A Mechanism for Vertebrate Hedgehog Signaling: Recruitment to Cilia and Dissociation of SuFu-Gli Protein Complexes

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In vertebrates, Hedgehog (Hh) signaling initiated in primary cilia activates the membrane protein Smoothened (Smo) and leads to activation of Gli proteins, the transcriptional effectors of the pathway. In the absence of signaling, Gli proteins are inhibited by the cytoplasmic protein Suppressor of Fused (SuFu). It is unclear how Hh activates Gli and whether it directly regulates SuFu. We find that Hh stimulation quickly recruits endogenous SuFu–Gli complexes to cilia, suggesting a model in which Smo activates Gli by relieving inhibition by SuFu. In support of this model, we find that Hh causes rapid dissociation of the SuFu–Gli complex, thus allowing Gli to enter the nucleus and activate transcription. Activation of protein kinase A (PKA), an inhibitor of Hh signaling, blocks ciliary localization of SuFu–Gli complexes, which in turn prevents their dissociation by signaling. Our results support a simple mechanism in which Hh signals at vertebrate cilia cause dissociation of inactive SuFu–Gli complexes, a process inhibited by PKA.

Introduction

The Hedgehog (Hh) cell–cell signaling pathway is conserved in animals and has critical roles in embryonic development, in the maintenance of adult stem cells, and in cancer (Lum and Beachy, 2004; Kalderon, 2005; Huangfu and Anderson, 2006; Rohatgi and Scott, 2007). In the resting state of Hh signaling, the transcriptional output of the pathway is kept off by the membrane protein Patched (Ptc), which inhibits the seven-spanner Smoothened (Smo; Alcedo et al., 1996). The Hh pathway is activated when the secreted protein Hh binds and inactivates Ptc (Marigo et al., 1996; Stone et al., 1996), thus relieving the inhibition exerted on Smo, which becomes active. Active Smo signals to the cytoplasm, leading to activation of the zinc finger transcription factors that control the output of the Hh pathway, Cubitus interruptus (Ci) in *Drosophila melanogaster* (Aza-Blanc et al., 1997; Ohlmeyer and Kalderon, 1998) and the Gli proteins (Gli1, 2, and 3) in vertebrates.

A unique feature of vertebrate Hh pathway is that primary cilia are essential for signal transduction (Huangfu and Anderson, 2005), and the initial membrane events occur at cilia. Ptc is located at the base of the primary cilium (Rohatgi et al., 2007), and binding of Hh to Ptc leads to activation and recruitment of Smo to the cilium (Corbit et al., 2005; Rohatgi et al., 2007). Through an unknown mechanism, active Smo at the cilium relays Hh signals to the cytoplasm, resulting in the activation of Gli2 and Gli3 (Ohlmeyer and Kalderon, 1998; Wang et al., 2000; Lipinski et al., 2006), which control transcription of Hh target genes (Alexandre et al., 1996; Ruiz i Altaba, 1998; Dai et al., 1999). Since the discovery that Ptc and Smo function at the vertebrate primary cilium, an important question has been to understand how signaling through these upstream components of the Hh pathway couples to activation of the downstream Gli proteins.

An early study showed that Gli proteins localize to cilia in vertebrate limb bud cells (Haycraft et al., 2005); however, the relationship between ciliary localization and the state of Hh signaling was not investigated. Recently, Gli2 and Gli3 were shown to be recruited to the tip of primary cilia upon Hh stimulation

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Abbreviations used in this paper: CHX, cycloheximide; Ci, cubitus interruptus; Cyc, cyclopamine; FSK, forskolin; Gli1HA, HA-tagged Gli1; Gli3-FL, full-length Gli3; GSK3, glycogen synthase kinase 3; Hh, Hedgehog; MBP, maltose-binding protein; MEF, mouse embryonic fibroblast; MT, microtubule; Noc, nocodazole; Ptc, Patched; Q-PCR, quantitative PCR; Shh, Sonic Hh; Smo, Smoothened; SuFu, Suppressor of Fused.

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(Chen et al., 2009; Kim et al., 2009; Wen et al., 2010), which is consistent with the idea that activation of Gli2 and Gli3 by Hh signaling occurs at cilia; however, the mechanism by which Gli proteins are activated at cilia has not been clarified.

In the cytoplasm of unstimulated cells, two major negative regulators ensure that the vertebrate Hh pathway is kept off. The first negative regulator is the Gli-binding protein Suppressor of Fused (SuFu), which in vertebrates is essential for repressing Hh signaling; in cells lacking SuFu, the Hh pathway is maximally activated in a ligand-independent manner (Cooper et al., 2005; Svärd et al., 2006). SuFu is thought to inhibit Gli proteins by preventing their nuclear translocation (Ding et al., 1999; Kogerman et al., 1999; Méthot and Basler, 2000). Interestingly, constitutive activation of the Hh pathway in the absence of SuFu is independent of cilia (Jia et al., 2009), suggesting that Hh signaling at cilia may activate Gli proteins by inhibiting SuFu.

The second major negative regulator of Hh signaling is PKA. In *Drosophila*, PKA phosphorylates Ci, and loss of PKA leads to Hh pathway activation (Jiang and Struhl, 1995; Lepage et al., 1995; Li et al., 1995; Price and Kalderon, 1999), whereas overexpression of PKA inhibits Hh signaling (Li et al., 1995). The inhibitory effect of PKA is conserved in vertebrate Hh signaling (Concordet et al., 1996; Epstein et al., 1996) and, interestingly, depends on SuFu (Svärd et al., 2006; Chen et al., 2009), suggesting that PKA might inhibit Gli proteins by modulating their interaction with SuFu.

Although SuFu is essential for inhibiting Gli in unstimulated cells, it is unclear whether Hh signaling regulates SuFu. In one model, SuFu is a simple buffer for Gli and is not regulated by Hh signaling. This model is consistent with a recent study (Chen et al., 2009), which found that Hh stimulation does not affect the interaction between overexpressed Gli2 and Gli3 and SuFu; however, the relevance of this result for normal Hh signaling is unclear given the nonphysiological levels of Gli and SuFu proteins produced by transient transfection. In another model, Hh signaling at cilia activates Gli proteins by relieving SuFu inhibition, resulting in Gli nuclear translocation and transcriptional activation. This simple model is consistent with at least two findings: (1) the Hh pathway is constitutively active in $SuFu^{-/-}$ cells independent of cilia (Chen et al., 2009; Jia et al., 2009), suggesting that active Smo at cilia might signal by inhibiting SuFu, and (2) activation of PKA by forskolin (FSK) inhibits its signaling by active Smo in cells that have SuFu (Wu et al., 2004) but cannot block constitutive signaling caused by loss of SuFu (Svärd et al., 2006; Chen et al., 2009), suggesting that Smo and PKA might exert their opposing effects on Hh signaling through SuFu.

To begin deciphering how active Smo at the cilium activates Gli proteins, we examined the behavior of endogenous SuFu, Gli2, and full-length Gli3 (Gli3-FL) in Hh-responsive mammalian cultured cells. Focusing on endogenous proteins avoided problems associated with misregulation of overexpressed proteins. Furthermore, we analyzed biochemically the effect of Hh signaling on endogenous SuFu–Gli protein complexes after brief Hh pathway stimulation to avoid any confounding secondary effects caused by prolonged pathway stimulation. Our results complement and extend the findings of a recent study (Humke et al., 2010) that described how Hh signaling leads to the dissociation of SuFu from Gli. Specifically, our study demonstrates that Hh stimulation through active Smo leads to the recruitment of endogenous SuFu–Gli complexes to cilia and causes the rapid dissociation of a defined SuFu–Gli complex. Activation of PKA blocks localization of SuFu–Gli complexes to cilia and inhibits their dissociation by Smo, providing an explanation for how PKA inhibits Hh signaling: by uncoupling Smo activation from dissociation of SuFu–Gli complexes. We propose that vertebrate Hh signals are transduced by active Smo at the primary cilium by dissociating inhibitory SuFu from Gli and that a protein complex that likely contains only SuFu and Gli forms the core of vertebrate Hh signal transduction downstream of Smo.

Results

Hh stimulation quickly recruits endogenous SuFu and Gli proteins to the cilium

Tagged SuFu and Gli proteins localize to primary cilia in vertebrate cells (Haycraft et al., 2005). To study the subcellular dynamics of SuFu and Gli during Hh signaling and to avoid expressing proteins at nonphysiological levels, we raised polyclonal antibodies that specifically detect endogenous mouse SuFu, Gli2, and Gli3 in Hh-responsive cells (Fig. S1). We first used these antibodies to examine how Sonic Hh (Shh) stimulation affects subcellular localization of endogenous SuFu, Gli2, and Gli3-FL (Fig. 1 A). Without Shh stimulation, low levels of SuFu, Gli2, and Gli3-FL were detected at cilia in NIH-3T3 cells and in mouse embryonic fibroblast (MEFs); in contrast, Smo was absent from cilia in the absence of Shh stimulation (Fig. 1, A and B; and see Table S1 for SuFu, Gli, and Smo behavior in all cell lines used in this study). Hh stimulation led to the dramatic increase in the localization of SuFu, Gli2, and Gli3-FL to cilia (Fig. 1 A), which is similar to that recently reported for endogenous or overexpressed Gli2 and Gli3 (Chen et al., 2009; Kim et al., 2009; Wen et al., 2010), and paralleling the recruitment of Smo to cilia (Rohatgi et al., 2007). A previous study (Chen et al., 2009) failed to detect a signal-dependent recruitment of SuFu to cilia; one reason for this discrepancy might be that our antibodies are more sensitive than the commercial antibodies used for SuFu detection. Our other findings (that SuFu and Gli form a complex and that SuFu localization to cilia is strictly dependent on Gli; see Figs. 4 and 5) are consistent with the Hh-stimulated recruitment of SuFu to cilia that we observed.

Recruitment of SuFu, Gli2, Gli3-FL, and Smo was very rapid: strong ciliary localization of all these proteins was seen in as little as 30 min after addition of Shh to cells. The number of cilia positive for SuFu, Gli2, Gli3-FL, and Smo continued to increase with time (Fig. 1 B). We conclude that, although low amounts of SuFu and Gli proteins are present at cilia in unstimulated cells, the ciliary levels of these proteins quickly rise upon Hh stimulation.

