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Dual function of suppressor of fused in Hh pathway activation and mouse spinal cord patterning

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ABSTRACT

The morphogen Sonic hedgehog, one of the Hedgehog (Hh) family of secreted proteins, plays a key role in patterning the mammalian spinal cord along its dorsoventral (D/V) axis through the activation of Glioma-associated oncogene (Gli) family of transcription factors. Suppressor of Fused (Sufu), a Gli-interacting protein, modulates the D/V patterning of the spinal cord by antagonizing Hh signaling. The molecular mechanisms underlying the function of Sufu in Hh pathway activation and spinal cord D/V patterning remain controversial, particularly in light of recent findings that Sufu protects Gli2 and Gli3 proteins from proteasomal degradation. In the current study, we show that Hh pathway activation and dorsal expansion of ventral spinal cord cell types in the absence of Sufu depend on the activator activities of all three Gli family proteins. We also show that Sufu plays a positive role in the maximal activation of Hh signaling that defines the ventral-most cell fate in the mammalian spinal cord, likely through protecting Gli2 and Gli3 proteins from degradation. Finally, by altering the level of Gli3 repressor on a background of reduced Gli activator activities, we reveal an important contribution of Gli3 repressor activity to the Hh pathway activation and the D/V patterning of the spinal cord.

Introduction

Sonic hedgehog (Shh), one of the Hedgehog (Hh) family of secreted signaling molecules, plays a key role in the generation of diverse neural progenitor cells along the dorsoventral (D/V) axis of the vertebrate spinal cord (Dessaud et al., 2008). Shh is initially produced by cells of the notochord, a mesodermal rod underlying the spinal cord, and induces cells at the ventral midline of the spinal cord to form the floor plate. The floor plate subsequently becomes an additional source of Shh. Shh from both the notochord and floor plate forms a ventral-to-dorsal gradient and regulates the formation of V3 interneurons, motor neurons, V2 and V1 interneurons. The specification and locations of these neurons and their progenitors are defined by the concentration of Shh. Specifically, V3 interneurons require higher Shh activity and are located right next to the floor plate, whereas motor neurons, V2 and V1 interneurons are defined at progressively lower Shh concentrations and are located in more lateral parts of the ventral spinal cord.

Shh elicits transcriptional responses in the spinal cord cells through a family of zinc-finger domain-containing transcription factors, the Glioma-associated oncogene family (Gli1, Gli2 and Gli3) (Matise and Joyner, 1999). In the absence of Shh, both Gli2 and Gli3 can be proteolytically processed into transcriptional repressors (Pan et al., 2006; Wang et al., 2000). Shh inhibits the processing of Gli2 and Gli3 and turns them into transcriptional activators. The processing of Gli2 is inefficient in vivo, hence it mainly exists as a transcriptional activator and is required for the formation of the floor plate and most V3 interneurons in the spinal cord (Ding et al., 1998; Matise et al., 1998; Pan et al., 2006). In contrast, Gli3 is efficiently processed into a transcriptional repressor, and loss of Gli3 does not affect patterning of the ventral spinal cord, but leads to a minor dorsal expansion of the cells near the D/V boundary (Persson et al., 2002; Wang et al., 2000). Interestingly, *Gli2;Gli3* double mutants exhibit more severe ventral spinal cord patterning defects than *Gli2* single mutants, suggesting redundant activator functions between Gli2 and Gli3 (Bai et al., 2004; Lei et al., 2004). Gli1 appears to be an obligate activator, but because its expression is dependent on Shh, Gli1 only plays a secondary role in enhancing Shh pathway activity in the ventral spinal cord after its initial activation by Gli2 and Gli3 (Bai et al., 2002; Park et al., 2000).

In mammals, one of the important negative regulators of Hh signaling and Gli activities is Suppressor of Fused (Sufu). Ablation of *Sufu* in mouse embryos leads to widespread activation of the Hh pathway (Cooper et al., 2005; Svard et al., 2006). Specifically, in the *Sufu* mutant spinal cord, ventral cell types such as the floor plate, V3 interneurons and motor neurons are greatly expanded dorsally at the expense of more dorsal cell types. Sufu physically associates with all three mammalian Gli proteins and inhibits Gli-mediated Hh pathway activation at least partially through sequestering full-length Gli proteins in the cytoplasm (Ding et al., 1999; Kogerman et al., 1999; Pearse et al.,

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1999; Stone et al., 1999). Both Sufu and Gli proteins are localized to the tips of the primary cilia, and it appears that Sufu traffics to the cilia in a Gli-dependent process (Haycraft et al., 2005; Tukachinsky et al., 2010; Zeng et al., 2010). Whether Sufu is dissociated from Gli proteins in the presence of Shh is not clear. One study reported sustained association between Sufu and Gli proteins (Chen et al., 2009). However, more recent studies suggest that activated Gli proteins are no longer associated with Sufu (Humke et al., 2010; Tukachinsky et al., 2010).

Paradoxically, despite the maximal activation of Hh signaling in *Sufu* mutants, the levels of Gli2 and Gli3 proteins decrease drastically in the absence of Sufu (Chen et al., 2009; Jia et al., 2009). This decrease in Gli2 and Gli3 protein levels appears to be the result of proteasomal degradation of these two proteins mediated by Spop/Cul3-containing ubiquitin ligase complex; and reducing Spop in *Sufu* mutant cells restores the levels of Gli2 and Gli3 proteins (Chen et al., 2009; Zhang et al., 2006, 2009). Two hypotheses have been proposed to interpret the apparent contradiction between Hh pathway activation and decrease in Gli2 and Gli3 protein levels in *Sufu* mutant embryos. One hypothesis is that the short-lived activator forms of Gli2 and Gli3, produced in the presence of Shh or in the absence of Sufu, directly activate the Hh pathway (Humke et al., 2010; Tukachinsky et al., 2010). The second hypothesis posits that the decrease in Gli2 and Gli3 proteins reduces the overall Gli repressor activity, de-repressing the expression of *Gli1* that activates Hh pathway in *Sufu* mutant embryos (Chen et al., 2009). These alternative explanations of the roles of Sufu in Hh signal transduction and mammalian spinal cord patterning have not been experimentally addressed.

In the current study, we address the roles of the three Gli family members in Hh pathway activation and spinal cord patterning in the absence of Sufu through a series of genetic analyses. We show that all three Gli proteins contribute to the ectopic activation of the Hh pathway and cell fate change in the *Sufu* mutant spinal cord. Interestingly, Gli1 and Gli2, but not Gli3, are required for the maximal activation of Hh signaling and the formation of the floor plate and V3 interneurons in the absence of Sufu. We further show that the activator activities of Gli2 and Gli3 are essential for Hh pathway activation in *Sufu* mutants because removing Sufu in the absence of Gli2 and Gli3, as in *Gli2;Gli3;Sufu* triple mutants, fails to activate Hh signaling and ventralize the spinal cord. Significantly, by reducing overall Gli activator activities, as in *Gli1;Sufu* and *Gli2^{3ki};Sufu* embryos, we reveal a positive role of *Sufu* in the maximal activation of Hh signaling. Finally, by lowering the overall Gli activator activity, we showed a repressive role of Gli3 in Hh signaling and the D/V patterning of the *Sufu* mutant spinal cord. Our data suggest that Sufu plays a negative role in Hh pathway activation and ventral spinal cord patterning both by direct inhibition of Gli2 and Gli3 activator activities and by maintaining the proper level of Gli repressors. In addition, Sufu plays a positive role in the maximal activation of Hh signaling, likely through its role in protecting Gli2 and Gli3 proteins from degradation.

Materials and methods

Ethics statement

All animal work conducted in this report is in accordance of national and international guidelines and was approved by IACUC (#29195 and #29214) at Penn State University.

Mouse strains

Sufu, Gli1^{lacKI}, Gli2^{lacKI}, Gli2^{3KI}, Gli3$^{Ex\text{-}J}$ mutant mice are kept on 129S2/SvPasCrl (*Charles River Lab*) background and genotyped as previously described (Bai and Joyner, 2001; Bai et al., 2002, 2004; Maynard et al., 2002; Svard et al., 2006). Mouse embryos at specified stages are dissected in phosphate buffered saline (PBS) and documented using a Zeiss Discovery microscope and a QImaging Micropublisher digital camera.

Immunohistochemistry

Embryos were fixed in 4% paraformaldehyde (PFA) at 4 °C for 1 h and processed for cryosection. The sections were incubated with primary antibodies at 4 °C overnight followed by a 2 hour incubation with fluorescently labeled secondary antibodies, and mounted with DABCO (Sigma-Aldrich). Pictures were taken using a Nikon E600 microscope and a QImaging Micropublisher Digital Camera.

Xgal histochemistry

Embryos were fixed in 4% PFA at 4 °C for 1 h, washed in PBS three times and incubated with 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (Xgal) at 37 °C overnight. Pictures were taken using a Zeiss Discovery microscope and a QImaging Micropublisher digital camera.

RNA in situ hybridization

Embryos were fixed in 4% PFA at 4 °C overnight, washed in DEPC-treated PBS and processed for cryosection. RNA in situ hybridization with Digoxigenin-labeled riboprobes against *Gli1* and *Ptch1* was performed on the transverse sections through the spinal cord according to the protocol originally described in Hoover et al. (2008). The photos were taken using a Nikon E600 microscope and a QImaging Micropublisher digital camera.

Western blot

E10.5 mouse embryos were dissected in cold PBS. Whole protein lysate was prepared from individual embryos. 20 μg of lysate from each sample was then loaded onto 7.5% SDS-PAGE gels and western blotting was performed as described (Wang et al., 2000). The rabbit polyclonal antibody against the N-terminal region of Gli3 was originally reported in Wang et al. (2000). Anti β-tubulin antibody (Sigma, T4026) was used as a loading control. The result of western blot was quantitated using NIH ImageJ.

