PERSPECTIVE

A house with many rooms: how the heart got its chambers with *foxn4*

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The vertebrate heart is initially formed from two lateral domains of cardiogenic mesoderm that fuse at the ventral midline to form a simple heart tube that is similar to the hearts or dorsal vessels of invertebrates. As development proceeds, this initial heart tube undergoes a series of morphogenetic rearrangements that subdivides the heart into the distinct chambers found in adult vertebrates. In this issue of *Genes & Development*, Chi et al. (2008) describe a novel transcriptional pathway required for separation of the outflow (ventricular) portion from the inflow (atrial) portion of the two-chambered zebrafish heart. This separation takes place at the atrioventricular (AV) canal, which forms the valves separating the ventricular and atrial compartments required for unidirectional blood flow, as well as the electrical conduction system required for proper cardiac rhythm. Chi et al. (2008) identify a member of the forkhead box (*fox*) family of transcription factors, *foxn4*, which works in concert with *tbx5* to direct the development of the AV boundary by regulating the expression of *tbx2b*. This study sheds new light on how the AV canal myocardium became specialized during vertebrate evolution to perform its function as both the gatekeeper of blood flow and regulator of cardiac rhythm.

Development and function of the AV canal and *foxn4*

The basic design of the cardiovascular system changes dramatically during evolution to accommodate the increased blood flow and pressure found in vertebrates. In *Drosophila*, the peristaltic contraction of a simple myocardial tube promotes the circulation of hemolymph, the insect version of blood, within an open circulatory system. While this is sufficient for invertebrate species, which have small surface area-to-volume ratios and a relatively low demand for oxygenated blood, vertebrate species require more complex circulatory loops that provide directional flow of oxygenated blood from gas exchange organs such as lungs or gills to the rest of the

organism, followed by the subsequent return of deoxygenated blood to the heart. The simplest form of this unidirectional blood flow is seen in the two-chambered fish heart. Blood returning from the body first enters a receiving chamber or atria, which then contracts to force blood into a larger pumping chamber or ventricle through a valve structure that is specialized to prevent retrograde flow. After filling, the ventricle contracts, forcing blood out of the heart into the aorta where it is first delivered to the gills for oxygenation before being circulated to the rest of the body.

The AV boundary, which separates the atrial and ventricular compartments, plays at least three critical roles in the establishment of unidirectional flow through the heart. First, both myocardial and endocardial cells within the AV boundary undergo characteristic cell shape changes that cause the heart tube to bend in this region and form a physical constriction between the atrial and ventricular compartments. Second, the endocardial cells within the AV boundary delaminate and migrate into the lumen, where they form the endocardial cushions that later differentiate into the AV valve. Third, while the waves of electrical stimulation and subsequent contractility begin at the inflow poles of both vertebrate and invertebrate hearts, cells within the AV canal of the vertebrate heart have fewer gap junctions and slow the transfer of electrical excitation, thus allowing for the sequential contraction of the atrial and ventricular compartments. Finally, it should be noted that AV canal defects, including valvular defects, comprise a large and important group of human congenital heart diseases whose underlying molecular etiology is still poorly understood.

In a study appearing in this issue of *Genes & Development*, Chi et al. (2008) report that the zebrafish mutant, *slipjig* (*slj*), exhibit severe defects in AV canal development, leading to cardiovascular failure in these mutants. Chi et al. (2008) mapped the mutation to the zebrafish ortholog of the *foxn4* gene, a member of the large *fox* family of transcription factors, which play key roles in regulation of cell specification, differentiation, and proliferation during development. Although the linear heart tube develops normally in *slj* mutants, neither myocardial nor endocardial cells undergo the characteristic cell

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shape changes within the AV boundary and the heart tube fails to loop. Furthermore, the endocardial cushions as well as their derivative valve structures develop abnormally and the normal delay in electrical conduction at the AV boundary fails to develop so that the heart continues to display peristaltic beating similar to that of the early heart tube in *sli* mutants. Taken together, these data suggest that *sli/foxn4* is required for AV boundary specification in the zebrafish heart.

