RESEARCH ARTICLE

The loss of Hh responsiveness by a non-ciliary Gli2 variant

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ABSTRACT

Hedgehog signaling is crucial for vertebrate development and physiology. Gli2, the primary effecter of Hedgehog signaling, localizes to the tip of the primary cilium, but the importance of its ciliary localization remains unclear. We address the roles of Gli2 ciliary localization by replacing endogenous Gli2 with Gli2ΔCLR, a Gli2 variant not localizing to the cilium. The resulting Gli2ΔCLR deficiencies and Gli2ΔCLRΔCLR/KI double mutants resemble Gli2 null and Gli2/Gli3 double mutants, respectively, suggesting the lack of Gli2ΔCLR activation in development. Significantly, Gli2ΔCLR cannot be activated either by pharmacological activation of Smo in vitro or by loss of Ptch1 in vivo. Finally, Gli2ΔCLR exhibits strong transcriptional activator activity in the absence of Sufu, suggesting that the lack of its activation in vivo results from a specific failure in relieving the inhibitory function of Sufu. Our results provide strong evidence that the ciliary localization of Gli2 is crucial for ciliary-dependent activation of Hedgehog signaling.

KEY WORDS: Cilium, Hedgehog signaling, Mouse, Spinal cord, Sufu

INTRODUCTION

The Hedgehog (Hh) family of secreted proteins mediates numerous inductive events crucial for the proliferation, differentiation and migration of cells in diverse species ranging from planarian to fruit flies and vertebrates (Briscoe and Thérond, 2013; Ye and Liu, 2011). Studies in the past decade suggest that Hh signaling in vertebrates requires the primary cilium, a solitary protrusion on the surface of most somatic cells implicated in a plethora of human genetic diseases (Goetz and Anderson, 2010; Nozawa et al., 2013). However, the molecular mechanisms underlying the connection between the cilium and Hh signal transduction remain enigmatic.

The vertebrate Hh family of proteins, including Shh, Ihh and Dhh, interacts with the Patched (Ptch) receptors, especially Ptch1 (Briscoe and Thérond, 2013). This interaction relieves the inhibition on a G protein-coupled receptor-like protein Smoethened (Smo), and inhibits the proteolytic processing of zinc-finger transcription factors Gli2 and Gli3 into repressors. Moreover, high levels of Hh pathway activation convert the full-length Gli2 and Gli3 into labile activators, possibly through dissociating them from their negative regulator suppressor of fused (Sufu) (Humke et al., 2010; Lin et al., 2014; Tukachinsky et al., 2010). A third member of the Gli family, Gli1, is expressed in response to the initial activation of the Hh pathway, bolstering the pathway activation to a higher level.

Hh pathway activation coincides with the exclusion of Ptch1 from, and concomitant accumulation of Smo in, the primary cilium (Corbit et al., 2005; Rohatgi et al., 2007). The ciliary accumulation of Smo appears to be essential, but not sufficient, for its activation (Corbit et al., 2005; Rohatgi et al., 2009; Wang et al., 2009; Wilson et al., 2009). All mammalian Gli proteins, as well as Sufu, localize to the tip of the cilium in a coordinated process, which is further enhanced by Hh signaling (Chen et al., 2009; Haycraft et al., 2005; Wen et al., 2010; Zeng et al., 2010b). Disturbing the cytoplasmic microtubule network or intraflagellar transport (IFT) has been shown to reduce Gli2 ciliary localization and coincidently disrupt Hh signaling (Keady et al., 2012; Kim et al., 2009). However, as cytoskeleton and IFT affect the localization of numerous other cellular components, these approaches have not adequately established a connection between Gli2 ciliary localization and Hh pathway activation.

The molecular mechanisms of Gli activation have been the focus of inquiry in recent years. CAMP-dependent protein kinase (PKA) is an essential negative regulator of Gli proteins (Niewiadomski et al., 2014; Tuson et al., 2011). However, the pathway activation in the absence of PKA is dependent on the cilium, suggesting that additional cilium-dependent mechanisms exist downstream of PKA inhibition to allow Gli activation (Tuson et al., 2011). By contrast, the removal of Sufu leads to Gli activation even in the absence of the cilium, and the dissociation between Sufu and Gli proteins appear to be dependent on the cilium (Chen et al., 2009; Humke et al., 2010; Jia et al., 2009). These results suggest that the release of Gli proteins from Sufu inhibition in the cilium is a major event in Gli activation.

Here, we report that a Gli2 variant, Gli2ΔCLR, fails to localize to the cilium, but retains intrinsic transcriptional activity and responds to Sufu inhibition. To determine whether the ciliary localization is required for Gli2 activation, we generated a Gli2ΔCLR knock-in mouse strain in which Gli2ΔCLR is transcribed in a similar pattern to endogenous Gli2. We show that Hh signaling is compromised in Gli2ΔCLRΔCLR homozygous and Gli2ΔCLRΔCLR/KI triple mutants, and is further reduced in Gli2ΔCLRΔCLR/KI, Gli3Δ double mutants, suggesting that Gli2ΔCLR is not properly activated in development. Supporting the hypothesis that Gli2 ciliary localization is required for its activation by the Hh pathway, both in vitro pharmacological activation of Smo and in vivo loss of Ptch1 mutation fail to activate Gli2ΔCLR. Finally, we demonstrate that Gli2ΔCLRΔCLR is as capable as endogenous Gli2 at activating Hh target genes in the absence of Sufu, suggesting that the lack of its activation in vivo results from a failure in its cilium-dependent release from Sufu inhibition by Hh signaling.

