Mediator MED23 cooperates with RUNX2 to drive osteoblast differentiation and bone development

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How lineage specifiers are regulated during development is an outstanding question, and the molecular regulation of osteogenic factor RUNX2 remains to be fully understood. Here we report that the Mediator subunit MED23 cooperates with RUNX2 to regulate osteoblast differentiation and bone development. Med23 deletion in mesenchymal stem cells or osteoblast precursors results in multiple bone defects similar to those observed in Runx2⁺⁻/⁻ mice. In vitro, Med23-deficient progenitor cells are refractory to osteoblast differentiation, and Med23 deficiency reduces Runx2-target gene activity without changing Runx2 expression. Mechanistically, MED23 binds to RUNX2 and modulates its transcriptional activity. Moreover, Med23 deficiency in osteoprogenitor cells exacerbates the skeletal abnormalities observed in Runx2⁺⁻/⁻ mice. Collectively, our results establish a genetic and physical interaction between RUNX2 and MED23, suggesting that MED23 constitutes a molecular node in the regulatory network of anabolic bone formation and related diseases.

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Mesenchymal stem cells (MSCs) are multipotent progenitors that can self-renew and differentiate into several distinct cell lineages, including osteoblasts, chondrocytes and adipocytes. Their differential potentials and intrinsic properties make MSCs of great interest for applications in a variety of cell-based therapies. Although Runx2 triggers MSCs to differentiate into osteoblasts, it remained unknown whether MED23 is also involved in the transcriptional output and bone formation rate in osteoblast differentiation. To further determine the function of MED23 in the skeletal system, we utilized micro-quantitative computed tomography (μ-CT) to compare skeletal elements isolated from Med23MSC−/− mice with corresponding elements from control littermates. As shown in Fig. 1f, 6-day-old Med23MSC−/− mice displayed hypomineralization of the calvaria compared with control mice. In addition, we found that 1-month-old Med23MSC−/− mice were osteopenic, with reduced bone mineral density (BMD) and bone volume per tissue volume (BV/TV) in the femoral trabecular bone relative to age-matched control littermates. Further analysis showed that the reduced trabecular number (Tb.N) of Med23MSC−/− mice was accompanied by decreased trabecular thickness (Tb.Th) and a reduction in the Tb.N compared with those of control mice (Fig. 1g,h). Consistent with the decreased BMD in Med23MSC−/− mice, an ELISA assay of PINP (N-terminal propeptide of type I procollagen), a marker of bone formation, revealed a reduced bone formation rate in Med23MSC−/− mice (Fig. 1i). In adults, bone is continuously remodelled by bone-resorbing osteoclasts and bone-forming osteoblasts. We performed a histological analysis and in vitro osteoclastogenesis experiment to exclude the possibility that the decreased bone density in the long bones of Med23MSC−/− mice could be attributed to increased osteoclast differentiation and/or activity. We found that osteoclast activity was comparable between Med23MSC−/− mice and their control littermates (Supplementary Fig. 2a,b). Moreover, the expression of osteoclastic genes did not change either (Supplementary Fig. 2c). Taken together, these results suggest an important role for Med23 in bone formation.

**Ablation of Med23 in preosteoblasts reduced bone formation.** To further determine whether the abnormal osteogenesis in Med23MSC−/− mice results from a primary defect in osteoblast development, we generated an osteoblast-specific Med23-deleted mouse model (Med23ob−/− mice) by crossing Med23ob+/− mice with osterix (Osx)-Cre mice, a line in which Cre expression is primarily restricted to osteoblast precursors (Fig. 2a). Western blot assays showed that Med23 decreased in the bone of both Med23ob+/− and Med23ob−/− mice (Supplementary Fig. 3b). Consistent with decline in protein of MED23, histological analysis by alizarin blue and alizarin red (ARS) staining showed defects in the skeleton occurring in both Med23ob+/− and Med23ob−/− newborns, and more severe in the latter, including abnormality in calvarial and clavicle ossification (Supplementary Fig. 3a). However, Osx-Cre transgenic mice have been found to manifest defects in bone phenotype, such as to delay bone mineralization and develop scapula calluses. To exclude such effect of Oss-Cre, we made a comparison between Med23ob−/− mice and Med23ob+/− control littermates that were both in the context of Oss-Cre. Again, Med23ob−/− mice developed runt and underweight compared with age-matched control littermates (Fig. 2b,c). Mice with Med23 deficiency in osteoblast reproduced the phenotype of Med23MSC−/− mice, including impaired membranous ossification of calvarial bones and dysplasia of the clavicles, although the sternum appeared normal. In the Med23ob−/− mice, the bone was obviously porous; this feature was more evident in the bones of the skull (Fig. 2d,e). μCT analysis
Further confirmed the osteopenic phenotype of Med23ob−/− mice (Fig. 2f,g). Likewise, relatively lower levels of serum PINP indicated a decreased bone formation rate in Med23ab−/− mice (Fig. 2h). Again, in vivo and in vitro osteoclastogenesis showed comparable levels of osteoclasts between Med23ab−/− mice and control littersmates (Supplementary Fig. 4a–c). Hence, the Med23ab−/− mice recapitulate the defects observed in Med23ob−/− mice with striking fidelity, supporting the conclusion that Med23 is necessary to the differentiation and function of committed osteoblast precursors.

