

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

Shu-Hsuan C. Hsu^{1,2}, Xiaoyun Zhang¹, Chunying Yu¹, Zhu Juan Li^{1,4}, Jay S. Wunder³, Chi-Chung Hui^{1,4} and Benjamin A. Alman^{1,2,5,*}

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 (Ptch1) 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 *Ptch1* 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 *Ptch1*-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*^{-/-} 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

¹Program in Developmental & Stem Cell Biology, Hospital for Sick Children, Toronto, Ontario, Canada. ²Institute of Medical Science, University of Toronto, Toronto, Ontario, Canada. ³Samuel Lunenfeld Research Institute and Department of Surgery, Mount Sinai Hospital, Toronto, Ontario, Canada. ⁴Department of Molecular Genetics, University of Toronto, Toronto, Ontario, Canada. ⁵Division of Orthopaedic Surgery and Department of Surgery, University of Toronto, Toronto, Ontario, Canada.

*Author for correspondence (benjamin.alman@sickkids.ca)

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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^{fl/fl}*) were generated by crossing *Col2a1-Cre* mice expressing Cre-recombinase under type II collagen regulatory elements specific to chondrocytes with *Sufu*-floxed mice containing loxP sites flanking exons 4 to exon 8 of *Sufu* (Pospisilik et al., 2010). Conditional *Ptch1*-deficient mice (*Col2a1-Cre;Ptch1^{fl/fl}*) were generated from *Ptch1*-floxed mice, which contain loxP sites flanking exon 3 of *Ptch1* (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 PCR, 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 were 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*, *Ptch1*, *Hhip1*, *Gli2*, *Gli3*, *Ihh*, *Kif7* and *Sufu* (Applied Biosystems). The gene expression levels between samples were analyzed using the 2^{-ΔΔCt} 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% CO₂ 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 8×10³ cells per cm².

Measuring protein turnover

Cells were cultured till reach confluency 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.

Immunofluorescence and in situ hybridization

Samples were fixed in 4% paraformaldehyde overnight, embedded in paraffin and sectioned. Sections (5 μm) of growth plate were cut parallel to the longitudinal axis of the bone. The sections were stained with a monoclonal antibody against acetylated α -tubulin (clone 6-11b-1, 1:1000; Sigma-Aldrich, Steinheim, Germany), and/or a monoclonal antibody against γ -tubulin (Dyomics647) (1:100; Abcam), and/or a monoclonal antibody against Sufu (1:100; Abcam), and/or a polyclonal antibody against Kif7 (1:100) at 4°C overnight. Secondary antibodies conjugated with Alexa Fluor 488 (1:100; Invitrogen) and TRITC (1:100; Jackson ImmunoResearch Laboratories) were applied for 45 minutes to detect the primary antibody. Finally the sections were mounted in 4',6-diamidino-2-phenylindole (DAPI) containing Vectashield (Vector Laboratories). Confocal images were acquired using a spinning disc confocal microscope. In situ hybridization of embryonic sections was performed as described previously (Mo et al., 1997).

Statistical analysis

For all of the data, the mean, 95% confidence interval and standard deviation were calculated for each condition, and Student's *t*-test was used to compare data sets. The threshold for statistical significance was $P < 0.05$.

RESULTS

Sufu is differentially expressed in the growth plate and is required for normal skeletal development

To determine whether Sufu might play a role in growth plate chondrocyte function, Sufu expression was examined in fetal limb cartilage. qRT-PCR analysis on microdissected sections of the growth plate and in situ hybridization were used to localize its RNA expression. Sufu is highly expressed in articular and resting chondrocytes (also known as reserve chondrocytes; the early stages of chondrocyte differentiation), whereas its expression is downregulated when cells undergo differentiation to hypertrophic chondrocytes (Fig. 1A; see Fig. S1A in the supplementary material). Immunohistochemical staining of fetal limbs revealed high level of Sufu protein expression in resting and proliferating chondrocytes, and that fewer than 10% of the prehypertrophic and hypertrophic chondrocytes are Sufu positive (Fig. 1C; see Fig. S1B in the supplementary material). The pattern of Sufu expression is similar to that of *Pthlh* (a Hh-regulated gene in the growth plate) but, by contrast, *Ihh* expression is highest in the prehypertrophic chondrocytes (Fig. 1B). Thus, Sufu is differentially expressed in the growth plate, with its highest level in early stages of chondrocyte differentiation.

