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C2cd3 is required for cilia formation and Hedgehog signaling in mouse

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Abstract

Cilia are essential for mammalian embryonic development as well as for the physiological activity of various adult organ systems. Despite the multiple crucial roles that cilia play, the mechanisms underlying ciliogenesis in mammals remain poorly understood. Taking a forward genetic approach, we have identified *Hearty* (*Hty*), a recessive lethal mouse mutant with multiple defects, including neural tube defects, abnormal dorsal-ventral patterning of the spinal cord, a defect in left-right axis determination and severe polydactyly (extra digits). By genetic mapping, sequence analysis of candidate genes and characterization of a second mutant allele, we identify *Hty* as *C2cd3*, a novel gene encoding a vertebrate-specific C2 domain-containing protein. Target gene expression and double-mutant analyses suggest that *C2cd3* is an essential regulator of intracellular transduction of the Hedgehog signal. Furthering a link between Hedgehog signaling and cilia function, we find that cilia formation and proteolytic processing of Gli3 are disrupted in *C2cd3* mutants. Finally, we observe C2cd3 protein at the basal body, consistent with its essential function in ciliogenesis. Interestingly, the human ortholog for this gene lies in proximity to the critical regions of Meckel-Gruber syndrome 2 (MKS2) and Joubert syndrome 2 (JBTS2), making it a potential candidate for these two human genetic disorders.

Keywords

Mouse; Cilia; Hedgehog signaling; Gli3; C2 domain; C2cd3; Embryonic patterning; Basal body

INTRODUCTION

Cilia and flagella are cell surface organelles with microtubule-based axonemal cores. Although these organelles have been known to biologists for centuries, only in the last five years has it been recognized that cilia are crucial for mammalian embryonic development as well as for the function of multiple adult organs (Pan et al., 2005). Many potential ciliary proteins have been identified in various species in recent years using biochemical,

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comparative genomic and proteomic methods. Nevertheless, the spectrum of factors required for the formation and/or function of cilia, as well as the molecular mechanisms underlying the regulation of cilia biogenesis, have yet to be fully revealed.

Two multiprotein complexes, the intraflagellar transport (IFT, complex A and B) complexes, are present in the green alga *Chlamydomonas reinhardtii* (Rosenbaum and Witman, 2002). The IFT complexes move within the flagella, suggesting that they are likely to be involved in the transportation of molecules inside the flagella. Mutations in protein components of the IFT complexes (the IFT proteins), as well as in the microtubule motor proteins kinesin II and cytoplasmic dynein, result in the degeneration of flagella, indicating that IFT is required for flagella formation (Pan et al., 2005).

Cilia have been implicated in the pathogenesis of many human genetic diseases, such as polycystic kidney disease (PKD), Bardet-Biedl syndrome (BBS), Meckel-Gruber syndrome (MKS) and Joubert's syndrome (JBTS) (Fliegauf et al., 2007). Most of the proteins known to be connected with these diseases are localized to the cilia or to the basal bodies, centrosome-like structures from which cilia originate. The identities of additional genes, such as those mutated in MKS2 (Roume et al., 1998) and JBTS2 (Valente et al., 2005) patients, are yet to be discovered.

The discovery that cilia play essential roles in signal transduction in multiple pathways, especially the Hedgehog (Hh) pathway, greatly advanced our understanding of both the function of cilia and the mechanism of intracellular signaling (Bisgrove and Yost, 2006). The Hh proteins, a family of secreted proteins, regulate the development of multiple organ systems in both vertebrates and invertebrates (Hooper and Scott, 2005). Loss of Hh signaling in mammals results in disruption of left-right asymmetry, loss of ventral cell fate in the central nervous system (CNS), loss of digits and many other defects (Chiang et al., 1996).

In *Drosophila*, Hh regulates the activities of the transcription factor Cubitus interruptus (Ci) (Methot and Basler, 2001). Ci is a dual-function protein that acts as both a transcriptional activator and repressor. In the absence of Hh, Ci is proteolytically processed into a transcriptional repressor that maintains repression of Hh target genes. When Hh is present, proteolytic processing of Ci is inhibited and Ci acts as a transcriptional activator that turns on the transcription of Hh target genes. The signal from Hh is transmitted to Ci through a signaling cascade that starts with the binding of Hh ligand to its cell surface receptor, Patched (Ptc). As a result, the G-protein-coupled receptor-like protein Smoothened (Smo) is activated, leading to the inhibition of Ci processing and activation of Ci activator function.

Many components of the mammalian Hh pathway serve similar functions to their *Drosophila* counterparts (Hooper and Scott, 2005). However, significant differences do exist. One difference is the duplication of most Hh pathway genes and their subsequent functional divergence in vertebrates. For example, there are three mammalian homologs of Ci, which constitute the Gli family (Gli1, Gli2 and Gli3). Gli1 does not appear to be subject to proteolytic processing. Therefore, Gli1 functions as a transcriptional activator only. Both Gli2 and Gli3 undergo proteolytic processing in vivo, but Gli3 is much more efficiently processed than Gli2, making it the major repressor (Pan et al., 2006; Wang et al., 2000). Hh pathway regulation between *Drosophila* and vertebrates is also divergent in that some vertebrate-specific Hh pathway components, such as Hip (Hhip – Mouse Genome Informatics) and Rab23, have been identified (Chuang and McMahon, 1999; Eggenschwiler et al., 2001).

In recent years, we and others have found that mouse and zebrafish mutants with cilia defects exhibit compromised Hh signaling (reviewed by Bisgrove and Yost, 2006; Tobin et

al., 2008). Our detailed analysis indicates that IFT-related proteins are crucial for both Gli activator and repressor functions (Liu et al., 2005). Recent protein localization studies suggest that multiple components of the mouse Hh signaling pathway are localized in the primary cilia (Corbit et al., 2005; Haycraft et al., 2005; Rohatgi et al., 2007). By contrast, *Drosophila* cilia mutants do not exhibit defects in Hh signaling (Han et al., 2003; Sarpal et al., 2003). Therefore, roles for cilia in Hh signal transduction are likely to be restricted to vertebrates.

Calcium signaling was first associated with cilia function with the discovery that the intracellular calcium level rises upon the bending of cilia on canine MDCK cells (Praetorius and Spring, 2001). It was later shown that polycystin 2 (PC2; Pkd2), a calcium-channel protein localized to the cilia, and its binding partner polycystin 1 (PC1; Pkd1), are essential for initiating the calcium influx that triggers the calcium level change in renal epithelia (Nauli et al., 2003). A PC2-dependent intracellular calcium surge was also observed on the left side of the embryonic node at E8, after node cilia-mediated nodal flow is initiated (McGrath et al., 2003). However, PC1 is not expressed in the node and is not required for establishing left-right asymmetry in the mouse, suggesting that the mechanism involved in opening the PC2 channel in the embryo is different from that in the kidney (Karcher et al., 2005). *Shh* might play a role in PC2 activation because overexpression of *Shh* leads to an increase in the intracellular calcium level on the left side of the node (Tanaka et al., 2005).

