

# Runx1 and Runx2 cooperate during sternal morphogenesis

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## SUMMARY

Chondrocyte differentiation is strictly regulated by various transcription factors, including Runx2 and Runx3; however, the physiological role of Runx1 in chondrocyte differentiation remains unknown. To examine the role of Runx1, we generated mesenchymal-cell-specific and chondrocyte-specific *Runx1*-deficient mice [*Prx1 Runx1<sup>fl/fl</sup>* mice and  $\alpha 1(II)$  *Runx1<sup>fl/fl</sup>* mice, respectively] to circumvent the embryonic lethality of *Runx1*-deficient mice. We then mated these mice with *Runx2* mutant mice to obtain mesenchymal-cell-specific or chondrocyte-specific *Runx1*; *Runx2* double-mutant mice [*Prx1* DKO mice and  $\alpha 1(II)$  DKO mice, respectively]. *Prx1 Runx1<sup>fl/fl</sup>* mice displayed a delay in sternal development and *Prx1* DKO mice completely lacked a sternum. By contrast,  $\alpha 1(II)$  *Runx1<sup>fl/fl</sup>* mice and  $\alpha 1(II)$  DKO mice did not show any abnormal sternal morphogenesis or chondrocyte differentiation. Notably, *Runx1*, *Runx2* and the *Prx1*-Cre transgene were co-expressed specifically in the sternum, which explains the observation that the abnormalities were limited to the sternum. Histologically, mesenchymal cells condensed normally in the prospective sternum of *Prx1* DKO mice; however, commitment to the chondrocyte lineage, which follows mesenchymal condensation, was significantly impaired. In situ hybridization analyses demonstrated that the expression of  $\alpha 1(II)$  collagen (*Col2a1* – Mouse Genome Informatics), *Sox5* and *Sox6* in the prospective sternum of *Prx1* DKO mice was severely attenuated, whereas *Sox9* expression was unchanged. Molecular analyses revealed that Runx1 and Runx2 induce the expression of *Sox5* and *Sox6*, which leads to the induction of  $\alpha 1(II)$  collagen expression via the direct regulation of promoter activity. Collectively, these results show that Runx1 and Runx2 cooperatively regulate sternal morphogenesis and the commitment of mesenchymal cells to become chondrocytes through the induction of *Sox5* and *Sox6*.

**KEY WORDS:** Runx, Chondrogenesis, Sternum, Mouse

## INTRODUCTION

Endochondral bone formation is a key regulatory event affecting several aspects of skeletal development, such as longitudinal growth and the formation of the thoracic cavity, which are essential for movement and respiration in vertebrates (de Crombrughe et al., 2001; Kronenberg, 2003; Karsenty et al., 2009). A crucial step in the process of endochondral bone formation is chondrocyte differentiation. The importance of this step is evident from the severe skeletal dysplasia diseases that result from mutations affecting chondrocyte differentiation (Ornitz, 2005).

Chondrocytes develop through a series of sequential steps (de Crombrughe et al., 2001; Kronenberg, 2003; Lefebvre and Smits, 2005). First, mesenchymal cells migrate to the location in which the

future skeleton will be formed (Hall and Miyake, 2000). They then gather close to each other to form mesenchymal condensations, after which they begin to produce chondrocyte-specific extracellular matrix components such as  $\alpha 1(II)$  collagen (*Col2a1* – Mouse Genome Informatics) (cartilagenous anlagen). At this stage they are recognized as chondroblasts. Finally, chondroblasts differentiate into proliferative chondrocytes, which eventually mature into hypertrophic chondrocytes.

Since the beginning of the 1990s, much progress has been made in understanding the transcriptional control of chondrogenesis (Lefebvre and Smits, 2005; Karsenty et al., 2009). *Sox9* is essential for the differentiation of mesenchymal cells into chondroblasts, as shown by the fact that mice lacking *Sox9* possess no chondrocytes (Bi et al., 1999; Akiyama et al., 2002). In addition, *Sox5* and *Sox6*, which are expressed after mesenchymal condensations are formed, induce the differentiation of chondroblasts into chondrocytes in combination with *Sox9* (Lefebvre et al., 1998; Ikeda et al., 2004). Indeed, *Sox5<sup>-/-</sup>*; *Sox6<sup>-/-</sup>* mice undergo normal mesenchymal condensation; however, these cells do not further differentiate into chondrocytes (Smits et al., 2001). Interestingly, the expression of *Sox5* and *Sox6* is abolished in mice lacking *Sox9*, in contrast to the normal expression of *Sox9* in *Sox5<sup>-/-</sup>*; *Sox6<sup>-/-</sup>* mice (Smits et al., 2001; Akiyama et al., 2002). This result indicates that *Sox9* is genetically upstream of *Sox5* and *Sox6*; however, the molecular mechanism for the induction of *Sox5* and *Sox6* remains to be elucidated.

Once cells are committed to the chondrocyte lineage, *Runx2*, a master regulator of osteoblast differentiation (Stein et al., 2004; Karsenty et al., 2009), induces chondrocyte hypertrophy directly

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through its expression in nonhypertrophic chondrocytes or represses chondrocyte hypertrophy through its expression in the bone collar (Takeda et al., 2001; Hinoi et al., 2006). Mice overexpressing *Runx2* in chondrocytes show ectopic chondrocyte hypertrophy in places where hypertrophic chondrocytes do not normally exist (Takeda et al., 2001; Sato et al., 2008). By contrast, *Runx2*<sup>-/-</sup> mice exhibit abnormal chondrocyte hypertrophy in most skeletal elements except the distal limbs (Komori et al., 1997). *Runx3*, another member of the Runx family, also regulates chondrocyte hypertrophy in cooperation with *Runx2* (Yoshida et al., 2004); however, the role of *Runx1* in chondrocyte differentiation in vivo has never been addressed. In this study we addressed the role of *Runx1* at various stages of chondrocyte differentiation using tissue- and stage-specific *Runx1*-deficient mice. Our results demonstrate that *Runx1*, in cooperation with *Runx2*, is essential for the commitment of mesenchymal cells to the chondrocyte lineage through the induction of *Sox5* and *Sox6*.

## MATERIALS AND METHODS

### Generation of *Runx1* conditional knockout mice

To generate *Runx1*-floxed mice, targeting vectors harboring *loxP* sites as well as a floxed neomycin resistance cassette were electroporated into embryonic stem (ES) cells (for details, see Fig. S1 in the supplementary material). ES cells containing the floxed allele (after *Neo<sup>R</sup>* removal) were injected into 129Sv/EV blastocysts to generate chimeric mice. *Runx1*<sup>f/+</sup> mice were crossed with *Prx1* (*Prrx1* – Mouse Genome Informatics) Cre or *α1(II)* Cre mice (Martin and Olson, 2000; Takeda et al., 2001) to generate *Prx1* Cre *Runx1*<sup>f/+</sup> mice or *α1(II)* Cre *Runx1*<sup>f/+</sup> mice, respectively, and their progeny were intercrossed to obtain *Prx1* Cre *Runx1*<sup>f/f</sup> mice or *α1(II)* Cre *Runx1*<sup>f/f</sup> mice. All genotypes were determined using PCR. *Runx2*<sup>-/-</sup> mice have been described previously (Otto et al., 1997). We maintained all mice under a 12-hour light:dark cycle with ad libitum access to regular food and water.

All animal experiments were performed with the approval of the Animal Study Committee of the Tokyo Medical and Dental University and conformed to relevant guidelines and laws.

