Kif7 promotes hedgehog signaling in growth plate chondrocytes by restricting the inhibitory function of Sufu

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SUMMARY
Proper regulation of Indian hedgehog (Ihh) signaling is vital for chondrocyte proliferation and differentiation in the growth plate. Its dysregulation causes skeletal dysplasia, osteoarthritis or cartilaginous neoplasia. Here, we show that Suppressor of fused (Sufu) and Kif7 are essential regulators of Ihh signaling. While Sufu acts as a negative regulator of Gli transcription factors, Kif7 functions both positively and negatively in chondrocytes. Kif7 plays a role in the turnover of Sufu and the exclusion of Sufu-Gli complexes from the primary cilium. Importantly, halving the dose of Sufu restores normal hedgehog pathway activity and chondrocyte development in Kif7-null mice, demonstrating that the positive role of Kif7 is to restrict the inhibitory activity of Sufu. Furthermore, Kif7 also inhibits Gli transcriptional activity in the chondrocytes when Sufu function is absent. Therefore, Kif7 regulates the activity of Gli transcription factors through both Sufu-dependent and -independent mechanisms.

KEY WORDS: Chondrogenesis, Indian hedgehog, Gli transcription factors, Mouse

INTRODUCTION
The precise regulation of chondrocyte proliferation and differentiation is crucial for normal bone growth. During endochondral bone development, growth plate chondrocyte differentiation is governed by the spatial and temporal regulation of a number of signaling pathways. Dysregulation of these processes during development is responsible for skeletal dysplasias, which are characterized by short stature (Karsenty et al., 2009). Inhibition of differentiation of growth plate chondrocytes can cause cartilaginous tumors (Bovee et al., 2010), and aberrant activation of signaling pathways normally involved in the regulation of growth plate chondrocytes is associated with osteoarthritis, a common degenerative joint disease (Lin et al., 2009). Therefore, abnormal chondrocyte proliferation and differentiation have profound negative effects on overall health.

Ihh, a member of the hedgehog (Hh) family of signaling molecules, regulates the transcriptional activity of Gli proteins through binding to its receptor patched 1 (Ptc1) and derepression of the signaling receptor smoothened (Smo). Three Gli zinc-finger proteins (Gli1-Gli3) are transcription factors that mediate Hh signaling in mammalian cells (Jiang and Hui, 2008). In mice, Gli2 and Gli3 are essential genes, whereas Gli1 is dispensable for embryonic development and encodes a secondary mediator of Hh signaling. Gli2 and Gli3 are the major transcriptional activator and repressor of the mammalian Hh pathway, respectively, although all three Gli proteins can activate the expression of Hh target genes, such as Ptc1 and Gli1 itself. Gli3 is processed efficiently by the proteasome into a C-terminally truncated transcriptional repressor. Through ill-defined mechanisms, Hh signaling blocks the proteolytic cleavage of Gli3 and promotes the transcriptional activator function of Gli2 and Gli3. Mutant mouse analysis indicates that Gli2 and Gli3 are involved in Ihh-dependent chondrocyte development. Mice that lack Ihh are characterized by reduced chondrocyte proliferation, an expanded hypertrophic zone in the growth plate and lack of ossification in endochondral bones (St-Jacques et al., 1999). Similar to Ihh knockout mice, Gli2 knockout mice show an expanded hypertrophic zone and reduced bone formation, suggesting that the Ihh mutant phenotype is in part due to a reduction of Gli2 activator function (Miao et al., 2004). Loss of Gli3 rescues the chondrocyte proliferation and differentiation defects in Ihh mutant mice, indicating that a major action of Ihh is to limit the repressor function of Gli3 in growth plate chondrocytes (Koziel et al., 2005). These observations indicate that Ihh-dependent regulation of Gli2 and Gli3 plays a crucial role in chondrocyte differentiation.