Additional studies suggest further roles for calcium during gastrulation and left-right axis determination. In chicken, a high level of extracellular calcium is observed on the left side of Hensen's node, and this asymmetric distribution of calcium is translated into asymmetry of the embryo through Notch signaling (Raya et al., 2004). Calcium waves are also observed in the zebrafish and frog organizers during gastrulation (reviewed by Webb and Miller, 2006). It has been suggested that calcium waves might be important for convergent extension movements (Wallingford et al., 2001). However, the roles for calcium in cilia formation have not been investigated, despite the fact that several calcium-binding proteins (e.g. calmodulin, calcineurin, centrin) are localized to the cilia or basal body.

In the current study, we have identified C2cd3, a novel vertebrate-specific C2 domain-containing protein, as an essential regulator of ciliogenesis in the mouse. Through the study of mouse mutants carrying two different loss-of-function alleles of this gene, we show that *C2cd3* is essential for mouse embryonic development through regulating the intracellular transduction of Hh signals and proteolytic processing of Gli3. Cilia biogenesis is severely disrupted in the absence of *C2cd3*. We speculate that C2cd3, a putative calcium-dependent lipid-binding protein that is localized at the basal body of cilia, mediates calcium-dependent vesicular transport and/or recruitment of proteins, including Hh pathway components, during cilia biogenesis. Therefore, the discovery of *C2cd3* might lead to a better understanding of the connection between calcium signaling, cilia formation and cilia-dependent signal transduction.

MATERIALS AND METHODS

Mouse strains

The original *Hty* mutant allele was genotyped based on linkage to flanking SSLP markers D7psu9615a (forward primer, 5′-CAGAAGGGCTTTCCATATTTTG-3′; reverse primer, 5′-ATCCTCAAGGCAGAGGGAGT-3′) and D7psu9682e (forward primer, 5′-TGGTGAAGAAGAAGGGACTTTT-3′; reverse primer, 5′-TGCCCAAGCAGGTTTTACAT-3′). Phenotypic analyses were carried out in congenic C3H/HeN mice (Charles River Laboratory). A second *Hty* mutant allele, designated $C2cd3^{GT}$, was kept on the same C3H/HeN background as the original *Hty* allele. $C2cd3^{GT}$

mice were genotyped by PCR, using primers to detect the *lacZ* gene (Liu et al., 1999) or using the same SSLP marker primers described above to distinguish the mutant chromosome derived from the ES cells (129/Ola origin) and the wild-type chromosome (C3H/HeN origin).

Bioinformatics

Information on the cDNA, exon-intron structure and open reading frames was obtained from the Ensembl database (http://mouse.ensembl.org). C2cd3 protein structure was predicted using the SMART program (http://smart.embl-heidelberg.de). Searches for C2cd3 orthologs were performed by BLAST search against NCBI (http://www.ncbi.nlm.nih.gov/BLAST), the Chlamy Center (http://www.chlamy.org), the Cilia proteome (http://www.ciliaproteome.org) (Gherman et al., 2006) and the ciliome (http://www.sfu.ca/~leroux/ciliome_home.htm) (Inglis et al., 2006), using predicted mouse C2cd3 protein sequence. The *C2cd3* gene-trap ES cell line (AG0177) was identified through a BLAST search against the database of the International Gene-Trap Consortium (http://www.genetrap.org) using the *C2cd3* cDNA sequence.

Analysis of *Hty* mutant embryos

For immunohistochemical studies, mouse embryos were fixed in 4% paraformaldehyde in PBS for 1 hour at room temperature, washed in PBS and processed for cryosections at 10 μm. The sections were incubated with primary and secondary (Cy3-conjugated) antibodies, each followed by a series of washes in PBS containing 0.1% Triton X-100 and 1% goat serum. The slides were mounted with DABCO (Sigma) and visualized using a Nikon E600 fluorescent microscope. The X-Gal staining on whole-mount embryos has been described previously (Liu et al., 1998).

Scanning electron microscopy (SEM)

E8.0 mouse embryos were fixed overnight in 2.5% glutaraldehyde, washed in PBS and dehydrated through an ethanol series. A small portion of the embryo was removed for genotyping before dehydration. The dehydrated samples were critical-point dried, mounted to metal mounds with the embryonic node facing up, sputter-coated with silver and visualized with a JEOL JSM 5400 SEM at the Penn State EM facility.

Mouse embryonic fibroblast (MEF) culture, transfection and visualization of cilia

E10.5 embryos were dissected in sterile PBS, the cells dissociated by passage through gauge-20 needles and then plated in DMEM supplemented with 10% fetal bovine serum (FBS), non-essential amino acids, sodium pyruvate, Glutamax (Invitrogen) and antibiotics, at 37°C and 5% CO_2. To visualize the cilia, MEFs were passed onto gelatin-coated glass coverslips one day before being transferred to medium containing 0.5% FBS for 48 hours. The cells were then labeled with antibodies against acetylated tubulin (Sigma, T7451) or γ-tubulin (Sigma, T5326), and visualized under a Nikon E600 microscope. C2cd3 was tagged with GFP by cloning into the pEGFPN1 and pEGFPC3 mammalian expression vectors (Clontech). Transient transfection of the C2cd3-GFP expression construct was carried out using Lipofectamine 2000 (Invitrogen).

Western blot

Protein lysate was prepared from individual E10.5 mouse embryos and 20 μg from each sample loaded onto 7% SDS-PAGE gels for western blotting as described (Wang et al., 2000). Anti-β-tubulin antibody (Sigma, T4026) was used as loading control. The result of the western blot was quantitated using ImageJ (NIH). Additional antibodies were obtained

from the Developmental Studies Hybridoma Bank: anti-Shh, anti-Foxa2, anti-Nkx2.2, anti-Isl1, anti-Lhx3, anti-En1, anti-Pax6 and anti-Pax7.

RESULTS

Hearty is a recessive mouse mutant with multiple defects in embryonic development

In a forward genetic screen for recessive mutations affecting mouse embryonic development, we identified *Hearty* (*Hty*; previously named *11A*) (Zohn et al., 2005), a recessive mouse mutation that disrupts embryonic development and causes embryonic lethality between E11 and E13 (Table 1). Some *Hty* homozygous mutants exhibit exencephaly in the midbrain and posterior forebrain, a twisted body axis and pericardial edema (hence the name *Hty*) (Fig. 1A,B; Table 2). The *Hty* mutants that undergo neural tube closure present with a characteristic tight mesencephalic flexure (Fig. 1C,D). The heart loops in a leftward direction in wild-type embryos, but turns rightward in some *Hty* mutants (Fig. 1C,D, insets). At E12.5, all *Hty* mutants exhibit severe polydactyly in all four limbs (7-9 digits) (Fig. 1E,F).

