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How insights from cardiovascular developmental biology have impacted the care of infants and children with congenital heart disease

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Abstract

To illustrate the impact developmental biology and genetics have already had on the clinical management of the million infants born worldwide each year with CHD, we have chosen three stories which have had particular relevance for pediatric cardiologists, cardiothoracic surgeons, cardiac anesthesiologists, and cardiac nurses. First, we show how Margaret Kirby's finding of the unexpected contribution of an ectodermal cell population – the cranial neural crest – to the aortic arch arteries and arterial pole of the embryonic avian heart provided a key impetus to the field of cardiovascular patterning. Recognition that a majority of patients affected by the neurocristopathy DiGeorge syndrome have a chromosome 22q11 deletion, have also spurred tremendous efforts to characterize the molecular mechanisms contributing to this pathology, assigning a major role to the transcription factor Tbx1. Second, synthesizing the work of the last two decades by many laboratories on a wide gamut of metazoans (invertebrates, tunicates, agnathans, teleosts, lungfish, amphibians, and amniotes), we review the >20 major modifications and additions to the ancient circulatory arrangement composed solely of a unicameral (one-chambered), contractile myocardial tube and a short proximal aorta. Two changes will be discussed in detail – the interposition of a second cardiac chamber in the circulation and the septation of the cardiac ventricle. By comparing the developmental genetic data of several model organisms, we can better understand the origin of the various components of the multicameral (multi-chambered) heart seen in humans. Third, Martina Brueckner's discovery that a faulty axonemal dynein was responsible for the phenotype of the *iv*/*iv* mouse (the first mammalian model of human heterotaxy) focused attention on the biology of cilia. We discuss how even the care of the complex cardiac and non-cardiac anomalies seen in

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heterotaxy syndrome, which have long seemed impervious to advancements in surgical and medical intensive care (Jacobs et al., 2011), may yet yield to strategies grounded in a better understanding of the cilium. The fact that all cardiac defects seen in patients with full-blown heterotaxy can also be seen in patients without obvious laterality defects hints at important roles for ciliary function not only in left-right axis specification but also in cardiovascular morphogenesis. These three developmental biology stories illustrate how the remaining unexplained mortality and morbidity of congenital heart disease can be solved.

Keywords

"neural crest"; "right ventricle"; "DiGeorge Syndrome"; "heterotaxy syndrome"; cilium; "congenital heart disease"

Introduction

In the 50 years since cardiothoracic surgeons began to correct congenital heart disease (CHD), which constitutes by far the most common fatal birth defect in the human newborn, early postoperative survival has improved from <5% to 95% in the USA (Welke et al., 2009). This dramatic turnaround in the fate of the 1 in 300 infants born with lethal cardiovascular developmental anomalies is one of the great successes of modern medicine (Norwood, 2010). However, there has been little additional progress in early survival since 1999, and long-term (20-year) outcomes have been unexpectedly poor for some subgroups, such as children with "functional single ventricle" - underdevelopment of one ventricular chamber (Khairy et al., 2008). As recently as 2008, the number of deaths from CHD was still double those from all forms of childhood cancer combined (http://www.heart.org/idc/groups/heart-public/@wcm/@sop/@smd/documents/downloadable/ucm_319830.pdf; http://www.cancer.org/acs/groups/content/@nho/documents/document/2008cafffinalsecuredpdf.pdf).

The available evidence suggests that the main reasons for the improvement plateau as well as the surprisingly high attrition among CHD patients over the long-term are: (a) the failure to understand the underlying basis for the CHD in most cases, including the presence of coexisting non-cardiac anomalies, (b) the fragmentary nature of current clinical databases (Welke et al., 2009; Williams, 2010) hampering our ability to correlate genotype with phenotype, and (c) the lack of a rigorous understanding of the performance characteristics of Francis Fontan's operation for "single ventricle" disorders. As last year was the 40th anniversary of Fontan's original report (Fontan and Baudet, 1971) describing a way to redirect the systemic venous flow directly into the pulmonary arteries while confining the pulmonary venous flow to the systemic arterial side of the circulation, the surgical treatment of univentricular disorders has been the subject of several recent reviews (Kreutzer et al., 2010; Chin et al., 2010) and will not be covered here.

The proportion of CHD cases likely to be genetic in etiology is at least one third (Formigari et al., 2009) and possibly far higher. Furthermore, in the case of conotruncal disorders occurring in DiGeorge syndrome, we already know that extracardiac anomalies play a major role in the early postoperative course (Ziolkowska et al., 2008; Shen et al., 2011) as well as in long-term outcome (Carotti et al., 2010). Hence, even with the anticipated availability of affordable whole genome sequencing within the next decade, characterizing the full phenotypic spectrum accompanying each genetic alteration will be necessary to optimize the short and long term care of individuals affected by CHD. Since the combination of full-body high-resolution imaging and serum proteomic profiling will remain prohibitively expensive

over that time period, a first step is to better understand genotype-phenotype correlations in model organisms. To illustrate the impact developmental biology and genetics have already had on the clinical management of the million infants born worldwide each year with CHD, we have chosen three stories which have had particular relevance for pediatric cardiologists, cardiothoracic surgeons, cardiac anesthesiologists, and cardiac nurses.

First, we show how Margaret Kirby's finding of the unexpected contribution of an ectodermal cell population – the cranial neural crest – to the aortic arch arteries and arterial pole of the embryonic avian heart provided a key impetus to the field of cardiovascular patterning. Recognition that a majority of patients affected by the neurocristopathy DiGeorge syndrome have a chromosome 22q11 deletion, have also spurred tremendous efforts to characterize the molecular mechanisms contributing to this pathology, assigning a major role to the transcription factor Tbx1. Second, synthesizing the work of the last two decades by many laboratories on a wide gamut of metazoans (invertebrates, tunicates, agnathans, teleosts, lungfish, amphibians, and amniotes), we review the >20 major modifications and additions to the ancient circulatory arrangement composed solely of a unicameral (one-chambered), contractile myocardial tube and a short proximal aorta. Two changes will be discussed in detail – the interposition of a second cardiac chamber in the circulation and the septation of the cardiac ventricle. By comparing the developmental genetic data of several model organisms, we can better understand the origin of the various components of the multicameral (multi-chambered) heart seen in humans. Third, Martina Brueckner's discovery (Supp et al., 1997) that a faulty axonemal dynein was responsible for the phenotype of the *iv*/*iv* mouse (the first mammalian model of human heterotaxy) focused attention on the biology of cilia. We discuss how even the care of the complex cardiac and non-cardiac anomalies seen in heterotaxy syndrome, which have long seemed impervious to advancements in surgical and medical intensive care (Jacobs et al., 2011), may yet yield to strategies grounded in a better understanding of the cilium. The fact that all cardiac defects seen in patients with full-blown heterotaxy can also be seen in patients without obvious laterality defects hints at important roles for ciliary function not only in left-right axis specification but also in cardiovascular morphogenesis. These three developmental biology stories illustrate how the remaining unexplained mortality and morbidity of congenital heart disease can be solved.

On the origin of the cardiac neural crest

Almost 30 years ago, Margaret Kirby and her colleagues published the first evidence linking neural crest (NC) and heart development (Kirby et al., 1983). In this seminal work, sixteen chick embryos were subjected to a bilateral ablation of the NC over somites 1 to 3, a region then referred to as the occipital NC. Among these embryos, fifteen developed aorticopulmonary septal defects, fourteen of which were classified as persistent truncus arteriosus (Kirby et al., 1983), a phenotype well known to occur in humans. This finding was supported by quail-chick chimeras experiments, demonstrating the specific contribution of grafted quail NC cells to the septum of the host chick outflow tract and to the tunica media of the aorta and pulmonary trunk (Kirby et al., 1983). This work was the starting point of the ongoing quest to understand the specific requirements of the cardiac NC in cardiovascular development and its role in the pathogenesis of CHD.

The fate-map of the mammalian cardiac NC took much longer to describe due to the lack of appropriate markers to follow the cardiac NC, and the difficulty of manipulating embryonic tissues in the developing mouse. In mammals, the cardiac NC was first mapped using a transgenic line in which Lac-Z expression was driven by a 6.5kb upstream genomic fragment of the *connexin 43* gene (Lo et al., 1997), which is transiently expressed in NC cells and their derivatives (Waldo et al., 1999). Three years later, *Cre-Lox* technology was

used to lineage labeled NC cells in *Wnt1-Cre* and *Pax3-Cre* mice (Jiang et al., 2000; Li et al., 2000). From these studies in chick and mouse embryos, it is now well established that the cardiac NC represents a subdivision of the cranial NC, originating from the dorsal neural tube between the middle of the otic placode to the caudal border of somite 4, corresponding to rhombomeres 6–8 of the hindbrain (Fig. 1A; reviewed in Brown and Baldwin, 2006; Snider et al., 2007; Hutson and Kirby, 2007). Cardiac NC cells migrate through pharyngeal arches 3, 4 and 6, and contribute to the pharyngeal glands, thymus, thyroid and parathyroid (Le Lievre and Le Douarin, 1975; Bockman and Kirby, 1984). They also form the smooth muscle layer of the great arteries and play an important role in the remodeling of the five pairs of bilaterally symmetric pharyngeal arch arteries (numbered 1, 2, 3, 4, and 6; the fifth pharyngeal arch arteries are absent in amniotes) that eventually develop into the ascending aorta, proximal subclavian, carotid, and pulmonary arteries as well as the ductus arteriosus (Waldo et al., 1996; Jiang et al., 2000). A subset of cardiac NC cells migrate into the outflow tract cushions of the heart, where they form the spiral aorticopulmonary septum, which divides the truncus into the aorta and pulmonary arteries, thereby separating the systemic and pulmonary circulations at the arterial pole (Kirby et al., 1983; Jiang et al., 2000). A recent study suggests that the NC cells populating the outflow tract cushions also promote semilunar valve leaflet remodeling later in gestation (Jain et al., 2011). The parasympathetic innervation to the heart is also partially derived from the cardiac NC (Kirby and Stewart, 1983; Pietri et al., 2003). Finally, a number of lineage studies have proposed that the mammalian cardiac NC contributes to the myocardium and epicardium, however these observations remain controversial (reviewed in Stoller and Epstein, 2005).

