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Developmental Biology 287 (2005) 378 – 389
DEVELOPMENTAL BIOLOGY
www.elsevier.com/locate/ydbio

Loss of the retrograde motor for IFT disrupts localization of Smo to cilia and prevents the expression of both activator and repressor functions of Gli

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Received for publication 15 June 2005, revised 24 August 2005, accepted 29 August 2005
Available online 17 October 2005

Abstract

Sonic Hedgehog (Shh) signals are transduced into nuclear ratios of Gli transcriptional activator versus repressor. The initial part of this process is accomplished by Shh acting through Patched (Ptc) to regulate Smoothened (Smo) activity. The mechanisms by which Ptc regulates Smo, and Smo activity is transduced to processing of Gli proteins remain unclear. Recently, a forward genetic approach in mice identified a role for intraflagellar transport (IFT) genes in Shh signal transduction, downstream of Patched (Ptc) and Rab23. Here, we show that the retrograde motor for IFT is required in the mouse for the phenotypic expression of both Gli activator and repressor function and for effective proteolytic processing of Gli3. Furthermore, we show that the localization of Smo to primary cilia is disrupted in mutants. These data indicate that primary cilia act as specialized signal transduction organelles required for coupling Smo activity to the biochemical processing of Gli3 protein.

Keywords: Primary cilia; Smoothened; Shh; Gli cortical patterning

Introduction

Shh is a member of the Hedgehog (Hh) family of evolutionarily conserved signaling molecules. Localized expression of Hh patterns the early embryo by regulating cell fate growth and differentiation decisions in a diverse array of tissues. The importance of proper Hh signaling to human health is clear; defective signaling has profound developmental consequences, in the form of holoprosencephaly for example, and inappropriate activation of Hh signaling in somatic tissues can lead to medulloblastoma, glioma, basal cell carcinoma or prostate cancer (Karhadkar et al., 2004; Ruiz i Altaba et al., 2004; Sanchez et al., 2004). Despite its importance both to human health and to our basic understanding of embryogenesis, information about some of the key steps in the Hh signal transduction mechanism, coupling of cell surface to intracellular events, has been difficult to obtain. Work in both vertebrate and invertebrate systems has outlined the signal transduction steps on both sides of this gap in our understanding. The result of the signal transduction mechanism is to translate extracellular levels of Hh into a gradient of Gli/Ci activator that decreases with distance from the Hh secreting cells and a corresponding gradient of a repressor that increases with distance (Wang et al., 2000a). In the absence of *Hh* signaling, the vertebrate Gli3 and the fly Cubitus interruptus (Ci) proteins are proteolytically processed to produce amino terminal fragments that function as transcriptional repressors (Aza-Blanc et al., 1997; Wang et al., 2000a). Shh binding to Ptc on the surface of the responding cell releases a tonic inhibition of

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doi:10.1016/j.ydbio.2005.08.050

Smo (Casali and Struhl, 2004; Hooper, 2003; Ingham et al., 2000; Marigo et al., 1996; Martin et al., 2001; Taipale et al., 2002). Hh signaling inhibits Gli3 and Ci processing leading to the accumulation of full-length proteins, an event that is associated with the appearance of Gli/Ci activator function. Stabilization of the full-length form, however, is not sufficient to produce a transcriptional activator, indicating that an event in addition to regulation ofGli3/Ci cleavage is also required (Methot and Basler, 1999; Ohlmeyer and Kalderon, 1998).

The step in the Hh signal transduction process that couples Smo activity to Gli/Ci processing is enigmatic. In *Drosophila*, signal transduction involves Costal2 (Cos2), a kinesin-related protein that has been suggested to function as a molecular router, controlling the subcellular localization of signal transduction components including Smo and Ci (Jia et al., 2003; Lum and Beachy, 2004; Monnier et al., 2002; Ogden et al., 2003; Robbins et al., 1997; Ruel et al., 2003; Sisson et al., 1997; Stegman et al., 2004; Wang et al., 2000b). The kinesin-related domain of Cos2 suggests that its function involves microtubule binding. ATP-dependent motor activity has not been demonstrated, however, so it is possible that movement along microtubules is not required.

Vertebrate homologs of Cos2 have only recently been identified and their function has only been analyzed in zebrafish where preliminary results are consistent with the role suggested by the fly (Tay et al., 2005). The involvement of IFT has recently emerged as another piece of this puzzle. IFT is a specialized process for moving material, for example axonemal precursors, into and out of cilia and flagella (reviewed in Rosenbaum and Witman, 2002). It has been most intensively studied in the alga, *Chlamydomonas reinhardtii*, but defects in IFT genes have highlighted the importance of cilia function in sensory neurons, kidney homeostasis and determination of the left–right body axis in vertebrate development. Recently, genes homologous to *Chlamydomonas* IFT genes were shown to be required for Hh signal transduction in the spinal cord (Huangfu et al., 2003). The analysis of mutant phenotypes and genetic epistasis indicates that IFT proteins function downstream of the Patched receptor and are required for activation of gene transcription in response to Shh. Among the ciliary genes implicated in Hh signaling is Kif3A, a component of kinesin motor that transports material into the cilia. Kif3A has roles outside of the cilia (Marszalek and Goldstein, 2000; Nishimura et al., 2004) and the function of the other IFT gene products is not as clearly understood, leaving open the possibility that the defects in Hh signaling are not due to their identified roles in cilial transport.

Materials and methods

Animals and genetic mapping

We identified both alleles of Dnchc2 in an genetic screen for recessive mutations disrupting cortical development at E14.5 (Zarbalis et al., 2004). The two alleles both showed linkage to the proximal end of chromosome 9 and a complementation test revealed that they were noncomplementing. Separate mapping crosses generated over 400 meioses of each allele for high-resolution meiotic mapping. This was performed using simple-sequence length polymorphism markers available from the Whitehead Institute for Biomedical Research, MIT (http://www.broad.mit.edu/resources.html) and additional polymorphic markers that we generated (primer sequences available upon request). Both mutations were mapped to an interval between 5534133 and 10263482 base pairs in the NCBI m33 mouse assembly (http://www.ensembl.org/Mus_musculus/). All animals used for analysis were crossed for at least 3 generations onto an FVB/NJ background.

