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The Hox gene *abdominal-A* specifies heart cell fate in the *Drosophila* dorsal vessel

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SUMMARY

The *Drosophila melanogaster* dorsal vessel is a linear organ that pumps blood through the body. Blood enters the dorsal vessel in a posterior chamber termed the heart, and is pumped in an anterior direction through a region of the dorsal vessel termed the aorta. Although the genes that specify dorsal vessel cell fate are well understood, there is still much to be learned concerning how cell fate in this linear tube is determined in an anteroposterior manner, either in *Drosophila* or in any other animal. We demonstrate that the formation of a morphologically and molecularly distinct heart depends crucially upon the homeotic segmentation gene *abdominal-A* (*abd-A*). *abd-A* expression in the dorsal vessel was detected only in the heart, and overexpression of *abd-A* induced heart fate in the aorta in a cell-autonomous manner. Mutation of *abd-A* resulted in a loss of heart-specific markers. We also demonstrate that *abd-A* and *sevenup* co-expression in cardial cells defined the location of ostia, or inflow tracts. Other genes of the *Bithorax Complex* do not appear to participate in heart specification, although high level expression of *Ultrabithorax* is capable of inducing a partial heart fate in the aorta. These findings for the first time demonstrate a specific involvement for Hox genes in patterning the muscular circulatory system, and suggest a mechanism of broad relevance for animal heart patterning.

Key words: Heart, Hox genes, *abdominal-A*, Bithorax Complex, *Drosophila*, Aorta, Dorsal vessel, Ostia, Ostium

INTRODUCTION

Many animals use a muscular heart to pump blood around the body. Despite the varied morphology of the heart in different animal systems, there are strong similarities in the early embryological events that occur as the heart is formed (reviewed by Bodmer, 1995). In *Drosophila*, the dorsal vessel forms as a linear tube from two rows of cells that converge at the dorsal midline. In vertebrates, a linear heart tube is initially formed at the ventral midline from two rows of converging cells. Although the *Drosophila* dorsal vessel functions throughout the life cycle as a linear tube, in many cases the vertebrate heart subsequently undergoes morphogenetic movements to realize its final form.

Consistent with the embryological similarities, the molecular mechanisms and transcriptional regulatory pathways that govern heart cell specification and development are also strongly conserved between different animal species (reviewed by Cripps and Olson, 2002). Heart cell fate is induced by signaling molecules of the transforming growth factor β family (Frasch, 1995; Schultheiss et al., 1997). These signals serve to activate genes encoding the Tinman/NKX family of homeodomain transcription factors, which are required for specification or morphogenesis of the functional heart (reviewed by Harvey, 1996).

Despite a strong understanding of the signaling and transcriptional events that specify the linear heart tube, there is still much to learn concerning how unique cell types are specified within this organ. Although numerous genes are known to be expressed in the heart tube at specific anteroposterior (AP) locations, the mechanisms governing this process remain largely unknown (Srivastava and Olson, 2000). To elucidate the mechanisms governing AP identity in the heart, we studied diversification in the *Drosophila* dorsal vessel. The dorsal vessel is composed of a linear tube spanning segments thoracic 2 (T2) to abdominal 8 (A8). From T2 to A5 the tube is narrow and is termed the aorta, whereas the posterior portion has a larger bore and is termed the heart. Additionally the heart is perforated by three pairs of valve-like ostia, which serve as inflow tracts for hemolymph (Rizki, 1978; Bodmer and Frasch, 1999).

We demonstrate that the location of the heart correlates precisely with the expression of the Hox segmentation gene *abdominal-A* (*abd-A*), and that heart cell identity and the formation of heart-specific structures depends upon *abd-A* function. These findings for the first time demonstrate a mechanism for AP patterning the developing animal heart tube.

MATERIALS AND METHODS

Drosophila culture

Drosophila were cultured on Carpenter's medium (Carpenter, 1950) at 25°C. The gal4 driver lines *69B-gal4*, *24B-gal4* and *twi-gal4*, and

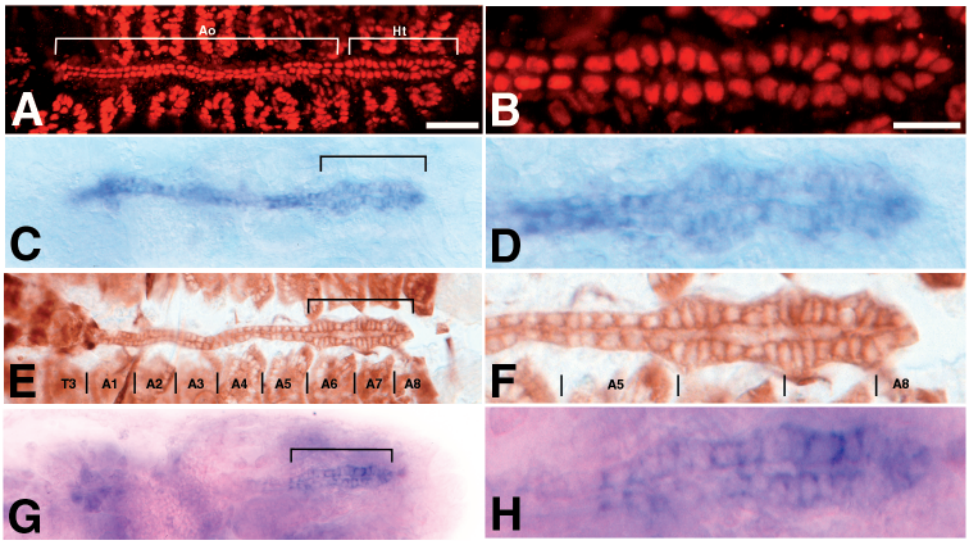

Fig. 1. The *Drosophila* dorsal vessel consists of a posterior heart and an anterior aorta, which can be distinguished on the basis of morphological and molecular criteria. (A,C,E,G) Low-magnification views of stage 16 embryos stained with various markers. (B,D,F,H) High-magnification views of same embryos. (A,B) MEF2 protein accumulation in the muscle cells of the embryo. The dorsal vessel consisted of two parallel rows of cells at the dorsal midline. Note that the distance between the two rows of MEF2-positive nuclei is greater in the heart (Ht) compared to the aorta (Ao). The pattern of *Hand* transcripts (C,D) and muscle MHC protein (E,F) in the cardial cells of the dorsal vessel can also be used to distinguish heart from aorta, and to visualize the heart lumen. Numbered body segments are indicated. (G,H) *Tina-1* transcripts in the dorsal vessel accumulated only in the heart. Scale bars: 50 μm for A,C,E,G; 25 μm for B,D,F,H. All pictures are dorsal views oriented with anterior towards the left. In C,E,G, the location of the heart is bracketed.

the UAS lines *UAS-Ubx* and UAS-*Abd-B* were obtained from the Bloomington Stock Center. *UAS-abd-A* was obtained from Alan Michelson (Harvard University, MA). The *TM3/svp-lacZ* line was obtained from James Skeath (Washington University School of Medicine, MO).The *abd-A* mutant stock *TM1/abd-A*^{MX1} and the *Ubx* mutant stock *TM1/Ubx*$^{9.22}$ *Sb*^{sbd} were from Bloomington and were used for mutant analysis. Homozygous mutants were identified based either upon reduced levels of UBX or ABD-A protein in double-labeling experiments, or upon the abnormal gut phenotypes characteristic of these mutants (Skaer, 1993).

