Inactivation of Chibby affects function of motile airway cilia

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Chibby (Cby) is a conserved component of the Wnt–β-catenin pathway. Cby physically interacts with β-catenin to repress its activation of transcription. To elucidate the function of Cby in vertebrates, we generated Cby−/− mice and found that after 2–3 d of weight loss, the majority of mice die before or around weaning. All Cby−/− mice develop rhinitis and sinusitis. When challenged with Pseudomonas aeruginosa isolates, Cby−/− mice are unable to clear the bacteria from the nasal cavity. Notably, Cby−/− mice exhibit a complete absence of mucociliary transport caused by a marked paucity of motile cilia in the nasal epithelium. Moreover, ultrastructural experiments reveal impaired basal body docking to the apical surface of multiciliated cells. In support of these phenotypes, endogenous Cby protein is localized at the base of cilia. As the phenotypes of Cby−/− mice bear striking similarities to primary ciliary dyskinesia, Cby−/− mice may prove to be a useful model for this condition.

Introduction

Chibby (Cby) was originally discovered as an inhibitor of the Wnt–β-catenin pathway (Takemaru et al., 2003). Cby inhibits Wnt–β-catenin signaling through two distinct mechanisms: by competing with T cell factor/lymphoid-enhancer factor transcription factors for binding β-catenin (Takemaru et al., 2003) and by facilitating nuclear export of β-catenin (Li et al., 2008). Another study demonstrated that Cby also functions in the cytoplasm as a binding partner and a mediator of intracellular trafficking of Polycystin-2 (Hidaka et al., 2004), which is a protein required for the proper function of primary cilia (Nauli et al., 2003). Interestingly, Polycystin-2 is also found in the membrane of motile cilia, suggesting that it may be important for the function of this distinct subclass of cilia (Pazour et al., 2005; Teilmann et al., 2006; Christensen et al., 2007).

Cilia are eukaryotic organelles that are traditionally classified according to their microtubule composition, with the 9 + 0 arrangements in primary cilia and the 9 + 2 arrangements in motile cilia, although this classification is an oversimplification (Kramer-Zucker et al., 2005; Ong and Wagner, 2005; Davis et al., 2006). Primary cilia are present on most mammalian cells and play critical roles in mechanosensation, photoreception, olfaction, and intracellular signaling (Singla and Reiter, 2006). Motile cilia are less ubiquitous and are found primarily in epithelial cells lining the airways, reproductive tracts, and the ependyma and choroid plexus in the brain (Eley et al., 2005). They are important in clearing mucus and debris from the airway, circulating cerebrospinal fluid in the brain, and possibly transporting the cumulus–oocyte complex in the female reproductive tract (Salathe, 2007). Defects in motile cilia lead to a wide spectrum of phenotypes, including abnormal left–right patterning, infertility, hydrocephalus, and respiratory infections (Badano et al., 2006; Salathe, 2007). Various combinations of these phenotypes are present in multiple diseases, including primary ciliary dyskinesia (PCD; Badano et al., 2006).

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Abbreviations used in this paper: BAC, bacterial artificial chromosome; Cby, Chibby; CF, cystic fibrosis; CFU, colony-forming unit; LB, Luria broth; MCT, mucociliary transport; PCD, primary ciliary dyskinesia; PMEF, primary mouse embryonic fibroblast; TEM, transmission EM.
PCD, which includes Kartagener syndrome and the immotile cilia syndrome, is a rare genetically heterogeneous disorder with an estimated prevalence rate of 1 in 15,000 to 1 in 60,000, although the number of diagnosed cases is lower as a result of variability in symptoms and similarity to acquired conditions (Coren et al., 2002). The most common genetic causes of PCD are mutations in the human DNAH1 and DNAH5 genes, which encode outer dynein arm components (Pennarun et al., 1999; Olbrich et al., 2002). Mutations in these genes are responsible for up to 40% of the known cases of PCD (Zariwala et al., 2007). A small percentage of patients with PCD were reported to have mutations in DNAH11, TXNDC3, RPGR, or the OFDI gene, whereas other causative genes remain unidentified (Bush and Ferkol, 2006; Livraghi and Randell, 2007). Recurrent respiratory infections, chronic otitis media, and partially penetrant situs inversus are the hallmarks of PCD (Meeks and Bush, 2000). Mortality and morbidity associated with PCD are primarily caused by repeated infections resulting in respiratory insufficiency.