To bypass the embryonic lethality of Sufu-null mice at E9.5 (Cooper et al., 2005; Svard et al., 2006), we generated chondrocyte-specific knockout mice (*Col2a1-Cre;Sufu^{fl/fl}*) 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;Sufu^{fl/fl}* 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,C in the supplementary material). Alcian Blue/Alizarin Red staining showed a significant reduction of bone ossification in *Col2a1-Cre;Sufu^{fl/fl}* 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;Ptch1^{fl/fl}*) 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 (twofold) 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 Gli 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^{-/-}* 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-*

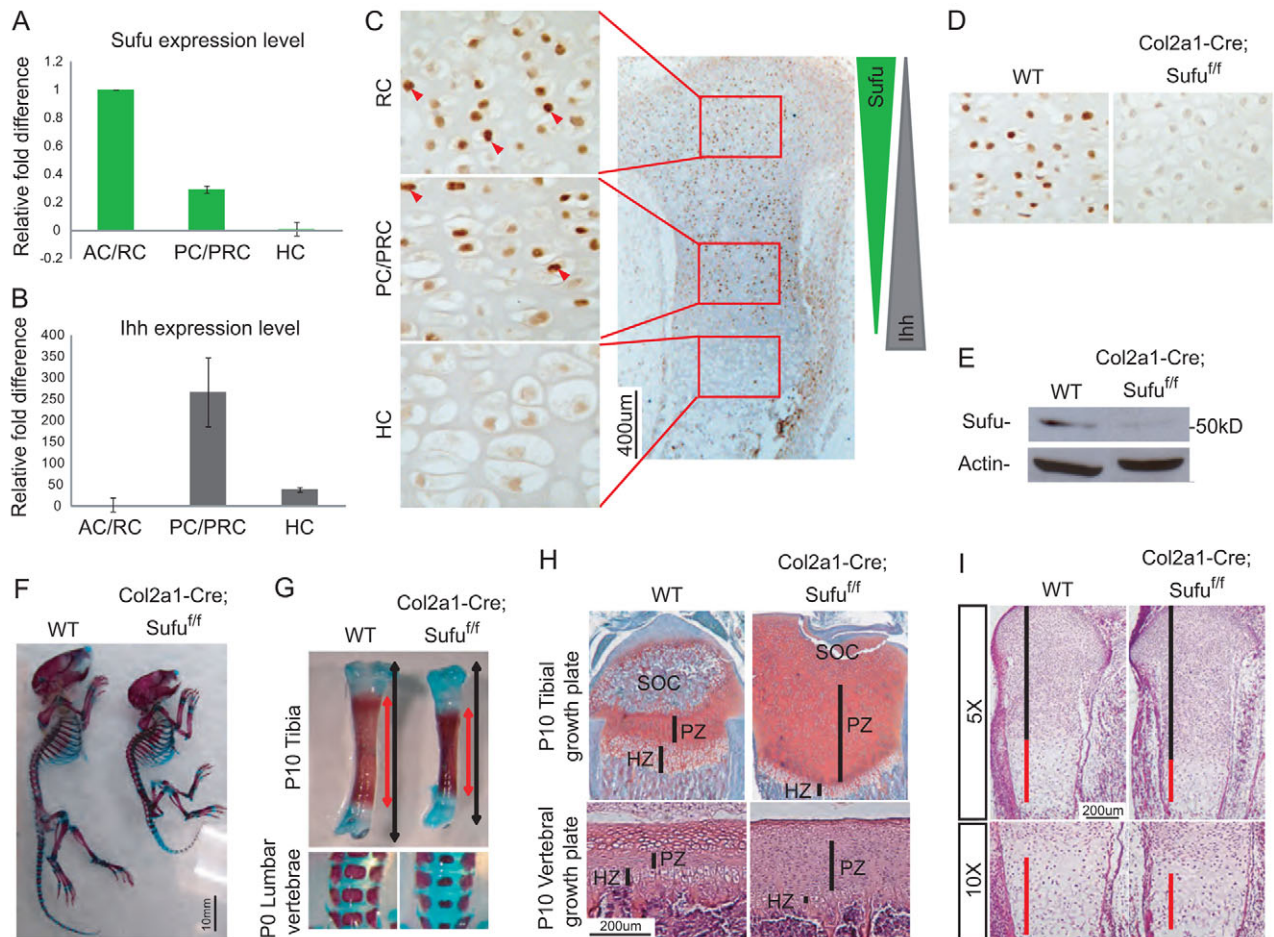


Fig. 1. *Sufu* is differentially expressed in the growth plate. (A,B) Expression of *Sufu* and *Ihh* in wild-type mouse growth plates at E18.5 assessed by qRT-PCR (AC/RC, articular chondrocytes/resting chondrocytes; PC/PRC, proliferating chondrocytes/prehypertrophic chondrocytes; HC, hypertrophic chondrocytes). Data are shown as means with 95% confidence intervals indicated ($n=3$). (C) Immunohistochemical analysis of E16.5 wild-type mouse growth plates showed that *Sufu* is highly expressed by in AC/RC and PC/PRC regions, and fewer than 10% of the HC are stained positive for *Sufu*. (D) A lack of immunohistochemical staining for *Sufu* was found in *Col2a1-Cre;Sufu^{ff}* growth plate, demonstrating efficient deletion of *Sufu* in chondrocytes. (E) Effective deletion of *Sufu* in growth plate chondrocytes was also verified using