Several heart defects are associated with cardiac NC ablation in birds and mouse embryos; however, conotruncal dysmorphologies are the most frequent (Kirby et al., 1983; 1985; Porras and Brown, 2008). They include persistent truncus arteriosus and tetralogy of Fallot (the abnormal positioning, or “overriding”, of the aorta over a large ventricular septal defect permitting persistent communication between the two ventricular chambers) (reviewed in Creazzo et al., 1998). These defects are also present in a number of mouse mutants with cardiac NC deficiencies, with one of the best-studied examples, the *Pax3* mouse mutant *Splotch* (Conway et al., 1997; Li et al, 1999; Epstein et al., 2000). *Pax3* is normally expressed in NC progenitors at the dorsal aspect of the neural tube. In the absence of Pax3 function, the NC progenitors pool fails to expand and to complete its migration resulting in scarce cardiac NC derivatives (Conway et al., 1997; Conway et al., 2000). Homozygous *Splotch* mice die *in utero* with persistent truncus arteriosus and pharyngeal arch patterning defects (Conway et al., 1997; Epstein et al., 2000).

Like the rest of the NC, cardiac NC cells migrate long distances to reach their final location and are extremely sensitive to influences from adjacent tissues. For that reason, deletion of genes involved in NC migration and patterning can also result in similar cardiac defects in a cell-autonomous or non-cell-autonomous manner. This is the case, for example, with the secreted ligand Semaphorin 3C (Sema3C), an important regulator of cardiac NC cell migration into the outflow tract. Sema3C is not expressed by cardiac NC cells but is expressed by the outflow tract myocardium where it acts as a chemoattractant for the cardiac NC. Targeted deletion of *Sema3C* in mice causes interrupted aortic arch and persistent truncus arteriosus (Feiner et al., 2001). The cardiac NC cells express the semaphorin receptor, PlexinA2, and *PlexinA2* mutant mouse embryos also display persistent truncus arteriosus and interrupted aortic arch (Brown et al., 2001).

Over the last 10 years, a fairly long list of molecules acting in a cell-autonomous or non-cell-autonomous fashion during cardiac NC development has been associated with cardiovascular defects in mouse mutants. These cardiac anomalies can be the result of altered migration, proliferation, survival, or differentiation of cardiac NC progenitors

(reviewed in Brown and Baldwin, 2006; Hutson and Kirby, 2007; Snider et al., 2007; Scholl and Kirby, 2009; Nelms and Labosky, 2010). These studies point to a very complex network of genes regulating the development of this cell population and its function in the morphogenesis of the cardiovascular system.

Cardiac NC and the clinical features of 22q11 deletion syndrome

Approximately 10% of patients with conotruncal defects carry a hemizygous microdeletion of Chromosome 22q11 (Lammer et al., 2009). More than half of patients with DiGeorge syndrome (DGS), a condition in which several derivatives of the cardiac NC are affected, have a chromosome 22q11 deletion (Greenberg, 1993). Clinical manifestations include heart defects, craniofacial dysmorphology with or without cleft palate, immunodeficiency and hypocalcemia due to thymus and parathyroid hypoplasia, respectively. Up to one-third of all individuals carrying the 22q11.2 deletion develop schizophrenia or schizoaffective disorder (Murphy, 2002; Karayiorgou, 2010). Similar deletions of the 22q11 region were found in patients with Velo-cardio-facial syndrome (VCFS) and conotruncal anomaly face syndrome (CAFS) (reviewed in Shprintzen, 2008), two other syndromes that share most of the phenotypic attributes of DGS, suggesting a common etiology. DGS, VCFS, and CAFS are now grouped together as the 22q11 deletion syndrome (reviewed in Yamagishi and Srivastava, 2003; Kobrinsky and Sullivan, 2007) with an estimated incidence of 1:4000 live births.

The range of defects seen in the 75% of 22q11 deletion syndrome patients with CHD include, (i) aberrant right subclavian artery (Fig. 2) in patients with left aortic arch; (ii) interruption of the aortic arch type B (Fig. 2), due to abnormal development of the left fourth pharyngeal arch artery, (iii) persistent truncus arteriosus usually accompanied by a large ventricular septal defect (reviewed in Momma, 2010), (iv) tetralogy of Fallot, and (v) tetralogy of Fallot with pulmonary atresia, characterized by malalignment of the great arteries over the ventricular chambers. Importantly, 22q11 deletion is not only a predictor of early postoperative hypocalcemia (Shen et al., 2011), but it is also an independent risk factor for late mortality in tetralogy of Fallot with pulmonary atresia (Carotti et al., 2010).

Around 90% of 22q11 deletion syndrome patients have a deleted region of ~3.0 Mb, which contains 60 genes, while the remaining patients have a nested deletion of ~1.5 Mb spanning a region of 35 genes (reviewed in Karayiorgou, 2010). Candidate genes to explain the phenotypes observed in hemizygous individuals include *HIRA*, encoding a WD40 repeat protein involved in chromatin remodeling (Wilming et al., 1997; Farrell et al., 1999), *UFD1L*, encoding a factor involved in ubiquitin-mediated protein processing (Pizzuti et al., 1997) and *CRKL*, encoding an adaptor protein that is implicated in the response to growth factor and focal adhesion signaling (Guris et al., 2001). The strongest candidate, however, is the transcription factor, *TBX1* (Chieffo et al., 1997). Since the 22q11.2 deletion is the commonest microdeletion syndrome encountered in humans (Botto et al., 2003), efforts to identify isolated *TBX1* deletions or *TBX1* point mutations in DGS patients without the large 1.5 Mb or 3.0 Mb 22q11 deletions were initially unsuccessful. In 2003, three *TBX1* point mutations were finally identified in a cohort of DGS patients in Japan (Yagi et al., 2003). These mutations were associated with the expected CHD, providing a strong link between TBX1 function, cardiovascular development and the etiology of DGS.

Homozygous *Tbx1* mutant mouse embryos have outflow tract septation defects and all the features of a severe DGS phenotype (Jerome and Papaioannou, 2001; Vitelli et al., 2002a). Haploinsufficiency of *Tbx1* does not account for all the clinical manifestations associated with 22q11 deletion syndrome; although these animals have aortic arch defects (Lindsay et al., 2001; Merscher et al., 2001), the craniofacial component of the disease is rarely

observed. By contrast, human patients with hemizygosity for 22q11 manifest both outflow tract and aortic arch defects as well as craniofacial malformations, suggesting inter-species and tissue-specific differences in *Tbx1* dosage sensitivity. It is also important to mention that the severity of the malformations in 22q11 deletion syndrome patients is highly variable, suggesting that other genetic and epigenetic modifiers are involved in the presentation of the disease.

The role of Tbx1 in 22q11 deletion syndrome

Tbx1 is a transcriptional activator that belongs to the T-box gene family, a class of genes implicated in several aspects of cardiac development (reviewed in Greulich et al., 2011). Because *Tbx1* is not expressed by premigratory cardiac NC cells or by migrating NC cells as they populate the pharyngeal arches, it is important to emphasize that the defects of NC-derived tissues in 22q11 deletion syndrome are occurring in a non-cell autonomous fashion. Instead, *Tbx1* is expressed in all other components of the pharyngeal arch ectoderm, endoderm and mesoderm (Lindsay et al., 2001; Jerome and Papaioannou, 2001; Merscher et al., 2001), as well as in the anterior-most region of the cardiogenic mesoderm, the precursor of the outflow tract (Xu et al., 2004). This expression pattern appears to be well conserved across species (Garg et al., 2001; Piotrowski et al., 2003; Ataliotis et al., 2005; Showell et al., 2006).

The tissue requirements for *Tbx1* have been analyzed using a variety of transgenic Cre lines and a conditional *Tbx1* allele to mediate tissue-specific inactivation of *Tbx1* in components of the pharyngeal arches and in the cardiogenic mesoderm (reviewed in Scambler, 2010). Pharyngeal mesoderm-specific deletion of *Tbx1* using *Mesp1-Cre* resulted in mouse mutants resembling *Tbx1*-nulls, with severe pharyngeal patterning and cardiovascular defects suggesting a fundamental role for Tbx1 in this tissue (Zhang et al., 2006). However, reactivation of *Tbx1* expression in the mesoderm of the mutants corrected the outflow tract septation defect but not the aortic arch phenotype (Zhang et al., 2006), suggesting that the aortic arch defects might be secondary to the pharyngeal segmentation defect. Conditional inactivation of *Tbx1* in the cardiogenic mesoderm with *Nkx2.5-Cre* recapitulates the outflow tract septation defect, but not the aortic arch patterning phenotype of *Tbx1* homozygote mutants (Xu et al., 2004). Therefore, it has been proposed that *Tbx1* is primarily required in the anterior-most region of the cardiogenic mesoderm to regulate the growth and morphogenesis of the outflow tract, which is a prerequisite for its proper colonization by the cardiac NC (Xu et al., 2004).

A number of studies have reported a genetic link between Fibroblast growth factor 8 (Fgf8), expressed in the pharyngeal endoderm, and the 22q11 phenotype (Abu-Issa et al., 2002; Frank et al., 2002; Vitelli et al., 2002b). Mouse mutants for Fgf8 exhibit the craniofacial and cardiovascular defects typically seen in 22q11 deletion syndrome patients (Frank et al., 2002). *Fgf8* expression is downregulated in the pharyngeal endoderm of *Tbx1* mutants, and double *Tbx1-Fgf8* heterozygote mutants have a higher incidence of aortic arch defects than either single heterozygote mutant (Vitelli et al., 2002b). These observations are consistent with a model in which *Tbx1* regulates *Fgf8* in the pharyngeal endoderm, which then signals to the adjacent cardiac NC cells to presumably promote their proliferation and/or survival (Abu-Issa et al., 2002; Zhang et al., 2006).

A more recent study has further refined this regulatory cascade providing novel information linking Tbx1 and Fgf8 (Guo et al., 2011). Double homozygote mutant mice for the transcription factor *Six1* and its coactivator *Eya1* exhibit a phenotype similar to *Tbx1* and *Fgf8* null embryos, including all the major craniofacial and cardiovascular defects seen in these mutants. *Six1/Eya1* double null mutants show reduced *Fgf8* expression in the

pharyngeal ectoderm and endoderm and in the cardiogenic mesoderm, lineages in which *Six1* and *Fgf8* are normally co-expressed. Altered cell proliferation and survival in all these tissues appear to be the cause of the cardiovascular phenotypes in *Six1*/*Eya1* mutants (Guo et al., 2011), similar to what was reported for Tbx1 and Fgf8 mouse mutants (Abu-Issa et al., 2002; Zhang et al., 2006). Using chromatin immunoprecipitation, the authors also demonstrate that the Six1/Eya1 transcription complex directly regulates *Fgf8* gene expression *in vivo* (Guo et al., 2011). Overall this study provides strong genetic evidence that a regulatory pathway consisting of *Tbx1* → *Six1/Eya1* → *Fgf8* is critical for the morphogenesis of cardiac and craniofacial structures. This suggests that mutations in *SIX1* and *EYA1* may contribute to the pathogenesis of 22q11 deletion-like syndromes (Guo et al., 2001), in addition to the well-documented association of *SIX1* and *EYA1* with branchio-oto-renal syndrome (reviewed in Kochhar et al., 2007).

