Foxp2 regulates anatomical features that may be relevant for vocal behaviors and bipedal locomotion

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Fundamental human traits, such as language and bipedalism, are associated with a range of anatomical adaptations in craniofacial shaping and skeletal remodeling. However, it is unclear how such morphological features arose during hominin evolution. FOXP2 is a brain-expressed transcription factor implicated in a rare disorder involving speech apraxia and language impairments. Analysis of its evolutionary history suggests that this gene may have contributed to the emergence of proficient spoken language. In the present study, through analyses of skeleton-specific knockout mice, we identified roles of *Foxp2* in skull shaping and bone remodeling. Selective ablation of *Foxp2* in cartilage disrupted pup vocalizations in a similar way to that of global *Foxp2* mutants, which may be due to pleiotropic effects on craniofacial morphogenesis. Our findings also indicate that Foxp2 helps to regulate strength and length of hind limbs and maintenance of joint cartilage and intervertebral discs, which are all anatomical features that are susceptible to adaptations for bipedal locomotion. In light of the known roles of Foxp2 in brain circuits that are important for motor skills and spoken language, we suggest that this gene may have been well placed to contribute to coevolution of neural and anatomical adaptations related to speech and bipedal locomotion.

Foxp2 | vocalization | bipedalism | cranial base | bone remodeling

Spoken language and bipedalism are two behavioral traits that distinguish humans from other living apes, each with a complex evolutionary history. The emergence of such derived traits was accompanied by various changes in skeletal anatomy. For example, as well as long-term increases in overall cranial capacity over the course of primate evolution, more recent alterations in skull shape occurred in our ancestors, changes that some hypothesize as important for language evolution (1, 2). Advances in genomics are uncovering genes of relevance for distinct human traits like language (3). In particular, disruptions of the FOXP2 transcription factor are implicated in a monogenic disorder involving childhood apraxia of speech (CAS) and expressive–receptive language impairments (4–7). The first etiological *FOXP2* mutation was identified in a family (KE) in which all affected members carried an R553H substitution within the Forkhead-box DNA-binding domain. In addition, mutations of *FOXP1*, the closest paralogue of *FOXP2*, cause a neurodevelopmental syndrome including speech and language impairments (8–11), partially overlapping with deficits associated with *FOXP2* variants in multiple different cases (12–14). The functions of Foxp2 in vocal behaviors have been assessed through analysis of ultrasonic vocalizations (USVs) in mouse models (15–20), or learned song in songbirds (21–23). Foxp2 is highly conserved across species, but underwent positive selection on the lineage that led to modern humans (24, 25). Two amino acid substitutions occurred in human *FOXP2* after splitting from our common ancestor with the chimpanzee. Investigations of these substitutions in partially humanized mice suggest they affect connectivity and plasticity of cortico-basal ganglia circuits, impacting learning mechanisms (26, 27).

Morphological correlation or covariation, a concept going as far back as Darwin's *On the Origin of Species*, is an essential driving force for evolution. The emergence of human speech involved not only neural changes, but also modifications in anatomical features of the vocal tract, including configuration of superficial vocal folds, trachea, and oral cavities. For instance, the importance of a relatively descended larynx for human speech has been a topic of much discussion (28). While multiple studies of Foxp2 have focused on neuronal functions, none have tested its potential contributions to vocal anatomical geometry. Of note, a comparison of transcriptional regulation by human and chimpanzee versions of FOXP2 reported enrichment of differential targets involved in craniofacial formation and cartilage development (29). Moreover, in a previous study, we demonstrated cooperative

Significance

Speech and bipedalism are key aspects of behavior that emerged during human evolution. *FOXP2*, a gene implicated in a human speech and language disorder, has been suggested to contribute to language evolution. Here, through knockout studies of mouse *Foxp2*, we show that this gene is not only important for neural circuits involved in vocal behaviors, it also helps regulate relevant anatomical substrates. We additionally demonstrate that *Foxp2* influences skeletal features that may be relevant for bipedal locomotion. Our findings raise the possibility that *FOXP2* might be important for anatomical features contributing to derived human traits, including speech and bipedalism.

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functions of *Foxp1/2* in regulating endochondral ossification during embryonic bone development (30).

Building on our demonstration of a Foxp2 role in embryonic bone development, and in light of prior hypothesized involvement of this gene in human evolution, we here used skeleton-specific loss-of-function analyses in mice to investigate how it might help regulate anatomy. Unexpectedly, skeletal *Foxp2* loss led to disruption of pup vocalizations, similar to phenotypes previously reported for global mutant or knockout lines (17, 31). Most interestingly, loss of *Foxp2* in skeletal tissue also led to pleiotropic deficits in skull shaping and bone strengthening. Our findings reveal regulatory roles of Foxp2 in helping build anatomic substrates that are important for vocal behaviors, and suggest that it might also be considered a candidate for skeletal adaptations relevant to bipedal locomotion.