RESULTS

Removing mouse Gli2 from the primary cilium without disrupting its intrinsic transcriptional activity and response to Sufu

One way to specifically test the role of Gli2 ciliary localization in Hh pathway activation is to determine whether a Gli2 variant that does not localize to the cilium can be activated by Hh signaling. To accomplish this goal, it is essential to make sure that the intrinsic transcriptional activity of Gli2 is not disrupted by the mutation that

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removes the protein from the cilium. The intrinsic transcriptional activity of Gli2 can be revealed by overexpression because previous studies indicated that overexpression of Gli1 or Gli2 overrides the inhibitory function of Sufu and activates Hh-target genes independent of the cilium (Chen et al., 2009; Jia et al., 2009). We have found that a central region of mouse Gli2 (residues 570 to 967, independent of the cilium (Chen et al., 2009; Jia et al., 2009). We inhibitory function of Sufu and activates Hh-target genes studies indicated that overexpression of Gli1 or Gli2 overrides the intrinsic transcriptional activity of Sufu and activates Hh-target genes.

Since the repressor, activator and DNA-binding domains were all intact in Gli2ΔCLR, we expected Gli2ΔCLR localized predominantly to the cytoplasm (Fig. 1B,D). We induced Hh signaling by treating the cells with Smo agonist (SAG). Although the efficient GFP-Gli2 ciliary localization in DMSO-treated cells (100% of 51 cells) prevented a further increase in the percentage of SAG-treated cells exhibiting GFP-Gli2 ciliary localization (100% of 50 cells), we did observe an increase in the amount of GFP-Gli2 at the ciliary tips when judged by the fluorescence intensity (Fig. 1B,C). By contrast, SAG treatment failed to induce the ciliary localization of GFP-Gli2ΔCLR (Fig. 1B,C). Interestingly, SAG treatment appeared to result in a slight decrease in the nuclear localization of GFP-Gli2, but in no appreciable change in the predominant distribution of GFP-Gli2ΔCLR in the cytoplasm (Fig. 1B,D).

Since the repressor, activator and DNA-binding domains were all intact in Gli2ΔCLR, we expected Gli2ΔCLR to retain intrinsic transcriptional activity. To test this, we overexpressed various amounts of GFP-tagged Gli2 and Gli2ΔCLR in Shh-L2 cells that stably express a Gli-responsive luciferase reporter gene (Taiapale et al., 2000), and found that GFP-Gli2ΔCLR activated the reporter gene expression more efficiently than GFP-Gli2, suggesting that the removal of CLR did not disrupt the intrinsic activator activity of Gli2 (Fig. 1E). The extra activity of GFP-Gli2ΔCLR could result from its increased stability because the CLR includes sites that are required for SCFβTRCP-mediated degradation of Gli2 (Pan et al., 2006). However, our immunoblot analysis suggested that the difference between the levels of GFP-Gli2ΔCLR and GFP-Gli2 (∼25%; Fig. 1F) could not explain the more than fivefold difference in activity. Therefore, we conclude that GFP-Gli2ΔCLR exhibits higher intrinsic transcriptional activator activity than GFP-Gli2.

Sufu is an essential negative regulator of Gli2 and cilium-dependent relief of Gli2 from Sufu inhibition is a crucial event in Hh pathway activation (Chen et al., 2009; Cooper et al., 2005; Hunke et al., 2010; Jia et al., 2009; Svärd et al., 2006; Tukachinsky et al., 2010). Consistent with our previous co-immunoprecipitation analysis that showed physical interaction between Sufu and Gli2ΔCLR (Zeng et al., 2010b), we found that overexpression of Sufu inhibits reporter expression activated by Gli2ΔCLR, suggesting that Gli2ΔCLR remains sensitive to Sufu inhibition (Fig. 1G).

**Replacement of Gli2 with Gli2ΔCLR in vivo through a knock-in approach**
As Gli2ΔCLR lacks ciliary localization, but retains its intrinsic transcriptional activity and its response to Sufu inhibition, we reasoned that Gli2ΔCLR could be used to test the hypothesis that the
ciliary localization of Gli2 was essential for Hh pathway activation. Previous studies have indicated that Gli overexpression overrides the inhibitory function of Sufu and activates Hh target genes independently of the cilium (Chen et al., 2009; Jia et al., 2009). Therefore, Gli2ΔCLR needs to be expressed at a near physiological level to address whether the lack of its ciliary localization affects its activation by Hh signaling. Therefore, we replaced endogenous Gli2 with Gli2ΔCLR knock-in alleles to enable drug selection of the correctly targeted ES cells. We derived the targeting constructs into the Gli2 locus through Southern hybridization and polymerase chain reaction (PCR) (supplementary material Fig. S1B-E).

