

Conserved Signaling Mechanisms in *Drosophila* Heart Development

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Abstract: Signal transduction through multiple distinct pathways regulates and orchestrates the numerous biological processes comprising heart development. This review outlines the roles of the FGFR, EGFR, Wnt, BMP, Notch, Hedgehog, Slit/Robo, and other signaling pathways during four sequential phases of *Drosophila* cardiogenesis—mesoderm migration, cardiac mesoderm establishment, differentiation of the cardiac mesoderm into distinct cardiac cell types, and morphogenesis of the heart and its lumen based on the proper positioning and cell shape changes of these differentiated cardiac cells—and illustrates how these same cardiogenic roles are conserved in vertebrates. Mechanisms bringing about the regulation and combinatorial integration of these diverse signaling pathways in *Drosophila* are also described. This synopsis of our present state of knowledge of conserved signaling pathways in *Drosophila* cardiogenesis and the means by which it was acquired should facilitate our understanding of and investigations into related processes in vertebrates. *Developmental Dynamics* 246:641–656, 2017. © 2017 Wiley Periodicals, Inc.

Key words: heart development; *Drosophila* cardiogenesis; cardiac mesoderm specification; cardiac morphogenesis; ostia formation; cardiac valve formation; signaling pathways; FGF signaling; Dpp/BMP signaling; Wnt signaling; Notch signaling; Hedgehog signaling; EGFR signaling; Slit-Robo signaling; Integrin mediated signaling; Pvr/VEGF signaling

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Introduction

Given that the heart first emerged in ancestral bilaterians over 500 million years ago (Romer, 1967; Moorman and Christoffels, 2003; Bishopric, 2005; Simoes-Costa et al., 2005; Ma et al., 2014), it is not surprising that the heart of the fruit fly *Drosophila melanogaster* exhibits remarkable similarities to that of vertebrates in terms of morphogenetic origins, structure, and regulatory mechanisms. In both *Drosophila* and vertebrate embryos, the heart originates from two bilaterally symmetrical rows of mesodermal cells that migrate most distally from the point of invagination during gastrulation, become committed to a cardiac fate, and ultimately fuse to form a heart tube at the midline (Bodmer, 1995). Subsequent looping and septa formation lead to the multiple-chambered heart in vertebrates while the *Drosophila* heart remains tubular, albeit divided by an intracardiac valve into a narrow anterior structure, termed the aorta, and a wider posterior structure, referred to as the heart proper (reviewed in Vogler and Bodmer, 2015; Rotstein and Paululat, 2016) (Fig. 1).

Proper heart development thus involves four sequential and integrated developmental processes: 1) the internal migration of a subset of mesodermal cells such that they reach locations where they are able to receive appropriate inductive signals, which in turn 2) enable them to become specified as the cardiac mesoderm (often termed the heart field or cardiogenic mesoderm in vertebrates), the precursor of the heart, 3) the refinement and differentiation of this cardiac mesoderm into the different cardiac cell types and tissues that comprise the heart, and 4) the ultimate positioning, arrangement, and changes in shape of these differentiated cardiac cells that complete the morphogenesis of the heart. The genes and proteins that mediate these developmental processes are also conserved, with considerable attention having already been focused on a core set of transcription factors—NK2, GATA, MEF2, T-box, LIM domain, and Hand proteins—which govern cardiac progenitor fate specification, cardiac morphogenesis, and the expression of downstream genes that bring about the differentiation of the distinct cell types and tissues of the heart (Bodmer and Venkatesh, 1998; Cripps and Olson, 2002; Olson, 2006; Tao and Schulz, 2007; Tao et al., 2007; Bodmer and Frasch, 2010; Meganathan et al., 2015; Vogler and Bodmer, 2015). This review will highlight the contributions of multiple signaling pathways that function both sequentially and in

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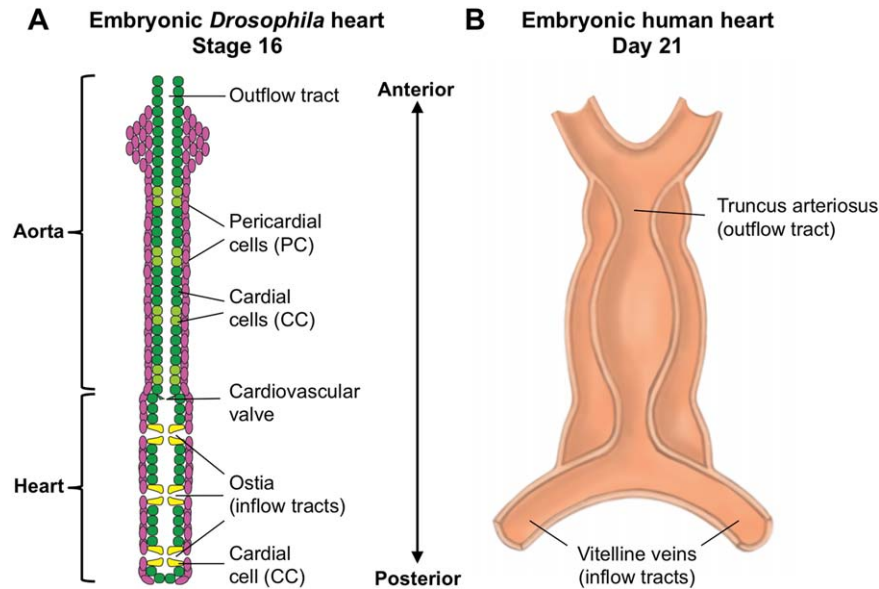


Fig. 1. The embryonic heart in *Drosophila* and humans. **A:** The tubular heart of a stage 16 *Drosophila* embryo, divided into a narrow anterior structure, termed the aorta, and a wider posterior structure, referred to as the heart proper. Both structures consist of an inner tube of contractile CCs (Tinman-Ladybird-CCs and Tinman-CCs are dark green; Seven up-CCs are light green and yellow) surrounded by an external sheath of non-muscle nephrocytic PCs (pink), all arranged metamericly in repeated units. Ostia (inflow tracts) are present only in the posterior compartment, three on each side, with two morphologically distinct Seven up-CCs (yellow) acting as a valve to regulate ingress through each ostium. **B:** The tubular single-chambered heart of a 21-day human embryo, prior to the looping and septation that will produce four chambers. Note that both hearts are organized as tubes along an anterior-posterior axis, with inflow tracts entering the heart at the posterior part of the organ, and circulatory fluid (hemolymph in *Drosophila*, blood in humans) being pumped anteriorward and exiting through the outflow tracts at the anterior.

concert to bring about cardiac development in *Drosophila*, and show that many of their cardiogenic roles are also conserved in vertebrates.

Mesoderm Migration

FGF Signaling in Mesoderm Migration

An essential prerequisite for the development of the heart is the internal migration of mesodermal cells after invagination at gastrulation. This movement ensures that a subset of mesodermal cells reaches stereotyped locations where the cells can receive appropriate position-specific inductive cues that determine their subsequent cardiac fates. In both flies and vertebrates, this migration is mediated by signaling through fibroblast growth factor receptors (FGFRs) expressed in these migrating cells.

In *Drosophila*, two FGF8-like ligands, *Pyramus* and *Thisbe*, are expressed during gastrulation in broad and dynamic patterns in the ectoderm (Gryzik and Muller, 2004; Stathopoulos et al., 2004). Mesodermal cells that have invaginated through the ventral furrow at gastrulation and express an FGFR named *Heartless* respond to these signals by migrating in a dorsolateral direction under the overlying ectoderm until they have formed a uniform monolayer that reaches as far as the dorsal layer of the epidermis (Fig. 2). The observations that 1) the activated form of the FGFR *Heartless* is detected specifically at the dorsolateral edges of the migrating mesoderm (Gabay et al., 1997), that 2) mesoderm migration is defective in embryos mutant for *pyramus*, *thisbe*, *heartless*, or genes such as *stumps* that encode components of the FGF signaling pathway downstream of *heartless* (Beiman et al., 1996; Gisselbrecht et al., 1996; Shishido et al., 1997;

Michelson et al., 1998a; Vincent et al., 1998; Imam et al., 1999; Gryzik and Muller, 2004; Stathopoulos et al., 2004), and that 3) these mutants also fail to develop dorsal mesodermal structures such as the heart, demonstrate that FGF signaling-mediated mesoderm migration at gastrulation is essential for proper cardiogenesis.