The analysis of mouse models has provided invaluable information on the roles of TBX1 in the pathogenesis of 22q11 deletion syndrome. While Tbx1 does not directly regulate cardiac NC development, Tbx1 plays a critical role in multiple tissues with which the cardiac NC interacts during its migration, and as such, Tbx1 has a major impact on the ultimate fate of these cells. Efforts in recent years have been focused on characterization of the targets and pathways regulated by Tbx1 and upstream regulators of *Tbx1* expression. This knowledge will be critical to understand the molecular mechanisms controlling the deployment of the cardiac NC and the conotruncal defects associated with 22q11 deletion syndrome. This is the starting point for understanding the risk factors for this class of human CHD.

Considerations on the evolution of the cardiac NC

While the cardiac NC has a critical function in outflow tract septation and in remodeling the aortic arch arteries in birds and mammals, its role in lower vertebrates is not as well understood. Zebrafish do not have separate systemic and pulmonary circulation and lack an outflow tract septum, but still possess a cardiac NC. Therefore, the existence of a cardiac NC population predated the need for a separated pulmonary circulation. In zebrafish, the cardiac NC arises from a much broader region of the hindbrain, anterior to the otic vesicle, and has been reported to contribute myocardial cells to all regions of the developing heart, including the outflow tract, atrium and ventricle (Sato and Yost, 2003; Sato et al., 2006; Li et al., 2003). With a single ventricular chamber, the separation of the pulmocutaneous and systemic blood is incomplete in amphibians, however, a spiral septum provides some separation at the level of the outflow tract (de Graaf, 1957; Farmer, 1999). A recent study using *Xenopus laevis* indicates, however, that the cardiac NC is not required for outflow tract septation. In this organism the spiral septum is exclusively derived from the anterior-most region of the cardiogenic mesoderm (Lee and Saint-Jeannet, 2011). In *Xenopus*, cardiac NC cells populate the aortic sac and arch arteries but halt their migration before entering the ouflow tract cushions.

How are these differences in the deployment of cardiac NC across species explained? With the transition from an aquatic to a terrestrial life came numerous changes in the cardio-respiratory system, including the complete separation of the pulmonary and systemic circulation. One can speculate that to provide species with the evolutionary advantage of a fully divided circulation, the NC was recruited into the outflow tract septum to complete the separation of the blood at the arterial pole of the heart. Since *Xenopus* is mostly aquatic, it may represent an exception among amphibians; therefore, it would be interesting to analyze the migration pattern of the cardiac NC in amphibian species that have a predominantly terrestrial lifestyle. If pulmonary respiration has been the evolutionary pressure to redirect migration of the cardiac NC into the outflow tract to complete the separation of the systemic and pulmonary circulations at the arterial pole, one would expect to find cardiac NC cells

infiltrating the outflow tract of terrestrial amphibians. The lungfish (*Protopterus*) is one of the few fish species that possess true lungs. Unlike most amphibians, lungfish have a more complete separation of oxygenated and deoxygenated blood through its cardiac cavities, with a partially septated ventricle and an outflow tract lined with two ridges fused at the distal end providing an incomplete division of the outflow tract (Icardo et al., 2005b). This is an organism in which the fate of the cardiac NC has not been investigated, however, its study may provide important clues on the underlying processes driving the evolution of this cell population.

The fate and the migration pattern of the cardiac NC appears to vary considerably among species, from a broad colonization of all regions of the heart including cardiomyocytes in zebrafish (Sato and Yost, 2003; Sato et al., 2006; Li et al., 2003), to a much more constrained migration that stops short of entering the outflow tract in *Xenopus* (Lee and Saint-Jeannet, 2011), to a narrowly targeted migration into the outflow tract cushions in chick and mouse (Kirby et al., 1983; Jiang et al., 2000) (Fig. 1B). Defining the factors that regulate the migration of cardiac NC cells once they exit the pharyngeal arches would be especially critical to understand these differences. Because *Xenopus* cardiac NC cells stop their migration before entering the outflow tract, *Xenopus* represents an excellent system in which to test factors that may promote the migration of these cells into the outflow tract cushions. Identification of such factors may provide important insights into the CHD resulting from abnormal deployment of the cardiac NC as seen in 22q11 deletion syndromes.

As indicated earlier the cardiac NC makes an important contribution to the autonomic innervation of the heart. The autonomic nervous system consists of two major divisions: the sympathetic and parasympathetic systems. The sympathetic system is organized in two bilateral chains of ganglia running parallel to the spinal cord. The parasympathetic system is lying in or near the innervated organs. In gnathostomes the autonomic innervation of the heart is almost entirely derived from the NC (reviewed in Hildreth et al, 2009). The sympathetic division comes from the trunk NC (Kirby et al., 1983; Jiang et al., 2000), while the parasympathetic system arises from the cardiac NC (Kirby and Stewart, 1983; Pietri et al., 2003), with additional contribution from the nodose placode providing sensory neurons to the distal ganglion of cranial nerve X or vagus nerve (D'Amico-Martel and Noden, 1983). The jawless vertebrates (agnathans), lamprey and hagfish, constitute the most basal vertebrate group, bearing many characteristics of the ancestral vertebrate. They possess NC cells and NC derivatives (McCauley and Bronner-Fraser 2003; Ota et al., 2007) but completely lack the sympathetic ganglia chains (Nicol, 1952; Haming et al., 2011). The lamprey is the first group of chordates with a vagal innervation of the heart, which is anatomically similar to the modern fish two-chambered heart. This innervation, however, differs from the rest of the vertebrates by being excitatory rather than inhibitory. The inhibitory action of the vagus nerve appeared with cartilagenous fishes, and the opposing excitatory sympathetic innervation evolved later with the emergence of bony fishes (reviewed in Burnstock 1969; Wang, 2011). Interestingly, unlike higher vertebrates, the vagal innervation of the heart in the lamprey is entirely placode derived (McCauley and Bronner-Fraser 2003), suggesting that the contribution of the cardiac NC to the parasympathetic division of the autonomic nervous system is a derived character in vertebrate evolution, which has presumably evolved in parallel with the specialization of the heart.

The rise of evolutionary developmental biology

While the chick (*Gallus gallus*) remained the dominant model system for the study of embryonic heart development for more than 10 years after Kirby's pioneering experiment, a

variety of other organisms have drawn investigators' attention since the early 1990s. Although invertebrates like *Drosophila melanogaster* (fruit fly) and *Ciona intestinalis* (sea squirt) and vertebrates like *Danio rerio* (zebrafish) and *Mus musculus* (mouse) have provided the bulk of the insights, lungfish species and amphibians (both anuran and urodele) have contributed in important ways as well. A more extensive history of the evolutionary approach to cardiac developmental biology, with particular focus on the development of the vasculature and the epicardium, was contributed by Pérez-Pomares et al. (2009). Nothing better illustrates the power of using a broad spectrum of animals to unravel the likely genetic and genomic drivers of a morphologically complex organ like the cardiovascular system than the saga of the transcription factor *Islet-1* (*Isl1*).

First identified as a protein which bound to a *cis*-regulatory element of the rat insulin I gene (Karlsson et al., 1990), Isl1 was rapidly recognized as playing a crucial role in motoneuron development. However, 8 years ago, Cai et al. (2003) reported that Isl1 was expressed in pharyngeal mesoderm and additionally required for proper heart development in the mouse embryo. *Isl1* persists postnatally in the outflow tract, right ventricle (RV), and right atrium of the mouse, and the *Isl1* null embryo dies at mid-gestation with a missing outflow tract and RV. Based on these observations, it was proposed that an *Isl1*-positive group of cells in the pharyngeal mesoderm contributing to the arterial pole of the heart evolved in amniotes specifically to give rise to the cardiac segments "associated with the pulmonary circulation" of air-breathing organisms, such as the RV (Olson, 2006). This *Isl1*-positive group of cells was given the name "the second heart field" (SHF) to distinguish them from progenitors of the left ventricle (LV) which were called "first heart field" (FHF). However, *Isl1* expression was also identified in the left atrium in the mouse postnatally as well as in a cardiac pluripotent cell population in both mouse and human (Bu et al., 2009). Of great interest to the field of regenerative medicine, the latter finding also argued for a broader and earlier embryonic function for the *Isl1* gene than just the fashioning of the outflow tract, RV, and right atrium. We would predict that not only should *Isl1* be among the earliest cardiac markers in the mouse embryo, but also that examination of *Isl1* in lower vertebrates and even invertebrates should reveal a required role for *Isl1* in cardiac progenitors.

Isl1 has indeed been identified in the precardiac mesoderm of mouse embryos (Cai et al., 2003; Prall et al., 2007). More importantly, analysis of the expression of *Isl1* homologs and their loss-of-function phenotypes in *Xenopus laevis* (Brade et al., 2007), zebrafish (de Pater et al., 2009), lamprey (Kokubo et al., 2010), and the fruit fly (Tao et al., 2007) confirms that *Isl1*'s role in the heart does not appear to be evolutionarily new. Since none of these animals has a septated ventricle, and since the fly has only one cardiac chamber, the evolutionarily ancient function of *Isl1* is in fact pancardiac, not "pulmonary circulation"-related.

In the zebrafish, in which atrial and ventricular precursors are situated near the margin in a ventral-to-dorsal configuration at 40% epiboly, just prior to the onset of gastrulation, fate mapping analysis at 44 hours post fertilization (hpf) reveals pharyngeal mesoderm progenitors at the dorsal-most extent of the ventricular "field" (Keegan et al., 2004) (Fig 3). Cells in this region of the blastula were also found to contribute to the outflow tract at 72 hpf by other investigators (Hami et al., 2011). By immunohistochemistry, Isl1 was observed in cardiac progenitors and pharyngeal mesoderm at 16 hpf as well as in pharyngeal mesoderm adjacent to the arterial pole at 24, 36, and 48, but not 72 hpf, and labeling of pharyngeal mesoderm with the lipophilic dye DiI allowed the tracking of descendants into the outflow tract at 48 and 72 hpf (Hami et al., 2011). By exploiting a photoconvertible fluorescent marker, Lazic and Scott (2011) showed that while the majority of addition of myocardial cells to the arterial pole occurred before 36 hpf, later addition of myocardial cells to the heart did occur and that the addition was exclusively to the arterial pole (Fig. 3). This late-added population of progenitors is *Mef2cb*-positive (Lazic and Scott, 2011) and *Ltbp3*-

positive (Zhou et al., 2011). These recent papers suggest both an early role for Isl1 as well as evolutionary conservation of late-added myocardial cells at the arterial pole.

