

# Chondrocytic Atf4 regulates osteoblast differentiation and function via *Ihh*

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## SUMMARY

Atf4 is a leucine zipper-containing transcription factor that activates osteocalcin (*Ocn*) in osteoblasts and indian hedgehog (*Ihh*) in chondrocytes. The relative contribution of Atf4 in chondrocytes and osteoblasts to the regulation of skeletal development and bone formation is poorly understood. Investigations of the *Atf4*<sup>−/−</sup>/*Col2a1-Atf4* mouse model, in which *Atf4* is selectively overexpressed in chondrocytes in an *Atf4*-null background, demonstrate that chondrocyte-derived Atf4 regulates osteogenesis during development and bone remodeling postnatally. *Atf4* overexpression in chondrocytes of the *Atf4*<sup>−/−</sup>/*Col2a1-Atf4* double mutants corrects the reduction in stature and limb in *Atf4*<sup>−/−</sup> embryos and rectifies the decrease in *Ihh* expression, Hh signaling, proliferation and accelerated hypertrophy that characterize the *Atf4*<sup>−/−</sup> developing growth plate cartilages. Unexpectedly, this genetic manipulation also restores the expression of osteoblastic marker genes, namely *Ocn* and bone sialoprotein, in *Atf4*<sup>−/−</sup> developing bones. In *Atf4*<sup>−/−</sup>/*Col2a1-Atf4* adult mice, all the defective bone parameters found in *Atf4*<sup>−/−</sup> mice, including bone volume, trabecular number and thickness, and bone formation rate, are rescued. In addition, the conditioned media of ex vivo cultures from wild-type or *Atf4*<sup>−/−</sup>/*Col2a1-Atf4*, but not *Atf4*<sup>−/−</sup> cartilage, corrects the differentiation defects of *Atf4*<sup>−/−</sup> bone marrow stromal cells and *Ihh*-blocking antibody eliminates this effect. Together, these data indicate that Atf4 in chondrocytes is required for normal *Ihh* expression and for its paracrine effect on osteoblast differentiation. Therefore, the cell-autonomous role of Atf4 in chondrocytes dominates the role of Atf4 in osteoblasts during development for the control of early osteogenesis and skeletal growth.

**KEY WORDS:** Atf4, Chondrocytes, Osteoblasts, Mouse

## INTRODUCTION

In vertebrate embryos at early stages of development, the skeleton consists of avascular cartilage that is gradually replaced by bone later during development, with the exception of areas that require resilient but flexible stiffening. These areas include the walls of larger respiratory passageways and the articular surfaces of joints. Bone, as a mineralized connective tissue, forms by one of the following two processes: replacement of pre-existing mesenchyme, which is defined as intramembranous ossification, or replacement of a pre-existing cartilage mold, which is defined as endochondral ossification. The latter process forms the majority of the bony elements in the body. In mice, ossification centers in the middle of diaphysis starts to form at embryonic day (E) 13 or 13 days post-coitum. Blood vessels invade in the midshaft of the cartilaginous mold of limb and vertebra (Hall, 1988; Reddi, 1994), which brings in precursors of osteoblasts and osteoclasts and transforms it into marrow (Maes et al., 2010; Karsenty and Wagner, 2002; Karsenty et al., 2009). The distal ends of the cartilage template are retained so that the outer surfaces become the articular joints. The inner areas of the cartilage at each of the two distal ends develop into the epiphyseal growth plate that contains four types of chondrocytes: resting, proliferative, prehypertrophic and hypertrophic chondrocytes (Karsenty and Wagner, 2002; Kronenberg, 2003; Karsenty et al., 2009).

Cartilage is not only an ontological precursor of bone, but is also an active participant in bone formation because it secretes local cytokines that regulate both the elongation of the cartilaginous rudiment and the formation of the bony skeleton. Indian hedgehog (*Ihh*) is one of the local cytokines that plays an indispensable role in controlling skeletal growth. In the developing cartilage, *Ihh* is expressed by prehypertrophic chondrocytes that have just exited the cell cycle and signals proliferative chondrocytes to divide and perichondrial mesenchymal cells to differentiate into osteoblasts. Thus, *Ihh* directs both longitudinal growth and bone collar formation. *Ihh*<sup>−/−</sup> mice exhibit a reduction in chondrocyte proliferation, impaired chondrocyte maturation, a delay in hypertrophic vascularization, and absence of osteoblasts (Vortkamp et al., 1996; St-Jacques et al., 1999; Chung et al., 2001; Long et al., 2001), establishing the role of *Ihh* in coupling chondrogenesis and osteogenesis during skeletal development. Importantly, deletion of *Ihh* specifically in chondrocytes leads to perinatal lethality and skeletal defects recapitulating *Ihh*<sup>−/−</sup> animals in which *Ihh* is globally eliminated (Razzaque et al., 2005), thus confirming that the function of *Ihh* is not restricted to the cartilage. Postnatally, *Ihh* is essential for maintaining growth plate and trabecular bone as conditional deletion of *Ihh* in chondrocytes after birth results in destruction of the articular surfaces in long bones and premature fusion of growth plates, as well as loss of trabecular bone over time (Maeda et al., 2007). Two osteoblast differentiation factors, Runx2 and Atf4, have been shown to activate *Ihh* expression and regulate chondrocyte proliferation and differentiation by binding directly to the *Ihh* proximal promoter (Yoshida et al., 2004; Wang et al., 2009). However, the role of these transcription factors in chondrocytes in controlling osteoblast differentiation and function has not been established.

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Atf4 is a leucine zipper-containing transcription factor and a member of the cAMP response element-binding protein (CREB) family. It was originally identified as a nuclear binding activity enriched in osteoblasts (Ducy and Karsenty, 1995; Schinke and Karsenty, 1999). Global deletion of *Atf4* in mice led to severe osteopenia, impaired osteoblast terminal differentiation, reduced *Ocn* expression and decreased type I collagen synthesis (Yang et al., 2004). Subsequent studies revealed that Atf4 plays an indispensable role in the regulation of chondrocyte proliferation and differentiation during skeletal development (Wang et al., 2009). *Atf4*<sup>-/-</sup> embryos and pups exhibit cartilage defects, characterized by a reduced and disorganized proliferative zone, decreased proliferation, expanded hypertrophic zone and reduced *Ihh* transcription and Hh signaling in chondrocytes (Wang et al., 2009). The latter two genetic studies established the role of Atf4 in osteoblasts and chondrocytes, respectively. However, the global nature of gene deletion in the *Atf4*<sup>-/-</sup> mouse model precluded the analysis of a putative role of Atf4 in chondrocytes on chondrocyte-osteoblast coupling during osteogenesis and skeletal development in vivo.

In this study, we created and analyzed *Atf4*<sup>-/-</sup>;*Col2a1-Atf4* mice, in which *Atf4* was overexpressed specifically in chondrocytes under the control of the promoter/enhancer of the mouse type II collagen  $\alpha 1$  chain (*Col2a1*) gene (Metsaranta et al., 1991; Metsaranta et al., 1995) in an *Atf4*<sup>-/-</sup> genetic background. *Atf4*<sup>-/-</sup>;*Col2a1-Atf4* double mutants lack *Atf4* in every cell type except for chondrocytes, providing a unique model to study the specific contribution of dysfunctional osteoblasts, chondrocytes or the combination of the two cell types to the skeletal abnormalities of *Atf4*<sup>-/-</sup> mutants. Analysis of this model provides evidence that chondrocyte-derived Atf4, via its transcriptional target *Ihh*, is required for proper osteoblast differentiation, function and bone mass acquisition.

## MATERIALS AND METHODS

### Animals

Animals were maintained in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were studied according to Institutional Animal Care and Use Committee approved protocols. *Col2a1-Atf4* transgene was constructed by inserting an entire *Atf4* locus in between a 3 kb fragment of the *Col2a1* chain gene promoter and its 3 kb chondrocyte-specific enhancer within the first intron (Metsaranta et al., 1995). Southern blot was performed using *Bam*HI/*Xho*I digested tail genomic DNA and an *Atf4* cDNA probe to select transgenic founders. *Atf4* cDNA probe for Southern blot was described previously (Wang et al., 2009). Two lines of transgenic mice containing high copy numbers of the *Col2a1-Atf4* were used for subsequent matings with wild type (WT) to maintain the transgenic lines or *Atf4*<sup>-/-</sup> mice to generate experimental animals. PCR was also performed for genotyping of transgenic mice using the primer pairs 5'-GCCTCGCTGCGCTTCGC-3' and 5'-GCTTAGGCCGGTGGGGTTG-3' and *Atf4* mutant mice as described (Yang et al., 2004).

### Quantitative real time RT-PCR (qRT-PCR)

Long bone cartilage and bone were isolated from P0 newborn pups by separating white articular cartilages and red midshaft marrow-containing bones under a dissecting microscope. Total RNA from tail, limb cartilage and long bones of indicated genotypes at indicated developmental stages was isolated using TRIzol (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocols. DNase I-treated RNA (2  $\mu$ g) was reverse-transcribed with 100 units of Superscript II plus RNase H<sup>-</sup> Reverse Transcriptase (Invitrogen) using 100  $\mu$ M random hexamer primers. qRT-PCR was performed using a standard TaqMan PCR kit protocol on an Applied Biosystems 7300 Sequence Detection System (Applied Biosystems, Carlsbad, CA, USA). Specific forward and reverse oligonucleotide primers

used for *Atf4* (Mm00515325\_m1), *Ihh* (Mm00439613\_m1), patched (Mm00436029\_m1), *Gli1* (Mm00494645\_m1), PTH/PTHrP receptor (*PPR*; *Pth1r* – Mouse Genome Informatics) (Mm00441046\_m1) were from Applied Biosystems. 18S rRNA was used as an internal control (QuantumRNA Classic 18S Internal Standard). For each genotype, at least three pups were analyzed and standard errors were calculated based on qRT-PCR results from at least three sample repetitions.

### Skeletal preparation and histology

Embryos and pups were isolated and skeletal preparation was performed according to standard protocols as described (Wang et al., 2009). For histology, embryos and postnatal day (P) 0 mice were fixed in 4% paraformaldehyde, embedded in paraplast and sectioned at 5  $\mu$ m. Sections were stained with Hematoxylin and Eosin (H&E). For each genotype, at least three embryos or mice were analyzed.

### In vivo proliferation assay

BrdU (5-bromo-2'-deoxyuridine) in vivo labeling of proliferative chondrocytes of embryos and newborn pups (0.1 mg/g of body weight) were performed. Embryos and pups were fixed, sectioned and stained by immunohistochemistry using a BrdU staining kit (Zymed Laboratories, San Francisco, CA, USA) as described previously (Wang et al., 2009).