In order to determine whether the reversed heart looping results from a defect in establishing proper left-right body patterning, we examined the expression of *Nodal* and *Lefty2*. *Nodal* and *Lefty2* are expressed asymmetrically in the left lateral plate mesoderm in wild-type E8.5 embryos (Fig. 1G,I) (Collignon et al., 1996; Meno et al., 1996). In *Hty* mutant embryos, both genes were expressed in both left and right lateral plate mesoderm, suggesting a loss of left-right asymmetry (Fig. 1H,J).

Hty encodes a C2 domain-containing protein

Through examination of 3692 meioses, we genetically mapped the *Hty* mutation to a ~700 kb region between 107.27 Mb and 107.97 Mb on mouse chromosome 7 (Fig. 2A). By sequence analysis of all 12 previously uncharacterized genes in this region, we found that *C2cd3* (C2 calcium-dependent domain-containing 3) contains a mutation in the first nucleotide of the fourth intron, changing a G to an A in *Hty* mutants (Fig. 2B). As the first nucleotide of an intron is essential for correct splicing of the preceding exon, we predicted that this point mutation would disrupt splicing of the *C2cd3* mRNA. Indeed, reverse transcriptase PCR (RT-PCR) using primers in the third and fifth exons of *C2cd3* revealed multiple abnormal transcripts in *Hty* mutants (Fig. 2C). Sequence analysis of these abnormal RT-PCR products indicated that the three longest transcripts (Fig. 2C, a-c) include two stop codons that were originally part of the fourth intron, and hence would encode a severely truncated C2cd3 protein (comprising the N-terminal 235 residues) (Fig. 2D). Two shorter transcripts (Fig. 2C, d,e) lack part of exon 4 and would encode C2cd3 proteins with small deletions (five residues in d, and 25 residues in e) in the N-terminal region (Fig. 2D; data not shown). We did not find any normal *C2cd3* transcript in the *Hty* mutants, even after two rounds of PCR amplification (data not shown).

The *C2cd3* locus spans 98 kb and includes 34 exons. The predicted full-length transcript is 7809 nt, encoding a 2322-residue cytoplasmic protein with five C2 domains (Fig. 2D). The C2 domain was named after the second functional domain of protein kinase C, which is required for its calcium-dependent lipid-binding capability (Nalefski and Falke, 1996). C2 domains were later identified in many proteins and found to mediate interactions not only with membrane lipids, but also with other proteins.

We found C2cd3 orthologs in all representative vertebrate species including human, rat, chicken, *Xenopus* and zebrafish, yet none has been previously characterized. We also discovered that no other mouse protein shares overall sequence similarity to C2cd3, suggesting that C2cd3 does not belong to a protein family. Interestingly, we did not find a

C2cd3 ortholog in *Drosophila*, *C. elegans* or in the flagellated green alga *Chlamydomonas*, suggesting that *C2cd3* is vertebrate specific.

By whole-mount RNA in situ hybridization using various regions of the *C2cd3* transcript as probes, we found that *C2cd3* is ubiquitously expressed in mouse embryos between E8.5 and E10.5 (Fig. 2E; data not shown), consistent with our observation that *Hty* mutants exhibit defects in the patterning of multiple tissues at E10.5 (see Fig. 1 and below).

A second *Hty* mutant allele exhibits similar developmental defects

In order to confirm that *C2cd3* is indeed the *Hty* gene, we generated another *C2cd3* mutant allele. We identified a gene-trap ES cell line (AG0177) that harbors a bacterial β-galactosidase (*lacZ*) insertion in the third intron of *C2cd3* (Fig. 2D). This *lacZ* insertion is predicted to ablate C2cd3 protein function by disrupting *C2cd3* transcription and splicing, resulting in a fusion protein between the N-terminal 161 residues of C2cd3 and β-galactosidase. We confirmed the *lacZ* insertion in the *C2cd3* gene by RT-PCR and sequence analysis (data not shown) and named this gene-trap allele $C2cd3^{GT}$.

We generated $C2cd3^{GT}$ carrier mice following blastocyst injection of the $C2cd3^{GT}$ ES cells. In a complementation assay, we bred a $C2cd3^{GT}$ carrier mouse with carriers of the original *Hty* mutant allele. The resulting transheterozygous ($C2cd3^{GT}$/*Hty*) embryos exhibited similar defects to *Hty* homozygous mutants (twisted body axis, pericardial edema, etc.), indicating that *C2cd3* is indeed the *Hty* gene (Fig. 2F). Subsequent analysis of the $C2cd3^{GT}$ homozygous mutants indicated that this allele exhibits similar, but more severe defects in embryonic development (Table 1 and below).

Dorsal-ventral patterning of the CNS is disrupted in *Hty* mutant embryos

In all *Hty* and $C2cd3^{GT}$ mutants with exencephaly, the basal plates of the midbrain are flat (Fig. 1B, inset), suggesting potential defects in ventral CNS patterning. Therefore, we examined dorsal-ventral (DV) patterning in the spinal cord at E10.5. *Shh* expression in the floor plate, the ventral-most region of the spinal cord, was absent in both *Hty* mutant alleles (Fig. 3A-C). Another floor plate marker gene, *Foxa2*, was also absent in the *Hty* mutant spinal cord, confirming the absence of the floor plate (data not shown). Despite the loss of *Shh* in the floor plate, *Shh* was expressed in the underlying notochord in both *Hty* mutant alleles, indicating that the loss of the floor plate is not due to the absence of inductive signals from the notochord (Fig. 3B,C). Immediately dorsal to the floor plate are V3 interneurons and their precursors that express *Nkx2.2* (Fig. 3D). In *Hty* mutants, the number of *Nkx2.2*-positive V3 interneurons appeared to be reduced and these cells were mislocalized to the ventral midline (Fig. 3E). *Isl1*-expressing motoneurons, which are normally located dorsal to the V3 interneurons (Fig. 3G), were expanded ventrally in *Hty* mutants, such that some *Isl1*-positive cells were found in the ventral midline of the spinal cord (Fig. 3H). Interestingly, the *Nkx2.2*-expressing V3 interneurons and most *Isl1*-expressing motoneurons failed to form in the $C2cd3^{GT}$ spinal cords (Fig. 3F,I). The loss of ventral cell types in the spinal cord of both *Hty* mutant alleles was accompanied by a ventral expansion of more lateral cell types, such as the *Lhx3*-expressing V2 interneurons (see Fig. 6; data not shown). *Pax6*, a gene normally expressed in the precursors of motoneurons and more dorsal cell types, was expressed throughout the DV aspect of the spinal cord of both *Hty* mutant alleles (Fig. 3J-L). These results indicate that *C2cd3* is required for normal patterning of the ventral CNS. The more severe loss of ventral cell types in the $C2cd3^{GT}$ spinal cord also suggests that *Hty* is likely to be a hypomorphic allele, possibly owing to residual activity of the mutant C2cd3 proteins with small in-frame deletions.

