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# Differential regulation of Gli proteins by Sufu in the lung affects PDGF signaling and myofibroblast development

**Chuwen Lin**[1], **Miao-Hsueh Chen**[1,2], **Erica Yao**[1], **Hai Song**[1], **Rhodora Gacayan**[1], **Chi-chung Hui**[3], and **Pao-Tien Chuang**[1,*]

[1]Cardiovascular Research Institute, University of California, San Francisco, CA 94158

[2]USDA/ARS Children's Nutrition Research Center, Department of Pediatrics, Baylor College of Medicine, Houston, TX 77030

[3]Program in Developmental and Stem Cell Biology, The Hospital for Sick Children, and Department of Molecular Genetics, University of Toronto, Toronto, Ontario, Canada

## Abstract

Mammalian Hedgehog (Hh) signaling relies on three Gli transcription factors to mediate Hh responses. This process is controlled in part by a major negative regulator, Sufu, through its effects on Gli protein level, distribution and activity. In this report, we showed that Sufu regulates Gli1 protein levels by antagonizing Numb/Itch. Otherwise, Numb/Itch would induce Gli1 protein degradation. This is in contrast to inhibition of Spop-mediated degradation of Gli2/3 by Sufu. Thus, controlling protein levels of all three Gli genes by Sufu is a conserved mechanism to modulate Hh responses albeit via distinct pathways. These findings in cell-based assays were further validated *in vivo*. In analyzing how Sufu controls Gli proteins in different tissues, we discovered that loss of *Sufu* in the lung exerts different effects on Hh target genes. Hh targets *Ptch1/Hhip* are upregulated in *Sufu*-deficient lungs, consistent with Hh pathway activation. Surprisingly, protein levels of Hh target Gli1 are reduced. We also found that myofibroblasts are absent from many prospective alveoli of *Sufu*-deficient lungs. Myofibroblast development is dependent on PDGF signaling. Interestingly, analysis of the *Pdgfra* promoter revealed a canonical Gli-binding site where Gli1 resides. These studies support a model in which loss of *Sufu* contributes to compromised *Pdgfra* activation and disrupts myofibroblast development in the lung. Our work illustrates the unappreciated complexity of Hh responses where distinct Hh targets could respond differently depending on the availability of Gli proteins that control their expression.

### Keywords

Hedgehog; Sufu; Gli; Lung; Myofibroblast; PDGF



*Correspondence should be addressed to: Pao-Tien Chuang: pao-tien.chuang@ucsf.edu.

## Introduction

Hedgehog (Hh) signaling controls key steps of development in most tissues and organs of invertebrates and vertebrates (Briscoe and Therond, 2013; Ingham et al., 2011; Wilson and Chuang, 2010). The unique cellular composition and morphological movement in individual tissues require distinct modes of Hh signaling. For example, in the mammalian neural tube and limb, Hh expression from a localized source, such as the notochord/floor plate and the zone of polarizing activity (ZPA), is known to exert dose-dependent long-range signaling effects on tissue patterning. By contrast, in several branching organs such as the lung, epithelial Hh signaling to the mesenchyme mediates critical aspects of epithelial-mesenchymal interactions that drive lung branching morphogenesis. Hh signaling thus generates different outputs in diverse tissues, which underlie cellular changes during tissue patterning. Uncovering the whole complement of Hh targets and how they control cellular changes in each tissue is required for understanding the development of a given tissue. This knowledge will also contribute to our mechanistic understanding of tissue regeneration and repair and cancer development, in which Hh signaling is frequently activated (Barakat et al., 2010; Bijlsma and Roelink, 2010; Scales and de Sauvage, 2009).

The Hh pathway has been extensively studied for two decades, culminating in a basic framework of mammalian Hh signal transduction that depends on Gli transcription factors (Gli1-3) to mediate Hh responses (Beachy et al., 2010; Chen and Jiang, 2013; Eggenschwiler and Anderson, 2007; Farzan et al., 2008; Hui and Angers, 2011; Rabinowitz and Vokes, 2012; Robbins et al., 2012; Ryan and Chiang, 2012; Wang et al., 2007). Gli3 (and to some extent Gli2) undergoes limited proteolysis in the absence of the Hh ligand to produce a transcriptional repressor (Pan et al., 2006; Wang et al., 2000). Hh signaling not only inhibits proteolysis of Gli proteins but also promotes the conversion of Gli proteins (primarily Gli2) into transcriptional activators. *Gli1*, like *Ptch1* and *Hhip*, is a transcriptional target of Hh signaling and Gli1 induction is believed to amplify Hh responses. The combinatorial effects of Gli activators and repressors likely mediate graded Hh responses in diverse tissues. In this regard, a large gap remains in our ability to correlate Hh signaling outputs with phenotypic outcomes since it is difficult to delineate the contributions of individual Gli protein or its processed form. This is further complicated by the differential expression and requirement of Gli proteins (Bai et al., 2004; Bowers et al., 2012; Cao et al., 2013; Ding et al., 1998; Matise et al., 1998) and their complex interactions in diverse tissues (Bowers et al., 2012; Liu et al., 2012).

One of the critical events in mammalian Hh signaling involves regulation of Gli by Suppressor of fused (Sufu), a major negative regulator. Studies of Sufu thus provide a unique opportunity to uncover the molecular mechanisms by which Gli proteins control Hh signaling. Sufu can sequester Gli proteins (Barnfield et al., 2005; Ding et al., 1999; Kogerman et al., 1999; Murone et al., 2000), regulate Gli2/3 protein levels (Chen et al., 2009; Jia et al., 2009; Wang et al., 2010), facilitate the production of Gli repressor and inhibit the generation of Gli activators (Humke et al., 2010; Tukachinsky et al., 2010). Perhaps all of these actions ensure the production of appropriate amounts of Gli activators and repressors as well as a pertinent Gli activator/repressor ratio necessary for tissue

development and homeostasis. The relative contribution of multiple effects of Sufu to Gli protein functions has not been clearly delineated.

A key aspect of Hh signaling is to turn on Hh target genes through Gli activators. In this report, we investigate how Sufu controls Gli1 protein levels both *in vitro* and *in vivo* (lungs). These studies not only revealed a conserved mechanism by which Sufu controls Gli protein levels but also led to the unexpected finding that Hh targets can exhibit different responses when the Hh pathway is activated. We speculate that this is because different combinations of Gli proteins are present in a particular tissue for activating Hh targets. Thus some Gli targets are upregulated while others are downregulated, depending on the availability of Gli proteins that control their expression in a specific tissue. This result reveals the complexity of Hh responses in diverse tissues and increases our understanding of how the Sufu/Gli circuitry controls Hh pathway activation.

## Materials and methods

### Animal husbandry

All mice were handled in accordance with the animal care policies of the UCSF Institutional Animal Care and Use Committee. Null and conditional alleles of *Sufu*, *Ptch1*$^{LacZ}$ (STOCK *Ptch1*$^{tm1Mps}$/J), *Dermo1*$^{Cre}$ (B6.129X1-$^{Twist2tm1.1(cre)Dor}$/J), *Gli1*$^{LacZ}$ (STOCK *Gli1*$^{tm2Alj}$/J) and *Pdgfra*$^{H2B\text{-}eGFP}$ (B6.129S4-*Pdgfra*$^{tm11(EGFP)Sor}$/J) mice have been previously described (Bai et al., 2002; Chen et al., 2009; Goodrich et al., 1997; Hamilton et al., 2003; Pospisilik et al., 2010; Yu et al., 2003).

*Sufu*$^{f}$ mice harboring loxP sites flanking exons 4–8 of *Sufu* have been previously reported (Chen et al., 2009). Mesenchyme-specific deletion of *Sufu* was achieved by generating *Sufu*$^{f/-}$; *Dermo1*$^{Cre/+}$ mice through crosses between *Dermo1*$^{Cre/+}$; *Sufu*$^{+/-}$ and *Sufu*$^{f/f}$ mice. Crosses were also set up to bring *Ptch1-LacZ* to *Sufu*$^{f/-}$; *Dermo1*$^{Cre/+}$ mice. Embryos were obtained from timed pregnancies.

### Histology and in situ hybridization

Embryos were harvested at indicated time points and the embryos or lungs were fixed in 4% paraformaldehyde (PFA) in PBS at 4°C overnight, embedded in paraffin and sectioned at 6 μm. Histological analysis and section *in situ* hybridization using $^{33}$P-labeled riboprobes were performed as reported (Chen et al., 2009).

### Standard molecular biology

Standard molecular biology techniques were performed as previously described (Nagy et al., 2003; Sambrook and Russell, 2001).

### Isolation of primary lung mesenchymal cells

To derive lung mesenchymal cells, mouse embryonic lungs from wild-type (wt) and *Sufu*$^{f/-}$; *Dermo1*$^{Cre/+}$ embryos were digested in 0.05% Trypsin/EDTA at 37°C for 10 minutes. Cells were then seeded into culture dishes and incubated at 37°C for 1 hour. Cells in suspension

were removed and lung mesenchymal cells that attached to the dishes were harvested for Western blotting, qPCR analysis or immortalization as described (Chen et al., 2009).

