Regulation of Sufu activity by p66β and Mycbp provides new insight into vertebrate Hedgehog signaling

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Control of Gli function by Suppressor of Fused (Sufu), a major negative regulator, is a key step in mammalian Hedgehog (Hh) signaling, but how this is achieved in the nucleus is unknown. We found that Hh signaling results in reduced Sufu protein levels and Sufu dissociation from Gli proteins in the nucleus, highlighting critical functions of Sufu in the nucleus. Through a proteomic approach, we identified several Sufu-interacting proteins, including p66β (a member of the NuRD [nucleosome remodeling and histone deacetylase] repressor complex) and Mycbp (a Myc-binding protein). p66β negatively and Mycbp positively regulate Hh signaling in cell-based assays and zebrafish. They function downstream from the membrane receptors, Patched and Smoothened, and the primary cilium. Sufu, p66β, Mycbp, and Gli are also detected on the promoters of Hh targets in a dynamic manner. Our results support a new model of Hh signaling in the nucleus. Sufu recruits p66β to block Gli-mediated Hh target gene expression. Meanwhile, Mycbp forms a complex with Gli and Sufu without Hh stimulation but remains inactive. Hh pathway activation leads to dissociation of Sufu/p66β from Gli, enabling Mycbp to promote Gli protein activity and Hh target gene expression. These studies provide novel insight into how Sufu controls Hh signaling in the nucleus.

[Keywords: Sufu; p66β; Mycbp; Gli; Hh signaling]

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signaling not only inhibits the production of Gli repressors but also facilitates the generation of Gli activators (GliA; largely derived from full-length Gli2) to activate Hh target genes, which include Ptc1, Gli1, and Hhip. Gli1 does not undergo limited proteolysis and only functions as a transcriptional activator. Both Gli2 and Gli3 can generate a repressor in the absence of Hh signaling, but Gli2 proteolysis is inefficient (Pan et al. 2006), and the Gli2R plays a minor role in vivo (Li et al. 2011). Similar to Gli2, full-length Gli3 can function as an activator, but the contribution of Gli3 activator to Hh pathway activity in vivo is insignificant [Bai et al. 2004].

Hh signal transduction in mammals uses the primary cilium [Eggerschwiler and Anderson 2007; Wong and Reiter 2008; Berbasi et al. 2009; DeRouen and Oro 2009; Goetz and Anderson 2010; Bay and Caspary 2012; Drummond 2012; Oh and Katsanis 2012, Kim and Dynlacht 2013; Nozawa et al. 2013; Mukhopadhyay and Rohatgi 2014; Nachury 2014], an evolutionarily conserved microtubule-based organelle analogous to the flagella found in single-celled eukaryotes such as Chlamydomonas reinhardtii [green algae]. All core components of vertebrate Hh signaling localize to the primary cilium in a dynamic manner (Corbit et al. 2005; Haycraft et al. 2005; Rohatgi et al. 2007; Chen et al. 2009; Endoh-Yamagami et al. 2009; Kim et al. 2009, Liem et al. 2009). However, correlating ciliary distribution and the movement of Hh pathway components with their biochemical functions in Hh signaling remains a daunting endeavor. A thorough characterization of the dynamic ciliary movement of Hh pathway components coupled with functional studies is required to address this important issue.

Sufu is a major negative regulator of mammalian Hh signaling. Loss of Sufu in mammals leads to global Hh pathway activation and early embryonic lethality (Cooper et al. 2005; Svard et al. 2006). Sufu thus provides a key tool to understand how Hh signaling controls target gene activity. It is known that Gli proteins execute their function in the nucleus. Sufu binds Gli proteins [Ding et al. 1999, Kogerman et al. 1999, Pearse et al. 1999, Stone et al. 1999], which display dynamic shuttling between the cytoplasm and nucleus [Kogerman et al. 1999, Kim et al. 2009, Humke et al. 2010]. We expect that an essential aspect of Sufu function must reside in its control of Gli activity in the nucleus. Surprisingly, our knowledge of Sufu/Gli activity in the nucleus is very limited. In contrast, Sufu function in the cytoplasm or on the primary cilium is better studied. Sufu has been shown to sequester Gli proteins in the cytoplasm [Ding et al. 1999, Kogerman et al. 1999, Murone et al. 2000, Barnfield et al. 2005], control Gli protein levels [Chen et al. 2009; Jia et al. 2009, Wang et al. 2010], and regulate the production of Gli repressors and activators [Humke et al. 2010, Tukachinsky et al. 2010]. Elucidating Sufu’s nuclear function would fill a major gap in our mechanistic understanding of Hh signaling.

Canonical Gli-binding sites [GliBs] have been identified in many Hh target genes. How various combinations of Gli activators and repressors control Hh target gene expression and confer graded Hh responses in the nucleus is a major unresolved issue in Hh signaling [Hui and Angers 2011, Rabinowitz and Vokes 2012, Falkenstein and Vokes 2014]. This task is particularly challenging because different tissues use a unique combination of Gli activator/repressor to produce specific Hh outputs [i.e., a specific set of Hh targets] necessary for patterning. This point is illustrated by the observation that the Gli2 activator plays a dominant role in neural tube development [Ding et al. 1998, Matise et al. 1998; Bai et al. 2004], while the Gli3R is a key determinant of limb patterning [Bowers et al. 2012, Cao et al. 2013], and a different group of Hh targets is activated accordingly. Moreover, complex interactions between various Gli proteins exist in both neural tube [Liu et al. 2012] and limb patterning [Bowers et al. 2012], and pinpointing the contribution of a given Gli protein is nontrivial.

The basic framework of Hh signaling is established through the identification and characterization of various Hh pathway components, many of which were initially identified by genetic screens in Drosophila. It has also become clear that while the fundamental aspects of Hh signaling are conserved across species, divergence in Hh pathway design has occurred during evolution [Wilson and Chuang 2010]. Moreover, mammalian Hh signaling relies on the primary cilium, while most fly cells do not contain a primary cilium [Kornberg 2014, KuzhandaiVel et al. 2014]. In this case, Hh regulators specific to the mammalian Hh pathway cannot be identified by genetic screens in flies. We envision that proteomic or genomic approaches will offer a powerful tool to uncover these components in order to gain new insight into the mechanism and evolution of Hh signaling [Evangelista et al. 2008, Hillman et al. 2011, Jacob et al. 2011].

Through a proteomic method, we identified two new Hh regulators: p66β and Mycbp (Myc-binding protein). Our results show that vertebrate p66β is a negative regulator of Hh signaling, while Mycbp is a positive modulator of Hh signaling. Both are used in conjunction with Sufu to control Hh target gene expression to produce graded Hh responses. In our model, in the absence of the Hh ligand, Gli (such as Gli2) is bound by Sufu, which recruits p66β to inhibit Gli protein-mediated activation of Hh targets. Hh pathway activation not only abolishes Sufu/p66β inhibition on Gli but also enables Mycbp to promote Gli-mediated Hh target gene expression. These advances represent an important step toward our understanding of how Sufu controls Gli activity in the nucleus.