To confirm that the levels of Gli2ΔCLR transcription in Gli2ΔCLR knock-in embryos were comparable with that of endogenous Gli2, we performed quantitative real-time reverse transcriptase PCR analysis (qRT-PCR) on embryonic day 9.5 (E9.5) embryos. We found that the level of Gli2ΔCLR mRNA in Gli2ΔCLRKI/ΔCLRKI embryos was nearly twice as much as that of endogenous Gli2 (Fig. 2B). Strikingly, immunoblot analysis of Gli2ΔCLRKI/ΔCLRKI embryos showed a ~12-fold increase in the level of Gli2ΔCLR protein compared with that of Gli2 in wild-type embryos (Fig. 2C). We subsequently removed neoR by breeding Gli2ΔCLRKI/− mice to a mouse line expressing Cre recombinase in the germline. The levels of the mRNA (~70% more than Gli2) and protein (~8.7-fold of Gli2) for Gli2ΔCLR in resulting Gli2ΔCLRKI/ΔCLRKI homozygous mutants were slightly lower than that in Gli2ΔCLRKI/ΔCLRKI mutant embryos, but the difference was not statistically significant (Fig. 2B,C).

To make the Gli2ΔCLR protein level more comparable with endogenous Gli2, we generated transheterozygous embryos by breeding Gli2ΔCLRΔΔLacI/ΔΔLacI or Gli2ΔCLRΔΔLacI/− heterozygotes to the carriers of a null allele of Gli2 (Gli2tm2Alj; Bai and Joyner, 2001). For simplicity, we will refer to it as Gli2ΔΔLacI. As expected, in Gli2ΔCLRΔΔLacI/ΔΔLacI and Gli2ΔCLRΔΔLacI/− transheterozygous embryos, Gli2ΔCLR was transcribed at a slightly lower (~80%) level than that of endogenous Gli2 in wild-type littermates (Fig. 2B). Consistently, the level of Gli2ΔCLR

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**Fig. 2. Replacing endogenous Gli2 with Gli2ΔCLR** (A) The two knock-in alleles used in this study. In Gli2ΔCLRKI, the open reading frame of Gli2ΔCLR, along with the 3′ untranslated region (UTR) of Gli2 and three tandem repeats of SV40 polyA sequence (pA), replaces 111 bp in the first coding exon of Gli2, followed by a neomycin expression cassette (neo) flanked with loxP sites. In Gli2ΔCLRΔLacI, recombination between the two loxP sites removes the neo cassette, leaving a single loxP site. (B) qRT-PCR analysis of RNA extracted from E9.5 whole embryos show comparable transcript levels between wild type and the Gli2ΔCLR knock-in alleles. n.s., P>0.05, unpaired Student’s t-test. m=3 embryos for each genotype. (C) Immunoblot analysis on E9.5 whole-embryo lysates shows an increase in the level of the Gli2ΔCLR protein in Gli2ΔCLRKI knock-in embryos compared with endogenous Gli2. *P<0.05, unpaired Student’s t-test. (D) Immunofluorescence analysis of starved mouse embryonic fibroblasts confirmed that Gli2ΔCLR did not localize to the cilium, even in the presence of Smo agonist (SAG). Cilia are labeled with acetylated α-Tubulin. Nuclei are labeled with DAPI. *P<0.05, χ²-test.
protein in the transheterozygotes is approximately four times as much as that of endogenous Gli2 protein in wild-type embryos (Fig. 2C).

We next examined the subcellular localization of Gli2CLR in Glia2CLR mice expressing mouse embryonic fibroblasts (MEFs) through immunofluorescence. Using an antibody against the N-terminal 416 amino acids of Gli2, we found that wild-type Gli2, but not Gli2CLR, localized to the tips of the primary cilium (Fig. 2D). Treating the wild-type cells with Smo agonist (SAG) resulted in an increase in Gli2 accumulation at the tips of the cilia (Fig. 2D). By contrast, Gli2CLR remains absent from the cilia upon SAG treatment (Fig. 2D).

Abnormal patterning and compromised Hh signaling in the Glia2CLR knock-in mutant spinal cords

Both Glia2CLR and Glia2CLR transheterozygous mice are viable and fertile, and do not exhibit any noticeable morphological or behavioral defects. However, we did not recover any Glia2CLR/GLA2CLR mice and Gli2CLR/Gli2CLR homozygotes or Gli2CLR/CLR transheterozygotes at weaning, suggesting that Gli2CLR was incompatible with postnatal survival.

At E10.5, Glia2CLR mice and Glia2CLR mice homozygous and Glia2CLR transheterozygous mutant embryos exhibit kinked mesencephalic flexure morphology (compare Fig. 3A-D) that is highly reminiscent of Gli2-null homozygous mutant embryos (Fig. 3E). Gli2 plays an important role in the induction of the floor plate and V3 interneurons of the mouse spinal cord (Ding et al., 1998; Matise et al., 1998). Therefore, we examined the ventral spinal cord patterning of these embryos to determine whether Gli2CLR exhibits full activity of Gli2 in development. Foxa2 was expressed strongly in the floor plate of wild-type spinal cord (Fig. 3F). We found fewer Foxa2-expressing cells in the Glia2CLR mice homozygous mutant spinal cords (Fig. 3G). Nkx2.2-expressing V3 interneuron progenitors were normally located lateral to the floor plate (Fig. 3K). A reduced number of Nkx2.2-expressing cells were present in the ventral midline of the Glia2CLR mice homozygous mutant spinal cords (Fig. 3L). Consistent with the reduction in the floor plate and V3 interneurons, Olig2-expressing motor neuron progenitors, which were dorsal to V3 interneurons in the wild-type spinal cord (Fig. 3P), were closer to the ventral midline of the Glia2CLR mice homozygous mutant spinal cords (Fig. 3Q). Although our qRT-PCR and immunoblot analyses showed slightly less Gli2CLR mRNA and Gli2CLR protein in Glia2CLR transheterozygous mice than in Glia2CLR homozygous mutants, the Gli2CLR homozygous spinal cords exhibit the same degree of reduction in floor plate and V3 interneurons, as well as the ventral expansion of the motor neuron domain (Fig. 3H,M,R).

