Temporal deletion of Arl13b reveals that a mispatterned neural tube corrects cell fate over time

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SUMMARY
Cilia are necessary for sonic hedgehog (Shh) signaling, which is required to pattern the neural tube. We know that ventral neural cell fates are defined by a specific cohort of transcription factors that are induced by distinct thresholds of Shh activity mediated by opposing gradients of Gli activator (GliA) and Gli repressor (GliR). Despite this understanding, the role of Shh as an instructive morphogen is viewed as increasingly complex, with current models integrating positive inputs in terms of ligand concentration and time, along with negative feedback via the downstream gene regulatory network. To investigate the relative contributions of the positive and negative inputs from Shh signaling in neural patterning, we took advantage of a protein that uncouples the regulation of GliA and GliR: the cilia protein ADP-ribosylation factor-like 13b (Arl13b). By deleting Arl13b in mouse, we induced low-level constitutive GliA function at specific developmental stages and defined a crucial period prior to E10.5 when shifts in the level of GliA cause cells to change their fate. Strikingly, we found that improperly patterned cells in these mice converted to the wild-type pattern by E12.5. We further showed that the recovery of patterning did not occur when we also deleted Gli3, the primary GliR in the neural tube, revealing a crucial role of Gli3 in the maintenance of neural patterning.

KEY WORDS: Arl13b, Cilia, Neural tube patterning, Sonic hedgehog, Mouse

INTRODUCTION
Cells in the ventral neural tube interpret Shh signaling levels over time to specify five different ventral cell fates (Briscoe et al., 2000; Briscoe et al., 1999; Chiang et al., 1996; Ericson et al., 1997a; Ericson et al., 1997b). Continuing study of the mechanism by which Shh patterns the neural tube has revealed increasing levels of complexity in this system. The initial conception was that Shh acts as a diffusible morphogen, whereby its activity is defined by its concentration at a given cell, which is a function of the distance of the cell from the morphogen source (Briscoe et al., 2000; Briscoe et al., 1999; Ericson et al., 1997a; Ericson et al., 1997b). However, as the tissue being patterned was simultaneously growing, the reliance of this model on distance was difficult to reconcile with the stable pattern observed. Hence, the current model incorporates the duration of Shh signaling in addition to concentration (Dessaud et al., 2010; Dessaud et al., 2007; Ribes et al., 2010). In this temporal adaptation model, cells become progressively less sensitive to Shh ligand as it induces expression of the negative regulator of the pathway Patched1 (Ptc1) (Chen and Struhl, 1996; Goodrich et al., 1997; Jeong and McMahon, 2005; Marigo and Tabin, 1996). This negative-feedback loop demands that cells be stimulated with higher concentrations of Shh over a longer period of time to achieve the highest Shh response. This implies that Shh signaling must be continuous and, indeed, when Shh signaling is not maintained, ventral neural cell fates are lost (Dessaud et al., 2010). Recent data show that the downstream gene regulatory network (GRN), induced by Shh activity, is also crucial for the ultimate cell fate decision in the neural tube, as it helps create a transcriptional circuit that can reinforce the ultimate fate of a cell (Balaskas et al., 2012). This transcriptional circuit insulates each cell from the normal fluctuations in Shh activity so that reliable patterning occurs.

Vertebrate hedgehog signaling requires the primary cilium, and components of the pathway are localized to cilia (Corbit et al., 2005; Haycraft et al., 2005; Huangfu et al., 2003; Rohatgi et al., 2007). Ptc1 is a Shh receptor and is localized to the cilium in the absence of Shh, whereas Smoothened (Smo) enters the cilium upon Shh stimulation (Corbit et al., 2005; Marigo and Tabin, 1996; Rohatgi et al., 2007). Gli2 and Gli3 proteins mediate the transcriptional response to Shh signaling and are processed to either an activator form (GliA) in the presence of Shh, or to a repressor form (GliR) without Shh ligand (Aza-Blanc et al., 2000; Ruiz i Altaba, 1998). Normally, the relative localization of Ptc1 and Smo shifts upon Shh stimulation, permitting Gli proteins to be enriched in cilia (Chen et al., 2009; Haycraft et al., 2005; Rohatgi et al., 2007; Tukachinsky et al., 2010).

Mouse mutants that lack specific intraflagellar transport (IFT) proteins do not have cilia, which results in an absence of ventral neural cell fates owing to a lack of both GliR and GliA function (Houde et al., 2006; Huangfu et al., 2003; Liu et al., 2005; May et al., 2005; Tran et al., 2008). By contrast, we have found that mouse mutants lacking the ciliary protein Arl13b [called Arl13bhnn(hnn)] exhibit constitutive low-level of Shh activity owing to loss of modulation of Gli2 activator. This defect in GliA function corresponds to the specification of progenitors of motoneurons (pMN cells) through most of the neural tube. However, in contrast to other mouse mutants that disrupt cilia, we found via both biochemical and genetic analyses that Gli3 repressor activity was unaffected in Arl13bhnn mutants (Caspary et al., 2007). Arl13bhnn mutants possess abnormal cilia in which components of Shh signaling are not regulated properly: Ptc1 and Smo localize to cilia regardless of Shh stimulation, and there is no Gli enrichment in cilia upon Shh stimulation (Larkins et al., 2011). This is consistent with the constitutive activation of Shh...
signaling in the Arl13b<sup>hnn</sup> mutant neural tube being Shh ligand independent (Caspany et al., 2007).