Results

The maximal activation of Hh pathway in Sufu mutants requires Gli1

Recent studies indicated that Gli2 and Gli3, but not Gli1, are subject to Spop/Cul3-mediated degradation in the absence of Sufu (Chen et al., 2009; Zhang et al., 2009). Therefore, it was hypothesized that ectopic *Gli1* expression, possibly as a result of decreased Gli repressor activity, underlies the widespread Hh pathway activation in *Sufu* mutants (Chen et al., 2009). An in vitro reporter assay suggested that Gli1 is required for the maximal activation of Hh target gene expression in the absence of *Sufu*. To address the contribution of Gli1 to Hh pathway activation in vivo, we characterized *Gli1;Sufu* double mutants. Consistent with previous reports (Bai et al., 2002; Park et al., 2000), there is no visible morphological difference between wild type (Fig. 1A) and *Gli1* homozygous mutants (Fig. 1B) at embryonic day (E) 10.5. *Sufu* mutants exhibit severe exencephaly, spina bifida, twisted body axis and underdevelopment of structures posterior to the forelimbs (Fig. 1C). Significantly, *Gli1;Sufu* double homozygous mutant embryos exhibit similar degree of exencephaly and spina bifida as *Sufu* homozygous mutants, but show more advanced posterior development and straight body axis, suggesting that loss of Gli1 may partially suppress the Hh pathway defects in *Sufu* mutants (Fig. 1D).

To analyze Hh pathway activity in greater detail, we compared the D/V patterning of the *Gli1;Sufu* double mutant spinal cord with that of *Sufu* single mutants. Previous studies suggested that there are subtle differences in the patterning mechanisms between the anterior

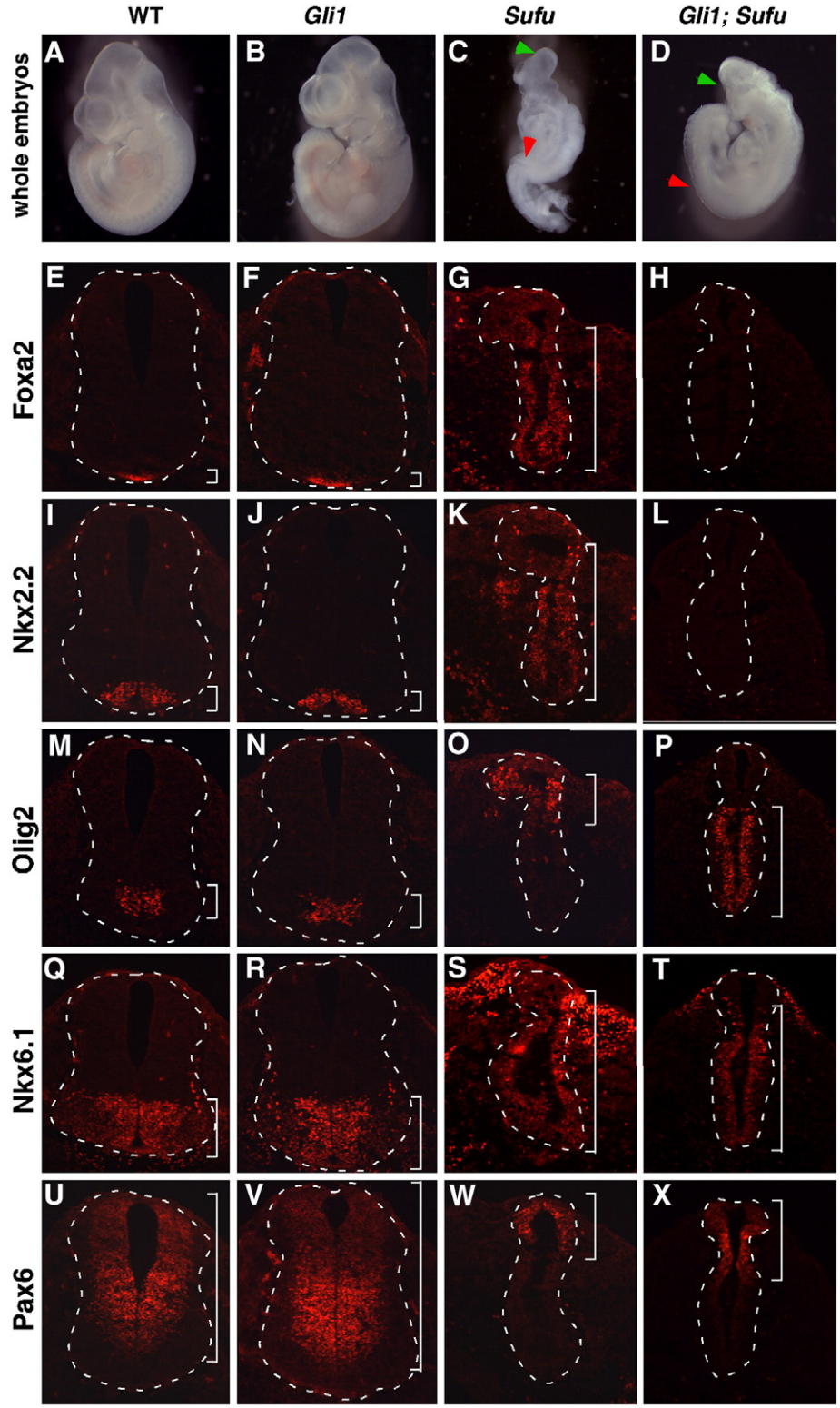


Fig. 1. The floor plate and V3 interneurons fail to form in *Gli1;Sufu* double mutant embryos. (A–D) Lateral views of E10.5 wild type (A), *Gli1* mutant (B), *Sufu* mutant (C), and *Gli1;Sufu* double mutant (D) embryos. Green arrowheads in C and D point to exencephaly and red arrowheads point to spina bifida. (E–X) Immunofluorescent images of the transverse sections of E10.5 spinal cords at the thoracic level. Dashed lines outline the spinal cords. Brackets indicate the domains of expression. (E) Foxa2 labels the floor plate in the wild type spinal cord. (F) Floor plate formation is not affected by loss of Gli1. (G) Foxa2 is found throughout the *Sufu* mutant spinal cord. (H) Foxa2 is absent in *Gli1;Sufu* double mutant spinal cord. (I) Nkx2.2 is found in V3 interneurons and their progenitors next to the floor plate in the wild type spinal cord. (J) V3 interneuron formation is normal in *Gli1* mutants. (K) Nkx2.2^{+} cells are throughout the spinal cord. (L) Nkx2.2 is absent in the *Gli1;Sufu* double mutant spinal cord. (M) Olig2 labels the progenitors for motor neurons, which are dorsal to V3 interneurons. (N) The motor neuron formation is normal in *Gli1* mutants. (O) The motor neuron domain is shifted to the dorsal region of the *Sufu* mutant spinal cord. (P) Olig2^{+} domain is expanded both dorsally and ventrally in the *Gli1;Sufu* double mutant spinal cord. (Q) Nkx6.1 labels progenitors for most ventral spinal cord cells including V2, V3 interneurons, motor neurons and floor plate. (R) Nkx6.1 domain is normal in the *Gli1* mutant spinal cord. (S and T) Nkx6.1 expression is found in all but the dorsal-most cells in the *Sufu* mutant (S) and *Gli1;Sufu* double mutant (T) spinal cord. (U) Pax6 labels progenitor cells for most spinal cord neurons except for the ventral-most V3 interneurons and the floor plate. (V) Pax6 domain is normal in *Gli1* mutants. (W) Pax6 expression is restricted to a few dorsal-most cells in the *Sufu* mutant spinal cord. (X) Pax6^{+} cells are restricted dorsally in the *Gli1;Sufu* double mutant spinal cord.

(thoracic) and posterior (lumbar) spinal cord (e.g. Motoyama et al., 2003). For both accuracy and completeness, throughout this study we examined the D/V patterning of the spinal cords at both axial levels. The results from the anterior spinal cords are shown in Figs. 1–3 and 5–7, and those from the posterior spinal cords are shown in Supplemental Figs. 1–4, 6 and 7.

