Multiple essential roles for primary cilia in heart development

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Abstract

Background: The primary cilium is a microtubule-based, plasma membrane-ensheathed protrusion projecting from the basal bodies of almost all cell types in the mammalian body. In the past several years a plethora of papers has indicated a crucial role for primary cilia in the development of a wide variety of organs. We have investigated heart development in cobblestone, a hypomorphic allele of the gene encoding the intraflagellar transport protein Ift88, and uncovered a number of the most common congenital heart defects seen in newborn humans.

Methods: We generated serial sections of mutant cobblestone and wild type embryos in the region encompassing the heart and the cardiac outflow tract. The sections were further processed to generate three-dimensional reconstructions of these structures, and immunofluorescence confocal microscopy, transmission electron microscopy, and in situ hybridization were used to examine signal transduction pathways in the relevant areas. Whole mount in situ hybridization was also employed for certain developmental markers.

Results: In addition to an enlarged pericardium and failure of both ventricular and atrial septum formation, the cobblestone mutants displayed manifold defects in outflow tract formation, including persistent truncus arteriosus, an overriding aorta, and abnormal transformation of the aortic arches. To discern the basis of these anomalies we examined both the maintenance of primary cilia as well as endogenous and migratory embryonic cell populations that contribute to the outflow tract and atrioventricular septa. The colonization of the embryonic heart by cardiac neural crest occurred normally in the cobblestone mutant, as did the expression of Sonic hedgehog. However, with the loss of primary cilia in the mutant hearts, there was a loss of both downstream Sonic hedgehog signaling and of Islet 1 expression in the second heart field, a derivative of the pharyngeal mesoderm. In addition, defects were recorded in development of atrial laterality and ventricular myocardiogenesis. Finally, we observed a reduction in expression of Bmp4 in the outflow tract, and complete loss of expression of both Bmp2 and Bmp4 in the atrioventricular endocardial cushions. Loss of BMP2/4 signaling may result in the observed proliferative defect in the endocardial cushions, which give rise to both the atrioventricular septa as well as to the septation of the outflow tract.

Conclusions: Taken together, our results potentially identify a novel link between Sonic hedgehog signaling at the primary cilium and BMP-dependent effects upon cardiogenesis. Our data further point to a potential linkage of atrioventricular septal defects, the most common congenital heart defects, to genes of the transport machinery or basal body of the cilia.

Keywords: Primary cilia, Heart, Outflow tract, Aorta, Pulmonary trunk, Endocardial cushions, AVSD, Nkx2.5, Pitx2c, Is11, Hand1, Alpha-actinin, Bmp2, Bmp4, Shh, Cardiac neural crest
Background

In the mouse cardiogenesis begins at embryonic day 7.5 (E7.5) by migration of the anterior lateral plate mesoderm towards the midline, where it forms a linear heart tube. This primary heart field gives rise to both the left and right atria and the left ventricle. The linear heart tube undergoes looping at E8.5, and during this stage a population of pharyngeal mesoderm-derived cells, the second heart field, contributes to myocardial as well as endocardial components of the right ventricle, the interventricular septum, the venous pole, and the base of the outflow tract (OFT) [1]. After birth, the task of directing oxygenated and deoxygenated blood to the systemic and pulmonary circulatory systems, respectively, is carried out by the OFT. In humans, congenital heart defects occur in almost 1% of newborns and defects in OFT development are some of the most severe heart abnormalities [2]. The OFT consists initially of cells from the heart tube but a major contribution is made later by cells of the anterior heart field and cardiac neural crest cells (cardiac NCC) [3]. Cardiac NCC represent a subpopulation of the cranial neural crest and originate from an area beginning around the middle of the otic placode to the caudal border of somite 3, corresponding to rhombomeres 6–8 [4]. Together these two populations generate a septated ascending aorta and pulmonary trunk during cardiogenesis.

At E10.5 the heart has acquired well-formed chambers, and cardiac NCC migrate to the OFT [5]. Cardiac NCC migrate into the endocardial cushions, which are derived from the heart endocardial layer, and together these two populations form the septum that divides the OFT into systemic and pulmonary outlets. The septum of the OFT starts as a shelf-like structure at the distal end of the OFT. The shelf develops between the fourth aortic arch artery (the future aorta) and sixth aortic arch artery (the future pulmonary trunk), and it expands proximally from the distal end. Cardiac NCC are important for a proper septation process of the OFT and for patterning of the great vessels [6,7]. Cardiac NCC also contribute to the septation process between the left and right ventricles and atria [8] that occurs between E10 and E13 [9]. By E14.5 the heart chambers are completely septated and are connected to the pulmonary trunk and aorta.

Cilia are 1 to 3 μm long protrusions of most cell types in vertebrates, which grow out from a centriole-based basal body located in the cytoplasm, bear a microtubule-based axoneme [10], and are coated by the plasma membrane. Primary cilia possess an intraflagellar transport system (IFT) for proteins and other cargo to travel in and out of the cilium. The IFT proteins act as scaffolds between the protein cargo and the motors kinesin-II and dynein, which are responsible for antero- and retrograde transport, respectively [11]. Primary cilia have recently been revealed as crucial for the development of a number of organs (reviewed in [12]). A clinical correlate of the developmental studies has been the re-definition of a wide variety of human syndromes as ‘ciliopathies,’ because the defective genes in question encode proteins that localize to the basal body or the cilium, including Bardet-Biedl, Meckel-Gruber, Alstrom, Joubert, orofaciodigital, and Senior-Loken syndromes, as well as nephronophthisis and polycystic kidney disease (PKD) (reviewed in [13]). In particular, primary cilia are essential for signal transduction of the Hedgehog (Hh) pathway. The three ligands of the Hh family bind to the inhibitory receptor Patched, which, in the absence of Hh ligands, localizes to primary cilia. Upon exposure to ligand, Patched and its ligand move out of the cilium and become internalized within the cytoplasm. In turn, the Hh signal-promoting receptor Smoothened enters into the cilium and executes a proper transduction of Hh signals [14,15].

