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

Jinling Liu, Westley Heydeck, Huiqing Zeng, Aimin Liu *

Department of Biology, Eberly College of Science, Center for Cellular Dynamics, Huck Institute of Life Sciences, The Pennsylvania State University, University Park, PA 16802, USA

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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.,

* Corresponding author at: The Penn State University, 201 Life Science Building, University Park, PA 16802, USA. Fax: +1 814 863 1357.
E-mail address: AXL25@psu.edu (A. Liu).

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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