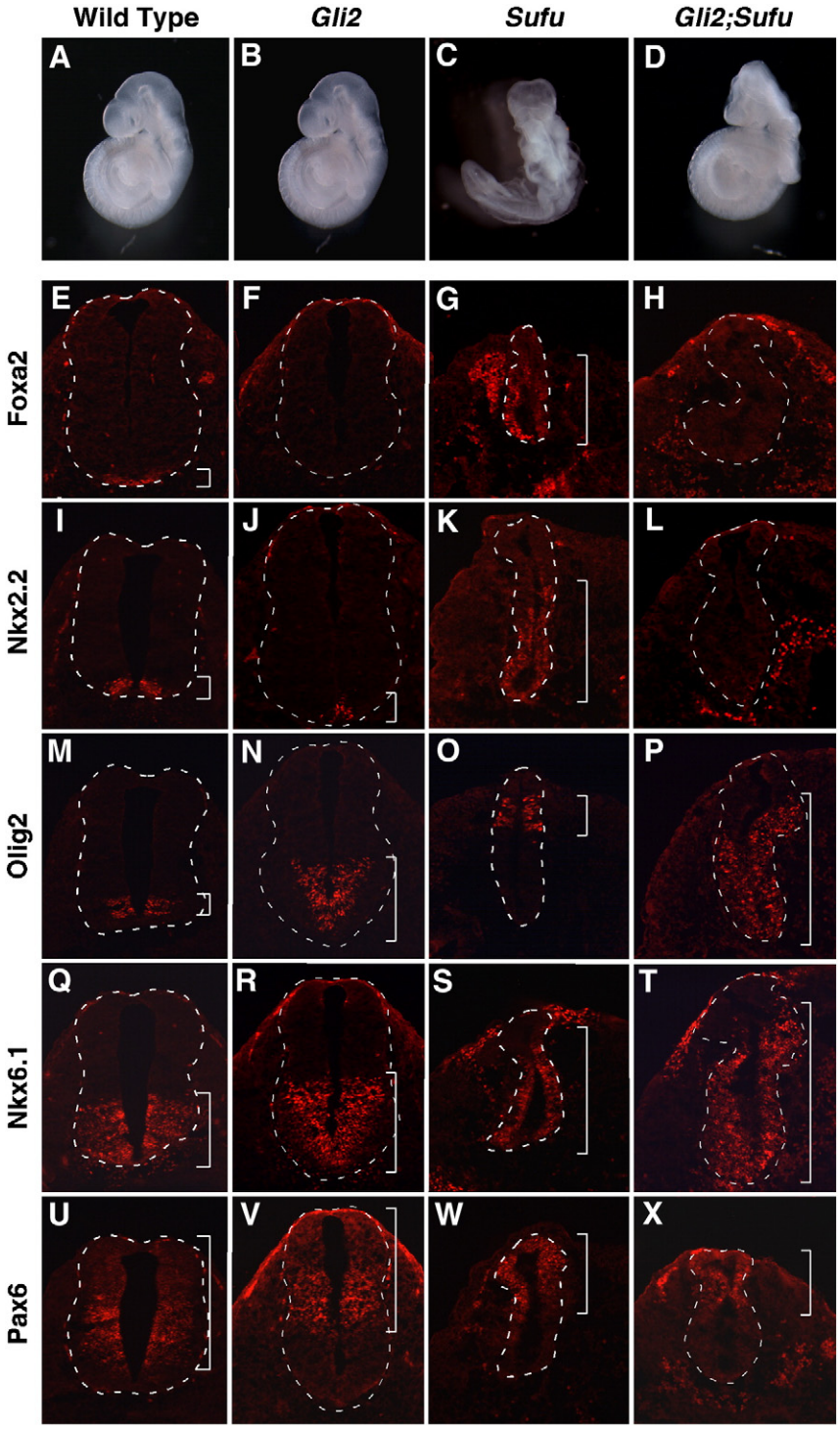


Fig. 2. The floor plate and V3 interneurons fail to form in *Gli2;Sufu* double mutant embryos. (A–D) Lateral views of E9.5 wild type (A), *Gli2* mutant (B), *Sufu* mutant (C), and *Gli2;Sufu* double mutant (D) embryos. (E–X) Immunofluorescent images of the transverse sections of E10.5 spinal cords at the thoracic level. Dashed lines outline the spinal cords. Brackets indicate the domains of expression. (E) Foxa2 labels the floor plate. (F) The floor plate is absent in the *Gli2* mutant spinal cord. (G) Foxa2 is found throughout the spinal cord in *Sufu* mutants. (H) Foxa2 is absent in the *Gli2;Sufu* double mutant spinal cord. (I) Nkx2.2 labels V3 interneurons and their progenitors. (J) A reduced number of V3 interneurons are present in the ventral midline of the *Gli2* mutant spinal cord. (K) The Nkx2.2 domain is dorsally expanded in the *Sufu* mutant spinal cord. (L) Nkx2.2^{+} cells are absent or greatly reduced in the *Gli2;Sufu* double mutant spinal cord. (M) Olig2 labels the progenitors for motor neurons. (N) The motor neuron domain is ventrally expanded in *Gli2* mutants. (O) The motor neuron domain is dorsally shifted in the *Sufu* mutant spinal cord. (P) The motor neuron domain is expanded both dorsally and ventrally in *Gli2;Sufu* double mutant spinal cord. (Q) Nkx6.1 labels most ventral progenitor cells including those for V2, V3 interneurons, motor neurons and the floor plate. (R) Nkx6.1 expression is normal in *Gli2* mutant spinal cord. (S and T) Nkx6.1 expression is expanded into the dorsal regions of the *Sufu* mutant (S) and *Gli2;Sufu* double mutant (T) spinal cord. (U) Pax6 labels most progenitor cells except for the ventral-most V3 interneurons and the floor plate. (V) Pax6 expression is normal in *Gli2* mutants. (W and X) Pax6 expression is restricted to the dorsal regions of the *Sufu* mutant (W) and *Gli2;Sufu* double mutant (X) spinal cord.

At E10.5, region-specific expression of a series of homeobox and basic helix-loop-helix transcription factor genes in the spinal cord progenitor domain defines the progenitors for various neuronal cell types (Dessaud et al., 2008). In the anterior spinal cord, *Foxa2* is expressed in the floor plate cells at the ventral midline of the spinal cord (Fig. 1E). Nkx2.2 labels progenitors for V3 interneurons

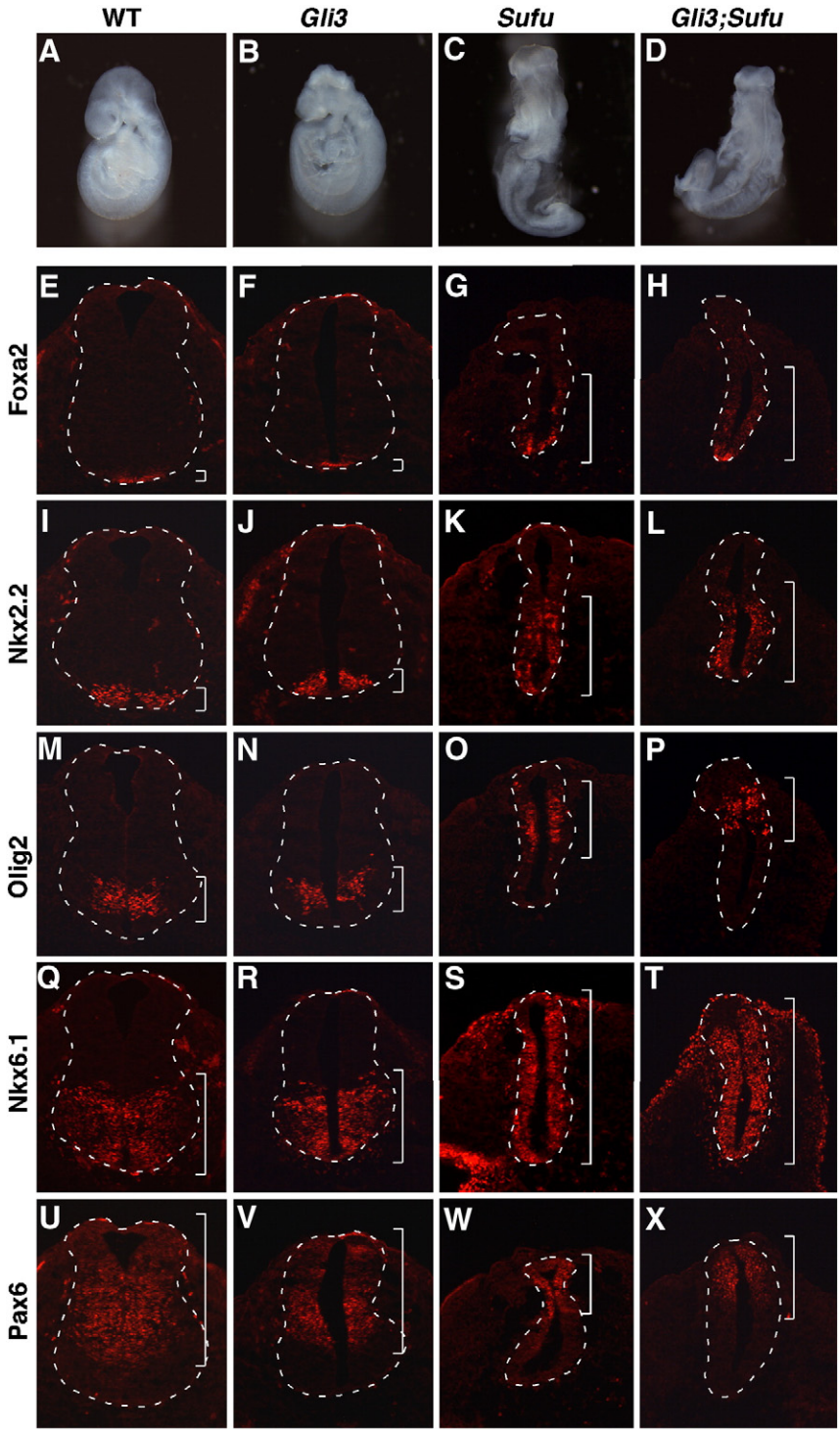


Fig. 3. Gli3 is not required for the activation of Hh signaling and the ventralization of the spinal cord in the absence of *Sufu*. (A–D) Lateral views of E9.5 wild type (A), *Gli3* mutant (B), *Sufu* mutant (C), and *Gli3;Sufu* double mutant (D) embryos. (E–X) Immunofluorescent images of the transverse sections of E10.5 spinal cords at the thoracic level. Dashed lines outline the spinal cords. Brackets indicate the domains of expression. (E) Foxa2 labels the floor plate. (F) Floor plate formation is not affected by loss of Gli3. (G) Foxa2 domain is dorsally expanded in the *Sufu* mutant spinal cord. (H) Foxa2 domain is dorsally expanded in the *Gli3;Sufu* double mutant spinal cord. (I) Nkx2.2 is found in V3 interneurons and their progenitors. (J) The Nkx2.2 domain is normal in *Gli3* mutants. (K and L) The Nkx2.2 domain is dorsally expanded in the *Sufu* mutant (K) and *Gli3;Sufu* double mutant (L) spinal cord. (M) Olig2 labels the progenitors for motor neurons. (N) The motor neuron formation is normal in *Gli3* mutants. (O and P) The motor neuron domain is shifted to the dorsal regions of the *Sufu* mutant (O) and *Gli3;Sufu* double mutant (P) spinal cord. (Q) Nkx6.1 labels progenitors for most ventral spinal cord cells including V2, V3 interneurons, motor neurons and the floor plate. (R) Nkx6.1 expression is normal in the *Gli3* mutant spinal cord. (S) Nkx6.1 expression is expanded to the entire *Sufu* mutant spinal cord. (T) Nkx6.1 expression is present in all but the dorsal-most cells of the *Gli3;Sufu* double mutant spinal cord. (U) Pax6 labels most progenitor cells except for the ventral-most V3 interneurons and the floor plate. (V) Pax6 is normal in *Gli3* mutants. (W) Pax6 expression is restricted to a few dorsal-most cells in the *Sufu* mutant spinal cord. (X) Pax6 is present in the dorsal-most cells of the *Gli3;Sufu* double mutant spinal cord.

immediately dorsal to the floor plate (Fig. 1I). *Olig2*-expressing progenitor cells give rise to motor neurons next to V3 interneurons (Fig. 1M). *Nkx6.1* is expressed in progenitors for most ventral spinal cord cells including the floor plate, V3 interneurons, motor neurons and V2 interneurons (Fig. 1Q). Pax6 is present in all spinal cord progenitor cells except for the ventral-most ones that give rise to V3 interneurons and the floor plate (Fig. 1U). Shh activates the expression of *Foxa2, Nkx2.2* and *Nkx6.1*, and represses the expression of *Pax6* in

