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Defective Decapentaplegic Signaling Results in Heart Overgrowth and Reduced Cardiac Output in Drosophila

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ABSTRACT

During germ-band extension, Decapentaplegic (Dpp) signals from the dorsal ectoderm to maintain Tinman (Tin) expression in the underlying mesoderm. This signal specifies the cardiac field, and homologous genes (BMP2/4 and Nkx2.5) perform this function in mammals. We showed previously that a second Dpp signal from the dorsal ectoderm restricts the number of pericardial cells expressing the transcription factor Zfh1. Here we report that, via Zfh1, the second Dpp signal restricts the number of Odd-skipped-expressing and the number of Tin-expressing pericardial cells. Dpp also represses Tin expression independently of Zfh1, implicating a feed-forward mechanism in the regulation of Tin pericardial cell number. In the adjacent dorsal muscles, Dpp has the opposite effect. Dpp maintains Krüppel and Even-skipped expression required for muscle development. Our data show that Dpp refines the cardiac field by limiting the number of pericardial cells. This maintains the boundary between pericardial and dorsal muscle cells and defines the size of the heart. In the absence of the second Dpp signal, pericardial cells overgrow and this significantly reduces larval cardiac output. Our study suggests the existence of a second round of BMP signaling in mammalian heart development and that perhaps defects in this signal play a role in congenital heart defects.

BONE *m*orphogenetic *p*roteins (BMPs), a subfamily of the transforming growth factor-β (TGF-β) family of secreted cytokines, are critical for the induction of cardiac mesoderm in both flies and mammals (Cripps and Olson 2002). It has been proposed that BMP ligands signal to the cardiac field multiple times to regulate embryonic heart development in both groups (Zaffran and Frasch 2002). Consistent with this suggestion, we showed that the Drosophila BMP family member Decapentaplegic (Dpp) signals from the dorsal ectoderm to the dorsal mesoderm for a second time (Johnson *et al.* 2003). We reported that the second round of Dpp signaling represses the expression of the transcription factor Zfh1.

To date, three studies of Zfh1 activity in mesoderm development have been reported. The first study showed that Zfh1 positively regulates Even-skipped (Eve) expression in a subset of heart cells. Subsequently, a Zfh1-binding site was identified in the Eve mesodermal enhancer and a mutational analysis showed that the site was active specifically in heart cells (Su *et al.* 1999; Knirr and Frasch 2001). Another study showed that misexpression of Zfh1 throughout the mesoderm disrupts the development of dorsal somatic muscles (Postigo *et al.* 1999).

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The Drosophila embryonic heart is composed of two major cell types: contractile cardial cells that form the heart tube and Zfh1-expressing pericardial cells that surround the cardial cells. Pericardial cells can be further divided into subpopulations on the basis of expression of specific genes, including Eve, Odd-skipped (Odd), and Tinman (Tin) (Su *et al.* 1999; Ward and Skeath 2000; Alvarez *et al.* 2003). Somatic dorsal muscle cells, positioned just ventral to the pericardial cells, express a unique set of genes and represent a third major cell type within the dorsal mesoderm.

A boundary exists between any two adjacent cell types with differing gene expression profiles (Irvine and Rauskolb 2001). Boundaries separate cells with differing fates, and proper boundary formation is a fundamental aspect of many developmental processes. The boundaries separating cardiac, pericardial, and dorsal muscle cells are established via a multistep process that initiates during germ-band extension. First, combinatorial Dpp, Wingless, and Hedgehog signaling from the dorsal ectoderm specifies the positions of heart precursors and dorsal muscle precursor cells (Xu *et al.* 1998; Halfon *et al.* 2000; Klinedinst and Bodmer 2003; Reim and Frasch 2005; Liu *et al.* 2006). Subsequent specification of heart *vs.* dorsal muscle fate requires the activity of Ras downstream of the epidermal growth factor and fibroblast growth factor receptors (Carmena *et al.* 1998a). Once specified, the precursor cells divide and populate the dorsal mesoderm. Notch-regulated asymmetric cell

divisions as well as cross-repressive interactions ensure the appropriate segregation of daughter cells into the cardiac, pericardial, and dorsal muscle domains (Carmena *et al.* 1998b; Ward and Skeath 2000; Jagla *et al.* 2002; Han and Bodmer 2003). Although boundary-forming mechanisms in the dorsal mesoderm have been characterized, little is known about the mechanisms that maintain these boundaries.

Here we report that a second Dpp signal from the dorsal ectoderm to the mesoderm maintains the boundary between pericardial and dorsal muscle cells. Specifically, loss of the second round of Dpp signaling expands the number of Odd-expressing and the number of Tin-expressing pericardial cells while simultaneously reducing the number of dorsal muscle cells expressing Krüppel and Eve. We show that Dpp maintains the dorsal muscle–pericardial cell boundary via two mechanisms: the restriction of cell proliferation and the regulation of gene expression critical for cell fate. Finally, we show that embryonic pericardial cell overgrowth resulting from the loss of this Dpp signal has a detrimental effect on the function of the larval heart. Larvae without this signal have significantly reduced cardiac output in comparison to wild type.

MATERIALS AND METHODS

Drosophila genetics: Fly stocks are as described: *In(2L)dpp^{d6}* and *In(2L)dpp^{d12}* (St. Johnston *et al.* 1990), *Df(2L)dpp^{d14}* (Segal and Gelbart 1985), *zfh1^{2}* (Lai *et al.* 1993), *CycA^{C8}* (Knoblich and Lehner 1993), *lmd^{1}* (Duan *et al.* 2001), 24B.Gal4 (Brand and Perrimon 1993), tinCΔ4.Gal4 (Lo and Frasch 2001), Prc.Gal4 (Chartier *et al.* 2002), LE.Gal4 (Glise and Noselli 1997), UAS.Dpp (Staehling-Hampton and Hoffmann 1994), UAS.CA-Tkv (Haerry *et al.* 1998), UAS.Zfh1.2B (FlyBase at http://flybase.bio.indiana.edu/), and HCH.GFP (Han and Olson 2005). All crosses were conducted at 25°. Standard methods were used to generate recombinant chromosomes when necessary and to identify homozygous mutant embryos (Johnson *et al.* 2003).

Immunohistochemistry and *in situ* hybridization: Immunohistochemistry was performed essentially as described (Johnson *et al.* 2003). The following primary antibodies were utilized: rabbit α-dMef2 (Bour *et al.* 1995), guinea pig α-Kr, (Kosman *et al.* 1998), rabbit α-muscle myosin (Kiehart and Feghali 1986), rabbit α-Odd (Ward and Coulter 2000), mouse α-Prc (Developmental Studies Hybridoma Bank), rabbit α-phosphohistone 3 (Sigma, St. Louis), rabbit α-phospho-Smad1 (Persson *et al.* 1998), rabbit α-Tin for Figures 4 and 5 and Table 1 (Yin and Frasch 1998), rabbit α-Tin for Figure 6 (Venkatesh *et al.* 2000), mouse α-Zfh1b (Lai *et al.* 1991), and rabbit α-lacZ (Organon Teknika, Malvern, PA). Secondary antibodies include biotinylated goat α-rabbit, α-mouse, and α-guinea pig (Vector Laboratories, Burlingame, CA); Alexa Fluor 488- and 633-conjugated goat α-rabbit, α-mouse, and α-guinea pig (Molecular Probes, Eugene, OR); and horseradish peroxidase (HRP)-conjugated goat α-rabbit (Molecular Probes). The Vectastain Elite kit (Vector Laboratories) was employed to detect biotinylated secondary antibodies and the TSA Amplification kit (Molecular Probes) was utilized to detect HRP-conjugated secondary antibodies. A *midline* cDNA (RE27439), inserted in pFLC-1, was obtained from the Drosophila Genomics Resource Center. A riboprobe was generated, after linearization with *Xho*I, using the Stratagene (La Jolla, CA) *in vitro* transcription kit. *In situ* hybridization was performed essentially as described (Lockwood and Bodmer 2002).

Cell counts and statistics: Cell identities were assigned on the basis of staining intensity and position along the dorsal ventral axis. Cell number quantification was performed as described (Johnson *et al.* 2003) except that embryos stage 13 and younger were viewed laterally to distinguish dorsal from lateral mesoderm. Statistical analysis of pericardial cell transcription factor expression utilized unilateral (stage 13) or bilateral (stage 15+) cell counts of the entire dorsal mesoderm with a minimum of five embryos assayed per genotype. For the quantification of dorsal muscle transcription factor expression, we assayed the number of nuclei per segment (minimum of 50 segments per genotype). Unpaired, two-tailed *t*-tests were used to determine whether the difference in the number of expressing cells between the two genotypes was statistically significant.

Larval heartbeat analysis: First instar larvae containing the heart expressing transgene HCH.GFP were collected 24–28 hr after egg lay and mounted on 60-mm petri plates coated with 0.1% poly-l-lysine (Ted Pella). Larval heartbeats were captured at room temperature for 2 min at a rate of 12 frames/sec with a Roper Cool SNAP ES digital camera (Roper Scientific). Image acquisition and processing was performed with MetaMorph 6.0/6.1 software (Universal Imaging). Custom software was constructed using C++ Borland Builder 6 Enterprise edition that provides a user interface for computer-assisted tracking of individual cells. A pair of cardial cells two to three cell diameters from the anterior end of the heart (a region also known as the aorta; Lovato *et al.* 2002; Sellin *et al.* 2006) and a second pair of cardial cells two to three cell diameters from the posterior end of the heart (a region also referred to as the heart proper; Lovato *et al.* 2002; Sellin *et al.* 2006) were tracked for each animal. The heartbeat of first instar larvae is discontinuous and tracking was performed only during active contractions. The position of each tracked cell was recorded and the distance between each pair of cells was calculated to determine diastolic and systolic distances for each heartbeat. Heart rate was also determined. The heart rate and pulse distance reported is an average of every heartbeat tracked in all animals of each genotype.

RESULTS

Dpp signaling specifically restricts the number of pericardial cells: Our previous study suggested that a second round of Dpp dorsal ectoderm-to-mesoderm signaling, stimulated by enhancers located in the *dpp* disk region, initiates during germ-band retraction (stage 12; Johnson *et al.* 2003). We refer to this as the second round of signaling because a distinct set of enhancers located in the *dpp* Haplo-insufficiency (Hin) region activates Dpp dorsal ectoderm-to-mesoderm signaling during germ-band extension (stage 8; Newfeld and Takaesu 2002). Further, our data and that of others (*e.g.*, Kato *et al.* 2004) revealed that *dpp* dorsal ectoderm expression driven by the Hin region enhancers persists long after germ-band retraction. These studies showed that Hin-region-driven *dpp* expression is sufficient for Dpp ectodermal functions such as dorsal closure and dorsal branch migration.

