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LETTERS

Rb regulates fate choice and lineage commitment *in vivo*

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Mutation of the retinoblastoma gene (*RB1*) tumour suppressor occurs in one-third of all human tumours and is particularly associated with retinoblastoma and osteosarcoma[1]. Numerous functions have been ascribed to the product of the human *RB1* gene, the retinoblastoma protein (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[1]. In addition, pRb has been shown *in vitro* to regulate several transcription factors that are master differentiation inducers[2]. 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 *in vitro*[3]. In contrast, pRb acts with E2F to suppress peroxisome proliferator-activated receptor γ subunit (PPAR-γ), the master activator of adipogenesis[4,5]. Because 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, so far, there is no evidence for this *in vivo*. Here we use mouse models to address this hypothesis in 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 *RB1* (70–90% of cases) and *TP53* (50–70% of cases) are strongly associated with human osteosarcoma[6,7]. To model osteosarcoma in the mouse, we crossed $Rb^{fl/fl}$ (ref. 8) and $p53^{fl/fl}$ (ref. 9) conditional mutant mice with a transgenic line, *Prx1-Cre*[10], which expresses Cre recombinase in uncommitted mesenchymal cells that contribute to bone, muscle and both white and brown adipose tissue (Supplementary Fig. 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 (Table 1). 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 (Table 1 and Fig. 1a): deletion of one *Rb* allele increased the frequency of osteosarcomas (to 92%), whereas mutation of both *Rb* alleles shifted the tumour spectrum away from osteosarcoma (now 18%) and towards hibernomas (91%; Supplementary Fig. 2). This propensity for brown-fat, as opposed to white-fat, tumours fits with previous studies showing that *Rb* loss promotes brown-fat over white-fat differentiation[11–13]. Genotyping confirmed that the tumour cells had undergone Cre-mediated recombination of *Rb* and/or *p53* (Fig. 1b and data not shown). Moreover, it showed that the *Prx1-Cre;*$Rb^{+/fl}$;$p53^{fl/fl}$ tumours consistently retained the wild-type *Rb* allele (Fig. 1b and data not shown). Thus *Rb* acts in a dose-dependent manner to modulate the spectrum of tumours arising from p53-deficient, uncommitted mesenchymal stem cells: osteosarcomas predominant in the presence of *Rb*, whereas loss of *Rb* 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[14], which uses *Osterix* promoter sequences to express Cre in the pre-oestoblast (Supplementary Fig. 1d). In this model[15], *Osx-Cre;*$p53^{fl/fl}$ mice specifically develop osteosarcoma (100%) whereas *Osx-Cre;*$Rb^{fl/fl}$;$p53^{fl/fl}$ mice develop osteosarcoma (53%), hibernomas (46%) and sarcomas (2%). We established cell lines from multiple (at least three) 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 (Fig. 2 and data not shown). The *Rb;p53* (*DKO*) osteosarcoma cell lines expressed messenger RNAs (mRNAs) that are characteristic of bone

Table 1 | Mesenchymal tumour distribution

Genotype			Tumour distribution (%)				Number of mice	Average latency (days ± SD)
Prx1	Rb	p53	RMS	SAR	OS	HIB		
+	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

Values are the percentage of animals analysed up to 24 months of age for *Prx1-Cre;Rb* and/or *p53* compound mutant animals. RMS, rhabdomyosarcomas; SAR, undifferentiated sarcomas; OS, osteosarcomas; HIB, hibernosarcomas.

