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Skeletal growth is enhanced by a shared role for SOX8 and SOX9 in promoting reserve chondrocyte commitment to columnar proliferation

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SOX8 was linked in a genome-wide association study to human height heritability, but roles in chondrocytes for this close relative of the master chondrogenic transcription factor SOX9 remain unknown. We undertook here to fill this knowledge gap. High-throughput assays demonstrate expression of human *SOX8* and mouse *Sox8* in growth plate cartilage. In situ assays show that *Sox8* is expressed at a similar level as *Sox9* in reserve and early columnar chondrocytes and turned off when *Sox9* expression peaks in late columnar and prehypertrophic chondrocytes. *Sox8*$^{-/-}$ mice and *Sox8*$^{fl/fl}$*Prx1Cre* and *Sox9*$^{fl/+}$*Prx1Cre* mice (inactivation in limb skeletal cells) have a normal or near normal skeletal size. In contrast, juvenile and adult *Sox8*$^{fl/fl}$*Sox9*$^{fl/+}$*Prx-1Cre* compound mutants exhibit a 15 to 20% shortening of long bones. Their growth plate reserve chondrocytes progress slowly toward the columnar stage, as witnessed by a delay in down-regulating *Pthlh* expression, in packing in columns and in elevating their proliferation rate. *SOX8* or *SOX9* overexpression in chondrocytes reveals not only that SOX8 can promote growth plate cell proliferation and differentiation, even upon inactivation of endogenous *Sox9*, but also that it is more efficient than SOX9, possibly due to greater protein stability. Altogether, these findings uncover a major role for SOX8 and SOX9 in promoting skeletal growth by stimulating commitment of growth plate reserve chondrocytes to actively proliferating columnar cells. Further, by showing that SOX8 is more chondrogenic than SOX9, they suggest that SOX8 could be preferred over SOX9 in therapies to promote cartilage formation or regeneration in developmental and degenerative cartilage diseases.

skeletal growth | SOX9 | growth plate cartilage | mouse model | development

Significance

This study investigates the importance of SOX8, a close relative of the master chondrogenic transcription factor SOX9, in growth plate cartilage, the tissue driving bone growth in development. Using mouse models, it shows that SOX8 and SOX9 are coexpressed in early-stage chondrocytes and promote cell progression from a reserve to actively proliferative stage. Further, it shows that SOX8 is more stable than SOX9 and outperforms it in regenerating cartilage lost upon inactivation of endogenous *Sox9*. These findings widen our understanding of the complex network of factors regulating skeletal growth. They validate a genome-wide association study proposition that SOX8 is a determinant of human height and identifies SOX8 as a potent tool to promote chondrogenesis in skeletal malformation and degenerative diseases.

Vertebrate bones fulfill major functions, including body shaping, internal organ protection, hematopoiesis homing, calcium and phosphate metabolism regulation, and hormonal regulation. Most form through endochondral ossification, i.e., replacement of a cartilage template that sculps their shape and size during development. Growth plates (GPs) are tissue engines within these templates that steer skeletal growth, primarily elongation, and induce ossification. Their chondrocytes (GPCs) follow a multistep differentiation program. The GP region closest to the joint is a resting or reserve zone (RZ). It features stem cells and round, low-proliferating, immature chondrocytes (1, 2). Upon entering the columnar zone (CZ), GPCs increase their proliferation rate and tightly pack in longitudinal queues. They subsequently slow down proliferation and eventually undergo postmitotic maturation, i.e., prehypertrophic, hypertrophic, and terminal differentiation. At this last step, they face the endochondral ossification front and die or become osteoblasts (3). GPC proliferation and hypertrophy are the main drivers of skeletal growth, along with extracellular matrix formation. These activities are governed by cell type-specific transcription factors, hormones, and local growth factors (4, 5). Differential control of GPCs generates anatomical diversity among bones, individuals, and species, and pathological alterations underlie a large spectrum of skeletal dysplasias (6). However, the factors involved and their functions and regulation are incompletely known. The present study helps fill this gap.

SOX8 is one of twenty transcription factors forming the SOX family (7). Based on sequence identity in their hallmark, a high-mobility-group (HMG)-type DNA-binding domain, SOX proteins distribute in eight groups, A to H. Most are pivotal determinants of cell fate and differentiation in discrete lineages such that they control altogether most biological processes (8). Accordingly, variants in many SOX genes cause severe diseases, called SOXopathies (9).

SOX8, SOX9, and SOX10 form the SOXE group. Having similar DNA-binding, homodimerization and transactivation domains, and overlapping expression patterns (10),

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they have distinct, additive, or redundant functions in several processes. All three contribute to specify cranial neural crest cells and to generate and maintain the nervous system (11–13). *SOX8* and *SOX9* participate in sex determination (14). *SOX9* heterozygosity causes disorders of sex development (DSDs) in XY individuals, and *SOX8* heterozygous or homozygous loss causes a DSD spectrum, including male infertility and primary ovarian insufficiency (15). In mice, *Sox9* is haploinsufficient in Sertoli cells, *Sox8* reinforces its function, and both genes maintain male fertility (16, 17). *SOX8* and *SOX9* may thus also share functions in other processes.

SOX9 is well known to be essential for chondrogenesis. Heterozygous variants cause campomelic dysplasia, a skeletal malformation syndrome affecting the entire endochondral skeleton (18–20). *SOX9* is expressed in the chondrocyte lineage from the multipotent progenitor stage. It secures commitment of progenitors and differentiated cells to the lineage, up-regulates many chondrocyte markers, and maintains cells alive (4, 8). Roles for SOX8 in chondrogenesis have been suggested in a few studies. Mice in which *lacZ* replaced *Sox8* showed locus activity in embryonic cartilage primordia (21); mouse *Sox8* and human *SOX8* were found expressed in chondrocytes in vitro, but weakly compared to *Sox9*/*SOX9*, and SOX8 activated chondrocyte-specific reporters, albeit less potently than SOX9 (10, 22); and *Sox8*-null mice were slightly smaller than control littermates (21). Interestingly, a significant score was obtained for a SNP located upstream of *SOX8* in a genome-wide association study (GWAS), leading to propose that *SOX8* may contribute to determining human height heritability (23). We used here mouse models to test this proposition. Our findings uncover that SOX8 is chondrogenic and participates in skeletal growth by sharing a function with SOX9 in promoting the commitment of reserve GPCs to actively proliferating columnar cells.

Results

Genomic, Proteomic, and Transcriptomic Data Advocate That SOX8 May Be a Skeletal Size Determinant. *SOX8* was proposed to contribute to human height based on a significant SNP (rs12597498) located in the *LMF1* intron 3 (23) (*SI Appendix*, Fig. S1*A*). *LMF1* encodes a lipase maturation factor, whose loss causes lipase deficiency with severe hypertriglyceridemia, but no known skeletal impact (24, 25). *LMF1* thus unlikely determines human height. The 500-kb interval encompassing rs12597498 contains 14 additional coding genes. Among them, *CCDC78*, *CACNA1H,* and *SOX8* have been associated with genetic disorders, but no skeletal disease or stature variation (15, 26–28). *SOX8* is the closest gene to *LMF1*. The two genes have their transcription start sites (TSS) separated by only 7.9 kb. rs12597498 is located 41 kb upstream of the *SOX8* TSS. Interestingly, three enhancers (E1, E3, and E4) conferring transgene activity in mouse embryos in discrete domains of the *Sox8* expression pattern were identified in *Lmf1* introns (29). Beside activity in other tissues, E4, located 7 kb from rs12597498, showed prominent activity in limb bud skeletogenic mesenchyme, and E3, located 41 kb from rs12597498, was active in facial skeletogenic mesenchyme. Mining of high-throughput transcriptomic assays revealed *SOX8* expression in human cartilage, including fetal growth plates (30, 31) (Fig. 1*A*). Its RNA level was twofold to fourfold lower than that of *SOX9* but at least as high as that of *SOX5* and *SOX6* (which form a chondrogenic trio with *SOX9*), whereas *SOX10* RNA was not detected. These data and the fact that *SOX8* encodes a protein closely related to SOX9 (*SI Appendix*, Fig. S1*B*) advocate that *SOX8* is the best candidate gene for height determination in the vicinity of rs12597498.

***Sox8* and *Sox9* Are Expressed at Similar Levels in Immature Growth Plate Chondrocytes.** We performed RNA in situ assays to define the expression pattern of *Sox8* in chondrogenesis. Mouse embryos at day 13.5 of gestation (E13.5) showed *Sox8* expression in differentiated chondrocytes of cartilage primordia, while *Sox9* expression was stronger and broader, including presumptive joints, perichondrium and both nascent and differentiated chondrocytes (*SI Appendix*, Fig. S1*C*). At E16.5, *Sox8* expression was low in incipient articular chondrocytes and in epiphyseal and early-CZ chondrocytes, while *Sox9* expression was strong in these cells and even stronger in late-CZ and prehypertrophic GPCs (*SI Appendix*, Fig. S1*D*). In juvenile and adult mice (P7 to P35), *Sox8* remained coexpressed with *Sox9* in epiphyseal, articular, and early columnar chondrocytes (*SI Appendix*, Fig. S1*E* and Fig. 1*B*). Noticeably, its RNA level looked similar to that of *Sox9* in reserve GPCs and faded in the upper and middle CZ, while *Sox9* expression peaked in late-CZ and prehypertrophic cells before fading in hypertrophic GPCs. Our single-cell transcriptome profiling of P19 pup epiphyses (32) consolidated these data, showing that *Sox8* expression was highest in *Pthlh*$^{+}$reserve/early-CZ GPCs, while *Sox9* expression was higher than *Sox8* expression in these cells and became even higher in more mature *Pthlh*-GPCs (Fig. 1*C*). These data support the proposition that *SOX8* could promote skeletal growth by sharing functions with SOX9 in RZ/early-CZ GPCs.

