FUNCTION OF ATF4 DURING ENDOCHONDRAL OSSIFICATION

By

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CHAPTER I

INTRODUCTION

Skeleton formation

Formation of the vertebrate skeleton requires the coordinated proliferation, migration, and differentiation of a variety of cell types, including chondrocyte, osteoblast, and osteo/chondroclasts (Karsenty et al., 2009). Chondrocytes are of mesenchymal origin, and they produce cartilage, a compliant connective tissue of the musculoskeletal system. Osteoblasts also differentiate from mesenchymal cells and build the bone. Osteo/chondroclasts are derived from bone marrow myelo-monocytic cells and responsible for digesting the bone/cartilage matrix. During mammalian development, the mesenchymal cells that form the skeleton are derived from distinct embryonic origins. Neural crest cells contribute to the skull and craniofacial bones, paraxial mesoderm contributes to both the craniofacial and the axial skeleton, and the lateral plate mesoderm supplies progenitor cells for the limb skeleton (Olsen et al., 2000) (Fig. 1). Despite the distinctly different embryonic lineages that contribute to skeletal formation, there are similarities in function and gene expression in the skeletal cells derived from these lineages; and moreover, these cells all participate in several phases of skeletogenesis. Initially, mesenchymal progenitor cells migrate to sites of skeletogenesis, then differentiate either directly into bone-forming osteoblasts (a process termed intermembranous bone formation, occurring primarily in the craniofacial skeleton in which there is no cartilaginous template for bone formation) or into chondrocytes, which

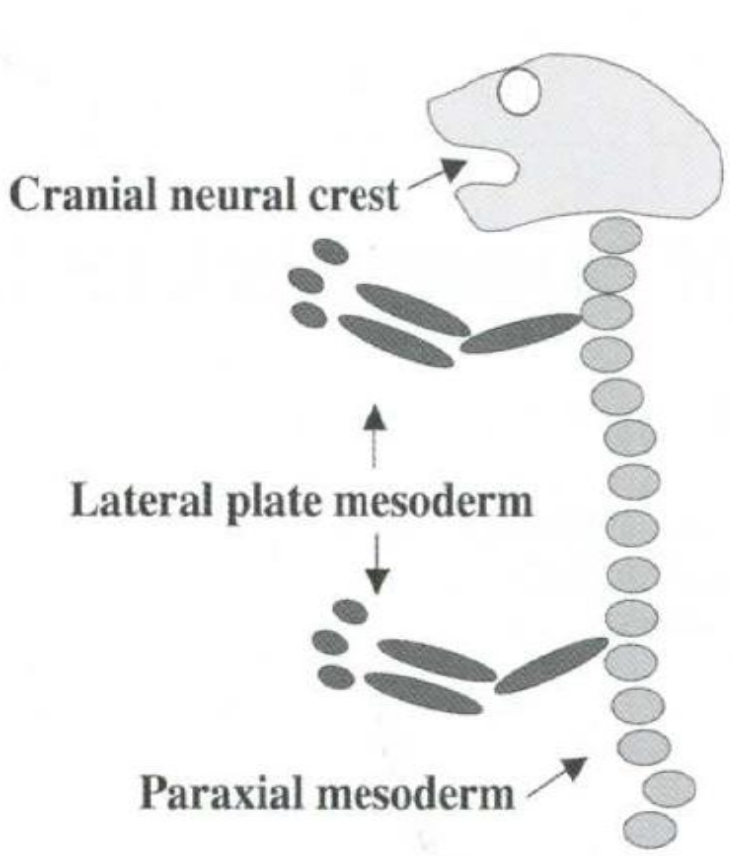

Figure 1. Progenitor cells of skeleton

Diagram illustrating the three sources of osteochondroprogenitor cells for the craniofacial (light gray), axial (intermediate gray), and limb skeleton (dark gray) **(Pourquié, 2009).**.

will go on to form a cartilaginous template for subsequent bone formation (Olsen et al., 2000).

Endochondral bone formation

Bone is a form of mineralized connective tissue in the skeleton of high vertebrates. There are two different processes to develop bones: intramembranous ossification and endochondral ossification. Some skeletal elements, such as flat bones of the skull, are formed by intramembranous ossification, whereby mesenchymal stem cells (MSCs) directly differentiate into osteoblasts that build the bone matrix. On the other hand, long bones, such as humerus, femur, tibia and most of the other bones in the body are formed by endochondral ossification, in which bone formation is intermediated by cartilage development (Karsenty et al., 2009). In endochondral ossification, the MSCs condense at the sites of the future skeletal elements (Fig. 2a), and differentiate into round chondrocytes, which secrete aggrecan and collagen type II into extracellular matrix (ECM) (Fig. 2b). These chondrocytes then proliferate and enlarge the size of cartilage elements. The cells in the center of the cartilage elements undergo a series of differentiation steps and become hypertrophic chondrocytes, which then secrete collagen type X into the ECM (Fig. 2c). The MSCs surrounding the cartilage elements flatten and differentiate into perichondrium, which will guide the future growth and the differentiation of the cartilage. In addition, the hypertrophic cells provide signals that induce osteogenesis in the abutted perichondrium, which then becomes periosteum (Fig. 2d). Finally, the hypertrophic chondrocytes undergo apoptosis, leaving the mineralized ECM as a scaffold for future osteogenesis. Hypertrophic chondrocytes also attract blood

vessels invading the cartilage mold, and recruit chondroclasts and osteoblasts. This cartilage ECM is digested by chondroclasts, and is replaced by a bone ECM rich in collagen type I secreted by osteoblasts in the primary ossification center (Fig. 2e) (Kronenberg, 2003).

The chondrocytes in the proximal and distal end of the skeletal elements continue to proliferate, and are separated by the primary ossification center into two growth plates. These plates contain resting, proliferative, prehypertrophic and hypertrophic chondrocytes sequentially arranged from articular end to the edge of primary ossification center of the skeletal elements (Fig. 2f, g). Each type of chondrocyte has different cell morphology and gene expression, and occupies in distinguished but connected zone in the growth plate (Fig. 3). Resting chondrocytes express *Collagen type II α 1* (*Col2α1*) and *Fibroblast growth factor receptor 1* (*Fgfr1*). They take round shapes, and have low proliferation. Proliferative chondrocytes are rapidly dividing flat cells, which express *Col2α1* and *Fgfr3.* They stack together linearly, forming the columnar structures in the proliferative zone. Prehypertrophic chondrocytes express *Indian hedgehog* (*Ihh*) and *PTHrP/PTH receptor* (*PPR*). They exit the rapid dividing stage, start to enlarge their sizes in a narrow prehypertrophic zone, and then become hypertrophic chondrocytes that express *Col10α1*(Fig. 3).

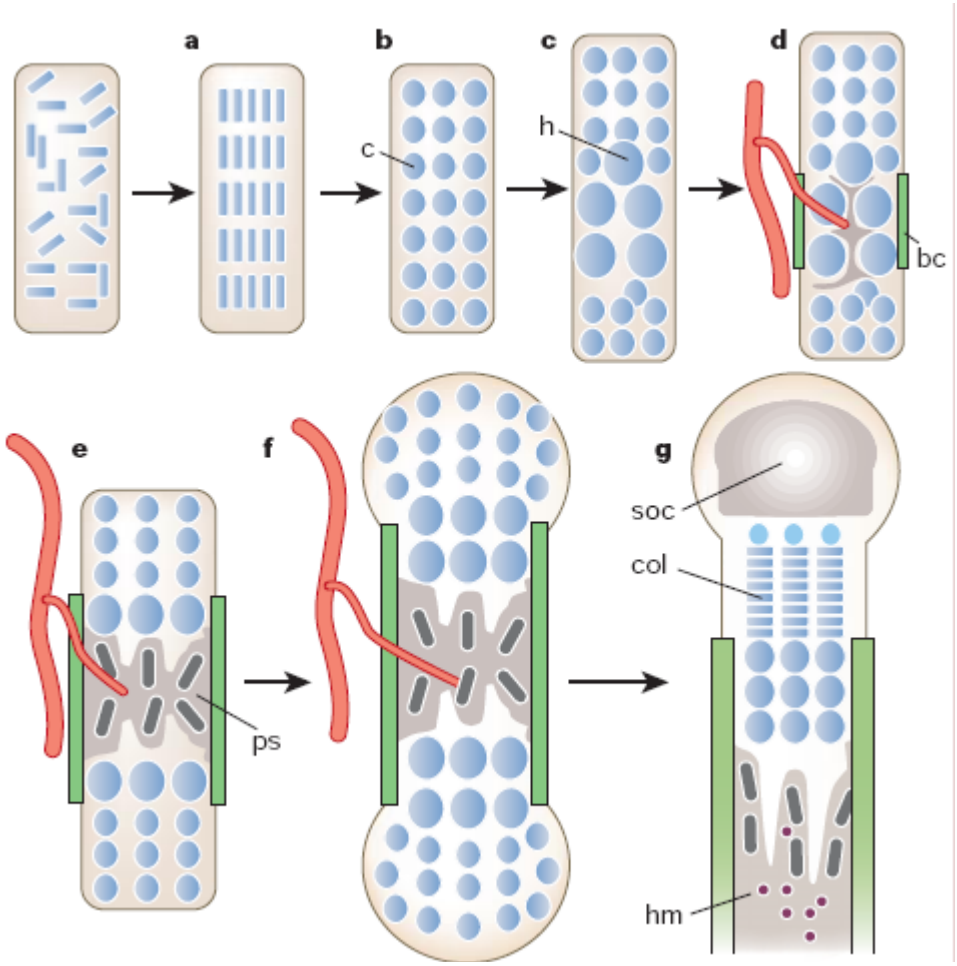
a
b
c
d
c
h
bc
e
f
g
ps
soc
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Figure 2. Endochondral bone formation (Kronenberg, 2003).

a, Mesenchymal cells condense.

b, Cells of condensations become chondrocytes (c).

c, Chondrocytes at the centre of condensation stop proliferating and become hypertrophic (h).

d, Perichondrial cells adjacent to hypertrophic chondrocytes become osteoblasts, forming bone collar (bc). Hypertrophic chondrocytes direct the formation of mineralized matrix, attract blood vessels, and undergo apoptosis.

e, Osteoblasts of primary spongiosa accompany vascular invasion, forming the primary spongiosa (ps).

f, Chondrocytes continue to proliferate, lengthening the bone. Osteoblasts of primary spongiosa are precursors of eventual trabecular bone; osteoblasts of bone collar become cortical bone.

g, At the end of the bone, the secondary ossification centre (soc) forms through cycles of chondrocyte hypertrophy, vascular invasion and osteoblast activity. The growth plate below the secondary centre of ossification forms orderly columns of proliferating chondrocytes (col). Haematopoietic marrow (hm) expands in marrow space along with stromal cells.

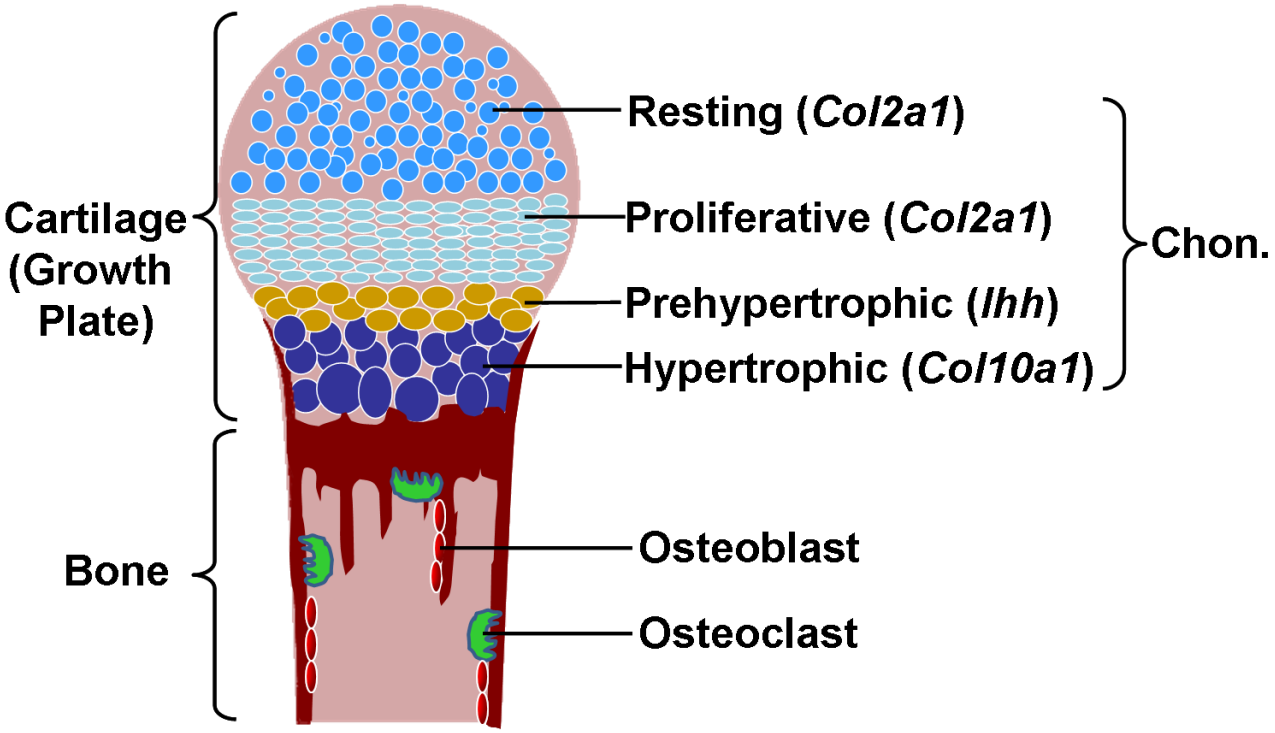

Fig. 3 Simplified diagram of chondrocyte differentiation in growth plate
Diagram showing the four different chondrocytes in the cartilage growth plate including resting, proliferative, prehypertrophic, and hypertrophic chondrocytes. In addition, osteoblasts build and Osteoclasts reabsorbs the bone.

Although the hypertrophic chondrocytes keep undergoing apoptosis and give space to trabecular bone growth, the hypertrophic cell layer is maintained by adding new hypertrophic cells differentiated from prehypertrophic cells, and prehypertrophic cells are renewed by proliferative cells. Thus, while trabecular bones replace cartilage, the proliferative chondrocytes maintain the length of the cartilage growth plates. This whole process leads to the longitudinal growth of skeletal elements (Kronenberg, 2003). Multiple factors and endogenous transcription factors regulate the growth and differentiation of chondrocytes to maintain the proper structure and function of growth plate, until closure of the epiphysis (growth plate fusion) at puberty. At the same time, osteogenesis is also regulated by many factors and endogenous transcription factors. Therefore, the simultaneous replacement of cartilage ECM by bone ECM at the interface of growth plate and primary ossification center requires orchestra of multiple signaling systems to coordinate chondrogenesis and osteogenesis during endochondral ossification. Indian hedgehog (Ihh) is one of these factors in skeletal development (Kronenberg, 2003).

Ihh and Hh signaling

Ihh is a secreted morphogen that belongs to the Hedgehog (Hh) proteins, which is involved in multiple developmental processes in both invertebrates and vertebrates. Hh is synthesized as a 45 kDa precursor, but is cleaved to an active 19 kDa N-terminal fragment, which is subsequently modified by attachment of cholesterol and palmitic acid. Secreted active Hh fragments can regulate cellular activities of neighboring and distant cells (Ingham and McMahon, 2001). In *Drosophila*, in which Hh was first discovered and is best understood, the long-range effects of Hh are facilitated by Hh-cholesterol

interactions with heparan sulfate proteoglycans (HSPG) in the surrounding extracellular matrix (Ingham and McMahon, 2001). Hedgehog proteins are conserved in vertebrates. There are three vertebrate Hh proteins: Desert hedgehog (Dhh), Sonic hedgehog (Shh), and Indian hedgehog (Ihh). All of them have unique sets of functions in regulation of different developmental processes. Dhh has the closest sequence similarity to the *Drosophila* Hh, and is essential for the development of peripheral nerves and spermatogenesis. Shh is involved in establishing lateral asymmetry, the anterior-posterior limb axis, and development of the central nervous system. Ihh is a master regulator of endochondral bone development (McMahon et al., 2003).

Hh signaling is a highly conserved pathway and essential for the patterning and morphogenesis of many different regions within the bodies of vertebrates and invertebrates (Ingham and McMahon, 2001). In the conventional model of Hh signal transduction (Fig. 4), Hh is bound by a receptor complex consisting of the 12-transmembrane receptor Patched (Ptc) and the 7-transmembrane receptor Smoothened (Smo). Two Ptc genes, Ptc1 and Ptc2, have been identified in vertebrates. They both bind Hh proteins with similar affinity, and both can interact with mammalian Smo. Ptc1 is widely expressed throughout the mouse embryo and serves as the extracellular receptor for multiple Hh proteins, and is itself upregulated by Hh signaling. Ptc2, on the other hand, is more discreetly expressed with high levels in the skin and spermatocytes where it is thought to act as the receptor for Dhh that is co-expressed in the testis (Carpenter et al., 1998). Ptc represses the intrinsic intracellular signaling activity of Smo.

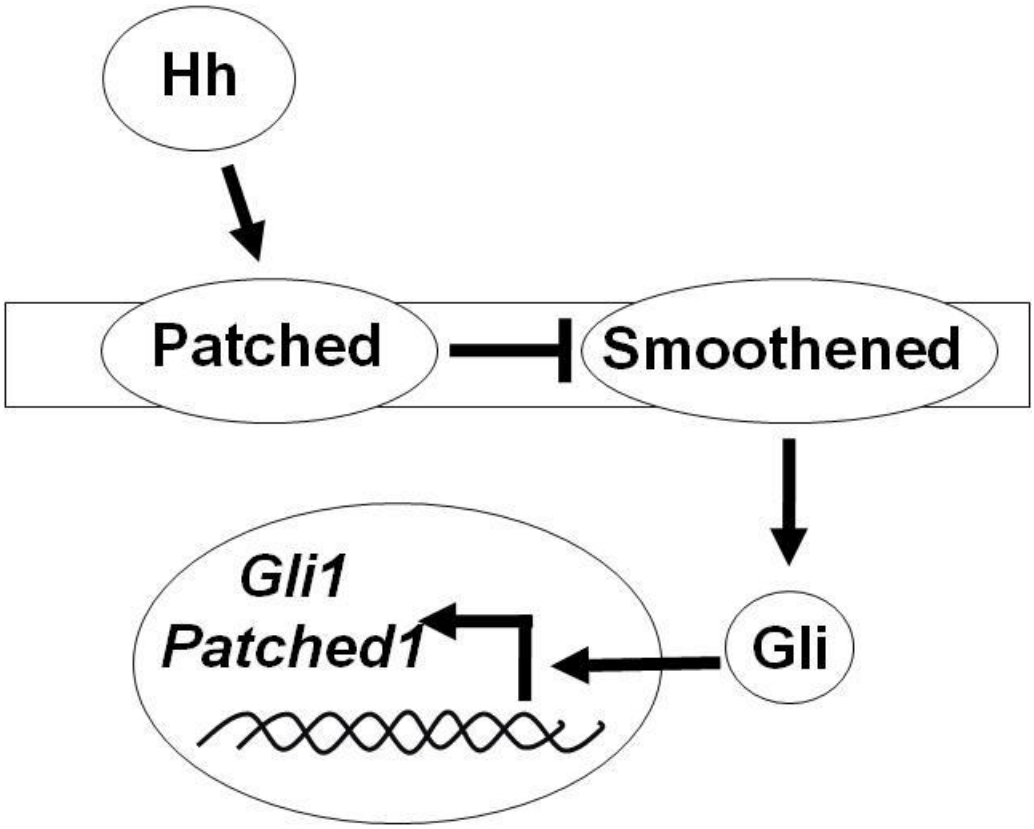

Figure 4. Hh signal transduction

Diagram showing Hh signaling pathway. Binding of Hh to Patched releases the repression of Smoothened, which, via a complex signaling cascade, alters the activity of the zinc finger transcription factor Gli. Activated Gli serves as a transcriptional activator of Hh target genes including Patched1 and Gli1.

Binding of Hh to Ptc releases the repression of Smo, which, via a complex signaling cascade, alters the activity of the zinc finger transcription factor Cubitus interruptus (Ci) (Lum and Beachy, 2004). Ci belongs to the Gli family of transcription factors. These proteins contain a domain of five conserved zinc fingers and a conserved C-terminal transactivation domain. In the absence of Hh signals, the 155 kDa Ci protein is phosphorylated and proteolytically processed into a truncated N-terminal repressor protein of 75 kDa containing the zinc fingers, which inhibits the expression of Hh target genes. Upon Hh signaling, phosphorylation and thus proteolytic processing is blocked, and full length Ci protein acts as a transcriptional activator of Hh target genes (Aza-Blanc et al., 1997); (Chen et al., 1998); (Jia et al., 2002); (Methot and Basler, 2001); (Price and Kalderon, 2002).

In vertebrates, intracellular Hh signaling activity is mediated through three Ci homologues and zinc finger transcription factors Gli1, Gli2, and Gli3 (Ingham and McMahon, 2001). Biochemical investigations indicate that, similar to Ci, Gli2 and Gli3 can be proteolytically processed into a truncated repressor form, whereas Gli1 lacks the protein kinase A recognition site necessary for phosphorylation and subsequent cleavage. Gli1 is therefore likely to function exclusively as an activator (Aza-Blanc et al., 2000); (Price and Kalderon, 2002); (von Mering and Basler, 1999). Gli2 and Gli3 can function as either transcriptional activators or inhibitors depending on the cellular context (Ruiz i Altaba et al., 2002).

Mutations in vertebrate Gli genes result in a range of different phenotypes, however the process of endochondral ossification is only mildly affected (Mo et al., 1997); (Park et al., 2000); (Schimmang et al., 1992). Whereas no bone phenotype has been detected in *Gli1*$^{-/-}$ mutants, loss of Gli2 or Gli3 results in a slight reduction in bone length. Analysis of *Gli2*$^{-/-}$;*Gli3*$^{+/-}$ compound mutants revealed a more severe phenotype indicating functional redundancy of Gli2 and Gli3 in controlling endochondral bone formation (Mo et al., 1997).

Gli3 acts mainly as a repressor but also can be an activator in skeletal development. One of the striking evidences for its repression function is provided by the analysis of *Shh*$^{-/-}$;*Gli3*$^{-/-}$ double mutants. Limbs of *Shh* mutants lack anterior-posterior polarity, and can only develop one digit. Loss of *Gli3* converts the Shh phenotype into the polydactylous limb phenotype of *Gli3*$^{-/-}$ mutants (Litingtung et al., 2002); (te Welscher et al., 2002). Shh seems thus to act mainly by opposing the repressive activities of Gli3. Other evidence for Gli3 as a repressor is from the analysis of *Ihh*$^{-/-}$;*Gli3*$^{-/-}$ double mutants. In the absence of Ihh signaling, Gli3 acts as a strong repressor, negatively controlling chondrocyte proliferation and inhibiting the expression of the two Ihh target genes, *Patched1* and *PTHrP*. Interestingly, loss of *Gli3* function in mice, which over expresses *Ihh* in chondrocytes, rescues the delayed onset of hypertrophic differentiation. This strongly suggests an activating role of Gli3 downstream of Ihh in activating PTHrP expression.

Ihh in endochondral bone formation

Ihh is the only member of the hedgehog (Hh) family secreted molecules that is expressed in chondrocytes during endochondral bone formation (Bitgood and McMahon, 1995; Karsenty et al., 2009). Ihh is an important regulator of the growth plate chondrocytes. It stimulates chondrocyte proliferation, enhances maturation of hypertrophy chondrocytes in the lower border of hypertrophic zone and prevents premature differentiation of chondrocytes in the higher border of hypertrophic zone (St-Jacques et al., 1999); (Long et al., 2001).

As shown in Fig. 5, Ihh is expressed and secreted by pre-hypertrophic chondrocytes preceding and overlapping with expression of PPR. Ihh either directly or indirectly induces PTHrP production from the periarticular perichondrium (Fig. 5(1)). PTHrP is able to diffuse to the PPR that is expressed by both proliferative and pre-hypertrophic chondrocytes. Activation of PPR in these cells delays their rate of differentiation into hypertrophic chondrocytes, and thereby inhibits the synthesis of Ihh by keeping chondrocyte in the proliferative state (Fig. 5(2)). This forms a negative feedback regulatory loop to control the pace of long bone growth during endochondral bone formation (Karp et al., 2000). This feedback loop is important for regulation of normal endochondral bone development. Disruption of any component of the system results in abnormal limb development. In $Ihh^{-/-}$ mice, PTHrP was not detected in the periarticular regions of cartilaginous structures, and chondrocyte differentiation was affected. Chondrocyte hypertrophic differentiation was initially delayed and later

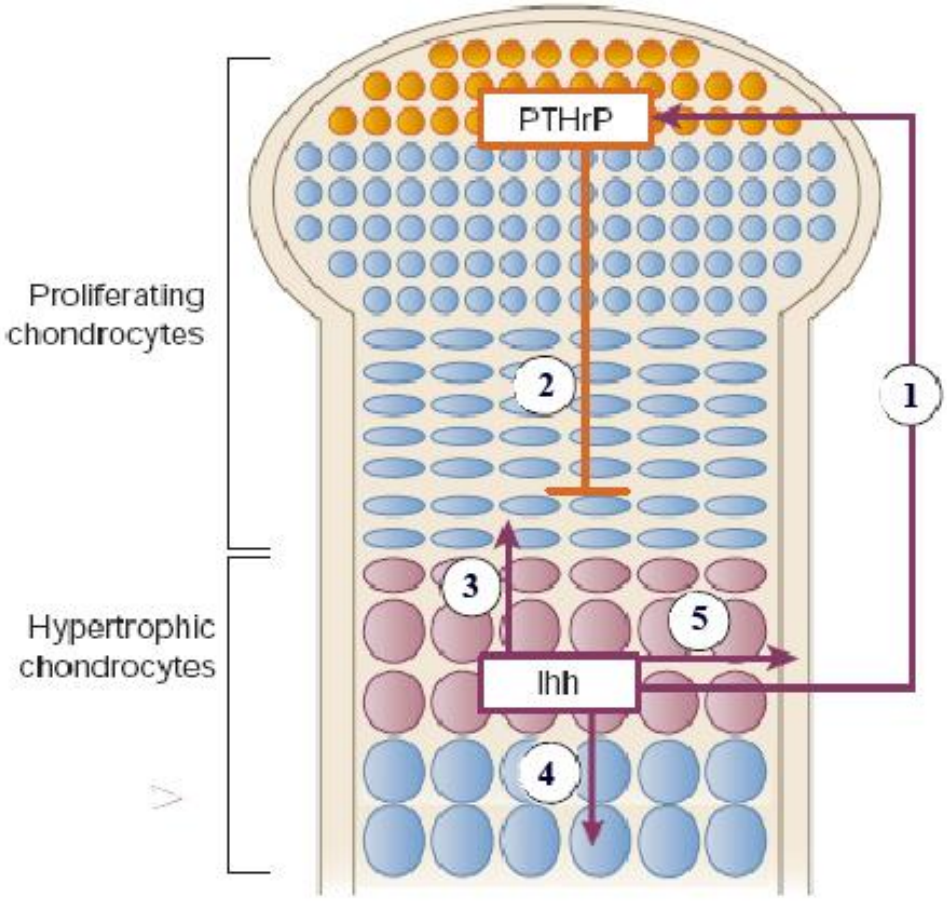

Figure 5. Indian hedgehog (Ihh)/parathyroid hormone-related protein (PTHrP) negative-feedback loop (Kronenberg, 2003)

(**1**) Ihh stimulates the production of PTHrP that secreted from perichondrial cells and chondrocytes at the ends of long bones. (**2**) PTHrP acts on receptors on proliferating chondrocytes to keep the chondrocytes proliferating and, thereby, to delay the production of Ihh. When the source of PTHrP production is sufficiently distant, then Ihh is produced. (**3**) The Ihh acts on its receptor on chondrocytes to increase the rate of proliferation. (**4**) Ihh promotes chondrocyte hypertrophy. (**5**) Ihh also acts on perichondrial cells to convert these cells into osteoblasts of the bone collar.

occurred at abnormal locations in *Ihh*$^{-/-}$ mice close to the epiphyseal ends of the bones, instead of at the center (St-Jacques et al., 1999). Expression of a constitutively active PPR in chondrocytes was able to correct the defects in chondrocyte hypertrophy in Ihh$^{-/-}$ mice (Karp et al., 2000).

In addition, Ihh stimulates chondrocyte proliferation by additional PTHrP-independent roles (Fig. 5(3)). *Ihh*$^{-/-}$ mice have shorter limbs as a result of decrease in chondrocyte proliferation. Both Ptc1 and Gli in *Ihh*$^{-/-}$ mice were expressed in proliferative chondrocytes adjacent to the pre-hypertrophic cells, indicating a direct role for Ihh in regulation of chondrocyte proliferation (St-Jacques et al., 1999). Further analysis showed that *Ihh*$^{-/-}$*;PTHrP*$^{-/-}$ double knockout mice are smaller than *PTHrP*$^{-/-}$ mice. *PTHrP*$^{-/-}$ mice had slightly shorter limbs than wild type mice, and the proportion of cells undergoing division in the proliferative chondrocyte zone was also smaller. However, double knockout mice had even shorter limbs than those of the *PTHrP*$^{-/-}$ mice, supporting the idea that Ihh regulates chondrocyte proliferation, independent of PTHrP. Furthermore, overexpression of a constitutively active PPR was unable to correct the growth defects of the *Ihh*$^{-/-}$ mice. The slightly decreased chondrocyte proliferation rate in *PTHrP*$^{-/-}$ mice suggests that PTHrP may also have the ability to promote proliferation or this may only be a secondary effect of PTHrP delaying chondrocyte hypertrophic differentiation (Karp et al., 2000).

The mechanism of how Ihh regulates chondrocyte proliferation is not completely understood. Nonetheless, chondrocyte-specific knockout of either *Ihh* or *Smo* in mice

resulted in a decrease in chondrocyte proliferation and a reduction of expression of cyclin D1, which promotes cell-cycle progression through G1/S phase transition (Long et al., 2001). Cyclin D1 is expressed at high levels in rapidly dividing columnar chondrocytes and at low levels in slowly dividing reserve chondrocytes (Yang et al., 2003). Studies in *Drosophila* showed that Hh signaling directly induces cyclin D transcription through Ci (Duman-Scheel et al., 2002). Similarly, Shh induces cyclin D expression in order to sustain cell cycle progression in mammalian neuronal precursor cells (Kenney and Rowitch, 2000). Altogether, these studies suggest that cyclin D mediates Ihh-dependent proliferative effects.

Moreover, Ihh signals independently of PTHrP to promote chondrocyte hypertrophy (Fig. 5(4)). Upregulation of Ihh signaling in the developing cartilage by treating $PTHrP^{-/-}$ limb explants with sonic hedgehog protein in vitro, or overexpressing Ihh in the cartilage of $PTHrP^{-/-}$ embryos or inactivating *patched1*, a negative regulator of Hh signaling, accelerated chondrocyte hypertrophy in the $PTHrP^{-/-}$ embryos. Conversely, when Hh signaling was blocked by cyclopamine or by removing *Smoothened* (*Smo*), a positive regulator of Hh signaling, chondrocyte hypertrophy was delayed in the $PTHrP^{-/-}$ embryo (Mak et al., 2008).

