarial Osteoblasts Preparation and Culture

Calvaria from embryonic day 18.5 embryos were removed and carefully cleaned in sterile PBS from contaminating tissue. Then treated with several rounds of collagenase/trypsin digestion at 37°C, and plated onto six-well plates for 2 days in αMEM with 10% fetal bovine serum and penicillin/streptomycin. For differentiation, $3.5x10^5$ cells were plated onto a well of a 6-well tissue culture plates. Upon reaching confluence, calvarial osteoblasts were treated with medium supplemented with 50 μg/mL of ascorbic acid and 10 mmol/L of β-glycerol-phosphate. Adenovirus (University of Iowa Gene Transfer Vector Core) was added to the medium at 100 plaque-forming units per cell and washed away

24 h later. To assay for calcium deposits, plates were stained with 1% alizarin red S solution (pH 5.0)

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Chapter 5

pRb represses tumor progression by reactivating the differentiation program.

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The author contributed to all text, graphs, and figures.

Abstract

Mutations in the *RB-1* tumor suppressor gene are commonly observed in retinoblastomas and osteosarcomas. To study the role of pRb in osteosarcoma, we have generated a mouse model in which *Rb* and/or *p53* are inactivated in the osteoblastic lineage. Our previous data show that loss of *Rb*, although insufficient to initiate tumorigenesis, strongly synergizes with *p53* mutations to enhance osteosarcoma development (Berman *et al.*, 2008). Here we further our studies by using cell lines derived from *Rb*;*p53*- and *p53*-mutant osteosarcomas to understand how loss of *Rb* cooperate with *p53* mutations in this malignancy and the therapeutic consequences of reactivating pRb in osteosarcomas *in vivo*. In accordance with our *in vivo* studies, downregulation of *Rb* in *p53*-mutant cells enhances their tumorigenicity *in vitro* and *in vivo* to a similar extend than the *Rb*;*p53*-deficient cells. On the other hand, re-expression of pRb in actively cycling *Rb;p53*-mutant osteosarcoma cells reduces their tumorigenic properties *in vitro*, suggesting that even in the absence of *p53*, pRb's tumor suppressive network can be reactivated in these cells. To explore this *in vivo*, we re-expressed pRb in *Rb;p53*-mutant osteosarcomas and find that reactivation of pRb halts tumor progression and strikingly promotes differentiation. This enhanced differentiation potential was confirmed by qPCR and chromatin immunoprecipitation analyses from tumor tissues. Together, our data uncovered a novel tumor suppressive function regulated by pRb, which is dependent on pRb's ability to modulate cellular differentiation.

Introduction

Inactivation of the *RB-1* and *TP53* pathways is considered a hallmark in cancer. However, the specific mechanisms, the consequences, and the order in which either of these pathways are inactivated during tumor development remain to be elucidated. Therefore, differentially modeling the order in which these two pathways are deregulated during tumor progression will provide insight into how tumors evolved as disease progresse. *In vivo* studies in a variety of mouse models showed that loss of *Rb* strongly synergizes with *p53* mutations to promote tumor development (Berman *et al.*, 2008; Zhou *et al.*, 2006; Walkley *et al.*, 2008; Williams *et al.*, 1994a). However, the specific cellular mechanism unleashed by losing *Rb* functions in an already established *p53* mutant background has not been reported. Likewise, the effect of losing *p53* tumor suppressive activity in the presence of a mutated *Rb* pathway has not been explored. Both *Rb* and *p53* modulate cellular processes that are essential to maintain genome integrity and prevent tumor progression. They both regulate the cell cycle, apoptosis, and cellular senescence as well as processes related to stem cell self-renewal and differentiation (Classon and Harlow, 2002; Sherr and McCormick, 2002).

Unlike *Rb*, *p53* germline homozygous mutant animals are viable and highly tumor prone (Jacks *et al.*, 1994; Donehower *et al.*, 1992). These properties allowed the generation of an elegant mouse model in which *p53* function can be restore *in vivo* from its endogenous locus. Studies using this system have shown

that restoration of *p53* functions, in *p53*-deficient tumors, promotes tumor regression in a context-dependent manner: While in hematopoietic malignancies restoration of *p53* promotes apoptosis, in solid tumors the *p53*-dependent tumor suppressive effect is through cellular senescence (Ventura *et al.*, 2007). These results clearly suggest that tumor suppressors' mode of action is indeed tissue specific. Similar studies have been conducted to reactivate *p53* function in hepatocellular carcinomas *in vivo* (Xue *et al.*, 2007). However, the tumor suppressive network regulated by pRb has not been explored *in vivo*. This is in part because homozygous mutant animals for *Rb* are not viable and the tumor spectrum observed in $Rb^{-/+}$ mutant animals is limited to neuroendocrine malignancies, which are difficult to detect (Williams *et al.*, 1994b). Much of our knowledge about how *Rb* inhibits tumor progression came from studies performed *in vitro*. Although theses experiments have provided valuable information about the biology of *Rb* as a tumor suppressor, they do not fully recapitulate the *in vivo* nature of the disease, impeding the generation of effective therapeutic approaches to treat *Rb*-deficient tumors.

In this study we analyzed the role of *Rb* in osteosarcomas from two different perspectives: First, we explored the tumorigenic advantages of inactivating the *Rb* pathway in an already established *p53*-null background and second, we examined the consequences of restoring pRb function in *Rb*- and *p53*-defficient osteosarcomas *in vivo*. Our data show that downregulation of *Rb* in a *p53*-null tumorigenic background enhances cellular proliferation, attachment independent growth and promotes tumor progression *in vivo*. Consistently, re-

expression of *Rb* function *in vivo* halts tumor progression and surprisingly promotes differentiation. Together our studies uncover a novel tumor suppressive pathway regulated by pRb *in vivo*, which is solely dependent on pRb's ability to modulate cellular differentiation.

Results

_Rb_-loss synergizes with _p53_-mutations in osteosarcomas _in vitro_ and _in vivo_.

To explore whether and how *Rb* loss synergizes with *p53* mutations *in vitro* and *in vivo* we have generated *p53* null, *Rb* wild type, murine osteosarcoma cell lines (*p53KO* OS) from a mouse model for osteosarcoma in which inactivation of *p53* occurs in the osteoblast lineage (Berman *et al.*, 2008). These cell lines were fully immortalized *in vitro* and capable of generating tumors *in vivo*, when injected into immunocompromised mice. Importantly, these secondary tumors were transplantable and pathologically indistinguishable from the parental tumor from which the cells were derived (data not shown). Thus, inactivation of the *p53* pathway is a sufficient oncogenic event to initiate osteosarcomas *in vivo* and the tumorigenic potential is fully retained after *p53KO* OS cells have been established in culture. Next, we investigated how inactivation of the pRb pathway, as a secondary oncogenic event, will affect the tumorigenic potential of these *p53KO* cells. We infected *p53KO* OS cells with retroviruses expressing either an *Rb* specific short hairpin RNA (*shRb-p53KO*) or a *luciferase* control hairpin (*shluc-p53KO*). In accordance with a role for *Rb* as a negative regulator of the cell cycle, we found that *shRb-p53KO* OS cells proliferate faster than *shluc-p53KO* OS cells, but similar to control *DKO* (*Osx1-Cre;Rb*$^{-/-}$*;p53*$^{-/-}$) OS cells (Figure 1A). Consistently, *shRb-p53KO* cells were able to generate bigger and more aggressive tumors than control *shluc-p53KO* OS cells, when injected into