Gessert and Kuhl (2009) showed robust *Isl1* expression in the cardiac progenitors at stage 12/13 in *Xenopus laevis* (late gastrula/ early neurula; roughly analogous to 15 hpf in zebrafish), continuing through stages 15/16 (neurula; similar to 18 hpf in zebrafish), 20 (end of neurulation; roughly equivalent to 22 hpf in zebrafish), and 24 (early tailbud; similar to 26 hpf in zebrafish). It then turns off at stage 24 in Tbx5-positive cells starting terminal differentiation but persists in a domain rostral to the Nkx2.5-positive cells; this rostral domain eventually contributes to the outflow tract.

In addition, a re-examination of the SHF and FHF in the mouse embryo suggests less disparity in *Isl1* expression than previously believed. Although cells descended from cardiovascular progenitors are heritably and irreversibly marked by recombination of Cre-activated reporter genes (Soriano, 1999), different floxed loci exhibit differential susceptibility to Cre recombination (Novak et al., 2000; Vooijs et al., 2001). A *Gata4*-based reporter, $Gata4^{flap}$, that was more susceptible to Cre recombination than a *Rosa26*-based reporter (Zhou et al., 2008), was recombined by $Isl1^{Cre}$ and $Nkx2\text{-}5^{Cre}$ in a substantially broader domain than previously reported using standard Cre-activated reporters (Ma et al., 2008). The expanded *Isl1* and *Nkx2-5* cardiac fate maps were remarkably similar, including in both cases extensive contributions to cardiomyocyte, endocardial, and smooth muscle lineages in all four cardiac chambers (Ma et al., 2008). Since *Isl1* is expressed in both left and right heart precursors, it does not qualitatively distinguish SHF and FHF progenitors (Ma et al., 2008), a point also emphasized by Pérez-Pomares et al. (2009). Recently, *Isl1* derivatives in the heart were also shown to be of neural crest origin (Engleka et al., 2012).

Hence, one potentially unifying model for vertebrates is that there is a common progenitor population in which all cells initially express *Isl1*, which is extinguished only in those cells starting to terminally differentiate while it remains active for a longer time period in those cells added to the arterial and venous poles of the heart. For chordates, the bHLH transcription factor *Mesp1* is upstream of *Isl1* and is the earliest marker of multipotent cardiovascular progenitors, descendants of which account for all components of the heart but a small portion of the ventricular conduction system and the outflow cushions (reviewed in Bondue and Blanpain, 2010). The outflow tract and the RV derive from a population of head mesoderm which also gives rise to craniofacial muscles (Lescroart et al., 2010; reviewed in Sambasivan et al., 2011). Even though the concept of two heart fields – one an add-on by air-breathing organisms and one evolutionarily more ancient – has proved to be erroneous, the underlying genetic and genomic basis for the evolution of form in the heart remains a fundamental question for the field of cardiovascular development.

By examining a range of organisms – invertebrates, urochordates, agnathans, teleosts, lungfish, amphibians, and amniotes – we can see at least twenty stepwise, major modifications to the ancient circulatory arrangement, seen in *Drosophila*. The dorsal vessel of the fly is composed of myocardial cells surrounded by nephrocytes (pericardial cells) (Weaver et al., 2009). Subsequent modifications are seen in the Table. Of these twenty morphological modifications, the two we will discuss in detail – the interposition of a second cardiac chamber between the time of tunicates and the appearance of agnathans; and the post-amphibian sculpting of two equal-sized compartments within the ventricular chamber by making one muscular trabecula taller than all others – have been genetically dissected in recent years. The new understanding of how changes in gene regulation can drive morphological change bears directly on complex human congenital heart disorders. While atrial aplasia has never been reported in humans and atrial hypoplasia is rare, ventricular hypoplasia occurs in >1000 newborns annually in the US with most cases

involving an unequal partitioning of the ventricular chamber, such that the LV is much smaller than normal while the chamber to the right of the ventricular septum appears much less affected (Fyler et al., 1980).

The emergence of a two-chambered pump

In the model tunicate *Ciona intestinalis*, the heart arises from the B7.5 blastomere pair. *Ci-Mesp*, the sole ortholog of the vertebrate *Mesp* genes, drives the expression of *Ets1/2* in the cells of this pair of blastomeres and all their descendants. (Hirano and Nishida 1997; Davidson and Levine 2003). Following two cell divisions, the rostral B7.5 lineages normally migrate anteriorly to form the heart, and the caudal B7.5 lineages migrate posteriorly to form muscle at the base of the tail (Fig. 4). Targeted expression of a constitutively activated form of Ets1/2, EtsVp16, causes both lineages to migrate into the head transforming the proximal tail muscle lineage into supernumerary heart cells, and nine percent of the juveniles contain beating hearts with two distinct myocardial compartments within a single pericardium (Davidson et al., 2006). Most dramatically, the two compartments are connected and function to drive blood flow efficiently through the juvenile body cavity (Fig. 4E–G). The independence of the two compartments is proved by periodic bouts of asynchronous beating (Davidson et al., 2006). This phenotype is distinct from cardia bifida, in that the two compartments are contained in a single pericardium and constitute a single tube (Davidson et al., 2006). This is an elegant illustration of how a change in the regulation of a transcription factor, rather than a change in its protein-coding region, could underlie a key transition in the emergence of dual-chambered hearts in a basal vertebrate such as the lamprey, namely the recruitment of additional heart precursor cells. All extant vertebrate species including man have hearts with at least two chambers connected in series.

Ventricular septation

The evolutionary record of another transcription factor, Tbx5, argues further that it is the more dorsal, morphologic left ventricle (LV), and not the ventral, morphologic RV, which is the more recent modification to the heart. In humans and other mammals, *Tbx5* expression correlates with the formation of the ventricular septum (Fig. 5A–D) - high in the left ventricle and low in the right, with a sharp boundary of expression exactly at the location where the septum forms (Koshiba-Takeuchi et al., 2009). The Tbx5 null mouse embryo has a severely hypoplastic LV (Bruneau et al., 2001). During early development in the turtle, an animal with only one ventricle, Tbx5 is expressed throughout its lone ventricular chamber. To prove that the level of Tbx5 is causal of ventricular septum formation rather than merely correlative, the Bruneau lab genetically engineered mice to express *Tbx5* at a moderate level throughout the developing heart, as in turtles. Offspring from these mice had only a single ventricle. By mimicking the turtle pattern of Tbx5, these investigators had created mouse hearts, which now resembled turtle hearts (Koshiba-Takeuchi et al., 2009). Therefore, a sharp line delineating an area of high expression of Tbx5 is critical to induce the formation of a ventricular septum, a precursor for the fashioning of a separate, specialized ventricular compartment (Fig. 5). For clinicians who have long judged chambers by their gross anatomical landmarks (e.g., assessment of whether apical trabecular pattern is fine or coarse), a modern, molecular definition of the morphologic LV is that it is the persistently Tbx5-positive and Isl1-negative chamber of the heart.

For the last 40 years, controversy has raged over whether an infant born with hypoplasia of the LV could ever be expected to have a normal lifespan and quality of life if the surgically reconstructed circulatory pattern required a dependence on the RV. Although patients with transposition of the great arteries (TGA) and large ventricular septal defect (VSD) treated with atrial inversion and VSD closure did experience ongoing attrition unrelated to sinus

node dysfunction, the evidence that patients with TGA alone suffered RV dysfunction has not been persuasive (reviewed in Chin et al., 2010). Moreover, although several analyses of Fontan survivors have identified having a morphological RV as a risk factor for poor long-term outcome, other reports disagree (reviewed in Chin et al., 2010).

In addition, it is instructive to examine the question: Are there long-lived univentricular organisms, with high-pressure circulations, which predate the transition to air-breathing and emergence of the LV? Sharks, including the dogfish shark and the whale shark, can live to 100 years (Mattingly et al., 2004; Bradshaw et al., 2007). The systolic blood pressure of shortfin mako sharks, even anesthetized, is 70 mmHg (Lai et al., 1997). They are entirely dependent on a single ventricle. The fiber arrangement and intramyocardial connective tissue architecture is remarkably similar to that of mammals (Sanchez-Quintana et al., 1996), as is their cardiac myosin heavy chain (Franco et al., 2002). By scanning electron microscopy and diffusion tensor magnetic resonance imaging, there is remarkably little difference in the fiber arrangement between the right and left ventricles of the human heart (Fernandez-Teran and Hurle, 1982; Rohmer et al., 2006).

If one defines the mammalian RV as the persistently Isl1-positive chamber, then the RV is really the ancient chamber. An Isl-positive cardiac chamber has existed at least as early as *Drosophila melanogaster*, in which the *islet* homolog (*tailup*) is absolutely required for heart development (Tao et al., 2007). The appearance of the LV or, more rigorously speaking, the two-ventricle configuration does not occur until a change in the spatial expression pattern of *Tbx5* occurs (Koshiba-Takeuchi et al., 2009), namely the new imposition of a late, sharp-edged gradient in Tbx5 such that the left part of the ventricular mass has high Tbx5 while the right part of the ventricular mass has low Tbx5. So the mammalian LV, the “Isl1-negative, persistently Tbx5-positive” chamber, is clearly a more recent evolutionary occurrence. There is no obvious *Tbx5* homolog in *Drosophila*, the closest being *omb* (*optomotor blind*), and *omb* is not expressed in the dorsal vessel (*Drosophila* heart) (Poeck et al., 1993).

Hence, although the LV clearly has outstanding performance characteristics (including inflow and outflow valves which are superior to those of the RV), the RV underwent 250 million years of refinement (the evolutionary distance between fly and fish) before air-breathing animals even arose. The RV is not “an afterthought”, added only to handle a second, low-pressure vascular bed, namely the lungs. It is the sole ventricular chamber for all fish species, amphibians, lizards, and turtles. The RV should not be assumed *a priori* to be incapable of sustaining the systemic circulation. If the morphologic RV in any individual human patient appears to perform poorly, the cause (whether genetic or acquired) is likely to be specific to that patient rather than a systematic inherent “RV design flaw”.

Finally, whether the emergence of a second atrium in anuran amphibians occurred by: (a) a change in the spatial expression of a single transcription factor which was sufficient to add a second, in-series cardiac chamber as in *Ciona* (Davidson et al., 2006), (b) a change in the spatial gradient of a transcription factor accentuating a ridge within a chamber as shown by Bruneau in the ventricle of the mouse (Koshiba-Takeuchi et al., 2009), or (c) via a Fgf-mediated reapportionment of fates of marginal progenitor cells at the blastula margin as recently proposed by Simões, based on work in zebrafish (Simões et al., 2011), is still an open question. However, the evolutionarily intermediate atrial septation steps evident in lungfish species (Icardo et al., 2005a) and urodele amphibians (Putnam and Parkerson, 1985) may favor Bruneau’s mechanism (Koshiba-Takeuchi et al., 2009).

