SHP2 regulates skeletal cell fate by modifying SOX9 expression and transcriptional activity

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Chondrocytes and osteoblasts differentiate from a common mesenchymal precursor, the osteochondroprogenitor (OCP), and help build the vertebrate skeleton. The signaling pathways that control lineage commitment for OCPs are incompletely understood. We asked whether the ubiquitously expressed protein-tyrosine phosphatase SHP2 (encoded by Ptpn11) affects skeletal lineage commitment by conditionally deleting Ptpn11 in mouse limb and head mesenchyme using “Cre-loxP”-mediated gene excision. SHP2-deficient mice have increased cartilage mass and deficient ossification, suggesting that SHP2-deficient OCPs become chondrocytes and not osteoblasts. Consistent with these observations, the expression of the master chondrogenic transcription factor SOX9 and its target genes Acan, Col2a1, and Col10a1 were increased in SHP2-deficient chondrocytes, as revealed by gene expression arrays, qRT-PCR, in situ hybridization, and immunostaining. Mechanistic studies demonstrate that SHP2 regulates OCP fate determination via the phosphorylation and SUMOylation of SOX9, mediated at least in part via the PKA signaling pathway. Our data indicate that SHP2 is critical for skeletal cell lineage differentiation and could thus be a pharmacologic target for bone and cartilage regeneration.

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INTRODUCTION

Vertebrate skeletal development occurs through intramembranous and endochondral ossification. Intramembranous ossification involves the direct differentiation of mesenchymal stem cells into osteoblasts and is responsible for the ossification of cranial bones and for appositional bone growth.1,2 Endochondral ossification requires the formation of cartilaginous anlagen and their subsequent replacement by osteoblasts, and contributes to longitudinal bone growth.3,4 During endochondral ossification, mesenchymal cells condense and then differentiate into early proliferating chondrocytes, which undergo further differentiation to establish a cartilage growth plate. Cells within growth plates are organized into distinct zones containing resting, proliferating, prehypertrophic, and hypertrophic chondrocytes. Hypertrophic chondrocytes undergo apoptosis and are replaced by osteoblasts or transdifferentiate into osteoblasts, which produce bone.5-10

Signaling molecules and transcription factors, including SOX911,12 β-Catenin13 and RUNX214,15 regulate skeletal development. The transcription factor SOX9 is a master regulator of chondrogenesis, essential for chondrocyte specification, proliferation, and early differentiation.2,16,17 SOX9 promotes the expression of important chondrocytic genes, including Col2a1, Col10a1, and Acan.11,16 Whereas SOX9 is critical for chondroid cell fate determination and chondrocytic differentiation, β-Catenin is a critical regulator of osteoblast differentiation and osteogenesis. Wnt ligand-induced β-Catenin signaling mediates major events in endochondral and intramembranous bone formation.18 Increased abundance of SOX9 or β-Catenin promote chondrogenesis or osteoblastogenesis, respectively.3,4,15,21

Transcription factor activity can be regulated at the transcriptional and post-translational levels. One post-translational mechanism, SUMOylation, tags proteins with small ubiquitin-like molecules (SUMO)22,23 that can alter the biological functions of their targets. Another post-translation regulatory modification is phosphorylation. SOX9 can be SUMOylated and phosphorylated.24,25 How SUMOylation is regulated remains elusive, but pathways involving the serine-threonine kinases ERK, ROCK, and PKA have been implicated.26-30

SHP2, encoded by PTPN11, is a ubiquitously expressed Src homology-2 domain-containing protein-tyrosine phosphatase. SHP2 is required for activation of the RAS/ERK pathway downstream of almost all receptor protein-tyrosine kinases (RTK) and...
cytokine and integrin receptors. There is evidence that SHP2 plays an important role in skeletal development. Notably, autosomal dominant mutations in PTPN11 cause Noonan and LEOPARD syndromes (NS and LS, respectively), which feature skeletal manifestations that can include pectus carinatum or LEOPARD syndromes (NS and LS, respectively), which feature autosomal dominant mutations in PTPN11 that are responsible for the skeletal manifestations that can include pectus carinatum or LEOPARD syndromes (NS and LS, respectively), which feature autosomal dominant mutations in PTPN11. There is evidence that SHP2 plays a crucial role in PRRX1-expressing OCPs in both endochondral and intramembranous bone formation.

RESULTS

SHP2 deficiency in limb and head mesenchyme affects skeletogenesis

To investigate the role of SHP2 in limb and head mesenchymal cells during early skeletogenesis, mice carrying Ptpn11 floxed (Ptpn11fl/fl) alleles were crossed to paired related homeobox 1 Tg(Prrx1-Cre) mice to generate Tg(Pprx1-Cre;Ptpn11fl/fl) (SHP2Prrx1KO), Tg(Prrx1-CreERT2;Ptpn11fl/fl) (SHP2Prrx1KO/ER) and Tg(Prrx1-CreERT2;p2C;Ptpn11fl/fl) (SHP2Prrx1KO/ER) mice. The Prrx1 promoter is active both in the undifferentiated mesenchyme of limb buds and in the peristome of adult mice. Therefore, in SHP2Prrx1KO and SHP2Prrx1KO/ER mice, Ptpn11 is specifically deleted in PRRX1-expressing mesenchymal osteochondroprogenitors (OCPs) and their progeny. The deletion efficiency of Ptpn11 floxed alleles in OCPs and their derivatives by Prrx1-Cre or Prrx1-CreERT2 was determined by Western blot analysis, which revealed that SHP2 abundance was reduced by >80% and >70% in purified OCPs and their derivatives from SHP2Prrx1KO or tamoxifen-treated SHP2Prrx1KO/ER mice, respectively, compared with those from SHP2Prrx1CTR and SHP2Prrx1CTR/ER controls (Fig. S1b). SHP2Prrx1CTR and SHP2Prrx1CTR/ER mice had no discernible phenotype, so subsequent analyses were focused on SHP2Prrx1KO and SHP2Prrx1KO/ER mice.