An analogous requirement of FGFR-mediated mesoderm migration for heart development is also observed in mammalian embryos. In mid-streak-staged gastrulating mouse embryos, FGFR1 is concentrated in the posterior mesoderm lateral to the primitive streak and is maintained in the migrating mesodermal wings (Yamaguchi et al., 1992). Analysis of chimeric embryos revealed that cells mutant for *Fgfr1* are defective in lateral migration; unlike their wild-type counterparts, they accumulate along the primitive streak and fail to contribute in any significant quantity to the migrating mesodermal wings (Rossant et al., 1997). Furthermore, while *Fgfr1* mutant cells have previously been shown to be capable of forming all types of mesoderm (Tam et al., 1997), in the chimeras they fail to reach the positions that would allow them to contribute to the heart, somites, gut, and cephalic mesenchyme (Rossant et al., 1997).

These results demonstrate that FGFR-mediated mesoderm migration is necessary for heart development in both *Drosophila* and vertebrates and raise the question of what signals induce these migrated cells to become specified as the cardiac mesoderm.

Establishing the Cardiac Mesoderm

In *Drosophila*, the specification of the dorsal mesoderm as the precursor of dorsal structures such as the heart can be followed

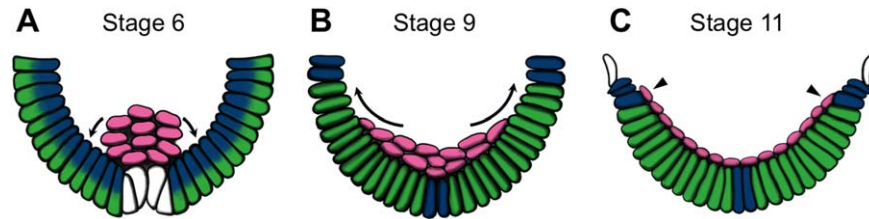


Fig. 2. Mesoderm migration in *Drosophila* cardiogenesis. **A–C:** Schematic cross-sections of *Drosophila* embryos at stages 6 (A), 9 (B), and 11 (C) showing the dorsolateral migration (arrows) of the FGFR Heartless-expressing mesodermal cells (pink) after invagination through the ventral furrow at gastrulation. Dynamic and broad expression patterns of the FGFRs Pyramus (blue) and Thisbe (green) along the ectoderm direct this migration (cells that are half green and half blue express both FGFRs). The dorsal-most cells at stage 11 (arrowheads) receive inductive signals to become specified as the cardiac mesoderm. Adapted from Kadam et al. (2009).

by examining the expression pattern of *tinman*, an NK homeodomain-encoding gene necessary for the development of dorsal mesodermal derivatives. *tinman* is originally expressed ubiquitously in the uncommitted migrating mesoderm but becomes restricted to the dorsal mesoderm only upon the latter's specification as a precursor of cardiac and other dorsal mesoderm derivatives in response to position-specific inductive signals (Bodmer et al., 1990; Azpiazu and Frasch, 1993; Bodmer, 1993).

BMP Signaling Specifies the Dorsal Mesoderm

At least two secreted factors expressed by the ectoderm act in a combinatorial fashion in *Drosophila* to induce a cardiogenic fate on the underlying migrated mesoderm. The first of these is Decapentaplegic (Dpp), a TGF- β bone morphogenetic protein (BMP) expressed by the dorsal ectoderm; the role of the second signal, the Wnt protein Wingless, is described in the next section. Three sets of observations demonstrate that signaling through Dpp induces the migrated mesoderm immediately underlying its expression domain in the ectoderm to become specified as the dorsal mesoderm: the precursor of the heart, visceral mesoderm, and dorsal somatic mesoderm. First, the ubiquitous expression of *tinman* throughout the trunk mesoderm and the T-box-encoding *Dorsocross* genes become restricted to only the dorsal mesoderm in direct contact with Dpp-expressing ectodermal cells (Frasch, 1995; Reim and Frasch, 2005). Second, loss-of-function mutations in genes encoding Dpp, its receptor Thickveins, or its downstream SMAD transcription factors, Mad and Medea, results in *tinman* expression fading shortly after gastrulation and a failure to develop dorsal (including cardiac) mesodermal derivatives (Frasch, 1995; Xu et al., 1998; Yin and Frasch, 1998). Finally, ectopic expression of either Dpp throughout the entire ectoderm or a constitutively active form of the Thickveins receptor throughout the entire mesoderm is able to induce *tinman* in the ventral mesoderm at a stage when its expression is restricted to only the dorsal mesoderm in wild-type embryos (Frasch, 1995; Yin and Frasch, 1998).

The role of Dpp in *Drosophila* cardiogenesis described previously led researchers to examine whether BMPs also induced cardiac mesoderm in vertebrates. At the stage when the mesodermal cells that will ultimately give rise to the heart are located in two bilateral regions in the anterolateral part of the chicken embryo, BMP4 and BMP7 are expressed by the adjacent ectoderm, while BMP2 and BMP5 are expressed by the adjacent endoderm (Schultheiss et al., 1997; Somi et al., 2004). Overlapping expression of these BMPs along the borders of these

cardiac precursors persists until and beyond both the onset of Nkx2.5 (the vertebrate ortholog of *tinman*) expression and the fusion of the cardiac precursors into the heart tube (Somi et al., 2004). While their overlapping expression patterns allow the BMPs to function redundantly during cardiogenesis, more than 16% of mouse embryos homozygous for a *Bmp2*-null mutation formed no heart cells at all (that could be detected by Nkx2.5 expression), while the remainder all exhibited defective heart development (Zhang and Bradley, 1996). Double-mutant mice homozygous for null mutations in both the *Bmp5* and *Bmp7* genes exhibited significantly delayed heart development compared to wild-type, with the double-mutant hearts also displaying disorganized heart morphology (Solloway and Robertson, 1999). Finally, BMP7, BMP2, and BMP4 were all able to induce Nkx2.5 expression in chicken embryo anterior medial mesoderm explant cultures, with the latter two also inducing complete cardiogenesis (Schultheiss et al., 1997). Collectively, these data indicate that BMPs induce vertebrate mesodermal cells to commit to a cardiac mesodermal fate in a manner analogous to that in flies.

Intersections of Dpp and Wnt Signaling Domains Specify the Cardiac Mesoderm

The dorsal mesodermal identity induced by Dpp in *Drosophila* gives rise to the visceral mesoderm and dorsal somatic muscles in addition to the heart, implying that an additional second signal must act in concert with Dpp to specify the more restricted cardiac mesoderm. Wingless, the *Drosophila* ortholog of the secreted glycoprotein Wnt1 (Baker, 1987), which is expressed in 15 transverse stripes along the trunk ectoderm overlying the mesoderm (van den Heuvel et al., 1989), functions as this second signal. Eliminating Wingless function using a temperature-sensitive *wingless* mutation at the time when *tinman* expression becomes restricted to the dorsal mesoderm results in a reduction of heart cells without any effect on the visceral mesoderm or dorsal somatic muscles (Wu et al., 1995). This decrease in heart cell number is accompanied by the loss of *tinman* expression in cardiac precursor cells. Cardiac precursor cells are also missing in embryos lacking both Frizzled and Frizzled2, the receptors of Wingless (Bhanot et al., 1999; Chen and Struhl, 1999); Dishevelled, a downstream component of the Wingless signal transduction pathway; or Armadillo (β -catenin), a transcription factor in the canonical Wnt pathway (Park et al., 1996). Finally, overexpression of Dishevelled is able to rescue defective heart development in *wingless* mutants (Park et al., 1996), indicating that signaling through the Wnt pathway is indeed essential for

Drosophila cardiogenesis. Taken together, these data show that segmentally arranged mesodermal cells underlying the intersections of Dpp and Wingless expression in the ectoderm become specified as the cardiac mesoderm (Lockwood and Bodmer, 2002). These cardiac progenitor cells then merge to ultimately form the continuous heart along the anterior-posterior axis (Borkowski et al., 1995).

The role of Wnt signaling in specifying cardiac progenitors in vertebrates is considerably more complex, with canonical Wnt signaling both inducing and suppressing cardiac differentiation, depending on the time of action. First, canonical Wnt signaling utilizing Wnt3a and β -catenin is necessary for mesoderm specification before gastrulation—Wnt3a- or β -catenin-deficient mouse embryos failed to generate mesodermal tissue (Liu et al., 1999; Huelsken et al., 2000), while early inhibition of canonical Wnt signaling in murine embryonic stem (ES) cells blocked the expression of mesodermal marker genes (Lindsley et al., 2006).