Given these data, it appears that the *dpp^{d6}* inversion (Figure 1A) prevents the augmentation of *dpp* expression

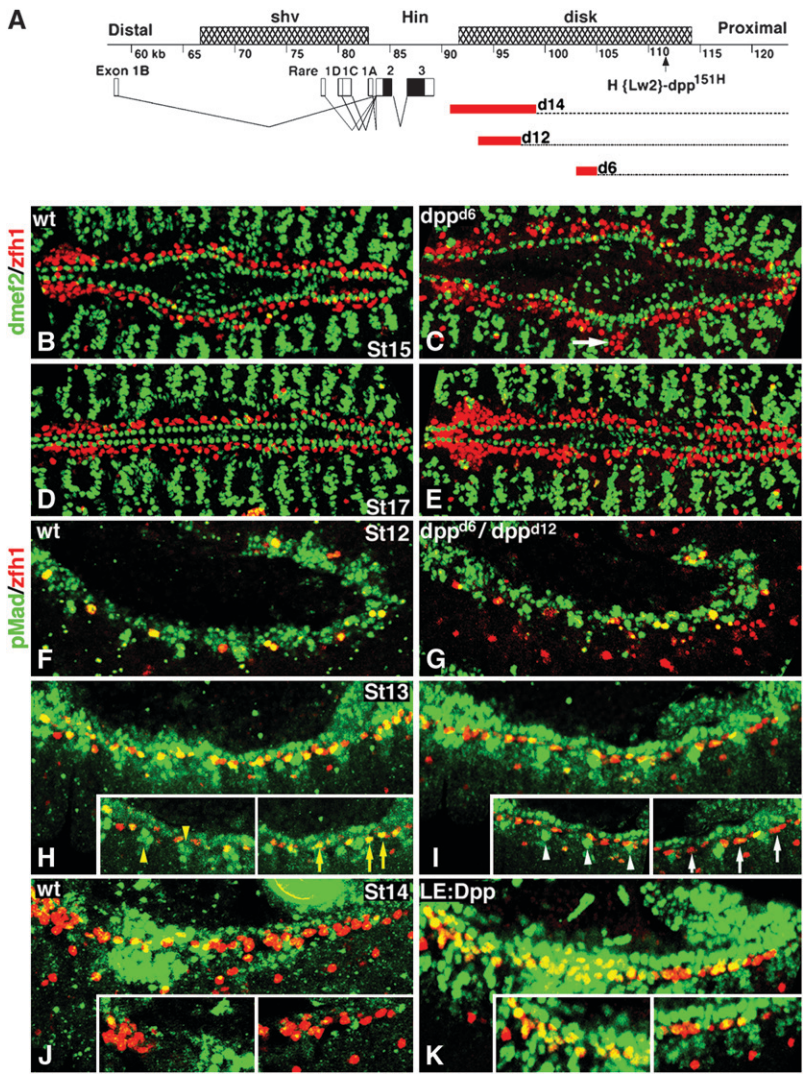

FIGURE 1.—*dpp* mutant embryos display pericardial but not cardiac cell overgrowth and have reduced pMad accumulation in the dorsal mesoderm. (A) Map of the *dpp* locus showing three genetically defined regions: shortvein (shv), Hin, and disk. The structures of *dpp* transcripts are shown with black boxes representing the open reading frame and open boxes corresponding to untranslated regions. *dpp* disk region mutations used in this study are the deficiency dpp^{d14} and the inversions dpp^{d12} and dpp^{d6}. Red boxes represent the degree of uncertainty in the position of the distal breakpoint associated with each aberration. The dpp^{151H} enhancer trap is also shown (JOHNSON *et al.* 2003). (B–E) Embryos double labeled for dMef2 (green) and Zfh1 (red). (B and C) Stage 15. (B) Wild type. (C) dpp^{d6} embryos contain a significantly greater number of Zfh1-expressing pericardial cells (see JOHNSON *et al.* 2003 for statistics) but show no change in the number of dMef2-expressing cells. Note some Zfh1-expressing cells are visible in the dorsal muscle domain (white arrow). (D and E). Stage 17. (D) Wild type. (E) dpp^{d6} embryos continue to display pericardial cell hyperplasia. (F–K) Merged scans of embryos double labeled for pMad (green) and Zfh1 (red). Insets are single mesodermal scans to exclude ectodermal pMad accumulation. (F and G) Stage 12. (F) Wild type. (G) dpp^{d12}/dpp^{d6}. pMad is detected in all Zfh1-expressing pericardial precursor cells in both genotypes. (H and I) Stage 13. (H) Wild type. pMad is detected in a majority of the Zfh1-expressing cells (yellow arrows) as well as in non-Zfh1-expressing dorsal mesoderm cells (yellow arrowheads). (I) dpp^{d6}/dpp^{d12}. pMad is not detected in a subset of Zfh1-expressing cells (white arrows). pMad is detected in fewer nonpericardial dorsal mesoderm cells (white arrowheads). (J and K) Stage 14. (J) Wild type. pMad is largely undetectable in Zfh1-expressing cells. (K) LE.Gal4:UAS.Dpp. Ectopic pMad accumulates in Zfh1-expressing and non-Zfh1-expressing dorsal mesoderm cells.

in the dorsal ectoderm during germ-band retraction that is normally provided by disk region enhancers. The presence of numerous mesodermal phenotypes in dpp^{d6} mutants (JOHNSON *et al.* 2003) suggests that the augmentation of *dpp* expression is necessary to boost Dpp dorsal ectoderm signals so that they can reach the underlying mesoderm. Perhaps there are barriers of distance or extracellular matrix density between these germ layers that must be overcome.

We continued our analysis of dpp^{d6} mutant phenotypes by clearly documenting that Dpp signals from the dorsal ectoderm during germ-band retraction act specifically on pericardial but not on cardiac cells (JOHNSON *et al.* 2003). First, we double labeled wild-type and dpp^{d6} embryos with anti-Zfh1 and anti-dMef2 antibodies. Zfh1 and dMef2 have mutually exclusive expression patterns: Zfh1 is expressed in all pericardial cells (LAI *et al.* 1991; WARD and SKEATH 2000), while dMef2 is expressed in all muscle cell lineages, including cardiac cells and dorsal muscle cells (LILLY *et al.* 1994; NGUYEN *et al.* 1994). dpp^{d6} mutants lack Dpp signals from the dorsal ectoderm during germ-band retraction. In dpp^{d6} embryos, the number of Zfh1-expressing pericardial cells is greater than that of wild type at stage 15 (Figure 1, B and C), indicating that Dpp normally represses Zfh1 expression. In JOHNSON *et al.* (2003), we showed utilizing *t*-tests that differences in Zfh1 cell number between wild-type and dpp^{d6} embryos are statistically significant. Alternatively, the number of dMef2-expressing cardiac cells in dpp^{d6} embryos is not different from wild type at stage 13 (*t*-test, $P = 0.224$) or stage 17 (*t*-test, $P = 0.149$).

We also noted that the expression domains of dMef2 and Zfh1 remain mutually exclusive in dpp^{d6} embryos although ectopic Zfh1-expressing cells are observed as lateral to their typical location in a region of the dorsal mesoderm usually associated with dorsal muscle cells (Figure 1C, white arrow). In stage 17 embryos, the expression of dMef2 and Zfh1 is mutually exclusive in both wild-type and dpp^{d6} embryos (Figure 1, D and E) although pericardial cell hyperplasia is still apparent in

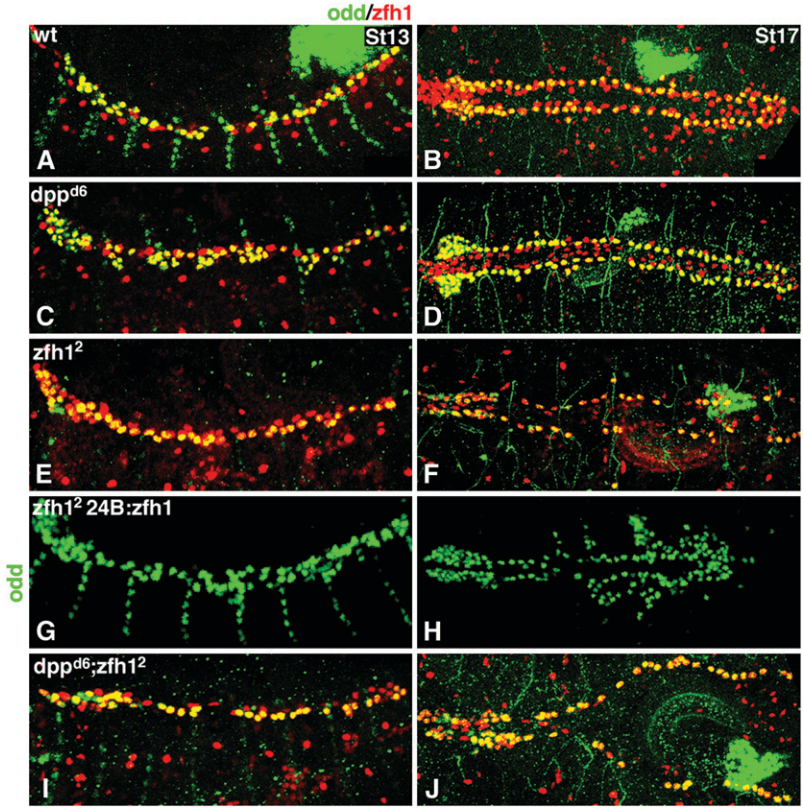

FIGURE 2.—Dpp restricts the number of Odd-skipped pericardial cells via *zfh1*. Stage 13 embryos in lateral view (A, C, E, G, and I) and stage 17 in dorsal view (B, D, F, H, and J) double labeled for Odd (green) and Zfh1 (red). See Table 1 for statistics. (A and B) Wild type. Odd is expressed in a subset of Zfh1-expressing pericardial cells. (C and D) dpp^{d6}. The number of Odd-expressing pericardial cells is significantly increased. All Odd pericardial cells co-express Zfh1. (E and F) $zfh1^2$. The number of Odd-expressing cells is comparable to wild type at stage 13 (E) but is significantly less than wild type by stage 17 (F). (G and H) 24B. Gal4:UAS.Zfh1; $zfh1^2$. Pan-mesodermal Zfh1 expression in $zfh1^2$ mutants not only rescues Odd expression in pericardial cells but also induces ectopic Odd-expressing cells in lateral regions of the mesoderm. (I and J) dpp^{d6}; $zfh1^2$. The number of Odd-expressing cells is comparable to wild type at stage 13 (I) but is significantly decreased by stage 17 (J), a phenocopy of $zfh1^2$ single mutants.

dpp^{d6} embryos. Taken together, these results show that Dpp does not regulate a cell fate choice between cardiac and pericardial cells but that Dpp specifically restricts the number of pericardial cells.

