Available online at www.sciencedirect.com

SCIENCE DIRECT

Developmental Biology 278 (2005) 459–472

DEVELOPMENTAL BIOLOGY

www.elsevier.com/locate/ydbio

The *Drosophila melanogaster* T-box genes *midline* and *H15* are conserved regulators of heart development

Cindy M. Miskolczi-McCallum, Rick J. Scavetta, Pia C. Svendsen, Kelly H. Soanes, William J. Brook*

Genes and Development Research Group, University of Calgary, 3330 Hospital Drive NW, Calgary Alberta, Canada T2N 4N1
Department of Biochemistry and Molecular Biology, University of Calgary, 3330 Hospital Drive NW, Calgary Alberta, Canada T2N 4N1

Received for publication 16 September 2004, revised 17 November 2004, accepted 17 November 2004
Available online 18 December 2004

Abstract

The *Drosophila melanogaster* genes *midline* and *H15* encode predicted T-box transcription factors homologous to vertebrate *Tbx20* genes. All identified vertebrate *Tbx20* genes are expressed in the embryonic heart and we find that both *midline* and *H15* are expressed in the cardioblasts of the dorsal vessel, the insect organ equivalent to the vertebrate heart. The *midline* mRNA is first detected in dorsal mesoderm at embryonic stage 12 in the two progenitors per hemisegment that will divide to give rise to all six cardioblasts. Expression of *H15* mRNA in the dorsal mesoderm is detected first in four to six cells per hemisegment at stage 13. The expression of *midline* and *H15* in the dorsal vessel is dependent on Wingless signaling and the transcription factors *tinman* and *pannier*. We find that the selection of two *midline*-expressing cells from a pool of competent progenitors is dependent on Notch signaling. Embryos deleted for both *midline* and *H15* have defects in the alignment of the cardioblasts and associated pericardial cells. Embryos null for *midline* have weaker and less penetrant phenotypes while embryos deficient for *H15* have morphologically normal hearts, suggesting that the two genes are partially redundant in heart development. Despite the dorsal vessel defects, embryos mutant for both *midline* and *H15* have normal numbers of cardioblasts, suggesting that cardiac cell fate specification is not disrupted. However, ectopic expression of *midline* in the dorsal mesoderm can lead to dramatic increases in the expression of cardiac markers, suggesting that *midline* and *H15* participate in cardiac fate specification and may normally act redundantly with other cardiogenic factors. Conservation of *Tbx20* expression and function in cardiac development lends further support for a common ancestral origin of the insect dorsal vessel and the vertebrate heart.

Keywords: Drosophila; T-box; Heart

Introduction

The insect heart, or dorsal vessel, is a simple muscular tube that pumps hemolymph and hematocytes through the open circulatory system. Heart development in vertebrates and dorsal vessel development have many features in common. During gastrulation, the trunk mesoderm of the fly invaginates along the ventral midline. The mesoderm flattens, forming a single cell layer inside the ectoderm, and migrates dorsally. The fly heart forms from bilateral rows of cells at the leading edge of the migrating mesoderm. The rows fuse along the dorsal midline forming a linear tube (Cripps and Olson, 2002; Zaffran and Frasch, 2002). A similar process initiates vertebrate heart development, where bilateral fields of mesoderm migrate and fuse to form a linear heart tube prior to the looping and subsequent morphogenesis that forms a multichambered heart (Bruneau, 2002). In addition to these morphological similarities, signaling from other germ layers is required for the cardiac potential of the mesoderm tube and a conserved transcription factor network specifies cardiac fate in both

* Corresponding author. Department of Biochemistry and Molecular Biology, University of Calgary, 3330 Hospital Drive NW, Calgary Alberta, Canada T2N 4N1. Fax: +1 403 270 0737.
E-mail address: brook@ucalgary.ca (W.J. Brook).

0012-1606/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2004.11.026

Drosophila and vertebrates. (Cripps and Olson, 2002; Zaffran and Frasch, 2002).

The *Drosophila* NK-2 class homeodomain transcription factor Tinman (Tin) is required for the specification of the dorsal mesoderm; heart development is completely abolished in *tinman* (*tin*) mutants (Azpiazu and Frasch, 1993; Bodmer, 1993). After mesoderm involution, *tin* is initially expressed in all mesoderm cells under the control of the bHLH factor Twist (Yin et al., 1997). During stages 9 and 10, *tin* becomes restricted to a broad domain in the dorsal trunk mesoderm through maintenance by signaling from overlying ectoderm, which secretes the BMP family protein Decapentaplegic (Dpp) (Frasch, 1995; Lockwood and Bodmer, 2002). By mid stage 11, *tin* expression is maintained in segmental clusters of dorsal mesoderm cells by ectoderm and mesoderm secreting the Wnt protein Wingless (Wg) (Lawrence et al., 1995; Lee and Frasch, 2000; Lockwood and Bodmer, 2002; Park et al., 1996). These *tin*-expressing domains contain the precursors for the heart including progenitors of both the contractile cardioblasts and the noncontractile pericardial cells (Alvarez et al., 2003; Han and Bodmer, 2003; Ward and Skeath, 2000). The dorsal vessel is not specified in the absence of *tin* function or if Dpp or Wg signal transduction is blocked (Frasch et al., 1995; Park et al., 1996).

Tin regulates cardiac gene expression in conjunction with the GATA factor Pannier (Pnr) (Gajewski et al., 1998, 1999, 2001). In stage 11, *pnr* is expressed in the mesoderm under the control of ectodermal Dpp signaling and mesodermal Tin (Gajewski et al., 2001; Herranz and Morata, 2001; Klinedinst and Bodmer, 2003). Null mutations in *pnr* greatly diminish heart development, and ectopic *pnr* expression is sufficient to induce excess cardiac fate (Alvarez et al., 2003; Gajewski et al., 1999; Klinedinst and Bodmer, 2003). Expression of *pnr* in the mesoderm is also required to maintain proper *tin* expression (Klinedinst and Bodmer, 2003). Ectopic expression of *tin* has little effect on heart formation, but in combination with *pnr*, ectopic *tin* has a synergistic effect on the ability of mesoderm to adopt cardiac fate (Gajewski et al., 1999, 2001; Klinedinst and Bodmer, 2003; Lockwood and Bodmer, 2002). This synergy is likely to occur at the level of the regulatory sequences of cardiac genes as Tin and Pnr form complexes in vitro and heart-specific enhancers require clusters of Tin and Pnr binding sites (Gajewski et al., 2001).

NK-2 homeodomain and GATA transcription factors are also critical for heart development in vertebrates. Loss of function for *Nkx2.5*, the vertebrate *tin* homologue, causes arrested heart development at the linear tube stage (Lyons et al., 1995; Tanaka et al., 1998). Furthermore, Nkx2.5 activates cardiac gene expression in a complex with GATA4 (Durocher et al., 1997). Genetic analysis of the roles of GATA factors in vertebrate heart development is complicated by potential genetic redundancy of *GATA4*, *5*, and *6* (Jiang and Evans, 1996; Laverriere et al., 1994), but several lines of evidence suggest that GATA factors are also required to establish cardiac fate (reviewed in Bruneau, 2002).

More recently, T-box transcription factors have been identified as regulators of vertebrate heart development that interact with both Nkx2.5 and GATA proteins. *Tbx5*, the gene responsible for Holt–Oram syndrome, is required for chamber formation (Bruneau et al., 2001). Mutations in *Tbx5*, *Nkx2.5*, and *GATA4* all cause similar heart defects in humans (Basson et al., 1997; Garg et al., 2003; Li et al., 1997; Schott et al., 1998) and Tbx5 interacts physically with both Nkx2.5 and GATA4 (Garg et al., 2003; Hiroi et al., 2001). Mutations in *GATA4* that abrogate the Tbx5–GATA4 physical interaction in vitro cause heart defects similar to those seen in *Tbx5* mutants, indicating that the physical interactions are functionally important (Garg et al., 2003). All pairwise combinations of Tbx5, Nkx2.5, and GATA4 activate gene expression synergistically in vitro (Durocher et al., 1997; Garg et al., 2003; Hiroi et al., 2001). The cardiac expression of another T-box family member, *Tbx20* (also called *Tbx12*), is conserved in all vertebrates (Ahn et al., 2000; Carson et al., 2000; Griffin et al., 2000; Iio et al., 2001; Kraus et al., 2001; Meins et al., 2000). Zebrafish embryos exhibit heart defects following injection with morpholino oligonucleotides directed against *H15-related-T-box* (*hrT*), a zebrafish *Tbx20* homolog (Szeto et al., 2002). Tbx20 shares some of the properties of Tbx5 in vitro, forming complexes with Nkx2.5 and GATA4 (Plageman and Yutzey, 2004; Stennard et al., 2003) and synergistically activating cardiac gene expression in some assays (Stennard et al., 2003). However, the relationship between Tbx20 and Tbx5 may be complex as *Tbx20* acts to inhibit *Tbx5* transcription in vivo (Szeto et al., 2002; Takeuchi et al., 2003) and in some assays can block transcriptional activation by Tbx5 in vitro (Plageman and Yutzey, 2004).

Clearly, T-box proteins are key regulators of heart development in vertebrates, which raises the question of whether T-box function is conserved in *Drosophila* dorsal vessel development. T-box genes have duplicated and diverged throughout metazoan evolution, acting in several developmental processes in a wide variety of organisms (Papaioannou and Silver, 1998; Wilson and Conlon, 2002). In the human and mouse genomes, there are 18 identified T-box genes representing five subfamilies, while the fly genome contains eight T-box genes representing four of the five subfamilies found in mice and humans (Reim et al., 2003). There is no clear *Tbx5* homolog in *Drosophila*, and the *Drosophila* gene most similar to *Tbx5*, *optomotor-blind* (*omb*), is not expressed in the dorsal vessel (Poeck et al., 1993). *Dorsocross 1*, *2*, and *3* (*Doc1*, *2*, and *3*), the *Tbx6* homologs, are expressed in a subset of cardioblasts. The *Doc* genes play a key role in the subdivision of the dorsal mesoderm (Hamaguchi et al., 2004; Reim et al., 2003), but it has not been shown if they play a role specifying cardiac fate and the expression of *Tbx6* is not conserved in vertebrate hearts. Therefore, the best candidates for *Droso-*

phila cardiogenic T-box genes are the *Tbx20* homologs *H15* (Brook and Cohen, 1996) and *midline* (*mid*) (Buescher et al., 2004). *H15–lacZ*, a *lacZ* enhancer trap inserted adjacent in the *H15 mid* locus, is expressed in all cardioblasts, the contractile myocardial cells of the dorsal vessel (Griffin et al., 2000).