Consistent with *foxn4* being required for zebrafish AV canal specification, several genes, including *tbx2b*, expressed in the AV boundary of wild-type hearts, fail to localize in *sli* mutants (Chi et al. 2008). *Tbx2* was previously shown to be required for AV canal and outflow tract development in the mouse (Harrelson et al. 2004). In the current report by Chi et al. (2008), morpholino knockdown of *tbx2b* in zebrafish phenocopies the loss of AV boundary formation and the defects with electrical conduction observed in *sli* mutants. *sli* mutants display expanded expression of AV canal markers such as *bmp4* and *versican* and expanded expression of the endocardial marker *notch1b*, all pointing to a loss of AV canal myocardial and endocardial specification or differentiation (Chi et al. 2008). Furthermore, the *tbx2b* promoter contains conserved binding sites for *foxn4* and another T-box family transcription factor *tbx5*. *foxn4* and *tbx5* both bind directly to the *tbx2b* promoter in electrophoretic mobility shift assays, and mutating the binding sites for either *foxn4* or *tbx5* abolishes the AV canal specific expression of GFP reporters under the control of the *tbx2b* promoter in transgenic zebrafish embryos. Chi et al. (2008) went even further to draw the link between *foxn4* and *tbx2b* by performing morpholino knockdowns of *tbx2b*. These morphants exhibited similar defects in AV canal formation and conduction delay as *sli/foxn4* mutants. The authors were rigorous in their studies and attempted to rescue *sli/foxn4* mutants with overexpression of *tbx2b*. Although this did rescue the *sli/foxn4* AV canal phenotype in a majority of embryos, such overexpression resulted in AV canal defects even in wild-type embryos, limiting the usefulness of this approach. Taken together, these data indicate that *foxn4* and *tbx5* act in concert to directly regulate *tbx2b* expression at the AV boundary.

Although other mutations have been shown to disrupt AV canal development, the zebrafish *sli/foxn4* mutation is unique in that it specifically ablates AV canal specification without disrupting myocardial development, and the initial linear heart tube appears to be unaffected. The report by Chi et al. (2008) therefore raises the intriguing possibility that *foxn4* was harnessed to direct the expression of targets, such as *tbx2b*, to promote the specialized differentiation of the AV canal myocardium during the evolutionary jump from the simple heart tube found in invertebrates to the multichambered hearts of vertebrates. While the *Drosophila* heart consists of a linear tube, there is differential gene expression along its anterior-posterior axis, and two *Drosophila* *tbx20* homologs recently have been shown to be required for this polarized gene expression (Miskolczi-McCallum et al. 2005;

Qian et al. 2005; Reim et al. 2005). A Fox gene has not yet been implicated in early *Drosophila* cardiac development, further supporting the hypothesis that members of this gene family including *foxn4* have been recruited to help define the AV boundary in vertebrates. The expression of *foxn4* to the AV boundary and its regulation of *tbx2b* therefore may have allowed a partially pre-existing set of positional cues, in the case of *tbx* factors, to be modified in order to define the AV boundary for its specialized functions (Fig. 1).

Fox genes and cardiac development

The current findings by Chi et al. (2008) are not the first to implicate Fox genes in the regulation of cardiac development. For example, *Foxp1* mutants display defects in the endocardial-mesenchymal transition (EMT) process that drives AV cushion development, in addition to defects in myocardial proliferation and differentiation and outflow tract septation (Wang et al. 2004). *Foxc1/c2* also regulate cardiac outflow tract septation, in part, through regulation of *Tbx1*, providing an

Figure 1. Development of a simple vessel into a two-chambered heart using *foxn4-tbx2b*. (A) The zebrafish heart begins as a simple vessel containing myocardial (red), epicardial (blue), and endocardial (green) cells. The zebrafish heart resembles the dorsal vessel in insects at this stage, except for the presence of epicardial cells in the vertebrate heart, and pumps blood primarily by peristaltic action. (B) Upon expression of *foxn4*, which activates *tbx2b* expression, the endocardial cells ingress inward, forming the AV canal, forming a valve, and causing a conduction delay required for setting up a unidirectional circulation. (C) Finally, the mature two-chambered heart with mature valves can circulate blood with high efficiency in a closed system. Arrows indicate both blood flow and direction of electrical conduction in the developing zebrafish heart.

