Regulators of Hedgehog signaling in chondrocytes: Sufu, Kif7, and primary cilium

By

Shu-Hsuan Claire Hsu

Doctor of Philosophy
Institute of Medical Science
University of Toronto

© Copyright by Shu-Hsuan Claire Hsu 2012
Regulators of Hedgehog signaling in chondrocytes: Sufu, Kif7, and primary cilium

Shu-Hsuan Claire Hsu

Doctor of Philosophy

Institute of Medical Science
University of Toronto

2012

Abstract

The Hedgehog (Hh) signaling pathway has received attention regarding its important role in embryonic development, however the mechanism by which pathway regulators, such as Suppressor of fused (Sufu), Kinesin family member 7 (Kif7), and primary cilium, mediate Hh signaling transduction is not entirely understood. The work presented here examines the roles of Sufu and Kif7 in regulating Hh signaling in growth plate chondrocytes, as well as how they mediate parathyroid hormone-like hormone (Pthlh) signaling during chondrocyte development. I show here that Sufu and Kif7 are essential regulators of Indian hedgehog (Ihh) signaling. While Sufu negatively regulates Gli transcription factors, Kif7 functions both positively and negatively in chondrocytes. Kif7 plays a role in Sufu protein degradation and the exclusion of Sufu-Gli complexes from the primary cilium. Importantly, halving the dosage of Sufu restores normal Hh pathway activity and chondrocyte development in Kif7-null mice, demonstrating that the positive role of Kif7 is to restrict the inhibitory function of Sufu. Furthermore, Kif7 exerts inhibitory function on Gli transcriptional activity in chondrocytes when Sufu function is absent. Therefore, Kif7 regulates the activity of Gli transcription factors through both Sufu-dependent and Sufu-independent mechanisms. I show that Sufu is crucial for mediating the negative effect of Pthlh
on Gli transcriptional activity and chondrocyte hypertrophic differentiation, whereas Kif7 and primary cilium are dispensable in this process. Although primary cilium is required for Hh ligand-mediated activation of Gli transcription, Pthlh negatively controls Gli transcriptional activity in a cilia-independent manner. The results of this work provide insight into how Hh signaling is regulated by Sufu and Kif7 in the context of primary cilium, but also suggest Sufu serves as an important link between Ihh and Pthlh signaling during growth plate chondrocyte development.
I sincerely thank my supervisor Dr. Benjamin Alman for his guidance and encouragement. Enormous thanks to Dr. Chi-Chung Hui for his invaluable suggestions and guidance. I am also very grateful to my committee members, Dr. David Bazett-Jones and Dr. Martin Post, for their comments and suggestions. Thanks to Xiaoyun Zhang from the Hui lab for helping me with the western analysis. Thanks to the entire Alman lab, past and present for their scientific and moral support. Thanks to my family and friends for their love, for cheering me on, and their patience with me these past few years. Finally thanks to my boyfriend Robert for his support and for putting a smile on my face when things did not work at the bench.
# Table of contents

Abstract ........................................................................................................................................ ii

Acknowledgments ................................................................................................................................ iv

Table of contents .......................................................................................................................... v

List of abbreviations .................................................................................................................... x

List of figures ..................................................................................................................................... xii

Chapter 1 Introduction .................................................................................................................. 1

1.1 Summary ................................................................................................................................... 2

1.2 Overview of growth plate development ..................................................................................... 3

1.3 Indian hedgehog (Ihh) and Parathyroid hormone-like hormone (Pthlh) signaling in growth plate development .................................................................................................. 4

1.3.1 Ihh/Pthlh feedback loop ........................................................................................................ 4

1.3.2 Pthlh-dependent and –independent effects of Ihh .................................................................. 4

1.3.3 Actions of Pthlh in the growth plate ...................................................................................... 5

1.4 Ihh and Pthlh signaling pathways regulate Gli transcription factors ........................................ 7

1.5 Regulators in Hedgehog (Hh) signaling pathway: Sufu and Kif7 ........................................... 9
Chapter 2 Kif7 promotes Hh signaling in growth plate chondrocytes by restricting the inhibitory function of Sufu

2.1 Summary

2.2 Introduction

2.3 Results

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

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

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

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

2.3.5 Removal of one copy of Sufu rescues the Kif7 mutant growth plate phenotype
2.3.6 Kif7 and Sufu share overlapping functions in Hh signaling during chondrocyte development

2.4 Discussion

2.5 Materials & Methods

2.6 Figures

2.7 References

Chapter 3 Sufu mediates the effect of Pthlh on chondrocyte differentiation in the growth plate

3.1 Summary

3.2 Introduction

3.3 Results

3.3.1 Sufu regulates the effect of Pthlh on chondrocyte differentiation

3.3.2 Kif7 is not required for Pthlh to inhibit chondrocyte differentiation

3.3.3 Pthlh regulates Sufu protein level

3.3.4 Sufu is required for the ability of Pthlh to process Gli transcription factors

3.4 Discussion

3.5 Materials & Methods
3.6 Figures ............................................................................................................. 87

3.7 References ....................................................................................................... 94

Chapter 4 Conclusions & future directions ......................................................... 97

4.1 Summary ........................................................................................................ 98

4.2 Sufu expression is tightly regulated by multiple factors in the growth plate ....... 99

4.2.1 Lessons from genetically modified mice and Pthlh activation ..................... 99

4.2.2 Transcriptional regulation of Sufu ............................................................... 100

4.2.3 How does Sufu regulate subsequent steps of endochondral ossification? ...... 101

4.2.4 Sufu and osteoarthritis ............................................................................... 102

4.3 The role of Sufu in Wnt/ß-catenin signaling pathway during chondrocyte development ............................................................................................................... 105

4.3.1 Wnt/ß-catenin signaling in skeletal development ....................................... 105

4.3.2 The role of Sufu in mediating Wnt/ ß-catenin signaling ................................. 106

4.4 Conclusions .................................................................................................... 109

4.5 Figures ............................................................................................................. 110

4.6 References ..................................................................................................... 112
Appendix A: Pthlh negatively regulates Hh signaling activity in chondrocytes through a cilia-independent mechanism

A.1 Summary

A.2 Introduction

A.3 Results

A.3.1 Ifi88ORPK/OPRK mice show skeletal defects and dysregulated Hh signaling in chondrocytes

A.3.2 Pthlh downregulates Col10a1 expression and Hh signaling activity through a cilia-independent mechanism in chondrocytes

A.3.3 Ciliary tip localization of Sufu is induced by Pthlh

A.4 Discussion

A.5 Materials & Methods

A.6 Figures

A.7 References
## List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>APC</td>
<td>Adenomatous polyposis coli gene product</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin immunoprecipitation</td>
</tr>
<tr>
<td>Ci</td>
<td>Cubitus interruptus</td>
</tr>
<tr>
<td>CHX</td>
<td>Cycloheximide</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole (DAPI)</td>
</tr>
<tr>
<td>Gli1</td>
<td>Glioma-associated oncogen family member 1</td>
</tr>
<tr>
<td>Gli2</td>
<td>Glioma-associated oncogen family member 2</td>
</tr>
<tr>
<td>Gli3</td>
<td>Glioma-associated oncogen family member 3</td>
</tr>
<tr>
<td>Gli3FL</td>
<td>Full-length of glioma-associated oncogene family member 3</td>
</tr>
<tr>
<td>Gli3R</td>
<td>C-terminally truncated transcriptional repressor form of glioma-associated oncogene family member 3</td>
</tr>
<tr>
<td>GSK3β</td>
<td>Glycogen synthase kinase 3β</td>
</tr>
<tr>
<td>Hh</td>
<td>Hedgehog</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and eosin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td>IFT</td>
<td>Intraflagellar transport</td>
</tr>
<tr>
<td>Ihh</td>
<td>Indian hedgehog</td>
</tr>
<tr>
<td>IP</td>
<td>Immunoprecipitate</td>
</tr>
<tr>
<td>Kif7</td>
<td>Kinesin family member 7</td>
</tr>
<tr>
<td>Lef</td>
<td>Lymphoid enhancer factor</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>Ptc1</td>
<td>Patched 1</td>
</tr>
<tr>
<td>Pthh</td>
<td>Parathyroid hormone-like hormone</td>
</tr>
<tr>
<td>PTHrP</td>
<td>Parathyroid hormone-related protein</td>
</tr>
<tr>
<td>PTHR1</td>
<td>Parathyroid hormone 1 receptor</td>
</tr>
<tr>
<td>Pur</td>
<td>Purmorphamine</td>
</tr>
<tr>
<td>Shh</td>
<td>Sonic hedgehog</td>
</tr>
<tr>
<td>Smo</td>
<td>Smoothened</td>
</tr>
<tr>
<td>Sufu</td>
<td>Suppressor of fused</td>
</tr>
<tr>
<td>TCF</td>
<td>Transcription factors T-cell factor</td>
</tr>
</tbody>
</table>
List of figures

FIGURE 1-1 Schematic representation of endochondral ossification. .......................... 15

FIGURE 1-2 IHH/Pthlh signaling regulation in the growth plate. ................................. 16

FIGURE 1-3 Hh signaling in vertebrates. ........................................................................ 18

FIGURE 2-1 Sufu is differentially expressed in the growth plate. ............................... 49

FIGURE 2-2 Generating chondrocyte-specific knockout of Sufu. ................................. 50

FIGURE 2-3 Skeletal phenotype analysis of Col2a1-Cre; Sufu<sup>fl/fl</sup>. ....................... 52

FIGURE 2-4 Sufu negatively regulates growth plate chondrocyte differentiation. ... 54

FIGURE 2-5 In situ hybridization analysis of Ptc1 and Col10a1 expression in various genotypes ........................................................................................................................................ 56

FIGURE 2-6 Kif7 inactivation resulted in opposite effects on chondrocyte proliferation and differentiation to those of Sufu inactivation. .............................. 57

FIGURE 2-7 Increased level of Sufu in Kif7-deicient chondrocytes. ............................ 59

FIGURE 2-8 Increased level of Sufu-Gli complexes in Kif7-deicient chondrocytes. .... 60

FIGURE 2-9 A dual function for Kif7 in Hh signaling modulation .................................. 62
FIGURE 2-10 Proposed model for how SUFU and Kif7 regulate HH signaling in growth plate chondrocytes. ................................................................. 64

FIGURE 3-1 SUFU is required for the negative effect of Pthlh on chondrocyte differentiation. ................................................................. 87

FIGURE 3-2 Kif7 is not required for the negative effect of Pthlh on chondrocyte differentiation. ................................................................. 89

FIGURE 3-3 Pthlh increases SUFU protein level. ................................................................. 90

FIGURE 3-4 Pthlh signaling activation promotes GLI2 and GLI3 protein processing via a SUFU-mediated manner. ................................................................. 91

FIGURE 3-5 Proposed model for how Pthlh regulates GLI transcriptional factors through a SUFU-mediated mechanism. ................................................................. 93

FIGURE 4-1 Proposed model by which SUFU protein stability serves as a common mechanism that regulates GLI-mediated transcription. ................................................................. 110

FIGURE 4-2 Proposed mechanism by which Pthlh and Ihh signaling coordinate SUFU expression in the growth plate. ................................................................. 111

FIGURE A-1 The primary cilia are required for normal embryonic skeletal development. ................................................................. 132
FIGURE A- 2 ASYMMETRICAL HYPERTROPHY PHENOTYPE IN IFT88ORPK/ORPK MICE IS NOT SEEN AT E18.5................................................................................................................................. 133

FIGURE A- 3 THE ROLE OF PRIMARY CILIA IN CHONDROCYTE HH LIGAND RESPONSIVENESS........ 134

FIGURE A- 4 THE ROLE OF PRIMARY CILIA IN MEDIATING PTHLH SIGNALING IN CHONDROCYTE DIFFERENTIATION. ................................................................................................................................................ 135

FIGURE A- 5 INCREASED SUFU CILIARY LOCALIZATION IN PTHLH-TREATED WILD-TYPE CHONDROCYTES.............................................................................................................................................. 136

FIGURE A- 6 A PROPOSED MODEL FOR CILIA-MEDIATED REGULATION OF GLI ACTIVITY............. 138
Chapter 1 Introduction
1.1 Summary

The work presented in the subsequent chapters is aimed at investigating Hh regulators, Sufu, Kif7 and the primary cilium, in mediating Hh signaling as well as Pthlh signaling during growth plate chondrocyte development. I examined the role of Sufu and Kif7 in regulating Hh signaling in developing growth plate, and also proposed a model by which Sufu and Kif7 exert distinct and overlapping functions in Hh pathway regulation. Furthermore, I examined how these Hh regulators mediate the effects of Pthlh in chondrocytes. By dissecting the roles that Sufu, Kif7 and the primary cilium play in regulating Hh and Pthlh signaling in the growth plate chondrocytes. I provided insight into the mechanism by which Hh signaling is regulated and highlighted Sufu as a common link between Ihh and Pthlh signaling in growth plate chondrocyte development.
1.2 Overview of growth plate development

Endochondral bone growth, which is the process of the cartilaginous skeletal template progressively being replaced by bone, is precisely regulated by a number of signaling pathways during development. Chondrocyte proliferation and differentiation are tightly controlled, and dysregulation of these processes causes chondrodysplasias (generalized skeletal malformation diseases), cartilaginous tumors, and osteoarthritis, a common degenerative joint disease. At the onset of endochondral bone formation, mesenchymal cells condense and form a template of the future skeletal element. This cartilage template is surrounded by the perichondrium, which is a thin layer of flattened fibroblastic cells. Centrally, cells differentiate to chondrocytes and proliferate and then mature from resting/low-proliferating chondrocytes into high-proliferating cells that typically arrange in columns. The proliferating chondrocytes then undergo differentiation successively into prehypertrophic and eventually hypertrophic chondrocytes. In parallel, cells in the perichondrium adjacent to the hypertrophic region differentiate into osteoblasts, which form the bone collar. The hypertrophic chondrocytes undergo programmed cell death, and osteoblasts invade along with blood vessels and osteoclasts, leading to the replacement of the cartilage template with trabecular bone (Kronenberg, 2003; Olsen et al., 2000; Provot and Schipani, 2005). This process is illustrated in Figure 1-1. These layers of chondrocytes proceeding in a staggered manner through steps of hypertrophic differentiation constitute the growth plate, which is responsible for the longitudinal growth of the bones during embryonic development and in the postnatal long bones.
1.3 Indian hedgehog (Ihh) and Parathyroid hormone-like hormone (Pthlh) signaling in growth plate development

1.3.1 Ihh/Pthlh feedback loop

A feedback loop formed by Ihh and Pthlh signaling functions to regulate the pace of growth plate chondrocyte differentiation (Karp et al., 2000; Kobayashi et al., 2002; Lanske et al., 1996; Minina et al., 2001). Ihh, which is expressed by prehypertrophic chondrocytes, stimulates Pthlh expression in the articular region of the growth plate. Pthlh, which is a secreted protein that acts on PTH/Pthlh receptor (PTHR1) bearing chondrocytes to maintain cells in a proliferative state, thereby delaying the production of Ihh by prehypertrophic chondrocytes (Kronenberg, 2003; Long et al., 2001) (Fig.1-2A). The levels of Ihh and Pthlh signaling synchronize and regulate the pool of proliferating chondrocytes and the pace of chondrocyte hypertrophic differentiation, thus determining the distance from the joint to which chondrocyte hypertrophic differentiation take place (Karaplis et al., 1994; Lanske et al., 1996; St-Jacques et al., 1999; Vortkamp et al., 1996).

