Development 130, 3027-3038

doi:10.1242/dev.00517

Gata factor Pannier is required to establish competence for heart progenitor formation

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Accepted 31 March 2003

SUMMARY

Inductive signaling is of pivotal importance for developmental patterns to form. In *Drosophila*, the transfer of TGFβ (Dpp) and Wnt (Wg) signaling information from the ectoderm to the underlying mesoderm induces cardiac-specific differentiation in the presence of Tinman, a mesoderm-specific homeobox transcription factor. We present evidence that the Gata transcription factor, Pannier, and its binding partner U-shaped, also a zinc-finger protein, cooperate in the process of heart development. Loss-of-function and germ layer-specific rescue experiments suggest that *pannier* provides an essential function in the mesoderm for initiation of cardiac-specific expression of *tinman* and for specification of the heart primordium. *u-shaped* also promotes heart development, but unlike *pannier*, only by maintaining *tinman* expression in the cardiogenic region. By contrast, pan-mesodermal overexpression of *pannier* ectopically expands *tinman* expression, whereas overexpression of *u-shaped* inhibits cardiogenesis. Both factors are also required for maintaining *dpp* expression after germ band retraction in the dorsal ectoderm. Thus, we propose that Pannier mediates as well as maintains the cardiogenic Dpp signal. In support, we find that manipulation of *pannier* activity in either germ layer affects cardiac specification, suggesting that its function is required in both the mesoderm and the ectoderm.

Key words: *Drosophila*, Heart, Cardiogenesis, Mesoderm, *pannier*, *u-shaped*, *tinman*, *dpp*, Gata factors

INTRODUCTION

In *Drosophila*, bilaterally symmetric heart progenitors are specified within the dorsal most region of the mesoderm. These progenitor cells then migrate to the dorsal midline where they form a linear heart tube consisting of two different cell types, the inner contractile myocardial cells and the outer pericardial cells (Rizki, 1978), subtypes of which have been identified based on gene expression, function and lineage relationships (Alvares et al., 2003; Han and Bodmer, 2003; Ponzielli et al., 2002; Lo et al., 2002). Several regulatory genes have been identified to be required for the specification of cardiac progenitors within the dorsal mesoderm, including the homeobox transcription factor Tinman (Tin), and the TGFβ and Wnt signaling molecules encoded by *dpp* and *wingless* (*wg*), respectively (Bodmer, 1993; Azpiazu and Frasch, 1993; Frasch, 1995; Wu et al., 1995; Park et al., 1996; Azpiazu et al., 1996; Riechmann et al., 1997). *wg*, *dpp* and *tin* are not only necessary for heart formation, but as overexpression studies suggest the spatial convergence of *wg* and *dpp* signaling on cells expressing *tin* is also sufficient for cardiac-specific differentiation (Lockwood and Bodmer, 2002). A mesodermal mediator of ectodermal *wg* signaling to the mesoderm is achieved by activation of a transcription factor encoded by *sloppy-paired* (Lee and Frasch, 2000), but it is not known if the cardiogenic *dpp* signal is also mediated indirectly.

There are striking molecular and developmental similarities between vertebrate and *Drosophila* heart development (Bodmer, 1995; Bodmer and Venkatesh, 1998; Bodmer and Frasch, 1999). Developmentally, both vertebrate and *Drosophila* hearts are formed from bilaterally symmetrical rows of mesodermal cells, which will eventually migrate to the midline, where they will fuse to form a linear heart tube. More importantly, *tin* and *dpp*, two factors that determine the initial formation of the *Drosophila* heart, also have vertebrate counterparts (Nkx2.5 and Bmp2/4, respectively) with a similar function in cardiogenesis (Harvey, 1996; Schultheiss et al., 1997). In contrast to *Drosophila*, canonical Wnt signaling in vertebrates needs to be prevented for promoting heart formation in the anterior lateral plate mesoderm (Schneider and Mercola, 2001; Marvin et al., 2001). However, the non-canonical Wnt pathway is required for heart formation in vertebrates (Pandur et al., 2002).

Six Gata transcription factors have been identified in vertebrates, characterized by two conserved DNA-binding zinc fingers (Evans and Felsenfeld, 1989; Tsai et al., 1989; Yamamoto et al., 1990). Gata1, Gata2 and Gata3 are largely expressed in hematopoietic stem cells (reviewed by Orkin, 1998), and Gata4, Gata5 and Gata6 are expressed in several mesoderm- and endoderm-derived tissues, including the developing heart (Arceci et al., 1993; Kelley et al., 1993; Heikinheimo et al., 1994; Laverriere et al., 1994; Jiang and

Evans, 1996; Morrisey et al., 1996), where they are thought to regulate cardiac-specific genes (Grepin et al., 1994; Ip et al., 1994; Durocher et al., 1997; Murphy et al., 1997) (reviewed by Molkentin, 2000). Gata4 is already expressed in the early cardiac crescent of the lateral plate mesoderm, and in mice deficient for Gata4, these heart primordia fail to migrate towards the midline where they normally fuse into the primitive heart tube (Molkentin et al., 1997; Kuo et al., 1997). Owing to these ventral closure defects, it has been difficult to discriminate between a direct role for Gata4 in heart formation and an indirect involvement via its function in ventral morphogenesis. Furthermore, Gata4, Gata5 and Gata6 may act in part redundantly, which may further occlude their cardiogenic potential. Consistent with the direct involvement of Gata4 in heart development is the congenital heart disease phenotype observed in individuals heterozygous for deletions of chromosome 8p23.1 region, which includes the *GATA4* gene (Pehlivan et al., 1999; Bhatia et al., 1999).

In vitro, Gata4 interacts with a wide array of proteins, including the Tinman homolog Nkx2.5, the bHLH protein Hand and the multiple zinc-finger protein Fog2 (Durocher et al., 1997; Sepulveda et al., 1998; Lee et al., 1998; Lu et al., 1999; Sepulveda et al., 2002; Svensson et al., 1999; Tevosian et al., 1999; Dai et al., 2002). Fog2 apparently modulates Gata-mediated transcriptional regulation not only as a repressor, but also as an activator, depending on the promoter and on cell type (Lu et al., 1999). Fog2 is co-expressed with Gata4 in embryonic and adult cardiomyocytes, and Fog2-deficient mice exhibit severe developmental heart defects, suggesting a direct cardiogenic requirement (Tevosian et al., 2000; Svensson et al., 2000). Moreover, these heart defects are rescued by cardiac-specific transgenic expression of Fog2, providing strong evidence for a cardiac autonomous function (Tevosian et al., 2000).