### Skeletal analysis, lacZ staining and whole-mount in situ hybridization analysis

For skeletal preparations, mice were dissected, fixed in 95% ethanol and stained with Alcian Blue and Alizarin Red according to standard protocols (McLeod, 1980). At least six mice were analyzed for each genotype. For lacZ staining, skinned and eviscerated animals were fixed in 1% paraformaldehyde and 0.2% glutaraldehyde in phosphate buffer at pH 7.3 and stained overnight with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) (Sato et al., 2008). Whole-mount in situ hybridization analysis was performed as previously described (Iseki et al., 1999).

### Histological and in situ hybridization analysis

Embryos and pups were fixed in 4% paraformaldehyde/PBS overnight at 4°C and processed for paraffin embedding, and 5 μm sections were cut. The sections were stained with Alcian Blue and Nuclear Fast Red. Peanut agglutinin (PNA) staining was performed as previously described (Delise et al., 2002). Briefly, sections were dewaxed, rehydrated, incubated with biotinylated PNA (Vector Laboratories) at 100 μg/ml and then washed with PBS. Bound PNA was detected using the TSA-Biotin System (Perkin Elmer) and DAB substrate (Vector Laboratories) according to the manufacturer's instructions. We analyzed six mice per genotype. We performed in situ hybridization analysis as previously described (Sato et al., 2007). The *Sox9*, *Sox5* and *Sox6* probes were obtained from Dr Haruhiko Akiyama (Kyoto University, Kyoto, Japan). The *α1(II)* collagen probe was previously described (Takeda et al., 2001). We stained sections hybridized with <sup>35</sup>S-labeled riboprobes. Hybridizations were performed overnight at 55°C, and washes were performed at 63°C. We analyzed three mice per genotype.

### Cell culture and transfection

HeLa and C3H101/2 cells were grown in DMEM with 10% FBS and 1% GPS. Lipofectamine LTX (Invitrogen) or Hyperfect (Qiagen) was used for transient DNA or siRNA transfection, respectively, according to the

manufacturer's instructions. *Runx1* bioactivity was verified by cotransfection of HeLa cells with a *Runx1*-responsive-*Sox6* promoter luciferase reporter construct, a Renilla luciferase expression plasmid and a *Runx1* expression vector. After 48 hours, luciferase activity was measured. The data are presented as the ratio of Firefly luciferase activity to Renilla luciferase activity, and the values are the mean of six independent transfection experiments. We performed all cell culture experiments in triplicate or quadruplicate wells and repeated the experiments at least four times.

### Quantitative real-time PCR analysis

Real-time PCR analysis was performed using total RNA treated with DNase I. We extracted RNA with Trizol (Invitrogen) and performed reverse transcription to synthesize cDNA according to the manufacturer's instructions (Sato et al., 2008). We performed quantitative analysis of gene expression using the Mx3000P Real-Time PCR System (Stratagene). The primer sequences are available upon request. We used *Gapdh* expression as an internal control.

### Electrophoretic mobility shift assay and chromatin immunoprecipitation assay

Nuclear extracts of *Runx1*- or *Runx2*-expressing COS cells were incubated with a labeled *Sox6* probe at 4°C for 40 minutes. For the super-shifting experiments, 1 μg of anti-Runx1 (C-19X) or Runx2 (M-70X) antibody (Santa Cruz Biotechnology) was added to the reaction. Chromatin immunoprecipitation (ChIP) was performed according to the manufacturer's instructions (Diagenode), using HeLa cells that had been transfected with Runx1-GFP or GFP as a control, untransfected C3H10T/12 cells and naïve mouse sternal anlagen. We used the following PCR primers; primers spanning the Runx-binding site of the human *Sox6* promoter (-585/-389): forward 5'-CCCCCGTTTTGGACAGTA-3' and reverse 5'-GAC-TCCCTGGCGAAGTCC-3'. Control primers not spanning the Runx-binding site (-1663/-1144): forward 5'-TCCGTGAAGTTCGAGGTAGG-3' and reverse 5'-GCGGCGGTAACAAGAGATAC-3'. Primers spanning the Runx-binding site of the mouse *Sox6* promoter (-498/-287): forward 5'-TCAGCAGTACACAAACCGAAGT-3' and reverse 5'-AGAGCCTAG-AGGGACGAAGGT-3'. Control primers not spanning the Runx-binding site (-1546/-1385): forward 5'-TTCCTCCCCTCTCTCCCTTT-3' and reverse 5'-CTCAAACCCAAGCAAGCAT-3'. We isolated sternal anlagen as previously described (Zhang et al., 2004) with modifications. Briefly, the ventral rib cage was dissected from embryonic day 12.5 (E12.5) mouse embryos and digested with Dispase (1 U/ml) (Invitrogen) for 10 minutes to remove soft tissue. The remaining sternal anlagen was then manually separated, collected and digested for 20 minutes to obtain single-cell suspensions.

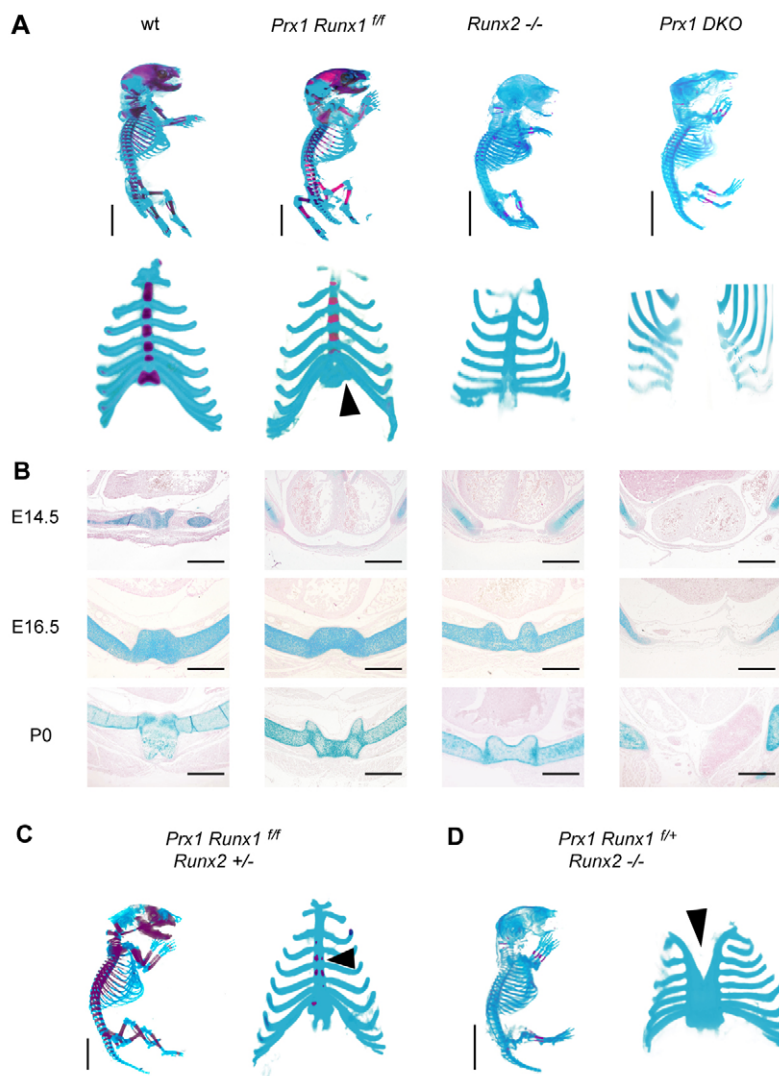
### Statistical analysis

All data are presented as the mean±s.d. (*n*=6 or more). Statistical significance was assessed using Student's *t*-test. Values were considered statistically significant at *P*<0.05. The results are representative of at least four individual experiments.

