Suppressor of Fused (Sufu) Mediates the Effect of Parathyroid Hormone-like Hormone (Pthlh) on Chondrocyte Differentiation in the Growth Plate*

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Background: Hh and Pthlh signaling pathways play important roles in regulating growth plate chondrocyte differentiation.

Results: Pthlh increases Sufu protein levels, and Sufu is required for the effect of Pthlh on chondrocyte differentiation.

Conclusion: Pthlh regulates chondrocyte differentiation and Gli activity in a Sufu-dependent manner.

Significance: The interaction between Pthlh, PKA, Sufu, and Gli transcriptional activities explains how Pthlh regulates chondrocyte differentiation.

Growth plate chondrocytes undergo a coordinated process of differentiation, regulating long bone growth. Parathyroid hormone-like hormone (Pthlh) inhibits hypertrophic differentiation in the growth plate chondrocytes and reduces Hedgehog (Hh) signaling. In mice lacking the Hh mediator Suppressor of fused (Sufu), Pthlh treatment resulted in the up-regulation of Hh activity and an increased number of hypertrophic chondrocytes. Furthermore, Pthlh increased Sufu protein levels, and in chondrocytes lacking Sufu, it was unable to process Hh-regulated Gli transcription factors. Pthlh regulates chondrocyte differentiation and Gli activity in a Sufu-dependent manner, with Sufu acting as a molecular switch in its regulation of differentiation.

Long bones grow through a process of endochondral ossification, in which growth plate cartilage undergoes a coordinated process of proliferation and differentiation, ultimately resulting in replacement by bone. Two signaling pathways that coordinate growth plate chondrocyte differentiation are the parathyroid hormone-like hormone (Pthlh; also called PTHrP) and Indian hedgehog (Ihh). Growth plate chondrocytes progress from a resting state through a proliferative, prehypertrophic, hypertrophic, and a terminal differentiation phase. Ihh is expressed in prehypertrophic chondrocytes, where it inhibits the onset of hypertrophic differentiation, in part by signaling periar-ticular chondrocytes to up-regulate the expression of Pthlh, which inhibits chondrocyte hypertrophic differentiation. Ihh and Pthlh act in a feedback loop, regulating each other’s activity, thus regulating the pace of chondrocyte differentiation. Data from Pthlh-deficient mice show that Ihh can also independently regulate the onset of hypertrophy. This independent function may play a more important role in postnatal growth, where Pthlh levels are lower than during fetal development (1–7).

Hedgehog (Hh) signaling is activated in response to an appropriate ligand binding to the Patched 1 (Ptch1) receptor. This binding leads to Smoothened (Smo) protein activation, ultimately resulting in the transcription of target genes. In Droso-sphila, Hh signaling activates the transcription factor Cubitus interruptus (Ci). In the absence of Hh binding, Ci is cleaved to a transcriptional repressor, whereas upon Hh binding, Smo activation allows uncleaved Ci to translocate into the nucleus, where it functions as a transcriptional activator. In mammals, there are three transcription factors that mediate Hh signaling: Gli1, Gli2, and Gli3. Data from studies in mice in the context of the development of major organ systems, including the limb, suggest that Gli1 is functionally redundant. Gli2 has not been demonstrated to be processed in vivo and, as such, functions as an activator, whereas Gli3 is processed similarly to Ci and can act as an activator or repressor of Hh-mediated transcriptional activation. Interestingly, Gli1 and Ptch1 are Hh-activated target genes in a variety of cell types and are frequently used to assay the level of Hh signaling activation. It has been speculated that their regulation plays a feedback role in controlling the level of Hh transcriptional activity (1–7).

Suppressor of fused (Sufu) and Kif7 are essential regulators of Hh signaling (8–11). Studies in knock-out mice show that Sufu acts as a repressor of the Hh signaling pathway, whereas Kif7 can function as either a positive or negative regulator of Hh signaling in chondrocytes. Kif7 positively regulates Hh signaling activity in part by regulating Sufu protein levels and also by excluding Sufu–Gli complexes from the primary cilium, allowing Gli proteins to enter the nucleus. Kif7 negatively regulates Hh signaling independent of Sufu (12). Sufu and Kif7 play important roles in regulating chondrocyte differentiation. They are both highly expressed by articular and resting chondrocytes.
in the growth plate, interestingly, the same region where Pthlh is expressed (12). Histological analysis of mice lacking Sufu in chondrocytes showed delayed formation of secondary ossification centers, expansion of the proliferating zone, and reduction of the hypertrophic zone. Kif7-null mice exhibit a reduction in the size of the proliferative zone and an expansion of the hypertrophic zone.