### In situ hybridization

Alternate sections of forelimb were used for histological analysis were hybridized with indicated probes as described previously (Wang et al., 2009). The probe for patched was from Dr Chin Chiang's laboratory (Vanderbilt University, Nashville, TN, USA).

### Microcomputed tomography ( $\mu$ CT) analysis

Femurs from 1-month-old and 3-month-old mice were collected and fixed overnight in 4% paraformaldehyde (pH 7.4) and then 70% ethanol. Distal femurs were scanned using a  $\mu$ CT imaging system (Scanco  $\mu$ CT 40; Scanco Medical, Bassersdorf, Switzerland). Tomographic cross-sectional images were acquired at 55 kV, medium resolution, and 100 slides. Contours were fitted to the outer perimeter of the distal femurs using the auto-contouring feature in the Scanco Software with the threshold of 220 mg hydroxyapatite/cm<sup>3</sup>.

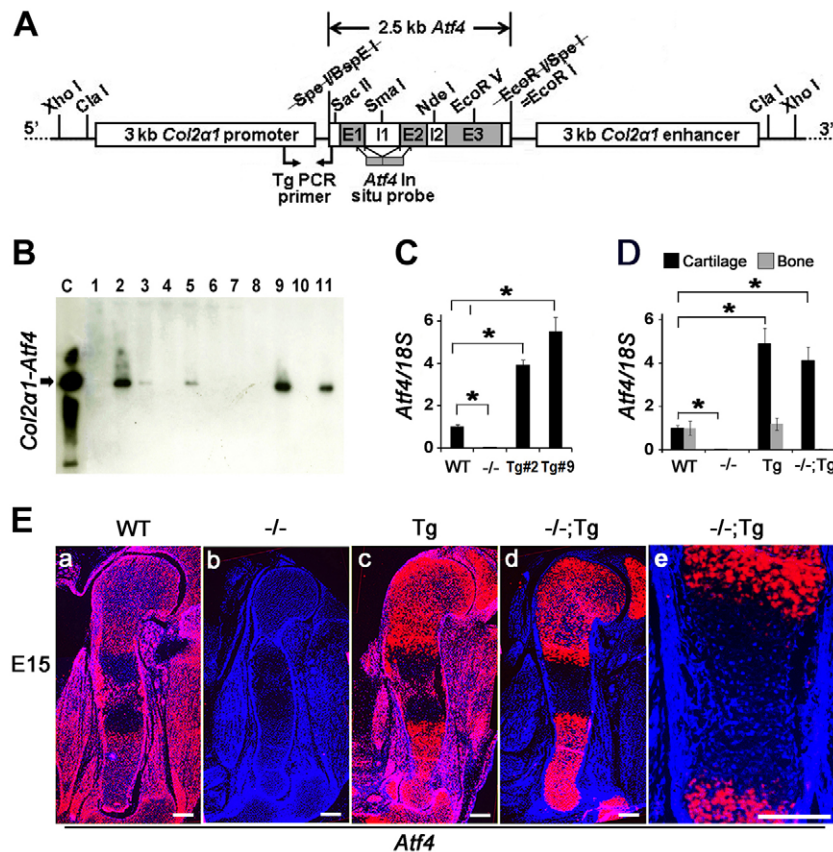
### Cartilage and bone marrow stromal cells culture

Cartilages isolated under a dissecting microscope from P0 to P2 newborn pups with the same genotype were pooled and cultured in  $\alpha$ MEM (6 mg cartilage/ml) containing 10% serum and 1% antibiotics. To harvest cartilage-conditioned media, 50% of the cartilage media filtered through cell culture inserts (pore size 1 microns, BD Falcon, Franklin Lakes, NJ, USA) was collected every two days and used as cartilage-conditioned medium (CM). An equal amount of fresh medium was added back to the organ culture. Bone marrow stromal cells (BMSCs) from 2-month-old mice were isolated from long bones by a centrifugation method as described (Dobson et al., 1999). BMSCs were plated in 6-well tissue culture plate with  $2 \times 10^5$  cells/well and  $4 \times 10^5$  cells/well for alkaline phosphatase-positive colony forming units (CFU-ap) and mineralized nodules (CFU-ob), respectively, following a standard protocol except that cartilage CM (100  $\mu$ l) was supplemented. For blocking Hh signaling, anti-Hh antibody (5E1, 2  $\mu$ g/ml; Developmental Studies Hybridoma Bank), cyclopamine (10  $\mu$ M) or anti-PTHrP antibody as a control (2  $\mu$ g/ml, a gift from Dr T. J. Martin, University of Melbourne, Australia) was added together with the CM into BMSC cultures. In a similar manner, purmorphamine (10  $\mu$ M) was added to stimulate Hh signaling. Alkaline phosphatase (ap) and calcified tissues were stained by ap activity staining and von Kossa staining as described previously (Yang et al., 2000; Lian et al., 2009). These experiments were repeated at least three times.

## RESULTS

### Generation of *Atf4*<sup>-/-</sup>;*Col2a1-Atf4* mice

A 2.5 kb sequence containing all three exons and two introns of the *Atf4* gene was inserted between the 3 kb promoter and 3 kb enhancer of the mouse type II collagen  $\alpha 1$  chain gene (*Col2a1*, Fig. 1A)



**Fig. 1. Generation of *Col2a1-Atf4* transgenic (Tg) mice.** (A) Schematic of the *Col2a1-Atf4* transgenic construct. Restriction sites, primers used for genotyping, and cDNA probe covering the coding region of the *Atf4* gene are indicated. E, exon; I, intron. (B) Southern hybridization of tail genomic DNA identified five founders (2, 3, 5, 9 and 11). (C) qRT-PCR of tail RNA revealed two transgenic lines (Tg#2 and Tg#9) overexpressing *Atf4*. Data were normalized to endogenous *Atf4* level in WT mice and 18S rRNA ( $n=3$ ). Error bars represent s.e.m.  $*P<0.05$  by paired Student's *t*-test. (D) qRT-PCR of cartilage and bone RNA demonstrated that *Atf4* is specifically overexpressed in cartilage but not in bone in *Col2a1-Atf4* (Tg) and *Atf4*<sup>-/-</sup>; *Col2a1-Atf4* (-/-;Tg, #9) mice. (E) In situ hybridization of E15 sections of humeri showing *Atf4* expression pattern. Note that *Atf4* expression is ubiquitous in WT (a) but absent in *Atf4*<sup>-/-</sup> (b) limb. The *Col2a1-Atf4* expression in *Col2a1-Atf4* (Tg, c) and *Atf4*<sup>-/-</sup>; *Col2a1-Atf4* (-/-;Tg, d) limbs is restricted to chondrocytes and at a higher level than that in WT limbs. (e) Higher magnification of the ossification center region of E15 *Atf4*<sup>-/-</sup>; *Col2a1-Atf4* humeral sections showing the absence of *Col11-Atf4* expression in osteoblasts.  $n=3$ . Scale bars: 0.2 mm.

(Metsaranta et al., 1991; Metsaranta et al., 1995). Among 28 mice screened by Southern hybridization of genomic DNA, five expressed the *Col2a1-Atf4* transgene (Fig. 1B). Male and female transgenic mice reproduced and transmitted the *Col2a1-Atf4* transgene to their progenies normally. The founders and their offspring showed no difference compared with their wild-type (WT) littermates in gross body size, weight and activity for up to 1 year and at least three generations of observation, suggesting that *Atf4* overexpression in chondrocytes does not affect skeletal development or metabolism. We selected and expanded two transgenic lines (2 and 9) that contained higher copy numbers of the transgene and thus expressed higher levels of *Atf4* in chondrocytes. Quantitative real time PCR (qRT-PCR), using total RNA from 6-week-old mouse tails, revealed that *Atf4* mRNA level was 4 and 5.5 times higher in lines 2 and 9, respectively, than the endogenous level in WT littermates (Fig. 1C). Both of these lines were used interchangeably to mate with *Atf4*<sup>+/-</sup> mice to produce *Atf4*<sup>+/-</sup>; *Col2a1-Atf4* mice that were then mated with *Atf4*<sup>+/-</sup> mice to obtain four types of experimental mouse littermates, WT, *Atf4*<sup>-/-</sup>, *Col2a1-Atf4* and *Atf4*<sup>-/-</sup>; *Col2a1-Atf4* mice at the expected percentages (12.5% each genotype). These results indicate that the *Col2a1-Atf4* transgene is not toxic, and is thus providing a useful tool for the study of the function of *Atf4* in chondrocytes.

### Selective chondrocytic *Atf4* expression in *Atf4*<sup>-/-</sup> mice

qRT-PCR using RNA samples isolated from cartilage and bone from P0 newborn pups confirmed that *Atf4* mRNA levels were significantly ( $P<0.05$ ) higher in *Col2a1-Atf4* and *Atf4*<sup>-/-</sup>; *Col2a1-Atf4* cartilage compared with the WT endogenous level in the cartilage and bone, with a 4.9-fold increase in *Col2a1-Atf4* and 4.1-fold increase in *Atf4*<sup>-/-</sup>; *Col2a1-Atf4* cartilage, respectively (Fig.

1D). To pinpoint the exact cell populations in which *Atf4* was overexpressed within the cartilage in the transgenic embryos, we performed in situ hybridization using E15, E17 and P0 limb sections and an *Atf4* cDNA probe that hybridizes with both endogenous and transgenic *Atf4* mRNA (Fig. 1A). As expected, *Atf4* mRNA was detected in WT humeri and surrounding tissues and was absent in all cell types in *Atf4*<sup>-/-</sup> humeri at E15 (Fig. 1Ea,b), E17 and P0 (supplementary material Fig. S1a,b,e,f). The expression pattern of *Atf4* in *Col2a1-Atf4* transgenic humeri was similar to that of WT humeri; however, the level of *Atf4* mRNA was visibly higher in epiphyseal growth plate chondrocytes where the endogenous *Col2a1* mRNA is highly expressed at E15 (Fig. 1Ea,c), E17 and P0 (supplementary material Fig. S1a,c,e,g). Cell specificity was also shown by an identical expression pattern of the *Col2a1-Atf4* transgene to endogenous *Col2a1* (Fig. 4Am) but not to endogenous *Atf4* in *Atf4*<sup>-/-</sup>; *Col2a1-Atf4* humeri at E15 (Fig. 1Ea,d), E17 and P0 (supplementary material Fig. S1a,d,e,h). No ectopic expression in hypertrophic chondrocytes, osteoblasts in the midshaft ossification centers, or cells within the perichondrium and periosteum areas was noted in E15 (Fig. 1Ee), E17 and P0 humeri (supplementary material Fig. S1). Together, these data demonstrate that the mouse *Col2a1* promoter/enhancer drives overexpression of *Atf4* exclusively in *Col2a1*-expressing chondrocytes.