## RESULTS

### Absence of a sternum in mice lacking *Runx1* in undifferentiated mesenchymal progenitor cells

*Runx1* is a pivotal transcription factor for the generation of hematopoietic stem cells (Ito, 2004), and *Runx1*-null mice die around E11.5, which is an insurmountable obstacle in the study of its role in chondrocyte differentiation. Therefore, we generated mesenchymal-cell-specific and chondrocyte-specific *Runx1*-deficient mice to examine the role of *Runx1* during different stages of chondrocyte differentiation. To this end, we first generated *Runx1*-floxed mice (*Runx1*<sup>f/f</sup> mice), in which exon 4 of the *Runx1* gene was flanked by *loxP* sites. This region encodes a runt domain, which is indispensable for DNA binding and the interaction with core binding factor beta, an essential partner for functional transcriptional activity (Ito, 2004) (see Fig. S1 in the supplementary



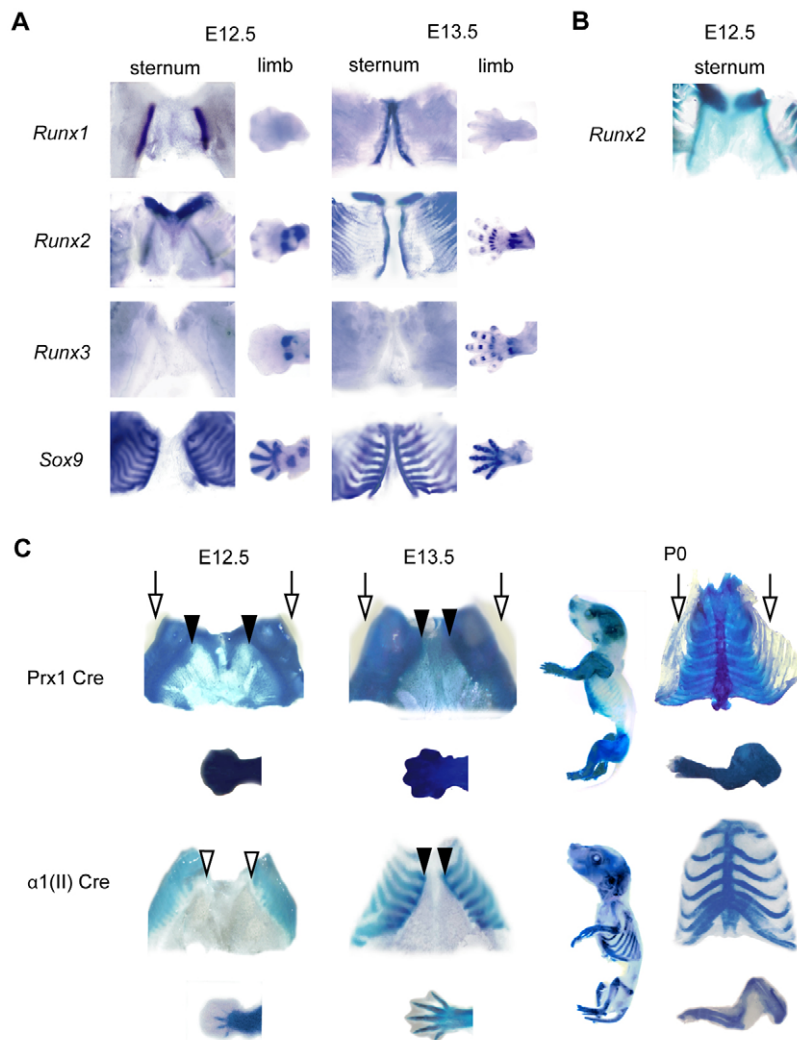
**Fig. 1. Sternal abnormality in mesenchymal-specific *Runx1*-deficient mice.** (A,C,D) Skeletal preparation of newborn mice. (A) Note the delay in calcification of the xiphoid process in *Prx1 Runx1<sup>fl/fl</sup>* mice (arrowhead) and the absence of the sternum in *Prx1 DKO* mice. By contrast, the sternal bars of *Runx2<sup>-/-</sup>* mice are fused by birth, yet their xiphoid processes are non-mineralized. (A,C) *Prx1 Runx1<sup>fl/fl</sup>/Runx2<sup>+/-</sup>* mice show an intermediate phenotype between *Prx1 Runx1<sup>fl/fl</sup>* mice and *Prx1 DKO* mice (arrowhead). (A,D) *Prx1 Runx1<sup>fl/fl</sup>/Runx2<sup>-/-</sup>* mice show an intermediate phenotype between *Runx2<sup>-/-</sup>* mice and *Prx1 DKO* mice (arrowhead). (B) Histological analysis of mouse embryo sternums. Axial section. Note the absence of the sternum and the protrusion of internal organs in *Prx1 DKO* mice. *Prx1 Runx1<sup>fl/fl</sup>* mice and *Prx1 DKO* mice denote *Prx1 Cre; Runx1<sup>fl/fl</sup>* mice and *Prx1 Cre; Runx1<sup>fl/fl</sup>; Runx2<sup>-/-</sup>* mice, respectively. Scale bars: 5 mm in A,C,D; 500  $\mu$ m in B.

material). We then inactivated *Runx1* in undifferentiated mesenchymal progenitor cells using *Prx1*-Cre transgenic (tg) mice to obtain *Prx1*-Cre tg/*Runx1<sup>fl/fl</sup>* mice (hereafter, *Prx1 Runx1<sup>fl/fl</sup>* mice) (Logan et al., 2002). The *Prx1*-Cre transgene is expressed in mesenchymal cells of the limbs, head and sternum (see Fig. 2C and data not shown) (Logan et al., 2002). *Prx1 Runx1<sup>fl/fl</sup>* mice were viable and were born in the expected Mendelian ratio. To study skeletal development, we first performed Alcian Blue/Alizarin Red staining of skeletal preparations. This analysis showed that in contrast to wild-type mice, the xiphoid process was not yet mineralized, i.e. was not stained by Alizarin Red in 91% of the newborn *Prx1 Runx1<sup>fl/fl</sup>* mice (21/23 newborns) (Fig. 1A). There was also a delay in sternal development at the age of 2 weeks (5/5 mice) (see Fig. S2 in the supplementary material); however, when the mice were analyzed at 3 weeks of age, this defect was not observed (4/5 mice) (see Fig. S2 in the supplementary material). No other skeletal elements of the *Prx1 Runx1<sup>fl/fl</sup>* mice showed any abnormalities, and no growth retardation was observed (Fig. 1A and data not shown). Importantly, the columnar structure in the growth plate of the femur of *Prx1 Runx1<sup>fl/fl</sup>* mice was indistinguishable from that of wild-type mice (data not shown). Taken at face value, these results suggest that *Runx1* alone, or in combination with other members of the *Runx* family of transcription factors, may play a transient role during the formation of the sternum. Because *Runx2* is also important for

chondrocyte differentiation (Takeda et al., 2001), we next determined whether *Runx2* was compensating for the loss of *Runx1* by breeding *Prx1 Runx1<sup>fl/fl</sup>* mice with *Runx2* mutant mice to generate *Prx1 Runx1<sup>fl/fl</sup>/Runx2<sup>-/-</sup>* mice (hereafter, *Prx1 DKO* mice). *Prx1 DKO* mice were non-viable, similar to *Runx2<sup>-/-</sup>* mice; therefore, the analysis was made on embryos and newborns. As previously shown, no mineralization was observed in most of the skeletal elements, including the sternum of *Runx2<sup>-/-</sup>* mice (Fig. 1A) (Komori et al., 1997). Surprisingly, skeletal preparations of newborn mice showed that all of the *Prx1 DKO* mice completely lacked sternums (9/9 newborns), and their internal organs were protruded, which is in sharp contrast to the normal sternal fusion seen in *Prx1 Runx1<sup>fl/fl</sup>* mice and *Runx2<sup>-/-</sup>* mice (Fig. 1A,B). These results suggest that *Runx1*, in cooperation with *Runx2*, regulates the development of the sternum.

