The Graded Response to Sonic Hedgehog Depends on Cilia Architecture

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SUMMARY
Several studies have linked cilia and Hedgehog signaling, but the precise roles of ciliary proteins in signal transduction remain enigmatic. Here we describe a mouse mutation, hennin (hnn), that causes coupled defects in cilia structure and Sonic hedgehog (Shh) signaling. The hnn mutant cilia are short with a specific defect in the structure of the ciliary axoneme, and the hnn neural tube shows a Shh-independent expansion of the domain of motor neuron progenitors. The hnn mutation is a null allele of Arl13b, a small GTPase of the Arf/Arl family, and the Arl13b protein is localized to cilia. Double mutant analysis indicates that Gli3 repressor activity is normal in hnn embryos, but Gli activators are constitutively active at low levels. Thus, normal structure of the ciliary axoneme is required for the cell to translate different levels of Shh ligand into differential regulation of the Gli transcription factors that implement Hedgehog signals.

INTRODUCTION
Nonmotile primary cilia are microtubule-based organelles present on nearly every interphase cell in vertebrates whose functions are only beginning to be understood (Scholey and Anderson, 2006; Wheatley, 1995; Wheatley et al., 1996). Some specialized nonmotile cilia such as olfactory cilia and photoreceptor outer segments act as chemosensory antennae, and others act as mechanosensors (Eley et al., 2005). Recent data argue that the broadly distributed, nonspecialized primary cilia are important for Hedgehog signal transduction. Cilia depend on intraflagellar transport (IFT) proteins for their assembly and maintenance (Scholey, 2003), and the ability to respond to Hedgehog ligands is lost in embryos that lack any one of six different IFT proteins (Houde et al., 2006; Huangfu and Anderson, 2005; Huangfu et al., 2003; Liu et al., 2005; May et al., 2005). Hedgehog-responsive cells are ciliated, and Hedgehog signaling depends on both the anterograde and retrograde IFT motors, which suggested that the cilium is an organelle required for Hh signaling (Huangfu and Anderson, 2005). This hypothesis was supported by the demonstration that several components of the classical Sonic hedgehog (Shh) signaling pathway are enriched in cilia. Smoothened (Smo), a transmembrane protein that is essential for Hh signaling, is enriched in the cilia of Hh responding cells in the mouse embryo (Corbit et al., 2005). The Gli transcription factors are the effectors of vertebrate Hh signaling. Overexpressed Gli1, Gli2, and Gli3 proteins are localized to cilia of primary limb bud cells (Haycraft et al., 2005). Endogenous Gli3 and Suppressor of Fused (SuFu), a negative regulator of Hedgehog signaling, have also been detected in limb bud cell cilia (Dunaeva et al., 2003; Haycraft et al., 2005; Merchant et al., 2004).

Patterning of the cell types in the ventral neural tube depends on a graded response to Shh ligand. Shh produced in the notochord moves to the ventral spinal cord, where it induces a series of ventral cell types in a concentration-dependent manner (Echelard et al., 1993). High concentrations of Shh induce the floor plate at the ventral midline of the neural tube, and progressively lower levels of Shh can specify a series of five different types of neurons in a concentration-dependent manner, including motor neurons and four different classes of interneurons (Briscoe et al., 2000; Ericson et al., 1997). Mutants that lack all Hh signaling do not specify a floor plate and lack all five classes of ventral neurons (Chiang et al., 1996; Litingtung and Chiang, 2000). The gradient of Shh activity in the spinal cord can be visualized in the graded expression pattern of Patched1 (Ptc1), a direct Shh target gene that is expressed in a ventral-to-dorsal gradient in the spinal cord (Eggerschwiler et al., 2001; Goodrich et al., 1997).