In order to assess whether the requirement for T-box transcription factors is conserved in heart and dorsal vessel development, we have examined the expression, regulation, and function of *mid* and *H15* in the development of the *Drosophila* dorsal vessel. We show that the expression of the genes correlates with the specification of cardioblast fate, the genes are required in a partially redundant fashion in heart morphogenesis, and that ectopic expression of *mid* is sufficient to cause dorsal mesoderm cells to acquire cardiac fate. Based on these results, we argue that *Tbx20* genes represent another component of the conserved transcription factor network acting in heart and dorsal vessel development.

Materials and methods

Drosophila stocks and genetics

Drosophila melanogaster strains were maintained at 25°C on cornmeal–yeast–agar media. Oregon-R was used as a wild-type strain. $H15^{x4}$, *Df(2L)x528*, *UAS-mid* (Buescher et al., in press), and $PZ[lac\text{-}Z;ry^{+}]H15$ (*H15–lacZ*) (Brook et al., 1993) were generated in this lab. Despite attempting several strategies, we have been unable to generate a *UAS–H15* construct. A stock containing tin^{EC40}, a null allele of *tin*, was a gift of Rolf Bodmer (University of Michigan). A strain containing *UAS–pannier* (*UAS–pnr*) was a gift of Bruce Reed (University of Toronto). The *B2-3-20* enhancer-trap line (Haag et al., 1999) was a gift of Roger Jacobs (McMaster University). Strains carrying *Df(2L) GpdhA*, Dl^{x}, wg^{cx4}, pnr^{vx6}, *UAS–Nicd*, *twi–GAL4*, and *How–GAL4* were obtained from the Bloomington Stock Center. For experiments requiring the identification of homozygous mutant embryos, mutations were balanced over either *CyO–wg–lacZ* or *TM6–lacZ* and homozygotes were detected by absence of β-galactosidase activity or immunoreactivity.

Immunohistochemistry and in situ hybridization

Mouse monoclonal antibodies for Engrailed (4D9) (used at a dilution of 1/10), Even-Skipped (2B8) (1/100), Fasciclin III (7G10) (1/50), and Wingless (Wg) (4D4) (1/10) were obtained from the Developmental Studies Hybridoma Bank at the University of Iowa. Mouse monoclonal antibodies for Zfh-1 (1/750) were a gift from Zhi Chun Lai (Pennsylvania State University). Rabbit antibodies for Myosin-Heavy-Chain (1/500) were a gift from Dan Kiehart (Duke University), rabbit antibodies for DMef2 (1/750) were a gift from Hanh Nguyen (Albert Einstein College), and rabbit antibodies for Tinman (1/500) were kindly provided by Rolf Bodmer (University of Michigan). Rabbit (Cappel) and mouse (Promega) anti-β-galactosidase were used at 1/1000. Immunostaining of embryos was performed using standard protocols (Sullivan et al., 2000) for all antibodies except Anti-Mhc, which was performed as described previously (Young et al., 1991). Fluorescent detection was performed with Alexafluor-labeled secondary antibodies (1/500; Molecular Probes, Inc.). Histochemical detection was performed with biotinylated secondary antibodies (1/500) (Jackson Labs) in conjunction with the Vectastain ABC Kit (Vector labs). In situ hybridization was performed using standard protocols (Tautz and Pfeifle, 1989). Simultaneous detection of mRNA and protein by in situ hybridization and immunostaining was performed using standard protocols (Wiellette and McGinnis, 1999).

Results

midline and H15 are expressed in cardioblasts

The dorsal vessel forms from bilateral rows of cardioblasts and pericardial cells. The cardioblasts form a line of cells at the leading edge of the dorsal mesoderm. Pericardial cells are arranged in adjacent ventral rows. During dorsal closure, the rows of cardioblasts and associated pericardial cells migrate dorsally and the cardioblasts fuse with their contralateral partners. The two rows of cardioblasts are located medially and fuse to form the lumen of the dorsal vessel and are flanked by rows of noncontractile pericardial cells located ventrally, laterally, and dorsolaterally (Figs. 1A, B, and K) (Haag et al., 1999). *H15* (Brook and Cohen, 1996) and *mid* (Buescher et al., 2004) are linked T-box genes (Fig. 2) that share 89% identity in their T-box domains. Previously, we demonstrated that the *H15–lacZ* enhancer-trap (Brook et al., 1993), inserted 3 kb 5′ of *H15*, shares the expression pattern of both *mid* and *H15* during ectodermal segmentation (Buescher et al., 2004). Griffin et al. (2000) reported that *H15–lacZ* was expressed in cardioblasts. The *mid* and *H15* expressions in the embryo are first detected in ventral ectodermal stripes and neuroblasts (Buescher et al., 2004). In the mesoderm, *mid* is first expressed during early stage 12, where it is detected in two cells per hemisegment in second thoracic through eighth abdominal segments (T2-A8) (Figs. 1C and D). During stages 12 and 13, the expression pattern evolves into clusters of cells (Figs. 1E and F) and then into a continuous row of cells in the dorsal mesoderm (Fig. 1G) that fuse dorsally (Fig. 1H). We first detect *H15* mRNA in the dorsal mesoderm in clusters of cells at stage 13, after which *H15* is expressed in the same pattern as *mid* but at lower levels (data not shown). To confirm that *mid* and *H15* are expressed in cardioblasts, we examined *mid* and *H15* mRNA in embryos carrying an enhancer trap inserted at the *seven-up* locus (*svp–lacZ*) (Mlodzik et al., 1990). *svp* is expressed in

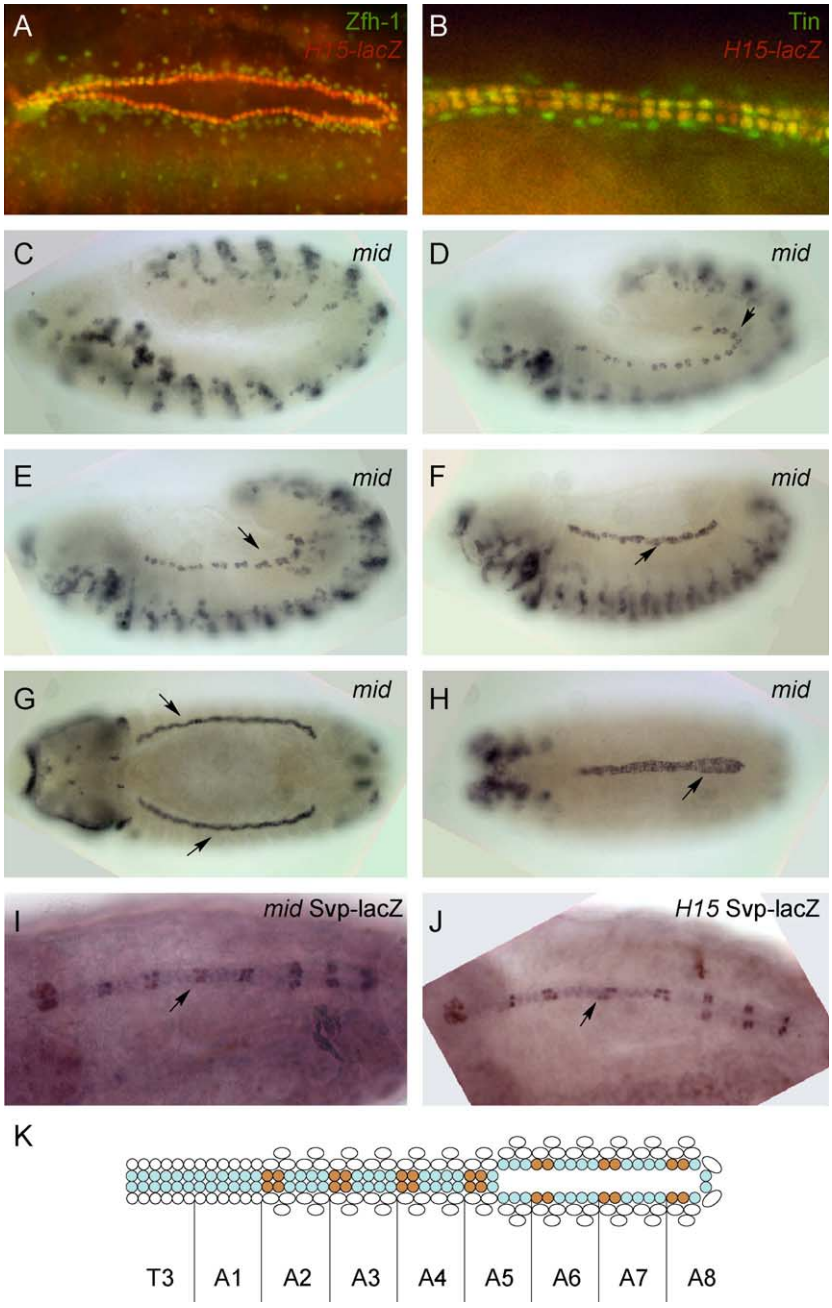

Fig. 1. *mid* and *H15* expression in the dorsal vessel. (A) *H15–lacZ* in cardioblasts detected with anti-β-galactosidase (red) and Zfh-1 in pericardial cells (green), in a stage 15 embryo, showing the two rows of cardioblasts in the process of dorsal migration. (B) *H15–lacZ* in cardioblasts detected with anti-β-galactosidase (red) and Tinman in pericardial cells and cardioblasts (green), in a stage 17 embryo shown at a higher magnification than the embryo in A. Four of every six cardioblasts in each hemisegment are labeled for both *H15–lacZ* and Tin (yellow), while the other two cardioblasts are labeled only with anti-β-galactosidase (red). Tin also labels several pericardial cells per hemisegment (green). (C) In situ hybridization showing *mid* expression predominantly in the ventral ectoderm and neuroblasts in a stage 11 embryo. (D) In early stage 12 embryos, *mid* mRNA is detected in the dorsal mesoderm in two cells per hemisegment in segments T2 through A8 (arrow). (E) In stage 12, clusters of approximately four *mid*-expressing cells are detected in each hemisegment (arrow). (F) In stage 13, groups of six *mid*-expressing cells per hemisegment begin to form a continuous row of cells in the dorsal mesoderm (arrow). (G) Dorsal view of a stage 14 embryo showing the bilateral rows of *mid*-expressing cells in the process of dorsal closure (arrows). (H) Dorsal view of a stage 17 embryo with *mid* expression in a fully closed dorsal vessel (arrow). (I) Colocalization of *mid* mRNA and β-galactosidase in cardioblasts of a stage 17 *svp–lacZ* embryo (arrow). (J) Colocalization of *H15* mRNA and β-galactosidase in cardioblasts of a stage 17 *svp–lacZ* embryo (arrow). (K) Diagram of gene expression in the dorsal vessel based on panels A, B, G, H and previous reports (Alvarez et al., 2003; Han and Bodmer, 2003; Ward and Skeath, 2000). All cardioblasts express *mid* and *H15*, while a subset also express *tin* (blue) or *svp–lacZ* (brown). Pericardial cells lie laterally (white inner row) or dorsolaterally (white, outer row). Four ventrally located pericardial cells per hemisegment are not shown.

