p53 inhibits SP7/Osterix activity in the transcriptional program of osteoblast differentiation

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Osteoblast differentiation is achieved by activating a transcriptional network in which *Dlx5*, *Runx2* and *Osx/SP7* have fundamental roles. The tumour suppressor p53 exerts a repressive effect on bone development and remodelling through an unknown mechanism that inhibits the osteoblast differentiation programme. Here we report a physical and functional interaction between *Osx* and *p53* gene products. Physical interaction was found between overexpressed proteins and involved a region adjacent to the OSX zinc fingers and the DNA-binding domain of p53. This interaction results in a p53-mediated repression of OSX transcriptional activity leading to a downregulation of the osteogenic programme. Moreover, we show that p53 is also able to repress key osteoblastic genes in *Runx2*-deficient osteoblasts. The ability of p53 to suppress osteogenesis is independent of its DNA recognition ability but requires a native conformation of p53, as a conformational missense mutant failed to inhibit OSX. Our data further demonstrates that p53 inhibits OSX binding to their responsive Sp1/GC-rich sites in the promoters of their osteogenic target genes, such as *IBSP* or *COL1A1*. Moreover, p53 interaction to OSX sequesters OSX from binding to DLX5. This competition blocks the ability of OSX to act as a cofactor of DLX5 to activate homeodomain-containing promoters. Altogether, our data support a model wherein p53 represses OSX–DNA binding and DLX5–OSX interaction, and thereby deregulates the osteogenic transcriptional network. This mechanism might have relevant roles in bone pathologies associated to osteosarcomas and ageing.
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Osteoblast differentiation is triggered by a variety of intra- and extracellular osteogenic signals, such as BMPs, IGFs and Wnts.[1] The process commences with a mesenchymal stem cell (MSC) precursor becoming an osteochondroprogenitor cell, which is later sequentially transformed into a mature osteoblast.

It is also well known that osteochondroprogenitor maturation and the subsequent conversion of pre-osteoblasts to mature osteoblasts is controlled by a network of specific transcription factors. Among these, *Runx2* and *Osterix/SP7* (*Osx*) have a critical role in osteogenesis.[2,3] *Runx2* deletion in mice causes a severe impairment in the development of the skeleton and *Runx2* mutations in humans are causative of cleidocranial dysplasia disorder.[4] Similarly, *Osx* deletion impairs the consecution of a mature osteoblast phenotype and was proven to be important in the maintenance of bone homeostasis, because its postnatal deletion causes loss of bone mass and bone defects.[5,6] Several studies also found that *SP7/Osx* mutations or SNPs are related to osteoporosis and *osteogenesis imperfecta*.[7,8] For these reasons, *Osx* and *Runx2* are mandatory for the development of the skeleton. Moreover, both cooperatively regulate the expression of key genes in bone biology forming a transcriptional complex.[9] OSX also acts as a necessary cofactor for DLX family of transcription factors.[10] Furthermore, these transcription factors are subjected to fine tuning by posttranscriptional regulation. For instance, MAP kinases phosphorylate DLX5, RUNX2 and OSX, leading to their activation.[11–13] These

studies highlighted the complexity of the transcription factor network, which controls the osteoblast differentiation process and bone development.

Maturation of MSCs to the osteoblastic phenotype is a multi-step process that requires cell expansion, differentiation and survival. The tumour suppressor p53 is considered a master regulator of proliferation and apoptosis. p53 activity helps to eliminate damaged cells, preventing tumorigenesis.[14] Furthermore, p53 has been linked to cell differentiation in a variety of cell types, such as neurons, muscular cells and osteoblasts.[15–17] Surprisingly, despite the key cellular functions of p53, *Trp53* knockout mice did not show major developmental defects. However, detailed studies demonstrated skeletal abnormalities in some animals, such as upper incisor fusion and craniofacial and limb malformations.[18] *Trp53* knockout mice are also characterized by a denser skeleton than their wild-type littermates and the *Trp53*-deficient bone marrow-derived MSCs (BM-MSCs) have a higher capacity to differentiate towards the osteoblastic fate.[17,19] The impact of p53 on the osteoblastic transcription factor network is also evident, as cells with the *Trp53* deletion overexpress *Osx* and osteogenic genes through an unknown mechanism.[17]

Previous studies suggested that *p53* deletion allows over-activation of the BMP pathway by mechanisms that involve changes in the expression of *Smad1* or *Smurf1*.[20,21] It has also been shown that p53 and RUNX2 proteins interact, blocking Runx2 transcriptional activity, and that p53 regulates *Runx2*

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expression levels by an miRNA-mediated mechanism.[22,23] Therefore, although the inhibitory role of p53 in bone formation is well established, little is yet known about the molecular mechanisms by which p53 exerts this function. Moreover, an in-depth understanding of the role of p53 in bone biology could have implications in the knowledge of pathologies associated with p53 signalling network alterations.

Our work focused in the identification of the molecular mechanisms by which p53 exerts a repressive effect over the osteoblast differentiation programme. We found, using either loss- or gain-of-function models, that p53 expression has a negative impact on the expression of osteoblast-specific transcription factors and their targets. Our work further demonstrated that the negative role of p53 is independent of p53 transcriptional activity but instead required physical interaction between OSX and p53 at the protein level. p53 prevented OSX from binding to Sp1/GC-rich sequences and blocked OSX from interacting with DLX5 and binding to homeodomain sequences.

Results

p53 downregulates osteoblastic gene expression. It has been previously established that p53 has an inhibitory role in osteoblast differentiation using mouse models.[17,24] There is also evidences suggesting that these phenotypes are cell autonomous, as the BM-MSCs from *p53*-deficient mice show enhanced differentiation *in vitro*.[19] To further characterize the molecular mechanism underlying these phenotypes, we first evaluated osteoblastic gene expression from primary osteoblasts obtained from *Trp53* knockout or wild-type mice. Absence of p53 results in upregulation of important genes implicated in bone development (Figure 1a). Importantly, two transcription factors with relevant roles in bone biology, *Dlx5* and *Osx*,[11,25] were 2-fold and 3.5-fold overexpressed, respectively. Furthermore, *Runx2* showed a slight upregulation at the mRNA level. OSX target genes were also upregulated in *Trp53* knockout osteoblasts, such as *Ibsp* (bone sialoprotein) or *Bglap* (osteocalcin).[9,26,27]

To confirm these results, we used the p53-inducible SaOs2 cell line SaOs2-p53TetOn, which only expresses p53 upon the addition of doxycycline (Figure 1b). Treatment with doxycycline for 24 h confirmed the p53-dependent inhibition of *OSX*, *DLX5*, *IBSP* and *COL1A1* expression. The upregulation of *BGLAP* expression after induction of p53 expression could be explained by direct binding of p53 to the known p53-responsive sequences in *BGLAP* promoter.[28,29] These results provide evidence of a p53-dependent downregulation of the expression of osteoblastic genes.