Immunohistochemistry and in situ hybridization

Antibody staining was performed as described (Patel, 1994). Primary antibodies and the concentrations used were: rabbit anti-MEF2, 1:1000 (Lilly et al., 1995); rabbit anti-myosin heavy-chain (MHC), 1:500 (Kiehart and Feghali, 1986); mouse anti-UBX, 1:50 (White and Wilcox, 1984); mouse anti-ABDA, 1:1000 (Macias et al., 1990); mouse anti-ABDB, 1:1000 (Celniker et al., 1989); and rabbit anti-Tin, 1:1000 (Yin et al., 1997). Fluorescent secondary antibodies were either Alexa 488 anti-mouse or Alexa 568 anti-rabbit (Molecular Probes, Seattle, WA) all used at a 1:2000 dilution. Non-fluorescent antibody detection was using the Vectastain Elite kit (Vector Laboratories, Burlingame, CA).

Confocal microscopy was performed using a BioRad MRC-600 confocal laser-scanning microscope using 488/568 nm excitation in the dual-channel mode (T1/T2A filter cubes, BioRad, Hercules, CA).

In situ hybridization was performed as described (O'Neill and Bier, 1994), using digoxigenin-labeled riboprobes (Roche Molecular Biologicals, Indianapolis, IN). To generate a probe for the *Hand* and *Tina-1* genes, primers were designed to amplify sequences from embryonic cDNA, based upon the exon structures for each gene described (Adams et al., 2000). Primer sequences for *Hand* were: 5′-CATGTTCGACATGAAACG-3′ and 5′-AAATATTATTTTTGC-AAAATATG-3′. Primer sequences for *Tina-1* were: 5′-CAAGAA-AATGGGCCAAGC-3′ and 5′-CCACGTTATTAGTTCTGG-3′. PCR products were cloned into the pGEM-T Easy vector (Promega, Madison, WI). Recombinant clones were sequenced for orientation and then sense and antisense riboprobes generated following linearization of the plasmid.

All samples were mounted in 80% (v/v) glycerol for photography. Samples were either prepared as whole mounts, or were filleted along the ventral midline with the viscera removed to allow detailed visualization of the dorsal vessel and the ostia. The size of the *svp*-expressing cells which formed the ostia were measured as follows. Embryos stained for expression of *tin* (in black) and *Myosin heavy-chain* (in brown) were filleted and photographed such that the muscular dorsal vessel and the *tin*-expressing cells were visible at 600× magnification. A 10 μm graduation slide was also photographed at the same magnification. The sizes of each non-Tin expressing cell (those cells of the dorsal vessel lacking black nuclei) were determined on projected images by measuring the distance from the luminal to the apical sides of the cell, and the measurements converted to actual μm values after measuring a projection of the calibration slide. Average cell sizes were then calculated for each cluster of *svp*-expressing cells along the AP axis in *y w* control embryos and in embryos ectopically expressing *abd-A*. Cell sizes from at least five animals were measured, and because there are four Svp cells at each AP location, sample sizes were relatively large ($15 \leq n \leq 24$).

RESULTS

The heart and aorta can be distinguished based upon morphological and molecular criteria

Markers that label the dorsal vessel can be used to illuminate the morphological differences between the heart and aorta. These markers include MEF2, which is detectable in all muscle cell nuclei (Lilly et al., 1995; Bour et al., 1995) (Fig. 1A,B); the basic helix-loop-helix factor *Hand*, which is expressed in cardial cells and some pericardial cells (Moore et al., 2000) (Fig. 1C,D); and muscle myosin heavy chain (MHC), which accumulates in all muscle cells (Kiehart and Feghali, 1986) (Fig. 1E,F). Heart cells have a larger volume compared with aorta cells, and the lumen in the heart is larger than in the aorta. However, no markers exist that distinguish at the molecular level between the heart and aorta.

To identify genes expressed in subpopulations of the dorsal vessel, we analyzed by in situ hybridization the expression of several muscle structural gene isoforms. We identified a novel member of the troponin-C superfamily termed *Tina-1* (for

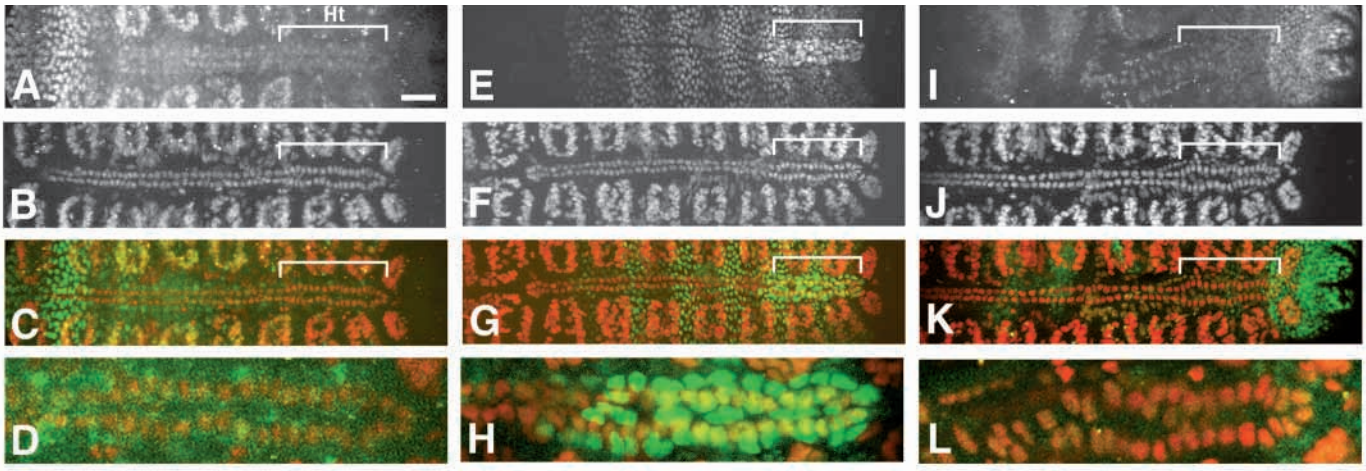

Fig. 2. Expression of *Bithorax-Complex* genes in the dorsal vessel. For each *BX-C* gene product, single-channel views of an embryo stained with an antibody to the *BX-C* product (A,E,I, top row) and the same embryo stained with an antibody to MEF2 (B,F,J, second row) are shown. These images are merged in the third row (C,G,K). The top three rows of micrographs represent combination of several individual *z*-series images. This is necessary to show the arrangement of the entire dorsal vessel. To confirm co-expression in the mesoderm, the lower row shows the combined images for only one or two optical sections (differing by 2 μm). (A) UBX accumulation. (B) MEF2 accumulation in the same embryo. (C) Merged image of A and B (UBX in green, MEF2 in red). (D) Merged image at limited optical sections to show co-expression in the mesoderm. UBX was detected broadly albeit at low levels in the dorsal vessel. (E) ABDA accumulation. (F) MEF2 accumulation in the same embryo. (G) Merged image of E and F (ABDA in green, MEF2 in red). (H) Merged image at limited optical sections to show co-expression in the mesoderm. ABDA was detected in cardial (yellow) and pericardial (green) cells of the heart region only. (I) ABDB accumulation. (J) MEF2 accumulation in the same embryo. (K) Merged image of I and J (ABDA in green, MEF2 in red). (L) Merged image at limited optical sections to show co-expression in the mesoderm. ABDB was detected only in the posterior four nuclei of the dorsal vessel. Scale bars: 50 μm for A-C,E-G,I-K; 8 μm for D,H,L. All micrographs are dorsal views of stage 16 embryos with the anterior towards the left. The location of the heart (Ht) is bracketed in the lower magnification images.