However, the next step in vertebrate cardiogenesis, the specification of multipotent cardiac progenitors from these mesodermal cells, is suppressed by canonical Wnt/ β -catenin signaling. In both chicken and *Xenopus* embryos, activation of the canonical Wnt pathway by ectopic expression of Wnt3a or Wnt8 suppressed cardiac differentiation as indicated by the downregulation of cardiac markers such *Nkr2.5*, while inhibition of the pathway through Wnt antagonists such as Crescent and Dkk-1 favored cardiac-specific gene expression (Marvin et al., 2001; Schneider and Mercola, 2001). Consistent with these results, β -catenin knockouts in mouse embryos also produced multiple ectopic hearts (Lickert et al., 2002). Collectively, these data indicate that the diffusion of Wnt antagonists from the Spemann organizer coupled with the inhibitory effect of canonical Wnt/ β -catenin signaling on cardiogenesis at this stage of development serves to delimit the domains of the cardiogenic mesoderm.

This biphasic role, both positive and negative, of canonical Wnt/ β -catenin signaling on cardiac specification depends on its time of action. This was elegantly illustrated in transgenic Zebrafish, where heat shock-induced expression of Wnt8 before gastrulation resulted in more cardiac progenitors than in wild-type embryos, whereas induced Wnt8 expression after gastrulation produced fewer cardiac progenitors (Ueno et al., 2007). Conversely, induced expression of the Wnt antagonist Dkk-1 before gastrulation inhibited cardiogenesis, while expression after gastrulation enhanced it (Ueno et al., 2007). A similar temporal biphasic effect was also seen in mouse ES cells, where treatment with Wnt ligands and inhibitors showed that Wnt/ β -catenin signaling at an earlier time point during differentiation led to increased cardiac gene expression and larger contractile areas within embryoid bodies, while canonical Wnt/ β -catenin signaling at a later time point had the reverse effect (Naito et al., 2006; Ueno et al., 2007).

Not all Wnts play an inhibitory role during this stage of cardiogenesis. In particular, non-canonical signaling through Wnt11 is essential for vertebrate cardiac specification of the mesodermal cells. Loss-of-function experiments showed that Wnt11 was required for cardiac gene expression and normal heart development in *Xenopus* embryos, while its overexpression was sufficient to induce contractile phenotypes in *Xenopus* explants (Pandur et al., 2002; Afouda et al., 2008). Further analysis showed that this cardiogenic activity of Wnt11 in *Xenopus* was mediated by a PKC- and JNK-mediated non-canonical Wnt pathway (Pandur

et al., 2002). Similar positive roles for Wnt11 in cardiac specification were also seen with both quail mesoderm and mouse ES cells (Eisenberg et al., 1997; Eisenberg and Eisenberg, 1999; Terami et al., 2004; Ueno et al., 2007).

Of note, this alternating biphasic role of canonical Wnt signaling and the incorporation of non-canonical Wnt signaling pathways is utilized in vertebrate cardiogenesis even after the specification of the cardiac mesoderm: Canonical Wnt/ β -catenin signaling brings about the proliferation of cardiac progenitor cells in the second heart field (Ai et al., 2007; Klaus et al., 2007; Kwon et al., 2007; Lin et al., 2007; Tian et al., 2010) but inhibits the subsequent terminal differentiation of cardiomyocytes (Brott and Sokol, 2005; Lavery et al., 2008; Zhu et al., 2008). The latter process is mediated instead by non-canonical Wnt signaling through Wnt11 (Garriock et al., 2005; Gessert et al., 2008). This elaborate step-by-step variation in Wnt signaling pathways in vertebrate cardiogenesis likely reflects the overall complexity of spatiotemporally integrating multiple processes such as the specification, proliferation, and differentiation of distinct cardiac progenitors from diverse ancestral and intermediate cell populations in different heart fields.

An Additional Role for FGF Signaling in Specifying the Dorsal Mesoderm

In addition to its initial role in bringing about mesoderm migration in *Drosophila* embryos, the FGFR Heartless has a second, later function in specifying the dorsal mesoderm. When this later function is disrupted by expressing a dominant negative version of the FGFR throughout the mesoderm at a stage when mesoderm migration is not affected, a reduction in the number of heart cells and dorsal somatic muscle cells, as detected by the expression of the Even skipped marker, is observed (Michelson et al., 1998b). Furthermore, ectopic overexpression of either of the FGF ligands Pyramus or Thisbe results in an increase in the number of these Even skipped-expressing cells (Kadam et al., 2009; Klingseisen et al., 2009). Thus, the induction of the dorsal (and hence, cardiac) mesoderm in *Drosophila* requires both Dpp and FGF signaling.

A similar requirement of both FGFs and BMPs is observed for cardiogenic induction in vertebrates. In both the chick and mouse embryos, cells fated to become cardiac cells are positioned close to FGF- and BMP-expressing cells (Garcia-Martinez and Schoenwolf, 1993; Crossley and Martin, 1995; Schultheiss et al., 1997; Shamim and Mason, 1999). Combined treatment with both FGFs and BMPs, but neither signal alone, promotes cardiogenesis in non-precardiac mesoderm explants from chick embryos (Lough et al., 1996; Barron et al., 2000). Ectopic application of FGF8 in regions of the chick embryo where BMP2 and/or BMP4 are already present results in expanded expression of cardiac markers such as *Nkx2.5* (Alsan and Schultheiss, 2002). Similarly, in the Zebrafish embryo, FGF8 is able to induce *Nkx2.5* expression in the presence of BMP2 (Reifers et al., 2000).

Signaling Through EGFR is Required for the Survival of the Cardiac Mesoderm

Once specified, signaling through the epidermal growth factor receptor (EGFR) is required for maintaining the cardiac mesoderm in *Drosophila*. Targeted overexpression of EGFR in the mesoderm results in an increase in the number of heart cells, suggesting a role in cardiac cell proliferation and/or maintenance (Grigorian

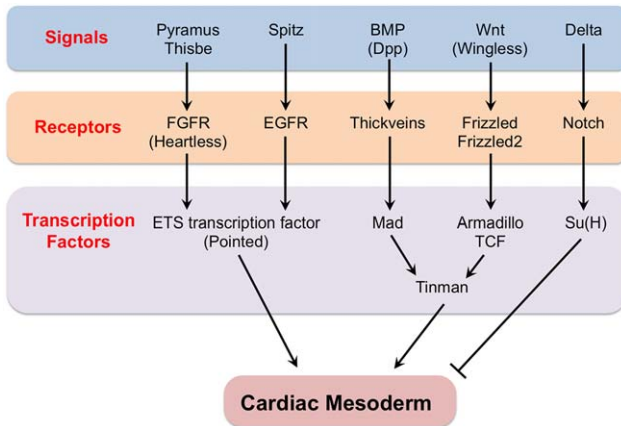


Fig. 3. Regulatory network of signaling pathways responsible for establishing the *Drosophila* cardiac mesoderm. Only the signaling ligands, their receptors, and the terminal transcription factors of these signal transduction pathways are shown.

et al., 2011). Loss-of-function mutations in *EGFR* result in an almost complete absence of most cardiac cell types, accompanied by significant amounts of cell death (Grigorian et al., 2011). These observations support the model that EGFR activity is required for the survival of the cardiac mesoderm during and after specification.

A similar role in cell survival for EGFR is observed in mice, albeit in vascular smooth muscle cells (VSMCs). Targeted inactivation of EGFR in VSMCs resulted in significantly higher lactate dehydrogenase (LDH) release, indicating reduced cell viability, as well as elevated caspase-3 activity, indicating an enhanced basal apoptosis rate (Schreier et al., 2011).

Notch Signaling Restricts Cardiac Mesodermal Fate

Signaling through Wingless, as described previously, positively regulates the cardiac mesoderm in *Drosophila*: Cells in the Dpp-induced dorsal mesoderm that also receive the Wingless signal become specified as cardiac progenitors. In contrast, signaling through the Notch receptor restricts the number of cardiac progenitors that are specified in the dorsal mesoderm (Carmena et al., 1998). Eliminating Notch using a temperature-sensitive allele during this cardiac mesoderm specification stage results in embryos with significantly larger numbers of every cardiac cell type (Hartenstein et al., 1992; Mandal et al., 2004).

