Antagonistic and Cooperative Actions of Kif7 and Sufu Define Graded Intracellular Gli Activities in Hedgehog Signaling

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Abstract
Graded Hedgehog (Hh) signaling governs the balance of Gli transcriptional activators and repressors to specify diverse ventral cell fates in the spinal cord. It remains unclear how distinct intracellular Gli activity is generated. Here, we demonstrate that Sufu acts universally as a negative regulator of Hh signaling, whereas Kif7 inhibits Gli activity in cooperation with, and independent of, Sufu. Together, they deter naive precursors from acquiring increasingly ventral identity. We show that Kif7 is also required to establish high intracellular Gli activity by antagonizing the Sufu-inhibition of Gli2. Strikingly, by abolishing the negative regulatory action of Sufu, diverse ventral cell fates can be specified in the absence of extracellular Hh signaling. These data suggest that Sufu is the primary regulator of graded Hh signaling and establish that the antagonistic and cooperative actions of Kif7 and Sufu are responsible for setting up distinct Gli activity in ventral cell fate specification.

Introduction
Sonic hedgehog (Shh) acts as a classical morphogen forming a ventro-to-dorsal signaling gradient to specify diverse cell fates in the spinal cord [1–5]. Shh first emanates from the notochord to induce the formation of floor plate (FP) cells, which then serve as a secondary source of Shh, and pattern the ventral neural tube into five neuronal progenitor populations, p0, p1, p2, pMN and p3 [2–4]. Increasing signaling activity, determined by the level and duration of Shh exposure, drives naive neuroepithelial cells to progressively more ventral neuronal cell fates. For example, Shh induces Olig2+ pMN precursors, which are programmed by additional Shh signaling to become Nkx2.2+ p3 cells. Furthermore, a temporal requirement of Shh signaling is involved in the induction of the non-neuronal FP cells. While FP induction depends initially on attaining high levels of Shh signaling, subsequent down-regulation of signaling in presumptive Foxa2+ FP cells is required for their differentiation into mature Shh+/Foxa2+ FP cells; however if this down-regulation is blocked, these cells adopt a p3 fate [5].

Mutant studies in mice illustrate that Shh signaling activity is governed by the balance of Gli activators and repressors [6]. In the absence of Shh, Patched1 (Pch1) inhibits Smoothened (Smo) to repress signaling through Gli3. Shh binding to Pch1 alleviates Smo inhibition and initiates signaling to promote Gli-dependent transcription. Gli2 is the main transcriptional activator, whereas Gli1 potentiates Shh signaling as a secondary activator. Proteolytic processing converts Gli3 into the major repressor of Shh signaling, though its full length form acts as an activator. In Shh−/− or Smo−/− neural tubes, p3 and pMN fates are not specified due to the absence of Shh signaling [7,8]. Elimination of Gli3 repressor function rescues the pMN, but not p3 or FP, fate in these mutants, indicating that the induction of p3 and FP fates depends on Gli activators rather than inactivation of Gli repressors [8,9]. Consistent with this notion, p3 and FP induction are largely compromised in Gli2−/− and Gli1−/−;Gli2−/− embryos [10,11]. Conversely, ectopic FP and p3 cells are induced in the neural tube of Pch1−/− embryos with elevated levels of Gli activators [12,13]. These studies together with the in vivo analysis of intracellular Gli activity [3,5,14,15] clearly unveil the importance of both Gli activators and repressors in the interpretation of the Shh gradient during ventral neural tube patterning. However, the mechanisms by which Shh signaling is converted into graded intracellular Gli activity are poorly understood.

Sufu and Kif7 are two key conserved regulators of Gli proteins [16–20]. They interact directly with Gli and control their processing, stabilization, as well as subcellular distribution [21–25]. Sufu−/− embryos exhibit a severely ventralized neural tube, whereas Kif7−/− embryos display a subtle phenotype with a slight expansion of the pMN domain. It is unknown whether Sufu and Kif7 function together during ventral patterning. Here, we show that they possess cooperative as well as antagonistic functions in...
the specification of graded Gli activity in the ventral neural tube. Strikingly, when Sufu function is eliminated, diverse ventral cell fates can be specified despite the absence of Shh signaling.