1.3.2 Pthlh-dependent and –independent effects of Ihh

Loss of Ihh results in severe dwarfism due to accelerated chondrocyte hypertrophic differentiation, reduced chondrocyte proliferation, and absence of osteoblasts in the bone collar (Chung et al., 2001; Lanske et al., 1996; Long et al., 2004; Long et al., 2001; Maeda et al., 2007; Razzaque et al., 2005; St-Jacques et al., 1999). Activation of Ihh signaling in Pthlh or PTHR1
mutant limbs does not result in a delay in chondrocyte hypertrophic differentiation, suggesting that the inhibitory effect of Ihh on chondrocyte hypertrophic differentiation is Pthlh-dependent (Fig.1-2A). However, Ihh mutant mice (Ihh<sup>−/−</sup>) exhibit a more severe reduction in the size of the bone compared with Pthlh (Pthlh<sup>−/−</sup>) or PTHR1 (PTHR1<sup>−/−</sup>) mutants, whereas expression of PTHR1 in Ihh mutant chondrocytes delays the accelerated onset of hypertrophic differentiation, but does not possess any effect on the proliferation defect observed in Ihh mutant mice (Karp et al., 2000). The data reveal a Pthlh-independent function of Ihh in chondrocyte proliferating (St-Jacques et al., 1999) (Fig.1-2A). Further studies reveal that Ihh stimulates chondrocyte proliferation in part through a cyclin D1-mediated mechanism (Duman-Scheel et al., 2002; Long et al., 2001). The absence of bone collar in the Ihh-deficient bones reveals a positive role of Ihh in regulating osteoblasts differentiation in the perichondrium. Interestingly, Ihh<sup>−/−</sup> mice do not exhibit severe defects in intramembranous bone formation (St-Jacques et al., 1999), which is another essential process of mammalian skeletal development that contains the direct conversion of mesenchymal tissue into bone. During later stages of osteoblast differentiation, inactivation of Ihh signaling does not result in defects in the osteoblast differentiation process. The data suggest that Ihh plays a role in initiating the differentiation of osteoblasts during endochondral skeletal development (Fig.1-2A).

1.3.3 Actions of Pthlh in the growth plate

The binding of Pthlh to its receptor, PTHR1, stimulates G protein activation, which leads to the production of cyclic AMP and activation of protein kinase A (PKA). The mechanism by which cyclic AMP and PKA maintain chondrocytes in a proliferative state and delay
chondrocyte hypertrophic differentiation is not completely understood. During mouse embryogenesis, Sox9 plays an important role in regulating chondrocyte differentiation (de Crombrugghe et al., 2001). Phosphorylation of Sox9 by Pthlh/PKA results in a more potent activator function in upregulating target genes (Huang et al., 2001); thus, Sox9 phosphorylation could contribute to the negative effect of Pthlh on chondrocyte hypertrophic differentiation. In addition to its inhibitory role in regulating chondrocyte hypertrophic differentiation, Pthlh also exhibits a stimulatory effect on chondrocyte proliferation. In contrast to the PKA-mediated action of Pthlh on chondrocyte hypertrophic differentiation, its pro-proliferative effect is not mimicked by PKA activation. Deletion of p57 results in a growth plate phenotype mimicking what was observed in Pthlh-null mice, including increased chondrocyte proliferation and delayed chondrocyte hypertrophic differentiation. Double knockout of Pthlh and p57 attenuates the growth plate defects in Pthlh-null mice (MacLean et al., 2004). The data suggest that the stimulatory effect of Pthlh on chondrocyte proliferation is mediated in part through decreasing p57 expression in the growth plate (MacLean et al., 2004).
1.4 Ihh and Pthlh signaling pathways regulate Gli transcription factors

Ihh, a member of the Hedgehog (Hh) family of signaling molecules, regulates Gli transcriptional activity through binding to its receptor Patched 1 (Ptc1) and derepressing the signal transducer Smoothened (Smo). Three Gli zinc finger proteins Gli1, Gli2 and Gli3 (glioma-associated oncogene family member 1, 2 and 3) are transcription factors mediating Hh pathway in mammalian cells (Jiang & Hui, 2008). Analysis of genetically modified mice show that Gli2 and Gli3 act as essential mediators in Hh signaling, whereas Gli1 is dispensable for embryonic development and acts as a secondary mediator of Hh signaling transduction (Bai et al., 2004; Ding et al., 1998; Mo et al., 1997; Motoyama et al., 1998; Park et al., 2000). Although all three Gli proteins can function as activators of the pathway, Gli2 and Gli3 are the major transcriptional activator and repressor of the mammalian Hh signaling, respectively (Bai et al., 2004; Buttitta et al., 2003; McDermott et al., 2005; Motoyama et al., 1998).

In the absence of Hh ligand, Gli3 is phosphorylated sequentially by PKA, glycogen synthase kinase 3β (GSK3β) and casein kinase 1 (CK1) and targeted to ubiquitin/proteasome-mediated proteolysis to generate a C-terminally truncated transcriptional repressor (Gli3R) (Tempe et al., 2006; Wang et al., 2000; Wang and Li, 2006). Gli2 is also phosphorylated by these kinases, although in the case of processing is inefficient and leads to mostly degradation by the proteasome (Pan et al., 2006). The specific domain in the C-terminus has been suggested to be responsible for the differential processing and degradation of Gli2 and Gli3 (Pan and Wang, 2007). Hh signaling activation blocks the proteolytic cleavage of Gli2 and promotes the activator function of Gli2 and Gli3. However the mechanism is undefined.
Data from mutant mouse analyses suggest that Gli2 and Gli3 are involved in Ihh-dependent growth plate chondrocyte development. Ihh-null mice show reduced chondrocyte proliferation, increased zone of hypertrophic chondrocytes and lack of ossification in endochondral bones (St-Jacques et al., 1999). Similarly, Gli2 knockout mice display expanded hypertrophic zone and reduced bone formation, suggesting that Gli2 functions as an activator in regulating Hh signaling in chondrocytes and reduced Gli2 function is in part responsible for the Ihh-null phenotype (Miao et al., 2004). Genetic studies in mice show that Gli3 functions as a major repressor regulating Hh signaling in growth plate chondrocytes. Loss of function of Gli3 rescues the growth plate developmental defects in Ihh-null mice, which supports the notion that Gli3 acts as a negative regulator of Hh-mediated signaling in chondrocytes and Ihh possess inhibitory effect on the repressor function of Gli3 in this process (Koziel et al., 2005).

Besides the Hh-mediated regulation, the Gli transcription factors may also be regulated through an Hh/Smo-independent mechanism. Studies of explants lacking the Gli genes suggest that Gli3, but not Gli2, is involved in the inhibitory effect of Pthlh on growth plate chondrocyte hypertrophic differentiation. Pthlh signaling activation inhibits Gli-mediated transcription through processing Gli3 via a PKA-dependent manner (Mau et al., 2007; Miao et al., 2004; Tukachinsky et al., 2010; Tuson et al., 2011; Vortkamp et al., 1996; Zeng et al., 2010 (Fig1-2B). These results suggest another level of feedback control between Ihh and Pthlh signaling and raise the possibility that the Gli transcription factors act as integrators of multiple signaling pathways during growth plate chondrocyte differentiation. However, our knowledge of the regulation of Gli-mediated pathway activity is still limited.
1.5 Regulators in Hedgehog (Hh) signaling pathway: Sufu and Kif7

In mammalian Hh signaling, Suppressor of fused (Sufu) and Kinesin family member 7 (Kif7) are two evolutionarily conserved regulators of Gli transcription factors. Deletion of Sufu results in embryonic lethality at E9.5 with severe ectopic Hh signaling activation, indicating Sufu functions as a major repressor of mammalian Hh pathway (Cooper et al., 2005; Svard et al., 2006). Western analysis revealed a severe reduction in the levels of Gli2 and full length Gli3 as well as a lack of Gli3 repressor in Sufu<sup>-/-</sup> embryos (Humke et al.; Wang et al., 2010), suggesting a role of Sufu in controlling Gli transcriptional repressor processing. However, the mechanism of action of Sufu remains controversial. Sufu interacts directly with all three Gli proteins and sequesters them in the cytoplasm in the absence of Hh ligand, which prevents Hh pathway activation (Barnfield et al., 2005; Ding et al., 1999; Kogerman et al., 1999; Murone et al., 2000). Sufu acts to recruit a co-repressor complex to inhibit Gli activator function (Cheng and Bishop, 2002) and promotes Gli3R formation through interacting with GSK3β (Kise et al., 2009). Recent studies further reveal a role for Sufu in maximal Gli-dependent transcriptional activation, suggesting that Sufu also acts positively in Hh signaling (Chen et al., 2009). Hh signaling activation promotes Sufu protein turnover via the ubiquitin-proteasome system (Yue et al., 2009). PKA and GSK3β phosphorylate Sufu at Ser-346 and Ser-342, respectively, and phosphorylation stabilizes Sufu against Hh signaling-induced degradation (Chen et al., 2011). Taken together, the data suggest that Sufu plays an important role in regulating Gli activities, and Sufu itself is tightly regulated to achieve a gradient level of Hh signaling activity during development.
In contrast, less is known about the action of Kif7. Loss of \textit{Kif7} results in perinatal death and phenotypes, such as polydactyly, mimicking Gli3 knockout mice (Chen et al., 2009; Cheung et al., 2009; Hui and Joyner, 1993). Kif7 also interacts with all three Gli proteins and elevated Gli2 and reduced Gli3 levels are found in \textit{Kif7}-null mice, suggesting that it acts as a negative regulator of the mammalian Hh pathway. \textit{Kif7}−/− embryos exhibit defects in floor plate development, which depends on maximal level of Hh pathway activation, indicating a potential positive role of Kif7 in Hh signaling (Cheung et al., 2009). The mechanism by which Sufu and Kif7 execute dual functions and/or whether they possess cooperative regulatory functions is not known.
1.6 The primary cilium in Hh signaling

In vertebrates, the primary cilium, which is a non-motile microtubule-based organelle, serves as a focal point in Hh signaling regulation (Corbit et al., 2005; Goetz and Anderson, 2010; Huangfu et al., 2003). The formation and maintenance of the primary cilium depend on intraflagellar transport (IFT) of protein complexes driven by kinesin and dynein motors for anterograde and retrograde IFT movements, respectively. Mutation in proteins involved in IFT, such as IFT88, IFT172, and the motor protein Kif3a, prevents functional cilia formation and attenuates Hh signaling (Huangfu et al., 2003). To date, many components of the Hh pathway are found to be localized to the cilia either in the absence of Hh ligand or during pathway activation (Corbit et al., 2005; Haycraft et al., 2005; Huangfu and Anderson, 2005; Liu et al., 2005). Ptch1 is localized to the primary cilia where it prevents the ciliary localization of Smo in the absence of Hh ligand. Binding of Hh ligand to Ptch1 induces its export from the cilia, which allows the ciliary translocation of Smo. Through an unknown mechanism, ciliary localization of Smo relays Hh signals to the cytoplasm, which leads to Gli-mediated transcription activation (Rohatgi et al., 2007) (Fig. 1-3).

Hh pathway activation in the absence of Sufu is independent of cilia (Chen et al., 2009; Jia et al., 2009), suggesting that Smo may activate Gli proteins at the cilia by inhibiting Sufu. PKA downregulates Smo-mediated Hh signaling activation via a Sufu-dependent mechanism (Chen et al., 2009; Svard et al., 2006; Wu et al., 2004), suggesting that Smo and PKA both modulate Hh signaling thorough Sufu. Therefore, a model by which Hh signaling activates Gli protein at the cilia through relieving the inhibitory function of Sufu, resulting in Gli-mediated
transcriptional activation was proposed. This model is supported by recent studies carried out in cultured fibroblasts, showing that Smo plays a positive role in dissociation of inhibitory Sufu-Gli complexes at the primary cilium upon Hh pathway activation. Furthermore, Gli2 and Gli3 are required to recruit Sufu to cilia, but Gli proteins can localize to cilia in the absence of Sufu (Humke et al., 2010; Tukachinsky et al., 2010). However, how Hh signaling and/or other signaling pathways regulate Sufu in the context of primary cilia is unknown. Mammalian Sufu bears four conserved cAMP-dependent PKA recognition sites, and phosphorylation by PKA and GSK3β stabilizes Sufu and promotes its tendency to stay longer at the ciliary tip (Chen et al., 2011) (Fig. 1-3). This stabilizing effect of PKA on Sufu is consistent with its inhibitory function on Hh signaling (Dai et al., 1999; Tempe et al., 2006). A carboxy-terminal region of Sufu interacts and recruits GSK3β for efficient Gli3 processing (Kise et al., 2009). It is plausible that Sufu, stabilized through phosphorylation by PKA and GSK3β, forms a complex with Gli2/3 and is transported into the primary cilium where further modification takes place, allowing formation of the truncated transcriptional repressors via proteolytic processing in the cytoplasm (Fig.1-3). We do not yet know how the integrity of the Sufu-Gli complex is maintained or how the phosphorylation state of Sufu and/or Gli regulates this complex. We do not yet know how Hh signaling promotes Sufu-Gli complexes dissociation or whether this process can take place outside of the primary cilium. Additionally, it will be interesting to know if Kif7, which shows dynamic ciliary localization (Liem et al., 2009), plays a regulatory role in the formation and/or dissociation of Sufu-Gli complexes in the primary cilium. Furthermore, during growth plate chondrocyte differentiation, Pthlh exerts regulatory functions on Gli proteins as well as Gli-mediated transcriptional activity, and it functions mainly through PKA activation. Thus, Sufu regulation may serve as a common and important regulatory mechanism between Ihh and Pthlh.
signaling during growth plate chondrocyte development.
1.7 Hypothesis & Specific aims

Proper regulation of Hh and Pthlh signaling is crucial in chondrogenesis. Hh signaling mediators, such as Sufu, Kif7, and primary cilium, may play important roles in controlling the process of chondrocyte differentiation. I hypothesize that Sufu, Kif7, and primary cilium play distinct and overlapping roles in regulating Hh signaling activity during chondrocyte development.