Generation of Mice Harboring *Sox8* Null/*lacZ* and Conditional Null Alleles. To generate mice with *Sox8* conditional null alleles, we recombined the *Sox8* locus in mouse embryonic stem (ES) cells with a premade targeting vector (*SI Appendix*, Fig. S2*A*). The resulting *Sox8*^{lacZNeoR} allele contained *Frt* sites flanking *IRES-lacZ* and *NeoR* cassettes, and *loxP* sites flanking the *NeoR* cassette and the *Sox8* exon 2. An ES clone harboring a properly recombined allele (*SI Appendix*, Fig. S2*B*) was used to generate mouse chimeras. Male chimeras were bred with *PrmCre* females to obtain *Sox8*$^{lacZNeoR/+}$*PrmCre* males (*SI Appendix*, Fig. S2*C*). The latter expressed Cre in their germline, allowing *Sox8*^{lacZNeo} conversion into *Sox8*^{lacZ}. *Sox8*$^{lacZ/+}$ progeny were used to derive *Sox8*$^{lacZ/lacZ}$ mice (*SI Appendix*, Fig. S2 *D* and *E*). We also bred chimeras with *Flpe*-expressing females to obtain progeny converting *Sox8*^{lacZNeo} into a *Sox8*^{fl} conditional null allele. *Sox8*$^{fl/+}$ mice were bred to derive *Sox8*$^{fl/fl}$ mice (*SI Appendix*, Fig. S2 *F* and *G*). We then generated *Sox8*$^{fl/+}$*PrmCre* males and bred them with wild-type females to obtain *Sox8*$^{+/-}$ mice, with which we derived *Sox8*$^{-/-}$ mice (*SI Appendix*, Fig. S2 *F* and *H*).

Sox8^{fl} was expected to produce the same RNA as *Sox8*$^{+}$ since it only differed from the latter by *Frt* and *loxP* sites inserted in intronic sequences. Accordingly, *Sox8*$^{fl/+}$ and *Sox8*$^{fl/fl}$ mice looked normal and had normal body weights and *Sox8* RNA levels (*SI Appendix*, Fig. S2*I*).

Sox8^{lacZ} and *Sox8*$^{-}$ encode SOX8 proteins truncated in the DNA-binding domain and lacking all downstream sequences, including the transactivation domains. The *Sox8*^{lacZ}-derived protein was predicted to end with a 26-residue-long exogenous sequence, and the *Sox8*$^{-}$-derived protein with a 58-residue-long tail resulting from a frameshift caused by splicing of exon 1 into exon 3 (*SI Appendix*, Fig. S2*J*). Although the mutant proteins retained an intact homodimerization domain, they were unlikely functional since SOXE homodimerization occurs only upon DNA binding (33, 34). Both *Sox8*^{lacZ} and *Sox8*- were thus expected to be null alleles.

Sox8^{lacZ} contained an internal ribosome entry site (IRES) to allow expression of E. coli β-galactosidase (*lacZ* product). X-gal (β-galactosidase substrate) staining of whole-mount fetuses and mouse sections showed that *lacZ* expression matched *Sox8* expression

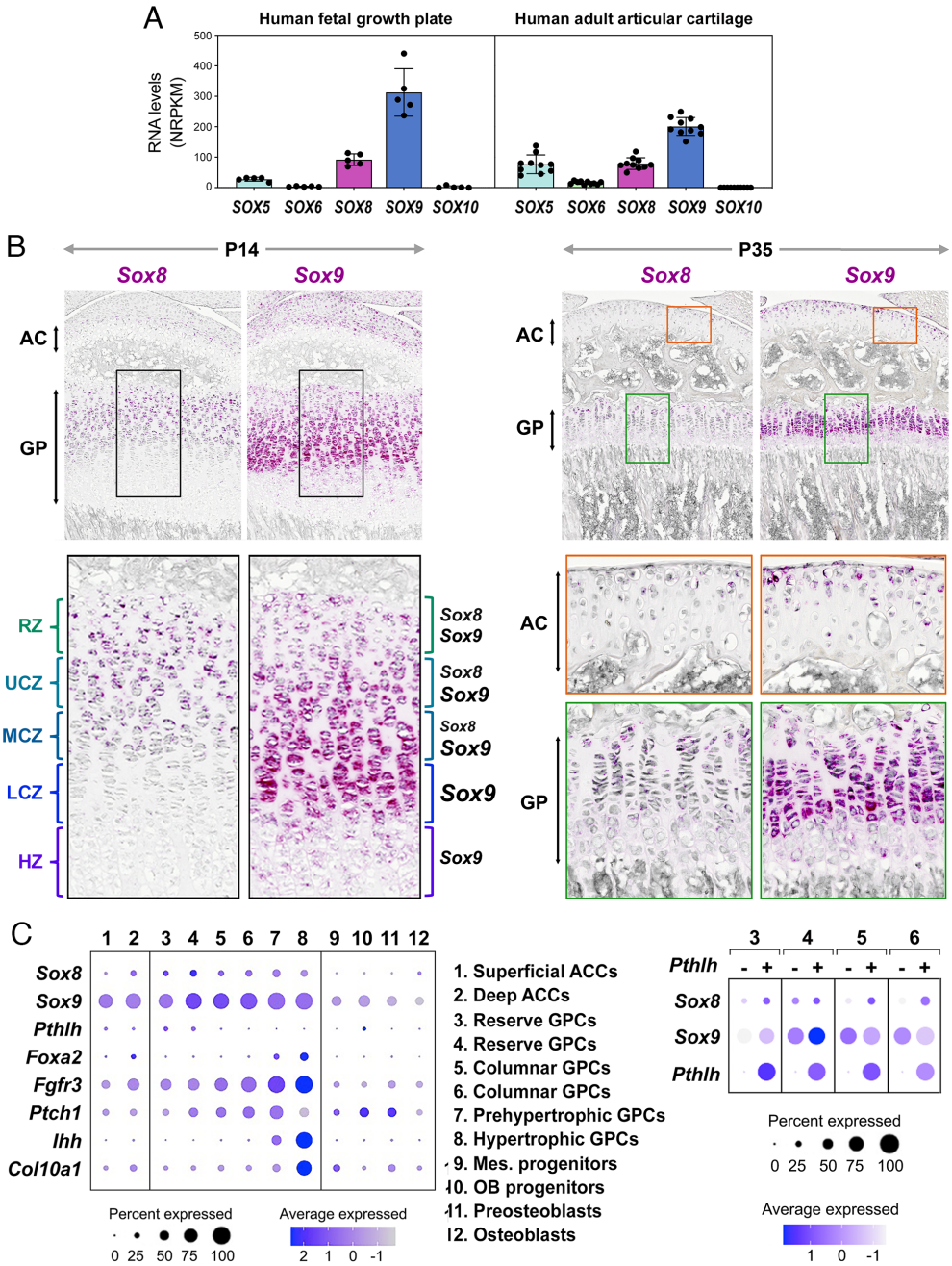

Fig. 1. SOX8 genomic, proteomic, and expression features. (*A*) Expression of SOXD (*SOX5* and *SOX6*) and SOXE (*SOX8*, *SOX9,* and *SOX10*) genes in human fetal cartilage growth plate and adult articular cartilage (GSE111358 and GSE162033 datasets, respectively). NRPKM, normalized reads per kilobase gene model per million total reads. (*B*) Representative in situ assays comparing *Sox8* and *Sox9* RNA levels in sections of tibia proximal GPs from juvenile (P14) and adult (P35) mice. Hematoxylin staining signal was desaturated with Adobe Photoshop to facilitate RNA visualization (magenta). *Top*, low-magnification images. AC, articular cartilage. GP, growth plate. *Bottom*, high-magnification images of boxed regions. At P14, GP zones are indicated on the left, and their relative *Sox8* and *Sox9* RNA levels are represented with different font sizes on the right. RZ, resting zone. UCZ, upper, MCZ, middle, and LCZ lower columnar zone. HZ, hypertrophic zone. (*C*) Dot plot of scRNA-seq data from femoral and tibial epiphyses of P19 mice (GSE162033 dataset). *Left*, *Sox8* and *Sox9* expression are compared to those of *Pthlh* (RZ/early-CZ marker), *Foxa2* (GP stem cells and pre/hypertrophic GPCs), *Fgfr3* (maturing GPCs), *Ptch1* (maturing GPCs, except hypertrophic cells), and *Ihh* and *Col10a1* (pre/hypertrophic GPCs). *Right*, *Sox8* and *Sox9* expression are compared in *Pthlh*$^-$ *and Pthlh*$^+$ subpopulations of RZ and CZ GPCs. Note that *Sox8* expression is higher in *Pthlh*$^+$than *Pthlh*$^-$ subpopulations. *Sox9* expression is higher in *Pthlh*$^+$than *Pthlh*$^-$ subpopulations of reserve GPCs but lower in *Pthlh*$^+$than *Pthlh*$^-$ subpopulations of columnar GPCs, in agreement with its increased expression as GPCs mature in CZ and HZ.

in E12.5 *Sox8*$^{lacZ/+}$ embryos (21, 35) (*SI Appendix*, Fig. S2*K*) and in GPs and skeletal muscle of *Sox8*$^{lacZ/lacZ}$ pups (*SI Appendix*, Fig. S2*L*).