SuFu, Gli2, and Gli3-FL show very similar "comet tail" patterns at the cilium, with the highest accumulation at the distal tip (Fig. 1 C). This pattern is different from that of Smo,

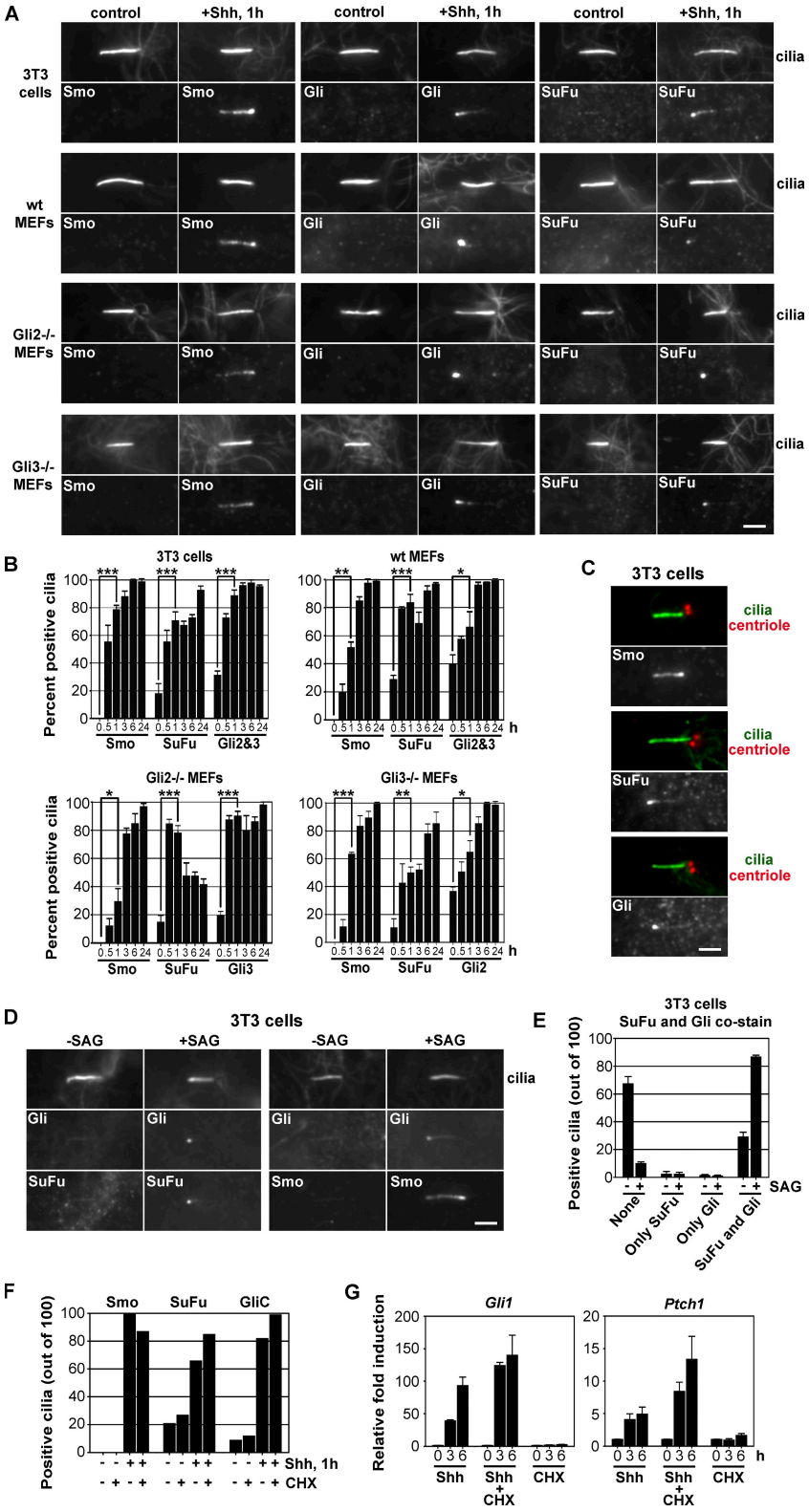

Figure 1. **Endogenous SuFu is rapidly recruited to primary cilia by Hh signaling, paralleling recruitment of endogenous Smo, Gli2, and Gli3-FL.** (A) Fluorescence micrographs of cilia from untreated cells or cells treated with Shh. Cilia were detected by staining against acetylated tubulin. Because the anti-GliC antibody detects both Gli2 and Gli3-FL, Gli2$^{-/-}$ and Gli3$^{-/-}$ MEFs are shown to demonstrate ciliary recruitment of Gli2 and Gli3-FL separately. The tip of the cilium points to the left. (B) Cells were treated with Shh for varying amounts of time, and ciliary recruitment of SuFu, Smo, Gli2, and Gli3-FL was determined. Asterisks indicate p-values for ciliary recruitment at 1 h compared with t = 0 (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). $P < 0.05$ for all later time points. (C) In NIH-3T3 cells stimulated with Shh for 1 h, SuFu and Gli proteins localize at the tip, whereas Smo localizes along the length of cilia. Cilia were stained as in A, and centrioles were stained with anti–γ-tubulin. (D) Endogenous SuFu and Gli proteins colocalize at the tips of primary cilia in SAG-treated NIH-3T3 cells. (left) Cilia costained for endogenous SuFu (rabbit antibody) and Gli (goat antibody). (right) Cilia costained for Smo (rabbit antibody) and Gli (goat antibody). (E) Cilia counts for the experiment in D (left). Endogenous SuFu and Gli colocalize both in the resting and stimulated states of the Hh pathway. (F) Recruitment of SuFu, Smo, and Gli to cilia in response to Hh stimulation does not require new protein synthesis. Ciliary localization was determined in NIH-3T3 cells treated or not with Shh in the presence or absence of CHX. (G) Inhibition of protein synthesis does not block the transcriptional output of the Hh pathway. Transcription of the direct transcriptional targets *Gli1* and *Ptch1* was assayed by Q-PCR after 3 and 6 h of stimulation with Shh in the presence or absence of CHX. wt, wild type. Error bars indicate mean ± SD for three independent counts. Bars, 2 µm.

which localizes along the entire length of the cilium, often at a higher level toward its base (Fig. 1, A and C). Identical results were obtained when the Hh pathway was activated by the oxysterols 20- and 25-hydroxycholesterol (Fig. S2 A; Corcoran and Scott, 2006; Dwyer et al., 2007) and by the synthetic Smo activator SAG (Fig. 1 D; Chen et al., 2002; Frank-Kamenetsky et al., 2002).

The similar localization pattern of SuFu and Gli at the tips of cilia and the fact that SuFu binds Gli proteins (Pearse et al., 1999) suggests that SuFu and Gli likely localize to the cilium as a complex. Costaining for endogenous SuFu and Gli (using a goat anti-Gli antibody; Fig. S1 E) shows identical patterns at cilia (Fig. 1 D); furthermore, SuFu and Gli always appear

together in cilia, both in the unstimulated and stimulated states of Hh signaling (Fig. 1 E). Thus, we propose that Hh stimulation quickly recruits SuFu–Gli complexes to cilia, suggesting that the molecular species to which the signal from active Smo is relayed might be the SuFu–Gli complex.

Recruitment of endogenous SuFu and Gli proteins to the cilium does not require new protein synthesis

Although the rapid recruitment of SuFu, Gli, and Smo suggests that it represents an immediate response to Hh activation, results from *Drosophila* cultured cells showed that protein synthesis is required for certain aspects of Hh signal transduction (Lum et al., 2003). In contrast to *Drosophila* cells, we find that in Shh-stimulated NIH-3T3 cells, inhibiting protein synthesis does not block the recruitment of endogenous SuFu, Gli, and Smo to cilia (Fig. 1 F; and Fig. S2, B and C) or the transcriptional activation of Hh target genes (Fig. 1 G). Also in contrast to *Drosophila* cells, we did not observe any change in the electrophoretic mobility of SuFu or SuFu levels upon stimulation of the Hh pathway in NIH-3T3 cells or MEFs (Fig. S4, A and B). Recruitment of SuFu and Gli protein to cilia is thus an immediate response to Hh stimulation.

Uncoupling ciliary recruitment of SuFu and Gli from the transcriptional response to Hh signaling: the role of dynamic microtubules (MTs)

Recruitment of SuFu, Gli, and Smo to cilia upon Shh stimulation is not affected when MTs are depolymerized with nocodazole (Noc; Fig. S2, D and E), suggesting that these proteins do not need dynamic MTs to arrive at the ciliary base. Noc does not disrupt the stable MTs of primary cilia (Fig. S2 F), suggesting that in the presence of Noc, motors such as Kif3a (Kovacs et al., 2008) and Kif7 (Cheung et al., 2009; Endoh-Yamagami et al., 2009; Liem et al., 2009), which were implicated in Hh signaling, can still move along ciliary MTs, explaining the proper SuFu, Gli, and Smo localization to cilia. Interestingly, Noc inhibits Hh transcriptional responses in a dose-dependent manner (Fig. S2 G). Thus, dynamic MTs are not required for recruitment of SuFu, Gli, and Smo to cilia but are required for the transcriptional output of the pathway. We speculate that dynamic MTs are required downstream of ciliary events, such as the transport of Gli from cilia to the nucleus (Kim et al., 2009; Humke et al., 2010).

Active Smo is required for the recruitment and continued maintenance of SuFu and Gli to cilia

Low levels of SuFu and Gli localize to cilia even in unstimulated cells and do not require Smo, as seen in $Smo^{-/-}$ MEFs (Fig. S3 A). Shh stimulation of $Smo^{-/-}$ MEFs does not increase ciliary SuFu and Gli, indicating that signal-dependent recruitment of SuFu and Gli requires Smo.

Active Smo translocates to cilia during normal Hh signaling, but inactive Smo can be pharmacologically forced to localize to cilia with the Smo inhibitor cyclopamine (Cyc; Rohatgi et al., 2009; Wang et al., 2009; Wilson et al., 2009). Thus, Smo might recruit SuFu and Gli to cilia irrespective of its activation state; alternatively, only active Smo recruits SuFu and Gli. To distinguish between these two alternatives, we compared SuFu, Gli, and Smo localization in cells treated with SAG (Chen et al., 2002; Frank-Kamenetsky et al., 2002) or Cyc (Taipale et al., 2000). Although both SAG and Cyc recruited Smo to cilia, SuFu and Gli were recruited only by SAG but not by Cyc (Fig. 2, A–C), demonstrating that only active Smo recruits SuFu and Gli to cilia.