western analysis. (F) P10 *Col2a1-Cre;Sufu^{ff}* mutant mice showed significant reduction in body length compared with wild-type littermates. (G) Alcian Blue/Alizarin Red staining showed a delay in ossification in the P10 tibial bones and P0 lumbar vertebrae of the *Col2a1-Cre;Sufu^{ff}* mice. (H) Safranin O staining revealed an expansion of the proliferative zone (PZ), a decrease in the length of the hypertrophic zone (HZ) and a delayed secondary ossification (SOC) formation in the tibia of P10 *Col2a1-Cre;Sufu^{ff}* mice (above). An increased length of the PZ and a reduction of the HZ were also found in the P10 mutant vertebral growth plates, as revealed by Hematoxylin and Eosin staining (below). (I) Hematoxylin and Eosin staining revealed an expansion of the proliferative zone, which is represented by the length from the end of the tibia to the beginning of the hypertrophic zone (black bar), and a reduction of the hypertrophic zone (red bar) in the *Sufu*-deficient tibial growth plates compared with wild-type littermates at E16.5. Lower panels show magnified images of the hypertrophic zone (red bar) seen in the upper panel. See also Figs S1, S2 and S4 in the supplementary material.

Cre;Kif7^{ff}). *Col2a1-Cre;Kif7^{ff}* 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;Kif7^{ff}* 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;Kif7^{ff}* 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 *Ihh* target genes, *Gli1* and *Ptch1*, as revealed by qRT-PCR analysis (Fig. 5D), and reduced *Ptch1* 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 *Ihh* 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

