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*Genes Dev.* 2009 23: 1843-1848

Access the most recent version at doi:[10.1101/gad.1838109](https://doi.org/10.1101/gad.1838109)

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## PERSPECTIVE

# Variations in Hedgehog signaling: divergence and perpetuation in Sufu regulation of Gli

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**The Hedgehog (Hh) proteins play a universal role in metazoan development. Nevertheless, fundamental differences exist between *Drosophila* and vertebrates in the transduction of the Hh signal, notably regarding the role of primary cilia in mammalian cells. In this issue of *Genes & Development*, Chen and colleagues (pp. 1910–1928) demonstrate that mouse Suppressor of fused (Sufu) regulates the stability of the transcription factors Gli2 and Gli3 by antagonizing the conserved Gli degradation device mediated by Hib/Spop in a cilia-independent manner.**

The Hedgehog (Hh) family of secreted proteins is involved in the developmental regulation of numerous tissues and organs. Initially identified in *Drosophila* (Nüsslein-Volhard and Wieschaus 1980), where it participates in the organization of the embryonic segments and adult appendages—such as wings and legs—Hh's role as an organizing molecule is highly conserved through evolution. One of its vertebrate counterparts, Sonic Hedgehog (Shh), has been studied extensively for its significant role in neural tube and limb patterning (McMahon et al. 2003). Dramatic consequences ensue from the deregulation of the Hh pathway either during development or adult life. For instance, a decrease in Shh signaling in the mammalian embryos led to cases of holoprosencephaly (failure of separation of the cephalic lobes associated with facial deformities). The recent broad interest in the Hh field certainly arose from the observation that postnatal uncontrolled pathway activation cause tragic effects: Mutations in the components of the Hh signaling pathway have been involved in the majority of cases of basal cell carcinoma, the most common cancer in humans. In addition to skin cancer, unrestrained pathway activation has been linked to the initiation or maintenance of numerous cancers, including medulloblastomas, chronic

myelogenous leukaemia, and multiple myeloma, as well as cancers affecting the pancreas and digestive tract (Beachy et al. 2004).

The importance of Hh as an organizing molecule is also apparent in the conservation of its signaling pathway. In both *Drosophila* and vertebrates, secreted Hh binds to its receptor, Patched (Ptc), and to its coreceptor, interference hedgehog (ihog/Cdo). It has been proposed that Ptc, which constitutively represses the Hh pathway, belongs to a proton gradient-driven transporter family and enzymatically inhibits the conserved transmembrane protein Smoothened (Smo) of the G-protein-coupled receptor (GPCR) family (Taipale et al. 2002; Corcoran and Scott 2006). In *Drosophila*, the binding of Hh to Ptc induces translocation of Smo to the plasma membrane, leading to the activation of Smo and of the downstream zinc finger transcription factors of the Gli protein family (McMahon et al. 2003). Whereas *Drosophila* has only one Gli homolog—named Cubitus interruptus (Ci)—multiple Gli transcription factors (Gli1, Gli2, and Gli3) interplay in the vertebrate Hh signaling pathway. Gli3 is processed efficiently into a repressor form (GliR), whereas Gli2 contributes largely as a primary activator (GliA). Gli1 is a transcriptional target of the pathway and acts as a transcriptional activator that reinforces GliA. In all species, activation of Hh signaling inhibits proteolytic processing of the Ci/Gli cytoplasmic proteins, and leads to the production of the transcriptional activator forms. The change in the balance between the activator and the repressor forms of the Gli proteins is of major importance in the cellular responses to Hh signal and is manifested as a combination of transcriptional derepression and activation (Ruiz i Altaba et al. 2007).

## Important divergences in the Hh pathway

Despite the apparent conservation of the fly and mammalian Hh pathways, there are important divergences concerning the relative contribution of conserved signaling components. In *Drosophila*, the Hh transduction is mediated by the interaction of Smo with a cytoplasmic signaling complex that includes the serine–threonine kinase Fused (Fu), the kinesin-like Costal-2 (Cos2), the

[*Keywords:* Hedgehog; signal transduction; evolution; primary cilium; Sufu; Gli]

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Article is online at <http://www.genesdev.org/cgi/doi/10.1101/gad.1838109>.