We next asked whether maintaining high levels of SuFu and Gli in cilia is continuously dependent on active Smo. We first activated Hh signaling by addition of Shh followed by Smo inhibition with Cyc; in this manner, Smo is inactivated without changing its ciliary localization. When Smo, SuFu, and Gli were recruited to cilia by Shh stimulation, addition of Cyc caused the levels of SuFu and Gli at the cilium to drop, whereas levels of Smo continued to rise (Fig. 2 D). Similar kinetics for the exit of SuFu and Gli from cilia were seen when cells were first stimulated with Shh followed by Smo inhibition with the small molecule inhibitor, SANT-1 (Fig. 2 E). Smo inhibited by SANT-1 exited cilia more rapidly than SuFu and Gli proteins. Collectively, these experiments demonstrate that active Smo at cilia is required for maintaining high levels of SuFu and Gli at cilia during Hh signaling.

Activation of PKA blocks ciliary trafficking of endogenous SuFu and Gli

PKA is a negative regulator of the Hh pathway, and FSK, which activates PKA, is a potent inhibitor of Hh signaling. Recently, FSK was shown to recruit Smo to the cilium without activation of Hh signaling (Wilson et al., 2009). Interestingly, FSK treatment abolishes the ciliary localization of SuFu and Gli in both unstimulated and Shh-stimulated cells (Fig. 3, A and B), correlating with a complete inhibition of the transcriptional response to Hh stimulation (Fig. 3 C). We next asked whether the effect of FSK on SuFu and Gli localization to cilia depends on Smo. SuFu and Gli localize to the tips of cilia in $Smo^{-/-}$ MEFs (Fig. S3 A), and FSK causes a strong decrease in ciliary SuFu and Gli (Fig. 3 D), demonstrating that FSK prevents SuFu–Gli ciliary localization independently of Smo.

One possible explanation for the dramatic inhibition of SuFu–Gli localization to cilia by FSK is an increased degradation of Gli proteins; indeed, FSK promotes partial proteolysis of overexpressed Gli2 and Gli3-FL (Pan et al., 2006; Wang and Li, 2006). In cells treated with FSK, endogenous SuFu levels do not change, and Gli3-FL levels decrease only modestly (much less than the decrease caused by Shh stimulation; Fig. 3 E), demonstrating that absence of SuFu–Gli from cilia in the presence of FSK is not caused by degradation of SuFu or Gli proteins. Another explanation is that FSK blocks ciliary localization of the SuFu–Gli complex by promoting its dissociation. We excluded this possibility using NIH-3T3 cells stably expressing a direct fusion between Gli1 and SuFu in which FSK completely abolishes ciliary localization of the fusion (Fig. 3 F) without significantly affecting its expression level (Fig. S3 B). This effect of FSK is mediated by PKA, as it is reversed by the small molecule inhibitor of PKA, H-89

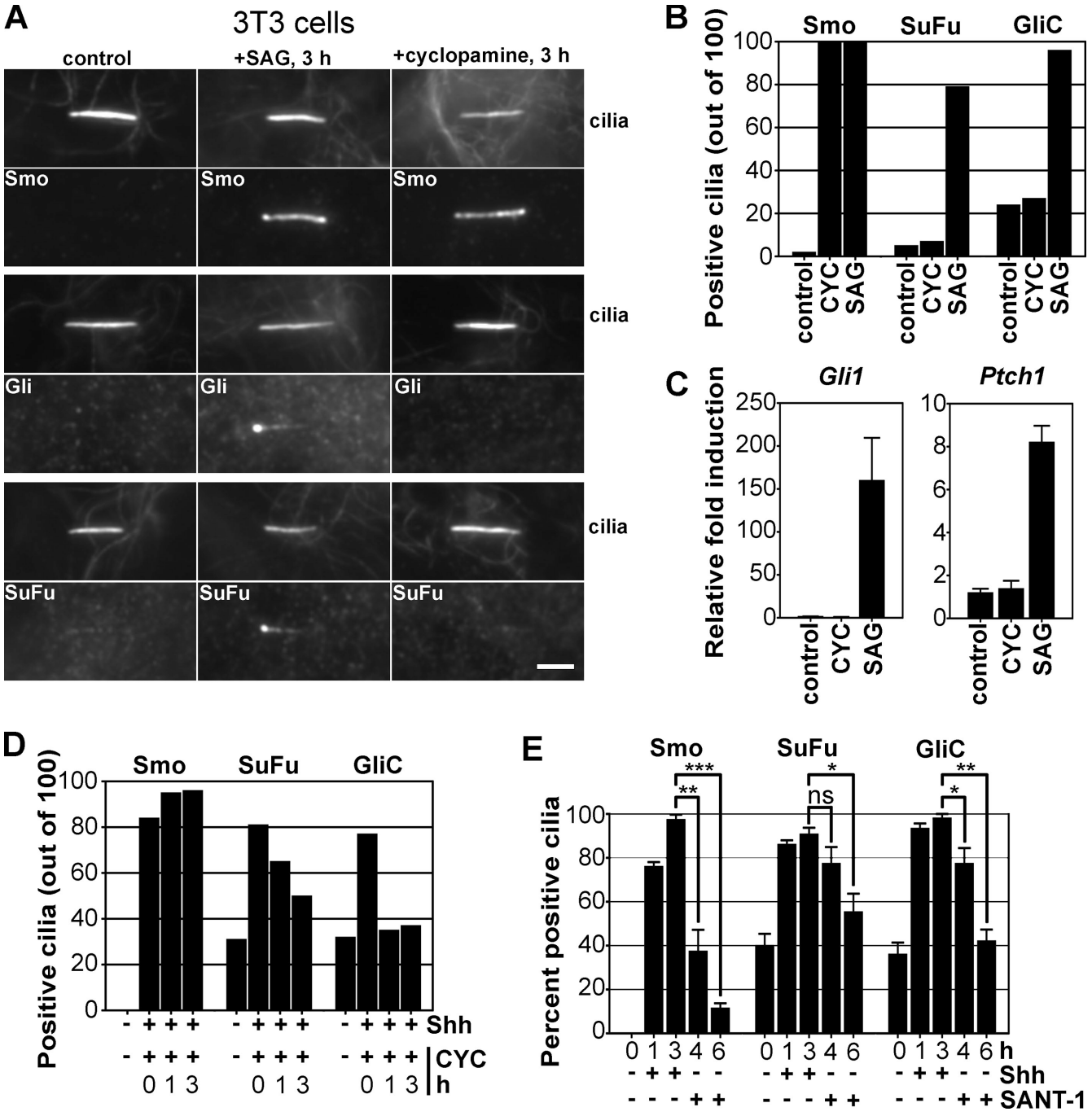

Figure 2. **Hh-dependent recruitment of SuFu and Gli proteins to cilia requires active Smo.** (A) NIH-3T3 cells were treated with the Smo agonist SAG or with the antagonist Cyc. SuFu and Gli are recruited to cilia by SAG but not by Cyc, although both SAG and Cyc recruit Smo to cilia. The tips of cilia point to the left. Bar, 2 µm. (B) Cilia counts for the experiment in A. (C) Q-PCR assay of Hh pathway target genes for the experiment in A. (D) Maintaining increased levels of SuFu and Gli at cilia is continuously dependent on active Smo. Cyc was added in the presence of Shh to NIH-3T3 cells prestimulated with Shh for 3 h. Ciliary localization was determined before and after 3 h of Shh stimulation and 1 and 3 h after Cyc addition. (E) NIH-3T3 cells were stimulated with Shh for 3 h followed by incubation with the Smo antagonist SANT-1 for 3 h. Ciliary localization of SuFu, Gli, and Smo was measured at the indicated times. $P < 0.002$ for the recruitment of Smo, SuFu, and Gli by Shh stimulation. P-values for exit from the cilium were calculated relative to ciliary localization after 3 h of Hh stimulation. Asterisks indicate the p-values for ciliary exit (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). Error bars indicate mean ± SD.

(Fig. S3 C). Furthermore, in FSK-treated cells, binding between endogenous SuFu and Gli3-FL is unaffected (see Fig. 5 I). We conclude that activation of PKA by FSK blocks ciliary trafficking of the SuFu–Gli complex, providing a pharmacological means for uncoupling recruitment of Smo to cilia from that of the SuFu–Gli complex.

Gli proteins are required to recruit SuFu to cilia, but Gli proteins can localize to cilia in the absence of SuFu

Because SuFu and Gli interact, we asked whether they require each other for ciliary localization by examining localization of Gli and SuFu in MEFs lacking SuFu and Gli proteins, respectively.

