Histone demethylase JMJD3 is required for osteoblast differentiation in mice

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JMJD3 (KDM6B) is an H3K27me3 demethylase and emerges as an important player in developmental processes. Although some evidence indicated the involvement of JMJD3 in osteoblast differentiation in vitro, its role as a whole in osteoblast differentiation and bone formation in vivo remains unknown. Here we showed that homozygous deletion of Jmjd3 resulted in severe delay of osteoblast differentiation and bone ossification in mice. By biochemical and genetical methods, we demonstrated that JMJD3 mediated RUNX2 transcriptional activity and cooperated with RUNX2 to promote osteoblast differentiation and bone formation in vivo. These results strongly demonstrated that JMJD3 is required for osteoblast differentiation and bone formation in mice.

During embryo development, bones form through two distinct processes: intramembranous and endochondral ossification. Some cranial bones and the lateral portion of the clavicles are formed by intramembranous ossification, during which mesenchymal progenitor cells directly differentiate into bone-forming osteoblasts. In contrast, endochondral ossification involves a cartilaginous template for future bone. Subsequently, this template is replaced following vascular invasion by bone cells. Meanwhile, the perichondral mesenchymal cells flanking the cartilaginous template differentiate into osteoblasts and form the periosteum or cortical bone. RUNX2 is a master regulator of osteoblast differentiation for both intramembranous and endochondral ossification. RUNX2 mutation in humans led to cleidocranial dysplasia, which is characterized by hypoplastic clavicles and delayed closure of the fontanelles. Runx2 null mice display a complete loss of both intramembranous and endochondral ossification with a blockage of osteoblast differentiation.

JMJD3 is a trimethylation of histone 3 at lysine 27 (H3K27me3) specific demethylase and counteracts Polycomb-mediated transcriptional repression. Jmjd3 knockout mice showed that JMJD3 was crucial for macrophage differentiation, lung development and neurogenesis in vivo. Recently, we observed that JMJD3 promoted chondrocyte proliferation and hypertrophy during endochondral bone formation in mice. Previously, some groups showed that JMJD3 was required for osteoblast differentiation in vitro. However, the role of JMJD3 in osteoblast differentiation and bone formation in vivo remains unknown. Here, we reported that JMJD3 promoted osteoblast differentiation and bone ossification in vivo. Moreover, we demonstrated that JMJD3 biochemically and genetically cooperates with RUNX2 to regulate osteoblast differentiation and bone formation in mice.

Results

**JMJD3 is required for intramembranous ossification in vivo.** Previously, we observed that JMJD3 is critical for chondrocyte proliferation and hypertrophy during endochondral formation. To examine
whether JMJD3 is also required for intramembranous ossification in vivo, we examined the membranous bones of E14.5–E18.5 littermates by alcian blue and alizarin S red staining. No obvious skeletal difference between WT and Jmjd3−/− mice was detected (data not shown). However, unlike wild-type embryos, which exhibited well calcified skeletons, homozygous mutant embryos showed continually less calcification in parietal bone from E14.5 to E18.5 (Fig. 1A). Detailed investigation revealed that Jmjd3−/− mice exhibited open fontanelles, less mineralized cranial bones and hypoplastic clavicles compared to WT littermates at E18.5 (Fig. 1B,C). These features suggested that JMJD3 is required for intramembraneous ossification in vivo.

JMJD3 is required for osteoblast differentiation during both intramembranous and endochondral bone formation. Since osteoblast maturation is essential for intramembranous ossification and endochondral bone formation, we next examined whether JMJD3 is required for osteoblast differentiation in vivo. Firstly, we examined the expressed level of JMJD3 in osteoblasts of E14.5, E16.5 and E18.5 parietal bones by western blot assays. The results showed that JMJD3 expression increased from E14.5 to E18.5 (Fig. 2A) and was primarily in the nuclei of osteoblasts (Fig. 2B). Subsequently, we performed histological investigations by von Kossa (Von) and alkaline phosphatase (AP) staining and observed much less mineralization and lower intensity of AP staining in the sections of parietal bones of Jmjd3−/− embryos compared to WT littermates (Fig. 2C). Furthermore, we also observed much less mineralization and lower intensity of AP staining in Jmjd3−/− long bones, such as in humerus (Fig. 2D). These results suggested JMJD3 is required for osteoblast maturation during both intramembranous and endochondral bone formation.