**Med23 is required for the osteoblast differentiation of MSCs.**

Next, we asked whether Med23 deficiency affects the osteogenic potential of MSCs in a cell-autonomous manner. Primary MSCs were isolated from the bone marrow of control and Med23MSC−/− littermates, and the deficiency of Med23 in the MSCs from Med23MSC−/− mice was confirmed by western blot assay (Fig. 3a). Med23 deficiency in MSCs does not appear to alter the cell viability or growth rate, indicated by comparable numbers of colony-forming unit fibroblasts (c.f.u.-F) (Fig. 3b). However, Med23−/− MSCs displayed markedly decreased alkaline phosphatase (ALP) activity and mineralization (Fig. 3c,d). In addition, the mRNA levels of osteogenic genes, such as osteocalcin (Ocn), were significantly downregulated in Med23MSC−/− cells (Fig. 3e), while Runx2, a key regulator in osteogenesis.
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mediated acute knockdown of Med23 in MSCs also resulted in decreased osteogenesis, as indicated by ALP and ARS staining (Supplementary Fig. 5a). Real-time PCR analysis showed that Med23 knockdown reduced the expression of osteogenic marker genes such as Osx, Alp, collagen type I (Col1a1) and Ocn. However, noticeably, the expression of Runx2, the master regulator for osteoblast differentiation, was unaffected by Med23 deficiency (Supplementary Fig. 5b).

Collectively, these results showed that Med23 is required for osteoblast differentiation. The key osteogenic genes downregulated in Med23-deficient cells are known targets of Runx2, suggesting that MED23 might regulate osteoblast differentiation via Runx2.

**Med23 regulates the gene network of bone development.** Despite our observation that the expression of Runx2, a master transcription factor for osteoblast differentiation, was not affected by Med23 deletion during osteogenesis, Med23MSC−/− mice appeared to phenocopy the skeletal defects of Runx2+/− mice, including delayed closure of the fontanelles, clavicular hypoplasia and decreased bone density10, suggesting that Med23 and Runx2 share a functional mechanism. To compare the effects of Med23 and Runx2 on global gene expression patterns, we performed RNA-seq with total RNA extracted from the calvaria of control and Med23MSC−/− littersmates, as well as WT and Runx2+/− littersmates. Globally, Med23MSC−/− versus control and Runx2+/− versus WT revealed a significantly positive correlation in global gene expression (Fig. 4a).

We next found that downregulated genes in Med23MSC−/− set presented in a similar pattern in Runx2+/− set (Fig. 4b). In more details, 438 genes were downregulated >1.5-fold in the Med23MSC−/− set compared with the corresponding control, and 504 genes were downregulated >1.5-fold in the Runx2+/− set compared with the control and a total of 124 genes overlapped between the 2 groups (Fig. 4c). These data further implied that these two molecules may function cooperatively. We then performed a gene ontology (GO) analysis of the set of 1.5-fold significantly downregulated genes from the Med23MSC−/− sample. The results showed that these genes were enriched for associations with bone development and ossification (Fig. 4d). We verified the expression of osteoblast-specific genes in the calvaria from control and Med23MSC−/− newborns by real-time PCR and found that osteogenic genes such as Osx, Alp, Col1a1 and Ocn

(Fig. 3a,e). Similarly, impaired osteogenesis was observed in Med23-deficient osteoblasts too (Supplementary Fig. 6). These results indicated that Med23 functioned necessarily in osteogenesis *in vitro*.