The three Gata factors found in *Drosophila* (*pannier*, *serpent* and *grain*) also play important developmental roles (Abel et al., 1993; Ramain et al., 1993; Winick et al., 1993; Lin et al., 1995; Heitzler et al., 1996; Rehorn et al., 1996; Sam et al., 1996; Riechmann et al., 1998; Gajewski et al., 1999; Brown and Castelli-Gair Hombria, 2000; Calleja et al., 2000; Herranz and Morata, 2001). *serpent* is required for endodermal gut development, mesodermal fat body formation and hematopoiesis. *grain* is involved in filzkorper and head skeleton morphogenesis. *pannier* (*pnr*) is best known for its requirement during embryonic and adult dorsal closure, and for dorsomedial patterning. The *Drosophila* counterpart of Fog2, U-shaped (Ush), can physically interact with Pnr, and (as with Gata4 and Fog2) this interaction is mediated by the N-terminal zinc finger of Pnr, which is thought to antagonize the role of Pnr as a transcriptional activator (Haenlin et al., 1997; Cubadda et al., 1997). At blastoderm, *pnr* and *ush* are expressed in response to the dorsal morphogen encoded by *dpp* (Winick et al., 1993; Jazwinska et al., 1999; Ashe et al., 2000), and are thought to be part of the process that subdivides the dorsal ectoderm (Herranz and Morata, 2001).

It has been proposed that *pnr* promotes myocardial as opposed to pericardial cell fates within the cardiac mesoderm (Gajewski et al., 1999; Gajewski et al., 2001) and that *ush* antagonizes this function (Fossett et al., 2000; Fossett et al., 2001). Recent lineage studies, however, have indicated that some heart progenitors give rise to mixed myocardial/pericardial progeny, but others do not (Park et al., 1998; Ward and Skeath, 2000; Han and Bodmer, 2003; Alvarez et al., 2003), raising the question of how *pnr* functions in different heart progenitor populations. We have re-examined the cardiogenic role of these two genes. We find that *pnr* is required for formation of all *tin*-expressing cardiac progenitors, and loss of *pnr* function results in loss of both myocardial and pericardial cell populations. By contrast, loss of *ush* function did not affect the initial expression of *tin* in the cardiac mesoderm, but is required for its maintenance of expression as well as for the correct differentiation of both myocardial and pericardial cells. Moreover, specific aspects of early cardiac differentiation were preferentially affected: most of the *seven-up* (*svp*)-expressing cells were absent in both mutants, more *ladybird* (*lbe*)-expressing cells were absent in *pnr* than in *ush* mutants, and the heart cells expressing *even-skipped* (*eve*) were only moderately affected in *pnr* and virtually not at all in *ush* mutants. Overexpression of *pnr* in the entire mesoderm produces ectopic *tin* expression, which is strongly antagonized by co-overexpression of *ush*, suggesting a dual role for *ush*: one that is necessary for cardiogenesis and another that counteracts *pnr* function. The heart phenotype of either mutant is rescued by mesoderm-specific expression of wild-type *pnr* or *ush* cDNA, respectively; and mesodermal expression of a dominant-negative form of *pnr* (*pnr*EnR) mimics the heart defects of *pnr* mutants when expressed in the mesoderm. Interestingly, dorsal ectodermal *dpp* expression fades after germband retraction in *pnr* mutants and cardiac differentiation is also compromised when *pnr*EnR is overexpressed in the ectoderm. Moreover, mesoderm-specific expression of *brinker* (*brk*), a repressor of *dpp* target genes (Jazwinska et al., 1999; Zhang et al., 2001), has a similar phenotype as *pnr* mutants or mesodermal *pnr*EnR expression, suggesting that *pnr* may be mediating, at least in part, the cardiogenic *dpp* signal in the mesoderm. Thus, we propose a dual role for *pnr* in heart development: (1) *pnr* functions as a mesodermal target and mediator of the ectodermally derived *dpp* signal by acting in concert with *tinman*; and (2) *pnr* is also required in the ectoderm for maintaining dorsal stripe *dpp* expression.

MATERIALS AND METHODS

Drosophila stocks

The following mutant stocks were used: *pnrVX6* is considered to be a null allele, because it contains a small deletion that eliminates all but nine amino acids of the *pnr*-coding region at the N terminus (Heitzler et al., 1996). *Df(2)ush^{rev18}* is a null allele that deletes the entire gene and some flanking genomic DNA (Cubadda et al., 1997). Misexpression of full-length transgenes was achieved using the Gal4-UAS system (Brand and Perrimon, 1993), using the following stocks: *UAS-pnr* (Haenlin et al., 1997), *UAS-ush* (Cubadda et al., 1997), *UAS-pnrD4* (Haenlin et al., 1997), *UAS-tin* (Ranganayakulu et al., 1998), *UAS-brk* (Jazwinska et al., 1999), *UAS-pnr*EnR (see below), da-Gal4 (Wodarz et al., 1995), ZKr-Gal4 (Frasch, 1995), 69B-Gal4, 24B-Gal4 (Brand and Perrimon, 1993), twi-Gal4 (Greig and Akam, 1993) and twi-Gal4;24B-Gal4 (Lockwood and Bodmer, 2002). twi-Gal4, 24B-Gal4 and twi-Gal4;24B-Gal4 drive expression of UAS constructs exclusively within the entire trunk mesoderm, without detectable expression in the ectoderm. twi-Gal4 initiates expression earlier (at least by stage 9) than 24B-Gal4 (stage 11). ZKr-Gal4 drives expression exclusively in the dorsolateral ectoderm, with highest levels in segments T3-A3, whereas 69B-Gal4 drives expression

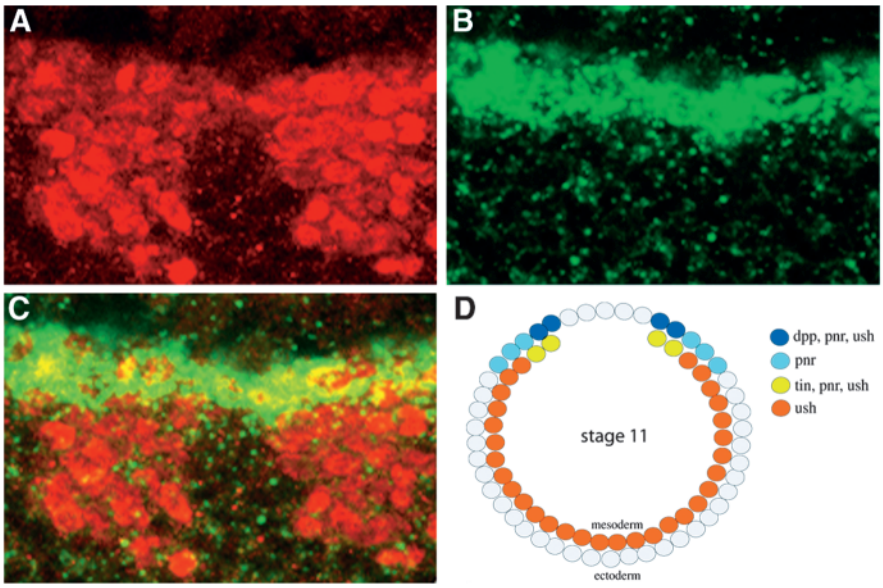


Fig. 1. Expression patterns of *pnr* and *ush* in stage 11 embryos. (A-C) Confocal optical section (2 μm) through the mesoderm of two abdominal segments double labeled for Mef2 (A,C) protein and *pnr* RNA (B,C). Note that *pnr* RNA is present at high levels in the cardiac mesoderm surrounding Mef2 labeled nuclei. (D) A wild-type embryo cross-section showing the relative patterns of *tin*, *pnr*, *ush* and *dpp* expression.

predominantly throughout the ectoderm but with less germ layer specificity than ZKr-Gal4. da-Gal4 drives expression ubiquitously. The following stocks were used for the rescue experiments:

UAS-pnr;pnrVX6/TM3-P[twi-lacZ]
twi-Gal4;pnrVX6/TM3-P[twi-lacZ]
UAS-pnr;pnrVX6,da-Gal4/TM3-P[ftz-lacZ]
Df(2)ush^{rev18}/CyO-P[wg-lacZ];UAS-ush
Df(2)ush^{rev18}/CyO-P[wg-lacZ];24B-Gal4
UAS-pnr;pnrVX6,ZKr-Gal4/TM3-P[ftz-lacZ]

All crosses were performed at 29°C. Combinations of transgene insertions were generated using standard genetic crosses. Oregon-R was used as the wild-type reference strain.