## Cell culture, transfections, and immunoprecipitation

Wild-type and *Sufu*$^{-/-}$ mouse embryonic fibroblasts (MEFs) (Chen et al., 2009) and lung mesenchymal cells were maintained as described (Chen et al., 2009).

HEK293T or lung mesenchymal cells were transfected with different combinations of pcDNA3-Gli1-3xFLAG, pcDNA3-Numb-Myc and pcDNA3-Sufu-Myc. 48 hrs post-transfection, cells were lysed with IP buffer (1% Triton X-100, 150mM NaCl, 50mM Tris-Cl, 1mM EDTA) with protease inhibitor cocktail (Roche). The lysates were cleared by centrifugation and the resultant supernatants were bound to 15 μl FLAG M2 agarose in 50% slurry (Sigma) overnight at 4°C. Beads were washed three times with IP buffer and eluted with SDS sample buffer for Western blot analysis. Cycloheximide (CHX) and MG132 were purchased from Sigma.

For Western blotting, samples were run on SDS-glycine gels and transferred to nitrocellulose membrane following standard procedures (Sambrook and Russell, 2001). The membrane was then blocked with Odyssey LI-COR Blocking Buffer (LI-COR), and incubated with primary antibodies overnight. After washes in TBST (0.1% Tween 20), the blots were incubated with IRDye 800CW–conjugated donkey anti-goat IgG, IR Dye 800CW–conjugated donkey anti-rabbit IgG, or IRDye-680LT–conjugated donkey anti-mouse IgG (LI-COR). The signals on the membranes were detected with the Odyssey infrared imaging system (LI-COR). The following primary antibodies were used: rabbit anti-Gli1 (Cell Signaling, 1:1,000), rabbit anti-FLAG (Sigma, 1:2,000), mouse anti-Myc 9E10 monoclonal antibody (1:100), mouse anti-α-tubulin (Sigma, 1:2,000), goat anti-Gli2 (R&D, 1:1,000), goat-anti-Gli3 (R&D, 1:1,000), rabbit anti-Pdgfra (Cell Signaling, 1:2,000).

## RNA extraction and qPCR analysis

The following primers for mouse genes were used for qPCR: *Gapdh* F: 5′ AGGTTGTCTCCTGCGACTTCA 3′; *Gapdh* R: 5′ CCAGGAAATGAGCTTGACAAAGTT 3′; *Ptch1* F: 5′ TGCTGTGCCTGTGGTCATCCTGATT 3′; *Ptch1* R: 5′ CAGAGCGAGCATAGCCCTGTGGTTC 3′; *Gli1* F: 5′ CCCATAGGGTCTCGGGGTCTCAAAC 3′; *Gli1* R: 5′ GGAGGACCTGCGGCTGACTGTGTAA 3′; *Numb* F: 5′ CGTAGCAATGCCTGTCCGTGAA 3′; *Numb* R: 5′ AGAGGCAGCACCAGAAGACTGA 3′; *Pdgfa* F: 5′ CTGGCTCGAAGTCAGATCCACA 3′; *Pdgfa* R: 5′ GACTTGTCTCCAAGGCATCCTC 3′; *Pdgfra* F: 5′ GCAGTTGCCTTACGACTCCAGA 3′; *Pdgfra* R: 5′ GGTTTGAGCATCTTCACAGCCAC 3′; *Pdgfrb* F: 5′ ACTACATCTCCAAAGGCAGCACCT 3′; *Pdgfrb* R: 5′ TGTAGAACTGGTCGTTCATGGGCA 3′; *Elastin* F: 5′ TCCTGGGATTGGAGGCATTGCA 3′; *Elastin* R: 5′ ACCAGGCACTAAACCTCCAGCA 3′.

### shRNA-mediated gene knockdown

shRNAs were designed using the pSicOligomaker program (Reynolds et al., 2004). Oligonucleotides encoding shRNAs were cloned into the pLentiLox3.7 vector. To generate lentiviral supernatant, HEK 293T cells were transfected with the pLentiLox3.7 vector carrying the insert and the packaging vectors pLP1, pLP2, and pLP/VSV-G using Lipofectamine 2000 (Life Technologies). Forty-eight hours post-transfection, supernatants were harvested. Wild-type or *Sufu*-deficient lung mesenchymal cells at 50% confluency were transduced with lentiviruses supplemented with 8 μg/mL polybrene. Upon reaching confluency, cells were harvested and the lysates were analyzed by Western blotting. The following 19-mer sequences were used for shRNA-mediated gene knockdown: mouse *Numb* (NM_001136075), 5′ GAAGATGTCACCCTTTAAA 3′ and 5′ GCAGACATTCCCTCAATAT 3′; mouse *Itch* (NM_001243712), 5′ GAAGCCAAGGTCAGTTAAA 3′ and 5′ GTACTTCTCAGTTGATAAA 3′; *GFP*, 5′ GCAGACCATTATCAACAAA 3′.

### Immunofluorescence and microscopy

Immunohistochemistry was performed following standard procedures (Ausubel et al., 2003). The primary antibodies used were: goat anti-CC10 (Santa Cruz, 1:500), rabbit anti-pro SP-C (Millipore, 1:400), hamster anti-T1α (Developmental Studies Hybridoma Bank, 1:200), mouse anti-Ki67 (BD Biosciences, 1:100), rabbit anti-phospho-Histone H3 (PH3) (Millipore, 1:200), mouse anti-smooth muscle actin (SMA) (Sigma, 1:1,000) and mouse anti-CD31 (PECAM-1) (BD Biosciences, 1:100). PECAM-1 staining was performed using the ABC kit (Vector Laboratories). Antibody against Ki67 required biotin-streptavidin amplification with the TSA kit (PerkinElmer) for optimal signal detection. Secondary antibodies and conjugates used were donkey anti-mouse Alexa Fluor 594 (Life Technologies, 1:2,000), donkey anti-rabbit Alexa Fluor 488 (Life Technologies, 1:2,000), biotinylated horse anti-mouse (Jackson ImmunoResearch Laboratories, 1:1,000) and DAPI (Sigma, 1:10,000).

### Luciferase assays

Analysis of transcription factor binding sites on *Pdgfra* promoters from different species was performed using ECR Browser (Ovcharenko et al., 2004) and rVISTA 2.0 (Loots and Ovcharenko, 2004). Mouse *Pdgfra* promoter fragments (−2473 to −2409 and −3027 to −1940; position zero marks the transcriptional start of *Pdgfra*) were cloned upstream of the firefly *luciferase* gene in a modified pGL-Basic vector (denoted pGL-TK), which contains a *thymidine kinase* (*TK*) minimal promoter. The consensus sequence of Gli binding site (GliBS) (5′GACCACCCA3′) within the *Pdgfra* promoter was altered to 5′GACTGAAGA3′ or deleted by site-directed mutagenesis. Both mutant constructs gave similar results.

These constructs were co-transfected with pRL-TK (Renilla) and cDNA encoding Gli1 into HEK293T cells. Cells were harvested 48 hours post-transfection for analysis using the Dual Luciferase Reporter Assay System (Promega).

### Chromatin immunoprecipitation (ChIP)

MEFs stably expressing Gli1-3xFLAG and Gli2-3xFLAG were used for ChIP analysis following established procedures (Collas, 2011; Weinmann and Farnham, 2002). ChIP was performed using the EZ-ChIP kit (Millipore) according to the manufacturer's manual. Briefly, cells were cross-linked in 1% formaldehyde and the DNA was sonicated into a range of 100–600 bp in size using a Bioruptor sonicator (Diagenode) for 5 cycles of 30 seconds on/30 seconds off. The extracts were precleared in BSA-blocked protein A/G beads and incubated with antibodies or IgG control overnight. Protein A/G beads were incubated with the lysate-antibody complex for 1 hour. After washes, the DNA was eluted and reverse-crosslinked at 65°C overnight. The DNA was purified and analyzed by qPCR. The antibodies used were mouse anti-FLAG M2 monoclonal antibody (Sigma) and normal mouse IgG (Santa Cruz).

For ChIP analysis, the following primers were used: *Pdgfra* promoter F: 5′ CTTGGCTAGGCACTGGCACTTGC 3′; *Pdgfra* promoter R: CCAGCCCAGTTCTTGCCCTGTTC; β-actin promoter F 5′AGAAGGACTCCTATGTGGGTGA 3′, β-actin promoter R 5′ACTGACCTGGGTCATCTTTTC 3′.

