Cilia and Ciliopathies in Congenital Heart Disease

Nikolai T. Klena, Brian C. Gibbs, and Cecilia W. Lo

Department of Developmental Biology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15201

Correspondence: cel36@pitt.edu

A central role for cilia in congenital heart disease (CHD) was recently identified in a large-scale mouse mutagenesis screen. Although the screen was phenotype-driven, the majority of genes recovered were cilia-related, suggesting that cilia play a central role in CHD pathogenesis. This partly reflects the role of cilia as a hub for cell signaling pathways regulating cardiovascular development. Consistent with this, many cilia-transduced cell signaling genes were also recovered, and genes regulating vesicular trafficking, a pathway essential for ciliogenesis and cell signaling. Interestingly, among CHD-cilia genes recovered, some regulate left–right patterning, indicating cardiac left–right asymmetry disturbance may play significant roles in CHD pathogenesis. Clinically, CHD patients show a high prevalence of ciliary dysfunction and show enrichment for de novo mutations in cilia-related pathways. Combined with the mouse findings, this would suggest CHD may be a new class of ciliopathy.

Congenital heart disease (CHD) is one of the most common birth defects, found in an estimated 1% of live births (Hoffman and Kaplan 2002). With advances in surgical palliation, most patients with CHD now survive their critical heart disease such that currently there are more adults with CHD than infants born with CHD each year (van der Bom et al. 2012). However, CHD patient prognosis is variable, with long-term outcome shown to be dependent on patient intrinsic factors rather than surgical parameters (Newburger et al. 2012; Marelli et al. 2016). This is likely driven by genetic factors, given CHD is highly associated with chromosomal anomalies (Fahed et al. 2013), and with copy number variants (Glessner et al. 2014). In addition, CHD has been

shown to have a high recurrence risk, with familial clustering indicating a genetic contribution (Gill et al. 2003; Oyen et al. 2009). The identification of the genetic causes of CHD may provide mechanistic insights that can help stratify patients for guiding the therapeutic management of their clinical care.

Investigations into the genetic causes of CHD in human clinical studies have been challenging given the high degree of genetic diversity in the human population. This has made a compelling case for pursuing the use of a systems genetic approach with large-scale forward genetic screens in animal models to investigate the genetic etiology of CHD. Although many animal models have provided invaluable insights into the developmental regulation of car-

N.T. Klena et al.

diovascular development, investigations into the genetic etiology of CHD must be conducted in a model system with the same four-chamber cardiac anatomy that is the substrate of human CHD. The mouse is one such model system, advantageous not only given its similar four-chamber cardiac anatomy, but also inbred mouse strains are readily available with genomes that are fully sequenced and annotated that would facilitate mutation recovery. Moreover, cardiovascular development in the mouse embryo is well studied, providing a strong foundation to interrogate the developmental and genetic etiology of CHD.

DEVELOPMENT OF THE CARDIOVASCULAR SYSTEM

Congenital heart defect is a structural birth defect arising from disruption of cardiovascular development. Formation of the four-chamber heart in mammals is orchestrated by the highly coordinated specification and migration of different cell populations in the embryo that together form the complex left–right asymmetric anatomy of the cardiovascular system. In the mouse embryo, ingression of cells through the primitive streak at E7.5 generates the anterior mesoderm forming the cardiac crescent—containing cells of the first heart field (FHF) and adjacent to it, the second heart field (SHF) (Fig. 1) (Buckingham 2016). Cells of the FHF migrate toward the midline, fusing to form the linear heart tube at E8.0 (Fig. 1). Pharyngeal mesoderm located anterior and medially continues to be added to the expanding heart tube, as the heart tube undergoes rightward looping at E8.5, delineating the primitive anlage of the left ventricle (LV) (Fig. 1). This is followed by addition of SHF cells to the anterior and posterior poles of the heart tube, giving rise to the outflow tract (OFT), right ventricle (RV), and most of the left and right atria (LA, RA) (Fig. 1).

Normal development of the heart also requires the contribution and activity of several other extracardiac cell lineages, including the cardiac neural crest cells derived from the dorsal hindbrain neural fold. The cardiac neural crest cells migrate into the cardiac OFT in two spiral

streams, helping to remodel the pharyngeal arch arteries and orchestrating OFT septation to form the two great arteries—the aorta and pulmonary artery (Kirby and Waldo 1990). The pharyngeal endoderm and ectoderm also play an important regulatory function in developmental patterning of the aortic arch arteries and the OFT. Dynamic processes mediating endocardial epithelial–mesenchyme transformation (EMT) lead to formation of the cushion mesenchyme that provides early valve function in the embryonic heart. These endocardial cushion tissues later remodel to form the mature leaflets of the outflow semilunar and atrioventricular valves (Fig. 1). Another extracardiac cell population required for heart development are the pro-epicardial cells that originate near the septum transversum. These cells migrate to the heart via the sinus venosus, delaminating onto the surface of the heart, and forming the epicardium that plays an essential role in development of the coronary arteries. Together, these diverse cell populations are recruited to orchestrate formation of the mammalian heart, an organ that is an unexpected mosaic of distinct cell lineages.

FOUR-CHAMBER HEART—THE ANATOMICAL SUBSTRATE FOR CONGENITAL HEART DISEASE

The cardiovascular system in mouse and human is adapted for breathing air, being comprised of four chambers organized into functionally distinct left versus right sides. This allows the formation of a separate pulmonary circuit that pumps deoxygenated blood from the body to the lungs via the RV and a systemic circuit pumping oxygenated blood from the lung to the body via the LV. This left–right asymmetric organization is critically dependent on appropriate patterning of the left–right body axis and entails formation of an atrial and ventricular septum separating the right versus left sides of the heart. This allows for compartmentalization of the heart into four chambers, LA versus RA and LV versus RV. This is coupled with septation of the OFT into two great arteries, the aorta, which is inserted into the LV and pulmonary artery into the RV, and formation of the atrio-

Figure 1. Diagram of mouse cardiovascular development. (A) Cardiac crescent formation containing first heart field (FHF) and second heart field (SHF) cells. (B) Cardiac crescent cells migrate toward the midline creating the linear heart tube with its arterial and venous poles and a primitive ventricle. (C) At E8.5, dextral looping of the heart tube leads to formation of the primitive atrial and ventricular chambers in the morphologically correct position. (D) At E9.5, the endocardial cushion cells pinch inward, creating the atrioventricular canal. At E9.5, the endocardial cushions form at the dorsal and ventral lumen of the atrial canal as the endocardial cells undergo epithelial to mesenchymal transition. Cardiac trabeculation initiates at E10.5, creating bundles of cardiomyocytes that extend into the primitive cardiac chambers. Septation initiates at E10.5, starting division of the chambers into the four-chamber anatomy. (E) At E10.5, the outflow tract (OFT) is remodeled leading to the primitive connection of the aorta and pulmonary artery from the primitive ventricle. (F) By E13.5, the heart is fully developed into four distinct chambers with appropriate aorta and pulmonary artery connections to the morphological left and right ventricles (RVs), respectively. Dark pink, FHF; light pink, SHF; light green, atrioventricular (AV) canal; dark blue, endocardial (EC) cushions; yellow green, septation; purple, trabeculations; yellow, OFT.

N.T. Klena et al.

ventricular and outflow valves that allow unidirectional blood flow. It is this complex left–right asymmetric developmental patterning of the cardiovascular anatomy that ensures efficient oxygenation of blood with air exchange via the lungs. The perturbation of this distinct four-chamber cardiac anatomy in CHD invariably results in neonatal mortality unless surgical intervention is provided to palliate the structural heart defects. Identifying the genetic causes of CHD may help elucidate the developmental processes contributing to CHD and suggests new avenues for prevention or intervention.

CENTRAL ROLE OF CILIA IN CARDIOVASCULAR DEVELOPMENT AND CONGENITAL HEART DISEASE

To elucidate the genetic etiology of CHD, a large-scale, near-saturation level forward genetic screen with ethylnitrosourea (ENU) chemical mutagenesis was conducted (Li et al. 2015b). This phenotype-driven cardiovascular screen used fetal echocardiography, a noninvasive imaging modality routinely used clinically for CHD diagnosis (Fig. 2). This allowed high detection sensitivity and specificity for CHD diagnosis and allowed the recovery of a wide spectrum of CHD in the mouse screen similar to those observed clinically (Figs. 2 and 3) (Liu et al. 2014). From ultrasound screening of ~100,000 mouse fetuses, we recovered >200 mutant mouse lines with a wide variety of CHD.