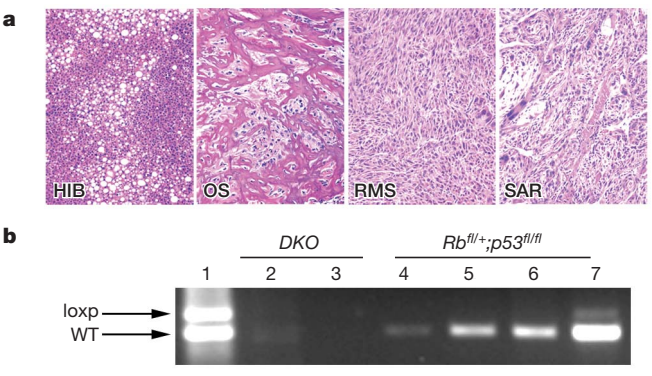

Figure 1 | *Rb* cooperates with *p53* and modulates mesenchymal tumour fate in a dose-dependent manner. a, Haematoxylin and eosin staining of representative sarcomas (×20 magnification). HIB, hibernoma; OS, osteosarcoma; RMS, rhabdomyosarcoma; SAR, undifferentiated sarcoma. **b**, PCR genotyping to detect *Rb* wild type (WT) and non-recombined conditional mutant (loxp) alleles in control $Rb^{fl/+}$;$p53^{fl/+}$ tissues (lane 1) or cell lines derived from *Prx1-Cre;*$Rb^{fl/fl}$;$p53^{fl/fl}$ (*DKO*) or *Prx1-Cre;*$Rb^{fl/+}$;$p53^{fl/fl}$ osteosarcomas. Cell lines were cultured for at least 20 passages before genotyping to eliminate stromal cell contribution.

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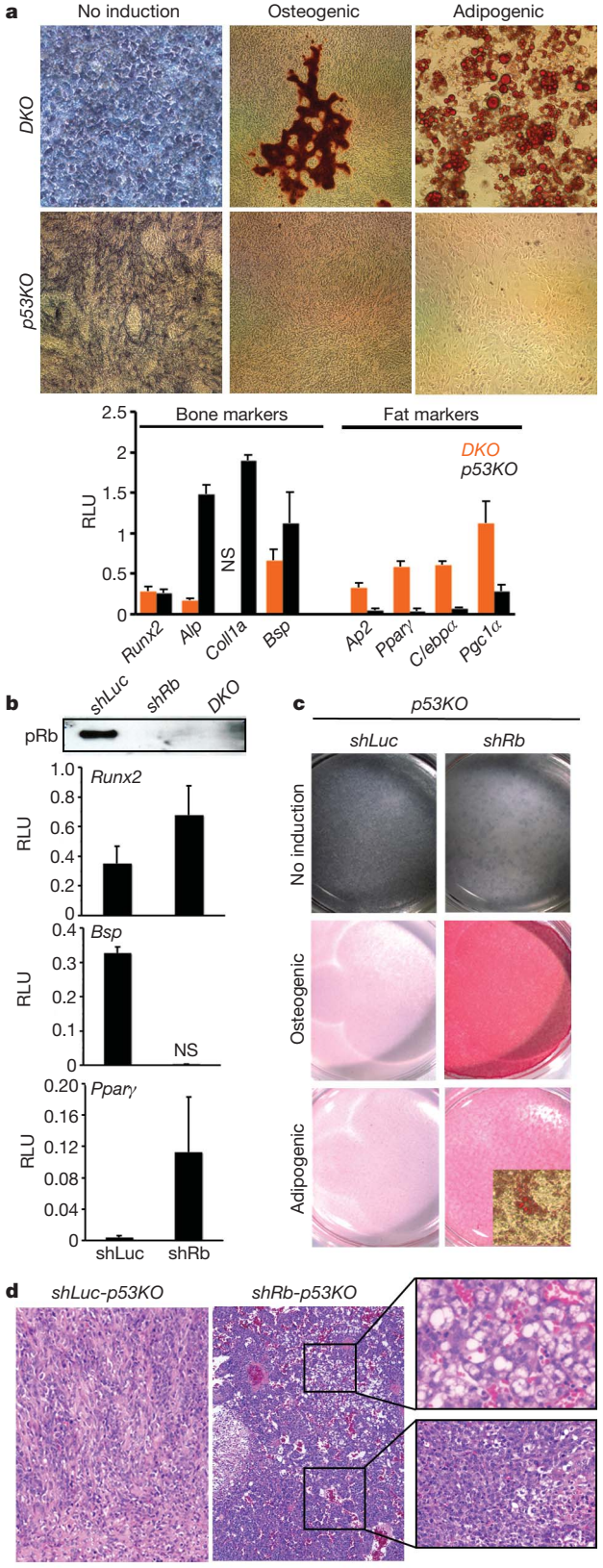