All vertebrate Hh signaling is mediated by the Gli family of transcription factors: Gli1, Gli2, and Gli3. Proteolytically processed forms of Gli proteins (Gli-repressor) can inhibit transcription of target genes in the absence of ligand, and full-length Gli proteins can be modified in the presence of Hh ligands (Gli-activator) to promote target gene transcription (Dai et al., 1999; Litingtung et al., 2002; Ruiz i Altaba, 1998; Sasaki et al., 1997; Wang et al., 2000). In the ventral neural tube Gli2 is predominantly an activator; Gli2 mutants lack the extreme ventral neural cell types that depend on high levels of Shh (Ding et al., 1998; Matise et al., 1998). Gli3 mutants have subtle defects in patterning of lateral cell types (Persson et al., 2002), but Gli3
repressor and activator activities are revealed in Gli3 mutants that also lack Shh, Smo, or Gli2 (Bai et al., 2004; Lei et al., 2004; Litintung and Chiang, 2000; Motoshima et al., 2003; Wijgerde et al., 2002). Shh controls the balance of the Gli activator and repressor, thereby generating a Gli activity gradient that is sufficient to define all ventral neural fates (Stamatakis et al., 2005). IFT proteins are required for both activity of Gli activator forms and for the proteolytic processing that generates Gli3 repressor (Huangfu and Anderson, 2005; May et al., 2005; Liu et al., 2005; Haycraft et al., 2005); as a result, IFT mutants lack all responses to Hh ligands.

Here we describe hennin, an ENU-induced mutation that has an unprecedented effect on dorsal-ventral patterning of the mouse neural tube. In hnn embryos, the ventrolateral domain of motor neuron progenitors is expanded at the expense of both the most ventral and the most dorsal neural cell types. We find that the mutation responsible for the hnn phenotype disrupts Arl13b, a member of the small GTPase superfamily. Arl13b (formerly called Ar21l) is also disrupted in the zebrafish scorpion mutant where it is required for the formation of kidney cilia (Sun et al., 2004), although no role in neural patterning was described. We find that the mouse Arl13b protein is localized to cilia, and in its absence, cilia are short and display a specific structural defect in the ciliary axoneme. The data indicate that production of the Gli activity gradient that defines the spatial organization of ventral neural cell types depends on cilia structure.

RESULTS

hennin Mutant Embryos Have Defects in Neural Tube Patterning, Limbs, and Eyes

hnn was identified in a recessive ENU screen that identified mutations that disrupted the morphology of the e9.5 mouse embryo (Garcia-Garcia et al., 2005). At that stage, hnn mutants showed several phenotypes, including an open neural tube in the head and caudal spinal cord, and randomized heart looping. hnn mutants survived until e13.5–14.5, when they also displayed abnormal eyes and axial polydactyly (Figures 1A–1E).

We tested whether the abnormal morphology of the hnn neural tube was caused by a disruption of patterning of cell types along the dorsal-ventral axis. Motor neurons are a Shh-dependent cell type and normally arise in a ventrolateral region of the neural tube. In the caudal neural tube of hnn mutants, the domain expressing the motor neuron markers HB9, Lhx3, and Is1/2 was expanded both ventrally and dorsally to include the ventral two-thirds of the neural tube (Figures 1H, 1M, 5B, 5C, 5E and 5F), a phenotype that has not been described in other mutants that affect neural patterning.

The increased number of motor neurons seen in hnn embryos (Figures 1H and 1M) could have been due to either overproliferation of motor neuron precursors or to an early expansion of the domain that gives rise to motor neurons. We examined expression of the cell cycle markers phospho-histone H3 and Ki67 and found they were indistinguishable in wild-type and hnn neural tubes from e8.5–e10.5 (see Figure S1 in the Supplemental Data available with this article online; data not shown). In contrast, Olig2, an early marker of motor neuron precursors, was expressed in an expanded domain of e9.5 hnn embryos (Figures 1G and 1L). Thus the hnn phenotype is the result of abnormal cell type specification rather than abnormal proliferation.

The Gradient of Sonic Hedgehog Activity Is Shallow in the hnn Neural Tube and Independent of Shh Ligand

Ptch1 is a direct transcriptional target of Shh signaling and therefore reflects Shh activity (Agre et al., 2004; Marigo et al., 1996). In wild-type embryos, there is a ventral-to-dorsal gradient of Ptch1 expression, which can be assayed by the expression of a Ptch1-lacZ reporter allele (Eggerschwiler et al., 2001). In hnn mutants, there was only a shallow gradient of Ptch1-lacZ expression: the level of Ptch1-lacZ at the ventral midline was lower than in wild-type embryos, and the domain of Ptch1-lacZ expression extended further dorsally than normal (Figures 1I and 1N).