To exclude developmental differences between control and Med23−/− primary MSCs, we infected wild-type primary bone marrow MSCs with retroviruses expressing either shRNAs targeting Med23 or control shRNA. Consistent with the differentiation difference between WT and Med23 KO primary MSCs, shRNA-mediated acute knockdown of Med23 in MSCs also resulted in decreased osteogenesis, as indicated by ALP and ARS staining (Supplementary Fig. 5a). Real-time PCR analysis showed that Med23 knockdown reduced the expression of osteogenic marker genes such as Osx, Alp, collagen type I (Col1a1) and Ocn. However, noticeably, the expression of Runx2, the master regulator for osteoblast differentiation, was unaffected by Med23 deficiency (Supplementary Fig. 5b).

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Med23 deficiency inhibits osteoblast differentiation. (a) Expression of Med23 and Runx2 in bone marrow-derived mesenchymal stem cells and calvarial osteoblasts from 6 to 8-week-old control and Med23MSC−/− mice. Isolated cells were expanded and analysed by western blotting. (b) c.f.u.-F assay for bone marrow cells from control and Med23MSC−/− littermates. Representative images of c.f.u.-Fs stained with crystal violet (left; scale bar, 0.5 cm) and quantification of c.f.u.-Fs (right, n=3 for each group). Data represent means ± s.d. t-test. (c) ALP staining of bone marrow mesenchymal stem cells after cultured for 7 days in osteogenic medium. Scale bar, 0.5 cm. (d) c.f.u.-ob assay for bone marrow of control and Med23MSC−/− littermates. Bone marrow cells were cultured in osteogenic medium for 21 days, followed by staining with Alizarin red (left; scale bar, 0.5 cm) and quantification (right, n=3 for each group). Data represent means ± s.d. t-test. **P<0.01, ***P<0.001. (e) The relative mRNA levels of Med23, Runx2, Osx, Alp and Ocn were quantified by RT-PCR. Data represent means ± s.d. All data represent means ± s.d. t-test, **P<0.01, ***P<0.001.

Med23 regulates Runx2 activity as a cofactor. We next set out to investigate whether Med23 controls the transcriptional activity of Runx2. To this end, we infected murine C3H10T1/2 embryonic fibroblasts with retroviruses expressing control or Med23 knockdown shRNAs and then transfected the stable cells with a luciferase reporter driven by the Ocn promoter (OC1050-Luc), which is a prototypical Runx2-target gene promoter that is widely used as a tool to study the molecular regulation of osteoblast development24. In the absence of MED23, RUNX2-dependent activation of the Ocn promoter was significantly inhibited (Fig. 5a). The Ocn promoter harbours two binding sites: OSE2 and OSE1, which are specifically bound by Runx2 and ATF4, respectively25. To discriminate the effects of Med23 deficiency on the two key factors, we performed the luciferase reporter assay with either 6XOSE2-Luc or 6XOSE1-Luc in control and Med23 shRNA knockdown cells. Med23 deficiency specifically impaired the Runx2-driven activation of 6XOSE2-Luc but did not affect the ATF4-driven activation of 6XOSE1-Luc, suggesting a specific connection between Med23 and Runx2 (Fig. 5b,c). To further verify the specific regulation of RUNX2 activity by Med23, we performed a titration experiment with low and high doses of Med23 co-transfected with the OC1050-Luc reporter, which showed that RUNX2 activity was repressed gradually with increasing dose of Med23 (Fig. 5d). The repressive effect on RUNX2 activity by overexpression of Med23 was also confirmed by 6XOSE2-Luc reporter system (Supplementary Fig. 7a). By contrast, overexpression of Med23 did not affect ATF4 activity (Fig. 5d and Supplementary Fig. 7b). RUNX2 activity was quenched by high level overexpression of MED23 probably in that the exogenous MED23 may compete with endogenous MED23 within the Mediator complex to prevent the transcription factor Runx2 from recruiting the Mediator complex.