Figure 2 | *Rb* regulates osteosarcoma cell-lineage plasticity *in vitro* and *in vivo*. The differentiation potential of three different *Osx-Cre;Rb*$^{fl/fl}$*:p53*$^{fl/fl}$ (*DKO*) and *Osx-Cre:p53*$^{fl/fl}$ (*p53KO*) osteosarcoma cell lines was assessed 0, 7, 14 or 21 days after addition of differentiation media. **a**, Representative staining for (left lane) alkaline phosphatase before differentiation induction, (middle lane) Alizarin red to detect bone mineralization 14 days after culture in osteogenic-induction media and (right lane) Oil red O to detect lipid droplets 14 days after culture in adipogenic-induction media. Expression of bone (*Runx2*, *Alp*, *Coll1a* and *Bsp*) and fat (*Ap2*, *Pparγ*, *C/ebpα* and *Pgc1α*) markers was assessed by qPCR of uninduced *DKO* (orange) and *p53KO* (black) osteosarcoma cells. Bars represent the mean of three independent experiments (± SD). NS, not significantly expressed. RLU, relative light units. **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 bone markers *Bsp* (also *Coll1a* and *Alp*, data not shown), and upregulation of fat markers *Pparγ* (also *Ap2* and *C/ebpα*, data not shown) without culture in differentiating media. Bars represent the mean of three independent experiments (± SD). **c**, The osteogenic and adipogenic potential of *shLuc*- and *shRb-p53KO* cell lines was assessed 0, 7, 14 and 21 days after differentiation induction by Alizarin red and Oil red O staining. A representative time point (14 days) is shown. **d**, Haematoxylin and eosin staining of representative tumours derived from *shLuc*- and *shRb-p53KO* cell lines injected subcutaneously into immunocompromised mice. *shRb-p53KO* osteosarcoma cells consistently (ten out of ten injections) yielded tumours that arose faster, and were more aggressive, than those arising from the parental *p53KO* osteosarcoma controls (ten injections). Moreover, the *shRb-p53KO* osteosarcoma-derived tumours were frequently (six out of ten injections) mixed lineage (top inset shows fat neoplasm; bottom inset shows bone/undifferentiated sarcoma), whereas the control *shLuc-p53KO* tumours were uniformly (ten out of ten injections) osteosarcomas. Additional analysis of these tumours (haematoxylin and eosin, Sirius red staining and Runx2 immunohistochemistry) is shown in Supplementary Fig. 4.

and fat differentiation (Fig. 2a). Indeed, their expression pattern more closely resembled that of mesenchymal stem cells than primary osteoblasts (Supplementary Fig. 3). Accordingly, culture in the appropriate differentiation media induced these *DKO* cells to adopt either the adipogenic or osteoblastic fate (Fig. 2a). In contrast, the *p53KO* osteosarcoma cell lines closely resembled pre-osteoblasts based on their gene expression patterns, but these cells were unable to differentiate into either bone or fat (Fig. 2a and Supplementary Fig. 3). Because this differentiation block occurs in the p53-null osteosarcoma cell lines, but not p53-deficient primary osteoblasts[16], it likely reflects their transformed state. We used these *p53KO* osteosarcoma 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*) short hairpin RNAs (shRNAs). pRb was readily detectable in *shLuc-p53KO*, but not *shRb-p53KO*, osteosarcoma cells (Fig. 2b). Strikingly, without addition of differentiation media, pRb knockdown downregulated the bone-specific mRNA *Bsp* and upregulated the fat regulator *Pparγ* (Fig. 2b). Accordingly, these *shRb-p53KO* osteosarcoma cells were now able to differentiate into either bone or fat *in vitro* (Fig. 2c). Moreover, when transplanted into nude mice, the *shRb-p53KO* osteosarcoma cells formed more aggressive tumours than the parental *p53KO* osteosarcoma 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* osteosarcoma cell lines (Fig. 2d, Supplementary Fig. 4 and data not shown). Thus pRb loss is sufficient to override the differentiation block of these p53-deficient tumour cell lines and expand their fate commitment to include the adipogenic state.