In mammalian Hh signaling, Sufu and Kif7 are two evolutionarily conserved regulators of Gli transcription factors (Wilson et al., 2009). In mice, Sufu is a major negative regulator of Hh signaling and inactivation of Sufu leads to embryonic lethality at E9.5 with severe ectopic Hh pathway activation similar to that observed in Ptc1-null embryos (Cooper et al., 2005; Svard et al., 2006). Sufu forms complexes with all three Gli proteins and inhibits their transcriptional activity (Barnfield et al., 2005; Ding et al., 1999). Recent studies in cultured fibroblasts have suggested that Hh signaling promotes the nuclear translocation and transcriptional activity of Gli2 and Gli3 through dissociation of cytoplasmic Sufu-Gli complexes (Humke et al., 2010; Tukachinsky et al., 2010). In addition, Sufu\textsuperscript{-/-} cells exhibit a drastic reduction in the levels of full-length Gli2 and Gli3, as well as a lack of Gli3 repressor, suggesting that it also plays a crucial role in the stabilization of Gli activators and the formation of Gli3 repressor (Humke et al., 2010; Wang et al., 2010). By contrast, less...
is known about the action of Kif7, which is a kinesin motor protein recently shown to play regulatory roles in mammalian Hh signaling. Kif7-null mice die at birth and exhibit a phenotype (Chen et al., 2009; Cheung et al., 2009) similar to that of Gli3-null mice (Hui and Joyner, 1993). Kif7-null embryos show mild ectopic Hh pathway activation with ectopic formation of ventral neurons in the spinal cord, as well as elevated Gli2 and reduced Gli3 levels, suggesting that it acts negatively in Hh signaling. Interestingly, Kif7 also functions positively in controlling Hh pathway activity (Cheung et al., 2009; Liem et al., 2009). For example, floor-plate development, which is induced by maximal level of Hh pathway activity, is compromised in the absence of Kif7 function (Adolphe et al., 2006; Cheung et al., 2009; Endoh-Yamagami et al., 2009). How Kif7 acts both negatively and positively in mammalian Hh signaling is not understood and whether Kif7 possesses cooperative regulatory function with Sufu is unclear.

The primary cilium acts as a focal point in the processing of Hh signaling (Corbit et al., 2005; Goetz and Anderson, 2010; Huangfu et al., 2003). Recent studies have suggested that, when the Hh pathway is activated, Smo promotes the dissociation of inhibitory Sufu-Gli complexes at the primary cilium (Tukachinsky et al., 2010). Kif7 has been shown to translocate to the tip of primary cilium in cultured fibroblasts upon pathway stimulation (Liem et al., 2009). However, it is not known whether Kif7 plays a regulatory role in the formation and/or dissociation of Sufu-Gli complexes in the primary cilium. In this study, we explored the roles of Sufu and Kif7 in Ihh-dependent chondrocyte development using genetically modified mice. Our results indicate that while Sufu is a major negative regulator of Hh pathway activity, Kif7 plays dual roles in the control of chondrocyte development. Intriguingly, Kif7 is localized to the ciliary tip of proliferating chondrocytes in vivo and appears to exclude Sufu-Gli complexes from the primary cilium. We speculate that Kif7 functions positively in Hh signaling to promote Smo-induced dissociation of Sufu-Gli complexes at the primary cilium.

**MATERIALS AND METHODS**

**Ethics statement**

A mouse protocol describing the experimental procedures used in the study was approved by the Animal Care Committee of The Hospital for Sick Children.

**Mice**

The generation of Kif7-deficient mice has been previously reported (Cheung et al., 2009). Conditional Sufu-deficient mice (Col2a1-Cre;Sufu<sup>fl</sup>) were generated by crossing Col2a1-Cre mice expressing Cre-recombinase under type II collagen regulatory elements specific to chondrocytes with Sufu<sup>loxP</sup> mice containing loxP sites flanking exons 4 to exon 8 of Sufu (Pospisilik et al., 2010). Conditional Ptc1-deficient mice (Col2a1-Cre;Ptc1<sup>fl</sup>) were generated from Ptc1<sup>loxP</sup> mice, which contain loxP sites flanking exon 3 of Ptc1 (Adolphe et al., 2006; Ellis et al., 2003). Embryonic mice were obtained from timed pregnancies and the genotypes of the various mice were determined as described (Cheung et al., 2009; Ding et al., 1999). In all cases, littermate mice were used as controls. The recombination efficiency in all conditional mutants was confirmed through Western analysis and by examining the Cre-drivers crossed with a Rosa-26 reporter line. All mice are on the 129/Sv background.

**Skeletal staining**

Mice were fixed in 95% ethanol after removal of skin and viscera. Bone samples were incubated in Alcian Blue solution (15% Alcian Blue in 80% ethanol and 20% glacial acetic acid) for 2-3 days at room temperature. Samples were then rehydrated and cleared in 1% KOH overnight or until clear. Samples were stained with Alizarin Red solution (7.5% Alizarin Red in 1% KOH) for 1-2 days and immersed in glycerol for storage (Mau et al., 2007).

**Microdissection of the growth plate**

Hindlimbs were obtained from E18.5 mouse embryos. Sections of the growth plate were dissected out using the assistance of a microscope followed by RNA isolation using Trizol reagent (Invitrogen). RNA concentration was determined by Nanodrop. cDNA was synthesized from 500 ng of total RNA using qScript cDNA SuperMix (Quanta Biosciences) for real-time PCR analysis.