Results

Cartilage-Specific Deletion of *Foxp2* Impairs Cranial Base Development. Cranial base morphogenesis is a major determinant of skull shaping (32). Basicranial skeletons, such as the sphenoid and basioccipital bones (Bos), are primarily formed through endochondral ossification. To test roles of Foxp2 in cranial base development, we firstly examined its expression in the synchondrosis joint—the unique growth plate sustaining endochondral ossification in sphenoid bones. We detected expression of Foxp2 protein, as well as its paralogue Foxp1, in mesenchymal progenitor cells in resting zone and/or perichondrium (white arrows in Fig. 1*A*). We then generated chondrocyte-specific *Foxp2* conditional knockout (cKO) mice by crossing a homozygous floxed line $Foxp2^{fl/fl}$ with *Col2-Cre*, which targeted cartilage in appendicular skeletons and partial craniofacial mesenchyme. We observed that craniofacial elements were consistently shortened in homozygous $Foxp2_{Col2}{}^{\Delta/\Delta}$ cKO mice compared with controls at postnatal day 10 (P10) and P30 stages (double-headed arrows in Fig. 1*B*). The skulls of $Foxp2_{Col2}{}^{\Delta/\Delta}$ mice were smaller in size than $Foxp2^{fl/fl}$ littermates at embryonic stage 15.5 days (E15.5) and P13 (Fig. 1*C*). Endochondral ossification of the Bo, the basisphenoid bone (Bs) and the nasal bone, were attenuated in $Foxp2_{Col2}{}^{\Delta/\Delta}$ mice at E15.5, as evidenced by diminished Alizarin red staining (arrows in Fig. 1*C*). In particular, broader funnel-shaped presphenoids were observed in $Foxp2_{Col2}{}^{\Delta/\Delta}$ mice at P13 (arrows in Fig. 1 *D* and *F*). Minor alterations in presphenoid morphology were also detected at P7 in $Foxp2^{R552H/+}$ mutant mice, which carry a point mutation matching that found in affected members of the KE family (Fig. 1 *E* and *G* and *SI Appendix*, Fig. S1*A*). Altered presphenoid morphology was much more evident in homozygous $Foxp2^{R552H/R552H}$ mice at P0 (arrows in Fig. 1*H* and *SI Appendix*, Fig. S1*B*). At the histological level, $Foxp2_{Col2}{}^{\Delta/\Delta}$ mice showed delayed chondrocyte hypertrophy and ossification within sphenooccipital synchondroses, as revealed by Safranin O staining and immunohistochemistry (IHC) and immunofluorescence (IF) examination using Col X, Osterix (Osx), and Foxp2 antibodies (*SI Appendix*, Figs. S1*C* and S2*A*). Collectively, these data indicate that Foxp2 is important for sphenoid development and cranial shaping.

Foxp1/2 redundantly regulate endochondral ossification during embryonic development (30). Therefore, we also examined the impact of Foxp1 on craniofacial development by generating cartilage-specific knockout mice. As observed in $Foxp2_{Col2}{}^{\Delta/\Delta}$ mice, homozygous $Foxp1_{Col2}{}^{\Delta/\Delta}$ mice had shorter nasal bones (*SI Appendix*, Fig. S2 *B* and *C*) and minor morphological deformities in their presphenoid bone (arrows in *SI Appendix*, Fig. S2 *D* and *E*), with similarities to features observed in cases of heterozygous human *FOXP1* disruption (10, 11). Then, we compared craniofacial shaping within the single ($Foxp1_{Col2}{}^{\Delta/\Delta}$ and $Foxp2_{Col2}{}^{\Delta/\Delta}$) and the double ($Foxp1/2_{Col2}{}^{\Delta/\Delta}$) cKO mice at E18.5. Shortening of nasal bones and vaulted skulls were evident in the $Foxp1/2_{Col2}{}^{\Delta/\Delta}$ double mutant compared with controls (*SI Appendix*, Fig. S2*F*, *Upper*). Defective sphenoid formation was more pronounced in $Foxp1/2_{Col2}{}^{\Delta/\Delta}$ double mutants than either single mutant or $Foxp1/2^{fl/fl}$ controls (yellow arrows in *SI Appendix*, Fig. S2*F*, *Lower*). The additive effect of double *Foxp1/2* deficiency on skull shaping was also observed in heterozygous knockout mice ($Foxp1^{fl/+}Foxp2^{fl/+}$, $Foxp1_{Col2}{}^{\Delta/+}Foxp2_{Col2}{}^{\Delta/+}$, $Foxp1_{Col2}{}^{\Delta/+}Foxp2_{Col2}{}^{\Delta/\Delta}$, and $Foxp1_{Col2}{}^{\Delta/\Delta}Foxp2_{Col2}{}^{\Delta/+}$) at P10 (*SI Appendix*, Fig. S2*G*). These results indicate that Foxp2 and Foxp1 regulate craniofacial development cooperatively.