There are three specific aims set out for this work:

Aim 1: What are the regulatory roles of Sufu and Kif7 in Hh signaling during growth plate chondrocyte development?

Aim 2: What is the role of Kif7 in the formation and/or dissociation of Sufu-Gli complexes?

Aim 3: What are the potential roles of Sufu, Kif7, and primary cilium in Pthlh action during chondrocyte development?
1.8 Figures

Figure 1-1 Schematic representation of endochondral ossification.

**Figure 1-1.** Schematic representation of endochondral ossification. (A) Mesenchymal cells condense to form the model of the bone. (B-C) Centrally, cells differentiate into chondrocytes and start to proliferate and undergo several steps of differentiation into hypertrophic chondrocytes. (D-E) Hypertrophic chondrocytes are postmitotic and express *Col10a1*. Osteoblasts invade along with blood vessels and osteoclasts to replace the cartilaginous template with trabecular bone. (F) Perichondrial cells adjacent to the hypertrophic chondrocytes become osteoblasts, which forms the bone collar. Postnatally, secondary ossification center forms at each end of the long bone.
Figure 1-2 Ihh/Pthlh signaling regulation in the growth plate.
Figure 1-2. Ihh/Pthlh signaling regulation in the growth plate. (A) Growth plate chondrocytes undergo a coordinated differentiation process, ending in chondrocyte hypertrophy and apoptosis. 1) Ihh, which is expressed by prehypertrophic chondrocytes, stimulates Pthlh expression at the periarticular region that functions to inhibit chondrocyte hypertrophic differentiation and Ihh expression (illustrated by 4.). 2) Ihh positively regulates chondrocyte proliferation (circled P) via a Pthlh-independent manner. 3) Ihh initiates osteoblast differentiation at the perichondrium adjacent to its expressing region. 4) Pthlh inhibits chondrocyte hypertrophic differentiation mainly through PKA activation. 5) Pthlh possesses stimulatory effect on chondrocyte proliferation. Solid lines show the regulation of Pthlh, and dashed lines show the regulation of Ihh. (B) Both Gli2 and Gli3 are involved in the regulation of chondrocyte hypertrophic differentiation. Pthlh negatively regulates this differentiation process through processing Gli3FL into its truncated form (Gli3R) via a PKA-dependent mechanism.
Figure 1-3 Hh signaling in vertebrates.

**Figure 1-3.** Hh signaling in vertebrates. In the absence of the Hh ligand, Ptch1 localizes to the primary cilium and prevents Smo ciliary accumulation. Phosphorylation by PKA and GSK3β stabilizes Sufu and promotes its tendency to stay longer at the ciliary tip. Sufu-Gli complexes traffic through the cilium at low level and promote Gli repressor (GliR) formation. Hh ligand binding to Ptch1 leads to the translocation of Smo into the cilium. Smo-mediated dissociation of Smo-Gli complexes results in Gli transcriptional activation.
1.9 References


Chapter 2 **Kif7 promotes Hh signaling in growth plate chondrocytes by restricting the inhibitory function of Sufu**

Shu-Hsuan C. Hsu, Xiaoyun Zhang, Chunying Yu, Zhu Juan Li, Jay S. Wunder, Chi-Chung Hui, and Benjamin A. Alman

All experiments and analysis described in this chapter were conducted by Shu-Hsuan C. Hsu with the exception of the western and in situ analysis by Xiaoyun Zhang and Chunying Yu. This study has been published in Development (Hsu et al., 2011).
2.1 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, I 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 dosage 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.
2.2 Introduction

The precise regulation of chondrocyte proliferation and differentiation is critical 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 Patched1 (Ptch1) and derepression of the signaling receptor Smoothened (Smo). Three Gli zinc finger proteins (Gli1-3) are transcription factors mediating Hh signaling in mammalian cells (Jiang & 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, though 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 lacking 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 critical 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 those 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 led to the suggestion 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 critical role in the stabilization of Gli activators and the formation of Gli3 repressor (Humke et al., 2010; Wang et al., 2010). In 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 unknown whether Kif7 plays a regulatory role in the formation and/or dissociation of Sufu-Gli complexes in the primary cilium. In this study, I 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. I speculate that Kif7 functions positively in Hh signaling to promote Smo-induced
dissociation of Sufu-Gli complexes at the primary cilium.
2.3 Results

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

To determine if Sufu might play a role in growth plate chondrocyte function, Sufu expression was examined in fetal limb cartilage. qRT-PCR analysis on micro-dissected 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), while its expression is downregulated when cells undergo differentiation to hypertrophic chondrocytes (Fig. 2-1A, D). 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. 2-1C, E). The pattern of Sufu expression is similar to that of Pthlh (an Hh regulated gene in the growth plate), but in contrast, Ihh expression is highest in the prehypertrophic chondrocytes (Fig. 2-1B). 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), I generated chondrocyte-specific knockout mice (Col2a1-Cre; Sufu<sup>fl/fl</sup>) 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<sup>fl/fl</sup> mice (Fig. 2-2A-C). 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. 2-2D-F). Alcian blue/Alizarin red staining showed a significant reduction of bone ossification in Col2a1-Cre; Sufu<sup>ff</sup> mice (Fig. 2-3A, D). 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. 2-3B). By Ki67 and Phospho-H3 immunostaining, a higher percentage of proliferating cells was found in the mutant growth plate (Fig. 2-3E, F; Fig. 2-6J). In contrast, immunostaining of active Caspase-3 and TUNEL analysis did not reveal a significant difference of apoptosis in the mutants (Fig. 2-3G). These data indicate that Sufu is required for normal endochondral skeletal development, where it regulates growth plate chondrocyte proliferation and differentiation.

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

Ptc1 inhibits Smo in the absence of Hh signals and acts as a negative regulator of Hh signaling. Chondrocyte-specific knockout of Ptc1 (Col2a1-Cre; Ptc1<sup>ff</sup>) 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. 2-3C; Fig. 2-4A-C). I found that the Sufu knockout phenotype is consistently milder than the Ptc1 knockout phenotype (Fig. 2-4A-C). To determine whether knockout of Sufu also results in Hh pathway activation in the chondrocytes, I performed qRT-PCR analysis. Knockout of Ptc1 leads to increased expression of Hh target genes, such as Gli1, Ptc1, and Hhip1 (Fig. 2-4D). Interestingly, I detected up-regulation of Ptc1 and Hhip1, but not Gli1, in Sufu-deficient
chondrocytes (Fig. 2-4D). Analysis by in situ hybridization 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 proliferative zone, *Sufu*-deficient mice exhibited higher levels of *Ptch1* transcripts also in the resting chondrocytes (Fig. 2-5A, C). Furthermore, loss of *Sufu* resulted in a reduction of the hypertrophic zone as verified by in situ hybridization analysis for collagen X expression (Fig. 2-5E, G). Western analysis revealed an increase (2-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. 2-4E). Together, these data suggest that Sufu, like Ptc1, functions as a negative regulator of Hh signaling in chondrocytes but its inactivation only leads to partial pathway activation.

2.3.3 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, I 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 down-regulated in proliferating, prehypertrophic, and hypertrophic chondrocytes demonstrated by qRT-PCR on micro-dissected sections of the growth plate, in situ hybridization, and immunostaining (Fig. 2-6A-C). To investigate whether it functions as a regulator of Ihh signaling, I 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, I found that Kif7-null mice exhibit a reduction in the size of the proliferative zone and an expansion of hypertrophic zone (Fig. 2-6D, F, G). To rule out the possibility that the growth plate phenotype is due to secondary effects caused by Kif7 inactivation in other cell types, I 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. 2-6E). No obvious phenotypic difference was found in tibial growth plates of P10 Kif7-deficient mice compared to their wild-type counterparts (Fig. 2-6H). 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. 2-6F, G; Fig. 2-5E, F) similar to those observed in Kif7/– mice. Furthermore, the growth plates of Kif7/– and Col2a1-Cre; Kif7f/f mice showed a reduction in cell proliferation demonstrated by Ki67 and Phospho-H3 immunostaining (Fig. 2-6I, J). 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 down-regulation of Hh target genes, Gli1 and Ptch1, as revealed by qRT-PCR analysis (Fig. 2-9D), and reduced Ptch1 mRNA expression in the proliferating region as illustrated by in situ hybridization analysis (Fig. 2-5A, B). 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.

2.3.4 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, I first examined their expression in Kif7 and Sufu mutant mice. While no difference in Kif7 protein or RNA levels was detected in Sufu-deficient chondrocytes (Fig. 2-7A, B), I found a substantial increase of Sufu protein levels in Kif7-deficient chondrocytes (Fig. 2-7C). To determine if this is caused by altered RNA expression or protein stability, I examined Sufu transcript levels using qRT-PCR (Fig. 2-7D) and Sufu protein levels using pulse chase experiments in the presence of cycloheximide (Fig. 2-7E, F). 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. 2-7G, H). 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 Gli2Δ;Gli3Δ 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. 2-8A, B). Similarly, Gli2 and Gli3 staining were rarely found in
the primary cilium of these chondrocytes (Fig. 2-8A, B). Both Sufu and Kif7 signals are specific since 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. 2-8A, C). Consistent with previous studies done 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. 2-8A, C), 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. 2-8A, D). Consistent with the notion that Sufu and Glis are transported to the cilia as a complex, Sufu is localized to the ciliary tip of ~90% of Kif7-deficient chondrocytes (Fig. 2-8A, D). 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. I 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. While 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. 2-8E,
F). These results indicate that, similar to those observed in cultured fibroblasts, Smo activation also promotes the dissociation of Sufu-Gli2 complexes in the chondrocytes.

2.3.5 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, I generated Col2a1-Cre; Sufu<sup>+/+</sup>; Kif7<sup>−/−</sup> mice. Strikingly, these mice develop a normal growth plate (Fig. 2-9A). The expansion of hypertrophic zone and the reduction of proliferative zone observed in Col2a1-Cre; Kif7<sup>−/−</sup> mice are almost completely rescued by the simultaneous removal of one dose of Sufu (Fig. 2-9A-C). Importantly, Gli1 and Ptch1 expression was also restored to near wild-type levels in Col2a1-Cre; Sufu<sup>+/+</sup>; Kif7<sup>−/−</sup> chondrocytes (Fig. 2-9D). 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 down-regulation of Sufu protein expression as well as the dissociation of Sufu-Gli complexes (Fig. 2-10A, B).

2.3.6 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. I generated Col2a1-Cre; Sufu<sup>i/f</sup> mice with additional deletion of one allele of Kif7 (Col2a1-Cre; Sufu<sup>i/f</sup>; Kif7<sup>+/a</sup>) as well as Sufu; Kif7 double knockout mice (Col2a1-Cre; Sufu<sup>i/f</sup>; Kif7<sup>−/−</sup>). While deletion of one allele of Kif7 in Sufu-deficient growth plate resulted in a similar phenotype as Col2a1-Cre; Sufu<sup>i/f</sup> mice, Col2a1-Cre; Sufu<sup>i/f</sup>; Kif7<sup>i/f</sup> mice showed a more severe phenotype (Fig. 2-9A-C; Fig. 2-5E, H), 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>i/f</sup> mice (Fig. 2-4A).

Importantly, I 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. 2-9D). By in situ hybridization, I 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 (Fig. 2-5A, C, D). Thus, in the absence of Sufu, Kif7 functions instead as a negative regulator of the Hh pathway suppressing the expression of Hh target genes. I 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. 2-10A, B).
2.4 Discussion

In this study, I 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 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 I provide genetic and molecular data indicating 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. 2-10A).

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. In 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. I showed that Sufu protein levels are elevated in Kif7-deficient chondrocytes and that reduction of Sufu gene dosage 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, I 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 down-regulation 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 critical 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, I found that Sufu is mostly excluded from the ciliary tip in wild-type chondrocytes, whereas Sufu appear 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 (due 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 I found that Kif7 localizes to the ciliary tip in both the resting and proliferating chondrocytes in vivo (data not shown). Since 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 likely 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 likely 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, I 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 critical 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 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, I show here a novel mechanism by which Sufu and Kif7 interact to mediate Hh signaling in the growth plate (Fig. 2-10, 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. 2-10Aa). Its role in the primary cilium allows for normal Sufu-Gli complex localization and processing (Fig. 2-10B). 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. 2-10Ab). 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. 2-10Ac). 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. 2-10Ad), showing that the regulation of Hh signaling by Kif7 and Sufu play critical roles in the growth plate.
2.5 Materials & 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 was previously reported (Cheung et al., 2009). Conditional *Sufu*-deficient mice (*Col2a1-Cre; Sufu*<sup>f/f</sup>) 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*<sup>f/f</sup>) 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 till 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 (H&E), Safranin O and Alcian blue staining were performed using standard techniques. Proliferation was
evaluated by immunostaining using antibodies against Ki-67 (DakoCytomation M7249) at a 1:50 dilution and Phospho-H3 (Sigma) at a 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 10X 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 (1:10000), 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). Precipitation of Sufu protein was performed using Dynabeads Protein A (Invitrogen following manufacture’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 $2^{\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001). Either GAPDH or β-actin (Applied Biosystems) was used as endogenous control for target gene normalization.

**Primary growth plate chondrocyte cultures**

Chondrocyte isolation protocol was modified from previous 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 (3mg/ml) for 45 min 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 type 4 solution containing chondrocytes was collected. Cells were washed with PBS and seeded at a density of $8 \times 10^3$ cells per cm$^2$.

**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 α-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-phenyldione (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 utilized to compare data sets. A p value of < 0.05 was used as a threshold for statistical significance.
2.6 Figures

Figure 2-1 Sufu is differentially expressed in the growth plate.