Here, we use a targeted conditional null allele of Arl13b to temporally control Shh signaling activity and experimentally uncouple GliA from GliR function in the mouse neural tube. Our analysis supplies in vivo evidence that cells in the mouse neural tube are sensitive to shifts in the level of functional GliA prior to E10.5. In contrast to the complete ablation of Shh signaling after the establishment of morphogen gradient, when ventral cell fates are initially specified and then lost (Dessaud et al., 2010), we show that mispatterned cells experiencing constitutive low levels of Shh signaling are able to correct their fates if the cells are initially exposed to a normal Shh gradient. As this phenotype contrasts with the Arl13b<sup>hnn</sup> germline null phenotype, our data define the timeframe during which Shh acts via GliA as an instructive morphogen. Strikingly, we found the recovery of patterning is Gli3 dependent, indicating GliR plays a crucial role in sustaining normal neural tube patterning.

MATERIALS AND METHODS

Mouse strains
All mouse work was performed under the approved guidelines of the Emory University IACUC. All mice were maintained on a C3H/HeJ background. Analysis was performed only after lines were crossed to C3H/HeJ for at least three generations. Mouse strains used were: Brn4-Cre [Tg (Pou3f4) 32Cren; from B. Crenshaw, Philadelphia, PA, USA], CAGG-CreER<sup>TM</sup> (JAX 004682), Ella-Cre (JAX 003724), Ptc1l-lucZ (D allele; from M. P. Scott, Stanford, CA, USA) and conditional Gli3 allele (JAX 008873). Genotyping was performed as previously described (Ahn et al., 2001; Goodrich et al., 1997; Hayashi and McMahon, 2002; Lakso et al., 1996).

Generation of conditional Arl13b allele

Three fragments of Arl13b containing exon 1, 2 and 3 were amplified from the bMQ55p06 (the Wellcome Trust Sanger Institute) and cloned into pFlexible (van der Weyden et al., 2005). Exon 2 of Arl13b was flanked with LoxP sites, so exon 2 could be deleted upon Cre recombination (supplementary material Fig. S1A). We screened 288 ES cell clones and identified 18 clones by Southern blotting that had undergone homologous recombination. Primers for genotyping the Arl13b<sup>hnn</sup> allele (forward, AGGCCATCTGCTGCTGCTG; reverse, GCATATACTTCCTGAAAATAA) amplify products of 526 bp, 679 bp and 109 bp for the wild-type, targeted and deleted alleles, respectively.

Phenotypic analysis

Immunofluorescence, RNA in situ hybridization, X-Gal staining and BrdU injection were carried out as previously described (Belo et al., 1997; Dubois et al., 2006; Horner and Caspary, 2011; Yamada et al., 1991). Primary antibodies used were rabbit anti-Olig2 (Chemicon AB9610, 1:300); mouse anti-FoxA2, -HB9, -Nkx2.2, -Nkx6.1 and -Shh (Developmental Studies Hybridoma Bank, 1:100); mouse anti-Cre (Sigma C7988, 1:500); rabbit anti-Smoothened (from K. V. Anderson, New York, USA; 1:500); mouse anti-acetylated α-tubulin (Sigma T6793, 1:2500); rabbit anti-Arl13b (serum, 1:1500) (Caspary et al., 2007); mouse anti-β-Tubulin (Roche 11770376001, 1:100); and rabbit anti-phospho-histone H3 (Millipore 06-570, 1:1000). Ptc1l probe was used for in situ hybridization (Goodrich et al., 1996). Control embryos used were: Cre-positive Arl13b<sup>hnn</sup>, Cre-negative Arl13b<sup>hnn</sup> or Cre-negative Arl13b<sup>hnn</sup>. Images were taken on a Leica DM6000B upright fluorescence microscope and processed using the SimplePCI program. Confocal images were taken on a Zeiss LSM510 META confocal at 63× with optical zoom and were processed by LSM Image browser.

Mouse embryonic fibroblasts (MEFs)

MEFs were isolated from E12.5 control Arl13b<sup>hnn</sup> and Arl13b<sup>hnn</sup>*CAGG-Cre* embryos, and grown on gelatin-coated plates. At confluence, MEFs were split into a six-well plate containing gelatin-coated cover slips at a density of 800,000 cells/well. MEFs were then serum-starved for 24 hours and tamosifen (2 μM, Sigma H7904) was added in serum-free media, 10% FBS-containing media or Shh-conditioned media (Larkins et al., 2011). Cover slips were collected at 24, 36, 42 and 48 hours, and MEFs were fixed and processed for antibody staining.

Quantitative real-time PCR

MEFs were isolated from E12.5 wild-type and Arl13b<sup>hnn</sup> embryos, and grown on gelatin-coated 10 cm plates. At confluence, MEFs were treated with either control 0.5% FBS-containing media or Shh-conditioned media (Larkins et al., 2011) for 24 hours. Whole RNA was extracted from MEFs using an RNeasy Mini Kit (Qiagen 79620) according to manufacturer’s instructions. Purified RNA was quantified using a NanoDrop ND-1000 spectrophotometer and reverse transcribed using the SuperScript III First-Strand Synthesis System (Invitrogen 18080) according to manufacturer’s instructions. Quantitative real-time PCR was performed using LightCycler 480 SYBR Green I Master (Roche 4707516001) in a BioRad CFX96 Real-Time PCR detection system. The following primers were used (5′-3′): Ptc1l (CCCTAACAAAAATTCACAACTGCTT and GCATATACTTCCTGAAACACTCTG); Gli1 (GCCCAACAAGTGCAAGTCGTTG and AAGGTGCGCTTGGTGGTTTCTTA); Gapdh (CGTCCTGGAGACCCAAATGGT and GAATTGCGTGTGAGTGGAGT) (Han et al., 2009). Each reaction was performed in triplicate. Ptc1l and Gli1 values were normalized to Gapdh within each sample. Statistical significance was evaluated by applying a two-factor ANOVA.