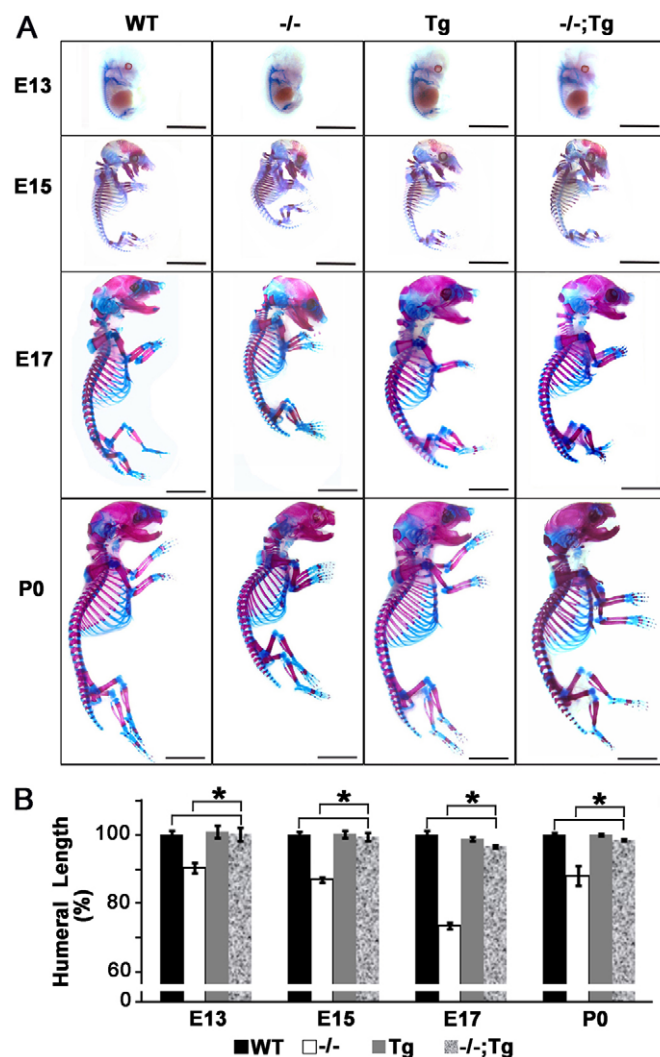
### Overexpression of *Atf4* in chondrocytes rescues the short stature of *Atf4*<sup>-/-</sup> mice

*Atf4*<sup>-/-</sup> embryos and mice have shorter long bones compared with their WT littermates, which is caused by defects in both cartilage and bone development (Yang et al., 2004; Wang et al., 2009). To examine the role of the *Col2a1-Atf4* transgene in chondrocytes and growth plate development, E13, E15 and E17 embryos as well as newborn



pups (P0) were analyzed by skeletal preparation followed by Alcian Blue and Alizarin Red staining of cartilage and bone, respectively. At E13, the skeleton of embryos of all four genotypes was cartilaginous as expected, indicated by lack of Alizarin Red-stained ossification centers (Fig. 2A). The visibly smaller skeleton of *Atf4*<sup>-/-</sup> embryos was not observed in the *Atf4*<sup>-/-</sup>; *Col2a1-Atf4* skeleton, which instead was comparable to WT and *Col2a1-Atf4* transgenic skeletons, suggesting that overexpression of *Atf4* in chondrocytes corrected the reduced cartilage growth in *Atf4*<sup>-/-</sup> embryos. At E15, the delayed formation of ossification centers in *Atf4*<sup>-/-</sup> skeletons was not observed in *Atf4*<sup>-/-</sup>; *Col2a1-Atf4* skeletons, which were still larger than *Atf4*<sup>-/-</sup> skeletons and the same size as WT and *Col2a1-Atf4* littermates. This held true for every later stage examined (Fig. 2A).

Quantitative measurement showed that the 8-30% reduction in E13, E15, E17 and P0 humeral length of *Atf4*<sup>-/-</sup> embryos and pups was completely rescued in *Atf4*<sup>-/-</sup>; *Col2a1-Atf4* mutants (Fig. 2B).



**Fig. 2. Overexpression of *Atf4* in chondrocytes rescues shortened statures in *Atf4*<sup>-/-</sup> mutant mice.** (A) Alizarin Red and Alcian Blue staining of skeletons of embryos and newborn pups at the indicated developmental stages. Scale bars: 0.5 mm. *n*=3. (B) Quantification of humeral length in embryos and pups of indicated genotypes at indicated developmental stages. Error bars represent s.e.m. *n*=3. \**P*<0.05 by paired Student's *t*-test. Tg, *Col2a1-Atf4*; -/-;Tg, *Atf4*<sup>-/-</sup>; *Col2a1-Atf4*.

None of the *Col2a1-Atf4* transgenic mutants was found to be larger than WT littermates at every stage examined (Fig. 2, Table 1), suggesting that forced expression of *Atf4* in chondrocytes to four to five times higher than the endogenous level does not cause excessive growth of *Atf4*<sup>-/-</sup> skeletons embryonically and postnatally. Collectively, these data support the notion that *Atf4* in chondrocytes is a crucial determinant of skeletal growth, and confirm that the dwarfism phenotype of *Atf4*<sup>-/-</sup> mice is caused by a developmental defect that is chondrocyte autonomous.

### Overexpression of *Atf4* in chondrocytes rescues the proliferation and differentiation defects of *Atf4*<sup>-/-</sup> growth plate chondrocytes

In developing cartilage, growth plate proliferative chondrocytes divide rapidly to form a stack of flattened cells, creating characteristic columns that are readily visible in E17 sections. In P0 growth plates, the stacks of chondrocytes appeared as long columns containing 20-30 cells, whereas in *Atf4*<sup>-/-</sup> growth plates these columns were short, disorganized and contained only 10-15 cells (Fig. 3A) (Wang et al., 2009). The 30% reduction in the length and the disorganization of the proliferative zone characteristic of *Atf4*<sup>-/-</sup> growth plates was not found in the *Atf4*<sup>-/-</sup>; *Col2a1-Atf4* growth plate (Fig. 3A,B). Furthermore, the 30% expansion in the hypertrophic zone of *Atf4*<sup>-/-</sup> humeral growth plate was also restored to normal in *Atf4*<sup>-/-</sup>; *Col2a1-Atf4* humeri (Fig. 3A,C). No lengthening of proliferative zone and shortening in hypertrophic zone of *Col2a1-Atf4* growth plates was observed compared with the WT growth plates (Fig. 3A-C). Accordingly, the decreased proliferation rate (10%) in *Atf4*<sup>-/-</sup> developing cartilage (Wang et al., 2009), as calculated by the percentage of BrdU-positive cells over total cells in proliferative zones, was corrected in *Atf4*<sup>-/-</sup>; *Col2a1-Atf4* limbs (20%), which was the same as that of *Col2a1-Atf4* (19%) and WT (19%) growth plates at E17 and P0 (Fig. 3D-F). Therefore, we conclude that overexpression of *Atf4* in *Col2a1*-expressing chondrocytes in *Atf4*<sup>-/-</sup> mutants rescues the proliferation and expanded hypertrophy defects but failed to cause overgrowth of properly formed WT growth plates.

### Overexpression of *Atf4* in chondrocytes restores Hh signaling in *Atf4*<sup>-/-</sup> cartilage and bone

We have previously shown that the expression of *Ihh* and Hh signaling target genes is decreased in *Atf4*<sup>-/-</sup> growth plates (Wang et al., 2009). To determine whether the *Col2a1-Atf4* transgene could restore *Ihh* expression in *Atf4*<sup>-/-</sup> growth plates, we performed in situ hybridization. Our data showed a comparable level of *Ihh* expression in prehypertrophic chondrocytes of WT, *Col2a1-Atf4* and *Atf4*<sup>-/-</sup>; *Col2a1-Atf4* humeri, which was stronger than in *Atf4*<sup>-/-</sup> humeri at every developmental stage examined, including E15 (Fig. 4Aa-d), E17 and P0 (supplementary material Fig. S2). *Ihh* expression was not detected in resting and proliferating chondrocytes in the *Col2a1-Atf4* or *Atf4*<sup>-/-</sup>; *Col2a1-Atf4* growth plate, suggesting that overexpression of *Atf4* in *Col2a1*-expressing chondrocytes is sufficient to rescue *Ihh* transcription in prehypertrophic cells where *Ihh* is expressed endogenously, but is not sufficient to induce *Ihh* ectopic expression in other chondrocyte populations in vivo.

The expression of patched1 (*Ptch1*) and *Gli1*, two direct downstream targets of Hh signaling during skeletal development (Alcedo and Noll, 1997; Mak et al., 2008), was also examined by in situ hybridization to determine whether Hh signaling was also rescued in *Atf4*<sup>-/-</sup>; *Col2a1-Atf4* embryos and pups. As expected, strong *Ptch1* expression was found in prehypertrophic

**Table 1. Total number of embryo and mice analyzed**

Genotype	Developmental stage						Total (340)	Percentage
	E13	E15	E17	P0	1M	3M		
+/+	4	9	9	15	5	7	49	14.4
+/-	5	15	20	14	9	17	80	23.5
-/-	3	8	9	6	5	5	36	10.6
+/-;Tg	4	10	5	9	6	7	41	12.1
+/-;Tg	6	19	13	23	14	15	90	26.5
-/-;Tg	3	8	7	10	9	7	44	12.9
-/-;Tg with reduced size	0	0	0	0	0	0	0	-

The number of embryos and mice of indicated developmental stages and genotypes is shown. Note that none of the *Atf4*<sup>-/-</sup>; *Col2a1-Atf4* (-/-;Tg) embryos and mice analyzed are smaller than WT (+/+) controls. Also, none of the *Col2a1-Atf4* (+/-;Tg) embryos and mice display larger body size than WT littermates.