**Figure 2-1.** 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 and 95% confidence intervals (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) In situ hybridization analysis of Sufu expression in wild-type mouse growth plate at E16.5. (E) Analysis of immunohistochemical studies. ~25% of
the cells in the AC/RC and PC/PRC regions are Sufu-positive. Less than 10% of the HC are Sufu-positive. Data are shown as means and 95% confidence intervals (n=3).

Figure 2-2 Generating chondrocyte-specific knockout of Sufu.
Figure 2-2. Generating chondrocyte-specific knockout of Sufu. (A) A lack of immunohistochemical staining for Sufu was found in Col2a1-Cre; Sufu<sup>f/f</sup> growth plate demonstrating efficient deletion of Sufu in chondrocytes. (B) Effective deletion of Sufu in growth plate chondrocytes was also verified using western analysis. (C) a and b: Immunohistochemical staining of Sufu in the growth plates of wild-type and Col2a1-Cre; Sufu<sup>f/f</sup> mice at E16.5; c and d: magnified images of the indicated areas in a and b, respectively (same images used in A). Due to the loss of glycogen and lipid during tissue preparation, the chondrocyte (Ch) display little more than the nucleus residing in a lacuna (L). (D) P10 Col2a1-Cre; Sufu<sup>f/f</sup> mutant mice showed significant reduction in body length compared with wild-type littermates. (E and F) Analysis of P10 Col2a1-Cre; Sufu<sup>f/f</sup> mutant mice showed significant reduction in body length and body weight compared with littermate wild-type controls. Data are shown as means with 95% confidence intervals indicated. (n=3). *P<0.05.
Figure 2-3 Skeletal phenotype analysis of Col2a1-Cre; Sufu<sup>fl/fl</sup>. 
Figure 2-3. Skeletal phenotype analysis of Col2a1-Cre; Sufu\textsuperscript{f/f}. (A) Alcian Blue/ Alizarin Red staining showed a delay in ossification in the P10 tibial bones and P0 lumbar vertebrae of the Col2a1-Cre; Sufu\textsuperscript{f/f} mice. (B) 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\textsuperscript{f/f} mice (upper panels). 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 H&E staining (lower panels). (C) H&E 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 panel: magnified images of the hypertrophic zone (red bar) seen in the upper panel. (D) Analysis of skeletal staining of Col2a1-Cre; Sufu\textsuperscript{f/f} mice exhibited delayed ossification demonstrated by the decreased percentage of the length of the mineralized zone to the length of the tibia. Data are shown as means with 95% confidence intervals indicated. (n=3). *P<0.05. (E and F) Deletion of Sufu in chondrocytes resulted in a significant increase in the percentage of Ki67-positive cells (indicated by red arrows at lower panel) in the E16.5 growth plate. Data are shown as means with 95% confidence intervals indicated. (n=3). *P<0.05. (G) No significant difference was found in apoptosis rate in the E16.5 Sufu-deficient growth plates demonstrated by TUNEL staining and active caspase-3 staining. Positive cells were indicated by red arrows. Data are shown as means with 95% confidence intervals indicated. (n=3). *P<0.05.
Figure 2-4 Sufu negatively regulates growth plate chondrocyte differentiation.
Figure 2-4. 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<sup>f/f</sup> embryos compared to wild-type littermates. An increased size of the proliferative zone, which 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<sup>f/f</sup> embryos. This phenotype resembles the phenotype displayed by deletion of Ptch1, another repressor of Hh signaling, although less severe. Data are shown as means and 95% confidence intervals (n=3). * p<0.05. (D) Expression of Hh target genes, Gli1, Ptch1, and Hhip1, were assessed by qRT-PCR. Data are shown as means and 95% confidence intervals (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).
Figure 2-5 In situ hybridization analysis of Ptch1 and Col10a1 expression in various genotypes.

(A-D) In situ hybridization for Ptch1 expression in the growth plates of wild-type, Col2a1-Cre; Sufu\textsuperscript{\textminus/}, Col2a1-Cre; Kif7\textsuperscript{\textminus/}, and Col2a1-Cre; Sufu\textsuperscript{\textminus/}; Kif7\textsuperscript{\textminus/} mice at E16.5; E-F: In situ hybridization for Col10a1 expression in the growth plates of wild-type, Col2a1-Cre; Sufu\textsuperscript{\textminus/}, Col2a1-Cre; Kif7\textsuperscript{\textminus/}, and Col2a1-Cre; Sufu\textsuperscript{\textminus/}; Kif7\textsuperscript{\textminus/} mice at E16.5. Studies using a sense probe as control showed no hybridization.
Figure 2-6 Kif7 inactivation resulted in opposite effects on chondrocyte proliferation and differentiation to those of Sufu inactivation.
Figure 2-6. *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 is highly expressed 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) In situ hybridization analysis of *Kif7* expression in the growth plate. (D) 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*<sup>−/−</sup>) compared to wild-type littermates. (E) Alcian Blue/ Alizarin Red staining of wild-type and *Col2a1-Cre; Kif7<sup>f/f</sup>* littermates at E18.5 and P10. *Col2a1-Cre; Kif7<sup>f/f</sup>* mice exhibited no gross abnormalities in skeletal development. (F, G) An increase in the length of the hypertrophic zone and a reduction in the length of the proliferative zone were found in *Kif7*<sup>−/−</sup> and *Col2a1-Cre; Kif7<sup>f/f</sup>* mice compared to wild-type littermates. Data are shown as means and 95% confidence intervals (n=3). * p<0.05. (H) H&E staining on the tibial sections of P10 wild-type and *Col2a1-Cre; Kif7<sup>f/f</sup>* mice (SOC: secondary ossification center; PZ: proliferative zone; HZ: hypertrophic zone). (I) A significant decrease in the percentage of proliferating cells was found in *Kif7*<sup>−/−</sup> and *Col2a1-Cre; Kif7<sup>f/f</sup>* growth plates as measured by immunohistochemical staining for Ki67. Data are shown as means and 95% confidence intervals (n=3). * p<0.05. (J) Sufu and kIf7 possess positive and negative effects on growth plate chondrocyte proliferation, respectively, demonstrated by immunohistochemical staining for Phospho H3.
Figure 2-7. Increased level of Sufu 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 level was found comparable with the wild-type controls. Data are shown as means and 95% confidence intervals (n=3). (C) A similar Kif7 protein level was found in Sufu-deficient chondrocytes. (D) The mRNA expression level of Kif7 remained comparable with the wild-type controls. (E, F) Western analysis of Sufu in wild-type and Kif7-deficient chondrocytes following 20µM cycloheximide (CHX) treatment. (G, H) Western analysis of Sufu in wild-type and Kif7-deficient chondrocytes following 25µM MG132 treatment. Actin was used as loading control.
Figure 2-8 Increased level of Sufu-Gli complexes in Kif7-deficient chondrocytes.
**Figure 2-8.** Increased level of Sufu-Gli complexes in *Kif7*-deficient chondrocytes. (A) Fluorescence micrographs of cilia from wild-type, *Col2a1-Cre; Sufu*\textsuperscript{ff}, and *Col2a1-Cre; Kif7*\textsuperscript{ff} growth plate chondrocytes. Gli2 (ciliary tip localization of Gli2 is indicated by an asterisk), Gli3 (ciliary tip localization of Gli3 is indicated by double asterisks), Sufu (ciliary base localization of Sufu is indicated by a yellow arrow; ciliary tip localization of Sufu is indicated by a green arrow), and Kif7 (ciliary tip localization of Kif7 is indicated by a white arrow) are detected in the green channel. Cilia are detected by staining against acetylated $\alpha$-tubulin (red channel). Centrioles are detected by staining against $\gamma$-tubulin (blue channel) to label the base of the cilia. (B-D) 25-50 ciliated cells from the proliferative zone of wild-type, *Sufu*-deficient and *Kif7*-deficient growth plates were examined for Gli2, Gli3, Sufu, and Kif7 ciliary localization. Data are shown as percentages. (E, F) 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 (*: 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 and 95% confidence intervals. ** p<0.01.
Figure 2-9 A dual function for Kif7 in Hh signaling modulation.
**Figure 2-9.** A dual function for Kif7 in Hh signaling modulation. (A-C) Histological and immunohistochemical analysis of the mouse tibial growth plates. Upper panel: Alcian blue staining on E16.5 tibial sections. Lower panel: Col10a1 immunostaining on E18.5 tibial sections (black bar: proliferative zone; red bar: hypertrophic zone). Data are shown as means and 95% confidence intervals (n=3). * p<0.05. (D) Expression of Gli1, Ptch1, and Hip1 in mouse growth plates of various mutants at E16.5 assessed by qRT-PCR. Data are shown as means and 95% confidence intervals (n=3-6). * p<0.05. See also Figure S1-3.
Figure 2-10 Proposed model for how Sufu and Kif7 regulate Hh signaling in growth plate chondrocytes.
**Figure 2-10.** 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 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 resulted 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.
2.7 References


Chapter 3  Sufu mediates the effect of Pthlh on chondrocyte differentiation in the growth plate

Shu-Hsuan C. Hsu, Xiaoyun Zhang, Chi-Chung Hui, and Benjamin A. Alman

All experiments and analysis described in this chapter were conducted by Shu-Hsuan C. Hsu with the exception of the western analysis by Xiaoyun Zhang. This study is submitted to EMBO Reports.
3.1 Summary

Growth plate chondrocytes undergo a coordinated process of proliferation and differentiation regulating normal long bone growth. Parathyroid hormone-like hormone (Pthlh) acts to regulate chondrocyte hypertrophic differentiation and the activation of hedgehog (Hh) signaling regulated Gli transcription factors. The mechanism by which Pthlh regulates Hh signaling is incompletely elucidated. Pthlh downregulates Gli transcriptional activity, and inhibits hypertrophic differentiation in the growth plate chondrocytes. In the absence of Suppressor of fused (Sufu), Pthlh treatment resulted in upregulated Hh activity and increased size of the hypertrophic zone. Pthlh treatment resulted in phosphorylation of Sufu and increased Sufu stability in wild-type chondrocytes through a protein kinase A (PKA)-mediated mechanism. In contrast, the regulation of Pthlh on Gli transcriptional activity and chondrocyte differentiation was independent of Kinesin family member 7 (Kif7), another mediator in the Hh signaling cascade. Thus, Sufu is a critical regulator in the ability of Pthlh to regulate Gli transcription activity and chondrocyte hypertrophic differentiation. Pthlh regulates Hh signaling activity and chondrocyte hypertrophy by promoting Sufu stability through PKA-mediated phosphorylation, allowing Gli2 degradation and Gli3 processing to a repressor form (Gli3R) in the growth plate chondrocytes.
3.2 Introduction

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 osteoblasts and new bone. Two signaling pathways that play a critical role coordinating growth plate chondrocyte differentiation are Pthlh (also called PTHrP) and Indian Hedgehog (Ihh). They act in a feedback loop, regulating each other's activity. Pthlh inhibits chondrocyte hypertrophic differentiation, while Ihh regulates the onset of hypertrophic differentiation, in part by signaling the periarticular chondrocytes to upregulate the expression of Pthlh. Ihh also regulates chondrocyte proliferation and induces ossification of the perichondrium in a Pthlh-independent manner. These two pathways interact with each other at several levels in their signaling cascades (Karp et al., 2000; Kobayashi et al., 2002; Lanske et al., 1996; Minina et al., 2001).

Hh signaling is activated in response to an appropriate Hh 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 Drosophila, Hh signaling activates the transcription factor Cubitus interruptus (Ci). In the absence of Hh binding, Ci is cleaved to a transcriptional repressor, while 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 (Jiang & Hui, 2008). 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 is not processed and as such functions as
an activator, while Gli3 is processed similar to Ci, and can act as an activator or repressor of Hh-mediated transcriptional activation (Bai et al., 2004; Ding et al., 1998; Mo et al., 1997; Motoyama et al., 1998; Park et al., 2000). I elucidated the role of Sufu and Kif7, essential regulators of Hh signaling, in the growth plate (Hsu et al., 2011; see also chapter 2). Sufu acts as a repressor of the Hh signaling pathway (Cooper et al., 2005; Svard et al., 2006), while Kif7 functions both positively and negatively in the Hh signaling (Chen et al., 2009; Cheung et al., 2009; Hui and Joyner, 1993). Kif7 positively regulates Hh signaling activity in part by regulating Sufu protein level and the exclusion of Sufu-Gli complexes from the primary cilium (Hsu et al., 2011). Thus, Sufu and Kif7 play important roles regulating Hh signaling activity and chondrocyte differentiation.

Previous studies show that Pthlh and the transcription factors Gli2 and Gli3 interact during growth plate chondrocyte differentiation and proliferation (Mau et al., 2007). Limb explant cultures showed that 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 regulates Gli-mediated transcription in cell cultures, and controls the level of the repressor form of Gli3 (Gli3R) in a PKA-dependent manner (Mau et al., 2007; Miao et al., 2004; Tukachinsky et al., 2010; Tuson et al., 2011; Vortkamp et al., 1996; Zeng et al., 2010). Thus, the interaction between Pthlh and Gli transcription factors plays an important role regulating growth plate chondrocyte differentiation.

Sufu and Kif7 are critical mediators of Hh signaling and the regulation of chondrocyte hypertrophic differentiation; they are highly expressed by articular and resting chondrocytes in the growth plate, the same region as where Pthlh is expressed (Hsu et al., 2011). This co-
expression pattern raises the possibility that Pthlh and these Hh signaling intermediates interact in these growth plate cells. Furthermore, since Sufu and Kif7 mediate Hh signaling and regulate chondrocyte hypertrophic differentiation, I examined how the interaction of Pthlh, Sufu, and Kif7 in the growth plate regulates chondrocyte hypertrophic differentiation.
3.3 Results

3.3.1 Sufu regulates the effect of Pthlh on chondrocyte differentiation

To determine if Sufu mediates the effect of Pthlh on growth plate chondrocyte differentiation, I examined the effects of Pthlh treatment on chondrocyte hypertrophic differentiation in E16.5 tibial explants from wild-type and Sufu-deficient mice (Col2a1-Cre; Sufu^f/f). I found that Pthlh treatment reduced the length of the hypertrophic zone (represented by Col10a1 staining) in explants from wild-type limbs (Fig. 3-1A, B). This finding is consistent with the known inhibitory role of Pthlh on chondrocyte hypertrophic differentiation. Unexpectedly, Pthlh treatment of Sufu-deficient explants resulted in an expansion of the hypertrophic zone (Fig. 3-1A, B). Thus, Sufu is required for the normal inhibitory effect of Pthlh on growth plate chondrocyte differentiation. Pthlh also possess a stimulatory effect on chondrocyte proliferation (Beier et al., 2001). I 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. 3-1A, C). Similar increase in the proliferative zone was observed in the Pthlh-treated Sufu-deficient limbs (Fig. 3-1A, C); showing that in contrast to its role in regulating chondrocyte hypertrophic differentiation, Sufu is not required for Pthlh to activate growth plate chondrocyte proliferation.

