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The role of *pygopus* in the differentiation of intra-cardiac valves in *Drosophila*

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Abstract

Cardiac valves serve an important function; they support unidirectional blood flow and prevent blood regurgitation. Wnt signaling plays an important role in the formation of mouse cardiac valves and cardiac valve proliferation in Zebrafish, but identification of the specific signaling components involved has not been addressed systematically. Of the components involved in Wnt signal transduction, *pygopus* (*pygo*), first identified as a core component of Wnt signaling in *Drosophila*, has yet to be investigated with respect to valve development and differentiation. Here, we take advantage of the *Drosophila* heart model to study the role of *pygo* in formation of valves between the cardiac chambers. We found that cardiac-specific *pygo* knockdown in the *Drosophila* heart causes dilation in the region of these cardiac valves, and their characteristic dense mesh of myofibrils does not form and resembles that of neighboring cardiomyocytes. In contrast, heart-specific knockdown of the transcription factors, *arm/β-Cat*, *lgs/BCL9* or *pan/TCF*, which mediate canonical Wnt signal transduction, shows a much weaker valve differentiation defect. Double-heterozygous combinations of mutants for *pygo* and the Wnt-signaling components have no additional effect on heart function compared to *pygo* heterozygotes alone. These results are consistent with the idea that *pygo* functions independently of canonical Wnt signaling in the differentiation of the adult inter-chamber cardiac valves.

Keywords

intra-cardiac valves; *pygo*; heart; Wnt; cardiomyocyte

Introduction

Cardiovascular malformations are common features of congenital heart disease, especially cardiac valve defects (Goldmuntz 2001; Loffredo 2000). In adults, cardiac valve defects result in blood regurgitation that is usually accompanied by weaker contractions, and they

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are a major cause of morbidity and mortality (Brickner *et al.* 2000). Therefore, understanding the molecular components involved in cardiac valve development and maintenance will be important to provide novel therapeutic strategies for treating cardiac valve disease.

Recently, significant advances have been made in the elucidation of regulatory pathways that control normal and abnormal cardiac valve development. The primitive linear heart tube in vertebrates is a two layered structure, composed of an inner endothelial layer and an outer myocardial layer. The myocardial layer will form the muscular walls of the heart while the endothelial layer comprises the endocardium (Abu-Issa and Kirby 2007; Brutsaert *et al.* 1996). Cells from specific regions of the endocardium will undergo epithelial-to-mesenchymal transformation (EMT) to form the endocardial cushions. The locally expanded cardiac cushion undergoes extensive remodeling to give rise to the valves (Combs and Yutzey 2009). Myocardial BMP2 expression increases hyaluronic acid deposition and promotes EMT in cushion-forming regions of the atrioventricular canal (AVC) and out flow tract (OFT) (Ma *et al.* 2005). EMT is also promoted by TGF- β signaling through transcription factor Slug (Romano and Runyan 2000), whereas Notch1 signaling through Snail promotes EMT by suppressing VE-cadherin expression (Niessen and Karsan 2008). Once the cushions are established, endocardial VEGF expression maintains endothelial cell proliferation and inhibits EMT (Dor *et al.* 2001).

In the *Drosophila* heart tube a pair of valve cells appears in each of three consecutive abdominal segments (A2–A4, see illustration in Fig. 1) that partition the fly heart into four chambers. They are part of the *tinman*-expressing working myocardium in these segments and their embryonic lineage has been elucidated (Alvarez *et al.* 2003; Han and Bodmer 2003; Lehmachner *et al.* 2012a; Molina and Cripps 2001). It is well established that the transcriptional networks and signaling pathways involved in the specification of the *Drosophila* heart are fundamentally conserved and have vertebrate counterparts with analogous cardiogenic functions (Bier and Bodmer 2004; Bodmer 1995; Bodmer and Frasch 2010; Cripps and Olson 2002). The adult valve cells have a distinct morphology compared to the neighboring cardiomyocytes due to a characteristically dense network of myofibrils, large intracellular cavities, as well as narrowing of the inner cardiac lumen between opposing valve cells (see (Lehmacher *et al.* 2012b) for detailed description). Although the *Drosophila* heart does not have an ‘endocardial’ lining or discernible endocardial cushion tissue, given the genetic conservation between flies and humans it is likely that there are similarities in valve development and/or function. This study sets the stage for a first comparison.

The specific activation of wingless (Wnt) signaling within the forming cardiac valves in mouse suggests a functional role for Wnt signaling in cardiac valve formation and function (Gitler *et al.* 2003). In Zebrafish, loss of Adenomatous polyposis coli (APC) function, an inhibitor of Wnt signaling, results in “un-looped” hearts and excessive endocardial cushion formation resulting from increased proliferation of endocardial cells, whereas overexpression of APC or Dickkopf-related protein 1 (DKK1) inhibits the formation of cardiac cushions (Hurlstone *et al.* 2003) and in chick, Wnt9a induced activation of Wnt/ β -catenin signaling causes proliferation of endocardial cells. The involvement of Wnt

signaling in the specification of the cardiac precursors was first demonstrated in *Drosophila* (Park et al. 1996; Wu et al. 1995). However, it is not known whether Wnt signaling plays a role in later aspects of heart development and differentiation in *Drosophila*.