Troponin C–akin-1; formerly *CG2803*) (Adams et al., 2000), whose expression in the dorsal vessel was detected at high levels only in the heart (Fig. 1G,H). *Tina-1* was also expressed in a subset of other cells, including the hindgut visceral mesoderm. The full expression pattern of this gene will be presented elsewhere (T. P. N., T. L. L. and R. M. C, unpublished). The identification of *Tina-1* as a heart-specific marker in the dorsal vessel permitted us to follow changes in heart versus aorta fate at both the morphological and molecular levels.

Expression of *Bithorax-Complex* genes in the dorsal vessel

To identify candidate genes involved in the segmentation of the dorsal vessel, we studied the expression of members of the *Bithorax Complex* (*BX-C*) of homeotic segmentation genes. The functions of these genes in controlling segmentation in the ectoderm and skeletal muscle precursors has been well documented (Lawrence and Morata, 1994; Grieg and Akam, 1993; Michelson, 1994). *BX-C* gene expression in the dorsal vessel was studied by double-staining embryos for the Hox gene product and the mesoderm marker MEF2, and examining the embryos by confocal microscopy (Fig. 2). As the dorsal vessel curves slightly along the dorsal midline of the embryo, it was necessary to combine several *z*-series images to illustrate in a single micrograph the expression pattern of each gene along the entire length of the dorsal vessel (Fig. 2A-C,E-G,I-K). As a result, some ectodermal *BX-C* gene expression is observed in addition to expression in the mesoderm. To demonstrate that the *BX-C* gene products were co-expressed with MEF2 in the muscular cardial cells of the dorsal vessel, high power images are also shown that represent only one or two optical sections combined, differing by 2μm (Fig. 2D,H,L).

All three *BX-C* gene products were detected in the dorsal vessel, albeit in strikingly different patterns. UBX was detected at low levels in dorsal vessel cells from A2 to the posterior tip of the heart, although expression was slightly lower in A5 to A8 (Fig. 2A-D). By contrast, ABDA protein was detected in cardial and pericardial cells in the heart region from A5 to A8 (Fig. 2E-H), and closer examination indicated that *abd-A* expression corresponded exactly to the cells forming the heart. ABDB was detected in the dorsal vessel in the most posterior four nuclei in A8, in which ABDA accumulation was reduced (Fig. 2I-L). Therefore ABDA seemed most likely to play a role in heart cell specification, and was chosen for further study. The expression of other *BX-C* genes in the dorsal vessel suggests that further structural and functional diversity also exists in this organ.

Together, *abd-A* and *Tina-1* represent the first two genes known whose expression patterns differentiate between the heart and the aorta cells of the dorsal vessel.

abd-A function controls dorsal vessel cell identity

To determine if *abd-A* functions as a selector gene in the *Drosophila* dorsal vessel to distinguish between heart and aorta cells we used the GAL4-UAS system (Brand and Perrimon, 1993) to express *abd-A* ectopically in different germ layers, and monitored the formation of the dorsal vessel by studying expression of *Mef2*, *Hand*, *Mhc* and the heart-specific marker *Tina-1* (Fig. 3). Initially, we induced *abd-A* using the ectodermal driver *69B-gal4* (Brand and Perrimon, 1993), to