Many forms of congenital heart disease may be ciliopathies

Another important question in cardiovascular developmental biology is how the vertebrate heart acquires its chirality. Although most organs are chiral, derived from the Greek *kheir* meaning "hand", in that they are not identical to, and cannot be superimposed onto, their mirror image, positioning of the heart tube with respect to the midline and its subsequent bending (looping) constitute the first morphological evidence of left-right asymmetry in the vertebrate embryo.

Although human lateralization disorders have been recognized for at least two centuries (Martin, 1826), only in the last two decades have genetic alterations responsible for their occurrence been identified. In 1997 Martina Brueckner showed that the locus of the *iv* mutation in mouse (the first mammalian model of incomplete lateralization, or heterotaxy, derived from the Greek *heteros* meaning "other" and *taxis*, meaning "order") encoded an axonemal dynein Lrd (Supp et al., 1997), for which the human homolog is DNAH11/DNAHC11. Since 40% of *iv* homozygotes have cardiovascular phenotypes resembling those seen in human heterotaxy patients (Icardo and Sanchez de Vega, 1991; Seo et al., 1992), the fact that *lrd* expression at embryonic day 7.5 was confined to the few hundred ciliated cells of the ventral surface of the node, the fluid-filled pit-shaped structure at the rostral end of the primitive streak, quickly focused investigators' attention on the cilium.

The cilium is an evolutionarily conserved ancient cellular organelle

Cilia are complex organelles composed of at least 600 different proteins (Pazour et al., 2005) organized around a microtubule based projection called the axoneme. While cilia can be motile or nonmotile, most nondividing cells harbor a single nonmotile primary cilium (Fig. 6; Pazour, 2004). The biological importance of the cilia is indicated by their high degree of conservation despite nearly 2 billion years of evolution. For example, motile cilia in the green alga *Chlamydomonas reinhardtii* and humans are nearly identical at the ultrastructural level, and the majority of the ciliary proteins identified in *Chlamydomonas* have clear homologues in humans (Pazour et al., 2005; 2006). The microtubules of the ciliary axoneme are templated from the basal body, a structure derived from the centriole, also a highly conserved organelle (Fig. 6). While the centriole is best known for its roles in mitosis and in organizing the interphase cytoskeleton, it is also postulated to be an important control center of the cell, integrating signals that regulate morphology, migration and proliferation (Satir and Christensen, 2007; Doxsey, 2001).

Given the complexity and high degree of evolutionary conservation of the cilia, it is perhaps not surprising that cilia assembly is mediated by an elaborate and highly conserved intraflagellar transport (IFT) system which carries ciliary component precursors from sites of synthesis in the cytoplasm to sites of assembly in the cilium (Fig. 6A). IFT transport also plays an essential role in the removal and turnover of proteins in the cilia (Rosenbaum and Witman, 2002; Scholey, 2003). In addition, recent studies show Bardet-Biedl syndrome (BBS) multiprotein complexes also play a role in ciliary assembly and turnover, possibly in transporting membrane proteins to the cilium (Nachury et al. 2007; Berbari et al., 2008), and to the proteasome (Gerdes et al. 2007).

The ciliary axoneme provides the scaffold for motor proteins required for ciliary motion, and also a multitude of other proteins required for sensory transduction of signals detected by the cilia. This includes ciliary membrane localized receptors, such as Patched and Smoothened involved in Hedgehog signaling, a pathway indispensible in cardiovascular development and cardiac septation (Goddeeris et al., 2008; Hoffman et al., 2009), as well as the polycystins which are involved in mechanosensory signal transduction and flow sensing, such as in endothelial cells and renal tubules (Nauli et al. 2003; 2006; Praetorius and Spring,

2001; 2005). Significantly, some of the proteins found in the cilia can traffic to the nucleus and alter transcriptional regulation. For example, the Gli3 transcription factor that mediates Shh signaling is sequestered in the cilia (Haycraft et al., 2005; Huangfu and Anderson, 2005). In fact, the pivotal role that the cilium plays in vertebrate Shh signaling (reviewed in Ingham et al., 2011) may well explain why the most prominent cardiovascular phenotypes reported so far involve not only the chirality of the venous pole but also septation of most portions of the heart - the atria (Hoffmann et al., 2009), the atrioventricular canal (Goddeeris et al., 2008), and the arterial pole (e.g., subpulmonary stenosis associated with double-outlet right ventricle, transposition of the great arteries, or tetralogy of Fallot; subaortic stenosis, sometimes associated with double-outlet right ventricle) (Hoffmann et al., 2009).

Polycystin-1, a protein associated with polycystic kidney disease, is localized to cilia and can form a complex with p100-Stat6 via its cytoplasmic tail. Upon changes in fluid flow, cleavage of the C-terminal cytoplasmic tail allows Stat6 to translocate to the nucleus (Low et al., 2006). The activity of other Stats such as Stat3 requires membrane- anchored polycystin-1 (Talbot et al., 2011). These observations suggest that sensory cilia integrate extracellular signals with changes in transcriptional regulation.

Cilia in development and human diseases

With rapid advances and insight into ciliary biology over the past decade, the cilium has gone from relative obscurity to being recognized as a key player both in vertebrate development and in human diseases. It has become apparent that defects in cilia underlie a large number of important human diseases collectively known as “ciliopathies”. Some ciliopathies such as Leber’s congenital amaurosis (LCA) type 6, characterized by both childhood-onset profound retinal dysfunction and nystagmus (involuntary eye movement) and attributed to mutations in *RPGRIP1*, cause defects in only one organ. Other ciliopathies like the Bardet-Biedl, Alström, Meckel-Gruber, Joubert’s, Orofaciodigital type 1, Senior-Løken, Jeune asphyxiating thoracic dystrophy, and short rib-polydactyly syndromes cause widespread disease with defects in a variety of organs. The anomalies caused by each of the syndromes vary, but cystic kidney disease, polydactyly, and laterality defects are commonly observed. Obesity, mental retardation, encephalocele, retinal degeneration, lung hypoplasia, deafness, and skeletal and facial abnormalities such as short-limb dwarfism and cleft palate, are more variably observed (Cardenas-Rodriguez and Badano, 2009; Baker and Beales, 2009). The defective genes in the syndromic ciliopathies tend to encode proteins required for basic functions of ciliary assembly and structure, such as basal body and ciliary axonemal proteins, as well as IFT and BBS proteins required for ciliogenesis.

Another class of syndromes known as primary ciliary dyskinesia (PCD), formerly known as Kartagener’s syndrome, is caused by mutations in genes required to produce ciliary force and consequently affect only motile cilia. These syndromes result in highly-penetrant laterality defects, male infertility due to immotile sperm, hydrocephaly, and recurrent respiratory infections (Satir and Christensen, 2007). Patients with PCD develop sinusitis, bronchitis, pneumonia, and eventually bronchiectasis (destruction of bronchial smooth muscle and elastic tissue resulting in dilated airways) due to mucociliary clearance defects resulting from immotile or dyskinetic cilia in the airway. Approximately 50% of PCD patients have the complete mirror-image reversal of visceral organ situs throughout the body, or *situs inversus totalis*. This reflects the dual requirement for motile cilia in both the specification of left-right patterning during embryonic development (Hirokawa et al., 2006) and in postnatal airway mucociliary clearance.

Virtually all motile cilia possess a central bundle of microtubules, called the axoneme, in which nine outer microtubule doublets (each doublet consisting of an A component with 13

protofilaments and a B component of 10 protofilaments) surround a central pair of singlet microtubules (Fig. 6B). Because ventral node cilia in the mouse embryo are missing the central doublet (i.e., have a "9+0" configuration of microtubule doublets seen in sensory cilia, rather than the "9+2" configuration typically seen in motile cilia), they were originally not believed to be motile. However, closer scrutiny showed that they do in fact move, despite their 9+0 arrangement of microtubules. In fact, uniquely among cilia, they rotate at 600 rpm (Okada et al., 1999). Whereas *iv* heterozygotes had cilia that rotated at 600 rpm, *iv* homozygotes had immotile cilia (Okada et al., 1999). Half of mouse embryos missing either *Kif3a* or *Kif3b*, components of the kinesin-2 molecular motor which, like dynein, is responsible for IFT along microtubules within cilia, had inverted heart chirality (Nonaka et al., 1998; Marszalek et al., 1999). Inspection of ventral node cells of *Kif3b* and *Kif3a* nulls revealed either sporadic, very short cilia or absent cilia (Nonaka et al., 1998; Marszalek et al., 1999).

Since it was hypothesized that a left-right asymmetric cascade of signaling molecules transmitting positional information from the midline node to lateral regions of the embryo was the way chirality was established during gastrulation, it remained to be explained how rotatory ciliary action could produce a consistently leftward (rather than a vortical) flow of signals within the node pit. In a remarkable series of experiments, the Hirokawa lab proved that a posterior tilt in the orientation of the cilia, as well as a difference in the viscous drag at the fluid surface compared with the base of the pit, is necessary and sufficient to cause the fluid within the node to move unidirectionally to the left (reviewed in Hirokawa et al., 2009). In one model, this flow sweeps signaling molecules or sheathed lipidic particles (termed "nodal vesicular" parcels) containing signaling molecules to the left side of the node (Tanaka et al., 2005). In another, termed the "two-cilia hypothesis", left-right specification further requires calcium signaling, transduced by polycystin-2 and the Pkd1-related protein Pkd1l1 (Field et al., 2011) in nonmotile sensory cilia localized in cells at the node periphery (McGrath and Brueckner, 2003). Finally, activation of a cascade of left-determining genes in the lateral plate mesoderm (the evolutionarily conserved nodal-Pitx2 pathway) completes the establishment of the left-right body axis (Shiratori and Hamada, 2006; Raya and Belmonte, 2006; Shen, 2007). Mutations in genes encoding proteins in the basal body, ciliary axoneme, or in BBS and IFT proteins have all been shown to cause laterality defects.