Results

**Hh signaling reduces Sufu protein levels in the nucleus**

To gain insight into Sufu’s function in the nucleus, we investigated whether Hh signaling alters Sufu protein levels in the nucleus as a possible means to modulate its activity. Hh treatment did not lead to obvious changes in total protein levels of Sufu. Since Sufu is more abundant in the cytoplasm than in the nucleus, we reasoned that any changes in nuclear Sufu protein levels in response to Hh signaling would be obscured by high levels of cytoplasmic Sufu. To test this idea, we fractionated cell lysates to
separate nuclear and cytoplasmic fractions from Hh-responsive mouse embryonic fibroblasts (MEFs) as previously described (Chen et al. 2009) and determined Sufu protein levels by Western blotting. The purity of the cytoplasmic and nuclear fractions was assessed by cytoplasmic- and nuclear-specific markers [Fig. 1A]. We found that Sufu protein levels were reduced by ~60% in the nuclear but not cytoplasmic fraction in response to Hh treatment [Fig. 1A]. Interestingly, reduction in nuclear Sufu protein levels could be restored by the addition of proteasome inhibitors [such as MG132] [Fig. 1B]. This suggests that control of Sufu protein levels in the nucleus is used to regulate Gli protein function.

**Hedgehog signaling leads to Sufu dissociation from Gli2 and Gli3 primarily in the nucleus**

We then asked whether Sufu activity is also regulated in the nucleus. Several groups reported reduced Gli3 levels (Humke et al. 2010; Tukachinsky et al. 2010; Wen et al. 2010) and dissociation of Sufu from Gli2 and Gli3 [Humke et al. 2010; Tukachinsky et al. 2010] upon Hh pathway activation. This conclusion was reached using total cell lysate from NIH3T3 cells for immunoprecipitation experiments [Humke et al. 2010; Tukachinsky et al. 2010]. We inspected in which subcellular compartment Sufu–Gli2 or Sufu–Gli3 dissociation occurs using lysates from MEFs and NIH3T3 cells. We isolated nuclear and cytoplasmic fractions as described above. We confirmed that Gli3 protein levels were reduced upon Hh stimulation [Supplemental Fig. S1A]. However, unlike Gli3, Hh signaling did not lead to reduced Gli2 protein levels [Supplemental Fig. S1A].

We then performed immunoprecipitation using either the cytoplasmic or nuclear fraction to test Sufu–Gli dissociation upon Hh pathway activation. While dissociation of Sufu–Gli3 can be detected to some degree in the cytoplasmic fraction, Sufu–Gli3 dissociation mainly occurs in the nuclear fraction [Fig. 1C; Supplemental Fig. S1B]. Similarly, we carried out nuclear–cytoplasmic fractionation and immunoprecipitation to examine the dissociation of Sufu and Gli2 upon Hh stimulation. Dissociation of Sufu–Gli2 was also found largely in the nuclear but not the cytoplasmic fraction in wild-type or Pch1<sup>+/−</sup> MEFS [Fig. 1C; Supplemental Figs. S1C, S2data not shown]. We noticed that dissociation between Sufu and Gli2/3 in the nuclear fraction could occur at 30 min after Hh stimulation [Fig. 1C], within the time frame of reported ciliary localization of Hh pathway components [Tukachinsky et al. 2010; Wen et al. 2010]. Sufu–Gli2/3 dissociation in the nuclear fraction was also detected at 6, 12, or 24 h after Hh activation when activation of Gli1 and reduction in Gli3R levels were apparent [Fig. 1C; Supplemental Fig. S3data not shown]. Together, these results reinforce the notion that important regulation of Gli activities by Sufu resides in the nucleus.

**Sufu mutants that fail to sequester Gli proteins in the cytoplasm could still repress Hh reporter activity**

To further explore Sufu’s function in the nucleus, we tested a few Sufu mutants for their ability to sequester Gli protein and inhibit Gli-mediated Hh responses. We produced Sufu<sup>N100</sup> and Sufu<sup>N212</sup> [Barnfield et al. 2005], which retain the first 100 and 212 amino acids of Sufu, respectively. Sufu<sup>N100</sup> and Sufu<sup>N212</sup> failed to sequester Gli proteins in the cytoplasm but were still capable of inhibiting Gli-
mediated Hh transcriptional responses in various cell lines, including MEFs, NIH3T3, C2C12, and C3H10T1/2 [Supplemental Figs. S4, S5; data not shown]. Readouts of Hh activity were determined by quantitative PCR (qPCR) analysis of Hh target gene expression [such as Gli1] or a standard Hh reporter assay [e.g., 8xGliBS-luc], in which a firefly luciferase [luc] reporter is placed under the control of Hh-responsive element 8xGliBS) (Sasaki et al. 1997). This result confirms and extends previous findings (Barnfield et al. 2005) and highlights a critical function of Sufu in controlling Gli activity in the nucleus that is independent of cytoplasmic sequestering. Taken together, our findings point to an essential role of Sufu in the nucleus in Hh signaling.

A proteomic approach to identify Sufu-interacting proteins uncovers p66β and Mycbp

In order to further understand the molecular mechanisms by which Sufu regulates Gli protein function, we took a proteomic approach to identify Sufu-interacting proteins other than the three Gli proteins. We generated a stable cell line in MEFs that expresses 3xFlag-Sufu and performed large-scale immunoprecipitation using anti-Flag antibodies (Fig. 2A). Endogenous Gli2 and Gli3 were present in the immunoprecipitates, validating our approach [Fig. 2B]. Immunoprecipitates were analyzed by mass spectrometry (Jager et al. 2011; Altelaar et al. 2013). We focused on nonstructural proteins with overrepresentations of peptides identified through mass spectrometry. They were good candidates for direct interactions with Sufu. We also treated MEFs expressing 3xFlag-Sufu with Hh-conditioned medium and performed a similar procedure to detect any changes in the levels and compositions of proteins that coimmunoprecipitated with Sufu.

We selected a number of Sufu-interacting proteins with overrepresentations of peptides in our proteomic analysis and systematically tested their ability to perturb Hh signaling through overexpression and knockdown studies. Two of the Sufu-interacting proteins, p66β and Mycbp, showed the most striking and robust effects on Hh signaling and thus are the primary focus of this study [Fig. 2C]. p66β, encoded by Gatad2b [GATA zinc finger

Both p66β and Mycbp physically interact with Sufu and are found in the nucleus

To confirm the physical interaction between Sufu and p66β/Mycbp, we expressed epitope-tagged Sufu, p66β, and Mycbp in HEK293T cells and performed immunoprecipitation using antibodies against the respective epitopes. We showed that Sufu immunoprecipitates contained p66β and vice versa [Fig. 2D; data not shown]. In addition, antibodies that pulled down endogenous Sufu in wild-type MEFs also coimmunoprecipitated endogenous p66β [Fig. 2E, bottom panels; Supplemental Fig. S6]. These results indicate physical interactions between Sufu and p66β either directly or indirectly through additional proteins. Interestingly, HDAC1 was coimmunoprecipitated with p66β and Sufu, but Sufu did not pull down other known components of the NuRD repressor [Fig. 2E, Supplemental Figs. S7, S8]. We also showed that Sufu and Mycbp were coimmunoprecipitated in cultured cells [Fig. 2D]. This result indicates that Sufu interacts with Mycbp directly or indirectly.

We examined the subcellular distribution of p66β and found that p66β protein was largely confined to the nucleus of Hh-responsive cells [Fig. 2F], consistent with its purported nuclear function. Similarly, Mycbp distribution could be found in the nucleus as previously documented [Fig. 2G; Furusawa et al. 2002].