The transcriptional adaptor protein encoded by *pygopus* (*pygo*) was first identified as a core component of Wnt signaling in embryonic patterning and other developmental processes in *Drosophila* (Belenkaya et al. 2002; Kramps et al. 2002; Parker et al. 2002; Thompson et al. 2002). Complete loss of *pygo* function in the developing fly embryo phenocopies loss of *wingless* and canonical Wnt signaling, but knockout of Pygo 1 and/or 2 in the mouse has only minor developmental effects and has not been studied in the adult heart (Schwab et al. 2007). Here, we examine the role of *pygo* in the formation of the adult cardiac valve in *Drosophila*. We find that cardiac-specific knock down (KD) of *pygo* during pupal cardiac remodeling prevents the proper differentiation of intra-cardiac valves from larval cardiomyocytes, which leads to defective valve structure and function. In contrast, disruption of canonical Wnt signaling by KD of other Wnt components, armadillo/b-catenin (*arm/β-cat*), *legless/BCL9* (*lgs/BCL9*) or pangolin/*T-cell factor* (*pan/TCF*), show much weaker defects in intra-cardiac valve differentiation than does *pygo* KD. Double-heterozygous combinations of mutants for *pygo* and the Wnt-signaling components have no additional effect on heart function compared to *pygo* heterozygotes alone. We conclude that *pygo* functions, at least in part, independently of Wnt signaling in the differentiation of the adult inter-chamber cardiac valves.

Results

1. cardiac specific *pygo* KD causes structural malformations of cardiac valves

In *Drosophila* adults, four pairs of *tinman*-expressing cardiomyocytes within each body segment derive from two embryonic cardiac progenitors (Han and Bodmer 2003; Lehmacher et al. 2012b). One pair of these cardiomyocytes in the abdominal segments A2, A3, and A4 differentiate into intra-cardiac valves, dividing the heart into four chambers (Fig. 1). We used the *hand4.2*-Gal4 driver (Han and Olson 2005) crossed to UAS-*pygo*-RNAi to specifically KD *pygo* in the heart and examined cardiac structure using phalloidin staining to identify F-actin within the heart tube. Valves are characterized by a dense myofibrillar network that can be visualized by F-actin staining (Fig. 2a, blue arrows) that makes them easily distinguishable from the adjacent cardiomyocytes (Fig. 2a, red arrows) (Lehmacher et al. 2012b; Monier 2005). Cardiac-specific *pygo* KD prevents characteristic valve cell differentiation, manifested by a lack of high-density myofibrils (Fig. 2b, blue arrows) compared to the adjacent myocardial cells (Fig. 2b, red arrows).

To gain further insight into the structural malformation in valves in response to *pygo* KD, we used Di-8-ANEPPS to stain the plasma membranes of cardiomyocytes in the heart tube. Di-8-ANEPPS fluorescence is localized to the membrane of excitable cells, including neurons and cardiac cells (Pucihar et al. 2009). In control hearts, valve cells stained with Di-8-ANEPPS (dashed white lines in Fig. 3A, and cross section in Fig. 3a') appear much thicker than adjacent cardiomyocytes (Fig. 3b'; locations of all cross sections are indicated by vertical yellow arrows). In addition, the inner cardiac diameter at the valve region is smaller than in the adjacent chamber (compare Fig. 3a' and b'). In contrast, *pygo* KD hearts

show no thickening of the heart wall in the valve regions (compare Fig. 3A and a' with Fig. 3C and c'). We were able to rescue these valve defects by heart-specific *pygo* overexpression in a *pygo* KD heart (*hand4.2>UASpygoRNAi*; *UASpygo*, Fig. 3D and d'). Together with the data obtained from phalloidin staining, our data suggest that cardiac-specific *pygo* KD causes defects in the structural differentiation of intra-cardiac valves in *Drosophila*.

We also examined the role of *pygo* in the formation of cardiac valve precursors, and observed that in *pygo* null mutant embryos (*pygo*^{s123}) (Thompson *et al.* 2002) stained with Dmef2 (Bour *et al.* 1995), the normal number of cardiomyocyte nuclei were formed, including those of the prospective intra-cardiac valve (Fig. S1). Therefore, we conclude that a zygotic *pygo* null mutation does not affect the initial formation of valve precursors, and the developmental effects of this mutation are likely masked by a maternal contribution.

2. Overexpression of *pygo* causes myofibrillar reorientation within the cardiac valve

The densely packed myofibrils that are normally found in adult myocardial cells are typically organized in a circumferential arrangement, including within the intra-cardiac valves (Fig. 4a). This arrangement is important for efficient heart contractions. Interestingly, we observed that cardiac overexpression of *pygo* causes the normally transverse arrangement of myofibrils to become more disorganized (Fig. 4b). Thus, both a decrease as well as an increase in *pygo* function within the heart results in cardiac valve abnormalities, indicating that it is the fine-tuned regulation of *pygo* that is important for the normal maturation of intra-cardiac valves in *Drosophila*.