Using exome-sequencing analysis, ~100 CHD-causing mutations were recovered in 61 genes, with more than half being cilia-related (Fig. 2) (Li et al. 2015). The cilia genes recovered included proteins localized in the cilia transition zone, basal body/centrosome, ciliary axoneme, and also multiprotein complexes in the cytoplasm required for cilia assembly (Fig. 2). Most of the proteins recovered are expressed in both motile (9 + 2) and primary cilia (9 + 0), such as components of the cilia transition zone. However, some genes encode proteins unique to motile cilia, such as the motor dyneins *Dnah5* and *Dnah11* localized in the outer dynein arm required for motile cilia function (Fig. 2). Many of these cilia protein components are known to

cause various human ciliopathies, mostly involving nonmotile, primary cilia defects, such as in Joubert syndrome (JBTS), Jeune syndrome, nephronophthisis, Meckel–Gruber syndrome, and others. The motile cilia mutations recovered in the screen are linked to the sinopulmonary disease primary ciliary dyskinesia (PCD). Although CHD is not an essential feature of ciliopathies, it is notable that the mutants we recovered were all based on having CHD phenotypes.

Further indicating the important role of cilia in CHD pathogenesis, we also recovered mutations in 12 CHD genes that are in cilia-transduced cell signaling pathways, including genes mediating sonic hedgehog (Shh), transforming growth factor β and bone morphogenetic proteins (TGF- β /BMPs), and Wnt signaling (Fig. 2). This enrichment of genes mediating cell signaling reflects the central role of cilia as a hub for signal transduction pathways essential to the regulation of key cardiovascular developmental processes. Also unexpected was the recovery of 10 CHD genes involved in vesicular trafficking. This included *Dynamin 2* and *Ap2b1* required for clathrin-mediated endocytosis, adaptin proteins *Ap1b1* and *Ap2b1*, and *Lrp1*, *Lrp2*, and *Snx17* mediating endocytic receptor recycling (Li et al. 2015b). Significantly, vesicular trafficking plays an essential role in cilia biology, with ciliogenesis initiated with capture of a ciliary vesicle by the mother centriole followed by docking of the basal body to the cell membrane and fusion of additional secondary vesicles that allow lengthening of the ciliary axoneme (Sorokin 1962; Kobayashi and Dynlacht 2011; Reiter et al. 2012). Vesicular trafficking and receptor recycling also play important roles in the regulation of cell signaling. Although the endocytic pathway was not previously known to play a role in CHD, its importance can be easily appreciated in the context of its role in regulating ciliogenesis and cilia-transduced cell signaling.

CILIA AND CILIA-TRANSDUCED CELL SIGNALING IN HEART DEVELOPMENT

The overall finding that the large majority of the CHD genes recovered were cilia or cilia-related was unexpected, given the screen was entirely

phenotype-driven. Hence, they point to a central role for cilia biology in regulating cardiovascular development and the pathogenesis of CHD. Primary cilia in the developing heart were first identified via electron microscopy in the chick, rabbit, mouse, and lizard embryos (Rash et al. 1969). These were observed only in nonmitotic cardiomyocytes or myoblasts,

Cite this article as *Cold Spring Harb Perspect Biol* 2017;9:a028266

N.T. Klena et al.

Figure 3. Congenital heart defects in a *Wdpcp* mutant and cilia localization. (A–H) Episcopic confocal histopathology showed a *Wdpcp*^{Cys40} mutant with an incomplete septum unevenly dividing the outflow tract (OFT) into one large and one small chamber, indicating pulmonary atresia (PAtr; black arrow in B). Also observed was an atrioventricular septal defect (AVSD; asterisk in D). Shown in A and C are comparable views of a control heart. In wild-type hearts (E), cardiomyocytes were observed in the OFT cushion (arrow), but in *Wdpcp*^{Cys40} mutants, cardiomyocytes were mostly absent in the cushion tissue (asterisk in F). Cardiomyocytes in outflow cushion of wild-type embryos (G) visualized with MF20 immunostaining showed polarized cell morphology with distinct elongated finger-like projections (asterisks) aligned with direction of cell migration and projecting into forming outflow septum (arrow in G). In contrast, in *Wdpcp*^{Cys40} mutant embryos (H), the cardiomyocytes showed rounded morphology without obvious cell polarity, nor the elongated cell projections seen in wild-type embryos. (From Cui et al. 2013; reprinted under the Creative Commons CCO public domain dedication.) (I–M) Immunofluorescence staining of cilia with acetylated tubulin (green) and γ -tubulin (red) antibodies (from data in Cui et al. 2013). Shown are the detection of cilia in the mouse embryonic node (I), and in the myocardium (J), outflow (OFT) cushions (K), and atrioventricular cushions (L) of wild-type E12.5 embryonic mouse heart. In contrast, cilia are missing in the atrioventricular cushion tissue of *Cc2d2a* mutant known to develop atrioventricular septal defect (adapted from data in Li et al. 2015b). Scale bars, 100 μ m (E, G).

atrial endocardial layer and more prominently in the endocardial cushion mesenchyme (Fig. 3K,L) and in the epicardium (Slough et al. 2008; Willaredt et al. 2012; Li et al. 2015b). A number of cilia-transduced cell signaling pathways have been shown to play essential roles in regulating cardiovascular development and may contribute to the pathogenesis of CHD. These include

Shh, TGF- β , BMP, and Wnt signaling. Four genes involved in Shh signaling were recovered from the mouse CHD screen, including *Sufu*, *Fuz*, *Tbc1d32*, and *Kif7* (Fig. 2). Also recovered were six genes involved in TGF- β /BMP signaling, including *Cfc1*, *Megf8*, *Tab1*, *Ltbp1*, *Smad6*, and *Pcsk5* and three mediating Wnt signaling—*Ptk7*, *Prickle1*, and *Fuz* (Fig. 2).

Role of Shh Signaling in Cardiac Development and CHD

Shh signaling is the best-described cilia-transduced cell signaling pathway. Numerous studies have shown that ablation of cilia can result in a drastic reduction of Shh signaling (Huangfu et al. 2003; Han et al. 2008; Goetz and Anderson 2010). During heart development, Shh is expressed in the pharyngeal endoderm and in the foregut endoderm adjacent to incoming SHF derivatives in the dorsal mesenchyme protrusion (Dyer and Kirby 2009). Shh knockout mice show atrial and atrioventricular septation defects, defects in OFT septation, and abnormal pharyngeal arch artery patterning (Washington Smoak et al. 2005). The outflow septation defects are characterized by the aorta shifted rightward overriding the septum, and with either pulmonary atresia or a hypoplastic pulmonary artery observed in conjunction with a variable degree of ventricular hypertrophy. This constellation of defects is reminiscent of tetralogy of Fallot (TOF), one of the most common complex CHD observed clinically (Washington Smoak et al. 2005). Using Cre targeted deletion analysis, it was shown that these outflow defects reflect a dual requirement for pharyngeal endodermal-derived Shh in the cardiac neural crest cells and the SHF derivatives (Goddeeris et al. 2007). These studies showed Shh signaling to the SHF and cardiac neural crest cells are required for OFT septation, but not for either OFT lengthening, or cushion formation, respectively. As the Shh knockout embryos showed a reduction in the number of SHF derivatives, this suggested a requirement for Shh in the specification of the SHF (Hildreth et al. 2009).

Role of Wnt Signaling in Cardiac Development and CHD

Primary cilia also play a role in the transduction of canonical and noncanonical Wnt signaling (Clevers 2006; MacDonald et al. 2009; Wallingford and Mitchell 2011; May-Simera and Kelley 2012) pathways that are also essential for normal heart development. One early evidence linking cilia with β -catenin-dependent canoni-

cal Wnt signaling was the observation that knockdown of basal body components bbs1, bbs4, and bbs6 resulted in several-fold increase in Wnt activity in zebrafish (Gerdes et al. 2007). The functional link between Wnt signaling and cilia was also shown by the observed localization of noncanonical Wnt/planar cell polarity (PCP) components, such as *Inversin*, *Dishevelled*, *Vangl2*, and *Wdpcp* in the basal body and/or ciliary axoneme (Fig. 4A,B) (Montcouquiol et al. 2003; May-Simera and Kelley 2012; Cui et al. 2013). Other studies also showed a role for cilia as a switch that can constrain canonical versus noncanonical Wnt signaling (Ross et al. 2005; Simons et al. 2005; Barrow et al. 2007; Gerdes et al. 2007; Corbit et al. 2008; Huang and Schier 2009; Stottmann et al. 2009; Lienkamp et al. 2012; Oh and Katsanis 2013). However, the precise mechanism by which cilia regulate Wnt signaling is not well understood.