Overexpression of p66β impairs Hh responses in cultured cells, while p66β knockdown results in enhanced Hh pathway activation in cell-based assays

As a first step toward understanding how p66β mediates Sufu function in Hh signaling, we overexpressed p66β in Hh-responsive cells [such as NIH3T3 and MEFs] and assessed its effect on Hh pathway activity. Readouts of Hh activity were determined by a standard Hh reporter assay or transcript levels of Hh target genes described above. Cotransfection of Gli1 or Gli2 together with Hh reporters resulted in Hh reporter activation [Fig. 3A]. We found that coexpression of p66β with Gli1 or Gli2 in this assay severely inhibited Gli-mediated Hh reporter activation or Hh target gene expression, an effect comparable with Sufu-mediated inhibition of Hh pathway activity [Fig. 3A; Supplemental Fig. S9]. Other signaling pathways such as Wnt signaling, assayed by the TOPFlash reporter [Molenaar et al. 1996], were unaffected by p66β overexpression [Fig. 3F], suggesting that p66β does not exert general inhibition of reporter activities. Conversely, shRNA-mediated knockdown [Hannon 2003] of p66β [Supplemental Figs. S10–S12] resulted in enhanced Hh responses. While p66β knockdown had a modest effect on Hh reporter activity in the absence of the Hh ligand, reduction in p66β enhanced Hh responses upon Hh stimulation [Fig. 3B; Supplemental Fig. S13]. These results are consistent with a negative role of p66β in controlling Hh signaling [Supplemental Figs. S14, S15].

Overexpression of Mycbp facilitates Hh responses, while knockdown of Mycbp compromises Hh responses

We took a similar approach to further understand how Mycbp controls Hh signaling by overexpressing Mycbp in Hh-responsive cells and assessing its effect on Hh pathway activity. Coexpression of Mycbp with Gli1 or Gli2 in this assay significantly promoted Gli-mediated Hh reporter activation or Hh target gene expression [Fig. 3C; Supplemental Fig. S9]. Again, Wnt responses were unaltered by Mycbp overexpression [Fig. 3F]. These findings are consistent with a positive role of Mycbp in Hh pathway activation. Since p66β and Mycbp exert opposite effects on Hh responses, we anticipate that p66β and Mycbp will antagonize each other in controlling Hh pathway activity. Indeed, when p66β and Mycbp were cotransfected in wild-type cells, Mycbp could partially reverse the inhibitory effects of p66β on Gli transcriptional activation, likely depending on the Mycbp/p66β ratio [Supplemental Figs. S15, S16].

We also determined the functional consequence of loss of Mycbp through shRNA-mediated knockdown [Hannon 2003] in Hh-responsive cells [Supplemental Fig. S10]. We showed that Hh pathway activation assayed by Hh reporter activity was severely compromised when Mycbp was knocked down [Fig. 3D]. Moreover, Hh target gene expression such as Gli1 was greatly reduced by Western blotting [Fig. 3E]. This further supports a positive role of Mycbp in enhancing Hh pathway activity.

Expression or knockdown of p66β and mycbp in zebrafish perturbs Hh signaling, consistent with their respective roles as negative and positive regulators of Hh signaling

To assess the in vivo function of p66β and mycbp, we used the zebrafish system as a readout of their in vivo activity. We injected p66β mRNA into zebrafish embryos and investigated the phenotypic consequences associated with p66β overexpression. We focused on the developing somites and fin buds, where Hh pathway perturbation leads to well-characterized phenotypes [Lewis et al. 1999; Neumann et al. 1999]. We found that p66β overexpression engendered U-shaped somites and reduced fin buds [Fig. 4A,B; Supplemental Fig. S17; data not shown], both
of which are associated with disruption of Hh signaling. Moreover, Hh target gene expression such as *ptch1* was also reduced (or lost) in fin buds when *p66* was overexpressed (Fig. 4C,D,F). Conversely, morpholino (MO)-mediated knockdown of *p66* enhanced *ptch1* expression (Fig. 4C, E,H). These results support our hypothesis that *p66* represses Hh responses.

Similarly, *mycbp* mRNA or MO was injected into zebrafish embryos, and Hh responses—including phenotypic outcomes and Hh target gene expression—were determined. We showed that overexpression of *mycbp* in zebrafish embryos resulted in an increase in Hh target gene expression [such as *ptch1*] in fin buds [Fig. 4C,E,G; Supplemental Fig. S17]. In contrast, *ptch1* expression was reduced in *mycbp* morphants [Fig. 4C,D,I]. This is consistent with a positive role of *mycbp* in Hh signaling. Taken together, these studies provide in vivo evidence to support *p66* and *Mycbp* as new regulators of vertebrate Hh signaling.

**p66* and *Mycbp* function downstream from *Ptc1*/Smo/cilia to mediate *Sufu* activity

Having established the effects of *p66* and *Mycbp* on Hh signaling, we performed similar assays using cell lines de-
ficient in various Hh pathway components (Chen et al. 2009) to reveal the relationship between p66ß, Mycbp, and other Hh components. We found that p66ß failed to inhibit Gli-mediated Hh reporter activity in Sufu-deficient MEFs (Fig. 5; Supplemental Fig. S18), suggesting that p66ß activity depends on Sufu. In contrast, p66ß was capable of inhibiting Gli-mediated Hh reporter activity in Ptch1- or Smo-deficient MEFs (Fig. 5; Supplemental Fig. S18), placing p66ß downstream from Ptch1 and Smo in controlling Hh signaling. Furthermore, p66ß also inhibited Gli-mediated Hh reporter activity in Kif3a-deficient MEFs (Fig. 5; Supplemental Fig. S18) in which primary cilia fail to form and Hh signaling cannot be transduced. This indicates that p66ß activity does not depend on the primary cilium.

Mycbp modestly promoted Gli-mediated Hh reporter activity in Sufu-deficient MEFs [Fig. 5]. We speculate that either Sufu or Gli can recruit Mycbp, and Mycbp remains inactive in the absence of the Hh ligand; Mycbp activity is required only when the Hh signal is transduced. Consistent with this, we found that knockdown of Mycbp in Sufu-deficient cells blunts Hh target gene expression (Supplemental Fig. S19). In addition, we found that full-length Gli proteins can interact with Mycbp in the absence of Sufu (Supplemental Fig. S20).

Mycbp was capable of stimulating Gli-mediated Hh reporter activity in Ptch1- or Smo-deficient MEFs (Fig. 5), suggesting that Mycbp functions downstream from Ptch1 and Smo. In addition, Mycbp also potenti-ated Gli-mediated Hh reporter activity in Kif3a-deficient MEFs [Fig. 5], indicating that Mycbp activity is independent of the primary cilium. Finally, overexpression of p66ß and Mycbp does not influence ciliary localization of Gli2 (data not shown). Together, these findings show that p66ß and Mycbp function downstream from Ptch1/Smo/cilia and likely at a similar step in the Hh pathway in mediating Sufu’s nuclear activity.

**Protein complex formation occurs between Sufu, Gli, and p66ß/Mycbp**

Since Sufu interacts with both p66ß and Gli, we surmise that Sufu, p66ß, and Gli may form a multicomponent protein complex, and Sufu bridges the interactions between p66ß and Gli. Consistent with this model, Gli2 immunoprecipitates from MEFs expressing 3xFlag-Gli2 contained both Sufu and p66ß [Fig. 6A, Supplemental Fig. S21], while communoprecipitation of p66ß–Gli2 failed to occur in Sufu+/− MEFs that express 3xFlag-Gli2 [Fig. 6B]. Similar interactions between full-length Gli3, Sufu, and p66ß were obtained in MEFs expressing 3xFlag-Gli3 [Fig. 6A,B, Supplemental Fig. S21]. This suggests that Sufu can recruit p66ß to control Gli activity. Using a parallel approach, we found that Mycbp bound to full-length p66ß.
p66β and Mycbp function downstream from membrane receptors Ptch1 and Smo and independently of the primary cilium. Note that Ptch1-deficient MEFs exhibit strong or even maximal Hh pathway activation in the absence of Hh ligands; Hh reporters can still be activated in Ptch1-deficient MEFs if Hh pathway components are exogenously overexpressed. By comparison, Smo-deficient or Kif3a-deficient MEFs are defective in Hh signal reception and transduction, and 8xGliBS-luc reporters can be activated only by exogenous nuclear Gli proteins in these cell lines. Gli1/2 were cotransfected with p66β or Mycbp into Smo- and Kif3a-deficient MEFs to assess the effects of p66β/Mycbp on Hh signaling. Otherwise, expression of p66β and Mycbp has no significant effect on Hh signaling in the uninduced state as discussed in the legend for Figure 3. [•] P < 0.05; [NS] not significant (unpaired Student’s t-test) [n number is indicated]. Similar results and conclusions were obtained by assessing the expression of endogenous Hh targets as the readouts of Hh signaling (Supplemental Fig. S18).