C2cd3 regulates Hh signal transduction in target cells

Hh signaling plays key roles in the regulation of ventral spinal cord patterning, digit formation and patterning of the left-right axis (Hooper and Scott, 2005), all of which are disrupted in *Hty* mutant embryos. The fact that ventral spinal cord patterning is disrupted in *Hty* mutants despite the presence of *Shh*-expressing notochord suggests that signal transduction downstream of Hh might be defective. In order to determine whether the *Hty* phenotype is associated with a disruption in Hh signal transduction, we examined the expression of *Ptch1*, one of the direct transcriptional targets of Hh signaling (Goodrich et al., 1997). Using a *Ptch1-lacZ* reporter mouse, we found that *Ptch1* expression is downregulated in multiple tissues, including the CNS and gut (Fig. 4A,B) and in the posterior region of the limb buds (Fig. 4E,F). The reduction of *Ptch1-lacZ* expression in the spinal cord was confirmed in sections (Fig. 4C,D). We next examined the expression of *Gli1*, another target gene of Hh signaling (Bai et al., 2004). *Gli1* expression was downregulated in all tissues of E10.5 *Hty* mutant embryos, including brain, otocysts (Fig. 4G,H), spinal cord (Fig. 4I,J) and limb buds (Fig. 4K,L). The reduction of *Ptch1* and *Gli1* expression in multiple tissues of *Hty* mutant embryos indicates that *C2cd3* is an important regulator of Hh signal transduction in the mouse.

To better understand the role of *C2cd3* in Hh signal transduction, we performed double-mutant analyses between *Hty* and known regulators of Hh signal transduction. *Rab23*, which encodes a small GTPase mutated in the mouse mutant *Open brain* (*Opb* and *Opb2*), negatively regulates Hh signaling in the mouse (Eggenschwiler et al., 2001). In the *Opb2* mutant spinal cord, Hh signaling is broadly activated, resulting in severe ventralization of the CNS. As a result, although the *Foxa2*-expressing floor plate remained ventrally restricted (Fig. 5A,B), other ventral cell types, such as the *Nkx2.2*-expressing V3 interneurons and their precursors, were dorsally expanded to occupy most of the spinal cord (Fig. 5E,F). By contrast, *Pax6*, which is expressed in the dorsal two-thirds of the wild-type spinal cord, was only expressed in the dorsal-most region of the spinal cord in *Opb2* mutants (Fig. 5I,J). In mouse embryos doubly mutant for *Hty* and *Rab23* (*Hty/Opb2*), as in *Hty* single mutants, the floor plate was missing (Fig. 5C,D) and a reduced number of *Nkx2.2*-expressing V3 interneurons was present in the ventral midline (Fig. 5G,H). *Pax6* was expressed throughout the spinal cord in both *Hty* single and *Hty/Opb2* double mutants (Fig. 5K,L). The indistinguishable spinal cord phenotype of *Hty* and *Hty/Opb2* double mutants suggests that *C2cd3* acts genetically downstream of *Rab23*. *Rab23* acts cell-autonomously to regulate the response of spinal cord cells to Hh and acts genetically downstream of *Ptch1* and *Smo* (Eggenschwiler and Anderson, 2000; Eggenschwiler et al., 2006). Therefore, *C2cd3* is likely to be an intracellular regulator of Hh signal transduction inside Hh target cells. Consistent with this proposal, a double-mutant analysis between *Hty* and *Ptch1* indicated that *C2cd3* is also genetically downstream of *Ptch1* in the Hh signal transduction pathway (see Fig. S1 in the supplementary material).

Cells in the spinal cord of *Hty* mutants are not responsive to Hh signaling

We next examined the impact of removing Shh ligand on the fates of the ventral spinal cord cells in *Hty* mutants. We first examined general DV patterning with *Pax7*, a marker gene expressed in the progenitors of all dorsal cell types (Fig. 6A). In *Shh* mutants, *Pax7* is misexpressed in all cells in the spinal cord (Fig. 6B). By contrast, in *Hty/Shh* double mutants, *Pax7* expression remained dorsally restricted, similar to what was observed in the wild type and in *Hty* single mutants (Fig. 6C,D). We then examined the formation of specific ventral spinal cord cell types. Motoneurons and V2 interneurons, which express the homeobox gene *Lhx3*, are located in the ventral-lateral regions of the wild-type spinal cord (Fig. 6E). Both cell types were absent in the *Shh* mutant spinal cord (Fig. 6F). In *Hty* single mutants (Fig. 6G) and *Hty/Shh* double mutants (Fig. 6H), *Lhx3* expression was present in

cells throughout the ventral spinal cord, indicating that complete removal of Shh ligand does not affect the *Hty* phenotype. Similarly, *En1*-expressing V1 interneurons, another ventral-lateral cell type in the spinal cord (Fig. 6I), were absent in *Shh* mutants (Fig. 6J), but present throughout the entire ventral spinal cord in both *Hty* single-mutant (Fig. 6K) and *Hty/Shh* double-mutant embryos (Fig. 6L). In conclusion, analysis of the *Hty/Shh* double mutants indicates that the spinal cord cells fail to respond to Hh signaling in the absence of C2cd3.

C2cd3 is required for ciliogenesis in the mouse

The multiple defects in mouse embryonic development and the disruption of Hh signaling in the *Hty* mutants closely resemble those seen in mutants for *Ift88* and *Ift52*, which exhibit severe loss of cilia [compare figs 3-5 in Liu et al. (Liu et al., 2005) with Figs 3-6 of this report]. Therefore, we sought to determine whether *C2cd3* also plays a role in the formation of cilia. Primary cilia (node cilia) are present on all cells of the ventral embryonic node in wild-type embryos (Fig. 7A). In *Hty* mutants, cilia failed to form in most cells in the node (Fig. 7B). In $C2cd3^{GT}$ mutant embryos, cilia were absent in nearly all node cells (Fig. 7C), suggesting that it is a more severe loss-of-function allele than the original *Hty* allele.

To further address whether *C2cd3* directly regulates the intrinsic capability of the cells to form primary cilia, we examined cilia formation in wild-type and *Hty* mutant mouse embryonic fibroblasts (MEFs) in culture. Wild-type cells kept in G0 for 48 hours developed primary cilia efficiently (Fig. 7D,F) (78% ciliated, n=373). By contrast, cilia formation in *Hty* mutant cells under identical conditions was greatly compromised (Fig. 7E,F) (21% ciliated, n=365). Therefore, C2cd3 is required intrinsically for the formation of cilia.

