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

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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.

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Introduction

The insect heart, or dorsal vessel, is a simple muscular tube that pumps hemolymph and hematocyes 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
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 (Fraser 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; Lavriere 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 (Pocek 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 Drosos-


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;Df(2L)x528, UAS–mid (Buescher et al., in press), and PZ;[lac-Z,ry¹]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 tinEC40, 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)xpdH2, Df(2L)xGpdH2, Df, wgEC40, pnrEC30, 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.). Histochernical 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) 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 is first expressed during early stage 12, but at lower levels (data not shown). To confirm that mid and H15 are expressed 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.
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 factors that confer cardiac fate. Indeed, the dorsal vessel expression of mid and H15 is completely lost in both wg<sup>vd</sup> and tin<sup>ex5</sup> 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<sup>ex5</sup> 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 V). 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 (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.
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 Dlx 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)x528 deletes genes beginning with H15 moving proximally, and 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').
pericardial cells, marked with Zfh-1 or Zth-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. $H15^{+/+}$/$Df(2L)GpdhA$ 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 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
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 \( H15_{\text{mid}} \) null embryos (121.5, \( n = 9 \)). The staining of Eve, a marker of two pericardial cells per hemisegment, was also normal in \( H15_{\text{mid}} \) null embryos, suggesting that \( \text{mid} \) 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 \( H15_{\text{mid}} \) null embryos compared to 50.9 cells labeled per gland (\( n = 14 \)) in wild-type embryos. Because \( \text{mid} \) and \( H15 \) are not expressed in the lymph gland primordia, this may be a nonautonomous effect of \( \text{mid} \) and \( H15 \) loss of function in the dorsal vessel.

**Ectopic expression of \( \text{mid} \) alters dorsal mesoderm fate**

The loss-of-function data indicate that \( \text{mid} \) 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 \( \text{mid} \) expression on cardiac development, we expressed \( \text{mid} \) with a \( UAS-\text{mid} \) construct (Buescher et al., 2004). Expressing \( UAS-\text{mid} \) under the control of \( \text{twi-GAL4} \) drives \( \text{mid} \) throughout the mesoderm beginning in gastrulation (Gajewski et al., 2001). We detected an increase in Tin-positive cells in the dorsal mesoderm of \( \text{twi-GAL4;UAS-\text{mid}} \) 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 \( \text{mid} \) in the mesoderm. We saw similar effects using \( \text{pnr-GAL4} \) (data not shown), which drives GAL4 in dorsal mesoderm and dorsal ectoderm, indicating that ectopic \( \text{mid} \) expression in dorsal mesoderm is sufficient to cause the observed effects.

The dramatic increase of Tin-positive cells caused by ectopic \( \text{mid} \) expression in the dorsal mesoderm led us to examine the effects of ectopic \( \text{mid} \) 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 \( \text{twi-GAL4;UAS-\text{mid}} \) embryos. In many embryos, we
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 twist–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-
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 cardioblast per hemisegement 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 (tm) 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 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.

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