Cbfβ deletion in mice recapitulates cleidocranial dysplasia and reveals multiple functions of Cbfβ required for skeletal development

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The pathogenesis of cleidocranial dysplasia (CCD) as well as the specific role of core binding factor β (Cbfβ) and the Runx-related transcription factor (RUNX)/Cbfβ complex in postnatal skeletogenesis remain unclear. We demonstrate that Cbfβ ablation in osteoblast precursors, differentiating chondrocytes, osteoblasts, and odontoblasts via Osterix-Cre, results in severe craniofacial dysplasia, skeletal dysplasia, abnormal teeth, and a phenotype recapitulating the clinical features of CCD. Cbfβ–/–Osterix-Cre mice have fewer proliferative and hypertrophic chondrocytes, fewer osteoblasts, and almost absent trabecular bone, indicating that Cbfβ may maintain trabecular bone formation through its function in hypertrophic chondrocytes and osteoblasts. Cbfβ–/–Collagen type 1, alpha 1 (Col1α1)–Cre mice show decreased bone mineralization and skeletal deformities, but no radical deformities in teeth, mandibles, or cartilage, indicating that osteoblast lineage-specific ablation of Cbfβ results in milder bone defects and less resemblance to CCD. Activating transcription factor 4 (Atf4) and Osterix protein levels in both mutant mice are dramatically reduced. ChIP assays show that Cbfβ directly associates with the promoter regions of Atf4 and Osterix. Our data further demonstrate that Cbfβ highly up-regulates the expression of Atf4 at the transcriptional regulation level. Overall, our genetic dissection approach revealed that Cbfβ plays an indispensable role in postnatal skeletal development and homeostasis in various skeletal cell types, at least partially by up-regulating the expression of Atf4 and Osterix. It also revealed that CCD may result from functional defects of the Runx2/Cbfβ heterodimeric complex in various skeletal cells. These insights into the role of Cbfβ in postnatal skeletogenesis and CCD pathogenesis may assist in the development of new therapies for CCD and osteoporosis.

The authors declare no conflict of interest.

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Significance

Cleidocranial dysplasia (CCD) is a hereditary human skeletal disease. Mutations in Runx-related transcription factor 2, which functions as a heterodimer with core binding factor β (Cbfβ), are found in most individuals with CCD. It has been suspected that Cbfβ may be responsible for other CCD cases. The pathogenesis of CCD and the role of Cbfβ in postnatal skeletogenesis remain unclear. There has been no animal model to study this disease. We demonstrate that ablation of Cbfβ in various skeletal cells results in severe craniofacial and skeletal dysplasia with the phenotype recapitulating clinical features of CCD. The findings from this study of Cbfβ in the skeleton provide insight into the role of Cbfβ in postnatal skeletogenesis and pathogenesis of CCD, which may assist in developing new therapies for CCD and osteoporosis.

Cbfα deletion in mice recapitulates cleidocranial dysplasia and reveals multiple functions of Cbfβ required for skeletal development

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Cbfβ/βOsx-Cre mice have decreased bone mineralization and skeletal deformities, resulting in a CCD-like phenotype. (A) Photographic analysis of 3-wk-old Cbfβ/βOsx-Cre (ff/ff) mice and WT (ff) mice. (B‒D) X-ray analysis of (B) femurs, (C) mandibles, and (D) clavicles. Yellow arrows in C and D indicate that Cbfβ/βOsx-Cre mice have a severe anterior open bite and mandibular retrognathism as well as hypoplastic/aplastic clavicles, respectively. (E and F) The μCT scans (E) and quantification (F) show that bones from Cbfβ/βOsx-Cre mice are smaller and less mineralized than those of WT. (G and H) Photographic analysis (G) and high magnification (H) of incisor tooth development. Yellow arrows indicate normal (left mouse), stunted (middle mouse), or abnormal (right mouse) tooth development. (I) High-magnification analysis of molar tooth development. (J) Photographic analysis shows defective, supernumerary-like teeth (red arrow) in Cbfβ/βOsx-Cre mice compared with WT. (K) All mice were genotyped by PCR from tail snip DNA. Reduced ossification (Fig. 1B). Cbfβ/βOsx-Cre mice also exhibited underdeveloped mandibles and mandibular retrognathism, resulting in a large gap inside the oral cavity and causing an anterior open bite, and severely affected teeth (Fig. 1C). X-ray analysis revealed hypoplasia/aplasia of clavicles and underdeveloped long bones, leading to severe deformities in the mutant mice (Fig. 1D). These skeletal defects persisted in 3- and 10-wk-old Cbfβ/βOsx-Cre mice (Fig. S1). Classical CCD is caused by RUNX2 haploinsufficiency. Our results showed that there was a milder CCD phenotype in Cbfβ cKO heterozygous mice and a more severe CCD phenotype in Cbfβ cKO homozygous mice (Fig. S1E). Microcomputed tomography (μCT) scan of femurs revealed a drastic decrease in bone density and an almost complete lack of trabecular bone in the mutant mice (Fig. 1E and F). Moreover, incisors were completely absent (Fig. 1G, middle mouse) or severely underdeveloped in 17-d-old Cbfβ/βOsx-Cre mice (Fig. 1G, right mouse). Higher magnification further illustrated the underdeveloped incisors (Fig. 1H) and underdeveloped molars in Cbfβ/βOsx-Cre mice (Fig. 1I). The teeth defects were still apparent in 10-d-old mutant mice, which also exhibited a supernumerary teeth-like phenotype, a clinical feature of CCD (Fig. 1J). The genotypes of the mice were confirmed by PCR (Fig. 1K) from tail snip DNA. These results showed that Cbfβ ablation in osteoblast precursors results in many clinical features of CCD, including short stature, hypoplastic/aplastic clavicles, and dental anomalies.