We also examined the consequences of reintroducing *Rb* into the *DKO* osteosarcoma cells. For this, we induced pRb in confluence-arrested *DKO* osteosarcoma cells using a doxocycline-inducible expression system (*DKO-Rb*$^{Dox\text{-}ON}$; Supplementary Fig. 5). Remarkably, pRb restoration caused the *DKO* osteosarcoma cells to adopt the differentiation state of the *p53KO* osteosarcoma cell lines within 2 days: it induced downregulation of adipogenic markers and upregulation of osteogenic markers, and the cells were unable to differentiate into fat (Supplementary Fig. 5). Thus removal or reintroduction 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*[4,5], and bind, and potentiate the transcriptional activity of, the osteogenic regulator RUNX2 (ref. 3). We hypothesized that pRb's role in these processes might underlie the

effect of *Rb* 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 (Fig. 3). First, we used chromatin-immunoprecipitation assays to investigate promoter regulation of *Pparγ* and representative Runx2-responsive genes *Coll1α* (Fig. 3a) and osteocalcin (*Oc*; data not shown). pRb-induction caused both pRb and E2f4, the predominant repressive E2F, to be recruited to the *Pparγ* promoter (Fig. 3a) and this correlated with *Pparγ* mRNA downregulation (Fig. 3a). Contemporaneously, pRb bound to *Coll1α* and *Oc* and this was accompanied by increased promoter occupancy of Runx2 and upregulation of *Coll1α* and *Oc* mRNAs (Fig. 3a and data not shown). Importantly, these changes in *Pparγ*, *Coll1α* and *Oc* regulation were all detected within 2 days of pRb

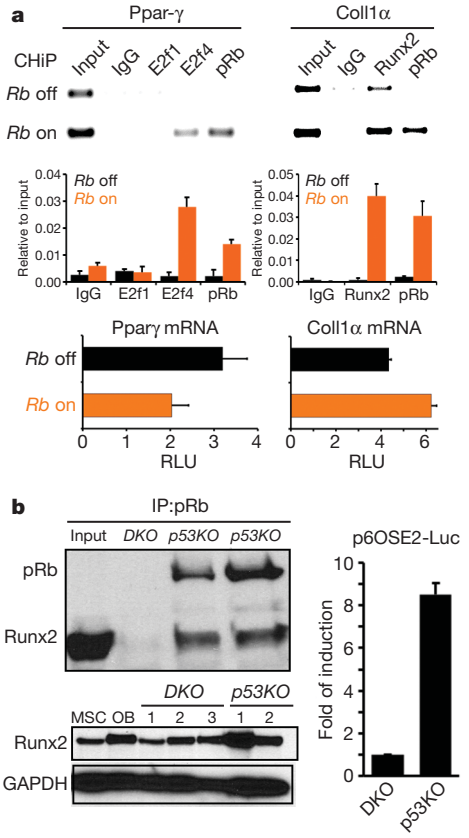

Figure 3 | pRb modulates the activity and the expression of the master lineage regulators Runx2 and Ppar-γ. a, The stable *DKO-Rb*$^{Dox\text{-}ON}$ osteosarcoma cells were generated by drug selection of pools of DKO cells transfected with the doxocyline-inducible construct pCW22-Rb. These were cultured for 2 days in the absence (*Rb* off) or presence (*Rb* on) of doxocycline and then analysed. 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 *Col1α*[20] and increased the binding of Runx2. These changes correlated with the downregulation of *Pparγ* mRNA and upregulation of Col1α mRNA, as judged by qPCR. Bars, the mean of three independent experiments (± SD). ChIP, chromatin immunoprecipitation **b**, Western blotting detected Runx2 in pRb-immunoprecipitates from *p53KO* osteosarcoma cell lines (left, top panel). Western blotting of whole-cell extracts confirmed that Runx2 was expressed in both *DKO* and *p53KO* osteosarcoma cell lines (left, bottom panel). Mesenchymal stem cells and osteoblasts were used as a positive control. Right panel: Runx2 transcriptional activity was shown to be higher in the *p53KO*- versus the *DKO* osteosarcoma cell lines as judged by activation of the artificial Runx2-responsive reporter p6OSE2-Luc. Results are the average of six independent samples. Error bars, s.e.m.