**Histological analysis and immunohistochemistry**

Samples were fixed in 4% paraformaldehyde overnight, embedded in paraffin and sectioned for histological evaluation. Sections were examined for Col10a1, a marker for hypertrophic growth plate chondrocytes, by immunohistochemistry using previously reported techniques and antibodies (Hu et al., 2006; Linsenmayer et al., 1988; Saika et al., 2004; Tiet et al., 2006; Wang et al., 2000). The proximal tibial growth plate was used for all analysis to minimize morphological variations due to anatomic location. Hematoxylin and Eosin, Safranin O and Alcian Blue staining were performed using standard techniques. Proliferation was evaluated by immunostaining using antibodies against Ki-67 (DakoCytomation M7249) at 1:50 dilution and phospho-H3 (Sigma) at 1:200 dilution at 4°C overnight. The proportion of positively stained cells was calculated in the proliferative zone of the growth plate by counting the number of positive and negative cells over 10× high powered field. Apoptotic cells were detected by using an antibody against active caspase 3 (Promega, Madison, WI, Cat # G7481) and TUNEL assay as previously reported (Tiet et al., 2006). Positively stained cells were analyzed in a similar manner as for Ki67 staining. Sufu protein was detected using a rabbit anti-mouse antibody (Santa Cruz Biotechnology, sc-28847) incubated at a 1:100 dilution at 4°C overnight.

**Western analysis and co-immunoprecipitation**

Western blot analysis was performed using standard protocols. Immunoblotting was performed overnight at 4°C with the following primary antibodies: Gli3 antibody from Santa Cruz Biotechnology (1:800), actin antibody from Oncogene (1:10,000), Sufu antibody (Meng et al., 2001) (1:3000), Kif7 antibody (Cheung et al., 2009) (1:1000) and Gli2 antibody (Hu et al., 2006) (1:1000), and phosphor-serine antibody from Cell Signaling (1:1000). Precipitation of Sufu protein was performed using Dynabeads Protein A (Invitrogen) by following the manufacturer’s protocol.

**Real-time quantitative PCR**

RNA isolated from at least three independent experiments was analyzed by qRT-PCR in triplicate for each treatment condition and primer set. The reactions were made up in TaqMan Universal PCR master mix (Applied Biosystems) with TaqMan Gene Expression Assays for mouse Gli1, Ptc1, Hhip1, Gli2, Gli3, Ihh, Kif7 and Sufu (Applied Biosystems). The gene expression levels between samples were analyzed using the 2<sup>-ΔΔCt</sup> method (Livak and Schmittgen, 2001). Either Gapdh or the β-actin gene (Applied Biosystems) was used as endogenous control for target gene normalization.

**Primary growth plate chondrocyte cultures**

Chondrocyte isolation protocol was modified from previously published methods (Gosset et al., 2008). Growth plates of the hindlimbs from E16.5 embryos were isolated and incubated in collagenase type 4 (Worthington) solution (3 mg/ml) for 45 minutes at 37°C incubator, under 5% CO<sub>2</sub> in a Petri dish. Soft tissues were detached by pipetting. The growth plates were placed in a clean Petri dish with 0.5 mg/ml collagenase type 4 solution and were incubated overnight at 37°C. Collagenase D solution containing chondrocytes was collected. Cells were washed with PBS and seeded at a density of 8×10<sup>3</sup> cells per cm<sup>2</sup>.

**Measuring protein turnover**

Cells were cultured till reach confluence before cycloheximide (20 μM) (Calbiochem) treatment to block de novo protein synthesis for the time indicated. At the end of each time point, cells were collected for western analysis.
Phenylindole (DAPI) containing Vectashield (Vector Laboratories).

- deficient tibial and vertebral growth plates (Fig. 1H). Using Sufu-specific knockout mice (Col2a1-Cre;Ptch1f/f) to investigate the role of Sufu in growth plate chondrocyte development. Immunohistochemical staining and western analysis demonstrated efficient deletion of Sufu in the growth plate of Col2a1-Cre;Sufuf/f mice (Fig. 1D,E; see Fig. S2A in the supplementary material). Chondrocyte-specific knockout of Sufu in mice resulted in perinatal death with a few exceptions that survived for 10 days. Body length and weight measurements demonstrated a 24% and 50% reduction in the mutants, respectively, when compared with wild-type littermates (Fig. 1F; see Fig. S2B in the supplementary material). Alcian Blue/Alizarin Red staining showed a significant reduction of bone ossification in Col2a1-Cre;Sufuf/f mice (Fig. 1G; see Fig. S2D in the supplementary material). Histological analysis revealed delayed formation of secondary ossification centers, expansion of proliferating zone as well as reduction of hypertrophic zone in the Sufu-deficient tibial and vertebral growth plates (Fig. 1H). Using Ki67 and phospho-H3 immunostaining, a higher percentage of proliferating cells was found in the mutant growth plate (see Fig. S2E,F and Fig. S4B in the supplementary material). By contrast, immunostaining of active caspase 3 and TUNEL analysis did not reveal a significant difference of apoptosis in the mutants (see Fig. S2G in the supplementary material). These data indicate that Sufu is required for normal endochondral skeletal development, where it regulates growth plate chondrocyte proliferation and differentiation.