The altered pattern of expression of Ptch-lacZ in hnn mutants indicated that the effective domain of Shh signaling in the neural tube was expanded while the highest level of response to Shh was lost. One possible explanation for this Ptch-lacZ expression pattern was that the Shh morphogen spread further from its source in the notochord. To test if there was an altered distribution of Shh ligand in hnn, we analyzed neural patterning in the Shh hnn double mutants. Patterning in the caudal neural tube of Shh hnn double mutants was indistinguishable from that in hnn single mutants (Figures 1J and 1O; data not shown); for example, motor neurons markers were expressed in the same expanded domain in the double as in the single mutants. As this indicates that hnn does not affect the distribution of Shh ligand in the neural tube, Arl13b must affect the Hedgehog signal transduction pathway at a point downstream of Shh ligand.

The hennin Mutation Disrupts Arl13b, which Encodes a Ciliary Protein

Using meiotic recombination, we mapped the hnn mutation to a 2 MB interval on mouse chromosome 16 that contained five predicted transcripts. We sequenced all five transcripts and found one mutation: a T-to-G transversion in the splice acceptor site of exon 2 of Arl13b (Figure 2A). RT-PCR analysis confirmed that the Arl13b transcripts in hnn did not contain exon 2. The remaining open reading frame lacked most of a predicted GTPase domain, including the four consensus nucleotide binding sites (Figure 2A).

Immunofluorescent staining revealed that Arl13b is expressed in the ventricular zone of the neural tube in a punctate pattern reminiscent of cilia (Experimental Procedures; Figures 2C and 3D). We confirmed the ciliary localization of Arl13b by double labeling with acetylated α-tubulin, which is enriched in the stable microtubules of the ciliary axoneme. Arl13b and acetylated α-tubulin colocalized in
the neural tube, primary mouse embryonic fibroblasts (PMEFs), and the e8.0 node (Figures 3A–3L), indicating that Arl13b is indeed enriched in cilia. To determine how the hnn splice site mutation, which eliminated exon 2, affected the protein, we performed immunofluorescence and western blotting in the hnn mutants with the antibody raised against the C-terminal half of Arl13b (Figures 2B–2D). Affinity-purified antibody specifically recognized the predicted 48 kDa band on a western blot of wild-type e10.5 whole embryo protein extract, as well as a larger band which is likely to be a posttranslationally modified Arl13b protein (see Experimental Procedures). Although a transcript including the C-terminal domain was detected in the hnn mutants by RT-PCR, both specific bands on the western were absent in extracts from hnn mutant embryos. In addition, no staining in cilia was detected in hnn mutant PMEFs by immunofluorescence (Figures 3J–3L). These results indicate that the hnn mutation is a null allele of the Arl13b gene.

Arl13b is a member of the Arl (ADP ribosylation factor [Arf] related) subfamily of the Ras superfamily of small GTPases. Specific members of the Arf/Arl group have been shown to be important for microtubule dynamics, lipid metabolism, or vesicle trafficking (Antoshechkin and Han, 2002; Hoyt et al., 1990; Kahn et al., 2006; Li et al., 2004; Radcliffe et al., 2000; Zhou et al., 2006), although the functions of most of the members of this group are not known. Arl13b is unusual among the Arf/Arl family, as most members are ~20 kDa in size and composed only of their Arf domain, whereas Arl13b is a 48 kDa
The Ciliary Axoneme Is Abnormal in hnn Mutants

Because Arl13b localized to cilia, we examined the structure of the cilia in the node of e8.0 embryos, which are relatively long and easy to visualize. Scanning electron microscopy (SEM) showed that hnn nodal cilia were approximately half the length of wild-type cilia (48% ± 18%); Figures 4A and 4B). In transmission electron microscopy (TEM) cross-sections, wild-type nodal cilia have nine peripheral doublet microtubules located around the circumference of the axoneme (Figures 4C and 4E). In wild-type cilia, the A-tubule of the doublet is composed of 13 tubulin protofilaments, and the B-tubule has 10 tubulin protofilaments and an 11th filament that connects the B-tubule to the A-tubule (Linch and Stephens, 2007; Tilney et al., 1973). All sections of hnn mutant nodal cilia showed disruptions of the outer doublet organization; in many outer doublets the B-tubule was not closed or attached to the A-tubule (Figures 4E and 4F). Thus Arl13b appears to have a specific role in the assembly of the outer doublet.