Cbfβ/βOsx-Cre mice were severely underdeveloped (Fig. 2A). However, some bones showed greater defects than other bones. The skull, calveria, and mandible were not only under-calciﬁed in the mutant mice, but also displayed patent fontanelles (Fig. 2B). Moreover, the forelimbs (Fig. 2C) and clavicles (Fig. 2D) were severely affected in the mutant mice, whereas, again, the vertebrae were largely unaffected (Fig. 2E). The mandibula, hyoid bone, thyroid cartilage, and cricoid cartilage in the newborn Cbfβ/βOsx-Cre mice were also underdeveloped due to decreased ossification (Fig. S2). Further, the underdevelopment of the hyoid bone was still apparent in 6-d-old Cbfβ/βOsx-Cre mice. Overall, the skeleton of the mutant mice was severely underdeveloped compared with normal littermates. The data suggest that the process of bone ossification was delayed in mutant mice. Notably, Cbfβ/βOsx-Cre mice had patent fontanelles, another common characteristic of CCD.

Cbfβ/βOsx-Cre newborn Tibiae Have Impaired Endochondral and Intramembranous Bone Ossification, and Goldner’s Trichrome Staining Revealed a Decrease in Osteoblasts Numbers Throughout Histomorphometric Analysis. To further analyze the growth retardation observed in the Cbfβ/βOsx-Cre mice, we performed hematoxylin/eosin (H&E), Von Kossa, Alcian blue, and Safranin O staining on tibia from newborn mice. Newborn Cbfβ/βOsx-Cre mice had decreased ossification and endochondral bone formation (Fig. 3A and B). Newborn Cbfβ/βOsx-Cre mice had decreased cartilage and underdeveloped growth zones, which may partially stem from the reduction in chondrocytes of the hypertrophic region of the growth plate in mutant mice (Fig. 3A–C). Furthermore, we found that the columns of proliferating and hypertrophic chondrocytes were less organized in Cbfβ/βOsx-Cre mice (Fig. 3C). We also found a decrease in intramembranous bone formation in Cbfβ/βOsx-Cre mice as assessed by H&E staining (Fig. 3D). Finally, Von Kossa and Fast red staining revealed a substantial decrease in calcium and a significantly shorter medullary cavity in 10-wk-old Cbfβ/βOsx-Cre mice compared with control mice (Fig. 3E). Collectively, these results demonstrate that Cbfβ/βOsx-Cre mice survive to adulthood and recapitulate the clinical features of CCD. Goldner’s Trichrome
staining revealed a decrease in bone density in Cbfβ<sup>f/f</sup>Osx-Cre (Fig. 3F) and Cbfβ<sup>f/f</sup>Col1α1-Cre mice (Fig. S3B). Notably, there was a significant decrease in bone volume and fewer osteoblasts in the Cbfβ<sup>f/f</sup>Osx-Cre mice (Fig. 3G), but the number of osteoclasts was not significantly affected (Fig. 3G). The tartrate-resistant acid phosphatase staining confirmed that the number of osteoclasts was not significantly affected in Cbfβ<sup>f/f</sup>Osx-Cre mice (Fig. S3A). Goldner’s Trichrome staining also showed that there was a significant decrease in bone volume and fewer osteoblasts (Fig. S3B), but the number of osteoclasts was not significantly affected in Cbfβ<sup>f/f</sup>Col1α1-Cre tibia sections (Fig. S3B and C).

**Cbfβ<sup>f/f</sup>Col1α1-Cre Mice Have Decreased Bone Mineralization and Skeletal Deformities but No Radical Deformities in Teeth, Mandibles, or Cartilage.** Because Cbfβ<sup>f/f</sup>Osx-Cre mice delete the Cbfβ gene in both osteoblasts and hypertrophic chondrocytes, we generated Cbfβ<sup>f/f</sup>Col1α1-Cre mice to observe the effect of Cbfβ specifically in the osteoblast lineage during postnatal bone development (Fig. 4). Similar to the Cbfβ<sup>f/f</sup>Osx-Cre mice (Fig. 1), bone formation was severely inhibited in Cbfβ<sup>f/f</sup>Col1α1-Cre mice, leading to shorter stature and decreased bone density compared with WT mice (Fig. 4A–C). Unlike Cbfβ<sup>f/f</sup>Osx-cre mice (Fig. 1), the clavicle (Fig. 4B), mandibles and teeth (Fig. 4D), and intramembranous bone formation (Fig. 4E) were not dramatically affected in Cbfβ<sup>f/f</sup>Col1α1-Cre mice. Safranin O staining revealed that there is a shortened growth plate, a decreased and disorganized proliferative zone, and a lack of hypertrophic chondrocytes in Cbfβ<sup>f/f</sup>Osx-Cre mutant mice (Fig. 4F). However, the growth plate development in Cbfβ<sup>f/f</sup>Col1α1-Cre mutant mice is normal compared with the WT mice control (Fig. 4G).