**Sufu acts as a negative regulator of Hh signaling during chondrocyte differentiation**

Ptch1 inhibits Smo in the absence of Hh signals and acts as a negative regulator of Hh signaling. Chondrocyte-specific knockout of Ptch1 (Col2a1-Cre;Ptch1f/f) leads to elevated Hh pathway activity and results in a phenotype similar to those observed in Sufu mutants, including an expansion of proliferative zone and a reduction of hypertrophic zone (Mak et al., 2008) (Fig. 1I, Fig. 2A-C). We found that the Sufu knockout phenotype is consistently milder than the Ptch1 knockout phenotype (Fig. 2A-C). To determine whether knockout of Sufu also results in Hh pathway activation in the chondrocytes, we performed qRT-PCR analysis. Knockout of Ptch1 leads to increased expression of Hh target genes, such as Gli1, Ptch1 and Hhip1 (Fig. 2D). Interestingly, we detected upregulation of Ptch1 and Hhip1, but not Gli1, in Sufu-deficient chondrocytes (Fig. 2D). In situ hybridization analysis revealed Ptch1 transcripts in the proliferating chondrocytes. In wild-type mice, cells adjacent to Ihh-producing prehypertrophic chondrocytes show highest levels of Ptch1 transcripts and Ptch1 expression decreases toward the end of the bone. In addition to the proliferative zone, Sufu-deficient mice exhibited higher levels of Ptch1 transcripts also in the resting chondrocytes (see Fig. S3A,C in the supplementary material). Furthermore, loss of Sufu resulted in a reduction of the hypertrophic zone as verified by in situ hybridization analysis for collagen X expression (see Fig. S3E,G in the supplementary material). Western analysis revealed an increase (two-fold) in the level of Gli2 protein as well as an increase (1.8-fold) in the ratio of full-length versus repressor form of Gli3 (Gli3FL;Gli3R) in Sufu-deficient chondrocytes (Fig. 2E). Together, these data suggest that Sufu, like Ptch1, functions as a negative regulator of Hh signaling in chondrocytes but its inactivation leads to only partial pathway activation.

**Loss of Kif7 in growth plate chondrocytes results in reduced Hh pathway activity**

As Sufu inactivation did not lead to a phenotype as severe as that of Ptch1 inactivation, we reasoned that other pathway components might cooperate with Sufu in regulating Hh signaling. One potential candidate is Kif7, which was recently shown to be a negative regulator of Gli1 transcription factors (Cheung et al., 2009). Kif7 is expressed at high levels in the articular/resting chondrocytes and its expression is drastically downregulated in proliferating, prehypertrophic and hypertrophic chondrocytes, as demonstrated by qRT-PCR on micro-dissected sections of the growth plate, in situ hybridization and immunostaining (Fig. 3A,B; see Fig. S4A in the supplementary material). To investigate whether it functions as a regulator of Ihh signaling, we examined the chondrocyte phenotype of Kif7-null mice (Cheung et al., 2009). Kif7 null mice die at birth and, as such, the growth plates of E16.5 mice were analyzed. Contrary to that observed in chondrocyte-specific Sufu knockout mice, we found that Kif7-null mice exhibit a reduction in the size of the proliferative zone and an expansion of hypertrophic zone (Fig. 3C,E,F). To rule out the possibility that the growth plate phenotype is due to secondary effects caused by Kif7 inactivation in other cells types, we generated chondrocyte-specific Kif7 knockout mice (Col2a1-Cre;Kif7f/f").
Col2a1-Cre;Kif7f/f mice appear normal and do not exhibit any gross defects (Fig. 3D). No obvious phenotypic difference was found in tibial growth plates of P10 Kif7-deficient mice compared with their wild-type counterparts (see Fig. S4C in the supplementary material). However, histological and in situ hybridization analyses of E16.5 Col2a1-Cre;Kif7f/f tibia revealed a reduction of proliferative zone and an expansion of hypertrophic zone (Fig. 3E,F; see Fig. S3E,F in the supplementary material) similar to those observed in Kif7–/– mice. Furthermore, the growth plates of Kif7–/– and Col2a1-Cre;Kif7f/f mice showed a reduction of cell proliferation demonstrated by Ki67 and phospho-H3 immunostaining (Fig. 3G; see Fig. S4B in the supplementary material). These results indicate that the effects of Kif7 inactivation on chondrocyte proliferation and differentiation are opposite to those of Sufu inactivation, and suggest that Kif7 acts as a positive regulator of Ihh signaling. Consistent with this notion, Kif7 inactivation leads to a downregulation of Hh target genes, Gli1 and Ptc1, as revealed by qRT-PCR analysis (Fig. 5D), and reduced Ptc1 mRNA expression in the proliferating region, as illustrated by in situ hybridization analysis (see Fig. S3A,B in the supplementary material). Therefore, in contrast to its role as a negative regulator of Hh signaling in early mouse embryos (Cheung et al., 2009), Kif7 functions positively in Ihh signaling during growth plate chondrocyte development.