It has previously been reported that, unlike most motile cilia, the motile cilia of the mouse node lack the central pair of microtubules (Takeda et al., 1999). It was therefore surprising that a central pair was clearly present in some sections of both wild-type and hnn mutant nodal cilia (Figures 4E and 4F); this finding would be consistent with the observation that there are motile and immotile cilia present in the node (McGrath et al., 2003) as well as recent data showing the presence of a central pair in rabbit nodal cilia (Feistel and Blum, 2006). We speculate that we observed central pairs because of the rapid fixation procedure used (see Experimental Procedures), as the central pair microtubules are more labile than the outer doublet (Behnke and Forer, 1967; Stephens, 1970). Because hnn cilia showed a specific defect in the B-tubule in sections that contained the labile central pair, we conclude that the electron micrographs reflected the true structure of the hnn axoneme.

The Role of Arl13b in Neural Patterning Depends on Cilia

The hnn mutant has striking defects in both cilia structure and activity of the Shh pathway. To test whether the defect in cilia structure could be responsible for the signaling defect, we analyzed embryos that lack both Arl13b and cilia. wimple (wim) mice lack the function of IFT172 and therefore have no cilia (Huangfu et al., 2003). Patterning of the caudal neural tube of wim and hnn wim double mutants was indistinguishable: there was no floor plate, no V3 in the floor plate and the motor neuron progenitor domain, was expanded both dorsally and ventrally in the hnn neural tube (Figure 5). Because Arl13b was localized in cilia and required for cilia structure and loss of Arl13b had no effect on neural patterning if cilia were not present, the simplest interpretation of the findings is that the primary function of Arl13b is in the construction of the ciliary axoneme, and that the correct structure of the axoneme is required for normal specification of cell types in the neural tube.

Arl13b Acts Downstream of Patched and Smo

To better define the role of Arl13b in neural patterning, we examined neural cell type specification in the hnn neural tube in greater detail. As described above (Figure 1), the domain of motor neurons was expanded both dorsally and ventrally in the hnn neural tube, paralleling the change in the expression pattern of Patch1. Shh, which is normally expressed in the notochord and induces the formation of the floor plate at the ventral midline of the neural tube, was expressed in the hnn notochord. However, markers of the floor plate, Shh and FoxA2, were not expressed in the neural tube (Figures 1F, 1K, 6A, and 6B). Nkx2.2, which is normally expressed in the p3 domain between the floor plate and the motor neuron progenitor domain, was detectable in hnn extracts. As the 60 kDa band is specific and many ARL proteins are posttranslationally modified, the ~60 kDa band is likely to represent a modified form of Arl13b. The 70 kD band is an unrelated crossreacting protein (see Experimental Procedures).
expressed across the ventral midline of hnn mutants and overlapped with expression of Olig2, a motor neuron progenitor marker (Figures 6E, 6F, 6I, and 6J). Pax6 is normally expressed at high levels in progenitors of lateral interneurons, but the Pax6 expression domain was shifted to the dorsal third of the hnn neural tube (Figures 1G, 1L, 5A, and 5D) and lateral Chx10- and En1-positive (V2 and V1) interneurons were shifted dorsally (data not shown).
Accompanying the shift of Pax6 to the dorsal neural tube, dorsal cell types were disrupted in hnn embryos: expression of Wnt1, a roof plate marker, was discontinuous in the caudal hnn embryo, and dorsal progenitors that normally express Math1 and Mash1 were not properly specified (Figure S2). Thus the expansion of the motor neuron domain in hnn embryos occurred at the expense of both more dorsal and more ventral neural cell types.

To help define how Arl13b affected Shh signaling, we generated double mutants that lacked both Arl13b and Smo, the membrane protein that promotes the response to Hh ligands. Smo mutants lack both Shh and Ihh signaling and have a slightly stronger effect on neural patterning than Shh mutants (Caspary et al., 2002; Zhang et al., 2001). Like hnn, the Smo hnn double mutants expressed Olig2 in the ventral two-thirds of the neural tube (Figures 6I, 6J, 6M, and 6N). Thus Arl13b must act at a step downstream of Smo in the cytoplasmic signal transduction pathway.