SHP2Prrx1KO mice were born at the expected Mendelian ratios and they were the same size as the SHP2Prrx1CTR littermates at birth, on average (48.8 ± 3.5) mm vs. (49.0 ± 4.2) mm long at P0.5, P = 0.96. By postpartum day 10 (P10) both the controls and knockouts had grown significantly (P < 0.001, both comparisons), however, the body length of SHP2Prrx1KO mice was only ~75% of SHP2Prrx1CTR controls [SHP2Prrx1CTR/SHP2Prrx1KO: (97.4 ± 5.4) mm vs. (74.4 ± 8.4) mm; n = 5, P < 0.001]. Most of the mutants died within 3 weeks after birth, likely due to respiratory failure. Other skeletal phenotypes included short and deformed forelimbs and hindlimbs, and pectus anomalies (carinatum or excavatum) (Fig. 1a, S1c). Interestingly, a few of the surviving SHP2Prrx1KO mice developed localized hypertrichosis on the forelimbs and hind limbs (Fig. 1a, bottom), which has been reported to be related to increased SOX9 expression in the hair follicle. Alcian blue and Alizarin red-stained skeletons in the mutants revealed split sternums, short appendicular bones and short tracheas (Fig. 1b). Compared with SHP2Prrx1CTR mice, the skulls of SHP2Prrx1KO mice were incompletely formed, with short and deformed forelimbs and hindlimbs, and concave-appearing ribcage (white arrow) (Figs. 1a, bottom), which has been reported to be related to increased SOX9 expression in the hair follicle.

Fig. 1 Mice lacking SHP2 in PRRX1-expressing osteochondroprogenitors (OCPs) display skeletal dysplasia and impaired ossification of multiple skeletal elements. a Representative photographs of 10-day-old SHP2Prrx1CTR and SHP2Prrx1KO mice. SHP2Prrx1KO mice have skeletal dwarfism, with short and deformed forelimbs and hindlimbs (black arrows), and concave-appearing ribcage (white arrow) (n = 5). A few SHP2Prrx1KO survivors grew long hairs surrounding both forelimbs and hindlimbs (black arrows) (n = 3). b Alcian blue/Alizarin red-stained skeletons of the entire skeleton, rib cage and sternum, forelimbs, hindlimbs, ilium, spine, trachea, and the skull of 7-day-old SHP2Prrx1CTR and SHP2Prrx1KO mice. Note that SHP2Prrx1KO mice have small ribcages, retarded ossification of sternum and skull, short and deformed forelimbs, hindlimbs, trachea, and metatarsal and phalangeal joint digits (n = 3).
SHP2 deficiency in OCPs promotes cell proliferation and chondrocytic differentiation
To begin to understand how SHP2 in OCPs regulates skeletogenesis, femur and tibia sections from 7-day-old SHP2_Prrx1CTR and SHP2_Prrx1KO mice were stained with hematoxylin and eosin (H&E) and Safranin O/fast green. The bones from SHP2_Prrx1CTR mice appeared normal, with distinct mineralized cortices and trabecular bone and hematopoietic cells occupying the cavity between the organized growth plate cartilage. By contrast, the bones of SHP2_Prrx1KO mice contained large epiphyseal cartilage masses, but no clearly ossified cortical nor trabecular bone. Instead, substantial numbers of chondrocytes remained in the regions that would have been the diaphyseal cortices of the femurs in SHP2_Prrx1KO mice, and there were persistent “cartilage islands” scattered throughout the marrow cavity (Fig. 2a). Moreover, the growth plates of SHP2_Prrx1KO mutants were profoundly affected. Rather than the typical, tightly-organized columns of chondrocytes seen in SHP2_Prrx1CTR mice, SHP2_Prrx1KO mutant growth plates were taller and much less organized. The growth plates in the mutant animals had a 2-fold increase in the height of epiphyseal cartilage and a 5-fold increase of the pre-hypertrophic and hypertrophic layers of the growth plate cartilage compared with controls (Fig. 2b, S2a). To rule out the possibility that impaired ossification in SHP2_Prrx1KO mice was due to the effect of SHP2 deficiency on osteoblast differentiation rather than OCP commitment, we deleted SHP2 in committed osteoblasts by crossing the Ptpn11 floxed allele to Tg(Bglap-Cre)วล mice, As Tg(Bglap-Cre) is expressed in committed osteoblasts, this deletion differentiates the roles for SHP2 in OCPs and fully differentiated osteoblastic cells. Importantly, Tg(Bglap-Cre/Ptpn11 Indy), mice had normal appearing trabecular and cortical bone at day P0.5 and by 8 weeks old (Fig. S10), which was not the case for Tg(Prrx1-Cre/Ptpn11 Indy) mice. These results strongly suggest that SHP2’s major role occurs during OCP commitment to the osteoblast lineage.

SHP2-deficient chondrocytes produce autocrine and paracrine signals. We therefore sought to determine whether the phenotype we observed in SHP2_Prrx1KO mice was due to the SHP2-deficient OCPs (i.e., whether it was OCP-autonomous) or whether these OCPs might be influencing other cells. To do so, we performed a cell lineage tracing study by crossing SHP2_Prrx1CTR and SHP2_Prrx1KO mice to cell membrane-targeted two-color fluorescent Cre reporter mice Rosa26 mTmG (R26 mTmG, expressing red fluorescent protein in cells prior to Cre exposure, and green fluorescent protein in Cre-expressing cells and their derivatives). As expected, GFP+ OCPs were found in a thin, continuous layer on the surface of mineralized bone cortex (periosteum) and in the
epiphyseal cartilage of SHP2 Prrx1CTR mice (Fig. 2c, left). By contrast, this cell population was scattered throughout what would have been the cortex and bone marrow compartments of the femurs and tibiae in SHP2Prrx1KO mice; no mature calcified cortical bone formed in these mice at postnatal day 10 (Fig. 2c, right; S2b). Coupled with their persistent cartilage phenotype, the localization of GFP+ cells to regions that should have been mineralized bone suggests that SHP2 deficiency resulted in the cell-autonomous differentiation of OCPs along the chondrocyte rather than osteoblast lineage. However, the possibility that altered paracrine signaling arising from SHP2-deficient OCPs contributes to the skeletal phenotypes cannot be conclusively excluded.