Furthermore, Ihh is also an important regulator of osteogenesis. Ihh acts on perichondrial cells to convert these cells into osteoblasts (Fig. 5(5)). $Ihh^{-/-}$ mice had no cortical bone development, suggesting that Ihh has a direct role in promoting osteoblast differentiation in the perichondrium (St-Jacques et al., 1999); (Long et al., 2001). Subsequent studies found that $Ihh^{-/-}$ mice had a thinner perichondrium, suggesting that

Ihh may regulate cell differentiation in the perichondrium, and therefore cortical bone defects may result from the lack of proper development of the perichondium (Colnot et al., 2005). *Ihh*$^{-/-}$ mice die in the fetus stage (St-Jacques et al., 1999). The early lethality of *Ihh*$^{-/-}$ mice hampered the understanding of the postnatal function of Ihh. Analysis of conditional knockout mice, in which Ihh was specifically deleted from postnatal chondrocytes, revealed that Ihh is essential for maintaining postnatal growth plate and trabecular bone (Maeda et al., 2007).

In short, Ihh is an essential regulator for both chondrocyte and osteoblast differentiation and function, during endochondral ossification.

Regulation of Ihh expression

The complexity of the temporal and spatial cooperation of chondrogenesis and osteogenesis during endochondral bone formation requires the delicate regulation on the involvement of growth factors and signaling. The expression of Ihh in growth plate chondrocytes is thus under both positive and negative regulations, which is supported by the following evidences.

Stimulators of Ihh Expression

First, BMPs play a role in regulating Ihh expression in pre-hypertrophic chondrocytes (Grimsrud et al., 2001). In vitro study showed that BMP7 increases Ihh mRNA levels. In addition, chromatin immunoprecipitation (ChIP)-based cloning methods show that the *Ihh* promoter region, with several putative BMP-responsive elements, is

able to bind to Smad 4, one of the downstream mediators of BMP signaling (Seki and Hata, 2004). These suggest that BMP signaling can directly upregulate Ihh mRNA, probably via Smad4. In the future, whether Smad4 is a transcriptional regulator of *Ihh* in vivo remain to be clarified.

Runx2 is one of the important transcription factors of *Ihh*. It belongs to the Runx family. *Runx2* deficient mice (*Runx2*$^{-/-}$) have no mineralized bones and are devoid of hypertrophic chondrocytes characterized by the absence of Ihh and collagen type X, suggesting that Runx2 was indispensible for both osteoblast and chondrocyte differentiation (Kim et al., 1999). Further studies discovered that Runx2 can control *Ihh* transcription by a direct manner (Yoshida et al., 2004): 1) overexpression of *Runx2* rescued *Ihh* expression in *Runx2*$^{-/-}$ mice, and 2) Runx2 could directly bind to three *Ihh* promoter regions and activate *Ihh* transcription. However, *Ihh* expression can still be detected in chondrocytes in *Runx2*$^{-/-}$ embryos at E12.5 and E13.5 (Yoshida et al., 2004), suggesting that some additional transcriptional factors are required for *Ihh* induction in *Runx2*$^{-/-}$ mice. Furthermore, it has been reported that BMP2 upregulates *Runx2* mRNA in chondrocytes (Takazawa et al., 2000). Therefore, BMP regulation of *Ihh* expression could be mediated by Runx2 transcription factors.

In addition, Wnt-signalling pathway has been shown to regulate *Ihh* expression in chondrocytes at E12.5 and E13.5 (Spater et al., 2006). There are 19 members of the mammalian family of lipid-modified Wnt glycoproteins (Gordon and Nusse, 2006); (Huang and He, 2008). These are thought to interact with at least three families of signal-

transducing receptors, the Frizzled, Ror, and Ryk receptors, as well as Lrp5/6 coreceptors. Wnt signaling activates several intracellular signaling pathways. The most well characterized one is the canonical Wnt pathway. It results in stabilization of a complex of beta-catenin with members of the Tcf/Lef family of DNA-binding proteins and transcriptional activation of targets through binding to *cis*-regulatory regions. Wnt9a is a temporal regulator of *Ihh* (Spater et al., 2006). Loss of *Wnt9a* results in transient downregulation of *Ihh* and reduced Hh-signaling activity at E12.5-E13.5, but *Wnt9a* is dispensable for *Ihh* expression from E14.5 onwards. Wnt9a, one of the 19 ligands of the Wnt-signaling pathway, could be signaling through the canonical Wnt-pathway (Guo et al., 2004). The canonical Wnt/ß-catenin pathway probably mediates regulation of *Ihh* expression in prehypertrophic chondrocytes by Wnt9a, because embryos double-heterozygous for *Wnt9a* and *ß-catenin* show reduced *Ihh* expression, and in vivo chromatin immunoprecipitation demonstrates a direct interaction between the ß-catenin/Lef1 complex and the *Ihh* promoter (Spater et al., 2006).

Inhibitors of Ihh Expression

FGFs negatively regulate *Ihh* expression. Early studies also suggested that FGFs inhibit chondrocyte differentiation (Iwata et al., 2000). FGFR3, a receptor of FGFs, is expressed in both proliferative and hypertrophic chondrocytes. FGFs bind to and activate this receptor and cause growth arrest in chondrocytes, leading to short limbs. Achondroplasia is the most common cause of dwarfism in humans, and it is caused by gain-of-function mutations in the fibroblast growth factor-receptor3 (FGFR3). Minina et

al. (2002) using an organ culture from embryonic limb explants, has demonstrated that FGF2 reduced the rate of chondrocyte proliferation, as well as promoting chondrocyte hypertrophic differentiation as indicated by the shorter distance between the joint and hypertrophic chondrocytes (Minina et al., 2002). However, the effect of FGFs in chondrocyte hypertrophy was not observed in *Ihh*-overexpressing transgenic mice. In addition, they showed that FGF2 reduced *Ihh* expression in pre-hypertrophic chondrocytes, suggesting that FGF2 by suppressing *Ihh* expression promotes chondrocyte hypertrophic differentiation. This conclusion was supported by other studies showing that transgenic mice expressing FGFR3 with a gain-of-function mutation had decreased *Ihh* and *PPR* expression (Chen et al., 2001). Taken together, these studies suggest that FGF signaling inhibits Ihh expression in order to suppress chondrocyte proliferation and promote chondrocyte hypertrophy. However, the mechanism by which FGF inhibits Ihh has not been established.

PTH or PTHrP stimulation of its receptors in prehypertrophic chondrocytes can inhibit *Ihh* expression. Yoshida et al. (2001) first demonstrated this in primary chicken chondrocytes showing that PTH directly down-regulated Ihh mRNA levels (Yoshida et al., 2001). Ihh regulation was mimicked by a cAMP analog, suggesting that PTH inhibits *Ihh* gene transcription, possibly via the cAMP/PKA pathway. There are several mechanisms by which stimulation of PKA may mediate Ihh inhibition. A putative cAMP response element was identified in the 5′ flanking region of *Ihh* that could mediate PPR regulation of *Ihh* transcription, however this has not yet been tested. Alternatively, Runx2

may be the focus of PTHrP inhibition of Ihh. Runx2 levels can be negatively regulated by PTHrP through activation of the cAMP/PKA pathway, resulting in decreased Runx2 expression in primary embryonic chick chondrocytes (Li et al., 2004). Since Runx2 has been shown to stimulate *Ihh* transcription, the PKA-stimulated loss of Runx2 would be expected to decrease *Ihh* transcription. This was supported by the fact that PTH inhibited Ihh in CFK2 cells by inhibition of ERK1/2 MAP kinase (Lai et al., 2005).

In a short summary, Runx2 is the only transcription factor that has been indentified that plays an essential role for activating *Ihh* transcription in vivo and directly binds the promoter region of *Ihh*. However, it seems that *Ihh* expression in the early stage before E13.5 does not require Runx2, suggesting that other transcription factors may involve in the direct regulation of *Ihh* transcription.

Properties and Functions of Atf4

Activating transcription factor 4 (Atf4) is a basic region-leucine zipper (bZip) transcription factor that belongs to the ATF/CREB (activating transcription factor/cyclic AMP response element binding protein) family, which has the consensus binding site cAMP responsive element (CRE) (Ameri and Harris, 2008). Activating Transcription Factor was first identified to bind the adenovirus early promoters E2, E3 and E4, which share a common core sequence “CGTCA” (Lee et al., 1987). Subsequent studies showed that the consensus binding site for ATF seems to be TGACGT (C/A) (G/A) (Lin and

Green, 1988). The cAMP responsive element binding protein (CREB) was first reported to bind the cAMP responsive element (CRE) on the somatostatin promoter (Montminy and Bilezikjian, 1987). Later the CRE consensus binding site was described as (TGACGTCA) (Deutsch et al., 1988). Thus, ATF and CREB members are classified as a family with a common DNA binding site. The CREB/ATF family members include ATF1, CREB/CREM, CREB314, CREB-H, ATF2, ATF3, ATF4, ATF6, ATF7, B-ATF and ATFX (also known as ATF5) (Persengiev and Green, 2003); (Hai and Hartman, 2001).

Expression of Atf4

Atf4 mRNA is ubiquitously expressed, whereas the protein is present only at very low levels (Vallejo et al., 1993) and hypoxia increases Atf4 protein without a concomitant increase in mRNA (Yukawa et al., 1999), suggesting that post-transcriptional mechanisms regulate Atf4 expression during stress.

The ATF4 protein contains two leucine zipper domains (N- and C-Leucine Zipper) for ATF4 homo/heterodimerization or interactions with other proteins, a basic region for DNA binding and a βTrCP recognition motif for the regulation of ATF4 at the protein stability level (Liang and Hai, 1997) (Fig. 6B). The protein is unstable. The βTrCP recognition motif is potentially essential for the regulation of ATF4 stability in response

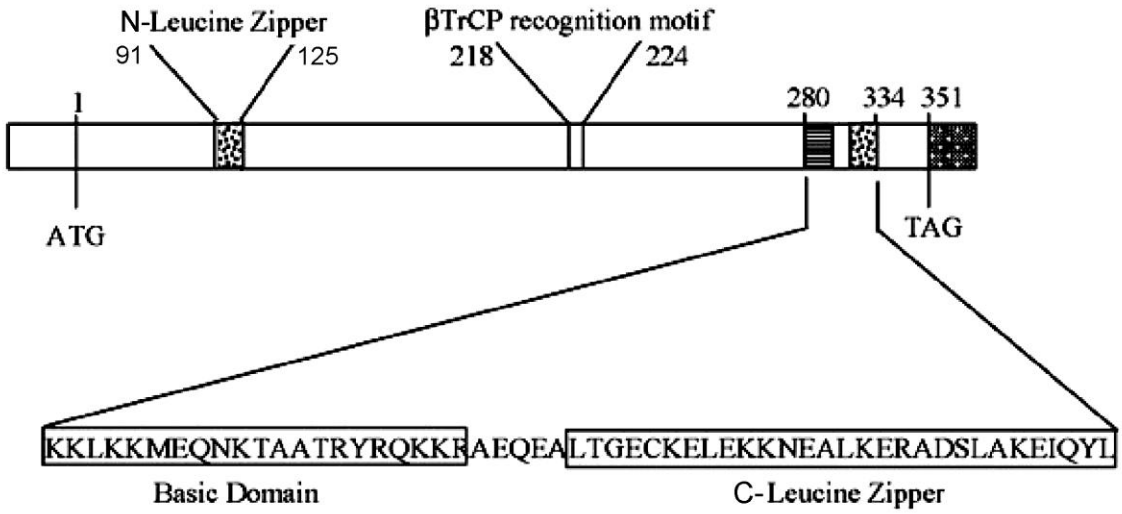

Figure 6. Schematic illustration of Atf4 protein primary structure

Schematic representation of the Atf4 structure indicating the N terminal and C terminal leucine zipper domains, βTrCP recognition, and basic DNA binding domain (Modified from (Ameri and Harris, 2008).

to stress, including hypoxic and anoxic insult. In fact, the ubiquitin ligase complex $SCF^{\beta TrCP}$ modulates ATF4 stability. ATF4 contains the βTrCP recognition motif DSGXX(X)S and when the serine of this motif is phosphorylated, it results in interaction with βTrCP and subsequent degradation by the proteasome (Lassot et al., 2001).

Function of Atf4

Atf4 protein can function as a transcriptional activator, as well as a repressor. It is also a protective gene regulating the adaptation of cells to stress factors such as anoxic insult, endoplasmic reticulum stress and oxidative stress. Atf4 plays an essential role in development, and is particularly required for proper skeletal (Yang et al., 2004), eye development (Hettmann et al., 2000), and is also involved in mammary gland development (Bagheri-Yarmand et al., 2003), as well as haematopoiesis (Masuoka and Townes, 2002). ATF4 is also a major factor in brain function, regulating synaptic plasticity and memory formation (Hoeffer and Klann, 2007); (Lai et al., 2008); (Ritter et al., 2004); (Vernon et al., 2001); (Chen et al., 2003). In addition, Atf4 is also a major factor in metabolic control and nutrition sensing, and thus plays a central role in managing the stress induced by amino acid imbalances (Palii et al., 2009); (Siu et al., 2002); (Wang et al., 2010a). Furthermore, Atf4 has also been recently implicated in extreme hypoxia/anoxia mediated metastasis (Ameri et al., 2004); (Bi et al., 2005).

Transcriptional regulator: Atf4 was originally described as a transcriptional repressor, negatively regulating transcription via the cAMP response element (CRE) of the *Human proenkephalin* (*PENK*) promoter (Karpinski et al., 1992). However, it is

viewed as an activator for transcription in a large number of genes, including: *Vascular endothelial growth factor* (*VEGF*) (Chin, 2008); (Malabanan et al., 2008); (Roybal et al., 2004); (Roybal et al., 2005); *Activating transcription factor 3* (*ATF3*) (Jiang et al., 2004); *Receptor activator of nuclear factor kappa B ligand* (*RANKL*), also known as tumor necrosis factor (ligand) superfamily, member 11 (TNFSF11) (Elefteriou et al., 2006); *Osteocalcin*, a marker gene for mature osteoblast (Yang et al., 2004); *CCAAT/enhancer-binding protein homologous protein* (*CHOP*), also known as DNA-damage-inducible transcript 3 (DDIT3) (Fawcett et al., 1999)

Brain function: Atf4 is a memory repressor that blocks the new expression of genes needed for memories, which appears to be a conserved mechanism. Decreasing the activity of Atf4 in mice or the sea slug Aplysia CREB2 (ApCREB2, the ortholog of ATF4) lowers the threshold for long-lasting changes in memory (Bartsch et al., 1995); (Chen et al., 2003).

Fetal liver hematopoiesis: A knockout m utation of *Atf4* has demonstrated severe fetal anemia in mice (Masuoka and Townes, 2002). *Atf4*$^{-/-}$ fetal livers are pale and hypoplastic, and the number of hematopoietic progenitors of multiple lineages is decreased more than 2 fold. Therefore, Atf4 is essential for the normal, high-level proliferation required for fetal-liver hematopoiesis.

Glucose metabolism: Atf4-deficient mice display significantly lower fat mass and blood glucose levels. Atf4 negatively regulates insulin secretion and decreases

sensitivity to insulin in liver, muscle, and fat cells. It achieves this function, in large part, through its expression in osteoblasts. Atf4 favored expression in osteoblasts of Esp, a receptor like protein tyrosine phosphatase termed OST-PTP4 that decreases the bioactivity of osteocalcin, despite its upregulation of this gene. Since activated osteocalcin is an osteoblast-specific secreted molecule that enhances secretion of and sensitivity to insulin, the osteoblast is actually an endocrine cell type that determines insulin secretion by β cells and insulin sensitivity in liver, muscle, and adipocytes (Yoshizawa et al., 2009).

Bone formation: In our lab, it had been found that Atf4 is required for the terminal differentiation of osteoblasts, and affects the osteoblast function by regulating the synthesis of Type I collagen, the main constituent of bone matrix (Yang et al., 2004). Atf4 favors amino acid import, and therefore is a critical determinant of the synthesis of proteins in osteoblasts. Type I collagen is the most abundant protein of the bone ECM, and therefore, Atf4 is a major regulator of bone formation. Consequently, Atf4-deficient mice are runted and harbor low bone mass, reduced osteoblast activity, decreased type I collagen synthesis, and inhibited osteocalcin and bone sialoprotein gene transcription. *Atf4* messager RNA is ubiquitously expressed. However Atf4 is a cell-specific transcription factor whose distribution is controlled to a large extent post-translationally (Yang and Karsenty, 2004). Subsequent studies found that Atf4 is present in the chondrocytes, $Atf4^{-/-}$ embryos showed developmental defects and reduction of *Ihh* expression in the cartilages, suggesting that Atf4 may play a role in the regulation of chondrogenesis by targeting *Ihh* expression during endochondral ossification.

Aims of the Dissertation

Atf4 deficient (*Atf4*$^{-/-}$) embryos and mice have smaller skeleton and shorter limbs comparing with their wt littermates (Fig. 7), indicating that they have defect in the long bone growth which is regulated by chondrocyte proliferation and differentiation in the growth plate. Preliminary analysis revealed several abnormalities in the growth plates of *Atf4*$^{-/-}$ mice, including a shorter proliferative chondrocyte zone, an expansion of the hypertrophic zone, and a decreased expression of *Ihh*. Furthermore, we found several putative Atf4 binding sites in the promoter of *Ihh* gene. Based on these preliminary data, we hypothesize that **Atf4 regulates chondrogenesis in the growth plate by targeting *Ihh* transcription**. To test this hypothesis, we propose the following specific aims:

1. To test whether Atf4 is required for the development of growth plate chondrocytes. The *Atf4*$^{-/-}$ mice have defects in skeleton growth, but when these defects start and which step of chondrogenesis is affected are unclear. To answer these questions, the defects of *Atf4*$^{-/-}$ growth plates were characterized in different developmental stage including E12, E14, E16, and at birth (P0). Immunohistochemistry was performed to test which type of chondrocyte expresses Atf4 in growth plate. Skeletal preparation examined the onset of the chondrogenesis defect. Histological analysis studied the morphological changes in the growth plate. In situ hybridization analysis examined the specific chondrocyte marker gene expression, including *Type II collagen*, *Type X collagen*, *PTH/PTHrP receptor*, *PTHrP*, *Ihh*, and *Gli1* to dissect the defects in particular chondrocyte cell type.

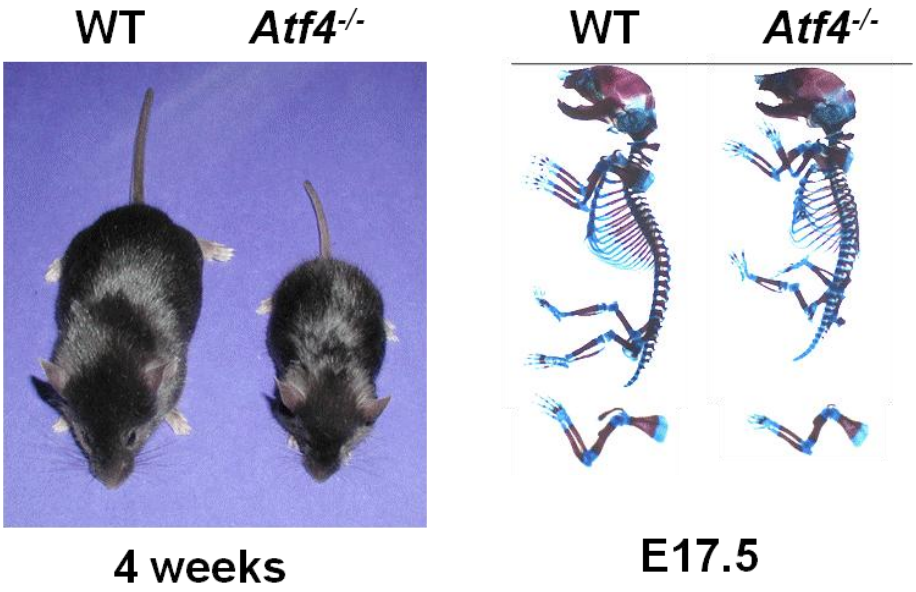

Figure 7. *Atf4*$^{-/-}$ mice are dwarf

Atf4$^{-/-}$ mice have developmental defect in skeleton characterizing by a small skeleton and short limb phenotype, suggesting that they have defects in cartilage growth plate.

2. To study how Atf4 regulates *Ihh* gene transcription during chondrogenesis. *Ihh* expression is dramatically decreased in *Atf4*$^{-/-}$ mice, suggesting that Atf4 as a transcription factor may regulate *Ihh* transcription. To test whether Atf4 can activate *Ihh* transcription, luciferase reporter constructs containing *Ihh* promoter fragment were generated and used for luciferase reporter assay. DNA transfection assays were used to test whether Atf4 has the transcriptional activity on *Ihh* promoter. To test whether Atf4 can directly bind to *Ihh* promoter, electrophoretic mobility shift assays (EMSA) were performed.

3. To test whether Atf4 regulate chondrogenesis via Hh signaling pathway. Hh signaling pathway was reactivated using pharmacological approaches to rescue the chondrocyte defects of *Atf4*$^{-/-}$ mice. Purmorphamine, which activates the Hedgehog pathway by targeting Smoothened, was used to test whether reactivation of Hh pathway in *Atf4*$^{-/-}$ mice can rescue the chondrocyte defects in limb culture assay.

4. To test the autonomous function of Atf4 in chondrocytes during endochondral ossification. Genetic approaches were used to control Atf4 gene expression specifically in chondrocytes. Taking advantage of the *Col2α1-Atf4* transgenic mice that were generated in our lab and have *Atf4* over-expression in chondrocytes driven by the promoter of the *Type II collagen*, these *Col2α1-Atf4* transgenic mice were crossed with *Atf4*$^{+/-}$ mice to generate *Atf4*$^{+/-}$;*Col2α1-Atf4* mice, then later *Atf4*$^{-/-}$;*Col2α1-Atf4* mice that were used to determine whether Atf4 autonomously regulates chondrocyte proliferation and differentiation via Ihh in vivo.

5. To test whether reactivating Hh signaling in vivo can rescue the endochondral ossification of *Atf4*$^{-/-}$ mice. Gli3 was removed to reactivate downstream Hh signaling in *Atf4*$^{-/-}$ mice. Gli3 acts as a repressor downstream of Ihh (Koziel et al., 2005). The *Gli3*$^{+/-}$ mice were crossed with *Atf4*$^{+/-}$ mice to get *Gli3*$^{+/-}$;*Atf4*$^{+/-}$ mice, which were crossed to generate *Gli3*$^{-/-}$;*Atf4*$^{-/-}$ mice. The double knock-out mice were used to test whether the defect of endochondral ossification in *Atf4*$^{-/-}$ mice can be rescued by removing the inhibition of Gli3 on Hh signaling.

CHAPTER II

ATF4 REGULATES CHONDROCYTE PROLIFERATION AND DIFFERENTIATION DURING ENDOCHONDRAL OSSIFICATION BY ACTIVATING *IHH* TRANSCRIPTION

Abstract

Activating transcription factor 4 (Atf4) is a leucine zip transcription factor. *Atf4* mutant (*Atf4*$^{-/-}$) mice show severe low bone mass and short stature phenotype. Atf4 is expressed in growth plate chondrocytes. *Atf4*$^{-/-}$ growth plate shows disturbed and shorten proliferative chondrocyte zone, expanded hypertrophic zone and decreased expression of *Indian hedgehog* (*Ihh*), whereas the expression of other chondrocyte marker genes, such as *type II collagen* (*Col2a1*), *PTH/PTHrP receptor* (*PPR*) and *type X collagen* (*Col10a1*), is normal. In addition, forced expression of Atf4 in chondrocytes induces endogenous *Ihh* mRNA, and Atf4 directly binds to the *Ihh* promoter and activates its transcription. Furthermore, Reactivation of Hh signaling pharmacologically in mouse limb explants corrects the *Atf4*$^{-/-}$ chondrocyte proliferation and short limb phenotypes. These results thus identify that Atf4 regulates chondrocyte proliferation and differentiation by targeting *Ihh* transcription.

Introduction

The skeleton starts to form at 10 days post-coitum (dpc) during mouse embryogenesis through intramembranous and endochondral ossification. The first step of skeletal development is mesenchymal stem cells condensation and patterning, which provides the mold for the future skeleton (Kaufman, 1992). The condensed mesenchymal cells then differentiate into osteoblasts directly during intramembranous ossification, or into chondrocytes to form the cartilage anlagen, which is eventually replaced by bone during endochondral ossification (Karsenty and Wagner, 2002). Endochondral ossification is crucial for skeletal growth in the developing vertebrate, as well as for skeletal repair in adults. It involves slowly proliferating, rounded, resting chondrocytes in the reserve zone acquiring cues to become rapidly dividing cells that are flattened and packed into columnar chondrocytes in the proliferating zone. Rapidly proliferating chondrocytes then stop dividing to progress to a transition stage of prehypertrophic chondrocytes, which quickly undergo differentiation (hypertrophy). Mature hypertrophic chondrocytes eventually die, allowing vascular invasion, a process that involves the entry of osteoclast and osteoblast precursors. Osteoclasts assist in the removal of cartilage matrix and osteoblasts use the remnants of cartilage matrix as a scaffold for the deposition of new bone matrix to form calcified bone. Therefore, endochondral ossification is a dynamic event that relies on chondrocyte proliferation and differentiation and is tightly regulated by systemic factors, locally secreted factors and transcription factors (reviewed by (Day and Yang, 2008); (de Crombrugghe et al., 1991); (Kronenberg, 2003); (Mackie et al., 2008); (Nilsson et al., 2005); (Ornitz, 2005); (Zuscik et al., 2008).

Indian hedgehog (Ihh) belongs to the hedgehog (Hh) family and is one of the aforementioned locally secreted factors required for mammalian skeletal development. By binding to its receptor patched, Hh ligands induce the release of patched inhibition of the smoothened, which allows activation of signaling events that promote cell proliferation (Alcedo and Noll, 1997) (Day and Yang, 2008). In the growth plate, Ihh is secreted by prehypertrophic chondrocytes and acts as a paracrine factor to promote adjacent chondrocyte proliferation. Ihh can also diffuse and reach cells in the articular perichondrium, where it induces the expression of parathyroid hormone related protein (PTHrP; Pthlh–Mouse Genome Informatics), which in turn inhibits chondrocyte hypertrophy and maintains the pool of proliferative chondrocytes (Chung et al., 2001); (Guo et al., 2006); (Karp et al., 2000); (Minina et al., 2001); (St-Jacques et al., 1999). Although the mechanism is not yet fully understood, this action of PTHrP to inhibit hypertrophy forms a negative-feedback regulatory loop on the production of Ihh, which controls the coordination between proliferation and differentiation of participating chondrocytes. Using genetic mouse models, Mak et al. demonstrated that Ihh also promotes chondrocyte hypertrophy in a PTHrP-independent manner (Mak et al., 2008). Given the crucial role that Hh signaling plays in the regulation of skeletal development, it is of interest to understand upstream signaling pathways that regulate *Ihh* at the transcriptional level.

Recent studies have identified several transcription factors that activate *Ihh* transcription. Runx proteins are a group of cell-specific transcription factors belonging to

the Runt family (see (Karsenty, 2001). Using genetic mouse models, (Yoshida et al., 2004). found that Runx2, with the assistance of Runx3, binds directly to the *Ihh* promoter and activates its expression (Yoshida et al., 2004). This was the first and thus far only characterization of a transcriptional mechanism involved in the regulation of chondrocyte proliferation and hypertrophy in vivo. Msx2, a homeodomain-containing protein that regulates cellular development in many tissues, including bone, teeth and neurons, has been shown to activate *Ihh* transcription in vitro (Amano et al., 2008); however, whether Msx2 is a transcriptional regulator of *Ihh* in vivo remains to be determined.

Atf4 is a leucine-zipper-containing transcription factor of the CREB family(Shaywitz and Greenberg, 1999). Using biochemical and genetic approaches, we have found that inactivation of *Atf4* in mice results in severe osteopenia, which is caused by a failure of *Atf4*$^{-/-}$ osteoblasts to achieve terminal differentiation and to synthesize type I collagen, the main constituent of bone matrix (Yang et al., 2004). In addition, *Atf4*$^{-/-}$ mice display dwarfism, suggesting a role of Atf4 in development of the growth plate chondrocyte. In this study, we identified Atf4 as a direct transcriptional activator of *Ihh* and thus a regulator of chondrocyte proliferation and differentiation.

Materials and Methods

Animals

Wild-type (WT) and *Atf4*$^{-/-}$ embryos and mice were obtained by crossing *Atf4*+/– mice. Zero dpc (E0) was defined by the morning the vaginal plug was found. *Atf4*

genotyping was performed by PCR using tail DNA (Masuoka and Townes, 2002). For each genotype, at least three embryos or mice were analyzed.

Atf4 expression

Primary chondrocytes were isolated by sequential digestion of rib cage cartilage from E14 embryos with collagenase D. Nuclear extracts isolated from the indicated sources were subjected to western blot analysis using an antibody against Atf4 (N127) (Yang and Karsenty, 2004). Immunohistochemistry was performed on paraffin-embedded sections

(5µm) of WT and *Atf4*$^{-/-}$ humeri. After deparaffinization and rehydration, antigens were retrieved by heating at 100°C for 10 minutes in Tris/EDTA buffer (pH 9.0) and immunostained with antibody N127. Sections were counterstained with Hematoxylin.

Skeletal preparation and histology

Skeletal preparation was according to standard protocols. For histology, embryos and P0 mice were fixed in 4% paraformaldehyde (PFA), embedded in Paraplast, and sectioned at 5µm. Slides were stained with Hematoxylin for nuclei, Alcian Blue for cartilage matrix, and Alizarin Red for bone matrix.

Microcomputed tomography (µCT) analysis

WT and *Atf4*$^{-/-}$ tibiae were collected and fixed overnight in 4% PFA (pH 7.4) and then 70% ethanol. Samples were scanned using a µCT system (ScancoµCT 40; Bassersdorf, Switzerland). Tomographic cross-sectional images of the proximal tibia

were acquired at 55 kV and 145 mA, at an isotropic voxel size of 12μm and an integration time of 250 milliseconds. Contours were fitted to the outer perimeter of the tibia beginning immediately distal to the growth plate and extending 1.2 mm distally using the auto-contouring feature in the Scanco Software with the threshold of 300 mg hydroxyapatite/cm3.

In vivo proliferation assay

For embryo limbs, pregnant mice received intraperitoneal injections of 0.1 mg 5-bromo-2'-deoxyuridine (BrdU)/g body weight and were sacrificed 2 hours later. For P0 pups, BrdU (0.1 mg/g body weight) was injected under the skin on the back of the neck 2 hours prior to sacrifice. In organ cultures, BrdU was added 1 hour prior to sample harvesting. Limbs were dissected and fixed in 4% PFA overnight at 4°C. After embedding and sectioning, BrdU was detected with a BrdU Staining Kit (Zymed Laboratories) following the manufacturer's procedure.