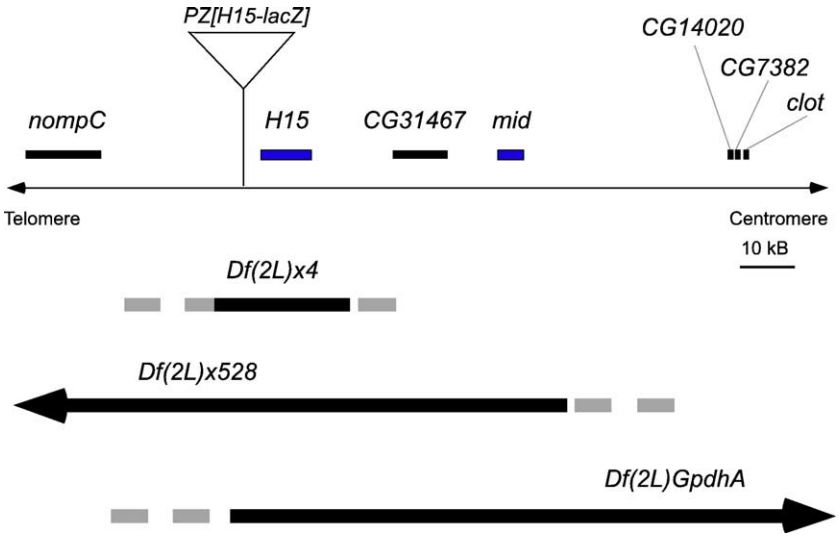

Fig. 2. Genomic map of the *H15 midline* locus. The positions of transcription units (boxes, introns not shown), defined for known and predicted genes in cytological region 25E, deficiencies used in this study (thick lines, gray dashes indicate endpoint uncertainty), and the *PZ[H15–lacZ]P* insertion are indicated. The map is oriented with the telomere to the left and centromere to the right. $H15^{x4}$ and *Df(2L)x528* are X-ray-induced *rosy* (*ry*) derivatives of *PZ[H15–lacZ]* (Buescher et al., 2004). *Df(2L)GpdhA* is described previously (Kotarski et al., 1983).

two cardioblasts per hemisegment (Gajewski et al., 2000; Lo and Frasch, 2001), and both *mid* and *H15* mRNA colocalize with *svp–lacZ*. As predicted for cardioblast-specific expression, each pair of *mid* and *H15 svp–lacZ*-expressing cells is separated by four cells expressing only *mid* and *H15* (Figs. 1I and J). Thus, the *mid* and *H15 mRNAs* are localized to cardioblasts in the mature dorsal vessel.

mid and H15 expression are dependent on cardiogenic factors

In order to determine where *mid* and *H15* fit in the genetic hierarchy controlling heart development, we examined their expression in several mutant backgrounds. The initiation of *mid* expression in the dorsal mesoderm in early stage 12 occurs after the expression of *tin* and *pnr* (Azpiazu and Frasch, 1993; Bodmer, 1993; Gajewski et al., 1999, 2001), as well as after the period of Wg signaling in the dorsal mesoderm (Lawrence et al., 1995; Lee and Frasch, 2000; Lockwood and Bodmer, 2002; Park et al., 1996), suggesting that *mid* and *H15* are regulated downstream of the factors that confer cardiac fate. Indeed, the dorsal vessel expression of *mid* and *H15* is completely lost in both wg^{cx4} and tin^{ec40} mutant embryos, which fail to specify dorsal mesoderm (Figs. 3B and C; data not shown). Embryos mutant for *pnr* have greatly decreased numbers of cardioblasts (Alvarez et al., 2003; Gajewski et al., 1999; Klinedinst and Bodmer, 2003). Accordingly, *mid* and *H15* expression is variably lost in pnr^{vx6} null mutant embryos, with most embryos completely lacking *mid* expression in the dorsal mesoderm (Fig. 3D; data not shown). Ectopic expression of *pnr* throughout the mesoderm using the GAL4/UAS system (Brand and Perrimon, 1993) is able to induce ectopic expression of *mid* and *H15* (Figs. 3E and F; data not shown). These results indicate that the initiation of *mid* and *H15* in the dorsal mesoderm is downstream of factors required for the specification of cardiac fate.

mid expression correlates with cardioblast specification

Unlike *tin* and *pnr*, which are expressed in broad domains throughout the dorsal mesoderm before becoming restricted to a subset of heart cells (Azpiazu and Frasch, 1993; Bodmer, 1993; Gajewski et al., 1999, 2001; Klinedinst and Bodmer, 2003), *mid* is initially activated in two cells per hemisegment. To determine where the cells arise, we examined the location of *mid*-expressing cells in the dorsal mesoderm relative to the ectodermal expression of the segmental markers Wg and Engrailed (En). Wg and En mark adjacent domains in ectoderm overlying the dorsal mesoderm with Wg labeling the row of cells just anterior to the En domain. The two *mid*-expressing cells arise in similar positions in the underlying mesoderm in each hemisegment. One cell located beneath the Wg stripe and the other lying beneath and slightly posteriorly of the En stripe (Figs. 4A, B, C, and C′). As stage 12 progresses, groups of three to six *mid*-expressing cells are located beneath or near the Wg and En stripes (Fig. 4D; data not shown), consistent with the two initial cells dividing and coalescing to form clusters. The clusters fuse to form a continuous line of cells with six cells per hemisegment (Fig. 4E). The progression of *mid* expression is consistent with previous mosaic analysis and experiments manipulating the cell cycle in the dorsal mesoderm cells (Alvarez et al., 2003; Han and Bodmer, 2003; Ward and Skeath, 2000). These studies provided indirect evidence that the six cardioblasts in each hemisegment arise from two progenitors that divide twice to give rise to all six cardioblasts and two of the pericardial cells.

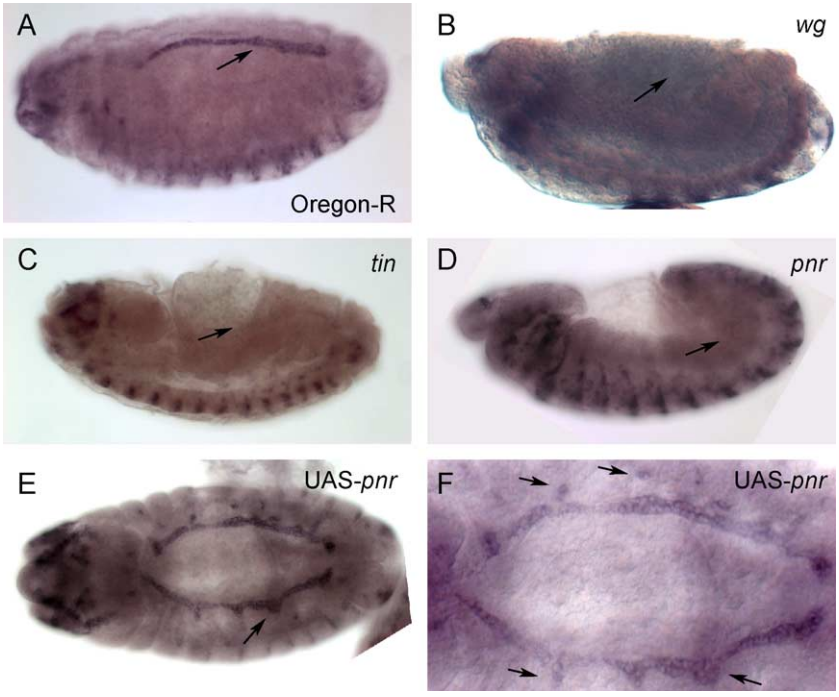

Fig. 3. The *midline* expression is lost in embryos mutant for cardiogenic factors. (A) *mid* dorsal vessel expression (arrow) detected by in situ hybridization. (B) Dorsal vessel *mid* expression is lost completely in embryos mutant for wg^{cx4} (arrow). (C). Dorsal vessel *mid* expression is lost completely in embryos mutant for tin^{ec40} (arrow). (D) Stage 12 pnr^{vx6} mutant with complete lack of *mid* expression in dorsal mesoderm (arrow). (E) Ectopic *pnr* expression in *twi–Gal4;UAS–mid* embryos induces ectopic *mid*-expressing cells (arrow). (F) Higher magnification of the embryo in E. Arrows indicate ectopic *mid* expression in lateral mesoderm cells and extra rows of *mid*-expressing cells in the dorsal vessel.