C2cd3 is required for the proteolytic processing of Gli3

Gli3 is proteolytically processed in vertebrates and Hh-regulated inhibition of Gli3 processing is a key event in vertebrate embryonic patterning (Wang et al., 2000). We have previously shown that mutations in mouse IFT genes lead to inefficient processing of Gli3 (Liu et al., 2005). In order to better understand the mechanisms underlying the roles of *C2cd3* in regulating Hh signaling and Gli activities, we examined Gli3 processing in *Hty* mutants by western blot, using an antibody against the N-terminus of Gli3 that interacts with both the full-length (Gli3-190) and processed short (Gli3-83) forms of Gli3 (Wang et al., 2000). As previously reported, in wild-type E10.5 embryos Gli3 is efficiently processed into its short repressor form (Gli3-83) (Fig. 8A, lanes 1 and 3). By contrast, in both *Hty* and $C2cd3^{GT}$ homozygous mutant embryos at the same stage, more Gli3 protein was present as unprocessed, full-length form (Gli3-190) (Fig. 8A, lanes 2 and 4). As a result, the ratio of Gli3-190/Gli3-83 was dramatically increased in the two *Hty* mutant alleles (from 0.36 in wild type to 1.34 in *Hty* and 1.47 in $C2cd3^{GT}$) (Fig. 8B). This suggests that *C2cd3* regulates Hh signaling (at least) partly through the regulation of Gli3 proteolytic processing.

C2cd3 protein is localized to the basal body of cilia

In order to better understand the roles of C2cd3 in cilia formation, we examined its subcellular localization by overexpressing C2cd3 tagged with green fluorescent protein at its C-terminus (C2cd3-GFP) in primary MEFs. By labeling ciliary axoneme with an anti-acetylated tubulin antibody, we discovered that C2cd3-GFP is present at one end of the cilia, presumably at the basal body (Fig. 9A). By labeling the two centrioles of the basal body with an anti-γ-tubulin antibody, we confirmed that C2cd3-GFP is indeed localized at the basal body (Fig. 9B). A similar basal body localization was observed when the GFP tag was added to the N-terminus of C2cd3 (data not shown).

DISCUSSION

In this study, we identified C2cd3, a novel C2 domain-containing protein specific to vertebrates, as an essential regulator of cilia formation, Hh signaling and mouse embryonic development. Through the characterization of two *C2cd3* mutant alleles (*Hty* and $C2cd3^{GT}$), we discovered that loss of *C2cd3* results in the disruption of embryonic patterning of multiple tissues, abnormal Hh signaling and inefficient processing of Gli3. A series of double-mutant analyses indicated that *C2cd3* regulates Hh signaling in target cells and is required for the target response to Shh ligand. We also found that mutations in *C2cd3* severely disrupt the formation of cilia of the ventral node, as well as cilia formation in cultured cells. Finally, we discovered that C2cd3 is localized to the basal body, consistent with its essential role in ciliogenesis. Intriguingly, although C2cd3 is conserved in vertebrates, it does not show homology to any other known protein involved in ciliogenesis.

C2cd3, cilia formation and cilia-related human genetic diseases

The formation of functional cilia is a complex process and requires a wide variety of genes. The lack of common structural features among proteins regulating cilia formation has hindered the discovery of these important regulators. Recent molecular screens utilizing comparative genomics and proteomics have identified dozens of potential novel regulators of cilia (Inglis et al., 2006). However, owing to the immensely complex nature of ciliogenic regulation, these screens have apparently failed to saturate the ‘ciliome’. The fact that C2cd3, which is specific to vertebrates, is not found in the current ciliome emphasizes the fact that our knowledge of ciliogenic regulation is far from complete.

The identification of novel regulators of cilia not only provides new insight into the molecular mechanism of cilia regulation, but is also invaluable in providing new animal models for cilia-related genetic diseases in human. A number of human genetic diseases, including BBS, MKS and JBTS, exhibit common symptoms, such as cystic kidney and polydactyly (Fliegauf et al., 2007). Many patients with these diseases carry mutations in genes encoding ciliary proteins. For many patients, the gene mutations have not been identified, although some have been mapped to specific chromosomal regions. Interestingly, the *C2cd3* ortholog in humans is in chromosomal region 11q13.4, close to the reported critical regions for MKS2 and JBTS2 (Roume et al., 1998; Valente et al., 2005). It will be interesting to determine whether the sequence or expression of the human *C2CD3* gene is disrupted in these patients.

An interesting question regarding the function of *C2cd3* is what molecular mechanism underlies its roles in cilia biogenesis. One possibility is that C2cd3 is a component of IFT particles. However, we have not been able to positively verify such a role. We have been unable to identify through extensive BLAST searches any known IFT protein in any species that has significant homology to C2cd3. In addition, the molecular mass of C2cd3 is predicted to be ~250 kDa, which is much larger than the reported size of any *Chlamydomonas* IFT proteins (Rosenbaum and Witman, 2002). Finally, we have not detected C2cd3 inside the cilia, suggesting that it might not be an integral part of the IFT complexes.

It has recently been reported that seven BBS proteins form another multiprotein complex called the BBSome (Nachury et al., 2007). The BBSome is localized to the primary cilia in ciliated cells and appears to be associated with the cell membrane, suggesting that it might be involved in vesicular transport. It will be interesting to determine whether C2cd3 plays a role in the function of the BBSome.

The C2 domain was originally identified as the calcium-dependent lipid-binding domain of protein kinase C (Nalefski and Falke, 1996). C2 domains have subsequently been found in other proteins, many of which are associated with the cell membrane and carry out a variety of functions. We speculate that C2cd3 might be involved in vesicular transport required for cilia biogenesis and/or in the transport of membrane-associated proteins. Alternatively, or additionally, some or all of its five C2 domains might mediate interactions with multiple proteins to form a multiprotein complex involved in cilia biogenesis. We are currently addressing these possibilities biochemically.

Hty, cilia and Hh signaling

Our previous studies indicate that IFT is required for Hh signal transduction in the mouse (Huangfu et al., 2003). More specifically, both the proteolytic processing and activation of the Gli transcription factors require IFT function (Liu et al., 2005). This is in contrast to *Drosophila*, in which the mechanism of IFT is largely conserved but only a small number of sensory neurons exhibit primary cilia and IFT is not required for Hh signaling (Witman, 2003). In the mouse, some Hh signaling components, such as Ptch1, Smo, Sufu (suppressor of fused), Gli1, Gli2 and Gli3, are localized to primary cilia (Corbit et al., 2005; Haycraft et al., 2005; Rohatgi et al., 2007). However, it is not clear whether the presence of cilia per se is enough for normal Hh signaling, or whether IFT participates directly in transporting Hh signaling components inside the cilia.