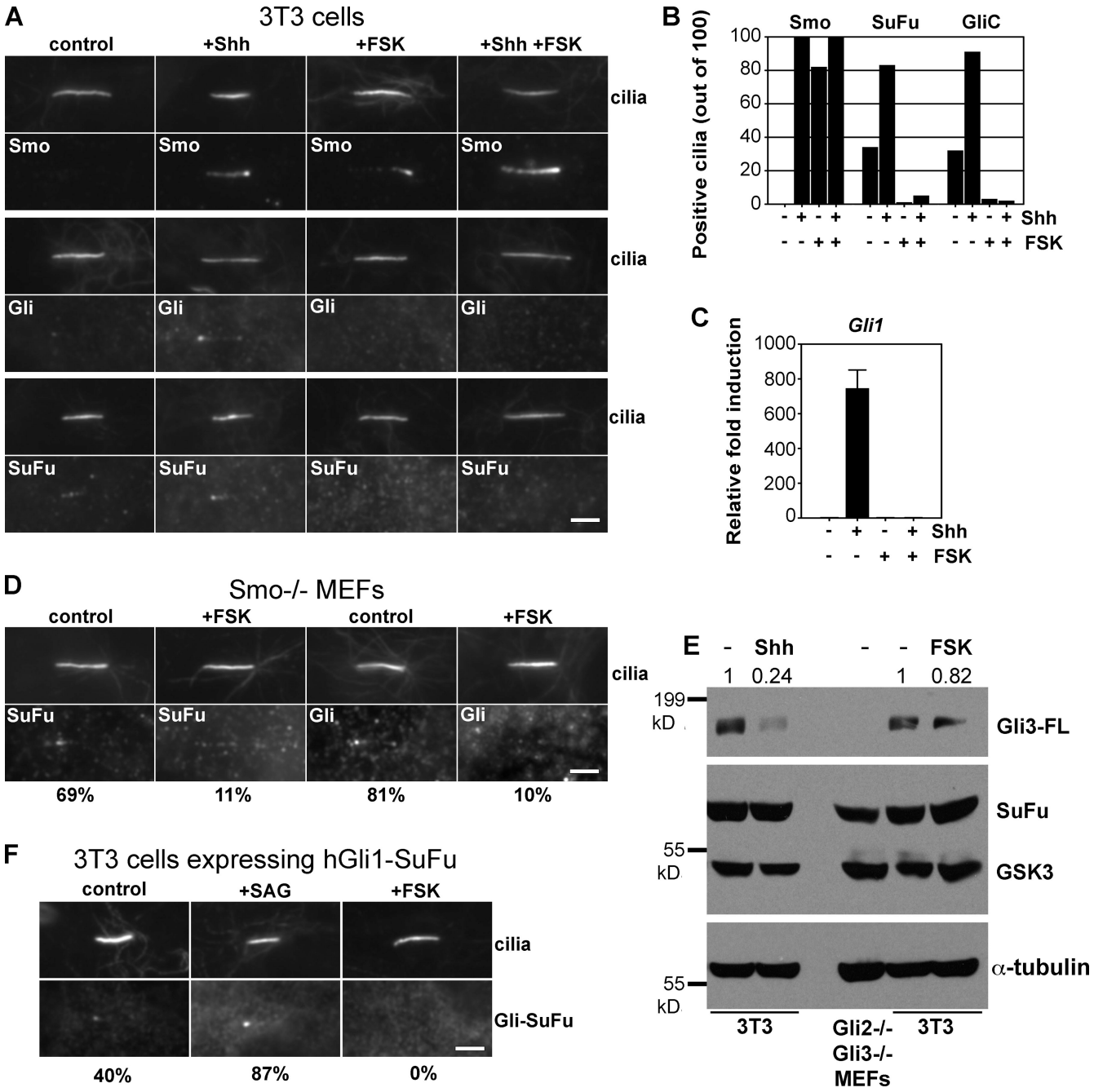

Figure 3. **Localization of endogenous SuFu and Gli to cilia is antagonized by PKA.** (A) Activation of PKA by FSK blocks localization of endogenous SuFu and Gli proteins to cilia. NIH-3T3 cells were treated with or without Shh and FSK. Shh, FSK, or Shh and FSK recruit Smo to the cilium; in contrast, endogenous SuFu and Gli are removed from cilia by FSK, both in the presence and absence of Shh stimulation. (B) Cilia counts for the experiment in A. (C) Q-PCR analysis of the experiment in A. Inhibition of SuFu and Gli ciliary localization by FSK correlates with complete inhibition of the transcriptional output of the Hh pathway. Error bar indicates mean ± SD. (D) FSK inhibits localization of SuFu and Gli to primary cilia in Smo$^{-/-}$ MEFs. Percentages shown indicate corresponding ciliary counts. (E) NIH-3T3 cells were incubated with or without Shh (left) or with or without FSK (right) followed by immunoblotting for SuFu, Gli3-FL, GSK3, and α-tubulin. (top) Numbers shown indicate the levels of Gli3-FL in each lane relative to α-tubulin. FSK treatment causes only a slight reduction in Gli3-FL, which is much smaller than the decrease caused by Shh. (F) NIH-3T3 cells stably expressing a Gli1–SuFu fusion were incubated with control media, SAG, or FSK. The Gli1–SuFu fusion localizes to cilia in unstimulated cells, and its localization is increased by SAG. FSK treatment completely blocks ciliary localization of the Gli1–SuFu fusion. Percentages shown indicate ciliary localization of the fusion. Bars, 2 μm.

Gli proteins are necessary for SuFu localization to cilia; in Gli2$^{-/-}$ Gli3$^{-/-}$ MEFs (Lipinski et al., 2006), SuFu is completely absent from cilia with or without Shh stimulation (Fig. 4, A and B), although SuFu levels are normal (Fig. S4 A); this excludes SuFu degradation as causing its absence from cilia in cells without Gli2 and 3. Importantly, Smo recruitment to cilia was normal in Gli2$^{-/-}$ Gli3$^{-/-}$ MEFs (Fig. 4, A and B), showing that ciliary transport and upstream Hh signaling were intact in these cells and that localization of Smo to cilia does not depend on SuFu and Gli proteins. Either Gli2 or Gli3 is sufficient to localize SuFu to cilia, as seen in Gli2$^{-/-}$ and Gli3$^{-/-}$ MEFs (Fig. 1, A and B). Collectively, these findings argue

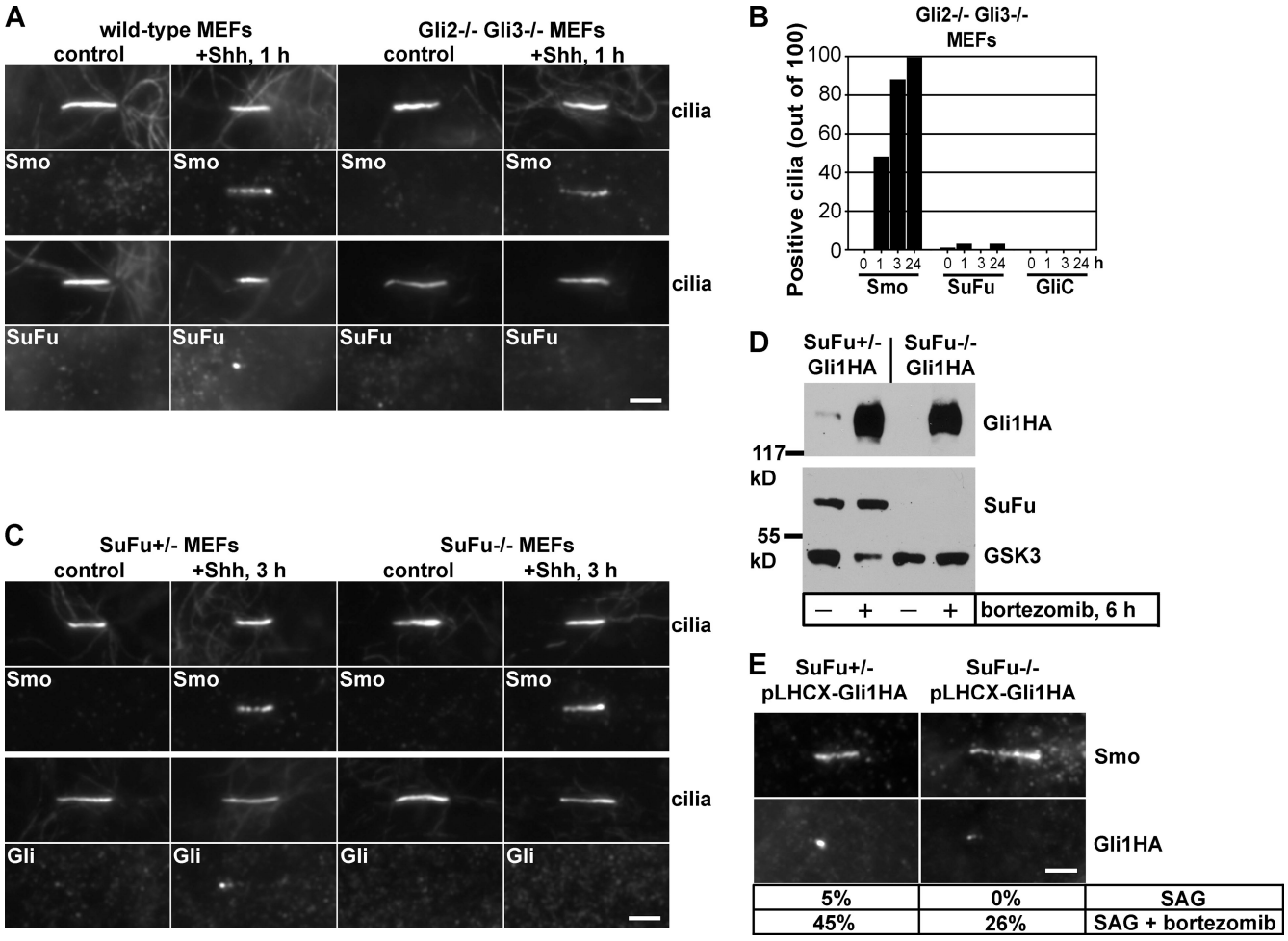

Figure 4. **Gli proteins are required to localize SuFu to cilia, but Gli proteins can localize to cilia in the absence of SuFu.** (A) Wild-type and Gli2$^{-/-}$ Gli3$^{-/-}$ MEFs were incubated with or without Shh. SuFu does not localize to cilia with or without Shh stimulation in Gli2$^{-/-}$ Gli3$^{-/-}$ MEFs, whereas Smo recruitment is normal. (B) Cilia counts for a time course of ciliary recruitment of Smo, SuFu, and Gli in Gli2$^{-/-}$ Gli3$^{-/-}$ MEFs stimulated with Shh. (C) SuFu$^{+/-}$ and SuFu$^{-/-}$ MEFs were stimulated or not with Shh. Endogenous Gli proteins do not localize to cilia with or without Shh stimulation in the absence of SuFu. Recruitment of Smo is normal. (D) Immunoblot of SuFu$^{-/-}$ and SuFu$^{+/-}$ MEFs stably expressing Gli1HA and treated with the proteasome inhibitor bortezomib. Proteasome inhibition allows SuFu$^{-/-}$ cells to accumulate Gli1HA to levels similar to those in the control SuFu$^{+/-}$ cells. (E) Stably expressed Gli1HA localizes to ciliary tips in SuFu$^{-/-}$ MEFs stimulated with SAG in the presence of bortezomib. Percentages shown indicate corresponding ciliary counts. Bars, 2 µm.

in favor of the recruitment of SuFu–Gli2 and SuFu–Gli3 complexes to cilia.