Research performed on ES cells suggests that Notch signaling also has a similar restrictive effect on cardiogenic mesoderm specification in vertebrates. Utilizing *Notch1* knockouts as well as activated and dominant negative versions of the mammalian homolog of Suppressor of Hairless [Su(H)], a critical transcription factor in the Notch pathway, investigators demonstrated that Notch signaling led to ES cells adopting neuroectodermal and not cardiogenic fates (Schroeder et al., 2003; Lowell et al., 2006; Nemir et al., 2006).

In summary, and as outlined in Figure 3, the convergence of positive regulatory inputs through the FGFR (Heartless), EGFR, Wnt (Wingless), and BMP (Dpp) signal transduction pathways, and negative regulation by the Notch signaling pathway, specify and maintain the cardiac mesoderm in *Drosophila*. And, as described above, orthologous counterparts of each of these

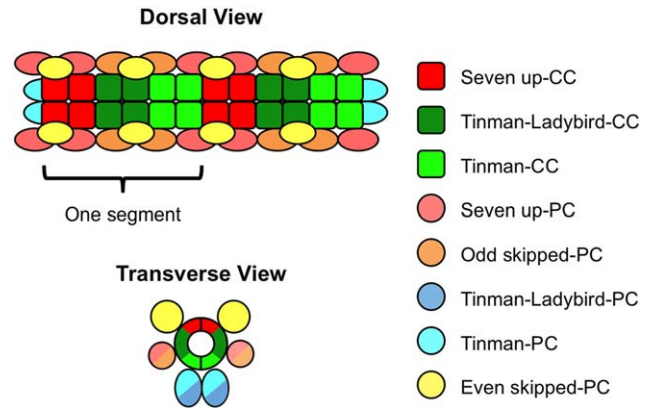


Fig. 4. Schematic diagram showing the stereotyped positions of the eight different cell types comprising the *Drosophila* heart. An individual heart segment, composed of two contralateral hemi-segments mirroring each other across the dorsal midline, is indicated.

signaling pathways, playing similar cardiogenic roles, are found in vertebrates.

Differentiation of the Cardiac Mesoderm Into Distinct Cardiac Cell Types

The cardiac progenitor cells comprising the *Drosophila* cardiac mesoderm undergo a stereotyped series of asymmetric and symmetric cell divisions to differentiate into two classes of heart cells: an inner row of contractile cardiac cells (CCs), and an external sheath of non-muscle nephrocytic pericardial cells (PCs) which surrounds the CCs. However, neither the CCs nor the PCs constitute a uniform population, being comprised of eight different cell types distinguishable by their distinct cell lineages and individual gene expression programs: Seven up-CCs, Tinman-Ladybird-CCs, Tinman-CCs, Seven up-PCs, Odd skipped-PCs, Even skipped-PCs, Tinman-Ladybird-PCs, and Tinman-PCs, all named for the transcription factors they express (Fig. 4) (Azpiazu and Frasch, 1993; Bodmer, 1993; Jagla et al., 1997; Ward and Skeath, 2000; Alvarez et al., 2003; Han and Bodmer, 2003). Some CCs become differentiated yet further to give rise to the valves that ensure that the circulatory fluid is pumped only anteriorward (Lehmacher et al., 2012) or to the valves that regulate the opening and closing of the ostia, channels that allow the *Drosophila* circulatory fluid to enter the heart (Rizki, 1978). These differentiation processes are also mediated by conserved signaling mechanisms.

Notch Signaling Brings About the Differentiation of the Pericardial Cells (PCs)

While signaling through the Notch pathway initially plays a suppressive role during the specification of the cardiac mesoderm, once the cardiac mesoderm has been established, Notch signaling is utilized again, this time for the differentiation of a subset of heart cells into PCs. When this later function of Notch signaling is inactivated after cardiac mesoderm specification using the temperature-sensitive *Notch* mutation or with mutations of its ligand Delta, supernumerary CCs are formed at the expense of PCs (Hartenstein et al., 1992; Mandal et al., 2004; Grigorian et al., 2011). Conversely, overexpression of an activated form of

Notch at this later stage converts CCs into PCs (Han and Bodmer, 2003; Ahmad et al., 2014).

In particular, this later function of Notch plays a critical role in determining the distinct fates of cardiac cell progeny produced by asymmetric cell divisions, thereby leading to cellular diversification. An antagonist of Notch activity, the membrane-associated protein Numb, becomes localized to one side of asymmetrically dividing progenitor cells and thus segregates primarily to only one of the two daughter cells (Rhyu et al., 1994; Spana and Doe, 1996; Ahmad et al., 2012). The daughter cell that inherits the bulk of the Numb protein is unable to respond to the Notch signal and therefore, in contrast to its pericardial sibling, adopts a myogenic or CC fate. In the cardiac mesoderm of embryos mutant for *numb*, however, Notch signaling is not antagonized in any of the progeny of asymmetric cell division, resulting in all of them becoming pericardial (Park et al., 1998; Ward and Skeath, 2000; Han and Bodmer, 2003).

Previous studies had shown that in the absence of Notch signaling, the transcription factor Su(H) forms a repressor complex with co-repressors such as Groucho or C-terminal binding protein that binds to the enhancers of target genes to prevent their transcription (Bray and Furiols, 2001; Barolo and Posakony, 2002; Bray and Bernard, 2010). The activation of Notch receptors by ligand binding produces a proteolytic cleavage that releases the Notch intracellular domain (N^{icd}) from the plasma membrane, allowing it to enter the nucleus, where it associates with Su(H), displaces the co-repressor, and converts the Su(H) complex from a transcriptional repressor into an activator. That prior knowledge, along with the observations that the expression of Delta, a ligand for Notch, is specifically restricted to CCs (Grigorian et al., 2011), and that Su(H) binding sites are enriched along the enhancers of genes specifically expressed in PCs compared to those in CCs (Ahmad et al., 2014), ultimately led to our understanding of the mechanism by which Notch signaling induces pericardial, as opposed to myocardial, CC fate (Fig. 5).

In CCs, PC-specific genes are repressed by the Su(H)-co-repressor complex. Delta, expressed by CCs, activates Notch receptor in the neighboring cells fated to become PCs, with the resulting N^{icd} associating with Su(H) and displacing the co-repressor. The resulting elimination of the repressor complex is sufficient to initiate transcription of PC-specific genes, leading these neighboring cells to adopt a pericardial fate. This mechanism was confirmed by examining the effects of eliminating either the Su(H) protein by RNAi or Su(H) binding by mutating its binding sites on the enhancers of PC genes, both of which led to presumptive CCs expressing PC-specific genes (Ahmad et al., 2014).

Intriguingly, Notch signaling in vertebrates also leads to cells in the established heart field adopting non-myocardial fates, suggesting that this later function of Notch in discriminating between distinct cardiac cell fates is also conserved. Conditional activation of Notch signaling in *Xenopus* embryos by injecting mRNA encoding an activated form of Su(H) leads to cells adopting a mesocardial or pericardial roof fate instead of a myocardial one (Rones et al., 2000). Similarly, constitutive activation of Notch by expressing N^{icd} in the developing chick heart promoted conduction cell differentiation and inhibited cardiomyocyte differentiation, while inactivating the Notch pathway by expression of a dominant negative version of Su(H) increased myocardial lineage markers at the expense of conduction cell markers (Chau et al., 2006).