important example of a *Fox–Tbx* link in mammalian cardiac development (Yamagishi et al. 2003; Seo and Kume 2006). *Foxp1*, *Foxc1*, and *Foxc2* are all expressed in the cardiac neural crest as well as in the secondary heart field (SHF), suggesting that these factors regulate important aspects of these cell lineages that are known to be required for proper outflow tract and right heart development (Shu et al. 2001; Yamagishi et al. 2003; Wang et al. 2004; Seo and Kume 2006). Thus, this gene family serves several critical roles in regulating diverse aspects of cardiac development, providing precedence for a role for *foxn4* in cardiac development.

A *Foxn4*-null mutation has been reported in the mouse (Christoffels et al. 2004). Although the investigators were primarily interested in the role for *Foxn4* in retinal neurogenesis, they did observe that *Foxn4* mutants died perinatally (Christoffels et al. 2004). Thus, it remains to be determined if *Foxn4* is required for AV boundary specification in the mouse. The current report by Chi et al. (2008) should spur an interest in re-examination of these mice for AV canal defects. Given the presence of multiple Fox genes in the heart, and in particular in the AV canal region, any future work must take into account the increased complexity, i.e., redundancy among these factors that likely exists in higher vertebrates required for directing morphogenesis of a more complex AV canal. This factor, among others, is one of the strengths of using the zebrafish system, as often there is less redundancy in the gene networks that regulate processes such as AV canal development.

Fox proteins bind to a conserved DNA-binding site through a highly conserved winged-helix DNA-binding domain (for review, see Lehmann et al. 2003). Fox genes have been further divided into subfamilies based on sequence homology outside of the DNA-binding domain (Kaestner et al. 2000). Members within these subfamilies often act redundantly in cell types where they are coexpressed. However, different subfamilies have different transcriptional regulatory characteristics. For example, *Foxc1/c2* are thought to be activators of gene transcription while *Foxp1* has been shown to repress gene transcription (Shu et al. 2001; Yamagishi et al. 2003). These data lead to an interesting hypothesis that there is a balance of Fox activation and repression that helps pattern the developing AV canal. Such a hypothesis is testable with additional work and the zebrafish model system, which is highly amenable to multiple gene knockdowns via morpholino oligonucleotide inhibition, will undoubtedly prove valuable for such studies.

A Fox–Tbx pathway in cardiac development

In their analysis of the *slf/foxn4* phenotype, Chi et al. (2008) showed that *tbx2b* expression was lost in the AV canal of the *slf* mutants. Tbx genes have been known for many years to play key roles in cardiac development including AV canal, ventricular growth, and outflow tract septation. In mice, *Tbx2* is required for proper AV canal development in part by repressing atrial and ventricular chamber-specific gene expression programs, keeping the

AV canal myocardium in a more plastic state (Habets et al. 2002; Christoffels et al. 2004; Harrelson et al. 2004; Cai et al. 2005). Moreover, *Tbx2* negatively regulates myocardial proliferation through repression of N-myc, which is required for myocardial proliferation (Cai et al. 2005). Thus, as with many other critical differentiation factors, *Tbx2* appears to promote AV myocardial differentiation at the expense of proliferation. Loss of *tbx2b* expression in *slf/foxn4* mutants may lead to increased proliferation in the AV canal, which could disrupt AV morphogenesis through improper expansion of AV myocardium. This might also explain the expansion of AV markers into the atria and ventricles including *notch1b*, *bmp4*, and *versican*.