We maintained all mouse lines in the C57BL/6 J genetic background and used them to determine whether SOX8 controls skeletal growth.

***Sox8* Has No Unique Functions That Substantially Affect Skeletal Length in Mice.** Previously generated *Sox8*-null mice (129 × C57BL/6J background), in which a *lacZ/NeoR* cassette replaced the *Sox8* coding region, were 30% lighter than wild-type littermates from weaning age onto adulthood (21). The naso-anal length of adults was slightly smaller (<10%), but long bone length was not reported. Our new *Sox8*$^{lacZ/lacZ}$ mice showed a weight deficit of 20% at weaning (3 wk) and in early adulthood (8 wk), whereas *Sox8*$^{-/-}$ mice had a normal weight at weaning, and a small (3 to 10%), insignificant weight deficit in adulthood (*SI Appendix*, Fig. S3*A*). *Sox8*$^{lacZ/lacZ}$ mice tended to have a 5 to 7% shorter naso-anal length, whereas *Sox8*$^{-/-}$ mice did not

(*SI Appendix*, Fig. S3*B*). The long bones (femurs, tibias, humeri, and ulnae) of all mice were of normal length at weaning, but humeri and femurs were 5 to 8% shorter in adulthood in both $Sox8^{lacZ/lacZ}$ and $Sox8^{-/-}$ mice (*SI Appendix*, Fig. S3*C*). Males and females were equally affected. According to their mild growth phenotype, both mutant types had histologically unremarkable GPs at 3 wk, except that the hypertrophic zone of $Sox8^{lacZ/lacZ}$ GPs was shorter than normal (*SI Appendix*, Fig. S3 *D* and *E*). This feature typically reflects an accelerated turnover of chondrocytes at the endochondral ossification front but is not a major skeleton growth driver if the cells reach a normal size and maintain it in several layers (36), as was the case of $Sox8^{lacZ/lacZ}$ and $Sox8^{-/-}$ mice (*SI Appendix*, Fig. S3*F*). At 8 wk, $Sox8^{lacZ/lacZ}$, $Sox8^{-/-}$ and control mice had similarly vestigial growth plates, indicating that *Sox8* is not necessary to keep growth plates open in adult mice (*SI Appendix*, Fig. S3*G*).

We concluded that the low weight of previously published $Sox8^{lacZNeoR/lacZNeoR}$ mice and our new $Sox8^{lacZ/lacZ}$ mice may be due to the *lacZ* knockin rather than *Sox8* inactivation, and that mouse *Sox8* does not have functions in skeletal growth that are unique, i.e., not compensated by another gene.

Sox8 and _Sox9_ Promote Skeletal Elongation in a Redundant Manner. To investigate whether *SOX8* and *SOX9* share functions in chondrogenesis, we generated mice harboring *Sox8* and/or *Sox9* (37) conditional null alleles and *Prx1Cre*, a transgene expressing Cre recombinase mainly in limb bud skeletogenic cells (38). A conditional inactivation approach was necessary because $Sox9^{+/-}$ mice die upon birth (39). $Sox9^{fl/+}Prx1Cre$ mice could be included in the study because they looked healthy throughout life, whereas $Sox9^{fl/fl}Prx1Cre$ mice were not included because they fail to form limbs (40). $Sox8^{fl/fl}Prx1Cre$ and $Sox9^{fl/+}Prx1Cre$ mice (single mutants) and $Sox8^{fl/fl}Sox9^{fl/+}Prx1Cre$ mice (compound mutants) had a normal body weight and trunk length at 3 and 8 wk (Fig. 2 *A* and *B*). $Sox8^{fl/fl}Prx1Cre$ mice had normal long bone lengths, and $Sox9^{fl/+}Prx1Cre$ mice had 4-8% shorter long bones (Fig. 2*C* and *SI Appendix*, Fig. S4). Interestingly, this shortening was exacerbated in $Sox8^{fl/fl}Sox9^{fl/+}Prx1Cre$ mice, reaching 14 to 20% at both 3 and 8 wk. These results thus revealed that *Sox8* and *Sox9* functionally interact to promote skeletal elongation, likely through redundant actions.

Sox8 and _Sox9_ Costimulate Commitment of Growth Plate Reserve Chondrocytes to the Columnar Stage. GPs looked histologically normal in $Sox8^{fl/fl}Prx1Cre$ and $Sox9^{fl/+}Prx1Cre$ juvenile mice (Fig. 3*A*). Interestingly, while the total length of GPs was normal in $Sox8^{fl/fl}Sox9^{fl/+}Prx1Cre$ mice, the RZ, as defined by round, loosely organized chondrocytes, was almost twice as long as normal, while the CZ was slightly shorter than normal. This suggested that SOX8 and SOX9 promote the transition of GPCs from RZ to CZ.

Quantification of EdU incorporation showed as expected that GPCs proliferated only in the GP upper half (i.e., RZ and CZ) (Fig. 3*B*). The overall level of cell proliferation in this region was lower in compound mutants (86% of controls), matching long bone shortness (85%). To determine whether cell proliferation was reduced evenly or not in this region, we compared the levels of EdU incorporation in four equal segments that approximated the RZ (RZ) and the upper (UCZ), middle (MCZ), and lower (LCZ) columnar zones. We found that control chondrocytes proliferated twice as fast in UCZ as in RZ and MCZ, and were almost growth-arrested in LCZ. $Sox8^{fl/fl}Prx1Cre$ and $Sox9^{fl/+}Prx1Cre$ GPCs had a proliferation rate in RZ reduced by 15 and 27%, respectively, while $Sox8^{fl/fl}Sox9^{fl/+}Prx1Cre$ GPCs reduced it by 50%. *Sox8*

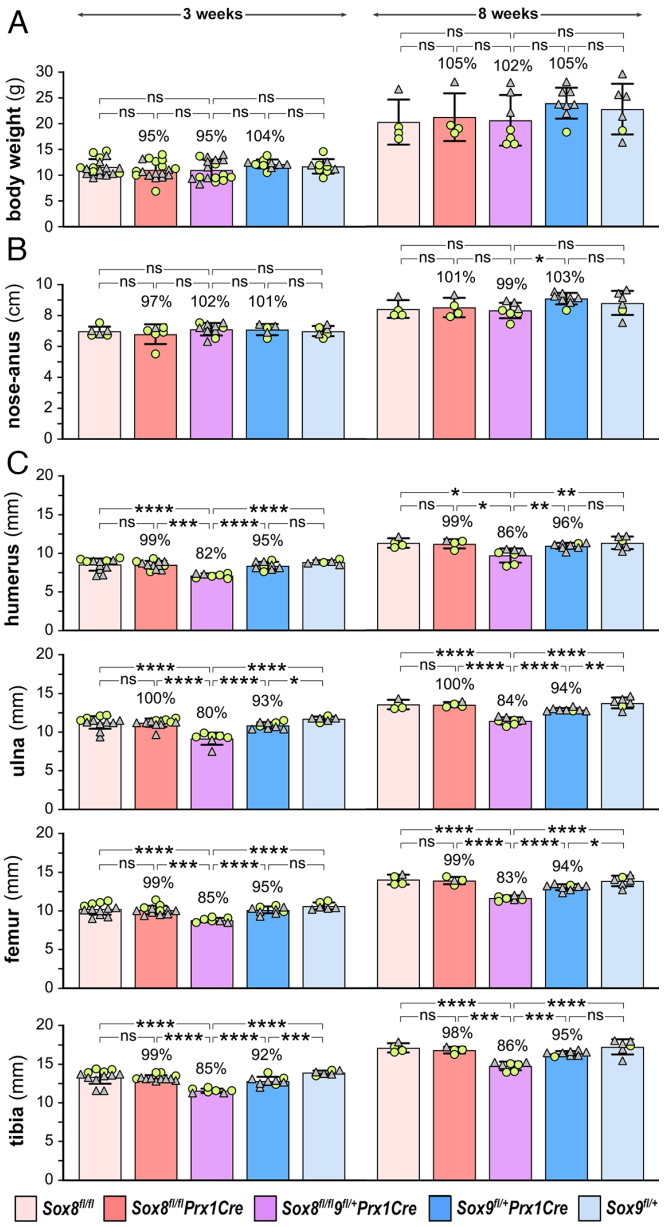

Fig. 2. Weights and sizes of mice with *Prx1Cre*-induced *Sox8* and *Sox9* inactivation. (*A*) Body weights of 3- and 8-wk-old mice. $Sox8^{fl/fl}$, $Sox8^{fl/fl}Prx1Cre$, and $Sox8^{fl/fl}Sox9^{fl/+}Prx1Cre$ mice were progeny of $Sox8^{fl/fl}Sox9^{fl/+}$ females and $Sox8^{fl/fl}Prx1Cre$ males. $Sox9^{fl/+}$ and $Sox9^{fl/+}Prx1Cre$ mice were progeny of $Sox9^{fl/fl}$ females and *Prx1Cre* males. Percentages of average values obtained for mutants relative to controls are indicated. Brackets, significance of differences between experimental groups detected using one-way ANOVA tests. $*P \le 0.05$; $**P \le 0.01$; $***P \le 0.001$; $****P \le 0.0001$; ns, nonsignificant. (*B*) Naso-anal lengths. (*C*) Long bone lengths. Of note, males are generally heavier than females at 8 wk, but long bones are of similar lengths in both sexes.