Suppressor of Fused (Sufu) protein, and Ci (Lum et al. 2003; Ruel et al. 2003). This protein complex regulates the subcellular localization and processing of Ci. In non-activated cells, the direct interaction of Cos2 with Ci is essential for targeting Ci for a proteasome-mediated proteolytic cleavage that generates the CiR form (see Fig. 1; Zhang et al. 2005). In cells under Hh influence, the activation of Smo leads to the phosphorylation of Cos2 by Fu, which antagonizes the negative function of Cos2 by alleviating the cytoplasmic sequestration and processing of Ci (Ruel et al. 2007). Consequently, Ci protein is transformed into a transcriptional activator (CiA) through an unknown mechanism.

In *Drosophila*, Sufu loss-of-function mutations were identified by their ability to suppress the lack of Fu phenotype (Preat 1992) but, intriguingly, Sufu-null mutant flies are viable, suggesting that Sufu is dispensable for Hh signaling. It nevertheless has been shown that restriction of Ci activity when Ci proteolysis is blocked is due to direct binding of Sufu on Ci (Smelkinson et al. 2007), suggesting that Sufu might act as an ultimate gatekeeper to prevent Ci nuclear translocation when other parallel inhibitory mechanisms are defective. But the mechanism by which Sufu inhibits Ci is still poorly understood and may involve not only regulation of Ci subcellular localization, but also Ci protein level and transcriptional activity in the nucleus (Ohlmeyer and Kalderon 1998; Méthot and Basler 2000; Wang et al. 2000). Nevertheless, based on the absence of phenotype in flies, it has been proposed previously that Sufu protein has a minor role in Hh transduction (Preat 1992).

In contrast, in mice, loss of Sufu function induced ventralization of the neural tube, characteristic of a constitutive activation of the Hh pathway (Cooper et al. 2005; Svärd et al. 2006). This phenotype resembles the phenotype displayed by absence of Ptc, another strong repressor of the pathway. Previous work by Chuang's laboratory and others (Chen et al. 2005; Merchant et al. 2005) led to the unexpected finding that loss of mouse Fu has no consequence on Hh signaling in mice. Furthermore, it has been demonstrated that the closest homologs of *Drosophila* Cos2—*kif7* and *kif27*—are not involved in Shh signaling, at least in mammalian fibroblast cultured cells (Varjosalo et al. 2006). Thus, the three proteins Fu, Cos2, and Sufu that provide the cytoplasmic core of the pathway in *Drosophila* are conserved in mammals; however, the mode of transduction has diverged.