induction and without addition of differentiation-inducing media. In addition, we found that Runx2 associated with pRb in the *p53KO*, but not the *DKO*, osteosarcoma cells, and that its transcriptional activity was eightfold higher in the former population than the latter (Fig. 3b). Thus the presence or absence of pRb directly modulates the levels and activity of Pparγ and Runx2 in accordance with the preferential commitment of our osteosarcoma 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, because 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*$^{fl/fl}$ at embryonic day 18.5. We brought these cells to confluence, to minimize the influence of altered proliferation, infected them with adenoviruses expressing Cre or a green fluorescent protein (GFP) control and then assayed differentiation. As expected, the control-infected osteoblasts were able to undergo osteogenesis but not adipogenesis (Fig. 4a and data not shown). Similarly, p53 loss had no effect on this fate commitment[16] (Fig. 4a). In stark contrast, deletion of *Rb*, either alone or with *p53*, allowed these cells to adopt either the bone or fat lineage (Fig. 4a and data not shown). This switch to multipotency correlated with the significant upregulation of adipogenic markers before the induction of differentiation (Fig. 4b). Thus pRb-loss is sufficient to alter the fate commitment in otherwise wild-type calvarial osteoblasts.

In vitro culture can modulate the plasticity of cells. Thus we examined the role of *Rb* in fate choice *in vivo*. For this, we used a third transgenic strain, *Meox2-Cre*, which expresses Cre in the embryo proper from embryonic day 6.5 (ref. 17). *Meox2-Cre;Rb*$^{fl/fl}$ embryos survived to birth[18]. We isolated wild-type (*Meox2-Cre;Rb*$^{+/+}$) and *Rb* mutant (*Meox2-Cre;Rb*$^{fl/fl}$) littermates at embryonic days 15.5 and 18.5 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 compared with wild-type embryos[19] (Fig. 4c). Moreover, quantitative PCR (qPCR) established that *Runx2* mRNA was present at appropriate levels in the *Rb* mutant calvarial osteoblasts at embryonic day 18.5, but there was a downregulation of other bone markers and a clear upregulation of fat-associated mRNAs (Fig. 4c). In parallel, we found that the level of brown fat dramatically increased in the *Rb* mutant compared with the wild-type controls at embryonic day 18.5 (Fig. 4d and Supplementary Fig. 6). Thus *Rb* loss in an, otherwise wild-type, 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*, irrespective of whether these cells are transformed or otherwise wild type. *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 exists 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 de-differentiation 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* osteosarcoma cells (Supplementary Fig. 3) and the broadening of the tumour 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 offer potential insight into the cell of origin for osteosarcomas. Specifically, given the high frequency of *RB1* mutations in human osteosarcoma, we were surprised to find that *Rb* mutation predisposes mesenchymal cells away from the osteoblastic state. Given this finding,