## Results

### Sufu regulates Gli1 protein levels by blocking proteasome-mediated protein degradation

Our prior studies showed that Gli1 protein levels are increased (likely due to transcriptional activation of Gli1) in *Sufu*-deficient mouse embryonic fibroblasts (MEFs) in contrast to the drastically reduced protein levels of Gli2 and Gli3 (Chen et al., 2009; Jia et al., 2009; Wang et al., 2010). Gli2 and Gli3 are subject to Spop-mediated ubiquitination and degradation, and Sufu antagonizes Spop function to preserve a pool of Gli2 and Gli3 (Chen et al., 2009; Wang et al., 2010). We also demonstrated that Gli1 is immune to Spop-mediated protein degradation (Chen et al., 2009; Zhang et al., 2009). In this study, we revisit the issue of whether Sufu controls Gli1 protein levels by examining Gli1 in $Sufu^{-/-}$ MEFs or *Sufu*-deficient mouse lung mesenchymal cells (their derivation are described below) treated with proteasome inhibitors (*e.g.*, MG132). To our surprise, Gli1 levels were further increased following MG132 treatment (Fig. 1A), similar to what was observed for Gli2 and Gli3. Since Numb was previously shown to activate the E3 ligase Itch, resulting in Gli1 ubiquitination and degradation (Di Marcotullio et al., 2011), we tested the idea that Sufu stabilizes Gli1 protein by antagonizing Numb/Itch, a mechanism distinct from Sufu control of Gli2/3 protein levels. We found that Gli1 protein levels were reduced upon Numb overexpression in cultured cells (Fig. 1B; supplementary material Fig. S1) and Sufu was capable of blocking Numb activity and restoring Gli1 protein levels (Fig. 1B; supplementary material Fig. S1). Conversely, knockdown of *Numb* or *Itch* in *Sufu*-deficient lung cells led to increased Gli1 protein levels (supplementary material Fig. S2) by eliminating Numb/Itch-mediated Gli1 degradation. Furthermore, Sufu overexpression decreased the binding between Numb and Gli1 (Fig. 1C; supplementary material Fig. S1). This suggests that Sufu controls Gli1 protein levels by antagonizing Numb/Itch (Fig. 1D). Interestingly, Drosophila Su(fu) was also capable of stabilizing Gli1 when expressed in cultured cells (supplementary

material Fig. S3). This supports the idea that control of Gli1 protein levels by Sufu is a conserved mechanism.

## Loss of Sufu in the mouse lung results in increased Hh signaling but reduced Gli1 protein levels

To further validate regulation of Gli1 protein levels by Sufu *in vivo*, we examined Gli1 protein in *Sufu* mutants. Since *Sufu* mutant embryos die at 9.5 *days post coitus* (*dpc*) before major organs develop, conditional inactivation of *Sufu* is required to investigate how Sufu/Gli interactions affect Hh signaling in various tissues. In this work, we explored tissues not previously examined and focused on the lung. We utilized the *Dermo1*$^{Cre}$ mouse line (Yu et al., 2003) to convert a conditional allele of *Sufu* (*Sufu*$^{f}$) into a null allele (*Sufu*$^{-}$) in the lung mesenchyme as well as the mesenchyme of other tissues. *Sufu*$^{f/-}$; *Dermo1*$^{Cre/+}$ mice are referred to as *Sufu*-deficient mice in this study. *Sufu*-deficient mice died soon after birth likely due to respiratory failure and defects in other tissues.

As a first step toward understanding how the loss of Sufu leads to lung defects, we examined Hh target gene expression in *Sufu*-deficient lungs by reporter activity, qPCR and Western blotting (Fig. 2A–D). For qPCR analysis of transcript levels or Western blotting, we also isolated lung mesenchyme since *Dermo1*$^{Cre}$ selectively inactivates *Sufu* in this compartment (data not shown). We found that *Ptch1* reporter activity (Fig. 2A, B) and *Ptch1*/*Hhip* mRNA levels (Fig. 2D) were increased in *Sufu*-deficient lungs, indicative of Hh pathway activation. Similar to *Sufu* mutant embryos, Gli2 and Gli3 protein levels were reduced in *Sufu*-deficient lungs (Fig. 2C). To our surprise, protein levels of Gli1, a Hh target, were reduced in *Sufu*-deficient lungs (Fig. 2C) even though *Gli1* mRNA levels were unaltered (Fig. 2D). This result contrasts with the expected outcome of global activation of Hh target genes, implying that our traditional view of Hh pathway activation is oversimplified.

We also tested whether reduced Gli protein levels were also present in other *Sufu*-deficient tissues and found that *Sufu* mutant hearts had increased *Gli1* mRNA but reduced Gli1 protein levels (Fig. 2C, D). This is in contrast to *Suf*u-deficient neural tubes where Gli1 activity is increased (Cooper et al., 2005; Svard et al., 2006), likely due to transcriptional activation of *Gli1*. Results from *in vitro* and *in vivo* studies on Sufu and Gli1 are consistent with a model in which regulation of Gli1 protein levels by Sufu is a conserved, general mechanism. This is achieved by antagonizing Numb through Sufu; otherwise, Numb would induce Gli1 degradation (supplementary material Fig. S4). Baseline Gli protein levels may differ in various tissues and cell lines depending on *Gli1* transcript levels. We suspect that in tissues where Gli1 protein levels are reduced in the absence of Sufu, Hh targets controlled by Gli1 proteins could be affected.

## Myofibroblast development is disrupted in Sufu-deficient lungs

To gain insight into how altered Hh target gene expression in *Sufu*-deficient lungs could impact lung development, we conducted a careful phenotypic analysis of *Sufu* mutant lungs at various stages of lung development (Fig. 3A–N). The phenotypes in *Sufu* mutant lungs were completely penetrant and showed little variation from animal to animal. Early patterns of epithelial branching seemed to be established properly in *Sufu*-deficient lungs, leading to

the correct number and positioning of lung lobes (Fig. 3D). However, defective morphogenesis in both the epithelium and mesenchyme became apparent as lung development proceeded (Fig. 3; supplementary material Fig. S5). At birth, *Sufu* mutant lungs were smaller in size and exhibited reduced branching morphogenesis associated with a compact mesenchyme (Fig. 3N).

Somewhat surprisingly, all the major epithelial cell types including Clara cells ($CC10^+$), ciliated cells (Ac-tubulin$^+$), pulmonary neuroendocrine cells ($CGRP^+$), alveolar type II ($SPC^+$) and type I ($T1\alpha^+$) cells (Morrisey and Hogan, 2010) were properly specified (Fig. 4C–H) in the absence of *Sufu*. Most mesenchymal cell types including the bronchial smooth muscle ($SMA^+$) (K, L) and blood vessels ($PECAM^+$) (M, N) were also properly produced. In addition, no apparent difference in the rate of cell proliferation (Fig. 4A, B, A′; supplementary material Fig. S6) or cell death (supplementary material Fig. S6) was detected in either the epithelium or mesenchyme between wild-type and *Sufu*-deficient lungs during lung development.

We noticed that *Sufu*-deficient lungs contained disorganized saccules, raising the possibility that alveolar development could be affected (Fig. 3N compared to 3M). In wild-type lungs, by 18.5 *dpc* (less than 24 hr before birth), myofibroblasts localized to the prospective alveoli (Fig. 4I) and participated in subsequent secondary septa formation during alveolar formation. By contrast, myofibroblasts were absent from many prospective alveoli of *Sufu*-deficient lungs (Fig. 4J) while smooth muscle cells surrounding the large airways or blood vessels could be detected (Fig. 4K, L). Moreover, reduced myofibroblasts in *Sufu*-deficient lungs were associated with reduced expression of *Elastin* (Fig. 4B′). This suggests that Hh pathway perturbation in the absence of *Sufu* leads to defective myofibroblast maturation and migration.

## Defective myofibroblast development in Sufu-deficient lungs is associated with reduced PDGF signaling

We first investigated potential perturbations of major signaling pathways in the absence of *Sufu*. The expression patterns and levels of components in the Fgf, Bmp and Wnt signaling pathways were similar between wt and *Sufu* mutant lungs (Fig. 4O–T and data not shown). We then focused on PDGF signaling since it has been shown to play a central role in myofibroblast development. *Pdgf* ligand or *Pdgf* receptor $\alpha$ (*Pdgfra*) knockout mice die without myofibroblasts or secondary septa (Bostrom et al., 1996; Sun et al., 2000). We surmised that defective myofibroblast development in *Sufu*-deficient lungs is caused by reduced PDGF signaling. To test this idea, we examined the expression of Pdgf ligand and receptor in wild-type and *Sufu*-deficient lungs by *in situ* hybridization, qPCR and Western blotting. We found that while *Pdgfa* expression was indistinguishable between wild-type and *Sufu*-deficient lungs (Fig. 4W, X and B′), *Pdgfra* expression was drastically reduced in *Sufu* mutant lungs (Fig. 4U, V and B′). By contrast, *Pdgfrb* expression was unaltered in *Sufu*-deficient lungs (Fig. 4B′). This suggests that disrupted Hh signaling in *Sufu* mutant lungs leads to reduced *Pdgfra* expression and consequently defective myofibroblast development.