Beginning around E12.5, the sternum starts to develop from mesenchymal cells as they form two cartilaginous processes called sternal bars (see Fig. S3 in the supplementary material) (Chen, 1952). Subsequently, these sternal bars migrate ventrally and fuse craniocaudally to form the sternum (see Fig. S3 in the supplementary material) (Chen, 1952). Histological analysis revealed that wild-type mice, *Prx1 Runx1<sup>fl/fl</sup>* mice and *Runx2<sup>-/-</sup>* mice had completed sternal fusion by birth (Fig. 1B). By contrast, there was no sign of sternal bar development in *Prx1 DKO* mice, even at birth (Fig. 1A,B). Interestingly, in *Prx1 Runx1<sup>fl/fl</sup>* mice and *Runx2<sup>-/-</sup>*





**Fig. 2. Expression of the Runx genes, the *Prx1*-Cre transgene and the  $\alpha 1(II)$  collagen-Cre transgene during skeletal development.** (A) Whole-mount in situ hybridization analysis of the Runx genes and *Sox9* in E12.5 and 13.5 mouse embryos. *Runx1* and *Runx2*, but not *Runx3*, are expressed in the prospective sternum. (B) *lacZ* staining analysis of *Runx2*<sup>+/-</sup> mice. Note the distinct expression of *Runx2* as shown by positive blue staining in the prospective sternum. (C) *lacZ* staining of *Prx1*-Cre; *Rosa26* reporter and  $\alpha 1(II)$ -Cre; *Rosa26* reporter embryos. Note that the *Prx1*-Cre transgene is expressed as early as E12.5 in the prospective sternum, whereas the  $\alpha 1(II)$ -Cre transgene is expressed only after E13.5. Expression of the *Prx1*-Cre transgene was also observed in the ventral, but not dorsal (arrows), rib cage from E12.5 to birth (P0). Arrowheads indicate the future sternum where the Cre transgenes are (black) or are not (white) expressed.

mice, sternal bar development was moderately delayed, with the two sternal bars remaining apart at E14.5, whereas the sternums of wild-type embryos at that stage had started to fuse, further indicating the complementary roles of *Runx1* and *Runx2* (Fig. 1B). In line with this finding, all of the *Prx1 Runx1*<sup>ff/+</sup>; *Runx2*<sup>-/-</sup> mice (6/6 mice) showed more severe abnormalities than *Runx2*<sup>-/-</sup> mice, and removal of one copy of *Runx2* from the *Prx1 Runx1*<sup>ff</sup> mice (i.e. 8/8 *Prx1 Runx1*<sup>ff</sup>/*Runx2*<sup>+/-</sup> mice) exaggerated the abnormality of *Prx1 Runx1*<sup>ff</sup> mice, even though the development of the sternum was normal in *Runx2*<sup>+/-</sup> mice (Fig. 1A,C,D; see Fig. S2 in the supplementary material). Taken together, these results demonstrate that *Runx1* and *Runx2* cooperatively regulate sternal development.

### Expression of Runx genes in skeletal elements

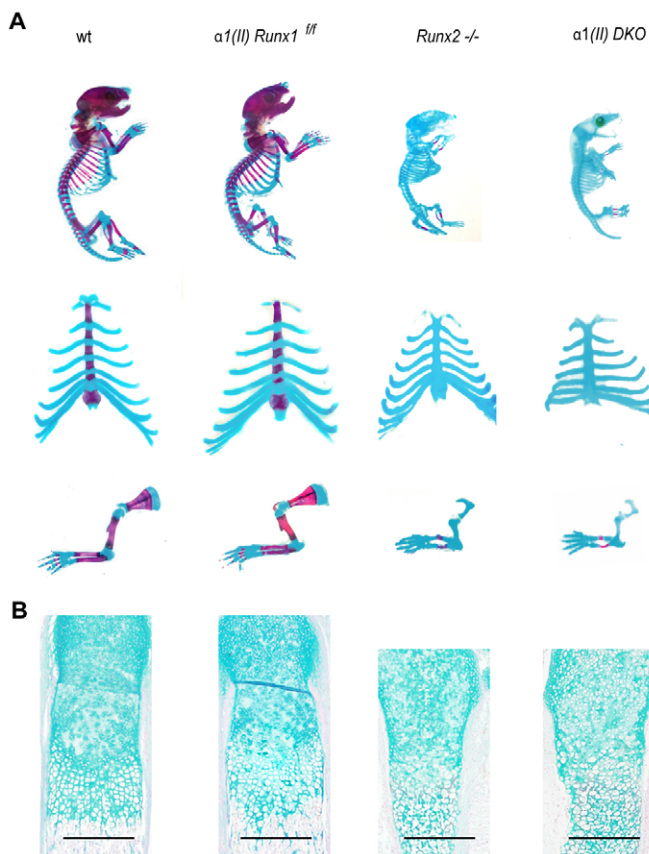
The sternum-restricted skeletal abnormalities in *Prx1* DKO mice prompted us to analyze the expression of the Runx genes and the *Prx1*-Cre transgene. As detected in whole-mount in situ hybridization analyses, *Runx1* was strongly expressed in the sternal bars at E12.5 and 13.5, and its expression decreased thereafter (Fig. 2A and data not shown). By contrast, little expression was detected in the limbs (Fig. 2A). *Runx2* was expressed in the sternal bars, clavicles, ribs and limbs (Fig. 2A). The expression of *Runx2* in the sternal bars was also verified using *lacZ* staining of *Runx2*<sup>+/-</sup> mice in which the *lacZ* allele had been inserted in the genomic *Runx2* locus (Fig. 2B) (Otto et al., 1997). By contrast, *Runx3* was expressed

only in the limbs and not in the sternum at E12.5 and 13.5 (Fig. 2A). To analyze the expression of the *Prx1*-Cre transgene, *Prx1* Cre tg mice were crossed with *Rosa26* reporter mice (Soriano, 1999). As detected using *lacZ* staining, expression of the transgene was observed in the sternal bars, ventral rib cage and limbs at E12.5 and thereafter by *lacZ* staining (Fig. 2C). Thus, the only common region where *Runx1* and the *Prx1*-Cre transgene were co-expressed was the sternum. This pattern of co-expression explains why the abnormalities in the *Prx1* DKO mice were restricted to the sternum. Interestingly, the distal long bones in *Runx2*<sup>-/-</sup> mice remained calcified after the deletion of *Runx1* in *Prx1* DKO mice (Fig. 1A), whereas they disappeared in *Runx2*<sup>-/-</sup>/*Runx3*<sup>-/-</sup> mice (Yoshida et al., 2004). This result suggests that distinct expression patterns define the specific role of each Runx gene in skeletal development.