Hty mutants display multiple embryonic developmental defects, including disruption of CNS and left-right patterning, polydactyly in the limbs and pericardial edema. Most of these defects can be explained by abnormal Hh signaling. Indeed, our Hh target gene expression analysis and genetic studies indicate that C2cd3 regulates Hh signaling, acting downstream of *Ptch1* and *Rab23*. The fact that C2cd3 is specific to vertebrates is intriguing as C2cd3 might be involved in transporting vertebrate-specific cargos such as Hh pathway components.

Finally, most mouse mutants with severe cilia defects show an apparent decrease in Gli3 processing, suggesting that efficient Gli3 processing requires cilia (e.g. Haycraft et al., 2005; Huangfu and Anderson, 2005; Liu et al., 2005). Our discovery that *C2cd3* is required for efficient processing of Gli3 is consistent with its essential role in cilia biogenesis. However, we do not have sufficient evidence to distinguish whether the inefficient processing of Gli3 in the *Hty* mutants results from the cilia defects, or whether C2cd3 plays a more direct role in Gli3 regulation.

It is paradoxical that despite the accumulation of full-length Gli3 protein (Gli3-190), the loss of ventral cell types in the spinal cord and downregulation of Hh target gene expression in the *Hty* mutants suggest a loss of Gli activator activity. We propose that Gli3-190 in *Hty* mutants is not in its active state and is likely to be subject to additional negative regulation. It has been shown in *Drosophila* that Sufu keeps Ci in a stable but inactive state (Ohlmeyer and Kalderon, 1998). Therefore, it will be of interest to investigate whether Sufu is responsible for the loss of Gli activator function in *Hty* and other cilia mutants.

Cilia and calcium signaling

Cilia are closely associated with intracellular calcium signaling in many contexts. Currently, it is generally believed that cilia play a role in regulating calcium levels in ciliated cells. In renal epithelial cells, bending of the primary cilia opens the calcium channel, PC2, allowing calcium to enter the cytoplasm (Wilson, 2004). In gastrulating mouse embryos, a calcium surge is observed on the left side of the embryonic node, which might be the consequence of cilia deformation-induced PC2 activation (McGrath et al., 2003). Alternatively, Shh might

play a role in PC2 activation, as overexpression of Shh leads to an increase in intracellular calcium levels on the left side of the node (Tanaka et al., 2005).

The presence of multiple C2 domains in the C2cd3 protein is likely to be key to an understanding of its molecular functions. It is possible that calcium plays important roles in C2cd3 function, as well as in cilia formation. In fact, many known ciliary proteins are calcium-binding proteins (e.g. calmodulin, calcineurin, centrin). Therefore, a close examination of the roles of calcium in cilia formation is needed.

Alternatively, calcium might be involved in the process of loading and unloading cargoes. One speculation is that C2cd3, as an adaptor protein between IFT complex and vesicular cargoes at the basal body, exhibits a high affinity for its cargoes in the presence of calcium, allowing their transportation to the base of cilia. A transient drop in calcium level at the destination, or the presence of a competing adaptor protein, could lead to the dissociation of C2cd3 from its cargoes, facilitating unloading.

In conclusion, we have discovered that *C2cd3* is a vertebrate-specific, novel, C2 domain-containing protein essential for Hh signaling, cilia formation and mouse embryonic development. The presence of calcium-dependent lipid-binding domains in C2cd3 suggests a potential role in vesicular transport. Finally, the proximity between the human *C2cd3* ortholog and certain cilia-related genetic disease loci on chromosome 11 makes it an interesting candidate for ciliopathy in humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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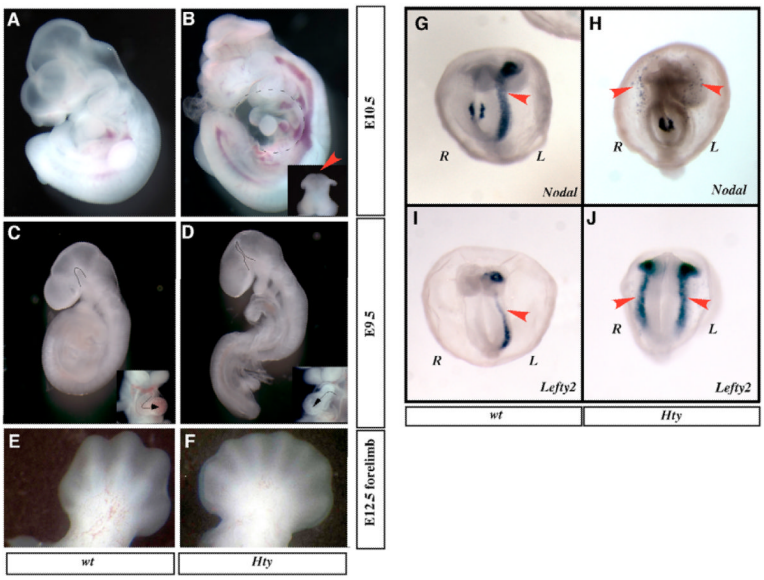

Fig. 1. Mouse *Hty* mutants exhibit multiple defects in embryonic development

(**A**,**B**) E10.5 wild-type (wt) (A) and *Hty* mutant (B) embryos showing exencephaly and pericardial edema (outlined by dashed line). Inset in B shows front view of the mutant head; note the flat basal plate of the brain (arrowhead). (**C**,**D**) E9.5 wild-type and *Hty* mutant embryos showing tight mesencephalic flexure in mutant embryos (outlined). Insets show heart looping in the two embryos (direction of heart looping indicated by arrows). (**E**,**F**) At E12.5, *Hty* mutant embryos exhibit severe polydactyly. Forelimbs are shown. (**G**,**H**) *Nodal* is normally expressed asymmetrically in left lateral plate mesoderm (G, arrowhead), but is expressed on both sides in *Hty* mutants (H). (**I**,**J**) *Lefty2* is normally expressed asymmetrically in left lateral plate mesoderm (I, arrowhead), but is expressed on both sides in *Hty* mutants (J). (G-J) In situ hybridization, ventral views. R, right; L, left.