Cooperative activation of the Ocn promoter by Med23 and RUNX2 led us to examine the physical interaction between the two molecules. We transfected 293T cells with tagged Runx2 and Med23 and performed a co-immunoprecipitation (co-IP) assay,
which revealed that MED23 physically associated with RUNX2 (Fig. 5e). Consistent with these results, endogenous RUNX2 and MED23 interacted in differentiated MC3T3E1 osteoblastic cells (Fig. 5f). The interaction with MED23 was mediated via the Runt and PST domains of RUNX2 (Supplementary Fig. 8a,b). GST pull-down assay implied that MED23/RUNX2 interaction was likely direct (Supplementary Fig. 8c). Moreover, immunofluorescence staining showed that these two proteins largely colocalized in the nucleus (Fig. 5g). As gene transcription involves a key step in which the activators work with the Mediator complex to recruit RNA Pol II to the gene promoters, we next used a chromatin IP (ChIP) assay to test whether Med23 deletion affected the recruitment of RNA Pol II by RUNX2 and the binding of RUNX2 to its target’s promoter. The result showed that the occupancy of Pol II at the promoter of Ocn, a direct target gene of Runx2, was markedly decreased in the absence of MED23 while RUNX2 occupancy did not alter, suggesting that Med23 deficiency impaired the recruitment of Pol II to the promoter of Runx2-target osteogenic marker genes (Fig. 5h). Taken together, these results suggest that the Mediator subunit MED23 acts as a coactivator of Runx2 to regulate osteoblast differentiation.
Figure 5 | Regulation of RUNX2 transcriptional activity by MED23. (a-c) Effect of Med23 deficiency on Runx2-mediated activation of the osteocalcin tested with luciferase expression report systems (a) OC1050-Luc, (b) 6XOSE2-Luc and (c) 6XOSE1-Luc. Luciferase assay was performed in control and Med23 knockdown C310T1/2 cells. n = 3 for each group, all data represent means ± s.d. t-test, ***P < 0.001 (d) Effects of an increasing amount of MED23 on transcriptional activity of Runx2 in C310T1/2 cells with the OC1050-Luc report system. Below is the western blot analysis of MED23 levels in the lysates. n = 3 for each group, all data represent means ± s.d. t-test. **P < 0.01, ***P < 0.001 (e) Co-immunoprecipitation (Co-IP) of MED23 with Flag-RUNX2. Flag-RUNX2 expressing plasmid was co-transfected with Myc-Med23 into 293T cells. Whole cell lysate was used for immunoprecipitation and then immunoblotting with indicated antibodies. (f) Physical interaction between endogenous RUNX2 and MED23. Co-IP experiment was performed in BMP2-stimulated MC3T3E1 cells. Whole cell lysate was used for immunoprecipitation with anti-RUNX2 or anti-MED23, followed by detection with indicated antibodies by western blot. (g) Co-localization assay in primary osteoblast cells. Cells were IF stained with antibodies against MED23 and RUNX2 after stimulated with BMP2 for 24 h. Scale bar, 10 μm. The scatter plot showed FITC and RRX emission intensities were plotted on x- and y-axes, respectively. Co-localization was analysed by the Velocity software, which showed the coefficient for co-localization of 0.786 ± 0.003 (Pearson’s correlation coefficient). (h,i) ChIP assay for occupation of RUNX2 (h) or Pol II (i) on the promoter of Osteocalcin. Immortalized WT and Med23−/− bone marrow-derived mesenchymal cells were cultured in osteogenic medium for 6 days, followed by fixation and lysisation. Chromatin from cell lysates was immunoprecipitated with anti-RUNX2 or anti-Pol II and quantified by RT–PCR. ‘Prom’ represents the promoter region and ‘Cod’ represents the coding region. n = 3 for each group, all data represent means ± s.d. t-test, **P < 0.01.
Med23 genetically interacts with Runx2 during osteogenesis. Based on the in vitro findings described above, we next questioned whether MED23 synergized with RUNX2 in vivo. We hypothesized that if MED23 acts together with RUNX2 to regulate osteoblast activity, then deletion of Med23 in parallel with Runx2 haploinsufficiency in vivo should aggravate the skeletal defects observed in Runx2+/– mice. To test this hypothesis and since disruption in single copy of Med23 did not reduce the protein level of MED23, we crossed Med23+/– mice with Med23+/–/Runx2+/– mice and analysed the skeletal phenotypes of E16.5 embryos by alcian blue/ARS double staining. As shown in Fig. 6a, both Med23+/–/Runx2+/– mice and Med23+/–/Runx2+/– mice showed the previously reported CCD-like skeletal abnormalities observed in Runx2+/– mice. To further investigate the genetic interaction between Med23 and Runx2, we performed in vivo experiments using compound mutant mice. Consistent with the reduced expression of Ocn in Med23-deficient mice, the expression of Ocn was decreased significantly in the Runx2+/–/Med23–/– compound mutant mice compared with the Runx2+/– and Med23+/– mice (Fig. 6c). Taken together, these data indicate a genetic interaction between Med23 and Runx2 and provide in vivo verification that MED23 can regulate osteoblast function via cooperation with RUNX2.