In mice, the noncanonical Wnt/PCP genes such as *Celsr*, *Frizzled3* (*Fzd3*), *Fzd6*, *Vangl1-2*, and *Dvl1-3* are highly expressed in the OFT (Etheridge et al. 2008; Paudyal et al. 2010). Mice with mutations in the PCP genes *Vangl2*, *Scrib* (Phillips et al. 2007), *Dvl 1, 2*, and *3* (Hamblet et al. 2002; Etheridge et al. 2008; Sinha et al. 2012), *Wdpcp* (Cui et al. 2013), and *Pk1* (Gibbs et al. 2016) show a spectrum of CHD phenotypes involving OFT malalignment and septation defects, such as double outlet RV (Fig. 2E,F), overriding aorta, pulmonary atresia (Fig. 3B), and persistent truncus arteriosus (Henderson et al. 2006; Cui et al. 2013; Boczonadi et al. 2014; Gibbs et al. 2016). These cardiac defects likely reflect a role for noncanonical Wnt/PCP pathway in regulating the polarized migration of cardiac neural crest and SHF derivatives (Tada and Smith 2000; Montcouquiol et al. 2003; Simons et al. 2005; Verzi et al. 2005; Cohen et al. 2007; Simons and Mlodzik 2008; Schlessinger et al. 2009; Gibbs et al. 2016). Consistent with this, examination of mouse embryonic fibroblasts derived from the *Wdpcp* or *Pk1* mutant embryos showed inability of the cells to polarize and engage in directional cell migration (Figs. 4C–G, 5K–N). In contrast to Shh deficiency, Wnt/PCP disruption caused failure of the OFT to appropriately lengthen

N.T. Klena et al.

Figure 4. Wdpcp is a cilia protein regulating cell polarity, directional cell migration, and the actin cytoskeleton. (A,B) IMCD3 cells immunostained with Wdpcp (green) and acetylated α -tubulin (red) antibodies show Wdpcp localization in the axoneme and ring-like structure (arrowhead) at the ciliary base. Localization of Wdpcp (red) in this ring-like structure is better seen with a 3D isosurface reconstruction, which also shows some colocalization of Septin-2 (green) with the Wdpcp ring. (C–G) In a wound-healing assay, control mouse embryonic fibroblasts (MEFs) (A) show good alignment with the direction of wound closure (indicated by white arrow). In contrast, *Wdpcp*^{Cys40} mutant MEFs (B) showed a disorganized distribution. These differences in cell polarity were also reflected in the Golgi orientation (white line drawn through the center of the Golgi stained green) (E,F). In wild-type MEFs, the Golgi (green) was mostly situated at the cell's leading edge (E,G), aligned with the direction of wound closure (white arrow), whereas the *Wdpcp*^{Cys40} mutant MEFs show randomized Golgi orientation (E,G). Scale bars, 20 μ m (A, B, C, D, F). (H–N) Confocal imaging of Sept2 (red) and Wdpcp (green) showed they are colocalized in actin stress fiber (phalloidin stained, blue) in wild-type MEFs (H–J), but in the *Wdpcp*^{Cys40} mutant MEFs, Wdpcp expression was lost (blue, L), whereas Sept2 immunostaining (red, K,M) showed the loss of colocalization with actin (blue) (K,L,M). (L) Wdpcp (green) is enriched at the cell cortex where actin filaments (phalloidin) insert into vinculin (red)-containing focal adhesions (N) in wild-type MEFs. (Adapted from Cui et al. 2013 under the Creative Commons CC0 public domain dedication.)

Figure 5. Shortened outflow tract (OFT) and defects in cell polarity and directional cell migration in the *Pk1^{Bj}* mutant. (A–D) E10.5 *Pk1^{Bj}* mutant embryo. B and D show shortened OFT as compared with that of heterozygous embryo (C,D). (E–H) Islet1 immunostaining show distribution of SHF cells in the dorsal pericardial wall of the OFT of control (E,G) and *Pk1^{Bj}* mutant embryos (F,H). Magnified views of region denoted by arrowheads in E and F revealed a cuboidal (H) rather than flat squamous (G) epithelial morphology in the homozygous mutant versus heterozygous embryo. (I,J) β-Catenin (green) and laminin (red) antibody staining of wild-type (I) and *Bj* mutant embryos (J) shown in the E10.5 *Bj* mutant embryo, marked disorganization of the epithelium in the transition zone (TZ) of the pericardial wall where SHF derivatives are found. Confocal imaging showed laminin (red) is localized basally (arrowhead I) in the TZ of the control embryo, but in the mutant embryo, it is localized apically (arrow) and basally (arrowhead J), indicating a loss of normal epithelial polarity. The distribution of β-catenin (green) remains at the cell surface in both the control and *Bj* mutant embryos. (K,L) Myocardialization defect in the OFT of *Pk1^{Bj}* mutants. Examination of the striated banding pattern from MF20 immunostain showed the developing myofilaments in the heart are closely aligned and oriented toward the direction of myocardialization in the wild-type E14.5 embryo (K), but in the *Bj* mutant, the myofilaments are sparse and are largely oriented perpendicular to the direction of myocardialization and septum formation (L). (M,N) Wound closure assay shows a defect in directional cell migration in *Pk1^{Bj}* mutant mouse embryonic fibroblasts (MEFs). The migration path of MEFs 8 h after wound scratch were well aligned with the direction of wound closure, but tortuous paths were observed with increased velocity for the *Pk1^{Bj}* mutant MEFs (M,N) (adapted from data in Gibbs et al. 2016). Scale bars, 0.5 μm (A, B); 50 μm (E); 20 μm (K).

N.T. Klena et al.

(Fig. 5A–D). In the *Pk1* mutant, the epithelial organization and apical-basal polarity of the SHF derivatives in the OFT are disrupted. This would suggest a defect in convergent-extension cell movement required for delamination of a cohesive epithelial sheet mediating OFT lengthening (Fig. 5E–J). This is followed later by a myocardialization defect of the OFT (Figs. 3E–H, 5K–L), that together with the shortened OFT likely account for the great artery malalignment defect in the *Pk1* mutant.

Role of TGF- β Signaling in Cardiac Development and CHD

A role for cilia in mediating TGF- β signaling was recently shown with the finding that ligand binding causes accumulation of TGF- β receptors at the base of the cilium, in a region known as the ciliary pocket (Clement et al. 2013). This triggers receptor-mediated endocytosis involving clathrin-coated vesicles, leading to downstream activation of SMAD phosphorylation (Clement et al. 2013). The essential role of TGF- β /BMP signaling in CHD is well described via in vitro and in vivo analyses of chick and mouse embryos, and also with the examination of knockout mouse models (Combs and Yutzey 2009; de Vlaming et al. 2012; Kruithof et al. 2012; von Gise and Pu 2012). These studies show TGF- β /BMP signaling has multiple roles in cardiovascular development that include the regulation of both endocardial EMT and endocardial cushion development (Potts and Runyan 1989; Camenisch et al. 2002). For example, early endocardial cushion development to acquire critical valve-like function requires BMP signaling in cardiac neural crest cells via the BMPRIA receptors (Nomura-Kitabayashi et al. 2009). A role for *Tgfb2* in OFT and aortic arch remodeling is indicated by the finding that *Tgfb2* knockout mice die perinatally with double outlet RV and interrupted aortic arch (Sanford et al. 1997).

The disturbance of TGF- β /BMP signaling is likely to play a major role in the valvular defects seen in mice harboring mutations disrupting clathrin-mediated endocytosis and endocytic receptor recycling (*Ap2b1*, *Dnm2*, *Ap1b1*,

Snx17, *LRP1*, *LRP2*). These endocytic mutants all show OFT malalignment and endocardial cushion defects, phenotypes reminiscent of those observed in mutants with disruption of TGF- β /BMP signaling (Li et al. 2015b). Similarly, mutations affecting cilia integrity in the endocardial cushions may cause disruption of cilia-transduced TGF- β /BMP signaling required for normal valve development. Thus, mutation in *Cc2d2a*, a cilia transition zone component, causes selective loss of cilia in the atrioventricular (AV) but not outflow cushions, and as might be expected, such mutants showed AV valve defects, while the outflow valves were spared (Fig. 3K–M).