Fox genes have been shown to regulate other Tbx genes during cardiac development. Yamagishi et al. (2003) showed that *Tbx1* expression in the pharyngeal endoderm and head mesenchyme is regulated by a Fox-dependent enhancer that is also sonic hedgehog (Shh)-responsive. In these studies, Shh was also required for expression of *Foxc1/c2* in the head mesenchyme, implicating a transcriptional loop where *Tbx1* is regulated by *Foxc1/c2*, which in turn are regulated by Shh, leading to proper development of tissues deriving from the head mesenchyme including the SHF. *Shh* mutants have defects related to improper remodeling of branchial arch arteries, but there are no reports of specific defects in AV canal development outside of the presence of ventricular septal defects and malrotation of the outflow tract, all of which could be attributed to the obvious defects in the cardiac neural crest (Washington Smoak et al. 2005). Although Chi et al. (2008) did not look at Shh expression or activity in the *slf/foxn4* mutants, it would be important to do so, since disruption of the Fox–Tbx axis of gene transcription may lead to loss of Shh in critical regions of the developing heart leading to the observed phenotype. However, since the role for neural crest and the actual existence of the SHF in zebrafish is debatable, the role for a *fox–tbx* axis may be restricted to the AV canal or primary myocardial cell lineages in zebrafish.

Using sophisticated imaging techniques, Chi et al. (2008) showed that *slf/foxn4* mutants had an absence of the critical conduction delay required for synchronous unidirectional blood flow. Although much of this could be attributed to the morphological defects, there is precedence for the role of Tbx genes in regulation of the cardiac conduction system. *Tbx2* and *Tbx3* have been shown to repress expression of connexins 40 and 43, which are gap junction proteins important for proper conduction system function (Christoffels et al. 2004). In contrast, *Tbx5* activates expression of connexins 40 and 43 (Bruneau et al. 2001; Hiroi et al. 2001). Moreover, patients with Holt–Oram syndrome, which is caused by mutations in *TBX5*, have severe conduction system anomalies as do *Tbx5* haploinsufficient mice (Basson et al. 1997; Bruneau et al. 2001). Thus, it will be important to determine whether haploinsufficient *slf/foxn4* zebrafish or *Foxn4* mouse mutants exhibit conduction system defects. This would help to directly link the *foxn4–tbx2b* axis in regulation of conduction system develop-

ment, and provide an important precedent for future studies exploring the role of other Fox gene mutants in conduction system development.

Fine-tuning AV canal development: implications for higher vertebrates

The present report from Chi et al. (2008) underscores the relationship between Fox and Tbx transcription factors in cardiac development. If the increasing complexity of the heart was driven in part by the acquisition of additional transcriptional and signaling networks required to properly pattern the additional chambers and regions, then the finding that a new Fox gene is involved in one of the most important early steps in cardiac chamber evolution is indeed important. However, several questions remain, including: How did additional chambers get added during evolution? Part of the answer could be the evolution of the SHF, which generates portions of the right side of the heart including parts of the AV canal and outflow tract (for review, see Kelly and Buckingham 2002). Acquiring the SHF may have led to the formation of additional chambers (right ventricle and right atria) and septation of the outflow tract, thus allowing the heart to further direct blood flow to the lungs in mammals that have a much higher metabolism than lower vertebrates, requiring more efficient circulatory perfusion. It is also important to consider the fact that in humans there are syndromes where either the right or left side of the heart is preferentially affected, including valve development. In Ebstein's anomaly, the tricuspid valve, which is the AV valve for the right ventricle, is malformed while the mitral valve, located in the AV region of the left ventricle, is relatively unaffected (for review, see Gurvitz and Stout 2007; Paranon and Acar 2008). Such left-right-specific defects may be indicative of additional molecular programs regulating left versus right AV canal development in mammals.

Future studies will be important in defining additional factors required for AV canal morphogenesis. Signaling pathways such as Wnt and BMP have been implicated in regulation of AV canal development, and whether Fox-Tbx factors act upstream or downstream from these pathways is sure to be a point of future investigations. Moreover, the fact that there are likely more Fox factors expressed in and important for mammalian AV canal development will likely require complex mouse genetics to define the full repertoire of Fox gene function in AV canal morphogenesis. Although these studies will require much time and effort, they will likely reveal the elegant complexity and importance of this process. More importantly, they will help to define the molecular mechanism of an important cause of congenital heart disease in humans.

Although the heart started as a simple tube contracting in a peristaltic manner, evolution has generated a remarkable organ with many chambers, each required for the high demands of mammalian metabolism. With the identification of a *foxn4-tbx2b* pathway for developmental regulation of that gate keeper of unidirectional

flow, the AV canal, we now have a better understanding of how the heart added new "rooms" to increase its efficiency as a pump in a closed circulatory system.

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