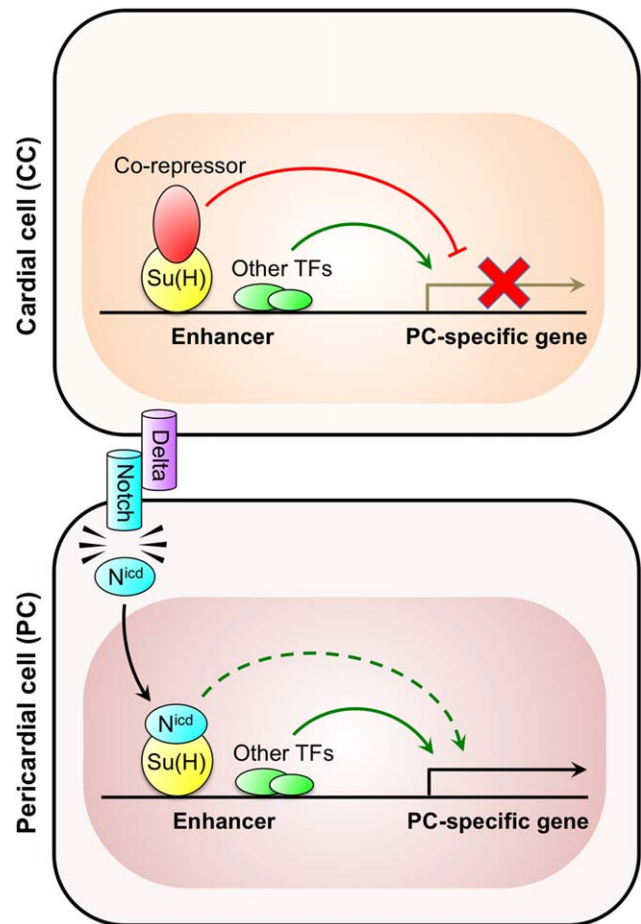


Fig. 5. Pericardial fate specification by the Notch signaling pathway. Schematic showing the regulation of PC-specific genes. Binding sites for the Su(H) transcription factor are enriched along the enhancers of PC-specific genes. In CCs, the enhancers of PC-specific genes are repressed by the binding of the Su(H)-co-repressor complex. However, the Delta ligand expressed specifically by the CCs activates the Notch receptor in the neighboring cells, with the resulting cleaved N^{icd} fragment associating with Su(H) and displacing the co-repressor. The resulting elimination of the repressor complex is sufficient to initiate transcription of the PC-specific genes due to the presence of other local activators, leading these neighboring cells to adopt a pericardial fate. Transcription of the PC-specific genes is further enhanced by the N^{icd} -Su(H) complex acting as an activator. Adapted from Ahmad et al. (2014).

Hedgehog Signaling Differentiates Cardiac Cells into Distinct Subtypes

The signaling molecule Hedgehog, expressed and secreted by the ectoderm in stripes adjacent to the Wingless-expressing cells, has three distinct functions in *Drosophila* cardiogenesis. First, it plays an indirect role in cardiac mesoderm specification by maintaining and reinforcing the Wingless expression from ectodermal cells necessary for the induction of cardiac progenitors (Park et al., 1996). These cardiac progenitor cells comprise non-overlapping subpopulations expressing distinct transcription factor-encoding genes such as *even skipped* and *ladybird* along each segment. The second function of Hedgehog signaling, in combination with the RAS pathway, is to specify and distinguish the *even skipped*-expressing subpopulation of cardiac progenitors from the *ladybird*-expressing subpopulation (Liu et al., 2006). Third, Hedgehog signaling initiates the differentiation of the CCs arising from the

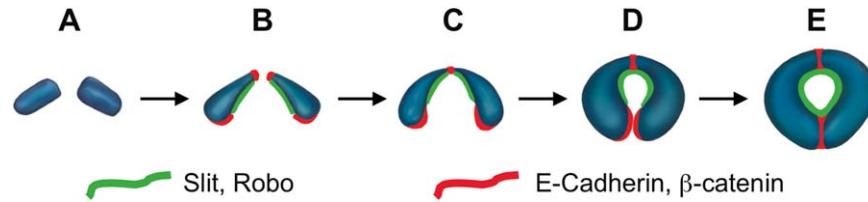


Fig. 6. Schematic representation of two CCs illustrating the temporal sequence of events during heart tube and lumen formation. **A:** At an early stage in migration, both CCs are cuboidal. **B:** As they approach one another, they become constricted along their dorsal domain. **C:** Each CC makes initial contact with its contralateral counterpart across the midline along this constricted dorsal domain. **D:** Each cell in these dorsally conjoined CC pairs then begins adopting a crescent shape. **E:** Ultimately, this shape change also allows the ventral domains of the CCs to join and form a tube that encloses an internal central lumen. Localization of Slit and Robo is shown in green; that of E-cadherin and β -catenin is shown in red. Adapted from Medioni et al. (2008) and Santiago-Martinez et al. (2008).

cardiac progenitors into distinct cell subtypes by bringing about the expression of the *seven up* gene in the anterior two pairs of CCs in a segment. Seven up inhibits the expression of *tinman* in these four cells, thus dividing each heart segment into two anterior pairs of Seven up-CCs and four posterior pairs of Tinman-Ladybird-CCs and Tinman-CCs (Fig. 4) (Gajewski et al., 2000; Lo and Frasch, 2001; Ponzielli et al., 2002). The Seven up-CCs subsequently differentiate into the valves regulating the opening and closing of the inflow tracts in the embryonic or adult heart (Molina and Cripps, 2001). Thus, Hedgehog, by specifying the Seven up-CCs, establishes heart segment polarity and the location and differentiation of the inflow tracts in *Drosophila*.

Similar requirements for Hedgehog signaling in both cardiac progenitor specification and subsequent differentiation are seen in vertebrates. In Zebrafish, Hedgehog specifies both the myocardial and endocardial progenitors and brings about the differentiation of the latter (Thomas et al., 2008; Wong et al., 2012). Furthermore, loss of function in mouse embryos of *Sonic hedgehog*, one of the three mammalian orthologs of the *hedgehog* gene in *Drosophila*, leads to defects in second heart field-derived cardiac structures, such as enlarged inflow tracts (Tsukui et al., 1999) and shortened and incorrectly positioned outflow tracts (Washington Smoak et al., 2005).

Migration, Positioning, Polarity, Adhesion, and Cell Shape Changes in Heart Tube Morphogenesis

The differentiating CCs and PCs from each side of the *Drosophila* embryo continue to migrate as attached rows of cells to the dorsal midline. In the process (Fig. 6), each CC first becomes constricted along its dorsal domain and makes initial contact with its contralateral counterpart across the midline along this constricted dorsal domain. Each cell in these dorsally conjoined CC pairs then adopts a crescent shape, thereby allowing the ventral domains of the CCs to also join and form a tube, which encloses an internal central lumen (Medioni et al., 2008). PCs that migrated along with the CCs flank and remain intimately connected with the resulting myocardial tube, and thus occupy positions somewhat farther from the dorsal midline. This final phase of heart tube morphogenesis thus entails the proper migration and positioning of CCs and PCs with respect to one another at the dorsal midline, adhesion between relevant cells, appropriate polarization of the CCs to determine leading edges during migration and contact between the relevant membrane domains, and cell shape changes that ensure tube and lumen formation. All these processes are also coordinated by conserved signaling pathways.

Signaling Through the Slit/Robo Pathway Mediates Migration, Positioning, Polarity, Adhesion, and Shape Change of Heart Cells

The highly conserved Slit protein and its receptors, Roundabout (Robo) and Roundabout 2 (Robo2) (Kramer et al., 2001; Wong et al., 2002), play critical roles in all of the processes described previously. Embryos lacking either *slit* or *robo2* function exhibit delayed migration of CCs and PCs toward the midline (MacMullin and Jacobs, 2006). The respective positions of both CCs and PCs are also defective in *slit*, *robo*, and *robo2* loss-of-function mutants. In embryos lacking *slit* function, the CCs fail to form two continuous rows of cells attached to each other at the midline with PCs flanking them; frequent gaps in both CC and PC rows are observed with the cells often forming inappropriate and ectopic clusters, suggesting defects in cell adhesion. Furthermore, many CCs fail to reach the dorsal midline, becoming inappropriately interspersed within the PCs. Similar phenotypes are seen in double mutants, which disrupt both *robo* and *robo2* functions, with relatively milder cardiac positioning and alignment defects being detected when only one receptor is eliminated (Qian et al., 2005; MacMullin and Jacobs, 2006; Santiago-Martinez et al., 2006).

Slit is expressed by the CCs and ultimately accumulates at the dorsal midline; Robo is expressed by both CCs and PCs; and Robo2 is expressed solely by the PCs (Qian et al., 2005; MacMullin and Jacobs, 2006; Santiago-Martinez et al., 2006). Misexpression studies showed that the correct migration, alignment, and positioning of both CCs and PCs were critically dependent on the specific localization of Slit and its receptors, and, further, that the correct localization of Slit required wild-type *robo* and *robo2* expression and functions, while that of Robo necessitated normal *slit* function (Qian et al., 2005; Santiago-Martinez et al., 2006).