The effects of Pthlh on normal growth plate chondrocytes is primarily mediated by PKA (Guo et al., 2002; Mau et al., 2007; Wang et al., 2000). A PKA-specific inhibitor H-89 abolished the effects of Pthlh on both wild-type and Col2a1-Cre; Sufu^f/f explants (Fig. 3-1A-C). Taken
together, the data suggest that Pthlh signaling inhibits chondrocyte hypertrophic differentiation through the activation of PKA, and it functions upstream of Sufu.

3.3.2 Kif7 is not required for Pthlh to inhibit chondrocyte differentiation

Kif7 plays a role in controlling Hh activity through restricting Sufu function in the growth plate chondrocytes (Hsu et al., 2011). To determine if Kif7 is required for the effect of Pthlh on chondrocyte differentiation, I performed tibial explants from E16.5 Col2a1-Cre; Kif7\textsuperscript{fr} embryos. Similar to wild-type explants, Pthlh exerts an inhibitory effect on chondrocyte hypertrophic differentiation in Kif7-deficient limbs (Fig. 3-2A, B), showing that Pthlh negatively regulates chondrocyte hypertrophic differentiation through a Kif7-independent mechanism. In growth plate chondrocytes, Kif7 demonstrates a negative function in Hh signaling in the absence of Sufu (Hsu et al., 2011). Since Kif7 plays a different role regulating Hh pathway activity in the absence of Sufu, I 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. 3-2A, B), similar to those observed in Col2a1-Cre; Sufu\textsuperscript{fr} explants (Fig. 3-1A, B), indicating that Sufu is indeed required for the inhibitory effect of Pthlh on chondrocyte hypertrophic differentiation.

3.3.3 Pthlh regulates Sufu protein level

Given the important role of Sufu in Pthlh-mediated regulation of chondrocyte hypertrophy, I set out to investigate how Pthlh and Sufu might interact. To investigate whether
Sufu is controlled in response to Pthlh signaling activation, I analyzed Sufu levels in Pthlh-treated wild-type explant cultures. In wild-type chondrocytes, Pthlh treatment resulted in a significant increased level of Sufu protein. The increase of Sufu in Pthlh-treated cells is likely due to post-translational regulation because the level of Sufu transcript in these cells remained comparable with that of the controls as determined by qRT-PCR analysis (Fig. 3-3A, B). As expected, Sufu protein was not found in cell lysates derived from Col2a1-Cre; Sufu<sup>−/−</sup> explants, demonstrating efficient deletion of Sufu in mutant chondrocytes (Fig. 3-3A).

Phosphorylation of Sufu protein by PKA can positively regulate its stability in cultured fibroblasts (Chen et al., 2011). Since Pthlh functions mainly through PKA in the growth plate chondrocytes, it is possible that Pthlh promotes phosphorylation of Sufu, leading to its stabilization. To determine whether Pthlh stimulates Sufu phosphorylation in chondrocytes, primary chondrocyte cultures were treated with Pthlh, and the level of serine phosphorylation of Sufu was quantified using western analysis of phosphor-serine followed by immunoprecipitation with Sufu-specific antibodies. There was a higher level of serine phosphorylation of Sufu in the Pthlh-treated chondrocytes compared to the untreated controls (data not shown). Taken together, these data suggest that, similar to those observed in cultured fibroblasts, Pthlh activation promotes phosphorylation of Sufu and stabilizes Sufu protein in the chondrocytes.

3.3.4 Sufu is required for the ability of Pthlh to process Gli transcription factors

One mechanism by which Pthlh negatively regulates Gli-mediated transcription is through processing of Gli3 (Mau et al., 2007). To investigate the interaction between Pthlh and Gli transcription factors, I examined the levels of Gli2, full-length Gli3 (Gli3FL), and truncated
repressor form of Gli3 (Gli3R) in wild-type and Col2a1-Cre; Sufu\textsuperscript{flf} growth plate chondrocytes with or without the treatment of Pthlh. qRT-PCR analysis revealed comparable levels of Gli2 and Gli3 mRNA in wild-type and Sufu-deficient chondrocytes (Fig. 3-4A). 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. 3-4B). Consistent with previous data, Western analysis revealed a decrease in the ratio of full-length versus repressor form of Gli3 (Gli3FL:Gli3R) upon Pthlh signaling activation in wild-type chondrocytes (Fig. 3-4C, D). However, Pthlh treatment resulted in an increase in the ratio of Gli3FL:Gli3R in chondrocytes lacking Sufu (Fig. 3-4C, D).

To determine whether Sufu influences Pthlh-mediated regulation of Hh signaling activity, primary chondrocytes from wild-type and Sufu-deficient mice were treated with Pthlh and the expression of Gli1 and Ptch1 was analyzed using qRT-PCR. Consistent with the repressor role of Sufu in Hh signaling pathway, significant increases in Gli1 and Ptch1 expression levels were found in Sufu-deficient chondrocytes. In wild-type cells, Pthlh treatment resulted in reduced expression level in Gli1 and Ptch1. In the absence of Sufu, Pthlh treatment poses a positive effect on Gli1 and Ptch1 expression (Fig. 3-4E, F), which suggests that Sufu is required for the inhibitory function of Pthlh on Hh signaling activity in the chondrocytes.
3.4 Discussion

Here, I 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 sequels of Pthlh stimulation of chondrocyte hypertrophic differentiation, it serves as a molecular switch in Pthlh function. In contrast to the role I found for Sufu, Kif7 is not required for Pthlh to influence chondrocyte hypertrophy in the growth plate. Thus, Sufu appears to be a critical link between Hh and Pthlh signaling in the control of chondrocyte hypertrophic differentiation, and a number of molecules play important roles regulating Sufu stability, which in turn is likely a central node in Hh signaling activation.

Phosphorylation of Sufu through PKA activation promotes Sufu protein stability (Chen et al., 2011). Here, I found that Pthlh increases Sufu protein level as well as its phosphorylation level. Thus, our data support the notion that phosphorylation stabilizes Sufu and Pthlh plays a role regulating this phosphorylation in the growth plate. Intriguingly, Kif7 functions positively in regulating Hh pathway activity through restricting Sufu level (Hsu et al., 2011). Therefore, the regulation of Sufu level may be a common mechanism regulating Hh-mediated transcription.

In the absence of Hh ligand, Sufu sequesters Gli proteins in the cytoplasm, preventing Hh pathway activation (Barnfield et al., 2005; Ding et al., 1999; Kogerman et al., 1999; Murone et al., 2000). Sufu also functions to promote full length Gli3 to be processed into its repressor form (Gli3R) in concert with GSK3β (Kise et al., 2009). Here, I found that the stimulatory effect of Pthlh on the formation of the Gli3R is absent in Sufu-deficient chondrocytes, which supports the notion that Sufu plays an important role in Gli3 protein processing. Although, Pthlh inhibits Gli
transcriptional activity and chondrocyte hypertrophy independent of Gli2 (Mau et al., 2007), I found that Pthlh promotes Gli2 degradation in wild-type chondrocytes. However, this effect of Pthlh on Gli2 is not seen in chondrocytes lacking Sufu, suggesting that Sufu is required for this process. Thus, these data suggest that Pthlh stimulates Gli2 degradation and the formation of Gli3R through a Sufu-dependent mechanism. Taken together, I propose a model by which Sufu mediates the effect of Pthlh signaling in the growth plate (Fig. 3-5). Pthlh positively regulates Sufu protein stability in part through phosphorylation and promotes Sufu-mediated Gli2 degradation and Gli3 protein processing, which inhibits chondrocyte hypertrophic differentiation.

Ihh and Pthlh maintain chondrocytes in a proliferative state and premature differentiation occurs in the absence of a functional Ihh/Pthlh feedback loop (Karp et al., 2000; Kobayashi et al., 2002; Lanske et al., 1996; Minina et al., 2001; Kronenberg, 2003; Long et al., 2001). However, what controls the transition from a proliferative state to terminal differentiation is not elucidated. Here, I found that Pthlh treatment delays hypertrophic differentiation in Kif7-deficient limb explants, but not in mutant limb explants lacking Sufu (Col2a-Cre;Sufu$^{ff}$ as well as Col2a-Cre;Sufu$^{ff}$;Kif7$^{ff}$). The requirement of Sufu for the effect of Pthlh on chondrocyte hypertrophic differentiation raises the possibility that Sufu serves as the molecular switch regulating the transition from proliferative to hypertrophic chondrocytes.

In contrast to the PKA-mediated action of Pthlh on chondrocyte hypertrophic differentiation, its stimulatory effect on chondrocyte proliferation is not mimicking by PKA activation. Notably, in the absence of Sufu, Pthlh treatment promotes chondrocyte proliferation and subsequently increases the number of growth plate chondrocytes. Thus, the stimulatory effect of Pthlh on chondrocyte proliferation is not dependent on Sufu, suggesting that Sufu is involved in the PKA-
mediated action of Pthlh. Taken together, Sufu appears to link Ihh and Pthlh signaling in the control of chondrocyte proliferation versus differentiation. Indeed, I showed here that Sufu protein expression is regulated by Pthlh. It is important to note that Sufu is highly expressed in the articular and resting chondrocytes (Hsu et al., 2011). It is plausible that phosphorylation of Sufu by Pthlh near the articular region acts to maintain the level of Sufu protein, thus, preventing Hh pathway activation mediated by Ihh that is expressed by the prehypertrophic chondrocytes. Therefore, the low level of Sufu in prehypertrophic and hypertrophic chondrocytes may be involved in determining the transition from proliferation to differentiation.

Furthermore, a germline mutation of Sufu has been identified in a family of Gorlin syndrome, which is characterized by a range of developmental abnormalities and increased risk of developing of basal cell carcinoma (Pastorino et al., 2009). The pathogenesis of basal cell carcinoma, which is the most common skin tumor, involves aberrant activation of the Hh signaling pathway (Epstein, 2008). Elevated Hh signaling in basal cell carcinomas can be attributed to loss of Ptch1, independent overexpression of Gli1 and Gli2 (Dahmane et al., 1997; Gailani et al., 1996; Grachtchouk et al., 2000; Reifenberger et al., 2005). In mice, deletion of one allele of Sufu resulted in a basal cell carcinoma-like neoplastic growth. Interestingly, Pthlh expression does not seem to be altered in basal cell carcinomas (Philbrick et al., 1996). Thus, it is tempting to speculate that Sufu functions as a tumor suppressor through mediating Gli processing downstream of Pthlh signaling activation in the skin. Therefore, proper regulation of Sufu-mediated Gli processing plays important role in both embryogenesis and tumorgenesis.
3.5 Materials & 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 was previously reported (Cheung et al., 2009). Conditional Sufu-deficient mice (Col2a1-Cre; Sufu<sup>f/f</sup>) 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. Conditional Ptch1-deficient mice (Col2a1-Cre; Ptch1<sup>f/f</sup>) 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. A mouse protocol describing the above experimental procedures was approved by the Animal Care Committee of The Hospital for Sick Children.
**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 till 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).

**Explant cultures**

Hindlimb organ cultures from E16.5 embryos were established as previously described (Ho et al., 2009; Lanske et al., 1996; Mau et al., 2007; Minina et al., 2001). Limbs were placed on a Nucleopore filter inside a well of 24-well tissue culture plate, with the limb at the liquid/air interface. The limb explants were then cultured for 3 days at 37°C in a 5% CO₂ incubator in DMEM containing 0.1% BSA, Vitamin C (50 mg/ml), and Antibiotics-Antimycotic Solution (Wisent). One limb from each embryo was treated with various agents whereas the other acted as a control. 10⁻⁷M Pthlh (Bachem, King of Prussia, Pennsylvania, USA) was added to activate Pthlh signaling. The PKA inhibitor, H-89 dihydrochloride (Alexia Biochemicals), was used at 20µM. These reagents were used alone or in combination for two 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 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. Safranin O staining was performed using standard techniques. Proliferation was evaluated by immunostaining using antibodies against Ki-67 and Phospho-H3 (DakoCytochemical M7249 (from Clone Tec-3) at a 1:50 dilution or (from Sigma) at a 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 10X 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 Biothechnology, sc-28847) incubated at a 1:100 dilution at 4 °C overnight.

Western analysis

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: 10000), Sufu antibody (Meng et
al., 2001), Kif7 antibody (Cheung et al., 2009), and Gli2 antibody (Hu et al., 2006), and phosphor-serine antibody from Cell Signaling (1:1000).

**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, Gli2, Gli3,* and *Sufu* (Applied Biosystems). The gene expression levels between samples were analyzed using the 2^{ΔΔCt} method (Livak and Schmittgen, 2001). Either *GAPDH* or *β-actin* (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 (3mg/ml) for 45 min 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 D 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.
Statistical analysis

For all of the data, the mean, 95% confidence interval, and standard deviation were calculated for each condition, and the Student’s $t$ test was utilized to compare data sets. The threshold for statistical significance was $p < 0.05$. 
3.6 Figures

Figure 3-1 Sufu is required for the negative effect of Pthlh on chondrocyte differentiation.
**Figure 3-1.** Sufu is required for the negative effect of Pthlh on chondrocyte differentiation. (A) Histological analysis of tibial explants from wild-type and *Col2a1-Cre; Sufu^{f/f}* mice in the absence or presence of Pthlh or Pthlh+H89. (B) Relative percentage difference of the length of the hypertrophic zone of Pthlh or Pthlh+H89 treated tibial explants compared to the controls. (C) Relative percentage difference of the proliferative zone of Pthlh or Pthlh+H89 treated tibial explants compared to the controls. Data are shown as means and 95% confidence intervals (n=3).
Figure 3-2 Kif7 is not required for the negative effect of Pthlh on chondrocyte differentiation.