Western blotting

E12.5 embryos were homogenized and protein concentration was determined by Bradford assay (BioRad 500-0006). Protein (50 μg) was separated on a 10% SDS-PAGE gel and transferred onto a nitrocellulose membrane (GE Healthcare RPN203D). The membrane was incubated with affinity-purified anti-Arl13b (1:1000) (Caspary et al., 2007) and anti-actin (Sigma A5060, 1:1000), then processed for enhanced chemiluminescence.

Quantitative analysis

Cells were counted by using ImageJ software, and the three color channels were separated, so Olig2-, HB9- or Hoechst-staining cells could be counted in their own channel. Two sections were counted for each E12.5 embryo. Counts of Olig2- and HB9-positive cells were normalized to the total number of cells in the neural tube. Three E12.5 embryos were counted for each genotype to obtain an average, and the error bars represent the s.d. Significant difference from normalized Olig2 or HB9 average of mutants compared with control was calculated by Student’s t-test. Arl13b-staining cilia in MEFs were counted using point selections in ImageJ software, and ten random pictures were taken from each condition to count the percentage of Arl13b-expressing cilia in total cell numbers as determined by Hoechst staining.

RESULTS

Modulation of Shh activity level and regulation of Smo localization

To alter Shh activity temporally in the neural tube during development without perturbing GliR, we generated a conditional null Arl13b allele (supplementary material Fig. S1). When we induced deletion in the germline, the conditional allele recapitulated the Arl13b<sup>hnn</sup> phenotype, indicating the same constitutive low-level Shh activity as in Arl13b<sup>hnn</sup> (supplementary material Fig. S1C-F). By combining a ubiquitous, tamosifen-inducible Cre line, CAGG-CreER<sup>TM</sup>, with the Arl13b<sup>hnn</sup> allele and...
injecting the pregnant dams with tamoxifen, we controlled the timing of Arl13b deletion in the embryos (Hayashi and McMahon, 2002) (supplementary material Fig. S1I). Via immunofluorescence, we saw a reduction of Arl13b starting 24 hours post-injection and a complete loss of Arl13b expression at 42 hours post-injection (supplementary material Fig. S2).

We previously showed that the expansion of pMN cells in the neural tube of Arl13bΔm mice is independent of Shh ligand, as Shh Arl13bΔm double mutants resemble Arl13bΔm single mutants (Caspar et al., 2007). However, it is unclear whether cells lacking Arl13b can increase their level of signaling if exposed to Shh ligand. To address this, we performed quantitative real-time PCR in wild-type and null Arl13bΔm mouse embryonic fibroblasts (MEFs) to detect transcriptional levels of two Shh target genes: Gli1 and Ptch1. In wild-type MEFs, we found Gli1 and Ptch1 transcription were induced after 24 hours of Shh stimulation (Fig. 1A). In Arl13bΔm MEFs, however, Gli1 and Ptch1 transcription remained at baseline levels regardless of Shh stimulation, indicating that, consistent with the in vivo phenotype, Arl13bΔm MEFs were not responsive to Shh in vitro (Fig. 1A).

To confirm that the temporal loss of Arl13b affected Shh signaling in the same mechanistic manner as constitutive loss of Arl13b, we turned to Arl13b deletion in cell culture with control Arl13bΔfloxed/floxed and Arl13bΔm/floxed, CAGG-Cre/+ (Arl13bΔCAGG-Cre) MEFs. We cultured the MEFs under two conditions: in serum-free media for 24 hours (to induce cilium formation), followed by tamoxifen treatment to delete Arl13b, or in serum-containing media with tamoxifen, which permits the cells to proliferate as they would in vivo. Consistent with what we had seen in vivo, we saw a slight decrease of Arl13b 24 hours after tamoxifen addition and an absence of Arl13b between 36 and 42 hours after tamoxifen treatment in Arl13bΔCAGG-Cre MEFs (supplementary material Fig. S3A-F,J-O,R). These data establish that Arl13b protein was abolished 42 hours after tamoxifen injection, implying that Arl13b-dependent phenotypes could be analyzed in vivo 2 days post-injection (supplementary material Fig. S3G,H,P,Q).

The germline deletion of the conditional allele recapitulated the Arl13bΔm neural patterning phenotype, arguing that the Arl13b conditional deletion globally affected Shh activity, as expected (supplementary material Fig. S1C-F). At the cellular level, this is due to a lack of regulation of key components of the pathway; Smo requires Shh stimulation to localize to cilia in wild-type MEFs, but was found in the cilia of Arl13bΔm MEFs regardless of Shh stimulation (Larkins et al., 2011). Therefore, we tested whether the conditional deletion of Arl13b affected ciliary Smo localization like the null allele. We examined Smo localization in control and Arl13bΔCAGG-Cre MEFs after treating cells with tamoxifen and either control or Shh-conditioned media for 36 hours (when Arl13b still remains) or 48 hours (when we could no longer detect Arl13b protein). As expected, we found that when Arl13b protein was present in control or in Arl13bΔCAGG-Cre MEFs at 36 hours, Smo localized to cilia only in the presence of Shh (Fig. 1B-I). By 48 hours in the control, we no longer found Smo in cilia, consistent with the observation that continued Shh response requires progressively increasing stimulation (Fig. 1J,K,N,O) (Dessaud et al., 2007). By contrast, Smo localized to cilia in Arl13bΔCAGG-Cre at 48 hours with or without Shh stimulation (Fig. 1L,M,P,Q). This indicates that temporal deletion of Arl13b results in the same loss of Shh-dependent Smo regulation observed in Arl13bΔm MEFs (Larkins et al., 2011); thus, the temporal deletion of Arl13b with CAGG-CreERT2 in vivo results in a constitutive low-level of Shh activity via the same mechanism we saw in the null allele.