The lesions of PCD highlight the importance of efficient mucociliary clearance, which depends on mucus production and mucociliary transport (MCT). In the normal airway, goblet cells produce mucus that traps particles and pathogens. Coordinated ciliary beating transports mucus and embedded particles toward the larynx to be swallowed (Cohen, 2006). In patients with PCD, abnormal motility of airway cilia leads to an inability to transport mucus. Mucus plaques obstruct the airway and serve as a nidus for many opportunistic bacteria, including *Pseudomonas aeruginosa*.

In this study, we describe germline inactivation of the *Cby* gene and the resulting respiratory phenotype, which is similar to the clinical features observed in PCD patients. *Cby*−/− mice develop upper respiratory infections and are unable to clear bacteria because of the absence of MCT. We show that the MCT defects result, at least in part, from a marked paucity of motile cilia in the nasal epithelium possibly caused by the abnormal transport or docking of basal bodies to the apical membrane. In agreement with this phenotype, we demonstrate that *Cby*−/− embryos lacked staining compared with heterozygous littermate controls (Fig. 1, compare A with C). *Cby*−/− embryos hybridized with the antisense probe were indistinguishable from control embryos stained with the sense probe (Fig. 1, compare C with D). Gross morphological evaluation of newborns did not reveal apparent defects. However, by 2 wk of age, *Cby*−/− pups were runted and demonstrated anemia, as indicated by decreased hematocrit on complete blood count analysis and reduced subcutaneous fat (Fig. 1 E and not depicted).

To better understand the association of *Cby* gene ablation with runting, we performed body mass and survival analysis of pups produced by *Cby*+/− parents. The survival analysis showed that *Cby*−/− mice stratified into two groups: the majority of them died by postnatal day (P) 25 (knockout-die), whereas a small percentage started to gain weight and survived for >18 mo (knockout-survive; Fig. 1, F and G). However, all surviving *Cby*−/− mice (7/32) continued to have low body mass compared with *Cby*+/− mice (Fig. 1 F). At all stages analyzed, *Cby*+/− mice were indistinguishable from *Cby*+/+ counterparts (unpublished data). These data demonstrate that the gross physiological effect of loss of *Cby* on mouse development results in lowered body mass followed by early postnatal lethality. However, the exact causes of their postnatal death remain unknown.

### Sinusitis and otitis in *Cby*−/− mice

Histopathological phenotyping demonstrated that all *Cby*−/− mice, both those that die postnatally and those that survive, develop sinusitis (Fig. 2, A and B). This presents itself as early as P7 and persists throughout the lifetime of the surviving adults. Mice with severe sinusitis also had otitis media (Fig. 2, C and D).

To further probe sinus function, we tested the susceptibility of *Cby*−/− mice to bacterial infection. We intranasally infected five *Cby*+/+, five *Cby*+/−, and six *Cby*+/+ mice with *P. aeruginosa*, a pathogenic bacterium frequently associated with chronic airway infection in patients with cystic fibrosis (CF) and PCD (Gibson et al., 2003). We found that at 72 h after infection, infected littermate controls efficiently cleared bacteria and had no sinus inflammation, as revealed by histological analysis of the upper airways (Fig. 2 E). In contrast, all *Cby*−/− mice had accumulated abundant mucus and bacterial debris, leading to severe inflammation and distorted morphology of sinuses (Fig. 2 F). Concurrent colony-forming unit (CFU) analysis revealed that both *Cby*−/− and control mice successfully cleared the bacteria from the lungs (Fig. 2 G). Therefore, bacterial challenge revealed further phenotypic abnormalities, as *Cby*−/− mice develop sinus and middle ear inflammation and fail to clear bacteria from the sinuses.