Conversely, we next asked whether SuFu is required for localizing Gli proteins to cilia. In SuFu$^{-/-}$ MEFs, Gli proteins do not localize to cilia with or without Shh stimulation, although Smo recruitment is normal (Fig. 4 C and Fig. S3 D). Localization of Gli to cilia was restored by stable expression of SuFu in SuFu$^{-/-}$ MEFs (Fig. S3 E). One explanation for the absence of Gli proteins from cilia in SuFu$^{-/-}$ cells is the dramatically reduced Gli levels in the absence of SuFu (Ohlmeyer and Kalderon, 1998; Chen et al., 2009). Indeed, in SuFu$^{-/-}$ MEFs, Gli3-FL is dramatically decreased compared with SuFu$^{+/-}$ MEFs (Fig. S3 F), and pharmacological inhibition of the proteasome only partially rescues Gli3-FL levels. To overcome the instability of Gli proteins, we generated SuFu$^{-/-}$ cells stably overexpressing HA-tagged Gli1 (Gli1HA), which we stabilized by proteasomal inhibition with bortezomib. This treatment allowed Gli1HA to accumulate in SuFu$^{-/-}$ MEFs to levels similar to those in the SuFu$^{+/-}$ MEFs (Fig. 4 D). Under these conditions, some Gli1HA can be detected in cilia of SuFu$^{-/-}$ MEFs (Fig. 4 E), demonstrating that at least Gli1 can localize to cilia in the absence of SuFu, as demonstrated for transiently transfected Gli proteins (Chen et al., 2009). In SuFu$^{-/-}$ cells, Gli1HA was concentrated in the nucleus, whereas in SuFu$^{+/-}$ cells it was excluded from the nucleus (Fig. S3 G), which is consistent with the proposed mechanism of SuFu inhibition by sequestering Gli proteins in the cytoplasm (Ding et al., 1999; Kogerman et al., 1999; Méthot and Basler, 2000). Nuclear accumulation of Gli1 in the absence of SuFu might also explain why ciliary levels of Gli1HA in SuFu$^{-/-}$ cells were lower than in SuFu$^{+/-}$ cells expressing comparable amounts of Gli1HA (Fig. 4 E).

Hh stimulation causes the rapid disappearance of a defined SuFu–Gli complex

Our cellular experiments of endogenous SuFu and Gli proteins suggested that active Smo at cilia relays the signal to cytoplasmic SuFu–Gli complexes. As SuFu blocks nuclear import of Gli

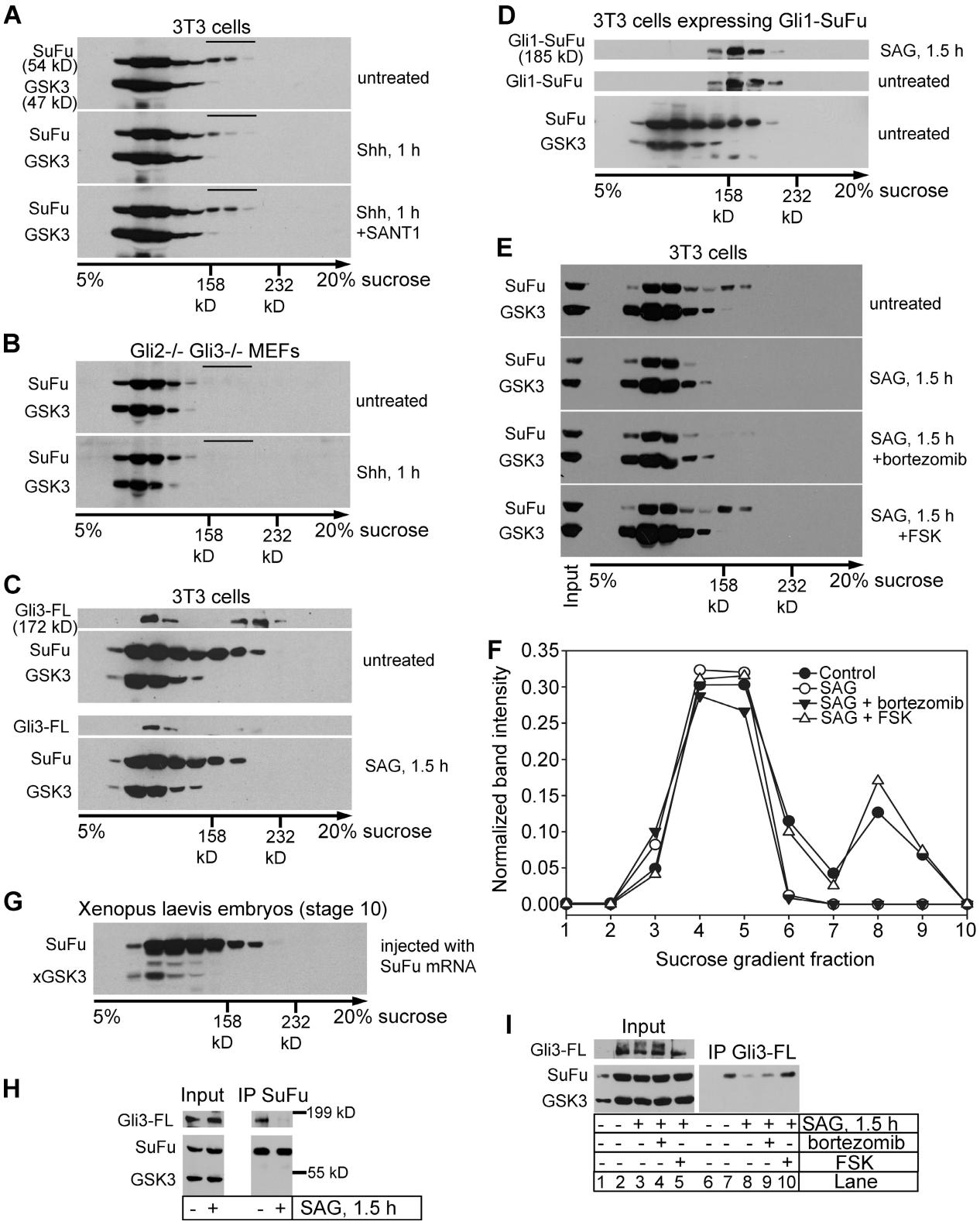

Figure 5. **Biochemical evidence that Hh pathway activation causes rapid dissociation of endogenous SuFu–Gli complexes.** (A–I) Endogenous SuFu–Gli complexes were analyzed by sucrose gradient centrifugation (A–G) and immunoprecipitation (H and I). (A) In untreated NIH-3T3 cells, the majority of endogenous SuFu (54 kD) exists as a monomer of similar size as the kinase GSK3-β (47 kD). A small fraction of SuFu from untreated cells forms a higher molecular mass complex (top, black lines), the level of which quickly drops in cells treated with Shh for 1 h (middle), an effect completely blocked if Smo is inhibited with 200 nM SANT-1 (bottom). The position in the gradient of two size markers run in parallel is shown below the Western blots (aldolase: molecular mass, 158 kD; Stokes radius, 48.1 Å; catalase: molecular mass, 232 kD; Stokes radius, 52.2 Å). (B) In Gli2$^{-/-}$ Gli3$^{-/-}$ MEFs, only the monomeric SuFu peak is seen by sucrose gradient centrifugation. Hh stimulation of Gli2$^{-/-}$ Gli3$^{-/-}$ MEFs does not change the size of the SuFu peak, although Smo is recruited to the cilia normally in these cells. (C) As in A, but cells were stimulated or not with SAG, and sucrose gradient fractions were immunoblotted for endogenous SuFu, GSK3, and Gli3-FL. The higher molecular mass SuFu peak overlaps with endogenous Gli3-FL in unstimulated cells. Acute Hh pathway

proteins, the major mechanistic question is how active Smo at the cilium modifies the SuFu–Gli complex to allow Gli activation and nuclear entry. Because Hh signaling can occur in the absence of new protein synthesis (Fig. 1, F and G), we hypothesized that signaling must regulate SuFu–Gli complexes post-translationally. To identify possible changes in endogenous SuFu–Gli complexes caused by Hh stimulation, we turned to measuring the size of native protein complexes by sucrose gradient centrifugation (Martin and Ames, 1961) of cellular lysates. Because prolonged Hh signaling causes a decrease in the level of Gli proteins (Fig. S4, A–C), we examined the effect of brief Hh stimulation (1–1.5 h). Given that SuFu and Gli proteins are recruited to cilia within 30 min or less, we reasoned that such a brief period of pathway activation should be sufficient to observe changes in SuFu–Gli complexes.

NIH-3T3 cells were stimulated or not with Shh for 1 h, after which they were lysed and SuFu was analyzed by sucrose gradient centrifugation. The majority of endogenous SuFu (54 kD) migrates as a small molecular mass peak (Fig. 5 A), which is similar in size and shape to the peak of glycogen synthase kinase 3 (GSK3; 47 kD). This hydrodynamic behavior indicates that most SuFu in cells is present as a monomer. In untreated cells, a small fraction of SuFu appears in fractions of higher Stokes radius (Fig. 5 A, top), which is consistent with SuFu associating with other proteins. Stimulating cells with Shh for 1 h causes the dramatic decrease of the higher molecular mass SuFu (Fig. 5 A, middle), an effect that is completely reversed by the small molecule Smo inhibitor SANT-1 (Fig. 5 A, bottom). In another experiment, a 1.5-h stimulation of NIH-3T3 cells with the Smo agonist, SAG, causes the complete disappearance of the high molecular mass SuFu complex (Fig. 5, E and F).

Two lines of evidence demonstrate that the high molecular mass SuFu species is a SuFu–Gli complex: (1) the SuFu complex is absent from Gli2$^{-/-}$ Gli3$^{-/-}$ MEFs (Lipinski et al., 2006) in which only monomeric SuFu is seen on sucrose gradients (Fig. 5 B, top). This also indicates that SuFu is dedicated to binding Gli proteins, and in their absence, SuFu does not stably associate with other proteins. Additionally, the size of endogenous SuFu in Gli2$^{-/-}$ Gli3$^{-/-}$ cells does not change upon Hh pathway stimulation (Fig. 5 B, bottom), indicating that signaling specifically couples to SuFu–Gli complexes and not to monomeric SuFu. (2) The high molecular mass SuFu complex overlaps with a Gli3-FL peak (Fig. 5 C, top), and Hh stimulation causes the simultaneous disappearance of the high molecular mass SuFu and Gli3-FL peaks (Fig. 5 C, bottom). Collectively, these data demonstrate that Hh stimulation causes the quick disappearance of the SuFu–Gli complex.

Although we do not know the shape of the SuFu–Gli complex and thus cannot determine its exact size, its migration on sucrose gradients is consistent with the calculated size of a 1:1 complex (mouse SuFu–Gli3-FL complex, 54 + 172 = 226 kD), suggesting that the complex might contain only one molecule of SuFu and Gli3-FL. To examine whether SuFu behavior is conserved in other vertebrate systems, we determined the sucrose gradient profile of SuFu expressed in *Xenopus laevis* embryos (Fig. 5 G) and found it very similar to that in NIH-3T3 cells, suggesting that SuFu forms complexes of a similar size with endogenous Gli proteins in *Xenopus* embryos.