Gene identification and sequencing

Three Ensembl transcripts (ENSMUST00000034501, ENSMUST00000034500 and ENSMUST00000048417) containing 38 exons orthologous to portions of the mRNA for rat Dnchc2 (NM_023024) were discovered within the genetic interval. BLASTing the mouse Ensembl genome with NM_023024 uncovered an additional 51 exons belonging to the mouse Dnchc2 gene. All 89 exons from mouse Dnchc2 were PCR amplified using genomic DNAs from wild-type, heterozygous and mutant animals for sequence analysis. In line A152, the only mutation discovered was a C to A transversion in exon 8 that creates a stop codon. In line 407, the only mutation found was a T to C transition in exon 46 that creates a missense codon. Sequencing was performed using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

In situ hybridization and antibody staining

Whole-mount in situ hybridization was carried out as previously described (Zarbalis et al., 2004) using digoxygenin-labeled antisense RNA probes. Immunohistochemical localization of proteins was performed as described (Briscoe et al., 2000). Primary cilia were visualized in the limb bud mesenchyme at E10.5 using a monoclonal antiacetylated tubulin antibody (clone 6-11B-1, #T6793, Sigma-Aldrich). Antibodies used to examine the spinal cord are as described (Ericson et al., 1997; Muller et al., 2002; Pierani et al., 1999; Tanabe et al., 1998; Vallstedt et al., 2001).

SEM

Electron microscopy was performed using a Phillips/FEI XL30 W TMP environmental SEM. The dorsolateral ventricular surface of the telencephalon at E10.5 was prepared and imaged as described (http://biology.berkeley.edu/EML/psem.html).

Western blotting

Western blotting was performed using an anti-Gli3 antibody as described (Wang et al., 2000a,b).

Confocal microscopy

Mouse embryos were fixed with 4% PFA for 1 h at 4°C, washed in PT (PBS + 0.2% Triton-X100), and blocked with PBT + 2% BSA + 1% goat serum for 1 h at 4°C. Primary antibodies were added in block buffer and incubated overnight at 4°C. Primary antibodies used in this study are ⟨-acetylated Tubulin (Sigma 6-11B-1, 1:1000), and rabbit ⟨-Smo (1:200). Two ⟨-Smo antibodies were generated against 15 and 16 amino acid peptides corresponding to conserved epitopes of the Smo amino-terminal extracellular domain. Both ⟨-Smo antibodies perform equivalently. After incubation with the primary antibody, cells were washed in PBT and incubated in a secondary block (PBT + 10% donkey serum + 2% BSA) at room temperature for 1 h, followed by incubation with secondary antibodies (1:400 donkey ⟨-rabbit IgG-Alexa 594 and donkey ⟨-mouse IgG-Alexa 488, Molecular Probes) in PBT + 2% BSA at room temperature for 1 h. After washes with PBT and a 10-min DAPI incubation, the samples were mounted and imaged with a Leica DMIRE2 confocal microscope. Images were processed using ImageJ and open source plugins (http://www.uhnresearch.ca/facilities/wcif/imagej).

Results

Identification of mutants with dorsoventral patterning defects in the forebrain

We recently carried out a forward genetic screen in the mouse designed to identify mutations that alter the development of the forebrain (Zarbalis et al., 2004). Two independent mutants with apparently identical phenotypes were identified in this screen. In both cases, the mutant was selected for further study because of severe defects in dorsoventral patterning of the forebrain at E14.5 (Figs. 1A–B). In addition to defective forebrain patterning, roughly half exhibit situs inversus, indicating a randomization of the left–right body axis (Figs. 1C–D) and all have polysyndactyly (Figs. 1E–F). Although the mutant phenotypes were initially observed at E14.5, most embryos die at E12.0 or earlier with pericardial edema and heart failure.

To gain greater insight into the nature of the forebrain defects, we used a set of markers that identify distinct domains along its dorsoventral axis. This analysis revealed a curious pattern of defects. The ventral domain identified by the expression of Nkx2.1 is reduced in size but still present (Figs. 2A–B). Moving dorsally, all the domains assayed are present but where inter-domain boundaries are ordinarily formed by regulatory interactions, apparent overlaps are detectable. For example, antagonistic interactions between the homeobox transcription factors Gsh2 and Pax6 establish the cortical–subcortical boundary (Toresson et al., 2000; Yun et al., 2001). In wild-type embryos, this boundary is readily detectable as the point at which the dorsal border of Gsh2 expression abuts the ventral edge of the high-level Pax6 expression domain (Fig. 2C). In the mutants, the Gsh2 domain overlaps with that of Pax6 and a clear distinction between cortical and subcortical territories cannot be seen (Fig. 2D).

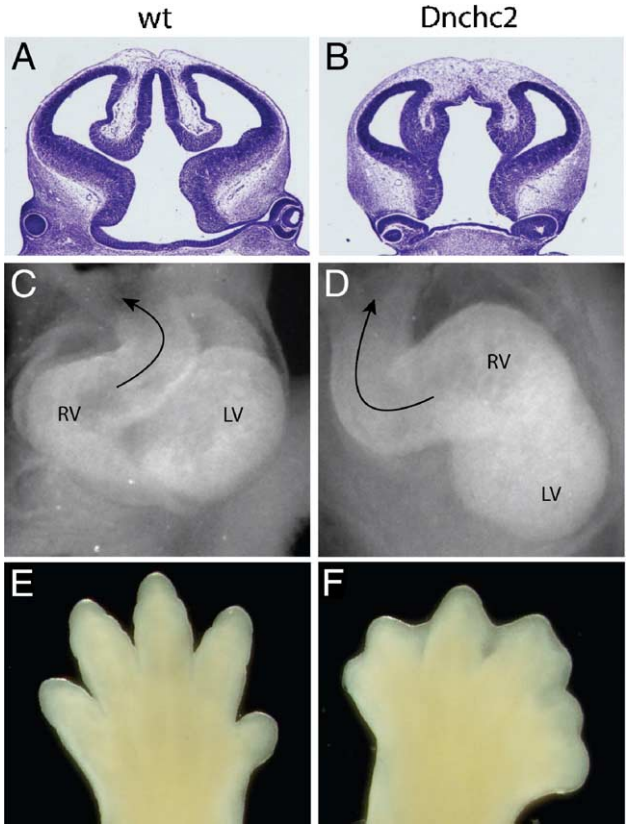


Fig. 1. Dnchc2 mutants have dorsoventral patterning defects, a randomized left–right axis and polysyndactyly. All mutants shown are from the Dnchc2^{W2502R} allele. (A and B) A loss of ventral tissue causing the eyes to be closer to the midline in coronal sections of E12.5 embryos, an indication of dorsoventral patterning defects in the forebrain. (C and D) Leftward looping of the heart in E10.5 mutant embryos (compare C and D) indicates situs inversus and improper determination of the left–right axis. (E and F) Mutant limbs are polysyndactylous with short, uniform digits at E14.5.