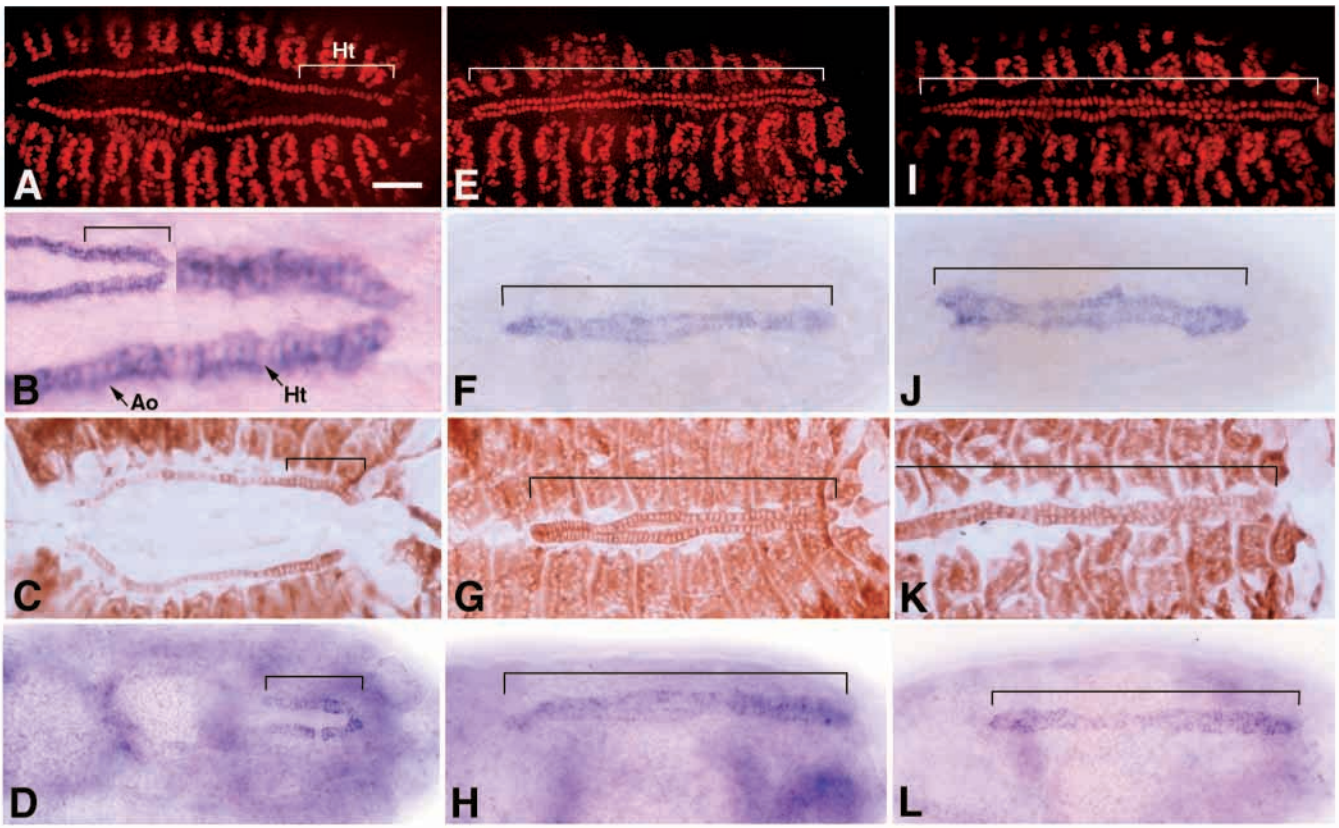

Fig. 3. Ectopic expression of *abd-A* in the mesoderm resulted in an aorta-to-heart transformation. (A-D) Ectopic expression of *abd-A* in the ectoderm did not affect heart cell identity. (A) MEF2 staining revealed that the dorsal vessel did not form normally; however, expression of *Hand* in the dorsal vessel was detectable (B, inset). (B) Closer examination of *Hand*-expression cells revealed that those at the posterior (Ht, the presumptive heart region) were larger than those more anterior (Ao, the presumptive aorta region), suggesting that heart cell identity had been retained. This was confirmed by staining for MHC (C), which showed stronger staining in the presumptive heart region. Heart identity at the molecular level was also retained, as determined by expression of *Tina-1* (D), which was restricted to the posterior of the dorsal vessel. Ectopic expression of *abd-A* in the mesoderm using the *24B-gal4* driver (E-H) or the *twi-gal4* driver (I-L) transformed the aorta into a heart. (E,I) MEF2 staining revealed a gap between the two rows of cardial cells throughout the length of the dorsal vessel suggesting the formation of a larger lumen than wild type. This was confirmed by examination of *Hand* expression in embryos (F,J), and by accumulation of muscle MHC (G,K). (H,L) *Tina-1*, the marker of heart cells in wild-type embryos showed an expansion of its expression domain throughout the dorsal vessel. Scale bars: 50 μm for all panels except B (15 μm). All micrographs are dorsal views of stage 16 embryos with anterior towards the left. The location of the heart is bracketed.

determine if expression of *abd-A* in an adjacent germ layer might affect dorsal vessel identity. These animals did not undergo dorsal closure to form a linear heart tube (Fig. 3A-D); however, we were still able to distinguish between heart and aorta cells. This was apparent when studying *Hand* expression, where the posterior cells of the dorsal vessel had a larger volume than the more rounded cells of the aorta (Fig. 3B), a characteristic of heart cells. In addition, MHC accumulated at higher levels in the presumptive heart region (Fig. 3C). Furthermore, *Tina-1* expression was restricted to the presumptive heart cells and was not detectable in the aorta (Fig. 3D). Clearly no alteration in dorsal vessel cell identity occurred upon ectopic ectodermal expression of *abd-A*.

By contrast, expression of *abd-A* in the mesoderm alone using either the *24B-gal4* driver or a *twist-gal4* driver (Brand and Perrimon, 1993; Baylies and Bate, 1996) resulted in a strong a transformation into heart cell fate for all dorsal vessel cells (Fig. 3E-L). There was a greater distance between MEF2-postive cells in the dorsal vessel, suggesting a large lumen running the length of the dorsal vessel (Fig. 3E,I). In addition, visualizing *Hand* expression and MHC accumulation indicated that most of the dorsal vessel cells assumed a larger volume characteristic of cells of the heart (Fig. 3F,G,J,K). Most striking was the appearance of *Tina-1* transcripts throughout the dorsal vessel, indicating that all the dorsal vessel cells had assumed a heart fate (Fig. 3H,L).

These results strongly suggested that ABDA plays an important instructive role in the dorsal vessel, directing cells to take on a heart fate. To confirm this, we studied heart formation in mutants lacking *abd-A* function, as this would be predicted to result in an heart-to-aorta transformation in the posterior region of the dorsal vessel. Many homozygous combinations of *abd-A* mutants do not complete development sufficiently to answer all of these questions; however, in the absence of *abd-A* function we saw phenotypes consistent with a loss of heart cell identity (Fig. 4). Despite the lack of dorsal closure, *Hand* expression persisted in the presumptive dorsal vessel cells; however, we

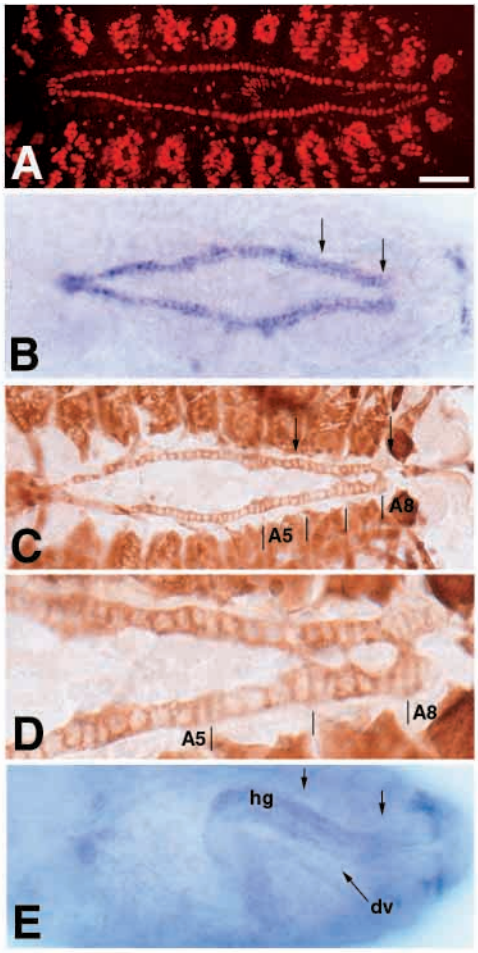

Fig. 4. *abd-A*^{MX1} homozygotes show no heart formation. Homozygous *abd-A* mutants were identified based upon a lack of reactivity to anti-ABDA antibody, or based upon abnormal morphology of the gut, and then stained for expression of the indicated genes. Small arrows bracket the cells that, in wild-type, would form the heart. (A) Anti-MEF2 staining demonstrated a lack of dorsal closure in the mutants. (B) *Hand* was expressed in the two rows of dorsal vessel cells; however, most cells had a uniform size and shape (compare with Fig. 3B, where the heart cells assumed an unique shape). (C,D) MHC accumulation in the dorsal vessel cells; once again, cell shape and levels of MHC accumulation along the length of the dorsal vessel were uniform. (E) *Tina-1* was not expressed in the dorsal vessel (one row of dorsal vessel cells in the presumptive heart forming region is indicated by dv), although expression in the hindgut (hg) was unaffected. Scale bars: 50 μm for A-C,E; 25 μm for D. All micrographs are dorsal views of stage 16 (or equivalent) embryos with anterior towards the left.

never saw any size dimorphism in these cells as was observed upon ectodermal expression of *abd-A* (in which the mutant individuals also failed to complete dorsal closure; Fig. 3B). Furthermore, there was no enrichment of MHC in the posterior group of dorsal vessel cells (Fig. 4C,D). *Tina-1* expression in the dorsal vessel was undetectable in the absence of *abd-A* function (Fig. 4E).

Taken together, the gain- and loss-of function experiments described here identify the homeotic selector gene *abd-A* as specifying heart cell identity in the *Drosophila* dorsal vessel.

Formation of ostia in ectopically generated heart

A unique characteristic of the *Drosophila* heart is the presence of inflow tracts termed ostia. There are three pairs of ostia located at the segmental boundaries of A5/A6, A6/A7 and A7/A8, and each ostium is visible in larvae as a broadening of the width of the heart, at the peak of which are small openings (Rizki, 1978; Bodmer and Frasch, 1999; Molina and Cripps, 2001). No ostia form in the aorta during embryonic or larval development. Recently, we demonstrated that the ostia which form at each segmental boundary develop from two pairs of cells expressing the orphan nuclear receptor gene *sevenup* (*svp*). The remaining four pairs of cardial cells in each segment express the homeobox-containing gene *tinman* (*tin*) and form the heart wall (Molina and Cripps, 2001).