3. *Pygo* KD causes significant dilation of cardiac valves

In adult *Drosophila*, the intra-cardiac valves (Fig. 5a, blue arrows) are characterized by apparent narrowing of the heart tube lumen compared to the lumen enclosed by adjacent cardiomyocytes (Fig. 5a, red arrows). In *pygo* KD hearts we observed only a slight narrowing of heart tube in the region of the valve cells (Fig. 5b, blue arrows) relative to the tube formed by the adjacent myocardial cells (Fig. 5b red arrows; see also Fig. 3). We quantified the degree of narrowing of heart tube by measuring the diastolic diameters of the intra-cardiac valve cells (valve diameter) and of adjacent myocardial cells (heart diameter). The valve/heart diameter ratio was significantly increased in *pygo* KD hearts, compared to controls (Fig. 5c, $p < 0.001$), indicating that loss of *Pygo* function causes significant dilation in the region of cardiac valve. Similar effects on valve dilation were observed using a second *pygo* RNAi line (Supplemental Figure 2b) suggesting that this phenotype is not due to off target effects. Significantly this dilation could be partially rescued by overexpression of *pygo* in the *pygo* KD hearts (*Hand 4.2-Gal4> UAS-pygo-RNAi*; *UAS-pygo*, Fig. 5c). These data indicate that the valve defects are specifically due to *pygo* KD and are heart autonomous. This valve dilation allows for a significant amount of hemolymph regurgitation and would be expected to reduce the efficiency of hemolymph circulation (Supplementary video 1).

4. *pygo* functions independently of Wnt signaling in the differentiation of intra-cardiac valve cells

pygo was originally identified as a critical component of canonical Wnt signaling that associates with *arm/β-Cat*, *lgs/BCL9*, and *pan/TCF* in the nucleus to active Wnt-responsive genes (Belenkaya *et al.* 2002; Kramps *et al.* 2002; Parker *et al.* 2002; Thompson *et al.* 2002). Thus, we wanted to determine whether or not the role of *pygo* in intra-cardiac valve differentiation is associated with canonical Wnt signaling. We therefore compared the valve diameter to heart diameter ratio in hearts from flies with cardiac-specific KD of the Wnt signaling components, *arm*, *lgs* and *TCF* and found that these KD hearts showed a slight but significant dilation of the valves (Fig. 6). However, this dilation was much smaller than for *pygo* KD hearts (Fig. 6). This suggests that the function of *pygo* in the differentiation of intra-cardiac valve cells may be, in part, independent of canonical Wnt signaling (see discussion).

5. *Pygo* does not genetically interact with other Canonical Wnt components during valve differentiation

To determine whether *pygo* regulates valve differentiation partially through Wnt signaling, we generated double heterozygotes combinations of *pygo*^{S123} with *arm*² or *pan*³ and found double heterozygotes *pygo*^{S123/arm}² or *pygo*^{S123/pan}³ exhibit no significant differences in the ratio of valve diameter to heart diameter compared to single heterozygotes *pygo*^{S123/+} (Supplementary fig. 3), indicating *arm* or *TCF* heterozygotes have no additional effects in valve differentiation over *pygo* heterozygotes alone. This finding further supports a canonical Wnt signaling-independent requirement for *pygo* in valve differentiation.

Discussion

Drosophila has been shown to be an attractive model for heart development, function and aging (Bodmer and Frasch 2010). Formation of the cardiac tubes of fruit flies and the initial linear heart of vertebrates are similar and are well conserved in terms of the regulatory genetic networks involved (Bier and Bodmer 2004; Bodmer 1995; Ocorr *et al.* 2007c). The formation of the intra-cardiac valves may also have aspects that are conserved. In mammals, the initial linear heart is composed of an outer layer of myocardium and inner lining of endocardial cells, separated by extensive extracellular matrix (ECM) referred to as the cardiac jelly. After rightward looping of the hearts, the cardiac jelly overlying the future AVC region expands to form the cardiac cushion where the endocardial cells undergo an EMT, proliferate and migrate into the cardiac jelly. Finally, the endocardial cushion area elongates and undergoes remodeling that eventually refines the primitive cushion into thin elongated valve leaflets (Armstrong and Bischoff 2004; Chang *et al.* 2004).

In *Drosophila*, the bilaterally aligned pairs of cardiomyocytes fuse at the dorsal midline to form the heart tube enclosing a lumen. Among them, one pair of cardiomyocytes in each of three consecutive abdominal segments (A2–4) form the intra-cardiac valves that partition the fly heart into four ‘chambers’. These cardiomyocyte-derived valve cells undergo changes in their shape and increase their myofibrillar content forming a thickening facing the luminal side of the tube. Whether these cardiac valves in *Drosophila* also have ‘endocardial’

properties, in addition to their myocardial function is not known. However, these cardiomyocytes contribute to the heart contractions and function as valves preventing regurgitation that may be functionally equivalent to the vertebrate cardiac cushion (Lehmacher *et al.* 2012a; Zeitouni *et al.* 2007).

The molecular pathways responsible for the cardiac valve may also have conserved aspects, as is the case for the overall process of heart formation (Bodmer 1995; Bodmer and Frasch 2010; Bodmer and Venkatesh 1998; Cripps and Olson 2002). Recent studies show that, for example, VEGF signaling in the formation of the cardiac valve in the mouse appears to have an analogous function in *Drosophila* (Chang *et al.* 2004; Zeitouni *et al.* 2007). We propose that even though *Drosophila* does not have an endocardial lineage separate from that of cardiomyocytes, it may nevertheless serve as a useful genetic model for research on the development and differentiation of cardiac valves.