Neural progenitors are sensitive to changes in Shh activity at E9.5, but not at E10.5

To investigate when cells are sensitive to changes in the level of GliA function, we first analyzed the consequences of removing Arl13b as neural patterning is established, at ~E9.5. By injecting tamoxifen at E7.75, we found a complete absence of Arl13b protein by E9.5, and refer to mice with this time of deletion as Arl13bΔm (Fig. 2Aii,H). In the caudal neural tube at E9.5, we found an expansion of Olig2 cells at the hindlimb level in the Arl13bΔm neural tube, although
the expansion was not as extensive as in Arl13b<sup>bnn</sup> embryos (Fig. 2E,1). We also saw that, compared with control embryos, the progenitor marker Nkx6.1 was expanded dorsally in the Arl13b<sup>AT9.5</sup> neural tube, albeit not as far dorsally as in Arl13b<sup>bnn</sup> embryos (see Fig. 5A",D",J"). Our observation that the patterning defects were not as severe in Arl13b<sup>AT9.5</sup> embryos as in Arl13b<sup>bnn</sup> embryos suggests that the competence of the cells in the neural tube to ectopic GliA is progressively lost.

Next, to examine whether cells are sensitive to changes in the level of GliA function at E10.5, we examined Arl13b<sup>AT10.5</sup> embryos, whose mothers were injected with tamoxifen at E8.5 (Fig. 2Aii,iii,LL). We found Olig2, HB9, Shh and Nkx2.2 expression was normal at E10.5, indicating the cells were no longer sensitive to changes in Shh activity and arguing that the cells are committed at E10.5 (Fig. 2K,M; supplementary material Fig. S4; data not shown). Thus, cells in the mouse neural tube are progressively less sensitive to changes in the level of GliA function for a developmental window after E8.5 and prior to E10.5, implying that Shh via GliA is not a potent instructive signal in vivo after E10.5.

**Rescue of neural tube patterning over time in Arl13b<sup>AT9.5</sup> embryos demonstrates an active role for low-level Shh activity**

The loss of cell fates over time in the Shh conditional mice indicated that Shh expression must be maintained for patterning to persist (Dessaud et al., 2010). Without Shh ligand, Gli protein is no longer activated but is instead cleaved to its repressor form, so it is unclear whether this phenotype is due to lack of activation or active repression. Previously, it has been difficult to separate these two possibilities because Gli protein can be processed into either activator or repressor, making it difficult to perturb GliA function without affecting GliR, and vice-versa (Aza-Blanc et al., 2000; Ruiz i Altaba, 1998). Unlike mutations in Gli2 or in Gli3, mutations in Arl13b enable us to uncouple the regulation of GliA from that of GliR (Caspari et al., 2007; Ding et al., 1998; Matise et al., 1998; Persson et al., 2002). To test the consequences of altering the level of GliA function while leaving GliR intact on ventral cell fates over time, we examined the Arl13b<sup>AT9.5</sup> embryos at several time points. Differentiated motoneurons are normally restricted to the ventrolateral neural tube, but are expanded in Arl13b<sup>bnn</sup>-null embryos from E10.5 through E12.5 (Fig. 3A,B,1,J). In E10.5 Arl13b<sup>AT9.5</sup> embryos, we detected an expansion of Olig2 and HB9 cells similar to the E10.5 Arl13b<sup>bnn</sup>-null phenotype (Fig. 3B,D). However, by E12.5 we found the same number of Olig2- and HB9-positive cells in Arl13b<sup>AT9.5</sup> embryos as in control embryos (Fig. 3Q). Furthermore, their expression domain resembled the wild-type pattern, not the Arl13b<sup>bnn</sup>-null embryo pattern (Fig. 31,J,L). This recovery of patterning from E10.5 to E12.5 was surprising because graded Shh does not direct different ventral neural cell fates after E10.5, according to the normal pattern we observed in Arl13b<sup>AT10.5</sup> embryos (Fig. 2K,M). Thus, whereas Shh activity must be maintained for normal neural cell fates to be specified, we find that the level of GliA function occurring in the absence of Arl13b, although abnormally regulated, permits initially mispatterned cells to be rescued to a wild-type fate over time.

This recovery of patterning was unexpected, so we investigated three potential artifacts that could stem from inducing deletion of Arl13b. A trivial explanation for the rescue of patterning we see is that Cre-induced deletion might not be complete. In such a scenario, wild-type cells might out-compete mutant cells over time. We ruled out this possibility by examining Arl13b expression via immunofluorescence (Fig. 4C,D,G,H) and western blotting (Fig. 3K). As well as by confirming Arl13b deletion via PCR of the deleted Arl13b<sup>bnn</sup> allele (Fig. 4L). In all cases, we could detect no protein or unrecombined DNA allele, indicating that the deletion was ubiquitous and no wild-type cells remained.

Alternatively, one could argue that the recovery is due to cells in the dorsal and ventral Arl13b<sup>AT9.5</sup> neural tube proliferating more than those within in the Olig2 domain. We eliminated this model by staining Arl13b<sup>AT9.5</sup> neural tubes with markers of cell proliferation: phospho-histone H3 and BrdU (supplementary material Fig. S5; data not shown). We found no proliferative differences from wild-type controls, indicating that differential proliferation cannot explain the patterning recovery. This is consistent with our previous data showing that loss of Arl13b does not impact cell proliferation (Caspari et al., 2007; Horner and Caspary, 2011).