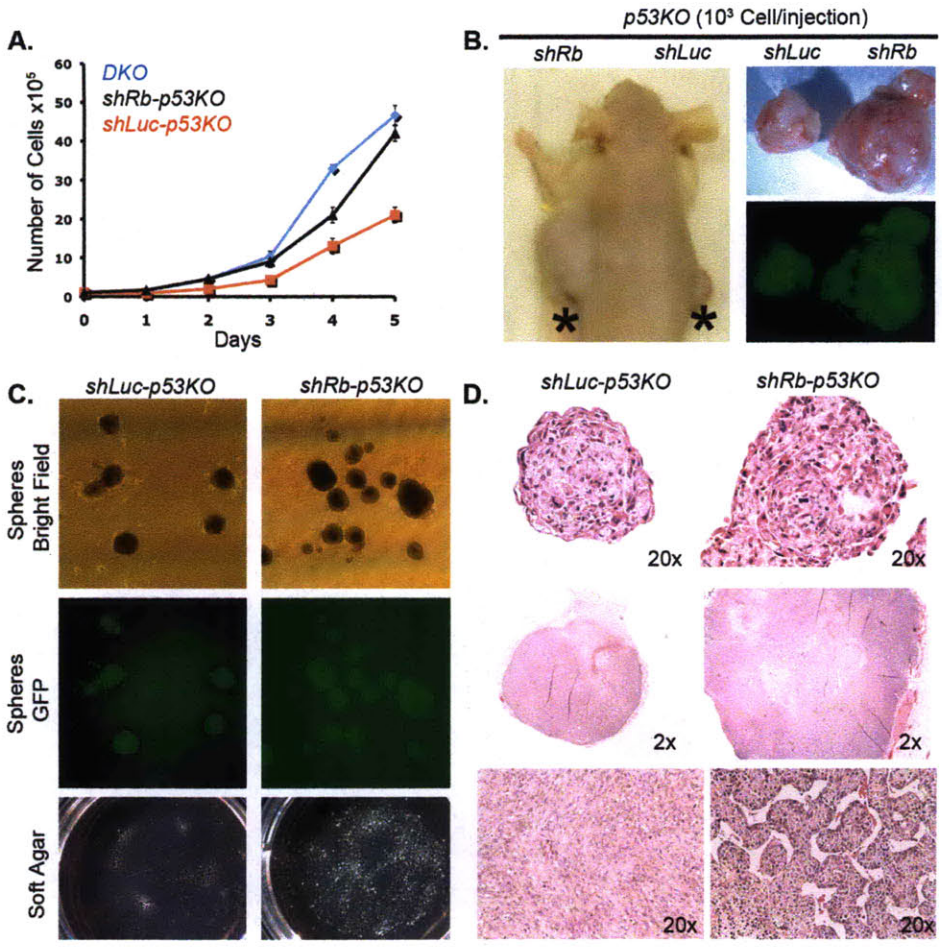

Figure 1: *Rb*-loss synergizes with *p53* mutations to promote tumorigenesis.

A. *p53KO* osteosarcoma cell lines were infected with retroviruses expressing either a short hairpin RNA against *Rb* or a control hairpin against *luciferase* (*shRb-p53KO* and *shLuc-p53KO* respectively. *shRb-p53KO* cells were able to proliferate faster than control *shLuc-p53KO,* but at a similar rate than *DKO* osteosarcoma cell lines. **B**. Injection of *shRb-p53KO* and *shLuc-p53KO* osteosarcoma cells subcutaneously into immunocompromised mice. GFP shows sustained expression of the hairpin. **C.** The ability of *shRb-p53KO* and *shLuc-p53KO* to grow in an attachment independent manner was assayed by sarcosphere formation (upper panel) and soft agar assay (lower panel). GFP confirm the expression of the hairpins (middle panel). **D**. Individual sarcospheres were injected into immunocompromised mice. Upper panel represent H+E staining of sarcosphere in tissue cultures. Middle and lower panels show H+E staining of representative tumors arising from injected sarcospheres.

immunocompromised mice (Figure 1B). This enhanced transformation is also reflected by the ability of *shRb-p53KO* cells to form colonies in semi solid media as well as to form bigger sarcospheres *in vitro* (Figure 1C). We further analyzed the tumorigenic potential of each individual sarcospheres by injecting single spheres into the flanks of immunocompromised mice. To our surprise, these spheres were able to form tumors faster than their respective cultured cells in monolayers. However, *shRb-p53KO* OS-derived sarcospheres give rise to bigger tumors than the ones generated from *shluc-p53KO* OS cells (Figure 1D). Together, these results and our previous work (Calo *et al.*, 2010) indicate that *Rb*-loss synergizes with *p53* mutations in osteosarcoma to enhance proliferation, attachment independent growth, and stemness.

***Rb*'s tumor suppressive network can be reestablished in the absence of *p53*.**

Our results are consistent with the fact that either loss or downregulation of the pRb pathway is considered a poor prognosis factor not only in osteosarcomas but also in multiple tumor types. However, we wanted to explore whether reactivation of *Rb* function in a *Rb;p53* deficient tumorigenic background would be sufficient to reactivate *Rb*'s tumor suppressive network or whether the phenotypes acquired once *Rb* and *p53* are inactivated would not be responsive to pRb. To do this, we engineered our *DKO* OS cell lines to express full-length murine *Rb*-cDNA downstream the tetracycline responsive elements (TRE). In this experimental setting we can control *Rb* expression by addition of the tetracycline