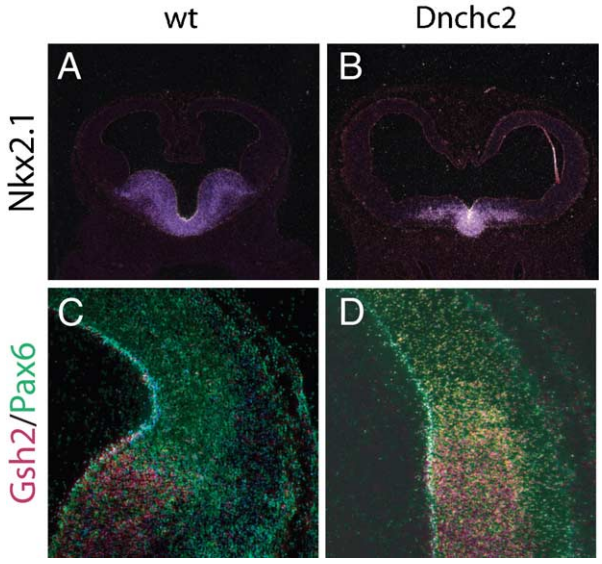


Fig. 2. Loss of ventral territories and dorsoventral boundaries. Analysis of markers reflecting dorsoventral patterning of the forebrain was carried out on sections of E12.5 wt and mutant littermates. The analysis shown is for the Dnchc2^{W2502R} allele. (A and B) Mutants have a reduction in the ventral midline domain that is defined by the expression of Nkx2.1 (C and D) The cortical–subcortical boundary region is shown as an overlay of Pax6 and Gsh2 expression assayed on adjacent sections. The expression of Gsh2 defines the subcortical telencephalon. The dorsal boundary is less distinct and appears to be shifted dorsally in the mutant. High-level expression of Pax6 defines the cortical telencephalon. The ventral boundary of expression is less distinct in the mutant. Pax6 expression is pseudo-colored in green and Gsh2 in red and the overlap produces shades of yellow. In the wild-type embryo, a sharp border is formed whereas extensive overlap is seen in the mutant.

Two independent mutations in the Dnchc2 gene

To determine the basis of the phenotypes, we mapped the mutations. Both were localized to the same small interval on chromosome 9, suggesting that they were two different alleles of the same gene. Sequencing of candidate genes in the nonrecombinant interval revealed a different mutation in the Dnchc2 gene in each of the two mutant lines (Fig. 3A). One of the mutations is an early stop codon whereas the other is a missense mutation in a conserved ATPase-activator (AAA) domain that is predicted to be required for motor activity (Fig.

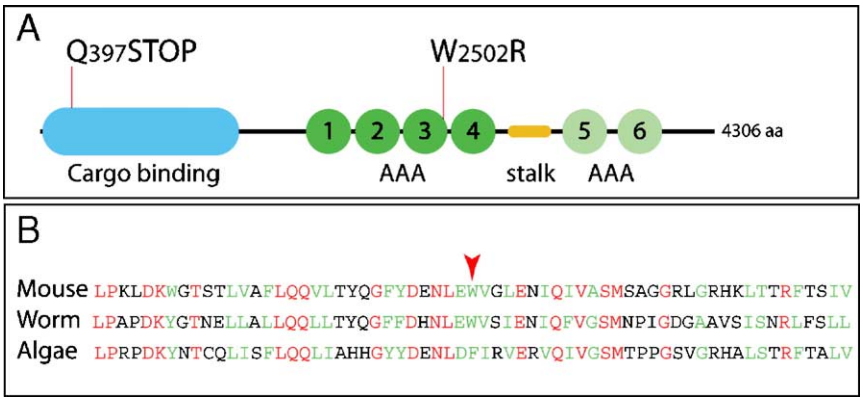


Fig. 3. Two independent point mutations in the mouse Dnchc2 gene. (A) The domain structure of Dnchc2 has an amino-terminal region involved in recognition of cargo and six AAA domains involved in motor activity. Two different alleles, an early nonsense mutation at amino acid position 397 (Dnchc2^{Q397Stop}) and a missense mutation in the third AAA domain at position 2502 (Dnchc2^{W2502R}), produce similar developmental defects. (B) Alignment of the mouse, *C. elegans* and *C. reinhardtii* Dnchc2 orthologues in the region surrounding the residue altered by the Dnchc2^{W2502R} mutation. The red arrowhead indicates the relevant tryptophan residue.

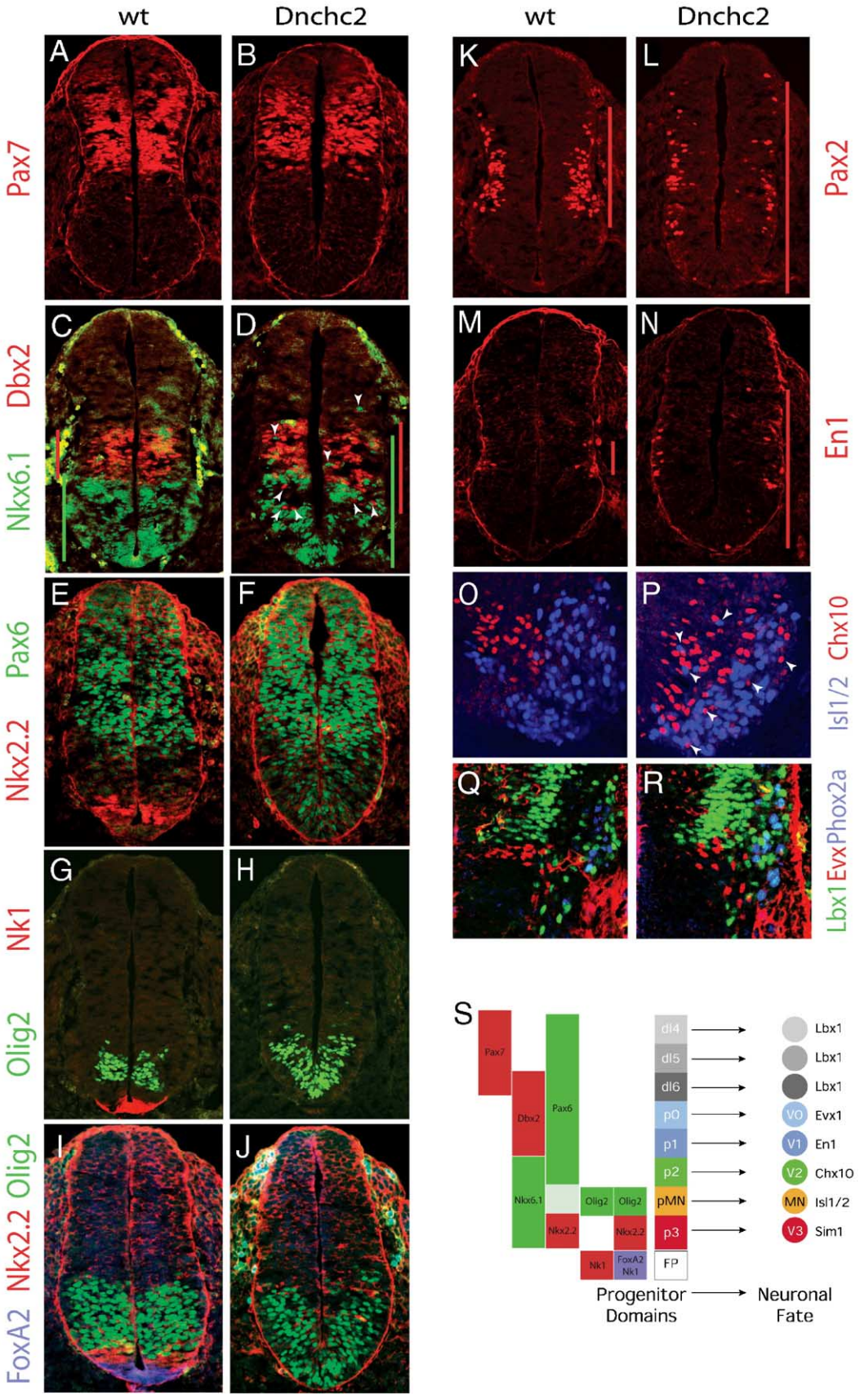
3B). Intercrosses between the two lines revealed a compound heterozygous phenotype that cannot be distinguished from that of the individual homozygotes. Given the apparently identical phenotype produced by the missense and the nonsense allele, we conclude that both are nulls.