TUNEL assay

Apoptotic cells in the growth plate of WT and *Atf4*$^{-/-}$ humeri were detected by in situ terminal deoxynucleotidyltransferase deoxyuridine triphosphate nick end labeling (TUNEL) assay using the In Situ Cell Death Detection Kit (Roche) following the manufacturer's instructions.

In situ hybridization

Alternate sections used for histological analysis were in situ hybridized for chondrocytic marker genes. Probes for type II collagen (*Col2a1*), *Ihh* and type X collagen *Col10a1* were as described previously (Ducy et al., 1997); (Takeda et al., 2001). The probe for *PPR* was from Dr T. J. Martin (University of Melbourne, Australia). The probe for *Gli1* was a mouse cDNA fragment (bp 968 to 1437), which was generated by RT-PCR using primers: forward, 5'-GAAGGAATTCGTGTGCCATT-3' and reverse, 5'-TCCAAGCTGG-ACAAGTCCTC-3'. Antisense cDNAs were used for riboprobe synthesis with RNA polymerases (Invitrogen) and [35S]uridine triphosphate (Perkin Elmer).

Establishment of Atf4-overexpressing chondrocytes

TMC23 chondrocytic cells (Xu et al., 1998) at 90% confluence were transfected with 50 ng Atf4 or Runx2 expression plasmid (pcDNA3.1-Atf4 or pcDNA3.1-Runx2) using Lipofectamine (Invitrogen). When cells reached 100% confluence, they were trypsinized and replated in αMEM containing G418 (400 μg/ml). G418-resistant clones were selected and maintained in G418-containing αMEM.

Electrophoretic mobility shift assay (EMSA)

Oligonucleotides of OSE1 in the osteocalcin gene 2 (*Bglap2*) promoter and of OSE1-like sequences (A1 to A9) in the *Ihh* promoter were synthesized. Annealed double-stranded oligonucleotides were labeled with [32P]dCTP and [32P]ATP and used as probes in EMSA, which was performed as described previously (Ducy and Karsenty,

1995); (Schinke and Karsenty, 1999) using purified recombinant Atf4 or nuclear extracts of primary chondrocytes.

Northern hybridization and real-time quantitative RT-PCR (qRT-PCR)

Total RNA was isolated using TRIzol (Invitrogen) and 10 μg from each sample was resolved in a 1% agarose gel, transferred onto nylon membrane, and hybridized with *Ihh*, *PTHrP* or *Gapdh* cDNA probes following standard protocols. qRT-PCR was performed using a standard TaqMan PCR Kit protocol on an Applied Biosystems 7300 machine. After treatment with DNase I, total RNA (2 μg) was reverse-transcribed with reverse transcriptase (Invitrogen) using 100 μM random hexamer primers. Specific oligonucleotide primers were from Applied Biosystems (*Ihh*, Mm00439613 m1; *PTHrP*, Mm00436056_g1; *Gli1*, Mm00494645_m1; *PPR*, Mm00441046_m1).

Construction of an *Ihh* luciferase reporter construct and mutagenesis

A 4.5 kb *Ihh* promoter fragment isolated from a bacterial artificial chromosomal clone, RP24-317G11 (Children's Hospital of Oakland Research Institute, Oakland, CA, USA) was inserted to a luciferase (Luc)-containing reporter vector. A serious of constructs containing 2.8kb, 2.2kb and 1.3 kb *Ihh* promoter fragment were generated by removal of the 5' end of the 4.5 kb fragment step by step via restriction digestion. Constructs containing 742 bp and 715 bp *Ihh* promoter fragments were cloned by PCR using the High Fidelity PCR System (Roche) or Pfu DNA polymerase (Stratagene) with the following primer sequences: for the 742 bp fragment, forward 5'-CTGAGAAAGGG-AATGTTGCC-3' and reverse 5'-GCGTGCTGTCCCCCTCGGCG-3'; for the 707 bp

fragment, forward 5'-AACTCGAGCACCAGGTTATGAATGACCT-3' and reverse 5'-GCGTGCTGTCCCCCTCGGCG-3'. Multimers (five copies) of the WT or mutated A9 oligonucleotides were made by ligation of *Bgl*II- and *Bam*HI-linked double-stranded oligos and digestion with *Bgl*II and *Bam*HI. For reporter constructs, ligated products were inserted (*Sma*I site) upstream of the TATA box (a 16 bp sequence) from the mouse osteocalcin gene 2 promoter (OG2-TATA box), which is followed by the *Luc* gene. All inserts were confirmed by DNA sequencing.

DNA transfection assay and mutagenesis

COS1 cells were plated at 5_104 cells/well in 24-well plates. After 18 hours, the cells were transfected with Lipofectamine. Each transfection contained per well, 0.25 μg of Ihh-Luc, 0.25 μg of Atf4 and 0.025 μg of β-galactosidase (β-gal) plasmids. Luc and β-gal assays were performed 24 hours post-transfection. Data presented are ratios of Luc/β-gal activity from at least three different experiments and each experiment was performed in triplicate for each DNA sample.

Organ culture and purmorphamine treatment

E14 limbs were freed of skin and muscles and cultured in BGJ-B medium with antibiotic/antimycotic (Life Technologies) and 0.5% BSA in a 24-well cell culture plate (Minina et al., 2001) for 4 days at 37°C in 5% CO2 and humidified atmosphere. Right limbs of each embryo were supplemented with 10 μM purmorphamine (Calbiochem) and left limbs of the same embryo were cultured with DMSO as a control. The experiments were repeated three times. Limb explants were photographed before and after culture and

then fixed and embedded in paraffin and sectioned at 5 μm for histological and in situ analyses.

Results

Atf4 is expressed in chondrocytes

To address the function of Atf4 during chondrogenesis, we first analyzed its expression pattern. In whole embryo nuclear extracts, Atf4 protein was detected from embryonic day (E) 9 (9 dpc) to E11. Atf4 was expressed at high levels from E12 to E14 and at low levels

from E15 to birth in nuclear extracts of isolated limbs (Fig. 1A). To further confirm that Atf4 was expressed in chondrocytes, nuclear extracts of primary chondrocytes isolated from pup ribs at post-natal day (P) 3 were examined. Atf4 was present at the same level as in primary osteoblasts isolated from P3 pup calvariae. Atf4 was not detected in primary fibroblasts of the same developmental stage (Fig. 1B). These results, together with the fact that Atf4 is not detectable in any other adult tissues except bone (Yang and Karsenty, 2004), eye and cartilage (Fig. 1C), suggest that Atf4 expression is broad in embryos during early development but becomes more restricted to skeletal tissues from E14 onward. Immunohistochemistry revealed that Atf4 was present in all growth plate chondrocytes, with high levels of expression in perichondrium, proliferative and prehypertrophic chondrocytes (Fig. 1D). Therefore, the expression pattern of Atf4 is consistent with a role in the regulation of chondrocyte biology.

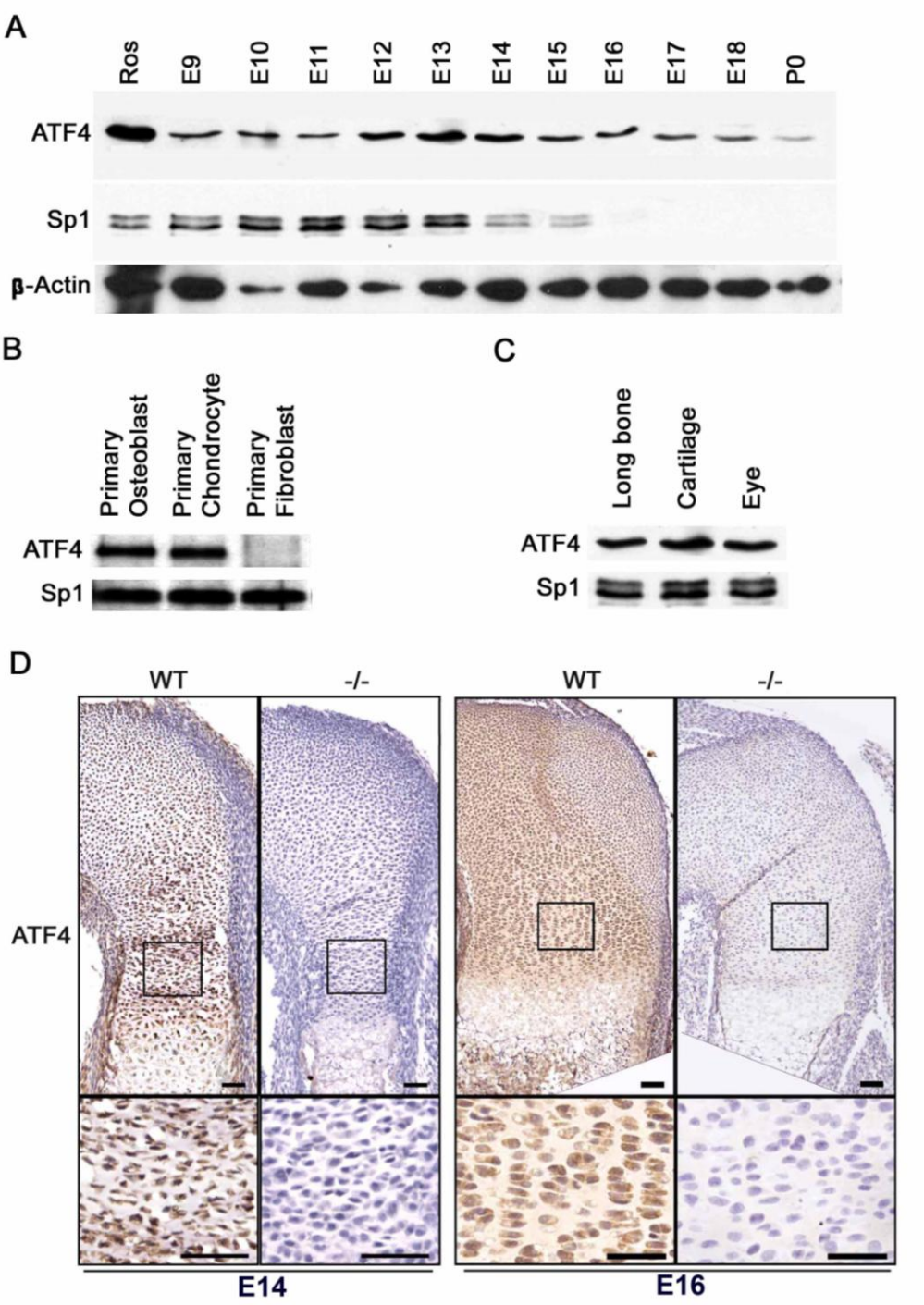

Fig. 1. Atf4 is expressed in chondrocytes.
(**A**)Western blot of nuclear extracts of mouse embryos (E9-11) and limbs (E12-P0) for Atf4. Sp1 and β-actin were used as a loading control. (**B**)Western blot of nuclear extracts from the indicated primary cells. (**C**)Western blot of nuclear extracts from the indicated tissues. (**D**)Immunohistochemistry of growth plate sections of E14 wild-type (WT) and mutant (*Atf4*$^{-/-}$) humeri. The boxed regions are magnified beneath. Scale bars: 50μm.

Dwarfism in *Atf4*$^{-/-}$ mice

In analyzing *Atf4*$^{-/-}$ mouse phenotypes we noticed a striking reduction in body size compared with their wild-type (WT) littermates (Fig. 2A). Quantification revealed a 50% reduction in body weight (9.53±1.55 g versus 20.25±1.33 g, P=0.014 by paired Student's t-test, n=4) and in femoral bone length (5.66±0.23 mm versus 10.55±0.08 mm, $P=3\times10^{-7}$, n=7) in 1-month-old mice (Fig. 2B,C), indicating severe limb dwarfism. To determine the onset of this phenotype, we examined embryos stained with Alizarin Red and Alcian Blue and measured their sizes from E9 to E18. The gross sizes of WT and *Atf4*$^{-/-}$ embryos were indistinguishable from E9 to E11 (data not shown), indicating that deletion of *Atf4* did not cause a general reduction in body size prior to the developmental stages during which the first bone elements are shaped. However, *Atf4*$^{-/-}$ embryos appeared smaller than WT littermates at E12, an early stage of chondrogenesis (Fig. 2D), and the small stature was persistent with an increasing penetrance from 55.6% at E12 and 85.6% at E14 to 100% at E16 and birth (Table 1). Given the severe decrease in limb length at adulthood (Fig. 2C), we focused our analysis on the long bones. At E12, the initial cartilaginous primordia of *Atf4*$^{-/-}$ humeri (black double-headed arrows in Fig. 2D) were smaller compared with WT littermates (red double-headed arrows in Fig. 2D), although the difference was not statistically significant (Table 2). From E13 to birth, the length of *Atf4*$^{-/-}$ humeri decreased by 7% at E13, 17% at E14, 16% at E16 and 10% at birth; this decrease was small but reproducible (Fig. 2D,E and Table 2). To assess the contribution of abnormal *Atf4*$^{-/-}$chondrocyte function to the short limb phenotype, the length of nonmineralized cartilage in the humerus was measured. A small but significant decrease

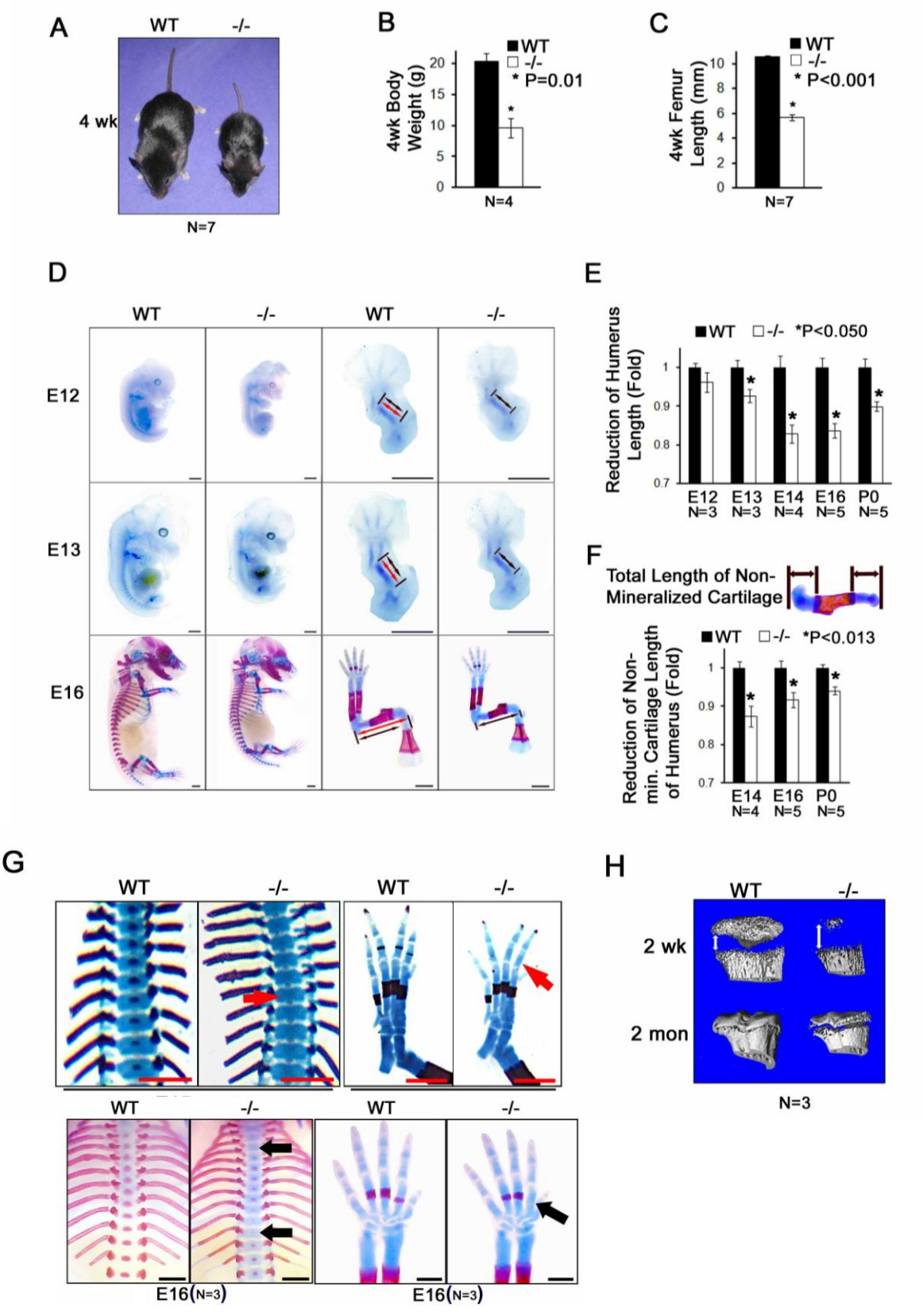
A
WT
-/-
4 wk
N=7
B
WT
-/-
* P=0.01
4wk Body Weight (g)
N=4
C
WT
-/-
* P<0.001
4wk Femur Length (mm)
N=7
D
WT
-/-
WT
-/-
E12
E13
E16
E
WT
-/-
*P<0.050
Reduction of Humerus Length (Fold)
E12 N=3
E13 N=3
E14 N=4
E16 N=5
P0 N=5
F
Total Length of Non-Mineralized Cartilage
WT
-/-
*P<0.013
Reduction of Non-min. Cartilage Length of Humerus (Fold)
E14 N=4
E16 N=5
P0 N=5
G
WT
-/-
WT
-/-
E16(N=3)
E16(N=3)
H
WT
-/-
2 wk
2 mon
N=3

Fig. 2. *Atf4*$^{-/-}$ embryos and mice exhibit dwarfism.

(**A**)One-month-old WT and *Atf4*$^{-/-}$ mice.

(**B**)Quantification of body weight of 1-month-old WT and *Atf4*$^{-/-}$ mice. Error bars indicate s.e.m.; n=4 mice of each genotype. P=0.01 by paired Student's t-test.

(**C**)Quantification of femur length of 1-monthold WT and *Atf4*$^{-/-}$ mice.

(**D**)Alizarin Red S and Alcian Blue stained skeletons of embryos and limbs at E12 (12 dpc), E13 and E16. Red and black double-headed arrows represent the length of WT and *Atf4*$^{-/-}$ humeri, respectively.

(**E**)Quantification of humerus length during development for WT and *Atf4*$^{-/-}$ mice. Error bars indicate s.e.m. of *Atf4*$^{-/-}$ humerus length normalized to WT humerus length.

(**F**)Quantification of non-mineralized cartilage length (top panel, double-headed arrows) in WT and *Atf4*$^{-/-}$ humerus during development. Error bars indicate s.e.m. of *Atf4*$^{-/-}$ normalized to WT humerus non-hypertrophic zone length.

(**G**)Alizarin Red S and Alcian Blue stained skeletons of embryos showing a delay in the formation of the primary ossification center in E15, E16 *Atf4*$^{-/-}$ thoracic vertebrae (left, arrow) and E17, E16 hindlimb digits (right, arrow).

(**H**) Microtomographic image showing a delay in formation of the secondary ossification center in *Atf4*$^{-/-}$ mice (as indicated by the double-headed arrow). Scale bars: 1 mm.

Table 1. Penetrance of *Atf4*$^{-/-}$ dwarfism

The number of embryos/pups assayed at each developmental stage is indicated, together with the number of *Atf4*$^{-/-}$ that show dwarfism.

	Developmental Stages (dpc)				
Genotype	**12**	**14**	**16**	**Newborn**	**Total (%)**
+/+	**17**	**7**	**10**	**14**	**48 (25.8)**
+/-	**30**	**27**	**10**	**34**	**101 (54.3)**
-/-	**9**	**7**	**9**	**12**	**37 (19.9)**
Dwarfism in -/-	**5**	**6**	**9**	**12**	
Penetrance (%)	**55.6**	**85.7**	**100**	**100**	

Table 2. Affect of *Atf4* mutation on total and non-mineralized cartilage lengths of humerus.

Length is shown as mean ± s.e.m., *P*-value by Student's *t*-test. *Significant; **strongly significant.

		Length (mm)		
Stages	**N**	**WT**	**-/-**	**P-valure**
Total Humerus				
E12	**4**	**0.645 ± 0.006**	**0.620 ± 0.015**	**0.095**
E13	**4**	**0.743 ± 0.011**	**0.688 ± 0.023**	**0.050 ***
E14	**4**	**1.765 ± 0.054**	**1.463 ± 0.041**	**0.023 ***
E16	**5**	**3.186 ± 0.078**	**2.664 ± 0.059**	**0.000079 ****
P0	**5**	**4.164 ± 0.092**	**3.744 ± 0.052**	**0.002 ****
Non-mineralized humerus				
E14	**4**	**1.307 ± 0.030**	**1.142 ± 0.048**	**0.013 ***
E16	**5**	**1.756 ± 0.061**	**1.629 ± 0.058**	**0.012 ****
P0	**5**	**1.515 ± 0.035**	**1.424 ± 0.042**	**0.003 ****

of 13% at E14, 7% at E16 and 6% at birth was found (Fig. 2F and Table 2). Together, these results suggest that Atf4 is required for chondrocyte function during skeletal development, but that the early condensation of mesenchymal cells to form the anlagen of the future bones and their differentiation into chondrocytes are independent of Atf4.

Delayed hypertrophic mineralization in *Atf4*$^{-/-}$ growth plates

Upon further detailed analysis of skeletal preparations, we found that the mineralization of chondrocytes was also affected by *Atf4* ablation. The first signs of mineralization, which appeared as a single focus of Alizarin Red staining in the center of cartilaginous elements, were detected at E14 in every skeletal element of WT hindlimbs, except for digit bones. However, this staining was only detected in the femur and tibia, but not ilium and fibula, of *Atf4*$^{-/-}$ embryos at this stage (data not shown). Extensive mineralization was detected in all long bones by E15 in WT embryos. However, at this stage, and even at E16 and E17, mineralization in *Atf4*$^{-/-}$ embryos remained absent in some vertebrae (Fig. 2G) and digits (Fig. 2G and see Fig. S1B in the supplementary material). In the post-natal growth plate, 3D-computed microtomographic analysis revealed the presence of a larger gap formed by non-mineralized chondrocyte matrix in *Atf4*$^{-/-}$ tibiae, as compared with WT littermates, at 2 weeks and 2 months of age (Fig. 2H). Together, these results demonstrate that deletion of *Atf4* causes a general delay in growth plate development and subsequent ossification. Therefore, Atf4 is indispensable for chondrocyte hypertrophic mineralization during endochondral ossification.

Proliferative chondrocyte disorganization and delayed hypertrophy in Atf4$^{-/-}$ growth plates

To further understand the cause of the *Atf4*$^{-/-}$ mutant phenotypes, we performed histological analyses on developing long bones of the forelimb. At E14, the hypertrophic zone in the center of the WT humerus shaft contained enlarged cells, which were surrounded by invading vasculature and a newly formed cortical bone collar in the perichondrium. The hypertrophic zone in *Atf4*$^{-/-}$ humeri was shorter (Fig. 3A, brackets), suggesting a delay in hypertrophy. In the *Atf4*$^{-/-}$ perichondrium, the cortical bone collar started to form (Fig. 3A, black arrows) and vascular invasion occurred (Fig. 3Aa,b, arrowheads), despite the reduction in the number of enlarged hypertrophic chondrocytes. These data demonstrate that Atf4 is not required for the onset of angiogenesis that initiates osteogenesis and bone collar formation, which is consistent with our previous observations (Yang et al., 2004). Furthermore, at E16, compared with the columnar pattern formed by the long and organized stacks of actively proliferating chondrocytes in the WT, the stacks were short and disorganized in *Atf4*$^{-/-}$ growth plates (Fig. 3B, bottom panels). At birth, columnar chondrocyte stacks were visible but remained disorganized in *Atf4*$^{-/-}$ growth plates (Fig. 3C, bottom panels). The length of the reserve zones was reduced by 13.5% at E16 and 8.3% at birth in *Atf4*$^{-/-}$ growth plates, and the length of the proliferative zones was decreased by 12% at E16 and 11% at birth compared with WT controls (Fig. 3D,E). Unexpectedly, despite the overall shortening and the delay in appearance of hypertrophic mineralization in the growth plate of developing *Atf4*$^{-/-}$ long bones, the length of the hypertrophic zone was increased by 12% at E16 and remained

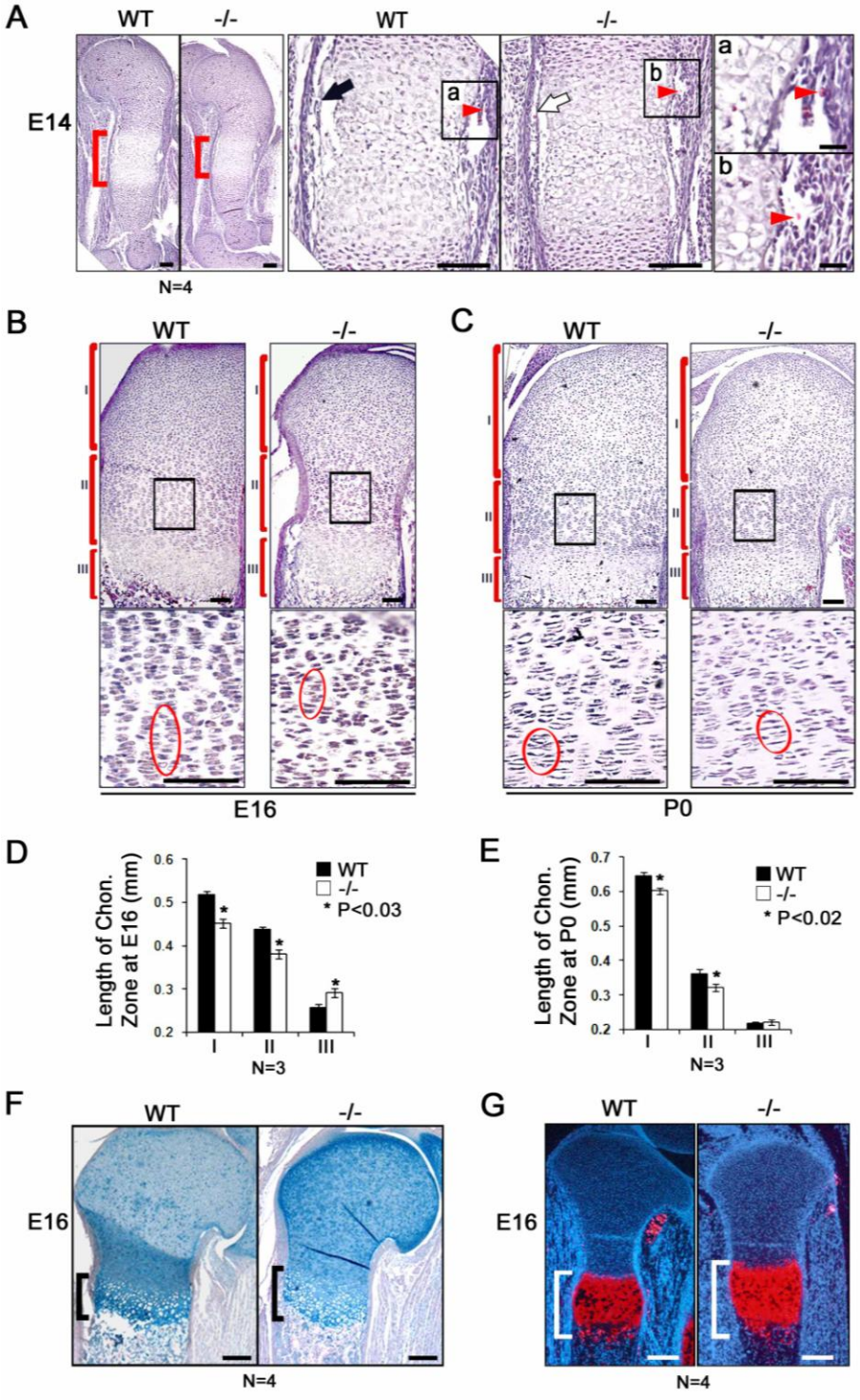
A
WT
-/-
WT
-/-
E14
a
b
a
b
N=4
B
WT
-/-
I
II
III
E16
C
WT
-/-
I
II
III
P0
D
Length of Chon. Zone at E16 (mm)
0.6
0.5
0.4
0.3
0.2
WT
-/-
* P<0.03
*
*
*
I
II
III
N=3
E
Length of Chon. Zone at P0 (mm)
0.7
0.6
0.5
0.4
0.3
0.2
WT
-/-
* P<0.02
*
*
I
II
III
N=3
F
WT
-/-
E16
N=4
G
WT
-/-
E16
N=4

Fig. 3. Decreased proliferation zone and expanded hypertrophic zone of *Atf4*$^{-/-}$ growth plate chondrocytes.

(**A**)Hematoxylin and Eosin (H&E) staining of sections through E14 WT and *Atf4*$^{-/-}$ mouse humerus. The center of the humeri (red brackets) are magnified in the middle pair of panels. The black arrow indicates newly formed cortical bone in the WT humerus, which is much thinner in the *Atf4*$^{-/-}$ humerus (white arrow).

(**a**,**b**)Higher magnification of the boxed regions of the humerus center showing vascular invasion (arrowheads) in WT and *Atf4*$^{-/-}$ humeri.

(**B**,**C**)H&E staining of E16 and P0 WT and *Atf4*$^{-/-}$ humerus sections. Reserve (I), proliferative (II) and hypertrophic (III) chondrocyte zones are indicated. Boxed regions showing proliferating chondrocytes are magnified beneath. The pattern of well-aligned columnar chondrocytes in the E16 WT proliferating zone is completely disorganized in the *Atf4*$^{-/-}$ growth plate (circled).

(**D**,**E**)Quantification of the length of the different chondrocyte zones. Error bars indicate s.e.m. In E, P □0.1 for III. Statistical analysis was performed by paired Student's *t*-test.

(**F**)Alizarin Red and Alcian Blue staining of growth plates of the radius showing that the hypertrophic zone (bracket) in *Atf4*$^{-/-}$ growth plates is expanded compared with its WT littermates at E16.

(**G**)In situ hybridization showing that the zone of *Col10a1*-expressing chondrocytes (red) is increased compared with WT control. Scale bars: 0.1 mm in A; 0.02 mm in Aa,b; 0.2 mm in F,G.

slightly extended at birth (Fig. 3B-F), which was confirmed by expanded zones of *Col10a1*-expressing chondrocytes (Fig. 3G). Collectively, these results demonstrate that Atf4 is required to induce timely hypertrophy at an early stage (i.e. E14), but to inhibit premature hypertrophy at later stages (i.e. E16 and P0) during skeletal development.