The effect of Pthlh on growth plate chondrocyte differentiation and proliferation is dependent in part on the transcription factors Gli2 and Gli3 (13). In limb explant cultures, Pthlh treatment inhibited Col10a1 expression, a marker of growth plate chondrocyte differentiation, and increased chondrocyte proliferation. This effect was substantially enhanced in Gli2+/− limbs, was absent in Gli3−/− limbs, and was only partially inhibited by Hh ligand blockade. Pthlh negatively regulated Gli-mediated transcription in cell cultures and regulated the level of the repressor form of Gli3 in a PKA-dependent manner. Thus, the interaction between Pthlh and Gli3 plays an important role in regulating growth plate chondrocyte differentiation.

PKA, the main effector of Pthlh in chondrocytes, can stimulate Sufu protein stability through phosphorylation (12, 14). This raises the possibility that Pthlh and the Hh signaling intermediates Sufu and Kif7 interact in growth plate cells, regulating chondrocyte hypertrophic differentiation. Here, we examined how the interaction of Pthlh, Sufu, and Kif7 in the growth plate regulates Hh signaling transcriptional activation and chondrocyte differentiation.

**EXPERIMENTAL PROCEDURES**

**Mice**—The generation of Kif7-deficient mice was reported previously (11). Conditional Sufu-deficient mice (Col2a1-Cre; Sufu<sup>fl/fl</sup>) were generated by crossing Col2a1-Cre mice expressing Cre recombinase under the control of type II collagen regulatory elements specific to chondrocytes with Sufu-floxed mice containing loxP sites flanking exons 4–8 of Sufu. Embryonic mice were obtained from timed pregnancies, and the genotypes of the various mice were determined as described (11, 15). In all cases, littermate mice were used as controls. The recombination efficiency in all conditional mutants was confirmed by PCR, Western analysis, and examination of the Cre drivers crossed with a Rosa-26 reporter line. All mice are on the 129/Sv background. A mouse protocol describing the above experimental procedures was approved by the Animal Care Committee of The Hospital for Sick Children.

**Explant Cultures**—Hind limb organ cultures from 16.5-day post-coitum embryos were established as described previously (3, 4, 13, 16). Limbs were placed on a Nucleopore filter inside a Petri dish. Embryonic mice were treated with various agents, whereas the other served as a control. 10<sup>−7</sup> M Pthlh (Bachem, King of Prussia, PA) was added to activate Pthlh signaling. The PKA inhibitor H-89 dihydrochloride (Alexis Biochemicals) was used at 20 μM. These reagents were used alone or in combination for 2 consecutive days, with media changed each day, replenishing the specific agent utilized. Each explant experiment was undertaken three times or more using limbs from three different fetal mice and littermate controls.

**Histological Analysis and Immunohistochemistry**—Samples were fixed overnight in 4% paraformaldehyde, 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 (17–21). The proximal tibial growth plate was used for all analyses to minimize morphological variations due to anatomic location. Safranin O staining was performed using standard techniques.

**Western Analysis**—Western blot analysis was performed using standard protocols. Immunoblotting was performed overnight at 4°C with the following primary antibodies: anti-Gli3 antibody (1:800; Santa Cruz Biotechnology), anti-actin antibody (1:10,000; Oncogene), anti-Sufu antibody (22), anti-Gli2 antibody (18), and anti-phospho-Sufu antibody (Signalway Antibody, Nanjing, China) (14). Real-time Quantitative PCR—RNA isolated from at least three independent experiments was analyzed by quantitative RT-PCR (qRT-PCR)<sup>2</sup> 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, Gli2, Gli3, Smo, and Sufu (Applied Biosystems). The gene expression levels between samples were analyzed using the 2<sup>−ΔΔCt</sup> method (24). Either Gapdh or β-actin (Applied Biosystems) was used as an endogenous control for target gene normalization.

**Primary Growth Plate Chondrocyte Cultures and Pulse-Chase Assay**—The chondrocyte isolation protocol was modified from previously published methods (25). Growth plates of hind limbs from 16.5-day post-coitum embryos were isolated and incubated in collagenase type 4 solution (3 mg/ml; Worthington) for 45 min at 37°C 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 D solution and incubated overnight at 37°C. Collagenase type 4 solution containing chondrocytes was collected. Cells were washed with PBS and seeded at a density of 8 × 10<sup>5</sup> cells/cm<sup>2</sup>. Primary cells were cultured until they reached confluence before cycloheximide treatment (20 μM; Calbiochem) to block de novo protein synthesis for the duration of the experiment. Transcend<sup>TM</sup> protein fluorescent labeling of proteins (Promega) was performed according to the manufacturer’s instructions to pulse label new protein synthesis for 12 h. The amount of labeled Sufu protein was detect after 12 and 24 h in the cell cultures with or without Pthlh treatment. Actin protein levels were used as a loading control.