Dominant-negative Pannier

The dominant-negative *pnr* (*UAS-pnr*EnR) was constructed according to the strategy described by Fu et al. (Fu et al., 1998). Basically the construct contains the repressor domain from *engrailed* (EnR, amino acid 2-298) (Jaynes and O'Farrell, 1991; Smith and Jaynes, 1996; Tolkunova et al., 1998) and the two N-terminal zinc-finger domains from *pnr* (amino acid 153-293) (Ramain et al., 1993). The *pnr* zinc-finger domains were PCR amplified from the full-length *pnr* cDNA (5′ primer, CATCTCGAGATGCAGTTCTACTCGCCAAACGCC; 3′primer, GCTCTAGACTACCTCCAAAGTGGAGCCTGTTC) and inserted into *Xho*I- and *Xba*I-digested pUAST vector already containing the EnR domain (Fu et al., 1998; Han et al., 2002). Transgenic flies were generated as previously described (Brand and Perrimon, 1993).

Immunohistochemistry and in situ hybridization

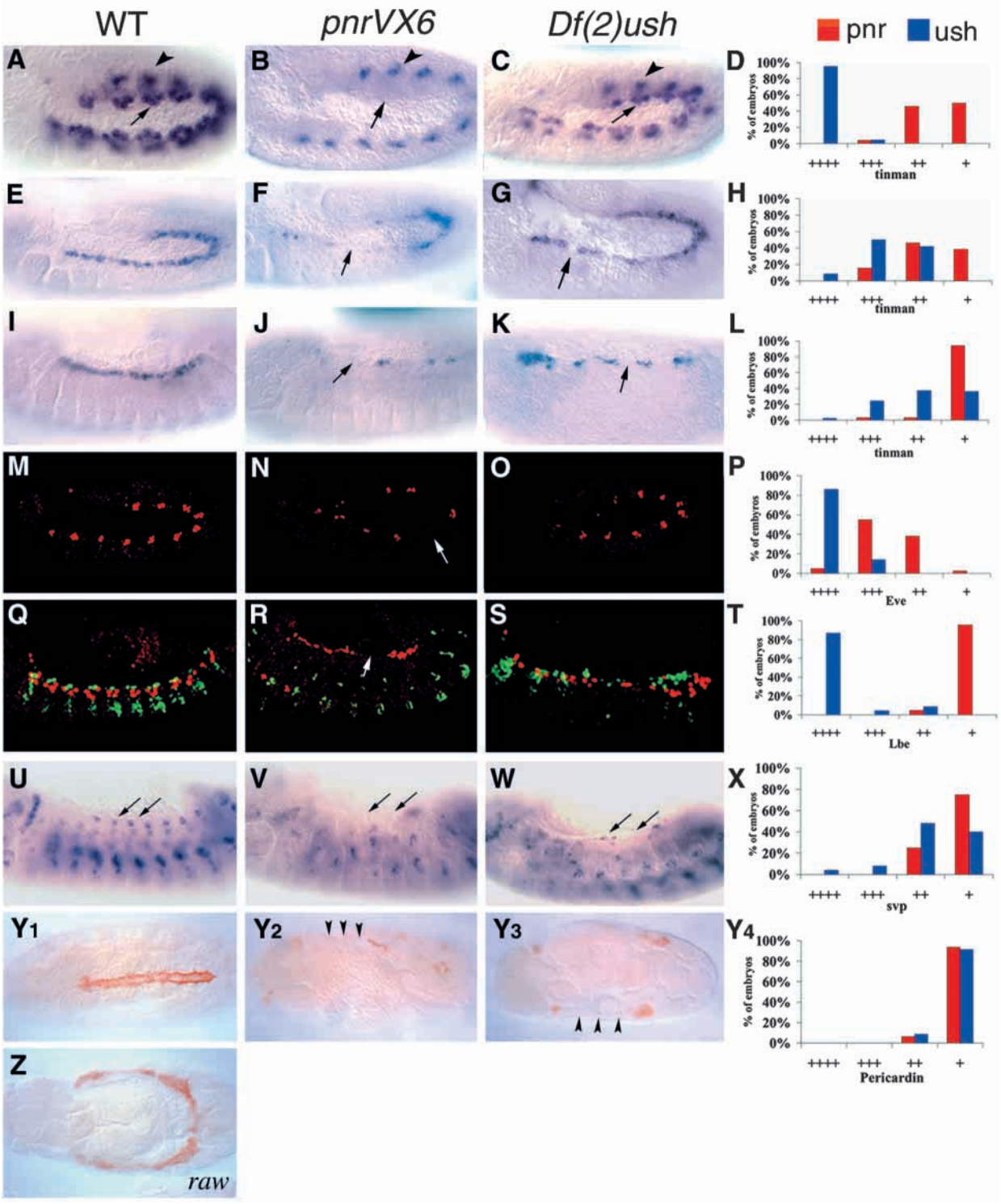
Immunohistochemistry and in situ hybridization were performed as described (Wu et al., 1995), except that Cy3- or FITC-conjugated secondary antibodies (The Jackson Laboratory) were used for fluorescent confocal microscopy. Fluorescent in situ double labeling was performed as described (Knirr et al., 1999). For Lbe staining the TSA Plus Fluorescence System was used (Perkin Elmer). Embryos were mounted in VectaShield (Vector Laboratories). Fluorescent embryo staining was analyzed by using a Zeiss LSM510 confocal microscope. Primary antibodies were used at the following dilutions: rabbit anti-Eve, 1:300 (Frasch et al., 1987); mouse anti-PC 1:10 (Yarnitzky and Volk, 1995); mouse anti-Lbe 1:40 (Jagla et al., 1997); and rabbit anti-Mef2 1:2000 (Lilly et al., 1995). Biotinylated secondary antibodies (Vector Laboratories) were used at 1:200. The following RNA probes were used: the *dpp* probe was generated from the 2.9 kb *dpp* E55 fragment (Padgett et al., 1987), the *tin* probe from a 1.7 kb insert (Bodmer et al., 1990), the *svp* probe from a 3.1 kb insert (Mlodzik et al., 1990), the *pnr* probe from a 1.6 kb fragment (Ramain et al., 1993) and the *Hand* probe from a 0.5 kb insert (Moore et al., 2000).

For expression analysis, 25-50 embryos were used as a sample size. Embryos were placed in categories based on expression: +, less than 1/4 staining or expression when compared with wild type; ++, 1/4 to 1/2; +++, 1/2 to 3/4; ++++, 3/4. When ZKr-Gal4 was used, only the segmentsT3-A3 were assayed.