The recessive mouse mutant cobblestone (cbs) bears a hypomorphic allele of the gene Ift88 [16], leading to a reduction of Ift88 mRNA and protein levels in cbs embryos by 70% to 80% compared to wild type littermates. Ift88, a component of the IFT system, is essential for the formation and maintenance of primary cilia [17]. Cobblestone mutants show a wide range of defects in the central nervous system that are at least partially explained by a deficiency in Sonic hedgehog (Shh) signaling [16]. This prompted us to examine cardiogenesis in this mutant mouse line, as Shh signaling contributes to several important aspects of heart development, including atrioventricular and OFT septation [18-23]. We show that cbs embryos display several malformations in cardiac development between E12.5 and E16.5, including interventricular- and interatrial septum defects, persistent truncus arteriosus, arteria lusoria, and pericardial hyperplasia. In this paper, we provide evidence that loss of cardiac cilia correlated with deficiencies in the expression of genes known to be important for OFT and atrioventricular septation, and we present a mechanistic explanation for these phenotypes.

Methods

Mouse lines

All experiments were conducted according to the guidelines of the state of Baden-Württemberg, Germany. Cobblestone mice were generated as described [16]. Twelve noon of the day of the vaginal plug was assigned the date embryonic day 0.5 (E0.5). For all embryonic stages before E12.5, somite-matched embryos were compared for analysis as follows: E9.5: 21 to 29 somites, E10.5: 35 to 39 somites; E11.5: 45 somites [24]. Genomic DNA was isolated from embryonic and adult tissue...
as described [25]. For PCR genotyping the following primers were utilized (D14Mit121 F: 5‘-TTG ACA TCT GGA TAT GAC AAT GC-3‘; D14Mit121 R: 5‘-TGT GCA TGT TGG TGT ACA TG-3‘; D14Mit259 F: 5‘-TGG TGT CTC CTT CGG AAT TT-3‘; D14Mit259 R: 5‘-TAA ATG TAA AAG GTA AAG GCA ATG G-3‘) and PCR products were resolved using 12% acrylamide gel electrophoresis.

Transmission electron microscopy
E12.5 mouse embryos were collected in cold PBS and fixed overnight (O/N) at 4°C with 2.5% glutaraldehyde in 0.1 M PIPES buffer, pH 7.6, containing 2% polyvinylpyrrolidone (MW 25000, Merck, Darmstadt, Germany). Transverse sections (300 μm-thick) of the trunk were prepared using a vibratome (D.S.K. Microslicer DTK-1000, Dosaka EM, Kyoto, Japan). For enhancement of membrane staining, samples were incubated in alkaline diaminobenzidine hydrochloride medium as described previously [26] for 60 minutes, and postfixed with 1.5% osmium tetroxide containing 1.5% potassium ferrocyanide for one hour followed by an additional one hour osmification with 1.5% osmium tetroxide in 0.1 M sodium cacodylate buffer. Finally, the slices were stained en bloc in 1% uranyl acetate for 30 minutes, dehydrated through a graded ethanol series and embedded in Epon 812 (Fluka 45345). Series of semithin sections were stained with a modified Richardson methylene blue-azure II solution and used for selection of corresponding stained areas in wild type hearts. Ultrathin sections were stained in 1% uranyl acetate for 1 hour, followed by an additional 1 hour staining in 0.1% Eosin, rinsed several times in dH2O, dehydrated in an ascending ethanol series, treated for 2x 5 minutes with xylol, and embedded in Entellan (Merck).

Hematoxylin & eosin (H & E) staining
Mouse embryos of different stages (E12.5 to E16.5) were fixed O/N with freshly-prepared 4% paraformaldehyde (PFA) in 0.1 M PBS at room temperature (RT), dehydrated in a graded ethanol series and embedded in paraffin. Serial paraffin sections (510 μm-thick) were treated for 2x 5 minutes with xylol, rehydrated in a descending ethanol series, and washed with distilled H2O (dH2O). The sections were stained with hematoxylin for 8 to 10 minutes, rinsed briefly in dH2O and 15 minutes in tap water. The sections were briefly rinsed in dH2O before staining in 0.1% Eosin, rinsed several times in dH2O, dehydrated in an ascending ethanol series, treated for 2x 5 minutes with xylol, and embedded in Entellan (Merck).

Three-dimensional OFT reconstruction
The OpenCAR software ([27]; available from http://opencar.ulster.ac.uk) was used for the three-dimensional reconstruction of the outflow tract of E14.5 old wild type and mutant embryos. The reconstruction was undertaken as previously described [28] with the following modifications. Light micrographs of H & E stained serial sections of the thorax were aligned manually using prominent structures in two adjacent images as landmarks. Subsequently, the individual vasculature was manually traced in each section, which resulted in a set of contours representing each of the components of the outflow tract. The contours were three-dimensionally reconstructed by applying the Delaunay method [27], generating renderings that were then exported. Amira 5.3.1 software (Visage Imaging, Richmond, Australia) was used for visualization of the exported three-dimensional reconstructed structures.