ROLE OF CILIA IN SPECIFICATION OF CELL POLARITY AND POLARIZED CELL MIGRATION

Some cilia proteins may help regulate cardiovascular development through cross talk, directly or indirectly, with the cytoskeleton to specify cell polarity and directional cell migration, morphogenetic cell movements, and epithelial–mesenchyme cell transformation. Given the basal body is a microtubule organizing center that can regulate nucleation and organization of microtubule outgrowth, one concept that has emerged is that cilia may regulate the cytoskeleton through dynamic interactions with PCP components and, in this manner, specify cell polarity and polarized cell migration (Figs. 4 and 5) (Wallingford and Mitchell 2011; May-Simera and Kelley 2012). These dynamic cell processes may help to direct the long-distance migration of multiple extracardiac cell populations to the embryonic heart that are required for normal heart development. This includes cells from the SHF, neural crest cells, and the pro-epicardial cells. In addition, cilia directed reorganization of the actin cytoskeleton may also contribute to the regulation of EMT, such as required for the emergence of cardiac neural crest cells from the dorsal neural fold, endocardial EMT mediating formation of the cardiac cushions and valves, or epicardial EMT that generate the epicardially derived cells forming the coronary vessels. These developmental pro-

cesses involving dynamic reorganization of the cytoskeleton is impacted by cilia and, in conjunction with cilia-transduced cell signaling, may help orchestrate development of the cardiovascular system.

Although the role of cilia in the regulation of cell polarity and directional cell migration in the cardiovascular development is well described in the context of OFT morphogenesis (see above), the precise mechanism and role of the cilia in modulating cell polarity is less understood. In this regard, it is worth pointing out that *Wdpcp*, a PCP component also known as Fritz, is localized not only in the cilia, but it is also colocalized with septins in the cilia (Kim et al. 2010; Cui et al. 2013) and in the actin cytoskeleton (Kim et al. 2010; Cui et al. 2013). In mouse embryonic fibroblast (MEF) cells deficient in *Wdpcp*, a marked reorganization of the actin cytoskeleton is observed (Fig. 4H–I), and this is associated with altered focal contacts (Fig. 4N) inability to establish cell polarity and engage in directional cell migration (Fig. 4C–G). Similar studies of MEFs harboring a mutation in the PCP component *Pk1* also showed a similar loss of cell polarity and defect in directional cell migration (Fig. 5M,N) (Gibbs et al. 2016). Together, these findings suggest that cilia mutations may cause CHD not only via the disruption of cilia-transduced cell signaling, but cilia mutations also may disrupt the cytoarchitecture and perturb the establishment of cell polarity, polarized cell migration, and/or EMT.

CILIA IN LEFT–RIGHT PATTERNING AND CONGENITAL HEART DISEASE

The enrichment of cilia genes was also notable in that it included a subset of genes that caused CHD in conjunction with left–right patterning defects. This likely reflects the known requirement for cilia in left–right patterning, with previous studies indicating that motile cilia at the embryonic node is required to break symmetry (Fig. 3I) (Hirokawa et al. 2009; Nakamura and Hamada 2012). Analysis of motile cilia mutant mice revealed CHD is typically observed in conjunction with heterotaxy, the randomization of left–right patterning (Tan et al. 2007). This is

consistent with the well-described clinical association of complex CHD with heterotaxy (Lin et al. 2014). As the heart is the most left–right asymmetric organ, and this asymmetry is essential for efficient oxygenation of blood, it is perhaps not surprising that left–right patterning defects may play a major role in CHD pathogenesis.

Among 34 cilia mutations recovered causing laterality defects, 22 genes perturbed the primary cilia (*Cc2d2a*, *Anks6*, *Nek8*, *Mks1*, *Cep290*, *Bicc1*) versus 12 genes that disrupted motile cilia (*Dnah5*, *Dnah11*, *Dnai1*, *Daw1*, *Armc4*, *Ccdc151*, *Drc1*, *Ccdc39*, *Dyxc1x1*, *Dnaaf3*) (Li et al. 2015b). The latter genes are known to cause PCD, a ciliopathy that is autosomal recessive (Collins et al. 2014; Horani et al. 2014; Lobo et al. 2015). In PCD, immotile/dyskinetic cilia in the airway cause mucociliary clearance defects that can lead to severe sinopulmonary disease. Approximately half of PCD patients show situs solitus, half situs inversus totalis, and varying numbers up to 8% may show CHD with heterotaxy (Kennedy et al. 2007; Shapiro et al. 2014). The disturbance of laterality with PCD reflects the essential role of motile cilia in left–right patterning. Studies in the PCD mutant mouse models showed each PCD mutation can give rise to three phenotypes—approximately half with situs solitus or situs inversus and half with heterotaxy, with complex CHD observed only with heterotaxy (Tan et al. 2007). Although the heterotaxy mutants mostly die prenatally or neonatally from the CHD, mutants with situs solitus or inversus are largely viable postnatally without CHD. Videomicroscopy showed most of these PCD mutants have immotile cilia in the embryonic node, even as half of the mutants show normal or inverted concordant situs that indicate the breaking of symmetry.

These striking observations suggest that motile cilia are not absolutely required for breaking symmetry, nor for left–right axis specification, although motile cilia are clearly required for high-fidelity situs solitus specification. As CHD is only seen with heterotaxy, this provides a clue that patterning of the cardiovascular system may occur very early in de-

N.T. Klena et al.

velopment, at the time the left–right body axis is specified. Even as these findings show that motile cilia play an important role in left–right patterning, the recovery of 24 mutations affecting primary cilia suggests nonmotile cilia also play an essential role in laterality specification (Li et al. 2015b). Previous studies suggested a two-cilia hypothesis in which motile cilia at the node generated right to left flow (for additional information, see Shinohara and Hamada 2016). This is proposed to trigger mechanosensory transduction of primary cilia in the perinodal crown cells, causing left-sided calcium release that is propagated into the surrounding lateral plate mesoderm, causing the breaking of symmetry (Nonaka et al. 2002; McGrath et al. 2003; Bruekner 2007; Yoshida et al. 2012). However, this model has been called into question recently given the failure to detect cilia-mediated mechanosensation and calcium release (Delling et al. 2016).

A role for primary cilia in left–right patterning could be easily understood nevertheless without invoking mechanosensation, because Shh and TGF- β signaling, both cilia-transduced pathways, play important roles in left–right patterning. Although *Shh* knockout mice do not show overt laterality defects, they show LA isomerism (Hildreth et al. 2009). Furthermore, the single outflow vessel seen in the *Shh* knockout mouse is said to represent pulmonary atresia, as the single great artery shows *Pitx2c*, indicating a left-sided identity (Washington Smoak et al. 2005). It is interesting to note in chick embryos where Shh plays a much more primary role in left–right patterning, the experimental manipulation of left–right expression of Shh can cause CHD, confirming its importance of left–right patterning in the pathogenesis of CHD (Levin et al. 1995). Signaling mediated by the TGF- β family of growth factors, including nodal, *lefty1*, and *lefty2*, are well described to specify the left–right axis. This nodal signaling cascade is believed to propagate left–right specification initiated at the node. How mutations affecting primary cilia may contribute to the disruption of left–right patterning is not known, but it is thought to cause disturbance in the propagation of this nodal signaling cascade.

CILIARY DYSFUNCTION AND CILIOME MUTATIONS IN CHD PATIENTS

The unexpected enrichment for mutations in cilia-related (ciliome) genes and genes involved in endocytic trafficking and in cilia-transduced cell signaling (Shh, WNT/PCP, TGF- β) in the mouse mutagenesis screen point to a central role for cilia in CHD pathogenesis. To assess the relevance of these findings to human CHD, we investigated the findings from exome-sequencing analysis of CHD patients by the Pediatric Cardiac Genomics Consortium (PCGC) (Zaidi et al. 2013). In this analysis, the focus was on examining de novo predicted pathogenic coding variants. Although the PCGC publication focused on the recovery of de novo variants in a number of chromatin-modifying genes, interestingly, we noted among the 28 de novo damaging mutations identified in the PCGC CHD patient cohort, 13 or nearly half were in genes associated with pathways identified in the mouse forward genetic screen—that is, ciliogenesis, endocytic trafficking, and cilia-transduced cell signaling (SHH, WNT, TGF- β) (Table 1), with *LRP2* being a gene recovered in both the PCGC CHD patients and the mouse CHD mutants recovered in our screen. We also noted the recovery in the PCGC cohort of a de novo variant in *Pitx2*, a gene known to play an essential role in left–right patterning, supporting an important role for left–right patterning disturbance in CHD pathogenesis.