Previous work has shown that loss of Notch signaling results in a fourfold increase in cardioblasts (Hartenstein et al., 1992). To test whether the expression of *mid* is dependent on the Notch pathway, we first blocked Notch signaling with a loss-of-function mutation in the ligand *Delta* (*Dl*). We observed 7–10 *mid*-expressing cells in each hemisegment in early stage 12 Dl^{x} mutant embryos. Each cluster of *mid*-expressing cells is associated with an ectodermal En stripe, although clusters are occasionally missing or fused (Figs. 4F and F′). To ectopically activate the Notch pathway, we expressed the intracellular domain of Notch (Nicd) throughout the mesoderm using the GAL4/UAS system. In *twi–GAL4;UAS–Nicd* embryos, *mid* expression in the dorsal mesoderm of stage 12 embryos was often absent (Figs. 4G and H). These results suggest that the *mid*-expressing cardioblast precursors are selected from a single pool of competent progenitors by Notch-mediated lateral inhibition.

mid and H15 act partially redundantly in dorsal vessel morphogenesis

Because *mid* and *H15* expression coincides with cardioblast fate, we tested whether *mid* and *H15* are required for cardioblast formation and dorsal vessel development. Because the two genes are very closely linked, we used a pair of overlapping deficiencies to generate embryos deleted for both *mid* and *H15* (Fig. 2). *Df(2L)GpdhA* (Kotarski et al., 1983) deletes genes beginning with *H15* moving proximally, and *Df(2L)x528* deletes genes beginning with *mid* and moving distally (Buescher et al., 2004). *Df(2L)x528/Df(2L)GpdhA* (*H15 mid* null) animals are embryonic lethal and have ventral segmentation defects due to the early essential role that we have previously demonstrated (Buescher et al., 2004). It is important to note that the expression and effects of *mid* and *H15* in segmentation are restricted to the ventral ectoderm (Buescher et al., 2004) and thus the loss of *mid* and *H15* does not affect the segment polarity gene expression in the dorsal ectoderm that is required for the proper induction of heart precursors (Buescher et al., 2004). The region deleted by both deficiencies contains only *H15*, *midline*, and a predicted gene of unknown function, *CG31467* (Gadfly). We have probed embryos with *CG31467* anti-sense RNA probes and detected no hybridization and thus *CG31467* is unlikely to contribute to dorsal vessel development (data not shown).

To characterize the effects of *mid* and *H15* loss of function on heart development, we compared mutant and control embryos labeled with antibodies to Tin (Bodmer, 1993) and Zfh-1 (Lai et al., 1991). At the end of morphogenesis, each cardioblast pairs with a cardioblast in the opposite row to form the central tube of the dorsal vessel (Haag et al., 1999). Four out of six cardioblasts pairs in each segment are labeled with Tin and these can be seen as ordered groups of four pairs of labeled cells separated by two pairs of unlabelled cells (Figs. 1B and 5A, A′). Rows of

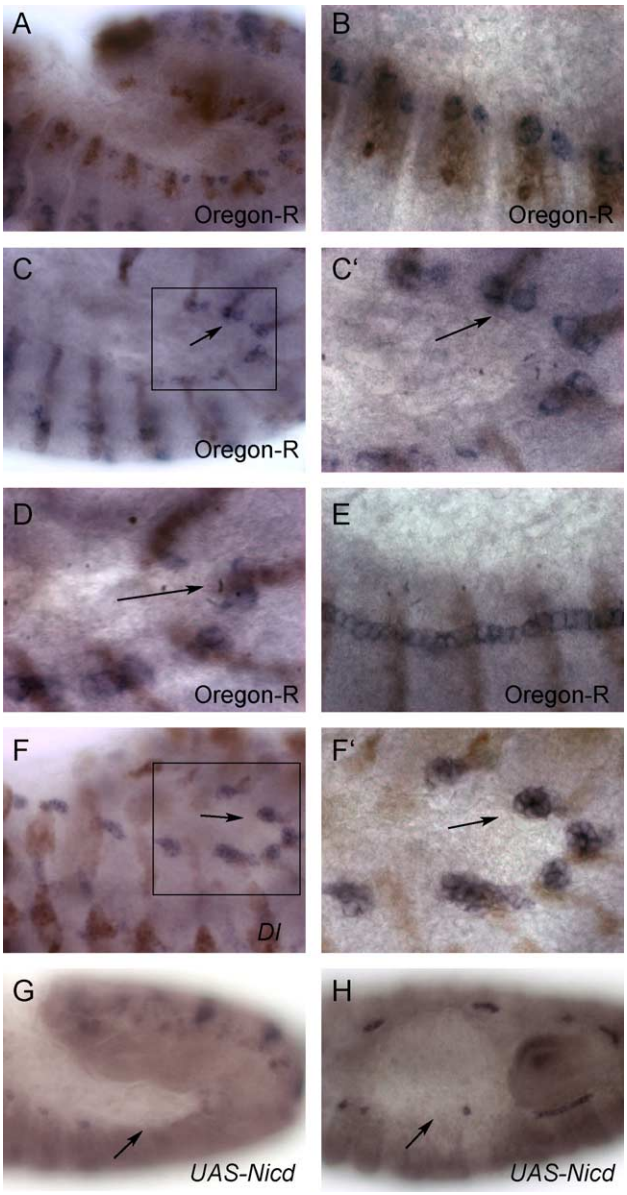

Fig. 4. Selection of *midline*-expressing cells from the dorsal mesoderm. (A) Stage 12 embryo double labeled with *mid* in situ hybridization (blue) and Wg antibody. One *mid*-expressing cell is located beneath the Wg secreting ectoderm in every hemisegment. (B) Higher magnification of an older stage 12 embryo labeled with *mid* in situ hybridization (blue) and Wg antibody. (C and C′) The *mid* in situ hybridization (blue) in the dorsal mesoderm of stage 12 embryos is found in consistent position relative to the ectodermal staining of En (brown). The cells in segment A7 are indicated with an arrow. Box in C shows the approximate location of panel C′. (D) *mid* expression is next seen in clusters of three to six cells in stage 12 (segment A7 indicated with arrow). (E) The clusters fuse to form a continuous row of cardioblasts with six cells per hemisegment in stage 13. (F and F′) Stage 12 Dl^{x} mutant embryo with single clusters of 7–10 *mid*-expressing cells found in each hemisegment (arrows indicate segment A7). Box in F shows the approximate location of panel F′. (G) Activation of the Notch pathway in all mesodermal cells of stage 12 *twi–GAL4;UAS–Nicd* embryos suppresses *mid* expression (dorsal mesoderm missing *mid*-expressing cells indicated with an arrow). (H) Stage 14 *twi–GAL4;UAS–Nicd* embryo showing gaps in dorsal vessel *mid* expression (arrow).

pericardial cells, marked with Zfh-1 or Zfh-1 and Tin, flank the central rows of cardioblasts (Figs. 5A and A′). In virtually all *H15 mid* null embryos, the pairing of the Tin^{+} cardioblasts was abnormal with some of the cells pairing and others not in proper alignment (Figs. 5B and B′). In some cases, the central tube of the dorsal vessel was twisted with one row of cardioblasts lying beneath the other. Pericardial cells were often displaced either laterally or medially from their normal location, possibly as a secondary consequence of defects in cardioblast alignment. The overall disorganization was most severe in the middle of the dorsal vessel, in abdominal segments 3, 4, and 5.

In order to test the individual contribution of *mid* and *H15* to the dorsal vessel phenotype, we examined embryos mutant for one gene but not the other. $H15^{x4}/Df(2L)GpdhA$ embryos are deleted for both copies of *H15* and one copy of *mid* (*H15* null) and showed no heart defects (Fig. 5C). In contrast, embryos homozygous for the null mutation mid^{1} (Buescher et al., 2004; Nüsslein-Volhard et al., 1984) have a variable heart phenotype. Typically, 50–60% of mid^{1} homozygous embryos exhibit an overall disorganization of the dorsal vessel as indicated by Zfh-1 and Tin double labeling (Figs. 5E and E′) while the rest have fairly normal heart morphology (Fig. 5D). The phenotypes are similar to but weaker than the phenotype seen in *H15 mid* null embryos. These results suggest that *mid* and *H15* play critical, partially redundant roles in the morphogenesis of the dorsal vessel with *mid* having a greater role but with loss of *H15* increasing the penetrance and expressivity of the defects. We have demonstrated a similar relative requirement for *mid* and *H15* in ectoderm segmentation (Buescher et al., 2004).

Dorsal vessel cell specification in H15 mid null embryos

To test whether *mid* and *H15* were required for cell fate specification in the dorsal vessel, we examined the expression of several cardioblast and pericardial cell markers in both wild-type embryos and in *H15 mid* null embryos. *DMef2* encodes a MADS box transcription factor expressed in most muscle cells and is specific to cardioblasts in the heart (Nguyen et al., 1994). In wild-type embryos, Dmef2 marks 52 cardioblasts in each half of the heart (Gajewski et al., 1999). In *H15 mid* null embryos, the wild-type number of Dmef2 cells is found (51.8, $n = 11$), indicating that cardioblasts are properly formed in the absence of *mid* and *H15* function (Figs. 6A and B). The expression of *B2-3-20*, a *lacZ* enhancer trap that marks cardioblasts (Haag et al., 1999), and Myosin Heavy Chain (MHC) (Young et al., 1991), a marker of muscle differentiation, were also found to be normal in *H15 mid* null embryos (Figs. 6C–H), as was the expression of the *Hand* mRNA, a marker of all cardioblasts and pericardial cells (not shown) (Kolsch and Paululat, 2002). These results suggest that *mid* and *H15* are not essential for cardioblast specification. We also examined pericardial cells in wild-type and *H15 mid* null embryos. We counted Zfh-1 labeling in confocal