**Cbfβ Deficiency Affects Chondrocyte Proliferation and Maturation in Cbfβ<sup>f/f</sup>Osx-Cre Mice.** Further examination of the impact of the Cbfβ deletion in chondrocytes through proliferating cell nuclear antigen (PCNA) staining revealed that there is a reduction in proliferative chondrocytes in both the resting and proliferation zones of the Cbfβ<sup>f/f</sup>Osx-Cre but not in the Cbfβ<sup>f/f</sup>Col1α1-Cre mice (Fig. S4A). This is due to the fact that Cbfβ<sup>f/f</sup>Osx-Cre mice excise the Cbfβ gene in odontoblasts, osteoblasts, and chondrocytes, whereas Cbfβ<sup>f/f</sup>Col1α1-Cre mice only delete Cbfβ in the osteoblast lineage. Importantly, this finding indicates that Cbfβ is required for chondrocyte differentiation and the subsequent

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**Fig. 3.** Cbfβ<sup>f/f</sup>Osx-Cre newborn tibiae have impaired endochondral bone osification, and Goldner’s Trichrome stain revealed a decrease in osteoblast numbers. (A–C) H&E staining (A), Von Kossa and Alcian blue staining (B), and Safranin O staining (C) of tibia from newborn Cbfβ<sup>f/f</sup>Osx-Cre (ff/∆) and WT (ff) mice. Yellow arrows in A and C indicate that Cbfβ<sup>f/f</sup>Osx-Cre mice have a reduced bone collar and fewer hypertrophic chondrocytes in the growth plate, respectively. (D) H&E staining shows defective intramembranous bone formation in newborn mutant mice compared with WT. (E) Von Kossa staining of tibia from 10-wk-old Cbfβ<sup>f/f</sup>Osx-Cre and WT bones. (F) Goldner’s Trichrome stain of hard-tissue sections of tibia from 3-wk-old Cbfβ<sup>f/f</sup>Osx-Cre (ff/∆) and WT (ff) mice. For histological detail of trabeculae, the bottom row shows a higher magnification of areas in yellow boxes. (G) Quantification of the data shown in F.
and Cbfβ of newborn mice). Immunostaining analysis revealed that Runx2 expression (Fig. 5B) of prehypertrophic chondrocytes (Fig. S4A) and Runx3 expression was mainly detected in hypertrophic zone, and Runx3 expression was mainly detected in hypertrophic and prehypertrophic chondrocytes (Fig. S4 A–C). Interestingly, expression of Runx1, Runx2, and Runx3 showed no variation between WT and mutant mice (Fig. S4 A–C). Immunostaining analysis of newborn mice showed a drastic reduction in the expression of Collagen X (ColX), which is a marker of hypertrophic chondrocytes, in the growth plates of Cbfββf/fOsx-Cre mice (Fig. 5C). These results demonstrate that chondrocyte proliferation and maturation are dependent on Cbfβ’s function.

**Cbfβ Deficiency in Primary Calvarial Cells Cultured from Cbfββf/fOsx-Cre and Cbfββf/fCol1α1-Cre Mice Inhibits Osteoblastogenesis.** We investigated the impact of Cbfβ deletion on osteoblastogenesis. Calvarial cells from Cbfββf/fOsx-Cre (Fig. 6A) and Cbfββf/fCol1α1-Cre mice (Fig. S5A) after 14 d of culture showed reduced alkaline phosphatase (ALP), indicating a decreased number of osteoblasts in the mutant cells. The reduction in mineralization observed in Cbfββf/fOsx-Cre was characterized by Von Kossa staining after 21 d of culture (Fig. 6A). GeneChip analysis indicated that Atf4 mRNA expression in mouse C57/BL6 WT calvarial cells on day 7 and day 21 of osteoblastogenesis is similar to that of secreted phosphoprotein 1 (Spp1), ALP liver/bone/kidney (Alpl), and Col1a1, whereas osteocalcin (OCN) has mRNA expression levels that were much higher (Fig. 6B). Further microarray data analysis revealed that Atf4 mRNA expression in mouse calvarial cells increased more than 10-fold on culture day 21 (Fig. 6B). The dramatic changes in Atf4 mRNA expression were confirmed by quantitative RT-PCR using Cbfββf/fOsx-Cre, Cbfββf/fCol1α1-Cre, and WT mouse calvarial cells during osteoblastogenesis (Fig. 6C, Fig. S5 B and C, and Fig. S6). Western blot was used for Cbfββf/fCol1α1-Cre and WT mouse calvarial cells cultured from Cbfββf/fOsx-Cre and Cbfββf/fCol1α1-Cre mice to investigate the impact of Cbfβ deletion on osteoblastogenesis. (A) Calvarial cells from Cbfββf/fOsx-Cre (ff/Δ) and WT (ff) newborn mice were applied to osteoblastogenesis assays. (B) GeneChip analysis of the expression of Atf4, Ocn, Spp1, Alpl, and Col1a1 in mouse calvarial cells. (C) qPCR analysis of the mRNA expression level of Atf4 in mouse calvarial cells after culturing in the osteoblast differentiation media. (D) Protein expression levels were analyzed by Western blot analysis. (E) qPCR analysis of mRNA expression levels of Col1a1, Spp1, Runx2, Sox9, OPG, RANKL, and OCN in calvaria-derived osteoblasts from Cbfββf/fOsx-Cre (ff/Δ) and WT (ff) mice. Results are expressed as mean ± SD, n ≥ 6 in each group. *P < 0.05, **P < 0.01, ***P < 0.005.

*Fig. 5.* Cbfβ deficiency decreases chondrocyte proliferation, reduces expression of Cbfβ and Osterix, and impairs chondrocyte hypertrophy in Cbfββf/fOsx-Cre mice. (A and B) PCNA staining for cellular proliferation (A) and immunohistochemistry (IHC) staining with anti-Cbfβ, anti-Runx2, and anti-Osterix antibodies (B) of tibial paraffin sections from 4-wk-old Cbfββf/fOsx-Cre (ff/Δ), Cbfββf/fCol1α1-Cre (ff/Δ), and WT (ff) mice. (Insets) The magnified images of the red boxed areas, which show a decrease in chondrocyte proliferation in Cbfββf/fOsx-Cre mice and reduced expression of Cbfβ and Osterix in both mutant mice compared with WT. (C) Immunofluorescence staining of the tibia from newborn Cbfββf/fOsx-Cre (ff/Δ) and WT (ff) mice.