Another possibility to account for the recovery of patterning is that, in contrast to Arl13b<sup>bnn</sup> embryos that never expressed Shh in...
Arl13b deletion that would allow the initial Shh activity gradient to be established, but would eliminate Shh expression in the floor plate. We achieved this by injecting tamoxifen at E7.5, 6 hours before the injection that generated the Arl13b<sup>ΔE9.5</sup> embryos, and called these embryos Arl13b<sup>ΔE9.5</sup> (Fig. 2Aii). Shh was absent in the floor plate in the Arl13b<sup>ΔE9.5</sup> embryos at E9.5, as in Arl13b<sup>ΔE9.5</sup> embryos (Fig. 2E’, G’). We did find an expansion of the Olig2-positive domain at E9.5 and E10.5 in the Arl13b<sup>ΔE9.5</sup> embryos (Fig. 2G; Fig. 3C); however, by E12.5, Olig2 was restricted to its normal wild-type domain (Fig. 3K). The equivalent recovery of patterning by E12.5 in Arl13b<sup>ΔE9.5</sup> as in Arl13b<sup>ΔE9.5</sup> embryos indicates that, as long as the initial gradient of Shh activity is induced, Shh expression in the floor plate of Arl13b<sup>ΔE9.5</sup> embryos cannot explain the recovery.

**Recovery of patterning is complete**

To further characterize the patterning phenotypes and the extent of recovery in Arl13b<sup>ΔE9.25</sup> and Arl13b<sup>ΔE9.5</sup> embryos, we examined other markers affected in the Arl13bh<sup>ΔE9.5</sup>-null neural tube. Nkx6.1 marks all ventral progenitors, which are divided into subdomains demarcated by FoxA2 in the floor plate, Nkx2.2 in the p3 cells and Olig2 in the pMN cells (Fig. 5A-C'). In Arl13bh<sup>ΔE9.5</sup> embryos, we rarely saw FoxA2-expressing cells; Nkx2.2-expressing cells were specified across the ventral midline, intermingled with Olig2 cells; and Nkx6.1 cells were expanded into dorsal neural tube (Fig. 5D-F'). In both Arl13b<sup>ΔE9.25</sup> and Arl13b<sup>ΔE9.5</sup> caudal neural tubes at E9.5, FoxA2 was present, consistent with the establishment of the Shh activity gradient inducing FoxA2 expression (Fig. 5G,J); however, by E10.5, there were FoxA2-positive cells in more dorsal positions than normal, which is likely to reflect the shift in the level of GliA function due to the loss of Arl13b (Fig. 5H,K). Nkx2.2 and Nkx6.1 expression expanded further dorsally in both the Arl13b<sup>ΔE9.25</sup> and Arl13b<sup>ΔE9.5</sup> caudal neural tube, albeit not as far dorsally as in Arl13bh<sup>ΔE9.5</sup> (Fig. 5G', H', I', J', K', K'). When we examined these markers in E12.5 Arl13b<sup>ΔE9.25</sup> and Arl13b<sup>ΔE9.5</sup> embryos, we found they were similar to control embryos, evidence that the pattern was restored (Fig. 5L-L').

In addition to the cell fates, we monitored the Shh activity gradient in Arl13b<sup>ΔE9.25</sup> and Arl13b<sup>ΔE9.5</sup> caudal neural tube by examining Ptch1 expression. Normally, a steep Shh activity gradient is visible starting at E8.5, and it is maintained at E9.5 and E10.5; we previously showed that Ptch1 expression in Arl13bh<sup>ΔE9.5</sup> is dorsally expanded and not in a gradient (Caspy et al., 2007). We found Ptch1-lacZ reporter expression at E10.5 in the Arl13b<sup>ΔE9.25</sup> caudal neural tube resembled that of Arl13bh<sup>ΔE9.5</sup> at E10.5, consistent with the cell fates we observed (Fig. 4B). Similarly, by E12.5, when the recovery appeared complete, we found no difference in Ptch1 expression between the wild-type and Arl13b<sup>ΔE9.5</sup> caudal neural tubes using either the Ptch1-lacZ allele or Ptch1 in situ hybridization (Fig. 4E,F,I,J). This suggests that in Arl13b<sup>ΔE9.25</sup> and Arl13b<sup>ΔE9.5</sup> caudal neural tubes by E12.5, the gradient of Shh target gene transcriptional activity in the neural tube is normal. Taken together, the recovery of the cell fates and the recovery of the Shh activity gradient in the Arl13b<sup>ΔE9.25</sup> and Arl13b<sup>ΔE9.5</sup> caudal neural tubes indicate that, as long as the Shh activity gradient was initially established, improperly patterned cells along the dorsal-ventral axis were rescued in the absence of Arl13b.

**The recovery of patterning in Arl13b<sup>ΔE9.25</sup> and Arl13b<sup>ΔE9.5</sup> neural tubes is Gli3 dependent**

By conditionally deleting Arl13b, we were able to modulate the level of GliA function while leaving GliR function intact, raising...
the possibility that GliR could be responsible for the recovery of patterning we observed. To test this directly, we conditionally deleted Gli3, which acts predominantly as a repressor in the neural tube, along with Arl13b at E9.25. Gli3 mutants display normal patterning of ventral markers, such as FoxA2, Nkx2.2, Olig2 and HB9, and only disrupt a few cell fates in the p0-p2 domains (Persson et al., 2002). In contrast to the relatively normal pattern we observed in single mutant Arl13bΔE9.25 neural tubes at E12.5, in double mutant Arl13bΔE9.25 Gli3ΔE9.25 neural tubes, we saw abnormal patterning: Nkx2.2 cells were concentrated in the p3 domain, but some were expressed dorsal to Olig2 cells (Fig. 6G,H); Olig2 and patterning: Nkx2.2 cells were concentrated in the p3 domain, but were expressed dorsal to Olig2 cells (Fig. 6G,H); Olig2 and Nkx6.1 cells were scattered to more dorsal positions compared with the domain in E12.5 Arl13bΔE9.25 neural tubes (Fig. 6H,J,L); and HB9 motoneurons were expanded dorsally (Fig. 6J). The overall patterning was less severe than in Arl13bΔE9.25 double mutant embryos at E9.5 and observed an expansion of neural tubes, along with deleted patterning we observed. To test this directly, we conditionally deleted Ptch1, as it is uniformly expressed in E12.5 control (E) and Arl13bΔE9.5 (F) ventral neural tube. (I,J) Ptch1 mRNA is expressed along the ventricular zone of E12.5 control (I) and Arl13bΔE9.5 (J) ventral neural tubes. White bracket indicates strong expression of Ptch1. (K) Western blot shows the absence of a 60 kDa Arl13b band in Arl13bΔE9.5. (L) PCR shows there is no conditional Arl13b allele DNA (679 bp), whereas a deleted band (109 bp) can be detected in Arl13bΔE9.5. A 526 bp band indicates the endogenous Arl13b allele. Controls used were either Cre-positive Arl13bΔfloxed/+, Cre-negative Arl13bΔfloxed/+ or Cre-negative Arl13bΔfloxed/floxed.