For the three-dimensional OFT reconstructions shown in Figure 1 and Additional file 2, serial sections were photographed and printed and transferred to balsa wood discs (1:60 magnification). The resulting models were then photographed and sketched with close reference to the photographs and the original micrographs. The vertical axis was artificially exaggerated by a factor of three in order to simplify understanding.

Immunohistochemical analysis
E10.5 to E12.5 old mouse embryos were collected in cold 0.1 M PBS, and embryonic tail samples were collected separately for DNA extraction and genotyping. The embryos were fixed O/N at 4°C in 4% PFA in 0.1 M PBS. After rinsing in 0.1 M PBS for 2 × 30 minutes the embryos were treated in an ascending sucrose series (10%, 20%, and 30% in 0.1 M PBS) and mounted in optimal cutting temperature (OCT) embedding medium (Leica Microsystems, Wetzlar, Germany). Stainings of 10 μm to 12 μm cryosections were performed as described [29] with the following primary antibodies: anti-acetylated alpha tubulin (clone 6-11B-1, Sigma, Munich, Germany) 1:1000; anti-gamma tubulin (clone GTU-88, Sigma) 1:1000; anti-phosphorylated histone H3 (Ser10, rabbit 06 –570; Millipore Corporation, Schwalbach am Taunus, Germany) 1:200; anti-α-actinin (clone EA-53, Sigma) 1:2000; anti-Apoptotic Ift88 (ab42497, goat polyclonal, Abcam plc, Cambridge, England) 1:500; anti-AP2α (clone 3B5, Developmental Studies Hybridoma Bank, Iowa City, Iowa, USA) 1:50; anti-α-actinin (clone EA-53, Sigma) 1:2000; anti-Ar13b (kind gift of Tamara Caspary, Atlanta, Georgia, USA) 1:1500; anti-apoptotic Ift88 (ab42497, goat polyclonal, Abcam plc, Cambridge, England) 1:500; anti-Ift13b (clone 39.4D5, Developmental Studies Hybridoma Bank) 1:1000. Secondary antibodies were employed as described [29], with the exception that for colabeling experiments to detect cilia, the anti-acetylated alpha tubulin and the anti-gamma tubulin antibodies were detected with isotype-specific anti-mouse immunoglobulin G1 (IgG1) and IgG2b secondary antibodies, respectively. All secondary antibodies were purchased from Invitrogen, Darmstadt, Germany.
Statistical analysis
To analyze the data presented in Figure 2, Student’s t-test was used, using two tailed, two-sample equal variance settings in Excel (Microsoft Office 2010).

In situ hybridization

In situ hybridizations on cryosections were performed as described [31]. In situ hybridizations on paraffin sections were performed as described [16]. Probes: Bmp2 & Bmp4 [32], Pitx2c (kindly provided by Axel Schweickert).

Whole mount in situ hybridization (WISH)

E10.5 mouse embryos were collected in cold 0.1 M PBS, and embryonic tail samples were collected separately for PCR genotyping. The embryos were fixed O/N at 4°C in 4% PFA in 0.1 M PBS. PFA was washed out with 0.1 M PBS, the embryos were dehydrated in an ascending methanol series, kept O/N in 100% methanol at −20°C, and then rehydrated in a descending methanol series. The embryos were washed 2× with 1× PBST (0.1 M PBS; 0.1% Tween-20) for 5 minutes, bleached for 1 hour with 6% H2O2 in 1× PBST, washed 3× 5 minutes with 1× PBST, treated with proteinase K (10 μg/ml) for 15 minutes, washed once for 10 minutes with 2 mg/ml glycine in 1× PBST, washed 2× 5 minutes with 1× PBST, postfixed for 20 minutes in 4% PFA and 0.2% glutaraldehyde in 1× PBST, and washed 2× 5 minutes in 1× PBST. The embryos were treated for a slow equilibration in the hybridization solution by incubating them 1× 10 minutes in a 1:1 mixture of hybridization solution/1× PBST, 1× 10 minutes in hybridization solution, 1× 60 minutes in hybridization solution at 70°C. The hybridization solution was replaced with fresh hybridization solution together with digoxigenin-labeled antisense-RNA probes and incubated O/N at 70°C. The embryos were washed 3× 30 minutes in prewarmed solution I (50% formamide, 5× SSC, pH 4.5; 1% SDS) at 70°C, 3× 30 minutes in prewarmed solution III (50% formamide; 2× SSC, pH 4.5) at 65°C, 3× 5 minutes in 1× TBST (25 mM Tris, pH 7.5; 150 mM NaCl; 2 mM KCl; 0.1% Tween-20) at RT, and subsequently incubated 1× 2 hours in blocking buffer (10% heat-inactivated sheep serum; 0.1% Boehringer Mannheim blocking reagent; 1× TBST) at RT. After blocking, the anti-digoxigenin-AP antibody/Fab fragments (Roche, Mannheim, Germany) diluted in fresh blocking buffer were added to the embryos and incubated O/N at 4°C. The embryos were washed 3× 5 minutes and 6× 1 hour in 1× TBST at RT, O/N in 1× TBST.
Figure 2 (See legend on next page.)
at 4°C, 3x 10 minutes in NTMT (100 mM NaCl; 100 mM Tris--HCl, pH 9.5; 50 mM MgCl2; 0.1% Tween-20; 2 mM levamisole) at RT, and then developed in the dark in reaction mix (NBT/BCIP stock solution (Roche) diluted 1:50 in NTMT). When the reaction was judged complete, the embryos were washed 1x 10 minutes in PBST, postfixed 10 minutes in 4% PFA at RT, washed 3x 5 minutes in 0.1 M PBS, and stored in 0.1 M PBS at 4°C. For documentation, embryos were cleared in 100% glycerol and photographed with an AZ100 Multizoom microscope (Nikon). Probes: Shh, Ptch1 [16], Sox10 (kindly provided by Peter J. Scambler), Hand1 ([33], kindly provided by Eric Olson), Nkx2.5 (kindly provided by Thomas Braun), Gli1 (kindly provided by Sandra Blaess).