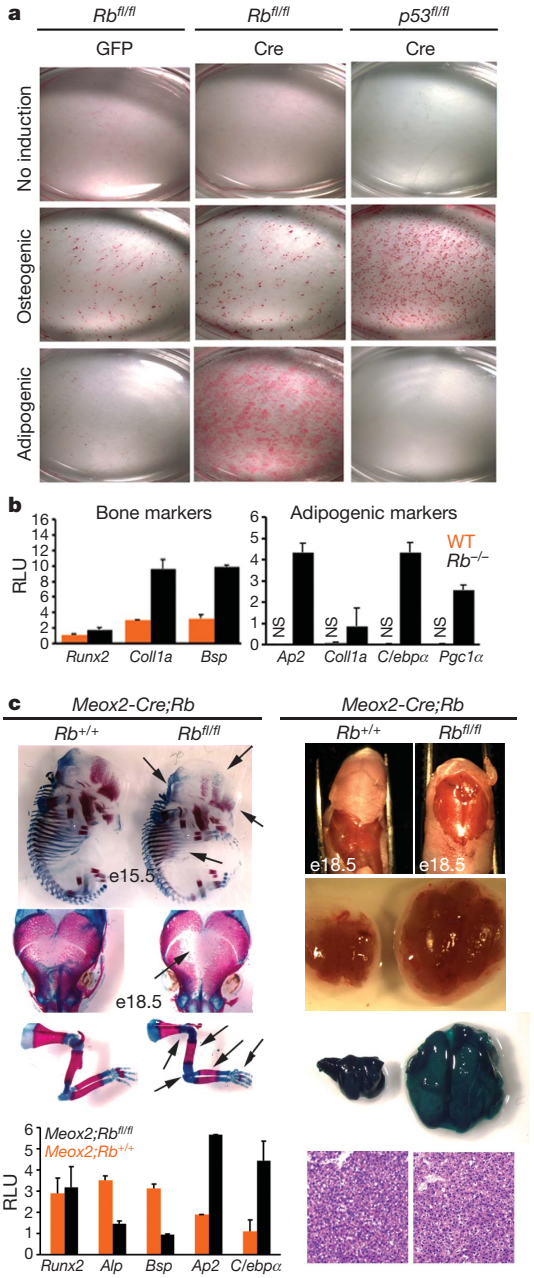

Figure 4 | *Rb* maintains the osteoblastic fate commitment in normal osteoblasts and regulates fate choice during normal development *in vivo*. **a**, Calvarial osteoblasts were prepared from $Rb^{fl/fl}$ or $p53^{fl/fl}$ embryos at embryonic day 18.5 and infected with Ad-GFP or Ad-Cre at P1. Five days later, the cells were induced with differentiation media and assayed for osteogenesis and adipogenesis at 0, 14 and 25 days by staining with Alizarin red and Oil red O. A representative time point (25 days) is shown. **b**, qPCR was also used to assess osteogenic and adipogenic markers in the uninduced $Rb^{fl/fl}$ (WT) versus $Rb^{fl/fl}$ + Ad-Cre ($Rb^{-/-}$) osteoblasts. Bars, the mean of three independent experiments (± SD). **c**, Alizarin red (bone mineralization) and Alcian blue (cartilage) staining of skeletons at embryonic day 15.5 (e15.5) (top panel), calvaria at embryonic day 18.5 (e18.5) (middle panel) and limbs at embryonic day 18.5 (bottom panel) from *Meox2-Cre;*$Rb^{+/+}$ and *Meox2-Cre;*$Rb^{fl/fl}$ littermate embryos. Arrows, 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 *Meox2-Cre;*$Rb^{+/+}$ and *Meox2-Cre;*$Rb^{fl/fl}$ embryos at embryonic day 18.5. Bars, the mean of three embryos arising in two independent crosses (± SD). **d**, Brown adipose tissue was dissected from the backs of *Meox2-Cre;*$Rb^{fl/fl}$ embryos ($n = 10$) and their *Meox2-Cre;*$Rb^{+/+}$ littermate controls. All ten 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 brown adipose tissue (third panel). Haematoxylin and eosin staining of brown adipose tissue (bottom panel).

we speculate that *RB1* mutant osteosarcomas are likely to arise from more committed osteoblastic lineages than from uncommitted mesenchymal progenitors. In this setting, *RB1* loss could enable de-differentiation and thereby synergize with other mutations to promote tumorigenesis.

METHODS SUMMARY

Animal maintenance and histological analyses. Animal procedures followed protocols approved by Massachusetts Institute of Technology's Committee on Animal Care. The $Rb^{fl/fl}$ (ref. 8), $p53^{fl/fl}$ (ref. 9), *Osx1-GFP::Cre*[14], *Prx1-Cre*[10], *Meox2-Cre*[17] and *Rosa26-LSL-lacZ* (Jackson Labs) animals were maintained on a mixed genetic background. The transplant assays were conducted in NOD/SCID mice using 10^4 cells. Protocols for tissue sectioning and skeletal staining are described in Methods.

Osteosarcoma cell lines and primary osteoblast generation and analysis. Osteoblast cell lines and primary osteoblasts were generated and analysed as described in Methods or previously[19]. Knockdown of *Rb* in the *p53KO* osteosarcoma cells was achieved using the pMLP-miR30-based short hairpin. *Rb* targeted sequence: CACGGACGTGTGAACTTATATA, Luc control: GAGCTC CCGTGAATTGGAATCC. Adenoviruses expressing Cre or GFP were provided by the University 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 Protein Assay Reagent (Pierce). 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 G. Karsenty.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions E.C. conducted all the experiments with assistance from J.A.Q.-E. in the Rb re-introduction study, P.S.D. and S.D.B. in the generation and analysis of compound mutant mouse strains and S.N. for the LSL-LacZ;Prx1-Cre embryo analysis. E.C. and J.A.L. were responsible for conceiving this study, interpreting the data and manuscript preparation.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to J.A.L. (e-mail: jalees@mit.edu).