Gli2 or Gli3 protein in addition to Sufu [Fig. 6C], providing evidence to support the presence of a Sufu/Mycbp/Gli protein complex. It is interesting to note that no significant interaction was detected between Gli repressors and p66β or Mycbp [Supplemental Fig. S21].

Since p66β protein levels do not appear to alter upon Hh stimulation [Fig. 2E], we envision two possible scenarios for p66β action. Hh pathway activation could lead to dissociation of the Sufu/p66β protein complex from Gli, thus terminating the inhibitory effects on Gli proteins. Alternatively, Hh signaling may result in disruption of the Sufu/p66β protein complex. To distinguish between these possibilities, we performed communoprecipitation experiments using cell lysates from Hh-responsive cells expressing p66β, Sufu, and Gli. Cells were also treated with Hh-conditioned medium or agonists to activate the Hh pathway. We found that p66β communoprecipitated with Sufu without Hh stimulation, and p66β/Sufu interaction was not altered by Hh stimulation [Fig. 2E; Supplemental Fig. S6]. Moreover, p66β dissociates from Gli2 [but not Sufu] upon Hh pathway activation in communoprecipitation experiments [Supplemental Fig. S22]. In conjunction with our demonstration of Sufu dissociation from Gli2/3 in the nucleus upon Hh activation [Fig. 1C; Supplemental Fig. S1], these results suggest that Sufu mediates the interaction between p66β and Gli, and the entire Sufu/p66β protein complex is released from Gli when the Hh pathway is activated.

**Sufu and p66β occupy the promoter of Hh-responsive genes in a Hh-dependent manner**

To further understand how Sufu and p66β control Hh signaling, we asked whether Sufu and p66β could occupy the promoter of Hh-responsive genes, many of which contain canonical GliBSs. This would provide mechanistic insight into how p66β modulates Sufu activity in controlling Hh signaling.

We first assessed the ability of Sufu and Gli proteins to recognize the GliBS. We used double-stranded oligonucleotides that contain a GliBS [denoted OligoGliBS] [Pan et al. 2006] for communoprecipitation. Oligonucleotides that contain the mutant GliBS (OligoGliBSΔ) were used as a control. We showed that endogenous Sufu, Gli2, Gli3, and Gli3R were immunoprecipitated by OligoGliBS but not OligoGliBSΔ in the absence of Hh ligand stimulation [Fig. 6D]. This provides strong evidence to support the notion that a Sufu–Gli protein complex can interact with the GliBS. Interestingly, Sufu failed to be immunoprecipitated by OligoGliBS in Gli2−/− or Gli3−/− MEFs [Fig. 6E; Zeng et al. 2010] in which all three Gli proteins are absent, since Gli1 expression relies on active Hh signaling via Gli2/3 [Bai et al. 2004]. This is consistent with the idea that Sufu association with the GliBS depends on Gli proteins. Hh stimulation led to decreased association between Sufu and the GliBS as well as between Gli3R and the GliBS, while the amount of Gli2 and full-length Gli3 pulled down by the GliBS was significantly increased [Fig. 6D]. This supports the notion of Sufu dissociation from Gli proteins upon Hh pathway activation. Given the interactions between Sufu, p66β, and Gli, these findings suggest that Sufu can bridge p66β and Gli interactions on Hh-responsive promoters.

To further test this idea, we performed chromatin immunoprecipitation (ChIP) assays [Collas 2011] on Gli1, Gli2, Sufu, and p66β. Epitope [Gli1, Gli2, Sufu, and p66β were expressed in Hh-responsive cells, and ChIP analysis was performed on known Hh promoters.
target genes (e.g., *Ptch1*, *Gli1*, and *Hhip*) (Goodrich et al. 1996; Lee et al. 1997; Chuang and McMahon 1999), the promoter of which contains canonical GliBSs. We showed that Gli1, Gli2, and Sufu proteins were enriched on the promoters of Hh-responsive genes (Fig. 6F; Supplemental Fig. S23), consistent with results from immunoprecipitation studies using GliBS oligonucleotides. Importantly, p66β was also enriched on Hh-responsive promoters (Fig. 6F; Supplemental Fig. S23). This was the first demonstration of the presence of Sufu and p66β on the promoter of Hh-responsive genes. p66β's chromatin association was abolished in Sufu-deficient MEFs (Supplemental Fig. S23), again consistent with the model in which Sufu recruits responsive genes. p66β to Hh target gene promoters. These results suggest that Sufu, Gli, and p66β are present at the GliBSs of Hh-responsive genes prior to Hh pathway activation. In this way, Sufu recruits p66β to inhibit Gli-mediated Hh gene expression. This model predicts that Hh pathway activation would relieve the inhibition of Sufu/p66β on Gli proteins on Hh target gene promoters. Indeed, ChIP analysis revealed diminished association of both Sufu and p66β with Hh promoters upon Hh stimulation (Fig. 6F; Supplemental Fig. S23). These studies yield novel insights into the dynamic interactions of Gli, Sufu, and p66β on the promoter in the process of Hh pathway activation.

Enhanced interactions between Mycβp and Gli during Hh signal transduction

Our studies show that p66β inhibits Hh responses, while Mycβp promotes Hh responses. Since both p66β and Mycβp interact with Sufu, this led to our hypothesis that Mycβp can potentiate Gli activity once Hh pathway activation removes Sufu/p66β from Gli proteins. To further test this idea, we studied the interaction between Mycβp, Sufu, and Gli proteins during Hh pathway activation. We found that Mycβp dissociated from Sufu upon Hh treatment, while Mycβp interaction with full-length Gli2 and Gli3 was enhanced upon Hh pathway activation (Fig. 6C).

![Figure 6](https://genesdev.cshlp.org/content/files/figures/255532950.jpg)