The defects in floor plate and V3 interneuron induction in the Glia2CLR mice and Glia2CLR mice homozygous mutant spinal cords were similar, but not as severe as the Gli2−/− mutant spinal cord, in which Foxa2 expression was largely absent (Fig. 3J) and Nkx2.2-expressing cells were greatly reduced in number (Fig. 3O), whereas Olig2 expression was expanded to the ventral midline (Fig. 3T; Ding et al., 1998; Matise et al., 1998). We reasoned that the difference might result from the higher than normal levels of the Gli2CLR protein in these mutants. To test this hypothesis, we examined the spinal cord development in Glia2CLR mice and Glia2CLR transheterozygous mutants, in which the levels of Gli2CLR protein were more similar to that of Gli2 in wild-type embryos. Indeed, these Glia2CLR transheterozygous mutants exhibit smaller floor plate and V3 interneuron domains resembling those in Glia2−/− homozygous...
mutants (Fig. 3L,N). Consequently, the motor neuron progenitor domain expands further into the ventral-most region of the spinal cord (Fig. 3S).

The ventral patterning defects in Gli2^{CLRKi/CLRki}; Gli2^{CLRKi/CLRki} homozygous and Gli2^{CLRKi/CLRki} transheterozygous mutant spinal cords suggested that Hh signaling was compromised due to the failure in Gli2^{CLR} activation. To determine the level of Hh pathway activation directly, we examined the expression of Gli1, a direct transcriptional target gene of Hh signaling. Through qRT-PCR, we reported, the reduction in Hh pathway activation (Fig. 3U). To better reveal endogenous Gli2, in studies for the rest of this paper.

The Hh receptor Ptch1 and its downstream target Smo both localize to the primary cilium, and Smo activation is dependent on its ciliary localization. Activated Smo fails to activate Gli2

Gli2

transheterozygous and Gli2^{−/−} mutant embryos, suggesting a reduction in Hh pathway activation (Fig. 3U). To better reveal Gli1 expression in the developing embryos, we examined the expression of a lacZ reporter gene inserted in the Gli1 locus (Bai et al., 2002). As reported, the lacZ expression nicely recapitulated the expression pattern of Gli1 in the ventral CNS, posterior limb buds and the gut (Fig. 3V). In Gli2^{CLRKi/CLRki} homozygous mutant embryos, the reporter expression was greatly decreased, especially in the midbrain and anterior hindbrain regions (Fig. 3W). In summary, the Gli1 expression, suggest that Gli2^{CLR} fails to activate Hh signaling efficiently in embryonic development.

The more severe disruption of Hh signaling in the transheterozygous mutants (Gli2^{CLRKi/CLRki} and Gli2^{CLRKi/CLRki}) than the homozygous mutants (Gli2^{CLRKi/CLRki} and Gli2^{CLRKi/CLRki}) suggested that the levels of Gli2^{CLR} protein in these mutants had a noticeable impact on Hh target gene activation. To minimize this impact, we used the transheterozygous mutants, in which the level of Gli2^{CLR} protein was more similar to that of endogenous Gli2, in studies for the rest of this paper.

**Gli2^{CLRKi};Gli3^{−/−} double mutants resemble Gli2^{+/−};Gli3^{−/−} double mutants**

Although Gli2^{CLR} knock-in mutants resemble Gli2-null mutants, the presence of small numbers of Foxa2-expressing floor-plate cells in the spinal cords of these mutants suggested that Hh pathway was slightly more activated in Gli2^{CLR} knock-in mutants than in Gli2-null mutants. One explanation for this minor difference is that the higher level of Gli2^{CLR} protein may sequester negative regulators such as suppressor of fused (Sufu), lowering the threshold for Gli3 activator to activate Hh target genes. To test whether this is true, we removed Gli3 from Gli2^{CLRKi/CLRki} mutants to obtain Gli2^{CLRKi/CLRki};Gli3^{−/−} double mutants. At E9.5, these Gli2^{CLRKi/CLRki};Gli3^{−/−} double mutants (Fig. 4B) exhibit exencephaly, like Gli2^{+/−};Gli3^{−/−} double mutants (Fig. 4C). The expression of Foxa2 (Fig. 4E) and Nkx2.2 (Fig. 4H) was absent in the Gli2^{CLRKi/CLRki};Gli3^{−/−} double mutant spinal cord, similar to Gli2^{+/−};Gli3^{−/−} double mutants (Fig. 4F,I). Olig2 and Pax6, which were excluded from the ventral-most region of the wild-type spinal cord (Fig. 4J,M), were expressed in the ventral spinal cord of both Gli2^{CLRKi/CLRki};Gli3^{+/−} (Fig. 4K,N) and Gli2^{+/−};Gli3^{−/−} (Fig. 4L,O) double mutants. The identical morphological and spinal cord patterning defects in Gli2^{CLRKi/CLRki};Gli3^{−/−} and Gli2^{+/−};Gli3^{−/−} double mutants further suggests that Gli2^{CLR} is incapable of transducing the Hh signal that is crucial for the neural tube development, and the Gli3 activator likely underlies the partial activation of Hh signaling in Gli2^{CLR} knock-in mutants.