*Fig. 6.* Cbfβ deficiency in primary calvarial cells cultured from Cbfββf/fOsx-Cre and Cbfββf/fCol1α1-Cre mice inhibits osteoblastogenesis. (A) Calvarial cells from Cbfββf/fOsx-Cre (ff/Δ) and WT (ff) newborn mice were applied to osteoblastogenesis assays. (B) GeneChip analysis of the expression of Atf4, Ocn, Spp1, Alpl, and Col1a1 in mouse calvarial cells. (C) qPCR analysis of the mRNA expression level of Atf4 in mouse calvarial cells after culturing in the osteoblast differentiation media. (D) Protein expression levels were analyzed by Western blot analysis. (E) qPCR analysis of mRNA expression levels of Col1a1, Spp1, Runx2, Sox9, OPG, RANKL, and OCN in calvaria-derived osteoblasts from Cbfββf/fOsx-Cre (ff/Δ) and WT (ff) mice. Results are expressed as mean ± SD, n ≥ 6 in each group. *P < 0.05, **P < 0.01, ***P < 0.005.
to analyze the expression of several key factors that influence osteoblast functions in Cbfβ\(^{-}\)/Osx-Cre and Cbfβ\(^{-}\)/Col1a1-Cre mice. Cbfβ deficiency reduced the expression of Cbfβ, Osx, Col1a1, and Atf4, but not Runx2 (Fig. 6D). In contrast, the expression of SRY (sex determining region Y)-box 9 (Sox9) was increased in Cbfβ\(^{-}\)-deficient calvaria-derived osteoblasts (Fig. 6D). Taken together, these results demonstrate that Cbfβ deletion impacts chondrocyte and osteoblast differentiation by affecting the expression of critical downstream genes at the mRNA level. Notably, on day 14, Cbfβ knockdown reduced the expression of critical downstream genes at the mRNA level. Notably, on day 14, Cbfβ knockdown reduced the expression of Cbfβ and Sox9, indicating that Runx binding sites 13–15 in the promoter region (Fig. S7). The promoter luciferase assay showed that luciferase activity driven by Atf4 promoters was very low in the absence of Cbfβ (Fig. S8). Luciferase activity was highest when driven by the longest Atf4 promoter fragment (−4051/+80) and less high when driven by the Atf4 promoter fragment (−3109/+80 and −1676/+80) in WT cells, but not in the mutant cells. Interestingly, about 70% of the full Luciferase activity driven by the longest Atf4 promoter fragment (−4051/+80) still remains with the Atf4 promoter fragment (−500/+80) in WT cells, but not in the mutant cells. This indicates that the Atf4 promoter region (−500/+80) containing Runx binding sites 5–7 is critical for Cbfβ regulation of Atf4 gene expression (Figs. S7 and S8). Consistently, the primer 3a amplifying regions near Runx binding sites 5–7 in the Atf4 promoter gave out the highest value in ChIP assays (Fig. 7C), indicating that the Cbfβ/RUNX complex may bind Runx binding sites 5–7 to up-regulate Atf4 gene expression. There was also a decrease in luciferase expression driven by the Runx2 promoter (−1580/+80 and −900/+80) in the absence of Cbfβ. However, Runx2 expression does not vary between WT and mutant cells (Fig. 7D and Fig. S6A). This indicates that there may be some other mechanism driving the steady expression of Runx2 in Cbfβ knockout mice and cells, probably working on a distant end of the Runx2 promoter (before −1580). In conclusion, we believe that Cbfβ directly associates with the Osx and Atf4 promoter regions and regulates their expression. To confirm these Western and real-time RT-PCR results in calvarial culture experiments, we used protein directly isolated from calvaria (Fig. 7D and Fig. S6). The Western blot result is consistent with that from the calvarial culture experiment result (Fig. 6D and Fig. 7D).

**Discussion**

**CCD May Result from a Functional Defect of the Runx2/Cbfβ Heterodimeric Complex in Various Cell Types.** Our data show that Cbfβ\(^{-}\)/Osx-Cre mice provide a unique CCD model recapitulating most of the characteristics of human CCD (i.e., wide/open fontanels, midface retrusion, abnormal dentition, severe clavicular hypoplasia, and hand/paw abnormalities). The CCD phenotype of Cbfβ\(^{-}\)/Osx-Cre mice indicates that multiple functions of Cbfβ are required for skeletal development and/or Hoxa5 in postnatal skeletogenesis. The fact that the Runx2 protein level was not changed in Cbfβ\(^{-}\)/Osx-Cre cKO mice and that Cbfβ\(^{-}\)/Osx-Cre mice exhibit a CCD-like phenotype (Fig. 1) supports the notion that CCD may result from a functional defect of the Runx2/Cbfβ heterodimeric complex in various cell types. It also indicates that, in terms of the pathogenesis of CCD, Cbfβ deficiency may be equivalent to RUNX2 haploinsufficiency as it relates to the function of the Runx2/Cbfβ complex in skeletogenesis.