Reduced chondrocyte proliferation in Atf4$^{-/-}$ long bones

Before hypertrophy, longitudinal cartilage growth relies on fast division of proliferative chondrocytes and slow division of reserve chondrocytes. After hypertrophy and subsequent ossification, longitudinal skeletal growth is dependent on proliferation of both

chondrocytes within the growth plate and osteoblasts within the bone (St-Jacques et al., 1999). The decrease in the reserve and proliferative chondrocyte zone length in *Atf4*$^{-/-}$ long bones led us to hypothesize that *Atf4* might be a regulator of chondrocyte proliferation. To address this, we performed in vivo BrdU incorporation assays and found that the chondrocyte proliferative index was significantly decreased in proliferating chondrocytes in *Atf4*$^{-/-}$ E16 embryos and P0 pups (Fig. 4A-C), in agreement with the observed reduction in proliferative zone size (Figs 2,3). Therefore, Atf4 is required to maintain the high rate of chondrocyte proliferation in the rapidly growing long bones. In addition, in situ TUNEL assay revealed that there was no difference in the number of apoptotic cells between WT and mutant samples from E16 humeri, although there was an increased number of apoptotic hypertrophic chondrocytes in the center of the reserve zone in P0 *Atf4*$^{-/-}$ cartilage (Fig. 4D, green). This result rules out any contribution by delayed apoptosis to the hypertrophic expansion in E16 and P0 *Atf4*$^{-/-}$ growth plates.

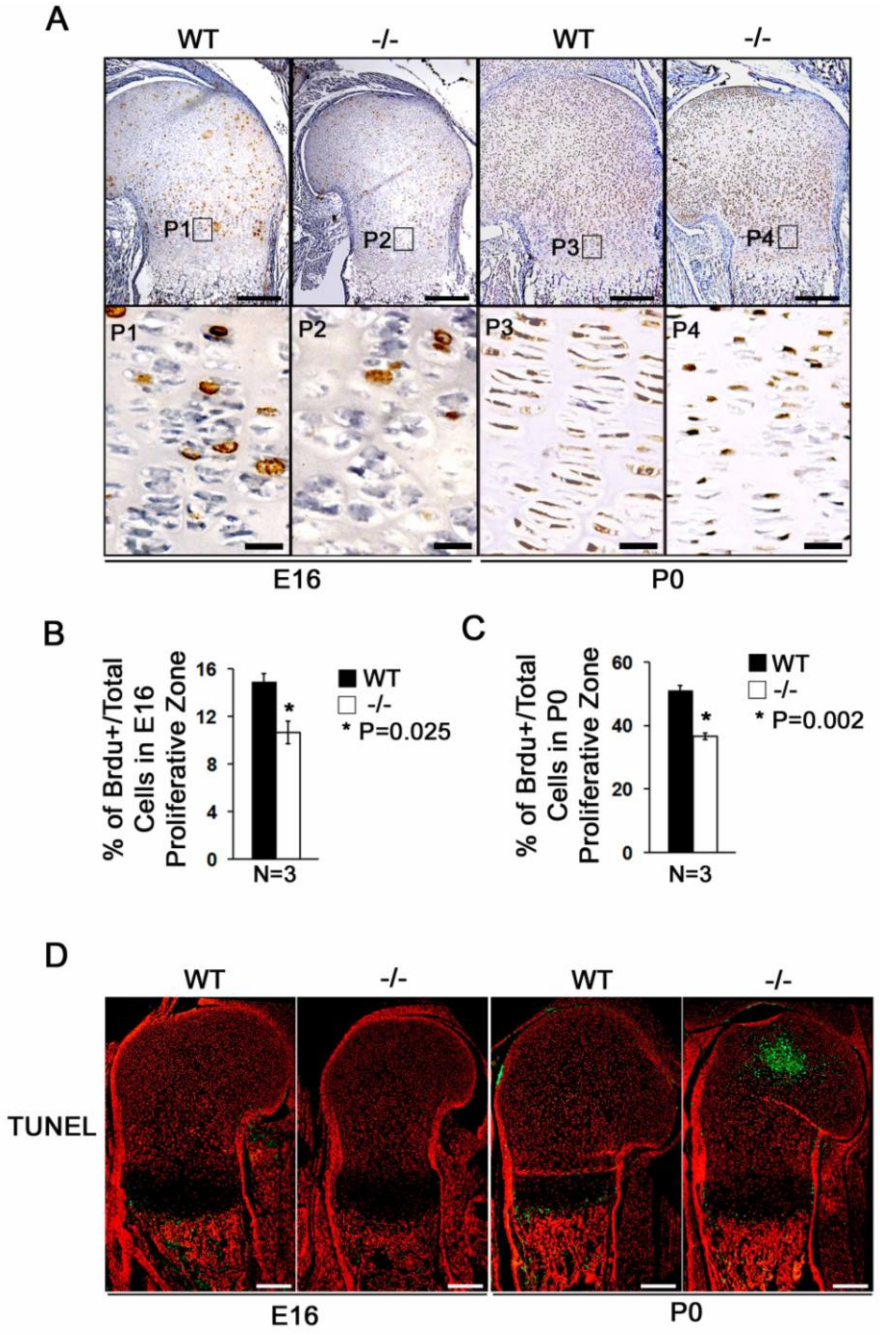
A
WT
-/-
WT
-/-
P1
P2
P3
P4
P1
P2
P3
P4
E16
P0
B
% of Brdu+/Total Cells in E16 Proliferative Zone
16
12
8
4
0
*
N=3
WT
-/-
* P=0.025
C
% of Brdu+/Total Cells in P0 Proliferative Zone
60
40
20
0
*
N=3
WT
-/-
* P=0.002
D
WT
-/-
WT
-/-
TUNEL
E16
P0

Figure 4. *Atf4* is required for chondrocyte proliferation.

(**A**) BrdU immunohistochemistry of E16 and P0 WT and *Atf4*$^{-/-}$ mouse humerus sections. Boxed regions (P1-P4) are magnified beneath, showing that there are fewer BrdU-positive (brown) proliferative chondrocytes in *Atf4*$^{-/-}$ growth plates in E16 embryos and P0 pups.

(**B**,**C**)Quantification of proliferation rate in proliferating chondrocytes, represented by the ratio of BrdU-positive cells normalized to total cells in WT and *Atf4*$^{-/-}$ E16 (B) and P0 (C) humeri. Error bars indicate s.e.m.

(**D**) TUNEL assay. In E16 WT and *Atf4*$^{-/-}$ humerus, no apoptotic cells are present in the cartilage, but some TUNEL-positive cells (green) appear in the primary spongiosa. At P0, there are abundant apoptotic cells in the secondary ossification center of the *Atf4*$^{-/-}$ humerus, but not in the WT. There are also some TUNEL-positive cells in primary spongiosa and hypertrophic chondrocyte zones in both WT and *Atf4*$^{-/-}$ growth plate. *N*=5. Scale bars: 0.2 mm in A,D; 0.02 mm in P1-P4 of A.

Atf4 is required for *Ihh* expression in chondrocytes

To understand the molecular mechanisms that mediate Atf4 function, and particularly to identify potential Atf4 targets, we performed in situ hybridization using chondrocyte markers. At E14, *Ihh* was expressed in prehypertrophic chondrocytes and its expression level increased at E16 and P0 in WT humeri (Fig. 5Aac). At E16, a low level of *Ihh* expression was found in osteoblasts in the center of WT long bones. By contrast, *Ihh* expression was not detectable at E14 and barely detectable at E16 in *Atf4*$^{-/-}$ humeri. Although detectable in *Atf4*$^{-/-}$ humeri at P0, the *Ihh* expression level was substantially lower than that in WT counterparts (Fig. 5Aa'-c'). These results indicate that *Atf4* deletion leads to a delay in the onset of, and a decrease in, *Ihh* expression. Consistently, the expression of transcription factor *Gli1*, a target and effector of Hh signaling, was decreased in perichondrium and proliferative chondrocytes in *Atf4*$^{-/-}$ humeri at all stages examined (Fig. 5Ad-f'). In E14 WT and *Atf4*$^{-/-}$ humeri, similar expression levels of PTH/PTHrP receptor (*PPR*; *Pth1r* – Mouse Genome Informatics) were detected in prehypertrophic chondrocytes, the same domain in which *Ihh* was expressed (Fig. 5Ag,g'). Interestingly, *PPR* expression transiently expanded to cells of the periosteum and to osteoblasts at E16, yet diminished in osteoblasts at birth in both WT and *Atf4*$^{-/-}$ humeri (Fig. 5Ah-I').

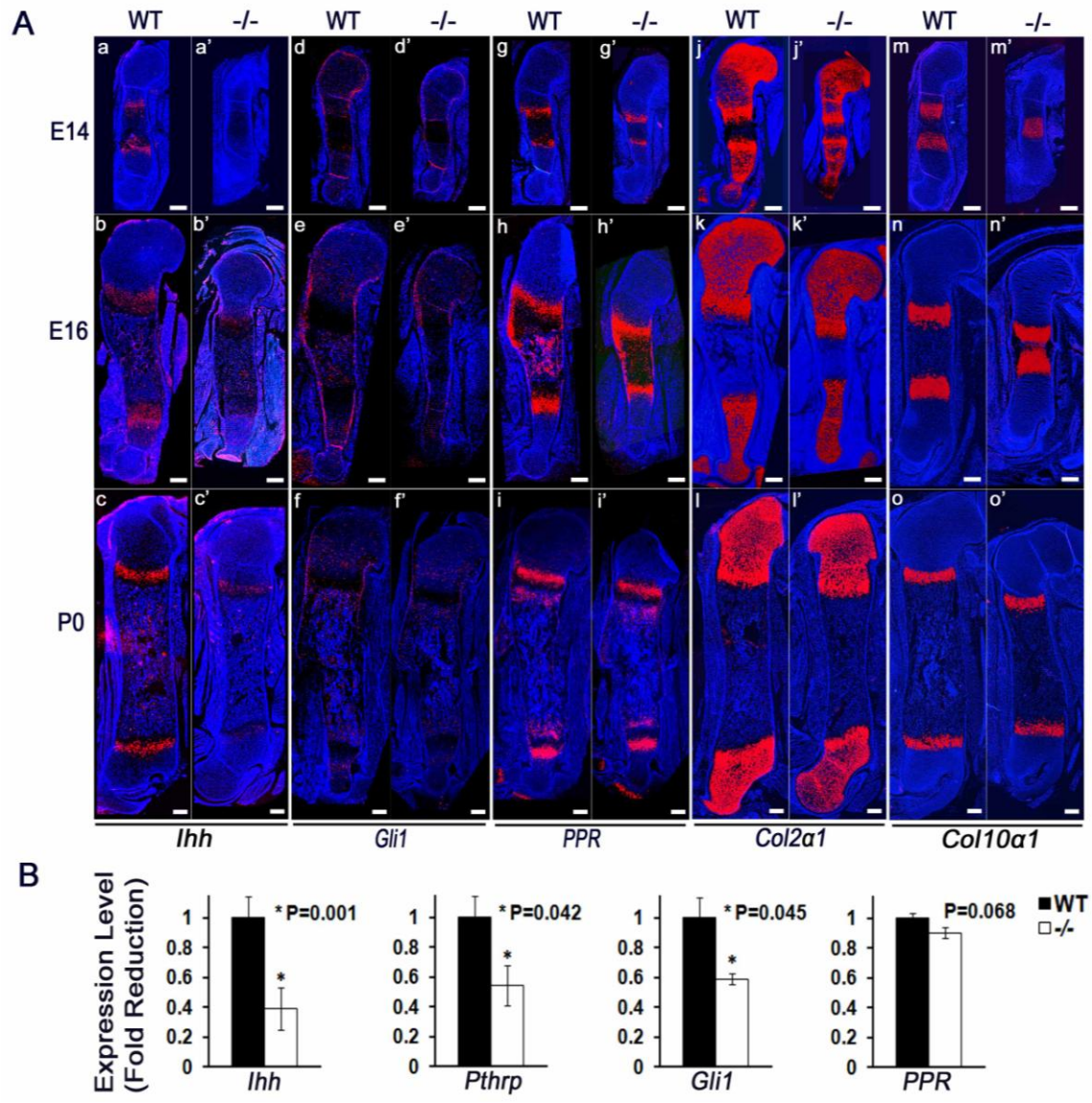

Fig. 5. *Ihh* expression is decreased in *Atf4*$^{-/-}$ cartilage.

(**Aa-o**') In situ hybridization of sections of E14, E16 and P0 WT and *Atf4*$^{-/-}$ mouse humeri. Note the decrease in *Ihh* and *Gli1*, but normal *PPR* (*Pth1r*), expression in *Atf4*$^{-/-}$ growth plates. Although the *Col2a1*-positive zones are shorter and the *Col10a1*-positive zones are slightly longer than their WT counterparts at every stage examined, the expression of *Col2a1* and *Col10a1* was unchanged in *Atf4*$^{-/-}$ growth plates. Scale bars: 0.2 mm.

(**B**) qRT-PCR analysis showing decreased levels of *Ihh*, *PTHrP* (*Pthlh*) and *Gli1* and normal levels of *PPR* mRNA in E14 *Atf4*$^{-/-}$ cartilage. Data are normalized to expression levels in WT cartilage and 18S rRNA (n=3).

There was no difference in *Col2a1* expression between WT and *Atf4*$^{-/-}$ humeri, although the zone of *Col2a1*-expressing chondrocytes was shorter in *Atf4*$^{-/-}$ bones (Fig. 5Aj-l'). At E14 in WT humeri, *Col10a1*-expressing hypertrophic chondrocytes had already separated into two distinct zones, whereas they appeared to be one single mass at the center in *Atf4*$^{-/-}$ humeri (Fig. 5Am,m'). There were no differences in the *Col10a1* expression level between WT and mutant humeri at any of the stages examined (Fig. 5Am-o'). However, at E16, mutant *Col10a1*-expressing hypertrophic chondrocyte zones were slightly expanded compared with their WT counterparts (Fig. 5An,n'). At birth, *Col10a1*-expressing hypertrophic chondrocyte zones remained unchanged, or were expanded, if one takes into account the overall length of the humeri (Fig. 5Al,l'). The pattern of *Col10a1* expression was consistent with the delay in long-bone mineralization and with the transient expansion of the hypertrophic zone at E16 (Figs 2,3).

Since the level of *Ihh* transcripts, as well as that of its downstream target gene *Gli1*, appeared to be reduced in the absence of *Atf4*, as judged by in situ hybridization, we further quantified the expression of these genes. qRT-PCR results confirmed a 60% decrease in *Ihh* expression in E14 *Atf4*$^{-/-}$ cartilage. Consistently, we observed a decrease in the expression of *PTHrP* (45%) and *Gli1* (41%), two downstream targets of the Hh signaling (Fig. 5B). Furthermore, and consistent with the in situ hybridization results, the expression of *PPR* was normal in E14 *Atf4*$^{-/-}$ cartilage. Taken together, these data strongly suggest that Atf4 is specifically required for *Ihh* expression in chondrocytes in vivo and that the decrease in *Ihh* expression is not a generalized consequence of *Atf4* deficiency.

Atf4 directly regulates *Ihh* transcription

To test whether *Ihh* is a direct transcriptional target of Atf4, we examined whether overexpression of Atf4 could affect endogenous *Ihh* mRNA levels in TMC23 chondrocytes (Xu et al., 1998). Atf4 overexpression induced endogenous *Ihh* expression to an extent similar to that induced by Runx2 overexpression (Fig. 6A). To address whether this function of Atf4 was direct, we co-transfected Ihh reporter constructs (pIhh-1.3-Luc and pIhh-742-Luc) individually with an Atf4 expression plasmid into COS1 cells. Both reporter constructs were transactivated by Atf4 to a similar extent (Fig. 6B), suggesting that the 742 bp promoter fragment of *Ihh* might contain an Atf4 binding site(s).

We examined the 742 bp promoter fragment of *Ihh* and located nine putative Atf4 binding sites, named A1 to A9 (Fig. 6C). EMSA revealed that Atf4 bound strongly to A9, but not to the other eight probes (Fig. 6D). Consistently, Atf4 failed to transactivate luciferase activity on pIhh-715-Luc, a construct in which A9 is deleted (Fig. 6B). Furthermore, Atf4 transactivated a reporter containing five repeats of A9 (pII5xA9-Luc) more than 5-fold (Fig. 6E), yet did not activate a reporter containing five copies of a mutant A9 sequence (PII5xmA9-Luc) that binds Atf4 with ten times lower affinity (Fig. 6F,G).

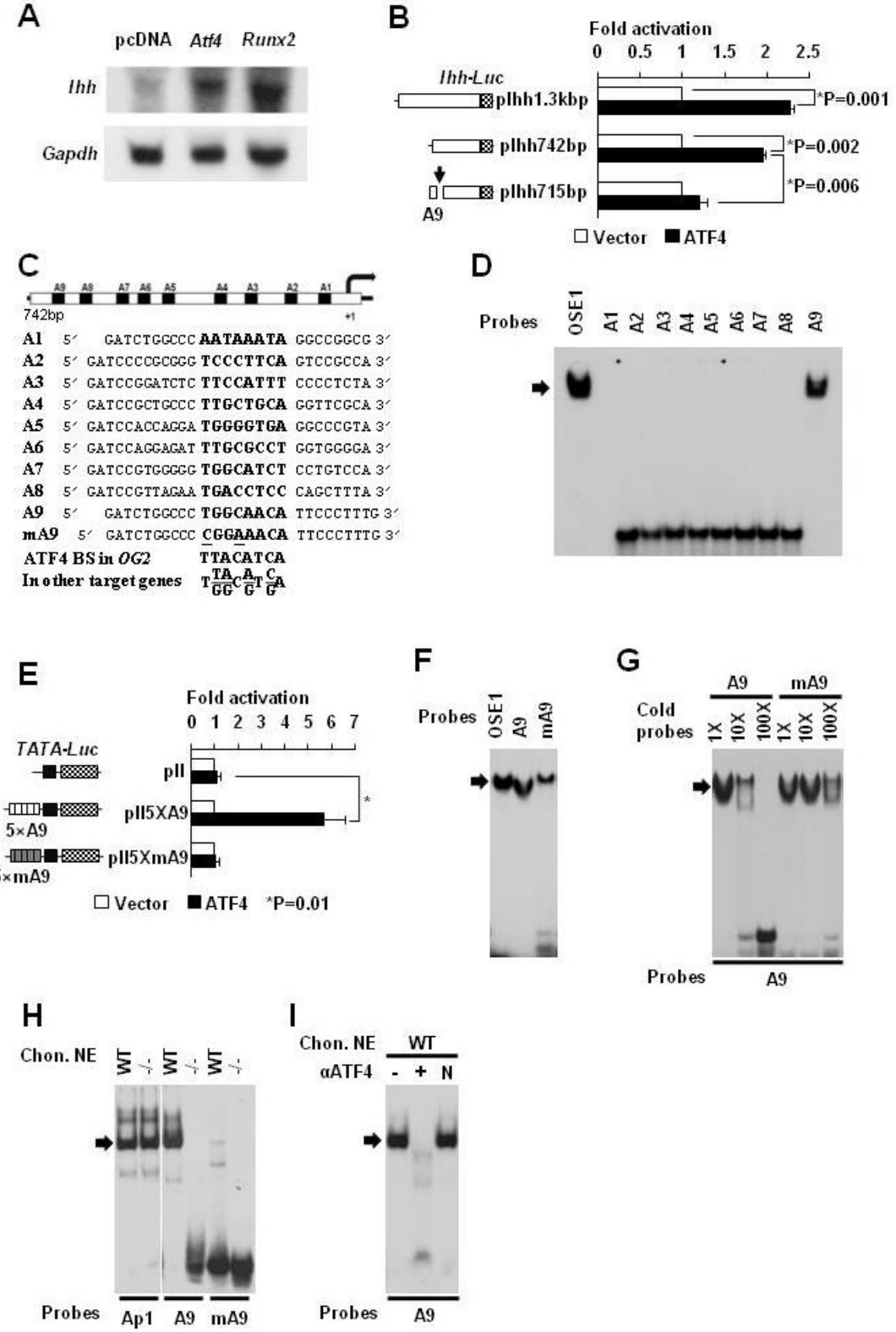
A
pcDNA
Atf4
Runx2
Ihh
Gapdh
B
Fold activation
0 0.5 1 1.5 2 2.5
Ihh-Luc
pIhh1.3kbp
*P=0.001
pIhh742bp
*P=0.002
pIhh715bp
*P=0.006
A9
Vector
ATF4
C
A9 A8 A7 A6 A5 A4 A3 A2 A1
742bp
+1
A1 5′ GATCTGGCCC AATAAATA GGCCGGCG 3′
A2 5′ GATCCCCGCGGG TCCCTTCA GTCCGCCA 3′
A3 5′ GATCCGGATCTC TTCCATTT CCCCTCTA 3′
A4 5′ GATCCGCTGCCC TTGCTGCA GGTTCGCA 3′
A5 5′ GATCCACCAGGA TGGGGTGA GGCCCGTA 3′
A6 5′ GATCCAGGAGAT TTGCGCCT GGTGGGGA 3′
A7 5′ GATCCGTGGGGG TGGCATCT CCTGTCCA 3′
A8 5′ GATCCGTTAGAA TGACCTCC CAGCTTTA 3′
A9 5′ GATCTGGCCC TGGCAACA TTCCCTTTG 3′
mA9 5′ GATCTGGCCC CGGAAACA TTCCCTTTG 3′
ATF4 BS in OG2 TTACATCA
In other target genes T(TA/GG)C(A/G)T(C/G)A
D
Probes
OSE1 A1 A2 A3 A4 A5 A6 A7 A8 A9
E
Fold activation
0 1 2 3 4 5 6 7
TATA-Luc
pII
pII5XA9
5×A9
pII5XmA9
5×mA9
*
Vector
ATF4
*P=0.01
F
Probes
OSE1 A9 mA9
G
A9
mA9
Cold probes
1X 10X 100X 1X 10X 100X
Probes
A9
H
Chon. NE
WT -/- WT -/- WT -/-
Probes
Ap1 A9 mA9
I
Chon. NE
WT
αATF4
- + N
Probes
A9

Fig. 6. Atf4 binds to the *Ihh* promoter to activate transcription.

(**A**) Northern blot analysis showing that Atf4 and Runx2 stimulate endogenous *Ihh* mRNA expression in TMC23 cells. *Gapdh* serves as a loading control.

(**B**) Atf4 activates *Ihh* promoter reporters in COS1 cells. Co-transfection assay showing that Atf4 activates *Ihh* promoter reporters (pIhh-1.3kbp and pIhh-742bp, as shown to left) but not the construct containing a mutated A9 (arrowhead), the Atf4 binding site.

(**C**) Sequences of nine putative Atf4 binding sites (A1-A9) in the proximate 742 bp promoter region of the mouse *Ihh* gene. Core sequences are indicated in bold, being identical to those within the Atf4 consensus sequence [i.e. TTACATCA, OSE1 in osteocalcin (*OG2*) and T(T/G)(A/G)C(A/G)T(C/G)A in other Atf4 target genes].

(**D**) Atf4 binds to A9 in the *Ihh* promoter. EMSA using the nine 32P-labeled putative Atf4 binding sites, A1-A9, as probes with purified Histagged Atf4 recombinant protein. OSE1 serves as a positive control. Arrow, Aft4-probe complex.

(**E**) A9 mediates Atf4 transactivation of *Ihh*. p5XA9-Luc and p5XmA9-Luc contain five copies of WT and mutant A9 [which binds Atf4 only weakly (see F,G)], respectively, linked to a TATAbox vector.

(**F**) EMSA using 32P-labeled probes at equal counts per minute of OSE1, A9 and A9 mutant (A9mut). Note the large amount of unbound A9mut probe at the bottom of the gel.

(**G**) Competition EMSAs. Fold molar excess of unlabeled double-stranded DNA competitor over labeled probe is indicated.

(**H**) Endogenous Atf4 binds to A9. The Ap1 probe, the cJun/cFos binding site, was used as a control for nuclear extract quality. (**I**) Supershift EMSAs showing that an antibody against Atf4 inhibits the binding of endogenous Atf4 to A9 (lane +). N, unrelated antibody.

EMSA revealed that endogenous Atf4 from WT, but not *Atf4*$^{-/-}$, primary chondrocytes bound to A9 (Fig. 6H). In addition, Atf4 antibody inhibited protein-A9 complex formation, whereas an unrelated control antibody had no effect (Fig. 6I). Taken together, these results confirm that the A9 sequence of the *Ihh* promoter serves as an Atf4 binding site, mediating Atf4 transactivation of *Ihh* transcription.

An Ihh agonist partially restores the length of *Atf4*$^{-/-}$ limbs

Since *Col2a1-Ihh* transgenic mice were not viable, we designed a rescue experiment based on an organ culture system. Limb explants from WT and *Atf4*$^{-/-}$ E14 embryos were cultured in the presence or absence of purmorphamine, a synthetic compound that directly targets Smoothened to activate Hh signaling (Sinha and Chen, 2006). At the beginning of the culture (t0), the primary ossification center was formed in WT ulna, but not in WT radii. As expected, no visible ossification center was present in mutant long bones at this stage (Fig. 7A, arrowheads). After 4 days of growth (t4), ossification centers were observed in both the ulna and radii in both genotypes (Fig. 7A, arrowheads), indicating a normal sequence of limb development in this organ culture system.

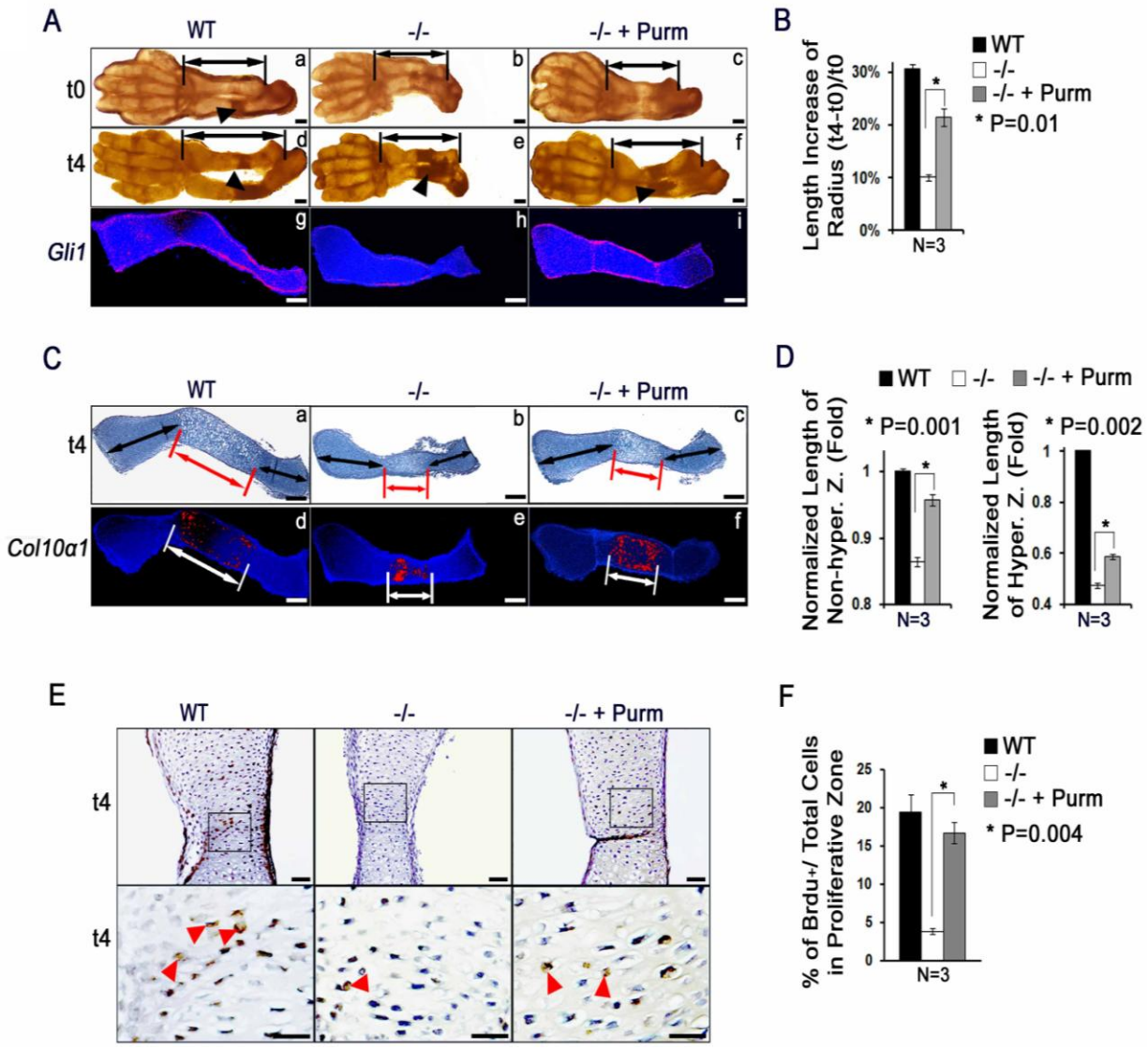
A
WT
-/-
-/- + Purm
t0
t4
Gli1
a
b
c
d
e
f
g
h
i
B
Length Increase of Radius (t4-t0)/t0
30%
20%
10%
0%
N=3
WT
-/-
-/- + Purm
* P=0.01
C
WT
-/-
-/- + Purm
t4
Col10a1
D
WT
-/-
-/- + Purm
Normalized Length of Non-hyper. Z. (Fold)
* P=0.001
1
0.9
0.8
N=3
Normalized Length of Hyper. Z. (Fold)
* P=0.002
1
0.8
0.6
0.4
N=3
E
WT
-/-
-/- + Purm
t4
t4
F
% of Brdu+/ Total Cells in Proliferative Zone
25
20
15
10
5
0
N=3
WT
-/-
-/- + Purm
* P=0.004

Fig. 7. Reactivation of Hh signaling by purmorphamine rescues limb defects in *Atf4*$^{-/-}$ embryos.

(**A**) Purmorphamine, an agonist of the Hh signaling pathway, restores the length of the *Atf4*$^{-/-}$ forelimb in organ cultures. (**a-f**) Limb explants at the beginning (t0, a-c) and the end (4 days, t4, d-f) of culture. *N*=3. Purmorphamine partially rescued the longitudinal growth of the *Atf4*$^{-/-}$ radius (double-headed arrow, f) as compared with the control (e). Arrowheads indicate BrdU-positive cells. (**g-i**) In situ hybridization of *Gli1* expression in WT (g), vehicle-treated *Atf4*$^{-/-}$ (h), and purmorphamine-treated *Atf4*$^{-/-}$ (i) radii. *N*=3. Purmorphamine restored *Gli1* expression in *Atf4*$^{-/-}$ radii (i) to a level similar to that in WT controls (h), indicating activation of Hh signaling in *Atf4*$^{-/-}$ explants.

(**B**) Quantification of the increase in radius length upon purmorphamine treatment of WT and *Atf4*$^{-/-}$ limbs. The percentage increase in radius growth after 4 days in culture is shown. *P*=0.01 by paired Student's *t*-test.

(**C**) Purmorphamine partially corrects the delayed chondrocyte hypertrophy in *Atf4*$^{-/-}$ growth plates in organ culture. (**a-c**) Alcian Blue, Alizarin Red and Hematoxylin staining of radius sections of 4-day cultures. (**d-f**) In situ hybridization for *Col10a1* in purmorphamine-treated and vehicle-treated limb explants. *N*=3.

(**D**) Quantification of the relative length of the non-hypertrophic and hypertrophic zones upon purmorphamine treatment of WT and *Atf4*$^{-/-}$ radii. Error bars indicate s.e.m. of the length of the non-hypertrophic (left) and hypertrophic (right) zones normalized to the respective WT controls at 4 days in culture.