**Results**

Primary cilia are present in developmentally-important regions of the embryonic heart

Cilia have been recently reported to be present throughout developing embryonic heart [34], using an immuno-fluorescent approach. However, an ultrastructural analysis is obligatory to definitively determine whether these cilia are primary or motile. Embryonic wild type mouse heart was examined using transmission electron microscopy (TEM) upon transverse sections of E12.5 embryonic trunk. Cilia, with lengths varying between 1.0 μm to 2.0 μm, frequently occurred in both the atrioventricular and conotruncal endocardial cushions (Figure 1). Cilia in the cushion mesenchyme were often found in ciliary pockets of varying depths (Figures 1B, C, F) and showed a random orientation within the extracellular matrix of the cushion, whereas endocardial cells oriented their primary cilia to the lumen of the inflow and outflow tract, respectively (Figure 1E). Transverse sections revealed a '9 + 0' morphology in the proximal cilium (Figure 1C), and longitudinal sections revealed an absence of a central microtubule doublet (Figures 1B, E, F), confirming them as primary cilia. No cilia bearing a '9 + 2' ultrastructure, indicative of motile cilia, were found in the embryonic heart.

The *Ift88* hypomorph cobblestone displays multiple heart defects

Homozygous *cbs* mutant E14.5 (Figures 3, 4A to C) and E16.5 (Figure 4D to E) embryos were analyzed in detail using computer-generated three-dimensional reconstructions (Additional file 1) and serial sections (Figures 3, 4, Additional file 2) of the thorax and compared to their wild type littermates. The following defects were recorded. 1) Persistent truncus arteriosus (PTA) including absence of a ductus arteriosus (Figure 3E to G, Figure 4D, E, n = 4/5). 2) Transposition of the common trunk arising from the right ventricle (Figure 3E, Figure 4D, E, n = 4/5). 3) Failure of correct formation of the tracheoesophageal septum (common for greet tube, Figure 3B to G, Figure 4A, D to F, n = 5/5) results in esophageal atresia and a tracheoesophageal fistula at the tracheal bifurcation. 4) Bilateral hypoplasia of the lungs (Figure 4A to C, n = 5/5). 5) The lungs were supplied by a common trunk arising as the first branch of the common truncus arteriosus, giving rise to the right and left pulmonary arteries that descended anterior to the common for greet tube (Figure 3F, G, n = 4/5). 6) Abnormal origin of the right subclavian artery, arising as the fourth branch of the aortic arch and passing posterior to the common for greet tube (Figure 3E and Figure 4D (Arteria lusoria), n = 2/5). 7) Both atrial (Figure 4B, C, F) and ventricular septal defects (VSD, pans membranacea, Figure 4C, F, n = 5/5). 8) Gross enlargement of the pericardial sac (Figure 4C, F, n = 12/12). 9) Hypoplasia of the myocardium, resulting in smaller atria and ventricles (Figure 3A to C, n = 2/5). 10) A single case of a double aortic arch [see Additional file 2]. Cardiovascular defects observed were seen in all examined embryos between E14.5 and E16.5 (for example, AVSD, n = 5). Since 80% of the embryos die by E14.5 (n = 34 litters), and almost all by E16.5 (n = 25 litters), detailed examination of the heart defects at later stages proved impossible.

Primary cilia are deficient in the developing heart of the *cbs* mutant

Many reports of mouse mutants with defects in ciliary proteins have reported either a loss of (for example, [35])
or morphological abnormalities (for example, [36]) in the cilia. In order to investigate the presence of primary cilia in the developing heart, we labeled the ciliary axoneme with an antibody against acetylated α-tubulin and the basal body with an antibody recognizing γ-tubulin. We observed a substantial loss of primary cilia in both the atrioventricular endocardial cushions (Figure 2A, D, 48.1 ± 10.1% reduction, n = 3, P < 0.01, Student’s t test) as well as the pericardium (Figure 2F, 36.0 ± 12.0% reduction, n = 5, P < 0.001, Student’s t test) of E12.5 heart, expressed as the number of cells with identifiable primary cilia per 1000 μm². To account for differences in cell density between wild type and mutant embryos, DAPI-stained nuclei were counted and found to be identical (endocardial cushions: wild type: 18.7 ± 2.3 cells/1000 μm²; cbs/cbs: 19.5 ± 3.4 cells/1000 μm², n = 10, P > 0.5). High magnification imaging of the primary cilia remaining in the cbs mutant appeared normal (Figure 2B), using antibodies recognizing Arl13b to mark the ciliary axoneme [36] and γ-tubulin to mark the basal bodies. In both the endocardial cushions as well as the pericardium, a reduction in the number of primary cilia was accompanied by an increase in the number of cells bearing basal bodies with no adjacent acetylated α-tubulin-positive ciliary axoneme (Figure 2A, E, G). These findings indicate that the fraction of cells bearing cilia decreased in the population, leading to an increase in the fraction of unciliated cells, with basal bodies but no ciliary axoneme. To see if the loss of cilia was accompanied by a change in proliferation, we employed an antibody that recognizes phosphorylated-histone H3, which becomes phosphorylated specifically during mitosis [37]. We observed a 48.2 ± 10.4% reduction in the number of mitotic cells (Figure 2C, E, n = 3, P < 0.001, Student’s t test) in the endocardial cushions. We did not observe any
PH3-positive cells bearing cilia in either wild type or mutant hearts.