chondrocytes, in cells of the perichondrium and periosteum, and in midshaft osteoblasts in WT growth plates at E15 (Fig. 4Ae), E17 and P0 (supplementary material Fig. S2). The expression level of *Ptch1* was noticeably decreased in *Atf4*<sup>-/-</sup> growth plates (Fig. 4Af), but rescued to normal levels by the *Col2a1-Atf4* transgene in *Atf4*<sup>-/-</sup>; *Col2a1-Atf4* humeri at every developmental stage examined (Fig. 4Ae-h; supplementary material Fig. S2). Similarly, the decreased *Gli1* expression seen in *Atf4*<sup>-/-</sup> humeri was not observed in *Atf4*<sup>-/-</sup>; *Col2a1-Atf4* humeri (Fig. 4Ai-l; supplementary material Fig. S2). As controls, chondrocyte markers, such as *Col2a1* in resting, proliferative and prehypertrophic chondrocytes, *Col10a1* in hypertrophic chondrocytes, and PTHrP receptor (*PPR*) in prehypertrophic chondrocytes, periosteum and osteoblasts (Wang et al., 2009), remained unchanged in *Col2a1-Atf4* and *Atf4*<sup>-/-</sup>; *Col2a1-Atf4* humeri compared with WT and *Atf4*<sup>-/-</sup> controls (Fig. 4Am-x; supplementary material Fig. S2). Quantitative measurements by qRT-PCR demonstrated that the 50% decrease in the expression of *Ihh*, *Ptch1* and *Gli1* in *Atf4*<sup>-/-</sup> cartilage was restored in *Atf4*<sup>-/-</sup>; *Col2a1-Atf4* cartilages to a level comparable to that of WT and *Col2a1-Atf4* cartilages (Fig. 4B-D), thus confirming the in situ hybridization results. Based on these results, we conclude that overexpression of *Atf4* in chondrocytes rescues Hh signaling in *Atf4*<sup>-/-</sup> mice.

As *Ptch1*, *Gli1* and *PPR* are also expressed in osteoblasts, we then examined the expression of these genes in bones by qRT-PCR. In agreement with the absence of *Ihh* mRNA signal in humeri at all the developmental stages examined by in situ hybridization (Fig. 4Aa-d; supplementary material Fig. S2), *Ihh* mRNA level in bone was 90% lower than that in cartilage of all four genotype pups examined (Fig. 4B). The expression of the Hh downstream genes *Ptch1* and *Gli1* was 50% and 70% lower, respectively, in bone than in cartilage of WT newborns. *Ptch1* and *Gli1* expression decreased about 50% in *Atf4*<sup>-/-</sup> bones compared with the WT and *Col2a1-Atf4* bones, suggesting defective Hh signaling in *Atf4*<sup>-/-</sup> bones. Interestingly, this Hh signaling defect was corrected in *Atf4*<sup>-/-</sup>; *Col2a1-Atf4* bones (Fig. 4B-D), showing that the *Col2a1-Atf4* transgene in chondrocytes rescued impaired Hh signaling in *Atf4*<sup>-/-</sup> bones. The expression of *PPR* in cartilages and bones was similar in all four genotypes examined (Fig. 4E), suggesting a specific effect of the *Col2a1-Atf4* transgene in rescuing the Hh signaling defects present in *Atf4*<sup>-/-</sup> skeletons. Collectively, these results indicate that selective overexpression of *Atf4* in chondrocytes restores *Ihh* expression in cartilage and Hh signaling in both cartilage and bone of the *Atf4*<sup>-/-</sup>; *Col2a1-Atf4* developing skeletons. Thus, these results confirm that *Atf4* plays a chondrocyte-autonomous role in growth plate development, and suggest that *Atf4* in chondrocytes might positively and indirectly regulate bone development and osteogenesis.

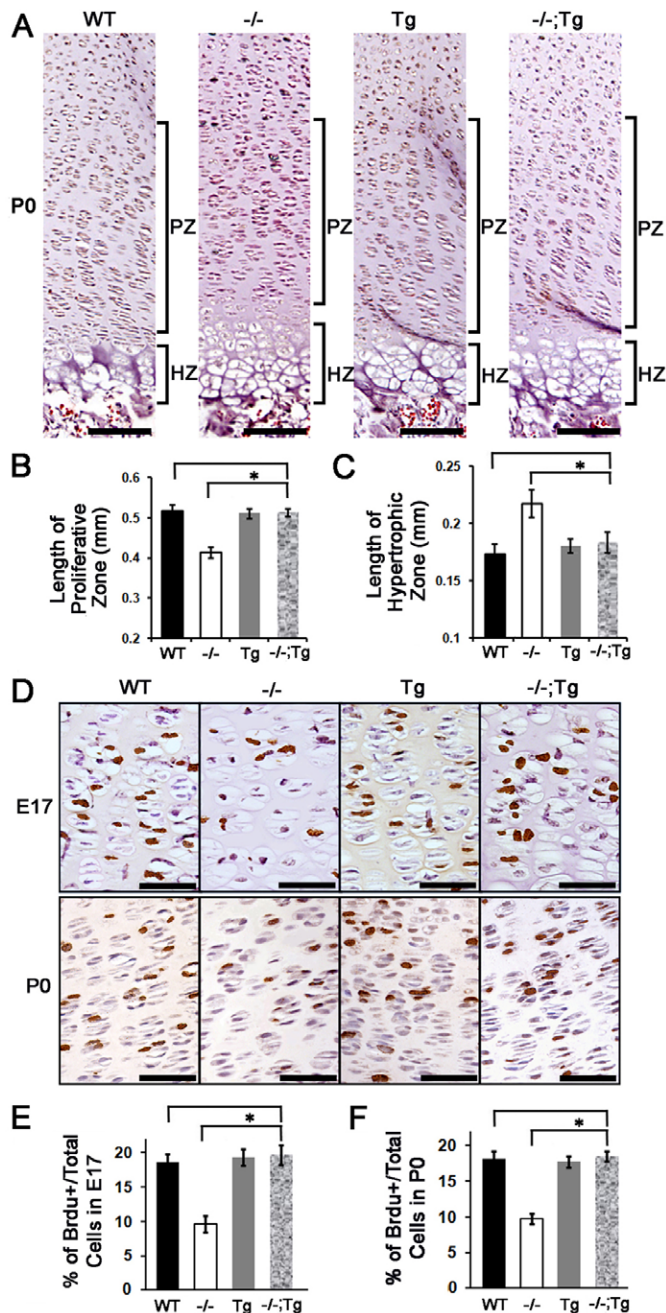
### Overexpression of *Atf4* in chondrocytes rescues defective osteogenesis in *Atf4*<sup>-/-</sup> embryos

The growth of the skeleton after E14 relies on the proliferation and differentiation of both chondrocytes and osteoblasts as primary ossification centers start to form in the midshaft of long bones and vertebrae. The observation of a complete skeletal size rescue in *Atf4*<sup>-/-</sup>; *Col2a1-Atf4* embryos at E15 and E17 and pups at P0 (Fig. 2) alluded to a possible beneficial effect of the *Col2a1-Atf4* transgene in both chondrocytes and osteoblasts. To test this hypothesis, we first examined the expression of two genetic markers of mature osteoblasts, osteocalcin (*Ocn*; *Bglap* – Mouse Genome Informatics) and bone sialoprotein (*Bsp*) by in situ hybridization. As expected, the expression of both *Ocn* and *Bsp* was almost absent in the periosteum and trabecular bone in E15 *Atf4*<sup>-/-</sup> humeri but was strong in WT controls (Fig. 5Aa,b,e,f), E17 and P0 (supplementary material Fig. S3). The level and pattern of *Ocn* and *Bsp* expression in the *Col2a1-Atf4* transgenic humeri were similar to those in WT controls at every developmental stage examined (Fig. 5Aa,c,e,g; supplementary material Fig. S3), indicating that *Atf4* overexpression in chondrocytes does not affect osteoblast differentiation in a WT background. Unexpectedly, however, *Ocn* and *Bsp* expression levels in *Atf4*<sup>-/-</sup>; *Col2a1-Atf4* humeri were much higher than those in *Atf4*<sup>-/-</sup> humeri and were comparable with those in WT and *Col2a1-Atf4* transgenic humeri (Fig. 5Ad,h; supplementary material Fig. S3), indicating that overexpression of *Atf4* in chondrocytes rescues the osteoblast differentiation defect in *Atf4*<sup>-/-</sup> humeri. Furthermore, in contrast to the severely delayed formation of primary ossification centers in the midshafts in E15 *Atf4*<sup>-/-</sup> humeri, well-developed ossification centers with two times larger mineralized areas were observed in *Atf4*<sup>-/-</sup>; *Col2a1-Atf4* humeri, similar to those observed in WT and *Col2a1-Atf4* humeri (Fig. 5Ai-l; supplementary material Fig. S3). These results provide the first line of evidence that *Atf4* in chondrocytes controls osteogenesis during skeletal development.

### Overexpression of *Atf4* in chondrocytes rescues the defects in bone formation in *Atf4*<sup>-/-</sup> adult mice

To investigate whether overexpression of *Atf4* in *Atf4*<sup>-/-</sup> chondrocytes could rescue bone defects beyond birth in *Atf4*<sup>-/-</sup> mice, we examined adult mice from 1- to 6-months-old. Among all the mice examined, none of the *Atf4*<sup>-/-</sup>; *Col2a1-Atf4* mutants was smaller than the WT littermates, as measured by nose-to-tail base length from 1- to 3-months-old. The penetrance of this genetic rescue in adult mice was also 100% (Table 1). Consistent with what was observed in embryos and pups, *Col2a1-Atf4* transgenic mice displayed the same body size as WT littermates, indicating that overexpression of *Atf4* in chondrocytes is not sufficient to cause the overgrowth of bone. To determine whether the osteoblast





**Fig. 3. Overexpression of *Atf4* in chondrocytes restores growth plate chondrocyte defects in *Atf4*<sup>-/-</sup> mice.** (A) H&E staining of humeral sections of indicated genotypes with proliferative chondrocyte zones (PZ) and hypertrophic chondrocyte zones (HZ) of growth plate chondrocytes are indicated. Scale bars: 0.1 mm. (B,C) Quantification of the length of the PZ and HZ in growth plates of indicated genotypes. Error bars represent s.e.m. *n*=3. \**P*<0.05 by paired Student's *t*-test. (D) BrdU immunohistochemistry of humeral sections showing BrdU-positive (brown) proliferating growth plate chondrocytes at indicated developmental stages. Scale bars: 0.05 mm. (E,F) Quantification of the BrdU immunohistochemistry results shown in D. Error bars represent s.e.m. *n*=3. \**P*<0.05 by paired Student's *t*-test. Tg, *Col2a1-Atf4*; -/-;Tg, *Atf4*<sup>-/-</sup>; *Col2a1-Atf4*.