RESULTS

pnr and *ush* are required for both myocardial and pericardial cell formation

pnr and *ush* are both expressed in the mesoderm at the time of cardiac mesoderm formation (Fig. 1), in addition to their expression in the dorsal ectoderm. Mesodermal expression of *pnr* is restricted to the dorsal cardiogenic margin, whereas *ush* extends more laterally (Fig. 1D) (Gajewski et al., 1999; Fossett et al., 2000). In order to assess the requirement for *pnr* and *ush* in initiating cardiac mesoderm and cardiac cell type-specific differentiation, we first examined *tin* expression at progressively later developmental stages in null mutants for both *pnr* and *ush*. During mid-stage 11, *tin* is expressed segmentally in two regions of the mesoderm (Fig. 2A). The dorsal clusters of cells correspond to the cardiac precursor cells, whereas the lateral clusters will become part of the visceral mesoderm. In same stage *pnr* mutant embryos, *tin* expression is dramatically reduced in the clusters that correspond to the cardiac precursors, indicating that cardiogenesis is not being initiated (Fig. 2B,D). *tin* expression in the visceral mesodermal clusters, as well as *tin* expression earlier in development, is unaffected, suggesting the heart is a focal point for *pnr* function, which is consistent with its cardiac-restricted expression in the mesoderm (Fig. 1) (Gajewski et al., 1999). By contrast, *ush* mutant embryos initially seem to exhibit normal *tin* expression (Fig. 2C,D). At later stages, when *tin* expression is solely restricted to the heart



cells, *ush* mutants display a progressively more severe reduction in *tin* expression, approaching the phenotype of *pnr* mutants (Fig. 2E-L). Thus, both *pnr* and *ush* are required for heart-specific *tin* expression, although *ush* seems to be initially dispensable.

Even though *tin* is initially expressed in all heart progenitors, its expression is later turned off in some specific lineages, but continues to be expressed in many myocardial and pericardial cells (Bodmer, 1993; Ward and Skeath, 2000; Venkatesh et al., 2000; Han et al., 2002). To determine which heart cells are affected in *pnr* and *ush* mutants, we examined mutant embryos with various markers. *eve*, for example, is co-expressed with *tin* in 11 clusters of heart progenitors (Fig. 2M), and these lineages give rise to a subset of pericardial cells (Frasch et al., 1987). *eve* expression is only moderately reduced in *pnr* and hardly at all in *ush* mutants at early as well as later stages (Fig. 2M-S; note, however, the patterning defects at progressively later stages in Fig. 2R,S). By contrast, the *lbe*-expressing heart

Fig. 2. *pnr* and *ush* are required for myocardial and pericardial cell formation. (A-L) *tin* expression. (A-D) Mid-stage 11. (A) Wild-type embryo expressing *tin* segmentally in two clusters of cells. The dorsal clusters (arrow) correspond to the cardiac precursors and the lateral clusters (arrowhead) correspond to visceral mesoderm. (B) *pnr* mutant embryo exhibiting normal *tin* expression only in the lateral clusters (arrowhead), but not in dorsal clusters (arrow). (C) *ush* mutant expressing *tin* normally. (D) Histogram of *tin* expression in the heart progenitors of *pnr* and *ush* mutants at mid-stage 11. (E-H) Late-stage 11. (E) Wild-type embryos expressing *tin* in the cardiac mesoderm. (F) *pnr* and (G) *ush* mutants exhibiting a reduction in *tin* expression (arrow). (H) Histogram of *tin* expression in the heart progenitors of *pnr* and *ush* mutants at late-stage 11. (I-L) Stage 13 embryos. (J) *pnr* and (K) *ush* mutants exhibiting reduced *tin* expression (arrow). (L) Histogram of *tin* expression in the heart progenitors of *pnr* and *ush* mutants at stage 13. (M-P) Late stage 11 embryos stained for Eve. (M) Wild-type embryo expressing Eve in 11 clusters of cells. (N) In *pnr* mutants, the number of Eve cells is reduced (arrow). (O) In *ush* mutants, Eve stained cardiac clusters are indistinguishable from wild type. (P) Histogram of Eve expression in late stage 11 *pnr* and *ush* mutants. (Q-T) Stage 13 embryos stained for Eve (red) and Lbe (green). (Q) Wild-type embryo. (R) *pnr* mutant embryo exhibiting dramatically reduced Lbe staining and moderately reduced Eve staining (arrow). (S) *ush* mutant embryo exhibiting near normal amounts of Lbe and Eve staining, although the segmental pattern is perturbed (compounded by defects in germ band retraction). (T) Histogram of Lbe expression in stage 13 *pnr* and *ush* mutants. (U-X) Stage 13 embryos expressing *svp* RNA in the cardiac mesoderm (indicated by arrows). (U) Wild type. (V) *pnr* and (W) *ush* mutant embryos exhibiting severely reduced *svp* expression in the heart. (X) Histogram of *svp* expression in stage 13 *pnr* and *ush* mutants. (Y_1-Z) Dorsal view of stage 16 embryos stained for the late pericardial cell marker Pericardin (Yarnitzky and Volk, 1995; Chartier et al., 2002). *pnr*, *ush* and *raw* mutants do not complete dorsal closure. (Y_1) Wild type. (Y_2) *pnr* and (Y_3) *ush* mutants exhibiting a severe decrease in pericardial cells (arrowheads). (Y_4) Histogram of Pericardin expression in stage 16 *pnr* and *ush* mutants. (Z) Dorsal open *raw* mutant exhibiting an excess in pericardial cell staining.

progenitors, which produce both myocardial and pericardial cells, are dramatically reduced in *pnr* but less so in *ush* mutants (Fig. 2Q-T). Moreover, the *svp*-expressing cells, which also give rise to a mixed lineage, but cease to co-express *tin* at later stages, are dramatically reduced in both mutants (Fig. 2U-X). Thus, all lineage markers we assayed are reduced in both mutants, but each is affected with disproportional severity, which is consistent with the idea that the formation of each cell type has a direct requirement for *pnr* and *ush*.