Further supporting a central role for cilia in the pathogenesis of CHD are clinical studies showing a high prevalence of ciliary dysfunction in CHD patients (Nakhleh et al. 2012; Garrod et al. 2014). Given that respiratory complications are among the biggest postsurgical complications for CHD patients, we previously hypothesized that some CHD patients with respiratory complications may have undiagnosed PCD. These studies were initiated with an examination of CHD patients with heterotaxy. Nasal scrapes were conducted and video microscopy was used to examine cilia motility in the nasal epithelium. This analysis showed a high prevalence of ciliary dysfunction in CHD patients with heterotaxy. The ciliary motion defects

Table 1. Functional annotation for 13 PCGC patients with de novo mutations

Patient ID	CHD type ^a	Gene	Mutation	Gene function annotation
1-00638	CTD	<i>FBN2</i>	p.D2191N	TGF- β signaling
1-02020	HTX	<i>SMAD2</i>	p.IVS12 + 1G > A	TGF- β signaling
1-02621	HTX	<i>SMAD2</i>	p.W244C	TGF- β signaling
1-00197	LVO	<i>BCL9</i>	p.M1395K	Wnt signaling
1-01828	CTD	<i>DAPK3</i>	p.P193L	Wnt signaling
1-01138	LVO	<i>USP34</i>	p.L432P	Wnt signaling
1-00802	LVO	<i>PTCH1</i>	p.R831Q	Shh signaling/ciliome
1-02598	HTX	<i>LRP2^b</i>	p.E4372K	Shh signaling/endocytic trafficking
1-01913	Other	<i>RAB10</i>	p.N112S	Endocytic trafficking
1-00750	LVO	<i>HUWE1</i>	p.R3219C	Ciliome
1-01151	CTD	<i>SUV420H1</i>	p.R143C	Ciliome
1-00853	CTD	<i>WDR5</i>	p.K7Q	Ciliome
1-02952	LVO	<i>PITX2</i>	p.A47V	Laterality-related

Based on exome-sequencing analysis of congenital heart disease (CHD) patients by Pediatric Cardiac Genomics Consortium (Data from Zaidi et al. 2013).

^aCTD, Conotruncal defect; HTX, heterotaxy; LVO, left ventricular obstruction.

^bLRP2 is an endocytic gene also recovered from our mouse screen.

observed span a spectrum that included some showing dyskinetic ciliary motion to slow or even immotile cilia. Overall, >40% of the patients showed ciliary dysfunction (Nakhleh et al. 2012). Although this was associated with an enrichment for coding variants in PCD genes, no patient was either homozygous or compound heterozygous for any PCD gene mutations. Thus, although CHD patients with heterotaxy are at high risk for ciliary dysfunction, these patients largely do not have PCD. Since this initial study, a large study has been conducted comprising >200 patients with CHD of a broad spectrum, mostly without heterotaxy. This analysis showed a similar high prevalence of ciliary dysfunction and this was correlated with increased risk of having PCD-related respiratory symptoms (Garrod et al. 2014). Together, these findings suggest ciliary dysfunction is commonly associated with CHD in the human population.

Although these studies focused on assessing motile cilia function, we note many cilia genes are expressed in both motile and primary cilia. Hence, the high prevalence of ciliary dysfunction in CHD patients may reflect not only the perturbation of motile cilia genes, but also genes required for primary cilia function. Indeed, we recently showed a patient harboring compound heterozygous mutations in *WDR35* causing

Sensenbrenner syndrome, a ciliopathy thought to affect only the primary cilia, showed motile cilia dysfunction. Pulmonary function assessments indicated obstructive airway disease that suggested possible mucociliary clearance defects in the airway (Li et al. 2015a). Indeed, several clinical studies have shown an increase in respiratory symptoms and disease in patients with other ciliopathies thought to affect only the primary cilia, indicating the distinction between ciliopathies involving motile versus primary cilia may not be so clear cut (Tobin and Beales 2009). These findings suggest further studies are warranted to assess ciliopathy patients of a wide spectrum for potential pulmonary complications, especially for those who will undergo high-risk surgeries, such as those involving cardiopulmonary bypass.

CONGENITAL HEART DISEASE AND CILIOPATHIES

It is notable that many cilia genes recovered in the mouse forward genetic screen for CHD-causing mutations are genes clinically known to cause various human ciliopathies. This includes not only motile cilia genes associated with PCD, but also cilia genes linked to various ciliopathies thought to affect the primary cilia,

N.T. Klena et al.

such as in JBTS, polycystic kidney disease, acrocallosal syndrome, hydroelethalus, Leber congenital amaurosis, Meckel–Gruber syndrome, Bardet–Biedl syndrome, etc. (Li et al. 2015b). While in our mouse screen, ciliopathy genes were recovered based on mutations causing CHD phenotypes, clinically these ciliopathies are not commonly associated with CHD. This may reflect ascertainment bias given that the patient population represent only human fetuses that can survive to term and, hence, are less likely to have severe cardiac anomalies. Indeed, clinical studies of aborted or stillborn fetuses have shown that the human fetal population has more than ten times higher incidence of CHD as compared with those in the clinical patient population (Hoffman and Kaplan 2002). Consistent with this, most of the CHD ciliopathy mutants recovered from our screen were inviable to term and were harvested preterm after in utero phenotyping by fetal echocardiography. On the flip side, there is undoubtedly ascertainment bias in our screen in the recovery of mutations in ciliopathy genes that specifically can cause CHD. That different ciliopathy mutations may have varying levels of penetrance for CHD phenotypes is suggested by observations of our mutant *Hug* (Damerla et al. 2015). This mutant has a mutation in *Jbts17*, a gene encoding a cilia transition zone protein known to cause JBTS (Srour et al. 2012). *Hug* mutants show cerebellar defects expected for JBTS and they also can show CHD comprising of pulmonary atresia. However, the CHD phenotype is incomplete in penetrance, as some *Hug* mutants show no heart defects (Damerla et al. 2015). These observations suggest that different mutations in the same ciliopathy gene may generate different phenotypic outcome and this perhaps can be further modified by the genetic background of the individual.

In light of these observations, we suggest that, clinically, CHD may be considered a structural birth defect related to ciliopathies. However, unlike other ciliopathies, which are relatively rare (<1 in 10,000) and with a Mendelian recessive inheritance, the much higher prevalence of CHD (up to 1%) and its sporadic occurrence would suggest the contribution of cilia-related

or ciliome genes in CHD will be multigenic and highly genetically heterogeneous. Such complex genetics is expected to reflect the complexity of cilia biology in which sequence variants found among different “ciliome” genes may affect the function of large multiprotein complexes that regulate ciliogenesis and cilia structure and function. Given that there are hundreds of ciliome genes that contribute to cilia assembly and cilia structure and function, it is perhaps not surprising that CHD patients are observed to have a high prevalence of ciliary dysfunction. While the CHD genes recovered from the mouse screen were by design recessive mutations, we expect mutations in these same genes can contribute to more complex genetic models of disease. Such complex genetics may also contribute to classic ciliopathies, as there are clinical reports of PCD patients and patients with other ciliopathies that have no homozygous or compound heterozygous ciliopathy mutations, but instead show multiple heterozygous mutations in known PCD or other ciliopathy genes (de Pontual et al. 2009; Li et al. 2016). A future challenge is to develop an effective bioinformatics pipeline for modeling and interrogating such complex genetics and assess the contribution of ciliome mutations in the pathogenesis of CHD and other structural birth defects.

CONCLUSIONS

CHDs are the most common structural birth defects, and despite its prevalence, the genetic etiology of CHD remains poorly understood. Interrogations into the genetic landscape for CHD using a large-scale forward genetic screen in mice unveiled a central role for ciliome genes in the pathogenesis of CHD. These studies suggest the perturbation of cilia and cilia-transduced cell signaling pathways may play a central role in the pathogenesis of CHD. The future challenge is to clinically translate these findings in mice to patients with CHD. The finding of a high prevalence of ciliary dysfunction in CHD patients and the enrichment of de novo pathogenic variants in cilia and cilia-related pathways in CHD patients would suggest such studies will be fruitful and may provide the basis for

stratifying patients to optimize the clinical management of patient care. The recent finding of primary cilia in the endothelial cells of the aorta regulating anti-atherosclerotic responses also point to a potential role for cilia in adult cardiac disease (Dinsmore and Reiter 2016). Further work in the future will be needed to clarify the role of cilia biology in human CHD and perhaps other cardiovascular diseases, and with such insights may come new avenues of therapeutic intervention to improve the outcome for patients with critical heart disease.

REFERENCES

*Reference is also in this collection.