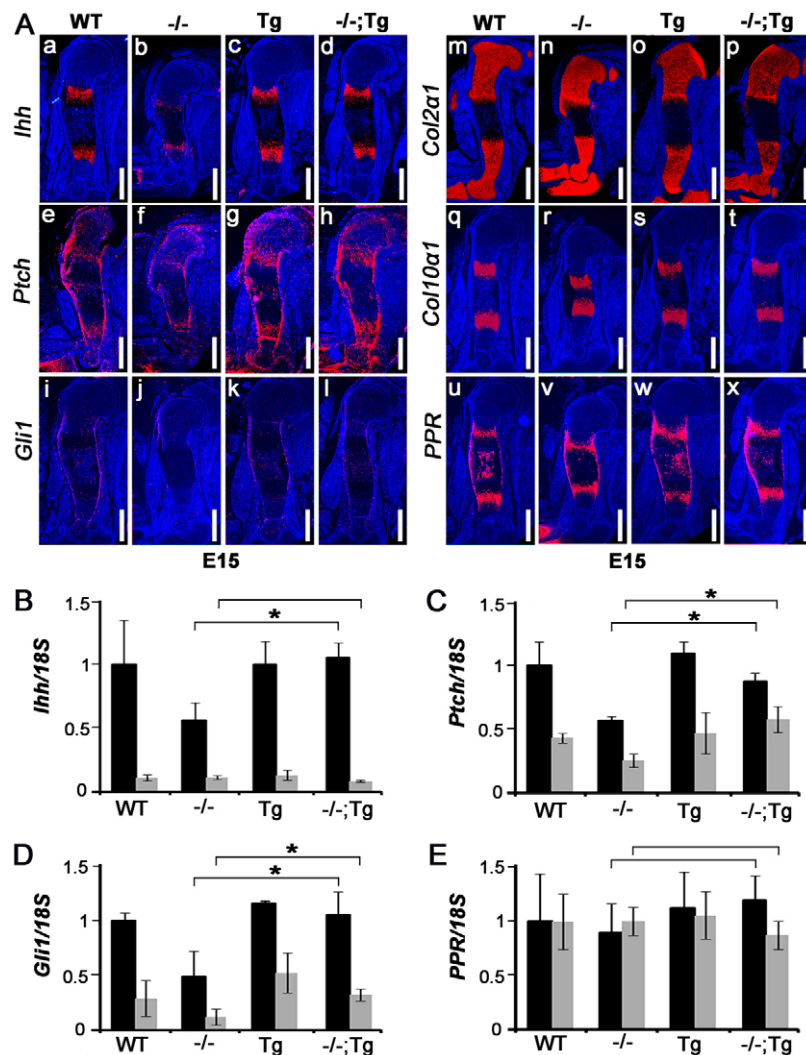
differentiation and bone mass defects in *Atf4*<sup>-/-</sup> mice were corrected by overexpression of *Atf4* in chondrocytes in vivo, we measured trabecular bone mass in distal femurs by 3D microcomputed

tomography (μCT). As expected, and in agreement with the low bone mass in *Atf4*<sup>-/-</sup> vertebrae at all ages examined (Yang et al., 2004), bone mass, as measured by a ratio of bone volume over total tissue volume (BV/TV), was 0.09±0.003% in *Atf4*<sup>-/-</sup> femurs of 1-month-old mice, which was significantly (*P*<0.05) lower than the ratio of 0.23±0.009% in *Atf4*<sup>-/-</sup>; *Col2a1-Atf4*, 0.26±0.005% in WT, and 0.27±0.008% in *Col2a1-Atf4* littermate controls (Fig. 5D,E). The rescue in BV/TV by the *Col2a1-Atf4* transgene was also observed at 3 months of age with a BV/TV of 0.26±0.020% in WT, 0.27±0.020% in *Col2a1-Atf4*, 0.27±0.024% in *Atf4*<sup>-/-</sup>; *Col2a1-Atf4* and 0.13±0.034% in *Atf4*<sup>-/-</sup> mice (Fig. 5D,F).

Histomorphometric analysis of vertebrae in 3-month-old mice confirmed that the decreased BV/TV in *Atf4*<sup>-/-</sup> mice was restored to normal levels in *Atf4*<sup>-/-</sup>; *Col2a1-Atf4* femurs (Table 2). Importantly, the markedly reduced bone formation rate over bone surface (BFR/BS) in *Atf4*<sup>-/-</sup> mice (0.43±0.07 in *Atf4*<sup>-/-</sup> vs 0.72±0.06 in WT, *P*<0.05) was normalized in *Atf4*<sup>-/-</sup>; *Col2a1-Atf4* mutants (0.64±0.09). BFR/BS in *Col2a1-Atf4* transgenic mice (0.65±0.13) was similar to that of WT mice. These data indicate that overexpression of *Atf4* in chondrocytes rescues the suboptimal osteoblast terminal differentiation and activity in *Atf4*<sup>-/-</sup> mice (Yang et al., 2004). The ratio of osteoblast number over bone surface (Ob#/BS) was normal in *Atf4*<sup>-/-</sup> mutants compared with WT littermates, which is consistent with our previous finding (Yang et al., 2004). Ob#/BS was also normal in *Col2a1-Atf4* and *Atf4*<sup>-/-</sup>; *Col2a1-Atf4* vertebrae, indicating that *Atf4* overexpression in chondrocytes is not sufficient to induce osteoblast proliferation. Lastly, the osteoclast number (Oc#/BS) and the osteoclast-covered bone surface over total bone surface (Oc.S/BS) in *Atf4*<sup>-/-</sup> vertebrae were higher than that of WT, *Col2a1-Atf4* and *Atf4*<sup>-/-</sup>; *Col2a1-Atf4* vertebrae, but these differences were not statistically significant (*P*>0.5, Table 2). Therefore, our data indicate that *Atf4* overexpression in chondrocytes promotes osteoblast differentiation and bone formation but does not significantly affect osteoclastogenesis. This finding is important as it suggests that the loss of *Atf4* in osteoblasts can be compensated for by overexpression of *Atf4* in chondrocytes.

### Overexpression of *Atf4* in chondrocytes corrects defective Hh signaling in *Atf4*<sup>-/-</sup> bone marrow stromal cells

That chondrocytic *Atf4* rescued the bone defects in *Atf4*<sup>-/-</sup> mice suggested that diffusible factors in cartilage of *Atf4*<sup>-/-</sup>; *Col2a1-Atf4* double mutants could make up for the loss of *Atf4* in osteoblasts to support proper osteoblast differentiation and bone formation. To address this hypothesis, we utilized an ex vivo system in which the conditioned media (CM) of WT, *Atf4*<sup>-/-</sup> and *Atf4*<sup>-/-</sup>; *Col2a1-Atf4* cartilage explants was collected and supplemented to the osteogenic media of WT, *Atf4*<sup>-/-</sup> and *Col2a1-Atf4* bone marrow stromal cell (BMSC) cultures. Without supplementation with cartilage CM, an 80% decrease in the number of alkaline phosphatase-positive colony forming units (CFU-ap) and mineralized nodules (CFU-ob) in *Atf4*<sup>-/-</sup> BMSC cultures compared with WT BMSC cultures was observed (Fig. 6A,B). This indicates that there is an intrinsic osteoblast differentiation defect in *Atf4*<sup>-/-</sup> BMSCs. Supplementation of WT cartilage CM led to a 45% increase in CFU-ap and CFU-ob formation in WT BMSC cultures. Interestingly, supplementation of *Atf4*<sup>-/-</sup> cartilage CM caused also a 62% increase in CFU-ap and CFU-ob formation in *Atf4*<sup>-/-</sup> BMSC cultures. These results reveal that cells in cartilage can exert a potent paracrine and stimulatory effect on osteoblast differentiation of BMSC progenitors. Importantly, supplementation of WT or



**Fig. 4. Overexpression of *Atf4* in chondrocytes corrects the reduced expression of *Ihh* and its target genes in *Atf4*<sup>-/-</sup> mice. (Aa-x)** In situ hybridization of E15 humeral sections. Note that the decrease in *Ihh*, *Ptch* and *Gli1* expression in *Atf4*<sup>-/-</sup> humeri was completely rescued in *Atf4*<sup>-/-</sup>;*Col2a1-Atf4* humeri, whereas the expression levels of *Col2a1*, *Col10a1* and *PPR* were not affected.  $n=3$ . Scale bars: 0.5 mm. **(B-E)** qRT-PCR analysis showing expression levels of *Ihh*, *Ptch*, *Gli1* and *PPR* in P0 cartilages (black bars) and bones (gray bars). Error bars represent s.e.m.  $N=3$ . \* $P<0.05$  by paired Student's *t*-test. Tg, *Col2a1-Atf4*; *-/-*;Tg, *Atf4*<sup>-/-</sup>;*Col2a1-Atf4*.

*Atf4*<sup>-/-</sup>;*Col2a1-Atf4* cartilage CM resulted in an 86% increase in CFU-ap and CFU-ob formation in *Atf4*<sup>-/-</sup> BMSC cultures (Fig. 6A,B). This demonstrates that WT or *Atf4*<sup>-/-</sup>;*Col2a1-Atf4* CM contains an osteogenic diffusible factor(s), which is not present in *Atf4*<sup>-/-</sup> cartilage CM and which is responsible for the complete rescue of the osteoblast differentiation defect of *Atf4*<sup>-/-</sup> BMSCs.