The Dnchc2 gene encodes an atypical cytoplasmic dynein heavy chain of unknown function. Its apparent orthologs in *C. reinhardtii* and *C. elegans* are essential components of the retrograde motor that transports material out of cilia (Pazour et al., 1999; Signor et al., 1999). In both species, the role of this dynein appears to be specific for IFT. Genes that are required for IFT and for the formation of cilia have previously been implicated in Shh signaling in the mouse (Huangfu et al., 2003; Rana et al., 2004). The constellation of defects that are produced by the two Dnchc2 alleles are consistent with defective cilia function but neither polysyndactyly nor the pattern of forebrain defects can be explained by a loss of Shh signaling as predicted by the previous analysis of a mutations in other IFT genes.

Defective patterning of the spinal cord in Dnchc2 mutants

To gain more insight into the nature of the defects in the Dnchc2 mutants, we analyzed patterning of the spinal cord where the role of Shh signaling in establishing progenitor domains is well established (Fig. 4). Analysis of a set of markers delineating domains in the ventral spinal cord revealed dorsoventral patterning defects that are most severe ventrally. In situ hybridization showed normal expression of Shh in the notochord (see Supplementary Fig. 1) indicating that the dorsoventral defects resulted from downstream signal transduction defects. In the dorsal spinal cord, progenitor domains are present but progenitors are inappropriately intermingled between domains, something that is only rarely seen in the wild-type (Figs. 4A–D). More severe defects in the ventral spinal cord are apparent from the absence of the floorplate and the ventral-most progenitor domain, p3 (Figs. 4E–J). The next-most dorsal domain, pMN is present and spans the ventral midline, occupying the territory vacated by the floor-plate and p3. The analysis of neuronal fates supports the picture painted by the analysis of progenitors. Each type of neuron is spread across a wider dorsoventral swath and is not segregated from other neuronal types as in the wild-type (Figs. 4K–N). This intermingling is seen clearly in the case of the V2 interneurons and motorneurons (Figs. 4O–P) and is apparent but less severe in more dorsal cell types (Figs. 4Q–R).

This pattern of defects can be compared to that seen with several other mutations that affect components of the Shh signaling pathway. A *Gli1, Gli2* double mutant for example is deficient in almost all Gli transcriptional activator function. The lack of Gli activator function leads to loss of the floorplate and the three most ventral progenitor domains (Bai et al., 2002, 2004). The loss of ventral domains is at least partly the result of unopposed Gli repressor activity as removal of all Gli activity leads to restoration of all but the floorplate and the most ventral progenitor domain in a phenotype that is similar to that seen here (Litingtung and Chiang, 2000; Meyer and Roelink, 2003; Motoyama et al., 2003; Persson et al., 2002). A partial loss of Gli activator function, for example in a Gli2 mutant, also causes the loss of the floorplate and most of p3 (Motoyama et al., 2003; Matise et al., 1998). Thus, the Dnchc2 spinal cord has patterning defects that are consistent with defects in Shh signaling as has been reported for other IFT genes. Normal expression of Shh in the notochord and Gli2 and Gli3 in the spinal cord (see Supplementary Fig. 1) supports the interpretation that the patterning defects are due to a downstream signal transduction defect in this case as well. Unlike the previously reported phenotypes, however, the Dnchc2 phenotype does not correspond to the phenotype seen with a severe loss of Gli activator function as might be expected for an essential component of Shh signal transduction mechanism but rather is more consistent with either a partial loss of Gli activator or with stronger effects on the ability to produce both Gli activator and Gli repressor function.


wt
Dnchc2
wt
Dnchc2
A
B
K
L
Pax7
Pax2
C
D
M
N
Dbx2
Nkx6.1
En1
E
F
O
P
Pax6
Nkx2.2
Isl1/2
Chx10
G
H
Q
R
Nk1
Olig2
Lbx1
Evx
Phox2a
I
J
Nkx2.2
Olig2
FoxA2
S
Pax7
Dbx2
Pax6
Nkx6.1
Olig2
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Lbx1
Lbx1
Lbx1
V0
Evx1
V1
En1
V2
Chx10
MN
Isl1/2
V3
Sim1
Progenitor Domains
Neuronal Fate