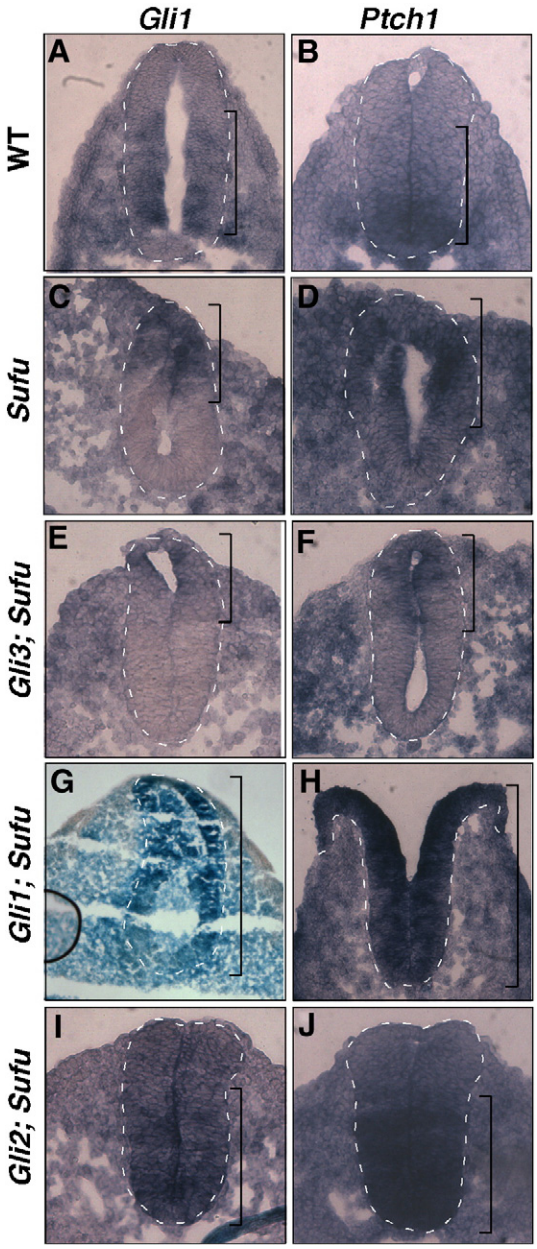


Fig. 4. The roles of Sufu and Gli proteins in the regulation of *Gli1* and *Ptch1* expression in the developing spinal cords. (A, B) Both *Gli1* and *Ptch1* are expressed in a ventral-to-dorsal gradient in the ventral spinal cord but their expression is excluded from the ventral-most cells of the spinal cord. (C, D) The expression domains of *Gli1* and *Ptch1* are shifted to the dorsal region of the *Sufu* mutant spinal cord. (E, F) The expression of both Gli1 and Ptch1 are dorsally restricted in the *Gli3;Sufu* double mutant spinal cords. (G, H) Both *Gli1* (indicated by a *Gli1-lacZ* reporter) and *Ptch1* are expressed throughout the spinal cords in *Gli1;Sufu* double mutants. (I, J) Both *Gli1* and *Ptch1* are expressed in the entire spinal cord, but the expression is much stronger ventrally. The sample shown in G is a transverse section of an E9.5 embryo at the spinal cord level with Xgal staining. Other samples shown are transverse sections of E9.5 embryos at the spinal cord level processed for RNA in situ hybridization. White dashed lines outline the spinal cords and the brackets indicate the domains of gene expression in the spinal cords.

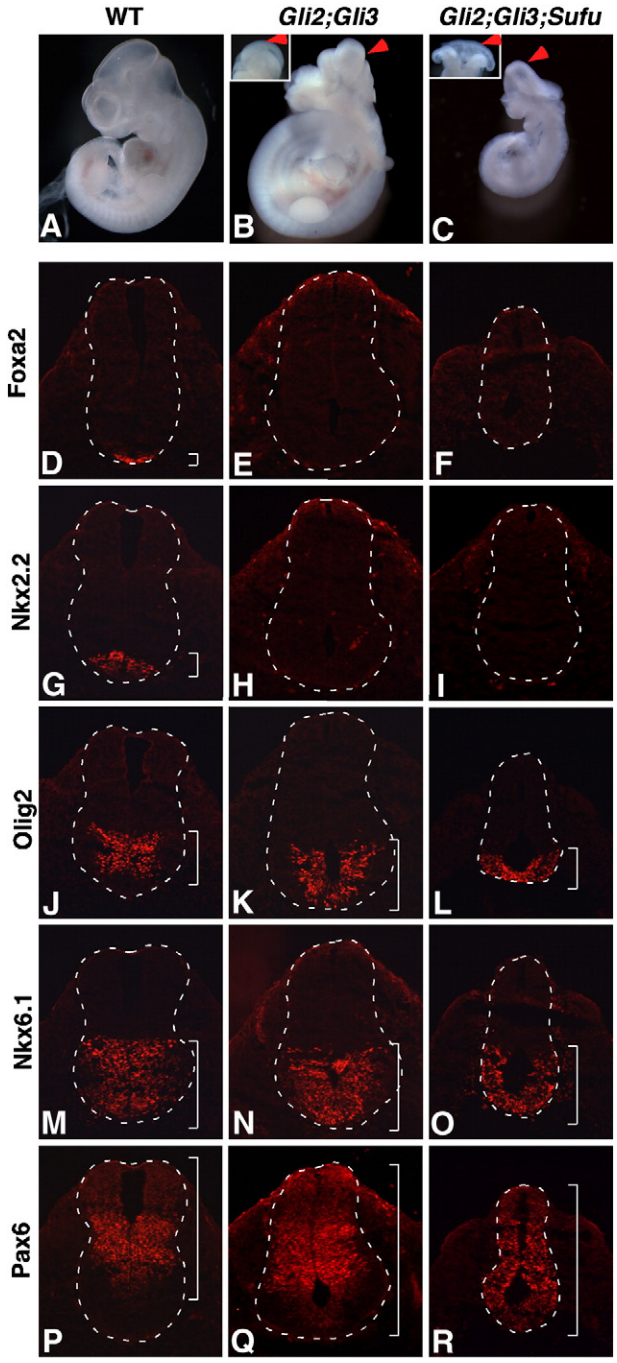


Fig. 5. Hh pathway is not activated in *Gli2;Gli3;Sufu* triple mutants. (A–C) Lateral views of E10.5 wild type (A), *Gli2;Gli3* double mutant (B) and *Gli2;Gli3;Sufu* triple mutant (C) embryos. Insets in B and C show the front views of the brains. Arrowheads point to the elevated floor of the midbrain. (D–R) Immunofluorescent images of the transverse sections of E10.5 spinal cords at the thoracic level. Dashed lines outline the spinal cords. Brackets indicate the domains of expression. (D) Foxa2 labels the floor plate. (E) The floor plate is absent in *Gli2;Gli3* double mutants. (F) Foxa2 is absent in *Gli2;Gli3;Sufu* triple mutants. (G) Nkx2.2 labels V3 interneurons and their progenitors. (H) Nkx2.2 expression is absent in the *Gli2;Gli3* double mutant spinal cord. (I) Nkx2.2 expression is absent in the *Gli2;Gli3;Sufu* triple mutant spinal cord at the thoracic level. (J) Olig2 labels the progenitors for motor neurons. (K and L) The motor neuron domain is expanded ventrally in the *Gli2;Gli3* double mutant (K) and *Gli2;Gli3;Sufu* triple mutant (L) spinal cord. (M) Nkx6.1 labels progenitors for V2, V3 interneurons, motor neurons and the floor plate. (N and O) Nkx6.1 expression is normal in the *Gli2;Gli3* double mutant (N) and *Gli2;Gli3;Sufu* triple mutant (O) spinal cord. (P) Pax6 labels most progenitor cells except for the ventral-most V3 interneurons and the floor plate. (Q and R) Pax6 expression is expanded ventrally in the *Gli2;Gli3* double mutant (Q) and *Gli2;Gli3;Sufu* triple mutant (R) spinal cord.