As previous studies had identified the involvement of the osteogenic BMP pathway on p53's effects, we next focused on this signalling pathway and its modulation by p53. As previously reported, p53 deletion results in a slight upregulation of *Smad1* mRNA, as well as the BMP-target gene *Id1* in primary osteoblasts [16,30] (Supplementary Figure 1A, left panel). Interestingly, an induction of *Smad7* was found in p53 knockout osteoblast. In accordance a 1.4-fold upregulation of *SMAD7* expression was obtained after induction of p53
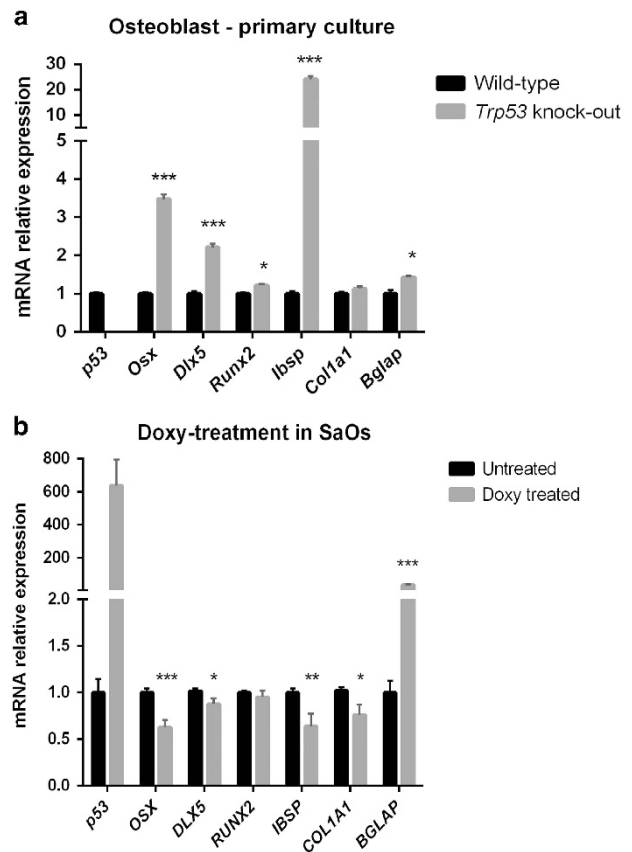
Figure 1 p53 protein inhibits osteogenic differentiation transcriptional programme. (**a**) mRNA expression levels of primary osteoblasts from wild-type or *Trp53* knockout mice grown for 3 days in osteogenic differentiation medium, and (**b**) SaOs2-p53TetOn were treated for 24 h with doxycycline 2 nM in 1% FBS medium. *Bglap* indicates *Osteocalcin* gene mRNA. mRNAs were measured by RT-qPCR, normalized to *TBP* and expressed as relative expression $\pm$ S.E.M. of at least three independent experiments (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, using the Student's *t*-test)

in SaOs2 cells, but no substantial changes in the expression of *SMAD1* or *ID1* (Supplementary Figure 1A, right panel).

SaOs2-p53TetOn cells were also incubated with doxycycline for 16 h and treated with 2 nM BMP2 for 2 h. Our results failed to detect significant differences in pSMAD1/5/8 levels in either primary osteoblasts or the SaOs2-p53TetOn model (Supplementary Figures 1B and C). Moreover, expression of genes such as *JUNB*, *SMAD7* or *DLX5*, which are direct targets of Smad-transcription factors, was independent of p53 induction and only *ID1* displayed a minor downregulation in the doxycycline-treated condition (Supplementary Figure 1D). Thus, these minor negative effects of p53 on the BMP pathway likely could not explain the changes observed in osteogenic gene expression.

p53 physically interacts with Osx. In order to identify the mechanisms involved in inhibition of osteogenic gene expression by p53 we focused on two drivers of bone development, RUNX2 and OSX. As a mechanism by which p53 inhibits RUNX2 through physical interaction has been described previously in a DNA-damage context,[22] we also tested the capacity of p53 to bind to RUNX2 and OSX. To
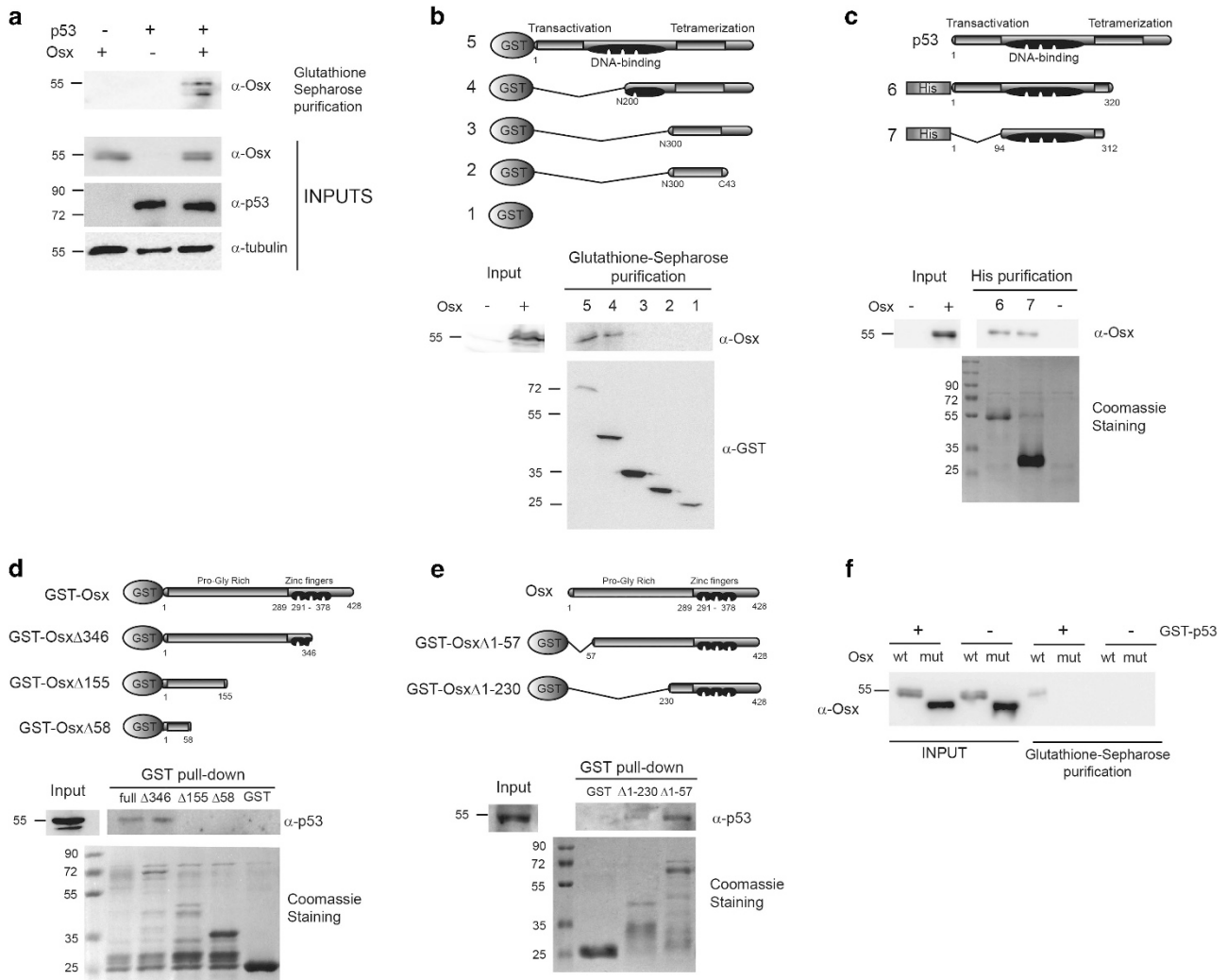
Figure 2 p53 protein physically interacts with OSX. (**a**) SaOs2 cells were infected with retroviral expression vectors for human Osx and/or GST-tagged human p53. Extracts from cells and Glutathione-Sepharose co-precipitated proteins were visualised by immunoblotting using anti-p53 and anti-Osx antibodies. (**b**) Extracts from HEK293T cells transfected with *Osx* expression vector in combination with the indicated GST-p53-chimeric protein were incubated with Glutathione-Sepharose beads for 1 h. Co-precipitated proteins were identified by immunoblotting using anti-OSX antibody. (**c–e**) Cell extracts were incubated with the indicated chimeric protein bound to Glutathione-Sepharose (**d** and **e**) or Ni^{2+} (**c**) beads overnight. Interacting proteins were identified by immunoblotting using anti-p53 or anti-OSX antibodies. (**f**) Lysates from HEK293T cells expressing murine wild-type *Osx* or the double mutant S73A/S77A in combination with the p53-chimeric protein or GST were incubated with Glutathione-Sepharose beads. Interacting proteins were identified by immunoblotting using anti-OSX antibody