- Barrow JR, Howell WD, Rule M, Hayashi S, Thomas KR, Capecchi MR, McMahon AP. 2007. Wnt3 signaling in the epiblast is required for proper orientation of the anteroposterior axis. *Dev Biol* **312**: 312–320.
- Boczonadi V, Gillespie R, Keenan I, Ramsbottom SA, Donald-Wilson C, Al Nazer M, Humbert P, Schwarz RJ, Chaudhry B, Henderson DJ. 2014. Scrib:Rac1 interactions are required for the morphogenesis of the ventricular myocardium. *Cardiovasc Res* **104**: 103–115.
- Brueckner M. 2007. Heterotaxia, congenital heart disease, and primary ciliary dyskinesia. *Circulation* **115**: 2793–2795.
- Buckingham M. 2016. First and second heart field. In *Congenital heart diseases: The broken heart: Clinical features, human genetics and molecular pathways* (ed. Rickert-Sperling S, Kelly GR, Driscoll JD), pp. 25–40. Springer, Vienna.
- Camenisch TD, Molin DG, Person A, Runyan RB, Gittenberger-de Groot AC, McDonald JA, Klewer SE. 2002. Temporal and distinct TGF- β ligand requirements during mouse and avian endocardial cushion morphogenesis. *Dev Biol* **248**: 170–181.
- Clement CA, Ajbro KD, Koefoed K, Vestergaard ML, Veland IR, Henriques de Jesus MP, Pedersen LB, Benmerah A, Andersen CY, Larsen LA, et al. 2013. TGF- β signaling is associated with endocytosis at the pocket region of the primary cilium. *Cell Rep* **3**: 1806–1814.
- Clevers H. 2006. Wnt/ β -catenin signaling in development and disease. *Cell* **127**: 469–480.
- Cohen ED, Wang Z, Lepore JJ, Lu MM, Taketo MM, Epstein DJ, Morrissey EE. 2007. Wnt/ β -catenin signaling promotes expansion of Isl-1-positive cardiac progenitor cells through regulation of FGF signaling. *J Clin Invest* **117**: 1794–1804.
- Collins SA, Walker WT, Lucas JS. 2014. Genetic testing in the diagnosis of primary ciliary dyskinesia: State-of-the-art and future perspectives. *J Clin Med* **3**: 491–503.
- Combs MD, Yutzy KE. 2009. Heart valve development: Regulatory networks in development and disease. *Circ Res* **105**: 408–421.
- Corbit KC, Shyer AE, Dowdle WE, Gaulden J, Singla V, Chen MH, Chuang PT, Reiter JE. 2008. Kif3a constrains β -catenin-dependent Wnt signalling through dual ciliary and non-ciliary mechanisms. *Nat Cell Biol* **10**: 70–76.
- Cui C, Chatterjee B, Lozito TP, Zhang Z, Francis RJ, Yagi H, Swanhart LM, Sanker S, Francis D, Yu Q, et al. 2013. Wdpcp, a PCP protein required for ciliogenesis, regulates directional cell migration and cell polarity by direct modulation of the actin cytoskeleton. *PLoS Biol* **11**: e1001720.
- Damerla RR, Cui C, Gabriel GC, Liu X, Craig B, Gibbs BC, Francis R, Li Y, Chatterjee B, San Agustin JT, et al. 2015. Novel *Jbts17* mutant mouse model of Joubert syndrome with cilia transition zone defects and cerebellar and other ciliopathy related anomalies. *Hum Mol Genet* **24**: 3994–4005.
- Delling M, Indzhykulyan AA, Liu X, Li Y, Xie T, Corey DP, Clapham DE. 2016. Primary cilia are not calcium-responsive mechanosensors. *Nature* **531**: 656–660.
- de Pontual L, Zaghloul NA, Thomas S, Davis EE, Mcgaughey DM, Dollfus H, Baumann C, Bessling SL, Babarit C, Pelet A, et al. 2009. Epistasis between *RET* and *BBS* mutations modulates enteric innervation and causes syndromic Hirschsprung disease. *Proc Natl Acad Sci* **106**: 13921–13926.
- de Vlaming A, Sauls K, Hajdu Z, Visconti RP, Mehesz AN, Levine RA, Slaugenhaupt SA, Hagege A, Chester AH, Markwald RR, et al. 2012. Atrioventricular valve development: New perspectives on an old theme. *Differentiation* **84**: 103–116.
- Dinsmore C, Reiter JE. 2016. Endothelial primary cilia inhibit atherosclerosis. *EMBO Rep* **17**: 156–166.
- Dyer LA, Kirby ML. 2009. Sonic Hedgehog maintains proliferation in secondary heart field progenitors and is required for normal arterial pole formation. *Dev Biol* **330**: 305–317.
- Etheridge SL, Ray S, Li S, Hamblet NS, Lijam N, Tsang M, Greer J, Kardos N, Wang J, Sussman DJ, et al. 2008. Murine dishevelled 3 functions in redundant pathways with dishevelled 1 and 2 in normal cardiac outflow tract, co-chlea, and neural tube development. *PLoS Genet* **4**: e1000259.
- Fahed AC, Gelb BD, Seidman JG, Seidman CE. 2013. Genetics of congenital heart disease: The glass half empty. *Circ Res* **112**: 707–720.
- Garrod AS, Zahid M, Tian X, Francis RJ, Khalifa O, Devine W, Gabriel GC, Leatherbury L, Lo CW. 2014. Airway ciliary dysfunction and sinopulmonary symptoms in patients with congenital heart disease. *Ann Am Thorac Soc* **11**: 1426–1432.
- Gerdes JM, Liu Y, Zaghloul NA, Leitch CC, Lawson SS, Kato M, Beachy PA, Beales PL, DeMartino GN, Fisher S, et al. 2007. Disruption of the basal body compromises proteasomal function and perturbs intracellular Wnt response. *Nat Genet* **39**: 1350–1360.
- Gibbs BC, Damerla RR, Vladar EK, Chatterjee B, Wan Y, Liu X, Cui C, Gabriel GC, Zahid M, Yagi H, et al. 2016. Prickle1 mutation causes planar cell polarity and directional cell migration defects associated with cardiac outflow tract anomalies and other structural birth defects. *Biol Open* **5**: 323–335.

N.T. Klena et al.