By stage 16, dorsal closure is complete and the linear heart tube has assembled beneath the dorsal midline. A general marker for pericardial cells shows a severe reduction in these cells in both mutants (Fig. 2Y_1-Y_4). As *pnr* and *ush* mutants fail to undergo dorsal closure, we wanted to determine if this process was a prerequisite for cardiac cell-type specification, by perhaps causing heart defects indirectly. As a test of this hypothesis, we examined another dorsal closure mutant, *raw* (Byars et al., 1999), in which we observe pericardial cell staining that is normal or in excess along the dorsal mesoderm (Fig. 2Z). This increase in cardiac differentiation is probably due to an excess in *dpp* signaling. Thus, a dorsal open phenotype in itself is insufficient to compromise cardiac differentiation.

pnr can activate but not efficiently maintain ectopic *tin* expression

Analysis of *pnr* and *ush* mutants suggests that both genes functions are required for heart formation. In order to explore their functional relationship in heart development further, we performed overexpression studies. When *pnr* is expressed throughout the mesoderm, *tin* expression is no longer confined to the heart precursors by late stage 11, but is expanded laterally throughout the mesoderm, suggesting that *pnr* is sufficient to ectopically initiate *tin* expression within the mesoderm (Fig. 3A,B). This is in contrast to mesodermal overexpression of *tin*, which does not seem to cause significant initiation of cardiogenesis without spatially intersecting with *dpp* (and *wg*) signaling (Lockwood and Bodmer, 2002). Much of this lateral expansion of *tin* driven by ectopic *pnr* does not persist beyond stage 13, where ectopic *tin* is reduced to small ventrolateral cell clusters (Fig. 3H). These results suggest that *pnr* can activate early ectopic expression of *tin*, but by itself is insufficient to maintain it at significant levels.

ush is likely to play a dual role in heart development

As *ush* is required to maintain *tin* expression in the heart-forming region, we wanted to see if *ush* can also provide a maintenance role ectopically. Pan-mesodermal *ush* expression, however, does not cause an expansion but rather a reduction of cardiac-specific *tin* expression (Fig. 3C,I), similar to *ush* loss of function (Fig. 2G,K). These findings suggest that a correct amount of *ush* activity is crucial for heart development, which is consistent with a model in which Ush and Pnr act in a multiprotein complex. To examine this idea further, we co-overexpressed both genes throughout the mesoderm. Similar to overexpressing *ush* alone, co-overexpression results in a reduction in *tin* expression (Fig. 3D,J), unlike what is observed with overexpression of *pnr* alone, suggesting that excess *ush* inhibits the level of *tin* activation by *pnr* in normal as well as ectopic locations. This repressor function of *ush* is reminiscent of its role in adult mechanosensory bristle formation and thorax development (Cubadda et al., 1997; Sato and Saigo, 2000; Tomoyasu et al., 2000). These results further support the idea that the appropriate level of *ush* activity is crucial for correct heart development.

Previous data suggest that Ush exerts its inhibitory activity by binding to the N-terminal zinc finger of Pnr, an interaction that is blocked in the allele *pnrD4*, which has an amino acid substitution in this domain and thereby abolishes Ush binding to Pnr (Haenlin et al., 1997). When we overexpressed this gain-of-function allele of *pnr* in the mesoderm, we also observed ectopic induction of ventrolateral *tin* expression in late stage 11 embryos (Fig. 3E), as with overexpression of the wild-type form of *pnr* (Fig. 3B). At later stages, however, ectopic *tin* levels increase dramatically in the ventrolateral mesoderm and exceed those of wild-type *pnr* mesodermal overexpression (Fig. 3H,K). Unlike co-overexpression of wild-type *pnr* and *ush*, using *pnrD4* in conjunction with *ush* does not cause a *ush*-like phenotype but rather one like *pnrD4*, which produces ectopic *tin* expression (Fig. 3L), suggesting that *ush* is unable to inhibit the gain of function of this *pnr* allele. Taken together, these data are consistent with a dual function of *ush*: (1) a positive role in maintaining *tin* expression within the cardiogenic region and (2) a negative role in limiting the level