### Normal bioelectric and impaired MCT in *Cby*−/− mice

Defects in bacterial clearance can have multiple etiologies, as illustrated by PCD and CF. Although respiratory phenotypes...
To address potential ion channel deficiencies in Cby<sup>−/−</sup> mice, we determined Na<sup>+</sup> absorption in response to amiloride and Cl<sup>−</sup> secretion in response to UTP and forskolin in tracheas from Cby<sup>+/+</sup> and Cby<sup>−/−</sup> mice. The tracheal bioelectrics were identical between the two genotypes (Fig. 2 H), indicating that ion channel function was not affected. These results argue against the involvement of ion channel defects in the airway pathology of Cby<sup>−/−</sup> mice.

Next, we examined ciliary function by measuring MCT in nasopharyngeal cavities. As expected, control mice exhibited normal MCT of endogenous particles in the nasal cavity. Strikingly, observed in PCD and CF patients are quite similar, the molecular and cellular mechanisms underlying these phenotypes differ. As previously mentioned, abnormal airway cilia structure or motility underlies PCD phenotypes (Livraghi and Randell, 2007). In contrast, human patients with CF caused by mutations in the CF transmembrane conductance regulator exhibit abnormal ion and water transport secondary to defects in Cl<sup>−</sup> conductance. This leads to depletion of the periciliary aqueous layer in the respiratory system with accumulation of abnormally viscous mucus that adheres to the epithelial surfaces and cannot be transported by cilia (Livraghi and Randell, 2007).

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Defects in MCT may be caused either by abnormal cilia structure or by reduced numbers of cilia. To examine cilia structure, we analyzed nasopharyngeal cilia by transmission EM (TEM). The cilia of the control mice generated a steady flow of mucus, whereas, in three of the four Cby−/− mice studied, mucus movements were completely absent (compare Video 1 with Video 2). In the fourth Cby−/− mouse, we observed small islands of limited mucus movement, but this was not sufficient to generate detectable MCT. Thus, Cby−/− mice develop sinusitis and otitis media because of a failure of the mucociliary defense system in the nasal passages and possibly in the eustachian tube.

Cilia paucity in Cby−/− mice

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Cilia from Cby<sup>−/−</sup> mice exhibit apparently normal ciliary ultrastructure with a 9 + 2 microtubular arrangement and the presence of outer dynein arms (Fig. 3, A and B). Basal bodies in Cby<sup>−/−</sup> mice also show normal nine-triplet structure (Fig. 3, A and B). Similarly, we did not detect any apparent defects in the structure of bronchial cilia from Cby<sup>−/−</sup> mice (Fig. S2, A and B). In the course of our TEM experiments, we noticed that Cby<sup>−/−</sup> mice have an abnormally low abundance of cilia in their nasopharynx (Fig. 3, compare C with D). A marked paucity of cilia was also observed upon analysis of Cby<sup>−/−</sup> nasopharyngeal tissue at P0 (Fig. S2, C and D), ruling out the possibility that loss of cilia is secondary to chronic inflammation. To verify our observation, we performed scanning EM analysis of proximal lung airways from adult Cby<sup>−/−</sup> and Cby<sup>+/+</sup> mice. We found that the number of cilia is dramatically decreased in the Cby<sup>−/−</sup> tissue when compared with that from Cby<sup>+/+</sup> controls (Fig. 3, E and F).
Cby protein is expressed in ciliated cells in the developing lung airways and esophagus at E18.5 (Fig. S2, E–G). Additionally, endogenous Cby was detected at the base of primary cilia in cultured MDCK2 cells (Fig. 4, G–I). We also noted that Cby colocalizes with the centrosomal marker γ-tubulin (Fig. 4, J–L).