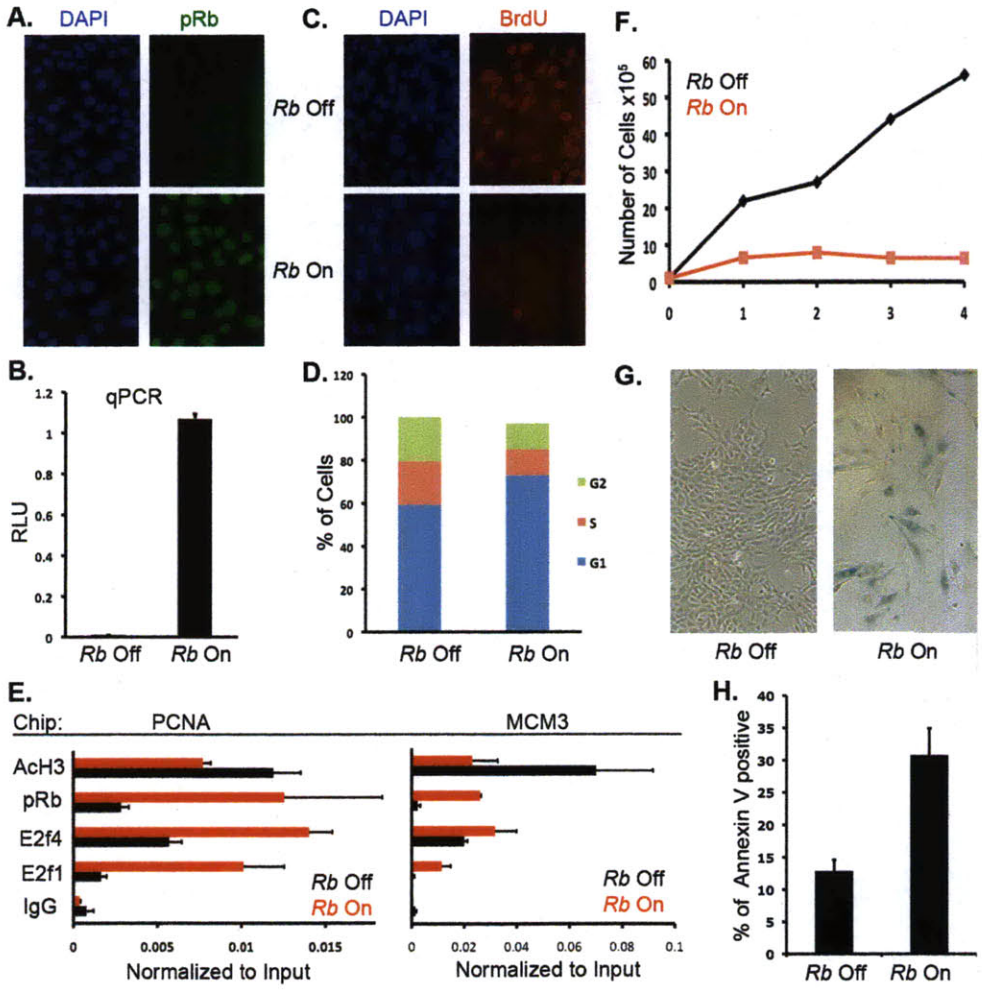

Figure 2: Re-expression of pRb in cycling DKO osteosarcoma cell lines.

A-B. *DKO* osteosarcoma cell lines were engineered to express *Rb* under the control of tetracycline responsive elements (*DKO-Rb*$^{Dox\text{-}On}$). *Rb* re-expression was confirmed by imunofluorescense and qPCR analyses. **C-D**. Analyses of the cell cycle phenotype 48 hours after Dox treatment was measure by the ability of *DKO-Rb*$^{Dox\text{-}On}$ cell to incorporate BrdU and to undergo cell cycle arrest in the G1 phase of the cell cycle as judge by PI-FACS profile. **E**. Chromatin-immunoprecipitaton experiments for two-representative E2f cell cycle related target genes show recruitment of pRb as well as E2fs to these promotes and these correlates with a decrease in the acetylation mark for histone 3. **F-G**. Sustained expression of pRb in *DKO-Rb*$^{Dox\text{-}On}$ cells result in irreversible cell cycle arrest and cellular senescence. **H**. AnnexinV FACS profile for the detection of apoptotic cells. Error bars represent the STDEV for three independent experiments.

analog, doxycycline (Dox). We generated stable clones from these inducible *DKO* OS cells (*DKO-Rb*$^{Dox\ On}$) and validated *Rb* expression upon Dox treatment by qPCR, immunofluorescence, and immunoprecipitation (Figure 2A-B and data not shown). As a proof of principle, we first assayed for the ability of *DKO-Rb*$^{Dox\ On}$ cells to undergo cell cycle arrest upon re-expression of pRb. In fact, 48h after Dox treatment *DKO-Rb*$^{Dox\ On}$ underwent cell cycle exit as judged by the inability of the cells to incorporate BrdU and by accumulation of cells in the G1 phase of the cell cycle as measured by immunofluorescence and FACS analyses, respectively (Figure 2C-D). Consistently, by chromatin immunoprecipitation experiments we observed a decrease in the acetylation status of histone 3 (AcH3) at promoters of cell cycle genes and this was accompanied by recruitment of pRb and an increase in promoter occupancy by E2f4, which is the major repressive E2f (Figure 2E). To test whether this cell cycle arrest upon reintroduction of pRb is transient or permanent, we followed the growth of *DKO-Rb*$^{Dox\ On}$ cells for 6 additional days after Dox treatment. In this context, *DKO-Rb*$^{Dox\ On}$ cells showed reduced growth capacity during this time course and do not re-entered the cell cycle (Figure 2F). Thus pRb restoration, as expected, induces a permanent cell cycle arrest in a p53 pro-tumorigenic background.

Besides cell cycle control, *Rb* also regulates other cellular processes such as cellular senescence (Chicas *et al.*) and more recently we have shown that pRb also promotes apoptosis in highly proliferating cells (Ianari *et al.*, 2009). To explore these phenotypes in our system, we cultured our *DKO-Rb*$^{Dox\ On}$ cells at low density and treated them with Dox for either 48 hours to assay for apoptosis

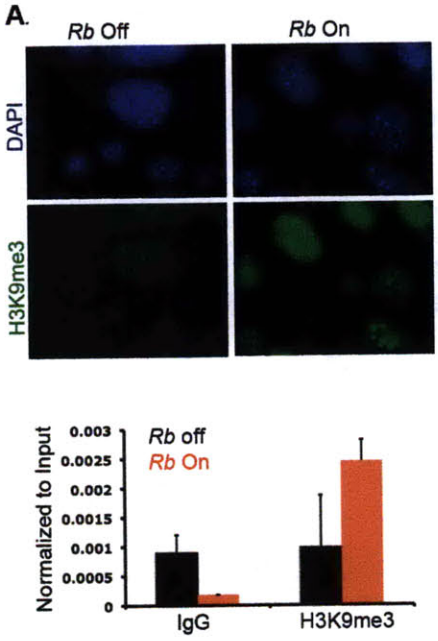

Figure 3. Re-expression of pRb in cycling cells induces heterochromatin formation.

A. H3K9me3 immunofluorescence upon Rb reintroduction shows formation of heterochromatin foci. This was comfirmed by chromatin immunopreciptation experiments for H3K9me3 mark at promoter of cell cycle genes. Chip data forThe Mcm3 promoter is shown here.

or for 10 days to assay for cellular senescence. We were surprised to find that even in the absence of *p53*, pRb re-expression was able to induce both apoptosis and cellular senescence (Figure 2G-H). This senescence phenotype correlates with accumulation of cells in G1 and an increase in the H3K9me3 at promoters of cell cycle related genes (Figure 3). Based on these findings we speculate that in highly proliferating cells pRb acts as a molecular switch to control the balance between senescence and apoptosis. We hypothesized that re-expression of pRb enforces cell cycle arrest by promoting histone deacetylation at promoters of cell cycle related genes, which then lead to cellular senescence. However, cells that fail to respond to the G1 arrest and continue to proliferate will instead undergo apoptosis.

Restoration of pRb *in vivo* halts tumor progression and promotes differentiation.

Given that re-expression of *Rb* in our *DKO-Rb*$^{\text{Dox On}}$ cells efficiently reestablished *Rb*'s tumor suppressive network, we wanted to explorer the consequences of reactivating pRb in tumors *in vivo*. To do that, we injected $5x10^6$ *DKO-Rb*$^{\text{Dox On}}$ cells into the flank of immunocompromised mice and waited for tumor formation. Once the tumors reached the size of ~ $0.5cm^3$ we fed half of the animals with Dox, while the remaining ones were maintained on a regular diet (control animals). We measured the tumor volume 0, 3, 7, and 10 days after Dox treatment (Figure 4A). Under these experimental conditions we observed that reactivation of pRb halts tumor progression, while control tumors continue to

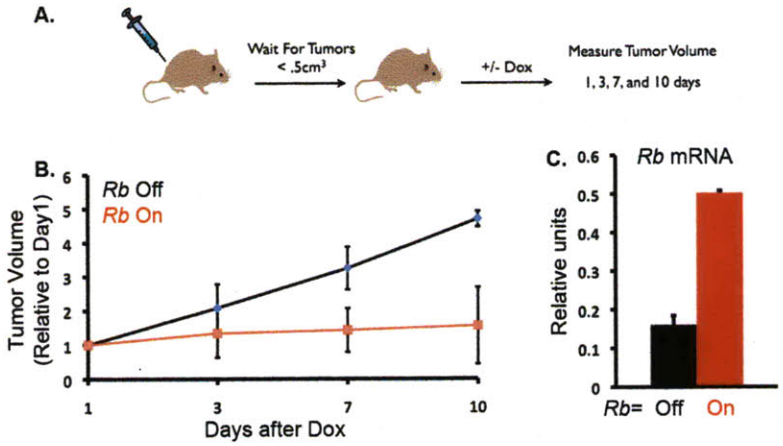

Figure 4: Re-introduction of pRb halts tumor progression *in vivo*.