- Gill HK, Splitt M, Sharland GK, Simpson JM. 2003. Patterns of recurrence of congenital heart disease: An analysis of 6,640 consecutive pregnancies evaluated by detailed fetal echocardiography. *J Am Coll Cardiol* **42**: 923–929.
- Glessner JT, Bick AG, Ito K, Homys JG, Rodriguez-Murillo L, Fromer M, Mazaika E, Vardarajan B, Italia M, Leipzig J, et al. 2014. Increased frequency of de novo copy number variants in congenital heart disease by integrative analysis of single nucleotide polymorphism array and exome sequence data. *Circ Res* **115**: 884–896.
- Goddeeris MM, Schwartz R, Klingensmith J, Meyers EN. 2007. Independent requirements for Hedgehog signaling by both the anterior heart field and neural crest cells for outflow tract development. *Development* **134**: 1593–1604.
- Goetz SC, Anderson KV. 2010. The primary cilium: A signalling centre during vertebrate development. *Nat Rev Genet* **11**: 331–344.
- Hamblet NS, Lijam N, Ruiz-Lozano P, Wang J, Yang Y, Luo Z, Mei L, Chien KR, Sussman DJ, Wynshaw-Boris A. 2002. Dishevelled 2 is essential for cardiac outflow tract development, somite segmentation and neural tube closure. *Development* **129**: 5827–5838.
- Han YG, Spassky N, Romaguera-Ros M, Garcia-Verdugo JM, Aguilar A, Schneider-Maunoury S, Alvarez-Buylla A. 2008. Hedgehog signaling and primary cilia are required for the formation of adult neural stem cells. *Nat Neurosci* **11**: 277–284.
- Henderson DJ, Phillips HM, Chaudhry B. 2006. Vang-like 2 and noncanonical Wnt signaling in outflow tract development. *Trends Cardiovasc Med* **16**: 38–45.
- Hildreth V, Webb S, Chaudhry B, Peat JD, Phillips HM, Brown N, Anderson RH, Henderson DJ. 2009. Left cardiac isomerism in the Sonic Hedgehog null mouse. *J Anat* **214**: 894–904.
- Hirokawa N, Tanaka Y, Okada Y. 2009. Left–right determination: Involvement of molecular motor KIF3, cilia, and nodal flow. *Cold Spring Harb Perspect Biol* **1**: a000802.
- Hoffman JI, Kaplan S. 2002. The incidence of congenital heart disease. *J Am Coll Cardiol* **39**: 1890–1900.
- Horani A, Brody SL, Ferkol TW. 2014. Picking up speed: Advances in the genetics of primary ciliary dyskinesia. *Pediatr Res* **75**: 158–164.
- Huang P, Schier AF. 2009. Dampened Hedgehog signaling but normal Wnt signaling in zebrafish without cilia. *Development* **136**: 3089–3098.
- Huangfu D, Liu A, Rakeman AS, Murcia NS, Niswander L, Anderson KV. 2003. Hedgehog signalling in the mouse requires intraflagellar transport proteins. *Nature* **426**: 83–87.
- Kennedy MP, Omran H, Leigh MW, Dell S, Morgan L, Molina PL, Robinson BV, Minnix SL, Olbrich H, Severin T, et al. 2007. Congenital heart disease and other heterotaxic defects in a large cohort of patients with primary ciliary dyskinesia. *Circulation* **115**: 2814–2821.
- Kim SK, Shindo A, Park TJ, Oh EC, Ghosh S, Gray RS, Lewis RA, Johnson CA, Attie-Bittach T, Katsanis N, et al. 2010. Planar cell polarity acts through septins to control collective cell movement and ciliogenesis. *Science* **329**: 1337–1340.
- Kirby ML, Waldo KL. 1990. Role of neural crest in congenital heart disease. *Circulation* **82**: 332–340.
- Kobayashi T, Dynlacht BD. 2011. Regulating the transition from centriole to basal body. *J Cell Biol* **193**: 435–444.
- Kruithof BP, Duim SN, Moerkamp AT, Goumans MJ. 2012. TGF- β and BMP signaling in cardiac cushion formation: Lessons from mice and chicken. *Differentiation* **84**: 89–102.
- Levin M, Johnson RL, Stern CD, Kuehn M, Tabin C. 1995. A molecular pathway determining left–right asymmetry in chick embryogenesis. *Cell* **82**: 803–814.
- Li Y, Garrod AS, Madan-Khetarpal S, Sreedher G, McGuire M, Yagi H, Klena NT, Gabriel GC, Khalifa O, Zahid M, et al. 2015a. Respiratory motile cilia dysfunction in a patient with cranioectodermal dysplasia. *Am J Med Genet A* **167A**: 2188–2196.
- Li Y, Klena NT, Gabriel GC, Liu X, Kim AJ, Lemke K, Chen Y, Chatterjee B, Devine W, Damerla RR, et al. 2015b. Global genetic analysis in mice unveils central role for cilia in congenital heart disease. *Nature* **521**: 520–524.
- Li Y, Yagi H, Onuoha EO, Damerla RR, Francis R, Furutani Y, Tariq M, King SM, Hendricks G, Cui C, et al. 2016. *DNAH6* and its interactions with PCD genes in heterotaxy and primary ciliary dyskinesia. *PLoS Genet* **12**: e1005821.
- Lienkamp S, Ganner A, Walz G. 2012. Inversin, Wnt signaling and primary cilia. *Differentiation* **83**: S49–S55.
- Lin AE, Krikov S, Riehle-Colarusso T, Frias JL, Belmont J, Anderka M, Geva T, Getz KD, Botto LD; National Birth Defects Prevention S. 2014. Laterality defects in the national birth defects prevention study (1998–2007): Birth prevalence and descriptive epidemiology. *Am J Med Genet A* **164**: 2581–2591.
- Liu X, Francis R, Kim AJ, Ramirez R, Chen G, Subramanian R, Anderton S, Kim Y, Wong L, Morgan J, et al. 2014. Interrogating congenital heart defects with noninvasive fetal echocardiography in a mouse forward genetic screen. *Circ Cardiovasc Imaging* **7**: 31–42.
- Lobo J, Zariwala MA, Noone PG. 2015. Primary ciliary dyskinesia. *Semin Respir Crit Care Med* **36**: 169–179.
- MacDonald BT, Tamai K, He X. 2009. Wnt/ β -catenin signaling: Components, mechanisms, and diseases. *Dev Cell* **17**: 9–26.
- Marelli A, Miller SB, Marino BS, Jefferson AL, Newburger JW. 2016. Brain in congenital heart disease across the lifespan: The cumulative burden of injury. *Circulation* **133**: 1951–1962.
- May-Simera HL, Kelley MW. 2012. Cilia, Wnt signaling, and the cytoskeleton. *Cilia* **1**: 7.
- McGrath J, Somlo S, Makova S, Tian X, Brueckner M. 2003. Two populations of node monocilia initiate left–right asymmetry in the mouse. *Cell* **114**: 61–73.
- Montcouquiol M, Rachel RA, Lanford PJ, Copeland NG, Jenkins NA, Kelley MW. 2003. Identification of *Vangl2* and *Scrib1* as planar polarity genes in mammals. *Nature* **423**: 173–177.
- Nakamura T, Hamada H. 2012. Left–right patterning: Conserved and divergent mechanisms. *Development* **139**: 3257–3262.
- Nakhleh N, Francis R, Giese RA, Tian X, Li Y, Zariwala MA, Yagi H, Khalifa O, Kureshi S, Chatterjee B, et al. 2012.