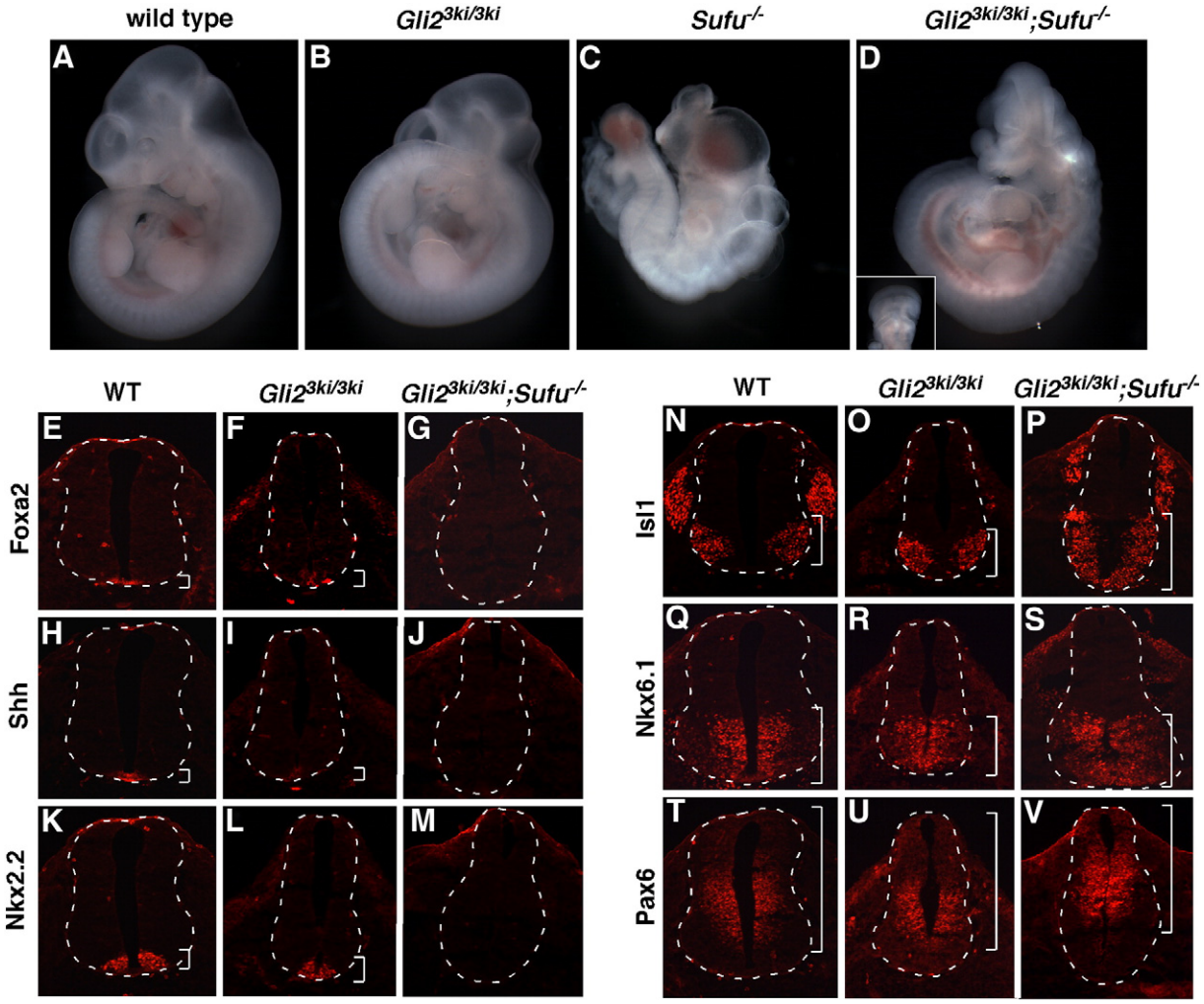


Fig. 6. Sufu plays a positive role in Hh signaling during mouse spinal cord dorsoventral patterning. (A–D) Lateral views of E10.5 wild type (A), *Gli2*^{3ki} homozygous mutant (B), *Sufu* mutant (C) and *Gli2*^{3ki}*;Sufu* double mutant (D) embryos. Inset in D shows the front view of the brain. (E–V) Immunofluorescent images of the transverse sections of E10.5 spinal cords at the thoracic level. Dashed lines outline the spinal cords. Brackets indicate the domains of expression. (E) Foxa2 labels the floor plate. (F) A few Foxa2^{+} cells are present in the *Gli2*^{3ki} mutant spinal cord. (G) Foxa2 is absent in the *Gli2*^{3ki}*;Sufu* double mutant spinal cord. (H) Shh protein is found on the surface of the cells in the floor plate. (I) A few Shh^{+} cells are present in the *Gli2*^{3ki} mutant spinal cord. (J) Shh is absent in the *Gli2*^{3ki}*;Sufu* double mutant spinal cord. (K) Nkx2.2 is found in V3 interneurons and their progenitors. (L) A few V3 interneurons are present in *Gli2*^{3ki} mutants. (M) Nkx2.2^{+} cells are absent in the *Gli2*^{3ki}*;Sufu* double mutant spinal cord. (N) Isl1 labels differentiated motor neurons. (O) In the anterior *Gli2*^{3ki} mutant spinal cord, Isl1 domain is expanded ventrally but is still excluded from the ventral midline. (P) In *Gli2*^{3ki}*;Sufu* double mutant spinal cord, Isl1 expression is no longer excluded from ventral midline. Its expression is also expanded dorsally. (Q) Nkx6.1 labels most ventral cells including V2, V3 interneurons, motor neurons and the floor plate. (R and S) Nkx6.1 expression is normal in *Gli2*^{3ki} mutant (R) and *Gli2*^{3ki}*;Sufu* double mutant (S) spinal cords. (T) Pax6 labels most progenitor cells except for the ventral-most V3 interneurons and floor plate. (U) Pax6 domain is normal in *Gli2*^{3ki} mutant spinal cord. (V) The domain of strong Pax6 expression in *Gli2*^{3ki}*;Sufu* double mutant spinal cord is slightly more dorsally restricted than wild type, but a few cells in the ventral region show weak Pax6 expression.

the ventral spinal cord (Dessaud et al., 2008). *Olig2* expression appears to be dependent on low levels of Shh but is inhibited by the highest level of Hh pathway activation.

Consistent with our morphological analysis and previous reports, all the spinal cord progenitor cell types we examined are generated in their normal locations in *Gli1* homozygous mutants (Fig. 1F, J, N, R and V) (Bai et al., 2002; Park et al., 2000). In contrast, the *Sufu* mutant spinal cord is severely ventralized, with floor plate (Foxa2^{+}; Nkx6.1^{+}) and V3 interneurons (Nkx2.2^{+}; Nkx6.1^{+}) expanded into the dorsal region of the spinal cord (Fig. 1G, K and S). The domain of motor neurons (Olig2^{+}) is shifted to the dorsal–lateral region of the spinal cord (Fig. 1O), supporting the idea of a dorsally expanded Hh pathway activity gradient. It also indicates that some D/V positional information still exists in the *Sufu* mutant spinal cord. Consistently, *Pax6* expression is also restricted to the dorsal-most part of the spinal cord in *Sufu* mutants (Fig. 1W).

In all *Gli1;Sufu* double mutant embryos examined, the dorsal boundaries of the expression domains of *Olig2* (n = 46 sections from 6 embryos) and *Nkx6.1* (n = 14 sections from 2 embryos) are shifted dorsally compared to those in wild type and *Gli1* mutant littermates (Fig. 1P and T), suggesting a widespread Hh pathway activation. *Pax6* expression is also more dorsally restricted in *Gli1;Sufu* double mutants than in wild type spinal cords (Fig. 1X; n = 11 sections from 2 embryos). As Gli1 activity is eliminated in these embryos, these results suggest that ectopic activation of Gli1 is not solely responsible for the ectopic activation of Hh signaling and ventralization of the spinal cord in *Sufu* mutants.

Interestingly, despite the widespread Hh pathway activation and dorsal expansion of ventral cell types such as motor neurons and possibly V2 interneurons, cell types that require the maximal activation of the Hh pathway, such as the floor plate and V3 interneurons, are greatly reduced or fail to form in the *Gli1;Sufu* double mutant spinal cord (Fig. 1H and L and data not shown; a total of 3 embryos were examined, among which 5 sections showed greatly reduced floor plate whereas 19 sections showed no floor plate. As to V3 interneurons, we found no V3 interneurons in 10 sections and greatly reduced

number of V3 interneurons in 15 sections). Consistent with the idea that Hh signaling is not maximally activated, the expression of *Olig2* is no longer excluded from the ventral-most region of the spinal cord in *Gli1;Sufu* double mutants (Fig. 1P; n = 46 sections from 6 embryos). From these observations we conclude that Gli1 is required for the maximal activation of the Hh pathway, but not for the low-to-intermediate levels of Hh signaling in *Sufu* mutants.

D/V patterning of the posterior (lumbar level) spinal cords in *Gli1, Sufu* mutants and *Gli1;Sufu* double mutants is similar to that of the anterior spinal cord, except that the loss of floor plate and V3 interneurons was found in all sections examined (Supplemental Fig. 1; n = 28 sections for floor plate and n = 26 sections for V3 interneurons from 3 embryos). In addition, the posterior part of neural tube fails to close in *Sufu* mutants and *Gli1;Sufu* double mutants (Fig. 1C, D and Supplemental Fig. 1).

Maximal Hh pathway activation in Sufu mutants requires the activator function of Gli2

The dorsal expansion of the *Olig2* and *Nkx6.1* expression domains in the *Gli1;Sufu* double mutant spinal cord suggests that the other two Gli family members may contribute to the ectopic activation of the Hh pathway in *Sufu* mutants. To address the roles of Gli2 and Gli3 in Hh pathway activation and spinal cord ventralization in *Sufu* mutants, we generated *Gli2;Sufu* and *Gli3;Sufu* double mutants, as well as *Gli2;Gli3;Sufu* triple mutants. We first characterized *Gli2;Sufu* double mutants. Morphologically, *Gli2* homozygous mutants exhibit variable mild defects ranging from a slightly tighter mesencephalic flexure to midbrain exencephaly at E9.5 (Fig. 2A, B and data not shown). The *Gli2;Sufu* double homozygous mutant embryos are of normal size and exhibit less severe defects in craniofacial and posterior development than *Sufu* single mutants, suggesting that loss of Gli2 partially suppresses the *Sufu* mutant phenotype (Fig. 2C and D). However, *Gli2;Sufu* double mutants exhibit more frequent and more severe exencephaly than *Gli2* single mutants, suggesting that Gli2 activation does not account for all ectopic Hh pathway activation in *Sufu* mutants.

To better understand the roles of Gli2 in Hh pathway activation and spinal cord patterning in the absence of Sufu, we examined the D/V patterning of the spinal cords in *Gli2;Sufu* double mutants. In the *Gli2* homozygous mutant spinal cords, the floor plate and most V3 interneurons are missing and the domain of motor neurons is expanded ventrally (Fig. 2F, J and N). More dorsal regions of the spinal cords are not affected by the loss of Gli2 (Fig. 2R and V). Interestingly, the floor plate and V3 interneurons, two cell types requiring the highest levels of Hh signaling, fail to form or greatly reduced in *Gli2;Sufu* double mutants, suggesting that Gli2 activator activity is critical for maximal activation of the Hh pathway in the absence of Sufu (Fig. 2H and L; among 4 embryos examined, floor plate is absent in 24 sections, and present in reduced numbers in 6 sections; V3 interneurons are absent in 16 sections and greatly reduced in 15 sections). Consistently, motor neurons are present in the ventral-most region of the spinal cord, indicating a lack of repression from high levels of Hh activities (Fig. 2P; n = 30 sections from 4 embryos). On the other hand, lower levels of Hh signaling activities are present and ectopically activated in *Gli2;Sufu* double mutant spinal cord, because motor neurons ($Olig2^+$ and $Nkx6.1^+$) are still expanded dorsally (Fig. 2P and T; for $Olig2^+$ cells, n = 30 sections from 4 embryos; for $Nkx6.1^+$ cells, n = 19 sections from 3 embryos), and the expression domain of *Pax6* is more dorsally restricted compared to wild type (Fig. 2X; n = 16 sections from 2 embryos). These data suggest that the activator function of Gli2 is also responsible for the activation of high levels of Hh signaling in *Sufu* mutants, but is not required for the ectopic activation of lower levels of Hh signaling.

The same requirement for Gli2 in the maximal activation, but not lower level activation, of Hh signaling is also revealed in the posterior regions of the spinal cord (Supplemental Fig. 2).

Removing Gli3 does not change spinal cord patterning in Sufu mutants

Some previous studies suggested that in addition to strong repressor activity, Gli3 also exhibits activator activity in certain contexts (Bai et al., 2004; Lei et al., 2004; Motoyama et al., 2003). We therefore addressed the possibility that Gli3 activator activity contributes to the Hh pathway activation in *Sufu* mutants. We first investigated whether Gli3 is required for Hh pathway activation in the absence of Sufu by characterizing *Gli3;Sufu* double mutants. At E9.5, *Gli3* mutants exhibit frequent exencephaly in the midbrain and anterior hindbrain, but are otherwise similar to their wild type littermates morphologically (Fig. 3A and B). *Gli3;Sufu* double mutant embryos exhibit severe exencephaly, spina bifida, a twisted body and underdevelopment of the posterior part of the body, similar to *Sufu* single mutants (Fig. 3C and D). The failure to suppress the *Sufu* mutant phenotype by removing Gli3 suggests that Hh pathway activation does not require Gli3 in the absence of *Sufu*.