assess this, we retrovirally infected SaOs2 cells with OSX and GST-tagged p53 expression vectors. OSX co-purified with p53, suggesting that they interact in osteogenic cells (Figure 2a). To map the involved interaction sites, we expressed *Osx* in combination with different truncated GST-p53 expression vectors in mammalian cells. OSX was able to bind to the N-terminal region of p53 protein in intact cells (Figure 2b). We further pinpoint the p53 domain involved in such interaction by studying OSX binding to recombinant N-terminal truncated p53 (Figure 2c). Altogether, the data demonstrate that the p53 DNA-binding domain is sufficient for physical interaction with OSX. We also verified which domain of OSX was involved in the interaction. Our results showed that p53 requires a region that encompasses part of the transactivation domain proximal to the OSX zinc fingers

(Figures 2d and e). We also determined whether the activation status of OSX influences its interaction with p53. A less active form of OSX (the OSX S73A/S77A mutant, which cannot be phosphorylated by MAP kinases) showed lower interaction with p53 in intact cells (Figure 2f).

Similar assays with GST-p53 expression vectors showed that RUNX2 was able to bind to the N-terminal domain of p53 in mammalian cells (Supplementary Figure 2A). Recombinant truncated RUNX2 proteins showed that RUNX2 requires the PST region to physically interact with p53 (Supplementary Figure 2B).

Osx transcriptional activity is inhibited by interaction with p53. We next assessed the effect of p53 interaction on OSX transcriptional activity. We evaluated the expression
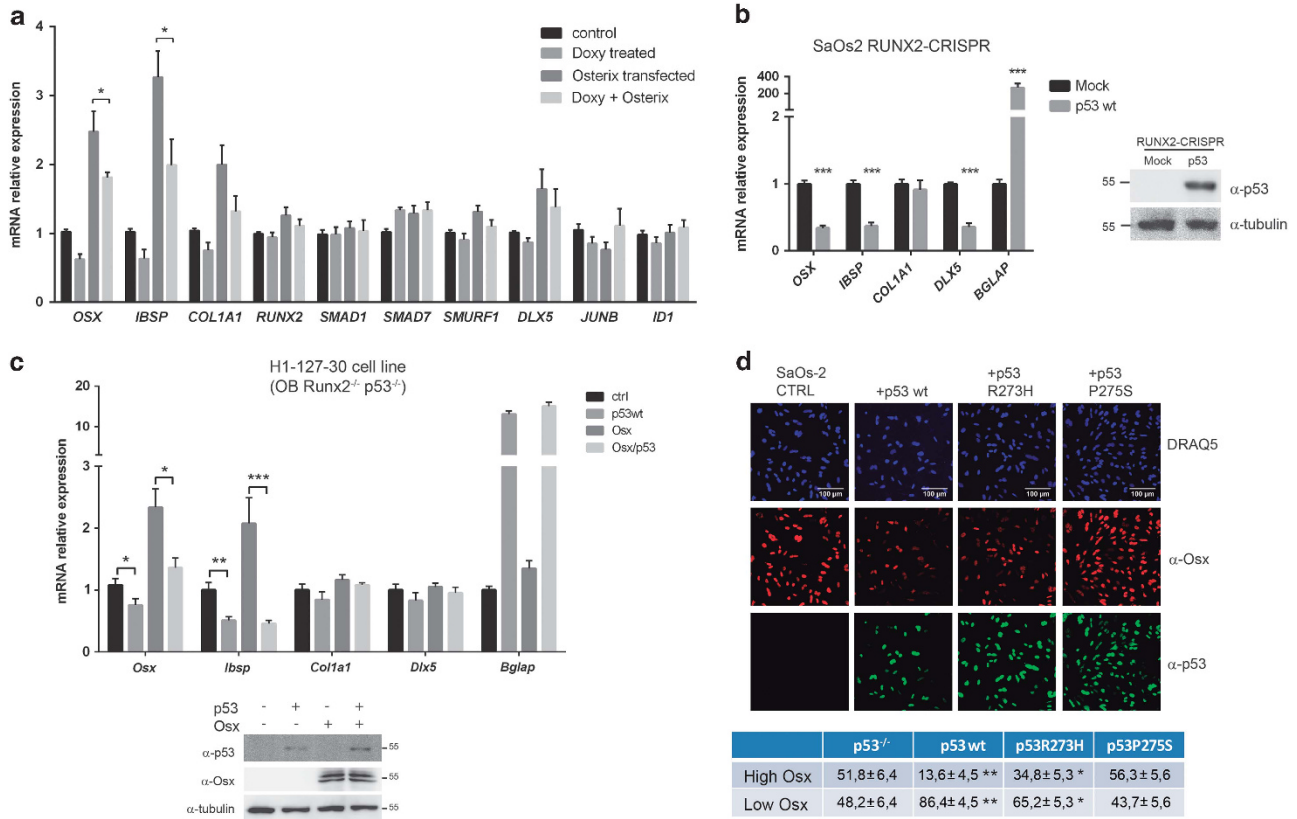
Figure 3 p53 regulates osteoblastic gene expression inhibiting OSX transcriptional activity. (**a**) mRNA expression levels in SaOs2-p53TetOn treated for 24 h with 2 nM doxycycline and/or transfected with *Osx* expression vector. mRNA was measured by qRT-PCR, normalized to *TBP* and expressed as relative expression ± S.E.M. of at least three independent experiments. (**b**) mRNA expression levels in SaOs2 *RUNX2*-CRISPR cells retrovirally infected with murine p53 expression vector. Right panel shows a western blotting to verify p53 expression. (**c**) H1-127-30 osteoblasts generated from the double *Runx2* and *Trp53* knockout mice were infected with retroviral expression vectors for murine p53 and OSX. mRNAs were measured by RT-qPCR, normalized to *TBP* and expressed as relative expression ± S.E.M. from six independent experiments. (**d**) Immunocytofluorescence of SaOs2 cells infected with retroviral expression vectors carrying murine p53 wild type, human p53R273H and murine p53P275S. Immunostaining was performed with anti-OSX and anti-p53 antibodies and nuclei visualized using DRAQ5. Quantification of OSX relative expression levels (cut-off point at < 150 OSX intensity) are expressed as percentage of cells. Statistics were performed against the p53 knockout condition. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ using the Student's *t*-test)