**Indispensable Role of Hypertrophic Chondrocytes in Endochondral Bone Formation.** The role of hypertrophic chondrocytes in endochondral bone formation is a long-standing question. Our results showed that there is a shortened growth plate, a decreased and disorganized proliferative zone, and a reduction in hypertrophic chondrocytes in Cbfβ\(^{-}\)/Osx-Cre mutant mice. However, growth plate development in Cbfβ\(^{-}\)/Col1a1-Cre mutant mice is normal compared with the WT control. These phenotypes of the mutant mice provided a unique opportunity to address the role of hypertrophic chondrocytes in endochondral bone formation. Our results indicate that Cbfβ maintains trabecular bone formation through its function in chondrocytes. Based on the severe endochondral bone defects in Cbfβ\(^{-}\)/Osx-Cre mutant mice (Figs. 1 and 3) and the mild endochondral bone defects in Cbfβ\(^{-}\)/Col1a1-Cre mutant mice (Fig. 4), we conclude that the role of hypertrophic chondrocytes in endochondral bone formation is indispensable.
We hypothesize that the lack of trabecular bone is not the result of a lack of osteoblast precursors and preosteoblasts but may be a result of a lack of the factor(s) (such as Ihh) secreted by prehypertrophic or hypertrophic chondrocytes, although the mechanism of this dependence remains to be identified.

*Cbfβ* Plays an Indispensable Role in Postnatal Skeletal Development and Homeostasis by Up-Regulating the Expression of *Atf4* and *Osterix*. *Cbfβ* deficiency reduced the expression of several key factors that mediate osteoblast formation and/or function (e.g., Osx and Atf4). This suggests that *Cbfβ* may have a role in promoting the commitment of osteoblast precursors into the osteoblast lineage. Yang et al. reported that *Atf4* is a critical regulator of osteoblast differentiation and function (15). However, *Atf4* is regulated at the transcriptional regulation level remains unclear. Our study shows that *Cbfβ* associates with the promoter regions of *Osx* and *Atf4* and highly up-regulates the expression of *Atf4* at the transcriptional regulation level as shown by ChIP assay, microarray analysis, quantitative PCR (qPCR) analysis, and promoter reporter assay. We also found that *Cbfβ* is crucial for the later stages of chondrocyte differentiation as its deletion affects chondrocyte maturation and the formation of the growth plate.

The Clinical Features of *Ccd* Are Recapitulated in *Cbfβ*<sup>−/−</sup>*Osx-Cre* Mice. Because Runx2 functions as a heterodimer with *Cbfβ*, it has been suspected that *Cbfβ* may be responsible for some cases of *Ccd*. Although no *Cbfβ* mutation has yet been identified in classical *Ccd* patients, our *Cbfβ*<sup>−/−</sup>*Osx-Cre* mouse models support the notion that search genetic alterations in the *Cbfβ* gene may be responsible for *Ccd* in those patients with no *Runx2* mutation. Our results are in agreement with that of previous studies reporting that Runx2 deficiency causes an arrest in tooth development (16) and that Osx is necessary for odontoblast differentiation (13). These findings provide great insight into the pathogenesis of *Ccd* and the role of *Cbfβ* in both postnatal skeletal and tooth development. The insights resulting from this study may assist in the development of novel treatments for *Ccd* and other bone diseases.

Materials and Methods

Animal Experimentation and Generation of *Cbfβ* cKO Mice. *Cbfβ*<sup>−/−</sup> mice (Jackson Laboratory, strain name B6.129P2-Cbfβ<sup>−/−<sup>dataref></sup></sup>mutai/j)) were crossed with skeletal tissue cell (including osteoblast precursors, osteoblasts, chondrocytes, and odontoblasts)-specific *Osx*<sup>-/-</sup> mice (12) [TgSp7-TTA,terOE-EGFPre1/Amc, Mouse Genome Informatics] or osteoblast-specific *Col1α1-Cre* mice. Their progeny were crossed with *Cbfβ*<sup>−/−</sup> mice to obtain *Cbfβ*<sup>−/−</sup>*Col1α1-Cre* mice or *Cbfβ*<sup>−/−</sup>*Osx-Cre* mice. In our study, we only use one copy of *Osx-Cre* (*Cbfβ*<sup>−/−</sup>*Osx-Cre*) in the *Cko* mutation. We used *Cbfβ*<sup>−/−</sup>*mice and *Osx-Cre*<sup>-/-</sup> mice as controls. Mouse CS/BL6 WT calvarial cells were also used. All research procedures using mice were approved by the University of Alabama at Birmingham (UAB) Animal Care and Use Committee and conformed to the National Institutes of Health guidelines.

Skeletal Analysis, Tissue Preparation, and Histology Stains. Histomorphometric samples were processed as nondecalciﬁed hard-tissue sections. Bone parameters were quantitated via 6 µm sections obtained from 3-wk-old mice. For paraffin sections, samples were decalcified and dehydrated in ethanol, cleared in xylene, embedded in paraffin, and sectioned at 6 µm with Leica microtome and mounted on SuperFrost Plus slides (Fisher). Histological analysis was performed including staining with Alcian blue, safranin O, and H&E using paraffin sections.

ChIP and Promoter Analyses. Cells were derived from calvaria of newborn WT mice and mutant mice. Osx, Runx2, and *Atf4* promoter sequences were analyzed for putative Runx binding sites with PROMO3.0 (http://alggen.lsi.upc.es) using version 8.3 of the TRANSFAC database. *Cbfβ* was performed using monoclonal anti-Cbfβ antibody (sc-20693X) and DNA extraction, and qPCR was performed. The Osx, Runx2, and *Atf4* promoters were ampliﬁed using PCR from BAC clones provided by the BACPAC Resource Center at Children’s Hospital Oakland Research Institute. These ampliﬁed fragments were cleaved and ligated into the pGL3 vector from Promega. The calvarial cells were cotransfected with each construct with the amount of 100 ng per well as well as pSv–β-galactosidase construct with the amount of 50 ng per well (Promega) and incubated for 6–8 h. The culture medium was replaced with osteogenic medium and cultured for 2 d. Luciferase activity was measured using a Steady-Glo luciferase assay system (Promega cat. no. E2510). pSV–β-galactosidase activity was measured using a β-galactosidase Enzyme Assay system (Promega cat. no. E2000).