### Pdgfra is a direct transcriptional target of Gli1

We investigated the molecular mechanisms by which loss of *Sufu* leads to reduced *Pdgfra* expression. Since a reduction in *Pdgfra* expression in *Sufu*-deficient lungs is associated with decreased Gli1 protein levels (despite global Hh pathway activation) and the expression of Pdgfra and Gli1 overlaps along the alveolar wall (Fig. 4Y, Z) and the secondary septum (arrows in Fig. 4Y, Z), we speculate that *Pdgfra* could be a direct target of Gli1. Indeed, we identified a canonical Gli-binding site (GliBS) in the mouse *Pdgfra* (but not *Pdgfrb*) promoter (Fig. 5A). A GliBS was also found in a similar location of the *Pdgfra* promoter in several vertebrate species (Fig. 5A). This is consistent with our model in which Gli1 controls *Pdgfra* expression. Reduced Gli1 protein levels in *Sufu*-deficient lungs could contribute to compromised *Pdgfra* promoter activation. By contrast, other Hh targets controlled by Gli2 and Gli3 are activated.

We further tested this idea by performing reporter assays using *Pdgfra-luc* in which a *Pdgfra* promoter fragment is placed upstream of the *luciferase* (*luc*) reporter (Fig. 5B). Gli1 was co-transfected with *Pdgfra-luc* in MEFs and tested for its ability to activate *Pdgfra-luc*. A *Pdgfra* fragment in which the canonical Gli-binding site is mutated (denoted as *PdgfraΔGliBS*) was used as a control (Fig. 5B). We showed that the addition of Gli1 increased *Pdgfra-luc* activity but failed to activate *PdgfraΔGliBS-luc* (Fig. 5B). By contrast, Gli2 has low activity in activating the *Pdgfra* promoter *in vitro* (supplementary material Fig. S7). Finally, Hh stimulation led to increased expression levels of *Pdgfra* but not *Pdgfrb* in lung mesenchymal cells (supplementary material Fig. S8), suggesting a connection between Hh and PDGF signaling.

To determine whether the endogenous *Pdgfra* promoter is occupied by Gli1, we performed chromatin immunoprecipitation (ChIP) analysis (Weinmann and Farnham, 2002) on MEFs expressing FLAG-tagged Gli1 and Gli2. Cell lysate from lung mesenchymal cells was immunoprecipitated using FLAG antibodies. We amplified *Pdgfra* genomic regions using FLAG immunoprecipitates and found significant enrichment of Gli1 (but not Gli2) on the *Pdgfra* genomic fragment (Fig. 5C). These results suggest that *Pdgfra* is a direct target of Gli1.

## Discussion

In this study, we discover that Sufu stabilizes Gli1 protein levels by antagonizing Numb/Itch-mediated protein degradation. Together with previous work that demonstrates control of Gli2/3 protein levels by Sufu (Chen et al., 2009; Jia et al., 2009; Wang et al., 2010), our new finding indicates that regulation of Gli protein levels by Sufu is a major general mechanism in modulating Hh responses. Interestingly, our analysis of *Sufu*-deficient lungs led to the unexpected observation that while canonical Hh targets such as *Ptch1* are upregulated, *Pdgfra*, a direct target of Gli1, is downregulated. We propose that Hh targets do not respond uniformly to Hh signaling under different genetic perturbations and this could also vary from tissue to tissue. We speculate that this may be due to differential levels of individual Gli proteins that control common and distinct sets of Hh targets in a given tissue (Fig. 5D). As a result, some Hh targets are activated while others are concomitantly downregulated. Perhaps this enables Hh signaling to generate complex outputs by regulating the availability

of different Gli proteins. Testing and generalizing this hypothesis in diverse tissues will significantly increase our understanding of how Hh signaling leads to diverse phenotypic consequences in homeostasis and disease.

## Control of Gli protein levels by Sufu

It was previously reported that Sufu controls protein levels of Gli2 and Gli3 by antagonizing Spop-mediated ubiquitination and degradation (Chen et al., 2009; Wang et al., 2010). In addition, Drosophila Su(fu) also controls protein levels of Ci (Gli homolog) by antagonizing HIB/roadkill (Spop homolog) (Zhang et al., 2006). However, Gli1 is immune to Spop-mediated regulation (Chen et al., 2009; Zhang et al., 2009). In this study, we showed that Sufu counters the effects of Numb/Itch-mediated Gli1 protein degradation. Thus, control of Gli protein levels by Sufu is a conserved mechanism employed to modulate Hh responses.

It is unclear how two regulatory circuitries were evolved to differentially control Gli1 and Gli2/3 protein levels mediated by Sufu respectively. In this regard, it is interesting to note that Drosophila Su(fu) not only can substitute for mammalian Sufu and partially restore Gli2/3 protein levels in cell-based assays (Chen et al., 2009), it is also able to stabilize Gli1 protein levels in a similar assay. This suggests that a similar interface of molecular interactions may exist between Sufu-Spop and Sufu-Numb. Alternatively, fly Su(fu) may not have exploited the Numb-Itch pathway for controlling Ci protein levels.

Gli1 is part of a positive feedback loop of Hh responses since Gli1 is an early transcriptional target of Hh signaling. Control of Gli1 protein levels by Sufu may provide a mechanism to dampen Hh signaling when Sufu becomes inactive either by Hh signaling or in disease states. Reduced Gli1 protein levels due to loss of Sufu would limit transcriptional activity mediated by Gli1. This resembles Sufu's effects on Gli2/3 protein levels, part of which are reflected by the failure to maximally activate Hh signaling in the absence of Sufu (Chen et al., 2009; Liu et al., 2012). In considering the consequence of loss of Sufu on Gli1 protein levels in various tissues, it is important to take into consideration *Gli1* transcription. For instance, Gli1 is transcriptionally activated in the neural tube when the Hh pathway is activated (*e.g.*, loss of Sufu). We surmise that increased *Gli1* transcript levels in the neural tube would offset the effect of Gli1 protein degradation due to loss of Sufu; as a result, Gli1 protein levels are elevated. By contrast, transcriptional activation of *Gli1* in the lungs is not as prominent and thus *Sufu*-deficient lungs have reduced Gli1 protein levels. However, we cannot rule out the possibility that different tissues may have distinct rates of Gli1 degradation and this could also contribute to variations in Gli1 protein levels in diverse tissues.

## Epithelial-mesenchymal Hh signaling and lung development

*Sufu*-deficient lungs display overall upregulation of Hh signaling, resulting in a smaller lung with a compact mesenchyme. Somewhat surprisingly, the rate of cell proliferation during early lung development does not seem to be altered. Moreover, despite defective lung development in the absence of *Sufu*, all major lung cell types are properly specified. We postulate that abnormal mesenchymal development in the absence of *Sufu* disrupts epithelial-mesenchymal interactions, leading to defective epithelial branching.

Consequently, lung size is reduced. Uncovering the cellular and molecular basis of lung defects in *Sufu* mutants would require future studies that employ tools such as live imaging and whole-genome analysis. This would allow the identification of potential defects in cellular behaviors and genes and pathways involved in this process.

### Control of PDGF signaling and myofibroblast development

Removal of *Sufu* in the lung mesenchyme results in a reduced number of myofibroblasts. This could contribute to defective lung development and even perinatal lethality. Alveolar myofibroblasts play a key role in alveolus formation. They are contractile cells found in the alveolar interstitium during lung development and possess morphological and biochemical features intermediate between fibroblast and smooth muscle. Alveolar myofibroblasts appear to be derived from a population of mesenchymal cells that express the PDGF receptor. These cells subsequently spread to the walls of prospective terminal saccules to become future alveolar myofibroblasts. Alveolar myofibroblasts produce Elastin and participate in septal formation during alveologenesis. Indeed, reduced myofibroblasts in *Sufu*-deficient lungs are associated with reduced expression of *Elastin*.

We showed that *Sufu*-deficient lungs have reduced *Pdgfra* expression. Since PDGF signaling plays an essential role in myofibroblast development, our findings are consistent with the idea that downregulation of *Pdgfra* expression in *Sufu* mutant lungs likely results in a reduced myofibroblast number. Importantly, we demonstrated that *Pdgfra* is a direct target of Gli1. This allows us to propose a model in which reduced Gli1 protein levels in the absence of *Sufu* contribute to impaired *Pdgfra* expression and consequently myofibroblast development. Consistent with these observations, it was previously reported that *Pdgfra* is regulated by Gli1 in Hh-responsive cell lines (Xie et al., 2001). We also showed that Gli1 and Pdgfra co-localize extensively in the alveolar structure in postnatal lungs, further supporting the notion that Gli1 regulates *Pdgfra* expression. Future genetic studies that employ *Sufu* and *Pdgfra* mutants will further support a functional connection between Hh and PDGF signaling.