To address whether the apoptotic rate was changed we used an antibody recognizing cleaved, activated caspase-3, a processed protein that initiates the proteolytic cascade common to both intrinsic and extrinsic apoptotic pathways [38]. We detected no significant change in the apoptotic rate in cbs atrioventricular endocardial cushions, compared to wild type (Figure 2D, F, n = 2, P > 0.05, Student’s t test). Unexpectedly, in the pericardium both mitosis (285.8 ± 57.2%, n = 3, P < 0.001, Student’s t test) and apoptotic counts (642.9 ± 214.3%, n = 2, P < 0.001, Student’s t test) increased dramatically (Figure 2H).

Migration of cardiac neural crest cells into the developing heart is normal in the cbs mutant despite the absence of cilia on the migrating cells

The cardiovascular phenotypes resulting from cardiac neural crest ablation include PTA, overriding aorta, and double-outlet right ventricle [4]. As we had consistently observed these first two phenotypes in the cbs mutants, we decided to investigate the migration pattern of cardiac NCC in cbs mutants. First, we co-labeled migrating cardiac NCC with an antibody against the neurotrophin receptor p75 [39] (Figure 5). In both wild type (Figure 5A) and cbs/cbs (Figure 5C) E10.5 embryos, cardiac NCC migrating from the dorsal caudal hindbrain could be observed clustered lateral to the hindbrain wall. To examine cilia, we labeled the ciliary axoneme with an antibody against Ift88 and the basal body with an antibody recognizing gamma tubulin. p75-positive migrating cardiac NCC in wild type embryos clearly demonstrated cilia (Figure 5A, B). In the cbs/cbs embryos, migrating cardiac NCC also displayed cilia, although the number of ciliated cells was lower (Figure 5C, D). In the same embryos, cohorts of migrating cardiac NCC could be identified migrating along the third pharyngeal artery toward the heart tube in both wild type (Figure 5E) and cbs/cbs (Figure 5H) E10.5 embryos. Again, p75-positive migrating cardiac NCC in wild type embryos bore cilia (Figure 5F, G). In contrast, migrating cardiac NCC in cbs/cbs embryos did not elaborate cilia (Figure 5I, J). In order to use an independent marker for the ciliary axoneme, we employed an antibody recognizing Arl13b [36], colabeling for another marker of migrating cardiac NCC, the transcription factor AP2α [40]. Examination of
cells migrating along the third pharyngeal artery of E10.5 embryos revealed results identical to those seen with the anti-p75 antibody (Figure 6A to D). AP2α-positive cells could be found in the cbs/cbs embryos (Figure 6B), but the cells for the most part did not elaborate cilia (Figure 6D).

Secondly, we employed whole mount in situ hybridization (WISH) against mRNA of the transcription factor Sox10, which is strongly expressed in migrating neural crest cells [41]. WISH was performed on embryos between E10.5 and E11.5, the period in which cardiac NCC migrate to the heart [42]. A close analysis of the WISH results showed that the cardiac NCC in the cbs mutant embryos do in fact migrate from the pharyngeal arches to the heart tube, exhibiting an identical distribution pattern along the pharyngeal arteries (Figure 6E, F).
Downregulation of Shh signaling in the cbs mutant

To date, Shh is the best-documented signal transduction pathway to act through primary cilia [12]. At midgestation and with proximity to the heart tube, Shh is expressed in the ventral spinal cord and notochord, the pharyngeal mesoderm [43], and the pulmonary endoderm [21,44]. Inhibition of Shh signaling from the pharyngeal mesoderm [43], and the pulmonary endoderm [21,44]. In- 

hibition of Shh signaling from the pharyngeal mesoderm results in severe OFT abnormalities [20]. To examine the effect of the cbs mutation upon Shh signaling, we examined Shh expression using WISH upon E10.5 embryos. No differences in Shh expression were observed in the embryonic thorax, comparing cbs mutants to their wild type littermates (Figure 7A). The observed reduction in the number of primary cilia in the cbs mutant (Figure 4) should lead to a reduction in Shh signal transduction. This was indeed the case. Using WISH, we observed a strong reduction in the expression of two downstream targets of Shh signaling, Gli1 (Figure 7B) and Ptc1 (Figure 7C), in pharyngeal mesoderm and endoderm.