To further characterize the defects of osteoblast differentiation in vivo, we performed in situ hybridization with RNA probes of molecular marker for osteoblast differentiation at different stages. COL1A1, the most abundant protein in the bone matrix, is an early marker of osteoblast differentiation. In E13.5 and E15.5 embryos, in situ hybridization indicated that Coll1a1 mRNA level was much less in the perichondrium of Jmjd3−/− than that of WT humeri (Fig. 2Ea–d). At E18.5, the level of osteocalcin (Bglap), a late marker of osteoblast differentiation, markedly decreased in perichondral and periosteal osteoblasts of Jmjd3−/− embryos compared to WT littermates (Fig. 2Ee,f). These results revealed that defects of osteoblast differentiation occurred at both early and late developmental stages of Jmjd3−/− embryos. Consistently, the mRNA level of Fgfl8, a factor crucial for both early and late osteoblast differentiation, also obviously reduced in mutants compared with WT embryos (Fig. 2Eg,h).

In addition, we examined the differentiation potential of primary cultured preosteoblasts from cranial bones of E14.5 WT and Jmjd3−/− embryos in osseogenic medium. Alizarin S red and von Kossa staining
revealed obvious delay of osteoblast differentiation in $Jmjd3^{-/-}$ vs. WT osteoblasts (Fig. 2F). Similar results were observed in perichondral and periosteal osteoblasts derived from four limbs of E18.5 WT and $Jmjd3^{-/-}$ embryos (data not shown). Collectively, these results strongly demonstrated that JMJD3 is required for osteoblast differentiation.
JMJD3 regulates RUNX2 transcriptional activity during osteoblast differentiation. To explore the molecular mechanism of JMJD3 in osteoblast differentiation, we first analyzed expression of osteoblast-specific genes in WT and Jmjd3−/− embryos. RT-qPCR showed that the mRNA levels of Runx2, Sp7, Spai, Bglap, Col1a1 and Fgf18 in parietal osteoblasts were reduced in Jmjd3−/− embryos compared with WT mice (Fig. 3A). In contrast, the mRNA level of atf4 was not affected. To test whether JMJD3 directly regulates these genes transcription, we examined JMJD3 level at the promoters of Runx2 and Bglap by chromatin immunoprecipitation (ChiP) followed by qPCR analysis in primary osteoblasts. Indeed, JMJD3 was showed to strongly recruit to the promoters of the two genes (Fig. 3Ba,b). Consistently, the H3K27me3 level at the promoters of Runx2 and Bglap genes markedly increased in Jmjd3−/− compared with WT primary osteoblasts (Fig. 3Bc,d). These results clearly demonstrated that JMJD3 directly promoted Runx2 and Bglap transcription by erasing H3K27me3 in osteoblasts. This was consistent with a recent report that knockdown of Jmjd3 in MC3T3-E1 osteoblasts resulted in increased H3K27me3 level at the promoter regions of Runx224. In addition, western blot assay showed that knock-out of Jmjd3 increased the total level of H3K27me3 in primary osteoblasts (Fig. 3C). This indicated that JMJD3 might regulate more genes transcriptions for osteoblast differentiation.