A. Schematic representation of the re-activation experiment. *DKO-Rb*$^{\text{Dox On}}$ osteosarcoma cell lines were injected subcutaneously into immunocompromised animals. Once tumors developed (~0.5cm^3), animals were fed with Dox or not. **B**. Tumor volume was measure 1, 3, 7, and 10 days after treatment (n=10 animals per experimental conditions). To minimize variability, all the tumor volumes for each particular time point were normalized to the volume measured on day 1. **C**. The *Rb* mRNA was measured by qPCR from tumors harvested at 10 day after Dox treatment (n=10 animals per experimental condition). Error bars = the standard error.

growth (Figure 4B). To confirm that pRb was expressed in these tumors we measured *Rb* mRNA levels by qPCR in tumors tissues, and found that *Rb* was significantly expressed in Dox treated tumor compared to non-treated ones (Figure 4C). Thus reactivation of pRb halts tumor progression *in vivo*.

To explore the mechanism by which pRb inhibit tumor progression we performed histological analyses on restored and unrestored tumors (Figure 5A). We first tested for β-Gal-associated cellular senescence in freshly processed frozen tissue sections and find that cellular senescence is not the mechanism by which these tumors are not progressing, as we could not detect any β-Gal staining (data not shown). We then test whether apoptosis may be the underlying mechanism. Thus we performed immunohistochemical staining for cleaved caspase 3 and find that apoptosis is not playing any role in this system (Figure 5B). In accordance with the fact that the tumors stop progressing, we do observe a significant decrease in proliferation as judged by immunostaining for the nuclear antigent Kia67. We then assayed whether the decreased proliferative capacity corresponds to an increase in differentiation in *Rb* positive tumors. The rational behind this experiment is that we have previously reported that reintroduction of pRb in confluent arrested *DKO-Rb*$^{\text{Dox On}}$ cells promotes osteogenesis by binding to Runx2, which is the master regulator of the bone lineage (Calo *et al.*, 2010). Strikingly, we find high levels of nuclear Runx2 and Collagen matrix in *Rb* positive tumors compared to controls (Figure 5D-E), suggesting that reactivation of pRb in these tumors is promoting differentiation.

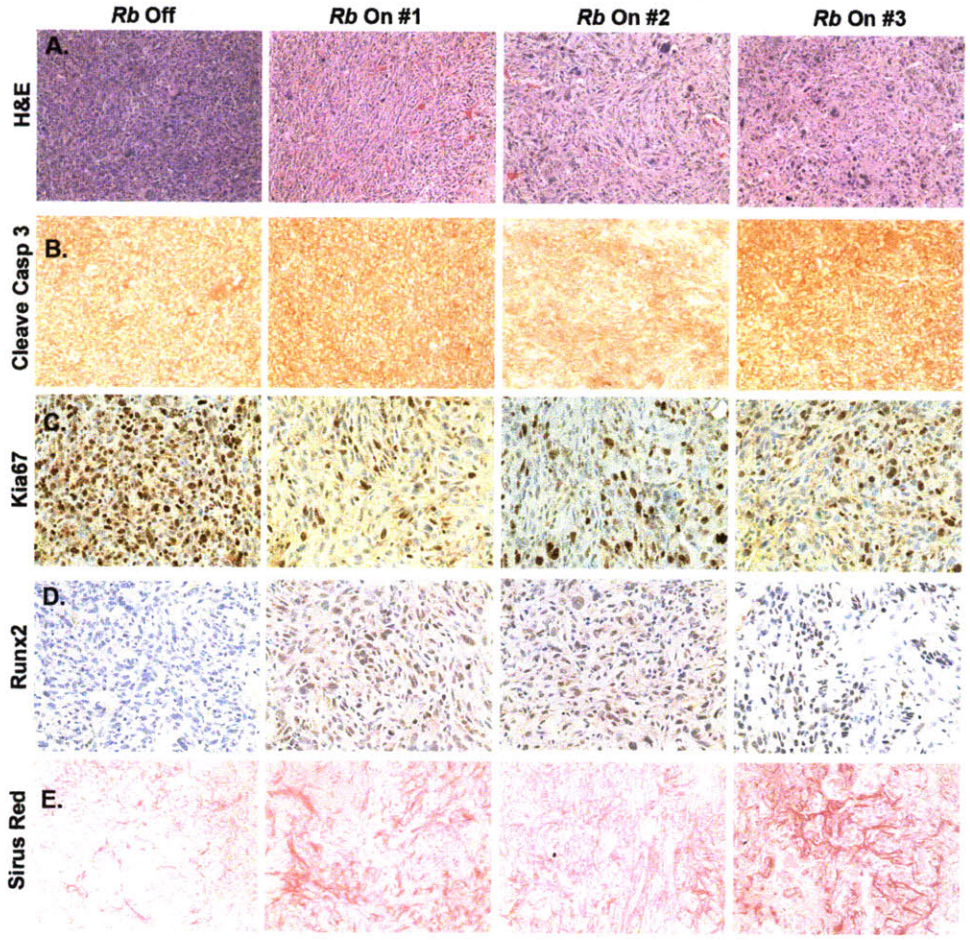

Figure 5: Re-expression of pRb promotes differentiation *in vivo*.

A. H+E staining of tumors in which pRb has been re-activates. **B**. Detection of apoptosis by immunohistochemistry for cleaved caspase 3. **C**. The proliferative status of the tumors was assayed by immunohistochemistry for the nuclear antigen Kia67. **D-E**. The differentiation grade of the tumors was analyzed by immunohistochemistry for Runx2 and Collagen deposition as judged by Sirius Red staining.

To confirm that pRb is promoting tumor-differentiation, we performed qPCR for the osteogenic differentiation markers *Runx2*, *Osterix*, *Collagen 1a*, and *Osteocalcin*, which are differentially expressed during bone formation (Figure 6). We observed that re-expression of pRb enhances the expression of the intermediate/late osteogenic markers, *Osterix*, *Collagen 1a*, and *Osteocalcin*, but has little effect on the early bone marker *Runx2* (Figure 6A). To explore whether this enhanced differentiation is a direct consequence of *Rb* re-expression, we performed chromatin immunoprecipitation from tumor tissues and found that pRb is recruited to the promoters of osteoblast specific genes *Collagen 1a* and *Osteocalcin*. In addition, we observed an increase in the acetylation status of histone 4 on these promoters, which is indicative of transcriptionally active chromatin (Figure 6B). Moreover, we performed the same experiments at promoters of the cell cycle related genes *Mcm3* and *Pcna*. Although we observed recruitment of pRb to these promoters, we do not detect any changes on AcH4 (Figure 6C) status. Together our data clearly shows that these tumors are indeed undergoing differentiation. This is in contrast to the phenotypes observed *in vitro* in actively dividing *DKO-Rb*$^{\text{Dox On}}$ in which cellular senescence and apoptosis are the main tumor suppressive pathways. Thus we conclude that in osteosarcomas reactivation of pRb halts tumor progression and promotes differentiation.