Statistical Analysis. All data are presented as the mean ± SD (n ≥ 6). The significance was assessed using Student t test. P values < 0.05 were considered significant. Data are expressed as mean ± SD, n ≥ 2, *P* < 0.05, **P** < 0.01, ***P*** < 0.001. The results are representative of at least four individual experiments. The analyses of the data were performed with the SPSS 16.0 software (SPSS Incorporation). Please see SI Materials and Methods for additional details.

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Supporting Information

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SI Materials and Methods

CbfβCreOssCre+/Generation. We only use one copy of Oss-Cre (CbfβCreOssCre+/ OssCre+/+) in the conditional knockout (cKO) mutation phenotype analysis. CbfβCreOssCre/+ mice were generated by crossing CbfβCre mice and CbfβCre−/− mice with OssCre/+ and CbfβCre−/− mice as the control. We did not find any delayed cortical bone growth or any other skeletal defect in the control mice.

Radiographic Procedures. For X-ray analysis, radiography was performed using the Faxitron Model MX-20 at 26 kV by the University of Alabama at Birmingham (UAB) Small Animal Bone Phenotyping Core associated with the Center for Metabolic Bone Disease. The microcomputed tomography analysis was performed to determine the bone mass of fixed femurs by the UAB Small Animal Bone Phenotyping Core associated with the Center for Metabolic Bone Disease.

Histomorphometric Analysis. Histomorphometric samples were processed as nondecalcified hard-tissue sections as described (1). Bone parameters were quantified via 6 μm sections obtained from 3-wk-old mice.

Skeletal Analysis. For skeletal preparations, mice were skinned, eviscerated, fixed in 95% (vol/vol) ethanol, cleared in acetone, stained with Alizarin red and/or Alcian blue stains, and sequentially cleared in 1% KOH. Cartilage and mineralized bone were characterized by different colors (blue and red, respectively) after the stain, according to standard protocols (2).

Tissue Preparation and Histology Stains. Femurs and tibiae of mice were harvested, skinned, and fixed in 4% (wt/vol) paraformaldehyde overnight. Samples were then dehydrated in ethanol solution and decalcified in 10% (wt/vol) EDTA for 1–4 wk. For paraffin sections, samples were dehydrated in ethanol, cleared in xylene, embedded in paraffin, and sectioned at 6 μm with Leica microtome and mounted on Superfrost Plus slides (Fisher).

For frozen sections, samples were infiltrated in 30% (wt/vol) sucrose, embedded in optimal cutting temperature compound, sectioned at 8 μm with a freezing microtome, and affixed to Superfrost Plus Gold slides (Fisher). Histological analysis was performed including staining with Alcian blue, safranin O, hematoxylin/eosin (H&E) (3), and Goldner’s trichrome stains (4) using paraffin sections.

TRAP Staining. Paraffin sections were stained using the Acid Phosphatase, Leukocyte [tartrate-resistant acid phosphatase (TRAP)] Kit (387A-1KT, Sigma) following the manufacturer’s instructions, counterstained with hematoxylin, dehydrated, and mounted. Data are included as graphs of osteoclasts per millimeter of bone perimeter.

Von Kossa Staining. Von Kossa staining was performed as follows. Cells were washed with Ca2+/Mg2+-free PBS and then fixed on slides in 10% (vol/vol) cold Neutral Formalin solution. We then added 2.5% (wt/vol) silver nitrate solution, and the slides were incubated under UV light for 5–10 min. After incubation, the unincorporated silver nitrate was removed by washing with 5% (wt/vol) sodium thiosulfate.

Proliferation Assay. To detect proliferating cells in culture, immunocytochemistry staining was performed according to the manufacturer’s instructions. Horseradish peroxidase-conjugated proliferating cell nuclear antigen (PCNA) antibodies (cat. no. 93–1143; Zymed Laboratories Inc.) and Vector DAB substrate (3,3′-diaminobenzidine) kits (cat. no. SK-4100; Vector Laboratories) were used.

Immunohistochemistry. For immunohistochemistry, samples were embedded in paraffin and sectioned as described previously. The Vector DAB substrate kit (cat. no. SK-4100; Vector Laboratories) was used along with secondary staining kits for mouse (on mouse) and rabbit (cat. no. BMK-2202 and PK-6101, respectively; Vector Laboratories) and primary antibodies for Osterix (ab22552; Abcam), Runx-related transcription factor 2 (RUNX2) (ab23981; Abcam), and Cbfβ (sc-56751; Santa Cruz).

Immunofluorescence. Samples were embedded in tissue freezing medium, and sections were cut at a thickness of 8 μm using a cryotome. Pictures were taken by Leica confocal microscopes (SP1) and a Zeiss fluorescence microscope (Zeiss Axiom Imager). The following antibodies were used: rabbit anti- Col11 (1:200; ab58632, Abcam), anti-Runx1 (ab35962, Abcam), Runm2 (pc-287, Merck/Millipore), and Runm3 (sc-30197, Santa Cruz).