**Activated Smo fails to activate Gli2^{CLR}**

The Hh receptor Ptc1 and its downstream target Smo both localize to the primary cilium, and Smo activation is dependent on its ciliary localization (Corbit et al., 2005; Rohatgi et al., 2007). Local interactions between Smo and a Ci (fly homolog of Gli2)-containing signaling complex mediate Hh signaling in *Drosophila* (Briscoe and Thérond, 2013). If local interaction between vertebrate Smo and Gli2 inside the cilia similarly underlies Gli2 activation by Smo, Smo activation would not result in the activation of the non-ciliary Gli2^{CLR}. To test this hypothesis, we treated the Gli2^{CLRKi/CLRki/CLRki} mutant MEFs with SAG. The SAG treatment induced similar ciliary localization of Smo in wild-type, Gli2^{+/−} and Gli2^{CLRKi/CLRki/CLRki} mutant MEFs (data not shown). It also greatly increased the expression of Hh target gene *Gli1*.
in wild-type MEFs (Fig. 5A). By contrast, the expression of Glil was only slightly increased in Gli2\(^{-}\) and Gli2\(^{ΔCLR}\) mutant MEFs, suggesting a disruption of the signal transduction from Smo to Gli2\(^{ΔCLR}\) (Fig. 5A).

To determine whether Smo activation resulted in the activation of Gli2\(^{ΔCLR}\) in vivo, we examined the morphology. Hh target gene expression and spinal cord patterning in Gli2\(^{ΔCLR}\)/Ptch1\(^{-}\) double mutants. At E9.5, Ptch1\(^{-}\) mutant embryos failed to turn and exhibited severe neural tube defects (Fig. 5B; Goodrich et al., 1997). The turning and neural tube defects were partially rescued in Gli2\(^{ΔCLR}\)/Ptch1\(^{-}\) and Gli2\(^{-}\)/Ptch1\(^{-}\) double mutants, and some double mutants were noticeably larger than Ptch1\(^{-}\) mutants (Fig. 5B). Hh target gene Glil was expressed in a ventral-to-dorsal gradient in the E9.5 wild-type spinal cord (Fig. 5C). However, as reported previously, Glil expression in the floor plate, the ventral-most part of the spinal cord, was downregulated by prolonged exposure to extremely high levels of Shh through a feedback mechanism (Ribes et al., 2010). Glil expression was downregulated in the entire Ptch1\(^{-}\) mutant spinal cord, consistent with very high levels of Hh pathway activation (Fig. 5C). In Gli2\(^{ΔCLR}\)/Ptch1\(^{-}\) double mutants, Glil expression was restored in the dorsal spinal cord, suggesting that Hh pathway activation was less than that in Ptch1\(^{-}\) mutants (Fig. 5C). Interestingly, the expression pattern of Glil in the Gli2\(^{ΔCLR}\)/Ptch1\(^{-}\) double mutant spinal cord was similar to that in Gli2\(^{ΔCLR}\)/Ptch1\(^{-}\) double mutants (Fig. 5C), suggesting that the reduction of Hh pathway activation in these mutants resulted from a disruption of Gli2\(^{ΔCLR}\) activation.

Consistent with the extreme activation of Hh signaling, Foxa2- and Nkx2.2-expressing cells spread throughout the Ptch1\(^{-}\) mutant spinal cord (Fig. 5D). The expression of Pax6 and Olig2 was absent or restricted to few cells in the dorsal-most part of the spinal cord (Fig. 5D). Interestingly, both Foxa2- and Nkx2.2-expressing cells were restricted to the ventral regions of the Gli2\(^{ΔCLR}\)/Ptch1\(^{-}\) double mutant spinal cords, suggesting that Hh pathway activation in these mutants was insufficient to support the expression of these two genes in the dorsal spinal cord (Fig. 5D). Consistent with a reduction of Hh pathway activation, Olig2 and Pax6 expression was restored in the dorsal two-thirds of the Gli2\(^{ΔCLR}\)/Ptch1\(^{-}\) double mutant spinal cord (Fig. 5D). These changes in spinal cord patterning in Gli2\(^{ΔCLR}\)/Ptch1\(^{-}\) double mutants were similar to those in Gli2\(^{-}\)/Ptch1\(^{-}\) double mutants (Fig. 5D), providing further support for the inability of Gli2\(^{ΔCLR}\) to respond to upstream Hh pathway activation in vivo.

**Gli2\(^{ΔCLR}\) is active in the absence of Sufu**

The results described thus far indicate that Gli2\(^{ΔCLR}\) does not respond to Hh pathway activation at the levels of Ptch1 or Smo. The parsimonious explanation for this phenomenon is that a local interaction at the ciliéum cannot be established between activated Smo and Gli2\(^{ΔCLR}\). Alternatively, the deletion of CLR may result in damage to the intrinsic transcriptional activity of Gli2 that failed to be detected in our in vitro overexpression analysis. To distinguish these two possibilities, we investigated whether Gli2\(^{ΔCLR}\) can be activated in the absence of Sufu, the inhibitory action of which on
Gli2 can be relieved only through a cilium-dependent process under physiological condition (Kim et al., 2009).