of genes implicated in the osteoblastic differentiation programme on SaOs2-p53TetOn cells overexpressing OSX in combination with doxycycline treatment (Figure 3a). Both controls and cells overexpressing OSX had a lower induction of OSX target genes (such as *OSX* itself and *IBSP*[27,31]) when p53 was expressed. The qPCR assay only detected endogenous *OSX* expression; thus, these data provide additional evidence of OSX regulation of its own promoter.[31] These observations suggest that OSX transcriptional activity is inhibited by p53.

p53 has been shown to repress RUNX2 function.[23] For this reason, we then analyzed whether the effects of p53 on osteogenic gene expression were derived from inhibition of RUNX2 or also depended on the inhibition of OSX transcriptional activity. Two distinct approaches were performed: first, generation of pools of SaOs2 cells, which are p53-deficient, where the third exon of the *RUNX2* gene was inactivated by CRISPR technology (Supplementary Figure 3A). Second, analysis of the osteoblast cell line H1-127-30 generated from the double *Runx2/p53* knockout mice.[32] The *RUNX2*-specific CRISPR deletion was confirmed by immunoblot, obtaining a pool of cells with 60% reduction of RUNX2 levels

(Supplementary Figure 3B). Expression of p53 in these cells still conferred a strong downregulation of osteogenic genes (Figure 3b). Similarly, re-expression of p53 in *Runx2*^{−/−} *p53*^{−/−} osteoblasts led to inhibition of *Osx* and *Ibsp* expression, and abolished their induction by ectopic OSX (Figure 3c). Altogether, these data suggest that the inhibitory effects of p53 on osteogenic gene expression depend not only on RUNX2 but also on OSX inhibition.

We also analyzed by immunofluorescence whether p53 could block OSX trafficking to the nucleus. First, we visualized that OSX expression in SaOs2-p53TetOn cells was localized in the nucleus regardless of p53 expression. Second, at the single-cell level the expression of p53 was negatively correlated with that of OSX (Supplementary Figure 4). To further confirm the negative correlation between p53 and OSX protein levels, we infected p53-deficient SaOs2 cells with retrovirus expressing murine wild-type p53, the human contact mutant p53R273H or the murine structural mutant p53P275S. Whereas the p53R273H changes the Arg273 involved in base recognition, p53P275S (corresponding to P278S in the human p53 gene) alters the structure of the its DNA-binding domain.[33–35] Expression of wild-type p53 or the p53R273H

mutant induced a strong downregulation of endogenous OSX expression, whereas the p53P275S mutant did not significantly changed OSX levels (Figure 3d).

Osx–DNA binding is blocked by p53 protein. A mechanism by which p53 could inhibit OSX transcriptional activity could be affecting its affinity for DNA. We evaluated the OSX ability to bind to Sp1/GC-rich sequences (which have been shown to be OSX responsive elements[12,26] by performing an oligo-pull-down assay using a sequence of the bone enhancer from the *Col1a1* promoter.[26] We expressed OSX in *Trp53* knockout osteoblasts using retrovirus and tested the affinity of OSX for the oligonucleotide. Co-infection with a murine wild-type p53 retrovirus led to a 60–70% lower OSX binding affinity for the Sp1/GC-rich sites (Figure 4a). We also co-infected the p53-deficient osteoblasts with the transcriptionally inactive human p53R273H mutant. Co-expression of this mutant also reduced OSX binding to the oligonucleotide, which refutes the possibility of a p53-dependent transcriptional mechanism underlying OSX binding inhibition (Figure 4a). These results *in vitro* were confirmed by chromatin immunoprecipitation (ChIP) in SaOs2, where expression of wild-type p53 reduced OSX and RUNX2 occupancy in the responsive regions of the promoter of their target genes *IBSP*, *COL1A1* and *BGLAP* (Figure 4b). Expression of the human p53R273H mutant also reduced recruitment of OSX to *IBSP*, *COL1A1* and *BGLAP* promoters, whereas OSX binding was not significantly reduced by p53P275S (Figure 4b).

In accordance with it, the expression levels of the OSX target genes were repressed by the presence of murine wild-type p53 and the human p53R273H contact mutant in *Trp53* knockout osteoblast (Figure 4c). However, p53P275S failed to induce repression of these genes. To verify the mechanism that confers differential inhibition of OSX activity by distinct p53 mutants, we analyzed the physical interaction of OSX with wild-type p53, and the R273H and P275S mutants. Purification of OSX from extracts of SaOs2 cells retrovirally expressing *Osx* and *p53* mutants demonstrate that physical interaction between OSX and p53P275S was reduced compared with the interaction with wild type and R273H mutant (Figure 4d). These results confirm that the effects on OSX transcriptional activity are not dependent on p53 transcriptional activity, but on protein-protein interaction. Together, these data suggest regulation of OSX transcriptional activity by p53 at protein level via blockage of the OSX interaction with their Sp1/GC-rich responsive sites.

The OSX interaction network is inhibited by p53. It has recently been described that OSX acts as a cofactor for the DLX transcription factor family.[10] As p53 binds close to the OSX zinc-finger domain implicated in the OSX–DLX5 interaction, we investigated the impact of p53 binding on the OSX–DLX5 complex. First, we analyzed whether p53 could disrupt the OSX–DLX5 complex. We performed GST-DLX5 pull-down assays using extracts from SaOs2-p53TetOn cells infected with murine wild-type p53 or the human R273H mutant. As previously described OSX bound DLX5,[10] but both wild type p53 and the R273H mutant disrupted this interaction (Figure 5a). Thus, p53 could likely inhibit OSX

transcriptional activity in promoters regulated by the OSX–DLX complex.