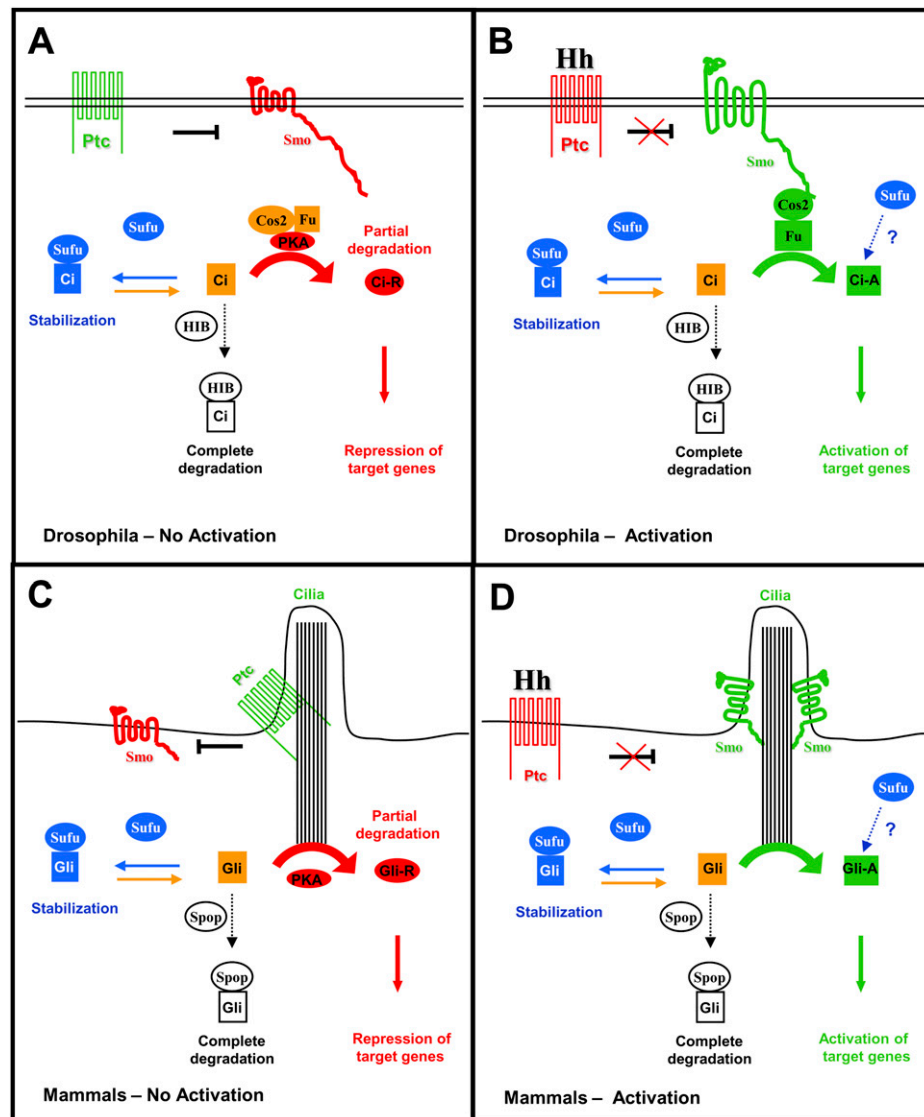
How could such conserved proteins have diverged? This divergence might be linked to the presence of a cellular compartment, the nonmotile primary cilium—present on most vertebrate cells (and not fly cells)—that is necessary for Shh signaling (Fig. 1). Indeed, the lack of the intraflagellar transport complex (IFT proteins)—involved in the assembly and activity of cilia through evolution—induces a defect in Shh activity, notably for the Shh-dependent ventralization of the mammalian neural tube. Colocalization of Shh and Ptc at primary cilia reinforces the idea that cilia might function as chemosensors for the detection of extracellular Shh (Rohatgi et al. 2007). In addition, the presence of cilia

correlates with Hh responsiveness. Indeed, cultured cells become Shh-responsive only at confluence, and cilia are detected only during this phase (Huangfu and Anderson 2006). The fact that *Drosophila* does not possess such a structure and that most Hh mammalian components analyzed by several laboratories are localized to the primary cilium led to the proposal that primary cilia provide a new cellular function that replaces the mode of action of the *Drosophila* cytoplasmic core (Cos2/Fu) of the Hh pathway (see Fig. 1). Indeed, IFT proteins transport cargo along the axoneural microtubules of the cilia and present an anterograde or retrograde motor activity, which are required for both GliA function and GliR formation. This led to the following model: IFT proteins might have an instructive role in the pathway and could provide a specific assembly site for components of the Hh pathway or participate in transporting key players of the signaling pathway within the primary cilium to provide full activation of the pathway. Smo relocalization and regulation in primary cilium upon pathway activation support this model (Rohatgi et al. 2009). The aim of the recent study from Chuang's laboratory (Chen et al. 2009) is to understand how mammalian Sufu functions independently of Fu and Cos2 and whether it requires cilia to regulate Gli function. By using mouse embryonic fibroblasts (MEFs) derived from knockout animals and mouse genetic epistatic experiments, Chen et al. (2009) provide several major contributions, which are discussed below.