and *Sox9* thus have additive roles in RZ, where their expression levels appear even. Similar but milder differences were seen in UCZ, but none in MCZ and LCZ, in line with progressive down-regulation of *Sox8* expression. Since GPC proliferation is a major skeletal growth driver, these data suggested that SOX8 and SOX9 promote skeleton elongation by stimulating RZ chondrocytes to commit to actively proliferating CZ cells. Slower proliferation may explain why $Sox8^{fl/fl}Sox9^{fl/+}Prx1Cre$ RZ/UCZ GPCs longed to pack in columns.

We previously showed that *Sox9* homozygous loss prompted GP closure by causing columnar GPCs to growth arrest and either die or undergo osteoblast-like conversion (32, 41). We found here in TUNEL assays that equally few GPCs were dying in 3-wk-old

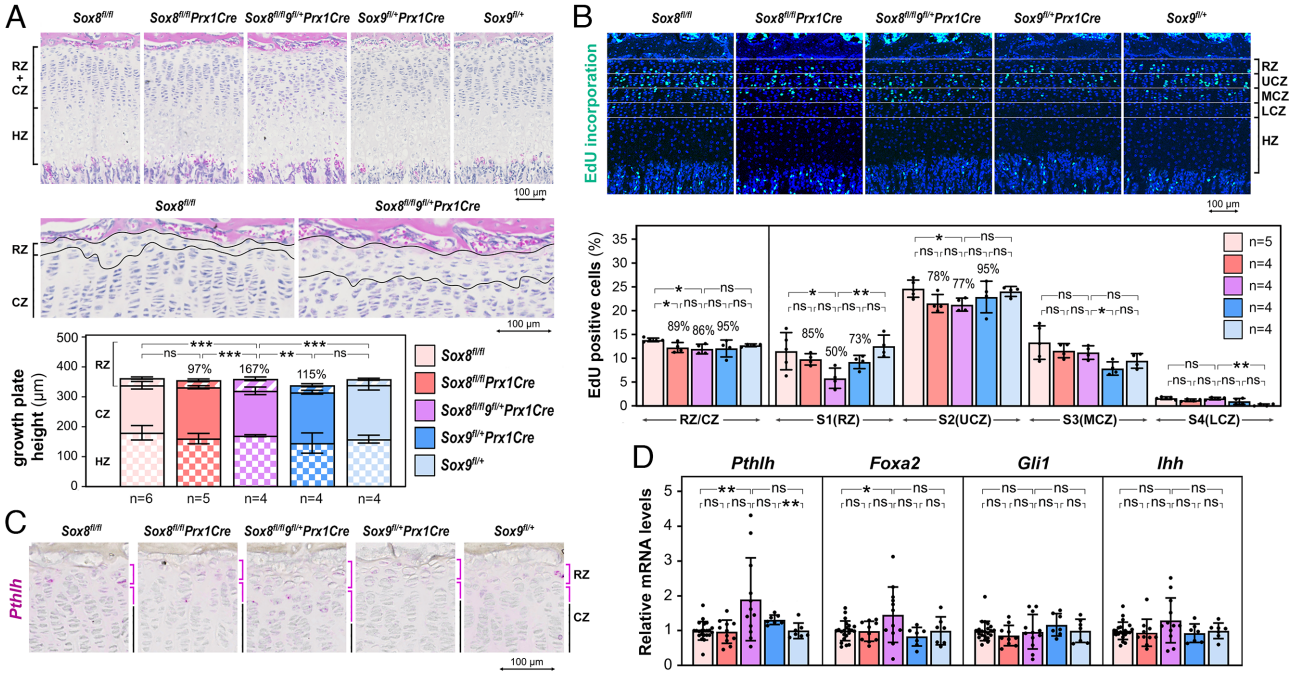

Fig. 3. Analysis of GPs from mice with *Prx1Cre*-induced *Sox8* and *Sox9* inactivation. (*A*) *Top*, representative H&E-stained sections of tibia proximal GPs at 3 wk. *Bottom*, high-magnification images of the RZ/UCZ. Graph, quantification of GP zones. Brackets contain *t* test significance for RZ height comparisons. No other zone height was significantly different among mouse groups. The average length of mutant RZ is shown as a percentage of the average length of the control RZ. (*B*) Cell proliferation assay in GPs of 3-wk-old mice. *Top*, representative pictures of EdU incorporation (green) in sections adjacent to those shown in (*A*). Cell nuclei are stained with DAPI (blue). The GP upper half is divided into RZ, UCZ, MCZ, and LCZ. *Bottom*, percentages of EdU$^+$ chondrocytes obtained in all (*Left*) and individual (*Right*) segments. Vertical brackets, SD (n = 4 to 5 mice). Percentages of control values and *P* values obtained in *t* tests are indicated. *$P \le 0.05$; **$P \le 0.01$; ns, nonsignificant. (*C*) Representative images of in situ assays of *Pthlh* RNA in tibia proximal GPs of 3-wk-old mice. Hematoxylin staining signal was desaturated with Adobe Photoshop to facilitate RNA visualization (magenta). (*D*) RT-qPCR assays of indicated RNAs in P21 mouse epiphyses. Values were normalized with *Hprt* RNA levels and are presented as fold-changes relative to controls (n = 7 to 22 mice). *P* values from *t* tests are indicated.

Sox8/*Sox9* single and compound mutants as in controls (*SI Appendix*, Fig. S5*A*) and observed on histology sections that GPs were similarly vestigial in 8-wk-old controls and *Sox8*/*Sox9* single and compound mutants (*SI Appendix*, Fig. S5*B*). Thus, while both alleles of *Sox9* are necessary to maintain actively proliferating GPCs in juveniles, *Sox8* is not necessary and one allele of *Sox9* is sufficient, even upon *Sox8* loss, to keep GPCs alive and GPs open in juvenile and adult mice.

To consolidate evidence that SOX8 and SOX9 promote the progression of reserve GPCs toward the columnar stage, we examined the expression of *Pthlh* (parathyroid hormone-related protein), which marks RZ and UCZ GPCs and is critical to keep these cells immature (2, 4, 42). In situ assays detected the *Pthlh* RNA in more GPC layers in *Sox8*$^{fl/fl}$*Sox9*$^{fl/+}$*Prx1Cre* than control and single mutant mice (Fig. 3*C*). Accordingly, RT-qPCR assays on whole GPs showed an increase of about twofold of the *Pthlh* RNA level in compound mutants compared to controls (Fig. 3*D*). A mild increase was also detected in the level of *Foxa2* RNA, which marks GP stem cells and hypertrophic GPCs (43). No expression change was seen for *Ihh*, which marks pre- and hypertrophic chondrocytes and is necessary for *Pthlh* expression, and for *Gli1*, an *Ihh* target in CZ GPCs. SOX8 and SOX9 thus down-regulate *Pthlh* expression as they foster GPC progression toward the columnar stage.

Generation of Mice Allowing Conditional Expression of Human SOX8 or SOX9 in Any Lineage. We generated gain-of-function mouse models to further assess and compare the chondrogenic properties of SOX8 and SOX9 in vivo. We used an established strategy to knock-in the human SOX8 and SOX9 coding sequences into the *Gt(ROSA)26Sor* locus (referred to as *R26* hereafter) (44, 45). The targeting vectors included a CAG hybrid promoter/enhancer (46) and loxP-flanked polyadenylation sites to express the knocked-in sequences upon Cre-mediated recombination (*SI Appendix*, Fig. S6*A*). To readily detect them, we tagged the SOX8 and SOX9 proteins with an N-terminal 3FLAG epitope and a linker sequence, which did not affect their levels and activities (10). We performed DNA recombination in C57BL/6J ES cells and used properly targeted clones (*SI Appendix*, Fig. S6*B*) to generate mouse chimeras and then derive mouse lines (*SI Appendix*, Fig. S6*C*). As expected, *R26*$^{SOX8/SOX8}$ and *R26*$^{SOX9/SOX9}$ primary chondrocytes expressed the 3FLAG-SOX proteins only following treatment with a *Cre*-expressing adenovirus (*SI Appendix*, Fig. S6*D*). Using antibodies against the FLAG epitope, SOX9 or both SOX8 and SOX9 (*SI Appendix*, Fig. S6*E*), we found that the cells exhibited less 3FLAG-SOX9 than endogenous SOX9 and more 3FLAG-SOX8 than 3FLAG-SOX9 and endogenous SOX8 (*SI Appendix*, Fig. S6*F*). We concluded that our new mouse lines should be suitable to force SOX8 and SOX9 expression in any desired lineage in vivo.