**Results**

**Sufu and Kif7 possess overlapping negative regulatory roles in Shh-dependent ventral neural patterning**

To investigate whether Kif7 and Sufu function together during ventral neural patterning, we generated **Kif7**°°/°°;Sufu°°/°° mice and examined the expression of Olig2 and Nkx2.2, which are markers of pMN and p3 cells respectively, at embryonic day (E) 9.5. While **Sufu**°°/°° embryos exhibit an increase of both Olig2°° cells (45% increase) and Nkx2.2°° cells (3-fold increase), **Kif7**°°/°° embryos only show a slight increase (25%) of Olig2°° cells (Figures 1A–1C and 1M). These results are consistent with previous observations that both Sufu and Kif7 act as negative regulators of Shh-dependent ventral neural patterning. The fact that Kif7 inactivation only results in a slight increase of Olig2°° cells, but not Nkx2.2°° cells, suggests that Kif7 is a weaker negative regulator than Sufu. In **Kif7°°/°°;Sufu°°/°°** embryos, we found a significant increase in the number of Nkx2.2°° cells (33% higher than that of **Sufu**°°/°° embryos) at the expense of Olig2°° cells (Figures 1D and 1M), suggesting that the removal of Kif7 in the Sufu°°/°° background drives more Olig2°° pMN precursors toward the Nkx2.2°° p3 fate. These results demonstrate that Sufu and Kif7 possess overlapping roles in the negative regulation of Shh signaling during ventral neural patterning.

**Opposing roles of Kif7 and Sufu in FP development**

Kif7 appears to act positively in Shh-dependent FP induction [17,18]. The number of Shh°°/Foxa2°° FP cells is reduced by 50% in E9.5 **Kif7**°°/°° embryos (Figures 1E–1F and 1M). In contrast, **Sufu**°°/°° embryos exhibit a drastic increase (3-fold) in the number of FP cells (Figures 1G–1G' and 1M), revealing a key role for Sufu in limiting Shh-dependent FP induction. These results illustrate that Kif7 and Sufu play opposing roles in FP development. Importantly, **Kif7°°/°°;Sufu°°/°°** embryos show a FP defect similar to that of **Sufu**°°/°° embryos (Figures 1G–H' and 1M). In situ hybridization analysis confirms that the expansion of the Shh...
Figure 2. Kif7 promotes ectopic FP induction by restricting Sufu in Ptc1 mutants. (A–D) Nkx2.2 and Olig2 expression shows an increased number of Olig2+ pMN cells by Kif7 inactivation in the Ptc1−/− background. (E–H) Shh and Foxa2 expression shows that Kif7 inactivation abolishes ectopic FP induction in Ptc1−/− embryos, which is restored by reducing one gene dosage of Sufu. Arrowheads indicate the dorsal limit of the neural tube. Scale bar, 25 μm. (I–L) In situ hybridization analysis of Shh RNA expression from the FP in anterior spinal cord sections. (M) Graphs indicate the number of FP cells, represented as the mean ± SEM, n=5.
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domain in these mutants is due to increased Shh RNA expression (Figures 1I–1L). Although Kif7 is positively involved in FP induction, it exerts no effect on ectopic FP development in Ptch1−/− embryos as revealed by the analysis of Kif7−/−;Sufu−/− embryos. These observations indicate that Kif7 is epistatic to Sufu in FP induction and suggest that the positive action of Kif7 in FP induction is mediated through the restriction of Sufu.

**Kif7 is required for ectopic FP induction in Ptch1 mutants**

Next, we examined how Kif7 acts positively in ectopic FP induction in Ptch1−/− mice, where Smo is constitutively active. Previously, we have shown that Shh+ FP cells are formed throughout the neural tube of E9.5 Ptch1−/− embryos [13] (see Figures 2A–2B and 2E–2F). Gli2 is the major activator of mammalian Shh signaling and is essential for FP development [10]. Consistent with this notion, removal of Gli2 function largely suppresses ectopic FP development in the Ptch1−/− neural tube [13]. If Kif7 acts as a positive regulator of Shh signaling, we expect that Ptch1−/−;Kif7−/− embryos should exhibit a FP phenotype similar to that of Ptch1−/−;Gli2−/− embryos. Indeed, removal of Kif7 function drastically reduces the number of Shh+/Foxa2+ FP cells by 90% in the Ptch1−/− background (Figures 2G–2G′ and 2M), suggesting that Kif7 is required to maintain robust Gli2 activator function for FP induction.