As mentioned before, OSX is able to regulate its own promoter[31] and our group had also previously identified DLX5 as an activator of the proximal promoter of *Osx*.[11] The *Osx* promoter − 114/ − 51 contains a Sp1/GC-rich box that could be a target for direct OSX binding, as well as a DLX5-binding homeobox. Luciferase assays using this *Osx* promoter region showed that both OSX and DLX5 activated the *Osx* promoter, and they showed an additive effect when expressed together (Figure 5b). More importantly, activation of the *Osx* promoter was repressed by p53. Mutation of either the homeobox or the Sp1/GC-rich sequences reduced the ability of DLX5 and OSX to activate transcription from the *Osx* promoter, suggesting that both sites are relevant for their function. To further discern whether the inhibition of p53 on the *Osx* promoter is through inhibition of OSX binding to the GC-rich box or instead by avoiding its role as a DLX5 cofactor, we performed an oligo-pull-down assay using the − 114/ − 51 *Osx* promoter. We tested the capacity of OSX and DLX5 to bind to the wild-type − 114/ − 51 sequence (pOsx HB-wt), or to the sequence with the homeobox site mutated (pOsx HB-mut) (Figure 5c). Assays from doxycycline-treated SaOs2-p53TetOn cells confirmed that OSX was able to bind to this promoter region probably using the Sp1/GC-rich box, besides binding to the homeobox, given that OSX was still able to bind to the HB-mut oligonucleotide. As expected, DLX5 bound to the HB-wt and showed lower affinity to the HB-mut oligonucleotide. The presence of p53 not only reduced OSX binding to both sequences but also blocked the binding of DLX5 to the homeobox site. These results were confirmed by chromatin-immunoprecipitation in SaOs2-p53TetOn cells, where induction of p53 strongly reduced DLX5 occupancy in the *Osx* promoter (Figure 5d). Therefore, binding of both DLX5 and OSX to their sites were inhibited by p53. Altogether, our results suggest a novel mechanism of OSX regulation, as summarized in Figure 6. p53 inhibits OSX transcriptional and osteogenic activity by binding close to its zinc-finger region, repressing its interaction with DNA and its transcriptional partners, such as DLX5.

Discussion

Cell differentiation and proliferation are usually thought of as antagonistic events in many cell types, with differentiation being only possible after blockage of proliferation. As a canonical tumour suppressor, p53 is supposed to inhibit proliferation but also promote differentiation.[36] However, the inhibitory role of p53 in osteogenic differentiation, in a cell-autonomous manner, is well established by mouse genetics and cell-based approaches.[19] Although the p53-null mouse shows enhanced bone mineral density, Mdm2-conditional knockout results in a severe impairment of bone formation.[17,24] Interestingly, MSCs derived from induced pluripotent stem cells from Li–Fraumeni patients carrying the transcriptionally inactive p53G245D mutation showed deficient osteoblast differentiation. Moreover, deletion of p53 in these iPS cells eliminated the osteogenic differentiation defect suggesting that osteogenic effects are derived from gain-of-function activities of mutant p53.[37]

Despite this evidence, the exact nature of the mechanisms that underlie these p53 effects remained unknown. Our work identifies these effects on osteoblast differentiation through repression of OSX activity by direct interaction with p53. Previous studies focused on the impact of the p53 family over osteogenic BMP signalling with interesting findings such as
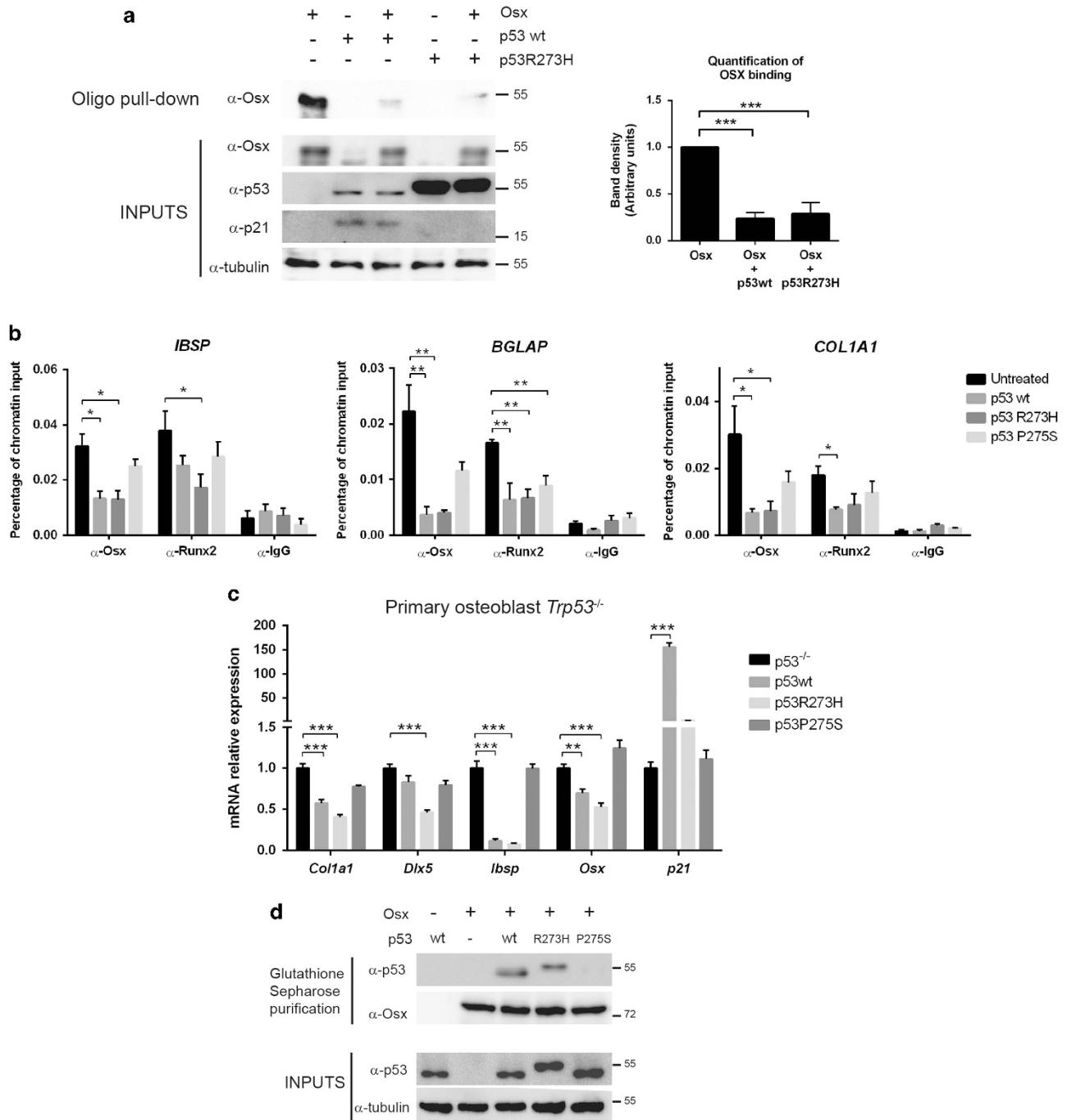