The endocardial cushion that provides physical support for mechanical function, serves as a scaffold and prevents the backflow of blood through the primitive heart tube in vertebrates (Camenisch *et al.* 2000; Schroeder *et al.* 2003). In mammals, the maturation of the endothelial cushion includes the appropriate synthesis and organization of the extracellular matrix (Peacock *et al.* 2008; Tan *et al.* 2011) which adopts a polarized configuration (Butcher *et al.* 2007; Canty *et al.* 2006; Sandbo and Dulin 2011). It has been demonstrated that actin-filaments are required for correct collagen fibril alignment and defects in this organization underlies cardiac valvular dystrophy in humans (Canty *et al.* 2006; Kyndt *et al.* 2007; Lardeux *et al.* 2011). In *Drosophila*, adult valve cardiomyocytes normally contain densely packed and precisely organized myofibrils, and they are distinguishable from the less dense myofibrillar meshwork within the neighboring cardiomyocytes (Lehmacher *et al.* 2012a; Zeitouni *et al.* 2007) (see also Figs. 3&5). Our observations show that cardiac-specific *pygo* KD causes decreased density and disorganization of myofibrillar structure in intra-cardiac valve cells, which causes a functional defect in the valve and problems with blood regurgitation. Whereas, cardiac overexpression of *pygo* results in a reorientation of myofibrils in the valves, which suggests that appropriate levels of *pygo* expression are important for the proper myofibrillar alignment and valve development and function. Changing these levels cause structural defects. In sum, we conclude that *pygo* plays an important role in intra-cardiac valve differentiation in *Drosophila*.

pygo has been shown to interact directly with *arm/β-Cat*, *lgs/BCL9* and *pan/TCF* in the nucleus as an essential component of Wnt signal transduction in *Drosophila*, and *pygo* loss of function phenocopies defective Wnt/*wingless* signaling (Belenkaya *et al.* 2002; Kramps *et al.* 2002; Parker *et al.* 2002; Thompson *et al.* 2002). Our results show that *pygo* KD has a much stronger effect on the dilation of intra-cardiac valves compared to knock down of the other canonical Wnt component (*arm*, *lgs*, and *TCF*) (Fig. 2b). A possible explanation is that the effects of the RNAi transgenes used to knock down *dTCF*, *arm* and *lgs* are weaker than that of the *pygo* RNAi line. However this is unlikely, since we have previously shown that KD of wnt signaling by these RNAi transgenes is considerably more effective than that of the *pygo* RNAi-line (Tang *et al.* 2013) in eye size assay (Parker *et al.* 2002) although we cannot completely rule out tissue specific differences such as dramatic differences in expression. In addition, we show that *pygo* does not genetically interact with Wnt

components *arm/β-Cat* and *pan/TCF* to affect the ratio of valve/heart diameter, which suggests a Wnt signaling-independent role for *pygo* during valve differentiation. Finally, the abnormal differentiation of the adult valves in cardiac *pygo* KD hearts is unlikely to be due to the effects of *pygo* KD during embryonic heart formation since the number of cardiac progenitor cells are unchanged, indicating the valve abnormality following *pygo* KD occurs during the final valve remodeling rather than during the formation of valve precursors. In support of these conclusions, Zeitouni et al reported that inhibition of canonical Wnt signaling, by cardiac expression of a dominant-negative *TCF* transgene, affects the formation of inflow tracts (ostia in flies), without an obvious effect on valve formation (Zeitouni *et al.* 2007). Thus, we propose that *pygo* may function independently of Wnt signaling in the differentiation of intra-cardiac valve cells. This is further supported by our recent finding that *pygo* likely also functions independently of Wnt signaling in controlling the overall functionality and contractility of the *Drosophila* heart (Tang et al, 2012). Our results are also consistent with previous observations suggesting that some aspects of *pygo* function are distinct from those mediating Wnt signaling (Parker *et al.* 2002; Song *et al.* 2007).

The cellular mechanism underlying the Wnt-independent *pygo* function during valve differentiation remains unexplored. Recent studies showed that an actin-binding protein, Filamin-A, plays a significant role in valve remodeling through stabilizing actin and subsequently promoting collagen compaction to develop specific valvular architecture, evidenced by the specific valve defects in the *filamin-A* knock-out mouse (Feng *et al.* 2006; Hart *et al.* 2006). Additionally, the Filamin-A mutation is related to human valve dystrophy (Kyndt *et al.* 2007; Lardeux *et al.* 2011). Thus, actin is important for the organization of the extracellular matrix allowing valves to adopt a polarized configuration that contributes to the final valve morphology. In flies, both *pygo* KD and *pygo* overexpressing hearts showed valve dilation and actin disorganization. It has been previously shown that the dynamics of actin organization is regulated by non-canonical Wnt/PCP signaling (Capelluto *et al.* 2002; Liu *et al.* 2008; Sato *et al.* 2006; Tanegashima *et al.* 2008) thus, *pygo* may be involved in or cooperate with Wnt/PCP signaling to regulate actin assembly. Increased signaling, via VEGF, BMP, Notch and TGF-β and their coordinate interaction is involved in valve remodeling (Lin *et al.* 2012) and it is possible that that *pygo* cooperates with these pathways to regulate valve differentiation. Our findings that *pygo* plays a role in the organization of actin containing filaments in the fly heart suggest that it may play a similar role in this process in vertebrates.