### Possible distinct and overlapping targets of Gli proteins

Whole genome ChIP-on-chip studies using FLAG-tagged Gli1 and Gli3 in neural tissues and the limb have identified a significant number of genes with bona fide Gli-binding sites (Vokes et al., 2007; Vokes et al., 2008). These studies will serve as the guide for similar efforts in other organs such as the lungs. We anticipate that different sets of Gli targets will be identified in various tissues. A key issue is to determine whether certain Hh targets are controlled by distinct Gli proteins. Certain Hh targets may possess unique binding sequences that can only be recognized by a particular Gli protein. Alternatively, all Gli-binding sites are degenerate and can recruit any Gli proteins. In this case, differential binding would be an outcome of different Gli protein levels. Isolation of distinct cell types from a given tissue for whole-genome ChIP analysis may provide an opportunity to further test these hypotheses.

Gli2 is active in *Sufu* mutants despite its low levels. However, Gli2 activator fails to elevate *Pdgfra* expression levels in *Sufu*-deficient lungs. *Gli1* mutants are viable (Park et al., 2000) and do not seem to display apparent defects in myofibroblast development (data not shown).

Reduced Gli1 protein levels thus are unlikely the sole cause of defective myofibroblast development in *Sufu*–deficient lungs. Instead, loss of *Sufu* likely perturbs other processes, which in conjunction with reduced Gli1 protein levels, lead to the disruption of myofibroblast development. However, it remains possible that reduced Gli2/3 activator levels in the absence of *Sufu* also contributes to reduced *Pdgfra* expression in the developing lungs although Gli2 is not detected on the *Pdgfra* promoter by ChIP analysis using MEFs (Fig. 5C). In this scenario, myofibroblast defects would be observed in mice deficient in multiple Gli proteins. Genetic studies that produce mice deficient in Sufu and one or multiple Gli proteins will be informative in revealing regulation of Gli proteins by Sufu in the lung.

Taken together, our studies highlight the complexity of regulating Gli protein functions in diverse tissues. They also form the basis of further studies to investigate how multiple Gli proteins are regulated at multiple levels to control the expression of a unique set of Hh targets in a given tissue.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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## References

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K. Current Protocols in Molecular Biology (Wiley). 2003

Bai CB, Auerbach W, Lee JS, Stephen D, Joyner AL. Gli2, but not Gli1, is required for initial Shh signaling and ectopic activation of the Shh pathway. Development. 2002; 129:4753–4761. [PubMed: 12361967]

Bai CB, Stephen D, Joyner AL. All mouse ventral spinal cord patterning by hedgehog is Gli dependent and involves an activator function of Gli3. Dev Cell. 2004; 6:103–115. [PubMed: 14723851]

Barakat MT, Humke EW, Scott MP. Learning from Jekyll to control Hyde: Hedgehog signaling in development and cancer. Trends Mol Med. 2010; 16:337–348. [PubMed: 20696410]

Barnfield PC, Zhang X, Thanabalasingham V, Yoshida M, Hui CC. Negative regulation of Gli1 and Gli2 activator function by Suppressor of fused through multiple mechanisms. Differentiation; research in biological diversity. 2005; 73:397–405.

Beachy PA, Hymowitz SG, Lazarus RA, Leahy DJ, Siebold C. Interactions between Hedgehog proteins and their binding partners come into view. Genes Dev. 2010; 24:2001–2012. [PubMed: 20844013]

Bijlsma MF, Roelink H. Non-cell-autonomous signaling by Shh in tumors: challenges and opportunities for therapeutic targets. Expert opinion on therapeutic targets. 2010; 14:693–702. [PubMed: 20515293]

Bostrom H, Willetts K, Pekny M, Leveen P, Lindahl P, Hedstrand H, Pekna M, Hellstrom M, Gebre-Medhin S, Schalling M, Nilsson M, Kurland S, Tornell J, Heath JK, Betsholtz C. PDGF-A signaling is a critical event in lung alveolar myofibroblast development and alveogenesis. Cell. 1996; 85:863–873. [PubMed: 8681381]

Bowers M, Eng L, Lao Z, Turnbull RK, Bao X, Riedel E, Mackem S, Joyner AL. Limb anterior-posterior polarity integrates activator and repressor functions of GLI2 as well as GLI3. Dev Biol. 2012; 370:110–124. [PubMed: 22841643]

Briscoe J, Therond PP. The mechanisms of Hedgehog signalling and its roles in development and disease. Nature reviews Molecular cell biology. 2013; 14:416–429.

Cao T, Wang C, Yang M, Wu C, Wang B. Mouse limbs expressing only the Gli3 repressor resemble those of Sonic hedgehog mutants. Dev Biol. 2013; 379:221–228. [PubMed: 23644062]

Chen MH, Wilson CW, Li YJ, Law KK, Lu CS, Gacayan R, Zhang X, Hui CC, Chuang PT. Cilium-independent regulation of Gli protein function by Sufu in Hedgehog signaling is evolutionarily conserved. Genes Dev. 2009; 23:1910–1928. [PubMed: 19684112]

Chen Y, Jiang J. Decoding the phosphorylation code in Hedgehog signal transduction. Cell research. 2013; 23:186–200. [PubMed: 23337587]

Collas P. A chromatin immunoprecipitation protocol for small cell numbers. Methods Mol Biol. 2011; 791:179–193. [PubMed: 21913080]

Cooper AF, Yu KP, Brueckner M, Brailey LL, Johnson L, McGrath JM, Bale AE. Cardiac and CNS defects in a mouse with targeted disruption of suppressor of fused. Development. 2005; 132:4407–4417. [PubMed: 16155214]

Di Marcotullio L, Greco A, Mazza D, Canettieri G, Pietrosanti L, Infante P, Coni S, Moretti M, De Smaele E, Ferretti E, Screpanti I, Gulino A. Numb activates the E3 ligase Itch to control Gli1 function through a novel degradation signal. Oncogene. 2011; 30:65–76. [PubMed: 20818436]

Ding Q, Fukami S, Meng X, Nishizaki Y, Zhang X, Sasaki H, Dlugosz A, Nakafuku M, Hui C. Mouse suppressor of fused is a negative regulator of sonic hedgehog signaling and alters the subcellular distribution of Gli1. Current biology: CB. 1999; 9:1119–1122. [PubMed: 10531011]

Ding Q, Motoyama J, Gasca S, Mo R, Sasaki H, Rossant J, Hui CC. Diminished Sonic hedgehog signaling and lack of floor plate differentiation in Gli2 mutant mice. Development. 1998; 125:2533–2543. [PubMed: 9636069]

Eggenschwiler JT, Anderson KV. Cilia and developmental signaling. Annu Rev Cell Dev Biol. 2007; 23:345–373. [PubMed: 17506691]

Farzan SF, Singh S, Schilling NS, Robbins DJ. The adventures of sonic hedgehog in development and repair. III. Hedgehog processing and biological activity. Am J Physiol Gastrointest Liver Physiol. 2008; 294:G844–849. [PubMed: 18239057]

Goodrich LV, Milenkovic L, Higgins KM, Scott MP. Altered neural cell fates and medulloblastoma in mouse patched mutants. Science. 1997; 277:1109–1113. [PubMed: 9262482]

Hamilton TG, Klinghoffer RA, Corrin PD, Soriano P. Evolutionary divergence of platelet-derived growth factor alpha receptor signaling mechanisms. Molecular and cellular biology. 2003; 23:4013–4025. [PubMed: 12748302]

Hui CC, Angers S. Gli proteins in development and disease. Annu Rev Cell Dev Biol. 2011; 27:513–537. [PubMed: 21801010]

Humke EW, Dorn KV, Milenkovic L, Scott MP, Rohatgi R. The output of Hedgehog signaling is controlled by the dynamic association between Suppressor of Fused and the Gli proteins. Genes Dev. 2010; 24:670–682. [PubMed: 20360384]

Ingham PW, Nakano Y, Seger C. Mechanisms and functions of Hedgehog signalling across the metazoa. Nature reviews Genetics. 2011; 12:393–406.

Jia J, Kolterud A, Zeng H, Hoover A, Teglund S, Toftgard R, Liu A. Suppressor of Fused inhibits mammalian Hedgehog signaling in the absence of cilia. Dev Biol. 2009; 330:452–460. [PubMed: 19371734]

Kogerman P, Grimm T, Kogerman L, Krause D, Unden AB, Sandstedt B, Toftgard R, Zaphiropoulos PG. Mammalian suppressor-of-fused modulates nuclear-cytoplasmic shuttling of Gli-1. Nature cell biology. 1999; 1:312–319.