As expected because Ptch1 acts upstream of Smo, and Smo appeared to act upstream of Arl13b, loss of Arl13b modified the Ptch1 phenotype. In Ptch1 mutant embryos, the entire neural plate is specified as floor plate, the most ventral cell type (Goodrich et al., 1997). In Ptch1 hnn double mutant embryos, floor plate cells (expressing FoxA2) and motor neuron precursors (expressing Olig2) were both specified, intermixed at all positions along the dorsal-ventral axis (Figures 6A–6D and 6I–6L). Thus loss of Arl13b must act at a step downstream of Smo in the cytoplasmic signal transduction pathway.

As in hnn single mutants, the domain of motor neurons and their progenitors expanded dorsally in the Gli2 hnn neural tube (Figure 7H and data not shown); thus the level of Gli2 activator required for the initial specification of

The hnn Phenotype Is Associated with Altered Gli Activity

Because the specification of the ventral neural cell types affected in hnn embryos depends on the Gli2 and Gli3 transcription factors, we investigated whether Arl13b was required for normal regulation of Gli2, Gli3, or both.

Gli2, the primary transcriptional activator for Hh signaling in the neural tube, is required for specification of the floor plate and the normal number of Nkx2.2-expressing V3 interneuron progenitors (Ding et al., 1998; Matise et al., 1998). hnn mutants lack a floor plate, which suggested that hnn embryos lacked the high level of Gli2 activator required for floor plate specification. The number of Nkx2.2-expressing cells was decreased in the Gli2 hnn double mutant compared to hnn single mutants (Figures 7B and 7F), which indicated that Gli2 had some activity in hnn mutants.

As in hnn single mutants, the domain of motor neurons and their progenitors expanded dorsally in the Gli2 hnn neural tube (Figure 7H and data not shown); thus the level of Gli2 activator required for the initial specification of
motor neurons was present in the hnn neural tube. However, there were fewer mature motor neurons in the expanded motor neuron domain of the Gli2 hnn double mutants than in hnn single mutants (Figures 7D and 7H). It has been shown that the activator functions of both Gli2 and Gli3 promote the differentiation of motor neurons (Bai et al., 2004; Lei et al., 2004). We therefore think it likely that the reduction in the number of mature motor neurons in Gli2 hnn double mutants reflects the loss of Gli2 coupled with decreased levels of Gli3 activator.

Double mutants that lacked both Gli3 and hnn showed a greater expansion of ventrolateral cell types than hnn single mutants: the motor neuron domain in the double mutants expanded to include the entire neural tube (Figures 7D and 7L). Similarly, the Nkx2.2 expression domain of Gli3 hnn double mutants expanded to include all but the most dorsal neural tube (Figure 7J). Thus in the absence of both Gli3 and Arl13b, there is essentially no patterning of cell types along the dorsal-ventral axis, and nearly all neural cells assumed one of two ventrolateral identities, that of motor neurons or V3 interneurons. The double mutant phenotype suggests that the residual polarity present in hnn embryos is due to asymmetric activity of Gli3 repressor, with less Gli3 repressor ventrally than dorsally.

In the absence of Shh, Gli3 is processed to make a transcriptional repressor that prevents transcription of target genes in the neural tube. Unprocessed Gli3 is present in cilia (Haycraft et al., 2005), and Gli3 processing is blocked in mutants that lack cilia due to IFT mutations (Haycraft et al., 2005; Huangfu and Anderson, 2005; Liu et al., 2005). However, processing of Gli3 appeared to be normal in hnn embryos (Figure 7), despite their abnormally structured cilia. Thus while normal activity of Gli2 requires Arl13b, Gli3 repressor activity does not depend on Arl13b.

DISCUSSION

The hennin Phenotype Is Due to Loss of Function of Arl13b

The hnn mutant was identified on the basis of its abnormal morphology and abnormal neural patterning. Several lines of evidence demonstrate that the hnn mutant phenotypes are the result of loss of function of Arl13b. The hnn mutation mapped to an interval containing only five genes, and a mutation was found only in Arl13b. This splice site mutation does not result in the production of stable Arl13b protein as demonstrated by the lack of protein in hnn mutants by both immunofluorescence and western blot analysis. Furthermore, the Arl13b protein is localized to cilia, and cilia are defective in the hnn mutant. Additional support for the role of Arl13b in cilia comes from the zebrafish scorpion mutation, which is caused by a retroviral insertion into the zebrafish ARL13B gene; the scorpion mutation causes cystic kidneys, and kidney cilia were not detectable in scorpion mutants (Sun et al., 2004).