Materials and methods

Drosophila stocks

Wild type controls, *w¹¹¹⁸*, *arm²*, *pan³* and *pygo* mutant with GPF balancer (*pygo^{s123}/TM6B,tub-GFP*) were obtained from the Bloomington stock center. UAS-*pygo*-RNAi (v100724 and v19693), UAS-*arm*-RNAi (v107344), UAS-*lgs*-RNAi (v5694), and UAS-*TCF*-RNAi (v3014) was obtained from the Vienna *Drosophila* RNAi Center (VDRC) (Dietzl *et al.* 2007). UAS-*pygo* was a generous gift from Konrad Basler. The heart-specific

driver *hand4.2-Gal4* (Han and Olson 2005), expressed in myocardial as well as pericardial cells, was kindly provided by Eric Olson.

hand4.2-Gal4 flies were crossed to UAS-*pygo*-RNAi line or UAS-*pygo* overexpression line and incubated at room temperature throughout development. Then the adult F1 progeny were collected after eclosion and aged for 1 week. The homozygous *pygo*^{s123} mutant embryos were selected against the balancer with GFP marker.

Semi-intact *Drosophila* heart preparation and diameters measurement

Flies were anesthetized with Flynep (Carolina Biological Supply Co.) for 2–5 min, and the head, ventral thorax, and ventral abdominal cuticle were removed, exposing the abdomen (Vogler and Ocorr 2009). All internal organs and any abdominal fat except the heart were removed. Only hearts with rhythmical beating, indicating that no damage had occurred, were used for the staining. Dissections were done under oxygenated, artificial hemolymph (Ocorr *et al.* 2007).

During the maximum diastole, the heart edges of both bilateral sides were identified and marked. Two pairs of marks are made at two points, the intra-cardiac valve region (called as valve diameter) and the adjacent chamber enclosed by myocardial cells (called as heart diameter), respectively. Then the diameters is calculated by SOHA program (Ocorr *et al.* 2009). The ratio of valve diameter to heart diameter show the degree of narrowing of heart tube in the region of valve cell.

Immunostaining of the adult *Drosophila* heart

Semi-intact drosophila hearts were fixed *in situ* with 4% paraformaldehyde/PBS for 20 min. Then the hearts were washed three times in PBST (0.1% Triton X-100/PBS). Primary antibodies (diluted with PBST) were incubated at 4°C overnight and washed three times in PBST at room temperature. Hearts were incubated with secondary antibodies for 2 hours and washed three times in PBST at room temperature. Hearts were mounted in VectaShield (Vector Laboratories). Images were acquired using an Apotome (Zeiss). Rabbit anti-nmr (1:3000, (Leal *et al.* 2009)), Fluorescent labeled Alexa 488-phalloidin (1:1000, Chemicon, Temecula, CA), and anti-rabbit-cy3(1:250, Jackson ImmunoResearch) were used (Alayari *et al.* 2009; Taghli-Lamallem *et al.* 2008).

Di-8-ANEPPS fluorescence is localized to the membrane of excitable cells, including single neurons, cardiac cells, and intact brains (Pucihar *et al.* 2009). Heart cell membranes are typically labeled by direct addition of Di-8-ANEPPS dye stock solution (1:300, Invitrogen) to the submerged semi-intact heart prep and incubation for 30 minutes. After incubation, hearts were washed up to three times in dye-free medium and then fixed *in situ* with 4% paraformaldehyde/PBS for 20 min.

RQ-PCR

RNA was extracted from 20–30 hearts using PicoPure™ RNA Isolation Kit (ARCTURUS). The samples were treated with DNaseI (Qiagen) to remove DNA. First strand cDNA was synthesized using SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen).

For polymerase chain reaction, we used LightCycler FastStart DNA Master^{PLUS} SYBR Green I (Roche applied science). The following primers were used. pygo forward: AGCCGTGTTCTGTGAATCCGGT, pygo reverse: TGGAAGACACGCACTTGTCGCA, rp49 forward: GTGCGCTTGTTCGATCCGTAACCG, rp49 reverse: CTAAGCTGTCCGACAAATG.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

- Abu-Issa R, Kirby ML. Heart field: from mesoderm to heart tube. *Annu Rev Cell Dev Biol.* 2007; 23:45–68. [PubMed: 17456019]
- Alayari NN, Vogler G, Taghli-Lamalle O, Ocorr K, Bodmer R, et al. Fluorescent labeling of *Drosophila* heart structures. *Journal of Visualized Experiments: JoVE.* 2009
- Alvarez AD, Shi W, Wilson BA, Skeath JB. pannier and pointedP2 act sequentially to regulate *Drosophila* heart development. *Development.* 2003; 130:3015–3026. [PubMed: 12756183]
- Armstrong EJ, Bischoff J. Heart valve development: endothelial cell signaling and differentiation. *Circulation research.* 2004; 95:459–470. [PubMed: 15345668]
- Belenkaya TY, Han C, Standley HJ, Lin X, Houston DW, et al. pygopus Encodes a nuclear protein essential for wingless/Wnt signaling. *Development.* 2002; 129:4089–4101. [PubMed: 12163411]
- Bier E, Bodmer R. *Drosophila*, an emerging model for cardiac disease. *Gene.* 2004; 342:1–11. [PubMed: 15527959]
- Bodmer R. Heart development in *Drosophila* and its relationship to vertebrate system. *Trends in cardiovascular medicine.* 1995; 5:21–28. [PubMed: 21232234]
- Bodmer R, Frasch M. Development and aging of the *Drosophila* heart. *Heart Development and Regeneration.* 2010; 2:47–86.
- Bodmer R, Venkatesh TV. Heart development in *Drosophila* and vertebrates: conservation of molecular mechanisms. *Dev Genet.* 1998; 22:181–186. [PubMed: 9621426]
- Bour BA, O'Brien MA, Lockwood WL, Goldstein ES, Bodmer R, et al. *Drosophila* MEF2, a transcription factor that is essential for myogenesis. *Genes & Development.* 1995; 9:730–741. [PubMed: 7729689]
- Brickner ME, Hillis LD, Lange RA. Congenital heart disease in adults. First of two parts. *N Engl J Med.* 2000; 342:256–263. [PubMed: 10648769]
- Brutsaert DL, De Keulenaer GW, Franssen P, Mohan P, Kaluza GL, et al. The cardiac endothelium: functional morphology, development, and physiology. *Prog Cardiovasc Dis.* 1996; 39:239–262. [PubMed: 8970576]
- Butcher JT, Norris RA, Hoffman S, Mjaatvedt CH, Markwald RR. Periostin promotes atrioventricular mesenchyme matrix invasion and remodeling mediated by integrin signaling through Rho/PI 3-kinase. *Dev Biol.* 2007; 302:256–266. [PubMed: 17070513]