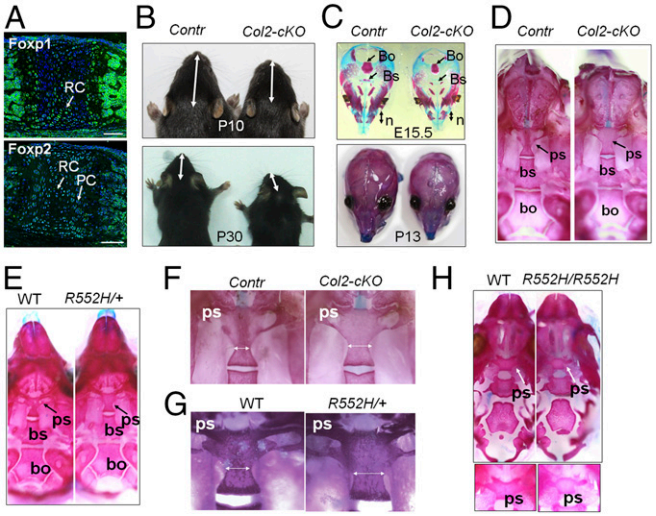

Fig. 1. Deletion of *Foxp2* in cartilage impairs craniofacial shaping. (*A*) IHC examinations detected the expression of Foxp1 and Foxp2 in different subsets of chondrocytes in intersphenoidal synchondrosis of sphenoid bones at P7. PC, proliferating chondrocytes; RC, resting chondrocytes. (Scale bar, 100 μm.) (*B*) Top view of heads of $Foxp2^{fl/fl}$ (Contr) and $Foxp2_{Col2}{}^{\Delta/\Delta}$ (*Col2-cKO*) mice at P10 and P30. (*C*) Top view of skull visualized by Alcian blue/Alizarin red staining at E15.5 and P13. (*D* and *E*) Top view of cranial bases of $Foxp2_{Col2}{}^{\Delta/\Delta}$ mice (*D*) at P13, and $Foxp2^{R552H/+}$ (*R552H/+*) mutant (*E*) at P7. (*F* and *G*) Enlarged view of presphenoid in *D* and *E*. Double arrow indicated the funnel position of presphenoid. (*H*) Altered morphology of presphenoid (arrow) in $Foxp2^{R552H/R552H}$ (*R552H/R552H*) mutant mice at P0. (*Lower*) Magnified presphenoid. Bs, basisphenoid; bo, basioccipital; ps, presphenoid.

Ablation of *Foxp2* in Cartilage Disrupts Pup USVs. Morphogenesis and elasticity of the larynx and vocal tract are rudimentary for animal sound production (33). In our observations, complete loss of *Foxp2* in $Foxp2_{Col2}{}^{\Delta/\Delta}$ cartilage tissue resulted in a minor perturbation of the morphogenesis of laryngeal thyroid and trachea cricoid cartilage, revealed by reduced Alcian blue staining in cricoid and trachea cartilage at P13 (Fig. 2*A*) and ectopic ventral expansion of the esophagus below the glottis (arrows in Fig. 2*B*). Subtle decreases in size of laryngeal cartilage were also observed in $Foxp2^{R552H/R552H}$ or $Foxp2^{R552H/+}$ mutant mice, at P0 and P7, respectively, as indicated by brackets in Fig. 2*C* and *SI Appendix*, Fig. S3. Meanwhile, development of trachea cartilage was relatively attenuated in homozygous $Foxp2^{R552H/R552H}$ mutant mice, as evidenced by Alcian blue staining (black arrows in Fig. 2 *C* and *D* and *SI Appendix*, Fig. S3).

We next examined the consequences of homozygous cartilage-specific *Foxp2* loss for mouse pup vocalizations. $Foxp2_{Col2}{}^{\Delta/\Delta}$ cKO mice at P10 were subjected to sound recording and spectrogram analyses. According to our bioacoustic analysis, $Foxp2_{Col2}{}^{\Delta/\Delta}$ pup calls were significantly perturbed compared with that of controls (Fig. 2*E*). Of note, approximately one-third of the $Foxp2_{Col2}{}^{\Delta/\Delta}$ pups presented no detectable calls. For the other two-thirds of $Foxp2_{Col2}{}^{\Delta/\Delta}$ mice, the call rate ($Z_{51} = 5.392$, $P < 0.01$) and the proportion of complex syllables, $t(34) = -3.237$, $P < 0.01$, were both significantly reduced in $Foxp2_{Col2}{}^{\Delta/\Delta}$ pups compared with