Liu J, Heydeck W, Zeng H, Liu A. Dual function of suppressor of fused in Hh pathway activation and mouse spinal cord patterning. Dev Biol. 2012; 362:141–153. [PubMed: 22182519]

Loots GG, Ovcharenko I. rVISTA 2.0: evolutionary analysis of transcription factor binding sites. Nucleic acids research. 2004; 32:W217–221. [PubMed: 15215384]

Matise MP, Epstein DJ, Park HL, Platt KA, Joyner AL. Gli2 is required for induction of floor plate and adjacent cells, but not most ventral neurons in the mouse central nervous system. Development. 1998; 125:2759–2770. [PubMed: 9655799]

Morrisey EE, Hogan BL. Preparing for the first breath: genetic and cellular mechanisms in lung development. Dev Cell. 2010; 18:8–23. [PubMed: 20152174]

Murone M, Luoh SM, Stone D, Li W, Gurney A, Armanini M, Grey C, Rosenthal A, de Sauvage FJ. Gli regulation by the opposing activities of fused and suppressor of fused. Nature cell biology. 2000; 2:310–312.

Nagy, A.; Gertsenstein, M.; Vintersten, K.; Behringer, R. Manipulating the Mouse Embryo: A Laboratory Manual. 3. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 2003.

Ovcharenko I, Nobrega MA, Loots GG, Stubbs L. ECR Browser: a tool for visualizing and accessing data from comparisons of multiple vertebrate genomes. Nucleic acids research. 2004; 32:W280–286. [PubMed: 15215395]

Pan Y, Bai CB, Joyner AL, Wang B. Sonic hedgehog signaling regulates Gli2 transcriptional activity by suppressing its processing and degradation. Molecular and cellular biology. 2006; 26:3365–3377. [PubMed: 16611981]

Park HL, Bai C, Platt KA, Matise MP, Beeghly A, Hui CC, Nakashima M, Joyner AL. Mouse Gli1 mutants are viable but have defects in SHH signaling in combination with a Gli2 mutation. Development. 2000; 127:1593–1605. [PubMed: 10725236]

Pospisilik JA, Schramek D, Schnidar H, Cronin SJ, Nehme NT, Zhang X, Knauf C, Cani PD, Aumayr K, Todoric J, Bayer M, Haschemi A, Puviindran V, Tar K, Orthofer M, Neely GG, Dietzl G, Manoukian A, Funovics M, Prager G, Wagner O, Ferrandon D, Aberger F, Hui CC, Esterbauer H, Penninger JM. Drosophila genome-wide obesity screen reveals hedgehog as a determinant of brown versus white adipose cell fate. Cell. 2010; 140:148–160. [PubMed: 20074523]

Rabinowitz AH, Vokes SA. Integration of the transcriptional networks regulating limb morphogenesis. Dev Biol. 2012; 368:165–180. [PubMed: 22683377]

Reynolds A, Leake D, Boese Q, Scaringe S, Marshall WS, Khvorova A. Rational siRNA design for RNA interference. Nature biotechnology. 2004; 22:326–330.

Robbins DJ, Fei DL, Riobo NA. The Hedgehog signal transduction network. Science signaling. 2012; 5:re6. [PubMed: 23074268]

Ryan KE, Chiang C. Hedgehog secretion and signal transduction in vertebrates. The Journal of biological chemistry. 2012; 287:17905–17913. [PubMed: 22474285]

Sambrook, J.; Russell, DW. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 2001.

Scales SJ, de Sauvage FJ. Mechanisms of Hedgehog pathway activation in cancer and implications for therapy. Trends in pharmacological sciences. 2009; 30:303–312. [PubMed: 19443052]

Sun T, Jayatilake D, Afink GB, Ataliotis P, Nister M, Richardson WD, Smith HK. A human YAC transgene rescues craniofacial and neural tube development in PDGFRalpha knockout mice and uncovers a role for PDGFRalpha in prenatal lung growth. Development. 2000; 127:4519–4529. [PubMed: 11023856]

Svard J, Heby-Henricson K, Persson-Lek M, Rozell B, Lauth M, Bergstrom A, Ericson J, Toftgard R, Teglund S. Genetic elimination of Suppressor of fused reveals an essential repressor function in the mammalian Hedgehog signaling pathway. Dev Cell. 2006; 10:187–197. [PubMed: 16459298]

Tukachinsky H, Lopez LV, Salic A. A mechanism for vertebrate Hedgehog signaling: recruitment to cilia and dissociation of SuFu-Gli protein complexes. J Cell Biol. 2010; 191:415–428. [PubMed: 20956384]

Vokes SA, Ji H, McCuine S, Tenzen T, Giles S, Zhong S, Longabaugh WJ, Davidson EH, Wong WH, McMahon AP. Genomic characterization of Gli-activator targets in sonic hedgehog-mediated neural patterning. Development. 2007; 134:1977–1989. [PubMed: 17442700]

Vokes SA, Ji H, Wong WH, McMahon AP. A genome-scale analysis of the cis-regulatory circuitry underlying sonic hedgehog-mediated patterning of the mammalian limb. Genes Dev. 2008; 22:2651–2663. [PubMed: 18832070]

Wang B, Fallon JF, Beachy PA. Hedgehog-regulated processing of Gli3 produces an anterior/posterior repressor gradient in the developing vertebrate limb. Cell. 2000; 100:423–434. [PubMed: 10693759]

Wang C, Pan Y, Wang B. Suppressor of fused and Spop regulate the stability, processing and function of Gli2 and Gli3 full-length activators but not their repressors. Development. 2010; 137:2001–2009. [PubMed: 20463034]

Wang Y, McMahon AP, Allen BL. Shifting paradigms in Hedgehog signaling. Current opinion in cell biology. 2007; 19:159–165. [PubMed: 17303409]

Weinmann AS, Farnham PJ. Identification of unknown target genes of human transcription factors using chromatin immunoprecipitation. Methods. 2002; 26:37–47. [PubMed: 12054903]

Wilson CW, Chuang PT. Mechanism and evolution of cytosolic Hedgehog signal transduction. Development. 2010; 137:2079–2094. [PubMed: 20530542]

Xie J, Aszterbaum M, Zhang X, Bonifas JM, Zachary C, Epstein E, McCormick F. A role of PDGFRalpha in basal cell carcinoma proliferation. Proceedings of the National Academy of Sciences of the United States of America. 2001; 98:9255–9259. [PubMed: 11481486]

Yu K, Xu J, Liu Z, Sosic D, Shao J, Olson EN, Towler DA, Ornitz DM. Conditional inactivation of FGF receptor 2 reveals an essential role for FGF signaling in the regulation of osteoblast function and bone growth. Development. 2003; 130:3063–3074. [PubMed: 12756187]

Zhang Q, Shi Q, Chen Y, Yue T, Li S, Wang B, Jiang J. Multiple Ser/Thr-rich degrons mediate the degradation of Ci/Gli by the Cul3-HIB/SPOP E3 ubiquitin ligase. Proceedings of the National Academy of Sciences of the United States of America. 2009; 106:21191–21196. [PubMed: 19955409]

Zhang Q, Zhang L, Wang B, Ou CY, Chien CT, Jiang J. A hedgehog-induced BTB protein modulates hedgehog signaling by degrading Ci/Gli transcription factor. Dev Cell. 2006; 10:719–729. [PubMed: 16740475]

- Sufu regulates Gli1 protein levels by antagonizing Gli1 degraders Numb/Itch
- Hh pathway is activated in *Sufu* mutant lungs but Gli1 protein levels are reduced
- *Sufu*-deficient lungs exhibit defective epithelial and mesenchymal development
- Disrupted myofibroblast development is associated with reduced *Pdgfra* expression
- *Pdgfra* is a direct target of Gli1, linking Hh and PDGF signaling