As reported previously, Sufu−/− mutants failed to turn and exhibited severe neural tube defects (Fig. 6A; Cooper et al., 2005; Svärd et al., 2006). There was no obvious rescue of these defects in Gli2ACLRKI−/−;Sufu−/− double mutants (Fig. 6A). The expression of Hh target gene Gli1 was restricted to the dorsal spinal cords of both Sufu−/− mutants and Gli2ACLRKI−/−;Sufu−/− double mutants (Fig. 6B), suggesting that Gli2ACLR is capable of activating target gene expression in the absence of Sufu. Furthermore, these results also suggest that the disruption of Gli2ACLR expression is shifted dorsally in the Gli2ACLRKI−/−;Sufu−/− double mutant and Gli2ACLRKI−/−;Sufu−/− double mutant spinal cords. (D) Immunoblot analysis shows decrease in both Gli2 and Gli2ACLR activation in Gli2ACLRKI−/−;Sufu−/− double mutants. (B) Transverse sections of E9.5 embryos processed for RNA in situ hybridization. The expression of Hh target gene Gli1 is dorsally restricted in Sufu−/− (n=3) and Gli2ACLRKI−/−;Sufu−/− double mutant (n=4) spinal cords. (C) Transverse sections of E9.5 embryos processed for immunofluorescent analysis. Shown are sections at the thoracic level. Similar patterns were observed in more posterior regions. The expression domains of Foxa2 and Nkx2.2 are expanded into the dorsal spinal cords of Sufu−/− mutant (n=4) and Gli2ACLRKI−/−;Sufu−/− double mutant (n=4) spinal cords. Olig2 expression is shifted dorsally in Sufu−/− mutant and Gli2ACLRKI−/−;Sufu−/− double mutant spinal cords. Pax6 expression is restricted to the dorsal-most part of Sufu−/− mutant and Gli2ACLRKI−/−;Sufu−/− double mutant spinal cords. (D) Immunoblot analysis shows decrease in both Gli2 and Gli2ACLR protein levels in the absence of Sufu. **P<0.01, n.s., P>0.05, unpaired Student’s t-test.

**DISCUSSION**

An essential role for the primary cilium in Hh pathway and Gli protein activation has been well established by numerous genetic studies in various vertebrate species in the past decade (Nozawa et al., 2013). However, the role of the ciliary localization of Gli
proteins in their activation has not yet been determined. One commonly taken approach to address this issue has been to disrupt the cellular and/or ciliary transport machinery. In one such study, the disruption of the cytoplasmic microtubule network through a drug treatment abolished the ciliary localization of Gli2 and disrupted the Hh signaling (Kim et al., 2009). Similarly, mutations in a microtubule motor protein Kif7, or in an intraflagellar transport protein Ift25, affected Gli2 ciliary localization and Hh pathway activation (Cheung et al., 2009; Endoh-Yamagami et al., 2009; He et al., 2014; Keady et al., 2012; Liem et al., 2009). One major concern with this approach is that additional proteins and processes affected by the disruption of cytoskeleton or transport machinery may contribute to the observed disruption of Hh signaling. Moreover, the Gli ciliary localization was only partially affected by many of these mutations, further complicating the interpretation of the results.

A more-targeted approach to study the role of the ciliary localization of Gli2 in its activation is to determine whether a non-ciliary variant of Gli2 can be activated by Hh signaling. It is crucial, however, to distinguish between the inability to respond to Hh pathway activation and the damage to the intrinsic transcriptional activity of the non-ciliary variant of Gli2. For example, a recent study found that the ciliary localization of a Gli2 variant lacking residues 852-1183 was greatly compromised compared with the full-length Gli2 (Santos and Reiter, 2014). However, this Gli2 variant acted as a transcriptional repressor when it was overexpressed, suggesting that the deletion has damaged its intrinsic transcriptional activator activity. Therefore, it could not be used to properly investigate the roles of the ciliary localization of Gli2 in its activation by Hh signaling.

In the current study, we address the relationship between Gli2 ciliary localization and activation using Gli2\textsuperscript{ΔCLR}, a non-ciliary variant of Gli2. Through the study of Gli2\textsuperscript{ΔCLR} knock-in mutant cells and embryos, we show that Gli2\textsuperscript{ΔCLR} fails to respond to Hh pathway activation at the levels of Ptch1 or Smo. To rule out the possibility that the intrinsic transcriptional activity of Gli2 is disrupted in Gli2\textsuperscript{ΔCLR} we first show that its overexpression is sufficient to activate a Gli-responsive reporter expression in cultured cells. In addition, we show that Gli2\textsuperscript{ΔCLR} can support the same level of Hh pathway activation as endogenous Gli2 in the absence of Sufu. Therefore, the lack of Gli2\textsuperscript{ΔCLR} activation in Gli2\textsuperscript{ΔCLR} knock-in mutant embryos likely results from the failure in cilium-dependent relief of the inhibitory action of Sufu, not the disruption of intrinsic transcriptional activity of Gli2.