Close examination of MHC-stained wild-type hearts from embryos indicated that the wall of the heart curved sharply outwards close to the segment borders (bracketed regions in Fig. 5A), whereas no such broadening occurred in the aorta (Fig. 5B). At these locations, two cardial cells were morphologically distinct in that they had oval-shaped nuclei, rather than the round nuclei of the remaining cells. Given the locations of these morphologically distinct cells close to the segmental boundary and the similarity of this structure to the organization of the larval heart, we reasoned that the sharp curves in the outer heart wall corresponded to the locations of the ostia. In support of this, we occasionally saw indentations at the tip of these cell pairs, suggesting that the heart wall was perforated at these locations. To confirm our identification of these cells as ostia, we double-stained wild-type embryos with an antibody to Tin (to identify the heart wall cell nuclei) and with an antibody to muscle MHC (to visualize the shape of the heart). The sharp curves in the heart wall corresponded to the locations of ostia, as they were formed by the non-Tin expressing population of cardial cells (Fig. 5C). In the aorta of wild-type embryos, *svp*-expressing cells were still detected; however, the wall of the aorta was uniform (Fig. 5D).

As ectopic mesodermal expression of *abd-A* resulted in ectopic heart formation, we studied these ectopic heart structures for the presence of cells forming ostia. In many cases, sharp curves in the wall of the heart tube in locations more anterior to those found in wild type indicated the presence of ectopic ostia, formed by cells more elongated than their neighbors (asterisks in Fig. 5E). Furthermore, by staining these embryos with anti-Tin and anti-MHC, as we did for wild type, we found that these elongated cells precisely corresponded to those expressing *svp* (Fig. 5F). Although it is difficult to visualize the openings of the ostia, the most likely conclusion from these observations is that ectopic ostia were formed in the presence of ectopic ABD-A. Furthermore, these ostia were positioned appropriately within the segment, only at the coincidence of *abd-A* expression and *svp* expression.

To quantify more precisely the alteration in Svp cell morphology upon the induction of ectopic heart structures, we determined the size of each *svp*-expressing cell by measuring the distance from the luminal surface of the Svp cells to the outer wall of the dorsal vessel. In wild-type embryos there are seven segmentally repeating groups of Svp cardial cells in the dorsal vessel, four cells in each group. To distinguish between groups located at unique positions along the AP axis, we refer the groups as S1 to S7, from anterior to posterior in the embryo.

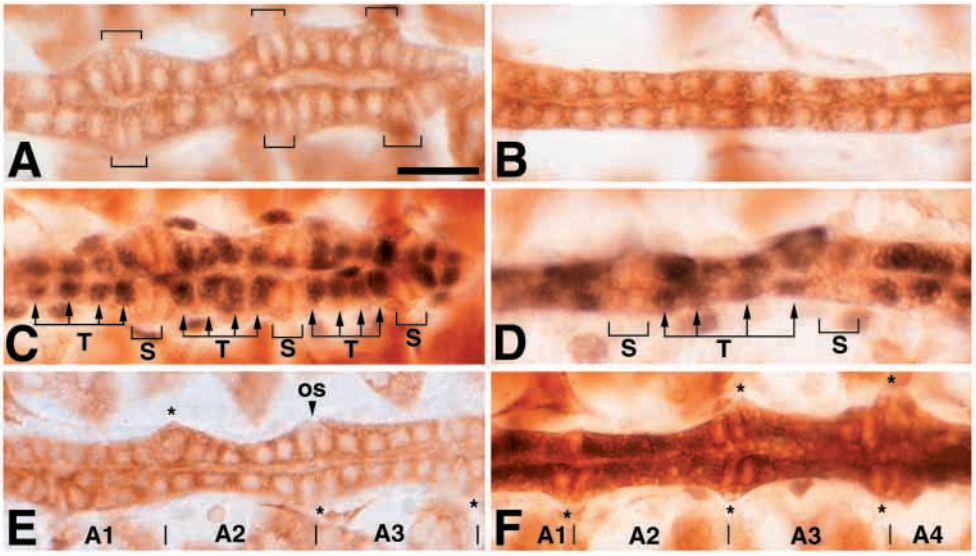

Fig. 5. Ectopic ostium formation occurs upon induction of ectopic heart. (A,B,E) Anti-MHC staining alone. (C,D,F) Anti-Tin staining in black, anti-MHC staining in brown. (A) In wild type, anti-MHC staining revealed the fine structure of the embryonic heart. At each segmental boundary, the heart was broader, and this structure was coincident with two pairs of cells with a unique morphology (bracketed). (B) In the aorta, dorsal vessel shape was uniform. (C) In wild type, the cells forming the broader region of the heart did not express *tin* and were therefore *svp*-expressing cells (S, bracketed), which formed the ostia. Expression of *tin* is indicated by T (arrows). (D) Although *svp* and *tin* expression were mutually exclusive in the aorta, *svp*-expressing cells were not distinguishable based upon the morphology of the vessel. (E) In *twi-gal4/+; UAS-abd-A/+* embryos, variations in dorsal vessel diameter similar to that found in the heart were seen in anterior locations (indicated by asterisks). Occasionally, tiny perforations in the heart wall were observed (os, arrowhead), consistent with the interpretation that ectopic ostia were being formed. (F) These ectopic ostia form from the *svp*-expressing population of cells, as the widening of the dorsal vessel occurred at locations where Tin was absent (asterisks). Segment boundaries are indicated in E and F to demonstrate that the broadening of the heart occurred at ectopic locations. All panels are dorsal views of stage 16 embryos oriented with anterior towards the left. Scale bar: 20 μm.

Thus, the Svp cells of clusters S1 to S4 do not form ostia in wild type, whereas S5 to S7 form the ostia of the heart.

In control embryos, clusters S1 to S4 contained cells measuring approximately 5 μm, whereas the Svp cells of the heart were significantly larger (7-8 μm; Fig. 6). Upon overexpression of *abd-A* in the mesoderm there was a large increase in the sizes of cells in groups S1 to S4, many of which were indistinguishable from those in the wild-type heart (Fig. 6). These results clearly show the effects of *abd-A* expression upon aorta cell fate, transforming Svp cells of the aorta into ostia.

Ubx function is not required for heart formation

The data above suggest strongly that autonomous action of *abd-A* in the dorsal vessel promotes heart cell fate at the expense of aorta cell fate. A formal alternative possibility is that a reduction in UBX levels could specify heart fate, as we have also shown that UBX levels are slightly lower in the heart versus the aorta (Fig. 2). Indeed, ectopic expression of *abd-A* in the mesoderm reduced UBX accumulation (Fig. 7A).

To determine if a loss of *Ubx* function could induce heart fate in the aorta, we studied dorsal vessel formation in $Ubx^{9.22}$ homozygotes (Fig. 7B-D). These mutant individuals frequently showed a range of defects in dorsal vessel development, including incomplete dorsal migration of the cardiac precursors, and more minor morphological defects in the dorsal vessel. However, staining for MHC accumulation (Fig. 7B) demonstrated that a clear distinction could still be detected between the heart and aorta in these individuals; the heart showed a larger lumen and cell size compared with more anterior cells in the dorsal vessel, and cells forming ostia could also be distinguished in the heart region only (Fig. 7B, arrow). Consistent with a failure of fate change, *Tina-1* expression was also confined to the heart in *Ubx* mutants (Fig. 7C). Interestingly, there was a slight increase in *abd-A* expression in cells of the aorta in *Ubx* mutants (Fig. 7D), however this was clearly insufficient to alter cell fate.