To ask whether SHP2 deficiency might also affect cell proliferation, as in other types of cells, we administered BrdU to 10-day-old SHP2Prrx1CTR and SHP2Prrx1KO mice for 4 h prior to euthanasia. Proximal tibia sections from these mice revealed an increase of BrdU-positive cells in the epiphyseal cartilage of SHP2Prrx1KO mice (34.4%), compared with SHP2Prrx1CTR controls (22.3%) (Fig. 2d). Similar findings were obtained via an in vitro BrdU labeling assay using chondroprogenitors isolated from SHP2Prrx1CTR and SHP2Prrx1KO mice (Fig. S2c); their viability was, however, comparable, as determined by annexin-V staining and flow cytometry analysis. Taken together, these experiments suggest that cell fate in OCPs is regulated by SHP2, as is the rate of growth plate chondrocyte proliferation and differentiation.
SHP2 differentially regulates chondrocytic gene expression

Given that SHP2 influences OCP cell fate, we next sought to define the genes and pathways that might be involved. To start, we used differential gene expression array analysis to identify transcripts that varied in abundance in SHP2-deficient OCPs and their derivatives, compared to wild-type controls. GFP⁺ OCPs and their derivatives from SHP2⁺⁺/CTR/R26⁺/+ and SHP2⁻⁻/KO/R26⁺/+ mice were purified by FACS. Preliminary characterization demonstrated that GFP⁺ OCPs and their derivatives express Prx1, Sox9 and mesenchymal cell surface markers SCA1, STRO1 and CD44, although SHP2 deficiency increased the expression of SCA1 and STRO1 (Fig. S3d). 100 ng of total RNA from each was analyzed using Affymetrix GeneChip Mouse Genome 2.0 Arrays. Transcripts that increased or decreased in abundance by >2-fold between SHP2⁺⁺/CTR and SHP2⁻⁻/KO mice, and had a P value <0.05 were considered to reflect significant alteration of expression. Of 953 transcripts with differential abundance in OCPs from SHP2⁻⁻/KO;R26⁺/+ and SHP2⁺⁺/CTR;R26⁺/+ mice, 397 increased, and 556 decreased. Genes encoding cytoplasmic proteins (12% increased; 20% decreased), ECM proteins (7% increased; 9% decreased),...
nuclear proteins and transcription factors (8% increased; 7% decreased), plasma membrane proteins (8% increased; 13% decreased) were particularly affected (Fig. 3a).

Chondrocytic and osteoblastogenic genes with altered abundance are shown in Fig. 3b. Among those transcripts that increased in abundance with SHP2 knockout, the greatest changes were in genes previously known to be critical to chondrogenesis and cartilage homeostasis, such as Acan, Col2a1, Col9a1, Col9a3, Col1a1, Comp, Matn1, Matn3, and Matn4. Most of these genes harbored binding sites for SOX9 (Fig. 3b asterisks; S4,58,69). Genes known to be involved in osteoblastogenesis, such as Mmp13 and Ptc1, however, were downregulated. The differential gene expression observations were confirmed by qRT-PCR analyses, which verified the upregulation of several chondrogenic genes transcripts, such as Acan, Col2a1, Col10a1, Sox9, Ihl1, Alpl, and PthrP in OCPs and their derivatives from SHP2 Prrx1KO;R26mTmG mice, compared to SHP2 Prrx1CTR;R26mTmG controls. By contrast, expression of the adipogenic marker Ppar-r and the osteogenic marker Col1a1 were comparable in the cells from these mice (Fig. 3c).

There are two potential interpretations for these findings: increased expression of chondrocytic transcripts in SHP2-deficient committed chondrocytes, and/or increased numbers of cells committed to the chondroid lineage. We hope to differentiate between these two possibilities in future experiments by employing single cell RNA sequencing methods.

To gain insight into the mechanism through which SHP2 regulates skeletogenesis, we used Ingenuity® Pathway Analysis (IPA®, Qiagen) to predict the pathways affected in the differential gene expression profiles from SHP2 Prrx1CTR;R26mTmG and SHP2 Prrx1KO;R26mTmG OCPs. The top 20 predicted signaling pathways are listed in Fig. 3d, and included the protein kinase A (PKA), RAS/ERK, WNT/β-CATENIN, integrin, p70S6 kinase, P3 kinase/AKT, PTEN, IGFl, and mTOR. Overall, pathways involving cell development processes and post-translational modification were the most frequently identified (Fig. 3p3). Many of these have been previously associated with skeletal development.

Enhanced chondrogenesis is associated with increased SOX9 abundance in SHP2 deficient KO (cKO) mice. Given the elevated abundance of several chondrogenic genes in OCPs and their derivatives from SHP2 Prrx1KO;R26mTmG mice, we next evaluated which cell population in the epiphyseal cartilage of SHP2 Prrx1KO;R26mTmG mice was affected by SHP2 deficiency in vivo. Entire tibiae were collected from 1.5-day-old SHP2 Prrx1CTR;R26mTmG and SHP2 Prrx1KO;R26mTmG mice. After fixation, frozen sections were subjected to in situ hybridizations to

![Fig. 5](image_url) **Fig. 5** SHP2 regulates SOX9 abundance by modifying its phosphorylation and SUMOylation. a) Diagrams showing the putative AGC family kinase phosphorylation motifs and SUMOylation sites on murine SOX9. b) Images of immunostained tibial growth plate and periosteal sections, demonstrating enhanced protein SUMOylation and phosphorylation of SOX9 in 7-day-old SHP2 Prrx1KO mice, compared with controls. Enlarged view of corresponding boxed areas in the periosteal regions and tibia growth plates are shown on the right (n = 3 for each genotype). c) Representative fluorescence microscopic images (top) and bar graphs of geometric mean values of SOX9 and SUMO1 abundance, as determined by flow cytometric analysis (bottom), demonstrating that the abundance of SOX9 (red) and protein SUMOylation (red) were increased in SHP2-deficient OCPs and their derivatives, compared with controls (n = 3 for each genotype, *P < 0.05, Student’s t test). d) Western blot analysis of total cell lysates (left and middle) and anti-SOX9 immunoprecipitates (right) demonstrating the elevated abundance and SUMOylation of endogenous SOX9 (red arrows) in SHP2-deficient (KO) vs. sufficient (CTR) OCPs that had been transiently transfected with pcDNA3/HASumo1 plasmids. Note the increase of overall protein SUMOylation in SHP2-deficient OCPs. e) Western blot analysis showing that blocking PKA activation in SHP2-deficient cKO chondroprogenitors with the inhibitor KT5720 compromises SOX9 phosphorylation, SUMOylation and its abundance. Images in d and e are representative of three experiments. Quantitative data relative to the controls are provided beneath each blot. TCL total cell lysate.
evaluate the mRNA abundance of Sox9, Acan, collagen types II (Col2a1) and X (Col10a1), and Mmp13, and by immunostaining to visualize Sox9 expression. Compared with SHP2pocr1;CTR;R26tmTmG mice, Sox9 transcript abundance was only slightly increased in the perichondrium and growth plate cartilage of SHP2pocr1;KO;R26tmTmG mice (Fig. 4a). However, immunostaining revealed significantly increased Sox9 levels in both of these cell types suggesting that the Sox9 protein persisted with SHP2 knockdown (Fig. 4b, arrows), with corresponding increases in the abundance of Acan, Col2a1, and Col10a1 mRNAs in the growth plate cartilage and periosteal cells of SHP2pocr1;KO;R26tmTmG mice, compared with SHP2pocr1;CTR;R26tmTmG controls (Fig. 4c, d; SS). No apparent difference in Mmp13 mRNA or MMP13 protein was detected in the growth plate cartilage of SHP2pocr1;CTR;R26tmTmG and SHP2pocr1;KO;R26tmTmG mice, whereas their levels were increased in the perichondral areas of SHP2pocr1;KO;R26tmTmG mice (Fig. 4c, d; arrows); SS). Consistent with these findings, cartilage and ossified endochondral bone comprised 82.6% and 17.4% of tibia length, respectively, in SHP2pocr1;KO;R26tmTmG mice, compared with 42.8% and 57.2% in SHP2pocr1;CTR;R26tmTmG mice (Fig. 4e). The increased abundance of Sox9, Acan, Col2a1, Col10a1, and Mmp13 in the epiphysis and perichondrial areas of SHP2pocr1;KO;R26tmTmG mice provides further support for our interpretation that SHP2 deficiency in OCPs causes enhanced chondrocytic differentiation mediated by Sox9.