Discussion
The present study provides several lines of evidence that Med23 is required for osteoblast differentiation and bone development. Homozygous loss of Med23 in mesenchymal progenitors resulted in prominent defects in bone development, including hypoplasia of the clavicles, retarded ossification of the cranial bones and reductions in the trabecular bone and overall bone mass. Furthermore, Med23 conditional knockout in preosteoblasts largely recapitulated the characteristics of Med23–/– mice. Phenotypic analysis showed that Med23 deficiency affected both intramembranous and endochondral bone formation. In vitro differentiation experiments indicated that the defective osteogenesis happened in a cell-autonomous manner. In contrast to the impaired osteoblast development, the presence of multinucleated osteoclasts in the mineralized cartilage matrix of Med23-deficient mice indicated that osteoclast differentiation occurred normally.

MSCs can differentiate into different lineages, including osteoblasts, adipocytes, chondrocytes and myocytes. Different
transcription factors have been shown to specify distinct cell fates. In addition to the function of Runx2 in osteoblast differentiation, PPARγ, Sox9 and MyoD drive MSCs to differentiate into adipocytes, chondrocytes and myocytes, respectively9,10,11. The role of the Mediator complex in PPARγ-mediated adipogenesis has been established by observing a direct interaction between MED1 and PPARγ9,11. For Sox9, studies from the zebrafish model indicated that Trap230/MED12 can function as a coactivator for SOX9 during cartilage development, with Trap230/Med12 mutant zebrafish strikingly resembling the Sox9 mutant phenotype9,10. Our previous study demonstrated that Med23 represses smooth muscle cell differentiation while facilitating adipocyte differentiation in multipotent MSCs9,10, indicating that a single Mediator complex subunit is able to regulate the differentiation of MSCs into various cell lineages, either positively or negatively, by cooperating with lineage-specific transcription factors such as Elk1 and Mal.

To date, there have been no reports of a direct role for the Mediator complex in RUNX2 function. Our study demonstrated that Med23 can act together with RUNX2 to drive osteoblast differentiation. To the best of our knowledge, this is the first report of a direct role for the Mediator complex in modulating RUNX2 function. We provide evidence that the Mediator complex subunit MED23 partially regulates the transcriptional activity of RUNX2 and is required for osteoblast differentiation and bone development. First, homozygous loss of Med23 in mesenchymal progenitors resembled the bone phenotype of Runx2 heterozygous mice, resulting in prominent defects in bone development, with hypoplasia of the clavicles, retarded ossification of the cranial bones and reduction in the trabecular bone and overall bone mass. Moreover, Med23 conditional knockout in preosteoblasts largely recapitulated the characteristics of Med23 heterozygous mice, with abnormalities in multiple skeletal elements. Interestingly, Med23 deficiency did not alter RUNX2 expression but instead caused the downregulation of multiple Runx2-regulated genes, such as Osx and Ocn. Reporter assays revealed that Med23 deficiency impaired Runx2 transcriptional activity but did not affect the activity of ATF4, another key regulator of osteoblast differentiation. Biochemical analyses further established the physical association between RUNX2 and MED23. Finally, Med23 deletion further aggravated the defective skeletal phenotype of Runx2+/− mice. In addition to the function in osteoblasts, Runx2 is also found to control the maturation of chondrocytes through Runx2 conditional knockout in chondrocytes by Col2a1-CreER10,30,31. Histological analysis of tibia showed that chondrocytes were arrested during terminal maturation in Med23 heterozygous mice compared with control littermates as evidenced by less endochondral ossification and smaller hypertrophic chondrocytes in Med23−/− mice (Supplementary Fig. 10a–c). All the results described above provide in vivo genetic evidence that MED23 is a cofactor of RUNX2.