**DISCUSSION**

Although the requirement of Shh morphogen for patterning the neural tube is clear, its precise mechanism of action has been more difficult to dissect. Shh acts as an extrinsic signal by establishing the GliA/GliR ratio to induce the expression of specific transcription factors (Briscoe et al., 2000; Briscoe et al., 1999; Chiang et al., 1996; Ericson et al., 1997a; Ericson et al., 1997b). Subsequently, the transcription factors regulated by Shh form a gene regulatory network (GRN) with both positive feed-forward and negative-feedback outputs (Briscoe et al., 1999; Ericson et al., 1997b; Novitch et al., 2001; Sander et al., 2000; Vallstedt et al., 2001). Recent work has proposed this GRN buffers the ultimate cell fate decision against temporary fluctuations in the extrinsic signal (Balaskas et al., 2012). Our previous genetic work established that Arl13b regulates only GliA and that loss of Arl13b leaves GliR intact (Caspary et al., 2012). Our analysis of Arl13bΔE9.5 allele. Controls used were either Cre-positive Arl13bΔfloxed/+ or Cre-negative Arl13bΔfloxed/floxed.

**The initial role of GliA as an instructive signal**

We define the in vivo window during which neural cell fates can respond to shifts in GliA to be prior to E10.5. Indeed, misregulation of GliA in Arl13bΔE9.5 and Arl13bΔE9.5 embryos results in Olig2 expansion in the neural tube similar to Arl13bΔE9.5 mutants, although the change in other ventral markers is not as dramatic. Later deletion of Arl13b results in less severe patterning defects, however (compare Fig. 3B-D with Fig. 5), indicating that the neural tube becomes progressively less sensitive to shifts in GliA over time, consistent with previous work showing the system requires...
increasing amounts of GliA inputs over time (Dessaud et al., 2007). Nonetheless, comparison of the germline null Arl13bhnn embryos with the Arl13bΔE9.25 and Arl13bΔE9.5 embryos underscores the crucial role of the initial Shh activity gradient in the mammalian neural tube (Fig. 7). At E12.5, abnormal patterning persists in Arl13bhnn mutants. In Arl13bΔE9.25 and Arl13bΔE9.5 embryos, however, the neural tube recovers its wild-type pattern by E12.5, suggesting that the initial establishment of the Shh activity gradient in Arl13bΔE9.25 and Arl13bΔE9.5 embryos provides the instructive signals that guide the ultimate fate of each cell (Fig. 7). This is in keeping with the prevailing view of Shh as an instructive morphogen.

Fig. 5. Floor-plate and p3 progenitor marker expression in different inducible Arl13b deletion caudal neural tubes. (A-C) FoxA2 is expressed in the floor plate (A,B,C), Nkx2.2 is specified in the p3 domain (A′,B′,C′) and Nkx6.1 is in the ventral neural tube in control embryos at E9.5 (A-A′), E10.5 (B-B′) and E12.5 (C-C′). White arrow in C indicates FoxA2-expressing cells. (D-F) FoxA2 is absent in Arl13bΔE9.25 at E9.5 (D), E10.5 (E) and E12.5 (F), whereas Nkx2.2 is expanded dorsally in all three developmental stages (D′,E′,F′). Nkx6.1 is specified in the whole neural tube at E9.5 (D′) and E10.5 (E′), and specified in the whole ventricular zone at E12.5 (F′). White arrow in F indicates the region that should be the floor plate. (G-L) Both FoxA2 and Nkx2.2 cells are expanded dorsally in Arl13bΔE9.25 and Arl13bΔE9.5 at E9.5 (G,G′,J,J′) and E10.5 (H,H′,K,K′). FoxA2 is restored at E12.5 in Arl13bΔE9.25 and Arl13bΔE9.5 (I,I′), Nkx2.2 is expressed normally at E12.5 in both Arl13bΔE9.25 and Arl13bΔE9.5 (I,I′), Nkx6.1 is expanded to the dorsal quarter of the neural tube at E9.5 and E10.5 (G′,J′,H′,K′), but restricted to the ventral ventricular zone at E12.5 (I′,I′). Nkx6.1 expression in motoneurons is similar to control in E12.5 Arl13bΔE9.25 (I), whereas fewer motoneurons express Nkx6.1 in Arl13bΔE9.5 (I′). White arrows in I and I′ show FoxA2-expressing cells.