**Sufu-Gli complexes are localized to the ciliary tip in the absence of Kif7**

To investigate whether loss of Kif7 affects the functional activity of Sufu in chondrocytes and vice versa, we first examined their expression in Kif7 and Sufu mutant mice. Although no difference...
in Kif7 protein or RNA levels was detected in Sufu-deficient chondrocytes (see Fig. S5A,B in the supplementary material), we found a substantial increase of Sufu protein levels in Kif7-deficient chondrocytes (Fig. 4A). To determine whether this is caused by altered RNA expression or protein stability, we examined Sufu transcript levels using qRT-PCR (Fig. 4B) and Sufu protein levels using pulse chase experiments in the presence of cycloheximide (Fig. 4C,D). These experiments demonstrated that elevated Sufu protein levels are due to increased stability in Kif7-deficient chondrocytes. Furthermore, treatment with MG132, an inhibitor of proteasome degradation, resulted in a higher level of Sufu protein in wild-type but not in Kif7-deficient chondrocytes, indicating that Sufu protein is rapidly degraded by the proteasome in wild-type and Sufu-deficient growth plates were assessed by western analysis. Actin was used as loading control. Sufu-deficient chondrocytes exhibited elevated Gli2 level and an increase in the Gli3FL::Gli3R ratio (*non-specific band). See also Fig. S3 in the supplementary material.

The observations that Smo activation promotes the dissociation of Sufu-Gli complexes at the primary cilium prompted us to investigate the ciliary localization of Sufu and Kif7 in the growth plate chondrocytes and to examine whether Kif7 influences Sufu-Gli complexes in their primary cilia. In Gli2<sup>−/−</sup>;Gli3<sup>−/−</sup> embryonic fibroblasts, where all Gli proteins are absent, Sufu is not detected in the cilia, even upon Shh stimulation, demonstrating that Gli proteins are necessary to recruit Sufu to the cilia (Tukachinsky et al., 2010; Zeng et al., 2010). Thus, Sufu immunostaining could serve as an indicator of Sufu-Gli complexes at the primary cilium. In proliferating chondrocytes (where the Hh pathway is active), Kif7 was mostly localized to the ciliary tip, whereas Sufu was found at the basal bodies in ~50% of these cells but was rarely detected at the ciliary tip (Fig. 4G,H). Similarly, Gli2 and Gli3 staining were rarely found in the primary cilium of these chondrocytes (Fig. 4G,H). Both Sufu and Kif7 signals are specific as they are absent in Sufu-deficient and Kif7-deficient chondrocytes, respectively. Together, these results indicate that there is very little Sufu-Gli2 or Sufu-Gli3 complexes present in the primary cilium of proliferating wild-type chondrocytes. Loss of Sufu has no apparent effects on the ciliary localization of Kif7 (Fig. 4G,I). Consistent with previous studies carried out in cultured fibroblasts (Hunke et al., 2010; Tukachinsky et al., 2010), Gli2 and Gli3 could be detected at the ciliary tip in ~10% of the Sufu-deficient chondrocytes (Fig. 4G,I). Both Sufu and Kif7 signals are specific as they are absent in Sufu-deficient and Kif7-deficient chondrocytes, respectively. Together, these results indicate that there is very little Sufu-Gli2 or Sufu-Gli3 complexes present in the primary cilium of proliferating wild-type chondrocytes. Loss of Sufu has no apparent effects on the ciliary localization of Kif7 (Fig. 4G,I). Consistent with previous studies carried out in cultured fibroblasts (Hunke et al., 2010; Tukachinsky et al., 2010), Gli2 and Gli3 could be detected at the ciliary tip in ~10% of the Sufu-deficient chondrocytes (Fig. 4G,I), suggesting that Gli proteins can translocate to the cilia independent of Sufu. Strikingly, in the absence of Kif7, ~20% and ~60% of proliferating chondrocytes showed Gli2 and Gli3 staining at the ciliary tip, respectively (Fig. 4G,J). Consistent with the notion that Sufu and Gli proteins are transported to the cilia as a complex, Sufu is localized to the ciliary tip of ~90% of Kif7-deficient chondrocytes (Fig. 4G,J). These observations indicate that both Sufu-Gli2 and Sufu-Gli3 complexes accumulate in
the primary cilium of Kif7-deficient chondrocytes, and suggest that when the Hh pathway is activated in chondrocytes, Kif7 plays a key role in excluding them from the primary cilia. We propose that Smo promotes the dissociation of Sufu-Gli complexes in the primary cilium through the action of Kif7.