METHODS

Mouse genotyping. Animal procedures followed protocols approved by Massachusetts Institute of Technology's Committee on Animal Care. The *Rb*$^{fl/fl}$ (ref. 8), *p53*$^{fl/fl}$ (ref. 9), *Osx1-GFP::Cre*[14], *Prx1-Cre*[10], *Meox2-Cre*[17] and *Rosa26-LSL-lacZ* (Jackson Labs) animals were maintained on a mixed genetic background. The *Rb* conditional band was detected using the primers 5′ lox: 5′-CTCTAGATCCTCTCATTCTTC-3′ and 3′ lox: 5′-CCTTGACCATAGCCC AGCAC-3′. Primer Rbcre3.2 (5′-GGTTAATGAAGGACTGGG-3′) was used 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′.

Tumour monitoring and analysis. The criteria for euthanasia (by CO_2 inhalation) were a total tumour burden of 2 cm^3, tumour ulceration/bleeding, signs of infection, respiratory distress, impaired mobility, at least 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 by an ethanol series before embedding in paraffin for sectioning. Tissues containing bone were decalcified in 0.46 M EDTA, 2.5% ammonium hydroxide, pH 7.2, for 2 weeks then processed for paraffin sectioning. All paraffin embedded sections were cut at 5 μm, dewaxed and stained with haematoxylin and eosin. Sirius red staining was performed by treating sections briefly stained with haematoxylin with 0.1% Sirius red in saturated picric acid (Electron Microscopy Sciences) for 1 h, washing in 5% v/v glacial acetic acid and then dehydrating in ethanol/xylene before mounting.

Immunohistochemistry. Runx2 immunohistochemistry was performed using a modified citric acid unmasking protocol. Briefly, slides had paraffin removed in xylene and were rehydrated through ethanol. Slides were boiled for 30 min in citrate buffer, pH 6.0, 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 next 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 Kit (Vector Laboratories). All samples were counterstained with haematoxylin.

Skeletal staining. Embryos were killed, 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 one more day. Tissue was cleared in 1% potassium hydroxide for several days and then stored in glycerol.

Generation of osteosarcoma cell lines. Osteosarcomas were dissected, minced, filtered through a 70-μm filter and plated in normal growth medium (10% FBS in DME, 1% P/S, L-glutamine) to generate the osteosarcoma cell lines. Cells were passaged as they reached confluence. Knockdown of *Rb* in the *p53KO* osteosarcoma cells was achieved using the pMLP-miR30-based short hairpin. *Rb* targeted sequence: CACGGACGTGTGAACTTATATA, Luc control: GAGCTCCCGTGA ATTGGAATCC. The transplant assays were conducted in NOD/SCID mice using 10^4 cells.

Calvarial osteoblast preparation and culture. Calvaria from embryos at embryonic day 18.5 were removed and cleaned in sterile PBS from contaminating tissue. They were 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% FBS and penicillin/streptomycin. For differentiation, 3.5×10^5 cells were plated onto a well of a six-well tissue culture plates. Upon reaching confluence, calvarial osteoblasts were treated with medium supplemented with 50 μg ml^{-1} of ascorbic acid and 10 mmol l^{-1} of β-glycerol-phosphate. Adenovirus expressing Cre or GFP (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).