- Camenisch TD, Spicer AP, Brehm-Gibson T, Biesterfeldt J, Augustine ML, et al. Disruption of hyaluronan synthase-2 abrogates normal cardiac morphogenesis and hyaluronan-mediated transformation of epithelium to mesenchyme. *J Clin Invest*. 2000; 106:349–360. [PubMed: 10930438]
- Canty EG, Starborg T, Lu Y, Humphries SM, Holmes DF, et al. Actin filaments are required for fibropositor-mediated collagen fibril alignment in tendon. *J Biol Chem*. 2006; 281:38592–38598. [PubMed: 17020878]
- Capelluto DG, Kutateladze TG, Habas R, Finkielstein CV, He X, et al. The DIX domain targets dishevelled to actin stress fibres and vesicular membranes. *Nature*. 2002; 419:726–729. [PubMed: 12384700]
- Chang CP, Neilson JR, Bayle JH, Gestwicki JE, Kuo A, et al. A field of myocardial-endocardial NFAT signaling underlies heart valve morphogenesis. *Cell*. 2004; 118:649–663. [PubMed: 15339668]
- Combs MD, Yutzey KE. Heart valve development: regulatory networks in development and disease. *Circulation research*. 2009; 105:408–421. [PubMed: 19713546]
- Cripps RM, Olson EN. Control of cardiac development by an evolutionarily conserved transcriptional network. *Developmental biology*. 2002; 246:14. [PubMed: 12027431]
- Dietzl G, Chen D, Schnorrer F, Su KC, Barinova Y, et al. A genome-wide transgenic RNAi library for conditional gene inactivation in *Drosophila*. *Nature*. 2007; 448:151–156. [PubMed: 17625558]
- Dor Y, Camenisch TD, Itin A, Fishman GI, McDonald JA, et al. A novel role for VEGF in endocardial cushion formation and its potential contribution to congenital heart defects. *Development*. 2001; 128:1531–1538. [PubMed: 11290292]
- Feng Y, Chen MH, Moskowitz IP, Mendonza AM, Vidali L, et al. Filamin A (FLNA) is required for cell-cell contact in vascular development and cardiac morphogenesis. *Proceedings of the National Academy of Sciences of the United States of America*. 2006; 103:19836–19841. [PubMed: 17172441]
- Gitler AD, Lu MM, Jiang YQ, Epstein JA, Gruber PJ. Molecular markers of cardiac endocardial cushion development. *Dev Dyn*. 2003; 228:643–650. [PubMed: 14648841]
- Goldmuntz E. The epidemiology and genetics of congenital heart disease. *Clin Perinatol*. 2001; 28:1–10. [PubMed: 11265502]
- Han Z, Bodmer R. Myogenic cells fates are antagonized by Notch only in asymmetric lineages of the *Drosophila* heart, with or without cell division. *Development*. 2003; 130:3039–3051. [PubMed: 12756185]
- Han Z, Olson EN. Hand is a direct target of Tinman and GATA factors during *Drosophila* cardiogenesis and hematopoiesis. *Development*. 2005; 132:3525–3536. [PubMed: 15975941]
- Hart AW, Morgan JE, Schneider J, West K, McKie L, et al. Cardiac malformations and midline skeletal defects in mice lacking filamin A. *Hum Mol Genet*. 2006; 15:2457–2467. [PubMed: 16825286]
- Hurlstone AF, Haramis AP, Wienholds E, Begthel H, Korving J, et al. The Wnt/beta-catenin pathway regulates cardiac valve formation. *Nature*. 2003; 425:633–637. [PubMed: 14534590]
- Kramps T, Peter O, Brunner E, Nellen D, Froesch B, et al. Wnt/wingless signaling requires BCL9/legless-mediated recruitment of pygopus to the nuclear beta-catenin-TCF complex. *Cell*. 2002; 109:47–60. [PubMed: 11955446]
- Kyndt F, Gueffet JP, Probst V, Jaafar P, Legendre A, et al. Mutations in the gene encoding filamin A as a cause for familial cardiac valvular dystrophy. *Circulation*. 2007; 115:40–49. [PubMed: 17190868]
- Lardeux A, Kyndt F, Lecointe S, Marec HL, Merot J, et al. Filamin-a-related myxomatous mitral valve dystrophy: genetic, echocardiographic and functional aspects. *J Cardiovasc Transl Res*. 2011; 4:748–756. [PubMed: 21773876]
- Leal SM, Qian L, Lacin H, Bodmer R, Skeath JB. Neuromancer1 and Neuromancer2 regulate cell fate specification in the developing embryonic CNS of *Drosophila melanogaster*. *Dev Biol*. 2009; 325:138–150. [PubMed: 19013145]
- Lehmacher C, Abeln B, Paululat A. The ultrastructure of *Drosophila* heart cells. *Arthropod Struct Dev*. 2012a; 41:459–474. [PubMed: 22426062]