Since RUNX2 has been shown to bind and activate its own promoter via a positive feedback loop20, and Sp7, Spai, Bglap and Col1a1 are target genes of RUNX225, we speculated that JMJD3 may act as a coactivator of RUNX2 in osteoblast differentiation. To test this possibility, we firstly tested whether JMJD3 biochemically interacts with RUNX2. Co-immunoprecipitation assays showed that RUNX2 really pull down JMJD3, or vice versa, in the primary culture of osteoblasts from E18.5 mice (Fig. 3D). In addition, immunofluorescence under confocal microscope indicated that JMJD3 co-localized with RUNX2 in the nuclei of primary osteoblasts (Fig. 3E). Next, to test whether JMJD3 can functionally cooperate with RUNX2 to regulate their target genes transcription, we performed luciferase reporter assays with Jmjd3 knockout mice. The results showed that JMJD3 quite obviously synergized with RUNX2 to potentiate the expression of Bglap. This synergistic effect was reduced by shRNA against Jmjd3 in a dose dependent manner (Fig. 3F). These results strongly supported that JMJD3 is a coactivator of RUNX2 in osteoblast differentiation in vivo and suggested that RUNX2 may recruit JMJD3 to target genes during bone formation. In order to test whether RUNX2 can affect JMJD3 binding to target genes during osteoblast differentiation, we examined JMJD3 occupancies at promoters of Runx2 and Bglap genes by ChiP-qPCR in WT and Runx2−/− primary osteoblasts from parietal bones. The results showed that the level of JMJD3 was reduced at the promoters of Runx2 and Bglap genes in Runx2−/− compared with WT osteoblasts (Fig. 3Ga,b). Consistently, the levels of H3K27me3 at the promoters of Runx2 and Bglap increased in Runx2−/− versus WT primary osteoblasts (Fig. 3Gc,d). These results supported that RUNX2 can recruit JMJD3 to target genes during osteoblast differentiation.

JMJD3 cooperates with RUNX2 to promote osteoblast differentiation in vivo. To examine whether JMJD3 cooperates with RUNX2 to promote osteoblast differentiation in vivo, we generated and analyzed Jmjd3−/−; Runx2−/− embryos. Skeletal preparations examination showed that Jmjd3−/−; Runx2−/− embryos displayed the least ossification in cranial bones (Fig. 4Ad,Ba) than WT, Jmjd3−/−, or Runx2−/− mice at E15.5. Von Kossa staining confirmed the most delay of ossification in perichondral zones of humerus of Jmjd3−/−; Runx2−/− embryos compared with other littermates at this stage (Fig. 4Al). In situ hybridization and RT-qPCR for Col1a1 and Bglap showed the least mRNA level of perichondrial osteoblasts of humerus of Jmjd3−/−; Runx2−/− embryos at E14.5 or E18.5 (Fig. 4Ap,t,Bb,c). Collectively, these results clearly demonstrated that JMJD3 genetically cooperates with RUNX2 to promote osteoblast maturation during both intramembraneous and endochondral ossification, which suggested that JMJD3 is a coactivator of RUNX2 in osteoblasts.