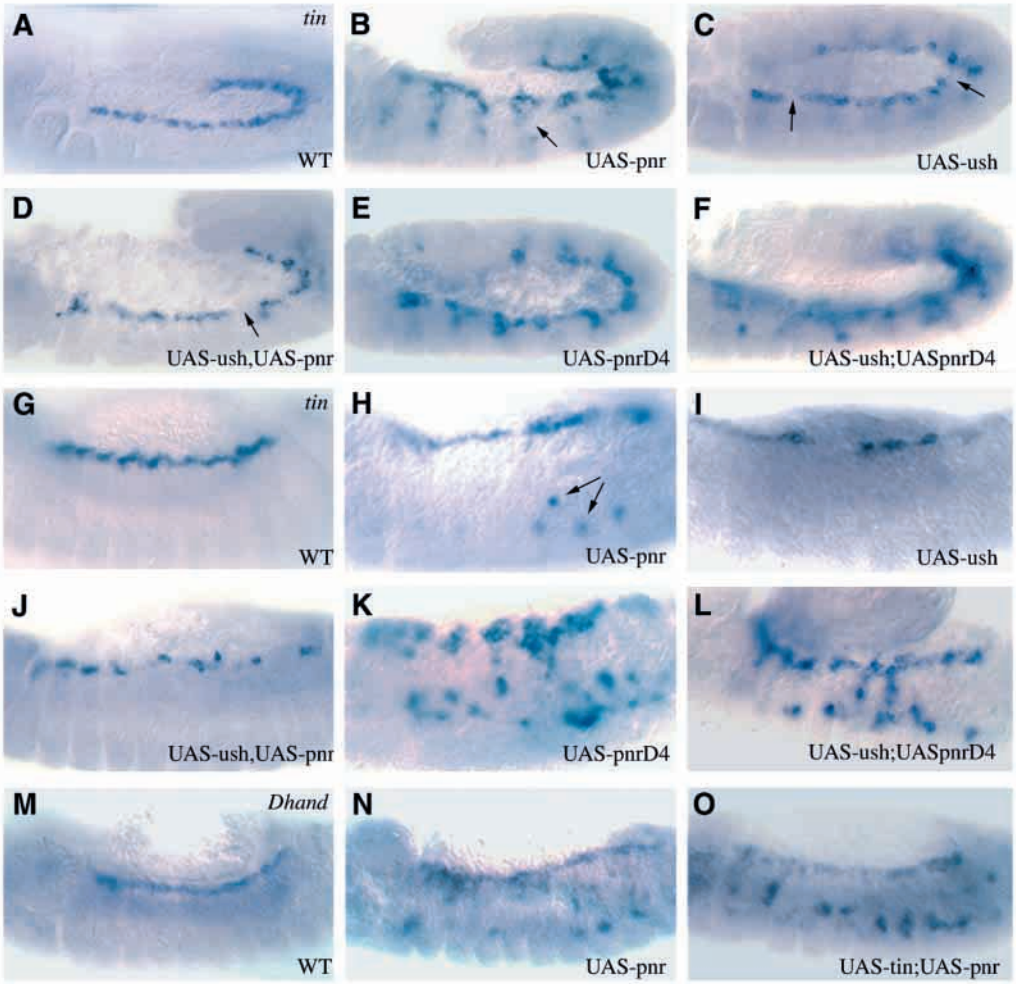


Fig. 3. Pan-mesodermal expression in progeny of the cross between twi-Gal4;24B-Gal4 driver and UAS-cDNA containing transgenic flies. (A-F) *tin* expression in late-stage 11 embryos. (A) Wild type. (B) *UAS-pnr* embryo shows ectopic expression in the ventrolateral mesoderm (arrow). (C) *UAS-ush* embryo shows unaltered or slightly reduced *tin* expression (arrows). (D) *UAS-ush,UAS-pnr* embryo shows a moderate reduction in *tin* expression (arrow). (E) *UAS-pnrD4* shows an increase in *tin* expression in the ventrolateral mesoderm, similar to *UAS-pnr* embryos (B). (F) *UAS-ush;UAS-pnrD4* embryo shows an increase in *tin* expression in the ventrolateral mesoderm, similar to *UAS-pnrD4* embryos (E). (G-L) *tin* expression in stage 13 embryos. (G) Wild type. (H) *UAS-pnr* embryo shows moderate ectopic expression in the ventrolateral mesoderm (arrows). (I) *UAS-ush* embryo shows a moderate reduction in *tin* expression. (J) *UAS-ush,UAS-pnr* embryo shows a similar decrease in *tin* expression as in *UAS-ush* embryos (I). (K) *UAS-pnrD4* embryo shows dramatic ectopic expression in the ventrolateral mesoderm. (L) *UAS-ush;UAS-pnrD4* embryo shows a similar increase in ectopic *tin* expression as in *UAS-pnrD4* embryos (K). (M-O) *Hand* expression in stage 13 embryos. (M) Wild type. (N) *UAS-pnr* embryo shows moderate ectopic expression in the ventrolateral mesoderm, as with *tin* (H). (O) *UAS-tin;UAS-pnr* embryo shows an increase in ectopic *Hand* expression in the ventrolateral mesoderm that is comparable with *tin* expression in embryos with mesodermal overexpression of *UAS-pnrD4* (K).

and spatial distribution of *pnr* activity (see Fig. 1D for normal patterns of expression).

To determine if *pnr* cooperated with *tin* in heart formation, we examined other markers of cardiac-specific differentiation. Similar to the presence of ectopic *tin* (Fig. 3H), ectopic expression of *Hand*, a general heart marker (Fig. 3M) (Kolsch and Paululat, 2002), is also observed ventrolaterally when *pnr* is induced throughout the mesoderm (Fig. 3N). Interestingly, more ectopic *Hand* expression is induced by co-overexpressing *pnr* as well as *tin* (Fig. 3O), similar to the extent of ectopic *tin* with pan-mesodermal *pnrD4* (Fig. 3K). This indicates that *pnr* and *tin* act synergistically in their ability to induce heart formation (overexpression of *tin* alone does not cause ectopic heart induction) (see Lockwood and Bodmer, 2002), and that the presence of 'activated' PnrD4 is sufficient to sustain heart formation.

pnr and *ush* are required within the mesoderm and ectoderm for heart development

It is well established that both ectodermal and mesodermal patterning information is required for heart development (Bodmer, 1993; Azpiazu and Frasch, 1993; Frasch, 1995; Wu et al., 1995; Park et al., 1996; Azpiazu et al., 1996; Lockwood and Bodmer, 2002). As *pnr* and *ush* are expressed in both of these germlayers (Fig. 1) (Winick et al., 1993; Heitzler et al., 1996; Calleja et al., 2000; Gajewski et al., 1999; Fosset et al., 2000; Herranz and Morata, 2001), it is possible they are required for heart development in either or both germlayers. We already showed that mesodermal overexpression of *pnr* and *ush* alters *tin* expression, demonstrating that these two genes can influence heart development within the mesoderm. In order to test for a specific germlayer requirement directly, we overexpressed these genes in the respective mutant background either in the mesoderm or the ectoderm (see Materials and Methods). We then assayed for restoration (i.e. rescue) of *tin* expression within the heart-forming mesoderm of these rescue embryos. When *pnr* or *ush* is rescued in the mesoderm specifically, 57% and 52% of the embryos, respectively, show cardiac-specific *tin* expression that is restored close to wild-type levels (Fig. 4A-C). The ubiquitous da-Gal4 driver confers

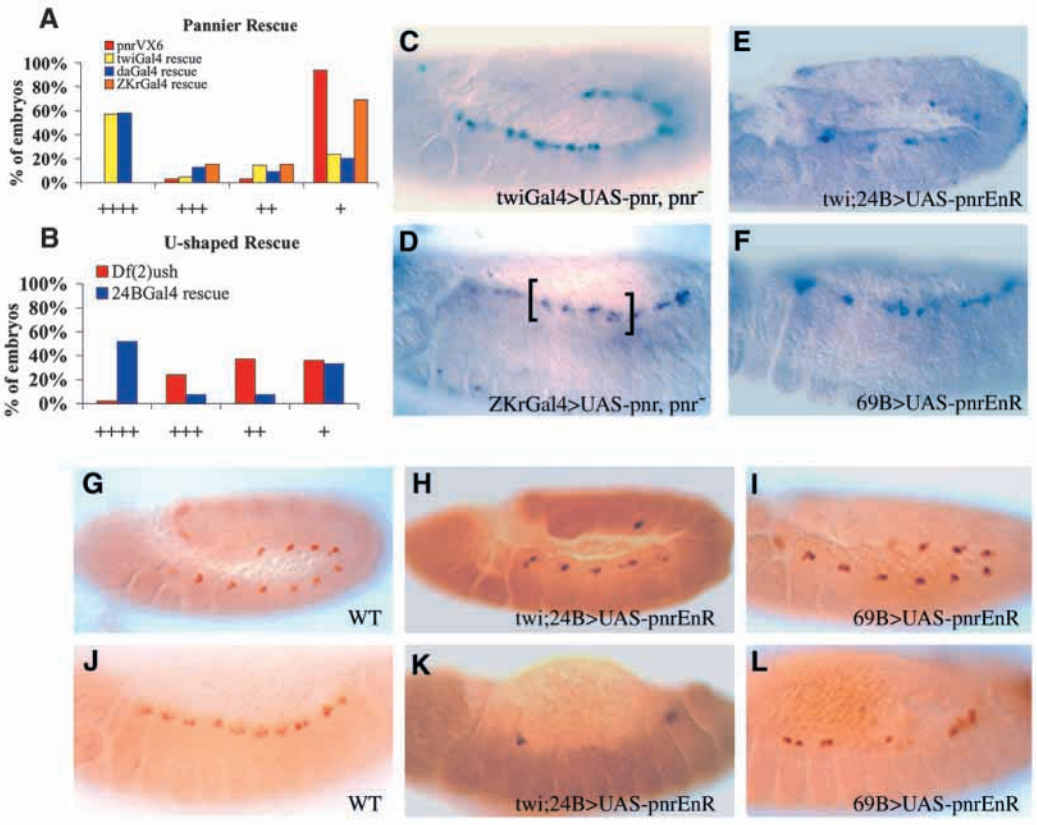


Fig. 4. Germ layer-specific requirement of *pnr* and *ush* for heart formation. (A,B) Histograms of *tin* expression in stage 13 *pnr* and *ush* mutants with ('rescue') or without mesodermal overexpression of wild-type cDNA for *pnr* and *ush*, respectively (see Materials and Methods). (A) Mesodermal (yellow) and ubiquitous (blue) *pnr* restores *tin* expression when compared with *pnr* mutants (red); however, ectodermal rescue (orange) moderately restores *tin* expression in a small percentage of embryos. (B) Mesodermal (blue) *ush* rescue also restores *tin* expression in a large proportion of embryos when compared with *ush* mutants (red). (C) *pnr* mesodermal rescued embryo shows restored *tin* expression, when compared with wild type (Fig. 2E). (D) *pnr* ectodermal rescued embryo exhibits moderately decreased *tin* expression (brackets indicate the embryonic domain affected with the ZKr-Gal4 driver). (E-L) Overexpression of *UAS-pnr*EnR (see Materials and Methods) in either the mesoderm or the ectoderm. *tin* (E,F) and Eve (G-L) expression. (E,G-I) Late stage 11. (F,J-L) Stage 13. (E) Mesodermal overexpression of *pnr*EnR causes a dramatic reduction in *tin* expression already at late stage 11. (F) Ectodermal overexpression causes a moderate reduction in *tin* expression that occurs only in later stage embryos. (G,J) Wild type. (H,K) Mesodermal overexpression of *pnr*EnR causes a decrease in mesodermal Eve, similar to *pnr* mutants (Fig. 2N). (I,L) Ectodermal overexpression causes a moderate reduction of Eve only in later stage embryos (L).