### Normal cartilage development in mice lacking Runx1 in committed chondrocytes

We next examined the role of *Runx1* in cells committed to the chondrocyte lineage after the formation of mesenchymal condensations by analyzing  $\alpha 1(II)$ -Cre tg/*Runx1*<sup>ff</sup> mice (hereafter,  $\alpha 1(II)$  *Runx1*<sup>ff</sup> mice) (Terpstra et al., 2003).

At birth and at all other stages analyzed, there were no overt abnormalities in the  $\alpha 1(II)$  *Runx1*<sup>ff</sup> mice (13/13  $\alpha 1(II)$  *Runx1*<sup>ff</sup> mice) (Fig. 3A). The length of the femur was comparable between wild-type and  $\alpha 1(II)$  *Runx1*<sup>ff</sup> mice (data not shown), and the



**Fig. 3. Normal chondrocyte differentiation in committed chondrocyte-specific *Runx1*-deficient mice.** (A) Skeletal preparations of newborn mice. (B) Histological sections of femurs from newborn mice. Sections were stained with Alcian Blue and Nuclear Fast Red stain; extracellular cartilage matrix is stained blue. There are no overt differences between wild-type and  $\alpha 1(II)$ -Cre;  $Runx1^{f/f}$  mice or between  $Runx2^{-/-}$  mice and  $\alpha 1(II)$ -Cre DKO mice.  $\alpha 1(II) Runx1^{f/f}$  mice and  $\alpha 1(II) DKO$  mice denote  $\alpha 1(II)$ -Cre;  $Runx1^{f/f}$  mice and  $\alpha 1(II)$ -Cre;  $Runx1^{f/f}; Runx2^{-/-}$  mice, respectively. Scale bars: 500  $\mu$ m.

columnar structure of the growth plate was indistinguishable (Fig. 3B). Moreover, the sternum was morphologically normal, and there was no sign of delay in mineralization or abnormalities in hypertrophic chondrocyte maturation, as observed by skeletal staining and histological analysis (Fig. 3A and data not shown), even though the  $\alpha 1(II)$ -Cre transgene was expressed in the sternum (Fig. 2C). This observation is in sharp contrast to the delayed mineralization of the sternum in  $Prx1 Runx1^{f/f}$  mice, further suggesting a requirement for Runx1 in early sternal development. We also generated  $\alpha 1(II) Runx1^{f/f}; Runx2^{-/-}$  mice (hereafter,  $\alpha 1(II) DKO$  mice); however, there were no overt differences between  $Runx2^{-/-}$  mice and  $\alpha 1(II) DKO$  mice (6/6  $\alpha 1(II) DKO$  mice) (Fig. 3A,B). Collectively, these results suggest that *Runx1* does not play a major role in chondrocyte differentiation once the cells are committed to becoming chondrocytes.

### Runx1 is essential for the differentiation of mesenchymal cells into chondrocytes

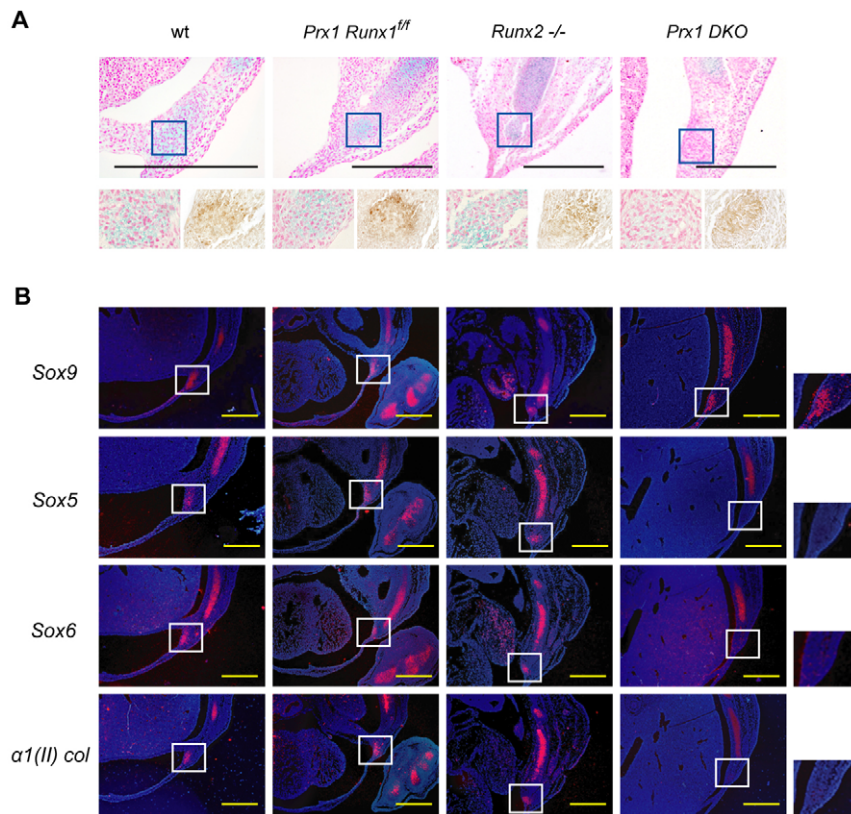
Although both the *Prx1*-Cre and the  $\alpha 1(II)$ -Cre transgenes were expressed in the sternum (Fig. 2C), only *Prx1* DKO mice developed a sternal defect, suggesting that Runx1 is important at a stage when

*Prx1* is expressed and  $\alpha 1(II)$  collagen is not, namely, early in chondrocyte development (Fig. 2C). Therefore, we focused our analysis on an early stage of sternal development. Around E12.5 the sternal rudiment first appears as a pair of mesodermal condensations in the dorsolateral body wall at the level of the upper ribs (see Fig. S3 in the supplementary material) (Chen, 1952). At E13.5, the sternal bars form more distinct structures: they are still located near the distal ends of the ribs, but a clear boundary is observed between the sternal bars and the rib ends (Fig. 4A; see Fig. S3 in the supplementary material) (Chen, 1952). In the sternal bars of E13.5 embryos, precartilaginous mesenchymal condensations were observed irrespective of deficiencies in Runx1 or Runx2, as evidenced by the dense packing of mesenchymal cells and also by PNA staining (Fig. 4A) (Delise et al., 2002). Importantly, weak Alcian Blue staining in *Prx1* DKO mice revealed that less cartilaginous matrices were produced in these mice compared with wild-type mice, indicating that chondrocyte differentiation was impaired (Fig. 4A). We next analyzed molecular markers of chondrocyte differentiation. Sox9, which is an essential transcription factor for chondrocyte differentiation and is also known as a marker of mesenchymal condensation (Lefebvre et al., 1998; Akiyama et al., 2002), was expressed in the sternal bars of *Prx1* DKO mice at a level comparable to that in wild-type mice, *Prx1 Runx1^{f/f} mice and  $Runx2^{-/-}$  mice. By contrast, the expression of Sox5 and Sox6, Sox9-dependent transcription factors necessary for chondrocyte differentiation (Smits et al., 2001), was significantly reduced in *Prx1* DKO mice (Fig. 4B). Furthermore, the expression of  $\alpha 1(II)$  collagen was reduced to background levels in *Prx1* DKO mice (Fig. 4B). Importantly, TUNEL staining revealed no apoptotic cell death, indicating that increased cell death was not responsible for the abnormal sternal development (see Fig. S4 in the supplementary material). This result is in agreement with the poor chondrocyte proliferation without an increase in the cell death of chondroblasts observed in  $Sox5^{-/-}; Sox6^{-/-}$  mice. Collectively, these results show that chondrocyte differentiation in *Prx1* DKO mice is hampered at the condensation stage, possibly as a result of impaired expression of *Sox5* and *Sox6*.*