Runx2 mutations are known to underlie human CCD, an autosomal-dominant heritable skeletal disease that is typically characterized by open or delayed closure of calvarial fontanelles and clavicle hypoplasia9,32,33. This phenotype can be reproduced in heterozygous Runx2 mutant mice10. More precisely, a 70% reduction in Runx2 levels also generates the CCD phenotype in mice24. Indeed, human bone marrow-derived MSCs with Med23 deficiency were retarded to differentiate into osteoblasts, which might imply that MED23 plays a role in human skeletal development and its mutation may relate to human bone diseases (Supplementary Fig. 9). In addition, the dysregulation of Runx2 activity through manipulation of numerous nuclear factors, for example, TAZ, MAF and Satb2, causes abnormal osteoblast differentiation and bone development11,35,36. These studies indicate that the Runx2-dependent transcriptional output is under fine surveillance via the cooperation of many factors. Our study demonstrated that the Mediator subunit MED23 acts as a cofactor of RUNX2 in the regulation of osteoblast differentiation and bone development, providing an insight into the regulation of RUNX2 activity and skeletal dysplasias such as CCD.

Methods

Mice. Med23−/− mice were generated by homologous recombination16,37. Briefly, the exons 5–7 were flanked by two loxP sites. After targeting vector delivery, ES cells were screened by PCR. Med23−/− mice were backcrossed with C57BL/6J mice for at least six generations. Ppx1−/− and Oss-Cre-ER mice were purchased from the Jackson Laboratory. Runx2+/− mice were kindly provided by Professor Laurie Glimcher’s lab. All mice were maintained under specific pathogen-free conditions. All animal experimental procedures were approved by the Institutional Animal Care and Research Advisory Committee of the Shanghai Institute of Biochemistry and Cell Biology.

Analysis of bone phenotypes. Skeletal preparations were double stained with alizarin red S and alcol blue. Briefly, embryos or newborns were eviscerated and the skin was removed. After fixation with 4% formaldehyde for 3 days, embryos or newborns were stained for 3 days in alizarin blue solution. Then they were fixed and cleared with 1% KOH/20% glycerol. For histological analysis, bone tissues were fixed in 4% paraformaldehyde (PFA) and then embedded in paraffin. For embryonic mice, 4-μm tissue sections were used for Von Kossa staining, DIG labelled in situ hybridisation (Roche) and immunohistochemical staining (Dako). For postnatal mice, bone tissues were fixed in 4% PFA and decalcified for 2 weeks prior to paraffin embedding. Tissue sections (4 μm) were used for TRAP staining according to the standard protocol.

Measurement of PINP concentrations. We determined serum concentrations of PINP using the mouse PINP ELIA kit (Immunodiagnostic Systems) according to the instructions provided.

Micro CT analysis. Mouse hind limbs were harvested, soft tissues were removed and the remaining tissues were stored in 70% ethanol. Scanning was performed with a SkyScan1076 instrument, and 36 slides (18 μm each) immediately below the growth plate in the distal metaphysis of the femur were used for quantification of the bone parameters.

Cell culture. C3H10T1/2 (ATCC) and 293T (ATCC) cells were maintained in DMEM containing 10% FBS. MG63-3E1 (ATCC) cells were maintained in Minimum Essential Medium α (MEM) containing 10% FBS. MSCs were isolated from newborns and cultured in α-MEM containing 15% FBS. All cells were cultured in a 5% CO2, humidified incubator at 37 °C. For the c.f.u.-F assay, 2 × 104 bone marrow-derived MSCs or 1 × 104 osteoblast cells from calvaria were plated on each well of a six-well plate, cultured for 2 weeks and then fixed in 4% PFA and stained with 0.5% crystal violet, followed by counting the stained colonies. For in vitro osteoblast differentiation, MSCs were cultured in osteogenic medium (10% FBS with 50 μg/ml ascorbic acid, 10 mM dexamethasone and 10 mM β-glycerophosphate) and subjected to ALP staining on day 7 and 9 ARS staining on day 21. Human bone marrow-derived MSCs were cultured and induced to differentiate into osteoblasts according to the protocol from ScienCell.