Identification of cilia defects in human heterotaxy

More recently, cilia mutations implicated in PCD has been shown to play a role not only in *situs inversus totalis* but also in heterotaxy, the randomization of left-right patterning, in both human and mouse (Kennedy et al.,2007; Tan et al., 2007). As heterotaxy and PCD are both rare phenotypes with an incidence of 1/10,000 to 15,000, this coincidental finding is unlikely to have occurred purely by chance. However, given the inherent genetic heterogeneity of the human population, it was not possible to ascertain whether the heterotaxy was elicited by the PCD-causing genetic lesion or some other lesions in the genetic background of the patients (Kennedy et al.,2007). Using inbred mutant mouse models, we were able to definitively answer this question. Thus, mice homozygous for a mutation in *Dnahc5*, a motor dynein gene whose human homolog *DNAH5* is commonly mutated in patients with PCD (with or without *situs inversus totalis*), exhibited a 40% incidence of heterotaxy (with 60% being situs solitus or situs inversus) (Fig. 7C). As the PCD mouse model is inbred and thus all animals are genetically identical except for the disease-causing mutation, we can conclude a single mutation can cause both PCD and also heterotaxy. It is significant to note in the *Dnahc5* mutant mouse embryos, cilia are retained in the embryonic node, but they are entirely immotile (Fig. 8A, B).

PCD mutant mice exhibiting heterotaxy all died prenatally or neonatally from complex structural heart defects. In contrast, mutants with *situs solitus* or *situs inversus totalis* were viable postnatally with no CHD. Clinically, half of PCD patients with heterotaxy also were found to have CHD such as double-outlet right ventricle, common atrioventricular canal, and systemic venous anomalies. Together, these observations suggest cilia play essential roles not only in left-right patterning but also in cardiovascular morphogenesis. They suggest the dynamic events that pattern left-right visceral organ situs are inextricably intertwined with the developmental processes that pattern left-right asymmetries of the heart. Overall, these observations suggest PCD patients and their families should be routinely screened for heterotaxy and for structural heart defects.

The observation that *Dnahc5* mutant mice with immotile cilia at the embryonic node are able to establish a consistent left-right body axis more than 50% of the time - either as normal *situs solitus* or mirror-image *situs inversus totalis* - suggests that motile cilia function is not absolutely required for breaking symmetry and specifying the left-right body axis. Since mouse mutants with no nodal cilia at all typically exhibit only heterotaxy, such as in the *Mks1* mutant mouse model of Meckel-Gruber syndrome (Fig. 8C–H) (Cui et al, 2010), this would argue the normal function of motile cilia at the node may involve an additional, non-motility-related function that may play a primary role in breaking symmetry and specifying the left-right body axis. It should be noted animals such as *Xenopus laevis* (reviewed in Vandenberg and Levin, 2010), chick (Gros et al., 2009), and pig (Gros et al., 2009) appear to use non-cilia-dependent strategies for breaking symmetry during development. It is interesting to note that *Mks1* null mutant fibroblast cells also showed defect in assembly of the primary cilia (Fig. 6D), but this is not observed in all cell types, suggesting possible differences in functional redundancies of proteins required for ciliogenesis.

Ciliary dysfunction affects the management and outcome of congenital heart disease in patients with heterotaxy

Human heterotaxy is usually associated with complex CHD, and such patients must undergo extensive cardiac surgical reconstruction. A number of studies have shown heterotaxy patients have unexplained worse outcomes, with higher postsurgical morbidity and mortality, even after adjusting for surgical complexity. For example, respiratory complications are common and often severe in heterotaxy patients (Swisher et al., 2009). Since sinusitis and bronchiectasis were included with *situs inversus totalis* in the original Kartagener's triad (now renamed as PCD), could the ciliary dysfunction in heterotaxy patients also contribute to pre- and post-operative respiratory complications leading to worse outcomes? In particular, it is unclear whether mutations that disrupt nodal cilia function underlying the laterality defects in heterotaxy patients also cause PCD-like mucociliary clearance defects. A small pilot study indeed showed 42% of CHD patients with heterotaxy had ciliary dysfunction similar to that observed in PCD patients (Nakhleh et al., 2012). Screening only by looking for normal-appearing axonemes is insufficient since *DNAH11*/*DNAHC11*/*Lrd* mutated cilia appear normal, even while demonstrating abnormal beating behavior on high-speed video microscopy (Schwabe et al., 2008). These findings suggest the possibility that undiagnosed ciliary dysfunction overlapping with that of PCD may contribute to respiratory complications and worse outcomes in heterotaxy patients (Swisher et al., 2011). While replication of these findings in a larger cohort together with outcome studies are needed to validate the potential mechanistic link between ciliary dysfunction and respiratory complications in CHD patients with heterotaxy, these findings show the potential value of insights into disease mechanism for improving patient care.

Patients with LCA type 10 are reported to have abnormal airway ciliary motility together with a clinical history of recurrent inflammatory diseases of the airway (Papon et al., 2010). These findings suggest increased respiratory disease associated with motile ciliary dysfunction and may have relevance for other ciliopathies. As the disease in LCA patients arises from a defect in the primary cilium (of photoreceptor cells), the connection to respiratory disease may be much broader, extending to disorders involving either motile or nonmotile cilia.

Summary

Although the progress in surgical reconstructive techniques in a half-century commonly called the Golden Age of Cardiology has been astounding, most of the remaining improvements in the care of patients with congenital heart disease will depend on an understanding of the developmental biology and genetics of the metazoan cardiovascular system. Even though these latter fields have blossomed only in the last 30 years, they have already yielded considerable insights relevant to clinicians, as exemplified by the above vignettes on outflow tract development, “single ventricle” disorders, and ciliopathies with or without heterotaxy. Over the next 30 years, progressively more powerful tools and databases will become available to the biomedical community. In addition, the fields of stem cell biology and regenerative medicine may well contribute to a second Golden Age of Cardiology.

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- 22q11 hemizygosity: aberrant right subclavian artery and interrupted aortic arch type B.
- One change in the regulation of *Ciona Ets1/2* is sufficient to convert a unicameral heart to a bicameral one.
- A sharp spatial gradient of Tbx5 is necessary and sufficient to septate the ventricle.
- Cilia mutations cause both heterotaxy and situs inversus totalis.
- Ciliary dysfunction affects the outcome of congenital heart disease in patients with heterotaxy.

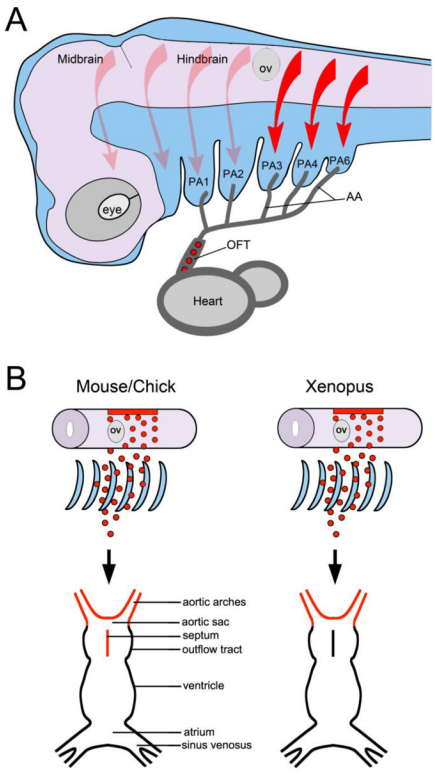


Figure 1. Cardiac NC migration and its contribution to the cardiovascular system

(A) In the mammalian embryo, NC cells delaminate form the dorsal neural tube and migrate in a stereotypical pattern. Based on their origin in the hindbrain (arrows) they populate individual pharyngeal arches (PA). The cardiac NC (red arrows) arises from a region posterior to the otic vesicle (OV) and migrates through pharyngeal arches 3, 4 and 6, along the aortic arch arteries (AA) and into the outflow tract (OFT) of the heart (red circles) to form the aorticopulmonary septum. There are no fifth pharyngeal arch arteries in amniotes. Lateral view, anterior to left, dorsal to top. (B) In chick and mouse the cardiac NC contributes to aortic arch arteries and forms the aorticopulmonary septum of the outflow tract. In *Xenopus,* cardiac NC cells populate the aortic sac and arch arteries, but do not colonize the outflow tract septum.

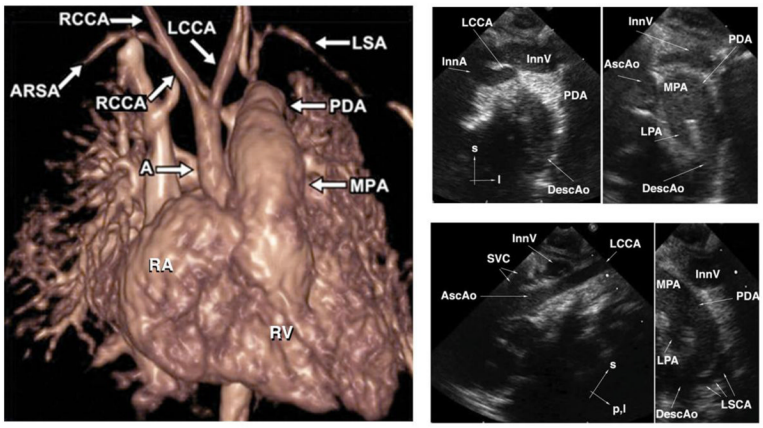


Figure 2. The two commonest cardiovascular phenotypes seen in 22q11 deletion syndrome are aberrant right subclavian artery and interrupted aortic arch type B

Left Panel: Volume-rendered image from gadolinium-enhanced 3D magnetic resonance angiography shows interruption of the left fourth aortic arch artery between the left common carotid artery (LCCA) and the left subclavian artery (LSA), a phenotype called type B interrupted aortic arch by clinicians. The right ventricle (RV) ejects blood into the main pulmonary artery (MPA). Without surgical reconstruction of the left fourth aortic arch, viability of the lower body is dependent on continued patency of the ductus arteriosus which allows blood in the MPA to supply the descending aorta, mitigating the effect of the arch interruption. The right subclavian artery in this patient is “aberrant” (ARSA) since it arises from the descending aorta, distal to both the ductus-descending aorta junction and the origin of the LSA. In the normal patient, the first brachiocephalic artery to arise from the aorta is an innominate artery which bifurcates into a RCCA and a RSA. A = ascending aorta; PDA = patent ductus arteriosus; RA = right atrium; RCCA = right common carotid artery. Used with permission from Frank et al., 2010. Right Panel: Suprasternal echocardiographic identification of interrupted aortic arch Type B. Upper left: Frontal (coronal) view showing the takeoffs of the innominate artery (InnA) and LCCA. No connection is seen with the distal aorta. Upper right: Slightly more dorsal (posterior) frontal view showing the MPA, large left-sided PDA, and left-sided upper descending aorta (DescAo). The ascending aorta (AscAo) is not in continuity with the descending aorta. Lower left: Left oblique view showing the LCCA takeoff and no discernible aortic arch. Lower right: sagittal view showing the origin of the left subclavian artery (LSCA) from the DescAo. InnV= innominate vein; l=left; LPA=left pulmonary artery; p, l = posterior and leftward; s=superior; SVC=superior vena cava. Other abbreviations as in Left Panel.