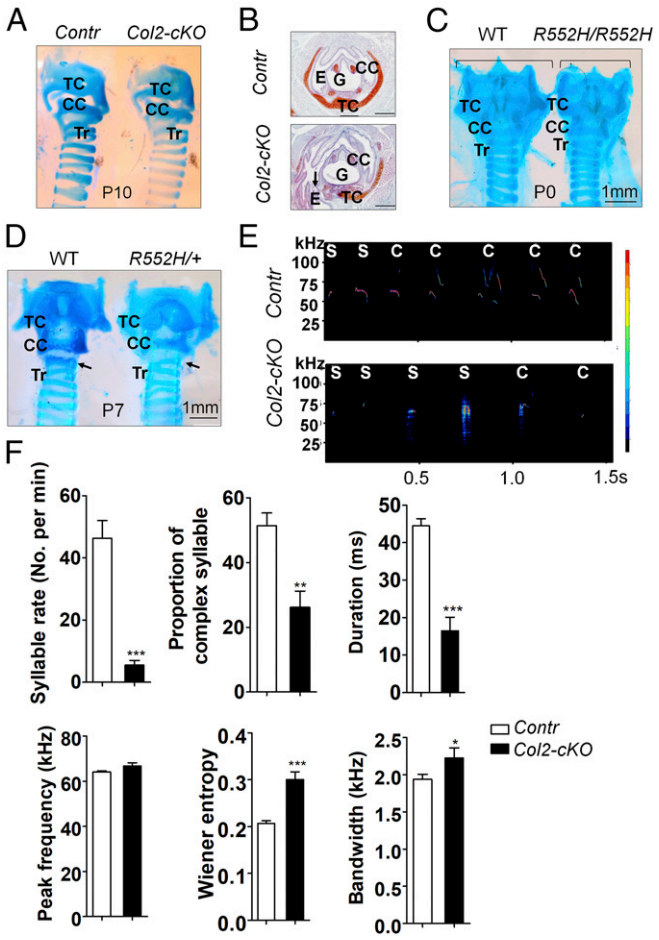

Fig. 2. Ablation of *Foxp2* in cartilage impairs USVs in pup calls. (*A*) Alcian blue staining of larynx cartilages from $Foxp2_{Col2}^{\Delta/\Delta}$ (*Col2-cKO*) mice. CC, cricoid cartilage; TC, thyroid cartilage; Tr, trachea cartilage. (*B*) Safranin O staining for the transverse sections of larynx at P10. (Scale bar, 500 μm.) E, esophagus; G, glottis. (*C* and *D*) Alcian blue staining of larynx cartilages from $Foxp2^{R552H/R552H}$ (*R552H/R552H*, *C*) at P0 and $Foxp2^{R552H/+}$ (*R552H/+*, *D*) mutant mice at P7. (*E*) Representative spectrograms of pup isolation calls in $Foxp2_{Col2}^{\Delta/\Delta}$ (*Col2-cKO*) mice at P10. The *y* axis indicates the frequency change of the USVs in the kilohertz range, whereas the *x* axis indicates time in seconds. Color depths in the sonograms represent relative intensity strength in decibels. C, complex syllable; S, simple syllable. (*F*) The sonic characteristics of pup calls, including syllable rate, proportion of complex syllables, syllable duration, peak frequency, wiener entropy, and bandwidth in $Foxp2^{fl/fl}$ (Contr) mice. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. $Foxp2^{fl/fl}$ mice, $n = 27$; $Foxp2_{Col2}^{\Delta/\Delta}$ knockouts, $n = 26$.

wild-type controls (Fig. 2*E*). Pup calls were also significantly shorter in duration, $t(42) = -3.691$, $P < 0.01$, broader in bandwidth, $t(42) = 2.093$, $P < 0.05$, and higher in entropy, $t(42) = 5.099$, $P < 0.01$, than those of controls (Fig. 2*F*). No significant differences were observed in the USVs of male and female cKO mice. Similar vocalization defects were observed in $Foxp1_{Col2}^{\Delta/\Delta}$ mice at P10 (*SI Appendix*, Fig. S4 *A* and *B*). Together these observations suggest that *Foxp2* is involved in regulating multiple aspects of vocal tract configuration, including morphological features of the trachea and larynx that are important for vocal production.

***Foxp2* Loss Perturbs Skull Integrity.** The interparietal bone is the boundary component between the parietal and occipital bones, which is considered to be a "hot spot" that is susceptible to cranial remodeling (34). Parietal and interparietal bones are formed in a process of intramembranous ossification. Foxp2 expression was detected in Osterix$^+$ skeletal progenitor cells in developing interparietal bones, as indicated by IHC examination of sections of skull from *Osx-GFP:Cre* embryos at E15.5 (arrow in *SI Appendix*, Fig. S5*A*). To investigate the contributions of *Foxp2* to skull vault development, we generated cKO mice with *Foxp2* deletion strictly in mesenchymal progenitor cells by crossing $Foxp2^{fl/fl}$ animals to a *Prx1-Cre* line. The $Foxp2_{Prx1}^{\Delta/\Delta}$ cKO mice were grossly indistinguishable from their wild-type littermates (*SI Appendix*, Fig. S5*B*), with significant deletion of *Foxp2* in mesenchymal stem cells (MSCs) from bone marrow (*SI Appendix*, Fig. S5 *C* and *D*). Loss of *Foxp2* from mesenchymal progenitors perturbed osteogenesis of interparietal bones (Fig. 3*A*), as evidenced by diminished Osx$^+$ osteoblasts at the suture (*SI Appendix*, Fig. S5*E*), and decreased expression of osteogenic genes (*Osx*, *Runx2*, *Col1a1*, and *Alp*) in mesenchymal progenitor cells (*SI Appendix*, Fig. S5*F*). Effects on lambdoid suture fusion were also observed in $Foxp2^{R552H/R552H}$ or $Foxp2^{R552H/+}$ perinatal mutant mice (Fig. 3 *B* and *C* and *SI Appendix*, Fig. S6). Attenuation in lambdoid suture closure was much more penetrant in $Foxp1/2_{Prx1}^{\Delta/\Delta}$ double knockout mice (Fig. 3*D*). Our findings suggest that Foxp2 helps to regulate posterior skull integrity, including interparietal bone development and lambdoid suture closure, by promoting osteogenic differentiation of MSCs.

Ablation of *Foxp2* Impairs Leg Gracility and Cartilage Maintenance. For appendicular long bones, postnatal elongation occurs at and depends on the growth plates, which progressively narrow down and ultimately disappear with age. Compared with control littermates, $Foxp2_{Prx1}^{\Delta/\Delta}$ femur bones were shortened in both males and females at 2 mo of age (Fig. 4 *A* and *B*). In cultures of MSCs prepared from wild-type bone marrow, Foxp2 showed overlapping expression with Nestin (*SI Appendix*, Fig. S7*A*). Chondrogenic differentiation of MSCs from $Foxp2_{Prx1}^{\Delta/\Delta}$ mutants was impaired compared with controls, as evaluated by Alcian blue staining and qPCR of chondrogenic markers (*SI Appendix*, Fig. S7 *B* and *C*). Consistent with this observation, growth plates in $Foxp2_{Prx1}^{\Delta/\Delta}$ femurs were narrower at 6 mo and manifested obvious signs of cessation/disruption at 12 mo (Fig. 4*C*). Thus, it appears that loss of *Foxp2* from mesenchymal progenitors leads to precocious arrest in the growth plate, partially accounting for the shortening of lower limbs. Given that our previous study showed that *Foxp2* sustains chondrocyte proliferation and protects from apoptosis in embryonic growth plates (30), the effect of *Foxp2* on chondrogenesis may underlie the defective maintenance of the postnatal growth plate.