### Sox6 is a molecular target of Runx1 during chondrogenesis

The decrease in *Sox5* and *Sox6* expression in *Prx1* DKO mice indicated that Runx1 and Runx2 might regulate the expression of *Sox5* and *Sox6* in chondrocytes. To address this possibility, we overexpressed *Runx1* or *Runx2* in the mesenchymal-chondrogenic cell line C3H10T1/2 to determine whether either protein induced the expression of *Sox5* or *Sox6*. Indeed, the expression of *Sox5* and *Sox6*, but not *Sox9*, was clearly induced by the overexpression of *Runx1* or *Runx2* (Fig. 5A). Moreover, both proteins upregulated  $\alpha 1(II)$  collagen expression, i.e. induced chondrogenesis (Fig. 5A). Surprisingly, ectopic expression of *Runx1* or *Runx2* in non-mesenchymal HeLa cells also induced *Sox5* and *Sox6* expression (data not shown). Collectively, these results demonstrate that Runx1 and Runx2 have a potent ability to induce chondrocyte differentiation. However, knockdown of *Runx1* and *Runx2* in mesenchymal-chondrogenic C3H10T1/2 cells significantly decreased *Sox5* and *Sox6* expression (Fig. 5B; see Fig. S5 in the supplementary material). We searched in silico for potential *Runx1*-binding sites in the *Sox6* promoter and found a consensus binding sequence for runt-related transcription factors (Fig. 5C). Of note, this binding site is conserved across several species (Fig. 5C). To study the role of this binding site in the Runx1- or Runx2-mediated induction of *Sox6*, we first performed an electrophoretic mobility





**Fig. 4. Normal condensation but abolished expression of Sox5 and Sox6 in the prospective sternum of Prx1-Cre DKO mice.**

(A) Histological analysis of axial sections of E13.5 mouse embryo prospective sternums. Upper and bottom left, Alcian Blue staining; bottom right, PNA staining. The bottom figures show high magnification of each corresponding rectangle in the upper panel. Note the PNA-positive mesenchymal condensations in Prx1 DKO mice. The diminished Alcian Blue staining in Prx1 DKO mice suggests the impaired accumulation of cartilage matrices. (B) In situ hybridization analysis of E13.5 mouse embryo prospective sternums (boxed area). In Prx1-Cre DKO mice, Sox9 expression in the prospective sternum (white box and higher magnification in the right panel) is not altered, whereas Sox5, Sox6 and  $\alpha 1(II)$  collagen expression in the same area are markedly decreased. Prx1 Runx1<sup>flf</sup> mice and Prx1 DKO mice denote Prx1-Cre; Runx1<sup>flf</sup> mice and Prx1-Cre; Runx1<sup>flf</sup>; Runx2<sup>-/-</sup> mice, respectively. Scale bars: 500  $\mu$ m.

shift assay (EMSA) using double-stranded oligonucleotides that covered the potential binding site as a probe. A protein-DNA complex was formed upon incubation of a nuclear extract from Runx1-expressing cells and the probe (Fig. 5D). This complex was not observed with mutated probes or with the addition of unlabeled probes (Fig. 5D). Moreover, an antibody against Runx1 supershifted this DNA-protein complex, demonstrating that Runx1 binds to the Sox6 promoter (Fig. 5D). Similar results were obtained using recombinant Runx2 protein and an anti-Runx2 antibody (Fig. 5D). We next performed ChIP. An antibody against Runx1 successfully immunoprecipitated the region containing the Runx1 binding site, whereas control serum did not, confirming that Runx1 binds to this site in vivo (Fig. 6A). We also performed ChIP analysis using an antibody against Runx2 and obtained similar results (Fig. 6A). Moreover, using untransfected C3H10T1/2 cells and naïve mouse primary sternal anlagen, we observed that endogenous Runx1 and Runx2 bind to the Sox6 promoter in vivo (Fig. 6A).

To address the importance of this site for Sox6 promoter activity, we performed DNA transfection experiments using a 0.5 kb Sox6 promoter construct containing a Runx binding site fused with a luciferase reporter (pSox6-luc) (Ikeda et al., 2007). Runx1 expression induced the luciferase expression 7.1-fold compared with the control, and mutation or deletion of the Runx binding site decreased expression by 50% (Fig. 6B). These results suggest that Runx1 and Runx2 regulate Sox6 promoter activity through this site. Collectively, these results clearly demonstrate that Sox6 is a bona fide transcriptional target of Runx1 and Runx2.

## DISCUSSION

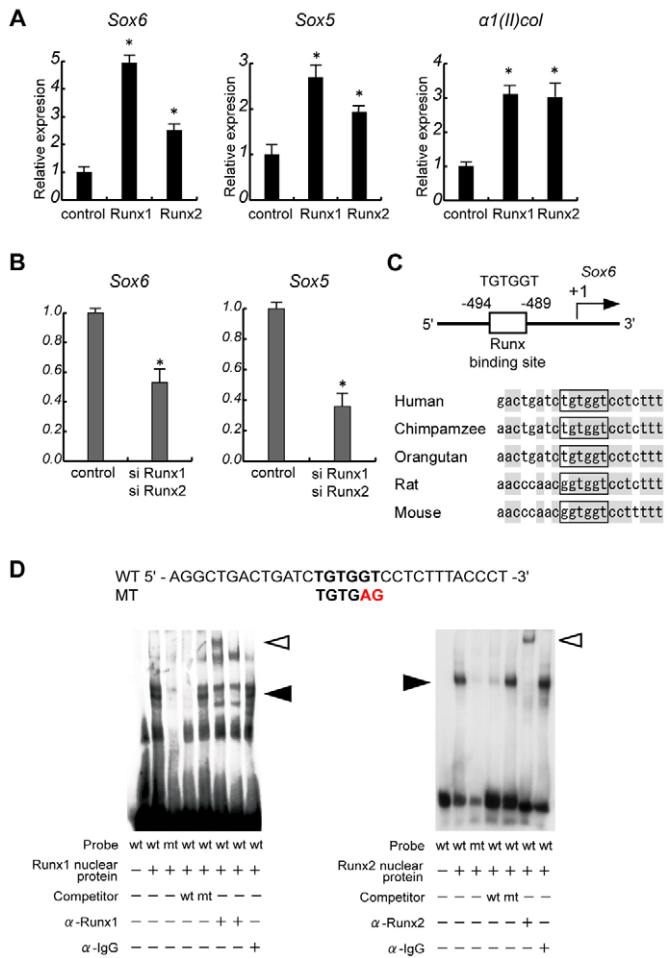
In this study we demonstrate that Runx1 and Runx2 act cooperatively to promote sternal morphogenesis. We also demonstrate that Runx1 is dispensable for chondrocyte maturation

once cells are committed to the chondrocyte lineage. Lastly, we found that Runx1 and Runx2 regulate the transcription of Sox5 and Sox6, leading to normal chondrogenesis and sternal development.