SHP2 regulates Sox9 abundance by modifying its phosphorylation and SUMOylation

With strong evidence that the fate of PRRX1-expressing OCPs is regulated by SHP2 via effects on Sox9 levels, we sought to explore the underlying mechanism. Sox9 abundance is tightly controlled by multiple signaling pathways, at both the transcriptional and post-translational levels. We focused on phosphorylation and SUMOylation, for several reasons. First, Sox9 is expressed in various tissue stem cells, including OCPs, and plays a crucial role in fate determination, survival and proliferation. Importantly, it has been reported that Sox9 is stabilized by phosphorylation and SUMOylation via the PKA signaling pathway. Second, pathway analysis of our array data predicted that PKA signaling is substantially affected by SHP2 deficiency in OCPs and their derivatives (Fig. 3d). And third, we found increased Sox9 abundance, but not Sox9 mRNA in SHP2pocr1;KO OCPs, which strongly suggested post-translational control.

There are 2 AGC family kinase consensus motifs and 2 SUMOylation sites in Sox9 (Fig. 5a), but Ser181 and Lys198 have been reported to be the primary phosphorylation and SUMOylation sites, respectively. Accordingly, immunostained tibia sections from 7-day-old mice for SUMO1 and pSOX9Ser181. We observed increased protein SUMOylation and Sox9 Ser181 phosphorylation (Fig. 5b) in the cells within the periosteal areas and growth plate cartilage of SHP2pocr1;KO mice, compared with SHP2pocr1;CTR controls. We confirmed these findings using FACs sorting for GFP+ OCPs and their derivatives from 1–3 day-old mice, and immunostaining with anti-SOX9 and -SUMO1 antibodies, respectively (Fig. 5c, top). The geometric means of the SOX9 and SUMO1 signals were significantly elevated upon Ptnp11 deletion (Fig. 5c, bottom). We obtained similar results using control and Ptnp11-knockdown ATDC5 cells that transiently expressed GFP-tagged SUMO1 and RFP-tagged human SOX9 (Fig. 5a).

The above experiments did not prove that the elevated protein SUMOylation in SHP2-deficient mice in vivo and in OCPs in vitro affected Sox9 abundance. To address this question, HA-tagged SUMO1 was transfected into SHP2-sufficient and deficient chondroprogenitors. Immunoprecipitation and western blot analyses demonstrated that Sox9 was indeed SUMOylated, and its SUMOylation was enhanced in the absence of SHP2. Most importantly, Sox9 protein abundance in SHP2-deficient chondroprogenitors was higher than in SHP2-sufficient cells (Fig. 5d).

Given that SOX9 SUMOylation is augmented by phosphorylation on Ser181 and the fact that the PKA signaling pathway ranked highly in the Ingenuity pathway analysis, we seemed likely that PKA would function downstream of SHP2 to regulate the phosphorylation and SUMOylation of SOX9. To test this hypothesis, groups of SHP2-sufficient and deficient chondroprogenitors were transfected with HA-tagged SUMO1. After 72 h of incubation, half of the transfected cells were exposed to the PKA inhibitor KT5720 for 6 h. Cells were then lysed, and SOX9 was immunoprecipitated and its phosphorylation and SUMOylation were evaluated by immunoblotting. PKA inhibition markedly compromised SOX9 phosphorylation (as judged by SOX9Ser181 phosphorylation) and SUMOylation in SHP2-deficient chondroprogenitors. KT5720 acts as a competitive antagonist of ATP at its binding site on the PKA catalytic subunit. Like other protein kinase inhibitors, KT5720 can have off-target effects and inhibits other protein kinases, such as ERK, when it exceeds its optimal dosage. We monitored ERK inhibition as a readout of PKA off-target effects in this study and found comparable pERK activation in KT5720-treated and untreated OCPs. These data together suggest that PKA activity in SHP2-deficient chondroid cells stabilizes SOX9 and enhances chondrogenesis (Fig. 5e).

SHP2 regulates lineage commitment of mesenchymal cells by tilting the balance of SOX9 and β-CATENIN expression

Having established a role for SHP2 in the regulation of SOX9 and chondrogenesis, we next examined how SHP2 deficiency in PRRX1-expressing OCPs affects osteogenesis in SHP2pocr1;KO mice. β-CATENIN activity is crucial for the differentiation of OCPs into bone cells, and mice lacking β-CATENIN in OCPs fail to form osteoblasts and calvarial bone. Furthermore, β-CATENIN protein abundance has been shown to be antagonistically regulated by SOX9. Given our finding of defective endochondral and intramembranous bone formation and elevated Sox9 abundance in SHP2pocr1;KO mice, we examined the effect of Ptnp11 deletion on β-CATENIN and Ctnnb1 abundance in the periosteal and bone cells of SHP2pocr1;CTR and SHP2pocr1;KO mice, compared with SHP2pocr1;CTR controls (Fig. 6a). We observed increased protein SUMOylation and Sox9 Ser181 phosphorylation (Fig. 5b) in the cells within the periosteal areas and growth plate cartilage of SHP2pocr1;KO mice, compared with SHP2pocr1;CTR controls. We confirmed these findings using FACs sorting for GFP+ OCPs and their derivatives from 1–3 day-old mice, and immunostaining with anti-SOX9 and -SUMO1 antibodies, respectively (Fig. 5c, top). The geometric means of the SOX9 and SUMO1 signals were significantly elevated upon Ptnp11 deletion (Fig. 5c, bottom). We obtained similar results using control and Ptnp11-knockdown ATDC5 cells that transiently expressed GFP-tagged SUMO1 and RFP-tagged human SOX9 (Fig. 5a).