The SuFu-Gli complex dissociates in response to Hh signaling

We considered two possibilities for the mechanism underlying the disappearance of the SuFu–Gli complex in response to Hh stimulation: (1) the SuFu–Gli complex disappears through proteolysis either of SuFu or Gli, and (2) the SuFu–Gli complex disappears as a result of dissociation. Our results support the idea that Hh stimulation causes the dissociation of the SuFu–Gli complex.

A recent study suggested that Hh signaling triggers the proteasomal degradation of SuFu in certain cancer cells (Yue et al., 2009). We find that in NIH-3T3 cells, neither the steady-state level nor the half-life of SuFu changes upon Shh stimulation (Fig. S4, A–E), suggesting that Hh signaling does not affect bulk SuFu levels or stability. However, it is conceivable that Hh signaling might stimulate degradation of the small fraction of SuFu in SuFu–Gli complexes but that the size of this pool is too small to detect. We excluded this possibility by blocking proteasomal degradation with the small molecule bortezomib (see below).

The levels of both Gli3-FL and Gli3-R (Fig. S4, A–C) and the half-life of Gli3-FL (Fig. S4, D and E) decrease after Hh pathway activation; thus, it is possible that the disappearance of the SuFu–Gli complex reflects the increased turnover of Gli caused by Hh signaling. The following results show that SuFu–Gli dissociation and not Gli degradation is responsible for the disappearance of the SuFu–Gli complex: (a) the SuFu–Gli complex disappears after as little as 1.5 h of SAG stimulation, which has little or no effect on Gli3-FL levels (Fig. 5, H and I), (b) the

stimulation causes the simultaneous disappearance of the overlapping, higher molecular mass SuFu and Gli3-FL peaks. (D) To prevent dissociation of SuFu from Gli, a direct fusion of Gli1 to SuFu was generated. NIH-3T3 cells stably expressing this Gli1–SuFu fusion were stimulated or not with SAG. The apparent size of the Gli1–SuFu fusion peak does not change upon Hh pathway activation. (E) Treatment of NIH-3T3 cells with SAG causes complete disappearance of the SuFu–Gli complex, which is not reversed by inhibition of the proteasome with bortezomib. In contrast, activation of PKA with FSK completely blocks SuFu–Gli dissociation induced by SAG stimulation. (F) Quantification of the experiment in E. The amount of SuFu in each fraction was measured relative to the amount of SuFu in the input lane. The first fraction represents the top of the sucrose gradient. (G) Mouse SuFu expressed in *Xenopus* embryos shows the same size distribution as endogenous SuFu in mammalian cultured cells, suggesting that SuFu forms a similar complex with endogenous Gli proteins in *Xenopus* embryos. (H) NIH-3T3 cells were incubated with or without SAG followed by immunoprecipitation (IP) with anti-SuFu antibodies. The level of Gli3-FL is similar in SAG-treated and untreated cells (left). Gli3-FL coimmunoprecipitates with SuFu only in untreated cells but not in SAG-stimulated cells (right), indicating that acute Hh pathway activation dissociates endogenous Gli3-FL from SuFu. (I) NIH-3T3 cells were incubated with control media, SAG, SAG and bortezomib, and SAG and FSK followed by immunoprecipitation with anti–Gli3-FL antibodies. Gli2$^{-/-}$ Gli3$^{-/-}$ MEFs were used as negative control (lanes 1 and 6). Endogenous SuFu does not coimmunoprecipitate with Gli3-FL in cells stimulated with SAG, although levels of Gli3-FL decrease only slightly. Proteasome inhibition by bortezomib (sufficient to abolish any decrease in the level of Gli3-FL) does not block dissociation of endogenous SuFu from Gli3-FL. In contrast, SAG-induced dissociation of SuFu from Gli3-FL is completely blocked by FSK.

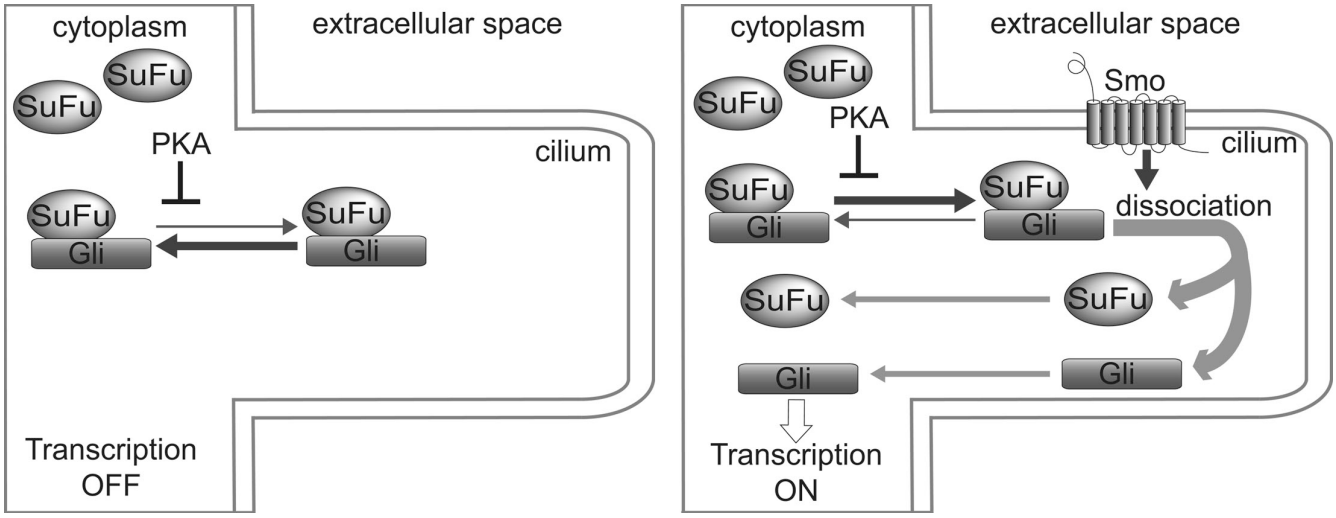

Figure 6. **A model for activation of Gli proteins during vertebrate Hh signaling.** In the resting state of the Hh pathway (left), SuFu forms inactive complexes with Gli2 and Gli3-FL, which are sequestered in the cytoplasm. Without Hh stimulation, SuFu–Gli complexes traffic to the primary cilium at a low level independently of Smo; this basal ciliary trafficking is antagonized by PKA. Hh pathway stimulation (right) leads to the translocation of active Smo to the cilium, which, in turn, recruits SuFu–Gli complexes. Active Smo at cilia causes the dissociation of SuFu from Gli. Monomeric SuFu and Gli leave the cilium followed by Gli nuclear translocation and activation of the transcriptional program of the Hh pathway. PKA antagonizes Hh signaling by blocking ciliary localization of SuFu–Gli complexes, thus preventing coupling between active Smo and dissociation of SuFu–Gli complexes.

SuFu–Gli complex disappears even when the proteasome is blocked with high levels of bortezomib (Fig. 5, E, F, and I), which are sufficient to completely block Gli3-FL degradation (Fig. S4 B), and (c) if dissociation is prevented by fusing SuFu and Gli1, the size of the stably expressed covalent SuFu–Gli1 complex no longer changes in response to Hh stimulation (Fig. 5 D).

Finally, we used immunoprecipitation of endogenous SuFu and Gli3-FL from NIH-3T3 cells to demonstrate dissociation of SuFu–Gli3-FL by Hh stimulation. The amount of Gli3-FL immunoprecipitated with SuFu from stimulated cells is dramatically reduced compared with untreated cells, although total Gli3-FL levels do not change appreciably during the 1.5-h stimulation time (Fig. 5 H). Conversely, the amount of SuFu immunoprecipitated with Gli3-FL is greatly decreased after acute Hh stimulation, an effect that is not reversed if Gli3-FL levels are stabilized by inhibition of the proteasome (Fig. 5 I).

In summary, Hh signaling causes the rapid dissociation of SuFu from Gli, suggesting a simple mechanism for relieving the inhibition of Gli by SuFu. We also conclude that Gli3-FL degradation during Hh signaling is not a cause but a consequence of dissociation from SuFu, which is consistent with the pronounced instability of Gli in cells lacking SuFu in spite of maximal activation of Gli target genes.

PKA inhibits SuFu-Gli complex dissociation: evidence that dissociation occurs at cilia

Activation of PKA by FSK potently inhibits Hh signaling, and we found that FSK completely blocks localization of the SuFu–Gli complex to cilia. Because FSK does not prevent recruitment of Smo to cilia by Hh stimulation, we used FSK to uncouple activation and recruitment of Smo to cilia from ciliary recruitment of SuFu–Gli. We then asked whether FSK affects dissociation of the SuFu–Gli complex caused by Hh stimulation. In cells treated with FSK, dissociation of endogenous SuFu–Gli3-FL by acute Hh stimulation is completely blocked (Fig. 5, E, F, and I). This result is consistent with a model in which dissociation of SuFu–Gli complexes by active Smo occurs at cilia; alternatively, FSK might independently inhibit both SuFu–Gli ciliary localization and dissociation. We favor the first model because it is consistent with inhibition of SuFu–Gli dissociation in Kif3a$^{-/-}$ cells in which ciliary localization of Smo is inhibited (Humke et al., 2010). Our findings also provide a new mechanism explaining the inhibition of Hh signaling by FSK and its strict dependence on SuFu (Svärd et al., 2006; Chen et al., 2009).

Discussion

A unique feature of the vertebrate Hh pathway is that primary cilia are critical for signal transduction (Huangfu and Anderson, 2005). The Hh ligand binds its receptor, Ptc, localized at the primary cilium (Rohatgi et al., 2007), leading to activation and recruitment of the seven-spanner Smo to the cilium (Corbit et al., 2005; Rohatgi et al., 2007), from where it signals to the cytoplasm to activate Gli proteins. In unstimulated cells, Gli proteins are kept inactive by the cytoplasmic protein SuFu. In vertebrate cells lacking SuFu, the Hh pathway is maximally active, independent of Smo (Cooper et al., 2005; Svärd et al., 2006), and independent of cilia (Jia et al., 2009). A simple model for vertebrate Hh signaling is that active Smo at the cilium inhibits SuFu to allow Gli activation; however, a major unanswered question has been if and how SuFu is regulated by Hh signaling.