The simultaneous loss of ciliary localization of Gli2\textsuperscript{ΔCLR} and the failure in the relief of the inhibitory action of Sufu on this non-ciliary variant of Gli2 strongly suggest that the ciliary localization of Gli2 is crucial for the Hh-dependent activation of this protein. However, we cannot formally rule out the possibility that the CLR may also be important for potential conformational changes of Gli2 that allow its dissociation from Sufu, which could be independent of the loss of Gli2 ciliary localization. To formally establish the causative relationship between the ciliary localization and activation of Gli2, it would be ideal to investigate whether restoring the ciliary localization of Gli2\textsuperscript{ΔCLR} with an unrelated ciliary-localization motif would allow normal Hh signaling. However, this could prove to be difficult because Gli2 appears to dynamically traffic through a special ciliary tip compartment, thus simply directing Gli2\textsuperscript{ΔCLR} localization to the cilium may not be sufficient to restore its response to Hh signaling (He et al., 2014).

Previous studies have shown that cilium-dependent regulation of Hh signaling was dependent on the proper levels of Gli proteins (Chen et al., 2009; Jia et al., 2009). Overexpression of Gli1 or Gli2 bypasses the inhibitory regulation of Sufu, thus activating the Hh pathway independently of the cilium. Therefore, we were concerned about the approximately fourfold difference between the Gli2\textsuperscript{ΔCLR} protein level in Gli2\textsuperscript{ΔCLR}KI/− transheterozygous mutant embryos and the Gli2 protein level in wild type. Interestingly, despite the increase in Gli2\textsuperscript{ΔCLR} protein levels, we observed decreased Hh signaling in Gli2\textsuperscript{ΔCLR}KI/− transheterozygous mutants. This suggests that Sufu is likely present in large excess compared with Gli proteins, as previously reported in fruit flies (Farzan et al., 2009). In effect, this increase in Gli2\textsuperscript{ΔCLR} protein levels helps to clarify that it is the lack of activation, rather than insufficient amount of protein, that underlies the Hh signaling defects in Gli2\textsuperscript{ΔCLR}KI/− mutants.

cAMP-dependent protein kinase (PKA) plays an essential negative role in Hh signaling (Tuson et al., 2011). A recent study showed that overexpressing a Gli2 variant lacking six PKA sites highly activates Hh pathway in cultured cells and chicken spinal cords (Niewiadomski et al., 2014). The non-ciliary Gli2\textsuperscript{ΔCLR} variant we studied here also lacks all six PKA sites, and yet it fails to activate Hh pathway in Gli2\textsuperscript{ΔCLR}KI mutant embryos. It was recently reported that Hh pathway activation in the absence of PKA remained cilium dependent (Tuson et al., 2011). Therefore, it is likely that even a PKA-insensitive Gli2 variant requires ciliary localization to be activated. Again, it is worth noting that the activity of any Gli2 variant has to be determined in a system in which the level of the Gli2 variant is not high enough to override the intricate regulatory mechanisms involving the cilium and Sufu, as we have demonstrated here and in previous studies (Chen et al., 2009; Jia et al., 2009).

![Fig. 7. The ciliary localization of Gli2 is essential for its activation. (A) In wild-type cells, activated Smo interacts with Gli2 and Sufu inside the cilium, releasing Gli2 from Sufu inhibition. (B) In Gli2\textsuperscript{ΔCLR} knock-in mutant cells, Gli2\textsuperscript{ΔCLR} fails to enter the cilium to interact with activated Smo, and remains inhibited by Sufu.](image)
It is surprising that Gli2ΔCLR exhibits higher intrinsic transcriptional activity than Gli2 in our luciferase reporter assay, despite its predominant cytoplasmic localization. It is possible that the removal of inhibitory post-translational modification may underlie this increase in transcriptional activity. For example, the acetylation of K518 in Gli1 has been shown to inhibit its transcriptional activity (Canettieri et al., 2010). The corresponding lysine in Gli2, K740, was removed in Gli2ΔCLR, which could contribute to the increase in its intrinsic transcriptional activity.

In summary, we show here that Hh signaling fails to activate a non-ciliary Gli2ΔCLR variant in vivo by relieving the inhibitory actions of Sufu. Based on our results, we propose that the ciliary localization of Gli2 allows its activation, likely through local interaction with activated Smo that relieves Gli2 from its inhibitor Sufu (Fig. 7A). However, Gli2ΔCLR is under constitutive inhibition by Sufu because it fails to travel to the cilium to interact with activated Smo (Fig. 7B). An exciting future direction would be to reveal the molecular events that directly lead to the activation of Gli2 once it is inside the cilium.

MATERIALS AND METHODS

Tissue culture, transfection and immunofluorescence
Mouse embryonic fibroblasts were prepared from E11.5 embryos as previously described (Jia et al., 2009). Cells were transfected with Lipofectamine 2000 (Life Technologies), JetPrime (Polyplus-Transfection) or PEI (Polysciences) according to the manufacturer’s recommendations. Immunofluorescence analyses were carried out as previously reported (Zeng et al., 2010b). After primary antibody incubation, membranes were incubated with IRD680- and IRD800-conjugated secondary antibodies (LI-COR), and scanned on a LI-COR Odyssey CLx imaging system. Quantitative analysis was performed using Image Studio software.