Figure 4 p53 blocks OSX binding to Sp1 elements. (**a**) Oligo-pull-down assay was performed using a biotinylated-oligonucleotide containing the *Col1a1* bone-enhancer sequence. The oligonucleotide was incubated with extracts from *Trp53* knockout primary osteoblasts retrovirally infected with the indicated expression vectors. Bar graph on the right shows the quantification of the OSX band after streptavidin-purification relative to their input levels. (**b**) ChIP from SaOs2 cells infected with retroviral expression vectors for murine p53 wild-type, human p53R273H and murine p53P275S. DNA–protein complexes were isolated with antibodies against Osx, Runx2 and IgG. Specific primers for the promoters of *IBSP*, *BGLAP* (Osteocalcin) and *COL1A1* bone-enhancer were used for qRT-PCR analysis. Results are represented relative to input chromatin. (**c**) *Trp53* knockout primary osteoblasts were retrovirally infected with murine p53 wild-type or p53P275S or human p53R273H mutant forms. *p21* gene was used as reporter of p53 transcriptional activity. mRNA was measured by qRT-PCR, normalized to *Tbp* and expressed as relative expression ± S.E.M. of at least three independent experiments (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, using Student's *t*-test). (**d**) SaOs2 cells were infected with retrovirus expression vectors for murine GST-tagged Osx and p53 wild-type and P275S from mouse or human p53R273H. Extracts from SaOs2 cells and Glutathione-Sepharose co-precipitated proteins were visualised by immunoblotting using anti-p53 and anti-Osx antibodies
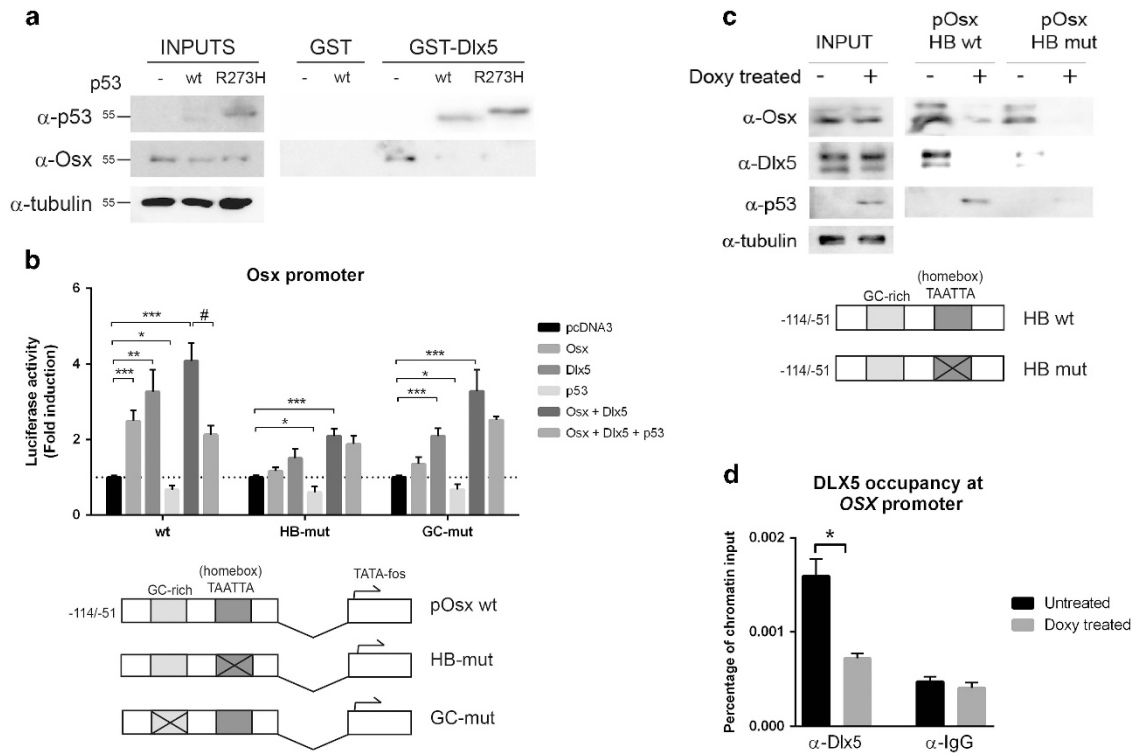
Figure 5 Inhibition of *Osx* promoter is mediated by p53 blocking DLX5-OSXx complex. (**a**) Extracts of SaOs2-p53TetOn retrovirally infected with the indicated p53-expression vectors were incubated with the indicated chimeric protein bound to Glutathione-Sepharose beads overnight. Interacting proteins were identified by immunoblotting using anti-Osx and anti-p53 antibodies. (**b**) SaOs2 cells were co-transfected with the indicated expression vectors, or empty pcDNA3 vector as control, and the murine − 114/ − 51 Osterix-promoter luciferase reporter, wild-type or with the indicated mutated versions. HB-mut is the reporter with the homeodomain box mutated; GC-mut indicates mutation in the Sp1/ GC-rich box.[11] Scheme of the − 114/ − 51 Osterix-promoter is shown. Luciferase activity was measured and normalized against β-galactosidase activity, and represented as fold induction compared with the pcDNA3 transfection condition. # is used to compare Osx+Dlx5 *versus* Osx+Dlx5+p53 conditions. (**c**) Oligo-pull down using biotinylated − 114/ − 51 Osterix-promoter sequence (pOsx HB wt) and its homeobox-mutated version (pOsx HB mut). Extracts from SaOs2-p53TetOn untreated or treated for 24 h with 2 nM doxycycline were used. Interacting proteins were identified by immunoblotting using anti-OSX, anti-DLX5 and anti-p53. (**d**) SaOs2-p53TetOn were treated or not with 2 nM doxycycline for 24 h. DNA-protein complexes were isolated with antibodies against DLX5 and IgG. Specific primers for the *Osx* promoter were used for qRT-PCR analysis. Results are represented relative to input chromatin from three independent experiments ($*P < 0.05$)
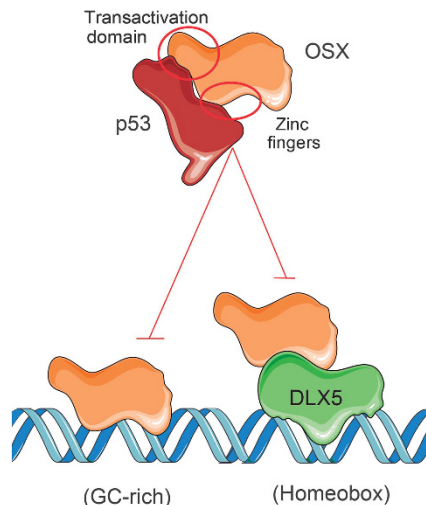