Several ciliopathy-associated proteins have been shown to localize to both centrosomes and basal bodies, including CEP290, which is involved in Joubert syndrome (Valente et al., 2006), and ALMS1, which is mutated in Alström syndrome (Li et al., 2007). Cby localization at the base of the cilia further supports our notion that Cby is directly involved in ciliogenesis.

Wnt–β-catenin signaling activity in Cby−/− mice
Cby acts as an antagonist of Wnt–β-catenin signaling in mamalian cultured cells and Drosophila melanogaster embryos (Takemaru et al., 2003). Thus, we evaluated the status of β-catenin–dependent transcription in nasal epithelia from Cby−/− mice and age-matched controls using quantitative PCR for the direct β-catenin targets Axin2 and CyclinD1. We observed a consistent increase in the expression of these genes in the Cby−/− tissue (Fig. 5 A). Similar results were obtained for primary mouse embryonic fibroblasts (PMEFs) derived from Cby−/− and Cby+/+ embryos (Fig. 5 B). These data are consistent with Cby

Collectively, we conclude that loss of Cby results in a paucity of cilia in respiratory tracts, which most likely accounts for the lack of MCT activity in Cby−/− mice.

Based on these data, we hypothesized that the ciliary defects in Cby−/− mice might be caused by altered docking or transport of basal bodies to the apical membrane. Through detailed EM analysis, we found that a significant number of basal bodies failed to position apically and dock at the plasma membrane in Cby−/− nasal ciliated cells (Fig. 3, G and H). This is not attributable to a general loss of apical–basal polarity in these cells because the apical marker ZO1 and actin filaments as well as the basolateral marker epithelial cadherin remained unaffected in Cby−/− mice (Fig. S3, M–R). As cilia extend from basal bodies, compromised basal body docking may explain, at least in part, the defective ciliogenesis in Cby−/− mice.

Cby protein is localized at the ciliary base
Given the involvement of Cby in cilia pathology, we asked whether Cby protein is localized in a manner consistent with this pathology. Double immunostaining with an antibody against acetylated α-tubulin (cilia marker) revealed that Cby is localized at the ciliary base in multiciliated cells of the adult Cby+/+ nasal epithelium (Fig. 4, A–C), whereas no Cby staining was detected in the Cby−/− tissue (Fig. 4, D–F). In agreement with this, Cby protein is expressed in ciliated cells in the developing lung airways and esophagus at E18.5 (Fig. S2, E–G). Additionally, endogenous Cby was detected at the base of primary cilia in cultured MDCK2 cells (Fig. 4, G–I). We also noted that Cby colocalizes with the centrosomal marker γ-tubulin (Fig. 4, J–L). Several ciliopathy-associated proteins have been shown to localize to both centrosomes and basal bodies, including CEP290, which is involved in Joubert syndrome (Valente et al., 2006), and ALMS1, which is mutated in Alström syndrome (Li et al., 2007). Cby localization at the base of the cilia further supports our notion that Cby is directly involved in ciliogenesis.

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Figure 4. Cellular localization of Cby protein. (A–F) Nasal epithelial sections from Cby+/+ (A–C) and Cby−/− (D–F) adult mice were double stained with antibodies against acetylated α-tubulin (ac-tubulin; A and D) and Cby (B and E), and the merged images of acetylated α-tubulin (red) and Cby (green) are shown [C and F]. (G–I) Ciliated MDCK2 cells were doubly immunostained with acetylated α-tubulin (G) and Cby (H) antibodies, and the merged image of acetylated α-tubulin (red) and Cby (green) is shown (I). Arrowheads point out Cby staining at the base of the cilia. Bars: (A–F) 6 µm; (G–I) 1 µm; (J–L) 5 µm.
being a β-catenin antagonist (Takemaru et al., 2003) and raise the question of whether Cby−/− mice display changes in the localization of Wnt–β-catenin pathway components such as adenomatosis polyposis coli, β-catenin, and Dishevelled, which have been shown to localize to the ciliary base (Gerdes et al., 2007; Corbit et al., 2008; Park et al., 2008). Therefore, we analyzed the localization of β-catenin and Dvl1 in nasal ciliated cells from Cby−/− mice and littermate controls. Lateral membranous β-catenin and apical Dvl1 localization remained unchanged in the absence of Cby (Fig. S3, A–L). Based on these results, it is not clear whether the ciliary phenotypes of Cby−/− mice are related to the modest elevation of Wnt–β-catenin target genes in these mice. These and other questions that seek to link the mechanisms of Wnt signaling to discrete subcellular compartments and structures await further research.