To determine whether Dpp acts directly on pericardial cells or indirectly through an intermediate, we looked for the presence of the phosphorylated form of the Dpp signal transducer Mad (pMad) in Zfh1-expressing cells. During germ-band retraction (stage 12) pMad is widely visible in the ectoderm and in a majority of Zfh1-expressing pericardial precursor cells in both wild-type and dpp^{d12}/dpp^{d6} embryos (Figure 1, F and G). In wild type, immediately following germ-band retraction (stage 13), the number of Zfh1-expressing cells increases and a majority of these cells continue to accumulate pMad (Figure 1H, yellow arrows). This observation is consistent with a previous report of pMad accumulation in the dorsal mesoderm (KNIRR and FRASCH 2001). In contrast, pMad is undetectable in a number of Zfh1-expressing cells in both the anterior and the posterior regions of the dorsal mesoderm in dpp^{d12}/dpp^{d6} embryos immediately following germ-band retraction (Figure 1I, white arrows). These pMad data demonstrate that pericardial cells respond to Dpp signals during stages 12 and 13 and that *dpp* disk region mutations abrogate this aspect of Dpp signaling.

In our previous study, we showed that overexpressing Dpp in the dorsal ectoderm following germ-band retraction using the driver LE.Gal4 causes a loss of Zfh1-expressing cells. If Dpp directly signals to pericardial cells, as suggested above, we would expect to see enhanced pMad expression in LE.Gal4:UAS.Dpp embryos as compared with wild type. Following complete germband retraction (stage 14) in wild-type embryos, pMad is detectable in just a few Zfh1-expressing cells (Figure 1J). In LE.Gal4:UAS.Dpp embryos at the same stage pMad is detected in many Zfh1-expressing cells (Figure 1K). Moreover, ectopic pMad is detected in lateral regions of the dorsal mesoderm in LE.Gal4:UAS.Dpp embryos (Figure 1K insets). These results demonstrate that pericardial cells in the dorsal mesoderm are targets of Dpp signaling during germ-band retraction.

Dpp restricts the number of Odd pericardial cells in a *zfh1*-dependent manner: Lineage tracing of pericardial-cell-specific transcription factors have identified three distinct cell types within the Zfh1-expressing pericardial cell population: Eve expressing, Odd expressing, and Tin expressing (SU *et al.* 1999; WARD and SKEATH 2000; ALVAREZ *et al.* 2003). To identify which pericardial cells responded to Dpp signals during germ-band retraction, we examined the expression of these genes in both *dpp* and *zfh1* mutant backgrounds. Our previous study showed that Eve-expressing pericardial cells are unaffected by *dpp* disk region mutations (JOHNSON *et al.* 2003). However, the number of Odd-expressing pericardial cells (OPCs) is significantly greater in dpp^{d6} stage 13 embryos than in wild type and these supernumerary OPCs persist throughout development (compare Figure 2, A and B, with Figure 2, C and D; Table 1). This result indicates that Dpp normally represses Odd expression.

TABLE 1

Odd-expressing and Tin-expressing cells in embryos with altered *dpp* or *zfh1* activity

Genotype	Mean no. of Odd-skipped expressing cells (SD)				Mean no. of Tinman-expressing cells (SD)			
	Stage 13[a]	*t*-test *vs.* wild type[b]	Stage 17[a]	*t*-test *vs.*wild type[b]	Stage 13[a]	*t*-test *vs.* wild type[b]	Stage 17[c]	*t*-test *vs.* wild type[b]
Wild type	44 (2.4)	—	82.8 (4.4)	—	73 (3.2)	—	127 (4.2)	—
dpp^{d6}	52.8 (5.2)	*0.014*	99.6 (15.3)	*0.021*	85.4 (4.3)	*0.002*	144.4 (7.7)	*<0.001*
LE:Dpp	43.4 (4.7)	0.821	73.8 (2.2)	*0.002*				
$zfh1^2$	46 (5.9)	0.447	71.9 (10.3)	*0.016*	59.9 (0.7)	*0.005*	106.8 (5.2)	*<0.001*
24B:zfh1; $zfh1^2$	60 (12.4)	*0.022*	127.8 (7.3)	*0.002*	92.0 (8.7)	*0.002*	162.2 (15.3)	*<0.001*
dpp^{d6}; $zfh1^2$	43.4 (2.3)	0.706	70 (6.5)	*0.018*	62.8 (4.5)	*0.006*	104.0 (3.6)	*<0.001*
prc:CA-Tkv					76.3 (3.3)	0.265	118.6 (12.7)	*0.027*
tinCΔ4:CA-Tkv					74.8 (1.2)	0.347	131.4 (4.9)	0.184

[a] Unilateral counts of a bilateral expression pattern.
[b] P-value (numbers in italic are statistically significant).
[c] Bilateral counts of a bilateral expression pattern.

Since Zfh1 is also expressed in all OPCs in dpp^{d6} embryos, we hypothesized that Dpp could restrict Odd expression by restricting the number of cells expressing *zfh1*. To test this hypothesis, we first assessed the number of OPCs in $zfh1^2$ embryos. We chose $zfh1^2$ because it is widely utilized and has been described as a genetic and protein null allele (*e.g.*, Lai *et al.* 1993). However, the exact nature of the $zfh1^2$ mutation is unknown. Homozygous $zfh1^2$ embryos show no staining with the anti-Zfh1-d antibody (Lai *et al.* 1993) generated against a fusion protein containing amino acids 648–775 of Zfh1 (Lai *et al.* 1991). Thus, it is possible that this allele encodes a truncated Zfh1 protein that is capable of partially fulfilling *zfh1* functions. Supporting this possibility, the range of mutant phenotypes seen in $zfh1^2$ embryos is highly variable (Lai *et al.* 1993). Further, in our hands, other Zfh1 antibodies (*e.g.*, Zfh1-a recognizing amino acids 1–561 and Zfh1-c recognizing amino acids 562–787; Lai *et al.* 1991) recognize a Zfh1 protein in $zfh1^2$ mutants (as shown in Figures 2 and 3). Nevertheless, $zfh1^2$ is the strongest mutant available and its use allows our data to be viewed in the context of other heart development studies.

Our analysis showed that $zfh1^2$ embryos contain the normal number of OPCs at stage 13 (Figure 2E), yet by stage 17 the number of OPCs is significantly decreased (Figure 2F), indicating that Zfh1 is a positively acting factor required to maintain the correct number of OPCs. This result is specific since the loss of OPCs in $zfh1^2$ mutants can be rescued by overexpressing Zfh1 using the pan-mesodermal driver 24B.Gal4 and ectopic OPCs are observed in lateral regions of the mesoderm when Zfh1 is misexpressed (Figure 2, G and H).

To determine if restriction of *zfh1* expression is sufficient for Dpp to specify the correct number of OPCs, we then assayed Odd expression in dpp^{d6}; $zfh1^2$ embryos. The number of OPCs in dpp^{d6}; $zfh1^2$ embryos resembles that of $zfh1^2$ embryos at stages 13 and 17 (Figure 2, I and J). Taken together, these results demonstrate that *zfh1* is epistatic to *dpp* in regulating OPC number and indicate that normally Dpp defines the number of OPCs by restricting *zfh1* expression.

Dpp restricts the number of Tin pericardial cells via a feed-forward mechanism: A second pericardial cell type, defined by Tin expression, is also responsive to Dpp signals during germ-band retraction. We noted that the number of Tin-expressing pericardial cells (TPC) in dpp^{d6} embryos is greater than that of wild type at stages 13 and 17 (compare Figure 3, A and B, with Figure 3, C and D; Table 1). Since *zfh1* is required downstream of Dpp to restrict the number of OPCs, we reasoned that a similar mechanism could be in place to restrict the number of TPCs. To test this hypothesis, we examined Tin expression in $zfh1^2$ embryos and found that the number of TPCs is significantly reduced (Figure 3, E and F). In addition, we found that this phenotype can be rescued by expressing Zfh1 in the mesoderm and that ectopic TPCs appear when Zfh1 is misexpressed in lateral regions of the mesoderm (Figure 3, G and H). These results indicate that Zfh1 is a positively acting factor required to maintain the correct number of TPCs. In dpp^{d6}; $zfh1^2$ embryos, the number of TPCs is significantly reduced by stage 13 (Figure 3, I and J), which phenocopies $zfh1^2$ embryos. These experiments demonstrate that *zfh1* is also epistatic to *dpp* in regulating the number of TPCs. Thus, Dpp restricts *zfh1* expression and, as a direct result, restricts the number of TPCs and OPCs.