Transient transfection and luciferase reporter assay. C3H10T1/2 cells were seeded overnight at 4 × 104 cells per well in a 24-well plate and transfected by Lipofectamine 2000 (Life Technologies) with a luciferase reporter plasmid and PRL-TK (Promega) along with various expression constructs, as indicated. All wells were supplemented with control empty expression vector plasmids to keep the total amount of DNA constant. At 36–48 h post transfection, the cells were harvested and subjected to dual-luciferase reporter assays according to the manufacturer’s protocol (Promega).

IP and immunoblotting. 293T cells were seeded at 6 × 105 cells per 10 cm dish and cultured overnight. At 36–48 h after transfection with Lipofectamine 2000, cells were harvested and lysed in lysis buffer (1% NP-40, 10% glycerol, 135 mM NaCl, 20 mM Tris, pH 8.0) supplemented with protease inhibitors. Lysates were subjected to IP with anti-Flag antibodies (M2, Sigma) at 4 °C overnight, followed by washing in lysis buffer, SDS-PAGE electrophoresis and immunoblotting with the indicated antibody (1:2,000, anti-Myc, Sigma). To investigate endogenous protein–protein interactions, MG63-3E1 cells were cultured for 4 days in α-MEM with 10% FBS.
supplemented with 100 ng/ml-1 BMP2, 50 μg/ml-1 ascorbic acid and 10 mM β-glycerophosphate. Two hours prior to harvest, MG132 (10 μM, Sigma) was added to all cultures. Harvested cells were subjected to lysis and IP with anti RUNX2 (Sigma), anti-MED23 (Novus) or control IgG, followed by washing in lysis buffer, SDS-PAGE electrophoresis and immunoblotting with the indicated antibody (Anti-MED23, 1:1,000, BD Biosciences; anti-Runx2, 1:1,000, MBL). Primary bone marrow MSCs or bone tissues were lysed in RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) in presence of protease inhibitors. Whole cell lysate were centrifuged and then subjected to western blots according to standard protocols. Unspotted results for all western blots are shown in Supplementary Fig. 11.

**In vitro interaction assay.** GST or GST-RUNX2 recombinant proteins were expressed in Escherichia coli BL21, followed by purification according to the instructions of the manufacturer (GE). His-Flag-MED23 was expressed using the Bac-to-Bac baculovirus expression system and purified by Ni-NTA agarose beads (Invitrogen). Purified GST or GST-RUNX2 was incubated with purified MED23 and subjected to GST pull-down experiment. The bound proteins were analysed by SDS–10% PAGE and immunoblotting with indicated antibodies (Anti-GST, 1:1,000, Santa Cruz; anti-MED23, 1:1,000).

**Immunofluorescence staining and co-localization analysis.** Primary osteoblast cells were plated on cover slips for 24 h in α-MEM with 10% FBS with BMP2 (100 ng ml-1) prior to staining. After removal of culture media, cells were washed with PBS, fixed with 4% PFA, permeabilized with 0.2% Triton X-100 for 10 min and then blocked with 2% BSA in PBS for 1 h. Anti-MED23 (1:100) and anti-RUNX2 (1:200) primary antibodies were diluted in the blocking solution and applied overnight at 4°C. After PBS wash for three times, secondary antibodies (Jackson Laboratory) were diluted (1:100) in blocking buffer and applied for 1 h at room temperature. After PBS wash for three times, nuclei were stained with DAPI (1,4-diamino-2phenylindole) (1,4,000) for 4 min. Co-localization analysis were performed using Velocity Software (PerkinElmer).

**Real-time PCR analysis.** Total RNA was isolated from bone tissue or cells with TRIzol reagent (Life Technologies). Complementary DNA was generated using M-MLV reverse transcription kit (Promega). Real-time PCR was conducted in 20 μl reactions with 1 μl cDNA and 5 μl SYBR Green (Invitrogen). Purified GST or GST-RUNX2 was incubated with purified MED23 and Bak-positive distributions of two data sets (Fig. 4a).

**Statistical analysis.** Statistical analyses were performed with two-tailed, unpaired Student’s t-test. Kolmogorov–Smirnov test was used to test for comparing cumulative distributions of two data sets (Fig 4a).

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Author contributions

Z.L. performed the majority of experiments and prepared the manuscript. G.Y. performed in situ hybridization experiments. X.Y., Y.X. and J.Y. analysed RNA-seq data. W.Z. and G.W. designed and supervised the project.

Additional information

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