**Statistical Analysis**—The mean, 95% confidence interval, and S.D. were calculated for each set of data, and Student’s t test was utilized to compare data sets. The threshold for statistical significance was p < 0.05.

**RESULTS**

**Pthlh Regulates Chondrocyte Differentiation in a Sufu-dependent Manner**—To determine whether Sufu mediates the effect of Pthlh on growth plate chondrocyte differentiation, we exam-

2 The abbreviation used is: qRT-PCR, quantitative RT-PCR.
ined the effects of Pthlh treatment on chondrocyte hypertrophic differentiation in embryonic day 16.5 tibial explants from wild-type and Sufu-deficient (Col2a1-Cre;Sufuf\textsuperscript{f/f}) mice. Mice lacking Sufu in chondrocytes show delayed formation of secondary ossification centers, expansion of the proliferating zone, and reduction of the hypertrophic zone compared with wild-type littermates. We found that Pthlh treatment reduced the length of the hypertrophic zone (which can be represented by Col10a1 staining) in explants from wild-type limbs (Fig. 1, A and B). This finding is consistent with the known inhibitory role of Pthlh in chondrocyte hypertrophic differentiation. Unexpectedly, Pthlh treatment of Sufu-deficient explants resulted in an expansion of the hypertrophic zone (Fig. 1, A and B). Pthlh also has a stimulatory effect on chondrocyte proliferation (26). We found that Pthlh treatment resulted in an expansion of the proliferative zone (represented by the length from the end of the tibia to the beginning of the hypertrophic zone) in wild-type limbs (Fig. 1, A and C). A similar increase in the proliferative zone was observed in the Pthlh-treated Sufu-deficient limbs (Fig. 1, A and C), showing that in contrast to its role in regulating chondrocyte differentiation, Sufu is not required for Pthlh to promote growth plate chondrocyte proliferation.

The effects of Pthlh on normal growth plate chondrocytes are mediated primarily by PKA (13, 21, 27). Treatment with the PKA inhibitor H-89 abolished the Pthlh effects on both wild-type and Col2a1-Cre;Sufuf\textsuperscript{f/f} explants (Fig. 1, A–C). Thus, PKA and Sufu are both required for Pthlh to inhibit chondrocyte hypertrophic differentiation, with PKA acting upstream of Sufu.

**Kif7 Is Not Required for Pthlh to Inhibit Chondrocyte Differentiation**—Kif7 controls Hh activity in part by restricting Sufu function in growth plate chondrocytes (12). To determine whether Kif7 is required for the effect of Pthlh on chondrocyte differentiation, we performed tibial explants from embryonic day 16.5 Col2a1-Cre;Kif7\textsuperscript{f/f} embryos. Kif7-null mice exhibited a reduction in the size of the proliferative zone and an expansion of the hypertrophic zone compared with wild-type littermates. Pthlh exerted an inhibitory effect on chondrocyte differentiation in Kif7-deficient limbs, similar to that observed in wild-type explants (Fig. 2, A and B). Thus, Pthlh negatively regulates chondrocyte differentiation through a Kif7-independent mechanism. In growth plate chondrocytes, Kif7 demonstrates a negative function on Hh signaling in the absence of Sufu (12). Because Kif7 plays a different role in regulating Hh pathway...
activity in the absence of Sufu, we examined mice lacking both Sufu and Kif7. Pthlh treatment resulted in an expansion of the hypertrophic zone in these mice lacking both Sufu and Kif7 (Fig. 2, A and B), similar to that observed in Col2a1-Cre;Sufu^fl/fl explants (Fig. 1, A and B), indicating that Sufu is required for the inhibitory effect of Pthlh on chondrocyte differentiation, independent of Kif7.