The pattern of Tin-expressing cells in dpp^{d6} embryos suggests that Dpp may also restrict TPC number independently of Zfh1. Tin is expressed in four of the six non-Zfh1-expressing cardiac cells per heart hemisegment in wild type (Ward and Skeath 2000; Lo and Frasch 2001) and dpp^{d6} embryos (Figure 3C). Interestingly, at stage 13, a subset of ectopic Tin-expressing cells in dpp^{d6} and dpp^{d6}; $zfh1^2$ embryos is located ventral to the cardiac cells in a domain usually associated with

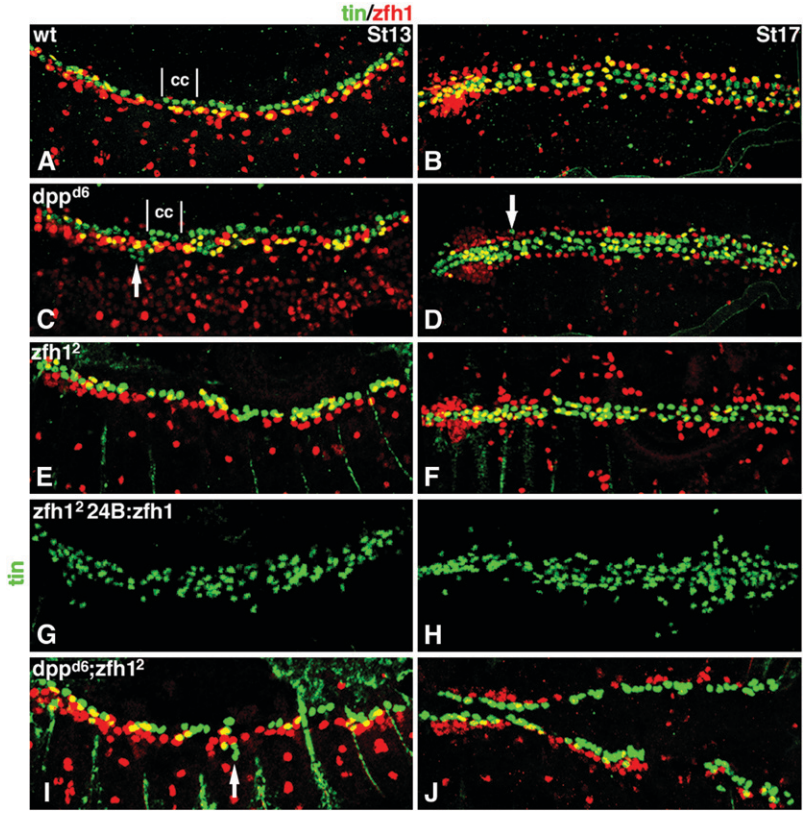

FIGURE 3.—Dpp restricts the number of Tin pericardial cells via *zfh1* and independently of *zfh1*. Stage and view as in Figure 2 for embryos double labeled for Tin (green) and Zfh1 (red). See Table 1 for statistics. (A and B) Wild type. Tin is expressed in a subset of Zfh1-expressing pericardial cells (yellow) and in a subset of cardial cells (cc). (C and D) dpp^{d6}. The number of Tin-expressing pericardial cells is significantly increased. Note that Tin-positive cells are located ventral to the Zfh1-expressing pericardial cells but they do not co-express Zfh1 (white arrows). (E and F) $zfh1^2$. The number of Tin-expressing cells is significantly fewer than wild type. (G and H) 24B.Gal4:UAS.Zfh1; $zfh1^2$. Pan-mesodermal Zfh1 expression in $zfh1^2$ mutants not only rescues Tin expression in pericardial cells but also induces ectopic Tin-expressing cells in lateral regions of the mesoderm. (I and J) dpp^{d6}; $zfh1^2$. The number of Tin-expressing cells is significantly decreased compared to wild type—a phenocopy of $zfh1^2$ single mutants. Some Tin-expressing cells positioned ventral to the Zfh1-expressing pericardial cells do not co-express Zfh1 in double-mutant embryos (white arrow in I).

pericardial or dorsal muscle cells, but these cells do not co-express Zfh1 (Figure 3, C and I). The position of the Tin-expressing cells in dpp^{d6} embryos plus the fact that dMef2-expressing cardiac cells are unaffected in dpp^{d6} embryos (Figure 1, D and E) indicate that the ectopic Tin-expressing cells in dpp^{d6} mutants are pericardial cells. The absence of Zfh1 expression in the ectopic Tin-expressing cells in dpp^{d6} and dpp^{d6}; $zfh1^2$ embryos further indicates that Dpp restricts the number of TPCs in a Zfh1-independent manner. Since *dpp* acts through Zfh1-dependent and Zfh1-independent mechanisms to restrict the number of TPCs, we conclude that *dpp* represses Tin expression via a feed-forward mechanism.

To further demonstrate that TPCs, and not Tin cardial cells, respond to Dpp signals, we expressed a constitutively active form of the Dpp type I receptor Thickveins (UAS.CA-Tkv) using two cell-type-specific drivers: the cardiac cell driver tinCΔ4.Gal4 (Figure 4A) and the pericardial cell driver prc.Gal4 (Figure 4B). Our experiments show that the wild-type Tin expression pattern (Figure 4C) is unaffected by expressing CA-Tkv specifically in cardiac cells (Figure 4D). In contrast, expressing CA-Tkv

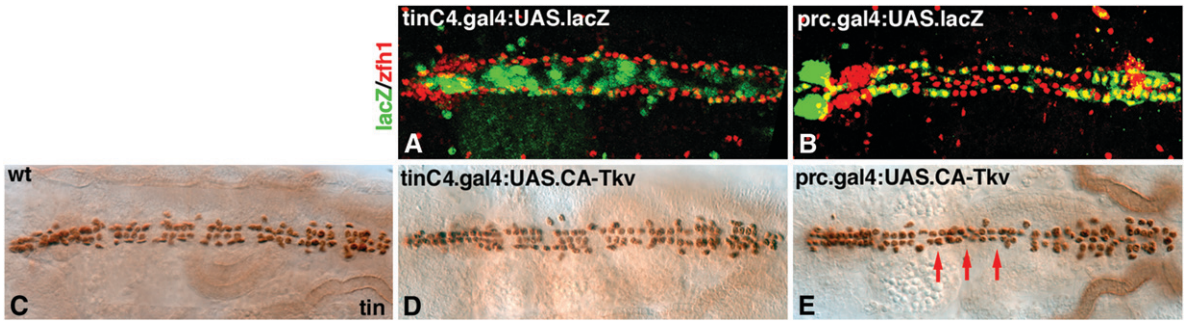

FIGURE 4.—Expressing activated Tkv in pericardial cells, but not in cardiac cells, reduces the number of cells expressing Tin. Dorsal view of stage 17 embryos. (A) tinCΔ4.Gal4 drives lacZ expression (green) in a majority of cardiac cells but not in Zfh1-expressing pericardial cells (red). (B) prc.gal4 drives lacZ expression in a subset of Zfh1-expressing pericardial cells. (C) Wild type Tin expression. (D) tinCΔ4.Gal4:UAS.CA-Tkv embryos do not show a significant change in the number of Tin-expressing cells. (E) prc.Gal4:UAS.CA-Tkv embryos show a significant decrease in the number of Tin-expressing cells (see Table 1 for statistics). Note that, in the region indicated by red arrows, only two rows (of presumably cardiac cells) instead of three or four rows in the comparable region of wild-type embryos are present.

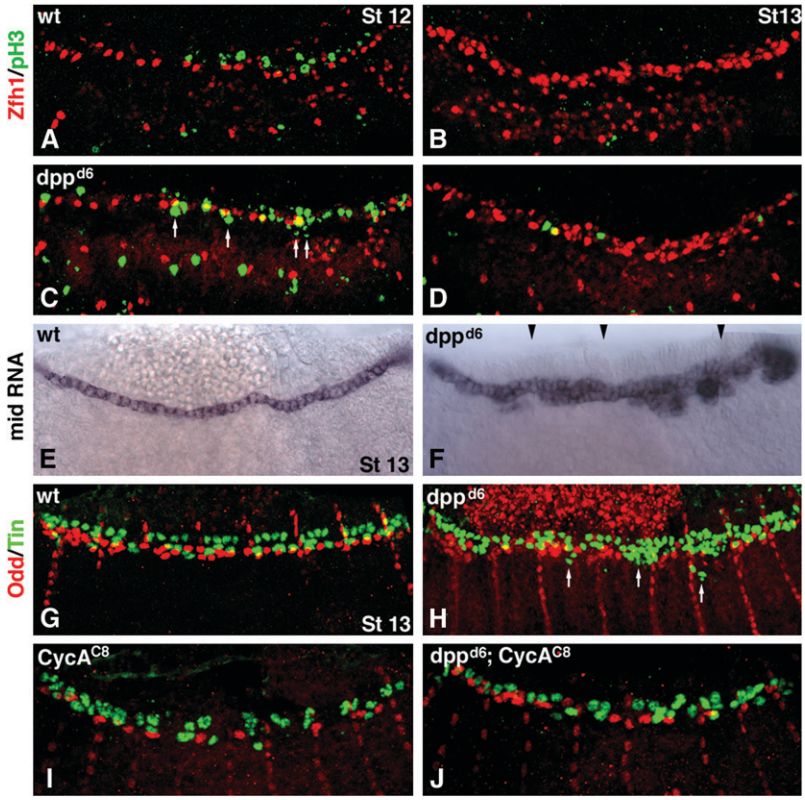

FIGURE 5.—Dpp signaling reduces cell proliferation in the dorsal mesoderm by restricting *mid* expression. (A–D) Merged mesodermal scans of embryos double labeled for Zfh1 (red) and the mitosis marker phospho-histone3 (pH3, green). (A) Wild-type stage 12. Note the limited cell proliferation (pH3 staining) dorsal to the Zfh1-expressing pericardial cells. (B) Wild-type stage 13. No cell proliferation in the dorsal mesoderm. (C) dpp^{d6} stage 12. Cell proliferation is apparent ventrally and medially to the Zfh1-expressing cells. White arrows identify ectopic pH3 staining. Note that apparent colocalization of Zfh1 and pH3 (yellow cells) is an artifact of merging scans. (D) dpp^{d6} stage 13. Cell proliferation persists. (E and F) *mid* RNA expression stage 13. (E) Wild type. *mid* expression is restricted to cardiac cells. (F) dpp^{d6}. *mid* expression expands laterally, specifically in the posterior dorsal mesoderm. This image is a composite of four images. Black arrowheads denote points of overlay. The embryo shown is an extreme example. (G–I) Stage 13 embryos double labeled for Tin (green) and Odd (red). (G) Wild type. (H) dpp^{d6}. (I) $CycA^{C8}$. (J) dpp^{d6}; $CycA^{C8}$. The number of Odd-expressing cells in $CycA^{C8}$ embryos is approximately half the number of wild type. However, the number of Odd-expressing cells in dpp^{d6}; $CycA^{C8}$ double mutants is greater than that observed in $CycA^{C8}$ embryos. In addition, none of these embryos co-express Tin and Odd. Numerous laterally displaced Tin-expressing cells (arrows in H) are observed in dpp^{d6} embryos.

in pericardial cells reduces the number of Tin-expressing cells in medial regions of the heart (Figure 4E; Table 1). The mild reduction in Tin expression observed in prc.Gal4:UAS.CA-Tkv embryos is due to the fact that prc.Gal4 drives expression in only a subset of pericardial cells and does not initiate until late stages of embryogenesis. Nonetheless, these studies strengthen our hypothesis that Dpp signals initiating during germ-band retraction specifically restrict the number of TPCs.

Dpp restricts cell proliferation in the dorsal mesoderm: The increases in pericardial cell number observed in *dpp* mutants could reflect changes in gene expression in a stable cell population or could be the result of changes in cell number. To distinguish between these possibilities, we assessed cell proliferation in the dorsal mesoderm during and after germ-band retraction. In wild-type embryos at stage 12, cell proliferation is very limited (Figure 5A) and completely absent by stage 13 (Figure 5B). In contrast, dpp^{d6} embryos show expanded cell proliferation during stage 12 particularly in mesoderm cells positioned immediately ventral to Zfh1-expressing cells (Figure 5C, arrows). Cell division in the dorsal mesoderm persists in dpp^{d6} embryos throughout stage 13 (Figure 5D). These results argue that Dpp restricts pericardial cell number by limiting cell proliferation in the dorsal mesoderm.