Cilia and Ciliopathies in Congenital Heart Disease

- High prevalence of respiratory ciliary dysfunction in congenital heart disease patients with heterotaxy. *Circulation* **125**: 2232–2242.
- Newburger JW, Sleeper LA, Bellinger DC, Goldberg CS, Tabbutt S, Lu M, Mussatto KA, Williams IA, Gustafson KE, Mital S, et al. 2012. Early developmental outcome in children with hypoplastic left heart syndrome and related anomalies: The single ventricle reconstruction trial. *Circulation* **125**: 2081–2091.
- Nomura-Kitabayashi A, Phoon CK, Kishigami S, Rosenthal J, Yamauchi Y, Abe K, Yamamura K, Samtani R, Lo CW, Mishina Y. 2009. Outflow tract cushions perform a critical valve-like function in the early embryonic heart requiring BMPRIA-mediated signaling in cardiac neural crest. *Am J Physiol Heart Circ Physiol* **297**: H1617–H1628.
- Nonaka S, Shiratori H, Saijoh Y, Hamada H. 2002. Determination of left–right patterning of the mouse embryo by artificial nodal flow. *Nature* **418**: 96–99.
- Oh EC, Katsanis N. 2013. Context-dependent regulation of Wnt signaling through the primary cilium. *J Am Soc Nephrol* **24**: 10–18.
- Oyen N, Poulsen G, Boyd HA, Wohlfahrt J, Jensen PK, Melbye M. 2009. Recurrence of congenital heart defects in families. *Circulation* **120**: 295–301.
- Paudyal A, Damrau C, Patterson VL, Ermakov A, Formstone C, Lalanne Z, Wells S, Lu X, Norris DP, Dean CH, et al. 2010. The novel mouse mutant, *chuzhoi*, has disruption of Ptk7 protein and exhibits defects in neural tube, heart and lung development and abnormal planar cell polarity in the ear. *BMC Dev Biol* **10**: 87.
- Phillips HM, Rhee HJ, Murdoch JN, Hildreth V, Peat JD, Anderson RH, Copp AJ, Chaudhry B, Henderson DJ. 2007. Disruption of planar cell polarity signaling results in congenital heart defects and cardiomyopathy attributable to early cardiomyocyte disorganization. *Circ Res* **101**: 137–145.
- Potts JD, Runyan RB. 1989. Epithelial-mesenchymal cell transformation in the embryonic heart can be mediated, in part, by transforming growth factor β . *Dev Biol* **134**: 392–401.
- Rash JE, Shay JW, Bieseke JJ. 1969. Cilia in cardiac differentiation. *J Ultrastruct Res* **29**: 470–484.
- Reiter JE, Blacque OE, Leroux MR. 2012. The base of the cilium: Roles for transition fibres and the transition zone in ciliary formation, maintenance and compartmentalization. *EMBO Rep* **13**: 608–618.
- Ross AJ, May-Simera H, Eichers ER, Kai M, Hill J, Jagger DJ, Leitch CC, Chapple JP, Munro PM, Fisher S, et al. 2005. Disruption of Bardet–Biedl syndrome ciliary proteins perturbs planar cell polarity in vertebrates. *Nat Genet* **37**: 1135–1140.
- Sanford LP, Ormsby I, Gittenberger-de Groot AC, Sariola H, Friedman R, Boivin GP, Cardell EL, Doetschman T. 1997. TGF- β 2 knockout mice have multiple developmental defects that are non-overlapping with other TGF- β knockout phenotypes. *Development* **124**: 2659–2670.
- Schlessinger K, Hall A, Tolwinski N. 2009. Wnt signaling pathways meet Rho GTPases. *Genes Dev* **23**: 265–277.
- Shapiro AJ, Davis SD, Ferkol T, Dell SD, Rosenfeld M, Olivier KN, Sagel SD, Milla C, Zariwala MA, Wolf W, et al. 2014. Laterality defects other than situs inversus totalis in primary ciliary dyskinesia: Insights into situs ambiguus and heterotaxy. *Chest* **146**: 1176–1186.
- Shen Y, Leatherbury L, Rosenthal J, Yu Q, Pappas MA, Wessels A, Lucas J, Siegfried B, Chatterjee B, Svenson K, et al. 2005. Cardiovascular phenotyping of fetal mice by noninvasive high-frequency ultrasound facilitates recovery of ENU-induced mutations causing congenital cardiac and extracardiac defects. *Physiol Genomics* **24**: 23–36.
- * Shinohara K, Hamada H. 2016. Cilia in left–right symmetry breaking. *Cold Spring Harb Perspect Biol* doi: 10.1101/cshperspect.a028282.
- Simons M, Mlodzik M. 2008. Planar cell polarity signaling: From fly development to human disease. *Annu Rev Genet* **42**: 517–540.
- Simons M, Gloy J, Ganner A, Bullerkotte A, Bashkurov M, Kronig C, Schermer B, Benzing T, Cabello OA, Jenny A, et al. 2005. Inversin, the gene product mutated in nephronophthisis type II, functions as a molecular switch between Wnt signaling pathways. *Nat Genet* **37**: 537–543.
- Sinha T, Wang B, Evans S, Wynshaw-Boris A, Wang J. 2012. Disheveled mediated planar cell polarity signaling is required in the second heart field lineage for outflow tract morphogenesis. *Dev Biol* **370**: 135–144.
- Slough J, Cooney L, Brueckner M. 2008. Monocilia in the embryonic mouse heart suggest a direct role for cilia in cardiac morphogenesis. *Dev Dyn* **237**: 2304–2314.
- Sorokin S. 1962. Centrioles and the formation of rudimentary cilia by fibroblasts and smooth muscle cells. *J Cell Biol* **15**: 363–377.
- Srouf M, Schwartzentruber J, Hamdan FF, Ospina LH, Patry L, Labuda D, Massicotte C, Dobrzyniecka S, Capo-Chichi JM, Papillon-Cavanagh S, et al. 2012. Mutations in *C5ORF42* cause Joubert syndrome in the French Canadian population. *Am J Hum Genet* **90**: 693–700.
- Stottmann RW, Tran PV, Turbe-Doan A, Beier DR. 2009. *Ttc21b* is required to restrict Sonic Hedgehog activity in the developing mouse forebrain. *Dev Biol* **335**: 166–178.
- Tada M, Smith JC. 2000. Xwnt11 is a target of *Xenopus* Brachyury: Regulation of gastrulation movements via Dishevelled, but not through the canonical Wnt pathway. *Development* **127**: 2227–2238.
- Tan SY, Rosenthal J, Zhao XQ, Francis RJ, Chatterjee B, Sabol SL, Linask KL, Bracero L, Connolly PS, Daniels MP, et al. 2007. Heterotaxy and complex structural heart defects in a mutant mouse model of primary ciliary dyskinesia. *J Clin Invest* **117**: 3742–3752.
- Tobin JL, Beales PL. 2009. The nonmotile ciliopathies. *Genet Med* **11**: 386–402.
- van der Bom T, Bouma BJ, Meijboom FJ, Zwinderman AH, Mulder BJ. 2012. The prevalence of adult congenital heart disease, results from a systematic review and evidence based calculation. *Am Heart J* **164**: 568–575.
- Verzi MP, McCulley DJ, De Val S, Dodou E, Black BL. 2005. The right ventricle, outflow tract, and ventricular septum comprise a restricted expression domain within

N.T. Klena et al.

- the secondary/anterior heart field. *Dev Biol* **287**: 134–145.
- von Gise A, Pu WT. 2012. Endocardial and epicardial epithelial to mesenchymal transitions in heart development and disease. *Circ Res* **110**: 1628–1645.
- Wallingford JB, Mitchell B. 2011. Strange as it may seem: The many links between Wnt signaling, planar cell polarity, and cilia. *Genes Dev* **25**: 201–213.
- Washington Smoak I, Byrd NA, Abu-Issa R, Goddeeris MM, Anderson R, Morris J, Yamamura K, Klingensmith J, Meyers EN. 2005. Sonic hedgehog is required for cardiac outflow tract and neural crest cell development. *Dev Biol* **283**: 357–372.
- Willaredt MA, Gorgas K, Gardner HA, Tucker KL. 2012. Multiple essential roles for primary cilia in heart development. *Cilia* **1**: 23.
- Yoshida S, Shiratori H, Kuo IY, Kawasumi A, Shinohara K, Nonaka S, Asai Y, Sasaki G, Belo JA, Sasaki H, et al. 2012. Cilia at the node of mouse embryos sense fluid flow for left–right determination via Pkd2. *Science* **338**: 226–231.
- Zaidi S, Choi M, Wakimoto H, Ma L, Jiang J, Overton JD, Romano-Adesman A, Bjornson RD, Breitbart RE, Brown KK, et al. 2013. De novo mutations in histone-modifying genes in congenital heart disease. *Nature* **498**: 220–223.

Cold Spring Harbor Perspectives in Biology

Cilia and Ciliopathies in Congenital Heart Disease

Nikolai T. Klena, Brian C. Gibbs and Cecilia W. Lo

Cold Spring Harb Perspect Biol 2017; doi: 10.1101/cshperspect.a028266 originally published online February 3, 2017

Subject Collection [Cilia](#)

Ciliary Mechanisms of Cyst Formation in Polycystic Kidney Disease

Ming Ma, Anna-Rachel Gallagher and Stefan Somlo

Photoreceptor Cilia and Retinal Ciliopathies

Kinga M. Bujakowska, Qin Liu and Eric A. Pierce

G-Protein-Coupled Receptor Signaling in Cilia

Kirk Mykytyn and Candice Askwith

Evolution of Cilia

David R. Mitchell

Transition Zone Migration: A Mechanism for Cytoplasmic Ciliogenesis and Postaxonemal Centriole Elongation

Tomer Avidor-Reiss, Andrew Ha and Marcus L. Basiri

Cilia and Obesity

Christian Vaisse, Jeremy F. Reiter and Nicolas F. Barbari

Posttranslational Modifications of Tubulin and Cilia

Dorota Wloga, Ewa Joachimiak, Panagiota Louka, et al.

Cilia in Left–Right Symmetry Breaking

Kyosuke Shinohara and Hiroshi Hamada

Discovery, Diagnosis, and Etiology of Craniofacial Ciliopathies

Elizabeth N. Schock and Samantha A. Brugmann

Axoneme Structure from Motile Cilia

Takashi Ishikawa

Cilia and Ciliopathies in Congenital Heart Disease

Nikolai T. Klena, Brian C. Gibbs and Cecilia W. Lo

Sperm Sensory Signaling

Dagmar Wachten, Jan F. Jikeli and U. Benjamin Kaupp

Primary Cilia and Coordination of Receptor Tyrosine Kinase (RTK) and Transforming Growth Factor β (TGF- β) Signaling

Søren T. Christensen, Stine K. Morthorst, Johanne B. Mogensen, et al.

Primary Cilia and Mammalian Hedgehog Signaling

Fiona Bangs and Kathryn V. Anderson

For additional articles in this collection, see <http://cshperspectives.cshlp.org/cgi/collection/>

**All Modifications and
Oligo Types Synthesized**

Long Oligos • Fluorescent • Chimeric • DNA • RNA • Antisense

Oligo Modifications?

Your wish is our command.

Radial Spokes—A Snapshot of the Motility Regulation, Assembly, and Evolution of Cilia and Flagella

Xiaoyan Zhu, Yi Liu and Pinfen Yang

Cilia and Mucociliary Clearance

Ximena M. Bustamante-Marin and Lawrence E. Ostrowski

For additional articles in this collection, see <http://cshperspectives.cshlp.org/cgi/collection/>

**All Modifications and
Oligo Types Synthesized**

Oligo Modifications?
Your wish is our command.

Long Oligos • Fluorescent • Chimeric • DNA • RNA • Antisense