similar levels of rescue (Fig. 4A), which suggests that forced mesodermal expression of these genes is sufficient to initiate proper heart formation. However, this interpretation does not exclude the possibility that ectodermal *pnr* and *ush* expression is also a contributor to heart-specific *tin* expression. Ectoderm-specific rescue of *pnr*, using ZKr-Gal4 (Frasch, 1995) (see Materials and Methods), restores a considerable amount of *tin* expression in a small but significant number of *pnr* mutant embryos (Fig. 4A,D), suggesting that *pnr* activity in the ectoderm can also contribute to cardiogenesis. Because the level of ectodermal rescue is low, we cannot rule out that this ectodermal driver also allows low levels of mesodermal expression, which may be sufficient to achieve considerable rescue. Nevertheless, these results are consistent with the hypothesis that *pnr* and *ush* are mediators of an ectodermal cardiogenic signal within both germlayers.

To test the idea further that *pnr* is normally required in both germ layers, we interfered with *pnr* activity by expressing a dominant-negative form of *pnr* (*pnr*EnR, see Materials and Methods) in the ectoderm or the mesoderm. When *pnr*EnR is expressed throughout the mesoderm, a dramatic decrease in *tin* expression is observed (Fig. 4E). When *pnr*EnR is expressed in the ectoderm using the 69B-Gal4 driver, which is broader but slightly less ectoderm-specific than ZKr-Gal4, early *tin* expression is undiminished (data not shown), but at later stages a moderate decrease is observed (Fig. 4F, typical of 10% of the embryos). Similar observations were obtained when assayed for Eve staining (Fig. 4G-L), except that Eve is affected less than *tin* at early stages, similar to *pnr* null mutants (Fig. 4E,H, compare with Fig. 2D,P). The fact that interference with *pnr* function predominantly in the ectoderm leads to a reduction in cardiac differentiation indicates strongly that *pnr* function is normally required not only in the mesoderm, but also in the ectodermal germlayer in order to achieve wild-type levels of cardiogenesis. As *pnr* codes for a transcription factor, its ectodermal role in heart formation is probably indirect, requiring induction across germlayers.

Maintaining *dpp* expression in the dorsal ectoderm requires *pnr* and *ush*

As previously described, the expression patterns of *pnr* and *ush* are initially broadly induced by *dpp* in the dorsal ectoderm (Winick et al., 1993; Ashe et al., 2000). Later, these expression patterns are further refined but continue to overlap spatially with ectodermal *dpp* (as well as *wg*) expression, but their genetic relationship at later stages is not known. The maintenance of *dpp* expression in a thin dorsal ectodermal stripe (Fig. 5A) is thought to be essential for controlling dorsal morphogenesis and closure by regulating a number of target genes (Winick et al., 1993; Heitzler et al., 1996; Calleja et al., 2000; Herranz and Morata, 2001). As *pnr* also exhibits an ectodermal requirement for heart development, we

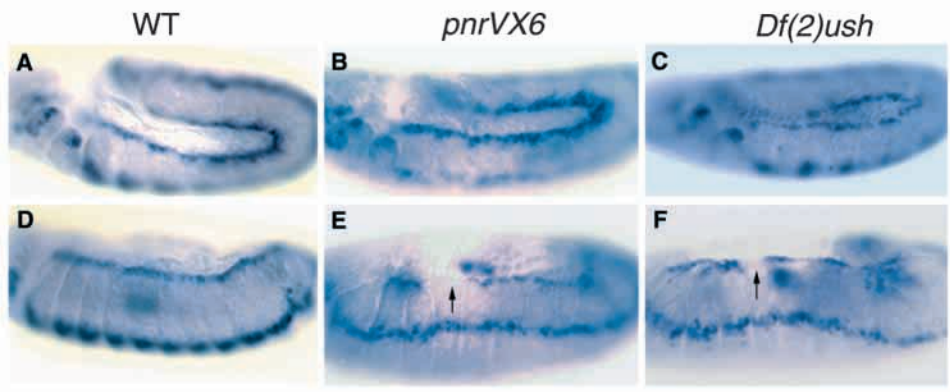


Fig. 5. *pnr* and *ush* mutants exhibit reduced *dpp* expression. (A-C) Stage 11. (D-F) Stage 13. (A,D) Wild-type *dpp* expression. *dpp* is expressed in two stripes, one along the dorsal edge of the ectoderm and the other more laterally. *pnr* and *ush* mutants exhibit normal *dpp* expression at stage 11 (B,C), but reduced expression at later stages (E,F). Note gaps in *dpp* expression (arrows).

hypothesized that *pnr* may be needed for maintaining late *dpp* expression (Herranz and Morata, 2001), which in turn contributes to the progression of cardiogenesis (Lockwood and Bodmer, 2002). A late role for *dpp* in maintaining cardiogenesis has been difficult to ascertain, because the stage 11 dorsal stripe expression could not be abolished easily or selectively. When we examined *dpp* expression in *pnr* and *ush* mutant embryos, we find that dorsal ectodermal stripe expression of *dpp* is present at stage 11, but is progressively reduced after germband retraction (Fig. 5). This finding is consistent with the idea that ectodermal *pnr/ush* function acts via maintenance of *dpp* in a dorsal stripe overlaying the forming heart. Thus, *pnr/ush* is likely to play a crucial role in a crossregulatory network of the cardiogenic function of *dpp*: first by mediating the early Dpp signal within the mesoderm and later by maintaining ectodermal *dpp* expression.