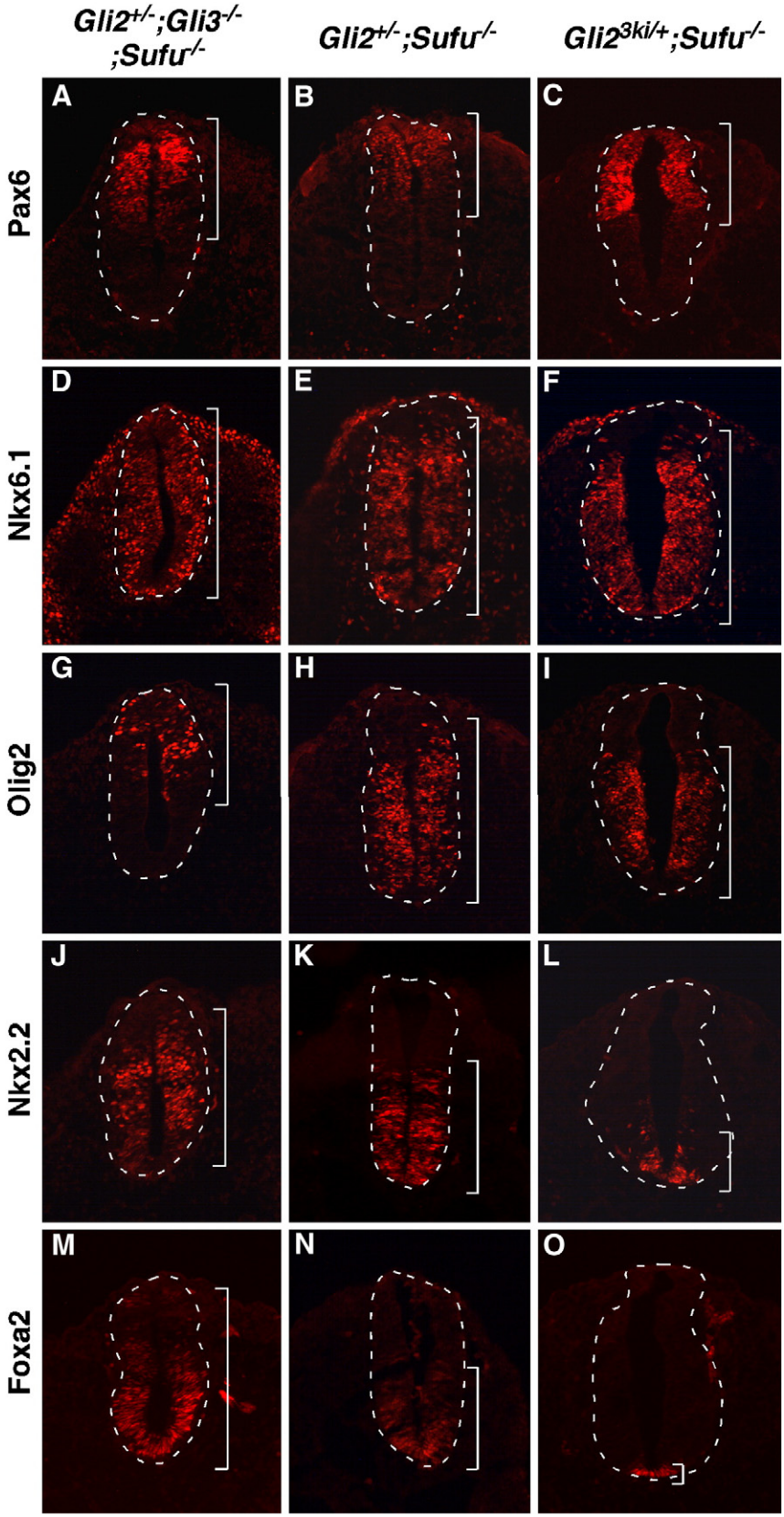
To investigate whether a subtle difference exists between *Sufu* mutants and *Gli3;Sufu* double mutants, we compared spinal cord D/V patterning in these two mutants. The formation of the floor plate, V2, V3 interneurons and motor neurons is not affected by loss of Gli3 alone (Fig. 3F, J, N, R and V). In the *Gli3;Sufu* double mutant spinal cord, the floor plate and V3 interneurons are dorsally expanded to the same degree as in *Sufu* mutants (Fig. 3G, H, K and L; n = 14 sections from 2 *Gli3;Sufu* embryos for both Foxa2 and Nkx2.2). Motor neurons are also shifted to a dorsal domain in both *Gli3;Sufu* double mutants and *Sufu* mutants (Fig. 3O and P; n = 14 sections from 2 *Gli3;Sufu* double mutant embryos for Olig2). The dorsal expansion of the Nkx6.1 domain and restriction of the Pax6 domain are also similar in *Gli3;Sufu* double mutants and *Sufu* single mutants (Fig. 3S, T, W and X; n = 14 sections from 2 *Gli3;Sufu* mutant embryos for Pax6 and Nkx6.1). The same observation was made in the posterior spinal cord (Supplemental Fig. 3). The lack of impact on the morphology and spinal cord patterning by removing Gli3 in the *Sufu* mutant embryos suggests that the Hh pathway activation in *Sufu* mutants does not require Gli3 when the other two Gli proteins are present.

The expression of Gli1 and Ptch1 responds to the loss of various Gli genes in the absence of Sufu

The expression of *Gli1* and *Ptch1* has been widely used as indicators of Hh pathway activation in both in vivo and in vitro studies. We thus examined the expression of these two genes in the wild

Fig. 7. The effect of Gli3 repressor on D/V patterning of the spinal cord in the absence of Sufu. Immunofluorescent images of the transverse sections of E10.5 spinal cords at the thoracic level. Dashed lines outline the spinal cords. Brackets indicate the domains of expression. (A) In $Gli2^{+/-}$;$Gli3^{-/-}$;$Sufu^{-/-}$ mutants, Pax6 is strongly expressed in the dorsal spinal cord, and weakly in some more ventrally located cells. (B) In $Gli2^{+/-}$;$Sufu^{-/-}$ mutants, strong Pax6 expression is still restricted to the dorsal spinal cord, but it appears that more ventral cells are expressing Pax6 at low levels. (C) In $Gli2^{3ki/+}$;$Sufu^{-/-}$ mutants, strong Pax6 expression is still restricted to the dorsal spinal cord, but it appears that more ventral cells are expressing Pax6 at low levels. (D) Nkx6.1 expression is throughout the $Gli2^{+/-}$;$Gli3^{-/-}$;$Sufu^{-/-}$ mutant spinal cord. (E) Nkx6.1 expression is excluded from the dorsal-most part of the $Gli2^{+/-}$;$Sufu^{-/-}$ mutant spinal cord. (F) Nkx6.1 expression is excluded from the dorsal-most part of the $Gli2^{3ki/+}$;$Sufu^{-/-}$ mutant spinal cord. (G) Olig2 is expressed in the dorsal half of the $Gli2^{+/-}$;$Gli3^{-/-}$;$Sufu^{-/-}$ mutant spinal cord. (H) Olig2 expression is excluded from the dorsal-most and ventral-most cells of the $Gli2^{+/-}$;$Sufu^{-/-}$ mutant spinal cord. (I) Olig2 expression is excluded from the dorsal-most and ventral-most cells of the $Gli2^{3ki/+}$;$Sufu^{-/-}$ mutant spinal cord. (J) Nkx2.2 is expressed in all but the dorsal-most region of the $Gli2^{+/-}$;$Gli3^{-/-}$;$Sufu^{-/-}$ mutant spinal cord. (K) Nkx2.2 expression is restricted to the ventral half of the $Gli2^{+/-}$;$Sufu^{-/-}$ mutant spinal cord. (L) In $Gli2^{3ki/+}$;$Sufu^{-/-}$ mutants, strong Nkx2.2 expression is found in the ventral midline of the spinal cord. A few cells weakly expressing Nkx2.2 are scattered in the ventral half of the spinal cord. (M) In $Gli2^{+/-}$;$Gli3^{-/-}$;$Sufu^{-/-}$ mutants, Foxa2 expression is found throughout the spinal cord. (N) In the $Gli2^{+/-}$;$Sufu^{-/-}$ mutant spinal cord, Foxa2 is restricted to the ventral half of the spinal cord. (O) In the $Gli2^{3ki/+}$;$Sufu^{-/-}$ mutant spinal cord, Foxa2 is only expressed in the floor plate.

type and various mutant spinal cords at E9.5. In the wild type spinal cord, both *Gli1* and *Ptch1* are expressed in a ventral to dorsal gradient (Fig. 4A and B). Notably, the expression of both genes is excluded from the ventral-most region of the spinal cord, which is consistent with a recent study suggesting that the expression of *Gli1* and *Ptch1* is downregulated in the floor plate in response to high concentration of Shh (Ribes et al., 2010). In *Sufu* mutants, the expression domains of *Gli1* and *Ptch1* are restricted to the dorsal parts of the spinal cord, consistent with the dorsal expansion of the floor plate (Fig. 4C and D; n = 15 sections from 2 embryos for *Gli1*; n = 9 sections from 2



embryos for *Ptch1*). Interestingly, this dorsal shift of the *Gli1* and *Ptch1* expression domains was also observed in *Ptch1* mutant spinal cord, which exhibits widespread Hh pathway activation (Motoyama et al., 2003). The removal of Gli3 does not have an obvious effect on *Gli1* and *Ptch1* expression as the *Gli3;Sufu* double mutant spinal cord has similar dorsally restricted *Gli1* and *Ptch1* expression to that in *Sufu* single mutants (Fig. 4E and F; n = 9 sections from 2 embryos for *Gli1* and n = 7 sections from 2 embryos for *Ptch1*).