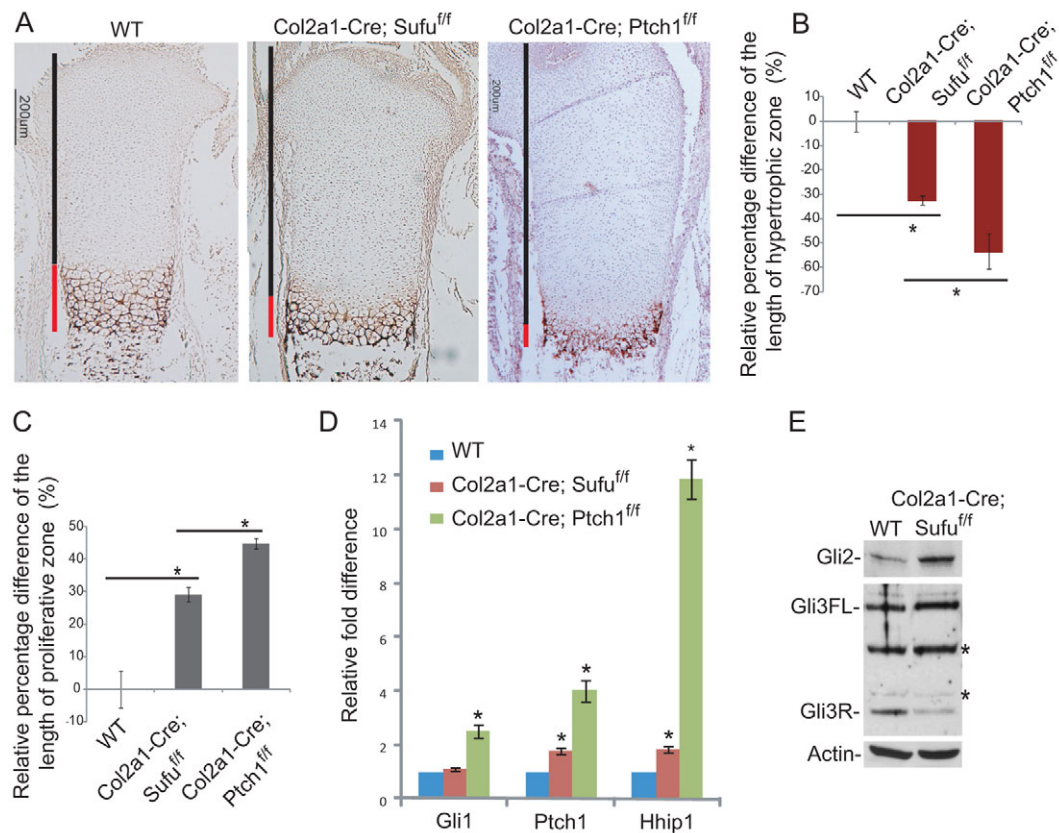


Fig. 2. Sufu negatively regulates growth plate chondrocyte differentiation. (A–C) Histological and immunohistochemical analysis of the E16.5 mouse tibia showed the length of the hypertrophic zone (red bar), which is characterized by Col10a1 staining, is reduced in *Col2a1-Cre;Sufu^{ff}* embryos compared with wild-type littermates. An increase in the size of the proliferative zone, which is represented by the length from the end of the tibia to the beginning of the Col10a1 expression zone (black bar), was found in the *Col2a1-Cre;Sufu^{ff}* embryos. This phenotype resembles the phenotype displayed by deletion of *Ptch1*, another repressor of Hh signaling, although less severe. Data are shown as means with 95% confidence intervals indicated ($n=3$). * $P<0.05$. (D) Expression of Hh target genes *Gli1*, *Ptch1* and *Hhip1* was assessed by qRT-PCR. Data are shown as means with 95% confidence intervals indicated ($n=3-6$). * $P<0.05$. (E) Protein levels of Gli2, full-length Gli3 (Gli3FL) and repressor form of Gli3 (Gli3R) 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.

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 chondrocytes (Fig. 4E,F). Together, these data suggest that Kif7 plays a role in the turnover of Sufu in wild-type chondrocytes.

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^{-/-};Gli3^{-/-}* 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 (Humke 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