Fig. 6. Ventral neural tube patterning is impaired in E12.5 Arl13b Gli3ΔE9.25. (A-F) Olig2 (A), Nkx2.2 (C) and Nkx6.1 (E) cells are expressed in pMN, p3 and ventral neural tube of Cre negative Arl13bfloxed/floxed Gli3floxed/+ (control) embryos at E9.5, whereas those ventral progenitors are expanded in Arl13b Gli3ΔE9.25 and Arl13bΔE9.5 at E9.5 (B,D,F) and E10.5 (H,H′,K,K′). Olig2 is restored at E12.5 in Arl13bΔE9.25 and Arl13bΔE9.5 (I,I′) and E12.5 in Arl13b Gli3ΔE9.25 (I′,I′). Although most Olig2 and Nkx2.2 cells are localized normally in Arl13b Gli3ΔE9.25, some cells are scattered to a more dorsal domain (H). In E12.5 Arl13b ΔGli3ΔE9.25 embryo, some Olig2 (red) and HB9 (green) cells are localized in more dorsal domains than their normal restricted domains (I), but expressed normally in control embryo (I). In E12.5 Arl13b ΔGli3ΔE9.25 embryo, some Olig2 (red) and HB9 (green) cells are localized in more dorsal domains than their normal restricted domains (I), but expressed normally in control embryo (I). (K,L) Nkx6.1 cells are mostly restricted to the ventricular zone of both control (K) and Arl13b ΔGli3ΔE9.25 (L) at E12.5, except some Nkx6.1 cells are localized more dorsally in Arl13b ΔGli3ΔE9.25 (L).
Recovery of patterning

The recovery of patterning we see in the Arl13b^ΔE9.25 and Arl13b^ΔE9.5 embryos is striking; the cells outside the normal pMN domain that expressed Olig2 at E9.5 switch to expressing the markers of the adjacent dorsal and ventral domains by E12.5. Two distinct models might explain this result. In the first model, the recovery may reflect the response of the cells to GliA over time. In this scenario, the cells would initially be mispatterned owing to the shift in GliA caused by loss of Arl13b, but the cells remain sufficiently plastic to be repatterned over time, provided Gli3 regulation is intact. Alternatively, the normal Shh gradient prior to Arl13b deletion might have turned on the appropriate target genes so that the downstream GRN is consolidated, but the cells remain sufficiently responsive to short-term fluctuations in GliA to induce the expression of Olig2; thus, the cells that ectopically express Olig2 at E9.5 would be respecified over time by the GRN established earlier in development. The latter is termed hysteresis, a memory of the initial signal, which has been argued for by recent work in neural patterning (Balaskas et al., 2012).

It remains unclear which model accounts for the recovery of patterning we observe or if both mechanisms are involved. The expansion of the Olig2 domain we observed in Arl13b^ΔE9.25 embryos was more pronounced than in Arl13b^ΔE9.5 embryos. The fact that earlier perturbation of the system results in more severe patterning defects could mean that GliA requires sufficient time to truly activate target genes and induce the downstream GRN, consistent with the first model. However, our analysis of the Arl13b Gli3^ΔE9.25 double mutants at E9.5 might point towards the second model (hysteresis). In the double mutants, loss of Gli3 effectively increases GliA function by removing the primary GliR in the neural tube, altering the GliA/GliR ratio. If the GRN is not consolidated by E9.5, we would have expected to see greater expansion of Olig2 expression in double mutants compared with single mutants owing to this shift in inputs. Instead, we saw that the Arl13b Gli3^ΔE9.25 double mutants resemble Arl13b^ΔE9.25 single mutants at E9.5, arguing that the functional level of ectopic GliA can be increased with no functional consequence to cell fate. Because we cannot measure...
levels of GliA directly, only the downstream transcriptional response, we cannot experimentally distinguish between these models. Regardless of whether the GliA inputs or the downstream GRN are responsible for the recovery, this capacity for recovery shows the robustness of the system that patterns the neural tube.

The role of Gli3 in neural patterning

Although Gli3 acts predominantly as a repressor of Shh target genes in the neural tube, its exact function remains enigmatic because Gli3 mutants display a relatively mild patterning phenotype (Persson et al., 2002). We saw mispatterning in the Arl13b ΔArl and Arl13b ΔArl9.5 embryos, indicating that GliIR could not initially compensate for the shift in GliA activity. By E12.5, however, we saw normal patterning only in the Arl13b ΔArl and Arl13b ΔArl9.5 single mutant embryos, and not in Arl13b Gli3 ΔArl9.5 double mutant embryos, demonstrating that the recovery depends on Gli3. This might be because the absence of Gli3 lowers the level of GliIR, and thus alters the balance of activation and repression such that the downstream transcriptional profile of cells is altered. Alternatively, our data raise the possibility that there might be temporal distinctions in the roles of GliA and GliR in neural patterning, whereby neural patterning is first most responsive to the positive inputs of Shh signaling, mediated by GliA, and subsequently requires the negative inputs, via GliR. Finally, we cannot rule out the possibility of other signaling pathways active in the neural tube being responsible for the recovery of patterning in a Gli3-dependent manner.

Most of the pathways known to play patterning roles in the neural tube, including bone morphogenetic protein (BMP), wingless (Wnt) and retinoic acid, interact with Shh signaling (Nishi et al., 2009). Retinoic acid signaling from the paraxial mesoderm works with Shh signaling to specify ventral progenitors (Novitch et al., 2003; Pierani et al., 1999; Wichterle et al., 2002). BMP antagonists in the ventral neural tube mediate a BMP activity gradient reciprocal to that of Shh (Liem et al., 2000; McMahon et al., 1998; Patten and Placzek, 2002). BMPs and Wnts are expressed at the dorsal midline of the neural tube and are needed for specifying dorsal progenitors (Chesnutt et al., 2004; Lee et al., 2000; Lee et al., 1998; Liem et al., 1997; Liem et al., 1995; Murayama et al., 2002; Parr et al., 1993; Timmer et al., 2002; Wine-Lee et al., 2004; Zechnier et al., 2007). Shh signaling interacts with both the Wnt and BMP pathways; Wnts directly regulate Gli3 repressor, and Gli3 activity can regulate canonical Wnt signaling (Alvarez-Medina et al., 2008; Ulloo et al., 2007; Yu et al., 2008). Furthermore, Wnt signaling regulates ventral neural fates through Gli3 in a time-dependent manner (Yu et al., 2008). Effectors of both BMP and Wnt signaling interact with Gli3 (Liu et al., 1998; Meyer and Roelink, 2003; Ulloo et al., 2007). Our finding that the recovery of patterning in Arl13b ΔArl and Arl13b ΔArl9.5 embryos is Gli3 dependent raises the possibility that one of these pathways could control the Gli3 repressor activity that is crucial to the recovery we saw.