(**E**) Purmorphamine increases chondrocyte proliferation in *Atf4*$^{-/-}$ growth plates. Immunohistochemistry of BrdU-labeled chondrocytes in limbs cultured for 4 days in the absence and presence of purmorphamine. The sections were counterstained with Hematoxylin. Boxed regions are magnified beneath to show BrdU-positive cells (arrowheads). *N*=3.

(**F**) Quantification of proliferation rate represented by the ratio of BrdU-positive cells (brown) to total cells upon purmorphamine treatment of WT and *Atf4*$^{-/-}$ radii. Error bars indicate s.e.m. Scale bars: 0.2 mm in A,C; 0.5 mm in E.

Hh signaling reactivation upon purmorphamine treatment was confirmed by increased *Gli1* expression in purmorphamine-treated *Atf4*$^{-/-}$ limbs as compared with vehicle-treated samples (Fig. 7Ah,i). Within 4 days of growth, the length of the radii increased by 31% in WT and by 21% in purmorphamine-treated *Atf4*$^{-/-}$limbs, whereas it increased only 10% in vehicle-treated mutant limbs (Fig. 7B). These results indicate that reactivation of Hh signaling improves the longitudinal growth of *Atf4*$^{-/-}$ limbs in culture. The increase in size in purmorphamine-treated *Atf4*$^{-/-}$ limbs was associated with elongated zones of proliferative and hypertrophic chondrocytes (Fig. 7Ca-c,D). In addition, purmorphamine reduced the delayed hypertrophy in *Atf4*$^{-/-}$ limbs, as shown by the increase in the *Col10a1*-expressing chondrocyte area in purmorphamine-treated versus vehicle-treated *Atf4*$^{-/-}$ radii (Fig. 7Ce,f,D). The BrdU proliferative index in proliferative chondrocytes (Fig. 7E, arrowheads) was significantly increased upon purmorphamine treatment of *Atf4*$^{-/-}$ limbs (Fig. 7F). These results indicate that activation of the Hh pathway in *Atf4*$^{-/-}$ limbs can partially rescue the defects in chondrocyte proliferation and hypertrophy characteristic of this mutant, further reinforcing the notion that Atf4 and Ihh lie in the same pathway for the regulation of limb development and long-bone growth.

Discussion

This study reveals the transcription factor Atf4 as a crucial regulator of chondrogenesis and identifies *Ihh* as a transcriptional target of Atf4 in chondrocytes. Mice lacking Atf4 exhibit dwarfism characterizing by markedly reduced growth plates, decreased chondrocyte proliferation and an abnormally expanded hypertrophic zone. These phenotypic abnormalities are similar to those of $Ihh^{-/-}$ mice (St-Jacques et al., 1999), which, together with the dramatic decrease in *Ihh* expression observed in $Atf4^{-/-}$ growth plates, indicate that Atf4 and Ihh lie in the same genetic pathway regulating chondrogenesis during skeletal development.

Distribution of Atf4 in chondrocytes

Atf4 was originally termed osteoblast-specific factor 1 (Osf1) because it was first identified as a specific binding activity of osteoblast nuclear extracts to OSE1, the osteoblast-specific element 1 found in the osteocalcin (*Bglap*) promoter (Ducy and Karsenty, 1995). Subsequent studies revealed that the cell specificity of Atf4 is regulated at the post-translational level (Yang and Karsenty, 2004). However, nuclear extracts of primary chondrocytes were not tested in previous studies, and the evidence for involvement of Atf4 in chondrogenesis was lacking. In this study, a systematic analysis of the expression pattern of Atf4 demonstrated that it is expressed at high levels in embryos, limbs and primary chondrocytes, supporting its role in chondrogenesis.

Atf4 as a novel transcriptional regulator of *Ihh*

A major finding of this study is that Atf4 directly regulates chondrocyte proliferation by affecting the transcription of *Ihh*, a molecule that plays crucial roles in both chondrogenesis and osteogenesis. We demonstrated, for the first time genetically and molecularly, that Atf4 acts as a direct transcriptional activator of *Ihh*, the expression of which is dramatically decreased in *Atf4* mutant mice. Atf4 binds and transactivates *Ihh* in chondrocytes and forced expression of Atf4 enhances endogenous *Ihh* mRNA synthesis. In organ cultures, reactivation of Hh signaling by a synthetic compound that bypasses the need for Ihh ligand almost completely rescues the proliferation defects and partially rescues the delay in hypertrophy in *Atf4*$^{-/-}$ limbs. As a result, the short limb phenotype in *Atf4*$^{-/-}$ animals was partially corrected. Together, these results strongly suggest that Atf4 and Ihh act in the same pathway to regulate chondrocyte proliferation and hypertrophy. The fact that the hypertrophic defect in *Atf4*$^{-/-}$ limbs was not fully rescued by purmorphamine might suggest that Atf4 regulates chondrocyte hypertrophy by an additional and Ihh-independent mechanism(s), or, more likely, it might reflect a limitation of the organ culture system.

The functions of Atf4 and Runx2, another transcriptional activator of *Ihh*, are not redundant as removal of *Atf4* or *Runx2* gives rise to severe chondrocyte proliferation or differentiation defects, respectively. Although our current study cannot dissect the relative contributions of Runx2 and Atf4 to the control of chondrogenesis, the fact that *Runx2*$^{-/-}$ animals completely lack hypertrophic chondrocytes in their skeletons (Komori et al., 1997); (Otto et al., 1997), and that the role of Runx2 is in the induction, rather than

inhibition, of chondrocyte hypertrophy (Takeda et al., 2001); (Ueta et al., 2001), suggest that the function of Runx2 in the control of chondrocyte biology could be Ihh-independent and antiproliferative.

In fact, a recent study showed that Runx2 inhibits chondrocyte proliferation and hypertrophy through regulation of *Fgf18* transcription in the perichondrium (Hinoi et al., 2006). It is unlikely that Atf4 regulates *Fgf18* directly because overexpressing Atf4 has no effect on luciferase activity driven by *Fgf18* promoter constructs in DNA co-transfection assays (Dr M. Naski, personal communication). Consistently, we observed no differences in the expression of *Fgf18* in WT and *Atf4*$^{-/-}$ cartilage (Fig. 8A). Other studies indicate that *Runx2* mRNA synthesis can be downregulated by PTH/PTHrP signaling (Guo et al., 2006), yet *Atf4* mRNA and protein are upregulated by parathyroid hormone (Yu et al., 2008), supporting the notion that Atf4 and Runx2 play different, but related, roles in the regulation of chondrogenesis.

Atf4 regulates chondrocyte differentiation

The fact that the hypertrophic chondrocyte zone in *Atf4*$^{-/-}$ long bones is transiently increased at E16 and then remains the same as in WT, and that *PTHrP* expression is downregulated in *Atf4* mutant growth plates, indicate that Atf4 plays a role in the control of hypertrophic chondrocyte differentiation. This provides indirect confirmation that *Ihh* expression is downregulated in *Atf4*$^{-/-}$ growth plates. PTHrP and its receptor, which lie downstream of Ihh signaling, play multiple roles in regulating chondrocyte proliferation and differentiation (Kronenberg, 2006). PTHrP acts as an inhibitor to prevent premature

chondrocyte hypertrophy (Karaplis et al., 1994), which ensures the maintenance of a pool of proliferative chondrocytes. PTHrP also directly stimulates chondrocyte proliferation in an organ culture system (Mau et al., 2007). However, it is unclear whether Atf4 is a direct transcriptional regulator of *PTHrP* or not. Our DNA transfection data suggest that the downregulation of *PTHrP* expression in *Atf4*$^{-/-}$ chondrocytes might be due to an indirect mechanism, most likely involving impaired Hh signaling, as: (1) Atf4 did not transactivate luciferase constructs driven by 4.5 or 1.1 kb *PTHrP* promoter fragments, whereas these two reporter constructs responded to Gli2, a known transcriptional activator of *PTHrP* (Sterling et al., 2006); (Zhao et al., 2006) (Fig. 8B,C); and (2) we could not locate any Atf4 binding consensus site within this promoter region.

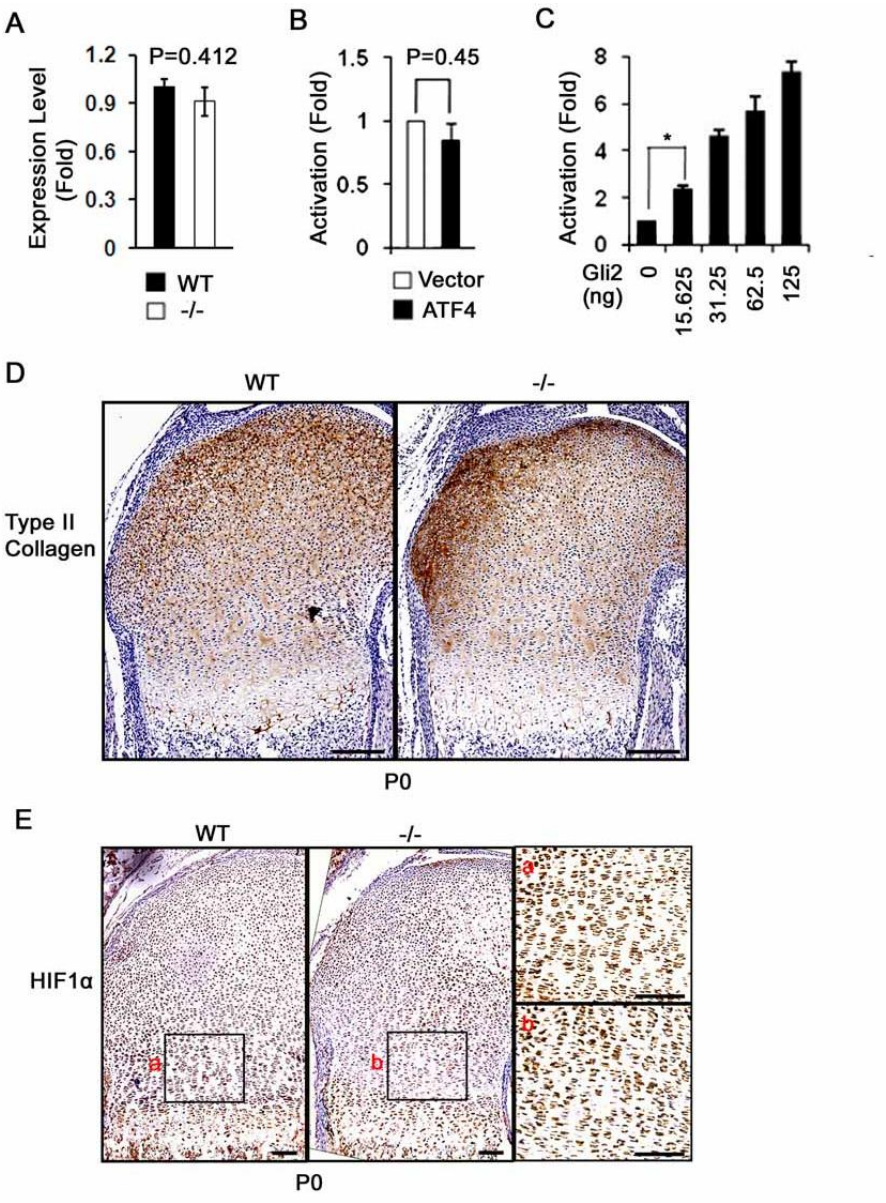
A
Expression Level (Fold)
1.2
0.9
0.6
0.3
0
P=0.412
WT
-/-
B
Activation (Fold)
1.5
1
0.5
0
P=0.45
Vector
ATF4
C
Activation (Fold)
8
6
4
2
0
*
Gli2 (ng)
0
15.625
31.25
62.5
125
D
WT
-/-
Type II Collagen
P0
E
WT
-/-
HIF1α
a
b
a'
b'
P0

Fig. 8. Atf4 is not required for *PTHrP* transcription and type II collagen and Hif1α synthesis.

(**A**) qRT-PCR showing a slight, but not significant, decrease in mRNA levels of *Fgf18* in P2 WT and *Atf4*$^{-/-}$ cartilage. Data are normalized to expression levels in WT cartilage and 18S rRNA expression (*n*=3).

(**B**) Atf4 fails to activate the *PTHrP* promoter in COS cells. DNA co-transfection was performed with a 1.1 kb *PTHrP* promoter-luciferase construct and an Atf4 expression vector. Statistical analysis by paired Student's *t*-test.

(**C**) Gli2 activates the *PTHrP* promoter in a dose-dependent manner in TMC23 cells. Cells were co-transfected with the same reporter construct as in Fig. S1A and the indicated amount of Gli2 expression vector. *Gli2 transactivated the *PTHrP* promoter significantly at lowest concentration.

(**D**) Immunohistochemistry for type II collagen (Col2α1) protein on P0 WT and *Atf4*$^{-/-}$ humerus growth plate sections. Representative images are displayed, showing similar type II collagen protein levels (brown) in the WT and mutants. Sections are counterstained with Hematoxylin. *n*=5. Scale bars: 0.2 mm.

(**E**) Immunohistochemistry for Hif1α protein on P0 WT and *Atf4*$^{-/-}$ humerus growth plate sections. (**a**,**b**) Magnified views of the boxed regions of reserve chondrocyte zones. HIF1α-positive cells are brown. Sections are counterstained with Hematoxylin. *n*=5. Scale bars: 0.1 mm.

Type II collagen secretion is normal in *Atf4*$^{-/-}$ chondrocytes

Given the indispensable role of Atf4 in the regulation of stress responses (Harding et al., 2000); (Harding et al., 2003); (Rutkowski and Kaufman, 2003) and in type I collagen synthesis in osteoblasts (Yang et al., 2004), Atf4 may be required for secretion of type II collagen in chondrocytes. Our immunohistochemistry results using a monoclonal antibody developed by Dr Thomas F. Linsenmayer (Tufts Medical School, Boston, MA, USA) revealed that type II collagen secretion is normal in *Atf4*$^{-/-}$ cartilage (Fig. 8D). Furthermore, we also observed a normal expression level of hypoxia-inducible factor 1 (Fig. 8E), a master regulator of oxygen homeostasis and a key element to cellular survival and adaptation and a regulator of chondrogenesis (Amarilio et al., 2007). These results cannot rule out the possibility that Atf4 plays a role in the response to oxygen level in chondrocytes, given the hypoxic environment in which chondrocytes exist.

This study reveals a novel mechanism by which Atf4 regulates chondrocyte proliferation and differentiation via upregulating *Ihh* expression. It remains unclear, however, what upstream signals regulate Atf4 expression and activity and what downstream effector molecules of Hh signaling are controlling chondrocyte proliferation and differentiation. Many extracellular regulators, including members of the FGF, BMP, Igf1, Wnt and PTHrP families, are reported to regulate chondrocyte proliferation and differentiation. Whether they act on chondrocytes by modifying Atf4 activity remains to be determined. Lastly, and importantly, it is of interest to establish whether the same mechanisms are responsible for the bone defects seen in the *Atf4*$^{-/-}$ mice.

CHAPTER III

ATF4 IN CHONDROCYTES REGULATES OSTEOBLAST DIFFERENTIATION AND FUNCTION VIA IHH

Abstract

Activating transcription factor 4 (Atf4) is required for chondrocyte proliferation and differentiation in growth plate. *Atf4* mutant mice (*Atf4*$^{-/-}$) display short stature and short limb phenotypes. To study the autonomous function of Atf4 in growth plate chondrocytes, *Atf4* was specifically overexpressed in chondrocytes in *Atf4*$^{-/-}$ background mice (*Atf4*$^{-/-}$;*Col2α1-Atf4*). Chondrocyte-specific overexpression of *Atf4* in *Atf4*$^{-/-}$ mice restores *Ihh* expression and Hh signaling, and completely rescues the cartilage defects present in *Atf4*$^{-/-}$ embryos, confirming a cell-autonomous function of Atf4 in chondrocytes. Unexpectedly, *Atf4* overexpression in chondrocytes also rescues the low bone mass of *Atf4*$^{-/-}$ mice, accompanied by rescued expression of osteogenic markers, including *osteocalcin* and *bone sialoprotein* in osteoblasts. Conditioned media from wildtype (WT) and *Atf4*$^{-/-}$;*Col2α1-Atf4* but not *Atf4*$^{-/-}$ cartilage cultures significantly increased the numbers of alkaline phosphatase-positive osteoblast progenitors and mineralized nodules in *Atf4*$^{-/-}$ primary bone marrow stromal cell cultures. Ihh blocking antibody abolished the effects of WT and *Atf4*$^{-/-}$; *Col2α1-Atf4* cartilage-conditioned media on the differentiation of *Atf4*$^{-/-}$ bone marrow stromal cells, indicating that Atf4 in chondrocytes indirectly regulates osteoblast differentiation via Ihh. Our study thus identifies a novel function of

Atf4 in chondrocytes for regulating both chondrogenesis and osteogenesis during endochondral ossification.

Introduction

In the early stage of mammalian skeleton development, mesenchymal stem cells differentiate into chondrocytes which build up a cartilage template for the skeleton. Most of the flexible cartilage is replaced by hard and rigid bone later, to support the increasing weight of body. Bone is a form of mineralized connective tissue in the skeleton. There are two different processes to develop bones: intramembranous ossification and endochondral ossification. Some skeletal elements, such as flat bones of the skull, are formed by intramembranous ossification, whereby mesenchymal stem cells (MSCs) directly differentiate into osteoblasts that build the bone matrix. On the other hand, long bones, such as humerus, femur, tibia and most of the rest of the bones in the body are formed by endochondral ossification, in which bone formation is intermediated by cartilage development (Karsenty et al., 2009). In endochondral ossification, the MSC*s* differentiate into chondrocytes which secrete collagen type II into extracellular matrix (ECM). Chondrocytes differentiate into resting, proliferative, prehypertrophic and hypertrophic chondrocytes sequentially. Each type of chondrocyte has different cell morphologies and occupies in different but connected zone in the growth plate. Resting chondrocytes have round shapes at the top of growth plate. Proliferative chondrocytes are rapid dividing flat cells, which stack together linearly forming the columnar structures in the proliferative zone. Prehypertrophic chondrocytes exit the rapid dividing stage, start to enlarge their sizes in a narrow prehypertrophic zone, and then become hypertrophic

chondrocytes. Hypertrophic chondrocytes enlarge their sizes extensively 3 to 5 folds, and secrete collagen type X into the ECM. Hypertrophic chondrocytes direct adjacent perichondrial cells to become osteoblasts that form a bone collar. Hypertrophic chondrocytes also attract blood vessels invading the cartilage mold, and recruit chondroclasts and osteoblasts. Hypertrophic chondrocytes then undergo apoptosis and leave behind a mineralized cartilage ECM. This cartilage ECM is digested by chondroclasts, and is replaced by a bone ECM rich in collagen type I secreted by osteoblasts in the primary ossification center. (Kronenberg, 2003). The complexity of endochondral ossification thus requires a proper transition of cartilage formation into bone formation, and a tight coordination of chondrocyte differentiation and osteoblast differentiation.

Ihh, a secreted molecule, is a master regulator for both chondrogenesis and osteogenesis during endochondral ossification. *Ihh* is the only member of the hedgehog (Hh) family that is specifically expressed in growth plate chondrocytes during endochondral bone formation (Bitgood and McMahon, 1995) (Karsenty et al., 2009). Ihh binds to its receptor Patched-1(Ptch1), a transmembrane protein, leading to the activation of Smoothened (Smo), a cell surface protein that activates Hh target gene expression, such as *Gli1* and *Ptch1*(Alcedo and Noll, 1997; Mak et al., 2008). *Ihh*-null mice die in the fetus stage, display abnormal chondrocyte proliferation and maturation, and lack mature osteoblasts (St-Jacques et al., 1999). This indicates that Ihh is crucial for endochondral ossification. The early lethality of *Ihh*-null mice hampered the understanding of the postnatal function of Ihh. Analysis of conditional knockout mice, in which Ihh was

specifically deleted from postnatal chondrocytes, revealed that Ihh is essential for maintaining postnatal growth plate and trabecular bone (Maeda et al., 2007). In short, Ihh is an essential regulator for both chondrocyte and osteoblast differentiation and function during endochondral ossification.

Atf4 is a leucine zipper-containing transcription factor of the cAMP response element-binding protein (CREB) family. Atf4-deficient (*Atf4*$^{-/-}$) mice show a low-bone-mass and short-stature phenotype, indicating this transcription factor plays a crucial role in both bone formation and bone longitudinal growth. Atf4 regulates osteoblast terminal differentiation, enhancing amino acid import and favoring synthesis of type I collagen. *Atf4* mRNA is ubiquitously expressed, but Atf4 protein determined by posttranslational regulation (Yang et al., 2004). Recently, we reported that Atf4 is also expressed in chondrocytes. *Atf4*$^{-/-}$ mice have defects in the cartilage growth plate, characterized by a reduced and disorganized proliferative chondrocyte zone, decreased chondrocyte proliferation, delayed chondrocyte hypertrophy, delayed hypertrophic mineralization, an expanded hypertrophic chondrocyte zone, and decreased *Indian hedgehog* (*Ihh*) transcription and Hedgehog (Hh) signaling. Atf4 directly binds and activates the *Ihh* promoter. Over-expression of *Atf4* in chondrocyte cell lines also increases *Ihh* expression. While Purmophamine, an Hh signaling activator, partially restores chondrocyte proliferation and differentiation (Wang et al., 2009). These results suggest that Atf4 regulates growth plate chondrocyte proliferation and differentiation by activating *Ihh* transcription. However, the global mutant of Atf4 in *Atf4*$^{-/-}$ mice interferes the understanding of the contribution of chondrocyte-autonomous function of Atf4 in

endochondral ossification. To overcome this limitation, we generated a transgenic mice *Col2α1-Atf4* mice with *Atf4* over-expressed in chondrocytes specifically driven by *Type II collagen alpha 1* (*Col2α1*) promoter/enhancer (Metsaranta et al., 1995). We then crossed *Col2α1-Atf4* mice onto the $Atf4^{-/-}$ background to generate $Atf4^{-/-}$;*Col2α1-Atf4* mice, which has Atf4 expressed only in chondrocytes and provide a novel model to study autonomous function of Atf4 in chondrocytes.

Materials and Methods

Animal

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. *Col2α1-Atf4* transgene was constructed by inserting an entire *Atf4* locus in between a 3 kb fragment of the *Col2α1* chain gene promoter and its 3 kb chondrocyte-specific enhancer within the first intron (Metsaranta et al., 1995). Southern blot was performed using BamHI/XhoI digested tail genomic DNA to select transgenic founders. Two lines of transgenic mice containing high copy numbers of the *Col2α1-Atf4* were used for subsequent mating with WT to maintain the transgenic lines or $Atf4^{+/-}$ mice to generate experimental animals. PCR was also performed for genotyping of transgenic or Atf4 mutant mice. The primers used to amplify the *Col2a1-Atf4* allele were a 5' primer within the *Col2a1* promoter region (5'-GCCTCGCTGCGCTTCGC-3') and a 3' primer from the 3' *Atf4* coding region (5'-GCTTAGGCCGGTGGGGGTTG-3'). The primers used to amplify the WT *Atf4* allele were a 5' primer within the 3' *Atf4* coding region and a 3' primer from the 3' *Atf4* un-transcriptional region (5' primer, 5'-GTAAGGCAAGG-GGGAAGAAG-3'; and 3' primer, 5'-ACCAGGTGGAAACAGTCCAG-3'). The primers used to amplify the $Atf4^{-/-}$ allele flanked the Neo allele (5' primer 5'-AGGATCTCCTGTCA-TCTCACCTTGCTCCTG-3' and 3' primer 5'-AAGAACTCGTCAAGAAGGCG-ATAGAAGGCG-3').

Quantitative real time RT-PCR (qPCR)

Total RNA from tail, limb cartilage and bones of indicated genotypes was isolated using TRIzol (Invitrogen) following the manufacturer's protocols. DNase I-treated RNA (2 µg) was reverse-transcribed with 100 units of Superscript II plus RNase H^- Reverse Transcriptase (Invitrogen) using 100 µM random hexamer primers. qPCR was performed using a standard TaqMan PCR kit protocol on an Applied Biosystems 7300 Sequence Detection System (Applied Biosystems). Specific forward and reverse oligonucleotide primers used for *Atf4* (Mm00515325_m1), *Ihh* (Mm00439613_m1), *Patched* (Mm00436029_m1), *Gli1* (Mm00494645_m1), *PTH/PTHrP receptor* (*PPR*) (Mm00441046_m1) were from Applied Biosystems. 18S rRNA was used as an internal control (QuantumRNA™ Classic 18S Internal Standard).

Skeletal preparation and histology

Embryos and pups were isolated and skeletal preparation was performed according to standard protocols. For histology, embryos and P0 mice were fixed in 4% paraformaldehyde, embedded in paraplast, and sectioned at 5µm. Sections were stained with Hematoxylin and Eosin (H&E). For each genotype, at least three embryos or mice were analyzed.

In vivo proliferation assay

5-bromo-2′-deoxyuridine (BrdU) 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 Labratories) as described previously (Wang et al., 2009).

In situ hybridization

Alternate sections of forelimb used for histological analysis were hybridized with in situ probes. (Wang et al., 2009) (Yang and Karsenty, 2004). Probes for type II collagen (*Col2a1*), *Ihh* and type X collagen *Col10a1* were as described previously (Ducy et al., 1997) (Takeda et al., 2001). The probe for *PPR* was from Dr T. J. Martin (University of Melbourne, Australia). The probe for *Gli1* was a mouse cDNA fragment (bp 968 to 1437), which was generated by RT-PCR using primers: forward, 5'-GAAGGAATTCGTGTGCCATT-3' and reverse, 5'-TCCAAGCTGGACAAGTCCTC-3'. Probe for *Patched* was from Dr. Chin Chiang's laboratory (Vanderbilt University). *Ocn* and *Bsp* probes as described previously (Yang and Karsenty, 2004). Antisense cDNAs were used for riboprobe synthesis with RNA polymerases (Invitrogen) and [35S]uridine triphosphate (Perkin Elmer).

Microcomputed tomography (μCT) 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 μCT imaging system (Scanco μCT 40; Scanco Medical, Bassersdorf, Switzerland). Tomographic cross sectional images were acquired at 55kV, medium resolution, and 100 slides. Contours were fit 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^3.

Cartilage and bone marrow stromal cells culture

Long bone cartilage was isolated from new born pups and cultured in with a αMEM (6 mg cartilage/ml) containing 10% serum and 1% antibiotics. 50% of the cartilage medium filtered through Cell Culture Inserts (BD Falcon™, pore size 1 microns) was collected every two days and used as cartilage conditioned medium (CM). 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 the long bone by centrifugation method as described (Dobson et al., 1999). BMSCs were plated in 6-well tissue culture plate with $2x10^5$ cells/well and $4x10^5$ cells/well respectively for CFU-ap and CFU-ob following standard protocol except that cartilage CM (100 μl) was supplemented. For blocking Hh signaling, anti-Hh antibody (5E1, 2 μg/ml) or cyclopamine (10 μM) was added together with the CM into BMSC cultures. In a similar manner, purmorphamine (10 μM) was added to stimulate Hh signaling.

Results

Generation of *Atf4*$^{-/-}$;*Col2α1-Atf4* mice overexpressing *Atf4* in chondrocytes

In order to understand the specific function of Atf4 in chondrocytes, we first generated transgenic mice which specifically over-express *Atf4* driven by the *Type II collagen* (*Col2α1*) gene promoter and enhancer in chondrocytes. The insertion of *Col2α1-Atf4* in the genome was examined by Southern hybridization analysis, 28 mice were screened, 5 mice (No. 2, 3, 5, 9, and 11) expressed *Col2α1-Atf4* transgene (Fig. 1A and data no shown). The bands of No. 2 and 9 are darker than No. 3, 5, and 11, indicating that they have more copies of *Col2α1-Atf4* inserted in the genome than others. The founders and their offsprings showed no difference compared to their wildtype (WT) littermates in gross body size, weight, and activity for up to 1 year and at least 3 generations of observation, suggesting that *Atf4* overexpression in chondrocytes does not affect skeletal development or metabolism. In order to get higher *Col2α1-Atf4* expression, we selected and expended 2 transgenic lines (2 and 9) that contained higher copy numbers of the transgene. Quantification real time PCR (qRT-PCR) showed that they have about 4 and 5.5 times of *Atf4* expression compared with the wildtype mice (WT) in tails (Fig. 1B). After observing 3 generations of their descendents including P0, 1 month and 3 month mice, we didn't find any abnormal skeletal growth, body weight, move activity or death in these transgenic mice compared with WT, and there were no difference between No. 2 and 9 (data no shown), suggesting that *Atf4* overexpression does not affect normal skeleton development. *Col2α1* is expressed specifically in cartilage, to test whether *Col2α1-Atf4* is specifically expressed in cartilage but no in bone, we isolated RNA of cartilage and bone from the limbs of WT, *Atf4*$^{-/-}$, *Col2α1-Atf4*, and *Atf4*$^{-/-}$;*Col2α1-Atf4*

littermates, and examined *Atf4* expression by qRT-PCR. The expression level of *Atf4* in *Col2α1-Atf4* and *Atf4*$^{-/-}$;*Col2α1-Atf4* cartilage of Po are 4.9 folds and 4.1 folds higher than their WT littermates, there is no *Atf4* expression in the *Atf4*$^{-/-}$ cartilage (Fig. 1C). There is no *Atf4* expression in the *Atf4*$^{-/-}$;*Col2α1-Atf4* bone and no overexpression of *Atf4* in the *Col2α1-Atf4* bone compared with WT (Fig. 1C), indicating that *Col2α1-Atf4* is only expressed in cartilage but not in bone. To further confirm the specificity of *Col2α1-Atf4* overexpression in chondrocytes, in situ hybridization was performed in the limb sections of 4 genotype littermates, using probe of *Atf4* and chondrocyte maker gene *Col2α1* as a control. The result showed that *Atf4* is ubiquitously expressed in every cell type in WT limb (Fig. 1Da). No *Atf4* expression in *Atf4*$^{-/-}$ limb (Fig. 1Db). *Atf4* is also expressed in every cell type in *Col2α1-Atf4* limb, and *Atf4* expression level in chondrocytes of *Col2α1-Atf4* is higher than that of WT (Fig. 1Dc), the location of chondrocytes is indicated by *Col2α1* expression (Fig 1De). *Atf4* is highly expressed only in chondrocytes, but not in other cells such as osteoblast and muscle cells in *Atf4*$^{-/-}$;*Col2α1-Atf4* limb (Fig 1Dd), indicating that *Col2α1-Atf4* is specifically over-expressed in the chondrocytes of *Atf4* mutant background. The specific overexpression of *Atf4* in chondrocytes allows us to further study its autonomous function in regulating chondrocyte proliferation and differentiation which drive bone longitudinal growth.