Overexpression of the Tbx family member *midline* (*mid*) induces cell proliferation and ectopic Tin expression in lateral regions of the dorsal mesoderm (QIAN *et al.* 2005). Further, these ectopic Tin-expressing cells do not express the cardiac cell marker Toll and are therefore likely to be Tin pericardial cells (REIM *et al.* 2005). Thus we reasoned that Dpp could repress cell proliferation and Tin pericardial cell expression independently of Zfh1 by restricting *mid* expression. To test this, we investigated *mid* expression in *dpp* and *zfh1* mutant embryos.

In wild-type embryos, *mid* is expressed solely in cardiac cells (Figure 5E) but in dpp^{d6} embryos, *mid* expression expands into ventral regions of the dorsal mesoderm, presumably into the pericardial or dorsal muscle domain (Figure 5F). In contrast, *mid* expression is unaltered in $zfh1^{2}$ and 24B:Zfh1 embryos (data not shown), indicating that Dpp regulates *mid* expression independently of Zfh1. We propose that the ventrally positioned TPCs observed in dpp^{d6} embryos (Figure 3C; white arrow) arise from misregulation of *mid*.

However, we were not yet fully convinced that cell proliferation could account for all ectopic pericardial cells observed in *dpp* mutants. We further tested this possibility in $CycA^{C8}$ embryos. Mitosis does not occur after maximum germ-band extension in these embryos and the number of OPCs in $CycA^{C8}$ embryos is approximately half the number of OPCs in wild-type embryos (HAN and BODMER 2003). We found that the number of OPCs in dpp^{d6}; $CycA^{C8}$ double mutants is greater than that observed in $CycA^{C8}$ embryos (Figure 5, G–J). Further

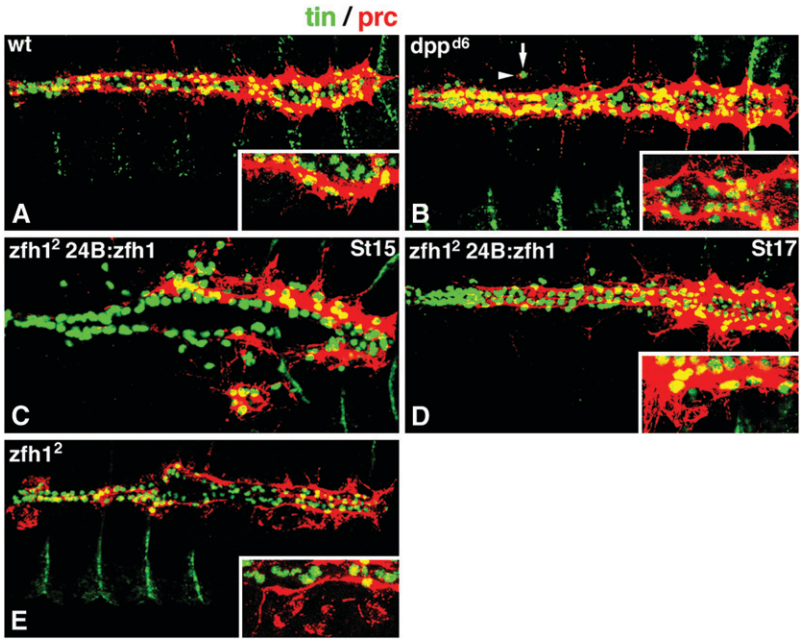

FIGURE 6.—Modifying Zfh1 expression alters Prc expression. Embryos double labeled for Prc (red) and Tin (green). Insets are high-magnification merged scans from the posterior regions of the heart. (A) Wild type stage 17. Prc is broadly expressed throughout the pericardial cell domain, most prominently in the posterior. (B) *dpp*^{d6} stage 17. Prc expression is expanded. Ectopic Tin-expressing cells (arrow) co-express Prc (arrowhead). (C) 24B.Gal4:UAS.Zfh1; *zfh1*^{2} stage 15 and (D) stage 17. Ventrally positioned Tin-expressing cells co-express Prc. (E) *zfh1*^{2} stage 17. Prc expression domain is greatly reduced.

cell counts and *t*-tests indicate that this difference is statistically significant (1.18 OPCs in *CycA*^{C8} *vs.* 1.90 OPCs in *dpp*^{d6}*; CycA*^{C8} per hemi-segment; *t*-test $P < 0.001$). This result indicates that Dpp inhibits cell proliferation and restricts pericardial-specific gene expression in dorsal mesoderm cells.

Lineage analyses have shown that TPCs and OPCs arise from separate precursor cells and that following germ-band retraction Tin and Odd are not co-expressed in dorsal mesoderm cells of wild-type embryos (Figure 5G). Since Dpp signals regulate cell proliferation and the number of TPCs and OPCs, we wanted to understand whether Dpp signals might play a role in maintaining the lineage identities of TPCs and OPCs. Therefore, we examined *dpp*^{d6} embryos double labeled for Tin and Odd expression. We found that, although the number of TPCs and OPCs is increased, co-expression of these proteins was not observed (Figure 5H). Further, co-expression of Tin and Odd was not observed in *dpp*^{d14} (not shown) or *CycA*^{C8} or *dpp*^{d6}*; CycA*^{C8} embryos (Figure 5, I and J). Thus, Dpp signals do not maintain TPC or OPC lineage identities.

***zfh1* expression is necessary and sufficient to induce pericardial cell fate in wild-type embryos but *zfh1* is bypassed in *dpp* and *lame duck* mutants:** Our experiments show that Zfh1 activity is required for the specification of two distinct pericardial cell types: OPCs and TPCs. Moreover, a previous study (SU *et al.* 1999) showed that Zfh1 also specifies a third cell type: Eve-expressing pericardial cells. Taken together, these findings suggested to us that in wild-type embryos Zfh1 expression in the dorsal mesoderm is sufficient to induce a pericardial cell fate. To test this hypothesis, we assayed the expression of the extracellular matrix protein Pericardin (Prc) in embryos misexpressing Zfh1. Prc is broadly expressed in, and then secreted from, pericardial cells, including OPCs and TPCs (Figure 6A), and appears to serve a pericardial-specific function (CHARTIER *et al.* 2002).

We found that ectopic expression of Zfh1 either in the excess pericardial cells of *dpp*^{d6} embryos or artificially in 24B.Gal4:UAS.Zfh1; *zfh1*^{2} embryos induced ectopic Prc expression (Figure 6, B–D). On the other hand, Prc expression is dramatically reduced, although not absent, in *zfh1*^{2} embryos (Figure 6E), perhaps as a result of residual *zfh1* activity in this mutant as discussed above. These results support the hypothesis that *zfh1* is a key regulator of pericardial cell fate. Moreover, since alterations in *zfh1* expression alter the expression of three pericardial cell proteins—Eve (SU *et al.* 1999), Odd, and Prc (this study)—we conclude that in wild-type embryos Zfh1 expression is necessary and sufficient to specify pericardial cell fates.

However, in *dpp*^{d6} mutant embryos, ectopic Tin-expressing cells that do not express Zfh1 are visible in positions normally reserved for dorsal muscle cells (Figure 3, C and D). Further, these ectopic Tin-expressing cells also express Prc (Figure 6B), indicating that they are pericardial cells. This implies that in *dpp*^{d6} mutants the requirement for Zfh1 in the specification of pericardial cell fate is bypassed. Our hypothesis regarding the origin of the ectopic pericardial cells, based on their location, is that they derive from the transformation of cells normally destined to become dorsal muscle cells—cells that do not normally express Zfh1.

To test this hypothesis, we examined *lame duck* (*lmd*) mutant embryos. *lmd* encodes a Gli-like transcription factor expressed in dorsal muscle precursors but not in cardiac or pericardial cells (DUAN *et al.* 2001). In *lmd*^{1} mutants, dorsal muscle precursors fail to develop into

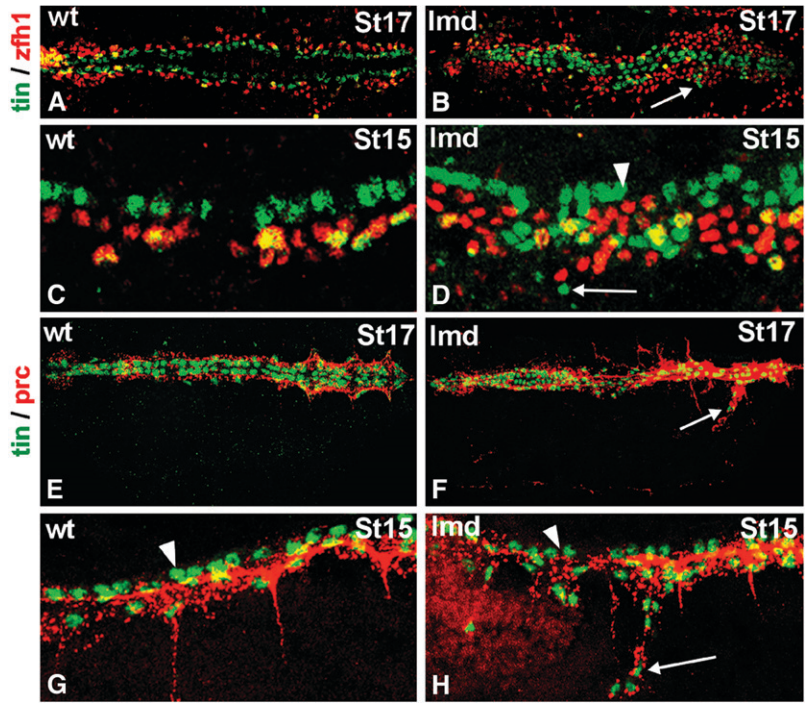

FIGURE 7.—Mutations in *lame duck* also display pericardial cell hyperplasia. (A–D) Embryos double labeled for Tin (green) and Zfh1 (red) at the indicated stages. (A and B) Dorsal (bilateral) view of stage 17 embryonic hearts at low magnification. (C and D) Lateral (unilateral) view of stage 15 embryonic hearts at high magnification. (A and C) In wild-type embryos, all Tin-positive pericardial cells co-express Zfh1 (yellow cells lateral to the green Tin-expressing cardiac cells). (B and D) In lmd^1 embryos, Tin-expressing cardiac cells look normal (cells indicated by an arrowhead in D). There are an excessive number of Zfh1-expressing cells and an excess of Tin-expressing cells, including many quite lateral to their normal position that do not co-express Zfh1 (cells indicated by an arrow in B and D). (E–H) Embryos double labeled for Tin (green) and Prc (red). (E and F) Dorsal (bilateral) view of stage 17 embryonic hearts at low magnification. (G and H) Lateral (unilateral) view of stage 15 embryonic hearts at intermediate magnification. (E and G) In wild-type embryos, all Tin-expressing pericardial cells express Prc but Tin-expressing cardiac cells do not (cardiac cells are indicated by an arrowhead in G and H). (F and H) In lmd^1 embryos, all Tin-expressing cells, except for Tin cardiac cells (arrowhead), also express Prc (cells indicated by an arrow).

fusion-competent myoblasts, leading to significant defects in dorsal muscle architecture (DUAN *et al.* 2001; RUIZ-GOMEZ *et al.* 2002). We examined *lmd* mutants because of the possibility that the missing muscle precursors were transformed into adjacent cell types—*e.g.*, into pericardial cells in dorsal regions.