To determine whether Ihh signaling promotes the dissociation of Sufu-Gli complexes in chondrocytes, primary chondrocyte cultures were treated with cyclopamine (Smo antagonist), purmorphamine (Smo agonist) or Shh, and Sufu-Gli2 complexes were quantified using western analysis of Gli2 followed by immunoprecipitation with Sufu-specific antibodies. There was a similar level of Gli2 in all samples. Although Gli2 levels were similar in the Sufu immunoprecipitate from control and cyclopamine-treated chondrocytes, there was a significant reduction of Gli2 levels in those of purmorphamine- and Shh-treated chondrocytes (Fig. 4K,L). These results indicate that, similar to those observed in cultured fibroblasts, Smo activation also promotes the dissociation of Sufu-Gli2 complexes in the chondrocytes.

Removal of one copy of Sufu rescues the Kif7 mutant growth plate phenotype

To investigate whether increased Sufu protein levels contribute to the reduced Hh pathway activity in Kif7-deficient chondrocytes, we generated Col2a1-Cre;Sufu<sup>fl/fl;</sup>Kif7<sup>fl/fl</sup> mice. Strikingly, these mice develop a normal growth plate (Fig. 5A). The expansion of hypertrophic zone and the reduction of proliferative zone observed in Col2a1-Cre;Kif7<sup>fl/fl</sup> mice is almost completely rescued by the simultaneous removal of one dose of Sufu (Fig. 5A-C). Importantly,
Gli1 and Ptc1 expression was also restored to near wild-type levels in Col2a1-Cre;Sufu<sup>f/f</sup>;Kif7<sup>f/f</sup> chondrocytes (Fig. 5D). These data clearly indicate that the reduced Hh pathway activity in Kif7-deficient chondrocytes is due to increased Sufu protein level and suggest that the positive role of Kif7 in Ihh signaling during chondrocyte differentiation is in part through the downregulation of Sufu protein expression as well as the dissociation of Sufu-Gli complexes (Fig. 6A,B).

**Kif7 and Sufu share overlapping functions in Hh signaling during chondrocyte development**

The co-expression of Sufu and Kif7 in articular/resting chondrocytes prompted us to analyze whether they possess additional overlapping functions in Ihh signaling and chondrocyte development. We generated Col2a1-Cre;Sufu<sup>f/f</sup> mice with additional deletion of one allele of Kif7 (Col2a1-Cre;Sufu<sup>f/f</sup>;Kif7<sup>f/+</sup>) as well as Sufu;Kif7 double knockout mice (Col2a1-
Although deletion of one allele of Kif7 in Sufu-deficient growth plate resulted in a similar phenotype as Col2a1-Cre; Sufu<sup>f</sup>f mice, Col2a1-Cre; Sufu<sup>f</sup>f; Kif7<sup>f</sup>f mice showed a more severe phenotype (Fig. 5A-C; see Fig. S3E-H in the supplementary material), including further reduction of hypertrophic zone and expansion of proliferative zone demonstrated by immunohistochemical staining and in situ hybridization analyses, similar to those observed in Col2a1-Cre; Ptch1<sup>f</sup>f mice (Fig. 2A).

Importantly, we found that inactivation of both Sufu and Kif7 leads to augmented expression of all three Hh target genes: Ptch1 and Hhip1, as well as Gli1 (Fig. 5D). Using in situ hybridization, we found that Ptch1 expression is elevated in both the resting and proliferative regions of Sufu; Kif7-deficient chondrocytes when compared with that of Sufu-deficient mice (see Fig. S3A-C in the supplementary material). Thus, in the absence of Sufu, Kif7 functions instead as a negative regulator of the Hh pathway suppressing the expression of Hh target genes. We examined the localization of Gli2 and Gli3 in the primary cilium of Sufu; Kif7-deficient chondrocytes. Interestingly, neither Gli2 nor Gli3 is localized to the ciliary tip of Sufu; Kif7-deficient chondrocytes, suggesting that Gli proteins cannot be processed in the cilium in the absence of both Sufu and Kif7 (data not shown). Together, these observations indicate that both Sufu and Kif7 could contribute to the negative regulation of Gli transcription factors and their concomitant deletion results in maximal Hh pathway activation, similar to that observed in cells lacking Ptch1. Therefore, Kif7 possesses both positive and negative roles in the regulation of Ihh signaling during chondrocyte development (Fig. 6A,B).