Based on the knowledge that *Ihh* is a transcriptional target gene of *Atf4* in chondrocytes and a crucial determinant of osteogenesis, as well as the observation that defective Hh signaling present in *Atf4*<sup>-/-</sup> bones was corrected in *Atf4*<sup>-/-</sup>;*Col2a1-Atf4* bones (Fig. 4), we tested whether *Ihh* was one of the osteogenic factors in WT or *Atf4*<sup>-/-</sup>;*Col2a1-Atf4* cartilage CM that promoted osteoblast differentiation of *Atf4*<sup>-/-</sup> BMSCs. We first modulated Hh signaling in the ex vivo system described above pharmacologically with cyclopamine, a Hh signaling blocker, and purmorphamine, a Hh signaling activator. Cyclopamine caused a 63% decrease in CFU-ap and CFU-ob formation in *Atf4*<sup>-/-</sup> BMSC cultures supplemented with *Atf4*<sup>-/-</sup>;*Col2a1-Atf4* cartilage CM. Conversely, and importantly, purmorphamine, but not Bmp2, completely restored CFU-ap and CFU-ob formation defect in *Atf4*<sup>-/-</sup> BMSC cultures supplemented with *Atf4*<sup>-/-</sup> cartilage CM (Fig. 6C,D). These data demonstrate that Hh signaling is required for *Atf4*<sup>-/-</sup> BMSCs to differentiate into mature osteoblasts. Next, direct blocking *Ihh* with the neutralizing antibody 5E1 but not an anti-PTHrP, reversed the

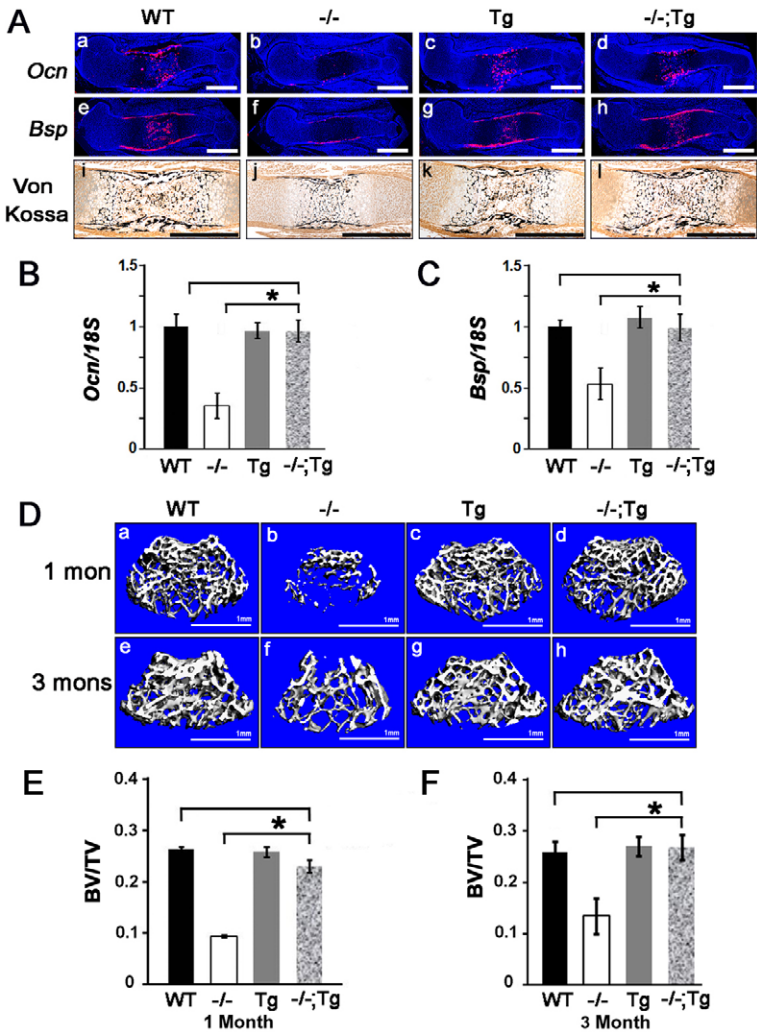
rescue of CFU-ap and CFU-ob formation in *Atf4*<sup>-/-</sup> BMSCs by supplementation of *Atf4*<sup>-/-</sup>;*Col2a1-Atf4* cartilage CM (Fig. 6C,D). Given that *Ihh* is not expressed in bones (Fig. 4A,B) and that *Ihh* is the only member of the Hh protein family expressed in chondrocytes (Bitgood and McMahon, 1995; Kronenberg, 2003), we conclude that *Ihh* is one of the major osteogenic factors differentially secreted by *Atf4*<sup>-/-</sup>;*Col2a1-Atf4* cartilage. Taken together, these data demonstrate that *Atf4* overexpression in *Atf4*<sup>-/-</sup>;*Col2a1-Atf4* cartilage, by increasing the production of *Ihh*, rescues the differentiation defect of *Atf4*<sup>-/-</sup> BMSC cultures.

## DISCUSSION

In this study we provide evidence that restoration of *Atf4* expression specifically in *Col2a1*-expressing chondrocytes in *Atf4*<sup>-/-</sup> mice rescues not only the growth defects, but also the osteogenesis defects and reduced bone mass observed in *Atf4*<sup>-/-</sup> mice. Therefore, this study identifies *Atf4* in chondrocytes as a crucial transcriptional regulator acting in a chondrocyte-autonomous fashion to regulate longitudinal growth of the skeleton and bone formation.

*Atf4* was originally identified as a nuclear binding activity specific to osteoblasts (Ducy and Karsenty, 1995; Schinke and Karsenty, 1999), and its global deletion in mice was shown to lead to severe osteopenia, impaired osteoblast terminal differentiation,





**Fig. 5. Overexpression of *Atf4* in chondrocytes restores osteoblast differentiation and bone formation in *Atf4*<sup>-/-</sup> mice.** (Aa-l) In situ hybridization of sections through E15 humeri showing that the decreased level of *Ocn* and *Bsp* in *Atf4*<sup>-/-</sup> bones was restored to normal level in *Atf4*<sup>-/-</sup>; *Col2a1-Atf4* bones. von Kossa staining of sections through E15 humeri showing rescued mineralization in *Atf4*<sup>-/-</sup>; *Col2a1-Atf4* primary ossification centers. Scale bars: 0.5 mm. (B,C) qRT-PCR analysis of P0 bones quantifying *Ocn* (B) and *Bsp* (C) expression. Error bars represent s.e.m. *n*=3. \**P*<0.05 by paired Student's *t*-test. (D) Microtomographic image showing rescued bone formation in 1- (1 mon) and 3-month (3 mons) *Atf4*<sup>-/-</sup>; *Col2a1-Atf4* femur heads. *n*=6. Scale bars: 1 mm. (E,F) Quantification of 1- and 3-month femur bone volume/tissue volume (BV/TV) measured by  $\mu$ CT. Error bars represent s.e.m. *N*=6. \**P*<0.05 by paired Student's *t*-test. Tg, *Col2a1-Atf4*; *-/-*;Tg, *Atf4*<sup>-/-</sup>; *Col2a1-Atf4*.

reduced osteocalcin expression and decreased type I collagen synthesis (Yang et al., 2004). Subsequent studies revealed that *Atf4* is also expressed in chondrocytes, in which it controls chondrocyte proliferation and differentiation during skeletal development by transcriptionally activating *Ihh* (Wang et al., 2009). These two studies have established the indispensable role of *Atf4* in osteoblasts and chondrocytes, respectively. However, the relative contribution of dysfunctional osteoblasts, chondrocytes or the combination of defects in these two cell types to the skeletal abnormalities observed in *Atf4*<sup>-/-</sup> mutants could not be defined in

this global *Atf4*<sup>-/-</sup> mouse model because *Atf4* is expressed in both cell types in equal abundance (Wang et al., 2009). By utilizing the *Atf4*<sup>-/-</sup>; *Col2a1-Atf4* model, we were able to restore the expression of *Atf4* specifically in chondrocytes, with osteoblasts remain deficient for *Atf4*. The bone elongation defects caused by dysfunctional growth plate chondrocytes in *Atf4*<sup>-/-</sup> cartilage were completely rescued by this genetic manipulation. These defects include shortened long bones and stature, disorganization of proliferating chondrocyte columns, shortening of the proliferating zone, a decrease in BrdU-positive chondrocyte number, expansion

**Table 2. Histomorphometric analysis of 3-month-old vertebrates**

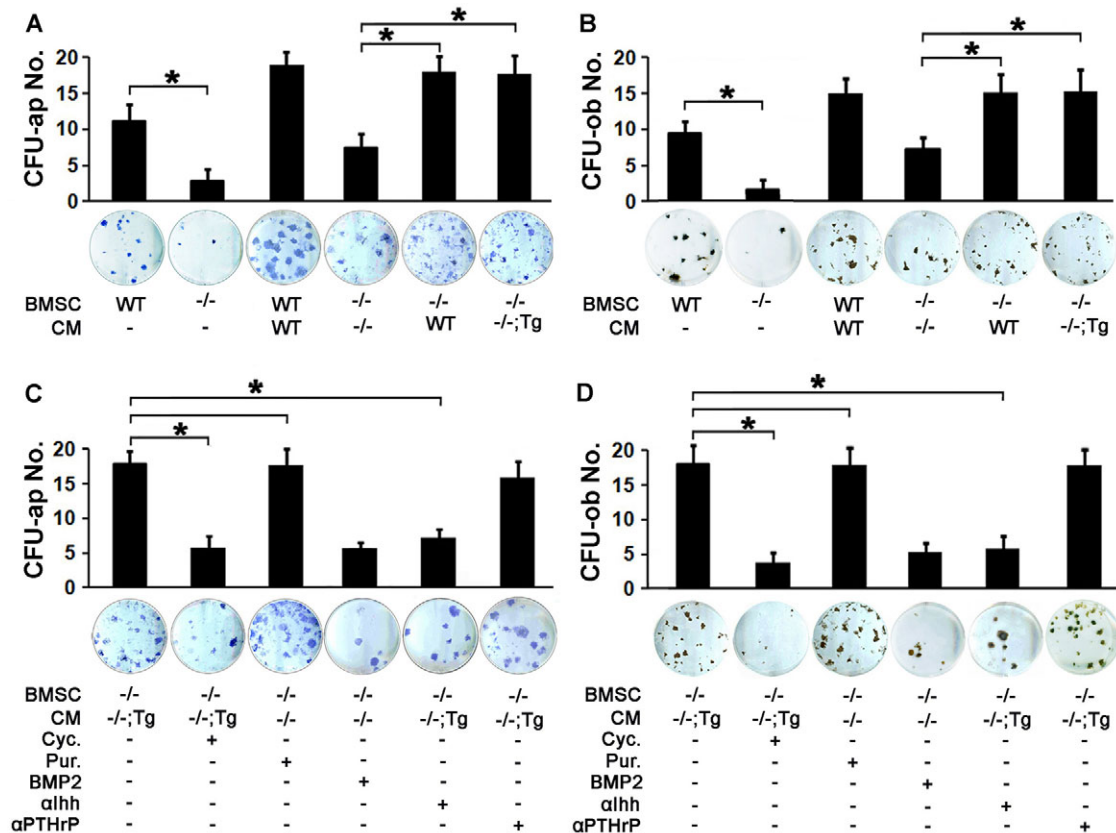
	WT	-/-	Tg	-/-;Tg
BV/TV	10.34±1.41	5.43±0.71*	10.58±1.51	9.10±0.60 <sup>‡</sup>
Tb.Th	22.88±1.78	19.05±1.43*	22.04±1.99	21.28±2.14 <sup>‡</sup>
Tb.N	4.48±0.48	2.87±0.33*	4.72±0.39	4.34±0.20 <sup>‡</sup>
BFR/BS	0.72±0.06	0.43±0.07*	0.65±0.13	0.64±0.09 <sup>‡</sup>
Ob.S/BS	7.49±0.85	7.36±1.84	7.42±1.84	8.23±1.10
Ob#/BS	6.86±0.68	6.75±1.31	7.88±1.00	7.73±0.63
Oc.S/BS	6.91±1.44	9.25±1.88	5.80±1.55	5.90±1.28
Oc#/BS	2.97±0.47	4.42±0.88	2.76±0.69	2.77±0.49

BV, bone volume; TV, tissue volume; Tb.Th, trabecular thickness; Tb.N, trabecular number; BFR, bone formation rate; BS, bone surface; Ob.S, osteoblast surface; Ob#, osteoblast number; Oc.S, osteoclast surface; Oc#, osteoclast number.