It has previously been reported that Runx1 induces chondrocyte differentiation in vitro (Wang et al., 2005). The physiological role of Runx1 in chondrocyte differentiation in vivo, however, including any temporal or spatial requirements, was not clear. This study presents the first in vivo evidence of the role of Runx1 in sternal development and chondrocyte differentiation and identifies Sox6 as a molecular target of Runx1 and Runx2 in this process (Fig. 6C).

## Molecular dissection of sternal morphogenesis

The sternum, like the forelimbs, develops from the lateral body wall, whereas the dorsal ribs develop from the axial mesoderm, specifically from somites and perichordal tissue. Indeed, the Prx1 gene, which is well known for its strong expression in the limbs, is also expressed in sternal rudiments but not in the dorsal ribs. Even though sternal malformations are sometimes observed in human genetic disorders and mutant mouse models (Wurst et al., 1994; Storm and Kingsley, 1996; Katagiri et al., 1998; Shalak et al., 2002), the complete absence of the sternum is a relatively rare occurrence (Shalak et al., 2002). Moreover, the genetic pathway regulating sternal development remains obscure. In this paper we identified a distinct role of the Runx family in sternal development. Interestingly, it has previously been reported that mesenchymal-specific Sox9-deficient mice (Prx1-Cre/Sox9<sup>fl</sup>) mice) completely lack the sternum (Akiyama et al., 2002). In addition, Sox5<sup>-/-</sup>; Sox6<sup>-/-</sup> mice have incomplete thoracic cage closure (Smits et al., 2004). Together with the complete absence of the sternum in Prx1 Runx1<sup>flf</sup>/Runx2<sup>-/-</sup> mice, these observations uncover a cascade of transcription factors necessary for sternal development. Although Sox9, Sox5 and Sox6 are expressed in all mesenchymal condensations (Akiyama et al., 2002; Smits et al., 2004), Runx1 is



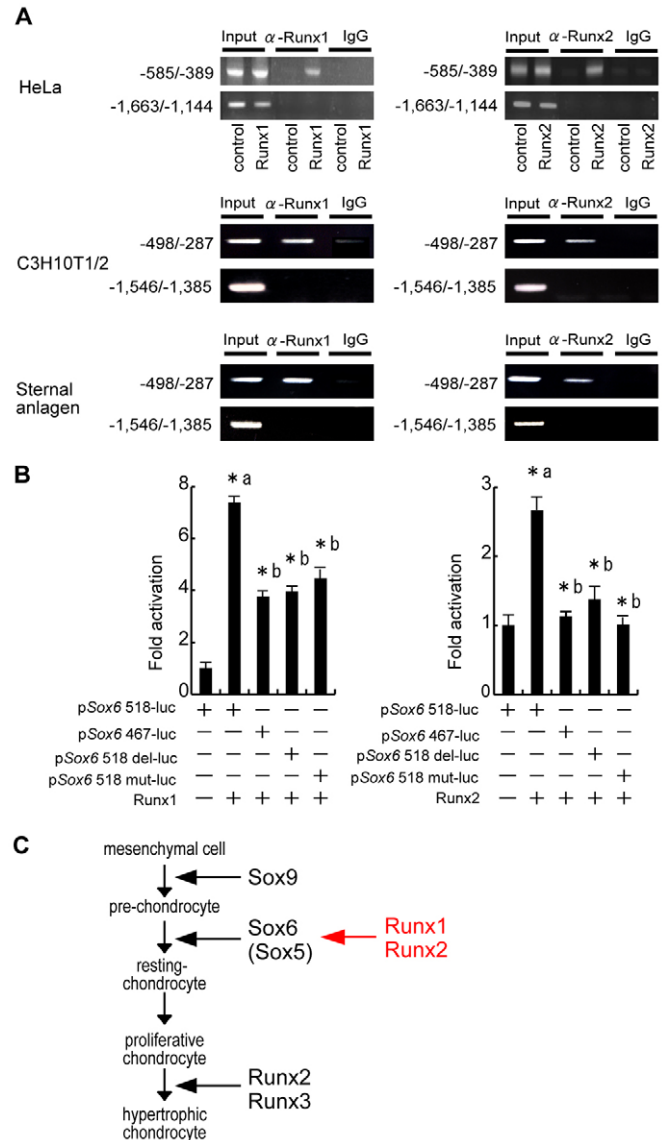
### Fig. 5. Sox6 is a molecular target of Runx1 and Runx2 in chondrocyte differentiation.

(A,B) Real-time PCR analysis. (A) Runx1 and Runx2 induced the expression of *Sox6* (left), *Sox5* (middle) and  $\alpha 1(II) collagen$  (right) in C3H10T1/2 cells. (B) The knockdown of *Runx1* and *Runx2* significantly decreased the expression of *Sox6* and *Sox5* in C3H10T1/2 cells. (C) Schematic representation of the putative Runx-binding site in the *Sox6* promoter (top). Comparison of the sequence of the putative Runx-binding site in the *Sox6* promoter across different species (bottom). Note the conservation of the binding site across species. (D) EMSA. Nuclear extracts (NEs) from *Runx1*- (left) or *Runx2*- (right) expressing COS cells formed a protein-DNA complex following incubation with oligonucleotides encompassing the Runx binding site in the *Sox6* promoter (black arrowheads). NEs incubated with antibodies against Runx1 or Runx2 showed supershifted bands (white arrowheads). \*,  $P < 0.05$  to control.

strongly and almost exclusively expressed in cells of the prospective sternum, suggesting that Runx1 is particularly involved in sternal morphogenesis. Analysis of the mutant mice generated for this study confirms this hypothesis and indicates that Runx1 is important in sternal morphogenesis by promoting chondrocyte differentiation. Currently, the molecular mechanisms of the sternum-specific expression of *Runx1* are unknown.

### Genetic hierarchy in chondrocyte lineage commitment

*Prx1 Runx1<sup>fl/fl</sup>/Runx2<sup>-/-</sup>* mice have a phenotype resembling that of *Prx1 Cre/Sox9<sup>fl/fl</sup>* mice in several respects but distinct in other ways (Akiyama et al., 2002). Both mutants lack expression of *Sox5* and



### Fig. 6. Sox6 is a molecular target of Runx1 and Runx2 in chondrocyte differentiation.

(A) ChIP assay. An antibody against Runx1 (left) or Runx2 (right) immunoprecipitated the Runx binding site of the human *Sox6* promoter (-494/-489) in HeLa cells overexpressing *Runx1* and *Runx2* (top). The same antibody also immunoprecipitated Runx binding site of the mouse *Sox6* promoter (-454/-449) in untransfected mesenchymal-chondrogenic C3H10T1/2 cells (middle) and naive mouse primary sternal anlagen (bottom). (B) *Sox6* promoter activity in HeLa cells. Runx1 (left) or Runx2 (right) significantly increased the activity of the 0.5 kb *Sox6* promoter (pSox6 518-luc). This induction was reduced by 50% by deleting (pSox6 467-luc, pSox6 518 del-luc) or mutating (pSox6 518 mut-luc) the Runx binding site in the promoter. \*a:  $P < 0.05$  to control. \*b:  $P < 0.05$  to pSox6 518-luc overexpressing Runx protein. (C) Proposed mechanism: Runx1 and Runx2 cooperatively upregulate *Sox5* and *Sox6* expression, which in turn induces chondrocyte differentiation.