DISCUSSION
In this study, we demonstrated distinct and overlapping functions of Sufu and Kif7 in Hh signaling during chondrocyte development. Recent studies established that Sufu and Kif7 are evolutionarily conserved regulators of Gli transcription factors and that they both play negative regulatory roles in Hh signaling during early mouse development.
embryogenesis (Cheung et al., 2009; Cooper et al., 2005; Svard et al., 2006). There is evidence suggesting that Sufu and Kif7 are also required for high levels of Hh pathway activity (Chen et al., 2009), but the underlying mechanism is not defined. Here, we provide genetic and molecular data to indicate that Sufu is a major negative regulator of the Hh pathway in the growth plate and that Kif7 plays dual roles in controlling Hh signaling and chondrocyte development (Fig. 6A).

Phenotypic analysis of conditional knockout mice indicates that Sufu and Kif7 normally play opposing roles in Hh signaling in the developing chondrocytes. Sufu-deficient chondrocytes showed augmented Hh pathway activity, increased proliferation and delayed differentiation. By contrast, Kif7-deficient chondrocytes exhibited lower Hh pathway activity, a decrease in proliferation and an expansion of the hypertrophic zone. This could be due to the negative effect of proliferation or the stimulatory effect on chondrocyte hypertrophy. We showed that Sufu protein levels are elevated in Kif7-deficient chondrocytes and that reduction of Sufu gene dose restores Hh pathway activity and growth plate development. These results suggest that a major role for Kif7 in the growth plate is to maintain Hh signaling activity by lowering the level of Sufu, the major negative regulator of the pathway. Importantly, Kif7 also plays a negative role in suppressing Hh signaling activity in Sufu-deficient chondrocytes, as revealed by further pathway augmentation in Sufu;Kif7-deficient chondrocytes. This partly explains why the Hh pathway is not fully activated in Sufu-deficient chondrocytes and why the Sufu knockout growth plate phenotype is not as severe as those of Ptch1 knockout mice (Mak et al., 2008).

Interestingly, we also found that Hh pathway activation in Sufu;Kif7-deficient chondrocytes is not as robust as those observed in Ptch1-deficient chondrocytes. This partly explains why the Sufu knockout growth plate phenotype is not as severe as those of Ptch1 knockout mice (Mak et al., 2008). Nevertheless, our data clearly revealed a cross-regulation of Sufu and Kif7 in Ihh signaling during chondrocyte development and demonstrated that Kif7 can positively modulate Hh pathway activity through downregulation of Sufu. These results also represent the first genetic evidence that Sufu and Kif7 play overlapping regulatory roles in the negative control of the Hh signaling pathway.

Hh pathway activation promotes degradation of Sufu in some cancer cell lines (Yue et al., 2009). Our results here show that Kif7 plays a key role in controlling the stability of Sufu protein in chondrocytes. Strikingly, removal of one copy of Sufu could restore normal chondrocyte development in Kif7-deficient growth plates, indicating that the control of Sufu protein/activity by Kif7 is a crucial regulatory step in chondrocyte proliferation and differentiation. Recent studies suggest that Hh stimulation promotes the dissociation of Sufu-Gli protein complexes at the ciliary tip and that this dissociation is important for Gli activation (Humke et al., 2010; Tukachinsky et al., 2010). Interestingly, we found that Sufu is mostly excluded from the ciliary tip in wild-type chondrocytes, whereas Sufu appears to be stabilized in the ciliary tip in the absence of Kif7. This is consistent with the observation that Hh pathway activity is reduced in Kif7-deficient chondrocytes (owing to inefficient dissociation of the Sufu-Gli complexes) and supports the notion that Kif7 plays a direct or indirect role in the Hh-stimulated dissociation of Sufu-Gli protein complexes and Gli activation. However, whether the ciliary tip localization contributes to the stabilization of Sufu in Kif7-deficient chondrocytes or is related to the increased Sufu protein level awaits further investigation. Nonetheless, our results clearly demonstrated that Sufu is a regulatory target of Kif7 in Ihh signaling during chondrocyte development.