Primary Cell Culture. Calvarial cells were isolated from newborn mice and seeded in cell culture dishes at a density of 3 × 10^5 cells/cm^2 as previously described (5). After growing to confluence, cells were induced to differentiate into osteoblasts using osteogenic medium and BGGb medium (Gibco, 12591) supplemented with 10% (vol/vol) FBS, 50 μg/mL L-ascorbic acid (Sigma, A4544), and 5 mM β-glycerolphosphate (Sigma, G9891). Osteoblastogenesis was analyzed by alkaline phosphatase (ALP) staining according to the manufacturer’s manual (sigma, A2356) on day 14. Osteoblast mineralization was examined by Von Kossa staining on day 21.

GeneChip Analysis. We analyzed independently prepared duplicate samples containing primary osteoblasts derived from the calvarial bone of C57/BL6 wild-type (WT) mice as previously described (3). TRZol reagent (Life Technologies) was used according to the manufacturer’s protocol to extract total RNA from C57/BL6 WT calvarial cells after culturing for 7 or 21 d. RNA profiling was performed by using the Affymetrix mouse 430 Plus 2.0 array as described (6). Replicate samples were averaged for reporting.

Promoter Analyses. To determine 5′-flanking regulatory activity, calvarial cells were cultured for 5 d in osteogenic medium, reseeded on 96-well plates, and transfected with constructs (Fig. S8) using Fugene6 reagent (Roche) as described (4). The calvarial cells were cotransfected with each construct (Fig. S8) using Fugene6 reagent (Roche) as described (4). The construct samples were cotransfected with each construct (Fig. S8) using Fugene6 reagent (Roche) as described (4). The luciferase activity in transfected cultures was standardized using a Steady-Glo luciferase assay system (Promega). PGL3 basic reporter vectors were used as controls (Promega). Luminescence was detected with a luminometer (BioTek Synergy 2). The luciferase activity in transfected cultures was standardized by normalization to β-galactosidase activity, and the protein concentration of cell extracts was determined using a protein assay kit (BioRad) as described (4, 7). In all experiments, constructs are tested in triplicate.

Quantitative Real-Time PCR Analysis. mRNA was extracted from cultured cells on days 7, 14, and 21 using TRIZol (Invitrogen) and then reverse-transcribed into cDNA according to the manu-
factor's manual (qScript cDNA Synthesis Kit, Quanta Bio-

Sciences Inc.). Expressions of osteoblastic marker genes were analyzed by quantitative real-time PCR (qRT-PCR) using the StepOne Real-Time PCR System (Life Technologies). Expression of Atf4, Col1α1, Spp1, Runx2, Sox9, OPG, RANKL, and OCN was analyzed. The primer sequences were available upon request.

Western Blot Analyses. Protein samples extracted from calvaria-derived osteoblasts were prepared in protein lysis buffer, resolved on SDS/PAGE, and electrotransferred onto nitrocellulose membranes. Immunoblotting was performed according to the manufacturer's instructions. Osteoblast- and chondrocyte-related regulators and marker genes including Cbfβ, Runx2, Col1α1, Osterix, ATF4, and Sox9 were detected using primary antibodies as follows: rabbit anti-Cbfβ (1:1,000; ab72696, Abcam), rabbit anti-Runx2 (1:2,000; ab23981, Abcam), rabbit anti-Col1α1 (1:1,000; ab34710, Abcam), rabbit anti-ATF4/Osterix (1:1,000; ab22552, Abcam), mouse anti-Atf4 (1:1,000; ab50546, Abcam), rabbit anti-sox9 (H-90) (1:1,000; sc-20095, Santa Cruz Biotechnology), and mouse anti-β-tubulin (1:100, cell lysates). Horseradish peroxidase-linked anti-rabbit IgG and horseradish peroxidase-linked anti-mouse IgG were purchased from Cell Signaling (nos. 7074 and 7076).

Chromatin Immunoprecipitation. Cells were derived from calvaria of newborn WT mice and mutant mice as described (8). Osx, Runx2, and Atf4 promoter sequences were analyzed for putative Runx binding sites with PROMO3.0 (http://alggen.lsi.upc.es/)


Fig. S1. Adult Cbfβff and Cbfβff Osx-Cre mice have decreased bone mineralization similar to Runx2+/− mice and severely defective clavicles. (A–C) X-ray analysis of the whole body (A), femurs (B), and clavicles (C) of 10-wk-old Cbfβff Osx-Cre (ff/Δ) and WT (ff) mice. Data showed severe defects in bone mineralization and skeletal development in the mutant mice compared with their WT littermates. Also, the clavicles of the mutant mice were drastically shorter. Arrows in A show that the calvaria and vertebral column were less calcified in the mutant mice compared with WT mice. Arrows in C show hypoplastic/aplastic clavicles in the mutant mice compared with WT mice. (D) X-ray analysis of 4-wk-old Runx2-heterozygous mutant (+/−) and WT (+/+) mice. There was decreased bone mineralization in the mutant mice compared with their WT cohorts. Arrows show that the calvaria and femurs were less calcified in the mutant mice and the mandibles were underdeveloped in the mutant mice. (E) X-ray analysis of 3-wk-old Cbfβff/f+(f/+, Cbfβff Osx-Cre (f/+Δ), and Cbfβff Osx-Cre (ff/Δ). There was a milder cleidocranial dysplasia (CCD) phenotype in Cbfβff Osx-Cre mice and a more severe CCD phenotype in Cbfβff Osx-Cre mice.
**Fig. S2.** *Cbfβ*<sup>*</sup><sup>*</sup>*Osx-Cre* mice have undercalcified mandible, hyoid bones, and thyroid cartilage. (A) Mandibula, hyoid bone, thyroid cartilage, and cricoid cartilage of *Cbfβ*<sup>*</sup><sup>*</sup>*Osx-Cre* (ff/Δ) and WT (ff) mice were stained by Alizarin red and Alcian blue staining at the newborn stage. (B) The hyoid bone of *Cbfβ*<sup>*</sup><sup>*</sup>*Osx-Cre* and WT mice were stained by Alizarin red and Alcian blue staining at 6 d of age. Arrows show decreased mineralization of the hyoid bone in the mutant mice compared with WT mice. *Cbfβ*<sup>*</sup><sup>*</sup>*Osx-Cre* mice had shorter mandibula and hyoid bones. Furthermore, the hyoid bone and thyroid cartilage of the mutant mice had reduced mineralization. These findings indicate that ablation of *Cbfβ* in the osteoblast precursors leads to defective postnatal bone development.