**Figure 6.** Complex formation between Sufu, p66β, Mycβp, and Gli proteins and their dynamic interactions on the promoters of Hh-responsive genes. (A) Western blot analysis of immunoprecipitates using lysates from MEFs expressing Flag-tagged Gli2 or Gli3. Endogenous p66β and Sufu were coimmunoprecipitated, consistent with the formation of a Sufu/p66β/Gli protein complex. (B) Western blot analysis of immunoprecipitates using lysates from Sufu-deficient MEFs expressing Flag-tagged Gli2 or Gli3. Endogenous p66β failed to be coimmunoprecipitated, suggesting that Sufu bridges the interaction between p66β and Gli protein. (C) Western blot analysis of immunoprecipitates using lysates from MEFs expressing Flag-tagged Mycβp. Endogenous Sufu, Gli2, and Gli3 were coimmunoprecipitated, implying the production of a Sufu/Mycβp/Gli protein complex. Moreover, Mycβp dissociated from Sufu upon Hh treatment, while Mycβp interaction with full-length Gli2 and Gli3 was enhanced upon Hh pathway activation. (*P < 0.05; **P < 0.01; [NS] not significant [unpaired Student’s t-test] n = 3). Mycβp/Gli interaction was increased upon Hh stimulation, but variations in immunoprecipitations affected the calculated statistical value. (D) Immunoprecipitation of Gli2, Gli3, and Sufu using oligonucleotides that contain a canonical GliBS or control oligonucleotides in which the GliBS is mutated (denoted as ΔGliBS). Sufu, full-length (FL) Gli2, full-length Gli3, and Gli3R were immunoprecipitated by a GliBS without Hh stimulation but not by the control ΔGliBS. Hh stimulation led to an increased binding of Gli2 and Gli3 to the GliBS, while association of Sufu or Gli3R with the GliBS was weakened. (E) Sufu failed to be immunoprecipitated by the GliBS in Gli2Δ/Gli3Δ MEFs, indicating that Sufu binding to the GliBS is dependent on Gli proteins. (F) ChIP analysis of Gli1, Gli2, Sufu, p66β, and Mycβp on the promoter of Hh-responsive genes such as *Ptch1*. Gli1, Gli2, Sufu, and p66β proteins were enriched on the *Ptch1* promoter by ChIP. Hh treatment led to the reduced presence of Sufu and p66β on the *Ptch1* promoter, while binding of Mycβp to the *Ptch1* promoter was enhanced. Interestingly, enhanced binding of Mycβp to Hh target gene promoters was abolished in Gli2Δ/Gli3Δ MEFs [Supplemental Fig. S24], suggesting that the promoter occupancy of Mycβp is dependent on Gli proteins. (*) P < 0.05 (unpaired Student’s t-test) n = 3).
We also performed ChIP analysis of Mycbp on the promoters of Hh target genes. In the presence of Hh signaling, Mycbp was enriched at the Hh promoters (Fig. 6F; Supplemental Fig. S23), and enhanced Mycbp binding was abolished in Gli2<sup>−/−</sup>; Gli3<sup>−/−</sup> MEFs [Supplemental Fig. S24]. This is in contrast to reduced binding of Sufu or p66<sup>β</sup> to Hh promoters by Hh pathway activation (Fig. 6F; Supplemental Fig. 23). These findings suggest that Hh signaling leads to dissociation of Sufu/p66<sup>β</sup> from Gli, allowing Mycbp/Gli to activate Hh target gene expression.

**Discussion**

Our studies on Sufu-interacting proteins p66<sup>β</sup> and Mycbp offer novel insight into the regulation of Hh target gene expression by Sufu and Gli in the nucleus. In particular, p66<sup>β</sup> and Mycbp modulate the process of how Sufu controls Gli protein function in the nucleus (Fig. 7). Our investigation thus provides a new framework for understanding Hh target gene expression and the production of graded Hh responses in diverse tissues, a key unresolved issue in Hh signaling.

The multiple roles of Sufu in controlling Gli protein functions

Extensive studies on Sufu uncovered its role in regulating Gli activities at several subcellular locales and multiple levels. Sufu can sequester Gli proteins [Ding et al. 1999; Kogerman et al. 1999; Murone et al. 2000; Barnfield et al. 2005], regulate Gli protein levels [Chen et al. 2009; Jia et al. 2009; Wang et al. 2010], promote the production of Gli repressors, and inhibit the generation of Gli activators [Humke et al. 2010; Tukachinsky et al. 2010]. Physical sequestration of Gli proteins by Sufu in the cytoplasm is traditionally viewed as a major mechanism of inhibiting Gli activities. Recently, Sufu was shown to control Gli protein levels through its antagonistic effects on Spop- or Numb-mediated Gli ubiquitination and degradation [Chen et al. 2009; Wang et al. 2010; Di Marzotullio et al. 2011; Lin et al. 2014]. In this way, Sufu preserves a pool of Gli protein that would be available to activate Hh target gene expression once the Hh signal is transduced. Interestingly, Sufu–Gli association gained a new level of complexity in light of the proposal in which Sufu inhibits Gli activity on the primary cilium [Humke et al. 2010; Tukachinsky et al. 2010]. In this model, Hh signaling leads to Sufu–Gli dissociation on the cilium, resulting in Gli activation and the production of a labile form of Gli, which is proposed to be derived from phosphorylated Gli proteins [Humke et al. 2010; Niewiadomski et al. 2014].

The relative contributions of various effects of Sufu to Gli protein activities have not been accurately assessed. In fact, it has not been unambiguously demonstrated where Sufu controls Gli activities. This is largely due to technical difficulty in selective inactivation of endogenous Sufu in a particular subcellular compartment and determining its functional consequence. The notion of a labile active form of Gli is consistent with prior work in *Drosophila* on Ci, the Gli homolog [Ohlmeyer and Kalderon 1998]. However, it should be noted that the addition of Hh ligands does not lead to reduced Gli2 protein levels [Supplemental Fig. S1; Kim et al. 2009]. Identifying Sufu mutants that affect unique aspects of Gli protein activities will provide critical tools to reveal the contributions of various Sufu/Gli interactions to Hh signaling.

While Sufu–Gli dissociation can be detected to some extent in the cytoplasm, the main site of Sufu–Gli dissociation seems to reside in the nucleus. The extent of dissociation between Gli2/3 and Sufu using nuclear fractions seems to be comparable with that using whole-cell lysates [Humke et al. 2010]. We speculate that changes in
Sufu and Gli distribution upon Hh activation are dynamic and quantitative, not “all or none.” In this model, a significant fraction of Sufu and Gli still associate with each other in the cytoplasm, perhaps as a reservoir. Consistent with this idea, Sufu and Gli accumulate in the primary cilium upon Hh stimulation, and, in fact, Sufu/Gli could still be detected in the cilium 24 h after Hh treatment.

Perhaps Sufu–Gli dissociation in different subcellular locales serves to execute Sufu’s multiple functions in Hh signaling. For instance, Sufu–Gli dissociation in the cytoplasm/cilium may blunt the generation of Gli repressors and lead to the production of Gli activators, while nuclear Sufu–Gli dissociation could free Gli proteins of their transcriptional repressors.

X-ray crystallographic structures of Sufu in a complex with a short Gli peptide (containing the Sufu-binding SYGH motif) revealed a clamp-like structure of Sufu that interacts with the Gli peptide (Cherry et al. 2013; Zhang et al. 2013). This raised the possibility that both the N-terminal and C-terminal domains of Sufu are required for Gli binding. However, previous studies have reported physical interactions between Sufu truncation mutants (e.g., the N-terminal fragment of Sufu) and Gli proteins (Merchant et al. 2004). Moreover, Gli2 [ΔSYGH] can still bind Sufu (Santos and Reiter 2014), suggesting the presence of multiple Sufu-binding domains in Gli2. It is possible that Sufu in a multiprotein complex in its native environment would display complex dynamic behaviors, and multiple domains of Sufu and Gli can interact with each other independently. In addition, Gli2 [ΔSYGH] is not sequestered in the cytoplasm and instead is enriched in the nucleus but does not lead to Hh pathway hyperactivation (Santos and Reiter 2014). This suggests that Sufu can inhibit Gli2 [ΔSYGH] activity in the nucleus, although it does not sequester Gli2 [ΔSYGH] in the cytoplasm. This is consistent with our finding that the sequestering function of Sufu can be separated from its ability to inhibit Gli-dependent transcription as revealed by the N-terminal fragments of Sufu (Sufu[ΔN100] and Sufu[ΔN212]). While Sufu[ΔN100] and Sufu[ΔN212] can bind Gli proteins, albeit less strongly than Sufu [Supplemental Fig. S5], and are potent in inhibiting Hh responses in cell-based assays, it is possible that overexpression augmented their inhibitory effects. The function of Sufu[N100] and Sufu[N212] would need to be assessed by introducing these mutants in vivo through gene targeting or genome editing.