Our analysis indicates that Kif7 acts upstream of Sufu during FP induction (Figure 1). To test whether activated Smo regulates Sufu through Kif7, we examined the effects of reduced Sufu gene dosage on FP induction in Ptch1−/−;Gli2−/− embryos. Consistent with this, Ptch1−/−;Kif7−/−;Sufu−/− embryos show a 6-fold increase in the number of FP cells when compared with Ptch1−/−;Sufu−/− embryos (Figures 2H–2J and 2L–2M). These observations support the model that Sufu is the major negative regulator of FP induction and that activated Smo restricts the inhibitory function of Sufu through Kif7.

Although Kif7 appears to act negatively in the induction of neuronal progenitors (Figure 1), a positive regulatory role for Kif7 is also unveiled in ventral neural patterning of Ptch1−/−;Kif7−/− embryos. Consistent with this, Ptch1−/−;Kif7−/−;Sufu−/− embryos show a dramatic increase in the number of Olig2+ cells and fewer Nkx2.2+ cells (Figures 2B–2C), suggesting that activated Smo is less efficient at driving cells towards the Nkx2.2+ p3 fate in the absence of Kif7. The fact that Ptch1−/−;Kif7−/−;Sufu−/− embryos show a reduction in the number of Olig2+ pMN cells further suggests the involvement of Sufu in limiting the conversion of pMN to p3 fate in Smo-active cells. Together, these observations indicate that when Smo is active, Kif7 functions positively in the induction of both non-neuronal FP and neuronal p3 cells.
Kif7 alleviates Sufu inhibition of Gli2-dependent FP development

Gli2 is essential for robust Shh signaling and FP induction [10]. We have previously demonstrated that reduction of one dose of Gli2 can exacerbate the FP phenotype of Kif72/2 mice [17] (see Figures 3A–C, 3F–H', 3L–N and 3K). To determine whether the absence of FP cells in Kif72/2;Gli2+/2 embryos is due to elevated inhibition by Sufu, we reduced the gene dosage of Sufu to test the possibility of restoring FP development. Indeed, FP cells are readily detected along the neural tube of Kif72/2;Gli2+/2;Sufu+/2 embryos (Figures 3J–J', 3O and 3K). Furthermore, when the Sufu dosage is reduced in Kif72/2;Gli2+/2 embryos, the number of Nkx2.2+p3 cells is restored to a level comparable to that found in Kif72/2 embryos (Figures 3B–3E). These results indicate that the requirement for Kif7 in promoting robust Gli2 activity can be bypassed by reducing the amount of Sufu and suggest that the primary function of Kif7 is to relieve the inhibitory effect of Sufu on Gli2 (Figure 3P).

Diverse ventral cell fates are specified despite the absence of graded Shh signaling when Sufu function is eliminated