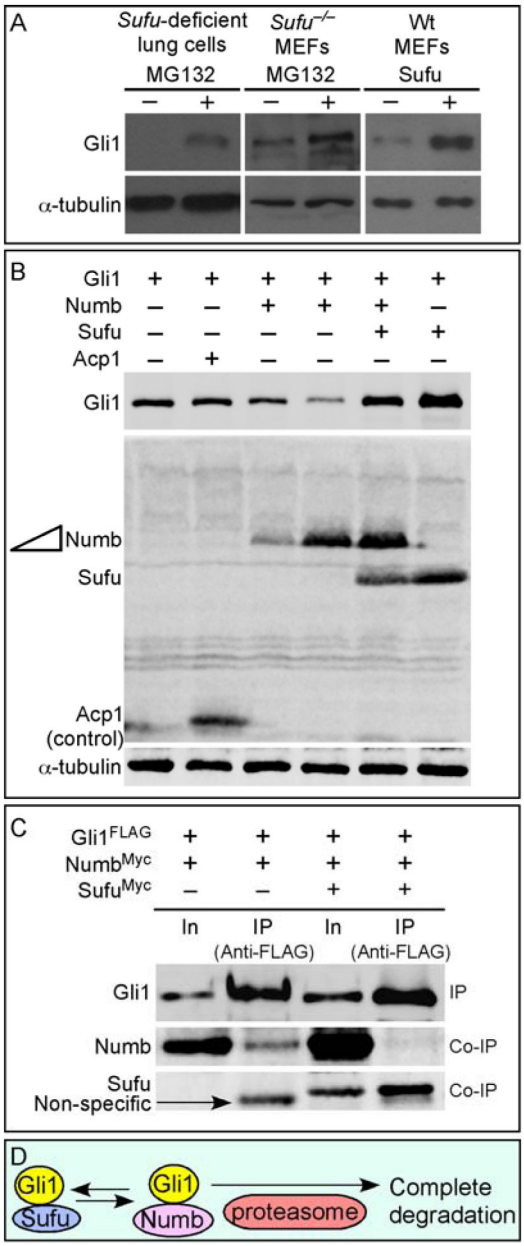


**Figure 1. Control of Gli1 protein levels by Sufu**

(A) Western blot analysis of lysates from *Sufu*-deficient (*Sufu*$^{f/-}$; *Dermo1*$^{Cre/+}$) lung cells or *Sufu* null (*Sufu*$^{-/-}$) MEFs treated with MG132 to block proteasome-mediated degradation. Endogenous Gli1 protein levels were elevated when protein degradation was inhibited in *Sufu* mutants. Similarly, protein levels of transfected Gli1 were increased when Sufu was co-expressed in wild-type (wt) MEFs. These results suggest that Sufu stabilizes Gli1 by preventing proteasome-dependent Gli1 degradation. Note that cycloheximide was added to block new protein synthesis in these studies. (B) Western blot analysis of lysates from HEK293T cells expressing various combinations of Gli1, Numb, Sufu and Acp1 (control). Numb expression resulted in reduction in Gli1 protein levels. This is consistent with

previous reports in which Numb was shown to activate the E3 ligase Itch, leading to Gli1 ubiquitination and degradation. Numb-induced Gli1 reduction was reversed when Sufu was co-expressed with Numb. Tubulin was used as the loading control. (C) Western blot analysis of immunoprecipitated $Gli1^{FLAG}$ from HEK293T cell lysates to test the competition between Sufu and Numb in binding to Gli1. Co-immunoprecipitated $Numb^{Myc}$ by Gli1 was significantly reduced when $Sufu^{Myc}$ was also pulled down by Gli1. (D) A model in which Sufu stabilizes Gli1 by inhibiting Numb-mediated protein degradation. In, input; IP, immunoprecipitation.

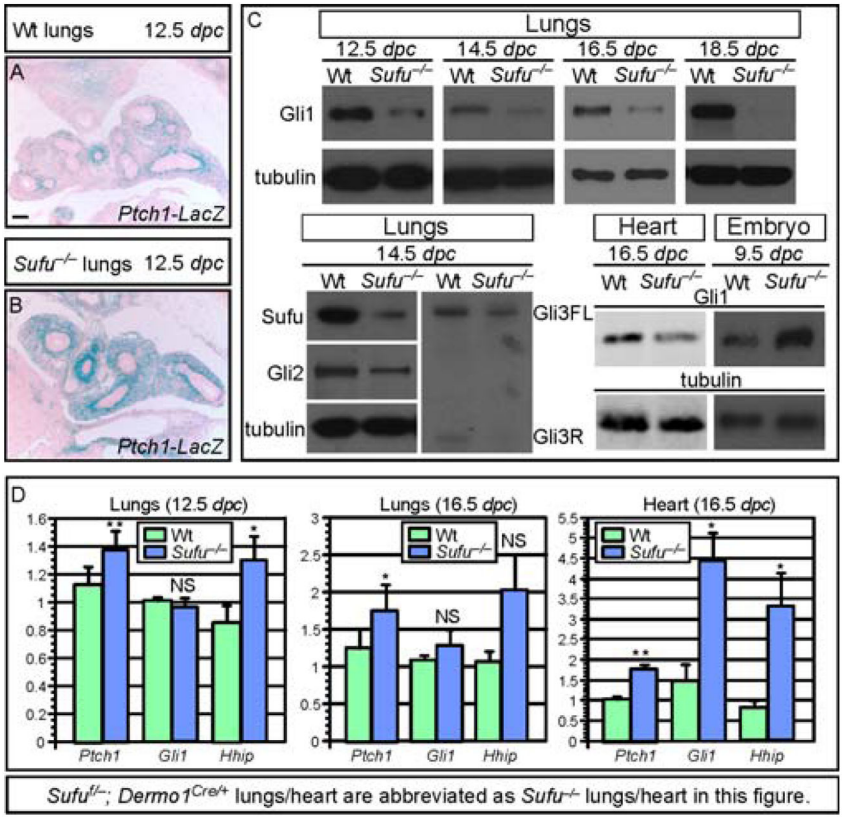


**Figure 2. Upregulation of Hh target gene expression with concomitant Gli1 protein reduction in the absence of Sufu**

(A–B) β-galactosidase staining of wild-type (wt) and *Sufu*-deficient (*Sufu*$^{f/-}$; *Dermo1*$^{Cre/+}$; *Ptch1*$^{LacZ/+}$) lungs to detect *Ptch1* expression in lung mesenchyme. *Ptch1-LacZ* expression was stronger and broader in *Sufu* mutants compared to wt. (C) Western blot analysis of endogenous Gli1 protein levels in wt and *Sufu* mutant lungs. Gli1 protein levels were decreased in *Sufu* mutant lungs collected at various stages of lung development. This resembles reduced protein levels of Gli2/3 in *Sufu* mutant lungs. Likewise, a reduction in Gli1 protein levels was also detected in *Sufu*-deficient hearts. (D) qPCR analysis of *Ptch1* and *Gli1* mRNA in wt and *Sufu* mutant lungs. *Ptch1* and *Hhip* mRNA levels were elevated in *Sufu* mutant lungs while *Gli1* mRNA levels were unaltered. This suggests that reduced Gli1 protein levels in *Sufu*-deficient lungs are a result of loss of Sufu and not due to changes in *Gli1* transcript levels. All values are means ± standard deviation. * $P < 0.05$; ** $P < 0.01$; NS, not significant (unpaired Student's *t*-test) (n=6 for 12.5 *dpc* lungs; n=4 for 16.5 *dpc* lungs; n=4 for 16.5 *dpc* heart). Elevation of *Hhip* mRNA levels at 16.5 *dpc* was not statistically significant likely due to large variations in transcript levels among different samples. Note that *Sufu*$^{f/-}$; *Dermo1*$^{Cre/+}$ lungs/heart are abbreviated as *Sufu*$^{-/-}$ lungs/heart while *Sufu*$^{-/-}$ embryos represent *Sufu* null embryos in this figure. FL, full-length; R, repressor. Scale bar = 100 μm for A–B.

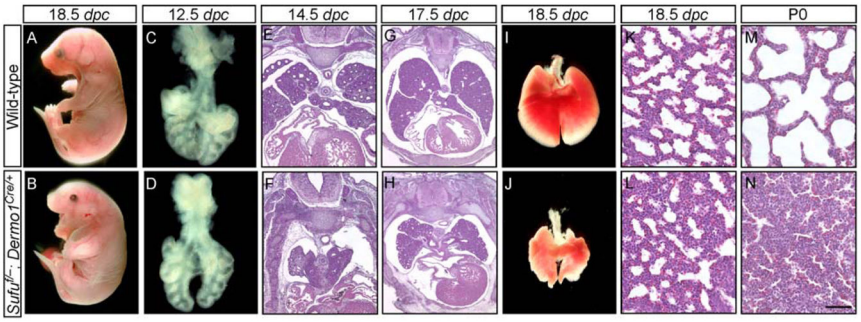


**Figure 3. Conditional inactivation of *Sufu* in lung mesenchyme**

(A–N) External morphology (A, B), dissected lungs (C, D, I, J) and histology (E–H, K–N) of lung sections from wild-type (wt) and $Sufu^{f/-}; Dermo1^{Cre/+}$ mouse embryos at various embryonic stages and postnatal (p) day 0 as indicated. More than 50 *Sufu* mutants were examined. The phenotypes in *Sufu* mutant lungs were completely penetrant and showed little variation from animal to animal. Epithelial and mesenchymal development was defective in *Sufu* mutants, resulting in a smaller sized lung with a compact mesenchyme. The length of the proximal-distal axis of *Sufu* mutant lungs is ~80% of that of wt lungs. *Sufu* mutant mice cannot expand their lungs and died a few hours after birth. Early patterns of epithelial branching appeared to be established properly in *Sufu*-deficient lungs, leading to the correct number and positioning of lung lobes. Epithelial branching at later stages of development was not as extensive in *Sufu*-deficient lungs compared to that in wt lungs. *dpc*, *days post coitus*. Scale bars: K–N, 50 μm.