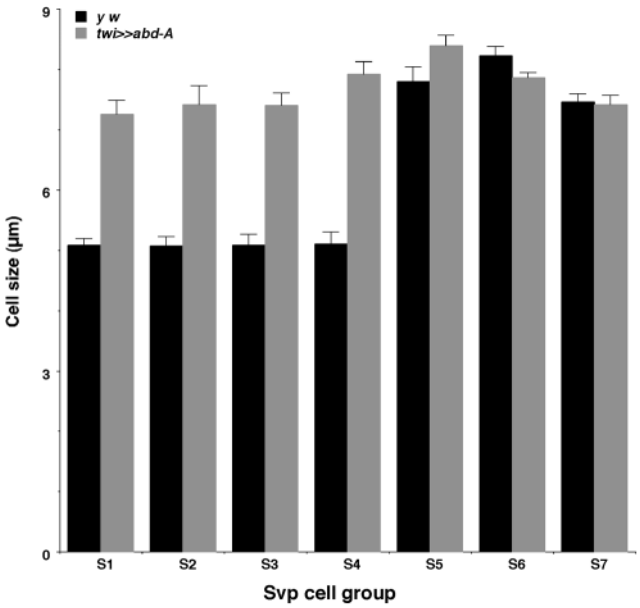

Fig. 6. *abd-A* expression induces an increase in Svp cell size in the aorta. Shown are the mean sizes (in μm) of the *svp*-expressing dorsal vessel cells of control *y w* embryos, and embryos in which *abd-A* is expressed throughout the mesoderm. S1 to S7 represent the segmentally repeating groups of Svp cells, from anterior to posterior. Error bars represent standard errors of the mean. Note that in *y w* the Svp cells that form the ostia (S5-S7) are larger than those in the aorta (S1-S4). Upon induction of ectopic heart, all Svp cells assume a larger average size.

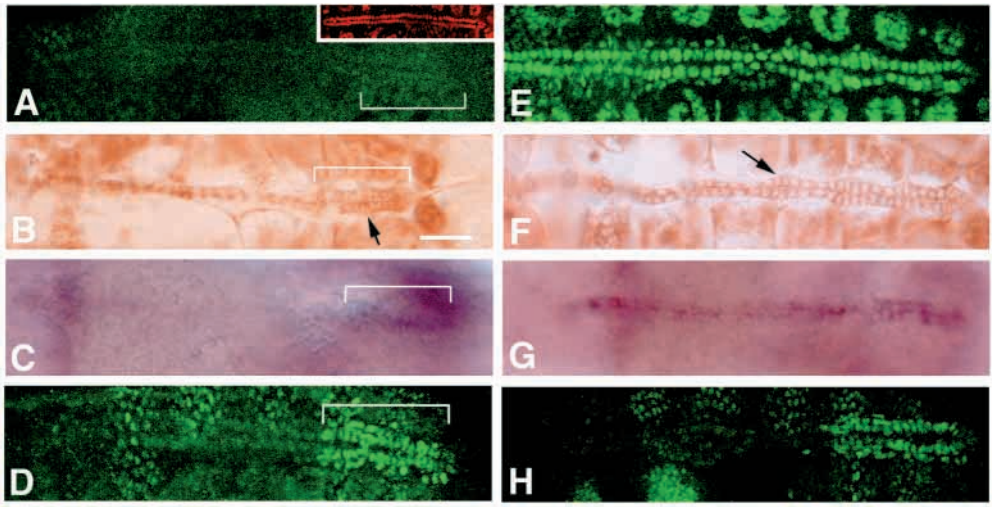

Fig. 7. *Ubx* is not required for heart specification, but can partially induce ectopic heart formation. (A) UBX levels (green) are reduced by ectopic expression of *abd-A*. Inset shows MEF2 accumulation (red) in the same sample at the same focal plane. (B-D) $Ubx^{9.22}$ homozygotes stained for MHC accumulation (B), *Tina-1* expression (C) and ABDA accumulation (D). Note that there is no effect of loss of *Ubx* function upon the specification of the heart, despite a slight increase in ABDA levels in the aorta. Arrow in B identifies one of the two ostia formed by S6. (E-H) Effects of ectopic expression of *Ubx* upon dorsal vessel cell identity. (E) UBX accumulation is at high levels throughout the mesoderm. (F) MHC accumulation indicates that the heart region is still formed in these embryos, and that additional ostia are occasionally observed in cells which would normally form the aorta (arrow). (G) *Tina-1* expression is now detected throughout the dorsal vessel. (H) *abd-A* expression is normal in these embryos. The normal location of the heart is bracketed in A-D. All panels are dorsal views of stage 16 embryos oriented with anterior towards the left. Scale bar: 50 μm.

We also studied the effects of high-level mesodermal expression of *Ubx* upon the formation of the heart, to determine if this gain-of-function assay might inhibit heart cell specification. Upon induction of *Ubx* expression by the mesodermal driver *24B-gal4*, UBX protein was detectable at high levels in the mesoderm (Fig. 7E). There was no inhibition of heart cell specification in segments A5-A8, as visualized by MHC accumulation, as might be predicted if UBX were an inhibitor of heart fate. However, we frequently observed an increase in cell size at the A4/A5 boundary, consistent with the formation of an ectopic ostium within the aorta (Fig. 7F, arrow). Furthermore, *Tina-1* expression in the dorsal vessel was also broadened upon ectopic expression of *Ubx* (Fig. 7G). These effects upon dorsal vessel morphology and fate were not due to alterations in the levels or expression of *abd-A*, which was still restricted to the heart (Fig. 7H). Taken together, these findings indicate that high levels of *Ubx* expression are capable of inducing some aspects of heart cell fate in more anterior locations of the dorsal vessel. Owing to a lack of markers for the aorta cell fate, we cannot determine if *Ubx* loss of function affects any aspects of aorta fate.

Ectopic expression of *Abd-B* inhibits cardiac and skeletal myogenesis

Given the restricted expression pattern of *Abd-B* in the dorsal vessel, we also studied the effects of ectopic mesodermal expression of this gene upon cardiac development. However, we were unable to define the role that this gene might play using these experiments, as forced expression of *Abd-B* caused severe defects in muscle development. In addition to massive derangements of the skeletal muscle pattern, no dorsal vessel cells were detected based upon MHC accumulation (Fig. 8A). By visualizing cardiac precursor cells using an anti-Tin antibody, we found that these defects in dorsal vessel formation