**Sufu Is Required for Pthlh-mediated Gli Processing**—One mechanism by which Pthlh negatively regulates Gli-mediated transcription is Gli3 processing (13). To investigate if Sufu plays a role in the interaction between Pthlh and Gli transcription factors, we examined the levels of Gli2, full-length Gli3, and a truncated repressor form of Gli3 in wild-type and Col2a1-Cre;Sufu^fl/fl growth plate chondrocytes with or without Pthlh treatment. qRT-PCR analysis showed comparable levels of Gli2 and Gli3 mRNAs in wild-type and Sufu-deficient chondrocytes (Fig. 3A). In wild-type limbs, Pthlh treatment resulted in a decreased level of Gli2. However, this effect was not seen in the Sufu-deficient chondrocytes (Fig. 3B). Consistent with previous data, Western analysis revealed a decrease in the ratio of full-length Gli3 to Gli3 truncated repressor upon Pthlh signaling activation in wild-type chondrocytes. However, in the absence of Sufu, Pthlh treatment resulted in an increase in the ratio of full-length Gli3 to Gli3 truncated repressor in Sufu-deficient chondrocytes (Fig. 3C).

To examine whether Sufu influences Pthlh-mediated regulation of Hh signaling activity, primary chondrocytes from wild-type and Sufu-deficient mice were treated with Pthlh, and expression of the Hh target genes Gli1 and Ptc1 was analyzed by qRT-PCR. Consistent with the repressor role of Sufu in the Hh signaling pathway, significant increases in Gli1 and Ptc1 expression levels were found in Sufu-deficient chondrocytes. In wild-type cells, Pthlh treatment resulted in reduced expression levels of Gli1 and Ptc1. In the absence of Sufu, Pthlh treatment posed a positive effect on Gli1 and Ptc1 expression (Fig. 3, D and E), which shows that Sufu is required for the inhibitory function of Pthlh on Hh signaling activity in chondrocytes. The level of expression of the Hh mediator Smo did not vary between any of the genotypes or with Pthlh treatment.

**Pthlh Regulates Sufu Protein Levels through a PKA-dependent Process**—Given the important role of Sufu in Pthlh-mediated regulation of chondrocyte hypertrophy, we investigated how Pthlh and Sufu might interact, analyzing Sufu levels in Pthlh-treated wild-type explant cultures. In wild-type chondrocytes, Pthlh treatment resulted in a significant increase in the protein level of Sufu (Fig. 4). The level of the Sufu transcript in these cells remained comparable with that in the controls as determined by qRT-PCR analysis (Fig. 4B). To determine whether Pthlh regulates Sufu protein stability, we examined the level of fluorophore-labeled Sufu protein, after the labeling reagents were removed from the cell cultures, with or without Pthlh added to the medium. There was a significantly higher level of Sufu protein 12 and 24 h after the pulse labeling when the cells were treated with Pthlh (Fig. 4C). Because the ubiquitin-mediated degradation of Sufu protein can be inhibited by phosphorylation at Ser-346 by PKA, we examined the ability of Pthlh to regulate this phosphorylation site. There was an increase in Ser-346-phosphorylated Sufu with Pthlh treatment (Fig. 4D). MG132 treatment, which inhibits ubiquitin-mediated degradation, caused an increase in Sufu protein levels, and treatment with Pthlh no longer altered Sufu protein levels in the presence of MG132 (Fig. 4E). Taken together, these data are consistent...
with the notion that Pthlh phosphorylates Sufu, preventing its ubiquitin-mediated degradation. As expected, Sufu protein was not found in cell lysates derived from \textit{Col2a1-Cre; Sufuf/f} explant cultures (Fig. 4A), demonstrating efficient deletion of \textit{Sufu} in mutant chondrocytes.

**DISCUSSION**

Here, we found that Sufu is specifically required for the inhibitory effect of Pthlh on chondrocyte hypertrophic differentiation. Because its absence results in a reversal of the sequel of Pthlh stimulation of chondrocyte hypertrophic differentiation, it serves as a molecular switch in Pthlh function. In contrast to the role we found for Sufu, Kif7 is not required for Pthlh to influence chondrocyte hypertrophy in the growth plate. Thus, Sufu is a critical link between Hh and Pthlh signaling in the control of chondrocyte hypertrophic differentiation.

Phosphorylation of Sufu protein by PKA can positively regulate its stability in cultured fibroblasts (14), and we found that Pthlh treatment similarly increased Sufu phosphorylation in growth plate chondrocytes, increasing the stability of the protein. Intriguingly, Kif7 functions positively in the regulation of Hh pathway activity through regulating Sufu levels (12). Thus, the regulation of Sufu levels may be a common mechanism controlling Hh-mediated transcriptional activity, and Sufu protein stability may be a central node in regulating Hh signaling in the growth plate.