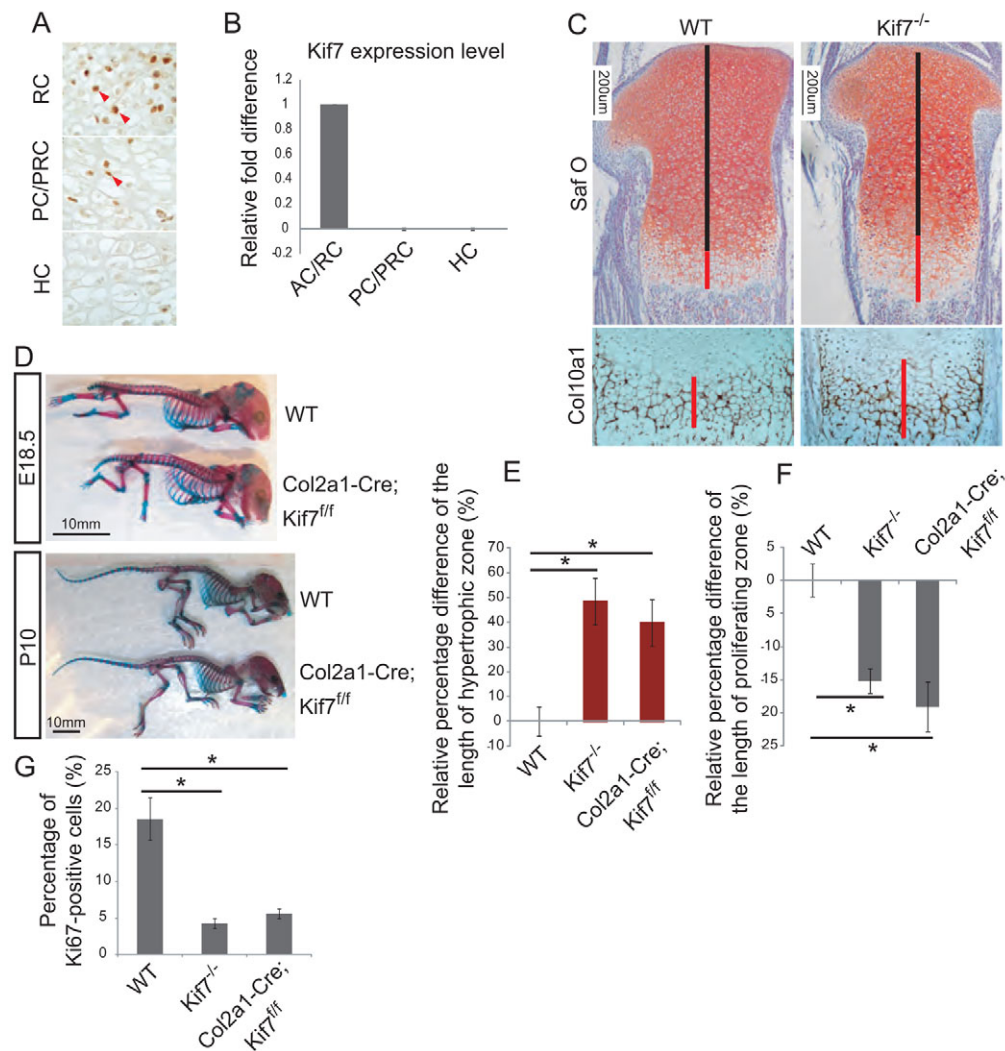


Fig. 3. *Kif7* inactivation resulted in opposite effects on chondrocyte proliferation and differentiation to those of *Sufu* inactivation.

(A) Immunohistochemical analysis of the tibia of E16.5 wild-type embryos showed that *Kif7* expressed at high levels in the AC/RC region, and its expression progressively decreases as chondrocytes differentiate. (B) Expression of *Kif7* in wild-type mouse growth plates at E16.5 assessed by qRT-PCR. Data are shown as means with 95% confidence intervals as indicated ($n=3$). (C) Safranin O and Col10a1 staining revealed an expansion of the hypertrophic zone (red bar), and reduced length of the proliferative zone (black bar) in the *Kif7* knockout mice (*Kif7^{-/-}*) compared with wild-type littermates. (D) Alcian Blue/Alizarin Red staining of wild-type and *Col2a1-Cre; Kif7^{fl/fl}* littermates at E18.5 and P10. *Col2a1-Cre; Kif7^{fl/fl}* embryos exhibited no gross abnormalities in skeletal development. (E, F) An increase in the length of the hypertrophic zone and a reduction in the length of the proliferative zone were found in *Kif7^{-/-}* and *Col2a1-Cre; Kif7^{fl/fl}* mice compared with wild-type littermates. Data are shown as means with 95% confidence intervals indicated ($n=3$). * $P<0.05$. (G) A significant decrease in the percentage of proliferating cells was found in *Kif7^{-/-}* and *Col2a1-Cre; Kif7^{fl/fl}* growth plates, as measured by immunohistochemical staining for Ki67. Data are shown as means with 95% confidence intervals indicated ($n=3$). * $P<0.05$. See also Figs S3 and S4 in the supplementary material.

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^{fl/+}; Kif7^{fl/fl}* 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^{fl/fl}* mice is almost completely rescued by the simultaneous removal of one dose of *Sufu* (Fig. 5A-C). Importantly,