(A) Immunohistochemical staining for Col10a1 and Safranin O staining on tibial explants of Col2a1-Cre; Kif7^{f/f} and Col2a1-Cre; Sufu^{f/f}; Kif7^{f/f} mice with or without Pthlh treatments. (B) Relative percentage difference of the length of the hypertrophic zone of Pthlh-treated tibial explants compared to the controls. Data are shown as means and 95% confidence intervals (n=3).
Figure 3-3 Pthlh increases Sufu protein level.

Figure 3-3. Pthlh increases Sufu protein level. (A) Pthlh treatment resulted in elevated level of Sufu protein in wild-type growth plate chondrocytes. Effective deletion of Sufu in chondrocytes was verified using 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 treatment. (Un: untreated). Data is shown as means and 95% confidence intervals. * p<0.05.
Figure 3-4 Pthlh signaling activation promotes Gli2 and Gli3 protein processing via a Sufu-mediated manner.
**Figure 3-4.** Pthlh signaling activation promotes Gli2 and Gli3 protein processing via a Sufu-mediated manner. (A) qRT-PCR analysis of the expression level of *Gli2* and *Gli3* in Wild-type and *Sufu*-deficient chondrocytes. (B) Western analysis for Gli2 of wild-type and *Sufu*-deficient chondrocytes with or without Pthlh. Actin was used as loading control. (C) Western analysis for full-length Gli3 (Gli3FL) and truncated Gli3 (Gli3R) of wild-type and *Sufu*-deficient chondrocytes with or without Pthlh treatments. (D) Graphical evaluation of the Gli3FL:Gli3R ratio in wild-type and *Sufu*-deficient chondrocytes with or without Pthlh treatment. (E) qRT-PCR analysis of *Gli1* expression in wild-type and *Sufu*-deficient growth plate chondrocytes with or without Pthlh treatment. (F) qRT-PCR analysis of *Ptch1* expression in wild-type and *Sufu*-deficient growth plate chondrocytes with or without Pthlh treatment. Data is shown as means and 95% confidence intervals. * p<0.05.
Figure 3-5 Proposed model for how Pthlh regulates Gli transcriptional factors through a Sufu-mediated mechanism.

**Figure 3-5.** Proposed model for how Pthlh regulates Gli transcriptional factors through a Sufu-mediated mechanism. Pthlh promotes Sufu protein level through phosphorylation, and facilitates Gli2 degradation and Gli3R formation, inhibiting chondrocyte hypertrophic differentiation in the growth plate.
3.7 References


Chapter 4 Conclusions & future directions
4.1 Summary

Although the signaling cascades in the regulation of the growth plate chondrocyte development have been identified, we know little about the precise mechanism by which they act. This study provides insight into a number of open questions in the Hh signaling field as well as into the development of growth plate; how is the dual function of Kif7 mediated, how does Kif7 control Sufu activity, how is Hh pathway activity regulated by Sufu and Kif7 in the context of primary cilium, and how is Sufu activity coupled to Pthlh signaling? In order to develop effective therapeutic approaches to target diseases caused by Hh pathway dysregulation, revealing the molecular basis underlying the Hh signaling cascade in various tissues will be necessary.
4.2 Sufu expression is tightly regulated by multiple factors in the growth plate

4.2.1 Lessons from genetically modified mice and Pthlh activation

In chapter 2, I confirmed that Sufu serves as a negative regulator of the Hh pathway, and it is differentially expressed in the growth plate. Kif7 promotes Hh signaling in chondrocytes by restricting the inhibitory function of Sufu in part through a proteasome-mediated pathway. Kif7 also negatively regulates Hh activity through a Sufu-independent mechanism. Previous studies in cultured fibroblasts have demonstrated that Hh signaling activation accelerates Sufu protein turnover through promoting its degradation in the proteasome (Yue et al., 2009). These data suggest that controlling Sufu protein stability serves as a common mechanism that connects the action of Smo to downstream Gli-mediated transcription (Fig. 4-1).

In chapter 3, I demonstrated that Pthlh increases Sufu protein levels possibly through phosphorylation (Fig. 3-3). Studies have shown that Sufu is phosphorylated at Ser-342 and Ser-346 by GSK3β and PKA, respectively, which leads to Sufu stabilization against Hh signaling-induced degradation (Chen et al., 2011). Although an increased level of phospho-serine upon Pthlh treatment was observed in this study, it will be necessary to confirm the phosphorylation site(s) of Sufu in those Pthlh-treated chondrocytes by examining their Ser-342 and Ser-346 phosphorylation levels. I found that Sufu is required for Pthlh to promote Gli2 degradation and the processing of Gli3, it is tempting to speculate that phosphorylation of Sufu by PKA stabilizes Sufu-Gli2/3 complexes, allowing proteolytic processing of Gli2 and Gli3. Further investigation of this issue requires examination of mice with mutated PKA phosphorylation site(s) of Sufu.
Thus, controlling Sufu activity by regulating its protein stability may again serve as a common mechanism between Ihh and Pthlh signaling in the developing growth plate (Fig. 4-2). In turn this gradual inactivation of Sufu with chondrocyte differentiation could establish a differential responsiveness of differentiating chondrocytes to various signaling cues in the growth plate, such as Ihh itself.

4.2.2 Transcriptional regulation of Sufu

The necessity for proper control of Sufu activity during chondrocyte differentiation raises the question of whether Sufu activity is also regulated by transcriptional mechanisms in the growth plate in addition to post-translational regulation. One potential candidate is Sox9, which is a key regulator for chondrogenesis, chondrocyte proliferation, and expression of cartilage matrix, such as Col2a1, Col9a1, Col11a1, Aggrekan. Sox9 also inhibits the transition of chondrocyte into hypertrophic chondrocyte and subsequent endochondral ossification. I hypothesize that Sox9 inhibits the expression of Sufu at the mRNA level. This hypothesis is supported by several lines of evidence. In the wild-type growth plate, Sox9 is expressed in resting and proliferating chondrocytes, with a maximum of expression in prehypertrophic chondrocyte, but its expression is abolished in hypertrophic chondrocytes (Zhao et al., 1997). Chondrocyte-specific knockout of Sox9 (Col2a1-Cre; Sox9<sup>fl/fl</sup>) results in severe chondrodysplasia, which is a characteristic of defective Hh signaling, as well as reduced expression of Ihh and its downstream target genes, Pthlh and Ptch1 (Akiyama et al., 2002). Furthermore, studies done in cultured multipotent neural precursor cells show that Sufu is a direct target gene of Sox10, which together with Sox8 and Sox9 belong to the SoxE family of transcription factors (Pozniak et al.,
Examination of Sufu transcripts via in situ hybridization in the \textit{Col2a1-Cre; Sox9^{ff}} mutant growth plate will provide preliminary information to this hypothesis. Identification of potential Sox9-binding sites at the promoter region of \textit{Sufu} through in silico analysis combined with chromatin immunoprecipitation (ChIP) will be necessary to further determine whether Sox9 modulates \textit{Sufu} expression level in chondrocytes.

4.2.3 How does Sufu regulate subsequent steps of endochondral ossification?

The lack of \textit{Sufu} expression in the hypertrophic region of the growth plate is of particular interest since it suggests that the downregulation of \textit{Sufu} might be permissive for subsequent transitional changes from the cartilage template to bone, such as cartilage absorption, angiogenesis, and trabecular bone formation. At the lower end of the hypertrophic zone in the growth plate, hypertrophic chondrocytes further mature to become terminally differentiated chondrocytes, a transition which is marked by upregulation of osteopontin (Farzan et al., 2008), \textit{Vegfa} (Gerber et al., 1999), and \textit{Mmp13} (Johansson et al., 1997). \textit{Vegfa} is required for capillary invasion into the hypertrophic zone in the diaphysis from the perichondrium (Carlevaro et al., 2000; Gerber et al., 1999; Maes et al., 2004; Zelzer et al., 2002). Mmp13, which is a matrix metalloproteinase, loosens up the matrix secreted by hypertrophic chondrocytes in order to facilitate blood vessel invasion (Inada et al., 2004; Johansson et al., 1997; Selvamurugan et al., 2004). In the \textit{Col2a1-Cre; Sufu^{ff}} mice, a delay in ossification was observed in both the tibial and vertebral growth plates (Fig. 2-2), suggesting that Sufu may play a role in cartilage vascularization. It will be interesting to generate transgenic mouse lines misexpressing \textit{Sufu} in hypertrophic chondrocytes under the \textit{Col10a1} promoter. In situ hybridization and qRT-PCR
studies will be needed to demonstrate the level of osteopontin, vegfa and Mmp13 expression in the growth plate as a result of Sufu overexpression.

4.2.4 Sufu and osteoarthritis

Activated Hh signaling in chondrocytes is associated with predisposition to the development of osteoarthritis and the level of Hh signaling activity in chondrocytes is associated with the severity of osteoarthritis (Lin et al., 2009). In this condition, articular chondrocytes undergo phenotypic and gene expression changes that are reminiscent of those that occur in the growth plate. Such a process at the articular surface could contribute to the onset of osteoarthritis.

However, the molecular mechanism underlying the Hh pathway activation in osteoarthritis development is unclear. I observed the expression of Sufu at the articular surface of wild-type tibia (Fig. 2-1C). It is tempting to speculate that, similar to what was observed in chondrocyte hypertrophic differentiation, downregulation of Sufu is required at the articular surface for the development of osteoarthritis. If this hypothesis is correct, the next question would be does overexpression of Sufu attenuate the progression of osteoarthritis in mice in vivo? The role of Sufu and Kif7 in the development of osteoarthritis has not been tested, but can be, as described below.

Consistent with the notion that Hh signaling activation is associated with a predisposition to osteoarthritis, reduced Alcian blue staining was observed in the superficial layers of the articular cartilage of Col2a1-Cre; Sufu^{ff} mice and further reduction was observed in the Col2a1-
Cre; Sufu<sup>f/f</sup>; Kif7<sup>f/f</sup> mice at E16.5. Sufu is negatively regulated by Kif7 in chondrocytes. Thus, in the absence of Kif7, the increased level of Sufu may be involved in maintaining chondrocyte characteristics at the articular surface. Indeed, Alcian blue-stained cartilage was observed distributed evenly at the articular surface of Kif7-deficient growth plates at E16.5. The next step to test the significance of Sufu and Kif7 in developing osteoarthritis would be to look at Sufu expression level in osteoarthritic cartilage samples from mouse and/or human. The perinatal lethality in Col2a1-Cre; Sufu<sup>f/f</sup> precludes comprehensive studies on the role of Sufu in osteoarthritis. To investigate whether Sufu inactivation predisposes mice to osteoarthritis, examination of the joint phenotype of genetically modified mice in which Sufu is deleted postnatally will be needed. Sufu level is negatively regulated by Kif7 in chondrocytes. Thus, the effect of overexpression of Sufu on developing osteoarthritis could be tested in Kif7-deficient mice (Col2a1-Cre; Kif7<sup>f/f</sup>). To generate conditional knockout of Sufu, Sufu<sup>f/f</sup> mice could be crossed with Col2a1-rtTA-Cre mice, which express Cre recombinase under Col2a1 regulatory elements inducible by doxycycline so that Sufu can be deleted in chondrocytes when we administer doxycycline (Grover and Roughley, 2006; Jeong et al., 2004). Doxycyclin inducible chondrocyte specific knockout of Sufu and Kif7 (Col2a1-rtTA-Cre; Sufu<sup>f/f</sup>; Kif7<sup>f/f</sup>) could be generated in the same manner. Radiographic and histological analysis could be used to assess the severity of osteoarthritis on wild-type, Col2a1-rtTA-Cre; Sufu<sup>f/f</sup>, Col2a1-rtTA-Cre; Sufu<sup>f/f</sup>; Kif7<sup>f/f</sup>, and Col2a1-Cre; Kif7<sup>f/f</sup> mice at 18-week-old. qRT-PCR analysis would be needed for genes known to be upregulated in osteoarthritis, such as Adamts5, Col10a1 and Mmp13 in all samples. Overall, it would be interesting to see if Sufu inactivation in articular chondrocytes signals the cells to undergo hypertrophic differentiation. If so, can Sufu
overexpression help to maintain their chondrocyte characteristics and therefore serve as a protective mechanism against cartilage degeneration over time?
4.3 The role of Sufu in Wnt/β-catenin signaling pathway during chondrocyte development

4.3.1 Wnt/β-catenin signaling in skeletal development

Similar to Ihh and Pthlh signaling, the Wnt/β-catenin pathway plays an important role in several aspects of endochondral ossification, such as chondrocyte and osteoblast differentiation. β-catenin is a major mediator of Wnt signaling (Kikuchi, 2000; Miller et al., 1999; Waltzer and Bienz, 1999) which has an important role in chondrocyte differentiation, as demonstrated by the growth plate phenotype in mutants lacking β-catenin in their chondrocytes. Studies have shown that overexpression of β-catenin inhibits the differentiation of chondrocyte progenitors, enhances hypertrophic chondrocyte differentiation and promotes the expression of markers for terminally differentiated chondrocytes, including MMP13 and vegfa (Hartmann and Tabin, 2000; Ryu et al., 2002). However, the precise function and regulation of β-catenin in chondrocyte differentiation still needs to be clarified.

β-catenin functions as a downstream intracellular signaling molecule in the canonical Wnt pathway, which regulates cell proliferation and cell differentiation, and cell migration in many aspects of embryogenesis (Huelsken and Birchmeier, 2001; Moon et al., 2002). A large multiprotein complex that includes axin and adenomatous polyposis coli gene product (APC) normally facilitates β-catenin phosphorylation by glycogen synthase kinase 3β (GSK3β) (Ikeda et al., 1998; Kishida et al., 1999; Liu et al., 2002). Phosphorylated β-catenin is ubiquitinated and targeted for degradation through the proteasome-mediated pathway (Kitagawa et al., 1999). In the absence of Wnt ligand, β-catenin phosphorylation and subsequent degradation are inhibited.
β-catenin then accumulates in the cell and translocates into the nucleus, where it binds to the transcription factors T-cell factor (TCF) and lymphoid enhancer factor (Lef), leading to the activation of pathway target genes. The molecular mechanism underlying the cross-talk between Ihh, Pthlh, and Wnt/β-catenin signaling pathways during chondrocyte differentiation remains elusive. Investigating the genetic interaction between Ihh, Pthlh, and Wnt/β-catenin signaling pathways will provide further insight into the means by which different signaling cascades orchestrate cartilage development.