Figure 6 Schematic representation of the proposed p53-OSX network: p53 blocks Osterix transcriptional activity by physical interaction. We suggest that p53-OSX interaction prevents OSX from binding to Sp1/GC-rich sequences, and also interferes in the formation of the OSX–DLX5 transcriptional complex, that responds to homeobox sequences

the p53-Smad1/5 physical interaction,[38] or Smad1 upregulation due to the lack of p53.[16,20] Our data suggest that these changes were of low magnitude. We also identified positive regulation of the gene expression of the Smad signalling inhibitor *Smad7*. These data are in agreement with data from epithelial cells, which establishes that p53 family members bind to the *Smad7* promoter through a p53-response element.[39]

As mentioned above, our work and previous studies revealed that p53 represses the expression of osteoblast-determining transcription factors independently of p53–DNA binding.[17,37] Our data further demonstrated the ability of p53 to inhibit the transcriptional osteogenic programme independent of p53–DNA binding on the basis of several lines of evidence. First, a transcriptionally inactive mutant form of human p53 (R273H) similarly blocked OSX function, whereas a murine conformational mutant (P275S) had lower inhibitory ability. Second, RUNX2-deficient osteoblasts were also sensitive to the inhibitory effects of p53. Third, p53 was able to repress binding to DNA and cooperative transcriptional effects of endogenous and ectopic OSX and DLX5.

Osx, *Runx2* and *Dlx5* are mandatory transcriptional regulators of the osteogenic process. Their gene products

physically interact and lead to a strong cooperation in the transcription of target genes and the production of a mineralized bone matrix.[9,10] Previous studies also depict an integrative feed-forward transcriptional network involving *Runx2*, *Osx* and *Dlx5*, in which they positively regulate each other´s transcription.[9,11,40–43] These fully integrated relation-ships suggest that any alteration in the function of any of them would result in a de-regulation of the whole network. Thus, the p53-mediated inhibition of OSX function could be an important factor in the maintenance of bone homeostasis under physiological conditions. p53 signalling mutations, which lead to high p53 protein expression levels,[44,45] would disturb the function of the *Dlx5-Runx-Osx* network with important consequences.

The DNA-binding domain of p53 interacts with an OSX domain adjacent to its zinc fingers. Similarly, we also mapped the PST transactivation domain of RUNX2 as its p53-interaction region. Interaction between p53 and OSX results in downregulation of *Osx* target genes involved in bone matrix maturation, such as *Ibsp*, *Col1a1* and *Osx* itself. The *Osx* proximal promoter, the first known molecular target of p53 inhibition,[17] is positively controlled through direct DNA binding by OSX and DLX5 proteins.[11,27,31] We determined that p53 interaction does not alter OSX localization but impairs OSX–DNA binding *in vitro* and *in vivo* to their canonical Sp1/GC-rich sites. Moreover, p53 also inhibits OSX–DLX5 transcriptional complex formation and binding to homeobox sequences. Importantly, the OSX–DLX5 complex co-occupy most the bone enhancer regions in genome-wide analysis and evolu-tionary analysis showed correlation with the evolution of skeletal formation.[10] As p53 and DLX5 share the region of interaction with OSX,[10] it is plausible to infer that higher levels of p53 would abrogate OSX–DLX5 complex formation. By blocking these two DNA-binding mechanisms of action of OSX, p53 breaks two important mechanisms of the osteoblast transcriptional programme.

Our findings that p53 has a transcriptionally independent role in OSX function suggest important physiopathological consequences. Proliferation and differentiation should co-exist in a coordinated manner to maintain tissue homeostasis over time. Besides its major role in the transcriptional control of proliferation and apoptosis, p53 might block osteogenic differentiation from mesenchymal precursors to allow its expansion and prevent their shortage from premature speci-fication. Deregulation of these networks would lead to osteoporosis by shortage of mesenchymal precursors and/or misdirected differentiation towards alternative fates such as adipocytes, which are both characteristic features of ageing.[46] Several reports indicate that loss of osteogenic potential and increased adipogenesis of bone progenitors during senes-cence is dependent on p53 and correlated with lower *Osx* expression.[47] Moreover, the shift from osteoblastogenesis to increased adiposity observed in osteopenias induced by ageing, accelerated senescence or ovariectomy correlated with upregulation of p53 levels.[46]

The relation between p53 mutations and osteosarcomagen-esis has been highlighted in several studies.[36,48,49] Osteo-sarcoma is largely composed of poorly differentiated cells[50] and the p53 loss-of-function is enough for the development of osteosarcoma.[51] Previous studies, aimed at detecting the origin of the osteosarcoma, point to dedifferentiated osteo-blastic cells, rather than MSCs.[36,37,49,50] In addition, most p53 mutations are missense and impair DNA binding, but give rise to the gain of new oncogenic functions.[52,53] Our data suggest a possible negative correlation between p53 and OSX protein levels that varies depending on p53 specific mutation (Figure 3d). Therefore, the definition of p53 status and its mutation in osteosarcomas likely could help us to understand its effect on osteogenic differentiation, and to better tackle the progression of the disease. Altogether, our data support a model where p53 represses OSX–DNA binding and OSX–DLX5 interaction and thereby deregulates the osteogenic transcriptional network.

Materials and Methods

Mouse model. *Trp53* knockout mice B6.129S2-*Trp53*tm1Tyj/J from Jackson Laboratories (Bar Harbor, ME, USA) were kindly provided by Dr J Martin-Caballero. Mice were housed under controlled conditions (12 h light/12 h dark cycle, 21 °C, 55% humidity) and fed *ad libitum* with water and a 14% protein diet (Teklad 2014, Harlan, Santa Perpètua de Mogoda, Barcelona, Spain). All animal experiments were performed in accordance with guidelines approved by the Ethical Committee for Animal Experimentation of the University of Barcelona and Generalitat de Catalunya (Spain).

Cell culture. SaOs2-p53TetOn, kindly provided by Dr R Bartrons, and HEK293T (American Type Culture Collection, Rockville, MD, USA) cell lines were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 1 mM pyruvate, 100 U/ml penicillin and 0.1 mg/ml streptomycin. The isolation of primary osteoblasts from calvaria was carried out as described previously.[54] Primary osteoblasts and H1-127-30 osteoblast cell line [32] were maintained in α-MEM supplemented with 10% FBS, 2 mM glutamine, 1 mM pyruvate, 100 U/ml penicillin and 0.1 mg/ml streptomycin. As osteogenic differentiation medium, osteoblast medium was supplemented with 50 μM ascorbic acid and 10 mM β-glyceropho-sphate. HEK293T cells were transiently transfected using polyethylenimine, and SaOs2 using Lipofectamine-LTX (Invitrogene). Retroviral infection in *Trp53* knockout osteoblasts, H1-127-30 and SaOs2 cells were performed as described previously.[55] Doxycycline treatment (2 nM) was carried out in media with 1% FBS. BMP2 (R&D, Minneapolis, MN, USA) was used at 2 nM.