Shh is also involved in early development in the establishment and maintenance of left/right asymmetry, which takes place initially at the embryonic node [45,46]. A loss of asymmetry can lead to the medical condition situs inversus, in which all or some of the internal organs are found on the opposite side of the body. In the mouse, this is manifested by a reversal of heart tube looping, which is found quite often in mutants in ciliary proteins [35,47,48]. The cbs mutant never displays reversed heart looping, but when crossed to a full deletion mutant of the ifit88 it does [16]. Subtler defects have recently been reported in congenital heart defects in humans that can be attributed to ciliary proteins, including OFT abnormalities and atrioventricular septal defects [49,50]. In order to investigate whether subtle laterality defects may occur in the cbs mutant, we utilized an in situ probe for Pitx2c [51,52], a heart-specific isoform of the Pitx2 gene. Pitx2c lies directly downstream of left/right determination pathways [53] and is important for atrioventricular development as well as OFT remodeling [54,55]. Examination of E10.5 embryos revealed an atrial expression of Pitx2c restricted to the left side of the heart (Figure 7D). In contrast, a bilateral atrial expression of Pitx2c was observed in cbs/cbs hearts (Figure 7D). This phenotype has also been reported in the Shh knock-out mouse model [56] and may reflect a left atrial isomerism.
Another reported consequence of a loss of Shh signaling in other mouse mutants is a remarkable increase in apoptotic rates in the pharyngeal mesoderm and endoderm and splanchnic mesoderm [20,22,23,43]. Thus, we examined apoptosis using an antibody recognizing cleaved, activated caspase in E10.5 embryos. Our data revealed very low levels of apoptosis in the pharyngeal mesoderm and endoderm and OFT of both wild type and cbs mutant embryos (Figure 7E). Finally, we examined primary cilia in the pharyngeal mesoderm using antibodies recognizing Arl13b to mark the ciliary axoneme [36] and γ-tubulin to mark the basal bodies. As in the atrioventricular endocardial cushions at E12.5 and migratory cardiac NCC at E10.5, we observed a reduction in the fraction of cells bearing cilia in cbs/cbs mutants, but the morphology of individual cilia in cbs/cbs mutants appeared normal (Figure 7F).

Defects in cardiac mesoderm differentiation and cardiomyogenesis in the cbs mutant
To examine differentiation of the first and second heart fields, we employed in situ probes against the genes Hand1 [57] (Figure 8A) and Nkx2.5 [58] (Figure 8B) and an antibody recognizing Islet1 [59] (Figure 8C), respectively. WISH analysis of E9.5 embryos revealed that Hand1 expression was found in the pharyngeal arches and restricted to the left side of the heart tube both in wild type and in cbs/cbs mutants (Figure 8A). Nkx2.5 expression was found throughout the heart tube at E9.5 in both wild type and cbs/cbs embryos but appeared reduced in expression levels in the cbs/cbs mutants (Figure 8B). However, this reduction in expression clearly did not prevent the proper left-sided expression of Hand1, in contrast to what is seen in Nkx2.5 mutant mice [60]. We next examined Islet1 expression, a downstream target of Shh signaling which is downregulated in the spinal cord of ciliary mutants [36,61,62]. More importantly, Islet1 is known to be crucial for the development of the second heart field and subsequent OFT formation [59]. Immunohistofluorescence with an anti-Islet1 antibody revealed a strong decrease in expression in the splanchnic mesoderm of cbs mutant embryos at E10.5 compared to wild type littermates (Figure 8C). To examine cardiomyogenesis, we employed an antibody recognizing the structural protein α-actinin (Figure 8D, E). At E10.5, α-actinin staining revealed a strong decrease in ventricular trabeculation in the cbs/cbs embryos (Figure 8D), as has
been previously reported for Ift88 [63] and other ciliary mutants, including Kif3a and Pkd2 [34]. However, at E12.5, trabeculation levels in the ventricle appeared to somewhat recover (Figure 8E).

Loss of Bmp2/4 expression in both OFT and endocardial cushions of the cbs mutant

Elimination of Islet1 expression in the Isl1 knockout mouse line leads not only to the loss of many second heart field-derived structures, including the OFT, but also to a loss of Bmp expression [59]. Therefore, we examined Bmp2 and Bmp4 expression in both embryonic OFT, where Bmp signaling is required for normal OFT development [64-69], as well as in atrioventricular endocardial cushions, where Bmp4 expression is crucial for normal atrioventricular septation [70]. In situ analysis of Bmp2 and Bmp4 expression in the OFT of E10.5 embryos revealed no change in Bmp2 expression in the cbs mutant (Figure 9A), but a strong reduction in the expression of Bmp4 (Figure 9B). In contrast, in the endocardial cushions, we observed a complete loss of Bmp2 (Figure 9C) and Bmp4 (Figure 9D) expression at E12.5 in the cbs mutants.

Discussion

Primary cilia and heart development

Defects in heart development in cilia mutants were reported well before cilia were realized to be essential for vertebrate organogenesis (reviewed in [12]). Pkd2 is a gene that, when mutated, causes autosomal dominant polycystic kidney disease and encodes a transmembrane protein localized to primary cilia. Mice with a targeted mutation in Pkd2 showed ventricular septum defects and to a lesser extent atrial septum defects [71]. In mice carrying a targeted mutation in Pkd1, whose gene product is also localized to primary cilia, atrioventricular septal defects (AVSD), disorganization and thinning of the myocardial wall and double-outlet right ventricles have been observed [72]. A targeted deletion of Ift88 resulted in a shortening of the distal truncus in the OFT, an apparent hypoplasia of endocardial cushions (ECC), and a reduction in ventricular trabeculation [63], but OFT septation defects and/or AVSD were not examined due to the early death of the embryos. Finally, a targeted deletion of Kif3a demonstrated an almost complete lack of ECC and a myocardial hypoplasia at E9.5 [34]. The complete knockouts of Kif3a and Ift88 die at E10.5 and E11.5, respectively, and so an analysis of downstream
effects of these deficiencies could not be performed. In addition, several ciliary mutants display an enlarged pericardium, including Pkd2 [71], Pkd1 [72], Kif3a [73], and Ift88 [63]. The abundance of gene mutants with similar phenotypes strongly suggests that the observed defects lie with deficiencies in ciliary function per se and not with potential non-ciliary roles of the individual proteins. Indeed, a loss of primary cilia in embryonic heart has been reported in the Kif3a mutant already at E9.5 [34] and in the Ift88 mutant at E11.5 [63]. Nevertheless, non-ciliary functions for Ift88 cannot be formally excluded and may contribute to the phenotypes reported here.