**Fig. 6. Proposed model for how Sufu and Kif7 regulate Hh signaling in growth plate chondrocytes.** (A) Schematic representation of Hh signaling regulation in chondrocytes. (a) In a wild-type cell, Sufu acts as a repressor in controlling Hh pathway activity. Kif7 possesses dual functions in regulating Gli transcription activity. (b) In the absence of Sufu, Kif7 acts as a repressor, resulting in a submaximal level of Hh pathway activation. (c) Loss of Kif7 results in increased Sufu activity, resulting in reduced Hh pathway activity. (d) Further augmentation of the Hh pathway activity is found in the absence of both Sufu and Kif7. (B) Schematic representation of Kif7-mediated dissociation of Sufu-Gli protein complexes at the primary cilium. Kif7 plays a functional role at the tip of the primary cilium in growth plate chondrocytes; it positively regulates Hh signaling activity via promoting the dissociation of Sufu-Gli complexes, leading to Gli-mediated transcriptional activation. Kif7 also function negatively in Hh signaling possibly through binding with Gli proteins in the cytoplasm.
Previous in vitro data show that Hh stimulation promotes the translocation of Kif7 to the tip of the cilia (Li et al., 2009). Here, we found that Kif7 localizes to the ciliary tip in both the resting and proliferating chondrocytes in vivo (data not shown). As the Hh pathway is inactive (as indicated by the lack of Hh target gene expression) in the resting zone, these results suggest that Kif7 might have a functional role in the primary cilium, even in the absence of Hh ligand activation. Such a role is probably important in the growth plate, allowing for the normal regulation of Gli proteins and other Hh pathway components, such as Sufu. This also hints that dynamic localization of Kif7 in the primary cilium itself is probably not the key control in regulating Hh ligand-mediated transcription in the growth plate chondrocytes. In embryonic fibroblasts, Gli2 and Gli3 do not localize to the cilium in the absence of Sufu (Chen et al., 2009). However, we found that in the Sufu-deficient chondrocytes, both Gli2 and Gli3 can still be found in the cilium. This supports the concept that Sufu is not required for the Gli proteins to localize to the cilia (Takahinsky et al., 2010) and also raises the possibility that other proteins or processes in the cilium are important for the regulation of Gli proteins, and their ability to regulate transcription.

During enchondral bone development, the coordinated differentiation of growth plate chondrocytes regulates the pace of long bone growth. Hh signaling plays a crucial role in this process, but how the cells are able to escape their proliferative state and undergo terminal differentiation is unclear. The proliferation and differentiation defects observed in Kif7- and Sufu-deficient mice suggest that Sufu and Kif7 expression plays an important role in how cells in various regions of the developing growth plate process Hh signals as they progress from the resting to hypertrophic zones. In wild-type mice, Sufu and Kif7 are highly expressed in the periarticular/resting region of the growth plate where minimal Ptch1 expression was observed. However, in the proliferating and hypertrophic regions, the level of expression of Sufu and Kif7 decreases and Ptch1 expression increases. Thus, the gradient of expression of Sufu and Kif7 negatively correlates with Ptch1 expression and Hh activity. Deletion of both Sufu and Kif7 resulted in a significant expansion of the Ptch1 expression, notably in the resting region of the growth plate, suggesting that the level of Sufu and Kif7 plays an important role in how cells process Hh signals in the growth plate. Intriguingly, the expression pattern of Sufu and Kif7 is very similar to the expression pattern of parathyroid hormone-like hormone protein (Pthhl; previously known as PthrP), which raises the possibility that interaction between those molecules may control how Pthhl regulates Hh signaling activity during development.

Taken together, we show here a novel mechanism by which Sufu and Kif7 interact to mediate Hh signaling in the growth plate (Fig. 6A,B). In wild-type chondrocytes, Kif7 positively regulates Gli-mediated transcription by downregulating Sufu protein levels, but also inhibits Gli-mediated transcription through a Sufu-independent mechanism (Fig. 6Aa). Its role in the primary cilium allows for normal Sufu-Gli complex localization and processing (Fig. 6B). In Sufu-deficient chondrocytes, the regulatory role of Kif7 on Sufu is lost; Gli protein localization in the cilium does not differ much from the situation in wild-type chondrocytes, resulting in a sub-maximal level of Hh pathway activation (Fig. 6Ab). In Kif7-deficient chondrocytes, Sufu acts unopposedly as a negative regulator of Gli-mediated transcription, and there is an increased level of Sufu-Gli complexes in the primary cilium, resulting in a greater inhibition of Hh signaling than in the wild-type situation (Fig. 6Ac). In the absence of both Sufu and Kif7, Gli proteins will not be bound to Sufu and prevented from becoming transcriptionally active. The inhibitory effect of Kif7 is relieved, leading to further augmentation of the Hh pathway activity (Fig. 6Ad), showing that the regulation of Hh signaling by Kif7 and Sufu play crucial roles in the growth plate.

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Competing interests statement
The authors declare no competing financial interests.

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