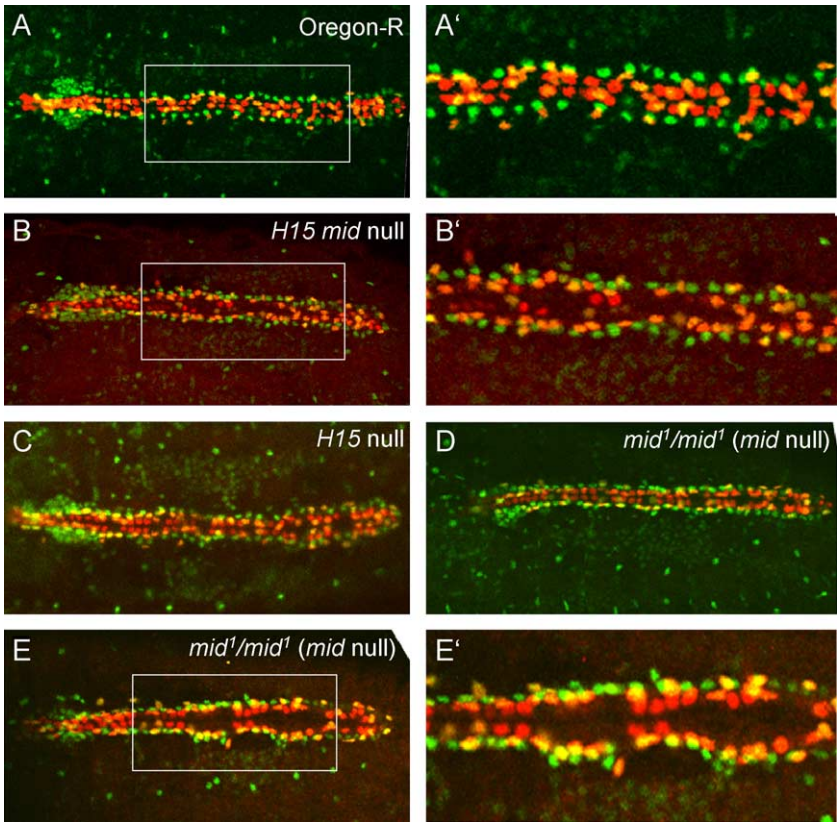

Fig. 5. Tin and Zfh-1 labeling of *mid* and *H15* double and single mutants. All panels show Tin (red) and Zfh-1 (green) labeling. Boxes in panels A, B, and E correspond to panels A′, B′, and E′, respectively. (A) Oregon-R control embryo at stage 17. (A′) Segments A3–A6 with Tin labeling in four ordered pairs of cardioblasts in each segment. (B and B′) Stage 17 embryo null for *mid* and *H15* (*Df(2L)x528/Df(2L)GpdhA*) show disruption of Tin-positive cardioblast pairing. (C) Embryos deficient for *H15* but carrying one copy of *mid* ($H15^{x4}$/*Df(2L)GpdhA*) have normal dorsal vessel organization. Embryos homozygous for mid^{1} have variable effects with some embryos appearing wild-type (D) and others exhibiting defects in the alignment of cardioblasts and the pericardial cells (E and E′).

slices from mutant and wild-type embryos to estimate pericardial cell numbers. In segments A2–A8, which are posterior of the lymph gland, we found similar numbers of Zfh-1-positive cells in wild-type (118.5, $n = 7$) and *H15 mid* null embryos (121.5, $n = 9$). The staining of Eve, a marker of two pericardial cells per hemisegment, was also normal in *H15 mid* null embryos, suggesting that *mid* and *H15* are not required for the generation of correct numbers of pericardial cells either (Figs. 6I and J). One difference we did note was in the lymph gland, a structure composed of Zfh-1-labeled cells that surround the two anterior-most segments of the dorsal vessel, T3 and A1 (Figs. 6K and L). We found an increase in Zfh-1-positive cells with 63.7 cells labeled per gland ($n = 11$) in *H15 mid* null embryos compared to 50.9 cells labeled per gland ($n = 14$) in wild-type embryos. Because *mid* and *H15* are not expressed in the lymph gland primordia, this may be a nonautonomous effect of *mid* and *H15* loss of function in the dorsal vessel.

Ectopic expression of mid alters dorsal mesoderm fate

The loss-of-function data indicate that *mid* and *H15* are required for heart morphogenesis but are not essential for the specification of cardiac fate. In order to assess the effect of ectopic *mid* expression on cardiac development, we expressed *mid* with a *UAS–mid* construct (Buescher et al., 2004). Expressing *UAS–mid* under the control of *twist–GAL4* drives *mid* throughout the mesoderm beginning in gastrulation (Gajewski et al., 2001). We detected an increase in Tin-positive cells in the dorsal mesoderm of *twi–GAL4;UAS–mid* embryos. By stage 13, a clear increase in the number of Tin-positive cells is visible (Figs. 7A and B, and data not shown), suggesting an increase in cardiac fate promoted by the ubiquitous expression of *mid* in the mesoderm. We saw similar effects using *pnr–GAL4* (data not shown), which drives GAL4 in dorsal mesoderm and dorsal ectoderm, indicating that ectopic *mid* expression in dorsal mesoderm is sufficient to cause the observed effects.

The dramatic increase of Tin-positive cells caused by ectopic *mid* expression in the dorsal mesoderm led us to examine the effects of ectopic *mid* expression on visceral mesoderm development, which is also derived from dorsal mesoderm. We examined the expression of Fasciclin III (Fas III) (Patel et al., 1987), a marker of visceral mesoderm in *twist–GAL4;UAS-mid* embryos. In many embryos, we

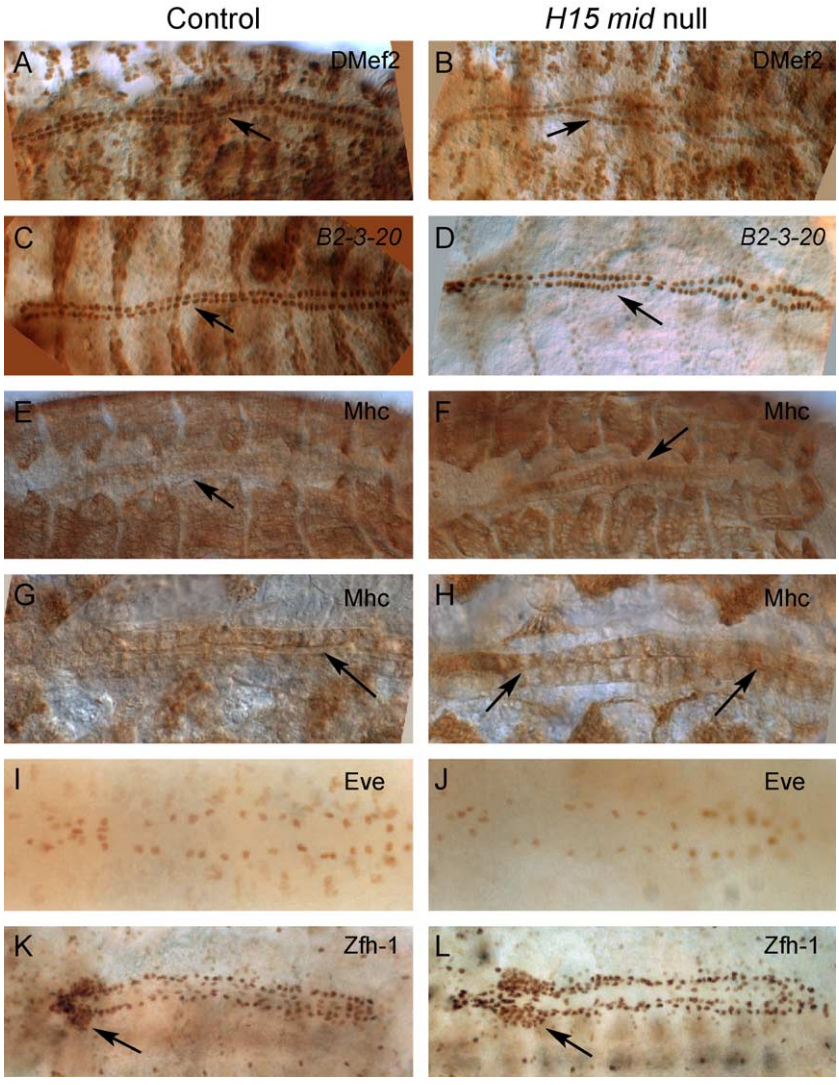

Fig. 6. Cell fate specification in *H15 mid* double mutant embryos. (A) Dmef2 labeling in stage 17 Oregon-R embryo. Cardioblasts are indicated with an arrow. (B) Dmef2 labeling in stage 17 *H15 mid* null embryo with misaligned cardioblasts (arrow). (C) β-gal labeling of control embryo (*Df(H15 mid)/CyO–wglacZ*) carrying the *B2-3-20* lacZ enhancer-trap that labels cardioblasts (arrow). Note that the strong ectodermal expression is due to the *wg–lacZ* enhancer-trap. (D) β-gal labeling of *H15 mid* null carrying the *B2-3-20* lacZ enhancer-trap showing misaligned cardioblasts (arrow). (E) Mhc labeling in a stage 17 Oregon-R embryo. Cardioblasts are indicated with an arrow. (F) Mhc labeling in stage 17 *H15 mid* null embryo. Cardioblasts are indicated with an arrow. (G) Mhc labeling of a stage 17 Oregon-R embryo shown at higher magnification. A clearly defined lumen is visible between the two rows of aligned cardioblasts. (H) Mhc labeling in a stage 17 *H15 mid* null embryo shown at higher magnification. The alignment of cardioblasts in this embryo is irregular with the lumen of the dorsal vessel not easily visible suggesting it is collapsed in parts of the dorsal vessel (arrows). (I) Eve labeling in a stage 17 Oregon-R embryo. (J) Eve labeling in a stage 17 *H15 mid* null embryo. (K) Zfh-1 labeling of a stage 17 Oregon-R embryo. Lymph gland indicated with arrow. (L) Zfh-1 labeling of a stage 17 *H15 mid* null embryo. Lymph gland indicated with arrow.

found reduced or gapped expression of Fas III, consistent with a loss of visceral mesoderm as a result of ectopic *mid* expression (Figs. 7E and F).