In addition, Slit/Robo signaling mediates heart lumen formation by regulating membrane polarity, adhesion, and the shape of the CCs. In wild-type CCs, the cell adhesion proteins E-cadherin and β -catenin are specifically localized at the membranes along the constricted dorsal domain, where the contralateral CCs make initial contact, and at the ventral domain, where they subsequently join after adopting a crescent shape. Cell-cell adhesion at these two junctional domains but not in the intervening luminal domain (where Slit and Robo are concentrated) ensures that a proper lumen is enclosed. However, in embryos lacking *slit* or *robo* function, both E-cadherin and β -catenin localization are expanded into the presumptive luminal domain, thus creating an extended region of cell-cell adhesion that prevents lumen encapsulation (Qian et al., 2005; Medioni et al., 2008; Santiago-Martinez et al., 2008). Furthermore, Slit and its receptors are also required for the initial constriction of CCs along their dorsal junctional domain, where the

cells make initial contact with their contralateral counterparts. In mutants lacking either Slit or both Robo and Robo2 receptors, the CCs remain rounded and come into contact along most of their apposing surfaces, including the presumptive luminal domain, thereby also blocking lumen formation (Medioni et al., 2008).

Similarly, expression of *Slit2*, *Slit3*, *Robo1*, and *Robo2* is detected in the developing mouse heart (Medioni et al., 2010), and mutational analysis has revealed functional roles for these genes in the correct migration and positioning of cells of the vertebrate cardiovascular system. In Zebrafish, both *slit2* and *robo1* are required during heart tube formation for the proper migration of endocardial cells (Fish et al., 2011); in mice, *Slit3*, *Robo1*, and *Robo2* are required for the proper development of the pericardium and the sinus horn myocardium, the alignment of caval veins, and the adhesion and migration of cardiac neural crest cells (Mommersteeg et al., 2013).

Integrins Operate in Concert With Slit and its Receptors to Bring About the Migration, Alignment, and Polarization of Differentiated Heart Cells in Addition to Lumen Formation

The transmembrane Integrin receptors Myospheroid (Mys) and Scab (Scb), their ligands, and their downstream intracellular messengers are also involved in the aspects of cardiac morphogenesis mediated by Slit/Robo signaling. Delays in CC and PC migration, errors in the alignment and positioning of these cells, and defective lumen encapsulation similar to those seen in *slit* loss-of-function mutants or *robo/robo2* double mutants are observed in embryos lacking these Integrins and their Laminin ligands (Yarnitzky and Volk, 1995; Stark et al., 1997; Martin et al., 1999; MacMullin and Jacobs, 2006; Vanderploeg et al., 2012). Furthermore, double-heterozygote assays in which the cardiac phenotypes of heterozygous *slit* mutations were found to be enhanced by reducing the functions of these Integrins, their Laminin and Collagen ligands, or their downstream signaling components such as Talin or Integrin Linked Kinase, indicated synergistic interactions and therefore likely cooperative signaling between the Slit/Robo- and Integrin-mediated pathways (MacMullin and Jacobs, 2006).

Consistent with their roles during CC migration and lumen encapsulation, these Integrins are predominantly localized at the presumptive luminal domain of the leading edges of the cells. They are required for the leading-edge motility necessary for the joining of contralateral CCs—extensions of lamellipodial and filopodial processes from the leading edge were significantly reduced in embryos lacking zygotic *mys* or *scb* function (Vanderploeg et al., 2012). These Integrins also mediate the localization of Slit and Robo proteins necessary for lumen formation at the presumptive luminal domain—in embryos mutant for the Integrin-coding genes, Slit and Robo are mislocalized and present at significantly reduced levels, and the lumen is consequently missing or defective (Vanderploeg et al., 2012).

The lumen encapsulation by the two juxtaposed CCs in the *Drosophila* heart shares considerable similarities with lumen development in the mammalian dorsal aorta, where two apposed endothelial cells undergo cell shape changes to give rise to an extracellular lumen (Strlic et al., 2009). Thus, it is worth noting that disrupting B1-Integrin function in vertebrates results in absent or defective vascular lumens reminiscent of the cardiac phenotypes of Integrin mutants in *Drosophila* (Drake et al., 1992; Zovein et al.,

2010). Similar contributions of Integrins to migration and adhesion during vertebrate vascular development are also seen (Davis and Senger, 2005; Hynes, 2007; Silva et al., 2008).

The Small GTPase Cdc42, the Non-muscle Myosin II Zipper, and the Formins dDAAM and Diaphanous Mediate Lumen Formation

The small GTPase Cdc42 plays a critical role in cardiac lumen formation in *Drosophila*. Contralateral CCs in embryos that lack *Cdc42* function exhibit defective cell shape changes during lumen formation, failing to make ventral contacts after the initial dorsal contacts. In particular, Cdc42 plays an integral role in the actomyosin network: Functioning in concert with dDAAM and Diaphanous, formins involved in actin polymerization, Cdc42 ensures the proper dynamic localization of the *Drosophila* non-muscle myosin II Zipper at the leading edge of the CCs that brings about the requisite cell shape changes for lumen formation (Vogler et al., 2014).

Cdc42 also plays critical roles in mammalian heart morphogenesis. The deletion of *Cdc42* in the developing mouse heart results in early embryonic lethality, and functional Cdc42 is required for leukocyte-inhibitory factor (LIF)-induced cell shape changes in cultured rat cardiomyocytes (Nagai et al., 2003; Maillet et al., 2009). Given the parallels described earlier between lumen formation in the *Drosophila* heart and the mammalian dorsal aorta, it is worth noting that *Cdc42* is also needed for lumen development in human endothelial cell cultures (Bayless and Davis, 2002; Koh et al., 2008).

Metamorphosis of the Embryonic/Larval *Drosophila* Heart Into The Adult Heart

During the pupal stage, the once-embryonic and subsequently larval heart tube undergoes extensive morphological changes to metamorphose into the adult heart (Curtis et al., 1999; Zikova et al., 2003; Monier et al., 2005). Most of the posterior wider region of the embryonic heart, referred to as the heart proper, is histolyzed, while the narrower, anterior part of the heart, referred to as the aorta, becomes widened. New ostia (inflow tracts) develop from the Seven up-CCs of what used to be the embryonic aorta, and three new intracardiac valves are formed (Molina and Cripps, 2001; Monier et al., 2005; Lehmacher et al., 2012). Finally, a subset of the syncytial alary muscles that connect the heart to the lateral exoskeleton dedifferentiates into mononucleate myoblasts and subsequently redifferentiates to form a new addition to the adult heart: a layer of longitudinal muscles associated with the heart tube along its ventral surface (Molina and Cripps, 2001; Lehmacher et al., 2012; Schaub et al., 2015).

Signaling through both the FGFR Heartless and Notch pathways was found to be necessary for the differentiation of the alary muscle-derived myoblasts (i.e., cells not originally part of the heart tube) into the ventral longitudinal muscles of the adult heart (Zeitouni et al., 2007; Schaub et al., 2015). Similarly, in vertebrates, FGF and Notch signaling mediate the proliferation and differentiation of cells from the second heart field—mesodermal cells that were not originally part of the initial heart tube but that contribute to tube extension and most of the cardiac structures (Reifers et al., 2000; Abu-Issa et al., 2002; Alsan and Schultheiss, 2002; Frank et al., 2002; Ilagan et al., 2006; Park et al., 2006; Chen et al., 2008; Park et al., 2008; Zhang et al., 2008; Kwon et al., 2009).

Furthermore, inhibition of the canonical Wnt/ β -catenin signaling pathway in *Drosophila* was found to be necessary for the transdifferentiation of the CCs of a segment of the wider embryonic heart proper into those of the narrower, posterior terminal chamber of the adult heart (Zeitouni et al., 2007). Of note, a similar reduction of canonical Wnt/ β -catenin signaling is required to bring about the terminal differentiation of cardiomyocytes in the vertebrate heart (Brott and Sokol, 2005; Lavery et al., 2008; Zhu et al., 2008).

Additional roles of Wnt and PDGF-VEGF signaling in the formation of ostial and intracardiac valves are discussed in the following section.