Limb patterning reflects loss of both Gli activator and repressor function

To clarify the function of Dnchc2, we turned our attention to the limb as a situation where the role of Shh in patterning is well understood (Fig. 5A). The defective limbs have polysyndactyly with poorly differentiated digit identity (Figs. 1E–F). Anterior polydactyly can be caused either by ectopic Hh signaling at the anterior margin of the limb (Lettice et al., 2002; Takahashi et al., 1998; Yang et al., 1998) or by loss of Gli repressor function (Litingtung et al., 2002; te Welscher et al., 2002). The other IFT genes that have been characterized as having roles in the Shh signaling pathway function downstream of Shh and Ptc so we first examined the expression of these two genes in mutant limb buds. Shh is expressed normally in mutant limbs whereas patched expression is drastically reduced (Figs. 5B–E). Ptc expression in the limb is a downstream response to Shh signaling (Goodrich et al., 1996) that is dependent upon the activity of Gli activator function. Gli activator function is provided by all three Gli genes but Gli1 appears to function solely as an activator and does not contribute to Gli repressor activity. Gli1 expression in the posterior portion of the limb bud is also a downstream response to Shh signaling that is dependent upon activator functions of Gli2 and Gli3 (Bai et al., 2004). In order to evaluate the potential for Gli activator function, we assessed the expression of each of the Gli genes. The early expression patterns of Gli2 and Gli3 are unaffected by Dnchc2 mutations (Figs. 5F–I, Supplementary Figs. 2E–H). In contrast, the expression of Gli1 is completely absent (Figs. 5L–M), similar to the loss of Ptc expression and consistent with the idea that Gli activator function is not being provided by Gli2 or Gli3 despite their normal patterns of mRNA expression. To test the idea that expression of Gli repressor function is defective, we examined the expression of Hoxd13 and gremlin. Both of these genes are ordinarily repressed by Gli3 in the anterior margin of the limb bud and are ectopically expressed when Gli repressor is absent (te Welscher et al., 2002). Dnchc2 mutations cause both of these genes to be ectopically expressed, indicating that Gli repressor function is reduced or absent. In summary, the analysis of limb patterning defects indicates that both Gli activator and Gli repressor activity are absent or significantly reduced. Polysyndactyly in these mutants is the result of a loss of Gli repressor activity in the anterior limb bud. Although Gli activator function does not have a significant role in determining the morphology of the limb bud (Park et al., 2000), its loss in the Dnchc2 mutants is readily apparent from the marker analysis presented here.

Gli3 processing is disrupted in mutant embryos

The limb phenotype provides strong support for the idea that Dnchc2 is required for the production of both activator and repressor forms of Gli. Together with previous analysis showing that IFT genes act downstream of Ptc function, this suggests that Dnchc2 may be required for coupling Smo activity to processing of Gli3 transcription factor. The repressor form of Gli3 (Gli3-83) results from the processing of full-length Gli3 protein (Gli3-190). Gli3-83 lacks almost an entire C-terminus of the full-length Gli3, and thus suppresses Shh target gene expression (Wang et al., 2000a,b). Gli3-190 constitutes activator function although it may need an additional unidentified event to be activated by Shh signaling. It is believed that the normal anterioposterior limb patterning is determined by the ratio of Gli3-190 versus Gli3-83, which is directly regulated by Shh signaling (Litingtung et al., 2002; Wang et al., 2000a). To directly address the question whether loss of both Gli3 repressor and activator function is at least in part due to the effect of Gli3 processing, we examined the levels of Gli3-190 and Gli3-83 in Dnchc2 mutant embryos by immunoblotting using a specific Gli3 antibody (Wang et al., 2000a,b). As predicted, the level of Gli3 full-length was significantly increased, while the level of processed form of Gli3 was markedly reduced in the mutant embryos as compared to those in wild-type embryos (Fig. 6A). As a result, the ratio between Gli3-83 versus Gli3-190 changes from nearly 8 to 1 in the wild-type embryos to less than 2 to 1 in the mutant embryos (Fig. 6B). These data demonstrate that Dnchc2 is required for the proper biochemical processing of Gli3 and provide a molecular mechanism to explain the polydactylous phenotype. Our results also suggest that the full-length Gli3 needs to be activated to function as an activator since the level of full-length Gli3 protein is increased in the mutant yet the protein is not active as measured by the marker gene expression of Shh signaling.

Cilia are present in tissues patterned by Shh

That *C. reinhardtii* and *C. elegans* orthologues of Dnchc2 function in retrograde cilial transport is well established (Pazour et al., 1999; Porter et al., 1999; Signor et al., 1999), providing

Fig. 4. Dorsoventral patterning defects in the spinal cord. (A–J) The analysis of markers for progenitor domains in E9.5 to E10.5 day spinal cords reveals a loss of ventral domains and milder defects in the dorsal spinal cord. (A–B) The Pax7 expression domain in the dorsal spinal cord appears relatively normal. (C–D) The Nkx6.1 (p1) and Dbx2 (p0) domains are also present but have intermingling of progenitors. Arrowheads indicate displaced progenitors in the mutant spinal cord. (E–F) The high-level Pax6 expression domain appears normal but the low level (MN) domain is elevated in mutants and extends to the ventral limit of the spinal cord, to a position normally occupied by the Nkx2.2 expressing p3 domain. (G–H) The expression of Olig2 (MN) extends to the ventral limit of the spinal cord and Nk1 (FP) expression is absent. (I–J) Expansion of the Olig2 domain to the ventral limit of the spinal cord at the expense of the p3 domain and the floorplate is demonstrated by triple labeling. Similar observations were made when neuronal markers were examined in E10.5 (K–N) or E11.5 (O–R) spinal cords. (K–L) Pax2 expressing interneurons are spread over a greater dorsoventral extent as indicated by the span of the red bar. (M–N) A similar phenotype is seen with En1 expressing V1 interneurons. (O–P) Populations of Chx10-expressing V2 interneurons and motorneurons are extensively mixed. Arrowheads indicate misplaced neurons. (Q–R) The more dorsal, Evx1 expressing V0 interneurons are displaced to a lesser degree. (S) Progenitor domains and neuronal cell types in the ventral spinal cord as defined by the markers used. No differences were seen in the phenotype produced by the two alleles. B, D, H, L and N are from the Q397STOP allele. F, J, P and R are from the W2502R allele.

strong support for the idea that the mouse gene product has a similar role. The addition of Dnchc2 to the list of cilia-related genes required for Shh signal transduction makes a compelling case for the idea that primary cilia are functioning as specialized signal transduction organelles. To make this interpretation, some evidence that Dnchc2's role in cilial transport is conserved is needed. Equally important would be the demonstration that primary cilia are present in the responding tissues. To obtain this information, we used scanning electron microscopy (SEM) to search for cilia in the neuroectoderm of both wild-type and mutant embryos. SEM of wild-type forebrain neuroectoderm provided the striking picture of single cilia projecting into the ventricle from the apical surface of neuroectodermal cells (Figs. 7A and C). Mutations that disrupt the Dnchc2 orthologue in *C. reinhardtii* produce short, bloated cilia which are filled with IFT particles that are transported in by the anterograde motor but cannot leave in the absence of the retrograde motor (Pazour et al., 1999; Porter et al., 1999). The cilia present in the neuroectoderm of Dnchc2 mutants have a profoundly different appearance than their wild-type counterparts (Figs. 7B and D). All of the cilia in mutant tissue are shorter and bloated along their entire length, often to the point of appearing spherical. This is remarkably similar to the flagella of retrograde motor mutants in *C. reinhardtii*.