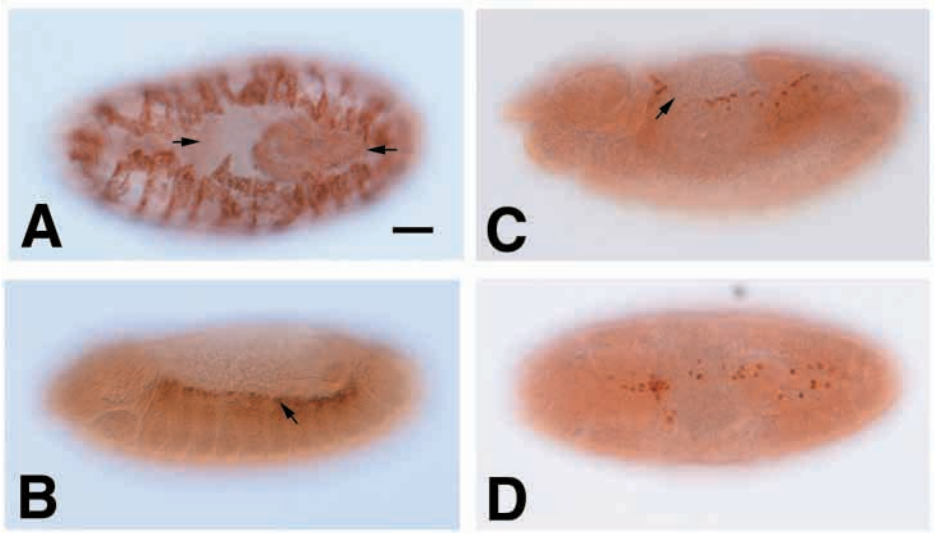

Fig. 8. Mesodermal expression of *Abd-B* inhibits muscle formation. (A) MHC accumulation demonstrates aberrations in the formation of skeletal muscles and a complete absence of MHC-expressing cells at the dorsal midline (between arrows). (B-D) Tin accumulation indicates that cardiac defects arise as a result of a loss of *tin*-expressing cardiac precursors. At stage 13 (B), the *tin*-expressing population is normal (arrow). By stage 14 (C), many Tin cells are absent. By stage 16 (D), very few Tin-positive cells are detectable in the embryo. (A,D) Dorsal views with anterior towards the left; (B,C) Sagittal views with anterior towards the left. Scale bar: 50 μm.

resulted from a loss of *tin*-expressing cells commencing around stage 13, such that by stage 16 very few Tin-positive cells were present (Fig. 8B-D). These results clearly indicated that inappropriate expression of *Abd-B* will inhibit cardiac and skeletal myogenesis. However, as ABDB is detectable in the mature heart tube at stage 16 (Fig. 2), the presence of ABDB cannot always be inhibitory to dorsal vessel formation. Defining a more clear role for *Abd-B* in the dorsal vessel must await the use of GAL4 lines with more restricted temporal or spatial patterns of expression.

DISCUSSION

Despite a detailed knowledge of the structure and function of the circulatory system in *Drosophila*, and recent descriptions of unique classes of cardial cells in the dorsal vessel (Bodmer and Frasch, 1999; Gajewski et al., 2000; Lo and Frasch, 2001), relatively little work has attempted to address the correlation between physiologically unique structures in the dorsal vessel and molecular markers for specific cell types. We have demonstrated that the morphologically distinct heart cells of *Drosophila* are distinguishable from the aorta in the expression of two genes: *Tina-1* and *abd-A*. Furthermore, we have shown that one of these genes, *abd-A*, functions autonomously in the mesoderm to specify heart cell fate. These findings are consistent with the roles of members of the *BX-C* as key regulators of cell fate along the AP axis (Lawrence and Morata, 1994).

Does the mechanism of AP heart patterning that we have uncovered in *Drosophila* apply to higher animals? The vertebrate heart initially forms as a linear tube in much the same manner as the *Drosophila* heart, and numerous genes are known to be expressed in unique domains along the AP axis in the developing vertebrate heart (Srivastava and Olson, 2000). However, there is much to learn concerning the factors that determine this AP pattern. Treatment of chick and zebrafish embryos with retinoic acid results in a loss of anterior heart structures and a broadening of the domain forming more posterior structures (Stainier and Fishman, 1992; Yutzey et al., 1994; Yutzey et al., 1995), suggesting that retinoic acid can influence the AP patterning of the heart. Retinoic acid also directly activates a number of Hox genes in the trunk of the embryo (reviewed by Krumlauf, 1994). Taking these findings together, it is tempting to speculate that Hox segmentation genes in vertebrates also function to control cell identity in the heart. In support of this are recent demonstrations of Hox gene expression in the developing heart (Searcy and Yutzey, 1998; Shin et al., 1998) and the finding that treatment of cardiogenic explants with retinoic acid can alter the expression of Hox genes (Searcy and Yutzey, 1998).

Our results also indicate that two distinct patterns of gene expression converge to control the differentiation of the *Drosophila* dorsal vessel. Superimposed upon the expression of *abd-A* in the heart segments, is the pattern of *tin*-expressing *versus svp*-expressing cells observed in cardial cells in every segment. Formation of the ostia in the heart occurs only at the intersection of *abd-A* expression and *svp* expression, and ectopic ostia form in the presence of ectopic ABD-A, but only in *svp*-expressing populations of cells.

Whether *svp* function is required for ostium formation in *Drosophila* remains to be determined. A vertebrate homolog of the Svp protein is chick ovalbumin upstream promoter transcription factor II (COUP-TF II) (Tsai and Tsai, 1997), which in mice is expressed in and is required for the formation of the atria and sinus venosus (Pereira et al., 1999). The atria and sinus venosus carry out functions in the mouse analogous to the ostia in *Drosophila*, acting as the inflow tracts for blood to enter the heart. It will be interesting to determine whether the homologous expression patterns of *svp* and *COUP-TF II* reflect an homologous function in development.

It is interesting that *Ubx* and *Abd-B* are also expressed in unique cells in the dorsal vessel. Although our loss-of-function experiments have not demonstrated a role for *Ubx* in the formation of the heart, it is still possible that *Ubx* plays a role in the specification of more anterior structures in the dorsal vessel. There are a number of cardial cells in an anterior location that do not express *Ubx*, suggesting an as-yet undetermined function for *Ubx* in the dorsal vessel. Along these lines, it is interesting to note that the domain of *Ubx* expression in the aorta roughly corresponds to the region of the dorsal vessel remodeled during pupal development to form the adult heart (Molina and Cripps, 2001). Furthermore, *Ubx* is required to repress lymph gland fate in the pericardial cells adjacent to the dorsal vessel (Mastick et al., 1995; Rodriguez et al., 1996), suggesting a broad requirement for members of the *BX-C* in patterning the dorsal vessel and its associated cells.

We have also shown that expression of *Ubx* throughout the mesoderm is capable of inducing a partial heart fate upon the cells of the aorta. This was apparent with the observation of single pairs of ectopic ostia, as well as an expansion of *Tina-1* expression throughout the dorsal vessel. Given the intensity of UBX accumulation in these embryos (compare Fig. 7E with Fig. 2A), it is likely that this function of UBX occurs at higher levels of expression than are normally found in the embryonic dorsal vessel. Furthermore, even at these high levels, the transformation to heart fate is only partial, compared with the apparently complete transformation effected by *abd-A* expression.

The similarity in function between UBX and ABDA illuminated in our gain-of-function experiments is not unusual given previous studies of these genes. Either gene can promote haltere formation (Casares et al., 1996), and both *Ubx* and *abd-A* expression have similar effects upon the patterning of somatic muscle precursors (Michelson, 1994). There are also examples where *Ubx* and *abd-A* influence different target genes (e.g. Brodu et al., 2002). It is therefore likely that the induction of a fully-functional heart in the dorsal vessel results from the activation of targets both specific to *abd-A* and common to *abd-A* and *Ubx*. A major challenge in the future will be to identify the various targets of both the HOX proteins and cardiac-restricted transcription factors which realize heart fate.

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REFERENCES

Adams, M. D., Celniker, S. E., Holt, R. A., Evans, C. A., Gocayne, J. D., Amanatides, P. G., Scherer, S. E., Li, P. W., Hoskins, R. A., Galle. R. F. et al. (2000). The genome sequence of *Drosophila melanogaster*. *Science* **287**, 2185-2195.

Baylies, M. K. and Bate, M. (1996). *twist*: a myogenic switch in Drosophila. *Science* **272**, 1481-1484.