WT, *Atf4*<sup>+/+</sup>; *-/-*, *Atf4*<sup>-/-</sup> mice; Tg, *Col2a1-Atf4* transgene; *-/-*;Tg, *Atf4*<sup>-/-</sup>; *Col2a1-Atf4* double mutants.

\**P*<0.05 by paired Student's *t*-test between WT and *-/-* mice; *n*=5. <sup>‡</sup>*P*<0.05 by paired Student's *t*-test between *-/-* and *-/-*;Tg mice; *n*=5.





**Fig. 6. Cartilage conditioned media contain osteogenic activities.** (A,B) Quantification of CFU-ap (A) and CFU-ob (B) formed in BMSC cultures under osteoblast differentiation condition. Note that *Atf4*<sup>-/-</sup> BMSC failed to properly differentiate into mature osteoblasts, which can be partially rescued by *Atf4*<sup>-/-</sup> cartilage CM and completely rescued by CM of WT and *Atf4*<sup>-/-</sup>; *Col2a1-Atf4* cartilages. Error bars represent s.e.m. *n*=3. \**P*<0.05 by paired Student's *t*-test. (C,D) Quantification of CFU-ap (C) and CFU-ob (D) formed in BMSC cultures in osteoblast differentiation assays. Note that purmorphamine (Pur.) increases CFU-ap and CFU-ob formed by *Atf4*<sup>-/-</sup> BMSCs supplemented with *Atf4*<sup>-/-</sup> cartilage CM and cyclopamine (Cyc.) and 5E1, but not anti-PTHrP, decrease the CFU-ap and CFU-ob formed by *Atf4*<sup>-/-</sup> BMSCs supplemented with *Atf4*<sup>-/-</sup>; *Col2a1-Atf4* cartilage CM. Error bars represent s.e.m. *N*=3. \**P*<0.05 by paired Student's *t*-test. Tg, *Col2a1-Atf4*; -/-;Tg, *Atf4*<sup>-/-</sup>; *Col2a1-Atf4*.

of the hypertrophic chondrocyte zone and decreased *Ihh* expression and Hh signaling (Figs 1, 2) (Wang et al., 2009). The complete correction of chondrogenesis in *Atf4*<sup>-/-</sup> mutants by the *Col2a1-Atf4* transgene thus demonstrates that the reduced stature of *Atf4*<sup>-/-</sup> mice is not caused by an endocrine imbalance, and that *Atf4* regulates growth plate development, chondrocyte proliferation and differentiation in vivo in a chondrocyte-autonomous manner.

A novel finding in this study is that the transcriptional activity of *Atf4* in chondrocytes controls osteogenesis during development and postnatal bone mass accrual. This conclusion is supported by the observation that *Atf4*<sup>-/-</sup>; *Col2a1-Atf4* mice have normal bone mass (Fig. 5), bone formation rate, trabecular bone thickness and trabecular numbers (Table 2) in contrast to *Atf4*<sup>-/-</sup> mice, and despite complete absence of endogenous or ectopic *Atf4* expression in osteoblasts (Fig. 1; supplementary material Fig. S1). It is important to note that, unlike the rat *Col2a1* promoter and enhancer (Nakamura et al., 2006; Chen et al., 2007; Hilton et al., 2007), the mouse *Col2a1* promoter and enhancer used to produce the *Col2a1-Atf4* transgenic construct in this study is chondrocyte-specific and does not show leakiness in any other tissues (Mukhopadhyay et al., 1995; Zhou et al., 1995; Ovchinnikov et al., 2000). This specificity is further confirmed by our in situ hybridization data showing that expression of the *Col2a1-Atf4* transgene was never detected in the periosteum, where osteoblasts progenitors are present, or in any

cells adjacent to *Col2a1*-expressing chondrocytes, at all the developmental stages analyzed (Fig. 1; supplementary material Figs S1, S2). These observations support the crucial role of *Atf4* in chondrocytes for the regulation of osteoblast differentiation and osteogenesis.

Based on our previous study (Wang et al., 2009) and on the ex vivo assays (Fig. 6) reported here, *Ihh* appears to be one of the main mediators of the effect of chondrocytes on osteogenesis that is missing in *Atf4*<sup>-/-</sup> cartilage. The role of Hh signaling in the regulation of osteoblast differentiation and function is supported by the observations that Hh signaling components, including *smoothed*, *patched* and *Gli* genes, are expressed in osteoblasts (Ohba et al., 2008; Joeng and Long, 2009) and deletion of *Ihh* in mice leads to bone defects in embryos and pups (St-Jacques et al., 1999). However, osteoblasts might not be a source of *Ihh* in vivo. Some in vitro studies suggested that *Ihh* is expressed in osteoblastic cell lines (Murakami et al., 1997; Jemtland et al., 2003), yet *Ihh* expression level is minimal, if not absent, in bone marrow osteoblasts in vivo (Fig. 2) (Chung et al., 2001; Wang et al., 2009). This strongly suggests that *Ihh* expression by osteoblastic cell lines was characteristic of immortalized transformed cells because *Ihh* is known to be upregulated in many tumor cells (Kayed et al., 2004; Xuan and Lin, 2009). Three main observations strongly suggest that the transcriptional activity of *Atf4* in chondrocytes is

required for proper osteogenesis, via its control of *Ihh* production. First, *Ihh* is specifically expressed by prehypertrophic chondrocytes but not in osteoblasts in vivo (Fig. 4) (Razzaque et al., 2005; Maeda et al., 2010). Second, the differentiation of *Atf4*<sup>-/-</sup> osteoblasts induced by the conditioned media of WT or *Atf4*<sup>-/-</sup>; *Col2a1-Atf4* cartilage can be blocked by neutralization of *Ihh* (Fig. 6). Lastly, deletion of *Ihh* specifically in *Col2a1*-expressing chondrocytes impairs osteoblast differentiation leading to loss of trabecular bone over time (Maeda et al., 2007). Additionally, our ex vivo data indicates that *Ihh* is not the only factor secreted by cartilage to promote osteogenesis, given that the WT and *Atf4*<sup>-/-</sup> cartilage CM stimulates the osteoblast differentiation of WT and *Atf4*<sup>-/-</sup> BMSC cultures, respectively (Fig. 6A,B). Additional factors, such as fibroblast growth factors, Bmps and Wnts, which are not direct transcriptional targets of *Atf4* and are produced by the cartilage, might contribute to the osteogenic activity observed in *Atf4*<sup>-/-</sup> cartilage CM.

The exact population of cells that chondrocyte-derived *Atf4* and *Ihh* target and the time point(s) during skeletal development at which this *Atf4*-*Ihh* effect occurs remain unclear and warrant further investigation. Mesenchymal progenitors before bone marrow formation might be the earliest target cells of chondrocyte-derived *Ihh*. Later during development, perichondrial and periosteal mesenchymal osteoblast progenitors might be relevant targets and they could contribute, at least partly, to the mechanism whereby *Atf4* and *Ihh* from chondrocytes indirectly control osteogenesis. It is also unclear whether chondrocyte-derived *Ihh* can act as a paracrine factor to control osteoblast differentiation in the primary spongiosa at later developmental stages once the marrow has formed. If it is biologically relevant, the regulation of *Ihh* by *Atf4* should be restricted to the bone growth period and cease or diminish once the growth plate has closed, because the source of *Ihh* then disappears. These speculative mechanisms will be best addressed by the use of conditional and inducible *Atf4* mutant mice.

The observation that overexpression of *Atf4* solely in chondrocytes rescues low bone mass in *Atf4*<sup>-/-</sup> mice (Figs 2-5) challenges the original notion that dysfunctional osteoblasts, characterized by impaired differentiation and collagen production (Yang et al., 2004), underlie this phenotype. This study supports the concept that bone mass accrual, which relies on osteoblast differentiation and activity, might be controlled, at least in part, by the transcriptional activity of *Atf4* in chondrocytes at early stages of development. The cell-autonomous role of *Atf4* in osteoblasts (Yang et al., 2004) might be secondary at these developmental stages, but might become more prominent in adults once the growth plate has closed, and contribute to the processes of bone remodeling, bone repair or glucose homeostasis (Hinoi et al., 2009; Yoshizawa et al., 2009) via its effects on *Ocn* production, amino acids import and stress responses (Harding et al., 2003; Sowa and Karsenty, 2007). Future studies comparing chondrocyte- and osteoblast-specific *Atf4* mutant mice will be instrumental to finding the answers to these questions.

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#### Competing interests statement

The authors declare no competing financial interests.

#### Author contributions

W.W. performed the majority of the research and drafted the paper. N.L. and Y.M. performed co-culture assays and part of the bone histology. L.L. generated and maintained the *Col2-Atf4* transgenic mice. R.C.G. helped genotyping the mice. F.E. participated in the design of experimental approaches and in the redaction of the manuscript. X.Y. supervised the research and wrote the manuscript.