As a consequence of bipedal locomotion, the articular cartilage in humans endures much more pressure than in other primates. Histological analyses of Safranin O-stained sections revealed

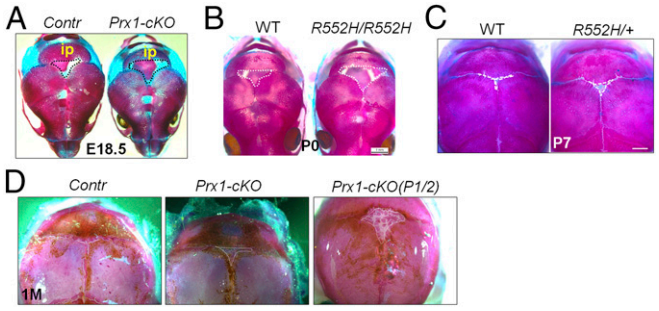

Fig. 3. Disruption of posterior skull integrity in *Foxp2* knockout mice. (*A*) Dorsal view of skulls of $Foxp2_{Prx1}^{\Delta/\Delta}$ mice (*Prx1-cKO*) at E18.5. Ip, interparietal bone. (*B* and *C*) Dorsal view of skulls of $Foxp2^{R552H/R552H}$ (*R552H/R552H*) mutant mice at P0 and $Foxp2^{R552H/+}$ mice (*R552H/+*) at P7. (*D*) Dorsal view of skulls of $Foxp1/2^{fl/fl}$, $Foxp2_{Prx1}^{\Delta/\Delta}$ (*Prx1-cKO*), and $Foxp1/2_{Prx1}^{\Delta/\Delta}$ [*Prx1-cKO* (*P1/2*)] mice at 1 mo of age. Dashed lines outline the lambdoid suture.

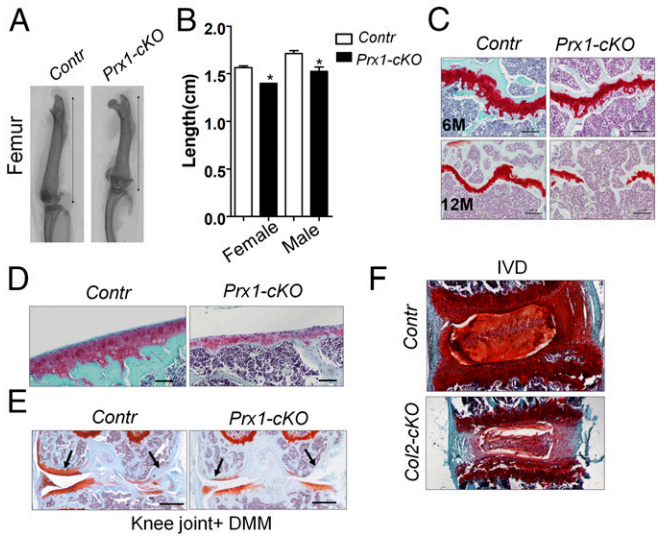

Fig. 4. Impaired articular cartilage integrity due to *Foxp2* loss. (*A*) Representative pictures of femur bones from $Foxp2^{fl/fl}$ (Contr) and $Foxp2_{Prx1}^{\Delta/\Delta}$ (*Prx1-cKO*) mice at 2 mo old. (*B*) Quantification of the length of femur bones in *A*. $n = 5$; $^*P < 0.05$. (*C*) Safranin O staining for growth plate in tibia bones from mice at 6 mo (*Upper*) and 12 mo (*Lower*) of age. (Scale bar, 500 μm.) (*D*) Representative pictures of articular cartilages from $Foxp2_{Prx1}^{\Delta/\Delta}$ (*Prx1-cKO*) mice at 6 mo of age. (*E*) Representative photographs of articular cartilages at knee joints from $Foxp2_{Prx1}^{\Delta/\Delta}$ (*Prx1-cKO*) 2-mo-old mice following 6-wk recovery from DMM surgery. (Scale bar, 100 μm.) (*F*) Representative pictures of intervertebral discs (IVDs) in lumbar vertebrates from $Foxp2_{Col2}^{\Delta/\Delta}$ (*Col2-cKO*) mice at 2 mo of age.

that $Foxp2_{Prx1}^{\Delta/\Delta}$ cKO animals manifested osteoarthritis (OA)-like pathology in their knee joints from the age of 6 mo, as well as reduction of superficial zones and proteoglycan content in distal femurs (Fig. 4*D*). Signs of OA in mutant knee joints were exacerbated by destabilization of the medial meniscus (DMM) at 2 mo of age (Fig. 4*E*). Interestingly, precocious signs of intervertebral disc (IVD) degeneration could be detected in the lumbar IVD of $Foxp2_{Col2}^{\Delta/\Delta}$ mutant at 2 mo of age, as evidenced by decreased Safranin O staining in annulus fibrosus (Fig. 4*F*).