Similarities to disease phenotypes

The airway phenotypes of Cby−/− mice bear strong similarities to those of human PCD patients. PCD is frequently associated with mutations in genes encoding dyneins, including Dnah1 and DNAH5 (Pennarun et al., 1999; Olbrich et al., 2002). Our quantitative PCR analysis demonstrated that inactivation of Cby did not significantly affect levels of Dnah1 and DNAH5 transcripts in nasal epithelial tissue (unpublished data).

The presence of sinusitis and otitis media and an inability to clear bacteria from the sinuses upon intranasal infection underlie similarities between the phenotypic defects found in Cby−/− mice and in human patients with PCD. This is especially evident when Cby−/− mice are exposed to bacterial challenge, exacerbating the already inflamed state of their upper airway. About half of PCD patients have laterality defects and many are subfertile (Meeks and Bush, 2000; Noone et al., 2004). To date, we have not observed left–right patterning defects such as situs inversus in Cby−/− mice, suggesting that Cby is not essential for proper structure and function of motile 9 + 0 cilia in the embryonic node. We noticed partially penetrant infertility in Cby−/− males (unpublished data). All of these observations signify similarities between phenotypes of Cby−/− mice and human patients with PCD.

Current animal models of PCD with respiratory pathology have abnormal ciliary structure (Ibanez-Tallon et al., 2002; Kobayashi et al., 2002). Although the majority of PCD patients also show defects in airway cilia structure, ~10% of atypical PCD cases show cilia with normal axoneme structure (Livraghi and Randell, 2007; Zarivala et al., 2007). This allows us to speculate that a subset of PCD may be caused by mutations in the Cby gene. Thus, Cby−/− mice might serve as a model for atypical cases of PCD. Given that the existing mouse models of PCD exhibit very high incidence of hydrocephalus and perinatal lethality, surviving Cby−/− mice may also be useful as an animal model for long-term studies of chronic upper airway infections (Livraghi and Randell, 2007).

Materials and methods

Northern blotting

Northern blots were purchased from OriGene and hybridized with a 32P-labeled full-length mouse Cby cDNA probe. The probe was synthesized by random priming using the DECAprime II kit (Applied Biosystems).

Generation of Cby−/− mice

A bacterial artificial chromosome (BAC) clone containing the Cby gene was isolated from the mouse 129 BAC library (Invitrogen). The BAC clone was characterized by restriction mapping and sequencing. An upstream 4.1-kb BamHI–Stul fragment and a downstream 4.1-kb XbaI–Smal fragment were subcloned into PGPneoFL2DTA targeting vector (a gift from P. Soriano, Fred Hutchinson Cancer Research Center, Seattle, WA) on either side of the neomycin resistance cassette to replace the whole Cby coding sequence. The linearized construct was electroporated into R1 embryonic stem cells, and transfectants resistant to G418 were screened for homologous recombination by Southern blot analysis. Of the 17 neomycin-resistant clones analyzed, 5 (29%) contained the correctly targeted Cby allele. Three independent targeted cell lines were separately injected into blastocysts by the transgenic mouse facility at the University of Washington. Chimeric males were crossed with female C57BL/6 mice, and germline transmission was detected by the presence of agouti-colored offspring and further confirmed by Southern blot analysis (Fig. S1 B). All three embryonic stem cell clones successfully underwent germline transmission, and all three mouse lines showed identical phenotypes. Mice characterized in this paper were backcrossed to C57BL/6 mice at least five times (n=5 generations). Genotyping was performed by PCR (Fig. S1 C); the primer sequences used for genotyping were P1, 5′-TGCCATTACACGAGAC- TAGACAG-3′; P2, 5′-AGACACACAGTGCAGAGGTAGG-3′; and P3, 5′-CTTCTAGAAGTTAGGACTCGG-3′.