This hypothesis was validated by the observation that there is widespread pericardial cell hyperplasia in *lmd* mutants (Figure 7, A–D). This aspect of the *lmd* mutant phenotype is more pronounced than the hyperplasia seen in *dpp* mutants. Both display an excess of Zfh1-expressing pericardial cells (compare Figures 3D, 6B, and 7B). Importantly for our hypothesis that *dpp* mutants bypass the Zfh1 requirement in pericardial cell fate specification, *lmd* mutants also have ectopic Tin-expressing cells ventral to their normal position that do not express Zfh1 (Figure 3, C and D; Figure 7, B and D). Further, the ectopic Tin-expressing cells in *dpp* and *lmd* mutants also express Prc and thus they must be pericardial cells (Figure 6B, Figure 7, F and H).

Overall, the data from *dpp* and *lmd* mutants suggest that the ectopic Tin- and Prc-expressing pericardial cells that do not express Zfh1 are derived from the cell fate transformation of dorsal muscle cells into pericardial cells at a relatively late stage of development.

Dpp maintains the boundary between pericardial cells and dorsal muscle cells: Our observation that loss of *dpp* or *lmd* results in an expanded pericardial cell domain led us to hypothesize that Dpp signals are specifically required to ensure that the dorsal muscle cells positioned just lateral to the pericardial cells are correctly specified. To test this hypothesis, we examined the expression of two dorsal muscle transcription factors, Kr and Eve, in *dpp* mutant embryos. In wild-type embryos, Kr expression becomes apparent in a pair of dorsal muscle precursors at the onset of germ-band retraction (RUIZ-GOMEZ *et al.* 1997). As the germ band retracts, Kr-expressing DA1 and DO1 founder cells fuse with neighboring cells to form multinucleate muscle precursors, and Kr expression concomitantly increases in the dorsal mesoderm (compare Figure 8A with 8B). Eve is expressed in DA1 founder cells and a subset of pericardial cells. Like Kr, Eve expression expands as DA1 founder cells begin to form multinucleate muscle precursors (CARMENA *et al.* 1998b). In *dpp* mutant embryos, Kr- and Eve-expressing muscle founder cells are specified correctly but neither Kr nor Eve expression expands at wild-type levels as the germ band retracts or after germ-band retraction (Figure 8, C and D; Table 2). These results indicate that Dpp signals ensure proper development of the dorsal musculature.

Since the absence of Dpp signals results in an expansion of the pericardial cell domain and a concomitant reduction in the expression of dorsal muscle transcription factors, we suspected that Dpp functions to maintain the pericardial–dorsal muscle cell boundary. We hypothesized that Dpp signals pattern the dorsal musculature by preventing pericardial cells from occupying ventral regions of the dorsal mesoderm. If this hypothesis is correct, then the presence of ectopic pericardial cells in the dorsal mesoderm alone should phenocopy the loss of Dpp signals. Indeed, misexpression of Zfh1

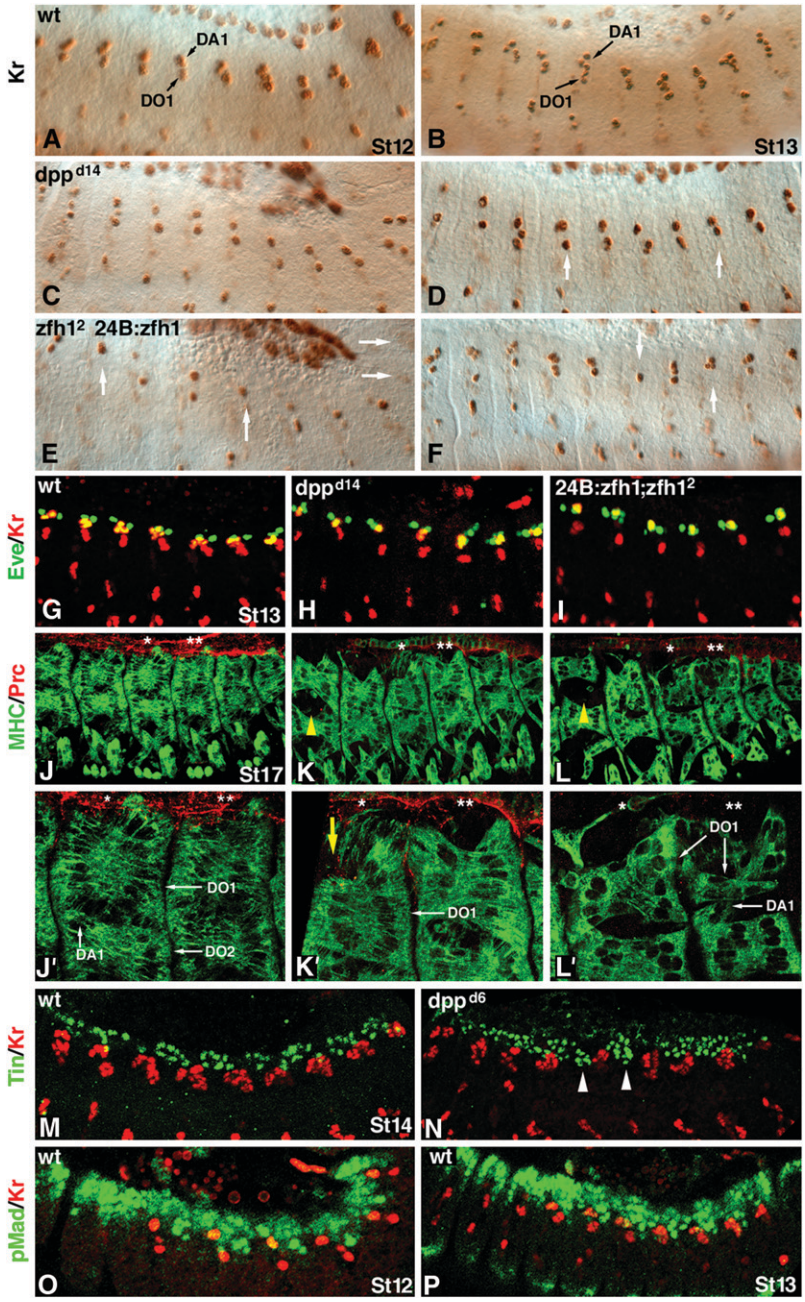

FIGURE 8.—Dpp indirectly maintains dorsal muscle cell fates. (A–F) Lateral views of embryos stained for Kr expression. See Table 2 for statistics. (A) Wild-type stage 12. Kr-expressing DA1 and DO1 muscle founder cells are shown. (B) Wild-type stage 13. An increased number of Kr-expressing nuclei are evident. (C) dpp^{d14} stage12 and (D) stage 13. Roughly half of the Kr-expressing cells per segment are present. White arrows in D identify segments with reduced Kr expression. (E) 24B.Gal4: UAS.Zfh1; $zfh1^2$ stage 12 and (F) stage 13. Kr expression is dramatically reduced. (G–I) Stage 13 embryos double labeled for Kr (red) and Eve (green). (G) Wild type. (H) dpp^{d14}. (I) 24B.Gal4: UAS.Zfh1; $zfh1^2$. Eve pericardial cells, two per segment, are present in all genotypes, but the number of Kr and Eve co-expressing dorsal muscle cells (in yellow) is considerably reduced in *dpp* and *zfh* mutants. (J–L) Stage 17 embryos double labeled for myosin heavy chain (green) and Prc (red). The DO1, DO2, and DA1 muscles are indicated with white arrows. Asterisks indicate the two segments viewed at high magnification in J′–L′. (J and J′) Wild type. The DO1 and DO2 muscles are tightly associated. The DO1 and DA1 muscles abut the pericardial cells. (K and K′) dpp^{d14}. The DO1 and DO2 muscles are loosely associated (yellow arrowhead in K) and the size of the DO1 muscles is reduced (yellow arrow in K′). (L and L′) 24B.Gal4: UAS.Zfh1; $zfh1^2$. The DO1 and DO2 muscles are loosely associated (yellow arrowhead in L), a subset of DA1 muscles is absent, and the DO1 muscles are reduced. (M and N) Stage 14 embryos labeled for Tin (green) and Kr (red). (M) Wild type. Tin-expressing cells are exclusively positioned dorsal to the Kr-expressing cells. (N) dpp^{d6}. There are Tin-expressing cells positioned ventral to the dorsal-most Kr-expressing cells (white arrowheads). (O and P) Stage 12 and 13 embryos labeled for pMad (green) and Kr (red). (O) Wild type. (P) dpp^{d6}. Co-labeling of Kr and pMad is not observed in either embryo, indicating that the effect of Dpp on Kr is indirect.

throughout the dorsal mesoderm reduces Kr expression in a manner very similar to that observed in *dpp* mutants (Figure 8, E and F; Table 2). Further, double-labeling experiments with Kr and Eve show that the reduction in Eve expression in *dpp* mutant embryos is due to the loss of Eve-expressing muscle founder cells and not due to changes in Eve pericardial cell number (Figure 8, G–I). Thus, the presence of ectopic pericardial cells in the dorsal mesoderm, via Zfh1 misexpression, also prevents the expression of these two dorsal muscle cell transcription factors.