Quantitative RT-PCR
RNA was extracted from embryos or cultured cells using a Nucleospin RNA miniprep kit (Macherey-Nagel). cDNA was synthesized using qScript cDNA SuperMix (Quanta Biosciences). PCR reactions were performed using a StepOnePlus Real-time PCR system (Applied Biosystems). All samples were analyzed in triplicate and normalized to the housekeeping gene β2-tubulin (Sigma-Aldrich, TT451, 1:1000), GFP (Life Technologies, A11122, 1:1000) and Gli2 (R&D Systems, AF3635, 1:500, raised against the N-terminal 416 amino acids of mouse Gli2).

Luciferase reporter assay
Shh-L2 cells (a gift from Dr L. Lum, UT Southwestern Medical Center, Dallas, TX, USA) in 24-well plates were transfected with GFP-Gli2, GFP-Gli2ΔCLR, or control empty GFP vector, alone or together with Sufu. Luciferase assays were performed 24 h after transfection using a dual-luciferase reporter assay system (Promega). Luminescence was measured in a Turner Biosystems 20/20luminometer. Firefly luciferase activities were normalized to those of Renilla luciferase.

Immunoblot analyses
Whole-cell protein lysates were prepared, separated on SDS polyacrylamide gel and transferred to nitrocellulose membrane according to a previously described protocol (Zeng et al., 2010b). After primary antibody incubation, membranes were incubated with IRD680- and IRD800-conjugated secondary antibodies (LI-COR), and scanned on a LI-COR Odyssey CLx imaging system. Quantitative analysis was performed using Image Studio software (LI-COR). Primary antibodies used were GFP (Life Technologies, A11122, 1:10,000), Gli2 (R&D Systems, AF3635, 1:1000) and β-tubulin (Sigma-Aldrich, T5201, 1:10,000).

Animals
E14 mouse embryonic stem (ES) cells (MMRRC) were transfected with linearized Gli2ΔCLRΔKIn targeting construct using a BioRad GenePulsor electroporation system as described previously (Zeng et al., 2010a). Genomic DNAs from G418-resistant ES cell clones were cut with Asp718 (Roche) and screened through Southern blot analyses using a 5′ external probe. Genomic DNAs from the targeted clones were cut with BamHI and screened again through Southern blot using a 3′ external probe. The homologous recombination events were further confirmed with PCR using locus-specific primers. Two targeted Gli2ΔCLRΔKIn ES cell clones were injected into C57BL/6 blastocysts (Charles River Labs). The resulting male chimeras and their male descendants were bred to wild-type 129 or C3H females (Charles River Labs). The genotypes of Gli2ΔCLRΔKIn mice and embryos were determined with PCR using previously described primers (Bai and Joyner, 2001).

Other mutant mouse strains used in this study include Gli1tm2.1Bu (Bai et al., 2002), Gli2tm2.1Alj (Bai and Joyner, 2001), Gli3−/− (Büscher et al., 1998), Ptc1tm1Mpo (Goodrich et al., 1997) and Sufumtm1Rbo (Svárd et al., 2006), and were genotyped as described. The use of the animals in this work was approved by the IACUC at the Penn State University (PA, USA).

Immunohistochemistry and RNA in situ hybridization
Immunohistochemistry using Cy3-labeled secondary antibodies as well as RNA in situ hybridization using DIG-labeled RNA probes were described by Liu et al. (2012). Antibodies used were Foxa2 (DHSB, 1:40), Nkx2.2 (DHSB, 1:20), Pax6 (DSHB, 1:500) and Olig2 (Millipore, AB9610, 1:1000).

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Competing interests
The authors declare no competing or financial interests.

Author contributions
A.L. designed the experiments. J.L. and H.Z. performed the experiments. J.L., H.Z. and A.L. analyzed the data and wrote the manuscript.

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Supplementary material
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References
function by Sufu in Hedgehog signaling is evolutionarily conserved. Genes Dev. 23, 1910-1928.


Supplemental Figure 1. Construction and verification of Gli2$^{\Delta CLR KIn}$ knock in mice. (A) In a gene-targeting construct, the coding region of Gli2$^{\Delta CLR}$, the 3’ UTR of Gli2 and three tandem repeats of SV40 polyA sequence (not shown), along with a Floxed neomycin resistance cassette, was inserted into the first coding exon of Gli2, flanked with a 4 kb 5’ homology arm and a 6kb 3’ homology arm. Through homologous recombination, Gli2$^{\Delta CLR}$ and floxed neo cassette was introduced into Gli2 locus to create Gli2$^{\Delta CLR KIn}$ allele. Gli2$^{\Delta CLR KI}$ allele was created by crossing Gli2$^{\Delta CLR KIn}$ mice with a Cre-expressing mouse strain. Non-coding exons are denoted as open boxes and coding exons are denoted as filled boxes. The triangles denote loxP sites. (B) ES cells harboring the predicted mutation were identified through Southern hybridization using a 5’ external probe. The wild type allele gives rise to a 10.9 kb product whereas mutant product should be ~ 6.5kb. (C) A secondary Southern hybridization screen using a 3’ external probe confirms the successful knock in event. This probe detects a 15.7 kb product in wild type and a 14.4 kb product in Gli2$^{\Delta CLR KIn}$ mutants after cutting the genomic DNA with BamHI. (D and E) The heterozygous carrier mice were identified with PCR reactions using 5’ (D, p1 and p2) and 3’ (E, p3 and p4) primers.