Because the *Gli1* transcript is absent in *Gli1;Sufu* double homozygous mutants, we took advantage of the *lacZ* reporter gene that was inserted into the first coding exon of *Gli1* and exhibits the same expression pattern as that of *Gli1* (Bai et al., 2002). We found that *Gli1-lacZ* is expressed throughout the D/V axis of the *Gli1;Sufu* double mutant spinal cord (Fig. 4G; n = 11 sections from 2 embryos). Similarly, *Ptch1* expression is also found in the entire spinal cord (Fig. 4H; n = 15 sections from 3 embryos). The ectopic expression of *Gli1* and *Ptch1* in the dorsal spinal cord is consistent with the dorsal expansion of the *Olig2* and *Nkx6.1* expression domains and confirms the ectopic Hh pathway activation in *Gli1;Sufu* double mutant spinal cord. The expression of *Gli1* and *Ptch1* in the ventral-most region of the spinal cord is consistent with the loss of floor plate and suggests the lack of maximal Hh pathway activation in *Gli1;Sufu* double mutants.

Similar to that in *Gli1;Sufu* double mutants, the expression of both *Gli1* and *Ptch1* is present in the ventral part of the *Gli2;Sufu* double mutant spinal cord, suggesting that the Hh pathway activation may not be high enough to inhibit *Gli1* and *Ptch1* expression and define the floor plate (Fig. 4I and J; n = 8 sections from 2 embryos for *Gli1*; n = 17 sections from 2 embryos for *Ptch1*). However, the expression of both genes in the dorsal-most regions of the spinal cord is downregulated in *Gli2;Sufu* double mutants, which appears to suggest that the absence of Gli2 has a more significant impact on Hh pathway activation than that of Gli1 in *Sufu* mutant spinal cord.

Gli2 and Gli3 activator activities underlie ectopic Hh pathway activation in Sufu mutants

Our double mutant analyses thus far did not identify a single Gli family member that is required for the ectopic activation of lower levels of Hh signaling and dorsal expansion of the motor neurons, although Gli1 and Gli2 are essential for the maximal activation of Hh signaling. This could be interpreted as functional redundancy in the activator activities of the three Gli family members. To address this possibility, we characterized *Gli2;Gli3;Sufu* triple mutants. Both *Gli2;Gli3* double mutants and *Gli2;Gli3;Sufu* triple mutants exhibit midbrain to anterior hindbrain exencephaly at E10.5 (Fig. 5B and C). Interestingly, in both mutants the floors of the midbrain are elevated (see insets in Fig. 5B and C). In the anterior part of the *Gli2;Gli3* double homozygous mutant spinal cords, the floor plate and V3 interneurons do not form, likely due to lack of Gli activator activities (Fig. 5E and H). Motor neuron progenitors ($Olig2^+$ and $Pax6^+$) consequently expand to the ventral midline (Fig. 5K and Q). The patterning of the more dorsal regions of the spinal cord appears to be normal, as the dorsal boundaries of Olig2 and Nkx6.1 expression domains are not altered in *Gli2;Gli3* double mutants (Fig. 5K and N). In characterizing the spinal cord D/V patterning in *Gli2;Gli3;Sufu* triple homozygous mutants, the most striking finding is the lack of ectopic Hh signaling. The floor plate is absent (Fig. 5F; n = 15 sections from 3 embryos). The V3 interneurons are absent (Fig. 5I; n = 8 sections from one embryo) or greatly reduced (data not shown; n = 7 sections from one embryo). The motor neurons are restricted to the ventral-most region of the spinal cords (Fig. 5L; n = 19 sections from 2 embryos). *Nkx6.1* is also restricted to its normal domain (Fig. 5O; n = 12 sections from 2 embryos). Pax6 expression is found throughout the spinal cord, similar to that in *Gli2;Gli3* double mutants (Fig. 5R; n = 12 sections from 2 embryos).

Similar expression patterns for these genes were seen in the posterior spinal cords, except that no V3 interneuron was present in any *Gli2;Gli3;Sufu* triple mutant sections (n = 12 sections from 2 embryos) (Supplemental Fig. 4).

In summary, our triple mutant analysis suggests that the ectopic activation of Hh signaling and ventralization of the spinal cord in *Sufu* mutants require redundant activator activities of Gli2 and Gli3.

Evidence for a positive role of Sufu in Hh pathway activation

One interesting observation we have made is the loss of the floor plate and V3 interneurons in the *Gli1;Sufu* double mutant, but not in the *Gli1* single mutant spinal cord (Fig. 1 and Supplemental Fig. 1). Because these ventral-most spinal cord cell types require the highest exposure to Shh, this observation suggests that maximal activation of the Hh pathway in the absence of Gli1 requires a positive function of Sufu (Dessaud et al., 2008). This positive function of Sufu has been predicted based on the observation that Sufu protects Gli2 and Gli3 from Spop-mediated degradation (Chen et al., 2009). Therefore, it is possible that the activator activities of Gli2 and Gli3 are sufficient to support maximal activation of the Hh pathway without Gli1. However, when Sufu is absent, the diminishing levels of Gli2 and Gli3 proteins may not be enough for the development of cell types requiring the maximal activation of the Hh pathway. Indeed, our Western blot analysis indicates that the levels of full-length (Gli3-190) and total (the full-length protein plus processed N-terminal product) Gli3 proteins are drastically lower in *Sufu* mutant and *Gli1;Sufu* double mutant embryos than those in wild type and *Gli1* mutant embryos (Supplemental Fig. 5).

To further address the positive role of Sufu, we took advantage of a $Gli2^{3ki}$ knock-in strain, in which the *Gli2* coding region is replaced with that of *Gli3*. A previous study showed that when being expressed from the *Gli2* locus, Gli3 can partially rescue the *Gli2* mutant phenotype, suggesting that Gli3 possesses an activator activity weaker than that of Gli2 (Bai et al., 2004). As reported, the $Gli2^{3ki}$ homozygous mutant embryos look indistinguishable from their wild type littermates (Fig. 6A and B). However, the $Gli2^{3ki}$*;Sufu* double mutant embryos are drastically different from *Sufu* mutants: the double mutants are of normal size, with well-developed trunk and tail region, as well as exencephaly similar to *Gli2;Gli3* double mutants (Fig. 6C and D). This suggests that Hh pathway activities are significantly dampened in these embryos. In the spinal cord, many V3 interneurons and a small number of floor plate cells are present in $Gli2^{3ki/3ki}$ homozygous mutants (Fig. 6F, I and L) (Bai et al., 2004). In addition, unlike *Gli2* null mutants, *Isl1*-expressing motor neurons are excluded from the ventral midline in the anterior $Gli2^{3ki}$ mutant spinal cord, suggesting that the activator activities of Gli3 can partially compensate for Gli2 in mediating Hh signaling (Fig. 6O). However, the number of floor plate cells is greatly reduced, and V3 interneurons are abnormally located to the ventral midline in $Gli2^{3ki}$ mutants, suggesting that the activator activity of Gli3 is not as strong as that of Gli2. The more dorsal regions of the spinal cords are not significantly altered (Fig. 6R and U).

Interestingly, both the floor plate (n = 10 sections from 2 embryos) and V3 interneurons (n = 6 sections from 2 embryos) are absent in $Gli2^{3ki}$*;Sufu* double mutants, concomitant with an expansion of motor neurons to the ventral midline of the spinal cord (n = 10 sections from 2 embryos) (Fig. 6G, J, M and P). The more dorsal parts of the spinal cords are normally patterned in $Gli2^{3ki}$*;Sufu* double mutants (Fig. 6S and V; n = 16 for Nkx6.1 and n = 10 for Pax6 from 2 embryos). The loss of floor plate and V3 interneurons was also seen in the posterior spinal cords of $Gli2^{3ki}$*;Sufu* double mutants (Supplemental Fig. 6). The complete loss of the floor plate and V3 interneurons in $Gli2^{3ki}$*;Sufu* double mutants but not in $Gli2^{3ki}$ single

mutants provides additional support for the hypothesis that Sufu plays a positive role in Hh signaling.

Replacing Gli2 with Gli3 dampens ectopic Hh signaling in the absence of Sufu

In the *Gli2^{3ki};Sufu* double mutant spinal cords, the motor neurons are not only expanded ventrally, but also expanded dorsally (Fig. 6P), indicating that the weak activator function of Gli3 can mediate low levels of Hh pathway activation in the absence of Sufu. However, a careful comparison with the *Gli2;Sufu* double mutant spinal cords reveals less Hh pathway activation in the *Gli2^{3ki};Sufu* double mutant spinal cords. First, the dorsal expansion of the motor neuron domain is less dramatic in *Gli2^{3ki};Sufu* double mutants (Fig. 6P; n = 10 sections from 2 embryos, compared to Fig. 2P). Second, the dorsal boundary of Nkx6.1 expression is almost completely restored in the anterior *Gli2^{3ki};Sufu* mutant spinal cord (Fig. 6S; n = 16 sections from 2 embryos). The expression of *Pax6* is also restored, albeit a little more dorsally restricted than normal, especially in the posterior region (Fig. 6V; n = 10 sections from 2 embryos, Supplemental Fig. 6). The most parsimonious explanation for this more efficient rescue of the ectopic Hh pathway activation defects in the *Gli2^{3ki};Sufu* double mutant spinal cord than that of *Gli2;Sufu* double mutants is that an increase in Gli3 repressor dosage dampens Hh pathway activity, emphasizing a role of Gli repressors in modulating Hh signaling and spinal cord D/V patterning in the context of *Sufu* mutants.