Strong and less massive legs have been suggested to represent evolutionary adaptations to improve walking economy (35). A key indicator for bone strength is stiffness, a parameter reflecting the deformation of bone under stress. We assessed the effects of *Foxp2* loss on bone strength at 2 mo of age by employing the three-point bending approach. According to the load-deformation curves, femurs from $Foxp2_{Prx1}^{\Delta/\Delta}$ knockouts had higher maximum load and yield load, but lower stiffness than wild-type littermates (*SI Appendix*, Fig. S7*D*). This finding suggests that *Foxp2* loss weakens long bone strength by impairing its bone material properties. Taken together, the data indicate that *Foxp2* helps maintain articular cartilage and IVD integrity, factors that are important for forging gracile but strong legs.

Foxp2 Regulates Bone Remodeling. To dissect the cellular basis of Foxp2 function in leg strengthening, we investigated its role in bone remodeling, including osteoblast-mediated bone formation and osteoclast-dependent bone resorption. In $Foxp2_{Prx1}^{\Delta/\Delta}$ mutant mice, μCT analyses revealed that trabecular bone volume, bone mineral density, thickness, and numbers were increased at 2 mo of age (Fig. 5*A* and *SI Appendix*, Fig. S7*E*). H&E staining of mutant femur sections displayed increased trabecular bone masses (*SI Appendix*, Fig. S7*F*). In addition, bone formation rate was still relatively reduced in $Foxp2_{Prx1}^{\Delta/\Delta}$ knockout mice, as quantified by dual calcein labeling (*SI Appendix*, Fig. S7 *G* and *H*). Consistent with that result, the osteogenic potency of *Foxp2*-deficient MSCs was impaired, indicated by a reduction in ALP and Alizarin red staining (Fig. 5*B*), as well as altered expression levels of osteoblast markers (*Alp*, *Col1a1*, *Runx2*, and *Osterix*; *SI Appendix*, Fig. S8*A*) during osteogenic induction. The above observations suggest that Foxp2 sustains MSC osteogenic differentiation.

Postnatal bone homeostasis is also affected by osteoclast-mediated bone resorption. We generated osteoclast-specific *Foxp2* cKO mice by crossing $Foxp2^{fl/fl}$ with a *Ctsk-Cre* line.