CRISPR-Cas9. The *RUNX2* gene was knocked down in SaOs2 cells using the system described by Ran *et al.*[56]. The gRNA sequence was designed to target the exon 3 of the *RUNX2* gene. The guide sequence was cloned into the pSpCas9(BB)-2A-Puro vector donated by R. Estevez and confirmed by sequencing. The primers used were: F:5′CACCGGCTGGTGCTCGGATCTACGG3′ and R:5′-AAACCCG TAGATCCGAGCACCAGCC-3′. SaOs2 cells were plated in six wells and transfected overnight. An empty vector without gRNA was used as negative control. Puromycin was added at 1 μg/ml and maintained three days for selection. The selected cells were tested for *RUNX2* gene deletion by endonuclease assay. The following primers were used for *RUNX2* amplification: F:5′-CAAACTTGATTTCTCACCTCCTCA-3′ and R:5′-TTCAAGGTAAGAGGCTACACCGC-3′. The positive pools were expanded and checked for RUNX2 knock-down by immunoblot.

Plasmids and reagents. Murine Osterix expression vectors were kindly provided by Dr B de Crombrugghe. The Osx S73A/S77A, GST-Osx, GST-OsxΔ346 and GST-OsxΔ155 GST-Dlx5 were previously described,[9,12] and GST-OsxΔ58, GST-OsxΔ1–57 and GST-OsxΔ1–230 were generated by double digestion from GST-Osx. GST-Runx2 and GST-Runx2Δ230 were kindly provided by Dr M Montecino. The GST-p53 and their derivatives were kindly provided by Dr Y Xiong. The human His-p53-(1–320) and His-p53-(94–312) were a gift from Dr Arrowsmith (Addgene plasmid 24864 and 24866, respectively). The pMXs-p53 and pMXs-p53P275S from mouse were a gift from Dr Yamanaka (Addgene plasmids 22725 and 22726, respectively).[57] The pMXs-p53R273H was generated from pLenti6/V5-p53_R273H (from Dr Futscher, Addgene plasmid 22934)[58] by subcloning the human p53R272H sequence into pMXs plasmid. Human pMXs-Osx was a gift from Dr O Mazda. The pBABE-GST-p53 retroviral expression vector

was generated from pEBG-GST-p53. The pBABE-GST-OSX was generated from pGEX-Osx.

Pull-down assays. For *in vitro* GST-pull down experiments, the assays were performed as described previously.[9] For analysis of the physical interaction assays in mammalian cells, the GST-p53 or the GST-Osx vectors were co-transfected with *Osx* or the distinct p53 mutant expression vectors in HEK293T or retrovirally infected in SaOs2 cells and cells were lysed after 48 h. Lysates were collected, centrifuged to eliminate debris and purified by binding to Glutathione-Sepharose beads for 1 h at 4 °C. Then, the beads were washed with washing buffer (50 mM Tris/HCl, 150 mM NaCl, 0.1% Igepal-CA630 plus protease and phosphatase inhibitors) three times. Bound proteins were detected by immunoblotting. For *in vitro* pull down of His-tagged proteins, all used buffers were supplemented with 10 mM imidazol and bound to Ni^{2+} resin for purification.

Luciferase assay. SaOs2 cells were cultured in 24-well plates and transfected for 8 h with Lipofectamine LTX with the indicated plasmids. Transfection efficiency was assessed by GFP expression. Luciferase activities were quantified 48 h post-transfection using the Luciferase assay system (Promega, Madison, WI, USA) and normalized using the β-Galactosidase Detection Kit II (Clontech, Mountain View, CA, USA).

Oligo-pull-down assays. Oligo-pull-down assays were carried out as previously described.[26] The pOsx HB-wt and pOsx HB-mut oligonucleotides were generated by PCR using biotinylated primers from the proximal Osterix murine promoter, and wild-type or homeobox-mutated $-114/-51$ *Osterix* promoter sequences as templates. The Sp1 oligonucleotide sequence was generated from the *Col1a1* promoter as previously described.[26]

Western blot assay. Identification of proteins, from cell extracts or pull-down assays, was performed by immunoblotting against phospho-Smad1/5/8, Smad1 (Cell Signaling 9743 and 13820, Danvers, MA, USA), Osx (Abcam 22552, Cambridge, UK), Runx2 (MBL D130-3, Woburn, MA, USA), p53 (Cell Signaling 48818), Id1 (C-20 sc-488) and p21 (C-19 sc-397) (Santa Cruz Biotechnology, Dallas, TX, USA) or α-tubulin (Sigma-Aldrich T5168, St. Louis, MO, USA), diluted at 1 : 1000. Antibodies against Dlx5 (C-20 sc-18152 and Y-20 sc-18151) (Santa Cruz Biotechnology) were used at 1 : 500. Immunoreactive bands were detected with horseradish-peroxidase-conjugated secondary antibodies and an ECL-kit (Biological Industries, Cromwell, CT, USA).

ChIP assay. ChIP assay was carried out as described in Artigas *et al*.[9] SaOs2 p53TetOn cells were cultured until confluence before overnight doxycycline treatment. SaOs2 were retrovirally infected with the distinct p53 mutant constructs. ChIP was carried out using 1 μg of the indicated antibodies: anti-Osx (Abcam), anti-Runx2 (MBL), anti-Dlx5 (C-20 sc-18152 and Y-20 sc-18151, 0.5 μg each) or anti-IgG (Upstate) as a control, and purified with 20 μl Magna ChIP Protein A+G Magnetic Beads (Millipore, Billerica, MA, USA). The DNA fragments were purified using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and analyzed by qRT-PCR. The primers used for the qRT-PCR analysis for *IBSP* and *COL1A1* were previously described.[9] *BGLAP* primers were R: 5′-CCGTAGGCCAAACCCCAG AGGATATGT-3′ and F: 5′-CTCTGCTTGAACCTATTTTAGGTCTCTGA-3′. *OSX* primers were: R: 5′-CCTGCTTCCCACCCCTTCCA-3′ and F: 5′-ATGAGGAGG GCGAGAGAGGG-3′.

Immunocytofluorescence. SaOs2 p53TetOn immunofluorescence was performed as previously described[9] using 1 : 100 p53 (Cell Signaling) and 1:250 anti-Osterix (Abcam).

qRT-PCR analysis. Total RNA was isolated from primary osteoblasts or SaOs2 cells using TRIsure reagent (Bioline, London, UK). Purified RNA was reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Quantitative PCRs were carried out using the ABI Prism 7900 HT Fast Real-Time PCR System and Taqman 5′-nuclease probes (Applied Biosystems). Designed Taqman assays (Applied Biosystems) were used to quantify gene expression. All transcripts were normalized to *Tbp* expression.

Statistical analysis. Statistical analysis was performed using the Student's *t*-test. Quantitative data are presented as means $\pm$ S.E.M. Differences were considered significant at $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$.

Conflict of Interest

The authors declare no conflict of interest.

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