- Lehmacher C, Abeln B, Paululat A. The ultrastructure of *Drosophila* heart cells. *Arthropod Structure & Development*. 2012b; 41:459–474. [PubMed: 22426062]
- Lin CJ, Lin CY, Chen CH, Zhou B, Chang CP. Partitioning the heart: mechanisms of cardiac septation and valve development. *Development*. 2012; 139:3277–3299. [PubMed: 22912411]
- Liu W, Sato A, Khadka D, Bharti R, Diaz H, et al. Mechanism of activation of the Formin protein Daam1. *Proceedings of the National Academy of Sciences*. 2008; 105:210–215.
- Loffredo CA. Epidemiology of cardiovascular malformations: prevalence and risk factors. *Am J Med Genet*. 2000; 97:319–325. [PubMed: 11376444]
- Ma L, Lu MF, Schwartz RJ, Martin JF. Bmp2 is essential for cardiac cushion epithelial-mesenchymal transition and myocardial patterning. *Development*. 2005; 132:5601–5611. [PubMed: 16314491]
- Molina MR, Cripps RM. Ostia, the inflow tracts of the *Drosophila* heart, develop from a genetically distinct subset of cardial cells. *Mech Dev*. 2001; 109:51–59. [PubMed: 11677052]
- Monier B. Steroid-dependent modification of Hox function drives myocyte reprogramming in the *Drosophila* heart. *Development*. 2005; 132:5283–5293. [PubMed: 16284119]
- Niessen K, Karsan A. Notch signaling in cardiac development. *Circulation research*. 2008; 102:1169–1181. [PubMed: 18497317]
- Ocorr K, Fink M, Cammarato A, Bernstein S, Bodmer R. Semi-automated Optical Heartbeat Analysis of small hearts. *J Vis Exp*. 2009
- Ocorr K, Perrin L, Lim HY, Qian L, Wu X, et al. Genetic control of heart function and aging in *Drosophila*. *Trends Cardiovasc Med*. 2007c; 17:177–182. [PubMed: 17574126]
- Ocorr K, Reeves NL, Wessells RJ, Fink M, Chen HS, et al. KCNQ potassium channel mutations cause cardiac arrhythmias in *Drosophila* that mimic the effects of aging. *Proceedings of the National Academy of Sciences of the United States of America*. 2007; 104:3943–3948. [PubMed: 17360457]
- Park M, Wu X, Golden K, Axelrod JD, Bodmer R. The wingless signaling pathway is directly involved in *Drosophila* heart development. *Dev Biol*. 1996; 177:104–116. [PubMed: 8660881]
- Parker DS, Jemison J, Cadigan KM. Pygopus, a nuclear PHD-finger protein required for Wingless signaling in *Drosophila*. *Development*. 2002; 129:2565–2576. [PubMed: 12015286]
- Peacock JD, Lu Y, Koch M, Kadler KE, Lincoln J. Temporal and spatial expression of collagens during murine atrioventricular heart valve development and maintenance. *Dev Dyn*. 2008; 237:3051–3058. [PubMed: 18816857]
- Pucihar G, Kotnik T, Miklavcic D. Measuring the induced membrane voltage with Di-8-ANEPPS. *J Vis Exp*. 2009
- Romano LA, Runyan RB. Slug is an essential target of TGFbeta2 signaling in the developing chicken heart. *Dev Biol*. 2000; 223:91–102. [PubMed: 10864463]
- Sandbo N, Dulin N. Actin cytoskeleton in myofibroblast differentiation: ultrastructure defining form and driving function. *Transl Res*. 2011; 158:181–196. [PubMed: 21925115]
- Sato A, Khadka DK, Liu W, Bharti R, Runnels LW, et al. Profilin is an effector for Daam1 in non-canonical Wnt signaling and is required for vertebrate gastrulation. *Development*. 2006; 133:4219–4231. [PubMed: 17021034]
- Schroeder JA, Jackson LF, Lee DC, Camenisch TD. Form and function of developing heart valves: coordination by extracellular matrix and growth factor signaling. *J Mol Med (Berl)*. 2003; 81:392–403. [PubMed: 12827270]
- Schwab KR, Patterson LT, Hartman HA, Song N, Lang RA, et al. Pygo1 and Pygo2 roles in Wnt signaling in mammalian kidney development. *BMC Biol*. 2007; 5:15. [PubMed: 17425782]
- Song N, Schwab KR, Patterson LT, Yamaguchi T, Lin X, et al. pygopus 2 has a crucial, Wnt pathway-independent function in lens induction. *Development*. 2007; 134:1873–1885. [PubMed: 17428831]
- Taghli-Lamalle O, Akasaka T, Hogg G, Nudel U, Yaffe D, et al. Dystrophin deficiency in *Drosophila* reduces lifespan and causes a dilated cardiomyopathy phenotype. *Aging Cell*. 2008; 7:237–249. [PubMed: 18221418]
- Tan H, Junor L, Price RL, Norris RA, Potts JD, et al. Expression and deposition of fibrous extracellular matrix proteins in cardiac valves during chick development. *Microsc Microanal*. 2011; 17:91–100. [PubMed: 21205426]