Ectopic mid expression promotes cardioblast and pericardial cell fate

In addition to the changes in *tin* and Fas III expression, ectopic *mid* expression driven by *pnr–GAL4* or *twi–GAL4* disrupts heart morphogenesis, often preventing the two halves of the heart from fusing and making it difficult to assess how ectopic *mid* affects specific cardiac lineages (Figs. 7C and D). However, when expressing *UAS–mid* under the control of *How–GAL4*, another driver with expression throughout mesoderm (Zaffran et al., 1997), we observed similar but weaker effects on Tin and Fas III distribution in stage 13 embryos compared to those observed for *twist–GAL4;UAS–mid* embryos (not shown). Furthermore, we see a range of defects in older embryos. In some embryos, there is a disruption of heart development similar to that seen with *twi–GAL4* and *pnr–GAL4*, but in most embryos, the two halves of the heart join together but have lateral branches and ectopic cells. The lateral branches contain Tin-positive cells, Zfh-1-positive cells, and Tin-

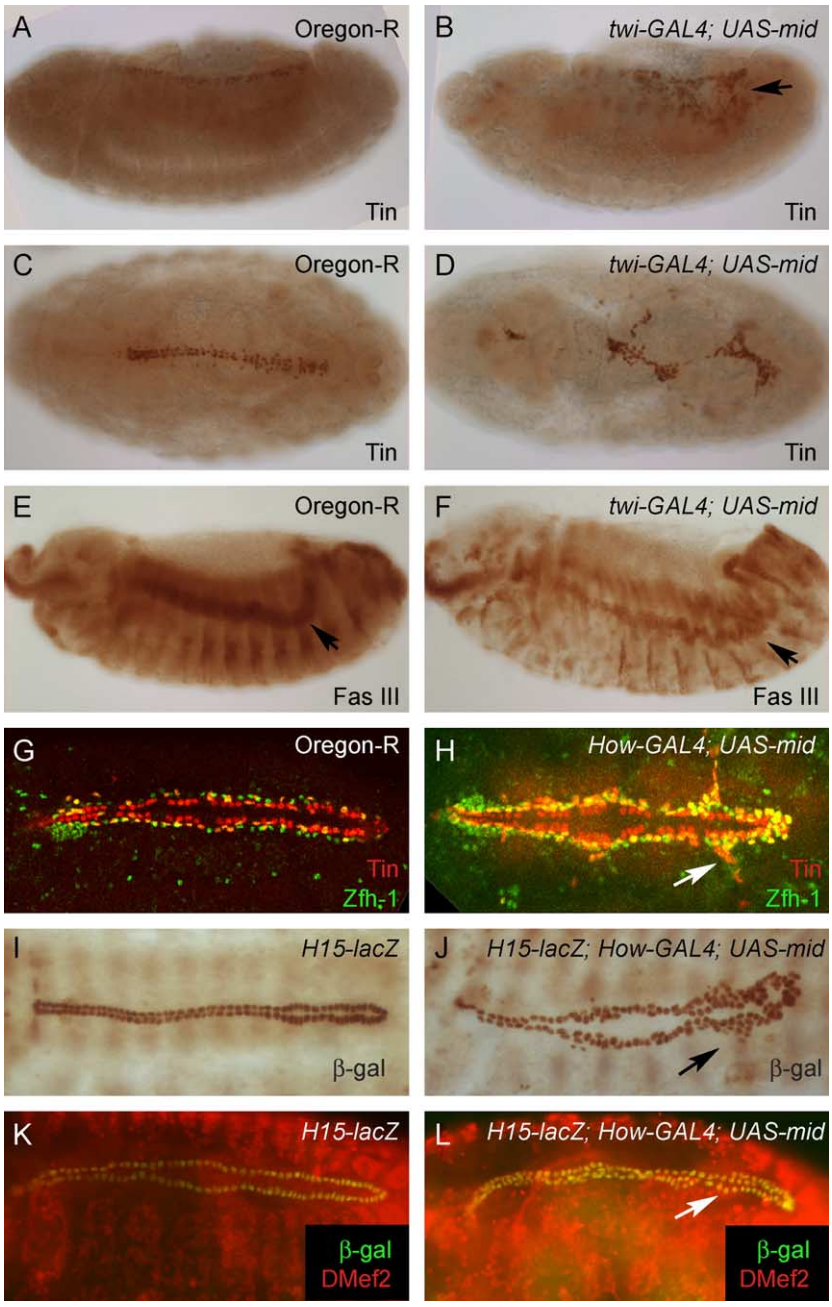

Fig. 7. Alteration of dorsal mesoderm fate in *twi–Gal4;UAS–mid* embryos. (A) Tin labeling in a stage 14 Oregon-R embryo. (B) Tin labeling in stage 14 *twi–GAL4;UAS–mid* embryo. Ectopic Tin expression in dorsal mesoderm is indicated with an arrow. (C) Tin labeling in a stage 17 Oregon-R embryo. (D) Disruption of dorsal vessel development indicated by Tin labeling in stage 17 *twi–GAL4;UAS–mid* embryo. (E) Fas III labeling in a stage 13 Oregon-R embryo. Visceral mesoderm expression is indicated by arrow. (F) Fas III labeling in stage 13 *twi–GAL4;UAS–mid* embryo. Reduced visceral mesoderm expression indicated by arrow. (G) Tin (red) and Zfh-1 (green) labeling of stage 16 wild-type embryo. (H) Tin (red) and Zfh-1 (green) labeling of stage 16 *How–GAL4;UAS–mid* embryo. Ectopic heart cells are indicated (arrow). (I) Anti-β-galactosidase labeling of stage 17 *H15–lacZ* embryo. (J) Anti-β-galactosidase labeling of a stage 17 *H15–lacZ*; *How–GAL4;UAS–mid* embryo. Ectopic cardioblasts indicated by arrow. (K) Anti-β-galactosidase (green) and Dmef2 (red) labeling of stage 17 *H15–lacZ* embryo. Cardioblasts appear yellow. (L) Anti-β-galactosidase (green) and Dmef2 (red) labeling of stage 17 *H15–lacZ;How–GAL4;UAS–mid* embryos. Cardioblasts appear yellow. Ectopic cardioblasts are indicated (arrow).

positive Zfh-1-positive cells (Fig. 7H). The ectopic Zfh-1-positive cells and Tin-positive Zfh-1-positive cells likely represent ectopic pericardial cells. The ectopic Tin-positive cells are likely to be ectopic cardioblasts. To confirm this, we examined β-galactosidase-labeled cardioblasts in *How–GAL4;UAS–mid* embryos carrying the *H15–lacZ* enhancer-trap. We observed ectopic cells labeled with β-galactosidase in branched patterns. In normal *H15–lacZ* embryos, we find 52 β-Gal-positive cardioblasts per side in the dorsal vessel. In *H15–lacZ;How–Gal4;UAS–mid* embryos, we find up to 30 extra cardioblasts per half dorsal vessel (Fig. 7I and J). Double labeling with Dmef2 (Fig. 7K and L) further

supports the identity of the ectopic cells as cardioblasts. In summary, the loss-of-function data indicate that *mid* and *H15* are not required for fate specification, while the gain-of-function data indicate that ectopic expression of *mid* in dorsal mesoderm is sufficient to induce ectopic cardiac fate.

Discussion

In this report, we provide evidence that T-box transcription factors play an essential role in the development of the dorsal vessel in *D. melanogaster*. The *Tbx20* homologs *mid* and *H15* are expressed in cardioblasts and are required in a partially redundant manner for dorsal vessel morphogenesis. Furthermore, the activation of *mid* in the dorsal mesoderm depends on cardiogenic factors and *mid* expression correlates with cardioblast specification. Finally, ectopic expression of *mid* induces cardiac fate in the dorsal mesoderm in a manner similar to the effects of ectopic *tin* and *pnr* expression (Gajewski et al., 1999, 2001; Klinedinst and Bodmer, 2003). Together, these results suggest an important role for *mid* and *H15* in the specification and differentiation of cardiac fate. Vertebrate T-box transcription factors are critical regulators of heart development. Tbx20 is essential for morphogenesis of the vertebrate heart and interacts with the Tin and Pnr homologs Nkx2.5 and GATA4 in gene regulation (Plageman and Yutzey, 2004; Stennard et al., 2003; Szeto et al., 2002). Thus, the previously reported conservation of a cardiac regulatory network, including the NK-2 homeodomain proteins and the GATA factors, may now be extended to include T-box transcription factors. Our findings offer further support for the proposal that, despite differing greatly in morphology, the vertebrate heart and the insect dorsal vessel may share a common evolutionary ancestor (reviewed in Cripps and Olson, 2002).

midline expression marks cardioblast progenitors

The timing and pattern of *mid* expression suggest that it is an early specific marker of cardioblasts. *mid* expression in the dorsal mesoderm is first observed in two cells per hemisegment in early stage 12, increasing to six cardioblasts per hemisegment throughout stages 12 and 13. Analysis of the cardioblast lineage with genetic mosaics and cell cycle manipulation indicates that the six cardioblasts in each hemisegment must derive from two progenitor cells (Alvarez et al., 2003; Han and Bodmer, 2003; Ward and Skeath, 2000). Furthermore, Notch signaling is required to restrict the number of cardioblasts in the dorsal vessel (Hartenstein et al., 1992), and we find that the number of cells expressing *mid* in the dorsal mesoderm is also restricted by Notch activation. Thus, our data are consistent with a model where *mid*-expressing cardioblast progenitors are normally selected from a cluster of competent cells by a signal or signals acting in opposition to Notch. The nature of this signal is unknown, although we note that the two *mid*-expressing cells arise in consistent positions relative to the ectodermal Wg and En stripes of cells and thus may respond to spatial cues emanating from the overlying ectoderm or arising from within the mesoderm.

mid and H15 act partially redundantly in heart morphogenesis

Both *mid* and *H15* are expressed in all cardioblasts from stage 13 onwards and the dorsal vessel is disorganized in *H15 mid* null embryos. Our analysis of *mid* and *H15* mutant embryos suggests that *mid* has the greater effect, causing some heart defects on its own while *H15* is nonessential. However, loss of both genes has a stronger phenotype, suggesting that *H15* augments the activity of *mid*. We find a similar relative requirement for the two genes in the segmentation of the embryonic ectoderm (Buescher et al., 2004). Normal numbers of cardioblasts, expressing markers of cardiac (*tin*) and muscle (*DMef2*) fate specification, as well as muscle differentiation (*Mhc*), are formed in *H15 mid* null embryos. The cardioblasts migrate dorsally but do not always align properly with their contralateral partners. The pericardial cells are often displaced laterally or medially, and the lymph gland is enlarged due to increased cell number. Given these defects and the restriction of *mid* and *H15* expression to cardioblasts, the essential function of *mid* and *H15* may be to regulate the later stages of cardioblast morphogenesis.

Understanding the essential function of *mid* and *H15* in dorsal vessel development awaits identifying the genes regulated by the two T-box transcription factors. Cardioblasts require specific cell polarity, adhesion, and migration in order to make the proper contacts with other cardioblasts and pericardial cells to form the tube of the dorsal vessel. Many cell adhesion molecules are expressed in cardioblasts and mutations in these genes cause dorsal vessel defects that are similar to those caused by loss of *mid* and *H15* (Haag et al., 1999; Martin et al., 1999; Stark et al., 1997). The misalignment of pericardial cells may be a secondary consequence of the defects in cardioblast morphogenesis. Similarly, the increase in lymph gland cell number is likely to be a nonautonomous effect as *mid* and *H15* are not expressed in the lymph gland primordia. Because this defect results from an increase in the lymph gland cell number, it is possible that *H15 mid* mutant embryos are defective in a signal from the dorsal vessel regulating cell proliferation in the lymph gland.