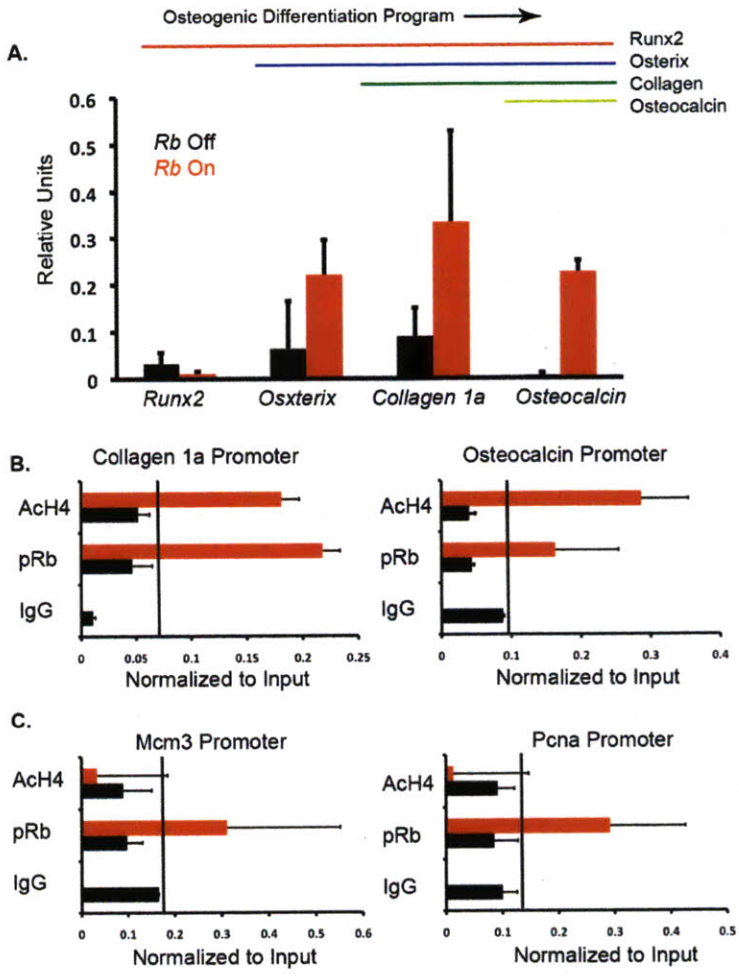

Figure 6: *Rb* re-expression promotes tumor differentiation *in vivo*.

A. Expression of bone specific genes in tumors in which *Rb* has been restored (red bars) and control tumors (black bars). The genes assayed are expressed at different time points during the osteogenic differentiation program. **B-C**. Chromatin immunoprecipitation analyses to assay for the presence of pRb and the active histone mark AcH4 at promoters of bone specific genes and E2f target cell cycle related genes. The vertical line across the plots indicates the level of background as judge by the IgG signal. Error bars represent three independent experiments

Discussion

Inactivation of the pRB pathway is commonly observed in most, if not all, human cancers and is often correlated with high-grade tumors and poor prognosis. However, the specific tumorigenic advantages for inactivating the Rb pathway remained to be elucidated. Our data show that downregulation of Rb in a p53 defficient tumorigenic background increases the transformation efficiency of these cells in vitro and in vivo. This is important because it shows that even in a tumorigenic setting the presence of pRb efficiently restrains uncontrolled cell growth, attachment independent growth and stemness. This is consistent with our previous studies in which Rb clearly synergizes with p53-loss to promote osteosarcoma tumor development in a dosage dependent manner (Berman et al., 2008). In this context both Rb and p53 were inactivated simultaneously in osteoprogenitor cells. However in human cancers, it would be very unlikely that these two pathways are inactivated at the same time. Instead, tumor suppressor pathways are deregulated at different stages as disease progresses. Our data suggest that inactivation of pRb, as a secondary oncogenic event, greatly enhances the tumorigenic potential of p53-mutant cells, which indicates that inactivation of the Rb pathway may be selected far during tumor's clonal evolution.

We also explored the therapeutic consequences of re-activating pRb function in osteosarcoma. Our rational for these experiments is that in tumors that retain pRB the protein sequence, for the most part, remains intact and instead, pRB is inactivated by phosphorylation. This is in contrast to TP53, in

which mutations in the DNA binding domain are the most frequent mode of inactivation. Thus, from a clinical perspective, reactivation of pRB seems to be a more feasible therapeutic approach than reactivating TP53 functions.

Consistent with previous studies, we show that pRb reactivation in actively dividing Rb;p53-deficient osteosarcoma cell lines induces cell cycle arrest, apoptosis, and cellular senescence in vitro. To our surprise, none of these tumor suppressive mechanisms was proven to be effective in vivo. Instead, we observed that reactivation of pRb in vivo halts tumor progression and promotes differentiation. These results are consistent with our previous studies conducted in osteoblasts in which pRb acts to control the balance between the multipotent and committed state. In that context, loss of Rb allows committed osteoblasts to differentiate into the bone and the fat lineages, while the presence of pRb maintains commitment by direct regulation of bone specific genes and the repression of the adipogenic transcriptional program (Calo et al., 2010). Notably, in our tumors pRb localizes at promoters of bone specific genes and this correlates with transcriptional activation of these promoters. Together, we have uncovered a novel tumors suppressive network regulated by pRb, which is fully dependent on pRb's ability to directly modulate cellular differentiation.

Experimental Procedures

Animal maintenance and histological analyses

Animal procedures followed protocols approved by MIT's Committee on Animal Care. Nude/SCID were purchase from Taconic. For tumor formation, animals were injected with tumor cell lines subcutaneously. The criteria for euthanasia (by CO2 inhalation) were a total tumor burden of $2cm^3$, tumor ulceration/bleeding, signs of infection, respiratory distress, impaired mobility, ≥20% reduction in body weight or general cachexia. Tissues were fixed in PBS with 3.7% formaldehyde. Prior to paraffin sectioning, soft tissues were dehydrated via an ethanol series, while bone-containing tissue was decalcified in 0.46M EDTA, 2.5% Ammonium Hydroxide pH 7.2.

Generation of osteosarcoma cell lines

Osteosarcomas were dissected, minced, filtered through a 70μm filter, and plated in normal growth medium (10% FBS in DME, 1% P/S, L-glutamine) to generate the OS cell lines. Cells were passaged as they reached confluence. For RNA purification, cells were rinsed 2x with PBS, and RNA extraction was performed using RNAeasy kit (Quiagen). First-strand cDNA was transcribed from 1 μg of RNA using Superscript III reverse transcriptase (Invitrogen) following the manufacturer's instructions. Quantitative RT-PCR with 20 to 100 ng cDNA was done using SYBR Green (Applied Biosystems). Reactions were run on the ABI Prism 7000 Sequence Detection System and analyzed using the 7000 SDS software. Primers used for qPCR are shown in Chapter 3 supplementary

materials. Knock-down of *Rb* in the *p53KO-OS* cells was achieved using the pMLP-miR30-based shorthairpin (*Rb* targeted sequence: CACGGACGTGTGAACTTATATA). Adenoviruses expressing Cre or GFP were provided by the U. of Iowa Gene Transfer Vector Core. Antibodies were from Santa Cruz Biotechnology [pRb (H-153), E2F1 (C-20), and E2F4 (C-20)], BD Pharmingen (pRb), Ambion (GAPDH) and MBL (Runx2).