To provide additional evidence that elevated Sox9 directly influences the skeletal phenotype in SHP2pocr1;KO mice, we used a genetic rescue approach. A single Sox9 floxed allele was bred to (SHP2pocr1;CTR;R26tmTmG and SHP2pocr1;KO;R26tmTmG) mice. After serial breeding we were able to generate lines of SHP2pocr1;CTR;R26tmTmG and SHP2pocr1;KO;R26tmTmG mice that were haploinsufficient for Sox9 in PRRX1-expressing cells (SHP2pocr1;CTR;Sox9+/−;R26tmTmG and SHP2pocr1;KO;Sox9+/−;R26tmTmG mice). With SHP2pocr1;CTR, SHP2pocr1;KO;R26tmTmG mice had no discernable phenotype. By contrast, haploinsufficiency of Sox9 in PRRX1-expressing cells in SHP2pocr1;KO mice (SHP2pocr1;KO;Sox9+/−;R26tmTmG) markedly restored endochondral ossification compared with SHP2pocr1;KO;Sox9+/−;R26tmTmG mice, which manifested as an increase of the primary ossification center and expression of Ctnnb1, Col1a1 and Ibsp in periosteum and spongiosa bone (Fig. 6a–c). Accordingly, the length of the epiphysial cartilage and primary ossification center was reduced and increased, respectively, in SHP2pocr1;KO;Sox9+/−;R26tmTmG mice (Fig. 6e, S6b). Collectively, these data suggest that elevated Sox9 in PRRX1-expressing cells of SHP2pocr1;KO mice plays a central role...
in chondroid cell development and abnormal endochondral ossification.

Mosaic SHP2 deficiency in PRRX1-expressing OCPs causes exostoses and enchondromas

SHP2 loss-of-function (LOF) mutations in humans are found in families segregating metachondromatosis, where a somatic second-hit mutation is postulated to be responsible for the development of cartilage lesions. In mice, homozygous SHP2 LOF mutations in COL2a1-expressing chondroid cells and the cathepsin K-expressing (CTSK) groove of Ranvier cells produce enchondromas (cartilaginous masses formed inside bone) and exostoses (outgrowth of cartilage capped masses on bone surfaces) similar to those found in metachondromatosis patients.35,37,39,58 In families segregating metachondromatosis, where a somatic second-hit (loss-of-heterozygosity, LOH) in murine OCPs might cause cartilaginous lesions by breeding mice with Ptpn11 floxed, null (Δk11), and R26mTmG reporter allele to Tg(Prx1-CreERT2) mice that express a tamoxifen-inducible Cre under the control of the Prx1 promoter.81 Pregnant dams were administered one low dose of TM at E13.5 (50 mg·kg⁻¹), and Tg(Prx1-CreERT2;Ptpn11Δk11;R26mTmG) (SHP2<sup>LOH/ER/mTmG</sup>) and Tg(Prx1-CreERT2;Ptpn11Δk11; R26mTmG) (SHP2<sup>CTR/ER/mTmG</sup>) offspring were identified postnatally and evaluated for the development of skeletal disease. SHP2<PBB>l<sup>CTR/ER/mTmG</sup></sup> and SHP2<sup>LOH/ER/mTmG</sup> mice were born at the expected Mendelian ratios and had normal gross appearance within the first 8 weeks after birth. By 11 weeks of age, however, 86% (12/14) of SHP2<sup>LOH/ER/mTmG</sup> mice had noticeable exostoses, mostly in the vertebrae and at the end of tubular bones (Fig. 7a). In addition, the growth plates of the radius and ulna were merged in SHP2<sup>LOH/ER/mTmG</sup> mice (Fig. 7b). By contrast, SHP2<sup>CTR/ER/mTmG</sup> mice appeared phenotypically normal. The Rosa26<sup>mTmG</sup> reporter study revealed that exostotic lesions in SHP2<sup>LOH/ER/mTmG</sup> mice were comprised of both green (recombined) and red (non-recombined) chondroid cells (Fig. 7c), suggesting that wild-type and SHP2-deficient chondroid cells participate in the growth of exostoses. Interestingly, SHP2<sup>CTR/ER/mTmG</sup> mice that received TM injection at postnatal week 2 had no apparent enchondromas or exostoses detectable by micro-CT when they were killed at post-natal week 12 (Fig. 7f). These data demonstrate that SHP2 has a critical time-dependent role in modulating the proliferation and chondrocytic differentiation of OCPs and that aberrant SHP2 signaling in OCPs can lead to neoplastic cell growth and cartilage tumor formation at certain developmental stages.

**DISCUSSION**

SHP2 is a ubiquitously expressed cytoplasmic protein-tyrosine phosphatase that has cellular context-specific roles in regulating the viability, proliferation, and differentiation of various cell types. Its role in skeletal development is particularly intriguing, as it is involved in the regulation of skeletal cell fate. SHP2 deficiency in PRRX1-expressing OCPs causes exostoses and enchondromas, which are cartilaginous masses formed inside bone and on the surface of bone, respectively. These lesions are often associated with genetic syndromes, such as metachondromatosis, where a second-hit mutation is thought to occur in somatic cells. In mice, homozygous SHP2 LOF mutations in COL2a1-expressing chondroid cells and the cathepsin K-expressing (CTSK) groove of Ranvier cells produce enchondromas and exostoses similar to those found in metachondromatosis patients.