Whether Sufu plays a key role in controlling Gli protein functions on Hh-responsive genes in the nucleus has not been extensively studied (Cheng and Bishop 2002). In this regard, our study represents a major step toward understanding Sufu’s nuclear function. While we focused on p66β and Mycbp in this study, other Sufu-interacting proteins discovered from our proteomic approach could shed light on Sufu function.

**Regulation of Gli activity by Sufu and p66β**

We showed that p66β negatively regulates Hh signaling in cell-based assays and zebrafish, and p66β relies on Sufu to mediate its inhibitory action on Hh signaling. p66β is a member of the NuRD repressor complex (Lai and Wade 2011; Allen et al. 2013), suggesting that p66β functions in the nucleus. This is further supported by the nuclear distribution of p66β, its presence on Hh promoters, and the independence of p66β activity on Ptch1/Smo and the primary cilium.

When p66β knockdown cells were treated with a high dose of Hh, induced Hh activity was similar to that in wild-type cells [Supplemental Fig. S13]. We reason that this was because Sufu dissociated from Gli (this occurs largely in the nucleus in our model) using high doses of Hh ligand. Under this condition, the level of p66β, whose function in Hh signaling depends on Sufu, would make no difference on Hh responses. Hence, p66β knockdown exerts little effect on Gli if Sufu/Gli dissociation has already occurred. In contrast, when cells are subjected to submaximal Hh stimulation, Sufu is not expected to completely dissociate from Gli in the nucleus. As expected, increased Hh responses were observed in p66β knockdown cells.

We showed that HDAC1 was coimmunoprecipitated with p66β and Sufu, but Sufu did not pull down other known components of the NuRD repressor. It is possible that interactions between p66β/Sufu and other NuRD components are weak or transient. However, it is also possible that the p66β/Sufu interaction represents a novel function of p66β independent of the NuRD complex. Interestingly, HDAC1 also exhibits NuRD-independent function (Canettieri et al. 2010). Whether p66β recruits additional components or relies on post-translational modifications of p66β and Gli proteins to control Hh signaling is unknown and would rely on future biochemical studies.

The phenotypes associated with the loss of p66β in mice are currently unavailable and would require the production of mutant mice and phenotypic analysis. Nevertheless, it is interesting to note that knockout mice of p66α die at 9.5 d post-coitum (dpc) [Marino and Nusse 2007]. The observation that the loss of p66α does not lead to global cell death at the earliest stage of embryonic development implies that components of the NuRD complex could be involved in specific cellular processes or interact with particular pathways during development. It is also plausible that functional redundancy exists between p66β and other components, and a better mechanistic understanding of p66β function is required for proper interpretation of the phenotypes.

**Regulation of Gli activity by Sufu and Mycbp**

We showed that Mycbp enhances Hh signaling in cultured cells and zebrafish. Mycbp was initially identified as a Myc-interacting protein and is known to enhance the activity of Myc [Taira et al. 1998]. Mycbp displays cell cycle-dependent shuttling between the nucleus and the cytoplasm [Furusawa et al. 2002]. In addition, overexpressed Mycbp can be detected in both the nucleus and cytoplasm. While Mycbp is present on the promoter of Hh-responsive genes, we cannot rule out its potential roles in cytoplasmic Hh signaling. In this regard, it is interesting to note that Mycbp could also be detected at the base of the primary cilium upon Hh stimulation.
during Hh target gene activation. For instance, while we
affected by Hh signaling. Consistent with this model, Hh
stimulation promotes dissociation of the Sufu/p66
interacts with Sufu and Gli in the absence of Hh signaling.
Gli protein function.
Regulation of Sufu activity at multiple levels ensures
this could contribute to the relief of Gli inhibition by Sufu.
Our coimmunoprecipitation studies suggest that Mycbp
and Gli proteins as a result of Hh signaling. Biochemical
binding between Mycbp and Gli. This would confer the
ability of Mycbp to stimulate Gli activity. How Mycbp is
inactive without Hh signaling and how Mycbp activity
is switched on upon Hh pathway activation remain un-
clear. We suspect that this could involve the recruitment of
other proteins or post-translational modifications of Mycbp
and Gli proteins as a result of Hh signaling. Biochemical
studies are required to address these critical issues.
We did not have the spatial or temporal resolution to
detect real-time changes of p66β and Mycbp on Hh
promoters. Consequently, we have no insight into the
dynamic behaviors of p66β and Mycbp on the chromatin
during Hh target gene activation. For instance, while we
show that Sufu/p66β are released from the Hh promoters
upon pathway activation, Sufu/p66β may subsequently
be recruited to Hh promoters during active transcription.
This could provide a means to efficiently turn off Hh
signaling. Gaining insight into these mechanistic issues
would require using new technologies to study the dynam-
ics of these proteins on Hh promoters during Hh pathway
activation. It is also important to note that the dynamic
interactions among p66β, Mycbp, and Gli proteins in
response to Hh signaling follow a quantitative and not an
all-or-none change. This may endow the system with the
ability to produce graded Hh responses.
It is possible that Sufu/p66β/Mycbp controls only a sub-
set of Hh targets or that multiple mechanisms are used to
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with the SAP18–mSin3 repressor complex (Cheng and
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p66β and Mycbp on the chromatin of Hh target genes
Our ChIP analysis revealed the presence of p66β and
Mycbp at the promoter region of Hh target genes. In addition, p66β
interactions with Gli depend on Sufu, while Sufu's presence on Hh promoters requires Gli
proteins. These results support our model in which Sufu
recruits p66β to inhibit Gli activity without Hh signaling.
We propose that Hh stimulation leads to dissociation of
the Sufu/p66β complex from Gli proteins and relieves Gli
inhibition, since Sufu/p66β dissociates from Gli proteins,
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affected by Hh signaling. Consistent with this model, Hh
pathway activation results in a concomitant reduction in
occupancy on Hh-responsive promoters for both Sufu and
p66β. In addition, we demonstrated that Sufu protein
levels in the nucleus decrease upon Hh activation, and
this could contribute to the relief of Gli inhibition by Sufu.
Regulation of Sufu activity at multiple levels ensures a
tight control of Gli protein function.

Our coimmunoprecipitation studies suggest that Mycbp
interacts with Sufu and Gli in the absence of Hh signaling.
This led to our model that Gli and/or Sufu recruits Mycbp
to Hh targets, but Mycbp remains inactive. When Hh
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with the SAP18–mSin3 repressor complex (Cheng and
Bishop 2002), although its relevance has not been validated
in vitro (Chen et al. 2009) or in vivo.

p66β and Mycbp in Hh signaling in diverse species
Our analysis of Sufu, p66β, and Mycbp in the mammalian
system suggests that these new Sufu regulators function in vertebrate Hh signaling. Whether they also play a role
in invertebrate Hh signaling is not known. Interestingly,
the Drosophila homolog of p66 was identified as a mod-
ifier of Wg signaling. Loss of p66 in fly wing and eye discs
does not have detectable phenotypes, a result attributed
to its redundancy with other histone deacetylase com-
plexes [Kon et al. 2005]. The biochemical function of Sufu
appears to be conserved in Hh signaling in different species.
Sufu sequesters Ci/Gli proteins and controls their protein
levels and repressor formation, and studies in Drosophila
also support a role of Su(fu) in suppressing nuclear ac-
itivity of Ci independent of its effects on nuclear import of
Ci [Wang et al. 2000b]. Nevertheless, Sufu could be
subjected to distinct modes of regulation in each organ-
ism. It is thus possible that p66β and Mycbp represent
vertebrate-specific regulators of Sufu and reflect pathway
divergence.