**Fig. S3.** TRAP staining of *Cbfβ*<sup>*</sup><sup>*</sup>*Osx-Cre*. (A) TRAP stain of femur plastic sections of newborn *Cbfβ*<sup>*</sup><sup>*</sup>*Osx-Cre* and WT (ff) mice. Osteoclast number is similar between WT and mutant mice. (B) Goldner’s Trichrome staining of tibial plastic sections of 4-wk-old *Cbfβ*<sup>*</sup><sup>*</sup>*Col1a1-Cre* (ff/Δ) and WT (ff) mice. For histological detail of trabeculae, the bottom row shows a higher magnification (yellow boxes). (C) Histomorphometric analysis and quantification of the data shown in A. *Cbfβ*<sup>*</sup>*Col1a1-Cre* mice had a significant reduction in trabecular bone and osteoblast numbers but not in osteoclast numbers. Data are expressed as mean ± SD, n ≥ 6, *P* < 0.05.
Fig. S4. Expression of Runx1, Runx2, and Runx3 is unchanged in chondrocytes of Cbfβ<sup>fl/fl</sup> Osx-Cre mice relative to WT mice. (A–C) Immunofluorescent staining for Runx1 (A), Runx2 (B), and Runx3 (C) on frozen sections of femur growth plates from newborn Cbfβ<sup>fl/fl</sup> Osx-Cre and WT mice. Bright field views were copresented. Hyp, hypertrophic zone; Pre, prehypertrophic zone; Prol, proliferating zone; R, resting zone; T, trabecular bone.
Primary calvarial cells cultured from Cbfβ^{ff} Col1α1-Cre mice have impaired osteoblastogenesis. (A) Calvarial cells from WT (ff) or Cbfβ^{ff} Col1α1-Cre (ff/Δ) newborn mice were submitted to osteoblastogenesis assays. Osteoblastogenesis was analyzed by ALP activity on day 14. (B and C) mRNA expression levels of Atf4, Col1α1, Spp1, Runx2, Sox9, and OCN were determined by qRT-PCR after culture in osteoblast differentiation media for 7 d (B) or 21 d (C). The results demonstrate that deletion of Cbfβ affects osteoblastogenesis in part by affecting the expression of osteoblast and chondrocyte genes. Data are normalized to the housekeeping gene, hypoxanthine-guanine phosphoribosyl transferase. Results are expressed as mean ± SD, n ≥ 6 in each group. *P < 0.05, **P < 0.01.
Fig. S6. Expression of osteoblast-related genes in calvarial cells in osteoblastogenic media conditions is attenuated in Cbfβf/f Osx-Cre– and Cbfβf/f Col1α1-Cre– derived cells relative to WT. (A) Quantification of protein expression, from Western blot (Fig. 7D), normalized to β-tubulin from the calvaria of Cbfβf/f Osx-Cre, Cbfβf/f Col1α1-Cre, and WT mice. (B) mRNA expression levels of Cbfβ, Runx1, Runx2, Runx3, Opn, Sox9, Atf4, and Osx in calvarial cells from Cbfβf/f Osx-Cre, Cbfβf/f Col1α1-Cre, and WT mice were determined by qRT-PCR after culturing in the osteoblast differentiation media for 14 d. Data were normalized to Gapdh. Results are expressed as means ± SD, n ≥ 6 in each group. *P < 0.05, **P < 0.001.

Fig. S7. Osx, Runx2, and Atf4 have predicted Runx/Cbf binding sites. Schematic display of Osx (−3000/+80) (A), Runx2 (−3000/+80) (B), and Atf4 (−4000/+80) (C) promoter regions. TSS (transcriptional start site), predicted Runx-binding sites, and ChiP primer positions (F, forward; R, reverse) are indicated in the figure.
Fig. S8. Expression driven by the Runx2 and Atf4 promoters is diminished in Cbfβf/f Osx-Cre chondrocytes. (A) Runx2 promoter fragments were inserted into the pGL3–basic vector. Calvarial cells were transfected with pGL3–control, pGL3–Runx2–1,580 bp, or pGL3–Runx2–900 bp. Luciferase was detected 48 h posttransfection. The signal was normalized to β-gal activity. (B) Atf4 promoter fragments were inserted into the pGL3–basic vector. Calvarial cells were transfected with pGL3–control, pGL3–Atf4–4,051 bp, pGL3–Atf4–3,109 bp, pGL3–ATF4–1,676 bp, or pGL3–ATF4–500 bp. β-gal expression plasmids were co-transfected as control. Luciferase was detected 48 h posttransfection. The signal was normalized to β-gal activity. Results are presented as mean ± SD, n ≥ 6 in each group. *P < 0.05, **P < 0.01, ***P < 0.005.