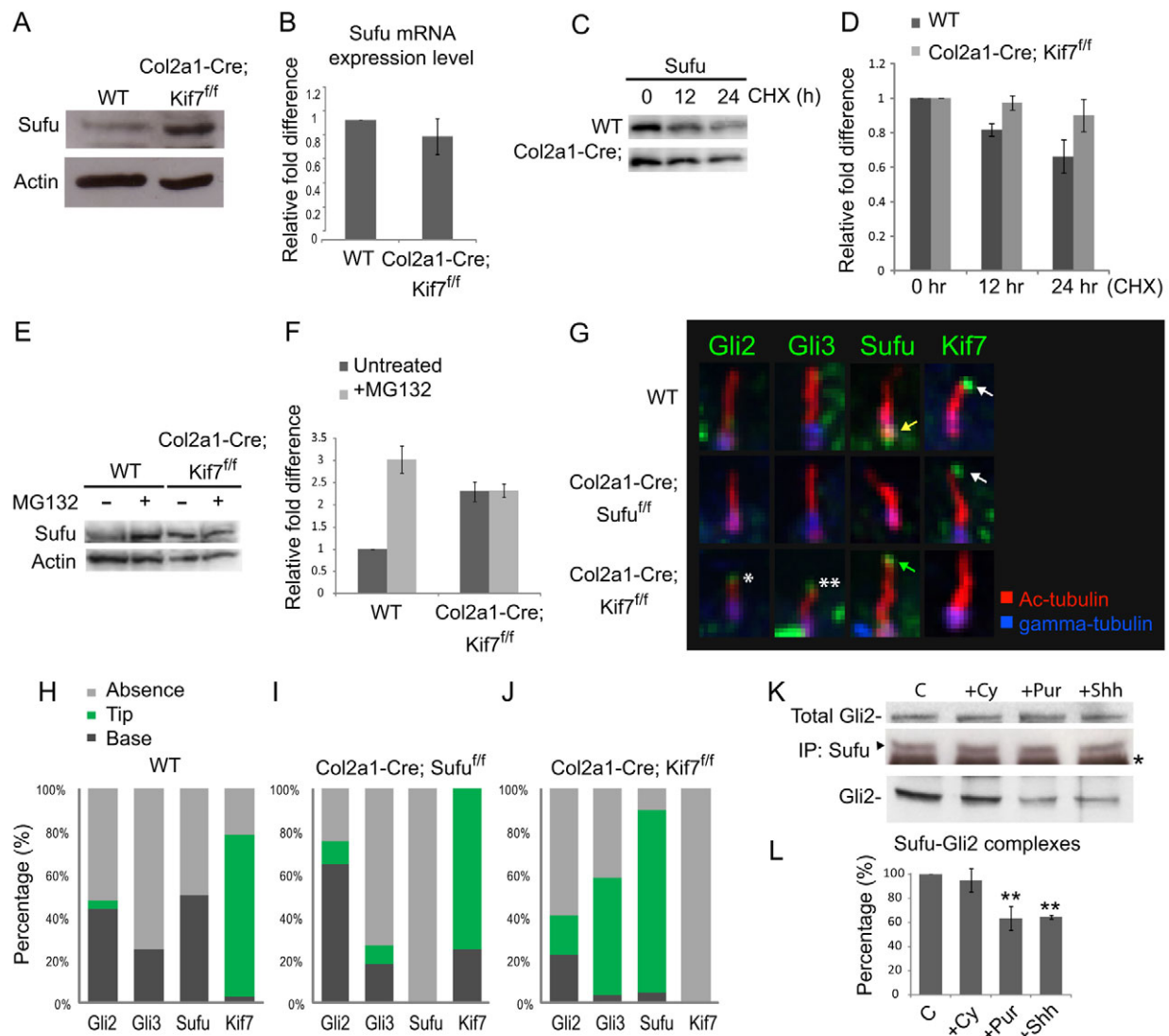


Fig. 4. Increased level of Sufu-Gli complexes in *Kif7*-deficient chondrocytes. (A) Sufu protein levels in wild-type and *Kif7*-deficient growth plates were assessed by western analysis. Actin was used as loading control. (B) *Sufu* mRNA expression levels were found to be comparable to the wild-type controls. Data are shown as means with 95% confidence intervals indicated ($n=3$). (C,D) Western analysis of Sufu in wild-type and *Kif7*-deficient chondrocytes following 20 μ M cycloheximide (CHX) treatment. (E,F) Western analysis of Sufu in wild-type and *Kif7*-deficient chondrocytes following 25 μ M MG132 treatment. Actin was used as loading control. (G) Fluorescence micrographs of cilia from wild-type, *Col2a1-Cre;Sufuff* and *Col2a1-Cre;Kif7ff* growth plate chondrocytes. Gli2 (ciliary tip localization of Gli2 indicated by an asterisk), Gli3 (ciliary tip localization of Gli3 indicated by double asterisks), Sufu (ciliary base localization of Sufu indicated by a yellow arrow; ciliary tip localization of Sufu indicated by a green arrow) and Kif7 (ciliary tip localization of Kif7 indicated by a white arrow) are detected in the green channel. Cilia are detected by staining against acetylated α -tubulin (red channel). Centrioles are detected by staining against γ -tubulin (blue channel) to label the base of the cilia. (H-J) Twenty-five to 50 ciliated cells from the proliferating zone of wild-type, *Sufuff* and *Kif7ff* growth plates were examined for Gli2, Gli3, Sufu and Kif7 ciliary localization. Data are shown as percentages. (K,L) Western analysis of total Gli2 protein and Gli2 protein immunoprecipitated (IP) with anti Sufu antibody from control (C), cyclopamine- (Cy), purmorphamine- (Pur) and Shh-treated primary chondrocyte cultures (asterisk indicates the rabbit IgG band). The level of total Gli2 is comparable in all samples. The level of Gli2 co-immunoprecipitated with Sufu is similar in cyclopamine-treated and control cells. Significantly less Gli2 co-immunoprecipitates with Sufu in purmorphamine- and Shh-treated chondrocyte cultures. Data are shown as means with 95% confidence intervals indicated. $**P<0.01$. See also Fig. S4 in the supplementary material.