p66β and Mycbp as potential targets of manipulating
Hh pathway activity
The identification of p66β and Mycbp as regulators of
Sufu and Gli activity in vertebrate Hh signaling also
provides new targets of modulating Hh activity. In particular, if the molecular interfaces between p66β and
Mycbp and Sufu/Gli could be identified, this would allow
screening of molecules that can manipulate Hh signaling
without affecting other processes in which p66β and
Mycbp may participate. Many disease processes may
result from unregulated Gli activity that could even be
independent of upstream Hh components. In this regard,
new modulators of Gli protein function, such as p66β and
Mycbp, offer a unique opportunity to develop new ther-
apies for diseases due to aberrant Gli activity.
Materials and methods

Cell lines and constructs
MEFs stably expressing 3xFlag-Sufu, GFP-Sufu<sub>N100</sub>, GFP-Sufu<sub>N212</sub>, 3xFlag-p66β, 3xFlag-Mycbp, 3xFlag-Gli2, and 3xFlag-Gli3 were generated through retroviral transduction as described [Chen et al. 2009]. Clones with stable expression of Sufu were confirmed by Western blotting. HEK293T and NIH3T3 cells and transformed MEF lines were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), penicillin/streptomycin, and L-glutamine (Life Technologies). Wild-type, Sufu<sup>−/−</sup>, Ptch1<sup>−/−</sup>, Gli2<sup>−/−</sup>, Gli3<sup>−/−</sup>, and Kif7<sup>−/−</sup> MEFs have been previously described [Chen et al. 2009] and were maintained in medium containing 500 μg/mL G418, Gli2<sup>−/−</sup>, Gli3<sup>−/−</sup> MEFs [Zeng et al. 2010] were kindly provided by Aimin Liu. Kif7<sup>−/−</sup> deficient embryos [Cheung et al. 2009] as previously reported [Chen et al. 2009].

Standard molecular biology was used to construct mouse Flag-Sufu<sub>N100</sub>, Flag-Sufu<sub>N212</sub>, Flag-p66β, Myc-p66β, Flag-Mycbp, and Myc-Mycbp. Flag-Sufu, Myc-Sufu, Flag-Gli1, Flag-Gli2, and Flag-Gli3 have been reported [Chen et al. 2009].

Full-length mouse and zebrafish p66β and Mycbp cDNAs were C-terminally tagged with 3xFlag, 6xMyc, or GFP and cloned into pcDNA3 or pcS2<sup>−</sup> for transient overexpression in cultured cells or zebrafish embryos and into pBabe-puro for retroviral overexpression in cultured cells. Mouse and zebrafish p66β and Mycbp cDNAs were also C-terminally tagged with GFP and cloned into pcS2<sup>−</sup> for transient expression in zebrafish embryos.

Affinity purification and mass spectrometry
Ten 10-cm plates of MEFs stably expressing Sufu-3xFlag were cultured to confluence. Cells were starved in 0.5% FBS/DMEM for 12 h and then switched to 0.5% FBS/DMEM with mock- or Shh-conditioned medium for 24 h. Cells were lysed in 1.5 mL of cold lysis buffer [50 mM Tris-Cl at pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P40 substitute, complete protease inhibitor [Roche], PhosSTOP [Roche]]. The lysate was cleared by centrifugation at 12,000 rpm for 10 min at 4°C. The supernatant was removed and bound to 20 μL of anti-Flag M2 beads (Sigma) overnight. The beads were washed four times with lysis buffer containing 0.2% Nonidet P40 substitute followed by three times with lysis buffer without detergents. Proteins were eluted with 40 μL of 50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1 mM EDTA containing 500 μg/mL 3xFlag peptide (ELIM), and 0.05% RapiGest (Waters). One microliter of the eluate was analyzed by Western blotting with anti-Flag [1:2000, Sigma], anti-Gli1 [1:500, R&D Systems], and anti-Gli3 (R&D, 1:500) antibodies. Ten microliters of eluate was analyzed by Coomassie Blue and 2 μL was analyzed by silver staining.

Mass spectrometry was performed essentially as described [Jager et al. 2011]. Briefly, for gel-free mass spectrometry, 10 μL of the eluate from immunoprecipitation was reduced, carboxamidomethylated, and digested with trypsin. For gel-based analysis, 30 μL of eluate was separated by 7% SDS-PAGE and stained with Coomassie Blue. The band of interest was cut out. Each gel piece was diced into small pieces, which were reduced, carboxamidomethylated, and digested with trypsin. All samples were analyzed on a Thermo Scientific LTQ Orbitrap XL mass spectrometer equipped with a nanoACQUITY UPLC (Waters) chromatography system and a nanoelectrospray source. The data-dependent mass spectrometer continuously collected a survey scan in the Orbitrap mass analyzer at 40,000 resolution with an automatic gain control (AGC) target of 1 × 10<sup>6</sup> followed by collision-induced dissociation (CID) tandem mass spectrometry scans of the 10 most abundant ions in the survey scan in the ion trap with an AGC target of 5000, a signal threshold of 1000, a 2.0-Da isolation width, and a 30-msec activation time at 35% normalized collision energy. Raw mass spectrometric data were converted into peak lists using Bioworks 3.3.1 SP1. The spectra were searched using Prospector version 5.3 [http://prospector.ucsf.edu] against a mouse-restricted UniProt database. Protein Prospector results were filtered by applying a minimum protein score of 22.0, a minimum peptide score of 15.0, a maximum protein E-value of 0.01, and a maximum peptide E-value of 0.05.

Nuclear–cytoplasmic fractionation

Subcellular fractionation was performed as reported [Chen et al. 2009]. The purity of the cytoplasmic and nuclear fractions was assessed by cytoplasmic- and nuclear-specific markers, including anti-tubulin [1:5000, Sigma] and anti-Lamin A [1:3000, Abcam]. The following antibodies were used for Western blotting of cytoplasmic and nuclear fractions: rabbit anti-Sufu [1:3000, Santa Cruz Biotechnology], goat anti-Gli2 [1:500, R&D Systems], and goat anti-Gli3 [1:500, R&D Systems].

Coimmunoprecipitation

Different combinations of Myc-Mycbp, Myc-p66β, Flag-Sufu, Flag-Gli1, and Flag-Gli2 were transfected into HEK293T cells by Lipofectamine 2000 [Life Technologies]. Cells were collected at 48 h post-transfection and lysed in immunoprecipitation buffer (1% Triton X-100, 150 mM NaCl, 50 mM Tris-Cl at pH 7.5, 1 mM EDTA, protease inhibitor cocktail [Roche], PhosSTOP [Roche]). The lysates were cleared by centrifugation at 12,000 rpm for 10 min at 4°C. The supernatant was removed and bound to 20 μL of anti-Flag M2 beads [Sigma] or anti-Myc beads [Santa Cruz Biotechnology] overnight at 4°C with constant rotation. The beads were washed three times with immunoprecipitation buffer and eluted with SDS sample buffer. Immunoprecipitates were analyzed by Western blotting using rabbit anti-Flag [1:2000, Sigma] and rabbit anti-Myc [1:2000, Sigma].

For immunoprecipitation of endogenous Sufu, Gli2, and Gli3, a 10-cm plate of confluent wild-type MEFs or NIH3T3 fibroblasts were starved in 0.5% FBS/DMEM for 12 h and then treated with Shh-conditioned medium or 100 mM SAG [Sigma] in 0.5% FBS/DMEM for the indicated time. Cells were lysed in immunoprecipitation buffer. The lysate was precleared by incubating with protein A or protein G beads, and primary antibody was subsequently added and incubated overnight at 4°C. Protein A [for anti-Sufu antibody] or protein G [for anti-Gli antibody] beads were added to the lysate. After incubation overnight at 4°C, the beads were washed with immunoprecipitation buffer and eluted with SDS sample buffer. The antibodies used were rabbit anti-Sufu [1:3000, Santa Cruz Biotechnology], rabbit anti-Gli2 [1:500, R&D Systems], and goat anti-Gli3 [1:500, R&D Systems].