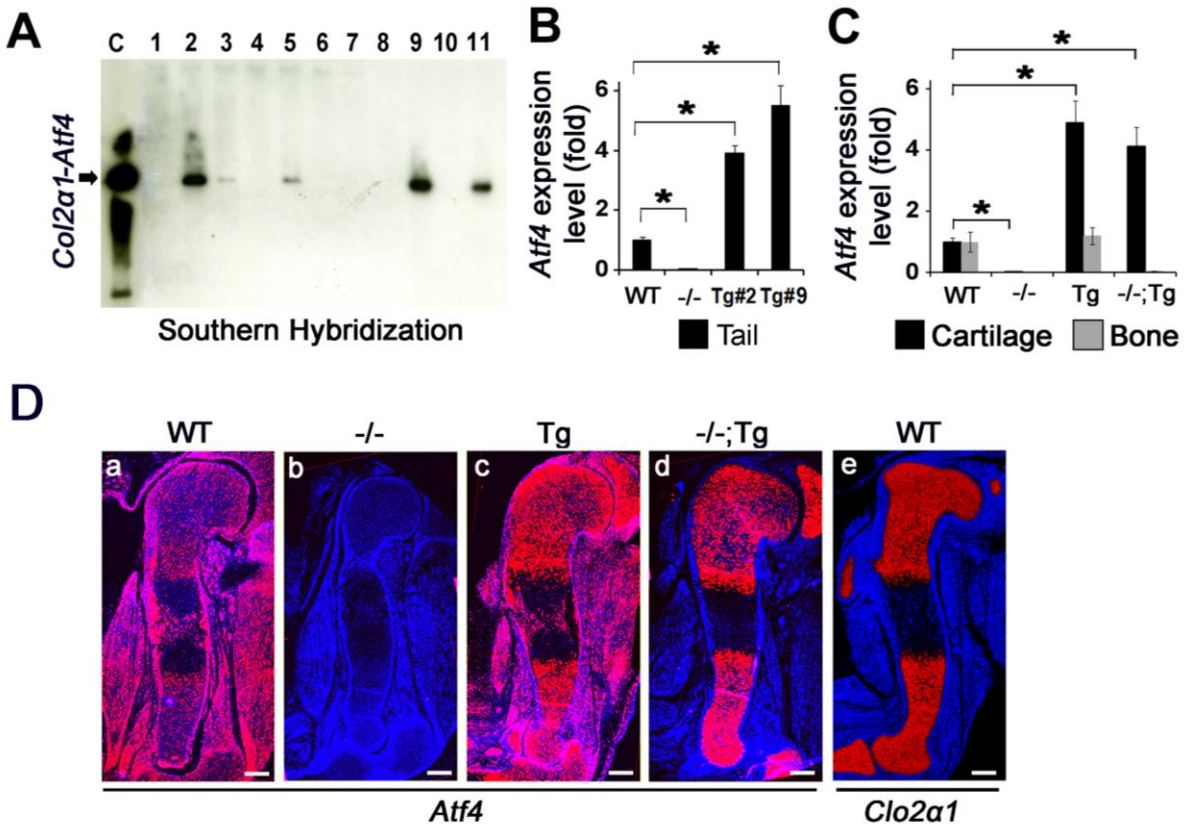

Fig. 1. ***Col2α1Atf4*** **transgenic (Tg) mice.**

(A) Southern hybridization of tail genomic DNA identified 5 founders (2, 3, 5, 9, and 11).

(B) qRT-PCR of tail RNA revealed two transgenic lines (#2 and #9) overexpressing *Atf4*. Data were normalized to endogenous *Atf4* level in WT mice and 18S rRNA (N=3). Error bars, standard error of the mean (SEM). *P<0.05 by paired student's t-test.

(C) qRT-PCR of cartilage and bone RNA demonstrated that *Atf4* is specifically overexpressed in cartilage but not in bone in *Col2α1-Atf4* (Tg) and *Atf4*$^{-/-}$*;Col2α1-Atf4* (-/-;Tg, #9) mice.

(D) 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*$^{-/-}$ (**b**) limb. The *Col2α1-Atf4* expression in *Col2α1-Atf4* (Tg, **c**) *Atf4*$^{-/-}$*;Col2α1-Atf4* (-/-;Tg, **d**) is restricted to *Col2α1*-expressing chondrocytes (**e**) and at a higher level than that in WT limbs. Scale bars: 0.2 mm.

Overexpression of *Atf4* in chondrocytes rescues short stature defect of *Atf4*$^{-/-}$ mice

Atf4$^{-/-}$ mice have smaller skeleton and shorter long bone compared with their WT littermates, which is caused by defects in both cartilage and bone development (Wang et al., 2009) (Yang and Karsenty, 2004). To test whether *Atf4* specific overexpression in chondrocytes affects skeleton development, the littermates of WT, *Atf4*$^{-/-}$, *Col2α1-Atf4*, and *Atf4*$^{-/-}$;*Col2α1-Atf4* were examined by skeleton preparation. The difference of skeletal size between WT and *Atf4*$^{-/-}$ is increased during development from E13, E15, E17 to P0, the difference is small in E13, then becomes obvious in E15, and is very striking in E17 and P0 (Fig. 2A). There is only blue staining in the skeleton in E13, exhibiting that there is no mineralized bone (red staining) in E13, and indicating that the small skeleton of E13 *Atf4*$^{-/-}$ is resulted only from chondrocyte defects. E13 *Col2α1-Atf4* has a similar size of blue skeleton as that of WT, indicating that overexpression of *Atf4* in chondrocytes does not affect the cartilage growth. The size of E13 *Atf4*$^{-/-}$;*Col2α1-Atf4* skeleton is restored to the normal as WT, indicating that *Atf4* overexpression in chondrocytes completely rescues the cartilage defect of *Atf4*$^{-/-}$ mice. Consistent with the observation in E13, there is no difference of the body size between *Col2α1-Atf4* and WT littermates in E15, E17, and P0, demonstrating that *Atf4* overexpression in chondrocytes does not affect the normal cartilage and bone growth in the skeleton. More importantly, the body size of *Atf4*$^{-/-}$;*Col2α1-Atf4* is as big as that of WT, and is obviously bigger than that of *Atf4*$^{-/-}$ mice in E15, E17, and P0 (Fig. 2A), indicating that *Atf4* overexpression in chondrocytes completely rescues the short stature defect of *Atf4*$^{-/-}$ mice.

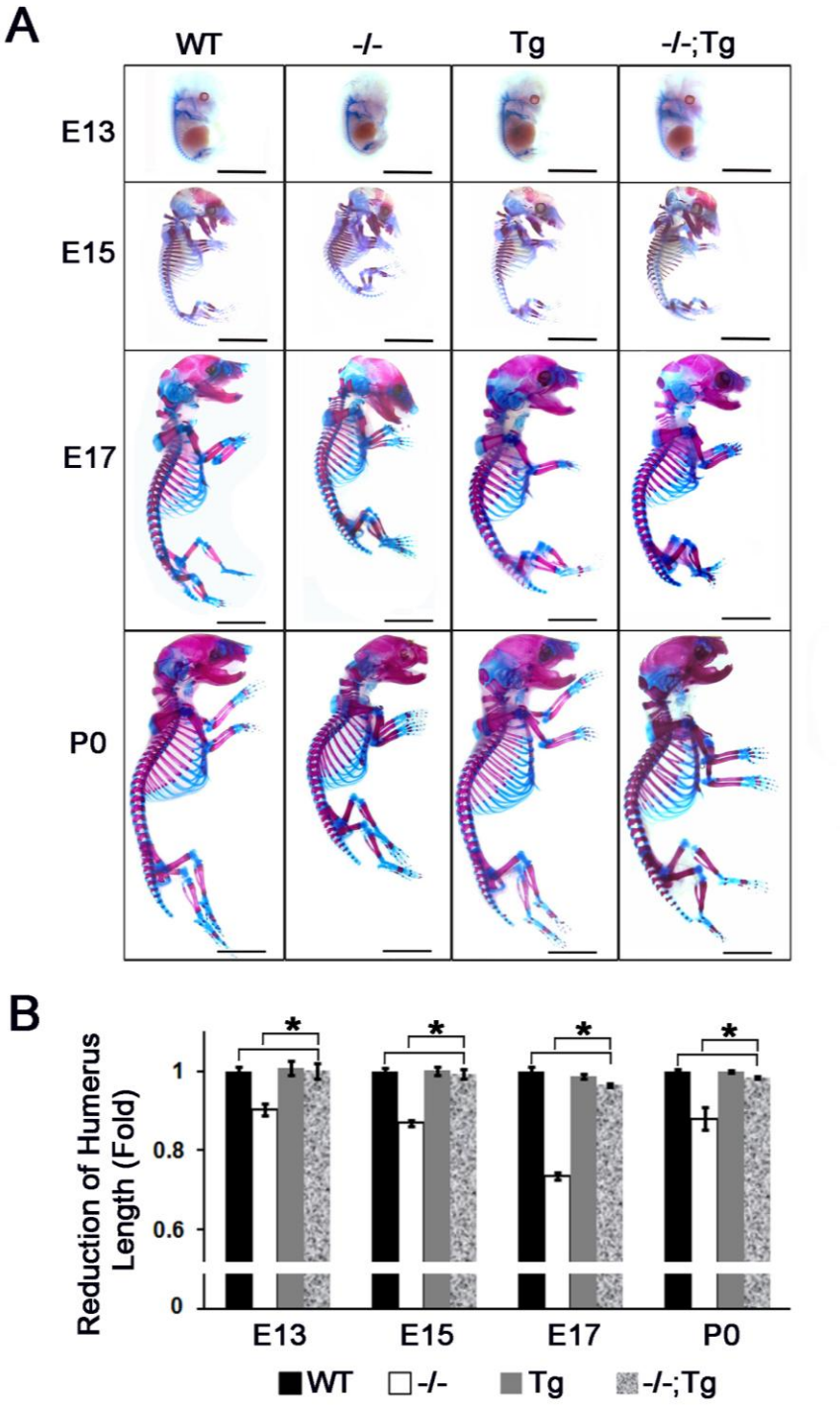

Fig. 2. Overexpression of *Atf4* in chondrocytes rescues shortened statures in *Atf4*$^{-/-}$ mutants. **(A)** Alizarin Red and Alcian Blue staining of skeletons of embryos at E13, E15, E17 and P0. Scale bars: 0.5mm. N=3. **(B)** Quantification of humerus length at indicated developmental stages. Error bars, SEM. N=3. * P<0.05 by paired student's t-test.

Table 3 Penetrance of *Atf4*$^{-/-}$*;Col2α1-Atf4* with normal status (Rescued).

The number of different developmental stage embryos and newborn (P0), 1 month (1M) and 3 months (3M) mice is indicated, together with the number of *Atf4*$^{-/-}$*;Col2α1-Atf4* (-/-;Tg) that show normal status. *Atf4*$^{+/+}$ (+/+), *Atf4*$^{+/-}$ (+/-), *Atf4*$^{-/-}$ (-/-), *Atf4*$^{+/+}$*;Col2α1-Atf4* (+/+;Tg), *Atf4*$^{+/-}$*;Col2α1-Atf4* (+/-;Tg).

	Developmental Stages							
Genotype	**E13**	**E15**	**E17**	**P0**	**1M**	**3M**	**Total (340)**	**%**
+/+	**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**
Rescued -/-;Tg	**3**	**8**	**7**	**10**	**9**	**7**	**44**	**12.9**
Penetrance	**100%**	**100%**	**100%**	**100%**	**100%**	**100%**		

To further examine whether overexpression of *Atf4* in chondrocytes affects longitudinal growth of long bone, we measured the length of humerus of 4 genotypes in E13, E15, E17, and P0. The result showed that the short humeri defect of *Atf4*$^{-/-}$ mice is completely rescued in *Atf4*$^{-/-}$;*Col2α1-Atf4* mice, which have the same humerus length as WT littermates in E13, E15, E17, and P0 (Fig. 2B). This indicates that specific overexpression of *Atf4* in chondrocytes completely rescues longitudinal growth of long bone in *Atf4*$^{-/-}$ mice. The penetrance of rescued *Atf4*$^{-/-}$;*Col2α1-Atf4* mice with a normal body size is 100% in E13, E15, E17, P0, 1 month and 3 months (Table 3), but none of the *Col2α1-Atf4* transgenic mutants were found larger than WT littermates, indicating that overexpression of *Atf4* in chondrocytes completely rescues but does not cause excessive growth of *Atf4*$^{-/-}$ skeletons in both embryonic and postnatal stages. These data demonstrate that Atf4 in chondrocytes is a critical determinant of skeleton growth.

Overexpression of *Atf4* in chondrocytes rescues the defects of *Atf4*$^{-/-}$ proliferative and hypertrophic chondrocytes

Atf4$^{-/-}$ mice have a shorter and disorganized proliferative chondrocyte zone, and an expanded hypertrophic chondrocyte zone in the growth plate (Wang et al., 2009). To test whether overexpression of *Atf4* in chondrocytes will affect the development and organization of chondrocyte zones, we performed histological analysis in the growth plate of newborn WT, *Atf4*$^{-/-}$, *Col2α1-Atf4*, and *Atf4*$^{-/-}$;*Col2α1-Atf4* littermates. H&E staining showed that the proliferative zones (PZ) of *Atf4*$^{-/-}$ mice are shorter than those of WT mice, but there is no distinguished difference of the PZ length among WT, *Col2α1-Atf4*, and *Atf4*$^{-/-}$;*Col2α1-Atf4* (Fig. 3 A, B). The cell columnar structures with proliferative

cell stacking together linearly in vertical direction in WT growth plates are severely disrupted in the *Atf4*$^{-/-}$ growth plates, but these columnar structures in *Col2α1-Atf4* and *Atf4*$^{-/-}$;*Col2α1-Atf4* mice are as normal as in WT mice (Fig. 3 A). In addition, the hypertrophic chondrocyte zones (HZ) of *Atf4*$^{-/-}$ mice are longer than those of WT mice, whereas this abnormality of HZ did not appear in *Col2α1-Atf4* and is completely rescued in *Atf4*$^{-/-}$;*Col2α1-Atf4* (Fig. 3 A, C). These results indicated that overexpression of *Atf4* in chondrocytes does not affect normal chondrocyte differentiation**,** and chondrocyte *Atf4* completely rescues the differentiation defects in *Atf4*$^{-/-}$ growth plate chondrocytes.

The short proliferative zone is correlative with the decrease of cell proliferation in *Atf4*$^{-/-}$ mice (Wang et al., 2009). To test whether overexpression of *Atf4* in chondrocytes will affect chondrocyte proliferation, in vivo proliferation assay with BrdU incorporation in replicating DNA was performed. Fig. 3D-F shows that the decrease of proliferation index of *Atf4*$^{-/-}$ mice is rescued to normal in *Atf4*$^{-/-}$;*Col2α1-Atf4* littermates, with no significant difference between *Col2α1-Atf4* and WT littermates (Fig. 3 D, E, F). These results demonstrated that overexpression of *Atf4* in chondrocytes does not affect normal chondrocyte proliferation**,** and chondrocyte *Atf4* rescues the proliferation defect in *Atf4*$^{-/-}$ growth plate chondrocytes.

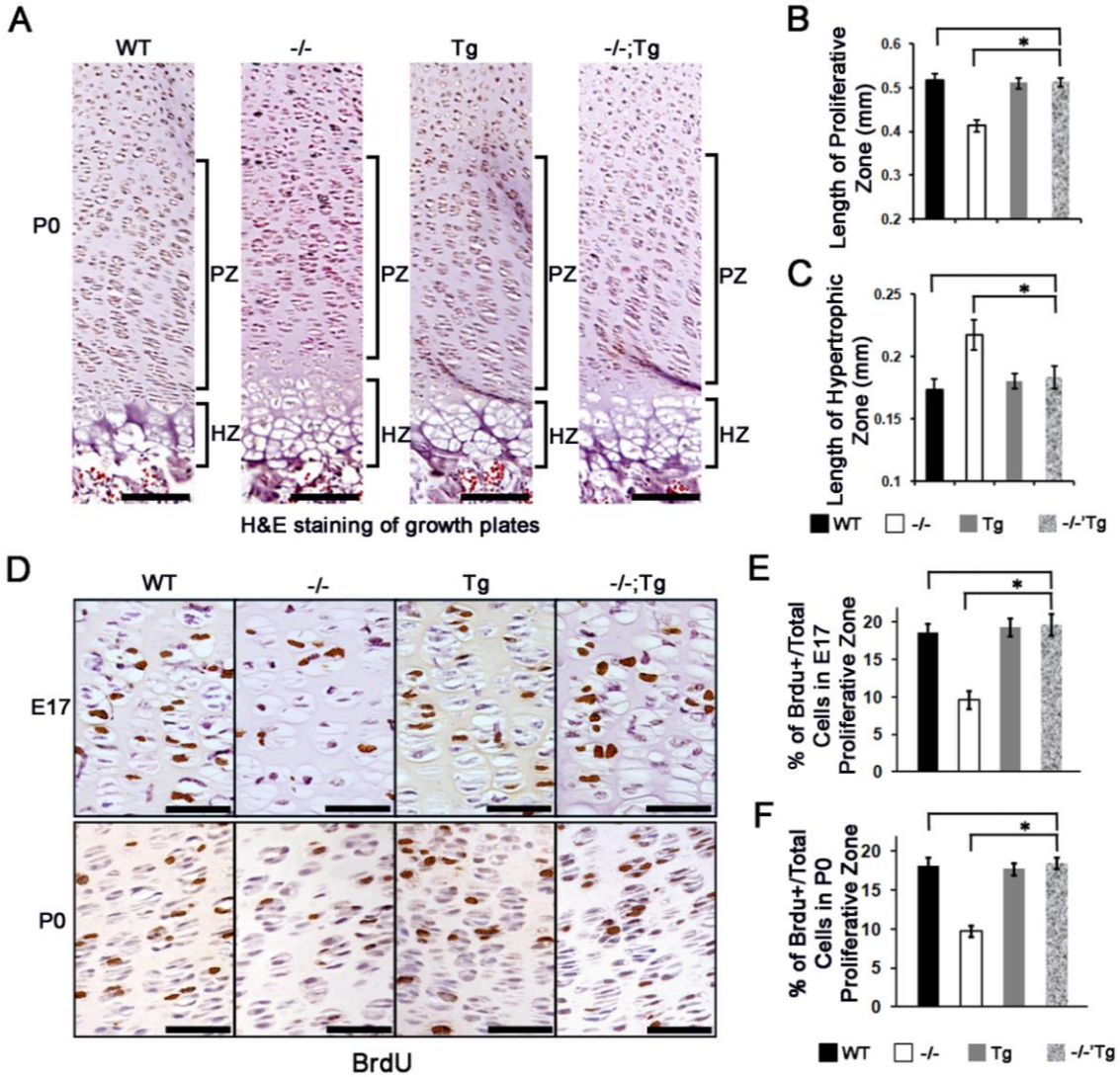
A
WT
-/-
Tg
-/-;Tg
P0
PZ
HZ
H&E staining of growth plates
B
Length of Proliferative Zone (mm)
0.6
0.5
0.4
0.3
0.2
*
C
Length of Hypertrophic Zone (mm)
0.25
0.2
0.15
0.1
*
WT
-/-
Tg
-/-;Tg
D
E17
P0
BrdU
E
% of Brdu+/Total Cells in E17 Proliferative Zone
20
15
10
5
0
*
F
% of Brdu+/Total Cells in P0 Proliferative Zone
20
15
10
5
0
*
WT
-/-
Tg
-/-;Tg

Fig. 3. Overexpression of *Atf4* in chondrocytes restores growth plate chondrdocyte defects in *Atf4*$^{-/-}$ mice.

(**A**) H&E staining of humerus sections. Proliferative chondrocyte zones (PZ) and hypertrophic chondrocyte zones (HZ) are indicated. Scale bars: 0.1mm.

(**B, C**) Quantification of the length of the PZ and HZ in growth plates. Error bars, SEM. N=3. * $P<0.05$ by paired student's t-test.

(**D**) BrdU immunohistochemistry of humerus sections showing BrdU-positive (brown) proliferating chondrocytes in growth plates. Scale bars: 0.05mm.

(**E, F**) Quantification of proliferation rate in proliferating chondrocytes. Error bars, SEM. N=3. * $P<0.05$ by paired student's t-test.

Overexpression of *Atf4* in chondrocytes restores Hh signaling in *Atf4*$^{-/-}$ chondrocytes and osteoblasts

Ihh is essential for chondrocytes proliferation and differentiation. *Atf4*$^{-/-}$ mice have decreased *Ihh* expression in prehypertrophic chondrocytes and decreased of Hh signal in chondrocytes of growth plates (Wang et al., 2009). To test whether *Col2α-Atf4* affects *Ihh* expression in chondrocytes, we performed in situ hybridization and found that *Ihh* is specifically expressed in prehypertrophic chondrocytes of all 4 genotypes (Fig. 4Aa-d). The expression of *Ihh* is dramatically decreased in *Atf4*$^{-/-}$ chondrocytes compared with WT chondrocytes (Fig. 4Aa,b). There is no significant difference of *Ihh* expression level between *Col2α1-Atf4* and WT chondrocytes (Fig. 4Ac). *Ihh* expression level is restored to normal in *Atf4*$^{-/-}$;*Col2α1-Atf4* mice as in WT littermates, and is much higher than that in *Atf4*$^{-/-}$ mice (Fig. 4Ad). These results exhibit that overexpression of *Atf4* in chondrocytes can rescue *Ihh* expression level in *Atf4*$^{-/-}$ background mice, but does not change *Ihh* expression level in *Col2α1-Atf4* mice with a WT background.

To test whether Hh signaling is also rescued in *Atf4*$^{-/-}$;*Col2α1-Atf4* mice, we examined Hh signaling downstream target *Patched1* (*Ptch*) and *Gli1* expression. *Ptch* is strongly expressed in E15 WT prehypertrophic chondrocytes and perichondrium, and weakly presents in osteoblasts (Fig. 4Ae). The expression level of *Ptch* is dramatically decreased in *Atf4*$^{-/-}$ prehypertrophic chondrocytes, perichondrium and osteoblasts (Fig. 4Af). Its level in *Col2α1-Atf4* prehypertrophic chondrocytes, perichondrium and osteoblasts is much higher than that in *Atf4*$^{-/-}$, but its signal intensity in *Col2α1-Atf4* is similar to WT in perichondrium and prehypertrophic chondrocytes and osteoblasts (Fig.

4Ag). This indicates that *Atf4* overexpression does not affect normal *Ptch* expression in the WT background. *Ptch* expression level in *Atf4*$^{-/-}$;*Col2α1-Atf4* is very similar to those in *Col2α1-Atf4* and WT, and is much higher than that in *Atf4*$^{-/-}$ (Fig. 4Ah), indicating that overexpression of *Atf4* in chondrocytes rescues *Ptch* expression in *Atf4*$^{-/-}$ mice. *Gli1* expression level is weaker than *Ptch*, and is mainly detected in perichondrium, periosteum, prehypertrophic chondrocytes, hypertrophic chondrocytes and osteoblasts in E15 WT (Fig. 4Ai), and *Gli1* expression is decreased in all these areas of *Atf4*$^{-/-}$ bone comparing with WT (Fig. 4Aj). *Gli1* expression level in *Col2α1-Atf4* mice is similar to WT (Fig. 4Ak). Its expression level in *Atf4*$^{-/-}$;*Col2α1-Atf4* mice is restored to normal as in WT (Fig. 4Al). These results indicate that overexpression of *Atf4* in chondrocytes rescues Hh signaling in *Atf4*$^{-/-}$ background mice, but does not affect normal Hh signaling in WT background mice.

To test whether overexpression of *Atf4* in chondrocytes specifically regulate *Ihh, Patch* and *Gli1* expression, we examined the expression of other important chondrocyte maker genes, including *Col2α1* in non-hypertrophic chondrocytes, *Col10α1* in hypertrophic chondrocytes, and *PPR* in prehypertrophic chondrocytes. The result turns out that the expression of *Col2α1, Col10α1* and *PPR* has not significant difference among these 4 genotypes (Fig. 4Am-x). These results confirm that Atf4 specifically regulating *Ihh* expression in prehypertrophic chondrocytes.

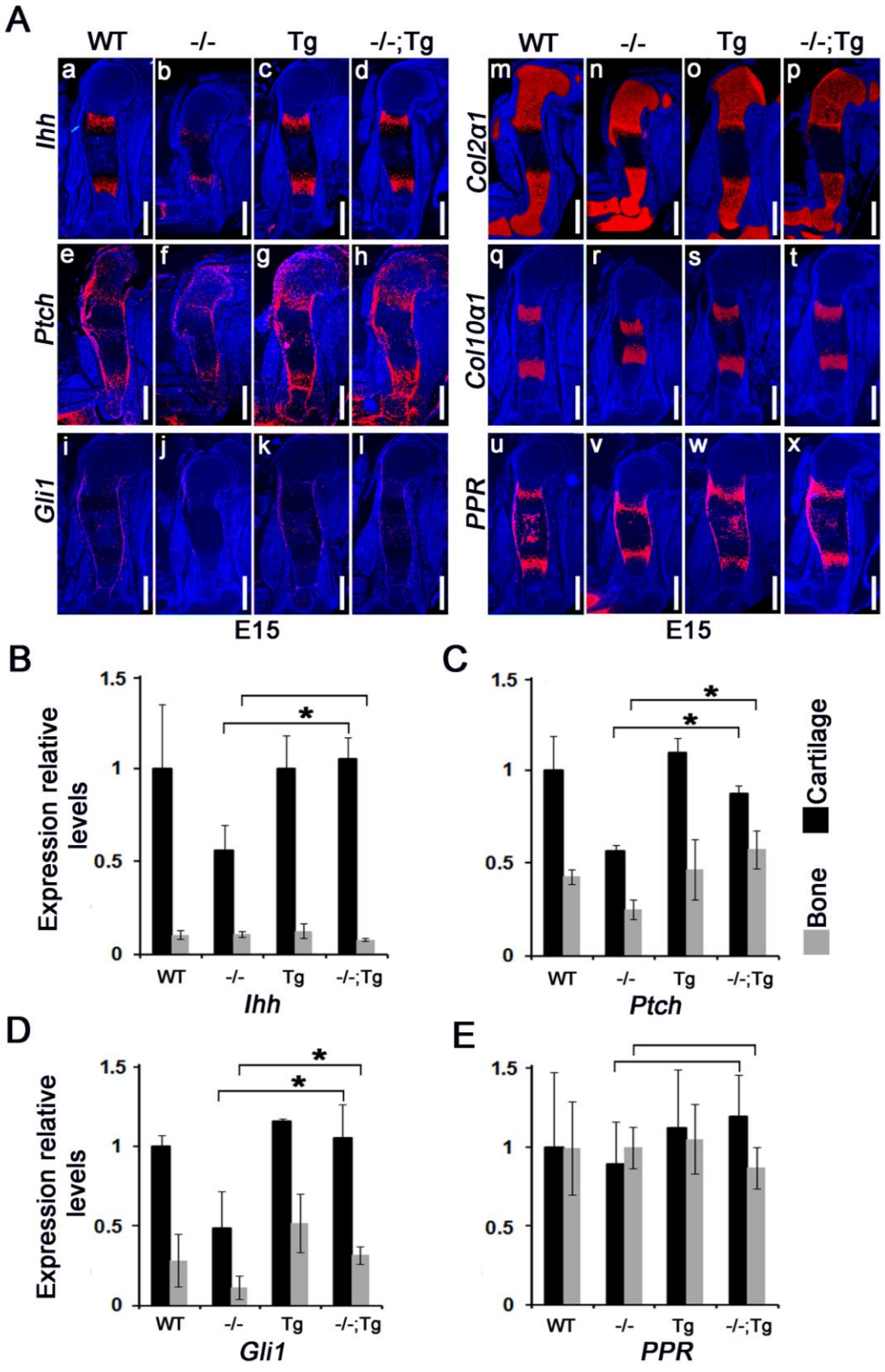
A
WT
-/-
Tg
-/-;Tg
Ihh
Ptch
Gli1
Col2a1
Col10a1
PPR
E15
B
C
D
E
Expression relative levels
1.5
1
0.5
0
*
Ihh
Ptch
Gli1
PPR
Cartilage
Bone

Fig. 4. Overexpression of *Atf4* in chondrocytes corrects the reduced expression of *Ihh* and its target genes in *Atf4*$^{-/-}$ mice.

(A) In situ hybridization of E15 humerus sections. Note that the decrease in *Ihh, Ptch* and *Gli1* expression in *Atf4*$^{-/-}$ humeri was completely recued in *Atf4*$^{-/-}$*;Col2α1-Atf4* humeri, whereas the expression levesl of *Col2α1***,** *Col10α1* and *PPR* were not affected. Scale bars: 0.5 mm.

(B-E) qRT-PCR analysis showing expression level of *Ihh, Ptch, Gli* and *PPR* in P0 cartilages and bones. Error bars, SEM. N=3. * $P<0.05$ by paired student's t-test.

Consistent with in situ results, qRT-PCR showed that the *Ihh* expression in cartilage is decreased in *Atf4*$^{-/-}$ and is rescued to normal in *Atf4*$^{-/-}$;*Col2α1-Atf4* (Fig. 4B). Although there is no detection of *Ihh* expression in bone area by in situ hybridization, qRT-PCR detected low level *Ihh* expression in bone. *Ihh* expression level in bone is similar among 4 genotypes and is about 10% of that in WT cartilage, indicating that *Ihh* expression in bone is not regulated by Atf4. The *Ptch* and *Gli1* expression levels have no significant difference in cartilage among WT, *Col2α1-Atf4*, and *Atf4*$^{-/-}$;*Col2α1-Atf4*, and are all higher than that in *Atf4*$^{-/-}$ (Fig. 4C, D), which is consistent with the results of in situ hybridization, confirming that overexpression of *Atf4* in chondrocyte rescues Hh signaling in *Atf4*$^{-/-}$;*Col2α1-Atf4* cartilage, but not affects Hh signaling in *Col2α1-Atf4* cartilage. Interestingly, even though *Atf4*$^{-/-}$ bone shows the same basic level of *Ihh* expression as those in WT, *Col2α1-Atf4* and *Atf4*$^{-/-}$;*Col2α1-Atf4* bone, the *Ptch* and *Gli1* expression levels are decreased in *Atf4*$^{-/-}$ bone, and these defects are also rescued in *Atf4*$^{-/-}$;*Col2α1-Atf4* bone. These results indicate that Hh signaling in bone is dominated by *Atf4* and *Ihh* expression in cartilage but not in bone. Finally, the expression level of *PPR* in cartilage and bone of all 4 genotypes was similar, indicating that Atf4 in chondrocytes specifically regulates *Ihh*, *Ptch* and *Gli1* expression but not *PPR* (Fig. 4D). These results demonstrate that overexpression of *Atf4* in chondrocytes rescues *Ihh* expression specifically in prehypertrophic chondrocytes, restores Hh signaling in both cartilage and bone of *Atf4*$^{-/-}$;*Col2α1-Atf4* mice, and does not affect the expression of other chondrocyte maker genes. Therefore, we conclude that Atf4 has a chondrocyte-autonomous function in growth plate development.