We found that the endogenous complex formed by SuFu and Gli proteins localizes to cilia and that this ciliary localization is strongly increased by Hh signaling through active Smo. This suggested that the Hh signal is transmitted from active Smo to the SuFu–Gli complex, leading to Gli activation. To determine the mechanism that activates Gli, we searched for biochemical changes of SuFu–Gli complexes caused by acute Hh

stimulation. SuFu is an abundant protein (we estimated its concentration in NIH-3T3 cells at ∼100 nM), and a small fraction of SuFu forms a complex with Gli in unstimulated cells, whereas most SuFu is monomeric. Hh stimulation leads to the rapid dissociation of the SuFu–Gli complex (Humke et al., 2010), suggesting a simple mechanism in which Gli activation is the consequence of relieving its inhibition by SuFu, which allows Gli to enter the nucleus (Fig. 6). We do not yet know whether SuFu dissociation from Gli is sufficient to activate Gli or if posttranslational changes are also required (Ohlmeyer and Kalderon, 1998) such as Gli phosphorylation (Humke et al., 2010). We also do not know whether all dissociation of the SuFu–Gli complex takes place at cilia or whether it also occurs in other parts of the cell. We propose that SuFu–Gli dissociation is the first step in a series of molecular events through which Gli proteins are activated by Hh signaling. This mechanism of vertebrate Hh signaling is reminiscent of Hh signal transduction in *Drosophila*, in which Hh stimulation causes the release of Ci complexes by decreasing the affinity of the atypical kinesin costal2 for MTs (Robbins et al., 1997).

Whether active Gli moves to the nucleus by itself or in complex with SuFu has been a matter of debate. We favor a model in which Gli enters the nucleus without SuFu for the following reasons: (a) SuFu blocks nuclear localization of overexpressed Gli (Barnfield et al., 2005), whereas Gli proteins are nuclear in the absence of SuFu (Humke et al., 2010; this study), (b) Hh stimulation causes the rapid dissociation of SuFu–Gli complexes, indicating that a critical step in generating active Gli is the removal of bound SuFu, and (c) SuFu is not required in the nucleus, as the transcriptional output of the Hh pathway is maximal in $SuFu^{-/-}$ cells (Svärd et al., 2006).

Recently, the BTB domain protein SPOP was suggested to antagonize the interaction between SuFu and Gli (Chen et al., 2009). However, SPOP does not localize to cilia (Chen et al., 2009), and loss of SPOP causes only a modest increase in the unstimulated transcription of Hh target genes (Wen et al., 2010), suggesting that although SPOP might play a role in Gli turnover, it likely does not regulate the SuFu–Gli complex during the initial Hh signaling events at the ciliary membrane.

The compartmentalization of vertebrate Hh signaling in primary cilia is accomplished through at least three, largely independent ciliary localization events: (1) localization of Ptc, which is independent of Smo (Rohatgi et al., 2007), (2) localization of Smo, which can be uncoupled from upstream components (Ptc and Hh), is independent of downstream components (SuFu and Gli), and is stimulated by PKA, and (3) localization of SuFu–Gli complexes, which is inhibited by PKA. We speculate that recruitment of SuFu–Gli complexes to cilia ensures that the signal from active Smo is channeled to Gli molecules inhibited by SuFu. If SuFu were recruited to cilia alone, it would compete with SuFu–Gli complexes and inhibit signaling because monomeric SuFu is present in a large excess over SuFu–Gli. SuFu–Gli complexes thus serve not only to keep Gli proteins inactive and stable but also to make them activatable by Hh signaling at the cilium.

Based on the size of the endogenous SuFu–Gli complex, we estimate that it might consist of only these two proteins. Thus, an unexpectedly simple protein complex lies at the core of vertebrate Hh signal transduction downstream of Smo. It will be important to understand how the integrity of the SuFu–Gli complex is maintained, how signaling stimulates its dissociation, and whether the posttranslational control of SuFu–Gli dissociation occurs at the levels of SuFu, Gli, or both. Additionally, it will be important to determine how SuFu–Gli complexes localize to cilia and how active Smo increases their ciliary localization.

The PKA activator FSK blocks the transcriptional output of the Hh pathway, although only in the presence of SuFu. We found that FSK abolishes the localization of the SuFu–Gli complex to cilia and its dissociation by Hh stimulation. We interpret these findings as follows: (a) dissociation of SuFu–Gli occurs at cilia during Hh signaling and is inhibited if SuFu–Gli cannot travel to the cilium, similar to inhibition of SuFu–Gli dissociation observed in $Kif3a^{-/-}$ cells (Humke et al., 2010), and (b) PKA controls trafficking of SuFu–Gli complexes to cilia independent of Smo, suggesting a novel mechanism for Hh inhibition by PKA. Although PKA localizes to the base of cilia (Barzi et al., 2010), whether Hh signaling regulates PKA remains unclear; one possibility is that local inhibition of PKA might allow coupling between active Smo and SuFu–Gli complexes at cilia. However, it is likely that additional events are required to transmit the signal from active Smo to the SuFu–Gli complex because pharmacological inhibition of PKA blocks rather than activates Hh signaling (unpublished data).

Of the three members of the Gli family of transcription factors, our study focused only on Gli2 and Gli3, which mediate the initial response to Hh stimulation. Gli1 is synthesized in response to Hh signaling (Ruiz i Altaba, 1998; Dai et al., 1999) and is part of a positive feedback loop that amplifies output of the pathway. Gli1 binds to and is inhibited by SuFu (Merchant et al., 2004; Chen et al., 2009). We envision that another role of SuFu is to inhibit newly synthesized Gli1 and that the SuFu–Gli1 complex has to pass through the cilium in the presence of active Smo in order for Gli1 to become active. This would ensure that the Hh pathway remains signal dependent even after prolonged stimulation and accumulation of Gli1 protein, avoiding runaway transcriptional activation.

Materials and methods

Cell culture and Hh pathway assays

NIH-3T3 cells were grown in DME supplemented with 10% bovine calf serum, penicillin, and streptomycin. MEFs were grown in DME supplemented with 10% fetal bovine serum, sodium pyruvate, nonessential amino acids, penicillin, and streptomycin. To assay Hh signaling, confluent cell cultures were starved for 24–48 h in starvation media (DME without serum for NIH-3T3 cells or with 0.2% fetal bovine serum for MEFs). The media were replaced with starvation media supplemented with the appropriate Hh pathway agonist, antagonist, or control vehicle. After incubation for the desired amount of time, cultures were processed for immunofluorescence or harvested for real-time PCR, Western blotting, immunoprecipitation, or sucrose gradient centrifugation.

Antibodies

Polyclonal antibodies against mouse Smo, SuFu, and Gli were generated in rabbits or goats (Cocalico Biologicals) and were affinity purified. The antibodies were tested for specificity by immunoblotting (on either overexpressed or endogenous proteins) and immunofluorescence on cells (against endogenous proteins; Fig. S1).

For the anti-Smo antibody, a fragment of the intracellular C-terminal domain of mouse Smo (amino acids 683–794) was expressed in bacteria as a soluble fusion with maltose-binding protein (MBP). Serum from rabbits immunized with this recombinant protein was depleted of anti-MBP antibodies, after which anti-Smo antibodies were affinity purified against the antigen immobilized on beads (Affigel 15; Bio-Rad Laboratories). To generate anti-SuFu antibodies, full-length mouse SuFu was expressed and purified from bacteria as an MBP fusion. The serum was affinity purified against a 6His-tagged EGFP fusion of mouse SuFu covalently attached to Affigel 15. To generate anti-Gli antibodies, two fragments of the human Gli3 protein (an N-terminal fragment consisting of amino acids 1–799 and a C-terminal fragment consisting of amino acids 1,061–1,599) were expressed in bacteria as insoluble 6His-MBP–tagged fusions. Inclusion bodies were isolated, separated by SDS-PAGE, and gel slices were used to immunize rabbits or goats (Cocalico Biologicals). The serum from rabbits immunized with the mixed recombinant Gli3 fragments was affinity purified successively against 6His-hGli3 (1–799) and 6His-hGli3 (1,061–1,599) to generate the anti-GliN and anti-GliC antibodies. On immunoblots, anti-GliN detects both full-length and processed Gli3, whereas anti-GliC only detects Gli3-FL. By immunofluorescence, anti-GliC strongly detects both Gli2 and Gli3-FL, whereas anti-GliN detects Gli3 strongly and Gli2 only weakly (Fig. S1). Anti-GliN and anti-GliC do not detect human or mouse Gli1 by either immunoblotting or immunofluorescence.

Real-time PCR assays of Hh pathway activity

Total cellular RNA was treated with DNase (Promega), purified, and cDNA was generated from 1 µg of total RNA using reverse transcription (Transcriptor; Roche) and random hexamers. *Gli1* and *Ptch1* gene expression was assayed by quantitative real-time PCR using SYBR green (FastStart; Roche) on a Rotor-Gene 6000 (Corbett Robotics). Relative gene expression was calculated using a two standard curve method in which each gene of interest was normalized to the ribosomal protein *L27* gene. The following sequences for gene-specific primers were used: *L27*, 5′-GTCGAGATGGGCAAGTTCAT-3′ and 5′-GCTTGGCGATCTTCTTCTTG-3′; *Gli1*, 5′-GGCCAATCACAAGTCAAGGT-3′ and 5′-TTCAGGAGGAGGGTACAACG-3′; and *Ptch1*, 5′-ACTGTCCAGCTACCCCAATG-3′ and 5′-CATCATGCCAAAGAGCTCAA-3′. Data represent SEM from three independent experiments.

Effect of protein synthesis inhibition on Hh signaling in NIH-3T3 cells

To determine whether ciliary recruitment and transcription activation by the Hh pathway require new protein synthesis, NIH-3T3 cells were starved overnight and incubated for 30 min in starvation media supplemented or not with cycloheximide (CHX; 50 µg/ml final). CHX-treated cells or controls were incubated with Shh in the presence or absence of CHX, respectively. Recruitment of Smo, SuFu, and Gli to cilia was assayed by immunofluorescence after 3 h of Shh stimulation. Expression of *Gli1* and *Ptch* genes was assayed by quantitative PCR (Q-PCR) after 0, 3, and 6 h of stimulation. To determine the degree of protein synthesis inhibition by CHX, cell cultures were starved for methionine by incubation for 2 h in methionine starvation media (DME without methionine). The cells were incubated for 30 min in methionine starvation media with or without 50 µg/ml CHX followed by incubation with or without CHX for 3 h in methionine starvation media supplemented with 35S-methionine (50 µCi/ml final). The cells were harvested, and 35S-labeled proteins were detected by SDS-PAGE and autoradiography. Protein synthesis was also measured by scintillation counting of 35S incorporated into TCA-insoluble material during the 3-h incubation period.