Both SOX8 and SOX9 Expression in the *Prx1Cre* Lineage Impair Cortical Bone Formation. As a first test of *R26*^{SOX8} and *R26*^{SOX9} in vivo, we generated mice harboring these alleles and *Prx1Cre*, which is active in the mesenchymal progenitors of appendicular chondrocytes and osteoblasts (38) as well as in mesoderm-derived skull progenitors (47). *R26*$^{SOX8/+}$*Prx1Cre* and *R26*$^{SOX9/+}$*Prx1Cre* mice looked healthy throughout life, whereas *R26*$^{SOX8/SOX8}$*Prx1Cre* and *R26*$^{SOX9/SOX9}$*Prx1Cre* pups showed a drastic health decline in the second week postnatally. Skeletal preparations showed that P13 heterozygotes of both types had a grossly normal skeleton, whereas homozygotes had defective skull vault osteogenesis, with open fontanels, and very short limbs, with midshaft bone fractures (*SI Appendix*, Fig. S7 *A*–*C*). Histology analysis showed fracture calluses and substantial bone cortex matrix deficiency that

explained the high rate of bone fractures (*SI Appendix*, Fig. S7*D*). These data aligned with reports that forced expression of SOX9 or SOX8 in the osteoblast lineage impaired osteogenesis by inhibiting cell differentiation (48, 49). They thus validated the functionality of $R26^{SOX8}$ and $R26^{SOX9}$.

Upon Overexpression, SOX8 Affects Growth Plate Chondrocytes Similarly but More Potently than SOX9. Histology analysis of P10 mouse tibias showed that the formation of secondary ossification centers was delayed in $R26^{SOX8/+}Prx1Cre$ and $R26^{SOX9/+}Prx1Cre$ mice and even more in $R26^{SOX8/SOX8}Prx1Cre$ and $R26^{SOX9/SOX9}Prx1Cre$ mice (Fig. 4*A*). The CZ looked smaller in homozygous mutants, and while the HZ was shorter in heterozygotes, it was longer in homozygotes, especially SOX8 mutants. About one-third of hypertrophic cells persisted twice as long as normal in SOX9 mutants, while most cells persisted up to three times longer in SOX8 mutants. A similar hypertrophic phenotype was reported when SOX9 was overexpressed using *Col10a1* regulatory elements (50), arguing that overexpressed SOX8 and SOX9 cell-autonomously prevented the cells from dying or converting into osteoblasts.

To compare the impacts of SOX8 and SOX9 overexpression specifically in GPCs, we generated $R26^{SOX8/SOX8}Acan^{CreER}$ and $R26^{SOX9/SOX9}Acan^{CreER}$ mice [expressing tamoxifen-inducible Cre from the *Acan* locus (51)]. We gave tamoxifen to mutant and control mice at 3 wk and analyzed GPs 1 wk later. Like SOXE homozygotes generated with *Prx1Cre*, those generated with $Acan^{CreER}$ had a shortened RZ/CZ and elongated HZ; this phenotype was stronger in SOX8- than SOX9-overexpressing mice; and hypertrophic chondrocytes had a normal size (Fig. 4*B*). An EdU incorporation assay showed that both SOX8- and SOX9-overexpressing GPCs tended to proliferate more slowly in RZ/CZ, especially as cells progressed toward hypertrophic maturation (Fig. 4*C*). This result aligned with the smaller RZ/CZ in mutants (Fig. 4*B*); increasing expression of *Sox9* as CZ GPCs progress toward the HZ (Fig. 1*B*); and reduced cell proliferation

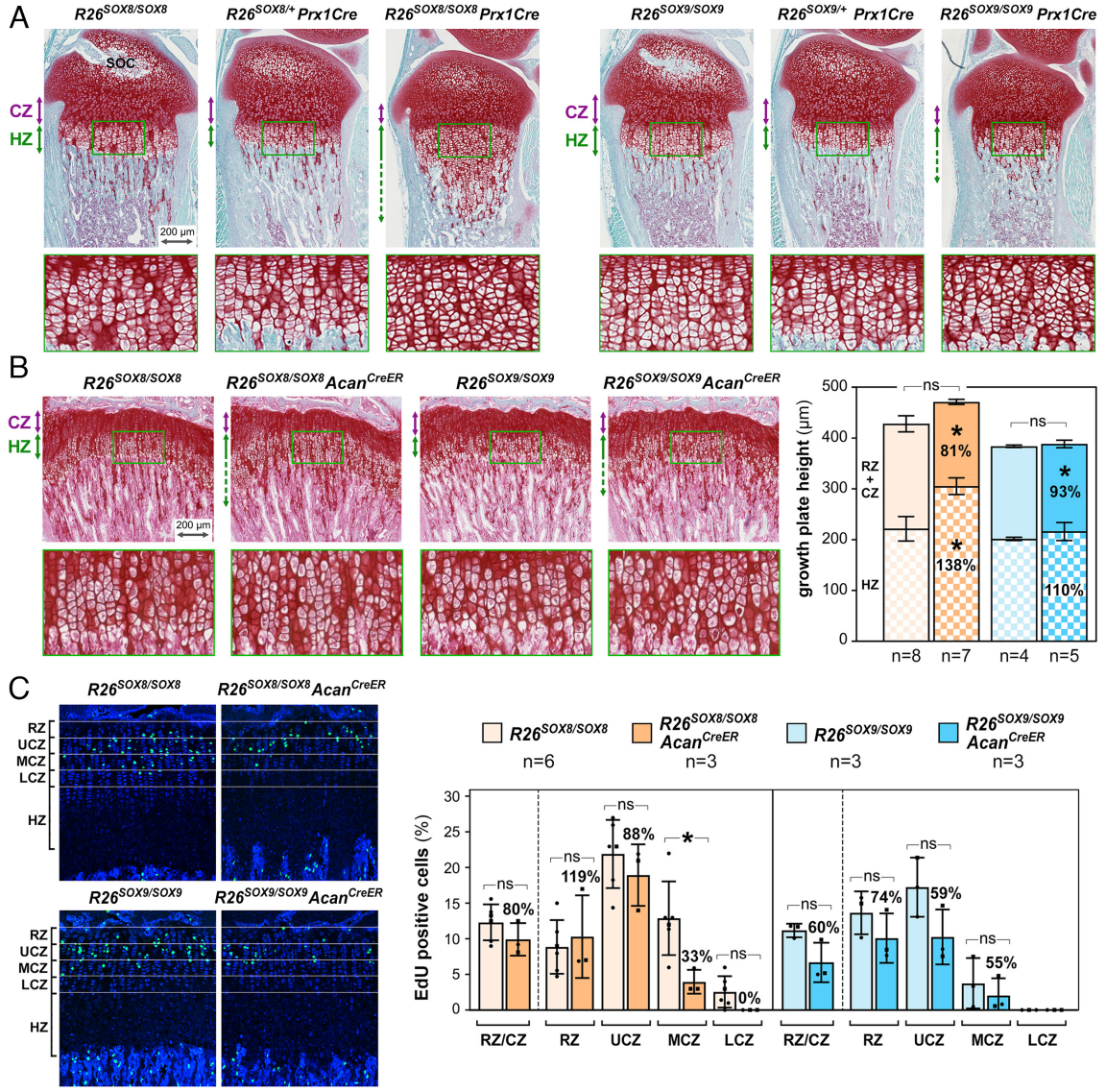

Fig. 4. Analysis of GPs from mice overexpressing SOX8 or SOX9 in skeletal cells. (*A*) *Top*, tibia proximal GPs of P10 littermates overexpressing SOX8 or SOX9 in the *Prx1Cre* lineage. Sections were stained with Safranin O (showing cartilage-specific matrix in red) and fast green. *Bottom*, high-magnification pictures of the HZ. The double-arrow green lines denoting the HZ are continuous where this zone is intact and dotted where it is partially replaced by bone. (*B*) *Top Left*, representative sections of tibia GPs collected 1 wk after P21 mice were injected with tamoxifen to overexpress SOX8 or SOX9 in the $Acan^{CreER}$ lineage. *Bottom Left*, high-magnification pictures of the HZ. *Right*, quantification of the GP zones. Brackets contain *t* test significance. (*C*) Cell proliferation assay. *Left*, representative pictures of EdU incorporation (green) in sections adjacent to those shown in (*B*). Cell nuclei are stained with DAPI (blue). The upper half of GPs is divided into four segments. *Right*, percentages of EdU^+ chondrocytes (n = 4 to 8 mice). Percentages of control values and *P* values from *t* tests are indicated.

reported when SOX9 was overexpressed in fetal chondrocytes using *Col2a1* regulatory sequences (40).

In conclusion, SOX8 and SOX9 overexpression showed that the two proteins are capable of exerting similar actions during GPC maturation, but SOX8 is more potent than SOX9.