Primary cilia are still present in the heart of Pkd2 mutants at E9.5 [34], yet this mutation displays AVSD later in development. Although the primary cilia were not quantitated in the Pkd2 mutant, it supports the crucial idea that ciliary function can be disrupted despite the presence of morphologically normal cilia, as seen also in the cbs mutant (Figure 2, [16]). The cbs mutant is a hypomorph, with expression levels of a normal Ift88 protein at 20% of wild type littermates [16]. The cbs mutants survive up to E16.5 because of remaining low levels of Ift88 expression and reach, therefore, the later stages of cardiogenesis, whereas Ift88 or Kif3a full
deletion mutants die before atrioventricular or OFT septation have taken place. Low levels of Ift88 clearly allow for the production of morphologically (Figure 2) and ultrastructurally [16] normal cilia in a subset of cells in the developing embryo. We have previously proposed that these residual numbers of cilia are sufficient to allow earlier developmental processes to take place normally, whereas later embryonic stages are compromised by a progressive loss of cilia [16]. Evidence for this ‘threshold hypothesis’ is provided by the observation that initial left-right determination is completely normal in the cbs mutant, but that a cross of the cbs allele to the full, targeted deletion of the Ift88 gene [74] results in a 50% incidence of situs inversus [16]. Given that the cbs mutants do continue to display cilia in a certain fraction of cells within the developing heart and adjacent pharyngeal arch tissue, we can only speculate as to how this manifests itself on the single-cell level. Given the importance of Ift88 for intraflagellar transport, we find it most likely that even those cells bearing cilia are diminished in their ability to properly transduce Shh signals from the environment. To prove this point, though, we would need to employ a Shh readout that displays single-cell resolution, and this would be a very useful tool for future experiments.

Primary cilia and the neural crest
Primary cilia and basal bodies have been previously implicated in the migration of NCC. Tobin et al. showed that craniofacial defects seen in patients with the ciliopathy Bardet-Biedl syndrome (BBS) could be ascribed to a deficit in the migration of the cranial neural crest to form aspects of the viscerocranium [75]. Examination of a conditional mouse mutant in which NCC were deficient for the ciliary protein Ki67a resulted in hypertelorism and frontonasal dysplasia [76]. As in our study, the authors observed normal migration of craniofacial neural crest but abnormalities after arrival at the migration target, in this case the facial prominences [76]. Although our results clearly show that cardiac NCC migration into the heart tube is taking place in the cbs mutants, despite the lack of cilia on migrating cardiac NCCs already within the pharyngeal arches, they do not necessarily contradict the reported role of BBS proteins in neural crest migration [75]. First, the presence or absence of primary cilia was not examined in either of the two aforementioned studies [75,76] during the actual migration process itself, as we have done here. Thus, it is unclear whether cilia are present or absent in migrating NCC in these mutant models. But even if primary cilia are lost in BBS mutants, where direct defects in migration per se were demonstrated, it does not necessarily mean that this is a ciliary phenomenon, as the BBS proteins are mostly localized to the basal body and may in any case have non-basal-body functions in this particular population. Second, there may be a difference in the control by primary cilia in neural crest migration from different axial levels. The cranial neural crest that populates the facial prominences arises from a more cranially-located position along the neural tube than cardiac NCC [77]. Also, in the cbs mutant we have observed no defects in either the formation or maturation of dorsal root ganglia (Bradford and Tucker, unpublished data), which arise from more caudally-located neural crest than cardiac NCC, suggesting that migration and later development of trunk neural crest is intact. Third, the results linking primary cilia and neural crest migration were reported for zebrafish [75], which may of course additionally reflect species-specific differences in the function of primary cilia. Finally, there may be subtle differences in neural crest migration patterns that we have not detected with our immunohistochemical and WISH analyses.

OFT defects have not been recorded for mutants with myocardial or endocardial-specific knockouts of Bmp2/4 receptors [64,78-80]. This suggests that Bmp2 and Bmp4 produced within the heart are acting upon the migratory cardiac NCC to effect proper OFT formation. Indeed, a neural crest-specific deletion of the Bmp receptors Alk3 (Bmpr1a) [69] or of Alk2 (Acvrl1) [66] demonstrated a defective colonization of the heart by cardiac NCC and a concomitant OFT defect. Since we do observe normal migration of cardiac NCC in the cbs mutant, this suggests that the heart defects observed lie at least in part in defective signaling of Bmp2/4 to post-migratory cardiac NCC cells. However, endogenous cells of the anterior heart field clearly also play a role in OFT septation, and are also responsive to Shh signaling [20]. Future experiments will further refine the roles of ciliary proteins in heart development with the use of conditional inactivation of ciliary genes using appropriate Cre deleter lines.