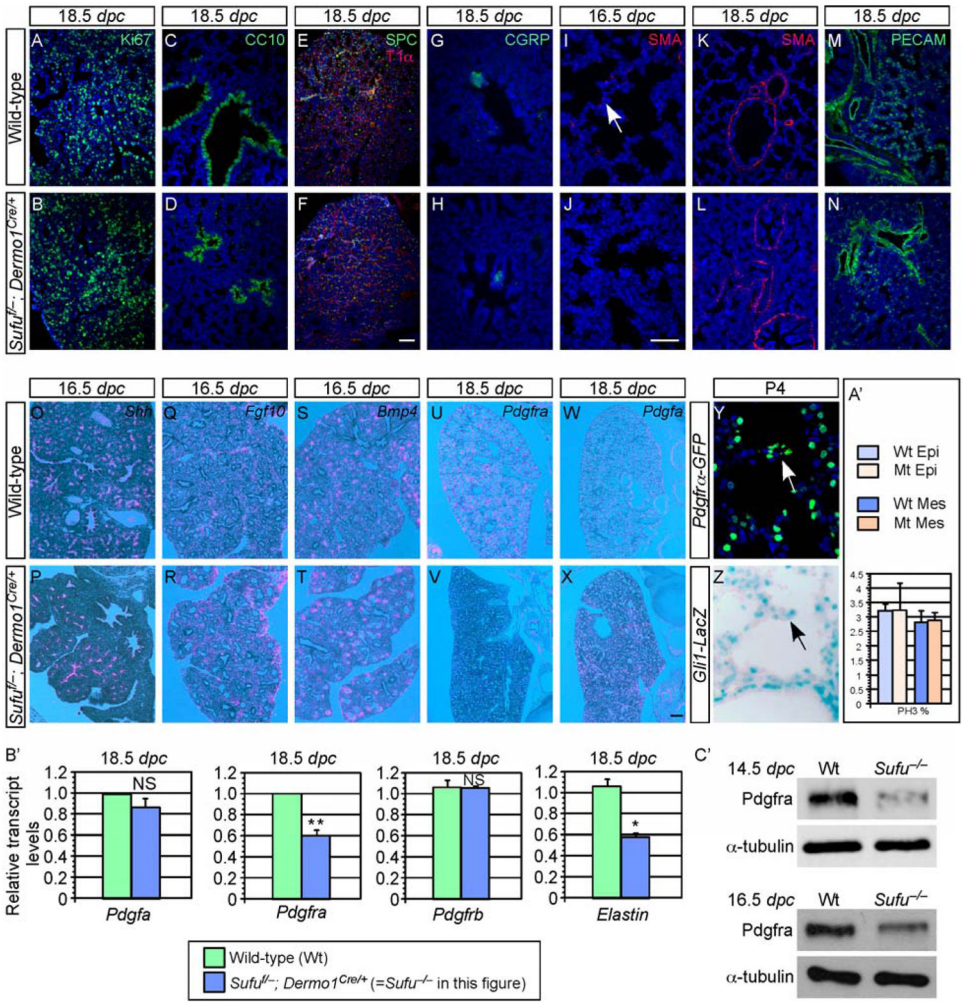


**Figure 4. Disruption of myofibroblast development in *Sufu*-deficient lungs**

(A–Z) Immunostaining (A–N, Y, Z) and *in situ* hybridization (O–X) of wild-type (wt) and *Sufu*-deficient (*Sufu*$^{f/-}$; *Dermo1*$^{Cre/+}$) lungs. No apparent difference in cell proliferation rate (judged by Ki67 and PH3 staining) or cell death was found between wt (A) and *Sufu* mutant (B) lungs. Quantification of cell proliferation in the epithelial and mesenchymal compartments at 12.5 *dpc* was shown in A′. Major epithelial cell types, including Clara cells (CC10$^+$) (C, D), alveolar type II (SPC$^+$), type I (T1α$^+$) cells (E, F) and pulmonary neuroendocrine cells (CGRP$^+$) (G, H) were properly specified in the absence of *Sufu*. Most mesenchymal cell types such as the bronchial smooth muscle (SMA$^+$) (K, L) and blood vessels (PECAM$^+$) (M, N) were also properly generated. By contrast, myofibroblasts (white arrow; marked by smooth muscle actin [SMA] staining) were greatly reduced in *Sufu* mutant lungs (I). This was associated with decreased *Pdgfra* expression (pink signal) (compare V to U) while *Pdgf* ligand expression was unaltered (compare X to W). In addition, the expression patterns and levels of *Shh* (O, P), *Fgf10* (Q, R) and *Bmp4* (S, T) or other components in Hh, Fgf and Bmp signaling were similar between wt and *Sufu* mutant lungs. Both Gli1 and Pdgfra were detected in the secondary septa of alveoli (arrows in Y, Z). (B′) qPCR analysis of *Pdgfa*, *Pdgfra*, *Pdgfrb* and *Elastin* mRNA in wt and *Sufu* mutant lungs.

*Pdgfra* mRNA levels were reduced in *Sufu* mutant lungs while *Pdgfa* and *Pdgfrb* mRNA levels were unaltered. This is consistent with results from *in situ* hybridization. *Elastin* transcript levels were also reduced in *Sufu* mutant lungs, consistent with defective myofibroblast development. All values are means ± standard deviation. * $P < 0.05$; ** $P < 0.01$; NS, not significant (unpaired Student's *t*-test) (n=3). (C′) Western blot of endogenous Pdgfra protein levels in wt and *Sufu* mutant lungs. Pdgfra protein levels were decreased in *Sufu* mutant lungs collected at various stages of lung development. This was likely due to reduced *Pdgfra* transcript levels in *Sufu* mutant lungs. *dpc*, *days post coitus*. Scale bars: A–F and K–N, 50 μm; G–J and Y–Z, 50 μm; O–X, 100 μm.

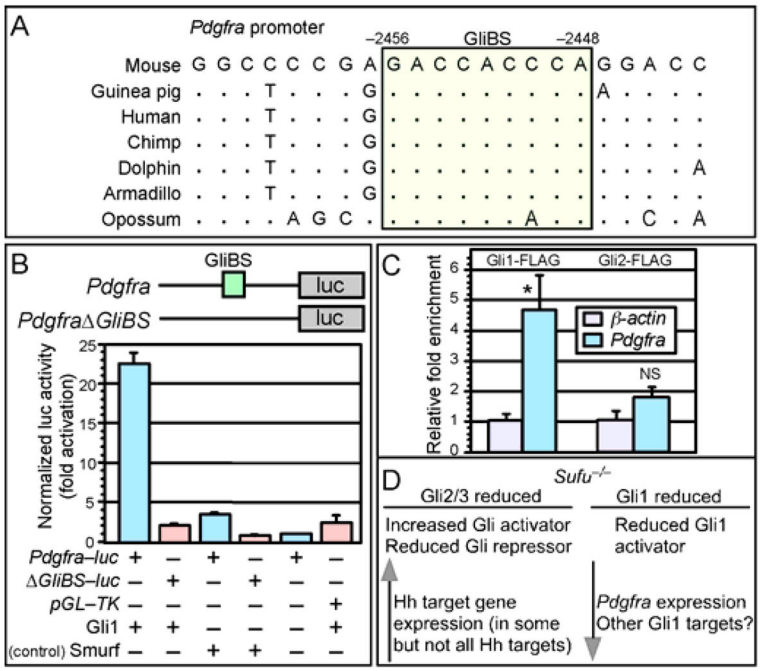


**Figure 5. Gli1 and regulation of *Pdgfra* promoter activity**

(A) Sequence analysis of the *Pdgfra* promoter from different species. A conserved canonical Gli-binding site (GliBS) is boxed and colored. The numbers represent distances from the transcriptional start site of *Pdgfra*, which is marked as position zero. (B) Schematic diagram depicting *Pdgfra-luc* reporter constructs in which mouse *Pdgfra* promoter fragments are placed upstream of *firefly luciferase* (*luc*). A canonical GliBS is present in the *Pdgfra* promoter and is mutated in the control construct *Pdgfra*Δ*GliBS-luc* (abbreviated as Δ*GliBS-luc* in the figure). Addition of Gli1 activated *Pdgfra-luc* and not *Pdgfra*Δ*GliBS-luc* in cell-based assays. Control nuclear protein Smurf did not induce *Pdgfra-luc* expression. (C) Gli1 but not Gli2 occupied the *Pdgfra* promoter by ChIP analysis using MEFs expressing FLAG-tagged Gli1 and Gli2. Gli-binding on the *Pdgfra* promoter was normalized to the *β-actin* control promoter. All values are means ± standard deviation. * $P < 0.05$; NS, not significant (unpaired Student's *t*-test) (n=3). (D) A model of differential regulation of Gli proteins by Sufu. Loss of *Sufu* results in reduced protein levels of all three Gli proteins. Reduction in Gli2/3 protein levels is associated with increased Gli activators and reduced Gli repressors. This would lead to overall Hh pathway activation and Hh target gene expression. By contrast, reduced protein levels of the constitutive activator Gli1 result in downregulation of Hh targets that primarily rely on Gli1 for their expression such as *Pdgfra* in the lung.