### A new role of mammalian Sufu in controlling Gli protein stability

It has been shown previously that overexpressed Gli protein localizes to the primary cilium. For the first time, using specific antibodies against Gli2 and Gli3 proteins, Chen et al. (2009) document the dynamic changes in endogenous Gli localization in MEFs and show that their levels in the cilium increased upon Shh pathway activation. Sufu is also present at the primary cilium, but its location is not regulated by the pathway. Interestingly, in MEFs derived from *Sufu*<sup>-/-</sup> animals, ciliary localization of Gli2 and Gli3 is abolished. But it is unlikely that Sufu regulates Gli location, because ciliary location of Glis can be rescued just by increasing their expression in *Sufu*<sup>-/-</sup> MEFs. Interestingly, the lack of Gli ciliary location in the absence of Sufu correlates with an overall decrease of the endogenous level of Gli2 and Gli3 (both full-length and repressor forms), revealing a new role of mammalian Sufu in controlling Gli protein stability.

Does Sufu regulate Gli level and activity within the primary cilium? Three lines of evidence lead to a resounding "no." First, Chuang's laboratory (Chen et al. 2009) used MEF cells lacking primary cilium (in which the IFT gene *Kif3A* has been knocked down), and show that Sufu regulation of Gli stability is independent of the primary cilium. Second, they performed epistatic analysis between *Kif3a*<sup>-/-</sup> mutant mice (in which Hh signaling in the neural tube is reduced) and *Sufu*-deficient mice (in which the pathway is constitutively activated). The phenotype of *Sufu*<sup>-/-</sup> *Kif3a*<sup>-/-</sup> embryos is similar to the



**Figure 1.** Stabilization, degradation, and activation of the Ci/Gli proteins by the Hh signaling pathway. (A) In *Drosophila*, the absence of Hh allows Ptc to inhibit Smo activity, and full-length Ci (Ci) activity is regulated by three different mechanisms, including phosphorylation, proteolysis, and stabilization. First, phosphorylation of Ci by multiple kinases, including PKA, targets Ci to the ubiquitin/proteasome-mediated partial proteolysis to generate a truncated repressor form of Ci (Ci-R). This step requires the kinesin-like protein Cos2, which acts as a molecular scaffold to bridge Ci and the kinases. Second, the activity of full-length Ci is also blocked by stoichiometric binding of Sufu, but is protected from Hib-dependent degradation. Third, the excess of Ci is controlled by Hib protein, which binds and completely degrades the full-length form of Ci. (B) Binding of Hh to Ptc stimulates Smo phosphorylation, which increases Smo cell surface concentration. The activation of Smo stimulates the associated Fu kinase activity and dissociates Ci from the Cos2 complex. This blocks PKA phosphorylation of Ci and subsequent processing. The Fu/Cos2 complex is also necessary to form the active Ci (Ci-A), but the mechanism of this activation, as its regulation by Sufu, is uncertain. (C) In mammals, in the absence of Hh, Ptc is present in the primary cilium and prevents Smo ciliary accumulation. PKA and others kinases phosphorylate Gli and target it for partial degradation to form the truncated repressor form of Gli (Gli-R). It is possible that the scaffolding role of Cos2 in *Drosophila* might have been replaced by the primary cilia. Independently of cilia, in the cytoplasm, the activity of full-length Gli is further blocked by Sufu, which appears to be the major mechanism for restricting Gli activity in mammals. In addition, Sufu protects Gli2 and Gli3 (but not Gli1) from degradation by Spop, the Hib mammalian homolog. (D) Binding of Hh to Ptc promotes the accumulation and activation of Smo in the cilium. Gli phosphorylation and processing are inhibited, and formation of the transcriptionally activated Gli (Gli-A) is triggered. This activation occurs in the cilia, but can also take place when both primary cilia and Sufu are lacking. The mechanism of Gli activation and Sufu regulation by Shh are not known. In both *Drosophila* and mammals, Sufu likely plays an important role in the nucleus, not indicated here.