#### Supplementary material

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#### References

- Alcedo, J. and Noll, M. (1997). Hedgehog and its patched-smoothened receptor complex: a novel signalling mechanism at the cell surface. *Biol. Chem.* **378**, 583-590.
- Bitgood, M. J. and McMahon, A. P. (1995). Hedgehog and BMP genes are coexpressed at many diverse sites of cell-cell interaction in the mouse embryo. *Dev. Biol.* **172**, 126-138.
- Chen, M., Lichtler, A. C., Sheu, T. J., Xie, C., Zhang, X., O'Keefe, R. J. and Chen, D. (2007). Generation of a transgenic mouse model with chondrocyte-specific and tamoxifen-inducible expression of Cre recombinase. *Genesis* **45**, 44-50.
- Chung, U. I., Schipani, E., McMahon, A. P. and Kronenberg, H. M. (2001). Indian hedgehog couples chondrogenesis to osteogenesis in endochondral bone development. *J. Clin. Invest.* **107**, 295-304.
- Dobson, K. R., Reading, L., Haberey, M., Marine, X. and Scutt, A. (1999). Centrifugal isolation of bone marrow from bone: an improved method for the recovery and quantitation of bone marrow osteoprogenitor cells from rat tibiae and femur. *Calcif. Tissue Int.* **65**, 411-413.
- Ducy, P. and Karsenty, G. (1995). Two distinct osteoblast-specific cis-acting elements control expression of a mouse osteocalcin gene. *Mol. Cell. Biol.* **15**, 1858-1869.
- Hall, B. K. (1988). The embryonic development of bone. *Am. Sci.* **76**, 174-181.
- Harding, H. P., Zhang, Y., Zeng, H., Novoa, I., Lu, P. D., Calfon, M., Sadri, N., Yun, C., Popko, B., Paules, R. et al. (2003). An integrated stress response regulates amino acid metabolism and resistance to oxidative stress. *Mol. Cell* **11**, 619-633.
- Hilton, M. J., Tu, X. and Long, F. (2007). Tamoxifen-inducible gene deletion reveals a distinct cell type associated with trabecular bone, and direct regulation of PTHrP expression and chondrocyte morphology by *Ihh* in growth region cartilage. *Dev. Biol.* **308**, 93-105.
- Hinoi, E., Gao, N., Jung, D. Y., Yadav, V., Yoshizawa, T., Kajimura, D., Myers, M. G., Jr, Chua, S. C., Jr, Wang, Q., Kim, J. K. et al. (2009). An Osteoblast-dependent mechanism contributes to the leptin regulation of insulin secretion. *Ann. New York Acad. Sci.* **1173** Suppl. 1, E20-E30.
- Jemtland, R., Divieti, P., Lee, K. and Segre, G. V. (2003). Hedgehog promotes primary osteoblast differentiation and increases PTHrP mRNA expression and iPTHrP secretion. *Bone* **32**, 611-620.
- Joeng, K. S. and Long, F. (2009). The Gli2 transcriptional activator is a crucial effector for *Ihh* signaling in osteoblast development and cartilage vascularization. *Development* **136**, 4177-4185.
- Karsenty, G. and Wagner, E. F. (2002). Reaching a genetic and molecular understanding of skeletal development. *Dev. Cell* **2**, 389-406.
- Karsenty, G., Kronenberg, H. M. and Settembre, C. (2009). Genetic control of bone formation. *Annu. Rev. Cell Dev. Biol.* **25**, 629-648.
- Kayed, H., Kleeff, J., Keleg, S., Guo, J., Ketterer, K., Berberat, P. O., Giese, N., Esposito, I., Giese, T., Buchler, M. W. et al. (2004). Indian hedgehog signaling pathway: expression and regulation in pancreatic cancer. *Int. J. Cancer* **110**, 668-676.
- Kronenberg, H. M. (2003). Developmental regulation of the growth plate. *Nature* **423**, 332-336.
- Lian, N., Wang, W., Li, L., Eleftheriou, F. and Yang, X. (2009). Vimentin inhibits ATF4-mediated osteocalcin transcription and osteoblast differentiation. *J. Biol. Chem.* **284**, 30518-30525.
- Long, F., Zhang, X. M., Karp, S., Yang, Y. and McMahon, A. P. (2001). Genetic manipulation of hedgehog signaling in the endochondral skeleton reveals a direct role in the regulation of chondrocyte proliferation. *Development* **128**, 5099-5108.
- Maeda, Y., Nakamura, E., Nguyen, M. T., Suva, L. J., Swain, F. L., Razzaque, M. S., Mackem, S. and Lanske, B. (2007). Indian Hedgehog produced by

- postnatal chondrocytes is essential for maintaining a growth plate and trabecular bone. *Proc. Natl. Acad. Sci. USA* **104**, 6382-6387.
- Maeda, Y., Schipani, E., Densmore, M. J. and Lanske, B.** (2010). Partial rescue of postnatal growth plate abnormalities in *lhh* mutants by expression of a constitutively active PTH/PTHrP receptor. *Bone* **46**, 472-478.
- Maes, C., Kobayashi, T., Selig, M. K., Torrekens, S., Roth, S. I., Mackem, S., Carmeliet, G. and Kronenberg, H. M.** (2010). Osteoblast precursors, but not mature osteoblasts, move into developing and fractured bones along with invading blood vessels. *Dev. Cell* **19**, 329-344.
- Mak, K. K., Kronenberg, H. M., Chuang, P. T., Mackem, S. and Yang, Y.** (2008). Indian hedgehog signals independently of PTHrP to promote chondrocyte hypertrophy. *Development* **135**, 1947-1956.
- Metsaranta, M., Toman, D., de Crombrughe, B. and Vuorio, E.** (1991). Mouse type II collagen gene. Complete nucleotide sequence, exon structure, and alternative splicing. *J. Biol. Chem.* **266**, 16862-16869.
- Metsaranta, M., Garofalo, S., Smith, C., Niederreither, K., de Crombrughe, B. and Vuorio, E.** (1995). Developmental expression of a type II collagen/beta-galactosidase fusion gene in transgenic mice. *Dev. Dyn.* **204**, 202-210.
- Mukhopadhyay, K., Lefebvre, V., Zhou, G., Garofalo, S., Kimura, J. H. and de Crombrughe, B.** (1995). Use of a new rat chondrosarcoma cell line to delineate a 119-base pair chondrocyte-specific enhancer element and to define active promoter segments in the mouse pro- $\alpha$  1(II) collagen gene. *J. Biol. Chem.* **270**, 27711-27719.
- Murakami, S., Nifuji, A. and Noda, M.** (1997). Expression of Indian hedgehog in osteoblasts and its posttranscriptional regulation by transforming growth factor- $\beta$ . *Endocrinology* **138**, 1972-1978.
- Nakamura, E., Nguyen, M. T. and Mackem, S.** (2006). Kinetics of tamoxifen-regulated Cre activity in mice using a cartilage-specific CreER(T) to assay temporal activity windows along the proximodistal limb skeleton. *Dev. Dyn.* **235**, 2603-2612.
- Ohba, S., Kawaguchi, H., Kugimiya, F., Ogasawara, T., Kawamura, N., Saito, T., Ikeda, T., Fujii, K., Miyajima, T., Kuramochi, A. et al.** (2008). Patched1 haploinsufficiency increases adult bone mass and modulates Gli3 repressor activity. *Dev. Cell* **14**, 689-699.
- Ovchinnikov, D. A., Deng, J. M., Ogunrinu, G. and Behringer, R. R.** (2000). Col2a1-directed expression of Cre recombinase in differentiating chondrocytes in transgenic mice. *Genesis* **26**, 145-146.
- Razzaque, M. S., Soegiarto, D. W., Chang, D., Long, F. and Lanske, B.** (2005). Conditional deletion of Indian hedgehog from collagen type 2 $\alpha$ 1-expressing cells results in abnormal endochondral bone formation. *J. Pathol.* **207**, 453-461.
- Reddi, A. H.** (1994). 'Bone and cartilage differentiation. *Curr. Opin. Genet. Dev.* **4**, 737-744.
- Schinke, T. and Karsenty, G.** (1999). Characterization of *Osf1*, an osteoblast-specific transcription factor binding to a critical cis-acting element in the mouse osteocalcin promoters. *J. Biol. Chem.* **274**, 30182-30189.
- Sowa, H. and Karsenty, G.** (2007). ATF4 is a key molecule linking food intake and skeletal development. *J. Musculoskelet. Neuronal Interact.* **7**, 326-327.
- St-Jacques, B., Hammerschmidt, M. and McMahon, A. P.** (1999). Indian hedgehog signaling regulates proliferation and differentiation of chondrocytes and is essential for bone formation. *Genes Dev.* **13**, 2072-2086.
- Vortkamp, A., Lee, K., Lanske, B., Segre, G. V., Kronenberg, H. M. and Tabin, C. J.** (1996). Regulation of rate of cartilage differentiation by Indian hedgehog and PTH-related protein. *Science* **273**, 613-622.
- Wang, W., Lian, N., Li, L., Moss, H. E., Wang, W., Perrien, D. S., Elefteriou, F. and Yang, X.** (2009). Atf4 regulates chondrocyte proliferation and differentiation during endochondral ossification by activating *lhh* transcription. *Development* **136**, 4143-4153.
- Xuan, Y. and Lin, Z.** (2009). Expression of Indian Hedgehog signaling molecules in breast cancer. *J. Cancer Res. Clin. Oncol.* **135**, 235-240.
- Yang, X., Ji, X., Shi, X. and Cao, X.** (2000). Smad1 domains interacting with Hoxc-8 induce osteoblast differentiation. *J. Biol. Chem.* **275**, 1065-1072.
- Yang, X., Matsuda, K., Bialek, P., Jacquot, S., Masuoka, H. C., Schinke, T., Li, L., Brancorsini, S., Sassone-Corsi, P., Townes, T. M. et al.** (2004). ATF4 is a substrate of RSK2 and an essential regulator of osteoblast biology; implication for Coffin-Lowry Syndrome. *Cell* **117**, 387-398.
- Yoshida, C. A., Yamamoto, H., Fujita, T., Furuichi, T., Ito, K., Inoue, K., Yamana, K., Zanma, A., Takada, K., Ito, Y. et al.** (2004). Runx2 and Runx3 are essential for chondrocyte maturation, and Runx2 regulates limb growth through induction of Indian hedgehog. *Genes Dev.* **18**, 952-963.
- Yoshizawa, T., Hinoi, E., Jung, D. Y., Kajimura, D., Ferron, M., Seo, J., Graff, J. M., Kim, J. K. and Karsenty, G.** (2009). The transcription factor ATF4 regulates glucose metabolism in mice through its expression in osteoblasts. *J. Clin. Invest.* **119**, 2807-2817.
- Zhou, G., Garofalo, S., Mukhopadhyay, K., Lefebvre, V., Smith, C. N., Eberspaecher, H. and de Crombrughe, B.** (1995). A 182 bp fragment of the mouse pro  $\alpha$  1(II) collagen gene is sufficient to direct chondrocyte expression in transgenic mice. *J. Cell Sci.* **108**, 3677-3684.