RNA analysis. For RNA purification, cells were rinsed twice with PBS, and RNA extraction was performed using RNAeasy Kit (Quiagen). First-strand complementary DNA was transcribed from 1 μg of RNA using Superscript III reverse transcriptase (Invitrogen) following the manufacturer's instructions. Quantitative PCR with reverse transcribed 20- to 100-ng complementary DNA was done using SYBR Green (Applied Biosystems). Reactions were run on the ABI Prism 7000 Sequence Detection System and analysed using the 7000 SDS software. Primers used for qPCR were the following: alkaline phosphatase (F) TCTCCAGACCCTGCAACCTC and (R) CATCCTGAGCAGACCTGGTC; Col1a1 (F) CGAGTCACACCGGAACTTGG and (R) GCAGGCAGGGCCAAT GTCTA; osteocalcin (F) CTCTGTCTCTCTGACCTCACAG and (R) CAGGT CCTAAATAGTGATACCG; osteopontin (F) TGCTTTTGCCTGTTTGGCAT and (R) TTCTGTGGCGCAAGGAGATT; Runx2 (F) TGAGATTTGTGGGCC GGA and (R) TCTGTGCCTTCTTGGTTCCC; Ap2 (F) ATCCCTTTGTGGG AACCTGGAA and (R) ACGCTGATGATCATGTTGGGCT; C/ebp α (F) CAAGAACAGCAACGAGTACCG and (R) GTCACTGGTCAACTCCAGCAC; Pparγ (F) GAGCTGACCCAATGGTTGCTG and (R) GCTTCAATCGGATGGT TCTTC; Srebp-1c (F) GGAGCCATGGATTGCACATT and (R) GCTTCCAGA GAGGAGGCCAG; Ucp-1 (F) AGCCGGCTTAATGACTGGAG and (R) TCTGTAGGCTGCCCAATGAAC; Pgc-1 (F) GTCCTCACAGAGACACTGGA and (R) TGGTTCTGAGTGCTAAGACC; Nbrf-1 (F) CGGCACCTAGCGCC CGG and (R) CGGCACCTAGCGCCCGG; MyoD (F) CGCCACTCCGGGACA TAG and (R) GAAGTCGTCTGCTGTCTCAAAGG; Prdm16 (F) GACCACGGT GAAGCCATTC and (R) GCGTGCATCCGCTTGTG; Taz (F) GTCACCAACA GTAGCTCAGATC and (R) AGTGATTACAGCCAGGTTAGAAAG; and Gapdh (F) CAAGGTGGCAGAGGCCTTT and (R) TCCAGCTGCTCAATGGACGC ATTT.

Immunoprecipitation, western blotting and reporter assays. For immunoprecipitations and immunoblotting, proteins were extracted with a Triton X-100-based buffer and quantified by the BCA Protein Assay Reagent (Pierce). 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 G. Karsenty.

Chromatin immunoprecipitation assay. Protein complexes were cross-linked to DNA by adding formaldehyde (Sigma) to live cells to a final concentration of 1%. After incubation for 10 min at 37 °C, glycine was added to give a final concentration of 0.125 M for 5 min. The cells were washed twice with PBS containing 1 mM PMSF, scraped and pelleted. Nuclei were extracted with a buffer (20 mM Tris pH 8, 3 mM $MgCl_2$, 20 mM KCl) containing protease inhibitors, pelleted by microcentrifugation and lysed with SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Trischloride pH 8.1) containing protease inhibitors. The resulting chromatin solution was sonicated to generate 500- to 1000-base-pair DNA fragments. After microcentrifugation, the supernatant was diluted 1:10 with dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-chloride, pH 8.1, 167 mM NaCl, containing protease inhibitors), precleared with blocked protein A-positive *Staphylococcus aureus* (Santa Cruz) and split into aliquots. These were incubated at 4 °C for 12–16 h with 5 μg antibody with rotation. Antibody–protein–DNA complexes were isolated by immunoprecipitation with blocked protein A-positive Staph A cells. After extensive washing, bound DNA fragments were eluted and analysed by quantitative PCR with reverse transcribed complementary DNA using the following primers: Pparγ proximal E2F site (F) ACGCGGAAGAAGAGACCT and (R) TCCTGTCAG AGTGTGACTTCTCCT; Pparγ distal E2F site (F) TCGCACTCAGAGCGG CAG and (R) AGGTCTCTTCCGCGTCCCT; Coll1a1 Runx2 site (F) TGCTTCC ACGTTTACAGCTCTAAAG and (R) GTCAGGAAAGGGTCATCTGTAGTCC; osteocalcin Runx2 site (F) GAGAGCACACAGTAGGAGTGGTGGAG and (R) TCCAGCATCCAGTAGCATTTATATCG.