Discussion
Osteoblast differentiation is essential for both intramembranous and endochondral ossification. Abnormality of this process will result in various bone diseases, such as osteoporosis or osteosclerosis22,23. It has been shown previously that H3K27 methyltransferase EZH2, a core subunit of PRC2 group, suppresses mesenchymal stem cells (MSCs) from differentiating into osteoblasts24, while WDR5, a core component of trithorax group (TrxG), promote osteoblasts25. These results implicate other PRC2 and trithorax proteins may also be involved in skeleton development. As an H3K27me3 demethylase, JMJD3 counteracts the transcription repression by Polycomb group (PcG) and biochemically interacts with the core subunits of TrxG, such as WDR5, RbBP5, ASH2L and BRG119,26. Thus, these biochemical features begged the question of the possible role of JMJD3 during osteoblast differentiation. Recently, Ye et al.19 showed that osteogenic differentiation of human bone marrow MSCs is positively regulated by JMJD3, while Yang et al.18 demonstrated that osteoblast differentiation is inhibited upon knocking-down Jmjd3 in vitro. In addition, both papers also showed by in vivo models that JMJD3 positively regulates osteogenic differentiation, either using a model injecting MSCs in which JMJD3 was silenced into mice, or by finding impaired local bone formation in adult mice upon local application of siRNA against Jmjd3 in the calvaria of living mice18. However, the detail roles of JMJD3 for osteoblast differentiation during both intramembranous and endochondral ossification is not clear. Here, using Jmjd3 knockout mice, we demonstrated that JMJD3 is required for osteoblast differentiation during both intramembranous and endochondral ossification. This conclusion was supported by the following reasons: (1)
Figure 3. JMJD3 regulates RUNX2 transcriptional activity during osteoblast differentiation. (A) RT-qPCR to determine the expression levels of Jmjd3, Runx2, Aft4, Sp7, Sp1, Bglap, Col1a1 and Fgf18 relative to GAPDH in parietal osteoblasts of E18.5 WT and Jmjd3−/− embryos. *p < 0.05, Error bar represents the SE of three independent experiments. (B) ChIP-qPCRs assays with antibodies specific for JMJD3 (a,b) or H3K27me3 (c,d) at the promoters of Runx2 (a,c) or Bglap (b,d) genes in WT and Jmjd3−/− primary parietal osteoblasts. Signals were shown as a percentage of the input. *p < 0.05, Error bar represents the SE of three independent experiments. (C) The total level of H3K27me3 in primary osteoblast of E18.5 WT and Jmjd3−/− parietal bones was examined by western blot assays. (D) Endogenous interaction of JMJD3 and RUNX2 was examined by Co-IP assays in the primary osteoblasts from E18.5 parietal bone. IP was performed as indicated. (E) Immunofluorescence under confocal microscope with anti-JMJD3 (green) and anti-RUNX2 (red) antibodies on the slides of primary osteoblasts of E18.5 embryos. Scale bar: 20 μm. (F) Luciferase reporter assays in HEK293 cells, which were transfected with the pGL3-basic reporter containing 2 kb Bglap promoter with or without expression plasmids encoding Runx2 (100 ng), Jmjd3 (100 ng) or sh-Jmjd3 in increasing amounts (50, 100 ng) as indicated. The basal luciferase activity for each reporter was calculated as 1 in y axis. *p < 0.05, **p < 0.01, Error bar represents the SE of three independent experiments. (G) ChIP-qPCRs assays with antibodies specific for JMJD3 (a,b) or H3K27me3 (c,d) at the promoters of Runx2 (a,c) or Bglap (b,d) genes in WT and Runx2−/− primary parietal osteoblasts. Signals were shown as a percentage of the input. *p < 0.05, Error bar represents the SE of three independent experiments.
Figure 4. JMJD3 cooperates with RUNX2 to promote osteoblast differentiation in vivo.
(A) Representative skeletal preparation, von Kossa staining and in situ hybridization with Col1a1 and Bglap probes of Jmjd3+/+; Runx2+/+; Jmjd3+/−; Runx2+/+; Jmjd3+/+; Runx2+/− and Jmjd3−/−; Runx2+/− mice. Red arrows: most delayed bone ossification in parietal bone and clavicles of Jmjd3+/−; Runx2+/− mice at E15.5. Black arrow: most delayed mineralization in perichondrium of humerus of Jmjd3+/−; Runx2+/− mice at E15.5. Blue arrow: most reduced Col1a1 mRNA level in perichondrium of humerus of Jmjd3+/−; Runx2+/− mice at E14.5. Purple arrow: most reduced Bglap mRNA level in perichondrium of humerus of Jmjd3+/−; Runx2+/− mice at E18.5. Littermates at each group were compared. n = 3. Scale bar: 1 mm in a–h; 200 μm in i–t. (B) Quantification of width of parietal bones (a), Col1a1 (b) and Bglap (c) mRNA level of humerus perichondrial osteoblasts of Jmjd3+/+; Runx2+/+, Jmjd3+/−; Runx2+/+; Jmjd3−/−; Runx2−/− and Jmjd3+/−; Runx2+/− embryos at E15.5 (a), E14.5 (b) or E18.5 (c) respectively. *p < 0.05. Error bar represents the SE of three independent experiments.
JMJD3 was expressed in osteoblasts, (2) Jmdj3 mutant mice displayed severe retardation of osteoblast differentiation by skeletal preparation and histological analysis, and (3) JMJD3 genetically and biochemically cooperated with RUNX2 to promote osteoblast differentiation and bone ossification in vivo. The existence of a fairly typical cleidocranial dysplasia phenotype in Jmdj3−/− embryos suggested that JMJD3 genetically associates with RUNX2 in vivo. Indeed, we detected that JMJD3 directly promotes Runx2 transcription in osteoblasts by CHIP-qPCR assay. This was consistent with a recent report that knockdown of Jmdj3 in MC3T3-E1 osteoblasts resulted in increased H3K27me3 level at the promoter regions of Runx2 forward and reverse; β-glycerolphosphate for 5 or 15 days. Cells were then stained for 