In the absence of a Hh ligand, Sufu sequesters Gli proteins in the cytoplasm, preventing Hh pathway activation (15, 28–30), and promotes processing of full-length Gli3 into its repressor form (31). Here, we found that the stimulatory effect of Pthlh on the formation of the Gli3 repressor form was absent in Sufu-deficient chondrocytes, thus providing additional support for the notion that Sufu plays a critical role in Gli3 protein processing. Pthlh also promoted Gli2 degradation in wild-type chondrocytes, and this effect was not seen in chondrocytes lacking Sufu. Taken together, the results suggest a model by which Pthlh positively regulates Sufu protein stability through PKA-mediated phosphorylation and promotes Sufu-mediated Gli2 degradation and Gli3 protein processing, ultimately inhibiting chondrocyte hypertrophic differentiation (Fig. 4F).

Through PKA, Pthlh could also have other effects on Hh signaling mediators. For instance, the level of Gli2 can be regulated by sumoylation, and PKA phosphorylation enhances Gli2 sumoylation, whereas Hh signaling inhibits it (32). Interestingly, we did not find that Pthlh in the absence of Sufu would regulate Gli2 levels, suggesting that this mechanism does not operate independently in growth plate chondrocytes. However, it does raise the possibility that there may be other mechanisms by which Pthlh and Hh signaling regulate each other. It is intriguing to speculate about the role of Sufu in such other PKA-mediated interactions, as in the regulation of Gli2 sumoylation.

Previous work shows that Sufu and Kif7 are highly expressed in the periarticular/resting region of the growth plate (12). Minimal Ptch1 expression is observed in this same region. However, in the proliferating and hypertrophic regions, the level of expression of Sufu and Kif7 decreases, and Ptch1 expression increases (12). Thus, the gradient of expression of Sufu and Kif7 negatively correlates with Ptch1 expression.
expression and Hh activity and, as such, mediates the consequences of Hh ligand activation. The phenotype of Sufu-deficient growth plates (12) suggests that down-regulation of Sufu is important for normal growth plate chondrocytes to undergo terminal differentiation, allowing cells to be maintained in a hypertrophic state.

In the normal growth plate, Pthlh is expressed in periartricular/resting cells, the same region as Sufu. Pthlh diffuses to other cells, such as those in hypertrophic growth plate chondrocytes, where Sufu is not expressed. Because Pthlh stabilizes Sufu, the expression of this ligand in the periartricular/resting region of the growth plate may act to further elevate Sufu protein levels, inhibiting the downstream transcriptional consequences of Hh ligand activation. This suggests that down-regulation of Sufu is required for normal chondrocyte differentiation, a notion supported by the finding that Sufu acts as a molecular switch in the effect of Pthlh on chondrocyte differentiation. Such a critical role for Hh signaling in chondrocyte differentiation is supported by data from Pthlh-deficient mice (7), suggesting that Hh signaling can independently regulate the onset of hypertrophy.

In the absence of Sufu, Pthlh and its regulation of PKA can have other effects on chondrocyte behavior and perhaps also on Hh-mediated signaling. For instance, Pthlh can regulate cell proliferation through mechanisms that may operate independently of Hh signaling (23, 26, 33). Such mechanisms may be responsible for the changes seen in the growth plate with Pthlh stimulation even in the absence of Sufu. The interactions between Pthlh, PKA, Sufu, and Gli transcriptional activities we observed in the growth plate may also play a role in other cell

**FIGURE 4.** Pthlh phosphorylates Sufu, resulting in inhibition of its degradation, and proposed model for how Pthlh acts through Sufu to regulate chondrocyte differentiation. A, Pthlh (PTH) treatment results in elevated levels of Sufu protein in wild-type growth plate chondrocytes. The effective deletion of Sufu in knock-out mouse chondrocytes was verified by Western analysis. Actin was used as loading control. Un, untreated. B, qRT-PCR analysis of Sufu expression levels in wild-type chondrocytes with or without Pthlh (PTHLH) treatment. C, pulse chase data showing that the rate of decline in Sufu protein slows with PTHLH treatment, a representative immunoblot and graphical data showing means and 95% confidence intervals are shown. D, Ser-346-phosphorylated Sufu levels increase with Pthlh treatment. Data are shown as means and 95% confidence intervals. *, significant difference from the control (untreated; p < 0.05). F, proposed model by which Pthlh promotes Sufu-dependent Gli2 degradation and Gli3 processing, ultimately inhibiting chondrocyte hypertrophic differentiation (circled D). E, treatment with MG132 inhibits Sufu degradation and PTH will not alter its Sufu level in the presence of MG132. A representative immunoblot and mean and 95% confidence intervals are given in a graphical form. F, Pthlh stimulates chondrocyte proliferation (circled P) in a Sufu-independent manner. Gli3R, Gli3 truncated repressor.
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types, e.g. in neural tumors, where PKA and Hh signaling are active and could interact to control cell behavior.

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