Gli1 and *Ptch1* expression was also restored to near wild-type levels in *Col2a1-Cre;Sufuff;Kif7ff* 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;Sufuff* mice with additional deletion of one allele of *Kif7* (*Col2a1-Cre;Sufuff;Kif7ff*) as well as *Sufu;Kif7* double knockout mice (*Col2a1-*

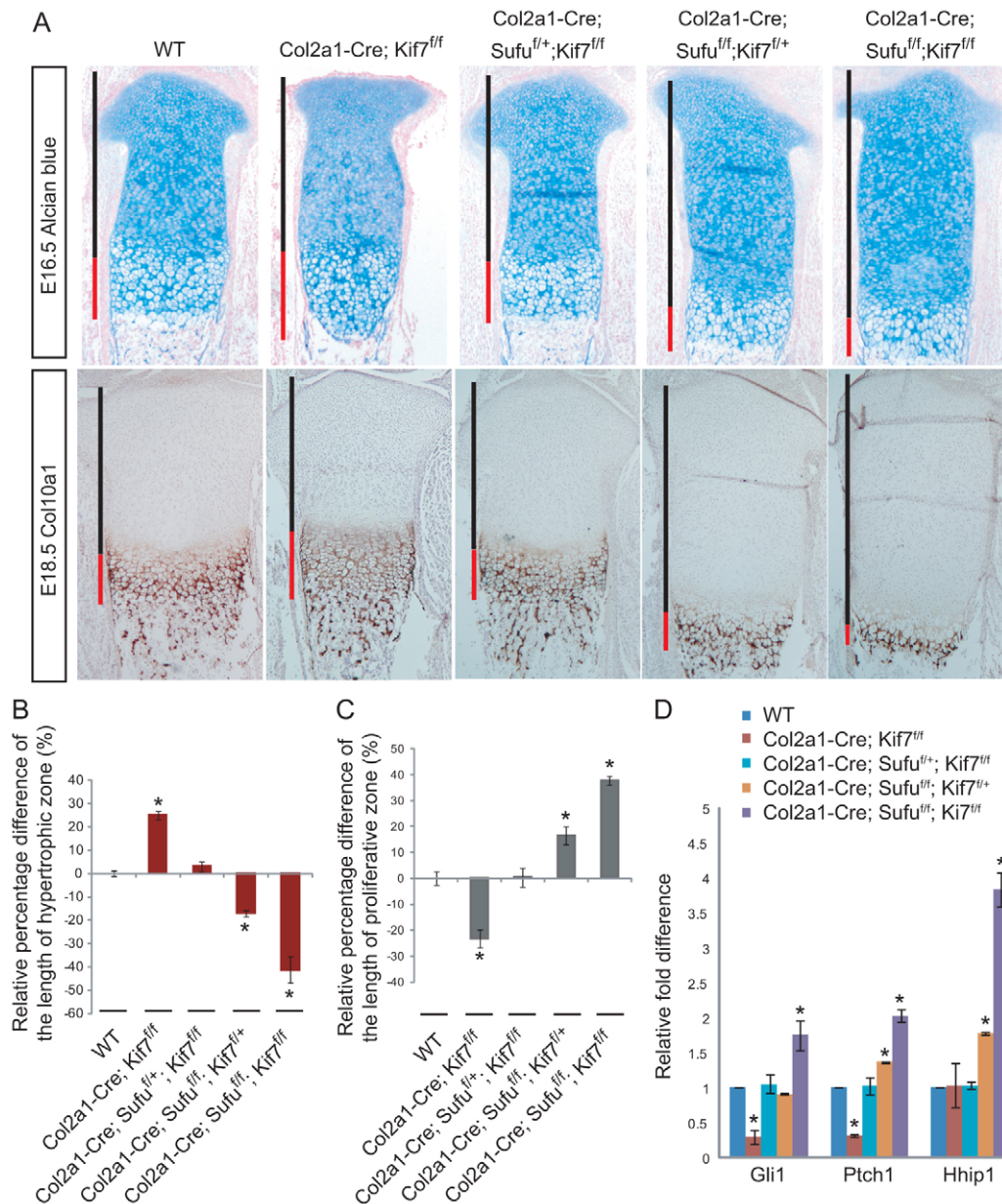


Fig. 5. A dual function for Kif7 in Hh signaling modulation. (A–C)

Histological and immunohistochemical analysis of the mouse tibial growth plates. Upper panels show Alcian Blue staining on E16.5 tibial sections; lower panels show Col10a1 immunostaining on E18.5 tibial sections (black bar, proliferative zone; red bar, hypertrophic zone). Data are shown as means with 95% confidence intervals indicated ($n=3$). * $P<0.05$. (D) Expression of *Gli1*, *Ptch1* and *Hhip1* in mouse growth plates of various mutants at E16.5 assessed by qRT-PCR. Data are shown as means with 95% confidence intervals indicated ($n=3-6$). * $P<0.05$. See also Fig S3 in the supplementary material.