Methods

Morphological and histology analysis. Jmdj3−/− and Runx2−/− mice was described previously. Whole-mount staining of skeletal preparation, histological analyses, alkaline phosphatase staining and in situ hybridization investigations were performed as described previously27. Briefly, the embryos were skinned, eviscerated, and fixed in 95% ethanol. After 4 days fixation in 95% ethanol, embryos were stained in Alcian blue solution overnight. After washing with 70% ethanol, the embryos were stained by Alizarin S red solution overnight and transferred into 1% KOH for 1 week. Lastly, embryos were transferred into 1% KOH/20% glycerol for 2 days and stored in 50% ethanol/50% glycerol. For histological analyses, embryos fixed in 10% formalin, processed, and embedded in paraffin or OCT (Tissue-Tek; Thermo Fisher Scientific). Serial sections were taken at 4 μm thickness. To detect mineral deposition, Von Kossa staining was performed and counterstained by alcan blue and nuclear fast red. Alkaline phosphatase staining was performed on 7 μm cryostat sections using alkaline phosphatase kit (Millpore) following the manufacturer’s instructions. For in situ hybridization, digoxigenin-11-UTP-labeled single-stranded RNA probes were prepared with a DIG RNA labeling kit (Roche) according to the manufacturer’s instructions. Fgf18 probe was from David Onnitz (Washington University School of Medicine) and Col1a1 and Bglap probes sequences were described before27,28. All animal procedures were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at Shanghai Institutes for Biological Sciences.

Primary osteoblasts culture and in vitro assays. Primary osteoblasts were obtained from the parietal bones or four limbs long bones of E14.5, E16.5 and E18.5 embryos by sequential collagenase A digestion at 37 °C as described29. Briefly, parietal bones or four limbs long bones were removed from the embryos under aseptic conditions and incubated at 37 °C in DMEM medium containing trypsin (0.5 mg/mL) for 10 min. Trypsin digestes were discarded and replaced with DMEM containing 1 mg/mL supplements with 10% fetal calf serum. To characterize the nature of the osteoblasts, the expression of RUNX2 was examined by western blot assays. For in vitro maturation assays, parietal osteoblasts from E14.5 embryos at confluence were cultured in differentiation medium (a-MEM containing 10% FCS, 50 μg/mL ascorbic acid, and 5 mmol/L β-glycerophosphate for 5 or 15 days. Cells were then stained for the presence of Alizarin S red and von Kossa staining. To characterize the nuclear localization of JMJD3 and RUNX2, double-labeled immunofluorescences with anti-JMJD3 (Rabbit Polyclonal, Abcam, ab85392) and anti-RUNX2 (mouse monoclonal, Abcam, ab76956) were performed on the slides of primary osteoblasts from E18.5 embryos according to standard protocols.

RNA isolation, reverse transcription, and real-time PCR. Parietal or perichondrial osteoblasts from E18.5 embryos were collected and lysed in TRIzol (Invitrogen) for RNA isolation following standard protocol. Reverse transcription and real-time PCR was performed as described27. The primer pairs used were as follows: Jmdj3, 5′-CAACTCCATCTGGCTTTACTG-3′ (forward) and 5′-CCCTTGCAACCAA- TTCCAG-3′ (reverse); Runx2, 5′-TGACATCCCC ATCCATCC AC-3′ (forward) and 5′-AGAAGTCAGAGGTGGCAGTG-3′ (reverse); Col1a1, 5′-TCCCAGAACACATCCTATCCATCA- TCAC-3′ (forward) and 5′-CTGTGCCCTGGCCTGAGTGG-3′ (reverse); Sp7, 5′-GGTCTTGA- GCCA AACTCTCCTC-3′ (forward) and 5′-TGGGAAAACCGCAGAATTGC-3′ (reverse); Spp1, 5′-GAATGCTGTGTCCTCTGAAG-3′ (forward) and 5′-ATTCCTGCCTCTC- GCATGG-3′ (reverse); Bglap, 5′-GCCACATTTTCTGTCTCCTC-3′ (forward) and 5′-CCGCTGGTCCTGGCCTGCG-3′ (forward); Atf4, 5′-CCAACTCTATGACCCACCTGAG-3′ (forward) and 5′-ACCTAGTGGCTGCTG-3′ (reverse); Fgf18, 5′-GGCCCATTTTCTGTCTCCTC-3′ (forward) and 5′-GATCCG