one displayed by *Sufu*<sup>-/-</sup> embryos. Accordingly, Gli2 and Gli3 protein levels are similarly reduced in *Sufu*<sup>-/-</sup> MEFs or in *Kif3a*<sup>-/-</sup> MEFs treated with shRNA for *Sufu*. Similar data were obtained with *Sufu*<sup>-/-</sup> MEFs expressing a dominant-negative (dn) form of another IFT protein, *Kif3b*. Third, overexpression of *Sufu* is able to suppress Gli-mediated Hh activation similarly in both wild-type MEFs and *Kif3a*<sup>-/-</sup> MEFs. Conclusions from Chuang's laboratory (Chen et al. 2009) nicely confirm a very recent similar approach developed by Jia et al. (2009), which showed that *Sufu* binding to and repression of Glis activity is observed in MEFs derived from IFT88-deficient embryos in which cilia formation is also disrupted. The *Sufu*/*Ift88* double-mutant embryos are also morphologically similar to the *Sufu* single mutant. Taken collectively, these data suggest that regulation of Gli protein level by *Sufu* can occur in the absence of cilia.

Based on these results, one could wonder: For which level of pathway activation is the primary cilium necessary then? Chen et al. (2009) adequately show that the elevated Hh signaling in *Ptch1*<sup>-/-</sup> MEFs is reduced by 80% when dn*Kif3b* is expressed. If we assume that in this later experiment ciliary function is defective, it clearly shows that, as for *Smo*, *Ptch1* function necessitates an intact primary cilium, but this is likely upstream of *Sufu* regulation of Glis.

#### Regulation of Gli protein stability by *Sufu* is conserved through evolution

If cilium integrity is not necessary for *Sufu* regulation of Glis, then one could hypothesize that the introduction of a form of vertebrate *Sufu* that does not localize to the cilium into *Sufu*<sup>-/-</sup> MEFs would restore Gli levels. Such *Sufu* variants are not available currently; however, to address this question, Chen et al. (2009) chose to manipulate *Sufu* from different species to challenge the conservation of this process. They show nicely that the cilium-independent regulation of Gli stability by *Sufu* seems to be evolutionarily conserved—zebrafish or *Drosophila* versions of *Sufu* can rescue stability, cilia localization, and activity of Gli2 and Gli3 in MEF cells lacking *Sufu* activity. Since neither zebrafish nor *Drosophila* versions of *Sufu* localize to cilia, it is likely that their rescue is independent of cilia regulation. To investigate the differences in cilium-dependent processes within Hh signal transduction, Chen et al. (2009) also assayed ciliary localization of *Smo* from different species in *Smo*<sup>-/-</sup> MEFs and show that zebrafish *Smo* localized to the cilium and rescued Hh signaling when MEFs are treated with Hh. On the contrary, *Drosophila* *Smo* failed to reach the cilium, and no Hh responses were observed. In a complementary experiment, it has been shown previously that mouse *Smo* cannot compensate for the lack of *Drosophila* *Smo* in insect cultured cells (De Rivoire et al. 2006). This makes sense since primary cilium regulation of the Hh signaling pathway has not been implemented in invertebrates and represents an evolutionary divergence.