Immunohistochemistry (IHC)

Runx2 and Kia67 IHC was performed using a modified citric acid unmasking protocol. Briefly, slides were deparaffinized in xylene and rehydrated through ethanol. Slides were boiled for 30 min in citrate buffer, pH 6.0, and then cooled in running tap water. Slides were then washed in PBS for 5 min followed by inactivation of endogenous peroxidases by incubation 0.5% H_2O_2 in methanol. Slides were blocked in 10% Goat Serum for 1 h at room temperature. Primary antibody (MBL anti-Runx2 Clone 8G5; anti-Kia67, BD Catalog #: 550609) was diluted 1:200 in PBS 0.15% Triton and incubated overnight at 4 °C. The following day, slides were washed three times in PBS. Secondary antibodies (Vectastatin ABC kits, Vector laboratories) were diluted 1:500 in PBS containing 0.4% Goat Serum and detected using a DAB substrate (Vector Laboratories). All samples were counterstained with hematoxylin.

Chromatin Immunoprecipitation assay

Protein complexes were cross-linked to DNA in living nuclei by adding

formaldehyde (Sigma, Inc.) to give a final concentration of 1%. After incubation for 10 min at 37 °C, crosslinking was stopped by addition of glycine to a final concentration of 0.125 M for 5 min. Cross-linked cells were washed twice with PBS containing PMSF 1mM (phenylmethylsulfonyl fluoride), scraped and pelleted. Nuclei were extracted with a 20mM Tris pH 8, 3mM MgCl2, 20 mM KCl buffer containing protease inhibitors, pelleted by microcentrifugation and lysed by incubation in SDS lysis buffer (1% sodium dodecyl sulfate, 10 mM EDTA, 50 mM Trischloride pH 8.1), containing protease inhibitors. The resulting chromatin solution was sonicated to generate 500-1000 bp DNA fragments. After microcentrifugation, the supernatant was diluted 1:10 with a dilution buffer (0.01% sodium dodecyl sulfate, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Trischloride pH 8.1, 167 mM NaCl, containing protease inhibitors), precleared with blocked protein A-positive Staph cells (Santa Cruz, Inc), and divided into aliquots. Five micrograms of the indicated antibodies was added to each aliquot and incubated for 12 to 16 hours at 4°C with rotation. Antibody-protein-DNA complexes were isolated by immunoprecipitation with blocked protein A-positive Staph A cells. Following extensive washing, bound DNA fragments were eluted and analyzed by Quantitative RT-PCR using primers shown in Chapter 3 supplementary materials.

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Chapter 6

Discussion

Discussion

This work derives from our efforts to model osteosarcoma in the mouse (Berman *et al.*, 2008a; Calo *et al.*, 2010). Osteosarcoma is the most common malignancy of bone tissues affecting mostly pediatric patients. Genetic analyses of bone tumors have linked mutations in the tumors suppressor genes *RB-1* and *TP53* with the development of this malignancy (Kansara and Thomas, 2007). Although osteosarcomas account for less than 1% of all human cancers, its high propensity to metastasize to the lungs makes this malignancy one of the most lethal and difficult to treat. Despite recent advances in anti-cancer therapy, the five-year survival rate for patients suffering this disease has not increased in the last two decades, clearly indicating the need to develop research and clinical approaches to fight this disease. However, as a first step in this task, we need to elucidate the molecular mechanisms and the genes responsible for osteosarcoma initiation, progression, and metastasis.

To better understand the mechanisms underlying osteosarcoma, we aimed to elucidate one of the most intriguing and yet unanswered question in cancer biology: Why is the *RB-1* pathway frequently inactivated in osteosarcomas? To address this question we decided to model osteosarcoma in the mouse by specifically targeting the pRb and/or p53 pathways in committed osteoblasts or mesenchymal stem cells (MSCs). These approaches have expanded our knowledge about the role of pRb not only in osteosarcoma but also in stem cell function. These findings are summarized below.

First, we have successfully generated a mouse model for osteosarcoma by targeting the *Rb* and *p53* pathways in committed osteoblasts. This is in fact the first mouse model for osteosarcoma in which pRb has been shown to play a role and the first in recapitulating most of the aspect observed in the human malignancy (Chapter 2; Berman *et al.*, 2008a). Second, we have narrowed the origin of pRb mutant osteosarcomas to committed osteoblasts by generating a second mouse model in which loss of *Rb* and *p53* is achieved the stem cell compartment (Chapter 3). These two models led us to discover that pRb acts as a molecular switch between the bone and brown fat lineages (Calo *et al.*, 2010). Third, by interrogating how downstream effectors of the pRb pathway influence osteosarcomagenesis we find that E2f1 promotes osteosarcoma tumor development by antagonizing osteogenic differentiation (Chapter 4). Finally, we closed this work by studying the therapeutic consequences of reactivating pRb in osteosarcomas *in vivo* and observed that re-expression of pRb function halts tumor progression and promotes differentiation (Chapter 5).

Thus, in our efforts to model osteosarcoma in the mouse, we have not only generated novel research tools to study this malignancy, but also expanded our knowledge into the role of pRb in MSCs differentiation and tumorigenesis. However, these studies opened more intriguing questions about the biology of pRb in mesenchymal tissue development and tumorigenesis; these questions and potential experimental approaches to address them are discussed in the following sections.

Part I: Role of pRB in Osteosarcoma

A. Generation of a mouse model for osteosarcoma

Multiple strategies have been employed to model osteosarcoma in the mouse. The initial models for this disease were generated using chemical- or radiation-induced lesion in animals (Ek *et al.*, 2006). These approaches were proven to be unpractical to study osteosarcoma due to their unpredictability and lack of reproducibility in tumor formation. Transplantation of mouse or human osteosarcoma cells into immunocompromised mice has also been reported as a system to study this malignancy (Kelly *et al.*, 2007). However, this approach is limited because the tumors are generated from cell lines established in tissue culture, are forced to develop in an ectopic environment, and do not take into consideration the role of the immune system during tumor progression (Becher and Holland, 2006; Sharpless and Depinho, 2006). Several genetically engineered mouse models, carrying null or transgenic alleles predisposed animals to osteosarcoma. These models are also not feasible because osteosarcoma development in these animals occurs with extremely low penetrance (Jacks *et al.*, 1994; Lang *et al.*, 2004; Olive *et al.*, 2004; Wang *et al.*, 1995). Osteoblasts restricted deletion of *p53* using the *Col3.6-Cre* transgenic mouse strain result in early onset osteosarcoma, however these animals also developed other non-mesenchymal tumors, rendering difficult the study of bones tumors (Lengner *et al.*, 2006). Thus, from a clinical perspective the models discussed above are unpractical because they do not recapitulate multiple aspects of the human disease.

We have overcome all these issues by generating a novel mouse model for osteosarcoma driven by osteoblasts restricted deletion of *Rb* and *p53*. In our mouse model the osteosarcomas develop with full penetrance, are osteoblastic in nature, highly undifferentiated, and able to undergo metastasis into the lung and the liver of the animals (Berman *et al.*, 2008a). Therefore, unlike the previously described mouse models for osteosarcoma, ours is clinically relevant and can be used as an aid in the investigation of the biology of this malignancy. For these reasons this model can be used as an assay for the preclinical development of anticancer drugs, and as a tool for discovering new clinical agents and assays.

B. Are there tumor-initiating cells in osteosarcoma?

The discovery of tumor initiating cells (TIC) completely revolutionized our understanding of cancer (Hope *et al.*, 2004). TIC were initially discovered in hematopoietic malignancies and since then, TICs have been identified in multiple tumor types (O'Brien *et al.*, 2010). Although, the existence of TIC is controversial, the rational behind this is that within a tumor there is a sub-population of cells with stem cell like properties. These cells are thought to be responsible for initiating/maintaining the bulk of the tumor. Interestingly, TICs are believe to acquire an aberrant and uncontrolled stem cell like phenotype that closely mimics normal tissue stem cells (Lobo *et al.*, 2007). Since there is no evidence that osteosarcomas can be initiated/maintained by TICs, we explored this possibility using our mouse model.