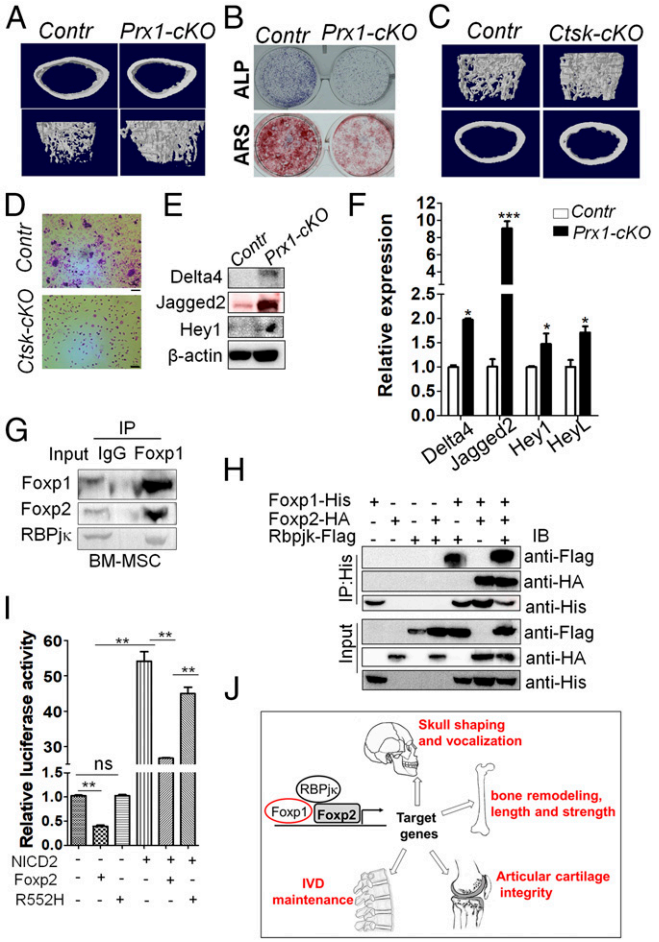

Fig. 5. Foxp2 controls bone remodeling in cooperation with Foxp1. (*A*) Representative images of 3D reconstruction of μCT analysis of $Foxp2_{Prx1}^{\Delta/\Delta}$ (*Prx1-cKO*) femur bones. (*Upper*) Cortical bone. (*Lower*) Trabecular bone. (*B*) ALP and Alizarin red staining following 14 d of osteogenic induction of MSCs from $Foxp2_{Prx1}^{\Delta/\Delta}$ (*Prx1-cKO*) mice at 2 mo of age. (*C*) Representative images of 3D reconstruction of μCT analysis of $Foxp2_{Ctsk}^{\Delta/\Delta}$ (*Ctsk-cKO*) femur bones at 2 mo of age. (*Upper*) Trabecular bone. (*Lower*) Cortical bone. (*D*) TRAP staining of osteoclastogenic cultures of bone marrow from $Foxp2_{Ctsk}^{\Delta/\Delta}$ (*Ctsk-cKO*) mice at 2 mo of age. (Scale bar, 250 μm.) (*E*) Western blotting detection of the expression of Notch-related proteins (Delta4, Jagged2, and Hey1) in MSCs from $Foxp2_{Prx1}^{\Delta/\Delta}$ (*Prx1-cKO*) mice. (*F*) qPCR assessment for expression of Notch-related marker genes (*Delta4*, *Jagged2*, *Hey1*, and *HeyL*) in bone marrow MSCs from $Foxp2_{Prx1}^{\Delta/\Delta}$ (*Prx1-cKO*) mice at 2 mo of age. $n = 3$. (*G* and *H*) Co-IP detected the in vivo interaction of Foxp1, Foxp2, and RBPjκ proteins in bone marrow MSCs, or in 293T cells transfected with the indicated plasmids. (*I*) Luciferase assay in 293T cells transfected with the indicated plasmids. Foxp2 repressed the transactivation of RBPjκ-Luc (containing RBPjκ DNA-binding sites in promoter region) by NICD2, whereas a Foxp2 missense mutation (R552H) alleviated the repressive function. $n = 3$. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; ns, not significant. (*J*) Diagrammatic summaries of the pleiotropic roles of Foxp2 in helping to regulate anatomical features involved in vocalization and bone strengthening. Foxp2 regulates skull shaping, vocalization, and bone remodeling by forming complexes with Foxp1 and RBPjκ proteins.

$Foxp2_{Ctsk}^{\Delta/\Delta}$ mice showed increased bone mass in both cortical and trabecular bones at 2 mo of age (Fig. 5*C* and *SI Appendix*, Fig. S8*C*). Osteoclast differentiation was impaired in $Foxp2_{Ctsk}^{\Delta/\Delta}$ bone marrow, as determined by tartrate-resistant acid phosphatase (TRAP) staining in osteoclastogenic cultures derived from mononuclear cells in bone marrow (Fig. 5*D*). This was coupled with down-regulation of prototypic osteoclastic genes (*c-Fos*, *Nfat2*, *Ctsk*, *Trap*, and *Rankl*) (*SI Appendix*, Fig. S8*B*). These findings suggest that Foxp2 promotes osteoclastogenesis in both a cell autonomous and nonautonomous manner. Collectively, our data show that Foxp2 helps to build strong bones by promoting bone remodeling with dual effect on bone formation and resorption.

Foxp2 Controls Bone Formation in Cooperation with Foxp1. As noted above, compound knockouts of *Foxp1* and *Foxp2* presented mostly additive defects in endochondral or intramembranous ossification (Fig. 3*D* and *SI Appendix*, Fig. S2). Our previous study demonstrated that Foxp1 promotes MSC osteogenic differentiation by repressing Notch signaling (36). $Foxp2_{Prx1}^{\Delta/\Delta}$ MSCs exhibited elevated expression of several Notch signaling members (e.g., Delta4, Jag2, Jag1, Hey1, and HeyL; Fig. 5 *E* and *F*). In terms of defective MSC osteogenic differentiation, $Foxp1/2_{Prx1}^{\Delta/\Delta}$ double knockout mice were more penetrant compared with either the *Foxp1* or *Foxp2* single knockouts (*SI Appendix*, Fig. S9). We further observed that Foxp2 interacted at the protein level with Foxp1 and RBPjκ in bone marrow MSCs, as judged by in vitro and in vivo coimmunoprecipitation (Co-IP) assays (Fig. 5 *G* and *H*). While Foxp2 repressed the activation of *Rbpjκ-Luc* via the intracellular domain of Notch (NICD2), a Foxp2 (R552H) version with a mutated DNA-binding domain relieved the repression (Fig. 5*I*). These findings suggest that Foxp2, in cooperation with Foxp1, promotes osteogenic differentiation of MSCs partially through repression of Notch signaling.

Discussion

To date, the majority of investigations into the genetic bases of vocal communication and language functions have focused on neural pathways (37). Here we used conditional knockouts in mice to extend the examination of Foxp2 function to skull shaping and long bone development. As shown in the model of Fig. 5*J*, our work suggests that Foxp2 exerts pleiotropic influences on skeletal development by helping to regulate: (*i*) skull shaping, including cranial base formation and interparietal bone development; (*ii*) vocal tract geometry, including the sphenoid bone and laryngeal cartilage, anatomical substrates that are important for speech; and (*iii*) development of gracile and strong hind limbs, and maintenance of cartilage integrity in knee joint and IVD. In sum, Foxp2 influences multiple skeletal features conferring susceptibility to anatomical variances in vocal production and, we speculate, maybe also bipedal locomotion.