Overexpressed SOX8 Is Chondrogenic on Its Own and Outperforms Overexpressed SOX9 in Rescuing Growth Plate Loss Due to Endogenous *Sox9* Inactivation. To determine whether SOX8 works as a complementary or redundant partner of SOX9 in GPCs, we compared mice in which we only inactivated *Sox9* in chondrocytes ($Sox9^{fl/fl}Acan^{CreER}$) or also overexpressed SOX8 ($R26^{SOX8/SOX8}Sox9^{fl/fl}Acan^{CreER}$) or SOX9 ($R26^{SOX9/SOX9}Sox9^{fl/fl}Acan^{CreER}$). Mice received tamoxifen at 3 wk and were analyzed 1 to 6 wk later. As described (32), *Sox9* inactivation caused GP closure and articular cartilage loss of specific proteoglycans within 2 wk (Fig. 5*A* and *SI Appendix*, Fig. S8). Forced expression of SOX8 or SOX9 provided little rescue in the first week, as GPs were still losing significant height, as GPCs were still dying massively, and as articular cartilage was still losing specific matrix components. SOX8 induced robust regeneration of GP cells and matrix in the following weeks, such that the GP was able to maintain its height for at least 6 wk while the control GP was shrinking to reach a similar height, corresponding to an adult, vestigial height. In contrast, SOX9 mildly consolidated the remaining GP tissue in the second week but did not prevent its subsequent loss. Accordingly, phospho-histone-3 immunostaining revealed active cell proliferation in regenerating $R26^{SOX8/SOX8}Sox9^{fl/fl}Acan^{CreER}$ cartilage but little in $R26^{SOX9/SOX9}Sox9^{fl/fl}Acan^{CreER}$ cartilage (Fig. 5*B*). $R26^{SOX8/SOX8}Sox9^{fl/fl}Acan^{CreER}$ GPCs did not form columns as they regenerated cartilage but nevertheless formed zones of immature and mature cells, as seen by cell morphology and *Pthlh* and *Ihh* expression (Fig. 5*C*). Thus, SOX8 is chondrogenic on its own (i.e., without SOX9) and even outperforms SOX9 in GPs.

SOX8 Is a More Stable Protein than SOX9. SOX8 may be more chondrogenic than SOX9 because of intrinsic protein properties. Our finding that SOX8 was less efficient than SOX9 in activating a cartilage-specific *Col2a1* reporter in vitro (10) does not exclude that SOX8 could be more potent than SOX9 in vivo. Another possibility is that SOX8 may be more stable than SOX9. Supporting it, the SOX8 protein level produced from the *R26* locus was always higher than that of SOX9 in primary $R26^{SOX8/SOX9}$ chondrocytes (*SI Appendix*, Fig. S6*F*). Furthermore, immunostaining of tissue sections confirmed a nearly complete loss of endogenous SOX9 in $Sox9^{fl/fl}Acan^{CreER}$ and $R26^{SOX8/SOX8}Sox9^{fl/fl}Acan^{CreER}$ GPCs 1 wk after tamoxifen treatment, and showed a weaker signal in

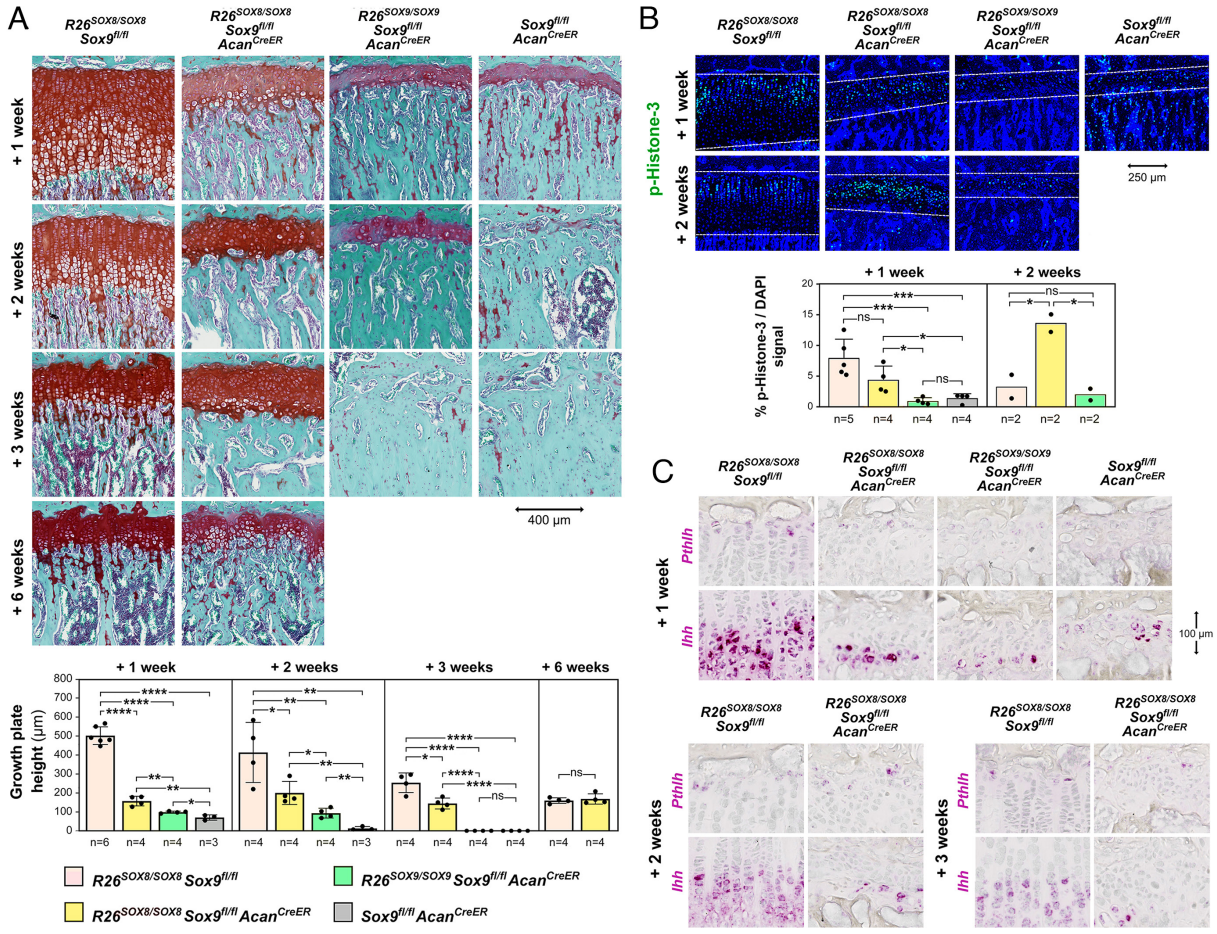

Fig. 5. SOX8 outperforms SOX9 in rescuing GP loss due to *Sox9* inactivation. (*A*) *Top*, representative images of tibia proximal GP sections from mice treated with tamoxifen at P21-24 and analyzed 1 to 6 wk later. Staining is with Safranin O and fast green. *Bottom*, quantification of average growth plate height. Each dot represents a distinct mouse. Statistical significance of differences between genotypes is indicated. *$P \le 0.05$; **$P \le 0.01$; ***$P \le 0.001$; ****$P \le 0.0001$; ns, nonsignificant. (*B*) Assessment of GPC proliferation by immunostaining of phospho-histone-3 (pH3). Mice treated as in panel (*A*). *Top*, representative pictures. Green, pH3 signal. Blue, DAPI signal. Dotted white lines delineate GPs. *Bottom*, percentages of pH3 signal relative to DAPI signal in the entire GPs. Data are presented as described for panel (*A*). (*C*) Representative images of in situ assays of *Pthlh* and *Ihh* RNAs in tibia proximal GPs from mice treated as described in panel (*A*) (n = 4). The hematoxylin counterstaining signal was desaturated, and the magenta signal was autocontrasted with Adobe Photoshop to help visualize RNA signals.

$R26^{SOX9/SOX9}Sox9^{fl/fl}Acan^{CreER}$ than control GPCs (Fig. 6*A*). This weaker signal explains the lack of chondrogenic rescue upon SOX9 overexpression. Interestingly, FLAG immunostaining showed a stronger signal in $R26^{SOX8/SOX8}Sox9^{fl/fl}Acan^{CreER}$ than $R26^{SOX9/SOX9}Sox9^{fl/fl}Acan^{CreER}$ GPCs, advocating that SOX8 is more stable than SOX9. To consolidate this notion, we compared the levels of the $R26^{SOX8}$ and $R26^{SOX9}$ RNAs and protein products in $R26^{SOX8/SOX9}Acan^{CreER}$ pup epiphyses. Even though the two *R26* alleles differed only by their SOXE sequences, the $R26^{SOX9}$ RNA level was about twice as high as that of $R26^{SOX8}$ (*SI Appendix*, Fig. S9*A*), while the SOX8 protein was 2.5-fold more abundant than the SOX9 protein (Fig. 6*B*). These data indicated that more SOX8 than SOX9 protein was present in GPCs from $R26^{SOXE}$ knockin mice, likely due to increased protein stability.