Requirement of active Smo for ciliary recruitment of SuFu and Gli proteins

Starved, confluent NIH-3T3 cells were incubated with or without 200 nM SAG or 10 µM Cyc. After 3 h, parallel cell cultures were either processed for immunofluorescence (to assay Smo, SuFu, and Gli recruitment to cilia) or Q-PCR (to assay *Gli1* and *Ptch1* transcription). To determine whether continued localization of SuFu and Gli proteins to cilia requires active Smo, confluent NIH-3T3 cells were first incubated in the absence or presence of Shh for 3 h to recruit Smo, SuFu, and Gli to cilia. 10 µM Cyc was added to Shh-stimulated cells, and ciliary localization of Smo, SuFu, and Gli was determined after the desired incubation time. To determine the effects of FSK, starved, confluent NIH-3T3 cells were treated overnight with control vehicle, Shh, 10 µM FSK (Sigma-Aldrich), or 10 µM FSK and Shh. Parallel cell cultures were processed for immunofluorescence or analyzed by Q-PCR. To reverse the effects of FSK, the small molecule PKA inhibitor H-89 (EMD) was used at 10 µM.

Immunoprecipitation

Affinity-purified anti-Gli3 and anti-SuFu antibodies were covalently attached to protein A beads (AffiPrep; Bio-Rad Laboratories) by cross-linking with dimethyl pimelimidate (Thermo Fisher Scientific). Confluent cell cultures were starved for 48 h followed by treatment for 1.5 h with or without 100 nM SAG, 2 µM bortezomib, or 20 µM FSK. The cells were lysed on ice in lysis buffer (20 mM Hepes, pH 7.5, 50 mM potassium chloride, and 1 mM magnesium chloride) with 0.5% digitonin in the presence of protease inhibitors (Complete; Roche). The lysate was clarified by centrifugation at 20,000 *g*, and the supernatant was incubated with antibody beads for 1.5 h at 4°C. The beads were washed in lysis buffer with 0.1% digitonin before elution in SDS-PAGE sample buffer and analysis by SDS-PAGE followed by immunoblotting.

Sucrose gradient centrifugation

12.8 mi linear sucrose gradients (5–20% sucrose) in XB buffer (10 mM Hepes, pH 7.5, 100 mM potassium chloride, 1 mM magnesium chloride, and 100 µM calcium chloride supplemented with protease inhibitors) were prepared using a gradient maker (BioComp) and were cooled to 4°C. Cells were treated and lysed as described for immunoprecipitation experiments, and a volume of 150 µl clarified lysate was layered on the top of the gradient. Gradients were centrifuged for 20 h at 4°C at 38,000 rpm in a rotor (SW-40; Beckman Coulter). The sucrose gradients were fractionated, and each fraction was precipitated with TCA. The TCA-precipitated proteins were analyzed by SDS-PAGE followed by immunoblotting for endogenous SuFu, Gli3, and GSK3. The sucrose gradients were calibrated using the molecular mass markers ovalbumin (molecular mass, 44 kD; Stokes radius, 30.5 A), aldolase (molecular mass, 158 kD; Stokes radius, 48.1 A), catalase (molecular mass, 232 kD; Stokes radius, 52.2 A), ferritin (molecular mass, 440 kD; Stokes radius, 61 A), and thyroglobulin (molecular mass, 669 kD; Stokes radius, 85 A).

Immunofluorescence

Cells grown on glass coverslips were fixed for 30 min at room temperature in PBS with 4% formaldehyde. The coverslips were rinsed with TBST (10 mM Tris, pH 7.5, 150 mM NaCl, and 0.2% Triton X-100), and nonspecific binding sites were blocked by incubation in TBST supplemented with 25 mg/ml BSA (TBST-BSA). The coverslips were incubated with primary antibodies diluted in TBST-BSA for 1 h at room temperature. Coverslips were washed with TBST, blocked again with TBST-BSA, and incubated with the appropriate secondary antibodies in TBST-BSA. After washing, the coverslips were mounted on glass slides in mounting media (0.5% *p*-phenylenediamine, 20 mM Tris, pH 8.8, and 90% glycerol). Affinity-purified primary antibodies against Smo, Gli3, and SuFu were used at a final concentration of 1–2 µg/ml. Mouse anti-acetylated tubulin, mouse anti–γ-tubulin, and mouse anti-Flag antibodies were purchased from Sigma-Aldrich. Alexa Fluor dye–conjugated secondary antibodies (Invitrogen) were used at a final concentration of 1 µg/ml. The immunostained cells were imaged by epifluorescence microscopy on an inverted microscope (TE2000U; Nikon) equipped with a digital camera (OrcaER; Hamamatsu Photonics) and a 100× Plan Apo 1.4 NA oil objective (Nikon). Images were collected using MetaMorph image acquisition software (Applied Precision). To measure ciliary localization of SuFu, Smo, and Gli, 150 cilia for each coverslip were identified by anti-acetylated tubulin staining and were scored visually for the presence or absence of SuFu, Smo, or Gli at the cilium. Data represent SD for groups of 50 cilia counted on different visual fields on the same coverslip. P-values for cilia counts were calculated using an unpaired, two-tailed *t* test, comparing each time point to t = 0. All experiments showing ciliary counts were repeated independently at least twice.

Immunoblotting

Cells were resuspended in TBS with protease inhibitors and were lysed with 1% Triton X-100 on ice for 20–30 min. The cell lysate was clarified by centrifugation for 30 min in a refrigerated microfuge at 20,000 *g*. The supernatant was collected, mixed with DTT (50 mM final) and 5× SDS-PAGE sample buffer, and separated by SDS-PAGE on 5–15% polyacrylamide gradient gels followed by transfer to nitrocellulose membranes. For immunoblotting, antibodies were used at a final concentration of 1 µg/ml in TBST with 5% nonfat dry milk.

Measurement of the half-life of endogenous SuFu by CHX chase

To determine whether activation of Hh signaling affects the half-life of endogenous SuFu, confluent, starved NIH-3T3 cells were pretreated for 15 min in DME with 50 µg/ml CHX. Parallel cultures were incubated with CHX in the presence or absence of 200 nM SAG in DME. At the indicated times, cells were harvested, and endogenous SuFu protein was detected by immunoblotting.

Noc treatment

To test whether MTs are required for recruitment of Smo, SuFu, and Gli to cilia and for the transcriptional responses of Hh signaling, confluent, starved NIH-3T3 cells were preincubated for 1 h with 0.25–5 µM Noc or

control vehicle. The cells were stimulated or not with Shh or with 200 nM SAG in the presence of the same Noc concentration as during preincubation. After 1 h, the cells were processed for immunofluorescence against Smo, SuFu, and Gli. Cilia were stained with the mouse anti-acetylated tubulin monoclonal antibody. To determine MT depolymerization, cells treated in parallel were stained with a mouse anti–α-tubulin antibody (DM1-α; Sigma-Aldrich). For Q-PCR analysis, cells were harvested after 2 h of incubation with or without Shh (or SAG) and in the absence or presence of the indicated concentration of Noc.

Shh, chemical agonists, and antagonists of the Hh pathway

Shh was produced in 293T cells by transient transfection of an expression plasmid encoding amino acids 1–198 of human Shh. Shh-conditioned media were harvested after 48 h, pooled, filter sterilized, and used in cellular assays usually diluted 1:4 in starvation media. Media conditioned by mock-transfected 293T cells were used as control, which had no effect on ciliary recruitment of Smo, Gli, or SuFu. The Smo agonist SAG was obtained from Axxora, the Smo antagonist SANT-1 was obtained from EMD, Cyc was obtained from LC Laboratories, and 20- and 25-hydroxycholesterol were obtained from Steraloids, Inc.

Pharmacological inhibition of the proteasome

To block ubiquitin-dependent proteolysis, confluent cells were starved for 24–48 h and pretreated with or without 2 µM bortezomib for 0.5–3 h. The cells were incubated with or without Hh pathway agonist in the presence or absence of 2 µM bortezomib for the desired amount of time. Parallel cultures were processed for immunofluorescent detection, Western blotting, Q-PCR, or sucrose gradient centrifugation.

Generation of stable cell lines

Constructs were generated in the retroviral vector pLHCX (Takara Bio Inc.), and retroviruses produced in 293T cells were used to infect NIH-3T3 cells or MEFs. Stably transduced lines were generated by hygromycin selection. Expression of the desired protein was confirmed by Western blotting and immunofluorescence. The retroviral constructs used in this study were (a) full-length mouse SuFu tagged at the C terminus with three copies of the Flag epitope, (b) full-length human Gli1 tagged at the C terminus with one copy of the HA epitope, and (c) a fusion between N-terminally Myc-tagged human Gli1 and mouse SuFu, which incorporates a flexible, 24–amino acid linker between Gli1 and SuFu.

Quantitation of endogenous SuFu levels in NIH-3T3 cells

The concentration of endogenous SuFu protein in NIH-3T3 cells was estimated by immunoblotting against serial dilutions of recombinant mouse SuFu expressed and purified from baculovirus-infected Sf9 cells.

***Xenopus* embryo injections**

Capped messenger RNA for mouse SuFu was generated in vitro using the Message Machine kit (Applied Biosystems). 100 pg SuFu mRNA in 10 nl water was injected per blastomere into both blastomeres of a stage 2 *Xenopus* embryo. 25 injected embryos were harvested at stage 10–11 (staged according to Nieuwkoop and Faber [1994]) and were homogenized on ice in 150 µl of XB buffer supplemented with 10 µg/ml cytochalasin B and protease inhibitors. The homogenate was clarified by centrifugation for 15 min at 20,000 *g* at 4°C. The supernatant was harvested and subjected to sucrose gradient centrifugation as described for lysates from cultured cells.

Online supplemental material

Fig. S1 shows the specificity of novel polyclonal antibodies used for immunofluorescence. Fig. S2 shows the effects of oxysterols, protein synthesis inhibition, and MT depolymerization. Fig. S3 provides supporting experiments characterizing $Smo^{-/-}$ MEFs, $SuFu^{-/-}$ MEFs, and NIH-3T3 cells expressing Gli1–SuFu fusion. Fig. S4 shows the levels of SuFu and Gli3 in cell lines used in this study assayed by immunoblotting. Table S1 summarizes immunolocalization of SuFu, Gli, and Smo in all cell lines used in this study under various conditions. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201004108/DC1.

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