The study by Zuo et al. demonstrates that SHP2 deficiency in PRRX1-expressing OCPs leads to abnormal endochondral ossification and cartilage lesions. The authors employed a mosaic SHP2 deficient model by breeding mice with Ptpn11 floxed, null (Δk11), and R26mTmG reporter allele to Tg(Prx1-CreERT2) mice. The tamoxifen-inducible Cre under the control of the Prx1 promoter was used to generate mosaic SHP2 deficiency in PRRX1-expressing OCPs. These mice were evaluated for the development of skeletal disease, and SHP2<sup>CTR/ER/mTmG</sup> and SHP2<sup>LOH/ER/mTmG</sup> mice were born at the expected Mendelian ratios and had normal gross appearance within the first 8 weeks after birth. By 11 weeks of age, 86% (12/14) of SHP2<sup>LOH/ER/mTmG</sup> mice had noticeable exostoses, mostly in the vertebrae and at the end of tubular bones. By contrast, SHP2<sup>CTR/ER/mTmG</sup> mice appeared phenotypically normal. The Rosa26<sup>mTmG</sup> reporter study revealed that exostotic lesions in SHP2<sup>LOH/ER/mTmG</sup> mice were comprised of both green (recombined) and red (non-recombined) chondroid cells. Interestingly, SHP2<sup>CTR/ER/mTmG</sup> mice that received TM injection at postnatal week 2 had no apparent enchondromas or exostoses detectable by micro-CT when they were killed at post-natal week 12. These data demonstrate that SHP2 has a critical time-dependent role in modulating the proliferation and chondrocytic differentiation of OCPs and that aberrant SHP2 signaling in OCPs can lead to neoplastic cell growth and cartilage tumor formation at certain developmental stages.
types of cells and tissues. In this study, we found that SHP2 influences skeletal phenotype through SOX9. SHP2 was specifically ablated in PRRX1-expressing osteochondroprogenitors (OCPs), a subpopulation of mesenchymal cells with the ability to form appendicular bone and cartilage. Mice lacking SHP2 in PRRX1-expressing OCPs displayed a broad range of skeletal abnormalities, including defective ossification of the skull and long bones, small and deformed concave- or convex-shaped ribcages, and short and deformed limbs and joints affecting the hip, knee, ankle and phalanges. Interestingly, a few of the surviving mice developed localized hypertrichosis on the forelimbs and hind limbs. Lineage tracing suggested that the skeletal phenotypes of SHP2 Prrx1 KO mice are OCP-autonomous. Gene arrays and pathway analyses revealed differential expression of 953 genes in SHP2-deficient OCPs, and more than 20 primary signaling pathways were affected substantially by SHP2 deficiency. Among significantly upregulated were genes known to be critical for chondrogenesis, such as Col2a1, Col10a1, Ihh, PthP, Acan, and Sox9, while genes involved in osteoblastogenesis, such as Sp7 and Cttnb1, decreased in abundance. The chondrocytic master transcription factor SOX9 was elevated in cells within the perilosteal areas and "cartilage islands" of the bone marrow compartment of SHP2prrx1KO mice. Most importantly, haploinsufficiency of SOX9 in the PRRX1-expressing cells partially rescued the defective endochondral ossification and epiphyseal cartilage development in SHP2prrx1KO mice.

Mechanistically, we found that SHP2 regulates SOX9 abundance in OCPs and chondrocytic cells by modulating the phosphorylation and SUMOylation of SOX9. Our data are consistent with previous reports that SOX9 contains target sites for the AGC family serine/threonine kinases PKA, RSK2, and ROCK. Moreover, SOX9 interacts physically with PPRX1, and phosphorylation of SOX9 at Ser64 and Ser181 by PKA modulates its transcriptional activity. Using conditional knockout mice and PRRX1-expressing chondroprogenitor cell lines, we found that SOX9 protein abundance was lowered when SHP2 was deficient. PKA inhibition with KT5720 lowered the amount of SOX9 in control and SHP2-deficient cells in vitro, suggesting that PKA regulation is downstream of SHP2. This observation was also supported by the localized hypertrichosis in some of the surviving SHP2Prrx1KO mice. It has been reported that increased abundance of SOX9, due to the deficiency of Trp1 in the hair follicle, accounts for the growth of long hairs. Given that Prx1-Cre activity has not been observed in limb ectoderm, the hypertrichotic phenotype in Tg(Ptpn11fl/fl;Prx1Cre) mice might be caused by miscommunication between PRRX1 + SHP2-deficient mesoderm cells and ectodermal cells within the hair follicle. Further studies will be required to delineate the specific cellular and molecular mechanisms behind these intriguing observations.

SUMOylation is a multistep enzymatic process that leads to the covalent attachment of a small ubiquitin-like modifier (SUMO) protein to a lysine residue of a target protein. This modification can regulate the activity, stability, subcellular localization, and the interaction of the modified protein with other proteins. SUMOylation is involved in a variety of cellular processes, including transcriptional regulation, protein degradation, and cell cycle control.

Fig. 7 Mosaic Ptpn11 deletion at E13.5 in Prx1-CreER2-expressing OCPs leads to osteochondromas and enchondromas. a High resolution plain radiographs showing the outgrowth of cartilaginous lesions (arrows) on the tail vertebrae, distal femur, distal tibia, radius and ulna (n = 14). i: SHP2CTR/ER/mTmG; ii, iii:SHP2LOH/ER/mTmG. b Images of tail vertebrae, distal femur, and ulna sections, stained with Alcian blue and nuclear fast red, demonstrating cartilaginous lesions (arrows) in SHP2KO/ER/mTmG mice, compared with SHP2CTR/ER/mTmG mice. Note that there is no clear separation between the growth plates of the radius and ulna in SHP2LOH/ER/mTmG mice. c Merged fluorescent microscopic images showing cells in which the Rosa26β-gal reporter allele has (green fluorescence) or has not (red fluorescence) been recombined by Cre recombinase in a SHP2LOH/ER/mTmG mouse. A large cartilage outgrowth (arrow adjacent to the intervertebral disc (IVD) and tail vertebral growth plate (GP) is shown. The GP and cartilage outgrowth are outlined (white dashed line). Note that green and red chondrocytes are observed in the outgrowth. Scale bars in a represent 4 mm; Scale bars in b and c represent 250 μm (For the studies in b and c, n = 3 for each genotype). d Proposed model of how SHP2 regulates chondrogenesis and endochondral bone formation by altering the expression of the transcription factors SOX9 and β-CATENIN. P, phosphorylation; S, SUMOylation.
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repertoire of interactions of targeted proteins. Two putative SUMOylation sites have been identified in murine Sox9—K61 and K396. Of these, K396 has been shown to be the primary SUMOylation site. We demonstrated that Sox9 SUMOylation is increased in SHP2-deficient OCPs and their derivatives, and that SUMOylation is associated with increased expression of Col2a1, Col10a1, Aggrecan, Ihh, and Pthr. SUMOylation of Sox9 on K396 affects its stability and activity, leading to an increase in both the abundance and transcriptional activity of Sox9. However, it has also been reported that Sox9 SUMOylation represses its transcriptional activity in 293 T cells in vitro. The reason for this discrepancy remains unclear, but it might reflect the two distinct cellular contexts and the reporter systems used.

Increasing evidence indicates that Sox9 levels and transcriptional activity are also regulated by the ubiquitin-proteasome-mediated degradation pathway. Inhibition of the 26 S proteasome by MG132 increases Sox9 activity. In addition, mutation of K398, the primary site for ubiquitination and SUMOylation of Sox9, increased Sox9 protein stability and transcriptional activity. Taken together, these data suggest a model where SUMO molecules compete with ubiquitin molecules to bind to K396. The balance between SOX ubiquitination and SUMOylation likely controls the chondrogenic processes.