The roles of Gli3 repressor activity revealed on a sensitized Gli2$^{+/-}$; Sufu$^{-/-}$ background

Our analyses of the double and triple homozygous mutants between *Sufu* and various *Gli* genes clearly indicate essential roles of the activator activities of Gli proteins in mediating both the maximal activation of Hh signaling in the ventral spinal cord and the ectopic activation of the Hh pathway in the dorsal spinal cord. However, we have not seen a major difference between *Gli3;Sufu* and *Sufu* mutants, which appears to argue against an essential role of (the reduction in) Gli repressors in Hh pathway activation in *Sufu* mutants. Alternatively, the Gli activator activities in *Sufu* mutants may be too strong for the roles of Gli repressor to be revealed. To sensitize the genetic background for the investigation of Gli repressor activities, we removed one copy of *Gli2* in *Sufu* mutants. In the anterior spinal cord of these *Gli2$^{+/-}$;Sufu$^{-/-}$* mutants, Pax6 is expressed in the dorsal-most region, and the expression of Nkx6.1 and Olig2 is found in all but the dorsal-most region (Fig. 7B, E and H; n = 8 sections from 2 embryos for each gene). The domains of Nkx2.2 and Foxa2 expression are expanded dorsally to occupy almost the ventral half of the spinal cord (Fig. 7K and N; n = 8 sections from 2 embryos for each gene). When we removed Gli3 from these mutants to make *Gli2$^{+/-}$;Gli3$^{-/-}$; Sufu$^{-/-}$* triple mutants, although Pax6 expression does not appear to change, cells expressing *Nkx6.1* and *Olig2* are both found in the dorsal-most region of the spinal cord (Fig. 7A, D and G; n = 8 sections from 2 embryos for each gene). The expression domains of *Nkx2.2* and *Foxa2* are also expanded more dorsally than those in *Gli2$^{+/-}$;Sufu$^{-/-}$* mutants (Fig. 7J and M; n = 8 sections from 2 embryos for each gene). More strikingly, Olig2 expression is repressed in the ventral spinal cord, suggesting a stronger activation of the Hh pathway when Gli3 repressor is removed (Fig. 7G; n = 8 sections from 2 embryos). On the other hand, when we introduce an extra copy of Gli3 into the *Gli2* locus to make *Gli2$^{3ki/+}$;Sufu$^{-/-}$*, we found that although the domains of Pax6 and Nkx6.1 expression are not altered, the dorsal expansion of Olig2 and Nkx2.2 is less severe than that in *Gli2$^{+/-}$; Sufu$^{-/-}$* mutant spinal cord (Fig. 7C, F, I and L; n = 8 sections from 2 embryos for each gene). Strikingly, ectopic Foxa2 expression is completely abolished in the anterior spinal cord of *Gli2$^{3ki/+}$;Sufu$^{-/-}$* mutants (Fig. 7O; n = 8 sections from 2 embryos). We observed similar, although less obvious, effects of manipulating the levels of Gli3 repressor in the posterior spinal cords of these mutants (Supplemental Fig. 7). Therefore, our characterization of these embryos with reduced Gli activator activities revealed an important contribution of the Gli repressor activities to the modulation of Hh pathway activation in the absence of *Sufu*.

Discussion

In the current study, we address the important question of how Sufu regulates Hh pathway activation in the mammalian spinal cord through its interaction with the activator and repressor forms of various Gli proteins. We first show that the activator activities of Gli1 and Gli2, but not Gli3, are required for the maximal activation of the Hh pathway in the ventral spinal cord of *Sufu* mutants. We then show that the ectopic activation of Hh signaling and ventralization of the dorsal spinal cord in *Sufu* mutants rely on the redundant activator function of Gli2 and Gli3. By reducing the overall activator activities of Gli proteins, we show that Sufu serves a positive function in maximizing Hh pathway activation in spinal cord development. Finally, we show that Gli repressors have an important role in modulating Hh pathway activation and spinal cord patterning in *Sufu* mutants by altering the Gli3 repressor level on a sensitized background. We conclude that the activation of Gli activators and the reduction of Gli repressor activities together lead to the activation of the Hh pathway in *Sufu* mutant embryos. Thus our studies reveal the specific and redundant functions of the Gli proteins and the action of Sufu in modulating these activities during early neural patterning.

Sufu inhibits Hh signaling by inhibition of the activator activities of all three Gli proteins

In vivo genetic studies revealed that the Hh pathway is broadly activated in *Sufu* mutant embryos (Cooper et al., 2005; Svard et al., 2006). However, the levels of Gli2 and Gli3 proteins, including the full-length and processed forms, are greatly reduced in the absence of Sufu (Chen et al., 2009; Jia et al., 2009). How do we reconcile this Hh pathway activation and the reduction of Gli proteins? One possibility is that in the absence of Sufu, Gli2 and Gli3 are turned into labile activators that may be sufficient to fully activate Hh signaling by themselves (Humke et al., 2010). Alternatively, as recently suggested, the reduction of Gli repressors, especially Gli3 repressor, may lead to deregulation of *Gli1*, which in turn activates Hh signaling (Chen et al., 2009).

Our genetic analyses suggest that the activator activities of all three Gli proteins contribute to Hh pathway activation in *Sufu* mutants. We determined that both Gli1 and Gli2 play essential roles in maximal activation of the Hh pathway in *Sufu* mutants. This is very interesting because it demonstrates that Sufu regulates high levels of Hh signaling both by directly inhibiting the activation of Gli2 (and likely Gli3), and by indirectly repressing the expression of *Gli1*. Gli3 is not essential for Hh pathway activation in *Sufu* mutants. However, a positive role for Gli3 in mediating Hh pathway activation in the absence of *Sufu* was revealed by the following observations. First, removing Gli2 in *Sufu* mutants does not abolish ectopic Hh pathway activation in the dorsal spinal cord. Although formally both Gli1 and Gli3 could underlie the ectopic Hh pathway activation in *Gli2; Sufu* double mutants, the complete loss of ectopic Hh pathway activation in *Gli2;Gli3;Sufu* triple mutants suggests that Gli3 is essential for the ectopic Hh pathway activation in *Gli2;Sufu* double mutants. In summary, these results indicate that the activator activities of the three Gli proteins are important for Hh pathway activation and spinal cord ventralization in *Sufu* mutants.

The expression of *Gli1* and *Ptch1* has been widely used as direct readouts of the Hh pathway historically. However, a recent study showed that although lower level of Hh signaling activates the

expression of these two target genes, the highest levels of exposure to Shh ligand in the floor plate cells inhibit their expression (Ribes et al., 2010). Based on this observation, our gene expression analysis showing the dorsal restriction of *Gli1* and *Ptch1* in *Sufu* mutant spinal cords, but not in *Gli1;Sufu* and *Gli2;Sufu* double mutant spinal cords, provides further support for the conclusion that both Gli1 and Gli2 are required for the maximal activation of Hh pathway in the *Sufu* mutant spinal cords.

A role for Gli3 repressor activity in D/V patterning of the Sufu mutant spinal cord

Although our data clearly indicated that direct activation of Gli2 and Gli3 activators is required for Hh pathway activation, it does not rule out the possibility that a decrease in Gli repressor levels also contributes to the Hh pathway activation in *Sufu* mutants. In fact, a recent study in which endogenous Gli3 is replaced with an obligate repressor form ($Gli3^{\Delta 699}$) partially restored D/V patterning of the spinal cord in *Sufu* mutants, suggesting that Hh pathway activation in *Sufu* mutants is subject to the repressive effect of Gli3 (Wang et al., 2010). The caveat of that study is that by replacing Gli3 with an obligate repressor form, it produced more Gli3 repressor than what is normally present in the cells. In our current study, we utilized both gain- and loss-of-function approaches to investigate the roles of Gli3 repressor in D/V patterning of the *Sufu* mutant spinal cords. We found that removing Gli3 from $Gli2^{+/-}$;$Sufu^{-/-}$ mutants exacerbates the ventralization of the spinal cord, suggesting that the dorsal expansion of ventral cell types in the *Sufu* mutant spinal cord is antagonized by remaining Gli3 repressor. On the other hand, replacing one copy or both copies of *Gli2* with *Gli3* restores the spinal cord D/V patterning more efficiently than simply removing *Gli2* in *Sufu* mutants, consistent with the conclusion that Hh pathway activation in *Sufu* mutants is indeed influenced by the level of Gli3 repressor. In summary, we conclude that Hh pathway activation in *Sufu* mutants is the combined result of activation of Gli2 and Gli3 activators and a simultaneous down regulation of Gli repressors.

A positive role of Sufu in Hh signaling

In addition to its role in inhibiting the activator activity of Gli proteins, Sufu protects Gli2 and Gli3 proteins from Spop-mediated proteasomal degradation (Chen et al., 2009; Zhang et al., 2006, 2009). It was proposed that by protecting Gli proteins from degradation, Sufu might play an additional positive role in Hh signaling (Chen et al., 2009). However, no genetic data suggested that this is the case in vivo. In the current study, we provide two pieces of evidence arguing that Sufu indeed plays a positive role in maximizing Hh pathway activation. First, we show that floor plate and V3 interneurons are present in *Gli1* mutants but not in *Gli1;Sufu* double mutants. Previous reports indicated that the levels of both Gli2 and Gli3 are greatly decreased in the absence of *Sufu* (Chen et al., 2009; Jia et al., 2009). We show that the level of Gli3 protein remains low in *Gli1;Sufu* double mutants compared to wild type or *Gli1* mutants (Supplemental Fig. 5). Although due to technical limitation, we have not been able to measure the level of Gli2 protein in *Gli1;Sufu* mutants, we speculate that its level should be lower than that in wild type and *Gli1* mutants. Therefore, it is possible that the combined activator activities of Gli2 and Gli3 are not sufficient to support the development of floor plate and V3 interneurons in *Gli1;Sufu* double mutants. Second, we show that floor plate and V3 interneurons, although reduced in number, are present in $Gli2^{3ki}$ homozygous mutants, but are absent in $Gli2^{3ki}$;*Sufu* double mutants. Again, we argue that in the absence of *Sufu*, the level of Gli3 activator in the embryos is not high enough for the formation of these ventral-most cell types. In summary, our data show that Sufu does play a positive role in Hh pathway activation by stabilizing Gli2 and Gli3, which can only be revealed when the overall Gli activator activities are reduced in the embryos.

How does Sufu exert this positive function in Hh signaling? Data in *Drosophila* and some studies in mammals indicated that Sufu remains associated with Gli proteins in the presence of Hh ligands (Chen et al., 2009; Sisson et al., 2006). It is conceivable that Hh pathway activation in the presence of Sufu leads to less efficient Gli degradation due to the protective function of Sufu. However, some recent studies using newly developed antibodies suggested that Hh ligands activate Gli proteins by inducing the dissociation between endogenous Gli and Sufu (Humke et al., 2010; Tukachinsky et al., 2010). This model appears to be at odds with our genetic data showing the positive function of Sufu because it proposes that Sufu no longer directly interacts with Gli proteins once the Hh pathway is activated. One possible scenario is that in the presence of Sufu, a pool of inactive Gli proteins are protected by Sufu and can be subsequently activated to allow continuous maximal activation of Hh signaling. In contrast, the Gli proteins are depleted quickly (or are not allowed to accumulate) without the protection of Sufu in *Sufu* mutants, preventing sustained maximal activation of Hh pathway. This effect becomes apparent when total Gli activator activities are further reduced, as in our *Gli1;Sufu* and $Gli2^{3ki}$;*Sufu* double mutants.

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