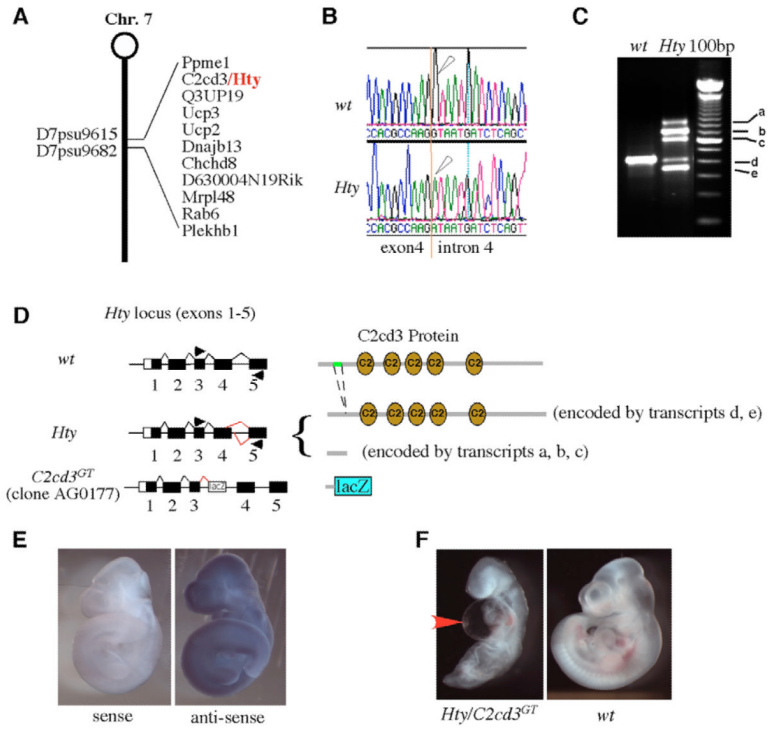

Fig. 2. *Hty* encodes a novel C2 domain-containing protein

(**A**) Genetic mapping of *Hty* to a 700 kb region on mouse chromosome 7. (**B**) A single nucleotide substitution (G to A; arrowheads) is present at the beginning of intron 4 of *C2cd3*. (**C**) RT-PCR using primers in exons 3 and 5 (arrowheads in D) of *C2cd3* shows multiple abnormal transcripts (a-e) in an E9.5 *Hty* mutant embryo. A 100-bp DNA ladder (100bp) is included for reference. (**D**) C2cd3 contains five C2 domains. Abnormal splicing in *Hty* mutants (in red) results in either a severe truncation or in-frame deletions. The gene-trap allele ($C2cd3^{GT}$, clone AG0177) is predicted to truncate the C2cd3 protein and fuses a β-galactosidase moiety (*lacZ*) to the remaining N-terminal fragment of C2cd3. The green region in the wild-type protein denotes the part of the C2cd3 protein that is not encoded by mutant transcripts d and e. (**E**) RNA in situ hybridization on E10.5 embryos using a sense (left) or an antisense (right) probe against *C2cd3*. (**F**) $Hty/C2cd3^{GT}$ transheterozygous embryos exhibit a similar phenotype to the original *Hty* mutants, confirming that *C2cd3* is indeed the *Hty* gene. Shown are an E10.5 transheterozygous embryo and its wild-type littermate. Arrowhead in F points to pericardial edema.

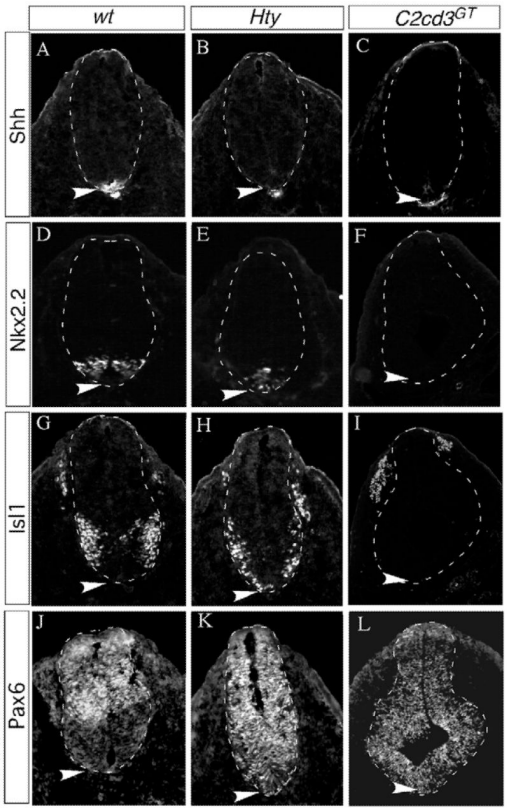

Fig. 3. Ventral spinal cord patterning is disrupted in both *Hty* mutant alleles

(**A-C**) The notochord, but not the floor plate, expresses *Shh* in *Hty* and *C2cd3*^{GT} mutants. (**D-F**) *Nkx2.2*-expressing V3 interneurons are mislocalized to the ventral midline in *Hty* mutants, and are absent in *C2cd3*^{GT} mutants. (**G-I**) *Isl1*-expressing motoneurons expand ventrally in *Hty* mutants, and are absent in *C2cd3*^{GT} mutants. (**J-L**) *Pax6* expression expands ventrally in *Hty* and *C2cd3*^{GT} mutants. Shown are immunofluorescent images of transverse sections at the forelimb level of E10.5 mouse embryos. Spinal cords are outlined by dashed lines. Arrowheads point to the ventral midline of the spinal cord, which corresponds to the floor plate in the wild-type embryos.

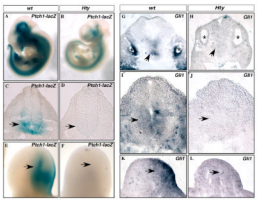

Fig. 4. Hh signaling is disrupted in *Hty* mutants

(**A**,**B**) *Ptch1-lacZ* is strongly expressed in Hh target cells in an E10.5 wild-type mouse embryo (A), but is greatly downregulated in an *Hty* embryo (B). (**C**,**D**) Transverse sections at the forelimb level to show *Ptch1-lacZ* expression in wild-type (C) and *Hty* mutant (D) embryos. (**E**,**F**) Dorsal views of E10.5 forelimb buds showing strong *Ptch1-lacZ* expression in the posterior region of the wild type (E), but no detectable expression in *Hty* (F; anterior is to the left). (**G-L**) *Gli1* is normally expressed in hindbrain, otocysts (G), spinal cord (I, hindlimb level) and limb buds (K). In *Hty* mutants, *Gli1* expression in all these tissues is greatly downregulated (H,J,L). Arrows point to expression of *Ptch1-lacZ* or *Gli1* in wild-type embryos and the corresponding locations in *Hty* mutant embryos where expression of both genes is greatly reduced. Asterisks in G and H indicate the otocysts.