In line with the speech and language disorders observed in people with heterozygous *FOXP2* mutations (i.e., with haploinsufficiency of the gene), prior studies of humans and animals have given substantial evidence that the gene is important for development and function of relevant brain circuits (38, 39). For example, neural investigations of mice with mutated *Foxp2* have identified significant effects on neurite outgrowth and synaptic plasticity of the corticostriatal and corticocerebellar circuits where it is typically expressed (40–42). The core behavioral phenotype associated with heterozygous disruptions of human *FOXP2* is still a matter of debate (39). The most obvious diagnostic feature is CAS, involving problems with the neural control of sequences of orofacial movements (6), and expressive skills are more profound than problems with receptive language and/or grammar. Recent work also points to cognitive deficits in phonological working memory in *FOXP2* mutation carriers in the KE family (43). Craniofacial and/or skeletal abnormalities have seldom been documented for human heterozygous *FOXP2* mutation cases. Interestingly, studies of people with *FOXP2* variants have anecdotally reported difficulties in infant feeding and coughing in a few cases (12, 13, 44, 45), which could feasibly relate to larynx cartilage changes. In the present study, cartilage-specific ablation of *Foxp2* in mouse pups disrupted the production of innate USVs, despite normal neural expression in key brain structures (*SI Appendix*, Fig. S10). The primary findings stem from homozygous skeleton-specific deletions of Foxp2. Thus, besides its important actions in the central nervous system and in vocal production learning (46), Foxp2 also helps to establish anatomical substrates important for vocal communication. On the other hand, our investigations also revealed that $Foxp2^{R552H}$ homozygous mutant mice showed alterations in presphenoid and larynx cartilage, although heterozygous mutants displayed only minor changes (*SI Appendix*, Figs. S1 and S3). The potential existence of subtle anatomical anomalies should be taken into consideration when dissecting the etiology of speech and language disorders.

Modifications of vocal tract morphology may have played roles in the emergence of human speech (33). Unlike speech, mouse vocalizations are not learned, but acoustic analysis of USVs is a commonly used tool for studying mice carrying mutations associated with communication disorders. Recent work has revealed a novel mechanism of USV production, a planar impinging air jet within the larynx (47). When epiglottis and thyroid cartilage in the larynx is damaged, the production of USVs may be blocked to varying degrees. In the present study, cartilage-specific ablation of *Foxp2* silenced around one-third of the knockout pups, which may correlate with their dysmorphogenesis of the larynx (Fig. 2 *A*–*D*). Moreover, a substantial reduction of USV syllable rates was observed in both $Foxp1_{Col2}^{\Delta/\Delta}$ and $Foxp2_{Col2}^{\Delta/\Delta}$ knockout pups (Fig. 2*F* and *SI Appendix*, Fig. S4*B*). However, the peak frequency, which is mostly regulated by laryngeal muscle motor and airflow pressure (48, 49), showed a significant increase in the $Foxp1_{Col2}^{\Delta/\Delta}$, but not $Foxp2_{Col2}^{\Delta/\Delta}$ knockout line. These findings also remind us to be cautious about using pup USVs to try to model human speech impairments (50).

FOXP1 and *FOXP2* show partially overlapping expression patterns in the brain, and heterozygous disruptions of these genes lead to a distinct yet overlapping spectrum of neurodevelopmental disorders (11). The phenotype associated with heterozygous *FOXP1* mutations is more severe and extensive, including global developmental delay, intellectual disability, autistic features and, notably, a number of documented craniofacial symptoms (9, 51). Interestingly, neuron-specific knockout of *Foxp1* in mice also impairs neonatal USVs (52, 53). In the present study, cartilage-specific knockout of *Foxp1* in mice led to impairment of cranial base formation and USVs, just as with knockout of *Foxp2*. In addition, loss of *Foxp1* and *Foxp2* displayed additive effects in skull shaping and bone formation (Fig. 3 and *SI Appendix*, Figs. S2 *F* and *G* and S9). Therefore, Foxp1 and Foxp2 cooperatively regulate craniofacial shaping.

Paleoanthropological evidence suggests that bipedalism emerged at an early stage of hominid evolution following the split from chimpanzee lineages (35). Two amino acid changes in *FOXP2* occurred on the lineage that led to modern humans, after splitting from the chimpanzee but before the divergence of Neandertals, and these changes have been considered as candidates for involvement in the evolution of speech (24, 26). We still know little about the genetic basis of bipedal gait, which is thought to provide advantages in strength and walking economy (35, 54). Given the coordination of osteogenesis and neurogenesis in shaping of the skull and brain (1), it is interesting to speculate on whether Foxp2 may have been relevant for bipedal evolution in early human history. Although we have not tested evolutionary changes in the present study, our findings suggest that Foxp2 may have been well placed to provide resources for adaptations in bone and cartilage that are relevant for human evolution. Firstly, Foxp2 helps regulate craniofacial shaping and skull integrity (Fig. 3), such as sphenooccipital synchondrosis and interparietal bone, which are major evolutionary sources of skull reshaping (55). Secondly, Foxp2 helps to forge gracile but strong

bones through its dual effects on bone remodeling (Figs. 4*A* and 5 *A*–*D*), improving walking economy and energy expenditure. Finally, Foxp2 sustains growth plate competency for elongation of hind limbs and helps maintain the integrity of knee joint articular cartilage and IVDs. All these features have the potential to protect bones from stress damage during bipedal striding. In summary, this study raises hypotheses about contributions of *FOXP2* to human evolution that can be empirically tested through studies of, for example, mice that have been humanized for this locus.

Materials and Methods

All animal experiments were performed according to the guidelines and approved by the ethical committee of Bio-X Institutes of Shanghai Jiao Tong University (SYXK 2011-0112). For skeletal morphological analysis, skeletal preparations for mice of different ages were made by Alcian blue/Alizarin red staining as previously reported. For μCT analysis, femurs were dissected from mice and fixed in 70% ethanol at 4 °C. μCT scanning of bones was performed on SkyScan 1176. A 3D model was reconstructed and structural indices were calculated using CTAn software, and the region of interest selected was 5 mm below growth plate of bones.

The details of other materials and methods can be found in *SI Appendix*.

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