Valve Formation

Wnt Signaling is Required for Ostia Valve Development

In both *Drosophila* and vertebrates, valves regulate the unidirectional flow of the circulatory fluid into the heart through the inflow tracts, and from the heart into the outflow tracts. Channels termed ostia serve as the inflow tracts in *Drosophila* (Rizki, 1978; Molina and Cripps, 2001). By undergoing a process of cellular constriction, extension out of the CC layer into the heart lumen, and partial delamination, the two Seven up-CCs that comprise each ostium achieve a distinct morphology that enables them to function as valves to regulate inflow into the heart. Signaling by Wnt4, which is expressed in these ostia cells at much higher levels than in other CCs, and Wingless, which is expressed specifically in the ostia-lining Seven up-CCs, are essential for proper development of the ostia. Embryos in which canonical Wnt signaling is disrupted with *Wnt4* mutations, *Wnt4* RNAi, *wingless* mutations, *wingless* RNAi, *frizzled* RNAi, *frizzled2* RNAi, or dominant negative versions of the downstream TCF transcription factor exhibit absence of Seven up-CCs or severe defects in ostia cell morphology, and thus in ostia formation (Tauc et al., 2012; Chen et al., 2016; Trujillo et al., 2016).

A similar role for Wnt signaling in valve formation is also seen in vertebrates. A subset of vertebrate endothelial/endocardial cells also undergoes constriction, extension, and delamination in an epithelial-mesenchymal transition (EMT) process as the cells leave the endothelial layer and migrate into the cardiac jelly to form the cardiac cushions that give rise to the cardiac valves (Armstrong and Bischoff, 2004). This early EMT process is associated with high levels of both Wnt gene expression and Wnt signaling in mouse, chicken, and Zebrafish embryos (Armstrong and Bischoff, 2004; Liebner et al., 2004; Alfieri et al., 2010; Moro et al., 2012). Furthermore, disruption of the Wnt signaling pathway with β -catenin mutations, β -catenin antagonists, Wnt antagonists, or Wnt inhibitors also blocks cushion formation and, thus, valve differentiation (Hurlstone et al., 2003; Liebner et al., 2004; Alfieri et al., 2010).

PDGF-VEGF Signaling is Required for Adult Heart Valve Formation

During metamorphosis in *Drosophila*, extensive remodeling of the CCs of the larval heart brings about the formation of the adult heart (Molina and Cripps, 2001; Monier et al., 2005). Programmed cell death eliminates most of what was the posterior larval heart chamber, new ostia differentiate in what used to be the larval aorta (anterior compartment), and three new pairs of valves form that ensure the circulatory fluid is pumped only anteriorward. PDGF

and VEGF receptor-related (Pvr), a receptor tyrosine kinase related to mammalian PDGF and VEGF receptors, is specifically expressed in the precursors of these new adult valves. Additionally, expression of a dominant negative version of Pvr in the heart partially repressed adult valve formation, while ectopic expression of a constitutionally activated form of Pvr occasionally induced additional ectopic valves, indicating a role for the PDGF-VEGF signaling pathway in adult cardiac valve formation (Zeitouni et al., 2007).

In vertebrates, signaling through the VEGF receptor is used to induce EMT-mediated cardiac cushions from the endocardium and ultimately gives rise to the cardiac valves (Armstrong and Bischoff, 2004). Ligands of the VEGF receptor become restricted to the endocardial cells in the cushion forming regions (Miquerol et al., 1999; Dor et al., 2001). Furthermore, blocking VEGF signaling with inhibitors of the VEGF receptor causes loss of valve differentiation markers along with structural and functional defects in cardiac valve development (Lee et al., 2006).

Pygopus is Required for Adult Heart Valve Differentiation

Another protein involved in *Drosophila* adult heart valve formation is Pygopus, which had previously been identified as a component of the canonical Wnt signaling pathway mediated by Wingless, Armadillo, and TCF (Belenkaya et al., 2002; Kramps et al., 2002; Parker et al., 2002). At the CCs comprising the intracardiac adult valves, the heart tube lumen is narrower and the myofibrillar network is denser than at the adjacent CCs. RNAi knockdown of *pygopus* results in both a lack of high-density myofibrils and an increase in the heart tube diameter at the valves. Furthermore, loss-of-function mutants of *pygopus* did not exhibit any defects in CC number, indicating that Pygopus played a role in cardiac valve differentiation, but not in specification. RNAi knockdown of other components of the Wingless signaling pathway also exhibited significant valve dilations, albeit not as severe as that in the *pygopus* knockdown, and no genetic interaction was detected between *pygopus* and the genes encoding these components in double-heterozygote assays (Tang et al., 2014). While this does not completely rule out the possibility that Pygopus might be mediating the differentiation of adult intracardiac valves through the canonical Wnt signaling pathway also utilized for valve formation in vertebrates (Armstrong and Bischoff, 2004; Liebner et al., 2004; Alfieri et al., 2010; Moro et al., 2012), it does raise the question of whether Pygopus could be using a different signaling pathway instead.

Signaling Pathways Utilized In Conserved Cardiogenic Processes Also Regulate Processes Unique to Vertebrate Cardiogenesis

While this review has focused primarily on signaling pathways mediating aspects of heart development that are common to both *Drosophila* and vertebrates in terms of origins, structure, morphogenesis, and regulatory mechanisms, there are certain features of vertebrate cardiogenesis that have no counterparts in the fly. Many of the signaling pathways that regulate the common conserved aspects of heart development, however, are also used to mediate these cardiogenic processes unique to vertebrates.

For example, both the *Drosophila* and the vertebrate embryonic hearts originate as linear tubes running along the anterior-posterior axis, with circulatory fluid entering the lower/posterior

part of the heart through inflow tracts and being pumped out from the upper/anterior region through the outflow tract (Fig. 1). However, in vertebrates, but not in *Drosophila*, looping of this originally linear tube leads to its anterior-posterior polarity being converted into right-left polarity, with the atria, the previously posterior region where blood enters the heart, becoming ultimately anterior to the presumptive ventricles, the part of the heart that pumps blood out (Manner, 2000). The direction of cardiac looping is dependent on the left-right asymmetry set up in vertebrate embryos by asymmetrical expression of the genes *Nodal*, *Lefty*, and *Pitx2* on the left side of the body, which in turn is mediated by the Notch, Hedgehog, Wnt, and BMP signaling pathways (Levin et al., 1995; Sampath et al., 1997; Boettger et al., 1999; Meyers and Martin, 1999; Schilling et al., 1999; Zhang et al., 2001; Krebs et al., 2003; Raya et al., 2003; Tsiarris and McMahon, 2009). The actual looping process itself is mediated by modulators of cytoskeletal changes such as the protein *Xin*, which in turn is induced by BMP2 signals (Wang et al., 1999).

Another unique aspect of vertebrate heart development is the separation of the undivided lumen of the looped heart tube into four distinct chambers. This entails the formation and fusion of endocardial cushions that divide the heart tube into right and left atrioventricular channels, the ventral growth of two atrial septa from the roof of the atrium into these endocardial cushions, and the development of the interventricular septum from the myocytes at the floor of the developing ventricles and extensions of the endocardial cushions (Schleich et al., 2013). Signaling through BMP and canonical Wnt pathways is necessary for forming the endocardial cushions by EMTs (Hurlstone et al., 2003; Liebner et al., 2004; Ma et al., 2005; Person et al., 2005; Rivera-Feliciano and Tabin, 2006), while the development and growth of the septa also require Hedgehog in addition to Wnt/ β -catenin and BMP signaling (Goddeeris et al., 2008; Tian et al., 2010; Briggs et al., 2013).

Yet another feature that distinguishes vertebrate cardiogenesis from that in *Drosophila* is the colonization of and contribution to the vertebrate heart by an extracardiac nonmesodermal progenitor population: the cardiac neural crest cells. Arising originally from the dorsal neural tube, the cardiac neural crest cells migrate through the caudal pharyngeal arches into the outflow tract, bringing about its septation, and contributing to endocardial cushions, valves, smooth muscle tissue, and, in the case of Zebrafish, the myocardium (Kirby and Waldo, 1995; Hutson and Kirby, 2003; Li et al., 2003; Sato and Yost, 2003). The migration of the cardiac neural crest cells is mediated by signaling through the Hedgehog, BMP, Slit-Robo, and non-canonical Wnt pathways (Kaartinen et al., 2004; De Calisto et al., 2005; Jia et al., 2005; Washington Smoak et al., 2005; Calmont et al., 2009), while their proliferation and survival involves Hedgehog, FGF, and primarily canonical but also non-canonical Wnt signaling (Gage et al., 1999; Brault et al., 2001; Abu-Issa et al., 2002; Hamblet et al., 2002; Kioussi et al., 2002; Goddeeris et al., 2007).