4.3.2 The role of Sufu in mediating Wnt/β-catenin signaling

Sufu and β-catenin are present in the same complex and Sufu can bind β-catenin and export it from the nucleus thereby negatively regulating TCF-dependent transcription in the human colon cancer SW480 cell line (Meng et al., 2001). Immunofluorescence studies have shown that whereas much of the endogenous β-catenin is localized in the cytoplasm of proliferating and prehypertrophic chondrocytes, nuclear localization of β-catenin was observed in hypertrophic chondrocytes (Enomoto-Iwamoto et al., 2002). In chapter 2 I described the expression pattern of Sufu in the growth plate. Sufu was absent in hypertrophic chondrocytes, where β-catenin resides in the nucleus. This result corroborates the notion that Sufu inhibits the nuclear localization of β-catenin. Thus, the downregulation of Sufu may be required for β-catenin nuclear localization in the hypertrophic chondrocytes. Consistent with an inhibitory role of Sufu in regulating Wnt/β-catenin signaling activity, I found a ~40% increase in β-catenin levels in Sufu-deficient chondrocytes demonstrated by western analysis. To clarify the role of Sufu in regulating β-catenin activity and cellular localization, it will be necessary to examine the
expression level of Wnt/β-catenin pathway target genes by qRT-PCR and to visualize the subcellular localization of β-catenin through immunohistochemical or immunofluorescence analysis in the Sufu-deficient growth plate chondrocytes.

In chapter 3 I showed that treatment of growth plate chondrocytes with Pthlh increases Sufu protein level through phosphorylation. Interestingly, phosphorylation of β-catenin at Ser-675 by PKA promotes β-catenin stability by inhibiting its ubiquitination (Hino et al., 2005). Thus, activation of Pthlh/PKA signaling increases the level of β-catenin, which interacts with Sufu. I speculate that Sufu plays a role in mediating the stabilization effect of Pthlh on β-catenin. To test that, western analysis was performed on lysates derived from wild-type and Sufu-deficient growth plates with or without Pthlh treatment. As expected, Pthlh treatment resulted in increased levels of β-catenin (~2-fold) in chondrocytes. However, this effect of Pthlh on β-catenin protein level is absent in the Sufu-deficient cells, suggesting that Sufu is required for the positive effect of Pthlh on the level of β-catenin. It is necessary to confirm the role of PKA as a major effector of Pthlh signaling activation in this process by analyzing the level of β-catenin in cells treated with Pthlh and H89, a PKA inhibitor. The mechanism by which Sufu mediates the stabilization of β-catenin is unknown. One possibility is that the Ser-675 residue of β-catenin may be masked by changes of its three-dimensional structure in the absence of Sufu. Alternatively, Sufu may be required for the cytoplasmic localization of β-catenin where it can be phosphorylated and stabilized.

Wnt/β-catenin signaling promotes chondrocyte hypertrophic differentiation, whereas Pthlh signaling inhibits it. Thus, activation of Pthlh in chondrocytes opposes Wnt/β-catenin signaling. Although Wnt/β-catenin signaling has been shown to regulate osteoblast differentiation from the perichondrium at late stage chondrocyte differentiation, the function of
high levels of β-catenin upon Pthlh/PKA stimulation in articular/resting and proliferating chondrocytes in vivo is unknown. This complicated pattern may allow regulatory cross-talk with other signaling pathways that are useful. One possible scenario is that Pthlh upregulates β-catenin and Sufu levels to ensure sufficient amounts of β-catenin throughout the course of chondrocyte hypertrophic differentiation; upregulation of Sufu levels may serve as an important mechanism by which cells maintain low Wnt signaling such that chondrocytes can differentiate. Later, in hypertrophic chondrocytes, Sufu inhibition is relieved, β-catenin translocates into the nucleus and activates expression of target genes, such as MMP13 and vegfa, which are essential for subsequent bone formation.

The involvement of Sufu in the regulation of Wnt/β-catenin signaling provides evidence that multiple levels of cross-talk exist between Ihh, Pthlh, and Wnt/β-catenin signaling. It also highlights the importance of Sufu as a general mediator for multiple signaling pathways during chondrocyte development. To this end, it will be interesting to further investigate the function of β-catenin in Sufu-expressing chondrocytes by generating Col2a1-Cre;Sufu^{9f}; Ctnb^{tm1Tak} mice, which express stabilized β-catenin in the absence of Sufu in growth plate chondrocytes.
4.4 Conclusions

Proper coordinate control of cell proliferation and differentiation through cell-cell signaling is a major strategy during endochondral ossification. The well-orchestrated cross-talk between genes spatially and temporally is essential to guide the patterning of each of the skeletal elements. I demonstrated that the molecular mechanism by which Sufu and Kif7 coordinate Ihh signaling and further pinpointed the role of Sufu in mediating Pthlh signaling in chondrocytes. These discoveries suggest that Sufu levels could be critical for cells to respond to multiple signaling pathways in chondrogenesis. Thus, understanding the mechanism underlying Sufu regulation may provide insight into cartilage regeneration in vitro. As other signaling pathways, such as Wnt/β-catenin signaling, also play important roles in controlling skeletal development, further investigation of how Sufu is integrated with various signaling pathways will help us to gain a full picture of the molecular regulatory network in this process.
Figure 4-1 Proposed model by which Sufu protein stability serves as a common mechanism that regulates Gli-mediated transcription.

Figure 4-1. Proposed model by which Sufu protein stability serves as a common mechanism that regulates Gli-mediated transcription. Sufu protein stability is negatively regulated by Smo-mediated Hh activation and Kif7 through a proteasome-mediated pathway. Phosphorylation of Sufu upon Pthlh signaling activation promotes Sufu stabilization resulted in reduced expression of Hh target genes.
Proposed mechanism by which Pthlh and Ihh signaling coordinate Sufu expression in the growth plate.

Figure 4-2. Proposed mechanism by which Pthlh and Ihh signaling coordinate Sufu level in the growth plate. Pthlh positively regulates Sufu expression through phosphorylation, and Ihh negatively regulates Sufu level thorough promoting its degradation. Sox9 may negatively regulate Sufu expression at the mRNA level.
4.6 References


McGlashan, S. R., Haycraft, C. J., Jensen, C. G., Yoder, B. K. and Poole, C. A. (2007). Articular cartilage and growth plate defects are associated with chondrocyte cytoskeletal...


Appendix A: Pthlh negatively regulates Hh signaling activity in chondrocytes through a cilia-independent mechanism

Shu-Hsuan C. Hsu, Xiaoyun Zhang, and Benjamin A. Alman

All experiments and analysis described here were conducted by Shu-Hsuan C. Hsu with the exception of the western analysis by Xiaoyun Zhang.
A.1 Summary

Chondrocyte proliferation and differentiation are tightly controlled by Indian hedgehog (Ihh) and parathyroid hormone-like hormone (Pthlh) signaling pathways in the growth plate. Pthlh is known to function as a negative regulator of chondrocyte hypertrophic differentiation and of the Hh signaling pathway. Smo-mediated Hh pathway activation depends on the primary cilium, which plays a positive role in regulating Hh activity in chondrocytes. Here I show that Pthlh functions in chondrocytes through a cilia-independent mechanism. The data suggest a model in which Pthlh promotes Sufu-Gli complexes accumulation at the cilium, resulting in reduced Hh pathway activity. However, in the absence of cilia, Pthlh treatment resulted in reduced Hh pathway activity, suggesting that the ciliary tip localization of Sufu-Gli complexes is not required for their ability to regulate transcription.
A.2 Introduction

Cilia are microtubule-based organelles that project from the surface of most eukaryotic cells (Davenport and Yoder, 2005; Pan et al., 2005; Satir and Christensen, 2007). The cilium extends from a basal body, which is a centriole-derived microtubule organizing center. Cilia are assembled and maintained through intraflagellar transport (IFT), a process involving anterograde and retrograde transportation of IFT particles, mediating by kinesin and dynein motors, respectively (Bisgrove and Yost, 2006; Davenport and Yoder, 2005; Pan et al., 2005; Satir and Christensen, 2007). Disruption of the expression and function of IFT particles, such as IFT88, results in the loss of cilia (Haycraft et al., 2007; Yoder et al., 2002). *Ift88<sup>ORP<sub>K/</sub>ORPK</sup> mouse*, which was developed from a large-scale transgene-induced insertional mutagenesis project at the Oak Ridge National Laboratory harbors in 1994, exhibits scruffy fur, growth retardation, and polydactyly (Moyer et al., 1994).

During growth plate development, Indian Hedgehog (Ihh) is secreted by the prehypertrophic chondrocytes and it regulates chondrocyte hypertrophic differentiation through upregulating parathyroid hormone-like hormone (Pthlh) expression at the periarticular region (Chung et al., 1998; St-Jacques et al., 1999; Vortkamp et al., 1996). Pthlh functions to maintain chondrocytes at the proliferative state and delay hypertrophic differentiation thus reducing the level of Ihh expression in prehypertrophic chondrocytes (Karaplis et al., 1994; Lanske et al., 1996). This negative feedback loop formed by Ihh and Pthlh plays an important role in coordinating chondrocyte proliferation and differentiation in the growth plate (Chung et al., 1998; Vortkamp et al., 1996).
In vertebrates, the effects of Hh signaling are mediated by three Gli transcription factors (Gli1-3). Upon Hh ligand binding to its receptor, patched 1 (Ptc1), Ptc1 inhibition on smoothened (Smo) is released, leading to activation of Gli-mediated transcription in the nucleus. Suppressor of fused (Sufu) and kinesin member 7 (Kif7) are two evolutionary conserved regulators of Gli transcription factors in mammalian Hh signaling (Wilson et al., 2009). During growth plate development, Sufu acts as a major negative regulator of Gli transcription factors whereas Kif7 functions both positively and negatively in chondrocytes (Hsu et al., 2011). The primary cilium serves as the focal point of hedgehog (Hh) signaling regulation in vertebrates. Important components of the Hh pathway, suppressor of fused (Sufu) and the Gli proteins, localize to cilia and Smo and Kif7 translocate to cilia upon pathway activation (Corbit et al., 2005; Haycraft et al., 2005). Recent studies carried out in cultured fibroblasts showed that Smo plays a positively role in dissociating of inhibitory Sufu-Gli complexes at the primary cilium upon Hh pathway activation. Therefore a model by which Hh signaling activates Gli protein at the cilia through relieving the inhibitory function of Sufu, leading to Gli-mediated transcription activation was proposed.

Studies of explants lacking Gli genes suggest the involvement of Gli3 in the inhibitory effect of Pthlh on growth plate chondrocyte differentiation. Pthlh represses Gli-mediated transcription via a protein kinase A (PKA)-dependent mechanism (Mau et al., 2007; Miao et al., 2004; Vortkamp et al., 1996). These data suggest that besides the Hh-mediated regulation, the Gli transcription factors may also be regulated by Pthlh signaling. However, the molecular mechanism as well as the role of cilia in the Pthlh-mediated regulation of Gli activity is unknown.
In this study, I explored the role of primary cilia in regulating the effect of Pthlh on Hh signaling activity during chondrocytes development using \( \text{Ift}^{\text{ORPK/ ORPK}} \) mice. My results indicate that the primary cilia are not required for the inhibitory effect of Pthlh on chondrocyte hypertrophic differentiation or Gli-mediated transcription activity. Sufu is localized to the ciliary tip of resting chondrocytes in vivo upon Pthlh stimulation, which is consistent with previous studies showing that phosphorylation of Sufu by PKA regulates its ciliary localization (Chen et al., 2011). However, the ciliary tip localization of Sufu is not required for the negative regulation of Pthlh on downstream Gli transcription activity.
A.3 Results

A.3.1 *Ift88*^{ORPK/ ORPK} mice show skeletal defects and dysregulated Hh signaling in chondrocytes

To investigate the role of primary cilia in growth plate chondrocyte development, I examined the skeletal phenotype of *Ift88*^{ORPK/ ORPK} mice. Consistent with previous studies, *Ift88*^{ORPK/ ORPK} mice survive into young adulthood and exhibit polydactyly and shortening of the bone in the limbs due to defects in endochondral bone formation (Fig. A-1). Histological analysis revealed asymmetric chondrocyte hypertrophy as well as a delay in vascularization of the primary ossification center in *Ift88*^{ORPK/ ORPK} mice compared with wild-type littermates (Fig. A-1). Notably, this asymmetric hypertrophy phenotype does recover with time, and two opposite growth plates were observed in the tibia of *Ift88*^{ORPK/ ORPK} mice at E18.5 (Fig. A-2). In support of the role of cilia in mediating Hh signaling during development, I found reduced *Ptch1* and *Gli1* mRNA expression in the cilia-deficient chondrocytes compared with wild-type controls. Activation of *Ptch1* and *Gli1* upon Hh ligand stimulation is lost in *Ift88*^{ORPK/ ORPK} chondrocytes, suggesting that cilia are required for Hh ligand responsiveness in chondrocytes (Fig. A-3).

A.3.2 Pthlh downregulates *Col10a1* expression and Hh signaling activity through a cilia-independent mechanism in chondrocytes

In chapter 2 I demonstrated that Pthlh represses Hh signaling activity in part through increasing Sufu levels and facilitating Gli2 degradation and Gli3R formation in chondrocytes.
However, questions remain regarding the role of primary cilia in mediating the effect of Pthlh on Hh activity regulation. In particular, the involvement of cilia in Pthlh signaling in chondrocytes needs to be tested. To begin to explore the role of primary cilia in mediating Pthlh signaling during chondrocyte differentiation, I performed Pthlh treatment on wild-type and Ifi88\textsuperscript{ORPK/ORPK} micromass cultures at various time points followed by qRT-PCR analysis for Col10a1 and Ptch1, as readouts for hypertrophy and Hh activity, respectively. The level of Col10a1 expression increased with time in wild-type cultures, demonstrating chondrocyte hypertrophy \textit{in vitro}. Deletion of Ifi88 resulted in reduced levels of Col10a1 expression in all three time points examined (Fig. A-4). Pthlh treatment resulted in a reduction in the Col10a1 expression levels in differentiated chondrocytes cultures (at Day 6 and 9) from both wild-type and Ifi88\textsuperscript{ORPK/ORPK} mice (Fig.A-4), suggesting that Pthlh inhibits Col10a1 expression in chondrocytes in a cilia-independent manner. In wild-type cultures, Ptch1 expression progressively increases with time, which mimics what was seen during chondrocyte differentiation in the growth plate. In the absence of Ifi88, Ptch1 expression is compromised, although increased Ptch1 expression can still be observed over time (Fig.A-4). Pthlh treatment resulted in decreased levels of Ptch1 expression in differentiated chondrocytes in both wild-type and Ifi88\textsuperscript{ORPK/ORPK} cultures (Fig.A-4). These data suggest that cilia are not required for the regulation of Gli transcription activity and chondrocyte hypertrophic differentiation by Pthlh.