Overexpression of *Atf4* in chondrocytes rescues the osteoblast differentiation and bone formation in *Atf4*$^{-/-}$ mice

Osteoblast differentiation and endochondral bone formation start in the primary ossification center which is triggered by chondrocyte hypertrophy. The fully rescue of bone and skeleton size of *Atf4*$^{-/-}$ mice in both embryonic and postnatal stages by *Col2α-Atf4* prompts us to further examine whether *Col2α-Atf4* affects osteoblast differentiation and bone formation. *Osteocalcin* (*Ocn*) and *Bone sialoprotein* (*Bsp*), specifically expressed in mature osteoblasts, are indicators for the presence of mature osteoblasts. In situ hybridization in E15 humerus showed that *Ocn* and *Bsp* expression are strong in cortical area and weak in trabecular area in WT humerus, indicating that there are more mature osteoblasts in the periosteum than in the trabecular area in E15 humerus (Fig. 5 Aa,e); *Ocn* expression is almost absent in *Atf4*$^{-/-}$ humerus, while *Bsp* expression is weakly present, indicative of less mature osteoblasts in *Atf4*$^{-/-}$ humerus (Fig. 5 Ab,f); *Ocn* and *Bsp* expression levels and patterns in *Col2α1-Atf4* humerus are similar to those in WT humerus (Fig. 5 Ac,g), with higher expression in cortical area and less expression in the trabecular area, and their expression levels are much higher than *Atf4*$^{-/-}$ humerus, representing that *Atf4* overexpression in chondrocytes does not affect osteoblast terminal differentiation in the WT background; unexpectedly, *Ocn* and *Bsp* expression levels and patterns in *Atf4*$^{-/-}$;*Col2α1-Atf4* humerus are also comparable with those of WT humerus (Fig. 5 Ad,h), and their expression levels are much higher than those of *Atf4*$^{-/-}$ humerus, demonstrating that overexpression of *Atf4* in chondrocytes rescues osteoblast terminal differentiation in *Atf4*$^{-/-}$ humerus. In addiction, Von Kossa staining showed that the mineral bone formation in the primary ossification center of *Atf4*$^{-/-}$;*Col2α1-Atf4* humerus

is rescued to normal as in WT humerus (Fig. 5 Ai,l), supporting that *Atf4* overexpression in chondrocytes indirectly rescues the bone formation defect of *Atf4*$^{-/-}$ E15 humerus.

To test whether postnatal bone formation of *Atf4*$^{-/-}$;*Col2α1-Atf4* mice is also affected by *Col2α-Atf4*, 1 month and 3 month bone mass of distal femurs were measured by microCT analysis (Fig. 5B), showing that the bone formation defects in *Atf4*$^{-/-}$ mice (Fig. 5Bb,f) are completely rescued by *Atf4* overexpression in chondrocytes in *Atf4*$^{-/-}$;*Col2α1-Atf4* mice (Fig. 5Bd,h). The quantification data of 1 month and 3 month bone mass, represented by bone volume/ total tissue volume (BV/TV), showed that the BV/TV of *Atf4*$^{-/-}$ mice is dramatically lower than that of WT mice, the BV/TV of *Col2α1-Atf4* mice is similar to that of WT mice, the BV/TV of *Atf4*$^{-/-}$;*Col2α1-Atf4* mice has no significant difference with that of WT mice, and the BV/TV of *Atf4*$^{-/-}$;*Col2α1-Atf4* mice is significantly higher than that of *Atf4*$^{-/-}$ mice (Fig. 5C, D). Histomorphometric analysis of 3 month old vertebrates confirms that the decreased BV/TV, trabeculae thickness (Tb.Th) and trabeculae number (Tb.N) in *Atf4*$^{-/-}$ mice are restored to normal level by overexpression *Atf4* in chondrocytes (Fig. E). *Atf4*$^{-/-}$ mice show a decreased bone formation rate over bone surface (BFR/BS), but a normal osteoblast number/bone surface (#Ob/BS) and a normal osteoblast surface/bone surface compared with WT (Table. 2). This indicates that the density of Atf4$^{-/-}$ osteoblast in bone surface is normal, but they have low activity for bone formation, which may be a result of lacking terminal differentiation. Overexpression Atf4 in chondrocytes also rescues the osteoblast bone formation rate (BFR/BS) in Atf4$^{-/-}$ mice, suggesting that chondrocyte-derived Atf4 indirectly rescues Atf4$^{-/-}$ osteoblast differentiation and function. Lastly, the number of

osteoclast (Oc #/BS) and osteoclast covered bone surface (Oc. S/BS) over bone surface were found non-significantly different among WT, $Atf4^{-/-}$ Col2α1-Atf4, and $Atf4^{-/-}$;Col2α1-Atf4 mice (Table 4), indicating that Atf4 overexpression in chondrocytes promotes osteoblast differentiation and bone formation but does not affect osteoclastogenesis.

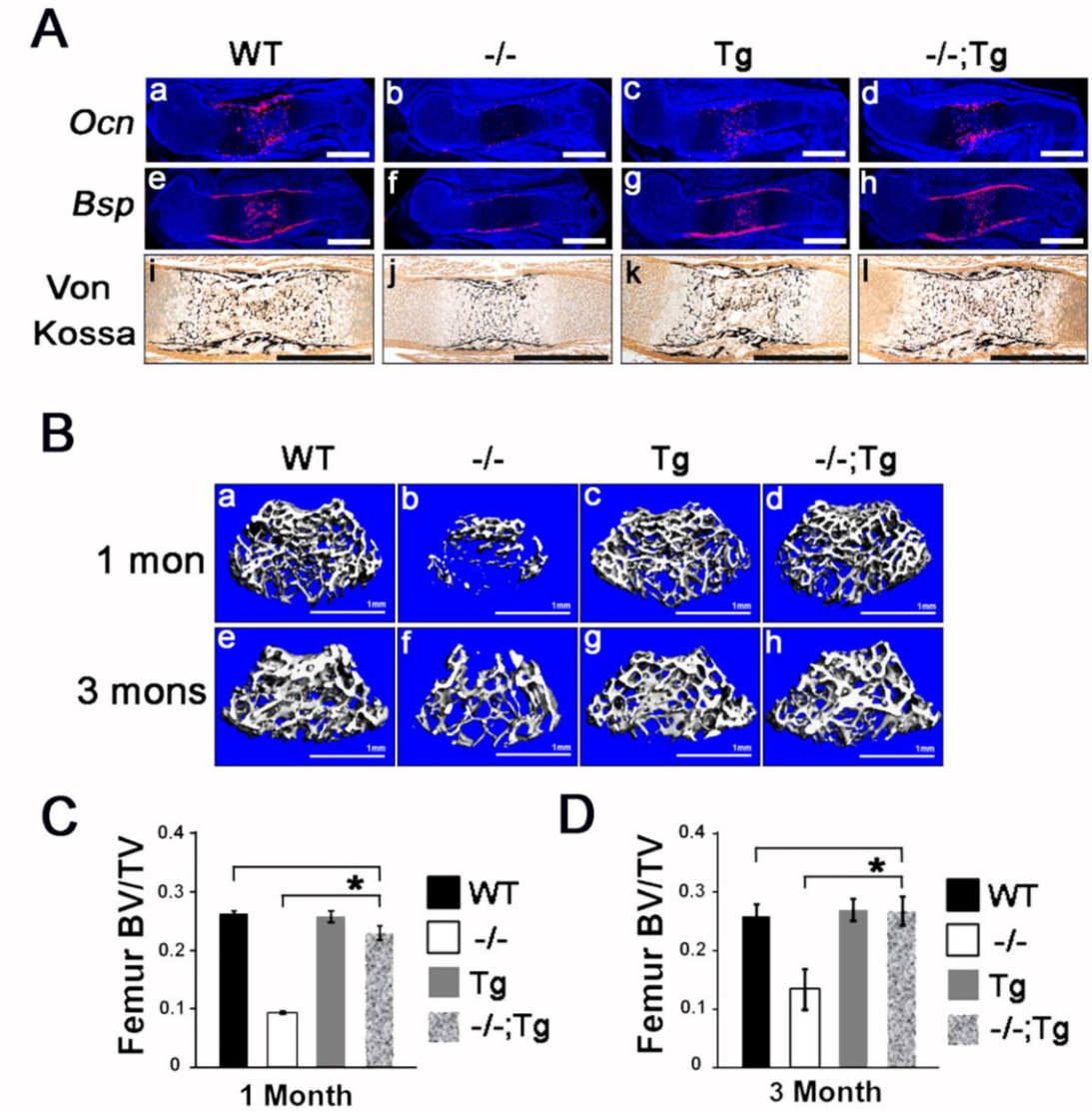

Figure 5. Overexpression of *Atf4* in chondrocytes restores osteoblast differentiation and bone formation in *Atf4*$^{-/-}$ mice.

(**A**) In situ hybridization of sections through E15 humeri showing that the decreased level of *Ocn* and *Bsp* in *Atf4*$^{-/-}$ bones was restored to normal level in *Atf4*$^{-/-}$*;Col2α1-Atf4* bones. von Kossa staining of sections through E15 humeri showing rescued mineralization in *Atf4*$^{-/-}$*;Col2α1-Atf4* primary ossification center. Scale bars: 0.5 mm. (**B**) Microtomographic image showing rescued bone formation in 1 month and 3 months *Atf4*$^{-/-}$*;Col2α1-Atf4* femur heads. (**C, D**) Quantification of 1 month and 3 months femur Bone volume/Tissue volume (BV/TV) measured by μCT. Error bars, SEM. N=6. * P<0.05 by paired student's t-test.

Table 4 Quantification of bone histomorphometric parameters.

Bone volume/Tissue volume (BV/TV), Trabecular thickness (Tb.Th), Trabacular number (Tb.N), bone formation rate/bone surface (BFR/BS), osteoblast surface/bone surface (Ob.S/BS), and osteoblast number/bone surface (#Ob/BS), osteoclast surface/bone surface (Oc.S/BS), and osteoclast number/bone surface (#Oc/BS) in 3 month vertebrates. *Atf4*$^{+/+}$ (WT), *Atf4*$^{-/-}$ (-/-), *Col2α1-Atf4* (Tg), *Atf4*$^{-/-}$*;Col2α1-Atf4* (-/-;Tg).

Parameter	WT	-/-	Tg	-/-;Tg
BV/TV	**10.34±1.41**	**5.43±0.71 ***	**10.58±1.51**	**9.10±0.60**
Tb.Th	**22.88±1.78**	**19.05±1.43 ***	**22.04±1.99**	**21.28±2.14**
Tb.N	**4.48±0.48**	**2.87±0.33 ***	**4.72±0.39**	**4.34±0.20**
BFR/BS	**0.72±0.06**	**0.43±0.07 ***	**0.65±0.13**	**0.64±0.09**
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**

Hh signaling in *Atf4*$^{-/-}$ osteoblasts is rescued by overexpressing *Atf4* in chondrocytes

To understand the mechanism by which chondrocyte-derived *Atf4* rescues osteoblast differentiation and bone formation of *Atf4*$^{-/-}$ mice, we hypothesized that one (or more) secreted factor from the cartilage of *Col2α1-Atf4* mice may diffuse into the bone area and stimulate osteoblast differentiation. To test this hypothesis, bone marrow stromal cells (BMCs) from 2 month old WT, *Atf4*$^{-/-}$, *Atf4*$^{-/-}$;*Col2α1-Atf4* mice were isolated, cultured and stimulated by cartilage conditional media (CM) of P0 WT, *Atf4*$^{-/-}$, *Atf4*$^{-/-}$;*Col2α1-Atf4* mice. Colony forming unit positive for alkaline phosphatase activity (CFU-ap) assay was performed to test early osteoblast differentiation, represented by the colony number of alkaline phosphatase-positive osteoblast progenitors (Blue). Colony forming unit-osteoblast assay (CFU-Ob) assay was performed to test terminal osteoblast differentiation, represented by the colony number of mineralized nodules (Black). The *Atf4*$^{-/-}$ BMCs with *Atf4*$^{-/-}$ CM have about 50% less of CFU-F and CFU-Ob colony number comparing with the WT BMCs with WT CM, WT CM and *Atf4*$^{-/-}$;*Col2α1-Atf4* CM completely restore the number of CFU-F and CFU-Ob colonies of *Atf4*$^{-/-}$ BMCs (Fig. 6 A), indicating that soluble factor(s) from the WT and *Atf4*$^{-/-}$;*Col2α1-Atf4* cartilage stimulates osteoblast differentiation. Since Hh signaling, which is required for endochondral bone formation, is rescued in *Atf4*$^{-/-}$;*Col2α1-Atf4* bone by *Col2α-Atf4*, we contemplated that activation of Hh signaling in BMCs by these secreted factors from cartilage may be required for osteoblast differentiation. To test this hypothesis, we modulated Hh signaling in BMCs culture by either blocking Hh signaling using Cyclophamine (Cyc) or enhancing Hh signaling using Purmophamine (Pur). Cyc blocked the rescuing activity of *Atf4*$^{-/-}$;*Col2α1-Atf4* CM, presenting by dramatically decreasing

CFU-F and CFU-Ob colony number of *Atf4*$^{-/-}$ BMCs (Fig. 6 B); Pur is sufficient to rescue the defect of *Atf4*$^{-/-}$ CM, showing by completely restoring CFU-F and CFU-Ob colony number of *Atf4*$^{-/-}$ BMCs (Fig. 6 B). Ihh is the only member of the Hh protein family expressed in chondrocyte.

To test whether Ihh is one of these secreted factors from *Atf4*$^{-/-}$;*Col2α1-Atf4* cartilage to rescue *Atf4*$^{-/-}$ osteoblast differentiation. Hh protein antibody 5E1 was used to block Ihh from *Atf4*$^{-/-}$;*Col2α1-Atf4* CM in *Atf4*$^{-/-}$ BMC cultures, showing that blocking Hh protein from cartilage inhibits the rescuing activity of *Atf4*$^{-/-}$;*Col2α1-Atf4* CM in *Atf4*$^{-/-}$ BMCs, the inhibition result of 5E1 is similar to that of Cyc (Fig. 6 C). These results demonstrate that chondrocyte-derived Atf4 directly regulates Ihh production in growth plate cartilage and indirectly regulates osteoblast differentiation in bone.

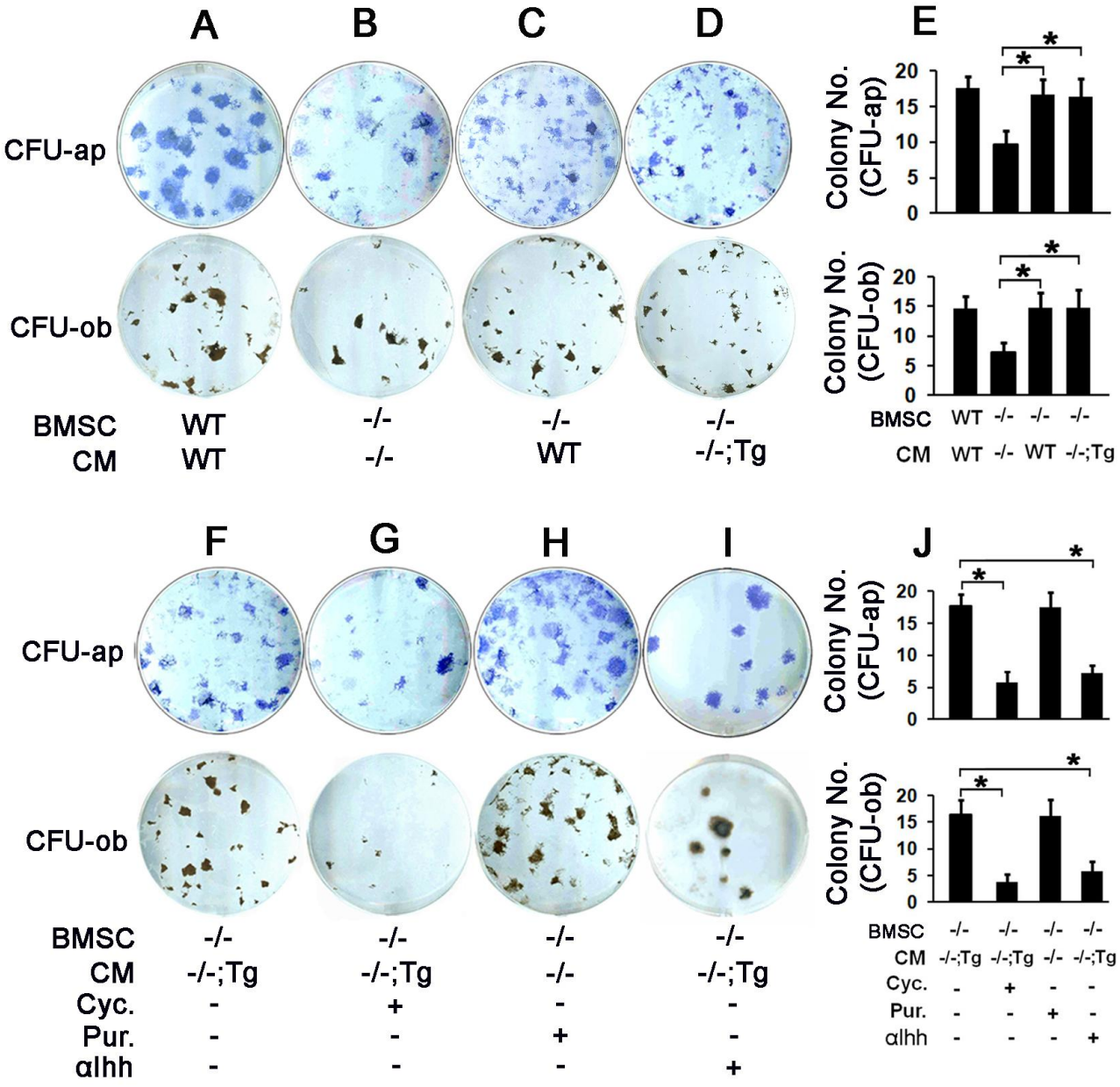
A
B
C
D
E
CFU-ap
CFU-ob
BMSC WT -/- -/- -/-
CM WT -/- WT -/-;Tg
Colony No. (CFU-ap)
Colony No. (CFU-ob)
0 5 10 15 20
BMSC WT -/- -/- -/-
CM WT -/- WT -/-;Tg
F
G
H
I
J
CFU-ap
CFU-ob
BMSC -/- -/- -/- -/-
CM -/-;Tg -/-;Tg -/- -/-;Tg
Cyc. - + - -
Pur. - - + -
αIhh - - - +
Colony No. (CFU-ap)
Colony No. (CFU-ob)
0 5 10 15 20
BMSC -/- -/- -/- -/-
CM -/-;Tg -/-;Tg -/- -/-;Tg
Cyc. - + - -
Pur. - - + -
αIhh - - - +

Fig. 6. *Col2α1-Atf4* cartilage conditioned media rescues osteoblast differentiation.

(**A**-**E**) Osteoblast differentiation assays showing that CM of WT and *Atf4*$^{-/-}$*;Col2α1-Atf4* cartilage increased the number of CFU-ap and CFU-ob in *Atf4*$^{-/-}$ BMSC cultures. Quantification of the number of CFU-ap and CFU-ob in each BMSC culture plate. Error bars, SEM. N=3. * P<0.05 by paired student's t-test.

(**F**-**J**) Osteoblast differentiation assays showing that cyclopamine (Cyc.) and 5E1 decreased the number of CFU-ap and CFU-ob formed by *Atf4*$^{-/-}$*;Col2α1-Atf4* BMSC cultures yet purmophamine (Pur.) increased the number of CFU-ap and CFU-ob formed by *Atf4*$^{-/-}$ BMSCs. Quantification data showing the colony number formed by BMSCs. Error bars, SEM. N=3. * P<0.05 by paired student's t-test.

Discussion

Chondrocyte-autonomous function of Atf4 regulating chondrocyte proliferation and differentiation

Atf4 mutant mice have a smaller skeleton and shorter limbs (Yang and Karsenty, 2004). Subsequent studies revealed that *Atf4* is expressed in chondrocytes, and controls chondrocyte proliferation and differentiation during skeletal development by transcriptionally activating *Ihh* (Wang et al., 2009). These indicate that *Atf4* in chondrocytes is necessary for chondrocyte proliferation and differentiation. However, it is not clear that whether autonomous function of *Atf4* in chondrocytes is enough to rescue the dwarfism in $Atf4^{-/-}$ mice. In this study, by generating a $Atf4^{-/-}$;*Col2α1-Atf4* mouse model, we can first ever to evaluate the sufficiency of chondrocyte-autonomous function of *Atf4* during skeleton development. The bone elongation defects caused by dysfunctional growth plate chondrocytes in $Atf4^{-/-}$ cartilage are completely rescued by this genetic manipulation. These defects include shortened long bones and stature, disorganization of proliferating chondrocyte columns, shortening of the proliferating zone, decreased in BrdU-positive chondrocyte number, expansion of the hypertrophic chondrocyte zone, decreased *Ihh* expression and Hh signaling (Figs. 2- 4). The complete correction of chondrogenesis in $Atf4^{-/-}$ mutants by the *Col2α1-Atf4* transgene thus demonstrates that Atf4 regulates chondrocyte proliferation and differentiation in vivo in a chondrocyte-autonomous manner.

Chondrocyte-derived Atf4 regulates osteoblast differentiation and function via Ihh

A novel finding in this study is that chondrocyte-derived Atf4 regulate osteogenesis in both developmental and postnatal stages. Despite complete absence of *Atf4* expression in osteoblasts or any other types of cells, specific overexpression of *Atf4* in chondrocytes restored the dramatically low bone mass, decreased bone formation rate, trabecular bone thickness, and trabecular numbers in *Atf4*$^{-/-}$ mice to normal in *Atf4*$^{-/-}$;*Col2α1-Atf4* mutants. Based on the observation that chondrocyte-specific overexpression of *Atf4* in *Atf4*$^{-/-}$ mice restores not only *Ihh* expression in chondrocytes but also Hh signaling in osteoblasts, and that the differentiation *Atf4*$^{-/-}$ osteoblasts induced by the conditioned medium of *Atf4*$^{-/-}$*;Col2α1-Atf4* cartilage can be blocked by a neutralizing Hh antibody, we propose that Atf4 in cartilage regulates the production of Ihh, which then diffuses into the bone marrow cavity to regulate osteoblast differentiation and function. In addition to our data, this hypothesis is also supported by the recent finding that deletion of *Ihh* specifically in *Col2α1*-expressing chondrocytes impairs osteoblast differentiation leading to the loss of trabecular bone over time (Maeda et al., 2007).

Chondrocyte-derived Atf4 regulates osteoblast differentiation and bone formation independent of osteoblast-derived Atf4

Atf4 is first discovered as an osteoblast-enriched transcription factor. Atf4 directly binds and activates the promoter of *Osteocalcin* that is the most osteoblast specific gene (Yang et al., 2004), depletion of *Atf4* in mice result in hindering osteoblast terminal differentiation and bone formation. Surprisingly, even thought *Atf4* is absent in osteoblasts in *Atf4*$^{-/-}$*;col2α1-Atf4* mice, overexpression of *Atf4* in chondrocytes rescues

Osteocalcin expression in *Atf4* mutant osteoblast, and restores osteoblast differentiation and bone formation to normal in *Atf4*$^{-/-}$*;col2α1-Atf4* mice. This implicates that there is an Atf4 independent signaling path way and transcription factor that regulate *Osteocalcin* expression in the absent of *Atf4*. In fact, there are two osteoblast-specific cis-acting elements in *Osteocalcin* promoter, termed OSE1 and OSE2. Atf4 binds to OSE1. Another transcription factor Runx2 binds to OSE2 (Ducy et al., 1997). *Runx2* is a master gene of osteoblast differentiation. *Runx2*-deficient mice have no endochondral and intramembranous bone formation without differentiated osteoblast (Komori et al., 1997; Otto et al., 1997). In vitro study showed that Ihh/Gli2 Signaling stimulates osteoblast differentiation by regulating Runx2 protein expression and function (Shimoyama et al., 2007). These findings suggest that overexpression of *Atf4* in chondrocytes indirectly regulates *Osteocalcin* expression and osteoblast differentiation via an Ihh-Runx2 path way. Further study by Western Blot analysis will help to examine whether the Runx2 protein level is increased in the osteoblasts of *Atf4*$^{-/-}$*;col2α1-Atf4* mice compared with *Atf4*$^{-/-}$ mice in the future. The fact that overexpression of *Atf4* in chondrocytes completely rescued osteoblast differentiation and bone formation in *Atf4*$^{-/-}$*;col2α1-Atf4* mice, indicates that chondrocyte-derived *Atf4* plays a dominant role in regulating osteoblast differentiation and bone formation in vivo.

In short, this study identifies a chondrocyte-autonomous function of Atf4 that directly regulates chondrocyte proliferation and differentiation, and indirectly regulates osteoblast differentiation via Ihh during endochondral ossification.

CHAPTER IV

GENERAL DISCUSSION AND FUTURE DIRECTION

Part I: General Discussion

Atf4$^{-/-}$ mice display smaller skeletons, shorter limbs and severe low bone mass phenotypes. Previous studies in our lab have demonstrated that Atf4 is essential for *Osteocalcin* expression, osteoblast terminal differentiation and bone formation. The fact that *Atf4*$^{-/-}$ mice have small skeletons and shorter limbs suggests that they have defects in the growth plate chondrocytes. However, how the Atf4 deficiency leads to these defects was not clear.

In this dissertation, I focused on studying the function of Atf4 in chondrogenesis to address this question. The result shows that Atf4 is expressed in chondrocytes and regulates chondrocyte proliferation and differentiation by directly controlling *Ihh* transcription during chondrogenesis. My further studies in *Atf4*$^{-/-}$*;Col2α1-Atf4* mice show that specific overexpression of *Atf4* in chondrocytes is sufficient to support normal chondrocyte proliferation and differentiation, confirming the autonomous function of Atf4 in chondrogenesis. In addition, overexpression of *Atf4* in chondrocytes completely restored the decreased *Osteocalcin* expression, the decreased osteoblast differentiation, and low bone mass in *Atf4*$^{-/-}$ mice to normal in *Atf4*$^{-/-}$;*Col2α1-Atf4* mice. Furthermore,

chondrocyte-specific overexpression of *Atf4* rescues Hh signaling in *Atf4*$^{-/-}$ osteoblasts. This rescue activity is repressed without Ihh as shown by the fact that the differentiation of *Atf4*$^{-/-}$ osteoblasts, induced by the conditioned medium of *Atf4*$^{-/-}$*;Col2α1-Atf4* cartilage, can be blocked by a neutralizing Hh antibody.

In a short summary, these studies demonstrate that Atf4 in chondrocytes directly regulates chondrocyte proliferation and differentiation, and indirectly regulates osteoblast differentiation and function via Ihh.

Function of Atf4 in chondrogenesis

Immunohistochemistry revealed that Atf4 is present in all growth plate chondrocytes, with high level of expression in proliferative and prehypertrophic chondrocytes, and low level of expression in resting and hypertrophic chondrocytes (Fig. 1D). This suggests that Atf4 has an important role in regulating chondrogenesis.

1. Atf4 is required for chondrocyte proliferation

Atf4$^{-/-}$ mice have a short proliferative chondrocyte zone with unorganized columnar structure and decreased proliferation of chondrocytes, indicating that Atf4 is indispensable for chondrocyte proliferation. Specific overexpression of *Atf4* in chondrocytes in *Atf4*$^{-/-}$*;Col2α1-Atf4* mice completely restored the imperfect chondrocyte proliferation and columnar structure to normal. This confirms the function of Atf4 in regulating chondrocyte proliferation. Ihh, expressed in prehypertrophic chondrocytes, is

an essential regulator of chondrocyte proliferation. *Ihh*$^{-/-}$ mice have a dramatic decrease of chondrocyte proliferation and lack organized columnar structure (St-Jacques et al., 1999). This present study showed that Atf4 regulates chondrocyte proliferation via Ihh. First, *Atf4*$^{-/-}$ mice showed a decrease of *Ihh* expression and Hh signaling in chondrocytes. Secondly, Atf4 directly binds to *Ihh* promoter and specifically activates its transcription. Thirdly, overexpression of *Atf4* in chondrocytes completely restored the expression of *Ihh* and Hh signaling in *Atf4*$^{-/-}$*;Col2α1-Atf4* mutants. Lastly, reactivation of Hh signaling by purmophamine, a compound that activates smoothened by pass of Ihh, almost completely rescued chondrocyte proliferation in *Atf4*$^{-/-}$ limbs. Altogether, these evidences demonstrate that Atf4 regulates chondrocyte proliferation via Ihh in prehypertrophic chondrocytes.

Interestingly, Atf4 is present not only in prehypertrophic chondrocytes, but also in proliferative chondrocytes. It is still a mystery whether Atf4 autonomously regulates cell proliferation in proliferative chondrocytes. The fact that reactivation of Hh signaling by purmophamine can not completely rescue chondrocyte proliferation in *Atf4*$^{-/-}$ limbs suggests that Atf4 may also regulate chondrocyte proliferation independent of Ihh. Conditional knockout Atf4 in proliferative chondrocytes may help to find out whether Atf4 autonomously regulates chondrocyte proliferation.

2. Atf4 is required for proper hypertrophy of chondrocytes

Atf4 prevents premature hypertrophy of proliferative chondrocytes. *Atf4*$^{-/-}$ mice have an expanded hypertrophic chondrocyte zone in E16 and P0 (Chapter II, Fig. 3). The

expanded hypertrophic chondrocyte zone in *Atf4*$^{-/-}$ mice is caused by the early hypertrophy of chondrocytes, but not the delay of the removal of hypertrophic chondrocytes, as indicated by a shorter non-hypertrophic chondrocyte zone in E16 (Chapter II, Fig. 3), and the normal cell apoptosis in the bottom of hypertrophic chondrocyte zone (Chapter II, Fig. 4). Overexpression of Atf4 in chondrocytes restores the expanded hypertrophic chondrocyte zone to normal in *Atf4*$^{-/-}$*;Col2α1-Atf4* mice (Chapter III, Fig. 3). Altogether, these indicate that Atf4 inhibits chondrocyte hypertrophy. The defect of early chondrocyte hypertrophy in *Atf4*$^{-/-}$ mice may be a result of the decrease of *Ihh* expression in chondrocytes. *Ihh*$^{-/-}$ mice have an expanded hypertrophic chondrocyte zone, as a result of early maturation and accelerated chondrocyte hypertrophy (St-Jacques et al., 1999). Ihh and PTHrP form a negative feedback loop in regulating chondrocyte hypertrophy. Ihh stimulates *PTHrP* expression, and chondrocyte proliferation. As a negative feedback, PTHrP delays the differentiation of proliferative chondrocytes into prehypertrophic chondrocytes and decreases the expression of *Ihh* (Kronenberg, 2006). The expression of *PTHrP* is decreased in *Atf4*$^{-/-}$ mice, but in vitro luciferase reporter assay showed that Atf4 does not activate *PTHrP* promoter, suggesting that Atf4 regulates *PTHrP* expression in an indirect manner via Ihh.