For immunoprecipitation of wild-type MEFs stably expressing Flag-tagged Sufu, Glil, Gli2, or Gli3 and Sufu<sup>−/−</sup> MEFs stably expressing Flag-tagged Gli2, Gli3, or Mycbp, cells were lysed in immunoprecipitation buffer. The lysate was centrifuged and then precleared by incubating with 30 μL of mouse IgG agarose beads [Sigma] in a 50% slurry for 1 h at 4°C. The lysate was subsequently incubated with 20 μL of Flag M2 agarose beads [Sigma] in a 50% slurry overnight at 4°C. The beads were washed rigorously in immunoprecipitation buffer and eluted with SDS sample buffer. Immunoprecipitates were analyzed by Western blotting using the following antibodies: rabbit anti-p66β [1:500, Millipore], rabbit anti-HDAC1 [1:500, Santa Cruz Biotechnology], rabbit anti-HDAC2 [1:1000, Santa Cruz Biotechnology], goat anti-Rbap46/48 [1:500, Santa Cruz Biotechnology], rabbit anti-
MBD2/3 (1:200, Santa Cruz Biotechnology), and goat anti-MTA1 (1:500, Santa Cruz Biotechnology).

**shRNA-mediated knockdown**

shRNAs were designed using pSilOligomaker (Reynolds et al., 2004), and oligonucleotides encoding shRNAs were cloned into the pLentiLox3.7 vector. Lentiviruses were produced as described (Chen et al. 2009). MEFs at 50% confluence were transduced with lentiviruses supplemented with 8 μg/mL polybrene. Upon reaching confluence, cells were starved in 0.5% FBS/DMEM for 12 h and switched to 0.5% FBS/DMEM/Shh-conditioned medium for another 24 h. The following 19-mer sequences were used for shRNA-mediated knockdown: mouse Mycbp [NM_019660], 5'-GCCCTTTGA-3' and 5'-GCCAGCTACCTTGA TTGTA-3'; mouse p66 [NM_139304], 5'-GCAAGTTGCTTC AAGGGTTA-3' and 5'-GCCAGGACCTTACAAAGCAA-3'; mouse Prmt5 [NM_01009818], 5'-GCAACGGAGTCCAGATATA-3' and reverse-transcribed to the first strand using FastStart SYBR Green master mix (Roche). Relative mRNA was extracted from NIH3T3 cells or MEFs using Trizol (Life Technologies) and reverse-transcribed to the first strand using Maxima Reverse Transcriptase (Thermo Scientific). Relative cDNA was quantified using TOPFlash: pRL-TK. Luciferase assays were performed as described (Chen et al. 2009).

**ChIP**

ChIP was performed using the EZ-ChIP kit (Upstate Biotechnology) 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 base pairs (bp) in size using a Bioruptor Sonicator (Diagenode) for five cycles of 30 sec on/30 sec off. The extracts were precleared in BSA-blocked protein G and incubated with antibodies or IgG control overnight. After washes, the DNA was eluted and reverse-cross-linked overnight at 65°C. 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 Biotechnology]. The primers for qPCR were mouse Ptc1 promoter [forward, 5'-TTATAAAGGCA GTGCCCAACAC-3'; reverse, 5'-ACGCATGTTTGCAAGATA GA-3'], mouse Gli1 promoter [forward, 5'-AGGAATGCTGTC ACGCCTA-3'; reverse, 5'-GGCAAAAGAGCTGGGACA-3'], mouse Hhip [Hip1] promoter [forward, 5'-AAAGCTTCGACACC TAAAT-3'; reverse, 5'-TTAAAGGGCCACTTGAAA-3'], and mouse β-actin promoter [forward, 5'-AGAAGGACTCTATG TGCGTGA-3'; reverse, 5'-AAGTGATTTGCCATCTTTC-3'].

**Zebrafish mRNA injection**

For mRNA synthesis, plasmids were linearized by NotI, and mRNA was synthesized using the SP6 mMESSAGE mMachine kit according to the manufacturer's manual [Ambion]. Three-hundred picograms of p66β-GFP, mycbp-6xMyc, and mycbp-GFP mRNA (in pCS2+ vector) was injected into one-cell zebrafish embryos [Langenbacher et al. 2012]. Embryos were collected at 24 h post-fertilization (hpf), 36 hpf, and 3.5 d post-fertilization. To avoid variations in expression levels, in some experiments, we also selected injected embryos expressing strong GFP at 8 and 20 hpf for subsequent analysis. Embryos were fixed at 32 hpf for whole-mount in situ hybridization using patched1 [ptch1] riboprobe and standard procedures. ptch1 riboprobe was synthesized using ptch1 cDNA cloned into pcS2. Mouse and zebrafish p66β and Mycbp yielded similar results, although zebrafish homologs produced more robust phenotypes. p66β or mycbp tagged with GFP at the 3' end produced results similar to those of untagged p66β or mycbp. Results in Figure 4 were derived from embryos injected with zebrafish p66β-GFP and mycbp-GFP mRNA.

**MO-mediated knockdown in zebrafish**

Zebrafish p66β antisense oligos (5'-CCTCCTCAGACATCCGC TCCATCCT-3' and mycbp antisense oligos 5'-GGATGGCG CATTACAGA-3') were synthesized by GeneTools, LLC. One nanogram of p66β MO was injected into one-cell stage zebrafish embryos, which were fixed at 36 hpf for in situ hybridization analysis. Similarly, 4 ng of mycbp MO was injected into one-cell stage embryos harvested at 36 hpf for in situ hybridization.

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lin et al.

construct: 8xGliBS-luc:pRL-TK using Lipofectamine 2000 (Life Technologies). For assessing Wnt pathway activation, NIH3T3 cells or MEFs were transfected with a 4:5:1 ratio of pcDNA3.1/Wnt3a expression construct: TOPFlash:pRL-TK. Luciferase assays were performed as described (Chen et al. 2009).

**Immunofluorescence**

Cells were fixed in 4% paraformaldehyde for 15 min at room temperature. Standard procedures for immunostaining were followed. The primary antibodies used were mouse anti-acetylated actin [1:2000, Sigma], rabbit anti-Flag [1:2000, Sigma], and rabbit anti-Myc [1:1000, Sigma]. Secondary antibodies and conjugates were used donkey anti-mouse Alexa Fluor 594 [1:2000, Life Technologies], donkey anti-rabbit Alexa Fluor 488 [1:2000, Life Technologies], and DAPI [1:10,000, Sigma].

**qPCR analysis**

RNA was extracted from NIH3T3 cells or MEFs using Trizol (Life Technologies) and reverse-transcribed to the first strand cDNA using Maxima Reverse Transcriptase (Thermo Scientific). Real-time qPCR was carried out in an ABI Prism 7900HT system using FastStart SYBR Green master mix (Roche). Relative gene expression data were analyzed using the 2^-ΔΔCT method (Schmittgen and Livak 2008). GAPDH was used for normalization of gene expression. The primers used were mouse Ptc1 (forward, 5'-TGGCTTGCCGTGTCACCTTGA-3'; reverse, 5'-CCAGCCAGCACCTACACCA-3'), mouse Gli1 (forward, 5'-CCCCATAGGCTGGGCTTCAAAAC-3'; reverse, 5'-GGAGGACTCGCTGGTACTGTGTA-3').
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