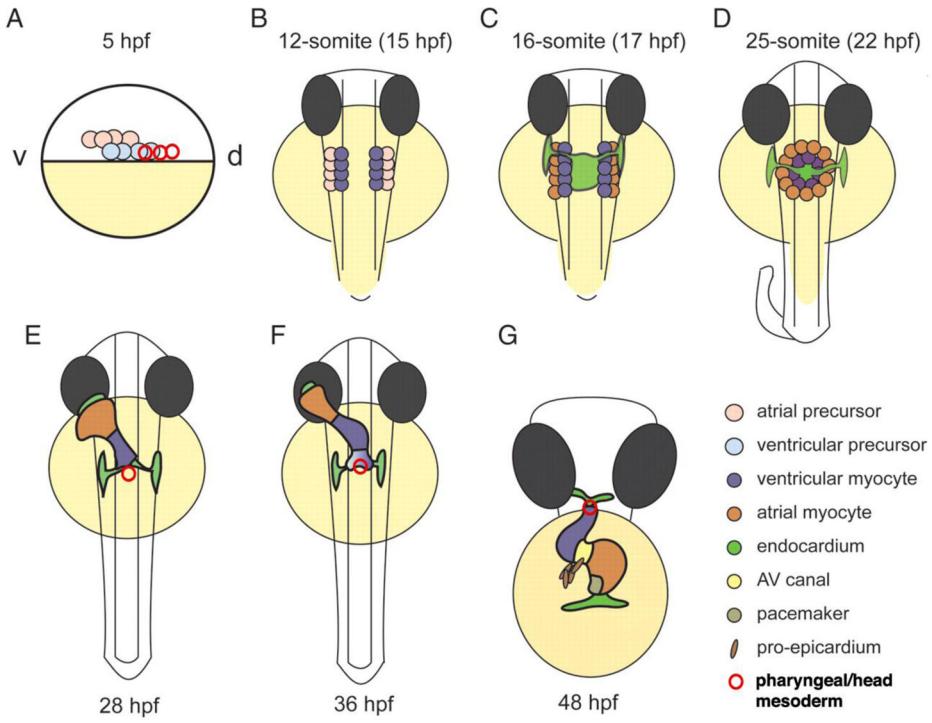


Figure 3. Stages of cardiac development in the zebrafish embryo

(**A**) Lateral view. At 5 h post fertilization (hpf), the blastula (white) covers approximately 50% of the large yolk cell (yellow). Cardiac progenitor cells are located bilaterally in the lateral marginal zone. Atrial progenitor cells (pink) are located more ventrally (v) than the ventricle progenitor cells (light blue) and pharyngeal mesoderm progenitors (red circles). During gastrulation, the cardiac progenitor cells move dorsally (d) towards the midline to end up in the anterior lateral plate mesoderm by the 12-somite stage (**B; B**–**F** are dorsal views). Pharyngeal mesoderm progenitors remain rostral to this region. (**C**) The endocardial cells (light green) are the first to migrate towards the midline, with the myocardial cells following slightly later. When the bilateral heart fields fuse at the midline, they form a shallow cardiac cone with the endocardial cells in the center, ventricular myocytes at the circumference and atrial myocytes at the periphery (**D**). Proliferation transforms the cardiac cone into a cardiac tube. The endocardium forms the inner lining of the myocardial tube. (**E**) By 28 hpf, the elongating heart tube has reoriented from the dorsal-ventral axis to the rostal-caudal axis, with the venous pole swinging to the left while the arterial pole remains fixed at the midline. New cardiomyocytes are added to the arterial pole from the pharyngeal/cranial mesoderm (red). At 36 hpf, cardiac looping is well underway, and the constriction at the position of the AV canal is obvious (**F**). The heart tube continuous to loop and forms an S-shaped loop (**G**, ventral view). Ellipsoid extracardiac pro-epicardial cells (brown) are located near the AV canal (yellow), from where they start to cover the myocardium with an epicardial layer. The pacemaker is present in the inner curvature of the atrium near the venous pole (dark green). This figure is modified from Bakkers (2011) and used with permission.

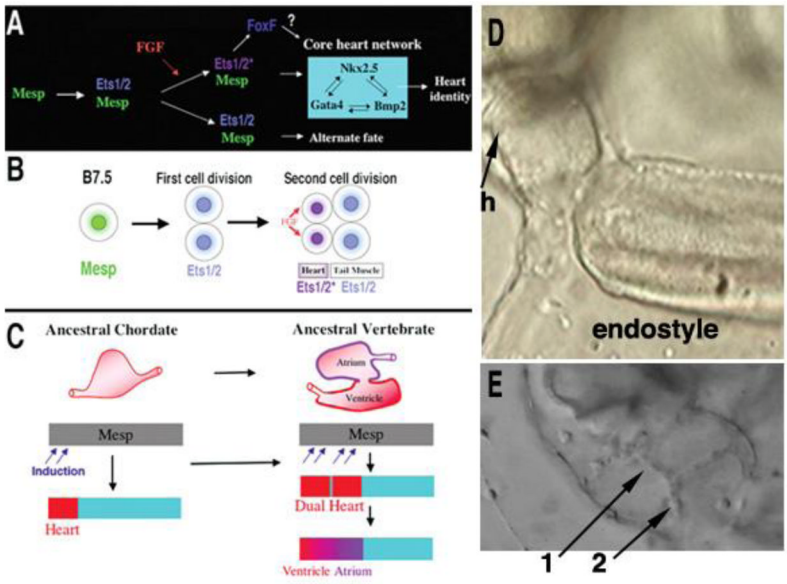


Figure 4. A single change in the regulation of the transcription factor *Ets1/2*, the *Ciona* homolog of *Ets1*, is sufficient to convert a unicameral (one-chambered) heart to a bicameral (two-chambered) heart

(**A**) Summary of the gene network controlling heart specification in *Ciona. Mesp* drives expression of *Ets1/2* in all descendants of the B7.5 blastomeres. (**B**) FGF signaling activates *Ets1/2* in the rostral daughters, leading to the expression of FoxF and ultimately to differentiation into heart myocytes. The caudal daughter cells become muscle at the base of the tail. (**C**) Diagram illustrating a model of chordate heart evolution suggested by the results of experimental manipulation of *Ets1/2* activation in *Ciona* embryos. (**D**) Control Mesp-GFP transgenic juveniles have a unicameral (one-chambered) heart (h). (**E**) In Mesp-EtsVp16 transgenic juveniles, expansion of *Ets1/2* induction within a broad heart field leads to a fate switch in the caudal B7.5 descendants and to the emergence of two hearts (denoted by **1** and **2**). The two hearts are connected to each other, since blood cells (in this still frame, one such blood cell can be seen exiting one chamber) can be easily be tracked visually. In basal vertebrates such as agnathans, this two-heart arrangement could then have been further patterned and modified to form one heart with two distinct chambers, an atrium and a ventricle with an atrio-ventricular valve (**C**). This composite figure is modified from a figure and still frames from two supplementary movies provided in Davidson et al. (2006) and is used with permission.

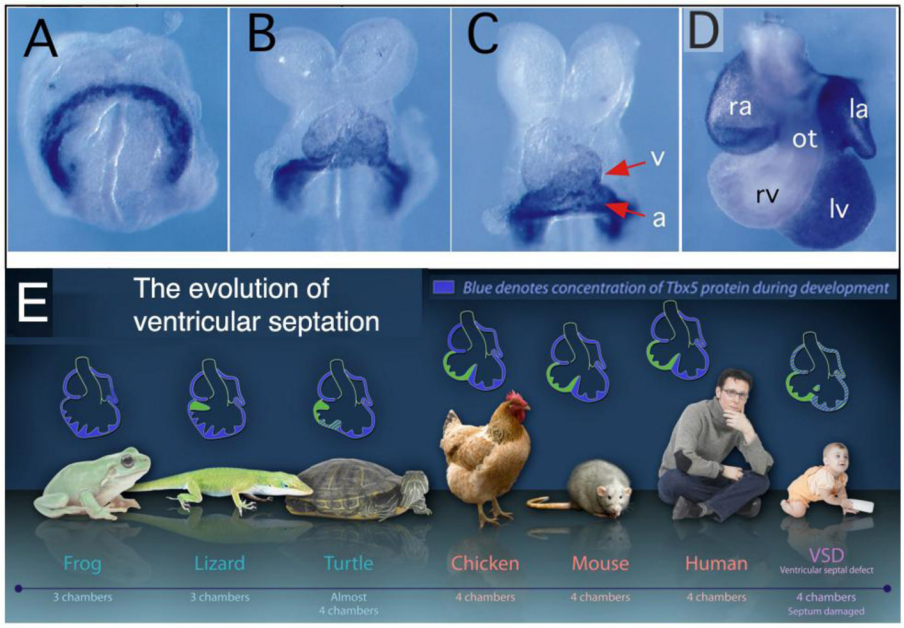


Figure 5. A sharp spatial gradient of Tbx5 is necessary and sufficient to septate the ventricle

(**A**) In the mouse, although the T-box transcription factor *Tbx5* is expressed in the cardiac crescent at embryonic day 8.0 (E8.0), it is rapidly extinguished by E8.5 except in the sinus venosus, atrium (a), and the portion of the ventricular chamber destined to be the left ventricle (v) (**B**). The embryo in Panel **C** is from slightly later on E8.5 than the embryo in Panel B. (**D**) E11.5 embryo. (**E**) In evolution, the emergence of a separated left ventricle correlates with the gradual spatial restriction of the expression of Tbx5. The infant shown with haploinsufficiency for TBX5 (Holt-Oram Syndrome) has muscular ventricular septal defects, holes in the trabecular septum. la=left atrium; lv=left ventricle; ot=outflow tract; ra=right atrium; rv=right ventricle. Panels **A** through **D** are from Bruneau et al. (1999) and are reproduced with permission. Panel **E** is modified from Zina Deretsky, National Science Foundation, after Benoit Bruneau, Gladstone Institute of Cardiovascular Disease (http://www.nsf.gov/news/news_images.jsp?cntn_id1/4115520&org1/4IOS).