The divergence downstream from *Smo* is still not well understood, and the precise role of vertebrate *Fu* and *Cos2*

is controversial (Tay et al. 2005; Varjosalo et al. 2006). Two homologs of *Cos2*—*Kif7* and *Kif27*—have been identified in mammals, whereas only one is present in zebra fish (*Kif7*). This suggests that duplication of ancestral *Cos2* in the vertebrate lineage might have occurred after emergence of the fish lineage (Wilson et al. 2009). Interestingly, morpholino knockdown experiments indicate that zebrafish *Kif7* acts like *Cos2*: as a negative regulator of the Hh pathway (Tay et al. 2005). However, no mouse knockout of *Kif7* has been developed yet to test for the conserved activity of *Cos2* in mouse. Another surprising observation from Chuang's laboratory (Wilson et al. 2009) was the demonstration that zebrafish *Fu* is not only necessary to control Hh signaling, but is also essential for construction of the central pair apparatus of motile cilia, which is structurally different from the primary cilium. Wilson et al. (2009) showed that, in the *Fu*<sup>-/-</sup> mouse, cells form primary cilia normally and Hh signaling is not affected, but motile ciliogenesis is. Despite this nonessential role of *Fu* in mammalian Hh signaling, it retains a physical association with *Kif27*. Thus, divergence within the Hh pathway between fish and mice might have led to a partitioning of functionality for *Fu* and *Kif7*, such that mouse *Fu* and *Kif27* are involved in motile ciliogenesis, while a compensatory *Fu* kinase, associated with *Kif7*, is necessary for Hh signaling. This model awaits the phenotypic analysis of mice deficient in *Kif7* and the kinases that have been shown to be necessary for Hh signaling in mammalian cells (Evangelista et al. 2008; Varjosalo et al. 2008). Alternatively, *Sufu* may have adopted a new function during evolution and could bypass the need for a *Fu*/kinesin pair. Indeed, the strong constitutive activation of Hh signaling in *Sufu*<sup>-/-</sup> mice indicates that *Sufu* has evolved as the major intracellular inhibitor of Gli activity (Svärd et al. 2006).

By which mechanism does *Sufu* regulate Gli2 and Gli3 levels? Chen et al. (2009) provide convincing data indicating that mouse *Sufu* regulates Gli2/3 stability by antagonizing the Spop protein of the BTB domain-containing protein family. Indeed, Chen et al. (2009) show that Spop binds to Gli2 and Gli3 and promotes their ubiquitination and degradation in a proteasome-dependent manner. The overexpression of *Sufu* appears to block Spop-dependent Gli protein reduction, and *Sufu*<sup>-/-</sup> MEFs treated with shRNA against Spop partially rescued Gli protein levels. How conserved is this mechanism? Well, it has been shown previously that the *Drosophila* counterpart of Spop, the Hib protein, forms a complex with Ci and Cul3 and promotes Ci ubiquitination and degradation (Zhang et al. 2006). Interestingly, in the same study, it has been shown in transgenic flies that Spop can rescue *Drosophila* Hib-dependent degradation of Ci, and that Gli2 and Gli3 transgenic proteins are subjected to degradation by Hib. Nevertheless, important differences in the Hib/Spop-Gli circuit exist between flies and mammals (see below).

One may wonder how the lack of *Sufu*—which induces a strong decrease in Gli levels—could lead to a constitutive activation of Hh signaling. Since binding of *Sufu* to

Gli2 and Gli3 represses their positive transcriptional activity, it is likely that the absence of Sufu leads to an increase in the Gli2/3 activator state. So, even though the bulk of the Gli protein level is strongly reduced, the quantity of GliA forms may increase and contribute to the constitutive activation of the pathway. It is also possible that, in absence of Sufu, GliR is less stable than the full-length Gli protein from which GliA is derived, leading to a change in the ratio between GliA and GliR, as suggested in a recent publication (Jia et al. 2009). Alternatively, since GliA reflects the collective activity of all three Gli proteins, it is possible that Gli1 may also contribute to GliA in Sufu<sup>-/-</sup> embryos. It is worth mentioning that Gli1, unlike Gli2 and Gli3, does not appear to be subject to Spop regulation. Since Gli1 expression is subjected to GliRep activity and is induced in the Sufu mutant, Chen et al. (2009) propose that the constitutive Hh signaling activity observed in the absence of Sufu results from increased levels of Gli1, triggered by Spop-mediated degradation of full-length Gli2/Gli3. The possibility that the overall level of GliA could be reduced in the absence of Sufu encouraged Chen et al. (2009) to re-evaluate the role of Sufu in the Hh signaling pathway. They observed that ligand-dependent pathway activation is reduced in Sufu<sup>-/-</sup> MEFs compared with wild-type MEFs, suggesting that Sufu is also required for maximal Hh signaling. This is contradictory to a previous study (Svärd et al. 2006) showing that Smo activation does not further induce Hh pathway activation in Sufu<sup>-/-</sup> MEFs, likely due to the fact that the pathway is already maximally activated. A detailed comparison between both studies will be needed to resolve this issue.