Cre;Sufu^{fl/fl};Kif7^{fl/fl}). Although deletion of one allele of *Kif7* in *Sufu*-deficient growth plate resulted in a similar phenotype as *Col2a1-Cre;Sufu^{fl/fl}* mice, *Col2a1-Cre;Sufu^{fl/fl};Kif7^{fl/fl}* 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^{fl/fl}* 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 proliferating regions of *Sufu;Kif7*-deficient mice when compared with that of *Sufu*-deficient mice (see Fig. S3A,C,D 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

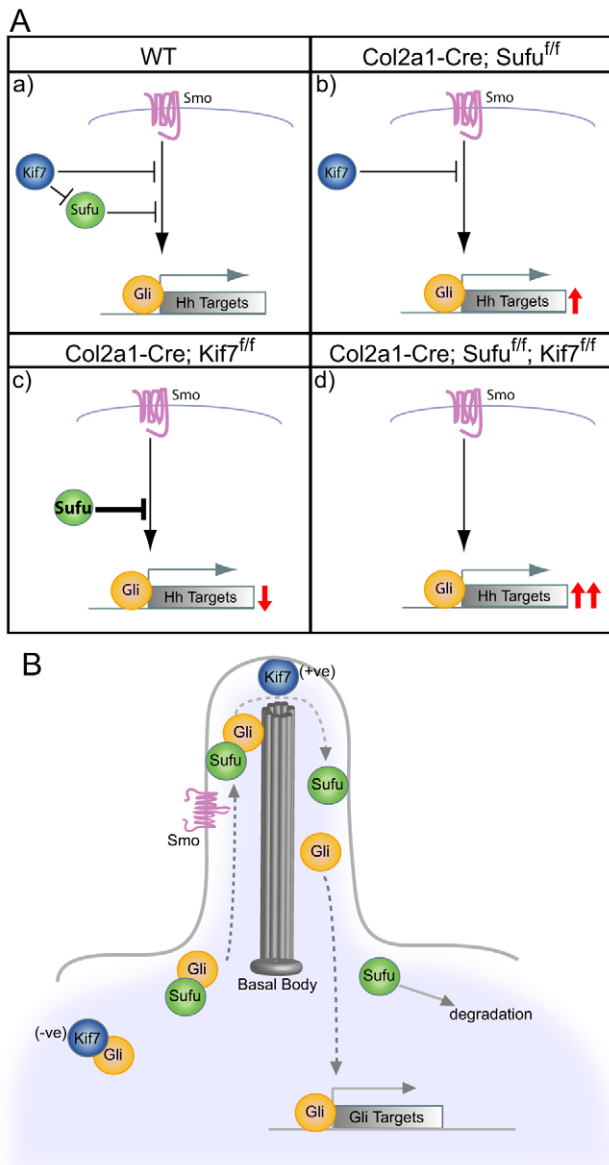


Fig. 6. Proposed model for how Sufu and Kif7 regulate Hh signaling in growth plate chondrocytes. (A) Schematic representation of Hh signaling regulation in chondrocytes. (a) In a wild-type cell, Sufu acts as a repressor in controlling Hh pathway activity. Kif7 possesses dual functions in regulating Gli 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.

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 Ihh signaling and chondrocyte development (Fig. 6A).

Phenotypic analysis of conditional knockout mice indicates that Sufu and Kif7 normally play opposing roles in Ihh 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. It remains to be determined whether this is due to the requirement of Sufu and/or Kif7 for full transcriptional activity of Gli proteins, or related to the ability of Ptch1 to sequester Hh ligands in the extracellular environment. 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 to date 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.

Previous *in vitro* data show that Hh stimulation promotes the translocation of Kif7 to the tip of the cilia (Liem 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 cilia. This supports the concept that Sufu is not required for the Gli proteins to localize to the cilia (Tukachinsky 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 endochondral 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 (Pthlh; previously known as PthrP), which raises the possibility that interaction between those molecules may control how Pthlh 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.

Supplementary material

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