The Function of Arl13b in Cilia Structure

The Arf/Arl family within the Ras superfamily of small GTPases is defined by sequence similarity and not by a common cellular function (Kahn et al., 2006). Of the 30 Arf/Arl mammalian proteins, several have been shown to affect vesicle trafficking, and three have been implicated in cilia and microtubule dynamics (Hoyt et al., 1990; Kahn et al., 2006; Van Valkenburgh et al., 2001; Vitale et al., 1998). Arl3 is localized to cilia (Zhou et al., 2006), and genetic and biochemical studies have suggested that Arl2 regulates microtubule assembly (Antoshechkin and Han, 2002; Bhamidipati et al., 2000; Cuvillier et al., 2000; Li et al., 2004; Radcliffe et al., 2000; Zhou et al., 2006). Arl3 regulates flagellar synthesis in flagellated protozoans (Cuvillier et al., 2000). Arl6 is mutated in human...
Bardet-Biedl Syndrome 3 (BBS3), a disease associated with defects in basal bodies and cilia (Fan et al., 2004). An Arl13b-related protein in C. elegans localizes to ciliated neurons (Fan et al., 2004), although this protein does not include the long C-terminal tail of Arl13b. The precise biochemical connections between Arl proteins and microtubules have not been defined, but the association of several Arl proteins with cilia suggests that some of these proteins regulate specific aspects of cilia structure.

We identified two defects in the structure of nodal cilia that lack Arl13b: they are half the normal length and the axoneme is asymmetrical, but Shh signaling must be intact as these animals are viable. Therefore, it is likely that the abnormal axoneme structure caused by loss of Arl13b, rather than shorter length of cilia, leads to the disruption of Shh signaling.

Mice that lack the transcription factor Rfx3 have shortened node cilia with normal ultrastructure (Bonnafe et al., 2004). These animals show randomized left/right asymmetry, but Shh signaling must be intact as these animals are viable. Therefore, it is likely that the abnormal axoneme structure caused by loss of Arl13b, rather than shorter length of cilia, leads to the disruption of Shh signaling.

The open B-tubule in the cilia of hnn mutants indicates that Arl13b is required for a specific aspect of axoneme structure. Structural and biochemical studies have identified several nontubulin proteins that are enriched at the junction of the A- and B-tubules of the outer doublet, and suggest that specific protein complexes are required to link the B-tubule to the A-tubule (Linck and Norrander, 2003; Nicastro et al., 2006; Stephens, 2000; Sui and Downing, 2006). Arl13b could be a protein in the 11th filament of the B-tubule (Linck and Stephens, 2007; Tilney et al., 1973), or it may regulate the assembly of a protein(s) that links the two tubules of the axonemal outer doublet.

**The Function of Arl13b in Hh Signaling**

The hnn phenotype does not correspond to either a simple decrease or increase in the activity of the Hh pathway, as the cells that require the highest Hh activity fail to be specified, while cells that require intermediate Hh activity are found in an expanded domain, a phenotype that has not been seen in other mouse mutants. It has been suggested that cilia may be important for signaling pathways other than the Hh pathway (Davis et al., 2006), so the complexity of the hnn phenotype could, in principle, reflect the disruption of more than one signaling pathway. Although we cannot rule out the possibility that the hnn mutation affects multiple signaling pathways, the altered patterning of neural cell types seen in hnn embryos parallels the expanded, shallow gradient of Ptc-hlacZ expression in the mutant neural tube, and Ptc-hlacZ expression is a direct readout of Hh pathway activity. We therefore conclude that the most parsimonious interpretation of the hnn phenotype is that loss of Arl13b specifically disrupts the Shh signaling pathway so that the pathway has an intermediate level of activity in an expanded domain.