SHP2 loss-of-function (LOF) mutations in humans cause the autosomal dominant, incompletely penetrant, cartilage tumor syndrome “metachondromatosis.” However, the cell-type(s) in which second hit Pttn11 mutation causes cartilage lesions remains unclear. We created a Pttn11 LOH mouse model in PRRX1-expressing cells, and found that SHP2 deficiency in PRX1+ OCPs was involved in the pathogenesis of cartilage lesions, indicating that SHP2 functions as a tumor suppressor in cartilage and is required in OCPs for cartilage development and homeostasis.

In summary, our data support a model in which SHP2 deficiency in OCPs leads to an increase in Sox9, although we do not exclude the possibility that other pathways also are involved. This increase in Sox9 either directs OCP cells towards the chondrocytic and away from the osteogenic lineage or delays their commitment to the osteogenic lineage (Fig. 7d). As a consequence of SHP2 deficiency, abnormal cartilage growths develop at sites where mineralized bone would normally form, including the periosteum and primary spongiosum. Our study suggests that other signaling pathway(s) in addition to the SHP2/PKA/Sox9 axis may also regulate the fate decision of OCPs. The ability to manipulate cell fate choice by modulating SHP2 activity suggests a new strategy therapeutically manipulate chondrogenesis in patients with a variety of cartilage-related disorders, ranging from tumors to degenerative diseases such as osteoarthritis. Given the “double-edged sword” effects of SHP2 deficiency on chondrogenesis and osteogenesis, caution also must be taken on the skeletal system with the use of SHP2 inhibition to treat neoplastic diseases.

**Materials and Methods**

Transgenic mice

Sox9 floxed (Sox9*), Ptn11 floxed (Ptn11*) and null alleles (ΔK11) were described previously. The Tg(Rosa26mTmG), Tg (Rosa26tm11aFlm), Tg (Prx1-Cre), Tg (Prx1-CreER), and Tg (Prx1-CreERKO) mice were also reported previously. PCR genotyping conditions for the Ptn1 mice were published. All assays were performed in triplicate. The antibodies and reagents were purchased from commercial sources: McAb against phospho(p)-ERK1/2, ERK1/2, and McAb against HA were from Cell Signaling Inc.; McAb against murine COL2a1 was from R&D Systems; McAb against Prrx1 was from Abcam; McAb against SUMO1, and pRFP-hSox9 were published. Antibodies and BrdU staining kit were obtained from BD Pharmingen. PE-conjugated, anti-CATENIN were purchased from Cell Signaling (MA). Anti-BrdU staining solutions were purchased from Poly Scientific. Antibodies and reagents

Polyclonal and monoclonal antibodies (PCAb and McAb) were purchased from commercial sources: PCAb against phospho(p)-ERK1/2, ERK1/2, and McAb against HA were from Cell Signaling Inc.; McAb against murine COL2a1 was from R&D Systems; PCAb against MMP13, Sox9 and COL10a1 were from Abcam; PCAb against SUMO1, and Shp2 were purchased from Invitrogen. Epitomics and UBI, respectively. PCAb against β-CATENIN were purchased from Cell Signaling (MA). Anti-BrdU antibodies and BrdU staining kit were obtained from BD Pharmingen. PE-conjugated, affinity purified PCAb against Sox9 were purchased from Bios. The PKA inhibitor KTS720 was purchased from Santa Cruz. PE-conjugated anti-Rabbit IgG was purchased from Cell Signaling. Alcian blue and Alizarin Red S staining solutions were purchased from Poly Scientific.

Histological analysis

To examine the effect of SHP2 deficiency on gross skeletal development, mice were eviscerated after euthanasia and fixed in 4% paraformaldehyde (PFA) overnight at 4°C. Fixed skeletons were stained with Alcian blue and Alizarin Red S staining solutions and were examined by X-ray imaging. Mice were analyzed and quantified at 37°C for 48 h. Undigested bony tissue was discarded by centrifugation and cultured in DMEM/F12 medium (1:1) (Invitrogen), supplemented with 10% FBS, 1% ampicillin and streptomycin (Invitrogen). After 2–3 passages, GFP + OCP and derivatives were enriched by FACS and used for total RNA isolation. SHP2-deficient and deficient chondroprogenitor cell lines were established by immortalizing PRRX1-expressing progenitors from 1 to 2-day-old SHP2Prrx1CTR;R26ZsgFi and SHP2Prrx1KO;R26ZsgFi mice with SV40 large T antigen and cultured at the indicated time points and used for X-ray, histological, biochemical and biological analyses. All transgenic mice were maintained on C57BL/6 background and studied in accordance with the Institutional Animal Care and Use Committee approved protocols.

Cells and DNA constructs

Primary PRRX1+ OCPs and their derivatives were isolated from 1 to 3-day-old pups. To maximize the yield of GFP+ OCPs and their derivatives, the epiphyseal portions of knee joints (including distal femurs and proximal tibia) were collected and incubated with trypsin-EDTA (0.25%, Invitrogen) at 37°C for 30 min. After washing with PBS twice, tissues were further incubated with hyaluronidase (2 mg·mL−1; Sigma) for 2 h and hyaluronidase/collagenase D mixture (1 mg·mL−1, Roche) for 4 h in DMEM at 37°C. Undigested bony tissue was discarded by filtration. OCPs and their derivatives were then collected by centrifugation and cultured in DMEM/F12 medium (1:1) (Invitrogen), supplemented with 10% FBS, 1% ampicillin and streptomycin (Invitrogen). After 2–3 passages, GFP + OCP and derivatives were enriched by FACS and used for total RNA isolation. SHP2-deficient and deficient chondroprogenitor cell lines were established by immortalizing PRRX1-expressing progenitors from 1 to 2-day-old SHP2Prrx1CTR;R26Zsg and SHP2Prrx1KO;R26Zsg mice with SV40 large T antigen and cultured in DMEM/F12 media supplemented with 10% FBS and 1% penicillin/streptomycin. Plasmids pcDNA3 HA-SUMO1, pEGFP-C1/ SUMO1, and pRFP-hSox9 were published and obtained from Dr. Riko Nishimura (Osaka University) and Dr. Akihiro Ito (RIKEN) in Japan, respectively.
SHP2<sub>Prrx1CTR/R26<sup>mtMg</sup></sub> and SHP2<sub>Prrx1KO/R26<sup>mtMg</sup></sub> mice and frozen section were used to visualize green fluorescent protein (GFP)-positive cells microscopically. DAPI was used to counterstain the nucleus. All fluorescent and phase contrast images were obtained using a Nikon digital fluorescence microscope and an Aperio slide scanner (Vista, CA). Immunostaining was carried out using Vectorstain ImmunPACT/DAB kit following the manufacturer's instructions.