In addition, Atf4 stimulates chondrocyte hypertrophy at the center of skeletal element in the early stage of development. This is supported by the evidence that *Atf4*$^{-/-}$ embryos have a delay of chondrocyte hypertrophy in the center of E14 humeri compared with WT (Chapter II, Fig. 3). In a limb culture system, reactivating Hh signaling by purmorphamine reduced the delayed hypertrophy in the center of *Atf4*$^{-/-}$ E14 limbs, as

shown by the increase in the length of *Col10a1*-expressing chondrocyte area in purmorphamine-treated versus vehicle-treated *Atf4*$^{-/-}$ radii (Chapter II, Fig. 7). These results suggest that Atf4 stimulates chondrocyte hypertrophy in the center of skeletal element via Ihh. Consistent with this finding, it has been reported that Ihh stimulates chondrocyte hypertrophy independent of PTHrP. Upregulation of Ihh signaling in the developing cartilage by treating *PTHrP*$^{-/-}$ limb explants with sonic hedgehog protein in vitro, or overexpressing Ihh in the cartilage of *PTHrP*$^{-/-}$ embryos, accelerated chondrocyte hypertrophy in the *PTHrP*$^{-/-}$ embryos. Conversely, when Hh signaling was blocked by cyclopamine or by removing *Smoothened* (*Smo*), a positive regulator of Hh signaling, chondrocyte hypertrophy was delayed in the *PTHrP*$^{-/-}$ embryo (Mak et al., 2008).

However, reactivating Hh signaling alone by purmorphamine cannot completely rescue the delayed hypertrophy in the center of *Atf4*$^{-/-}$ E14 limbs (Chapter II, Fig. 7), suggesting that Atf4 may also regulate chondrocyte hypertrophy through other mechanism independent of Ihh. Atf4 is expressed in hypertrophic chondrocytes, but its function in these cells is not clear. It had been reported that Atf4 regulate amino acid uptake and Type I collagen synthesis in osteoblast (Yang et al., 2004). Hypertrophic chondrocytes synthesize Type X collagen and secrete it into the extracellular matrix. Whether Atf4 plays a role in autonomously regulating Type X collagen synthesis and cell hypertrophy in chondrocytes can be found out in the future. Further studies by conditional knockout Atf4 in hypertrophic chondrocytes may help to find out the answer.

3. Atf4 directly regulates *Ihh* transcription in chondrocytes

This study identified that Atf4 in chondrocytes directly regulates the transcription of *Ihh*. Atf4 directly binds *Ihh* promoter and transactivates *Ihh* expression in chondrocytes. Overexpression of Atf4 in vitro enhances endogenous *Ihh* mRNA synthesis. Reactivation of Hh signaling by purmorphamine almost completely rescued the proliferation defects and partially rescues the length of *Atf4*$^{-/-}$ mutant limbs. Altogether, these results indicate that Atf4 and Ihh act in the same pathway to regulate chondrocyte proliferation and hypertrophy.

Atf4 is not sufficient to enhance Ihh expression in vivo. Overexpression of Atf4 in chondrocytes rescues the *Ihh* expression to normal as shown by comparing *Atf4*$^{-/-}$*;Col2α1-Atf4, Atf4*$^{-/-}$*, and WT* mice, but it cannot further increase *Ihh* expression. This implies that other transcription activator(s) is required, or, some inhibitor(s) is involved in regulating *Ihh* transcription. Runx2 is another transcription factor that can directly bind and activate *Ihh* transcription. *Runx2*$^{-/-}$ mice have some defects similar to the *Atf4*$^{-/-}$ mice. For example, they have a severely decreased *Ihh* expression and defects in chondrocyte proliferation. Nevertheless, *Runx2*$^{-/-}$ mice also have specific defect that is not present in *Atf4*$^{-/-}$ mice, such as completely lacking hypertrophic chondrocytes in their skeletons (Komori et al., 1997; Otto et al., 1997). These suggest that both Atf4 and Runx2 regulate *Ihh* expression, but they play different roles in the control of chondrocyte differentiation. On the other hand, Atf4 activates only short fragments (<1.3 kb) of *Ihh* promoter, suggesting that there is repressor(s) in the long fragments (>2.2 kb) of *Ihh* promoter (Fig. 1). What and how this repressor(s) regulates *Ihh* promoter is yet to be known. However, it

has been shown that PTHrP and FGF (fibroblast growth factor) signaling may play a role in inhibiting Ihh expression in chondrocytes. PTHrP and Ihh form a negative feedback loop in growth plate. Ihh stimulates *PTHrP* expression and chondrocyte proliferation. As a negative feedback, PTHrP delays the termination of chondrocyte proliferation and *Ihh* expression (Kronenberg, 2006). One other possibility is that *Ihh* expression is suppressed by FGF signaling. Depletion of Fgfr3, a target of FGFs, in mice resulted in an increase of *Ihh* expression and chondrocyte proliferation (Colvin et al., 1996; Deng et al., 1996). Conversely, transgenic mice expressing Fgfr3 with a gain-of-function mutation have decreased *Ihh* expression and the rate of proliferation of chondrocytes. This leads to shortened and disorganized chondrocyte columns in them (Chen et al., 2001; Naski et al., 1998). All put together, these findings suggest that the *Ihh* expression may be inhibited by PTHrP and FGF signaling. Further researches could be done on identifying which transcription factors that are downstream of PTHrP and FGF signaling can directly bind and repress *Ihh* promoter in chondrocytes.

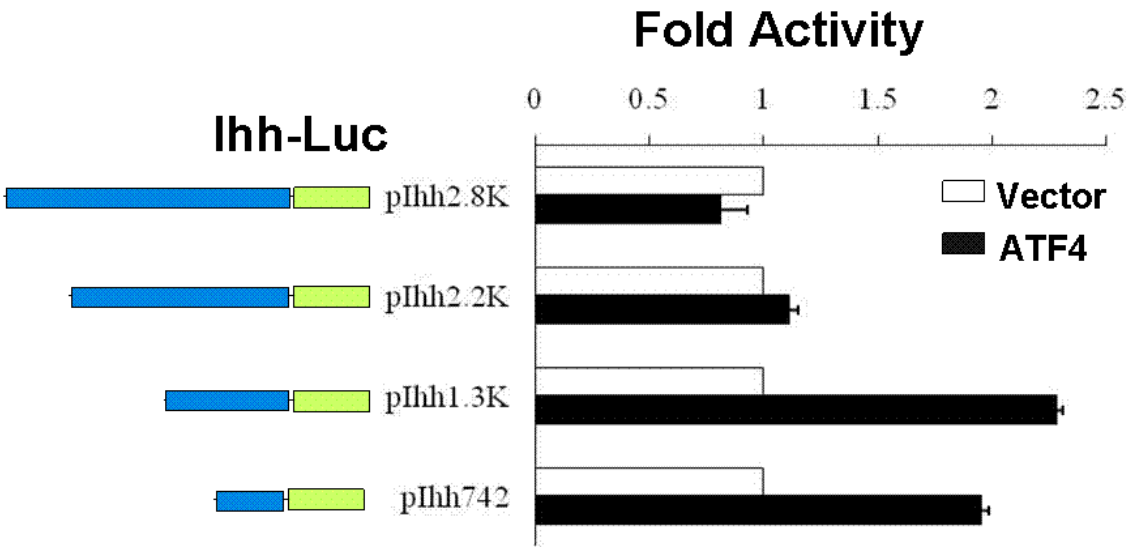

Fig. 1 The transcriptional repression on the long *Ihh* promoter fragments

Co-transfection assay in COS1 cells showing that Atf4 activates luciferase reporter constructs containing 1.3kbp and 742bp *Ihh* promoter fragments, but not the constructs containing 2.2kbp and 2.8kbp *Ihh* promoter fragments. This suggests that there is repressor(s) in the 2.2kbp and 2.8kbp fragments of *Ihh* promoter.

Function of Atf4 in osteogenesis

A previous study showed that Atf4 cell-autonomously regulates osteoblast differentiation and function. Atf4 is first discovered as an osteoblast-enriched transcription factor. Atf4 directly binds and activates the promoter of *Osteocalcin* that is an osteoblast specific gene (Yang et al., 2004). There are two osteoblast-specific cis-acting elements in *Osteocalcin* promoter, termed OSE1 and OSE2. Atf4 binds to OSE1. Another transcription factor Runx2 binds to OSE2 (Ducy et al., 1997). Atf4 enhances amino acid import and favors synthesis of type I collage in osteoblasts, although it does not affect expression of type I collagen genes (Yang et al., 2004). Atf4 global-deficient mice have a severe low bone mass phenotype, accompanied by the lack of terminal differentiation of osteoblasts (Yang et al., 2004).

The present study identified a novel function of chondrocyte-derived *Atf4* in regulating osteoblast differentiation. Overexpression of Atf4 in chondrocytes rescues *Osteocalcin* expression, osteoblast differentiation and bone formation in *Atf4*$^{-/-}$*;Col2α1-Atf4* mice which have no *Atf4* expression in any cell other than in the chondrocyte. These results demonstrate that chondrocyte-derived Atf4 indirectly regulates osteoblast differentiation. In addition, the differentiation of *Atf4*$^{-/-}$ osteoblasts induced by the conditioned medium of *Atf4*$^{-/-}$*;Col2α1-Atf4* cartilage can be blocked by a neutralizing Hh antibody. This suggests that Atf4 in cartilage regulates the production of Ihh, which then diffuses into the bone marrow cavity to regulate osteoblast differentiation and function. The function of Ihh in osteoblast differentiation has been identified by characterizing the *Ihh*$^{-/-}$ mice, which have no osteoblast development in endochondral bones (St-Jacques et

al., 1999). In addition, postnatal deletion of chondrocytic *Ihh* in tamoxifen-inducible conditional *col2α1-Cre ER*; floxed Ihh*-knockout mice leads to decreased osteoblast differentiation and loss of trabecular bone over time (Maeda et al., 2007). Furthermore, in vitro study showed that Ihh/Gli2 Signaling stimulates osteoblast differentiation by regulating Runx2 protein expression and function (Shimoyama et al., 2007). *Runx2* is a master gene of osteoblast differentiation. *Runx2*-deficient mice have no endochondral and intramembranous bone formation without differentiated osteoblast (Komori et al., 1997; Otto et al., 1997). Therefore, chondrocyte-derived *Atf4* may indirectly regulate osteoblast differentiation and function through Hh signaling and Runx2 pathway.

Based on the experiment results that *Atf4* overexpression in chondrocytes completely rescues osteoblast differentiation and bone formation in *Atf4*$^{-/-}$*;Col2α1-Atf4* mice (up to 3 month old), it is possible that chondrocyte-derived *Atf4* dominates over osteoblast-derived *Atf4* in regulating osteoblast differentiation and bone formation when there is enough Ihh from the cartilage diffusing into the bone marrow cavity. However, the cartilage growth plate will be closed and replaced by bone over time in adult mice. At this time, the autonomous function of *Atf4* in osteoblast may dominate the osteoblast differentiation and bone formation without the support of cartilage. If this is the case, the old *Atf4*$^{-/-}$*;Col2α1-Atf4* mice will decrease their bone mass faster than the WT mice over time. In the future, further studies on old *Atf4*$^{-/-}$*;Col2α1-Atf4* mice will help to test this hypothesis.

In summary, Atf4 in chondrocytes regulates chondrocyte proliferation and differentiation by directly controlling *Ihh* transcription, which diffuses into bone marrow cavity to stimulate osteoblast differentiation. Atf4 in osteoblasts further accelerates the osteoblast terminal differentiation. Thus, by controlling *Ihh* expression, Atf4 in chondrocytes couples chondrogenesis and osteogenesis to ensure a harmonious transition of cartilage to bone during endochondral ossification.

Part II: Future Direction

Other possible functions of Atf4 in chondrocytes

This study demonstrates that Atf4 regulates chondrocyte proliferation and differentiation by controlling *Ihh* transcription in prehypertrophic chondrocytes. However, the reactivation of Hh signaling by purmophamine can not completely rescue chondrocyte proliferation in $Atf4^{-/-}$ limbs. This suggests that Atf4 may also regulate chondrocyte proliferation independent of Ihh. Atf4 is expressed in proliferative chondrocytes, but its function in these cells is not clear. To test the cell autonomous function of Atf4 in proliferative chondrocytes, we can generate *Fgfr3-cre* transgenic mice that over-express specifically the Cre recombinase in proliferative chondrocytes and crossing with $Atf4^{flox/flox}$ mice to delete *Atf4* specifically in proliferative chondrocytes.

In addition, the autonomous function of Atf4 in hypertrophic chondrocytes is yet to be explored. Ihh, a downstream target of Atf4 in prehypertrophic chondrocytes, regulates chondrocyte hypertrophy. However, reactivating Hh signaling alone by purmorphamine can not completely rescue the delayed hypertrophy in the center of $Atf4^{-/-}$

E14 limbs. This suggests that Atf4 may regulate chondrocyte hypertrophy through other mechanism independent of Ihh, or pharmacological approach can not work as efficiently as endogenous gene. We cannot rule out the possibility that Atf4 in hypertrophic chondrocytes may directly regulate chondrocyte hypertrophy. Conditional knockout Atf4 by crossing *Collagen type X-cre* transgenic mice with $Atf4^{flox/flox}$ mice will delete *Atf4* specifically in hypertrophic chondrocytes and help to test this possibility in the future.

Furthermore, Atf4 is expressed in proliferative chondrocytes, whether Atf4 has direct function in resting chondrocytes is an area to be explored in the future. $Atf4^{-/-}$ mice have a lot of apoptotic cells in the secondary ossification center of the P0 humerus compared with WT (Chapter II, Fig. 4), suggesting that Atf4 may inhibit the premature and apoptosis of resting chondrocytes and delay the formation of secondary ossification center. To test the function of Atf4 in resting chondrocytes, we can generate *Fgfr1-cre* transgenic mice that over-express specifically the Cre recombinase in resting chondrocytes and crossing with $Atf4^{flox/flox}$ mice to delete *Atf4* specifically in resting chondrocytes.

Reactivation of Hh signaling in vivo to rescue the dwarfism of $Atf4^{-/-}$ mice

Aft4 regulates chondrocyte proliferation and differentiation via Ihh. $Atf4^{-/-}$ mice have decreased *Ihh* expression and Hh signaling. The reactivation of Hh signaling pharmacologically in mouse limb explants distinctly restores the $Atf4^{-/-}$ chondrocyte proliferation and long bone growth. However, it is not known whether the reactivation of Hh signaling in vivo is sufficient to rescue the endochondral ossification defects of $Atf4^{-/-}$

mice. Gli3, a zinc finger transcription factor, acts as a strong repressor of Ihh signals in regulating chondrocyte differentiation (Hilton et al., 2005; Koziel et al., 2005). It had been reported that the loss of Gli3 in *Ihh* mutants remarkably rescues the $Ihh^{-/-}$ phenotype. The $Ihh^{-/-}$ phenotype is characterized by decreased chondrocyte proliferation, accelerated onset of hypertrophic differentiation, and decreased Hh signaling and *PTHrP* expression (Hilton et al., 2005; Koziel et al., 2005). These characteristics are also present in $Atf4^{-/-}$ mice. Based on the above facts, we hypothesize that the loss of Gli3 may distinctly rescue the defects of $Atf4^{-/-}$ skeleton caused by decreasing of Hh signaling. To test this hypothesis, we first crossed $Gli3^{+/Xt\text{-}J}$ ($Gli3^{+/-}$) mice with $Atf4^{+/-}$ mice to generate $Gli3^{+/-}$;$Atf4^{+/-}$, and then crossed $Gli3^{+/-}$;$Atf4^{+/-}$ with $Gli3^{+/-}$;$Atf4^{+/-}$ to obtain $Gli3^{-/-}$;$Atf4^{-/-}$ mice which were used for further characterization.

1. Loss of Gli3 can not rescue Hh signaling in $Atf4^{-/-}$ chondrocytes

In situ hybridization data show that $Atf4^{-/-}$ E17 embryos have less *Ihh* expression compared with WT (Fig. 2 a, b). The removal of Gli3 does not change the expression of *Ihh* in $Gli3^{-/-}$ chondrocytes compared with WT (Fig. 2a, c). In addition, there is no difference of *Ihh* expression between $Gli3^{-/-}$ and $Atf4^{-/-}$;$Gli3^{-/-}$ growth plate chondrocytes (Fig. 1c, d), suggesting that Gli3 cannot affect *Ihh* expression.

Patch1 is a downstream target gene of Hh signaling. In situ hybridization analysis revealed that $Atf4^{-/-}$ embryos have low level expression of *Patch1* in growth plate chondrocytes in comparison with WT (Fig. 2 e, f), which is consistent with our previous finding (Chapter III). Loss of Gli3 has little effect on the expression of *Patch1* in $Gli3^{-/-}$

growth plate chondrocytes compared with WT (Fig. 2 e, g). There is a slight decrease in the expression of *Patch1* in *Atf4*$^{-/-}$;*Gli3*$^{-/-}$ growth plate chondrocytes in comparison with *Atf4*$^{-/-}$ embryos (Fig. 2 f, h). This suggests that the loss of Gli3 cannot rescue Hh signaling in *Atf4*$^{-/-}$ growth plate chondrocytes.

This result is different from that of the analysis on *Ihh*$^{-/-}$;*Gli3*$^{-/-}$ embryos, in which the loss of Gli3 in the absence of Ihh increases *Patched1* expression and partially rescues long bone growth (Koziel et al., 2005). In the absence of Hh signals, the Gli3 protein is phosphorylated and proteolytically processed into a truncated N-terminal repressor protein, which inhibits the expression of Hh target genes (Ohba et al., 2008). However, *Atf4*$^{-/-}$ mice still have low level of *Ihh* and *Patched1* expression (Fig. 2). With Hh signaling, phosphorylation and proteolytic processing are blocked and full length Gli3 protein acts as a transcriptional activator of Hh target genes (Bai et al., 2004; Wang et al., 2000). The present result shows that there is a slight decrease of *Patched1* expression in *Atf4*$^{-/-}$;*Gli3*$^{-/-}$ growth plate chondrocytes compared with *Atf4*$^{-/-}$ limbs (Fig. 2 f, h). This suggests that the activation function of Gli3 is dominant over the repression function of truncated Gli3 in *Atf4*$^{-/-}$ mice. Further analysis by Western Blot is necessary to detect whether the amount of full length Gli3 activator is more than that of truncated Gli3 repressor in *Atf4*$^{-/-}$ chondrocytes. In addition, in situ hybridization is not an accurate method to quantify the gene expression level. Quantification PCR and more repeated experiments are required to confirm this result in the future.

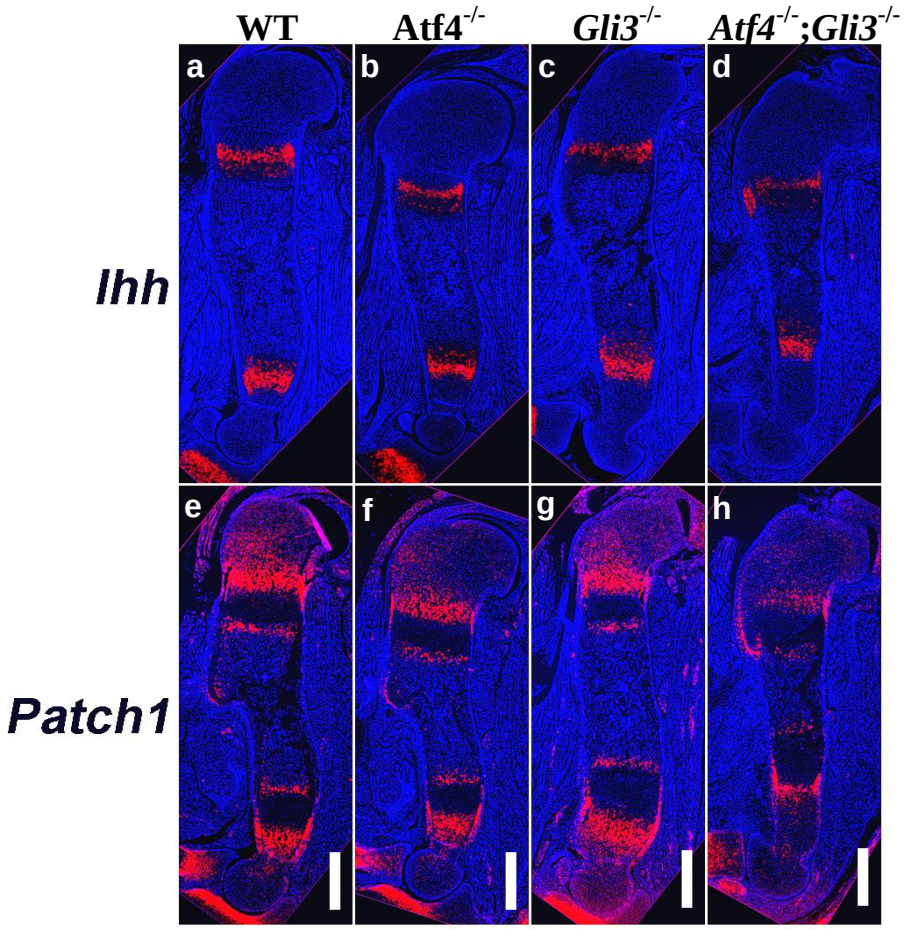

Figure 2. Loss of Gli3 does not rescue *Patch1* expression in *Atf4*$^{-/-}$ chondrocytes (N=1).

2. Loss of Gli3 expands hypertrophic chondrocyte zone

Col10α1 is expressed specifically in hypertrophic chondrocytes. There is no obvious difference of *Col10α1* expression level among WT, *Atf4*$^{-/-}$, *Gli3*$^{-/-}$ and *Atf4*$^{-/-}$;*Gli3*$^{-/-}$ embryos (Fig. 3 a, b, c, d). However, the loss of Gli3 expands the length of hypertrophic chondrocyte zone in the distal end of *Gli3*$^{-/-}$ humerus in comparison with WT (Fig. 3 a, c brackets). In addition, the loss of Gli3 also increases the length of hypertrophic chondrocyte zone in the distal end of *Atf4*$^{-/-}$;*Gli3*$^{-/-}$ humerus in comparison with *Atf4*$^{-/-}$ (Fig. 3 c, d brackets). These suggest that Gli3 inhibits the expansion of hypertrophic chondrocyte zone in the distal end of humeri, and this inhibition is independent of Atf4 in chondrocytes. Interestingly, loss of Gli3 has little effect on the length of hypertrophic chondrocyte zone in the proximal end of humeri (Fig. 3 a, b, c, d top), suggesting that the function of Gli3 is spatially controlled during chondrogenesis. More repeated experiments and zone length quantification need to be done to confirm these results in the future.

There are two possible mechanisms that can explain the extension of hypertrophic chondrocyte zone in the *Gli3*$^{-/-}$ and *Atf4*$^{-/-}$;*Gli3*$^{-/-}$ embryos. First, it can be a result of pre-maturation and early hypertrophy of chondrocytes. Second, it may be caused by the delay in the removal of late hypertrophic chondrocytes. If it is the first scenario, there will be a shorter non-hypertrophic chondrocyte zone in the *Gli3*$^{-/-}$ and *Gli3*$^{-/-}$;*Atf4*$^{-/-}$ embryos. *Col2α1* is the marker gene for non-hypertrophic chondrocytes. However, there is an increase in the length of non-hypertrophic chondrocyte zone in the distal end of *Gli3*$^{-/-}$ and *Atf4*$^{-/-}$;*Gli3*$^{-/-}$ humeri compared with WT and *Atf4*$^{-/-}$ humeri (Fig. 3 e, f, g, h brackets),

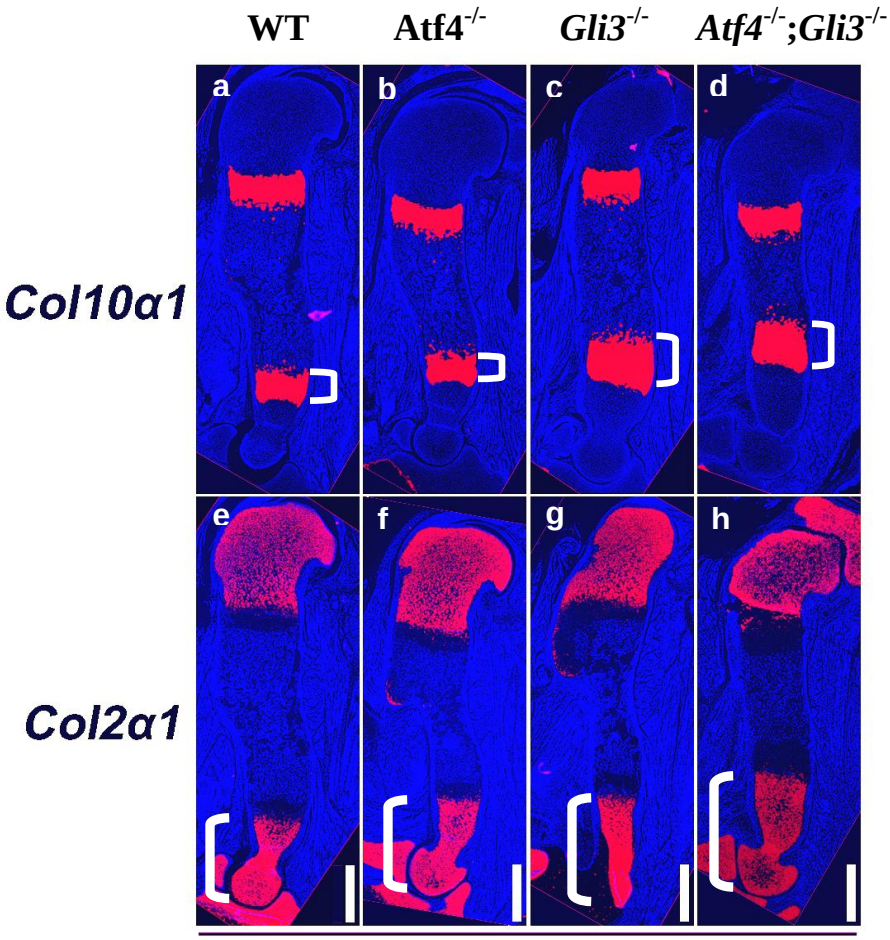

Figure 3. Loss of Gli3 expands hypertrophic chondrocyte zones (N=1).

suggesting that there is a delay in the removal of late hypertrophic chondrocytes instead of early hypertrophy of chondrocytes in *Gli3*$^{-/-}$ and *Gli3*$^{-/-}$;*Atf4*$^{-/-}$ embryos. More repeated experiments and zone length quantification can be done to confirm these data in the future.

If the extension of hypertrophic chondrocyte zone in the distal end of *Gli3*$^{-/-}$ and *Gli3*$^{-/-}$;*Atf4*$^{-/-}$ humeri is a result of delaying the removal of hypertrophic chondrocytes, this defect may be caused by the delay in terminal differentiation or apoptosis of hypertrophic chondrocytes. Terminal differentiation of hypertrophic chondrocyte can be detected by examining the expression of *matrix metalloproteinase 13* (*Mmp13*), which is a marker gene for terminal hypertrophic chondrocytes (Minina et al., 2005). Apoptosis of hypertrophic chondrocyte can be tested by cell apoptosis assay, such as TUNEL assay (Chapter II). In situ hybridization for *Mmp13* and TUNEL assay will help to test these hypotheses.

3. Other options to reactivate Hh signaling in the *Atf4*$^{-/-}$ mice

This study showed that the loss of Gli3 is not able to rescue Hh signaling, and cannot rescue the shorter bone defects in *Atf4*$^{-/-}$ mice. There are other options to reactivate Hh signaling in the *Atf4*$^{-/-}$ mice. The first option—Hh signaling can be activated by Gli2. Gli2 is an activator downstream of Hh signaling. Overexpression of *Gli2* in chondrocytes partially rescues the decreased proliferation, and delayed maturation of chondrocytes in *Ihh*$^{-/-}$ mice (Joeng and Long, 2009). It is possible that overexpression of *Gli2* specifically in chondrocytes may rescue the cartilage defects of *Atf4*$^{-/-}$ mice. The second option—Hh

signaling can be activated by the removal of Patched1. The removal of Patched1 had been shown to increase Hh signaling and enhance endochondral bone formation (Ohba et al., 2008). Future studies could be done by removing Patched1 from the chondrocytes to rescue the Hh signaling in *Atf4*$^{-/-}$ mice. The third option—Overexpressing Ihh in chondrocytes to activate Hh signaling in *Atf4*$^{-/-}$ mice. A UAS-Gal4 biogenic system (Crossing *Col2a1-Gal4* mice with *UAS-Ihh* mice) was used to overexpress Ihh gene in the cartilage of the *Col2α1-Ihh* mice (Yang et al., 2003). Crossing the *Col2α1-Ihh* mice with *Atf4*$^{-/-}$ mice can generate a compound mutant *UAS-Ihh;Col2a1-Gal4;Atf4*$^{-/-}$ to rescue the Hh signaling in *Atf4*$^{-/-}$ background mice.

Which one is dominant for osteoblast differentiation: chondrocyte-derived Atf4 or osteoblast-derived Atf4?

Both chondrocyte-derived Atf4 and osteoblast-derived Atf4 regulate osteoblast differentiation and function. Based on the results that *Atf4* overexpression in chondrocytes completely rescues osteoblast differentiation and bone formation in *Atf4*$^{-/-}$*;Col2α1-Atf4* mice, it seems that chondrocyte-derived *Atf4* dominates over osteoblast-derived *Atf4* in regulating osteoblast differentiation and bone formation. However, the overexpression of Atf4 in chondrocytes cannot represent the physiological function of Atf4 with a normal expression level in chondrocytes. Therefore, whether chondrocyte-derived *Atf4* or osteoblast-derived *Atf4* is dominant for regulating osteoblast differentiation and function is not clear. It is possible that they may contribute differently to the osteoblast differentiation in different stage of development. Conditional deletion of *Atf4* from chondrocytes or osteoblasts will be necessary to address this issue.

Atf4, Ihh and Hh signaling for postnatal bone repair

This study demonstrates that Atf4 in chondrocytes regulates both chondrogenesis and osteogenesis by directly controlling *Ihh* transcription during endochondral bone formation. Endochondral bone formation is important for both skeleton development and postnatal fracture healing process. After fracture, the bone repair undergoes a unique morphogenic process. The primary mode of that is through the formation of a cartilage scaffold, which is gradually replaced by bone. This process, to some extent, recapitulates the fundamental features of endochondral ossification in the developing limb (Ferguson et al., 1999). Ihh is essential for osteoblast differentiation during limb development (Chung et al., 2001; Long et al., 2004; St-Jacques et al., 1999) as well as postnatal bone formation (Maeda et al., 2007). It has also been reported that Ihh and Hh signaling play important roles in postnatal endochondral bone repair (Wang et al., 2010b). *Ihh* is expressed in hypertrophic chondrocytes in fracture callus associated with increased *Patched1*, *Gli1* and *PTHrP receptor* expression in the surrounding callus tissue (Vortkamp et al., 1998). Activation of Hh signaling in periosteal mesenchymal progenitors, by an adenovirus that expresses a secreted form of ShhN peptide, induces robust bone formation in vivo (Wang et al., 2010b). Inhibition of Hh signaling by a conditional deletion of Smoothened in repair callus results in significant reduction of bone callus formation during bone repair process (Wang et al., 2010b). Altogether, these results indicate that Ihh and Hh signaling are required for bone repair. Whether Atf4 regulates *Ihh* expression in the callus tissue, and whether the activity of Atf4, a hypoxia stress responsive factor (Ameri et al., 2004); (Bi et al., 2005), is enhanced under the

hypoxia condition in the bone fracture site, are yet to be known. More studies in this direction in the future will help us understand the bone repair process and provide more information for improving treatment efficiency.

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