Our data strongly suggests that osteosarcomas are propagated by a subpopulation of cells with tumor initiating potential (herein called OS TIC for osteosarcoma tumor initiating cells; Berman *et al.*, 2008a). We found the phenotypes that are hallmarks of TICs in cell lines derived from *Osx1-Cre;Rb*$^{fl/fl}$*;p53*$^{fl/fl}$ mutant osteosarcomas (*DKO OS*). First, we observed that these cells have elements of more primitive stem cells as they have the capacity of multilineage differentiation *in vivo*. Second, *DKO OS* cells expressed the stem cell associated antigen, Sca-1, which has been shown to enrich for TICs in multiple tumor types (Holmes and Stanford, 2007). Third, these cells have tumor reinitiating capacity *in vivo* and Sca-1^{high} expressing cells efficiently engrafted and generated tumors faster than the Sca1$^{low/-}$ cells. Importantly, these tumors were histologically indistinguishable from the parental osteosarcomas from where the tumor cells were derived.

Together, these experiments clearly indicate the existence of TICs in murine osteosarcomas. However, the lack of additional markers to better characterize OS TICs in our system made it impossible to bring these studies to completion. We tried to isolated OS TICs using several antibodies against cell surface markers previously identified in murine MSCs, unfortunately we did not find a combination of markers, other than Sca-1, that could further enriched for OS TICs. This is a barrier for us to translate these studies into the human disease because Sca-1 is a murine protein, which is not present in human cells. Thus, for the future identifying more markers for these cells will be beneficial not

only to study the biology of OS TICs in more detail, but also to help translate these findings to the human malignancy.

C. *Rb* loss is not sufficient to initiate osteosarcomas

The *RB-1* gene is inactivated in more than 50% of sporadic osteosarcomas. In addition, patients with familial retinoblastoma have 500-times higher incidence of developing osteosarcomas than the general population (Kansara and Thomas, 2007). These studies indicated that in humans loss of *RB-1* is a strong predictor of osteosarcoma tumor development. However, we found that in the mouse this is not the case. Specifically, we observed that deletion of *Rb* alone either in the stem cell compartment or in committed osteoblast did not result in osteosarcoma tumor development. In this section, I will discuss the additional experimental approaches that let us generate this conclusion and how we can reconcile these discrepancies.

The fact that, in our mouse models, the *Rb* single mutant animals did not develop osteosarcoma seems to be inconsistent with what is known about the human disease. There are three possible explanations for this discrepancy: First, the cell of origin for *Rb* mutant osteosarcomas is unknown, therefore it may be possible that inactivation of pRb in osteblastic compartments other than *Prx1*- and *Osx1-Cre* will result in the development of osteosarcomas. Alternatively, this discrepancy may reflect differences between the mouse and the human species. While *RB-1*$^{+/-}$ patients develop bilateral retinoblastoma and osteosarcomas, *Rb*$^{+/-}$ animals develop pituitary and thyroid tumors, never retinoblastoma or

osteosarcoma. Finally, it is possible that in the mouse, other pocket proteins (p107 or p130) compensate for the loss of *Rb*. This is supported by the fact that *Rb;p107*-null chimeras develop retinoblastoma and osteosarcomas at low frequencies. However, when we tried to recapitulate those findings in the context of the *Osx1-Cre* transgene none of the *Osx1-Cre;Rb*$^{fl/fl}$*;p107*$^{-/-}$ animals developed osteosarcomas (Lees and Landman unpublished data). These results support the notion that we are either not targeting the right compartment or that additional oncogenic mutations are required.

An experimental approach to better understand the role of pRb in osteosarcomas is to irradiate the *Osx1-Cre;Rb*$^{fl/fl}$ animals and test whether osteosarcomas will develop in this context. The rational behind this experiment is that patients with familial retinoblastoma develop osteosarcomas later in life. Therefore, to date it is not clear whether the osteosarcomas observed in these patients are initiated as secondary effect of the radiation used for the treatment of the childhood retinoblastoma. If osteosarcomas are observed they can be better characterized by sequencing the tumors to identify genes that cooperate with *Rb* loss to promote osteosarcoma in the mouse. If true, this finding will help clarify whether the incidence of osteosarcomas in patients with familial retinoblastoma is indeed radiation-induced. If osteosarcomas are not observed, then it is formally possible that *Rb* loss is not a transforming agent for murine osteosarcomas or that pRB is inactivated as a secondary oncogenic event in this malignancy. If true, this later statement will be consistent with our observation in the mouse in

which loss of *Rb* cooperate with mutations in *p53*, in a dosage dependent manner, to accelerate osteosarcoma tumor formation.

D. What makes *Rb* mutant osteoblasts more susceptible to oncogenic transformation?

Our *in vivo* analyses using the *Osx1-Cre* transgene revealed that *Rb* loss alone is insufficient to transform committed osteoblasts, but it synergizes with *p53* mutations to accelerate the onset of osteosarcoma. How does *Rb*-loss modulate the biology of committed osteoblasts to make them more susceptible to oncogenic transformation? The answer to this question came from the analyses of *Osx1-Cre;Rb*$^{fl/fl}$*;p53*$^{fl/fl}$ and *Osx1-Cre;p53*$^{fl/fl}$ derived osteosarcoma cell lines (herein called *DKO* OS and *p53KO* OS respectively). I mentioned that *DKO* OS cells have the capacity to undergo osteogenic and adipogenic differentiation *in vitro*. Therefore, we initially hypothesized that this stem cell "like" phenotype will be featured in all the osteosarcomas cell lines regardless of their starting genotype. However, this hypothesis did not hold to be true. To our surprise, we found that *p53KO* OS cells lines failed to undergo differentiation into either the bone or the fat lineages. Notably, *p53KO* osteosarcoma cells were Sca-1 positive and able to generate osteosarcomas *in vivo*. Thus, given that the only known genetic difference between *DKO* and *p53KO* OS cells is the absence of pRb in the former, we hypothesized that: *Rb* loss promotes cellular plasticity.

This hypothesis was further strengthening by several *in vivo* and *in vitro* studies. First, downregulation of *Rb* expression in *p53KO* OS cells reestablished

the multilineage differentiation program both *in vitro* and *in vivo*. Moreover, this *Rb*-dependent plasticity was not just restricted to transformed cells as *Rb* loss in primary osteoblasts was capable of rendering these cells multipotent *in vitro*. Thus, we conclude that *Rb*-loss enables plasticity by acting as a molecular switch between the multipotent and the committed state.

How does the loss of pRb promote cellular plasticity? One possibility is that *Rb* loss promotes dedifferentiation towards the multipotent state (model 1). If true, the tumorigenic consequences of losing *Rb* in committed osteoblast is to allow the cells to acquire an aberrant stem cell "like" phenotype. Alternatively, *Rb* loss promotes trans-differentiation to the adipogenic state (model 2). Evidence available in the literature only supports model 1. For example, studies in the *Drosophila* retina showed that *Rb* loss cooperate with mutations in the *hippo* pathway to promote photoreceptors dedifferentiation towards the multipotent state (Holmes and Stanford, 2007). In addition, mouse embryonic fibroblast that lack pocket protein function acquire an aberrant cancer stem cell phenotype *in vivo*, at least in part, by reactivating the embryonic stem cell program (Liu *et al.*, 2009). Finally, loss of *Rb* and $p19^{Arf}$ in terminally differentiated myoblasts promote cell cycle entry and dedifferentiation to a progenitor "like" state (Pajcini *et al.*, 2010). Our data also is more consistent with model 1. The fact that the gene expression profile of *DKO* OS cells more closely resembled MSCs than osteoblasts suggest that dedifferentiation has occurred. Consistently, the gene expression profile of *p53KO* cells resembles more osteoblasts than MSCs. Therefore, we believe that *Rb* loss enables dedifferentiation and this synergizes

with other mutations to promote tumorigenesis. However, more experiments need to be done in order to distinguish between these two models. Also, it will be important to investigate whether these findings translate to other tumor models.