Our results so far indicate that when Smo is activated, Kif7 regulation of Sufu’s inhibitory action on Gli2 is a critical step in Shh signaling. We next investigated the roles of Kif7 and Sufu in cells lacking Smo, in which the pathway is repressed and insensitive to Hh inputs. We have previously shown that Kif7 inactivation in the Smo2/2 background alleviates pathway inhibition, leading to the induction of Olig2+pMN cells in Smo2/2;Kif72/2 mice [17] (see Figures 4F and 4G). However, despite the abundance of Gli2 proteins in Smo2/2;Kif72/2 embryos [17], Gli2 rarely specifies Nkx2.2+p3 cells and is incapable of inducing FP without activated Smo (Figures 4G and 4K–K'). Strikingly, pMN, p3 and FP cells are all detected in Smo2/2;Sufu2/2 embryos (Figures 4D, 4H and 4L–L'). Though the number of pMN and p3 cells is higher than in wild type embryos (Figure 4M), the relative position of these neuronal progenitor and FP cells along the dorsoventral axis of the neural tube appears quite normal. Consistent with the observations that
Sufu controls Gli2 degradation and Gli3 processing [22,23,25], we found that Sufu+/− embryos exhibit low levels of Gli2185kD and little or no Gli383kD (Figure 4N). Importantly, Gli2185kD is restored to higher levels in Smo−/−;Sufu+/− embryos. These observations indicate that, in the absence of Sufu, diverse cell fates (pMN, p3 and FP) can be specified despite the absence of graded Shh signaling. Thus, a Shh signaling gradient is no longer necessary to drive graded intracellular Gli activity and cell fate specification. In the absence of Sufu, Gli3 repressor is not formed and Gli activators are not restricted by cytoplasmic Sufu-Gli complexes. Therefore, Gli activators become less efficient as denoted (i-iii), while robust Smo activation results in Kif7 antagonizing the inhibition of Gli activators by forming inhibitory complexes in the cytoplasm [24,25]. The molecular action of Kif7, a member of the kinesin motor proteins, is not well understood. The motor domain of Kif7 is important for its Shh-dependent translocation to the primary cilium [19]. When Smo is inactive or absent, Kif7 is critical for Gli3 repressor formation as loss of Kif7 restores the formation of pMN cells in Smo+/− embryos similar to those observed in Gli3−/−;Smo+/− embryos [8,17]. Our recent studies suggest that Kif7 restricts the ciliary localization of Sufu-Gli complexes in chondrocytes [20]. Here, we provide genetic evidence that Kif7 acts positively in Shh-dependent FP induction by alleviating the inhibitory action of Sufu on Gli2. We speculate that Kif7 mediates the action of activated Smo to promote the dissociation of inhibitory Sufu-Gli complexes. Contrary to this Sufu-dependent positive regulatory function, Kif7 acts negatively in Shh signaling via both Sufu-dependent and -independent mechanisms. We show here that more neuronal precursors adopt a p3 fate in Kif7+/−;Sufu−/− embryos than those in Sufu−/− embryos, indicating that loss of Kif7 augments Hh pathway activity in a Sufu−/− background. Further studies are needed to decipher these Sufu-dependent and -independent functions of Kif7 in the control of Gli activator and repressor.

Strikingly, inactivation of Sufu leads to the specification of diverse ventral cell fates in the absence of extracellular Shh signaling. In Smo−/−;Sufu−/− embryos, not only pMN, but p3 and FP fates are specified with relatively normal positions along the dorsoventral axis of the neural tube. Thus, positional information and distinct cell fates in the ventral neural tube can be conveyed without Sufu-dependent signaling when Sufu is absent. These observations suggest that Sufu is the key regulatory target of Shh signaling and that its regulated activity leads to distinct intracellular Gli activity and cell fate. In the absence of Sufu, Gli3 repressor is not formed and Gli activators are not restricted by cytoplasmic sequestration. In this situation, Smo activation is no longer required to promote Gli2-dependent formation of FP and p3 cells. In addition to Shh, retinoic acid and Tcf signaling also contribute to the patterning of the ventral neural tube [29–33]. It remains to be determined how these and other signaling pathways contribute to the regulation of Gli activators. Perhaps once Sufu regulation is alleviated, alternate pathways normally obscured by Shh can assume more prevalent roles in patterning, and through the cross-regulatory interactions of the Nkx2.2, Olig2 and Pax6 gene networks, can refine the precise patterning of these ventral progenitor domains [15].

**Discussion**

In this study, we establish that Kif7 and Sufu play distinct and overlapping functions in specifying graded Gli activity during Shh-dependent ventral neural tube patterning. We show that there are different requirements of Kif7 and Sufu for FP, p3 and pMN cell fate specification. Kif7 acts positively in Shh-dependent FP induction and negatively in the formation of pMN cells, while its dual functions are involved in p3 fate specification. In contrast, Sufu functions universally as a negative regulator of these Shh-induced cell fates. Importantly, in the complete absence of extracellular Shh signaling (i.e. in Smo−/− embryos), inactivation of Kif7 or Sufu can lead to specification of distinct ventral cell fates. We propose that, through acting on both Gli activators and repressors, Sufu and Kif7 function cooperatively to generate graded intracellular Gli activity (Figure 5).