Dnchc2 is also required in the limb bud, most likely in the mesenchyme, for proper patterning by Shh. To establish the basis for retrograde motor function, we searched for evidence of cilia in the mesenchymal cells of the developing limb. The irregular shape and absence of an obvious apical and basal surface of mesenchymal cells make it difficult to use SEM in this instance, so instead we immunostained tissue sections with antibodies recognizing acetylated tubulin. Cilia could be readily detected on mesenchymal cells of wild-type limb buds (Fig. 7E). Similar structures could not be identified in mutant mesenchyme (Fig. 7F), again consistent with the phenotype caused by mutations in *C. reinhardtii* where the accumulation of IFT particles is associated with a deficiency of recognizable axonemal tubulin in the cilia (Pazour et al., 1999; Porter et al., 1999).

Localization of Smo to cilia in the node is disrupted in mutants

The loss of both Gli activator and Gli repressor function and the inefficient proteolytic processing of Gli3 in mutants are

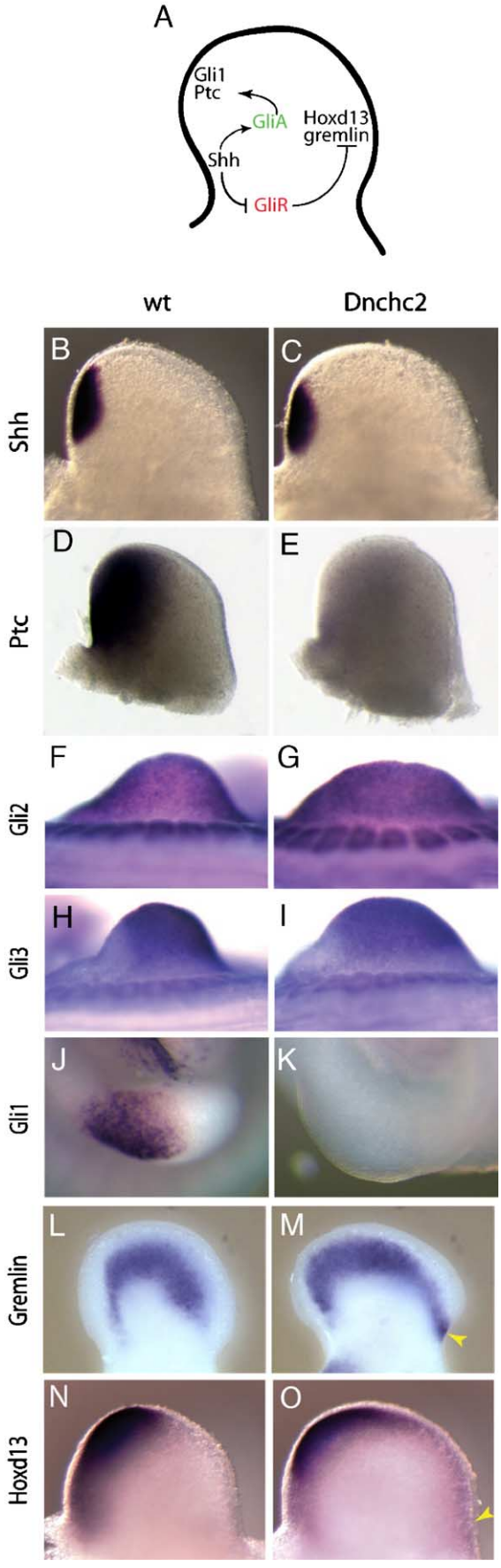


Fig. 5. Dnchc2 is required for the phenotypic expression of Gli activator and Gli repressor function in the limb. (A) Transcriptional activation function of Gli proteins (GliA) is required for the expression of Ptc and Gli1 in the posterior portion of the developing limb in response to Shh signaling. The transcriptional repressor function (GliR) prevents the expression of Hoxd13 and gremlin in the anterior limb bud. (B–C) Shh is expressed normally in the ZPA of mutants at E10.5. (D–E) Ptc-1 expression is dramatically downregulated throughout the posterior of the limb at E10.5. (F–G) Normal expression of Gli2 and (H–I) Gli3 in mutant limb buds at E9.5. (J–K) Gli1 is not expressed in E9.5 mutant limb buds. (L–M) Gremlin and (N–O) Hoxd13 expression extends to the anterior margin (yellow arrowheads in M and O) of mutant limb buds (at E11.5 and E10.5, respectively). The images shown are from the Q397STOP allele. Homozygotes for the W2502R allele have the same patterning defects (data not shown).

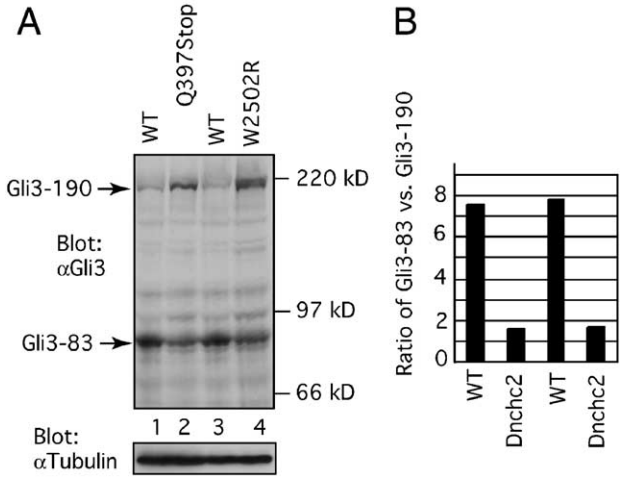


Fig. 6. Gli3 proteolysis is defective in Dnchc2 mutants. (A) Western blotting using polyclonal antisera against Gli3 (Wang et al., 2000a) was used to analyze total protein extracts from E10.5 embryos. Both Dnchc2 mutant alleles exhibit a marked accumulation of full-length Gli3 (Gli3-190). In addition, the generation of truncated repressor form of Gli3 (Gli3-83) is reduced. Immunoblotting with antitubulin antibody shows that similar amounts of protein lysates were used. (B) The overall ratio of Gli3-83 to Gli3-190 is reduced in mutants from both alleles by 4–5-fold.