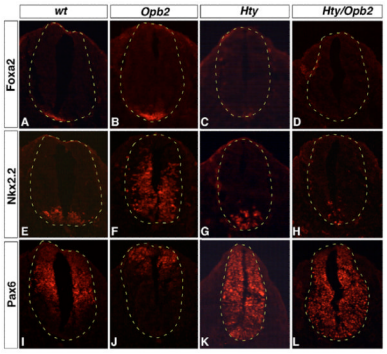

Fig. 5. *Hty* is downstream of *Rab23*

(**A**,**B**) *Foxa2* is expressed in the floor plate of the wild-type and *Opb2* (*Rab23*) mutant spinal cord. (**C**,**D**) *Foxa2* expression is absent in both *Hty* and *Hty/Opb2* mutant spinal cords. (**E**) *Nkx2.2* is expressed in V3 interneurons located adjacent to the floor plate in wild-type spinal cords. (**F**) In *Opb2* mutants, *Nkx2.2* expression is expanded dorsally to occupy most of the spinal cord. (**G**,**H**)In both *Hty* and *Hty/Opb2* mutants, a few *Nkx2.2*-expressing cells are located at the ventral midline. (**I**) *Pax6* is expressed in the dorsal two-thirds of the wild-type spinal cord. (**J**) In *Opb2* mutants, *Pax6* expression is restricted to a few cells in the dorsal-most region. (**K**,**L**) *Pax6*-expressing cells occupy the entire spinal cord in both *Hty* and *Hty/Opb2* mutants. Shown are immunofluorescent images of transverse sections at the forelimb level of E10.5 mouse embryos. Spinal cords are outlined with dashed lines.

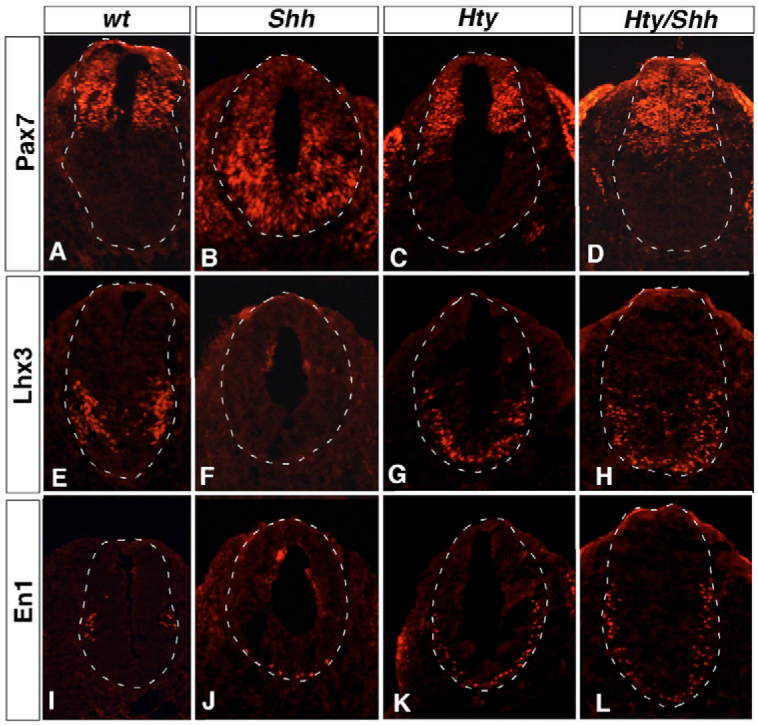

Fig. 6. Spinal cord cells do not respond to Shh in the absence of *C2cd3*

(**A**,**B**) *Pax7* is expressed in dorsal progenitor cells in wild-type spinal cord (A), but is expressed in all cells of *Shh* mutant spinal cord (B). (**C**,**D**) In both *Hty* and *Hty/Shh* double mutants, *Pax7* expression remains dorsally restricted. (**E**) *Lhx3* is expressed in V2 interneurons and motoneurons, which occupy the ventral-lateral region of wild-type spinal cord. (**F**) *Lhx3* expression is absent in the *Shh* mutant. (**G**,**H**)In both *Hty* and *Hty/Shh* mutant spinal cords, the *Lhx3* expression domain is expanded ventrally to include the ventral midline. (**I**) *En1* is expressed in V1 interneurons located in small lateral domains of wild-type spinal cord. (**J**) *En1* expression is absent in *Shh* mutants. (**K**,**L**) *En1*-expressing cells are scattered in the ventral half of *Hty* and *Hty/Shh* mutant spinal cords. Shown are immunofluorescent images of transverse sections at the forelimb level of E10.5 mouse embryos. Spinal cords are outlined with dashed lines.

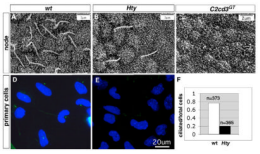

Fig. 7. Cilia formation is disrupted in *Hty* mutants

(**A**) SEM image of the ventral embryonic node of an E8.0 wild-type mouse embryo showing primary cilia on every cell. (**B**) In *Hty* mutants, most node cells do not have cilia. (**C**) In *C2cd3*^{GT} mutants, cilia are absent from nearly all cells. (**D**) Wild-type cells kept at G0 for 48 hours develop cilia efficiently. (**E**)Most *Hty* mutant cells kept at G0 for 48 hours fail to develop cilia. In D and E, cilia are stained green and nuclei are stained blue. (**F**) The ratio of ciliated cells versus total cells after 48 hours in G0. Approximately 78% of wild-type cells are ciliated, whereas only ~20% of *Hty* mutant cells are ciliated.

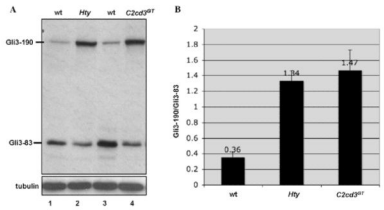

Fig. 8. C2cd3 regulates proteolytic processing of Gli3

(**A**) Western blot showing the levels of the full-length (Gli3-190) and processed (Gli3-83) forms of Gli3 in E10.5 wild-type, *Hty* and *C2cd3*^{GT} mutant mouse embryos. (**B**) The ratio between Gli3-190 and Gli3-83. The results were obtained from four independent experiments. The number above each bar indicates the Gli3-190/Gli3-83 value.

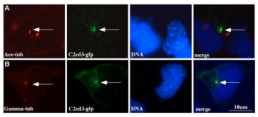

Fig. 9. C2cd3 is localized at the basal body

(**A**) C2cd3-GFP localized at one end of cilia labeled with antibody to acetylated tubulin (Ace-tub). (**B**) C2cd3-GFP is present at the basal body, around the centrioles labeled with antibody to γ-tubulin (Gamma-tub). Arrows point to the locations of C2cd3-GFP signals.

Table 1

Hty homozygous mutants are embryonic lethal

Alleles	Stage	Live embryos	Dead embryos	Mutant embryos	Ratio (%)
Hty	E10.5	243	7	53	21.81
	E11.5	83	7	20	22.22
	E12.5	25	6	3	9.67
	E13.5	14	3	0	0
C2cd3^{GT}	E10.5	121	0	29	23.97
	E11.5	28	1	3	10.34
	E12.5	10	4	0	0

Table 2

Hty homozygous mutant embryos exhibit multiple defects

Phenotype	Number of embryos
E10.5	
Total	93
Exencephaly	28
Tight mesencephalic flexure	65
Abnormal heart looping	30
Twisted body	93
Pericardial edema	93
E12.5	
Total	3
Polydactyly	3