E. What are the therapeutic consequences of re-expressing pRb in osteosarcomas?

Together our results are consistent with a model in which *Rb* is acting as a molecular switch between the multipotent and the committed states, which seems to be critical for tumor initiation. This model is further supported by our experiments described in Chapter 3 and 5 showing that downregulation of pRb, in *p53KO* cells, increased their tumorigenicity and reactivated the stem cell program. Thus we asked what are the consequences of reactivating pRb functions *in vitro* and *in vivo*. *In vitro*, we observed that reactivating pRb resulted in all the tumor suppressive properties already ascribed to this tumor suppressor gene: induction of cell cycle arrest, apoptosis, and cellular senescence (Burkhart and Sage, 2008). However, we found that *in vivo* cellular senescence and apoptosis do not seem to be playing an important role to suppress tumor progression. In fact, we found that reactivation of pRb in tumors *in situ* promotes cell cycle arrest and tumor differentiation. This data uncover a novel tumor suppressive network regulated by pRb, which is solely dependent on pRb's role to modulate commitment towards the differentiated state.

F. Conclusion

Overall our data uncover the tumor suppressive network regulated by pRb in osteosarcomas. We established that pRb plays a role in osteosarcoma when mutated in osteoblasts, but not in the stem cell compartment, indicating that *Rb* mutant osteosarcomas originate from committed osteoblasts. However, loss of *Rb* in committed osteoblasts is not capable alone to initiate tumorigenesis, but is sufficient to reprogram these cells to the multipotent state, which may serve as a precursor for further transformation. Consistently, reactivation of pRb in *Rb*-mutant osteosarcomas prevents tumor progression by reactivating the osteogenic differentiation program.

Part 2: *Rb* Regulates Mesenchymal Differentiation

A. *Rb* regulates fate choice between the bone and fat lineages

So far I have discussed how we used the mouse to uncover the mechanisms by which pRb modulates the biology of osteosarcomas. However the study of these mouse models also led us to explore the role of pRb in mesenchymal differentiation. One intriguing observation from our *in vivo* analyses is the fact that regardless of the targeted cellular compartment (whether is the MSCs or committed osteoblasts) *Rb* loss modulates the biology of the sarcomas arising from the *p53* mutant animals in two different ways: *Rb* decreases the average tumor free survival of the animals in a dosage dependent manner and it expanded the tumor spectrum to also include brown fat tumors. In the stem cell compartment, the presence or absence of pRb dictates tumor fate: osteosarcomas are the predominant tumor type in the presence of *Rb* and hibernomas in its absence, indicating that pRb may be modulating the fate between these two lineages *in vivo*. This possibility is supported by previous reports showing that pRb regulates master differentiation inducers for both of these lineages. pRb can inhibit adipogenesis by repressing Pparγ expression (Fajas *et al.*, 2002) and it acts as a cotranscriptional activator for Runx2 (Thomas *et al.*, 2001), the master regulator of osteogenesis. We further confirmed this experiments by using the *Meox-Cre* transgene, which expresses Cre recombinase in the embryo proper, allowing *Rb* mutant embryos to survive until birth (Wu *et al.*, 2003). We found that *Meox-Cre;Rb*$^{fl/fl}$ embryos indeed displayed defects in skeletal ossification and showed an expanded brown fat compartment

(Berman *et al.*, 2008b; Calo *et al.*, 2010). Thus our results showed that pRb modulated the fate between the bone and the brown fat lineages during normal development and tumorigenesis.

Our data also establish a clear role for pRB in determining the lineage commitment of pre-osteoblasts. This occurs both *in vitro* and *in vivo* and irrespective of whether these cells are transformed or otherwise wildtype. *In vivo*, *Rb*-loss disfavors osteogenesis to the extent that it can reduce the levels of calcified bone. Moreover, *Rb*-loss in pre-osteoblasts is sufficient to disfavour commitment to the osteogenic state and restores multipotency. Mechanistically, we showed that this phenotype reflects, at least in part, pRb's ability to promote Runx2 transcriptional activity and, at the same time, E2f-dependent repression of the master regulator of adipogenesis, *Pparγ*. This is consistent with our results shown in Chapter 5, in which loss of *E2f1* in *DKO* mutant animals reduces the incidence of brown fat tumors *in vivo* and the ability of *Rb* mutant osteoblasts to undergo adipogenesis *in vitro*. Thus the ability of *Rb* to promotes cellular plasticity, in fact, reflects a direct role for pRb's in maintaining lineage commitment.

B. Conclusion

Combined, our data show that pRb's role in modulating fate choice and lineage commitment is, in part, through the regulation of master differentiation inducers for both of these lineages. By exploring the contribution of other components of the pRb pathway during this event, we showed that *E2f1* loss represses *Rb*-

dependent plasticity both *in vivo* and *in vitro*. However, the specific mechanisms for this repression, whether is cell cycle dependent or direct influence of E2f1 during osteogenesis, remains to be elucidated. Similarly, upstream regulators of the pRb pathway may also play an important role during this process, therefore more experiment need to be performed to test this model.

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mas no la forma que el artista sueña.

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Publications:

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Saint Jude Children Research Hospital National Graduate Student Symposium. April 2010. "*Rb* Regulates Fate Choice and Lineage Commitment."

MIT's Independent Activity Period Special Seminar Series. January, 2010. "Role of *Rb* and *p53* during Mesenchymal Tumorigenesis."

Colrain Cancer Meeting. September 2008. "Metastatic Osteosarcoma Induced by Inactivation of *Rb* and *p53* in osteoblasts."

Poster Presentations

Eliezer Calo, Simona Nedelcu, Paul Danielian, Seth Berman, and Jacqueline A. Lees (January, 2010). *Role of Rb and p53 During Mesenchymal Tumorigenesis.* Poster session presented at the MIT Ludwig Cancer Center Annual Retreat.

Alison S. Landman, **Eliezer Calo**, Paul Danielian, Seth Berman, and Jacqueline A. Lees (January, 2009). *Mouse Model for Mesenchymal Neoplasia.* Poster session presented at the MIT Ludwig Cancer Center Annual Retreat.

Eliezer Calo, Shuyan Chen, Heather Rodriguez and Steven P. Bell. (November, 2005). *Mapping Cdt1 and Orc6 Interactions.* Poster session presented at the Annual Biomedical Research Conference for Minority Students.

Eliezer Calo, Brian P. Davis, and Clemens Richert. (November, 2004). *Synthesis of Modified Oligonucleotides for Immunomodulatory Testing.* Poster session presented at the Annual Biomedical Research Conference for Minority Students.

Eliezer Calo, Rosa V. Flores, and Fernando A Gonzalez. (March, 2004). *Expression of a functional P2Y2-EGFP in 13N1 Human Astrocytoma Cells.* Poster session presented at the 227th American Chemical Society National Meeting.