Although the shallow Ptc1 expression gradient and expanded domain of motor neurons in the hnn neural tube could arise if the Shh ligand were spread over a larger region of the neural tube, the phenotype of the Shh hnn double mutant shows that the hnn phenotype does not depend on the presence of Shh ligand. Furthermore, the double mutants with Ptc1 and Smo indicate that Arl13b acts downstream of Smo, the same step in the pathway that requires IFT proteins (Huangfu et al., 2003).

The hnn phenotype is reminiscent of that caused by loss of the zebrafish ijuana gene, a component of the zebrafish Hh pathway. The ijuana gene encodes DZIP1, a large protein with coiled-coil domains and a single zinc finger (Sekimizu et al., 2004; Wolff et al., 2004). hnn and ijuana mutants both show both a failure to activate the highest responses to Hh and an expanded domain of Hh response. Like hnn, the ijuana phenotype is ligand...
independent. It has been proposed that the complex phenotype of iguana embryos arises because DZIP1 controls nuclear import of both Gli activator and repressor forms, or that DZIP1 acts in the cytoplasm to modulate that activity of Sufu, a negative regulator of Hh signaling (Sekimizu et al., 2004; Wolff et al., 2004).

The neural tube of the mouse embryo provides a good context to understand phenotypes of the hnn phenotype because it is known that the identity of ventral neural cell types is defined by the ratio of Gli activator/Gli repressor and that changes in the effective level of either the activator or the repressor can alter the Gli activity gradient. The floor plate and the highest level of Ptc1 expression both depend on high levels of Gli2 activator (Ding et al., 1998; Eggenschwiler et al., 2006, 2001; Matise et al., 1998) and both are lost in hnn mutants, which indicates that the highest level of Gli2 activator is not achieved in hnn embryos. In contrast, the dorsal expansion of motor neuron progenitors and Ptc1 expression would correspond to an increase in the ratio of Gli activator/Gli repressor in lateral and dorsal neural cells. The dorsal expansion of motor neurons cannot be due simply to ectopic Gli2 activity, as the expanded domain is still present in hnn Gli2 double mutants. The dorsal expansion is therefore likely to be due to changes in the activity of both Gli2 and Gli3.

Gli3 processing appears to be normal in hnn embryos, even though IFT proteins (and therefore cilia) are required for normal Gli3 processing (Huangfu and Anderson, 2005; Liu et al., 2005; May et al., 2005). Although the hnn mutation affects neural tube patterning only at posterior positions, decreased Gli3 processing was detected in mutants that lack Rab23 (Eggenschwiler et al., 2006), which also affects patterning in only the caudal neural tube. Therefore, it appears that hnn cilia can promote Gli3 processing despite their abnormal structure. In addition, the strong enhancement of the hnn phenotype seen in hnn Gli3 double mutant embryos demonstrates that Gli3 repressor is functional in hnn embryos. The data are thus consistent with the hypothesis that neither Gli3 repressor production nor Gli3 repressor activity is affected by loss of Arl13b. We therefore conclude it is likely that the dorsal expansion of the motor neuron domain is due to ectopic activity of both Gli2 and Gli3 activators and that the hnn phenotype is the result of abnormal regulation of Gli activators, without changes in Gli3 repressor. This indicates that two cilia-dependent events, the events that promote formation of Gli activators and those that promote production of Gli3 repressor, can be regulated independently: the events that lead to formation of Gli3 repressor can occur in the normal cilia that form in the absence of Arl13b, but those that regulate the production of Gli activators cannot.

In mutants that lack cilia altogether, such as Ift172 and Ift88 mutants, no Gli activator is produced (Haycraft et al., 2005; Huangfu and Anderson, 2005; Liu et al., 2005). In contrast, in hnn mutants, it appears there is a constitutive low level of Gli activator in all cells in the neural tube. We therefore propose that there are two cilia-dependent steps in the formation of Gli activators. We propose that full-length Gli proteins are modified within the cilia in a Hh-independent process to have a low level of activator function, and the low-level activator is normally tethered in the cilia in the absence of ligand. The idea that there are ligand-independent steps in Hh signaling that depend on cilia is not novel: processing of Gli3 to make Gli3 repressor is also cilia dependent and occurs in the absence of ligand (Huangfu and Anderson, 2005). In response to Hh, full-length Gli proteins can be further modified to create high-level activator, which is then released from the cilium to the nucleus. In the abnormal cilia that lack Arl13b, the modification that produces full-length Gli protein with low-level activity takes place, but this low-level activator is not effectively tethered in the cilium and is released inappropriately to the nucleus in the absence of Hh ligand. This step retains a small amount of sensitivity to upstream signals in hnn mutant, as the phenotype of Ptc1 hnn double mutants is not identical to the hnn phenotype.