Gene expression array and quantitative RT-PCR analyses Total RNA was extracted using RNeasy kit (Qiagen) from short-term-expanded and FACs-enriched primary OCPs and their derivatives from 3-day-old SHP2<sub>Prrx1CTR/R26<sup>mtMg</sup></sub> and SHP2<sub>Prrx1KO/R26<sup>mtMg</sup></sub> mice, and analyzed for integrity by using an Agilent 2100 Bioanalyzer. For differential gene expression analysis, three RNA samples per mouse line were amplified using Invitrogen WT Expression Kit and hybridized to Affymetrix Mouse Gene ST 2.0 arrays. Ingenuity Pathway Analysis (IPA) Software from Invitrogen WT Expression kit and hybridized to Agilent 2100 Bioanalyzer. For differential gene expression (SHP2<sub>LOH/ER;/R26mTmG</sub>) and SHP2<sub>Prrx1CTR/R26<sup>mtMg</sup></sub>, three RNA samples per mouse line were amplified using Invitrogen WT Expression Kit and hybridized to Affymetrix Mouse Gene ST 2.0 arrays. Ingenuity Pathway Analysis (IPA) Software from Invitrogen was used for pathway analysis.

To validate the expression of differentially regulated genes on arrays, qRT-PCR was performed with RT<sup>2</sup>SYBR<sup>®</sup>Green qRT-PCR kit on a Bio-Rad CFX machine using cDNA that was synthesized using 1 µg total RNA with iScript<sup>™</sup> cDNA Synthesis Kit (Bio-Rad). All samples were normalized to Gapdh and gene expression data were presented as fold-increases or -decreases compared with controls. All primer sequences used for this study are available by request.

In situ hybridization Femurs and tibiae were collected from post-natal day 1.5 neonates. After fixation in 4% paraformaldehyde overnight, 7 µm cryostat sections were used for in situ hybridization with probes against murine Sox9, Acan, Col2<sub>1</sub>, Ctnnb1, Ibsp, and Mmp13. Hybridization and detection of hybridization signals were achieved using RNAscope HD-Brown kit per the manufacturer's instruction (Advanced Cell Diagnostics).

Flow cytometry analysis Primary OCPs and their derivatives from SHP2<sub>Prrx1CTR/R26<sup>mtMg</sup></sub> and SHP2<sub>Prrx1KO/R26<sup>mtMg</sup></sub> mice were either analyzed or purified by FACs for GFP<sup>+</sup> cells. Purified GFP<sup>+</sup> OCPs and their derivatives were fixed, permeabilized,<sup>68</sup> and stained with PE-conjugated anti-SOX9 or SUMO1 antibodies. All samples were subjected to FACS analysis in 4% paraformaldehyde overnight, 7 µm cryostat sections were used for in situ hybridization with probes against murine Sox9, Acan, Col2<sub>1</sub>, Ctnnb1, Ibsp, and Mmp13. Hybridization and detection of hybridization signals were achieved using RNAscope HD-Brown kit per the manufacturer's instruction (Advanced Cell Diagnostics).

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Immunoprecipitation and western blot analysis Cells were lysed in modified NP-40 lysis buffer [0.5% NP40, 150 mmol·L<sup>-1</sup> NaCl, 1 mmol·L<sup>-1</sup> EDTA, 50 mmol·L<sup>-1</sup> Tris (pH 7.4)], supplemented with a protease inhibitor cocktail (1 mmol·L<sup>-1</sup> PMSF, 10 mg·ml<sup>-1</sup> aprotonin, 0.5 mg·ml<sup>-1</sup> antipain, and 0.5 mg·ml<sup>-1</sup> pepstatin). Immunoprecipitations were performed on cleared lysates, as described previously.<sup>10</sup> For immunoblotting, cell lysates (30–50 µg) were resolved by SDS-PAGE, transferred to PVDF membranes, and incubated with primary antibodies for 2 h or overnight at 4 °C (according to the manufacturer's instructions), followed by HRP-conjugated secondary antibodies (Bio-Rad).

Induction of exostotic lesions in SHP2<sub>LOH/ER/R26<sup>mtMg</sup></sub> mice Timed matings were performed with 8 to 12-week-old females caged overnight with males, and vaginal plugs were checked the following morning. Fertilization was assumed to occur at midnight, and the time of plug identification was defined as E0.5. TM was administered to pregnant females at E13.5 (50 mg·kg<sup>-1</sup>) and pups with the genotypes Tg(Prrx1-CreER<sub>T2</sub>;<Ptpn11<sup>fl<sub>lox</sub></sup>-R26<sup>mtMg</sup></sub>) and Tg(Prrx1-CreER<sub>T2</sub>;<Ptpn11<sup>fl<sub>lox</sub></sup>-R26<sup>mtMg</sup></sub>;SHP2<sub>LOH/ER/R26<sup>mtMg</sup></sub>), were identified by PCR after birth. SHP2<sub>LOH/ER/R26<sup>mtMg</sup></sub> and SHP2<sub>Prrx1CTR/R26<sup>mtMg</sup></sub> mice were sacrificed at post-natal week

11 or 15 for radiographic and histologic analysis, as described earlier. X-ray images were taken immediately after euthanasia using a digital radiography system (MX-20, Faxitron Bioteics, LLC, Tucson, AZ).

Statistical analysis Statistical differences between groups were evaluated with Student's t tests or two-way ANOVAs followed by Holm-Sidak post hoc comparisons. P values < 0.05 were considered statistically significant. Analyses were performed by using Prism 3.0 (Graph-Pad, San Diego, CA), SigmaPlot (Systat Software, Inc. San Jose, CA) and Excel (Microsoft Inc., Redmond, WA).

Material availability All mouse lines, DNA constructs and cell lines are available upon request.

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AUTHOR CONTRIBUTIONS Conceived and designed the experiments: W.Y. and M.L.W. Critiqued the manuscript: M.G.E., Y.M.S., and B.G.N. Performed the experiments: R.M.K., C.Z., L.J.W, M.E.B., A.M.R., M.D., D.M.M, Q.W., C.S., and W.Y. Analyzed the data: R.M.K., M.E.B., C.Z., B.G.N., and W.Y. Wrote the paper: D.M.M and W.Y.

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