To test whether SOX8 is indeed more stable than SOX9, we used rat chondrosarcoma (RCS) cells, which have a stable GPC phenotype (52) and similar levels of *Sox8* and *Sox9* RNAs (*SI Appendix*, Fig. S9*B*). Of note, high conservation between mouse, rat, and human SOX8 and SOX9 allows extrapolation of data validity across species (10). RCS cell treatment with cycloheximide (5 μg/mL) to inhibit protein synthesis decreased the SOX9 protein level more than twofold within 2 h, but left the SOX8 protein level unchanged, even after 8 h (Fig. 6*C*). Treatment with MG-132 (10 μM) for 4 h, increased the SOX9 level (2.1-fold) more than the SOX8 level (1.5-fold), suggesting higher susceptibility of SOX9 than SOX8 to proteasomal degradation (Fig. 6*D*). Supporting this conclusion, treatment with LiCl (20 mM), which inhibits GSK3β and thereby stabilizes β-catenin and prompts SOX9 proteasomal degradation (53), decreased the SOX9 protein level by twofold in 24 h but did not affect the SOX8 protein level (Fig. 6*E*).

Together, these data suggest that higher stability explains, at least in part, why SOX8 is more chondrogenic than SOX9 in vivo.

Discussion

This study advances our understanding of the control of skeletal growth. It showed coexpression of SOX8 and SOX9 in upper GPCs and a shared role for these factors in promoting cell progression from the RZ to the CZ stage (Fig. 7). It also showed

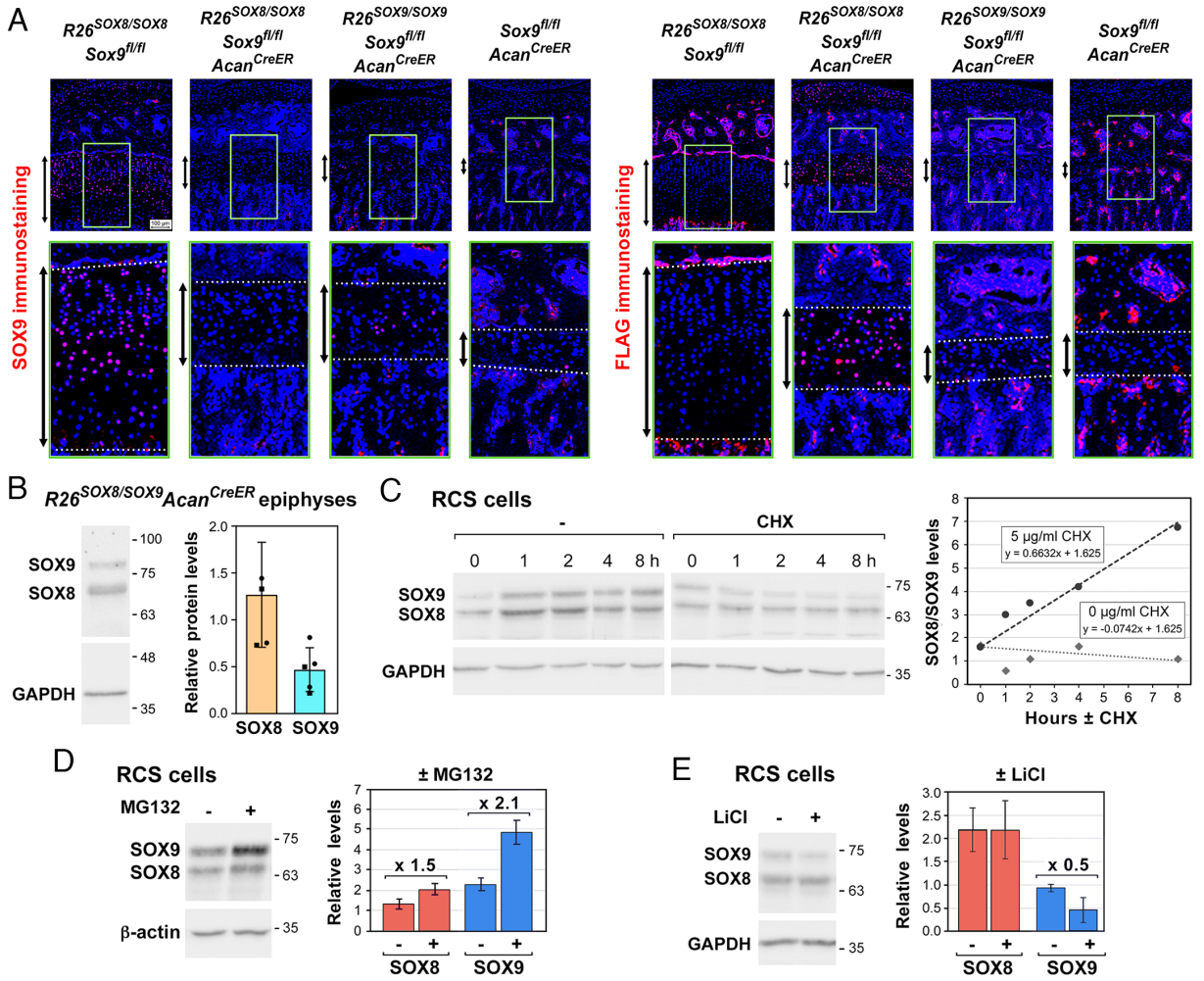

Fig. 6. SOX8 is more stable than SOX9. (*A*) SOX9 and FLAG immunostaining (red signals) of tibia proximal GPs of mice treated with tamoxifen at P21-P24 and analyzed 1 wk later. *Top*, low-magnification pictures. *Bottom*, high-magnification pictures of boxed areas. Double arrows and dotted white lines, GP height and boundaries, respectively. Nuclei were stained with DAPI. Note that the FLAG immunostaining assay shows signals in the bone marrow, suggesting that the mouse anti-FLAG antibody recognizes a nonspecific protein or that the anti-mouse secondary antibody recognizes endogenous IgG. (*B*) SOX8 and SOX9 levels in extracts from tibia and femur epiphyses of P12 $R26^{SOX8/SOX9}Acan^{CreER}$ pups (n = 5) treated with tamoxifen at P10-P11. *Left*, western blots hybridized with FLAG (*Top*) and GAPDH (*Bottom*) antibodies. *Right*, SOXE protein levels relative to GAPDH (n = 5). (*C*) SOX8 and SOX9 protein decay in RCS cells treated without or with 5 μg/mL cycloheximide (CHX) for 0 to 8 h. *Left*, western blots hybridized with SOX8 antibodies recognizing SOX8 and SOX9, and GAPDH antibodies. *Right*, quantification of SOX8/SOX9 protein levels. Linear curve fits are indicated. (*D*) SOX8 and SOX9 levels in cells treated without or with 10 μM MG132 for 4 h. *Left*, western blots hybridized with SOX8 and β-actin antibodies. *Right*, SOXE levels relative to β-actin (n = 3). (*E*) SOX8 and SOX9 levels in cells treated without or with 20 mM LiCl for 24 h. *Left*, westerns hybridized with SOX8 and GAPDH antibodies. *Right*, SOXE levels relative to GAPDH. All data are representative of two or more experiments.

that SOX8 is more chondrogenic than SOX9, possibly due to higher protein stability. These findings consolidate the proposition that SOX8 helps determine human height, add SOX8 to the SOX5/SOX6/SOX9 chondrogenic group, and suggest SOX8 as a SOX9 substitute in strategies to generate or regenerate cartilage.

New mouse models allowed us to identify shared functions of SOX8 and SOX9 in chondrogenesis and should likewise empower future studies in various processes. A first line of *Sox8*-null mice, which has *lacZ* inserted after a premature *Sox8* stop codon, phenocopies a previously generated line, where *lacZ* replaces the entire *Sox8* coding sequence (21). The mice exhibit weight reduction, previously attributed to small adipose stores. A second line ($Sox8^{-/-}$), only featuring the premature stop codon, does not have this phenotype. Since both lines were on the same genetic background, weight reduction in $Sox8^{lacZ/lacZ}$ mice cannot result from *Sox8* inactivation. It unlikely results from *lacZ*-encoded β-galactosidase expression either since it was not reported in other *lacZ* knock-ins. Rather, the *lacZ* knockin might alter the expression of a *Sox8* neighbor, possibly *Lmf1*, whose inactivation causes lipodystrophy (25). The $Sox8^{-/-}$ line and *Sox8* conditional null line ($Sox8^{fl/fl}$), from which $Sox8^{-/-}$ mice were derived, revealed roles for *Sox8* and *Sox9* in their common expression domain in GPs, giving confidence that these lines are reliable models for SOX8 studies. Mice with conditional gain-of-function alleles ($R26^{SOX8}$ and $R26^{SOX9}$) expressed SOX8 and SOX9 at similar levels as endogenous genes in GPCs and gave phenotypes consistent with known or predicted SOX8/SOX9 functions, supporting their suitability for more studies.