*Sox6* and subsequent chondrocyte differentiation; however, *Prx1 Cre/Sox9<sup>fl/fl</sup>* mice lack mesenchymal condensations (Akiyama et al., 2002), whereas *Prx1 Runx1<sup>fl/fl</sup>/Runx2<sup>-/-</sup>* mice develop clear mesenchymal condensations (Fig. 4A). Moreover, in *Prx1 Cre/Sox9<sup>fl/fl</sup>* mice, *Runx2* expression is abolished (Akiyama et al.,

2002). By contrast, *Sox9* expression is clearly observed in *Prx1 Runx1<sup>fl/fl</sup>/Runx2<sup>-/-</sup>* mice (Fig. 4B). In *Sox5<sup>-/-</sup>; Sox6<sup>-/-</sup>* mice, *Runx2* expression is observed in early mesenchymal condensations (Smits et al., 2001). By contrast, in the current study, the expression of *Sox5* and *Sox6* was downregulated in *Prx1 Runx1<sup>fl/fl</sup>/Runx2<sup>-/-</sup>* mice (Fig. 4B). Taken together, these findings suggest that *Runx1* and *Runx2* are located between *Sox9* and *Sox5/6* in the genetic cascade, although it is not clear whether *Runx1* and *Runx2* are direct targets of *Sox9*. In our hands, the overexpression of *Sox9* in HeLa cells or mesenchymal cell lines did not induce *Runx1* or *Runx2* expression (A.K. and S.T., unpublished observation), suggesting that the effect of *Sox9* on *Runx1* or *Runx2* expression is indirect. Because *Sox5* and *Sox6* lack an activation domain, it is essential for them to have co-regulators that transactivate their target genes (Lefebvre and Smits, 2005). Thus, it is tempting to hypothesize that *Runx1* and/or *Runx2* may be responsible for the transactivating function of *Sox5* and *Sox6*. Interestingly, whereas *Sox5<sup>-/-</sup>; Sox6<sup>-/-</sup>* mice have a defect in sternal development, *Sox5<sup>-/-</sup>; Sox6<sup>+/-</sup>* mice and *Sox5<sup>+/-</sup>; Sox6<sup>-/-</sup>* mice rather develop accelerated premature hypertrophy of sternal chondrocytes (Smits et al., 2004). Therefore, sternal mesenchymal cells require *Sox5* and *Sox6* for proper sternal development, but only at a low dose.

### Temporal but pivotal role of *Runx1* in sternal morphogenesis

In the current analysis, we demonstrate that the removal of both *Runx1* and *Runx2* early during skeletogenesis caused a complete loss of the sternum, whereas removal of *Runx1* and *Runx2* after the formation of mesenchymal condensations did not affect the development of the sternum. The fact that *Prx1 Runx1<sup>fl/fl</sup>/Runx2<sup>-/-</sup>* mice developed an intermediate phenotype between *Runx2<sup>-/-</sup>* mice and *Prx1 Runx1<sup>fl/fl</sup>/Runx2<sup>-/-</sup>* mice and that *Prx1 Runx1<sup>fl/fl</sup>/Runx2<sup>+/-</sup>* mice also developed an intermediate phenotype between *Prx1 Runx1<sup>fl/fl</sup>* mice and *Prx1 Runx1<sup>fl/fl</sup>/Runx2<sup>-/-</sup>* mice suggests that *Runx1* and *Runx2* have redundant roles in sternal morphogenesis and chondrocyte differentiation (Fig. 1A-D).

Interestingly, hypoxia induces chondrocyte differentiation and prevents hypertrophy. Moreover, mice deficient in HIF-1 $\alpha$ , an indispensable factor in the response to hypoxia, have a defect in chondroblast differentiation (Provot et al., 2007), although mesenchymal condensations develop normally, which is reminiscent of what occurs in *Prx1 Runx1<sup>fl/fl</sup>/Runx2<sup>-/-</sup>* mice. Moreover, *Runx1* is upregulated in hypoxic metatarsal culture, but interestingly, *Runx2* expression is downregulated in this same culture (Provot et al., 2007). Thus, these observations suggest a role of *Runx1* in hypoxia-induced chondrocyte differentiation.

### *Runx2* and chondrocyte lineage commitment

*Runx2* is expressed in mesenchymal cells and is then turned off in chondroblasts. Because *Runx2* is expressed throughout osteoblastic differentiation and is indispensable in the process, *Runx2* expression in mesenchymal cells has traditionally been considered to be essential for osteoblastic lineage commitment but not for chondrocyte lineage commitment (Ducy et al., 1997). In this study, however, we clearly demonstrated that removing *Runx2* in addition to *Runx1* results in the loss of chondrocyte lineage commitment, at least in the sternum (Fig. 1A, Fig. 4). Recently, the loss of  $\beta$ -catenin (Ctnnb1 – Mouse Genome Informatics) in mesenchymal condensations was shown to cause a lineage shift toward the chondrocyte lineage from the osteoblastic lineage (Day et al., 2005; Hill et al., 2005). Interestingly, the loss of  $\beta$ -catenin caused an increase in *Runx2*

and *Sox9* expression (Hill et al., 2005). Taken together, these observations suggest that, at the early mesenchymal condensation stage, *Runx2* works as a determinant for osteochondro progenitor cell development and not specifically as a determinant of osteoblast differentiation. It would be interesting to see whether the loss of  $\beta$ -catenin in mesenchymal cells also upregulates *Runx1*, which would result in the further induction of chondrocyte differentiation.

### Compensatory mechanism for the loss of *Runx1* and *Runx2* in chondrocyte differentiation in the limbs

In the current study, the abnormal chondrocyte lineage commitment in *Prx1 Runx1<sup>fl/fl</sup>/Runx2<sup>-/-</sup>* mice was restricted to the sternum (Fig. 1A,B), whereas chondrocyte differentiation was normal in the limbs (Fig. 1A, and data not shown). Because chondrocyte lineage commitment was normal throughout the bodies of *Runx2<sup>-/-</sup>*; *Runx3<sup>-/-</sup>* mice, despite a slight delay in early skeletal limb development, (Yoshida et al., 2004), it is possible that *Runx1*, *Runx2* and *Runx3* have redundant roles in limb chondrocyte development. Because *Runx1* expression was not observed in mesenchymal condensations in both the sternum and the limbs, however, it is likely that molecule(s) other than *Runx* proteins compensate for the loss of *Runx2* and *Runx3* in the skeletal limbs of *Runx2<sup>-/-</sup>*; *Runx3<sup>-/-</sup>* mice. To date, several transcription factors that regulate the commitment of mesenchymal cells to become chondrocytes have been identified (ten Berge et al., 1998; LeClair et al., 1999; Herbrand et al., 2002). Further elucidation is necessary to identify the factors that compensate for the absence of the *Runx* proteins.

### Acknowledgements

We would like to thank Dr G. Karsenty for helpful discussions, Dr H. Akiyama for providing the probes and J. Chen, R. Xu and C. Xu for superb technical assistance. This work was supported by a Grant-in-Aid for Fellows from the Japan Society for the Promotion of Science (JSPS) to Ms Kimura; by a Grant-in-Aid for Scientific Research grants from the Japan Society for the Promotion of Science to Drs Jinno, Ae, Shinomiya and Takeda; by NIH (RO1DE/HD 12324-12) to Dr Martin; and by Global Center of Excellence Program grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan to Dr Shinomiya. Deposited in PMC for release after 12 months.

### Competing interests statement

The authors declare no competing financial interests.

### Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.045005/-/DC1>

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