So, if not in the cilia, in which subcellular compartment would Sufu regulate Gli protein activity? This is still an open question, but regulation likely occurs at multiple levels, since Sufu is present in both the nucleus and cytoplasm in different cell types. Based on over-expression studies, it has been proposed that the role of Sufu in both flies and mammals is to retain Ci/Gli proteins in the cytoplasm, thereby preventing their nuclear translocation (Kogerman et al. 1999; Méthot and Basler 2000). Thus, one would expect to witness increased levels of Gli nuclear accumulation in the absence of Sufu. However, constitutive activation of Hh signaling observed in Sufu<sup>-/-</sup> MEFs is not accompanied by an increase of nuclear Gli1 (Svärd et al. 2006). Although conclusions based on Gli1 studies may not be applicable to Gli2 and Gli3 given their distinct properties, the main role of Sufu in mammals might be to mediate repression of Gli-dependent transcription directly in the nucleus. Indeed, physical interaction between Sufu and Gli2/3 is not affected by Hh signaling activation, and Sufu shuttles with Ci/Gli to form a complex with nuclear Gli bound to DNA (Kogerman et al. 1999; Sisson et al. 2006). Furthermore, it has been proposed that Sufu can repress Gli-mediated transcription by recruiting the Sin3–histone deacetylase (HDAC) corepressor complex to promoters containing Gli-binding elements (Cheng and Bishop 2002). In the present study, Chen et al. (2009) show that Spop has both cytoplasmic and nuclear localization, and

that both nuclear and cytoplasmic Gli2/3 protein levels are reduced in the absence of Sufu. So, it is conceivable that Sufu controls full-length Gli protein levels in the cytoplasm but also protects Gli proteins in the nucleus, where Hib/Spop proteins are present.

In the *Drosophila* wing imaginal disc, Hib expression depends on Hh signaling. Moreover, in eye imaginal discs, where Hh signals coordinate cell proliferation and differentiation, Hib is highly expressed in the differentiating cells to prevent aberrant Hh signaling activity and ensure normal eye development (Zhang et al. 2006). So, it is possible that, in mammals, Hh pathway activity needs to be turned off in differentiating cells to allow for their normal development, and Spop might represent a mechanism to terminate Hh signaling, as is the case in *Drosophila* eye development. It thus will be interesting to know whether Spop expression depends on Hh signaling in mouse embryonic development, and whether the association of Spop with Glis depends on the state of the Hh pathway. In both flies and mammals, defects of Ci/Gli degradation mechanisms potentiate the ability of Ci/Gli proteins to induce tissue overgrowth or tumor formation. Future studies should determine whether Spop acts as tumor suppressor and whether it contributes to Gli-dependent tumor formation. So, although important differences in Hh signaling between flies and mammals exist, as illustrated with the role of the primary cilium, the level of conservation of Hh signaling between species is still a topical question.

### Acknowledgments

We thank Katie Ayers and Rune Tofgard for helpful comments on the manuscript. Our research is supported by grants from La Ligue Nationale Contre Le Cancer "Equipe labellisée 2008."

### Note added in proof

During the proof correction of this perspective, two studies related the knockout of the kinesin Kif7 in mice showing that Kif7 is a critical regulator of Hh signaling in vivo (Cheung et al. 2009; Endoh-Yamagami et al. 2009).

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