Does mid play a role in cardiac fate specification?

The specification of cardiac fate in *Drosophila* is complex. No single factor has been identified that is both necessary and sufficient to induce mesoderm to form heart cells. Combined Dpp and Wg signaling are essential for heart development and can induce ectopic heart fate, but

only in *tin*-expressing cells (Lockwood and Bodmer, 2002). Tin is essential for the specification of dorsal mesoderm and directly regulates many genes expressed in cardioblasts and pericardial cells (Cripps and Olson, 2002). However, ectopic expression of *tin* has little effect on cardiac fate (Lockwood and Bodmer, 2002). By comparison, Pnr, a transcriptional cofactor of Tin, is able to induce extensive ectopic cardiac fate (Gajewski et al., 1999; Klinedinst and Bodmer, 2003). However, some cardioblasts still form in *pnr* null embryos, albeit in greatly reduced numbers, indicating that *pnr* is not absolutely essential for heart cells to form (Alvarez et al., 2003). We find a formally similar situation for *mid* in that it is also sufficient but not necessary (even in the absence of *H15*) for cardiac fate. Recently, it has been shown that the *Xenopus Tbx20* homolog interacts physically with GATA4 and Nkx2.5 (Plageman and Yutzey, 2004; Stennard et al., 2003). Furthermore, Tbx20 can have a synergistic effect on the activation of cardiac promoters in vitro with Nkx2.5 and GATA4 (Stennard et al., 2003). Thus, it is possible that Mid and H15 act as cofactors for Tin and Pnr in the regulation of cardiac fate specification in the fly. The expression of *mid* in the dorsal mesoderm is much more restricted than that of either *tin* or *pnr*, thus ectopic expression of *mid* may promote ectopic cardiac fate through interaction with Tin and Pnr. This interpretation is supported by the restriction of ectopic cardiac fate induced by *mid* to the dorsal mesoderm.

An interaction with Tin and Pnr may explain why *mid* induces ectopic cardioblasts, but it does not explain why *mid* and *H15*, or *pnr* for that matter, are not essential for cardioblast specification. A simple possibility is that another transcription factor may compensate for the absence of *mid* and *H15*. This could be either an unrelated factor or another T-box protein, for example, the Doc1, 2, and 3 Tbx6-like proteins (Hamaguchi et al., 2004; Reim et al., 2003), which are restricted to the *svp*-expressing cardioblasts (Lo and Frasch, 2001). Assessing *mid* and *H15* for redundancy with the *Doc* genes, which is not trivial because loss of function for *Doc* blocks embryonic development prior to heart morphogenesis (Hamaguchi et al., 2004; Reim et al., 2003), will be necessary to test this model. Another mechanism that may help compensate for the absence of *mid* and *H15* is redundancy with Tin and Pnr activity. Given the pairwise synergy of GATA4, Nkx2.5, and Tbx20 in transcriptional assays (Stennard et al., 2003), it may be that removal of any one *trans*-acting factor would be insufficient to completely block cardiac fate. Either Pnr or Mid could be sufficient to induce some cardiac fate as long as Tin or other factors are present. In this context, it is worth noting that removal of *tin*, which has the greatest effect, completely blocking cardioblast fate specification, also prevents the expression of *pnr* (Gajewski et al., 2001), *mid*, and *H15* (Fig. 3C). In contrast, *tin*, *mid* and *H15* are only partially reduced in *pnr* mutants (Klinedinst and Bodmer, 2003) (Fig. 3E), and *tin* and *pnr* are widely expressed in the dorsal mesoderm prior to the activation of *mid* and *H15* expression (Azpiazu and Frasch, 1993; Bodmer, 1993; Gajewski et al., 1999, 2001; Klinedinst and Bodmer, 2003). Analysis of genetic and molecular interactions between *tin*, *pnr*, *mid*, and *H15* will be necessary to test these models.

Acknowledgments

We thank Drs. Rolf Bodmer, Roger Jacobs, Dan Kiehart, Zhi Chun Lai, Hanh Nguyen, Bruce Reed, the Bloomington Indiana stock center, and the Developmental Studies Hybridoma Bank for sharing materials. We thank Drs. Paul Mains and Jim McGhee for a critical reading of the manuscript. This work was funded through operating grants from the CIHR (MOP14468 and MOP66981) and from a pilot project grant from the Faculty of Medicine, University of Calgary (Hughes/Miles). WJB is a Senior Scholar of the AHFMR; KHS and RJS were funded by the CIHR training program in Genetics, Child Development and Health (STP-53923).

References

Ahn, D.G., Ruvinsky, I., Oates, A.C., Silver, L.M., Ho, R.K., 2000. *Tbx20*, a new vertebrate T-box gene expressed in the cranial motor neurons and developing cardiovascular structures in zebrafish. Mech. Dev. 95, 253–258.

Alvarez, A.D., Shi, W., Wilson, B.A., Skeath, J.B., 2003. Pannier and pointedP2 act sequentially to regulate *Drosophila* heart development. Development 130, 3015–3026.

Azpiazu, N., Frasch, M., 1993. Tinman and bagpipe: two homeobox genes that determine cell fates in the dorsal mesoderm of *Drosophila*. Genes Dev. 7, 1325–1340.

Basson, C.T., Bachinsky, D.R., Lin, R.C., Levi, T., Elkins, J.A., Soults, J., Grayzel, D., Kroumpouzou, E., Traill, T.A., Leblanc-Straceski, J., Renault, B., Kucherlapati, R., Seidman, J.G., Seidman, C.E., 1997. Mutations in human TBX5 cause limb and cardiac malformation in Holt–Oram syndrome. Nat. Genet. 15, 30–35.

Bodmer, R., 1993. The gene *tinman* is required for specification of the heart and visceral muscles in *Drosophila*. Development 118, 719–729.

Brand, A., Perrimon, N., 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 118, 401–415.

Brook, W.J., Cohen, S.M., 1996. Antagonistic interactions between Wingless and Decapentaplegic responsible for dorsal–ventral pattern in the *Drosophila* leg. Science 273, 1373–1377.

Brook, W.J., Ostafichuk, L.M., Piorecky, J., Wilkinson, M.D., Hodgetts, D.J., Russell, M.A., 1993. Gene expression during imaginal disc regeneration detected using enhancer-sensitive P-elements. Development 117, 1287–1297.

Bruneau, B.G., 2002. Transcriptional regulation of vertebrate cardiac morphogenesis. Circ. Res. 90, 509–519.

Bruneau, B.G., Nemer, G., Schmitt, J.P., Charron, F., Robitaille, L., Caron, S., Conner, D.A., Gessler, M., Nemer, M., Seidman, C.E., Seidman, J.G., 2001. A murine model of Holt–Oram syndrome defines roles of the T-box transcription factor Tbx5 in cardiogenesis and disease. Cell 106, 709–721.

Buescher, M., Svendsen, P.C., Tio, M., Miscikolczi-McCallum, C.M., Tear, G., Brook, W.J., Chia, W., 2004. *Drosophila* T-box proteins break the symmetry of Hedgehog-dependent activation of Wingless. Curr. Biol. 14, 1694–1702.

