C2cd3 is required for cilia formation and Hedgehog signaling in mouse

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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 (JBT2S), making it a potential candidate for these two human genetic disorders.

KEY WORDS: 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, 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 (JBT) (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 JBT2S (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) (Metthot 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; Eggeschwiler 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
organisms during gastrulation (reviewed by Webb and Miller, 2006).
It has been suggested that calcium waves might be important for
cconvergent 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’-CAGAAGGGCTTCT-
CATATTTTG-3’; reverse primer, 5’-ATCCTCAAGCAGGAGGT-
3’ and D7psu9682e (forward primer, 5’-TGGGAAGAAGGG-
GACCTTT-3’; reverse primer, 5’-TGCCCAAGCGTGTGTTCACT-3’).
Phenotypic analyses were carried out in congenic C3H/HeN mice (Charles
River Laboratory). A second Hty mutant allele, designated C2cd3GT
was kept on the same C3H/HeN background as the original
Hty allele. C2cd3GT 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://
cilia.proteome.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%
parafomaldehyde 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% CO2. To
visualize the cilia, MEFS were passsed 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 pEGFP-C3 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 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 C2cd3GT.

We generated C2cd3GT carrier mice following blastocyst injection of the C2cd3GT ES cells. In a complementation assay, we bred a C2cd3GT carrier mouse with carriers of the original Hty mutant allele. The resulting transheterozygous (C2cd3GT/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 C2cd3GT 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 C2cd3GT 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 C2cd3GT spinal cords (Fig. 3J,L). 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 embryos. The more severe phenotype is likely to be a hypomorphic allele, possibly owing to reduced expression in the spinal cord also suggests that signal transduction downstream of Hh might be disrupted in Hty mutants despite the presence of Shh-expressing notochord. Therefore, we performed double-mutant analyses between Hty and known regulators of Hh signal transduction, 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 Lhx3-positive cells were found in the ventral midline of the spinal cord (Fig. 5A,B), other ventral cell types, such as the Lhx3-expressing V2 interneurons and most Isl1-expressing motoneurons failed to form in the C2cd3GT spinal cords (Fig. 3J,L). 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 C2cd3GT 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.

### Table 2. Hty homozygous mutant embryos exhibit multiple defects

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Number of embryos</th>
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<tr>
<td><strong>E10.5</strong></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>93</td>
</tr>
<tr>
<td>Exencephaly</td>
<td>28</td>
</tr>
<tr>
<td>Tight mesencephalic flexure</td>
<td>65</td>
</tr>
<tr>
<td>Abnormal heart looping</td>
<td>30</td>
</tr>
<tr>
<td>Twisted body</td>
<td>93</td>
</tr>
<tr>
<td>Pericardial edema</td>
<td>93</td>
</tr>
<tr>
<td><strong>E12.5</strong></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>3</td>
</tr>
<tr>
<td>Polydactyly</td>
<td>3</td>
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**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 Pch1, one of the direct transcriptional targets of Hh signaling (Goodrich et al., 1997). Using a Pch1-lacZ reporter mouse, we found that Pch1 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 Pch1-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. 4J,L) and limb buds (Fig. 4K,L). The reduction of Pch1 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. 5L). In mouse embryos doubly mutant for Hty and Rab23 (Hty/Opb2), as in Hty single mutants, the floor plate was missing (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 Ptc1 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 Ptc1 indicated that C2cd3 is also genetically downstream of Ptc1 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, Enl-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<sup>GT</sup> 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 C2cd3GT 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 C2cd3GT) (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 C2cd3GT), 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.

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 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 cords. (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.
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.

**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 C2cd3GT 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.
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Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/24/4049/DC1

References