Sufu possesses multiple molecular functions in the negative regulation of Shh signaling. It limits the nuclear translocation of Gli activators by forming inhibitory complexes in the cytoplasm [21,26,27] and also plays a major role in the processing of Gli3 into its repressor form [22,23]. Studies using cultured fibroblasts show that Smo activation promotes the dissociation of inhibitory cytoplasmic Sufu-Gli complexes [24,25]. The molecular action of Kif7, a member of the kinesin motor proteins, is not well understood. The motor domain of Kif7 is important for its Shh-dependent translocation to the primary cilium [19]. When Smo is inactive or absent, Kif7 is critical for Gli3 repressor formation as loss of Kif7 restores the formation of pMN cells in Smo+/− embryos similar to those observed in Gli3−/−;Smo+/− embryos [8,17]. Our recent studies suggest that Kif7 restricts the ciliary localization of Sufu-Gli complexes in chondrocytes [20]. Here, we provide genetic evidence that Kif7 acts positively in Shh-dependent FP induction by alleviating the inhibitory action of Sufu on Gli2. We speculate that Kif7 mediates the action of activated Smo to promote the dissociation of inhibitory Sufu-Gli complexes. Contrary to this Sufu-dependent positive regulatory function, Kif7 acts negatively in Shh signaling via both Sufu-dependent and -independent mechanisms. We show here that more neuronal precursors adopt a p3 fate in Kif7+/−;Sufu−/− embryos than those in Sufu−/− embryos, indicating that loss of Kif7 augments Hh pathway activity in a Sufu−/− background. Further studies are needed to decipher these Sufu-dependent and -independent functions of Kif7 in the control of Gli activator and repressor.

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**Materials and Methods**

**Ethics Statement**

All experimental procedures performed were approved by The Hospital for Sick Children Animal Care Committee.

**Mice**

Sufu mutant mice were generated by crossing NLS-Cre mice with ubiquitous Cre expression [provided by C. Lobe, University of Toronto] to Sufu-floxed mice [34]. Kif7 [17], Smo [35], Gli2 [36] and Pthkl [12] mutant mice were maintained in a CD-1 outbred background and genotyped as described.
Immunofluorescence
Embryos were harvested at E 9.5 and fixed in 4% paraformaldehyde (PFA) in PBS at 4°C overnight, then subjected to ethanol series dehydration, paraaffin embedding and sectioning at 7 μm. Immunohistochemistry on anterior spinal cord sections was performed as described [13]. Mouse anti-Nkx2.2 and anti-Foxa2 (HNF-3β, 1:20, Developmental Studies Hybridoma Bank), rabbit anti-Olig2 (1:300, Chemicon), rabbit anti-Pax6 (1:300, Covance), and rabbit anti-Shh (1:50, Santa Cruz) antibodies were used. Images were acquired with a Zeiss LSM510 META laser scanning confocal microscope.

Statistical Analysis
All analysis was performed using anterior spinal cord sections at the forelimb bud level in somite-matched E9.5 embryos (24±1 somite pairs), except Pitx1 single mutant embryos which arrest at 16–20 somite pairs. Cell fate quantification data were expressed and plotted as means ± standard error of the mean (SEM). Statistical analysis for multiple comparisons was completed using one-way ANOVA followed by the Bonferroni post-test (Figures 1M, 2M and 3K). Analysis for pair-wise comparisons was completed using Student’s t-Test (Figure 4M). The sample size and p-value for each Bonferroni comparison or Student’s t-Test are provided in the respective legends and figures.

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In situ hybridization
Whole-mount or section in situ hybridization was performed as described [10]. Embryos were fixed in 4% PFA in PBS at 4°C overnight, then subjected to methanol series dehydration. In situ hybridization was carried out with digoxigenin-11UTP-labeled RNA probes for Shh [36].

Western blot analysis
Embryos were snap-frozen, and sonicated in RIPA buffer as described [17]. Immunoblotting was performed with rabbit anti-Gli3 (Santa Cruz), and rabbit anti-Gli2 (amino acids 327–442) generated using standard protocols.

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Author Contributions
Conceived and designed the experiments: KL CCH. Performed the experiments: KL SM RM XZ VP. Analyzed the data: KL SM RM XZ. Wrote the paper: KL CCH.
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