consistent with the idea that Dnchc2 is required to couple Smo activity to processing of Gli3 protein. In *Drosophila*, activation of the Hh signaling pathway is associated with stabilization of Smo and its mobilization from internal membranes to the cell surface, a process that is critical for Smo to activate transcription of downstream target genes (Denef et al., 2000; Zhu et al., 2003). To determine if localization of Smo is affected in Dnchc2 mutants, we took advantage of recently identified antisera that allow detection of vertebrate Smo protein (Corbit et al., 2005). The node is a ciliated and Shh responsive tissue that is easily accessible and identifiable in early embryos and we chose to focus our attention there. Immunofluorescent detection of Smo in the node of wild-type embryos reveals widespread and punctate ctyoplasmic staining (Figs. 8A and G). In addition, significant localization to cilia is detectable as finger-like projections from the surface of nodal cells and co-staining with acetylated tubulin (Figs. 8A, C, E and G). In contrast, only punctate cytoplasmic staining is detectable in the node of mutant embryos indicating that the localization to the cilia is dependent upon Dnchc2 (Figs. 8B, D, F and H). As is the case with the limb bud mesenchyme, cilia are not detectable in the node of mutant embryos using antibodies recognizing acetylated tubulin. To determine if the lack of Smo localization to cilia was due to the absence of cilia in nodal cells, we examined the

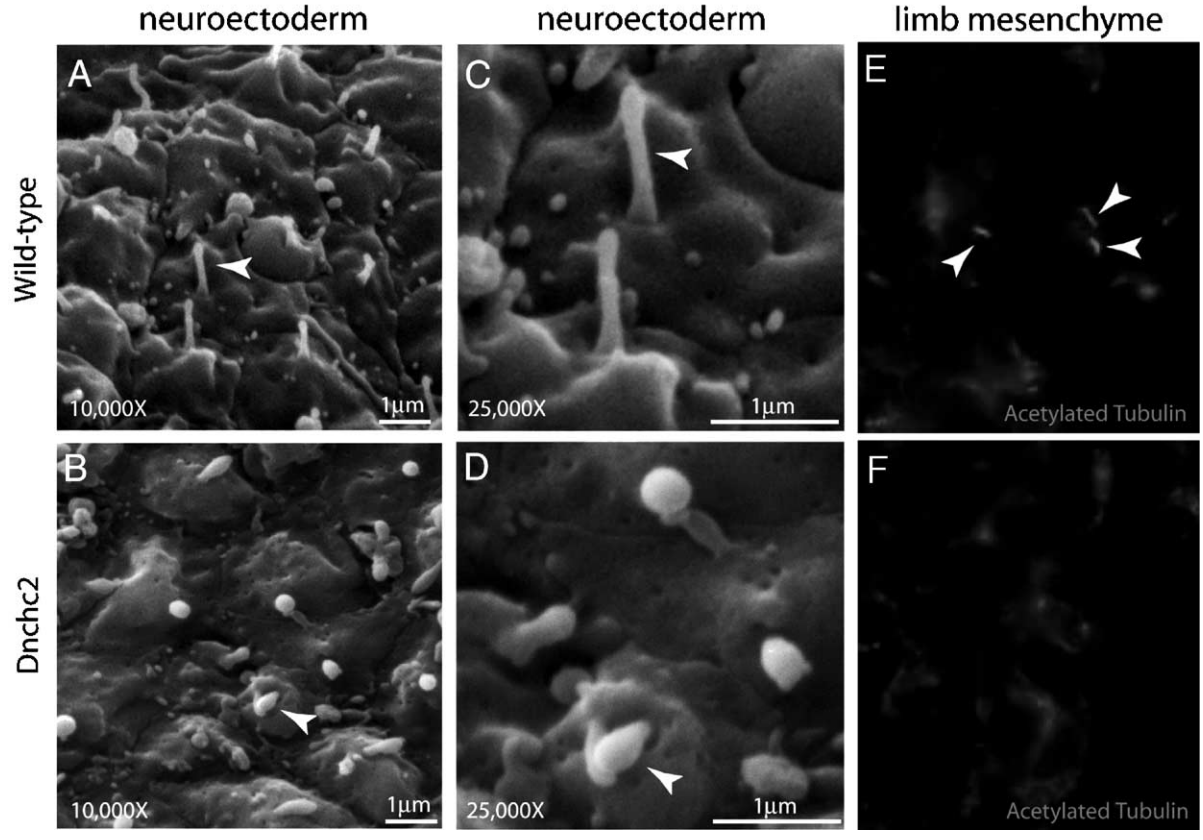
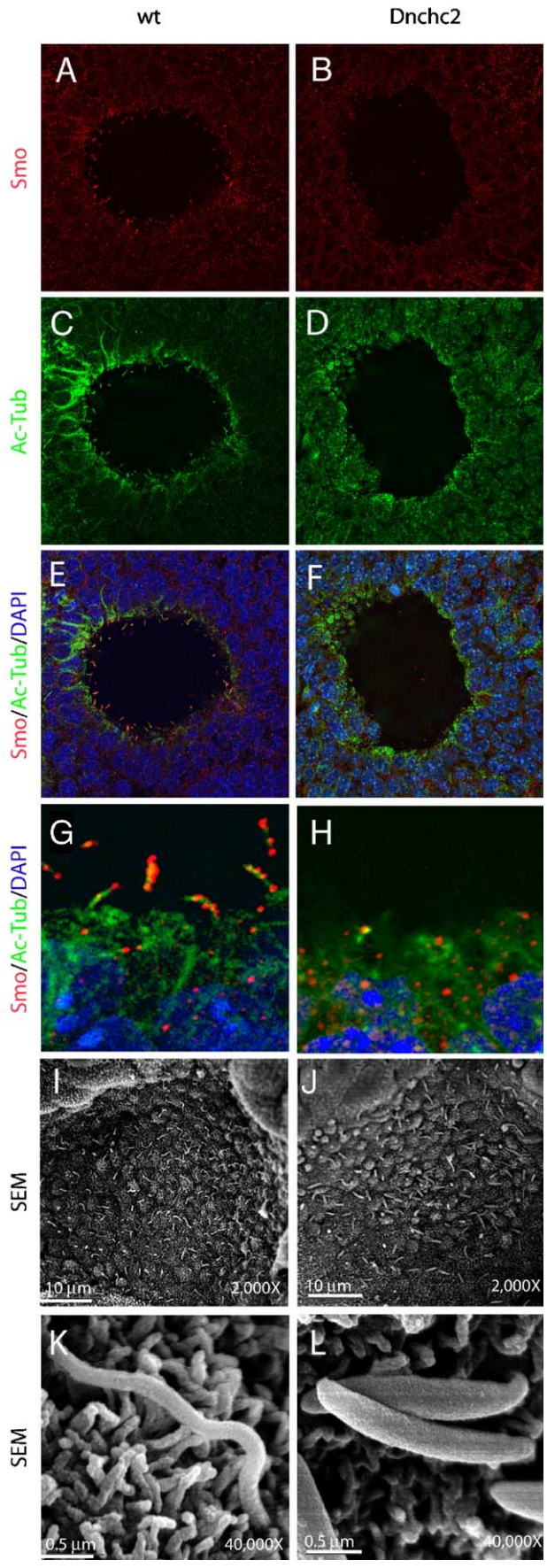