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GGATCGCTTG-3′ (reverse); Gapdh, 5′- CATCACAGCAACACAGAAGACC-3′ (forward) and 5′- ACCAGTAAGCTT- GCCATTGAG-3′ (reverse).

Co-immunoprecipitation and luciferase reporter assays. To detect the interaction between endogenous JMJD3 and RUNX2, primary parietal osteoblasts from E18.5 embryos were grown to confluence on 10 × 15 cm dishes in Dulbecco’s modified Eagle’s medium (DMEM) with β-glycerophosphate (5 mmol/L) and ascorbic acid (50 μg/mL). The cells were collected and lysed in buffer as described previously17. Protein lysate (5 μg) was incubated for 2 hours with 3 μg anti-JMJD3 (Abcam, ab85392) or anti-RUNX2 (Cell Signaling, #8486) antibodies followed by incubation overnight with 40 μl protein A/G Plus agarose (Santa Cruz Biotechnology, Inc.). Immunoprecipitated proteins were detected by Western blotting with the anti-JMJD3 and anti-RUNX2 antibodies.

Luciferase Reporter Assays in HEK293T was described previously17. Briefly, HEK293T cells were cultured in DMEM media supplemented with 10% fetal bovine serum. For transient transfection assays, HEK293T cells were plated in 48-well plate at 5 × 10^5/well overnight and were transfected with 100 ng pGL3-basic reporter containing Bglap promoter with or without expression plasmids encoding Runx2 (100 ng), Jmjd3 (100 ng) or Jmjd3-shRNA (sh-Jmjd3) in increasing amounts (50, 100 ng) and 10 ng pRL-Renilla (Promega) mixed with 1 μl Lipofectamine 2000 (Invitrogen). Cells were harvested 48 hours after transfection. For luciferase analysis, the cells were lysed according to the manufacturer’s instructions for the Dual-Luciferase Reporter assay (Promega).

Chromatin immunoprecipitation (ChIP). For ChIP assays, primary osteoblasts were cross linked with formaldehyde and sonicated into small chromatin fragments. Protein-DNA complexes were precipitated with rabbit anti-JMJD3 (Abcam, ab85392) or anti-H3K27me3 (Millipore, #07-449) antibodies. The purified DNA was quantified by real-time PCR with Maxima SYBR Green qPCR Master Mix. 2ΔΔCt method was used for relative quantification. The primer pairs around of transcriptional start site used were as follows: Runx2, 5′-TGAAACCAGATACCCCCGAG-3′ (forward) and 5′-CTCCCCACCTCACCCTCAG-3′ (reverse); Bglap, 5′-TGAACAGACATACCCCCCGAG-3′ (forward) and 5′-GCCCTGCTTG- TGTTGGAGAC-3′.

Statistical analyses. All quantitative data are presented as mean ± standard error (SE) with a minimum of three independent samples. Statistical significance is determined by two-tailed Student’s t-test.

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**Author Contributions**

C.D.C. and F.Z. designed the project. F.Z. carried out all experiments, if not otherwise stated. L.Y.X. generated the *Jmjd3* knockout mice. L.X.X. contributed to RNA *in situ* hybridization. Q.X. raises the mice. G.K. provided the *Runx2*+/− mice. G.K. provided important intellectual input and helped analyze the phenotype. C.D.C. and F.Z. wrote the manuscript, and all authors provided editorial input. All authors read and approved the final manuscript.

**Additional Information**

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