Two alternative views of the relationship between cilia and Hh signaling have been proposed (Scholey and Anderson, 2006). In the simpler view, cilia represent a site where Hh pathway components are enriched, and the high local concentration of the proteins allows efficient signaling transduction. Alternatively, dynamic trafficking within the cilia may allow a sequence of protein interactions that promote the activity of the pathway. The hnn phenotype supports the latter model, as it reveals a complexity of events that occur within the cilium.

EXPERIMENTAL PROCEDURES

Mouse Strains
We identified hnn in a screen for recessive N-ethyl, N-nitrosourea mutations that caused morphological defects at e9.5 (Garcia-Garcia et al., 2005). Other mouse strains used were: Shh, Ptc1+/−/−, Ptc1 (lacZ reporter, D allele, M.P. Scott, personal communication), Smo+/-, Rab23/-/-, wim, Gli2, and Gli3+/−/− (Caspari et al., 2002; Chiang et al., 1996; Eggenschwiler and Anderson, 2000; Goodrich et al., 1997; Huangfu et al., 2003; Hui and Joyner, 1993; Matise et al., 1998). All phenotypes were analyzed in the C3H background, and crosses and genotyping were performed as described (Caspari et al., 2002; Chiang et al., 1996; Eggenschwiler and Anderson, 2000; Goodrich et al., 1997; Huangfu et al., 2003; Hui and Joyner, 1993; Matise et al., 1998).

Genetic Mapping and Molecular Identification of hnn
MIT SSLP markers were used to map hnn on mouse chromosome 16. Additional polymorphic markers were generated for high-resolution mapping (http://mouse.ski.mskcc.org). In a mapping cross of 1283 opportunities for recombination, hnn was mapped to a 2.08 Mb interval between D16S20047 (D16S20047F: ATGGCCCTAATCGGTGCTGTCCTTT; D16S20047R: GGGACTGATCTTTTGCGAACA) and D16S20174 (D16S20174F: TGGTCATGCGATATGCTGAA; D16S20174R: GCTACTG TATTCTGTGTTCTGTGA). The only sequence change detected in the interval was a T-to-G substitution in the splice acceptor site of exon 2 of the Arl13b transcription unit.

Antibodies were raised in rabbit to a purified bacterially expressed protein of GST fused to amino acids 208–428 of Arl13b. Serum was used at 1:1500 dilution for immunohistochemistry. The serum was pre-absorbed against the fusion protein for western analysis and used at 1:5000 along with an anti-actin Ab (Sigma, 1:2000). Three bands were detected in e10.5 whole-embryo extracts and purified antigen
specifically competed away the 48 kDa and ~60 kDa bands, indicating that the remaining ~70 kDa band in hnn extracts is not specific.

Phenotypic Analysis
In situ hybridization and X-gal staining on whole-mount embryos, Aicin blue staining, immunofluorescence, and scanning electron microscopy (SEM) were done as previously described (Huangfu et al., 2003; Shen et al., 1997). For SEM, the embryos were viewed with a LEOP50 Field Emission Scanning Electron Microscope at 2.5 kV. For transmission electron microscopy (TEM), samples were dissected in fresh 2% paraformaldehyde/2.5% glutaraldehyde/0.1 M cacodylate buffer (pH 7.4) and fixed overnight at room temperature. After rinsing with 0.1 M cacodylate buffer, the embryos were postfixed with 1% OsO4/0.1 M cacodylate buffer for 1 hr on ice, dehydrated with a graded alcohol series and propylene oxide, and embedded in Durcupan (EM Sciences, Fort Washington, PA). Ultrathin sections were cut on a Reichert Ultracut E, poststained with Uranyl Acetate and Reynold's lead, and viewed with a JEOL CX100 at 80 kV.


References
Cilia Architecture and Sonic Hedgehog Signaling