To better understand the role of Dpp signals in patterning the dorsal musculature, we examined the size and position of somatic muscle fibers in embryos stained for muscle myosin. In wild-type embryos, muscle myosin identifies the dorsal-most DO1 and DA1 muscles positioned proximal to the pericardial cells (Figure 8J). DO2 muscles are positioned just ventral to the DO1 muscles. The size of some DO1 muscles, particularly in the sixth row of somatic muscles, is reduced in dpp^{d14} mutants, and the space between the DO1 and DO2 muscles is expanded (Figure 8K). Misexpressing Zfh1 in the dorsal mesoderm causes a similar phenotype: reduction in the size of DO1 muscles and dissociation of the DO1 and DO2 muscles (Figure 8L). In addition, the number and the size of the DA1 muscles are reduced in embryos

TABLE 2

Kr and Eve expression in embryos with altered *dpp* or *zfh1* activity

	Mean no. of Kr-expressing nuclei (SD)				Mean no. of Eve-expressing nuclei (SD)			
Genotype	Stage 12[a]	*t*-test *vs.* wild type[b]	Stage 13[a]	*t*-test *vs.* wild type[b]	Stage 12[a]	*t*-test *vs.* wild type[b]	Stage 13[a]	*t*-test *vs.* wild type[b]
Wild type	2.78 (0.76)	—	4.77 (1.22)	—	3.32 (0.65)	—	5.13 (0.97)	—
dpp^{d14}	2.43 (0.53)	*0.003*	2.29 (0.51)	*<0.001*	2.92 (0.90)	*0.006*	4.29 (1.13)	*0.001*
dpp^{d6}	2.38 (0.61)	*0.006*	3.96 (1.31)	*0.011*	3.10 (0.71)	0.173	4.32 (0.98)	*0.004*
$zfh1^2$	2.69 (0.95)	0.617	5.59 (1.06)	*<0.001*				
24B:zfh1; $zfh1^2$	1.60 (0.70)	*<0.001*	3.73 (2.09)	*<0.001*				

[a] Number of nuclei per hemi-segment in lateral view.
[b] P-value (numbers in italic are statistically significant).

misexpressing Zfh1. This result shows that increasing pericardial cell number simultaneously reduces the size and alters the pattern of the dorsal musculature.

We then reasoned that maintenance of the pericardial–dorsal muscle cell boundary might involve cross-repressive interactions between pericardial and dorsal muscle cells. Our data suggested that *zfh1* defines the pericardial cell domain and that, in the absence of *zfh1*, the dorsal muscle domain would expand. Indeed, following germ-band retraction, Kr expression is expanded in $zfh1^2$ embryos as compared with wild type (Table 2). Moreover, in wild-type embryos, the pericardial cell domain is tightly restricted to the region dorsal of the Kr-expressing dorsal muscle domain (Figure 8M). However, in dpp^{d6} embryos, pericardial cells are observed in the dorsal muscle domain (Figure 8N). These results argue that cross-repressive interactions between pericardial and dorsal muscle cells continue to maintain the pericardial–dorsal muscle cell boundary after the respective precursor cells have been specified.

To further characterize the mechanism by which Dpp maintains the pericardial–dorsal muscle cell boundary, we double labeled embryos for pMad and Kr. We found that in wild type pMad is barely detectable in Kr-expressing dorsal muscle cells during germ-band retraction (Figure 8O). Following germ-band retraction, pMad accumulation is absent from all Kr-expressing cells although high levels of pMad can be seen in cells adjacent to the Kr-expressing cells (Figure 8P). This demonstrates that Dpp does not signal to Kr-expressing dorsal muscle founder cells during germ-band retraction although Dpp might signal to the adjacent cells to restrict *zfh1* expression.

Embryonic pericardial cell overgrowth reduces larval cardiac output: To determine what physiological effect pericardial hyperplasia might have on cardiac function, we analyzed the heartbeat of wild-type and dpp^{d6} first instar larvae with live videomicroscopy. We studied the pulse distance traveled by pairs of cardiac cells each beat (the difference between the maximum separation at diastole and the minimum separation at systole) and the number of beats per second. In the anterior region of the heart (the aorta), we observed no difference in heart rate or pulse distance between wild-type and dpp^{d6} larvae (data not shown).

In the posterior region (the heart proper; Lovato *et al.* 2002, Sellin *et al.* 2006), dpp^{d6} larvae show a considerable reduction in the systolic distance (mutant cells do not approach each other as closely as wild type do) and the diastolic distance (mutant cells do not separate from each other as far as wild type do). The disparity is so pronounced that heartbeat tracings for wild-type and dpp^{d6} larvae are essentially nonoverlapping (Figure 9A). Quantifying the data, we found that dpp^{d6} larvae have a 44% reduction in the average pulse distance per beat (Figure 9B), an indication that the total amount of fluid moved per beat (beat volume) is significantly reduced. Alternatively, the heart rate of dpp^{d6} larvae in the posterior region is comparable to wild type (Figure 9B). We conclude that overall cardiac output, a function of beat volume and beat rate, is significantly reduced in dpp^{d6} larvae.

DISCUSSION

Our data are wholly consistent with the hypothesis that the dpp^{d6} inversion prevents the augmentation of *dpp* expression provided by disk region enhancers during germ-band retraction. The data further suggest that the augmentation of *dpp* expression is necessary to boost Dpp dorsal ectoderm signals such that they can reach the underlying mesoderm. Finally, we have shown that during germ-band retraction Dpp signals maintain the boundary between pericardial cells and dorsal muscle cells via two distinct mechanisms: the regulation of gene expression and the restriction of cell proliferation (see Figure 10 for a model). To regulate gene expression, Dpp signals directly to pericardial cells and restricts Odd and Tin expression in a *zfh1*-dependent manner. Dpp also limits Tin expression, independently of *zfh1*, by repressing the expression of *mid*, a stimulator of proliferation.

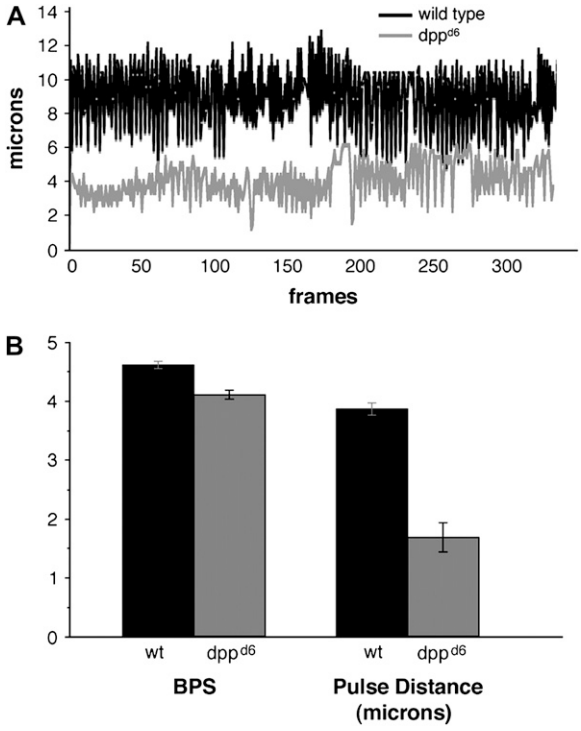

FIGURE 9.—Reduced cardiac output in *dpp* mutant larvae. (A) Representative traces showing the distance between a pair of cardiac cells in the posterior region of the heart over time in wild type (top) and dpp^{d6} (bottom) first instar larvae. One second corresponds to 12 frames. (B) Average number of beats per second (BPS) and pulse distance for wild type ($n = 3$) and dpp^{d6} ($n = 4$) first instar larvae. Pulse distance is the difference between the maximum diastolic position and the minimum systolic position for each heartbeat. Error bars signify the standard error of the mean.

Dpp restricts the number of cells derived from symmetrically dividing lineages: With respect to *zfh1*-dependent regulation, our data support the hypothesis that Dpp restricts Zfh1 expression to regulate the number of pericardial cells derived solely from symmetrically dividing lineages. Lineage analyses have identified both symmetric and asymmetric cell divisions of myogenic and pericardial precursor cells. Pericardial cells are derived from four separate lineages that arise from four distinct precursor cells. Asymmetric precursor cell divisions initiating between stages 8 and 10 give rise to the Odd-positive/Seven up (Svp)-positive pericardial cells and the Eve-positive/Tin-positive pericardial cells (EPCs) (Ward and Skeath 2000; Alvarez *et al.* 2003; Han and Bodmer 2003). On the other hand, symmetric division, initiating at the same stage, establishes the Odd-positive/Svp-negative pericardial cells (OPCs) and the Tin-positive/Eve-negative pericardial cells (TPCs). We show that *dpp* mutations do not affect the number of EPCs (Figure 8H) or the number of Odd-positive/Svp-positive cells (Johnson *et al.* 2003). However, embryos bearing *dpp* mutations show an increase in the number of OPCs (Figure 2) and TPCs (Figures 3 and 4). Therefore, the ectopic pericardial cells seen in *dpp* mutants derive from symmetrically dividing lineages.

Previous reports have shown that regulation of asymmetric cell division is a key mechanism in establishing boundaries among the various cell types in the dorsal mesoderm. For instance, in the absence of Numb, a Notch pathway antagonist, asymmetric progenitor cell division is abrogated and the number of Odd-positive/Svp-positive cells (Ward and Skeath 2000) and EPCs (Carmena *et al.* 1998b) increases at the expense of the Svp-expressing cardial cells and Eve-expressing dorsal muscle cells, respectively. Our study extends these observations by showing that pericardial cell types derived from symmetrically dividing lineages are also under strict regulatory control.

Dpp limits cell proliferation and the reactivation of Tin in a subset of pericardial cells: With respect to *zfh1*-dependent regulation of pericardial cell number, Dpp restricts cell proliferation and, in turn, Tin expression by limiting *mid* expression. In wild-type embryos, cell division in the dorsal mesoderm is largely complete by the early stages of germ-band retraction (stage 11), whereas in dpp^{d6} embryos cell proliferation in the dorsal mesoderm continues through stage 13 (Figure 5, C and D). Interestingly, the number of cells expressing Zfh1 increases from stage 12 to stage 13 in wild-type embryos in the absence of cell division (compare Figure 5A with 5B), demonstrating that patterning events subsequent to cell division regulate cell fate choices in the dorsal mesoderm. This hypothesis is supported by the fact that tracing pericardial cell lineages requires inducing mitotic clones by stage 8 (Alavarez *et al.* 2003). Therefore, the ectopically dividing mesoderm cells observed in dpp^{d6} embryos are derived from cells with the potential to become Tin-expressing cells.