Fig. 7. Primary cilia in the neuroectoderm and limb mesenchyme are defective in Dnchc2 mutants. Tissues in which Shh signal transduction is disrupted in Dnchc2 mutants were examined. (A–B) Scanning electron micrographs of the apical (ventricular) surface of E10.5 telencephalic neuroectoderm. (C–D) Higher magnification view of the cilia indicated by arrowheads. The short bloated phenotype caused by Dnchc2 mutations can be seen clearly in the cilia indicated by the arrowhead in panel D. (E–F) Immunofluorescent detection of acetylated tubulin reveals cilia (arrowheads) on wild-type mesenchyme that are not detectable on mutant mesenchymal cells. The images shown are from analysis of the Q397STOP allele. The W2502R allele produces similar ciliary defects (data not shown).



node of mutant embryos using scanning electron microscopy. Cilia with a characteristic short and bloated appearance are present in mutants (Figs. 8I–L) indicating that the defect in Smo localization results from defective transport and not from the absence of cilia.

Discussion

In *Drosophila*, it is clear that IFT genes, while present, do not function in Hh signal transduction (Han et al., 2003; Sarpal et al., 2003). Instead, it seems that Cos2 might play an analogous role. Although the role of Cos2 is far from clear, it appears that it plays an active role in scaffolding alternative signal transduction complexes necessary for the production of Ci activator or Ci repressor in response to extracellular Hh levels (Jia et al., 2003; Lum et al., 2003; Monnier et al., 2002; Ogden et al., 2003; Robbins et al., 1997; Ruel et al., 2003; Sisson et al., 1997; Stegman et al., 2004; Wang et al., 2000b). The role for Dnchc2 demonstrated here is clearly consistent with a similar function for IFT in Shh signal transduction. The idea that IFT function has replaced Cos2 function during evolution runs into trouble though, with the recent identification of zebrafish, mouse and human homologues of Cos2. Preliminary evidence suggests that the function of the zebrafish Cos2 homologue in Hh signaling has been conserved (Tay et al., 2005). This might mean that Cos2 functions together with IFT in vertebrates to regulate the formation of Hh signal transduction complexes. In flies, the contribution of IFT to these signal transduction events has been dispensed with or replaced by some as yet unidentified function. In this regard, it is intriguing that activation of the Hh pathway in flies, either by Hh itself, by loss of Ptc or by activating mutations in Smo, is associated with mobilization of Smo from intracellular stores to the cell surface (Denef et al., 2000; Zhu et al., 2003). It seems plausible that in vertebrates the critical event is mobilization to cilia rather than simply to the cell surface.

Previous analysis of IFT function in Shh signal transduction focused on the spinal cord rather than the limb (Huangfu et al., 2003). This analysis showed that while Gli activator function is abrogated, Gli repressor function is still present. This may represent a distinction between the role of Dnchc2 and the other IFT genes in Shh signaling. The nature of the cilia defects produced by Dnchc2 mutations is dramatically different than that produced by other IFT mutations. Nonetheless, mutations in IFT88 cause polydactyly, the basis of which has not been thoroughly investigated, suggesting that the differences may be relative rather than absolute. The recent results of Liu et al.

Fig. 8. Localization of Smo to nodal cilia is defective in mutants. Confocal sections through the node of late head fold stage embryos were used to examine the localization of Smo. (A–F) Polyclonal antisera recognizing Smo (Corbit et al., 2005) were used to detect its distribution. Punctate cytoplasmic staining is present in both wild-type and mutant embryos. The expression of Smo on cilia is only detectable in wild-type embryos. (I–L) That cilia are still present in the mutant node despite the absence of acetylated tubulin staining shown by scanning electron microscopy.

(2005) also support the idea that the apparent phenotypic differences can be explained by a combination of distinct requirements for Gli repressor and Gli activator in the two tissues in combination with the different roles of the various IFT components. The latter possibility points towards additional exciting insights into how this unusual signal transduction mechanism works that will come from further study of the precise roles of the numerous IFT components in the localization of Smo and other Hh pathway components.

The requirement for Dnchc2 in the proteolytic processing of Gli3 indicates that this pathway while often appearing to be a default choice, is instead regulated by components of the signal transduction apparatus that are also necessary for the production of Gli activator. This is consistent with the idea that retrograde transport in the cilia is required for the assembly of alternative signal transduction complexes. The loss of Smo localization to cilia in Dnchc2 mutant embryos strongly supports the idea that this is an essential event in the signal transduction process. The fact that missense mutations in Smo that prevent it from being localized to cilia also block its activity (Corbit et al., 2005), further substantiate this interpretation. At first glance though, the lack of Smo localization to the cilia raises questions as it does not seem consistent with the role of Dnchc2 as a motor that transports material *out* of the cilia. Smo localization is not constitutive, however, but is upregulated by exposure to Shh (Corbit et al., 2005). Presumably, in Dnchc2 mutant, cilia quickly become congested and anterograde transport is slowed or stopped and the cilia are then unable to respond to subsequent exposure to Shh by importing Smo.

It seems plausible that Ptc or Gli proteins are also transported into primary cilia, or to the pericentriolar material near the base of the cilium, where specialized activation or repression signaling complexes form, depending upon the extracellular ligand availability. The suggestion that Ptc functions to regulate vesicular trafficking of Smo is particularly intriguing in this context (Nakano et al., 2004; Strutt et al., 2001; Zhu et al., 2003) and the relationship between Ptc activity and Smo localization to the cilium is clearly an important area for future investigation.

Our interpretation of the role that IFT plays in Shh signaling has striking parallels with vertebrate phototransduction and olfaction. In each case, the localization of a seven transmembrane receptor, Smo, rhodopsin or an olfactory receptor, to the cilia is critical for signal transduction. This parallel is further supported by the activity-dependent internalization of Smo by β-arrestin (Chen et al., 2004). Regardless of how similar the function of cilia is in these different signal transduction processes, the dependence of Shh signal transduction on specialized motor proteins highlights the fact that specific subcellular compartmentalization is an essential event in response of the cell to this morphogen.

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2005.08.050.

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