A.3.3 Ciliary tip localization of Sufu is induced by Pthlh

In chapter 2 I demonstrated that Kif7 plays a role in the exclusion of Sufu-Gli complexes from the primary cilium. To investigate whether primary cilium is required for Sufu and Kif7
protein and/or RNA expression, I examined Sufu and Kif7 expression in Ift88\textsuperscript{ORPK/ORPK} mice. No significant difference in protein or RNA levels of Sufu and Kif7 was detected in Ift88\textsuperscript{ORPK/ORPK} cells (Fig A-5A, B), suggesting that primary cilia are not required for the expression of Sufu and Kif7. The observations that Pthlh represses Hh signaling activity independent of cilia prompted us to investigate the ciliary localization of Sufu and Kif7 upon Pthlh signaling activation in the presence or absence of primary cilium. Studies have shown that Sufu and Gli proteins are transported into the primary cilium as a complex (Hsu et al., 2011; Humke et al., 2010; Tukachinsky et al., 2010). In Gli2\textsuperscript{-/-}; Gli3\textsuperscript{-/-} embryonic fibroblasts, where all Gli proteins are absent, Sufu is not detected in the cilia, even upon Shh stimulation, suggesting that Gli proteins are necessary to recruit Sufu to the cilia (Tukachinsky et al., 2010; Zeng et al., 2010). Thus, the immunostaining of Sufu could serve as an indicator of Sufu-Gli complexes at the primary cilium.

In resting chondrocytes (where the Hh pathway is inactive), Kif7 was mostly localized to the ciliary tip, whereas Sufu was found at the basal bodies in ~75% of these cells but was rarely detected at the ciliary tip (Fig A-5C-E). Both Sufu and Kif7 signals are specific as they are absent in Sufu-deficient and Kif7-deficient chondrocytes, respectively (Fig. 2-8A). Together, these results indicate that there is very little Sufu-Gli complexes in the primary cilium of resting wild-type chondrocytes. Pthlh treatment resulted in a ~20% decrease in the ciliary tip localization of Kif7 (Fig. A-5C-E). Consistent with recent studies carried out in cultured fibroblasts showing that PKA phosphorylation of Sufu promotes its ciliary localization (Chen et al., 2011), Sufu was detected at the ciliary tip in ~50% of the Pthlh-treated wild-type chondrocytes (Fig. A-5C-E), suggesting that Pthlh stimulates Sufu ciliary tip localization possibility through promote phosphorylation of Sufu by PKA. Interestingly, in the absence of the primary cilium, ~80% of resting chondrocytes showed Sufu and Kif7 staining at the basal body
(Fig. A-5C, F, G). Although no change in the Sufu basal body localization was detected, a ~40% decrease of Kif7 basal body localization was found upon Pthlh stimulation (Fig. A-5C, F, G).

These observations suggest that Pthlh promotes Sufu-Gli complexes ciliary tip localization in wild-type chondrocytes, and the ciliary tip localization of Sufu-Gli complexes is not required for their downstream inhibitory function on Gli-mediated transcription activity. In chapter 3 I demonstrated that Kif7 is not required for the negative effect of Pthlh on growth plate chondrocytes. Thus, the changes in Kif7 ciliary localization may play a minor role in regulating the effect of Pthlh on chondrocyte Hh signaling activity.
A.4 Discussion

In chapter 2 I demonstrated distinct and overlapping functions of Sufu and Kif7 in Hh signaling during chondrocyte development. In chapter 3 I established that Sufu plays an important role in regulating the negative effect of Pthlh on chondrocyte hypertrophic differentiation, Gli proteins processing, and Hh signaling activity. There is evidence suggesting that the primary cilium serves as a focal point in Hh signaling regulation in vertebrates. Here, I provided data to indicate that Pthlh functions to inhibit chondrocyte hypertrophic differentiation and Hh signaling activity in chondrocytes through a cilia-independent mechanism.

Phenotypic analysis of Ift88\textsuperscript{ORPK/ORPK} mice indicates that the primary cilia are required for normal skeletal development. Ift88\textsuperscript{ORPK/ORPK} chondrocytes showed reduced Hh pathway activity and lack of response to Hh ligand stimulation compared to wild-type controls. Pthlh possess inhibitory effect on the expression of Col10a1 and Ptch1 in wild-type micromass cultures. This is consistent with its negative role in regulating chondrocyte hypertrophic differentiation and Hh signaling activity in the growth plate in vivo. Similarly, reduced Col10a1 and Ptch1 expression were observed upon Pthlh signaling activation in cilia-deficient (Ift88\textsuperscript{ORPK/ORPK}) micromass cultures. The data suggest that primary cilia are not required for Pthlh ligand responsiveness or the negative effect of Pthlh on chondrocyte differentiation and Hh activity regulation.

Hh pathway activation stimulates degradation of Sufu in some cancer cell lines (Yue et al., 2009). 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). In chapter 3 I showed that Pthlh plays a positive
role in controlling the stability of Sufu protein as well as regulating Gli2 protein degradation and Gli3 repressor (Gli3R) formation in the growth plate chondrocytes. Interestingly, here I 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 presence of Pthlh. This is consistent with the observation that Hh pathway activity is reduced in Pthlh-treated cells, owing to inefficient dissociation of the Sufu-Gli complexes. Sufu is found to be localized at the basal body in the absence of the primary cilium and there is no change in its basal body localization observed upon Pthlh activation. The data suggest that the ciliary tip localization is not required for the inhibitory effect of Sufu-Gli complexes in chondrocytes. However, whether the ciliary tip localization contributes to the stabilization of Sufu in Pthlh-treated chondrocytes or its related to the increased level of Sufu protein awaits further investigation. Nevertheless, results here demonstrated that Pthlh functions independent of primary cilia in chondrocytes.

Previous in vitro studies show that Hh ligand stimulation promotes Kif7 cilia tip translocation (Liem et al., 2009). In chapter 2 I found that Kif7 localizes to the ciliary tip in the proliferating chondrocytes in vivo. Here I reported that Kif7 localizes also to the ciliary tip in the resting chondrocytes where the Hh pathway is inactive (as indicated by the lack of Hh target gene expression). These results suggest that Kif7 might have a functional role in the primary cilium, even in the absence of Hh ligand activation which supports the proposed model in chapter 2 (Fig. 2-10B). In the cilia-deficient (Ifi88^{ORPK/ ORPK}) chondrocytes, Sufu localizes to the basal body in the absence or presence of Pthlh, suggesting that the ciliary tip localization is not the key control for the inhibitory function of Sufu-Gli complexes, and their ability to regulate transcription. This notion is consistent with the previous reports showing that Sufu functions independent of the primary cilium in controlling Gli protein levels (Chen et al., 2009). Taken
together, the data suggest that despite the importance of the primary cilium in controlling Hh pathway activation, the primary cilium is not required for Sufu-mediated negative regulation of Hh pathway activity in chondrocytes.

Recent studies have shown that PKA, the main mediator of Pthlh signaling in chondrocytes, localizes to the basal body in cultured fibroblasts (Barzi et al., 2010; Tuson et al., 2011). This colocalization of PKA and Sufu prompts me to propose a model for cilia-mediated regulation of Gli activity (Fig. A-6). In the presence of Hh ligand, Sufu-Gli complexes enter the cilium and are modified within the cilium, either by covalent modification or through interaction with other proteins such as Kif7, to allow dissociation of the complexes and activation of the Hh pathway. In the presence of Pthlh ligand, Sufu is phosphorylated by PKA; p-Sufu-Gli complexes enter the cilium. After exiting the cilium Gli2 complexed with Sufu is recognized and phosphorylated by PKA, which leads to Gli2 degradation. Phosphorylation by PKA targets Gli3 complexed with Sufu to proteasome-mediated processing into Gli3R.

Taken together, I show here a cilia-independent mechanism by which Pthlh mediates chondrocyte hypertrophic differentiation and Hh signaling activity regulation in chondrocytes. Therefore, I propose that in wild-type chondrocytes, Pthlh inhibits hypertrophic differentiation and Gli-mediated transcription. There is an increased level of Sufu-Gli complexes in the primary cilium upon Pthlh activation, resulting in an inhibition of Hh signaling. In the absence of the primary cilium, the inhibitory function of Sufu-Gli complexes remains. Pthlh treatment results in an increased level of Sufu-Gli complexes localized at the basal body, resulting in reduced Hh pathway activity.
A.5 Materials & 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

$Ift88^{\text{ORPK}} (Tg737^{\text{ORPK}})$ mutants were received from Dr. Yoder (The University of Alabama). The mice were genotyped as described previously (Yoder et al., 1996). In all cases, littermate mice were used as controls. A mouse protocol describing the above experimental procedures was approved by the Animal Care Committee of The Hospital for Sick Children.

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 till 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).

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 (H&E) staining were performed using standard techniques.

**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 Glil, Ptc1, and Col10a1 (Applied Biosystems). The gene expression levels between samples were analyzed using the $2^{\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001). Either GAPDH or β-actin (Applied Biosystems) was used as endogenous control for target gene normalization.

**Primary growth plate chondrocyte cultures**

Chondrocyte isolation protocol was modified from previous 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 (3mg/ml) for 45 min 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 type 4 solution containing chondrocytes was collected. Cells were washed with PBS and seeded at a density of 8x10³ cells per cm².

**Micromass cultures**

Fore- and hind-limbs were isolated from E11.5 mouse embryos and collected in growth medium (DMEM + 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin), washed twice in PBS, and digested in 0.1% trypsin and 0.1% collagenase D (Worthington Biochemical) in PBS at 37 °C for 20 min. 20 μl of a single cell suspension (2 × 10⁷ cells/ml) was plated in the center of 9.6 cm² dishes. The cultures were incubated at 37 °C in a humidified incubator with 5% CO₂ for 1 h, after which they were gently flooded with 2 ml of growth medium.

**Immunofluorescence**

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.

**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 utilized to compare data sets. A *p* value of < 0.05 was used as a threshold for statistical significance.
Figure A-1 The primary cilia are required for normal embryonic skeletal development.

**Figure A-1.** The primary cilia are required for normal embryonic skeletal development. Upper panel: Alcian blue/Alizarin red stained hind limbs from E16.5 wild-type and Ifi88^{ORPK/ ORPK} mice. Note polydactyly (arrowhead) and shortening of the long bones in the Ifi88^{ORPK/ ORPK} limb. Lower panel: H&E staining showed asymmetrical chondrocyte hypertrophy and delayed in primary ossification center formation in the Ifi88^{ORPK/ ORPK} mice compared to wild-type littermates at E16.5.
Figure A- 2 Asymmetrical hypertrophy phenotype in \textit{Ift88}^{OPK/OPK} mice is not seen at E18.5.

**Figure A-2.** Asymmetrical hypertrophy phenotype in \textit{Ift88}^{OPK/OPK} mice is not seen at E18.5. Histological and immunohistochemical analysis for Col10a1 expression of the \textit{Ift88}^{OPK/OPK} mouse tibia showed the asymmetrical hypertrophy phenotype observed at E16.5 is not seen at E18.5.
Figure A-3. The role of primary cilia in chondrocyte ligand responsiveness. Reduced level of *Ptch1* and *Gli1* expression was found in cilia-deficient chondrocytes assessed by qRT-PCR. Shh treatment resulted in increased *Ptch1* and *Gli1* mRNA expression in wild-type chondrocytes. In *cilia-deficient* chondrocytes, Shh treatment did not result in Hh signaling activation. Data are shown as means with 95% confidence intervals indicated (n=3). *P<0.05.
Figure A-4 The role of primary cilia in mediating Pthlh signaling in chondrocyte differentiation.

**Figure A-4.** The role of primary cilium in mediating Pthlh signaling in chondrocyte differentiation. Expression of *Col10a1* and *Ptch1* in micromass cultures of wild-type and *Ift88^{ORPK/ ORPK}* mice at various time points (Day3, Day6, and Day9) with or without Pthlh treatment was assessed by qRT-PCR. Data are shown as means with 95% confidence intervals indicated (n=2). *P*<0.05.
Figure A- 5 Increased Sufu ciliary localization in Pthlh-treated wild-type chondrocytes.
**Figure A-5.** Increased Sufu ciliary localization in Pthlh-treated wild-type chondrocytes. (A) Sufu and Kif7 protein levels in wild-type and Ift88ORPK/ORPK mice were assessed by western analysis. Actin was used as loading control. (B) Sufu and Kif7 mRNA expression levels in Ift88ORPK/ORPK mice were found to be comparable with wild-type controls. Data are shown as means with 95% confidence intervals indicated (n=3). (C) Fluorescence micrographs of cilia or basal bodies from wild-type and Ift88ORPK/ORPK growth plate chondrocytes in the present or absence of Pthlh treatment. Sufu (ciliary tip localization of Sufu is indicated by white arrow; basal body localization of Sufu is indicated by white asterisk) and Kif7 (ciliary tip localization of Kif7 is indicated by green arrow; basal body localization of Kif7 is indicated by green asterisk) 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. (D-G) 25 to 50 ciliated cells from the resting zone of wild-type and Ift88ORPK/ORPK growth plates with (+Pthlh) or without (Un: untreated) Pthlh treatment were examined. Data are shown as percentages.
Figure A-6 A proposed model for cilia-mediated regulation of Gli activity.

Figure A-6. A proposed model for cilia-mediated regulation of Gli activity. In this model, PKA-mediated Gli processing is determined by the appropriate Gli substrate, which becomes available only when Gli is complexed with phosphorylated Sufu (p-Sufu) in the cytoplasm. In the presence of Hh ligand, active Smo localizes to the cilia and dissociation of Sufu-Gli complexes in the cilium is promoted by Smo and Kif7; free Gli proteins enter the nucleus and activate the transcription. In the presence of Pthlh ligand, Sufu is phosphorylated by PKA and p-Sufu-Gli complexes enter the cilium. Gli2 complexed with p-Sufu is recognized and phosphorylated by PKA leading to Gli2 degradation. Gli3 complexed with p-Sufu is targeted for phosphorylation and proteasome-mediated processing into Gli3R.
A.7 References


140