During stage 12, *tin* expression is reactivated in a subset of cardiac cells in a *mid*-dependent fashion, suggesting that *tin* expression in precursor cells alone is not sufficient for specifying the ultimate fate of their daughter cells (Reim *et al.* 2005). Moreover, misexpression of *mid* results in both ectopic cell division and expanded *tin* expression (Qian *et al.* 2005). Lineage studies support the necessity of reactivating Tin by showing that a single precursor cell gives rise to two Tin-positive/Eve-negative pericardial cells and two siblings that do not express Tin (Alvarez *et al.* 2003). Thus *tin* is not reactivated in all subpopulations of pericardial cells. Our data suggest that, during stage12, Dpp prevents *tin* reactivation in cells occupying lateral regions of the dorsal mesoderm by limiting *mid* expression.

A pericardial cell–dorsal muscle cell boundary may be essential for myoblast fusion: Development of the dorsal musculature initiates when founder cells are specified in the mesoderm. These founder cells then fuse with neighboring cells to form syncitial myofibers. We found that, in the absence of Dpp, the pericardial

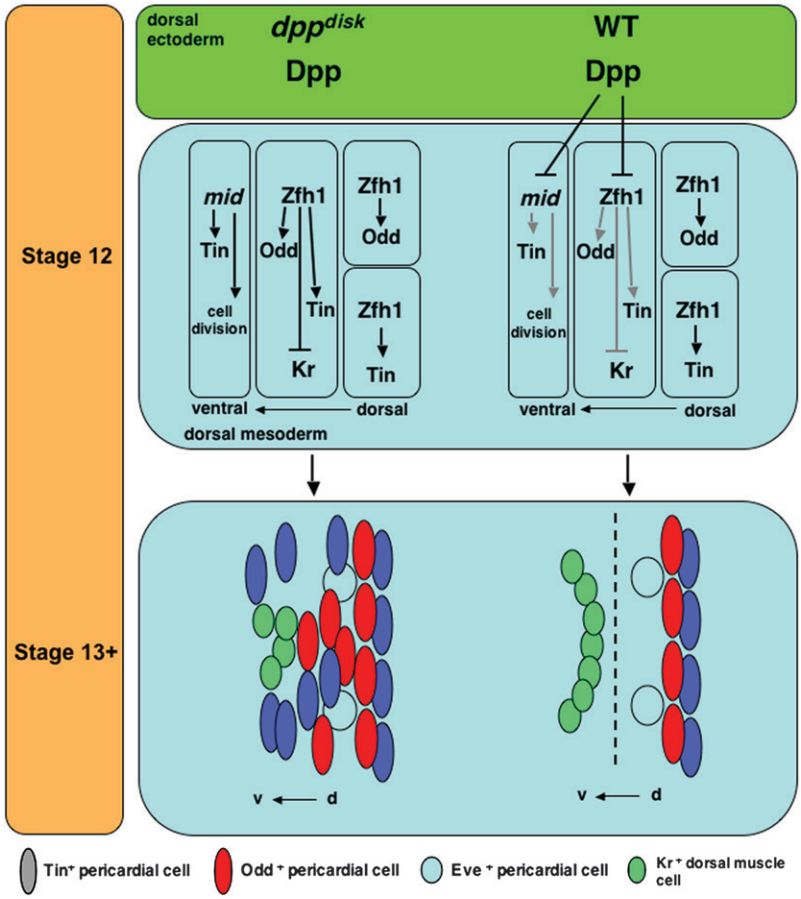

FIGURE 10.—Dpp signals pattern the embryonic dorsal mesoderm during germ-band retraction. dpp^{disk} at stage 12: in the absence of Dpp, we observed that (1) *mid* expression expands ventrally, drives cell proliferation, and induces ectopic Tin expression; (2) Zfh1 expression expands ventrally, inducing ectopic Tin and Odd expression and repressing Kr (and Eve) expression; and (3) pericardial cell specification of Odd and Tin by Zfh is unaffected. Wild type at stage 12: Dpp signals from the dorsal ectoderm restrict the expression of *mid* and Zfh1. dpp^{disk} at stage 13+: in the absence of Dpp, we observed that (1) pericardial cells populate ventral regions of the dorsal mesoderm normally occupied exclusively by dorsal muscle cells and (2) the inappropriate presence of pericardial cells reduces the expression of the dorsal muscle genes Kr (and Eve). It appears that there is a failure to maintain the pericardial cell–dorsal muscle cell boundary and that this failure results in an increase in heart size. Wild type (WT): Dpp maintains the boundary between pericardial and dorsal muscle cells (dashed line) by restricting the number of Tin- and Odd-expressing pericardial cells and by reducing cell division via the repression of *mid*.

cell domain expands into the dorsal muscle domain and reduces expression from the dorsal muscle genes Kr and Eve (Table 2). Since the separation between pericardial and dorsal muscle cells is lost in *dpp* mutant embryos, we conclude that Dpp maintains the pericardial–dorsal muscle cell boundary after it is established. Moreover, we find that reducing pericardial cell number increases Kr expression after germ-band retraction, suggesting that cross-repressive interactions between pericardial and dorsal muscle cells contribute to patterning of the dorsal mesoderm. The presence of ectopic pericardial cells in the dorsal mesoderm reduces the number of myofibers comprising the dorsal muscles (Figure 8, H and I) even though the dorsal muscle founder cells are, for the most part, correctly specified (Figure 8C). pMad does not accumulate in Kr-expressing founder cells yet Kr expression is significantly reduced in *dpp* mutant embryos. Therefore, changes in Kr and Eve expression observed in embryos with altered *dpp* or *zfh1* activity reflect alterations in the number of myoblast fusion events in the dorsal mesoderm.

Our data extend a previous study showing that misexpressing Zfh1 reduces dMef2 expression in somatic muscles (POSTIGO *et al.* 1999). Our study demonstrates that misexpression of Zfh1 induces ectopic pericardial cells and that the presence of pericardial cells in the dorsal muscle domain reduces myoblast fusion. Therefore, reduced dMef2 expression in embryos misexpressing Zfh1 is likely the result of reduced myoblast fusion and not of direct repression of dMef2 expression by Zfh1. Further, our analysis of *lmd* mutants that have reduced numbers of myoblasts revealed that they also contain an excessive number of pericardial cells. Together, these results suggest that maintaining the pericardial–dorsal muscle cell boundary requires Dpp-mediated cross-repressive interactions between these cell types. Thus, in the absence of Dpp, the transformation of dorsal muscle cells into pericardial cells reduces the number of myoblasts available for fusion.

Pericardial cell function: Experiments in the larvae of Drosophila and other insects suggested that pericardial cells act as nephrocytes that filter the hemolymph (CROSSLEY 1972). These studies also showed that pericardial cells secrete proteins into the hemolymph, suggesting that one pericardial cell function may be to provide short- or long-range signals. Consistent with this, reducing pericardial cell number reduces heart rate and increases the cardiac failure rate, suggesting that pericardial cells influence the development of cardiac cells (FUJIOKA *et al.* 2005).

Our study shows that pericardial cell hyperplasia reduces the luminal distance of the heart during systole as well as diastole, resulting in an overall decrease in average pulse distance of each contraction. However,

pericardial overgrowth does not alter heart rate, indicating that cardiac cells develop appropriately in the presence of ectopic pericardial cells. Our luminal measurements suggest a role for pericardial cells in the mechanics of heart function. One hypothesis for this is based on the fact that pericardial cell hyperplasia results in excess levels of Prc in the extracellular matrix (ECM) surrounding the heart (Figure 6). Prc is a collagen IV-like ECM protein secreted at high levels from pericardial cells (Chartier *et al.* 2002). In *dpp* mutants, excess Prc is seen predominantly in the posterior of the heart where we observe the pulse-distance reduction. We propose that Prc secreted by pericardial cells limits the width of the dorsal vessel at diastole and thus modulates the pulse distance of each heart contraction. Pericardial cell overgrowth would increase Prc deposition, thereby reducing the size of the diastolic heart and the pulse distance. Consistent with this hypothesis, excessive expression of ECM proteins, including collagen IV, was correlated with heart failure in patients presenting with end-stage cardiomyopathy (Schaper and Speiser 1992).

Functional conservation of BMP signaling during heart development: It is well documented that many of the early events driving Drosophila embryonic heart development have been conserved in vertebrates (Cripps and Olson 2002; Zaffran and Frasch 2002). Our data provide the first basis upon which to determine if Dpp regulation of Zfh1 or Tin late in heart development is also conserved.

Two orthologs of *zfh1*, Sip1 and Kheper, have been identified in vertebrates. Zebrafish embryos injected with the Dpp homolog BMP4 show reduced Kheper expression (Muraoka *et al.* 2000) while Xenopus embryos injected with the BMP antagonist Chordin display elevated Sip1 expression (Nitta *et al.* 2004). These results suggest the possibility that Dpp repression of *zfh1* expression may be conserved in vertebrates. In addition, mammalian Sip1 plays an essential role in heart development. In mice, Sip1 is expressed in neural crest cells (NCCs), paraxial mesoderm, and neuroectoderm. The subset of NCCs that express Sip1 give rise to the septum and large arteries of the heart. Sip1 knockout mice fail to form these NCCs (Van de Putte *et al.* 2003) and these mice die midway through gestation with numerous heart defects. Mice lacking the BMP receptors BMPRIA or ALK2 specifically in NCCs also display numerous cardiac phenotypes (Kaartinen *et al.* 2004; Stottmann *et al.* 2004). In conditional knockout of ALK2 in NCCs, abnormalities are seen in the heart's outflow tract, and conditional knockout of BMPRIA in NCCs results in heart failure and early embryonic lethality similar to Sip1 knockout mice. Thus BMP signals are required for proper specification of NCCs, and loss of BMP signaling in NCCs phenocopies Sip1 knockout mice to an extent. It is tempting to speculate that, as in Drosophila, BMP signals regulate the Zfh1 ortholog Sip1 to correctly specify NCCs and, in turn, to properly pattern the mammalian heart.

With regard to the conservation of late-stage Dpp regulation of Tin, a recent article describing a study of mice with a conditional knockout of Nkx2.5 where expression is missing only during late stages of heart development (post E14.5) is highly relevant (Prall *et al.* 2007). Utilizing rescue of Nkx2.5 mutant embryos with BMP-signaling-pathway components, the study identified a direct connection among BMP4 signaling, Nkx2.5 activity, and heart cell proliferation. As Nkx2.5 is the Tin homolog, BMP4 is the Dpp homolog, and the mutant phenotype (heart cell hyperplasia) is the same in both species, this suggests that this aspect of Dpp signaling is conserved in mammals. Together with our study, these results suggest that defects in late-stage BMP signaling may play a role in congenital heart defects.

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