Spatiotemporal Expression Patterns of Signaling Pathway Components Regulate Cardiogenic Processes

The diverse cardiogenic processes mediated by the distinct signaling mechanisms described in this review must be correctly orchestrated in time and space to bring about proper heart

development. Not surprisingly, in many instances, it is the regulation of the spatiotemporal expression patterns of the ligands of these signaling pathways that determines where and when the latter are activated. For example, it is the localized expression patterns of the FGF ligands *Pyramus* and *Thisbe* that ensure the dorsal migration of the mesodermal monolayer (Kadam et al., 2009; Klingseisen et al., 2009); of *Dpp* and *Wingless* that bring about the specification of the cells immediately underlying their intersection as the cardiac mesoderm (van den Heuvel et al., 1989; Frasch, 1995; Wu et al., 1995; Yin and Frasch, 1998; Lockwood and Bodmer, 2002); and of *Delta*, expressed by the CCs, that induces a pericardial fate on neighboring cells (Grigorian et al., 2011; Ahmad et al., 2014).

However, an observation often not fully appreciated is that the regulation of expression patterns of other components of the signaling pathways may also be used to determine the time and location of a particular cardiogenic process. One illustration of this involves the cells that will become specified as the cardiac mesoderm by the later FGFR-mediated signaling process once mesoderm migration is completed (Michelson et al., 1998b). These localized cells must continue to produce the FGFR *Heartless*, a feat mediated by a *heartless* enhancer driving expression specifically in the presumptive cardiac mesodermal cells (Ahmad et al., 2016).

Integrating Multiple Signals During Cardiogenesis

Given the number of distinct signaling pathways discussed so far, especially those involved in establishing the cardiac mesoderm, a particularly germane question is how the inputs from these diverse pathways are effectively combined to bring about proper heart development. An investigation into the regulation of *even skipped*, a gene expressed by a subset of cardiac progenitor cells, indicated that this integration is achieved at the transcriptional level in cardiac genes.

As illustrated in Figure 3, the signaling pathways involved in cardiac mesoderm specification act through downstream transcription factors: ETS domain transcription factors in the case of FGFR and EGFR, TCF in the case of *Wingless*, and *Mad* in the case of *Dpp*. A 312-bp minimal muscle and heart enhancer (MHE) of the *even skipped* gene that was sufficient to drive its cardiac mesodermal expression contained functional binding sites for ETS domain proteins, TCF, and *Mad*, in addition to those for *Twist* and *Tinman*, transcription factors ubiquitously expressed throughout the presumptive cardiac mesoderm (Halfon et al., 2000). These binding sites were critical for MHE function, since mutating individual sites significantly reduced reporter expression driven by the MHE. Furthermore, the transcription factors binding at these sites act in a synergistic manner, since mutating two distinct sites resulted in expression defects significantly more severe than the additive effects of mutating the sites independently. Collectively, these data demonstrate that cardiac genes can integrate the inputs from many distinct signaling pathways by combinatorial binding of multiple pathway-specific transcription factors to their enhancers. Many other examples of cardiac genes utilizing their enhancers to combine inputs from diverse signaling pathways have since been observed, indicating that this is a favored method for integration (Halfon et al., 2002; Han

et al., 2002; Philippakis et al., 2006; Zhu et al., 2012; Ahmad et al., 2014; Busser et al., 2015a).

Of note, the integration of signaling pathway inputs in establishing the cardiac mesoderm also provides an effective approach for identifying novel heart genes (Ahmad et al., 2012). As described previously, activation of the FGFR, EGFR, Dpp, and Wingless signaling pathways would produce more cardiac mesodermal cells, and thus elevate the expression of cardiac genes within the embryo, whereas signaling through Notch would result in fewer cardiac mesodermal cells and reduced levels of cardiac gene expression. Consequently, mutations or transgenes that inactivated or constitutively activated these signaling pathways would be expected to alter cardiac gene expression in a predictable manner. Genome-wide transcriptional expression profiles from wild-type embryos and multiple genetic backgrounds that perturbed individual signaling pathways in both these ways were thus obtained, and a statistical meta-analysis method fitted to a training set of 40 known cardiac mesodermal genes was used to rank all *Drosophila* genes by their likelihood of being expressed in the heart based on their collective behavior in these transcriptional expression profiles. In situ hybridization assays performed on the highly ranked genes in this meta-analysis identified 70 novel cardiac genes, with the cardiogenic roles of several being subsequently elucidated (Ahmad et al., 2012; Ahmad et al., 2014).

Perspectives

The leading causes of deaths worldwide are cardiovascular diseases (Go et al., 2014; Nichols et al., 2014). In particular, congenital heart disease (CHD) remains the most prevalently diagnosed birth defect, with a postnatal incidence of 0.75% and an incident rate of 10% in stillbirths (Hoffman, 1995; Hoffman and Kaplan, 2002; Reller et al., 2008; Marelli et al., 2014). Understanding the molecular and genetic defects underlying CHDs and cardiomyopathies is thus a pressing reason to aim for a complete and comprehensive analysis of heart development.

Multiple approaches have been used to study cardiogenesis. They include the use of cytogenetic analysis, linkage and association studies, copy number variation and DNA microarray analysis, and whole exome sequencing with CHD-affected individuals in humans (Fahed et al., 2013; Andersen et al., 2014; Digilio and Marino, 2016); of cell-based assays using either ES cells or induced pluripotent stem cells induced to differentiate along the cardiac lineage (Alexander and Bruneau, 2010); of vertebrate models such as the mouse, chicken, Zebrafish, and *Xenopus* (Bruneau, 2008; Staudt and Stainier, 2012; Kain et al., 2014; Duncan and Khokha, 2016; Grant et al., 2017); and, of course, of the invertebrate model *Drosophila* (Tao and Schulz, 2007; Vogler and Bodmer, 2015; Frasch, 2016; Lovato and Cripps, 2016; Rotstein and Paululat, 2016).

Each approach has its advantages and shortcomings, and a thorough understanding of heart development will require using all of them. As described previously, there are a few late vertebrate cardiogenic processes that are not recapitulated in *Drosophila*. But the relatively reduced level of complexity of the *Drosophila* heart compared to its vertebrate counterpart; the metamericly repeated and stereotyped arrangements of its cells that allow even subtle phenotypes to be detected and quantitated easily; its amenability to genetic analyses, such as the ability to disrupt any of its genes in any domain via RNAi and the ease of performing genetic interaction, epistasis, and rescue assays; the

ability to examine heart development at single cell resolution; the capacity of the fly to survive to the larval level of development even with a disrupted heart; and the fact that the relatively low level of gene duplication in *Drosophila* reduces redundancy issues associated with paralogs all serve to make it a particularly tractable system for studying cardiogenesis. The observation that not merely cardiogenic transcription factors but entire signaling pathways and the cardiogenic functions they mediate are conserved between *Drosophila* and vertebrate heart development further highlights the importance of utilizing investigations in *Drosophila* to enhance and facilitate our understanding of vertebrate cardiogenesis. Part of the power of *Drosophila* lies in being able to use its genetic tool kit to unravel these signaling pathways. This is particularly relevant in the context of the small number of genes identified as involved in CHD from human subjects: A significant fraction of these genes encode components of signaling pathways (Fahed et al., 2013; Andersen et al., 2014). They would be of little value if we were unable to elucidate the relevant cardiogenic signaling pathways that they contribute to based on research performed in *Drosophila* and other vertebrate models.

Similarly, attempts at understanding cardiogenesis using *Drosophila* should consider using integrative strategies that both leverage the distinct advantages of different systems and focus on genes and pathways likely to be playing critical roles in mammalian cardiogenesis, and thus be of particular relevance to human heart development. One elegant example of such an integrated approach utilized histone marks and gene expression profiles from human and mouse ES cells differentiating along the cardiac lineage to create an epigenetic signature to predict putative mammalian cardiogenic genes. The effects of knocking down their orthologs in the developing *Drosophila* cardiac mesoderm and heart were then examined to rapidly and efficiently assess whether these predicted genes actually possessed cardiogenic roles and determine which to analyze further (Busser et al., 2015b). Conversely, with more and more new genome-wide approaches for studying heart development being utilized in *Drosophila* (reviewed in Frasch, 2016), it has become even more important to explore and assess what the resulting findings can tell us about vertebrate, and ultimately human, cardiogenesis.

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