Previous studies showed SOX8 expression in human and mouse developing and adult cartilage but did not specify at which chondrocyte differentiation stages. We showed that while *Sox9* expression is already strong in embryonic chondroprogenitors, strong expression of *Sox8* is not detected until chondrocyte overt differentiation. The same is true for *Sox5* and *Sox6* (54, 55). Strikingly, *Sox8* and *Sox9* reach apparently similar expression levels in RZ and early-CZ cells, but *Sox8* is turned off when *Sox9* expression peaks in late-CZ and prehypertrophic cells. *Sox8* is thus active in a subset of the *Sox9* domain. Of note, *SOX10/Sox10*, the third SOXE gene, is not expressed in mammalian cartilage but is expressed in fish cartilage (56, 57). The three genes arose during evolution from a single ancestor (58). They conserved expression in neural crest but acquired divergent expression in downstream and other lineages. This likely occurred through gain or loss of tissue-specific enhancers. Several *Sox9* enhancers have been delineated that are active at distinct chondrocyte differentiation stages, but others remain unknown, including those specific for early-stage GPCs (59–61). The delineation of these enhancers and those directing *Sox8* expression would help understand the mechanisms regulating these cells and variants underlying skeletal variations and diseases.

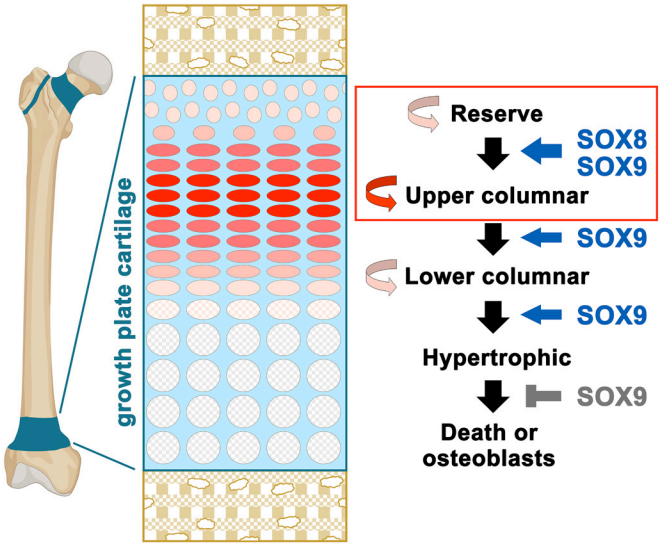

Fig. 7. Model highlighting the shared contribution of SOX8 and SOX9 to skeletal growth. *Left*, growth plates (blue) are cartilaginous domains in the bones of growing individuals that are responsible for skeletal elongation. The present study (red box) shows that reserve chondrocytes, which are round and weakly proliferative (pink circling arrow), are stimulated by coexpressed SOX8 and SOX9 to differentiate into highly proliferative cells (red circling arrow), which pack into longitudinal columns. Only SOX9 is expressed in further maturing chondrocytes, where it allows cell proliferation and subsequent hypertrophy rather than immediate death or osteoblastic conversion. By enhancing early-stage chondrocyte proliferation and subsequently supporting cell hypertrophy, SOX8/SOX9 and SOX9, respectively, promote the two main cellular events that drive skeletal growth.

Sox8/Sox9 compound mutant mice uncovered a concerted function of SOX8 and SOX9 in stimulating GPC progression from RZ to CZ. This process includes raising cell proliferation and repressing *Pthlh,* but its control is largely unknown. We predict that SOX8/SOX9 promote early-CZ cell proliferation by stimulating differentiation rather than cell cycle genes because differential effects of the factors on cell proliferation have been reported. SOX8/SOX9 overexpression was found in this study and previous ones to reduce late-CZ GPC proliferation and promote hypertrophy, while *Sox9* loss abruptly arrests CZ cell proliferation and induces posthypertrophic maturation. Direct repression of *Pthlh* by SOX8/SOX9 is unlikely as SOXE are proven transcriptional activators, not repressors. Further studies are thus needed to define the mechanisms regulating early GPCs, including the contributions of SOX8/SOX9.

Sox9 inactivation in skeletogenic cells precludes chondrogenesis (40) and inactivation in GPCs prompts cell apoptosis or osteoblastic conversion (32, 41), indicating that *Sox8* cannot compensate for *Sox9* loss. This can be explained by lower expression of *Sox8* than *Sox9* in chondroprogenitors and GPCs, and by the fact that *Sox8* expression, like *Sox5* expression, is reduced upon *Sox9* loss (32). Thus, SOX9 elevates its chondrogenic capabilities by reinforcing the expression of functional partners, as it does for its own expression (59, 62).

Our loss-of-function studies revealed a shared function of SOX8 and SOX9 in early-stage GPCs but did not differentiate complementary from redundant functions. Forcing SOX8 or SOX9 expression showed that SOX8 acted like SOX9 in late-CZ and HZ cells but was more potent. Thus, SOX8 is able not only to stimulate early-CZ cell progression but also to work together or in parallel with SOX9 at subsequent GPC stages. Evidence that unlike SOX5/SOX6, SOX8 does not empower SOX9 but is functionally similar and even superior was provided by showing that forcing SOX8 expression in *Sox9*-deprived GPCs resulted in more effective GP regeneration than forcing SOX9 expression. These reciprocal functions of the two factors can be explained by their highly similar functional domains. They may, however, not be able to fully substitute for one another in all conditions, as SOX10 replacement by SOX8 in the mouse revealed functional equivalence of the factors in some but not all lineages (63). We explained the higher potency of SOX8 over SOX9 in GPCs by higher stability of the protein, namely resistance to proteasomal degradation. The SOX9 C terminus was shown to sensitize the protein to proteasomal degradation, but the residues involved remain elusive (53). This region, which contains a transactivation domain, is only 52% similar between SOX8 and SOX9. Sequence divergence may

thus explain differences in transactivation ability and protein stability.

By ascertaining that both human *SOX8* and mouse *Sox8* are expressed in GPCs and by revealing that mouse *Sox8* fosters early-CZ cell proliferation, a driver of skeletal growth, our study strengthens the GWAS-derived proposition that *SOX8* may help determine human height (23). The fact that $Sox8^{-/-}$ and $Sox8^{fl/fl}Prx1Cre$ mice had normal trunk and limb lengths does not challenge this proposition but fuels evidence that many processes are more sensitive to gene expression dosage in humans than mice. This notion applies to skeletal growth since neither $Sox9^{+/-}$ fetuses (39) nor $Sox9^{fl/+}Prx1Cre$ postnatal mice show substantial skeletal length deficiency, even though human *SOX9* haploinsufficiency causes campomelic dysplasia, including dwarfism. This notion also applies to sex differentiation, where human *SOX9* haploinsufficiency frequently causes XY DSD, whereas mouse *Sox9* heterozygosity causes DSD only in a *Sox8*-null background (12, 64, 65) and *Sox8* is required along with *Sox9* for Sertoli cell maintenance (17). Thus, the fact that $Sox8^{fl/fl}Sox9^{fl/+}Prx1Cre$ mice, but not single mutants, exhibit marked skeletal shortness is sufficient to support the proposition that *SOX8* variants could affect human height. The height of humans with *SOX8* variants was not reported, but a deviation from the norm in these individuals could be difficult to assign to abnormal sex hormone levels or to intrinsic skeletal growth defects (15). It is worth noting that the GWAS for human height heritability was conducted with control individuals and that the rs12597498 SNP is located closer to enhancers active in skeletogenic tissues than to the *SOX8* coding sequence. Gonadal and skeletal enhancers of *SOX8* are likely distinct since this is the case for *SOX9* (59, 60, 66). Thus, height could be affected in humans with variants in *SOX8* skeletal enhancers without causing DSD.

In conclusion, this study pinpointed a role for SOX8 and SOX9 in early-stage GPCs that promotes skeletal growth. By increasing knowledge of mechanisms governing skeletogenesis, it also raises new questions on the molecular roles of SOX8 and SOX9 in early-stage GPCs and on the mechanisms driving the expression and stability of each factor in chondrocytes and other cells. Answering these questions in future studies should help better understand human variations and diseases and develop effective strategies to treat diseases.

Materials and Methods

Mice. Details for all animal models can be found in *SI Appendix*. Mice were used as approved by the Institutional Animal Care and Use Committees of the Cleveland Clinic and Children's Hospital of Philadelphia.

X-rays, Whole-Mount and Histology Assays. All assays to quantify mouse bone lengths and to analyze growth plates at tissue, cell, and molecular levels on tissue sections are described in *SI Appendix*.

RNA and Protein Assays. The methods used to isolate RNA and protein from mouse tissues and cultured cells and to analyze them by RT-qPCR or western blot can be found in *SI Appendix*.

Cell Cultures. RCS cell cultures and treatments are explained in *SI Appendix*.

Statistical Analyses. Differences between datasets were evaluated using two-tailed Student's *t* tests when comparing two experimental groups and one-way ANOVA tests when comparing multiple groups. Differences that reached $P \leq 0.05$ were considered significant.

Data, Materials, and Software Availability. GSE107649 RNA-seq data of human fetal growth plates (30), GSE111358 RNA-seq data of human adult articular cartilage (31), and GSE162033 single-cell RNA-seq data of juvenile mouse epiphyses (32) were accessed from the Gene Expression Omnibus (GEO) database. All other data are included in the manuscript and/or *SI Appendix*.

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