Carson, C.T., Kinzler, E.R., Parr, B.A., 2000. Tbx12, a novel T-box gene, is

expressed during early stages of heart and retinal development. Mech. Dev. 96, 137–140.
Cripps, R.M., Olson, E.N., 2002. Control of cardiac development by an evolutionarily conserved transcriptional network. Dev. Biol. 246, 14–28.
Durocher, D., Charron, F., Warren, R., Schwartz, R.J., Nemer, M., 1997. The cardiac transcription factors Nkx2-5 and GATA-4 are mutual cofactors. EMBO J. 16, 5687–5696.
Frasch, M., 1995. Induction of visceral and cardiac mesoderm by ectodermal Dpp in the early *Drosophila* embryo. Nature 374, 464–467.
Frasch, M., Chen, X., Lufkin, T., 1995. Evolutionary-conserved enhancers direct region-specific expression of the murine Hoxa-1 and Hoxa-2 loci in both mice and *Drosophila*. Development 121, 957–974.
Gajewski, K., Kim, Y., Choi, C.Y., Schulz, R.A., 1998. Combinatorial control of *Drosophila* mef2 gene expression in cardiac and somatic muscle cell lineages. Dev. Genes Evol. 208, 382–392.
Gajewski, K., Fossett, N., Molkentin, J.D., Schulz, R.A., 1999. The zinc finger proteins Pannier and GATA4 function as cardiogenic factors in *Drosophila*. Development 126, 5679–5688.
Gajewski, K., Choi, C.Y., Kim, Y., Schulz, R.A., 2000. Genetically distinct cardial cells within the *Drosophila* heart. Genesis 28, 36–43.
Gajewski, K., Zhang, Q., Choi, C.Y., Fossett, N., Dang, A., Kim, Y.H., Kim, Y., Schulz, R.A., 2001. Pannier is a transcriptional target and partner of tinman during *Drosophila* cardiogenesis. Dev. Biol. 233, 425–436.
Garg, V., Kathiriya, I.S., Barnes, R., Schluterman, M.K., King, I.N., Butler, C.A., Rothrock, C.R., Eapen, R.S., Hirayama-Yamada, K., Joo, K., Matsuoka, R., Cohen, J.C., Srivastava, D., 2003. GATA4 mutations cause human congenital heart defects and reveal an interaction with TBX5. Nature 424, 443–447.
Griffin, K.J., Stoller, J., Gibson, M., Chen, S., Yelon, D., Stainier, D.Y., Kimelman, D., 2000. A conserved role for *H15*-related T-Box transcription factors in zebrafish and *Drosophila* heart formation [in process citation]. Dev. Biol. 218, 235–247.
Haag, T.A., Haag, N.P., Lekven, A.C., Hartenstein, V., 1999. The role of cell adhesion molecules in *Drosophila* heart morphogenesis: faint sausage, shotgun/DE-cadherin, and laminin A are required for discrete stages in heart development. Dev. Biol. 208, 56–69.
Hamaguchi, T., Yabe, S., Uchiyama, H., Murakami, R., 2004. *Drosophila* Tbx6-related gene, *Dorsocross*, mediates high levels of Dpp and Scw signal required for the development of amnioserosa and wing disc primordium. Dev. Biol. 265, 355–368.
Han, Z., Bodmer, R., 2003. Myogenic cells fates are antagonized by Notch only in asymmetric lineages of the *Drosophila* heart, with or without cell division. Development 130, 3039–3051.
Hartenstein, A.Y., Rugendorff, A., Tepass, U., Hartenstein, V., 1992. The function of the neurogenic genes during epithelial development in the *Drosophila* embryo. Development 116, 1203–1220.
Herranz, H., Morata, G., 2001. The functions of pannier during *Drosophila* embryogenesis. Development 128, 4837–4846.
Hiroi, Y., Kudoh, S., Monzen, K., Ikeda, Y., Yazaki, Y., Nagai, R., Komuro, I., 2001. Tbx5 associates with Nkx2-5 and synergistically promotes cardiomyocyte differentiation. Nat. Genet. 28, 276–280.
Iio, A., Koide, M., Hidaka, K., Morisaki, T., 2001. Expression pattern of novel chick T-box gene, *Tbx20*. Dev. Genes Evol. 211, 559–562.
Jiang, Y., Evans, T., 1996. The Xenopus GATA-4/5/6 genes are associated with cardiac specification and can regulate cardiac-specific transcription during embryogenesis. Dev. Biol. 174, 258–270.
Klinedinst, S.L., Bodmer, R., 2003. Gata factor Pannier is required to establish competence for heart progenitor formation. Development 130, 3027–3038.
Kolsch, V., Paululat, A., 2002. The highly conserved cardiogenic bHLH factor Hand is specifically expressed in circular visceral muscle progenitor cells and in all cell types of the dorsal vessel during *Drosophila* embryogenesis. Dev. Genes Evol. 212, 473–485.
Kotarski, M.A., Pickert, S., MacIntyre, R.J., 1983. A cytogenetic analysis of the chromosomal region surrounding the alpha-glycerophosphate dehydrogenase locus of *Drosophila melanogaster*. Genetics 105, 371–386.
Kraus, F., Haenig, B., Kispert, A., 2001. Cloning and expression analysis of the mouse T-box gene *tbx20*. Mech. Dev. 100, 87–91.
Lai, Z.C., Fortini, M.E., Rubin, G.M., 1991. The embryonic expression patterns of zfh-1 and zfh-2, two *Drosophila* genes encoding novel zinc-finger homeodomain proteins. Mech. Dev. 34, 123–134.
Laverriere, A.C., MacNeill, C., Mueller, C., Poelmann, R.E., Burch, J.B., Evans, T., 1994. GATA-4/5/6, a subfamily of three transcription factors transcribed in developing heart and gut. J. Biol. Chem. 269, 23177–23184.
Lawrence, P.A., Bodmer, R., Vincent, J.P., 1995. Segmental patterning of heart precursors in *Drosophila*. Development 121, 4303–4308.
Lee, H.H., Frasch, M., 2000. Wingless effects mesoderm patterning and ectoderm segmentation events via induction of its downstream target sloppy paired. Development 127, 5497–5508.
Li, Q.Y., Newbury-Ecob, R.A., Terrett, J.A., Wilson, D.I., Curtis, A.R., Yi, C.H., Gebuhr, T., Bullen, P.J., Robson, S.C., Strachan, T., Bonnet, D., Lyonnet, S., Young, I.D., Raeburn, J.A., Buckler, A.J., Law, D.J., Brook, J.D., 1997. Holt–Oram syndrome is caused by mutations in TBX5, a member of the Brachyury (T) gene family. Nat. Genet. 15, 21–29.
Lo, P.C., Frasch, M., 2001. A role for the COUP-TF-related gene seven-up in the diversification of cardioblast identities in the dorsal vessel of *Drosophila*. Mech. Dev. 104, 49–60.
Lockwood, W., Bodmer, R., 2002. The patterns of Wingless, Decapentaplegic, and Tinman position the *Drosophila* heart. Mech. Dev. 114, 13.
Lyons, I., Parsons, L.M., Hartley, L., Li, R., Andrews, J.E., Robb, L., Harvey, R.P., 1995. Myogenic and morphogenetic defects in the heart tubes of murine embryos lacking the homeobox gene Nkx2-5. Genes Dev. 9, 1654–1666.
Martin, D., Zusman, S., Li, X., Williams, E.L., Khare, N., DaRocha, S., Chiquet-Ehrismann, R., Baumgartner, S., 1999. Wing blister, a new *Drosophila* laminin alpha chain required for cell adhesion and migration during embryonic and imaginal development. J. Cell Biol. 145, 191–201.
Meins, M., Henderson, D.J., Bhattacharya, S.S., Sowden, J.C., 2000. Characterization of the human *TBX20* gene, a new member of the T-Box gene family closely related to the *Drosophila H15* gene. Genomics 67, 317–332.
Mlodzik, M., Hiromi, Y., Weber, U., Goodman, C.S., Rubin, G.M., 1990. The *Drosophila* seven-up gene, a member of the steroid receptor gene superfamily, controls photoreceptor cell fates. Cell 60, 211–224.
Nguyen, H.T., Bodmer, R., Abmayr, S.M., McDermott, J.C., Spoerel, N.A., 1994. D-mef2: a *Drosophila* mesoderm-specific MADS box-containing gene with a biphasic expression profile during embryogenesis. Proc. Natl. Acad. Sci. U. S. A. 91, 7520–7524.
Nüsslein-Volhard, C., Wieschaus, E., Kluding, H., 1984. Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster*: I. Zygotic loci on the second chromosome. Wilhelm Roux's Arch. Dev. Biol. 193, 267–282.
Papaioannou, V.E., Silver, L.M., 1998. The T-box gene family. BioEssays 20, 9–19.
Park, M., Wu, X., Golden, K., Axelrod, J.D., Bodmer, R., 1996. The Wingless signaling pathway is directly involved in *Drosophila* heart development. Dev. Biol. 177, 104–116.
Patel, N.H., Snow, P., Goodman, C., 1987. Characterization and cloning of Fasciclin III: a glycoprotein expressed on a subset of neurons and axon pathways in *Drosophila*. Cell 48, 975–988.
Plageman Jr., T.F., Yutzey, K.E., 2004. Differential expression and function of *Tbx5* and *Tbx20* in cardiac development. J. Biol. Chem. 279, 19026–19034.
Poeck, B., Hofbauer, A., Pflugfelder, G.O., 1993. Expression of the *Drosophila* optomotor-blind gene transcript in neuronal and glial cells of the developing nervous system. Development 117, 1017–1029.
Reim, I., Lee, H.H., Frasch, M., 2003. The T-box-encoding *Dorsocross* genes function in amnioserosa development and the patterning of the

dorsolateral germ band downstream of Dpp. Development 130, 3187–3204.

Schott, J.J., Benson, D.W., Basson, C.T., Pease, W., Silberbach, G.M., Moak, J.P., Maron, B.J., Seidman, C.E., Seidman, J.G., 1998. Congenital heart disease caused by mutations in the transcription factor NKX2-5. Science 281, 108–111.

Stark, K.A., Yee, G.H., Roote, C.E., Williams, E.L., Zusman, S., Hynes, R.O., 1997. A novel alpha integrin subunit associates with betaPS and functions in tissue morphogenesis and movement during *Drosophila* development. Development 124, 4583–4594.

Stennard, F.A., Costa, M.W., Elliott, D.A., Rankin, S., Haast, S.J., Lai, D., McDonald, L.P., Niederreither, K., Dolle, P., Bruneau, B.G., Zorn, A.M., Harvey, R.P., 2003. Cardiac T-box factor *Tbx20* directly interacts with Nkx2-5, GATA4, and GATA5 in regulation of gene expression in the developing heart. Dev. Biol. 262, 206–224.

Sullivan, W., Ashburner, M., Hawley, R.S., 2000. *Drosophila* Protocols. Cold Spring Harbor Laboratory Press, Cold Srping Harbor.

Szeto, D.P., Griffin, K.J., Kimelman, D., 2002. HrT is required for cardiovascular development in zebrafish. Development 129, 5093–5101.

Takeuchi, J.K., Ohgi, M., Koshiba-Takeuchi, K., Shiratori, H., Sakaki, I., Ogura, K., Saijoh, Y., Ogura, T., 2003. Tbx5 specifies the left/right ventricles and ventricular septum position during cardiogenesis. Development 130, 5953–5964.

Tanaka, M., Kasahara, H., Bartunkova, S., Schinke, M., Komuro, I., Inagaki, H., Lee, Y., Lyons, G.E., Izumo, S., 1998. Vertebrate homologs of tinman and bagpipe: roles of the homeobox genes in cardiovascular development. Dev. Genet. 22, 239–249.

Tautz, D., Pfeifle, C., 1989. A non-radioactive in situ hybridization method for the localization of specific RNAs in *Drosophila* embryos reveals translational control of the segmentation gene hunchback. Chromosoma 98, 81–85.

Ward, E.J., Skeath, J.B., 2000. Characterization of a novel subset of cardiac cells and their progenitors in the *Drosophila* embryo. Development 127, 4959–4969.

Wiellette, E.L., McGinnis, W., 1999. Hox genes differentially regulate Serrate to generate segment-specific structures. Development 126, 1985–1995.

Wilson, V., Conlon, F.L., 2002. The T-box family. Genome Biol. 3008.1–3008.7 (reviews).

Yin, Z., Xu, X.L., Frasch, M., 1997. Regulation of the twist target gene tinman by modular *cis*-regulatory elements during early mesoderm development. Development 124, 4971–4982.

Young, P.E., Pesacreta, T.C., Kiehart, D.P., 1991. Dynamic changes in the distribution of cytoplasmic myosin during *Drosophila* embryogenesis. Development 111, 1–14.

Zaffran, S., Frasch, M., 2002. Early signals in cardiac development. Circ. Res. 91, 457–469.

Zaffran, S., Astier, M., Gratecos, D., Semeriva, M., 1997. The held out wings (how) *Drosophila* gene encodes a putative RNA-binding protein involved in the control of muscular and cardiac activity. Development 124, 2087–2098.