Shh and BMP signaling and OFT formation
Several lines of evidence point to an involvement of Shh in OFT formation. Both the Shh deletion mutant [23] as well as chicken embryos treated with the Smoothened inhibitor cyclopamine [18] displayed persistent truncus arteriosus and pulmonary atresia. In the mouse Shh mutant, these anomalies could largely be attributed to massive defects in the migration of cardiac NCC, a situation not seen in the cbs mutant. Both a conditional mutant in Smoothened, in which the gene is deleted in the Isl1-positive population [22], and a Shh conditional mutant crossed to the Nlx2.5:CRE deleter strain [20], displayed defects in OFT septation. Interestingly, cyclopamine treatment of embryonic chicken resulted in no defects in cardiac NCC migration but did result in OFT defects, suggesting that Shh signaling is also
Shh and BMP signaling and atrioventricular septum formation

As with OFT septum formation, several lines of evidence point to an involvement of Shh in atrioventricular septum formation. Late-term Shh mutants suffer from AVSD [23]. Also, a Smoothened conditional mutant crossed to the Islet1::CRE deleter strain displayed AVSD [22]. Shh activation does not seem to occur in the developing heart itself, but in extracardiac progenitor cells that migrate into the heart between E8.0 and E10.5 [19-21]. Deletion of the Smoothened receptor from these atrial septum progenitors [21] resulted in AVSD that looks very similar to the AVSD seen in the cbs mutant (Figures 3, 4).

In the mouse, both Bmp2 and Bmp4 are expressed in the myocardium of the atrioventricular canal [82,83]. A myocardium-specific deletion of a floxed Bmp4 allele under control of a cardiac Troponin T (TnT::Cre) deleter line showed that Bmp4 is essential for proper AV septation after ECC formation [70]. Interestingly, Bmp4 was not necessary for the formation of the ECC. At E12.5 in the cbs mutant, the formation and size of the ECC looks normal, but Bmp4 expression in the overlying myocardium is completely absent (Figure 8B, C). Our results clearly suggest that one basis for the AVSD in the cbs mutant mouse may be the absence of Bmp4 in the AV ECCs. Taken together, our results potentially identify a novel link between Shh signaling at the primary cilium and Bmp-dependent effects upon cardiogenesis.

Conclusions

In conclusion, we show for the first time that the ciliary protein Ift88 is essential for the permanent establishment of cardiac cilia, and that reduced expression levels of this protein result in congenital heart abnormalities in later embryogenesis, including OFT and AVSD. In consideration of the high incidence of AVSD in human congenital heart defects, the relatively small fraction of cases with a clear genetic cause, and the high variability in both penetrance and expressivity of these cases and in corresponding animal models [89], our data point to a potentially important linkage to genes of the IFT machinery or basal body of the cilia.

Note added in proof:

Primary cilia have previously been reported in the embryonic ventricle and pericardium of the chick and the mouse [90].

Additional files

Additional file 1: Three-dimensional reconstruction of the outflow tract in wild type and cbs/cbs mutants at E14.5. A schematic reconstruction of the OFT is shown, generated with the computer programs OpenCAR and Amira from serial sections of E14.5 thorax from wild type (+/+; A;C;E;G) and cbs/cbs (B;D;F;H) embryos. Views are shown from dorsal-cranial facing downward (A,B), from ventral facing dorsal (C,D), from lateral left (E,F) and from lateral right (G,H). The wild type is colored with arterial vessels in red, pulmonary trunk in blue, and the thoracic aorta in purple. The mutant reconstruction is colored in purple, reflecting the single outlet in the OFT. Abbreviations (alphabetically arranged): Aa, ascending aorta; Arc, aortic arch; At, thoracic aorta; Bt, brachiocephalic trunk; Da, ductus arteriosus; Lc, left common carotid artery; Lpa, left pulmonary artery; Lsa, left subclavian artery; Pt, pulmonary trunk; PTA, persistent truncus arteriosus; Rc, right common carotid artery; Rpa, right pulmonary artery; Rsa, right subclavian artery.
Abbreviations

ASD: Atrial septal defect; AVSD: Atrioventricular septal defect; BBS: Bardet-Biedl syndrome; BMP2: Bone morphogenetic protein 2; Bmp4: Bone morphogenetic protein 4; cardiac: NCC: Cardiac neural crest cells; cbs: cobblestone; DAPI: 4′,6-diamidino-2-phenylindole; DW: Distilled water; E: Embryonic day; ECC: Endocardial cushions; H & E: Hematoxylin and eosin; HH: Hedgehog; IFT: Intratragular transport system; N/O: Overnight; OFT: Outflow tract; PBS: Phosphate-buffered saline; PCR: Polymerase chain reaction; PFA: Paraformaldehyde; PKD: Polycystic kidney disease; PTA: Persistent truncus arteriosus; RT: Room temperature; SHh: Sonic hedgehog; TEM: Transmission electron microscopy; VSD: Ventricular septum defect; WISH: Whole mount in situ hybridization.

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

MAW conducted the experiments and analyzed the data. HARG and KG analyzed the data and criticized the manuscript. KLT conceived of the study, devised and conducted the experiments, analyzed the data, and wrote the manuscript. All authors read and approved the final manuscript.

Authors’ information

KLT and KG are experienced anatomists in the Institute of Anatomy at the University of Heidelberg and together have been performing ultrastructural and molecular analysis of mutants in ciliary function for the past nine years. MW is a graduate student in the laboratory of KLT, who has spent the past six years investigating the cbs mutation. HARG is a pathologist who has been consulting with KLT for 20 years on all matters of anatomy and mouse knockout analysis.

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