Our findings further define the critical time points when Shh signaling is required in neural patterning during development. Our data show that in the neural tube, the initial Shh activity gradient is ultimately instructive, yet inputs to the transcriptional circuit can be manipulated and perturbed in ways that can temporarily disrupt any cell fate decision. Our observation that initially mispatterned neural progenitors can recover a wild-type pattern over time highlights the functional transition from an activity gradient of Shh signaling to the role of the downstream transcriptional circuit in regulating cell fate decisions. In addition, our data reveal a crucial role for Gli3 in maintaining the balance of the feed-forward and negative-feedback signals that determine neural cell fate. Thus, our data provide in vivo evidence to support a model of a fundamental mechanism through which positive and negative effectors of Shh signaling function to properly specify and maintain cell fates in the mammalian spinal cord.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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Fig. S1. The generation of a conditional Arl13b allele. (A) Schematic shows that exon 2 of the wild-type Arl13b locus is flanked by loxP sites with a puromycin-resistance cassette. The hmn mutation (*) disrupts the splice site of exon 2 of Arl13b in Arl13b<sup>hmn</sup>. The puromycin-resistance cassette is removed by Flp recombinase, and exon 2 can be deleted upon Cre recombination. Genomic DNA is digested with BglII (Bgl) and BamHI (Bam), and probed with two unique external probes (black bars) to confirm homologous targeting. (B) The targeted locus creates a BamHI site that reduces a 13.5 kb BglII fragment into one 8.6 kb and one 7.5 kb fragment that can be detected by unique 5’ and 3’ external probes, respectively. (C-F) When Arl13b is deleted by germline-expressed Ella-Cre, the E10.5 Arl13b<sup>hmn</sup>Embryoscope<sup>Ella-Cre</sup> embryo shows exencephaly (E) and an expansion of Olig2 cells (F) that are identical to Arl13b<sup>hmn</sup> (C,D). (G-J) When a high dose of tamoxifen is injected at E6.5, Arl13b<sup>CAGG-Cre</sup> can recapitulate the null phenotype by embryo morphology (G,J) and the expansion of Olig2 cells (H,J) at E9.5.
Fig. S2. The rate of Arl13b protein turnover in vivo. (A,B) Arl13b can be detected in all cilia at E8.5 in control (A), whereas some cells do not have Arl13b in Arl13b<sup>ΔE9.5</sup> (B). (C-F) The expression of Arl13b can be observed along the ventricular zone in control embryos at E9.0 (C) and E9.25 (E). However, there is a dramatic decrease of Arl13b expression in Arl13b<sup>ΔE9.5</sup> at both stages (D,F).
Fig. S3. The kinetics of Arl13b deletion in conditional Arl13b knockout MEFs is similar to in vivo. (A-I) Arl13b (red) is localized in the cilium (stained by acetylated α-tubulin, green) in control MEFs under serum-free conditions (A,C,E,G). Arl13b expression in Arl13b\textsuperscript{ΔCAGG-Cre} MEFs is barely detected after tamoxifen treatment for 24 (B), 36 (D), 42 (F) or 48 (H) hours under serum-free conditions. The insets show Arl13b staining alone. Quantification of Arl13b-expressing cells in control (blue) and Arl13b\textsuperscript{ΔCAGG-Cre} (red) (I). (J-R) Arl13b (red) is colocalized with acetylated α-tubulin (green) in control MEFs under serum-containing conditions (J,L,N,P). The decrease in Arl13b expression is more dramatic in Arl13b\textsuperscript{ΔCAGG-Cre} MEFs that are cultured in serum-containing media for 24-48 hours after adding tamoxifen (K,M,O,Q). Quantification of Arl13b expression in control (blue) and Arl13b\textsuperscript{ΔCAGG-Cre} (red) MEFs (R). *P<0.05.
Fig. S4. Arl13b is still present at E10.5 when Cre expresses at E9.5. (A,B,D,E) Brn4-Cre (green) is expressed in the whole neural tube at E10.5 in *Arl13b*<sup>Brn4-Cre</sup> (D), but Arl13b can still be detected (E), even though *Brn4-Cre* begins to be expressed at E9.5. Control embryo does not contain Cre, but does express Arl13b in cilia (A,B). Hoechst (blue) stains nuclei. (C,F) E10.5 control (C) and *Arl13b*<sup>Brn4-Cre</sup> (F) caudal neural tubes show identical Olig2 (red) and Shh (green) expression.

Fig. S5. Proliferation is normal in the absence of Arl13b. (A-D) Phospho-histone H3 (red) is localized along the ventricular zone of neural tubes at E10.5 (A,C) and E11.5 (B,D), and there is no significant difference between Cre-negative *Arl13b<sup>floxed/floxed</sup>* or Cre-negative *Arl13b<sup>floxed/loxP</sup>* (A,B) and *Arl13b<sup>ΔE9.25</sup>* (C,D).