Bodmer, R. (1995). Heart development in Drosophila and its relationship to vertebrate systems. *Trends Cardiovasc. Med.* **5**, 21-27.

Bodmer, R. and Frasch, M. (1999). Genetic determination of Drosophila heart development. In *Heart Development* (ed. R. P. Harvey and N. Rosenthal), pp. 65-90. San Diego, CA: Academic Press.

Bour, B. A., O'Brien, M. A., Lockwood, W. L., Goldstein, E. S., Bodmer, R., Taghert, P. H., Abmayr, S. M. and Nguyen, H. T. (1995). Drosophila MEF2, a transcription factor that is essential for myogenesis. *Genes Dev.* **9**, 730-741.

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

Brodu, V., Elstob, P. R. and Gould, A. P. (2002). *Abdominal A* specifies one cell type in Drosophila by regulating one principal target gene. *Development* **129**, 2957-2963.

Carpenter, J. M. (1950). A new semisynthetic food medium for Drosophila. *Dros. Inform. Serv*. **24**, 96-97.

Casares, F., Calleja, M. and Sanchez-Herrero, E. (1996). Functional similarity in appendage specification by the *Ultrabithorax* and *abdominal-A* Drosophila HOX genes. *EMBO J.* **15**, 3934-3942.

Celniker, S. E., Keelan, D. J. and Lewis, E. B. (1989). The molecular genetics of the *Bithorax Complex* of Drosophila: characterization of the products of the *Abdominal-B* domain. *Genes Dev.* **3**, 1424-1436.

Cripps, R. M. and Olson, E. N. (2002). Control of cardiac development by an evolutionarily conserved transcriptional network. *Dev. Biol.* **246**, 14-28.

Frasch, M. (1995). Induction of visceral and cardiac mesoderm by ectodermal Dpp in the early Drosophila embryo. *Nature* **374**, 464-467.

Gajewski, K., Choi, C. Y., Kim, Y. and Schulz, R. A. (2000). Genetically distinct cardial cells within the Drosophila heart. *Genesis* **28**, 36-43.

Grieg, S. and Akam, M. (1993). Homeotic genes autonomously specify one aspect of pattern in the Drosophila mesoderm. *Nature* **362**, 630-632.

Harvey, R. P. (1996). NK-2 homeobox genes and heart development. *Dev. Biol.* **178**, 203-216.

Kiehart, D. P. and Feghali, R. (1986). Cytoplasmic myosin from *Drosophila melanogaster*. *J. Cell Biol.* **103**, 1517-1525.

Krumlauf, R. (1994). *Hox* genes in vertebrate development. *Cell* **78**, 191-201.

Lawrence, P. A. and Morata, G. (1994). Homeobox genes: their function in Drosophila segmentation and pattern formation. *Cell* **78**, 181-189.

Lilly, B., Zhao, B., Ranganayakulu, G., Paterson, B. M., Schulz, R. A. and Olson, E. N. (1995). Requirement of the MADS domain transcription factor D-MEF2 for muscle formation in Drosophila. *Science* **267**, 688-693.

Lo, P. C. H. and Frasch, M. (2001). A role for the COUP-TF-related gene sevenup in the diversification of cardioblast identities in the dorsal vessel of Drosophila. *Mech. Dev.* **104**, 49-60.

Macias, A., Casanova, J. and Morata, G. (1990). Expression and regulation of the *abd-A* gene of Drosophila. *Development* **110**, 1197-1207.

Mastick, G. S., McKay, R., Oligino, T., Donovan, K. and Lopez, A. J. (1995). Identification of target genes regulated by homeotic proteins in *Drosophila melanogaster* through genetic selection of Ultrabithorax protein-binding sites in yeast. *Genetics* **139,** 349-363.

Michelson, A. M. (1994). Muscle pattern diversification in Drosophila is determined by the autonomous function of homeotic genes in the embryonic mesoderm. *Development* **120**, 755-768.

Molina, M. R. and Cripps, R. M. (2001). Ostia, the inflow tracts of the Drosophila heart, develop from a genetically distinct subset of cardial cells. *Mech. Dev.* **109**, 51-59.

Moore, A. W., Barbel, S., Jan, L. Y. and Jan, Y. N. (2000). A genome-wide survey of basic helix-loop-helix factors in Drosophila. *Proc. Natl. Acad. Sci. USA* **97**, 10436-10441.

O'Neill, J. W. and Bier, E. (1994). Double-label in situ hybridization using biotin and digoxigenin-tagged RNA probes. *Biotechniques* **17**, 870-875.

Patel, N. (1994). Imaging neuronal subsets and other cell types in whole-mount Drosophila embryos and larvae using antibody probes. In *Methods in Cell Biology*, Vol. 44 (ed. E. A. Fyrberg, L. S. B. Golstein), pp. 445-487. Boston, MA: Academic Press.

Pereira, F. A., Qiu, Y., Zhou, G., Tsai, M.-J. and Tsai, S. Y. (1999). The orphan nuclear receptor COUP-TFII is required for angiogenesis and heart development. *Genes Dev.* **13**, 1037-1049.

Rizki, T. M. (1978). The circulatory system and associated cell s and tissues. In *The Genetics and Biology of* Drosophila, Vol. 2b (ed. M. Ashburner and T. R. F. Wright), pp 397-452. New York: Academic Press.

Rodriguez, A., Zhou, Z., Tang, M. L., Meller, S., Chen, J., Bellen, H. and Kimbrell, D. A. (1996). Identification of immune system and response genes, and novel mutations causing melanotic tumor formation in *Drosophila melanogaster*. *Genetics* **143**, 929-940.

Schultheiss, T. M., Burch, J. B. and Lassar, A. B. (1997). A role for bone morphogenetic proteins in the induction of cardiac myogenesis. *Genes Dev.* **11**, 451-462.

Searcy, R. D. and Yutzey, K. E. (1998). Analysis of Hox gene expression during early avian heart development. *Dev. Dyn.* **213**, 82-91.

Shin, C., Chang, H. H., Koh, K. S., Park, H. W. and Kim, M. H. (1998). Differential expression of the HoxA-9 gene during mouse development. *Korean J. Genetics* **20**, 91-101.

Skaer, H. (1993). The alimentary canal. In *The Development of* Drosophila melanogaster, Vol. II (ed. M. Bate and A. Martinez Arias), pp. 941-1012. New York: Cold Spring Harbor Laboratory Press.

Srivastava, D. and Olson, E. N. (2000). A genetic blueprint for cardiac development. *Nature* **407**, 221-226.

Stainier, D. Y. R. and Fishman, M. C. (1992). Patterning the zebrafish heart tube: acquisition of of anteroposterior polarity. *Dev. Biol.* **153**, 91-101.

Tsai, S. Y. and Tsai, M. J. (1997). Chick ovalbumin upstream promoter-transcription factors (COUP-TFs): coming of age. *Endocrinol. Rev.* **18**, 229-240.

White, R. A. H. and Wilcox, M. (1984). Protein products of the *Bithorax Complex* in Drosophila. *Cell* **39**, 163-171.

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

Yutzey, K. E., Rhee, J. T. and Bader, D. (1994). Expression of the atrial-specific myosin heavy-chain AMHC1 and the establishment of anteroposterior polarity in the developing chick heart. *Development* **120**, 871-883.

Yutzey, K. E., Gannon, M. and Bader, D. (1995). Diversification of cardiomyogenic cell lineages *in vitro*. *Dev. Biol.* **170**, 531-541.