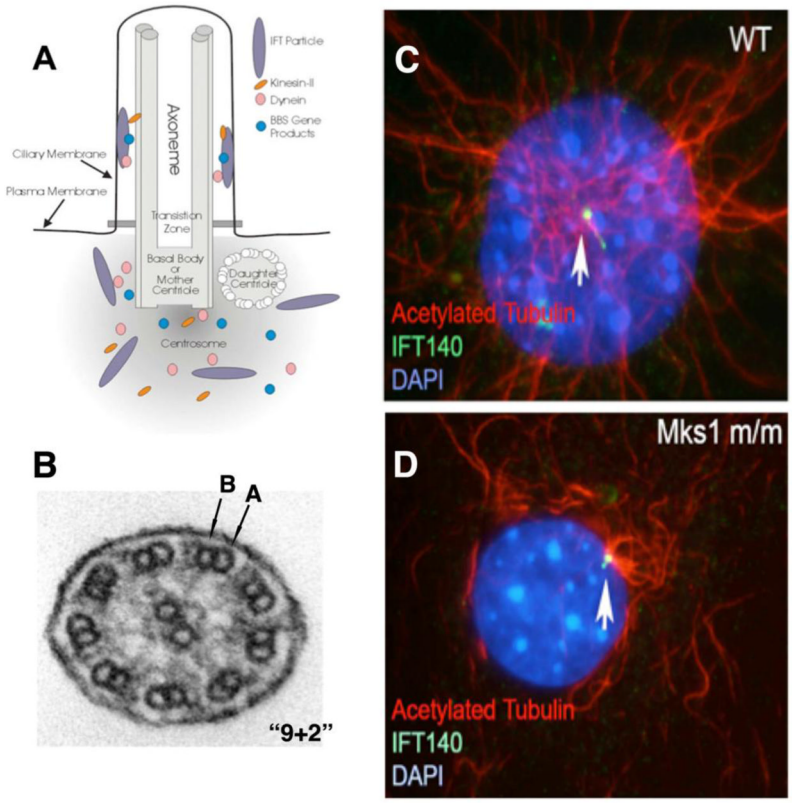


Figure 6. Mechanism of ciliogenesis

(A). Schematic diagram showing the transport of proteins into and out of the cilia via intraflagellar transport (IFT) particles mediated by kinesin-II and dynein, respectively. Note the cilium is built on a basal body template that is the mother centriole, with ciliogenesis also requiring BBS gene products. Diagram provided courtesy of Dr. Gregory Pazour (Pazour, 2004). (B). Cross sectional view of motile, tracheal airway cilium, showing the 9+2 arrangement of microtubule doublets (each doublet consisting of an A component with 13 protofilaments and a B component of 10 protofilaments) surrounding a central pair of singlets.

(C, D). *Mks1* null mutant fibroblast cells exhibit a primary cilia defect. *Mks1* encodes a protein localized to the centrosome and basal body. In wild-type (WT) mouse embryonic fibroblasts (C), ciliary axoneme is delineated by antibody to acetylated tubulin (red), with the tip and base stained by antibody to IFT140 (green). In contrast, *Mks1* null mutant (D) fibroblasts showed IFT140 (green) staining but no obvious cilium can be detected.

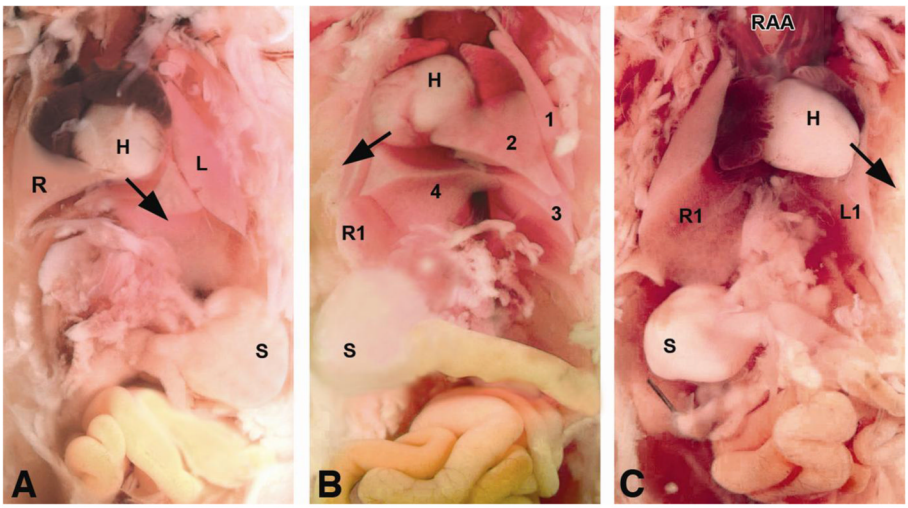


Figure 7. Situs anomalies in *Dnahc5 mouse* mutants

(**A**) Situs solitus with levocardia. The right lung (R) has 4 lobes, and the left lung (L) has 1. Arrow indicates direction of heart (H) apex. The stomach (S) is on the left. (**B**) Situs inversus totalis with dextrocardia. Note 4 left (labeled 1, 2, 3, and 4) and 1 right (R1) lung lobes, with stomach on the right. (**C** and **D**) Heterotaxy with levocardia. Note right aortic arch (RAA), 1 lung lobe on each side (R1 and L1), and stomach on the right (**C**). Reprinted from Tan et al., 2007.

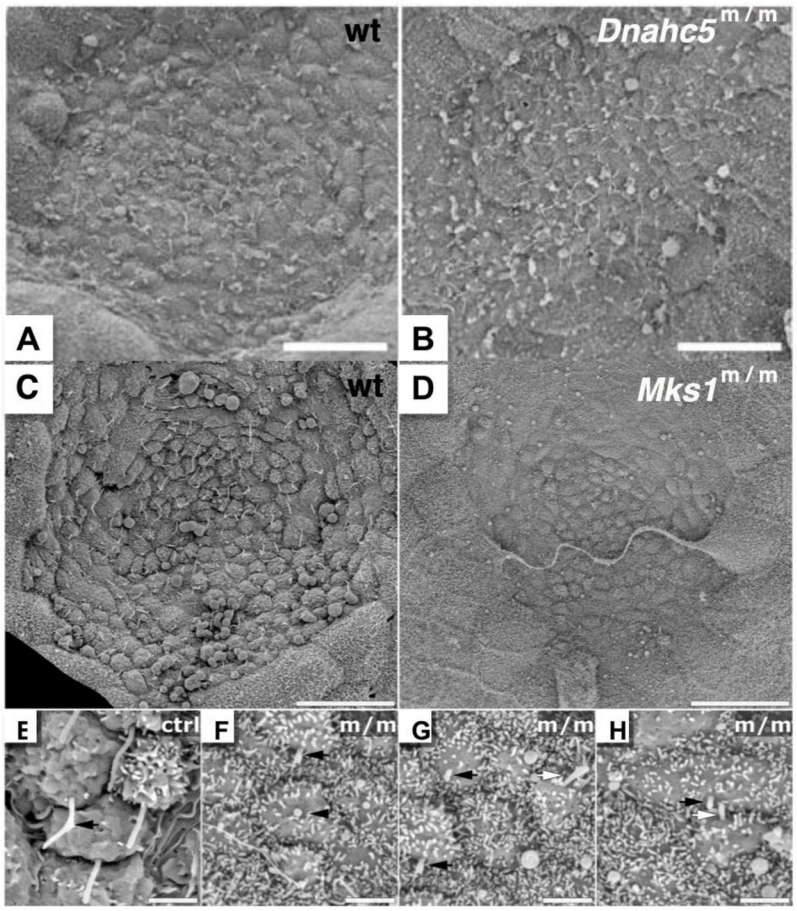
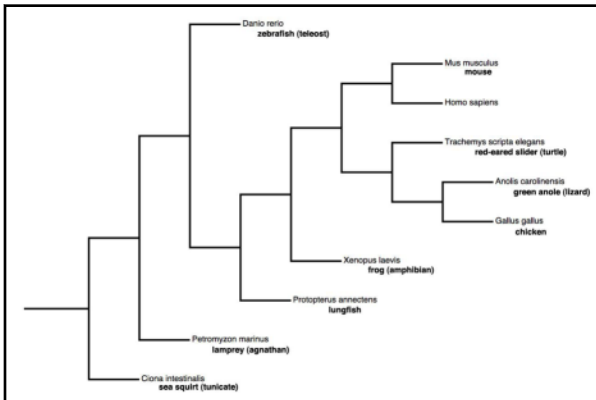


Figure 8. Nodal cilia in two mutant mouse models with laterality defects

In the early mouse embryo, cells in the embryonic node have a single motile cilia (A, C). In the *Dnahc5* PCD mutant mouse model, cilia in the embryonic node are retained, but the cilia are immotile (B). Homozygous *Dnahc5* mutants can exhibit situs solitus, situs inversus or heterotaxy. In comparison, in the Meckel-Gruber syndrome (MKS) mouse model harboring a mutation in *Mks1*, cells at the node do not have cilia (D, with magnified views in F–H, as compared to magnified view of cilia in wild-type node in E). *Mks1* mutants exhibit heterotaxy exclusively. Panels A, B are reprinted from Tan et al., 2007; Panels C–H are reprinted from Cui et al., 2010.

Table

Chordate phylogeny and twenty major modifications to the ancient circulatory arrangement seen in *Drosophila melanogaster.*



Organism	Modifications	References
tunicates	Addition of a vasculature	Davidson, 2007
agnathans	Appearance of a separate endocardial cell layer	Hatta, 1897
	Appearance of desmosome as a myocyte linker	Grosskurth et al., 2008
	Interposition of a second cardiac chamber	Kokubo et al., 2010
	the sculpting of a valve between those two chambers providing for unidirectional circulation	Tahara et al., 1988
	the emergence of the epicardium (visceral pericardium) from the pronephric external glomerulus	Pombal et al., 2008; reviewed in Pérez-Pomares et al., 2009
	the appearance of two myocardial zones (trabecular and compact	
	development of neural crest cells required for normal assembly of the ventral aorta	Newth, 1956
teleosts	the appearance of coronary circulation	Tota et al., 1983
	chiral, rather than ventral-dorsal, looping of the heart tube	Senior, 1909
	the eventual reliance on the gut-derived swimbladder as not only a buoyancy organ but also as a respiratory organ	Graham, 1997
lungfishes	complete co-option of the swimbladder as a lung communicating with the systemic circulation by means of a pulmonary vein passing across the roof of the sinus venosus to the atria divided only at their caudalmost extent	Icardo et al., 2005a
	emergence of outflow tract ridges	(Icardo et al., 2005b
	appearance of pulmonary arteries as branches from the sixth aortic arch arteries bilaterally	Johansen and Hanson, 1968
amphibians	septation of the pharynx to yield a trachea and bronchi	
	complete separation of the pulmonary venous return and the systemic venous return	
	various degrees of atrial septation	Putnam and Parkerson, 1985
amniotes	progressive separation of the streams at the arterial pole	
	“walling off” of the left ventricle (LV)	Koshiba-Takeuchi et al., 2009
	decrease in the LV’s trabecular/compact zone ratio with increasing workloads	Eliason et al., 2011