Mice were fed irradiated Picolab Rodent Diet 20 (PMI Nutrition International) and reverse osmosis water. All supplies entering animal rooms were autoclaved, and rooms were maintained at 70–74°F and 45–55% humidity, with 28 air changes/h and a 12-h light/12-h dark cycle. All
animal procedures were approved by the University of Washington Animal Care and Use Committee.

In situ hybridization
Whole mount in situ hybridizations were performed as previously described [Cygan et al., 1997].

Histological analysis
Mice were euthanized by CO$_2$ asphyxiation, and the trachea was isolated, and studied under short circuit current conditions (Grubb et al., 1994). The tissue was bathed bilaterally in Krebs Ringer bicarbonate buffer, and postfixed in 1% osmium tetroxide in Sorenson's buffer. Samples were then embedded in epoxy resin (Poly/Bed812, Polysciences), and 90-nm sections were cut, stained with 7% uranyl acetate/0.3% lead citrate, and viewed using an electron microscope (EM900; Carl Zeiss, Inc.).

RNA extraction and real-time RT-PCR
Total RNA was purified from PMFs and from nasopharyngeal epithelia using the RNasy Mini kit (Qiagen), with DNase digestion performed with the DNase-free DNase kit (Qiagen). First-strand cDNA synthesis and PCR amplification were performed with 100 ng of RNA template using the iScript One Step RT-PCR kit with SYBR Green (Bio-Rad Laboratories) and the MiniOpticon real-time PCR detection system (Bio-Rad Laboratories) according to the manufacturer's instructions. The primer pairs used were CyclinD1 (5'-GGTCGGCAAGCTTACATCCG-3' and 5'-GGTCTATACCTTGTTG-3'), Axin2 (5'-TCCCCACCTGGAATGGAA-3' and 5'-AACATGCGGAACCTCCGTG-3'), and ARBP (5'-TGTGGACAC-GGGACATTT-3' and 5'-CGAGGGAACAGTGGGTA-3'). The level of transcripts for ARBP was used as an internal standard. Samples were analyzed in triplicate.

Immunostaining
For immunofluorescence staining, MDCK2 cells were grown to confluency and then serum starved for 48 h to induce ciliation. Cells were fixed either in 4% PFA or in ice-cold methanol, washed in PBS, permeabilized with 0.1% Triton X-100 in PBS, and incubated with primary and secondary antibodies. The nasal epithelium was removed and processed as for routine histological analysis, and sections were incubated with primary and secondary antibodies. Stained sections were mounted with ProLong Gold antifade reagent with DAPI (Invitrogen). Images were acquired at room temperature using a confocal microscope (SP1/MP; Leica) equipped with 40x Plan-Apochromat NA 1.25 oil immersion, 100x Plan-Apochromat NA 1.40 oil immersion, and 63x Plan-Apochromat NA 1.20 water immersion objectives and confocal software (Leica). Images were analyzed and three-dimensionally visualized using Imaris software (Bitplane) and assembled in confocal format in confosoft. Primary antibodies used were anti-α-catenin, anti-β-catenin, and 2 compare MCT in the nasopharyngeal cavities of adult mice.

Online supplemental material
Fig. S1 shows gene targeting at the mouse Cby locus. Fig. S2 shows the axonal structure of adult bronchial cilia, a paucity of nasal cilia in newborn mice, and Cby expression in embryonic lung and esophageal epithelia at E18.5. Fig. S3 shows immunostaining for β-catenin, Dvl1, and apical–basal markers in the adult nasal epithelium. Videos 1 and 2 compare MCT in the nasopharyngeal cavities of adult Cby$^{+/+}$ and Cby$^{-/-}$ mice. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200809144/DC1.

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