- Tanegashima K, Zhao H, Dawid IB. WGEF activates Rho in the Wnt-PCP pathway and controls convergent extension in *Xenopus* gastrulation. *The EMBO journal*. 2008; 27:606–617. [PubMed: 18256687]
- Thompson B, Townsley F, Rosin-Arbesfeld R, Musisi H, Bienz M. A new nuclear component of the Wnt signalling pathway. *Nat Cell Biol*. 2002; 4:367–373. [PubMed: 11988739]
- Vogler G, Ocorr K. Visualizing the beating heart in *Drosophila*. *Journal of Visualized Experiments: JoVE*. 2009
- Wu X, Golden K, Bodmer R. Heart development in *Drosophila* requires the segment polarity gene wingless. *Dev Biol*. 1995; 169:619–628. [PubMed: 7781903]
- Zeitouni B, Senatore S, Severac D, Aknin C, Semeriva M, et al. Signalling pathways involved in adult heart formation revealed by gene expression profiling in *Drosophila*. *PLoS Genet*. 2007; 3:1907–1921. [PubMed: 17937502]

Figure 1. Schematic representation of *Drosophila* adult heart and their ultrastructure
Three valves are formed in the three consecutive abdominal segments in A2–A4 (black arrows). The valve cells are characterized by a high density of myofibrils and a narrowing of inner heart lumen (Lehmacher *et al.* 2012a).

Figure 2. *pygo* KD alters myofibrillar organization

Hearts were stained with phalloidin (green) to identify actin filaments and Nmr (red) to identify cardiac nuclei (as in Qian et al. 2008). (a) In wild type hearts, intra-cardiac valve cells are characterized by a narrowing of the heart tube and concomitant increasing of myofibril contents compared to neighboring myocardial cells (blue arrows). (b) *pygo* KD hearts showed no obvious increase in myofibrillar density within the valve cells (blue arrows), and their diameter is similar to that of neighboring myocardial cells (red arrows).

Figure 3. Cardiac-specific *pygo* KD causes valve defects that can be rescued by *pygo* OE
 Di-8-ANEPPS fluorescence was used to visualize the membranes of myocardial cells. (A & B) A portion of a Z stack from a wild type heart showing one cardiac chamber. (C) Comparable heart chamber from a cardiac- specific *pygo* KD fly (*hand 4.2>UAS pygo RNAi*). (D) Comparable heart chamber from a *pygo* rescue heart (*hand 4.2>UAS pygo RNAi; UAS-pygo*). Arrows indicate the positions where x and y cross sections were made through the entire stack. The location of valve cells are indicated by dotted lines. (a') Vertical cross section showing the heart lumen at the location of the intra-cardiac valve (outlined by dashed lines). (b') Vertical cross section showing the heart lumen at the location of the widest part of the heart chamber, note the reduction in luminal space at the valve. (c') Vertical cross section through the valve region (outlined by dashed lines) of a *pygo* KD heart shows a much larger luminal space and a thinner myocardial layer than for wt. (d') Vertical cross section through the valve region (outlined by dashed lines) in the *pygo* rescue heart shows a smaller luminal space and thicker surrounding myocardial tissue than in the *pygo* KD. (a'') Horizontal cross section through the wt heart show the open cardiac chamber (indicated by an asterisk) and myocardial tissue at the location of the valve (white arrow). (c'') Horizontal cross section through a *pygo* KD heart shows a dilated cardiac chamber and little myocardial tissue at the location of the valve. (d'') Horizontal cross section through a *pygo* rescue heart shows a smaller heart chamber and more myocardial tissue in the valve region compared to the *pygo* KD heart.

Figure 4. Overexpression of *pygo* causes disorganization of myofibrils

Hearts were stained with phalloidin (green) to identify actin filaments and Nmr (red) to identify cardiac nuclei (as in Qian et al. 2008). (a) The obvious circumferential orientation typical of wild type heart valves (a, yellow arrow) is lost in response to (b) cardiac-specific overexpression of *pygo*, which results in mis-orientation of myofibrils within intra-cardiac valve cells (b, yellow arrow).

Figure 5. *pygo* KD causes heart valve dilation in *Drosophila*

(a) Image of the heart chamber in the third abdominal segment in a 1 week control fly (taken from Supplemental Video 1) and (b) in a 1 week *pygo* KD fly (taken from supplemental Video 2). *pygo* KD hearts are much less constricted at the location of the valve cells (a', blue arrow) compared to control hearts (a, blue arrow). Scale bar is 50 μ M. (c) Heart diameters were measured at the intra-cardiac valve region (valve diameter, blue arrow) and the adjacent chamber enclosed by myocardial cells (heart diameter, red arrow) in hearts. Mean ratios of valve diameter/heart diameter are graphed. In contrast to controls, this ratio significantly increased in *pygo* KD and this increase can be partially reversed by overexpressing *pygo*.

Figure 6. Pygopus plays a role in adult heart morphology independently Wnt signaling
 Graph shows the mean ratio of heart diameter to valve diameter in hearts where components of the Wnt signaling pathway(*pygo*, *TCF*, *arm* and *lgs*) have been specifically knocked down. Note that this ratio is significantly increased in response to heart-specific *pygo* KD, whereas *TCF*, *arm* or *lgs* KD show a significantly weaker phenotype. Significance was determined using 1-way ANOVA and Newman-Keuls Multiple Comparison Test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).