To test if the immediate target genes of the cardiogenic Dpp signal transduction pathway are activated within the mesoderm or in the ectoderm or both, we examined the cardiogenic role of *brk*, a transcriptional repressor of *dpp* targets (Sivasankaran et al., 2000; Kirkpatrick et al., 2001; Rushlow et al., 2001; Saller and Bienz, 2001; Zhang et al., 2001). When *brk* is expressed throughout the mesoderm, there is a considerable reduction in cardiogenesis as assayed by *tin* and *eve* expression (Fig. 6A-D), similar to what is observed in *pnr* mutants and mesodermal expression of *pnr*EnR. This suggests that mesodermal *pnr* maybe a primary target of the cardiogenic Dpp signal. Moreover, *brk* overexpression in the ectoderm with the early onset ZKr-Gal4 driver selectively reduces cardiac-specific *tin* expression as early as stage 11 (Fig. 6E, compare with Fig. 2B). This is unlikely to be due solely to an elimination of ectodermal *pnr* expression, which causes a weaker and later-onset reduction of cardiac *tin* (Fig. 4F). Therefore, we examined if *dpp* expression itself is inhibited by ZKr-Gal4>*UAS-brk*. Indeed, *dpp* expression is significantly reduced within the ZKr-Gal4 expression domain already at stage 11 (Fig. 6G), which is much earlier than is the case in *pnr* mutants, suggesting that *dpp* is a direct target of *brk*. By contrast, when *brk* is overexpressed with the later onset ectodermal driver, 69B-Gal4, *tin* expression appears to be reduced later and only slightly (Fig. 6F),

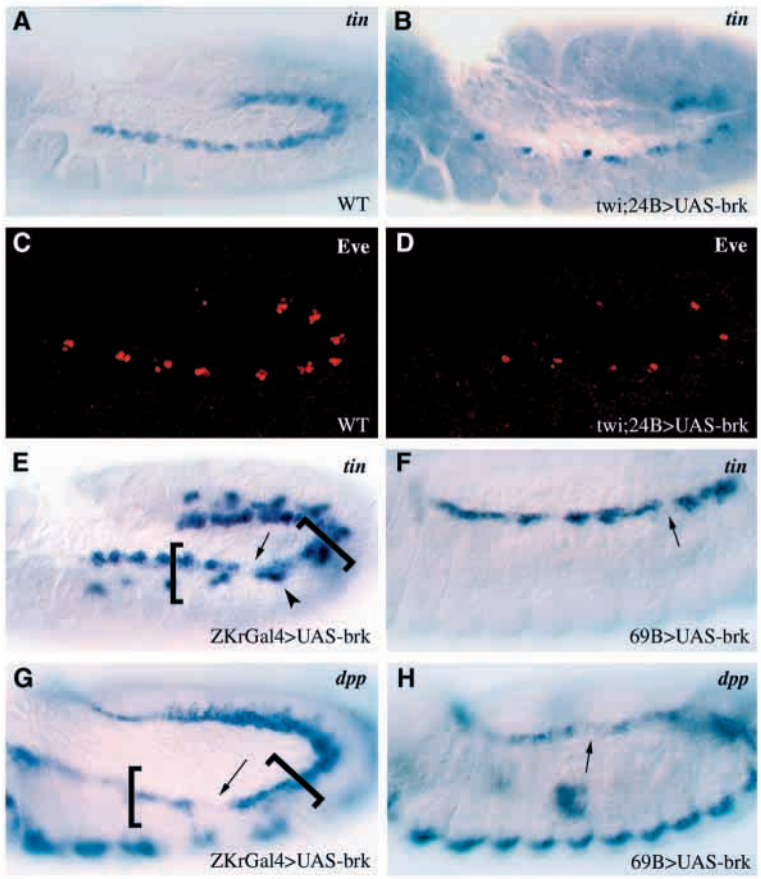


Fig. 6. Mesodermal or ectodermal overexpression of *brk* causes a decrease in *tin* (A,B,E,F), Eve (C,D) and *dpp* (G,H) expression. (A,C) Late stage 11 wild-type embryos. (B,D) Late stage 11 twi-Gal4;24B-Gal4>*UAS-brk* embryos exhibiting a severe reduction in *tin* expression (B) and in the number of Eve clusters (D). (E) Mid-stage 11 ZKr-Gal4>*UAS-brk* embryo exhibiting a selective reduction of cardiac (arrow), but not visceral (arrowhead) *tin* expression in the domain affected by the ZKr-Gal4 driver (brackets). (F) 69B-Gal4>*UAS-brk* embryo showing a slight reduction in cardiac *tin* expression (arrow) at stage 13, but not at earlier stages (data not shown). (G) Mid-stage 11 ZKr-Gal4>*UAS-brk* embryo exhibiting a selective reduction of dorsal ectodermal *dpp* expression (arrow) in the Kr domain (brackets). (H) 69B-Gal4>*UAS-brk* embryo showing a moderate reduction in dorsal ectodermal *dpp* expression (arrow) at stage 13, but not at earlier stages (data not shown).

accompanied by a weak and late reduction of *dpp* expression (Fig. 6H). These data suggest that the Dpp pathway directly affects targets in the mesoderm, and that *pnr/ush* (along with *tin*) are likely mediators and effectors of *dpp* signaling that is necessary for proper heart development (illustrated in Fig. 7).

DISCUSSION

It has been previously reported that *pnr* promotes myocardial cell fates and opposes that of the Eve pericardial cells (Gajewski et al., 1999), whereas the function of *ush* was to limit the development of both by interfering with dorsal spreading of the ventrally invaginated mesoderm (Fossett et al., 2000). In this study, we present evidence that *pnr* and *ush* are part of the initiation and maintenance process of cardiogenesis, respectively, and that they are required for the formation of both myocardial and pericardial cell fates. In *pnr* mutants, *tin* expression is normal until early stage 11, but by mid- to late-stage 11 becomes dramatically reduced along the dorsal mesodermal edge, where the heart precursors normally form, indicating a failure to specify cardiac mesoderm. By contrast, cardiac *tin* expression in *ush* mutants appears normal initially, and only later begins to exhibit a pronounced decrease in *tin* expression, considerably after dorsal mesodermal migration is complete, unlike what was observed in migration-defective *heartless* mutants (Gisselbrecht et al., 1996), indicating *ush* is involved in maintaining cardiac differentiation.

Even though *tin* expression is dramatically reduced in early and late stage *pnr* and *ush* mutants, respectively, cardiac subtype-specific gene expression is not affected equally. In stage 13 embryos, *eve*-, *lbe*- and *svp*-expressing cells were more affected in *pnr* than in *ush* mutants, presumably because the reduction in cardiac *tin* expression occurs earlier in *pnr* than in *ush* mutants. The largest difference in susceptibility to *pnr* relative to *ush* was observed with *lbe* expression. Of the three cardiac cell type-specific markers, Eve is the least sensitive to *pnr* loss-of-function. We speculate that this difference may be due to direct versus indirect (via *tin*) regulation of the relevant enhancers by *pnr*.

Ectopic ventrolateral *tin* expression is observed when *pnr* is overexpressed in the mesoderm. This expansion in *tin* expression is reminiscent to what is observed when *dpp* is expressed throughout the mesoderm (Lockwood and Bodmer, 2002). This raises the question of whether *pnr* directly regulates *tin* expression, or indirectly through *dpp* (or both). As shown previously, global overexpression of *pnr* causes ectopic *dpp* expression in the ectoderm (Herranz and Morata, 2001). However, we find that pan-mesodermal overexpression of *pnr* does not cause an expansion of *dpp* expression in the mesoderm or the ectoderm (data not shown). This suggests that *pnr* must be able to activate the expression of *tin* either by itself or with some other factors, excluding *dpp*, in this overexpression assay. This does exclude the possibility that normally *pnr* and ectodermal Dpp signaling could act in parallel to activate *tin* expression in the heart primordial (see below). The ability of *pnr* to activate *tin* is likely to be direct, as a heart-specific enhancer of *tin* (Venkatesh et al., 2000) contains several consensus Gata sites (M. Liu and R.B., unpublished). As shown by transcription assays (Gajewski et al., 2001), *pnr* is also a likely direct target of *tin*, suggesting

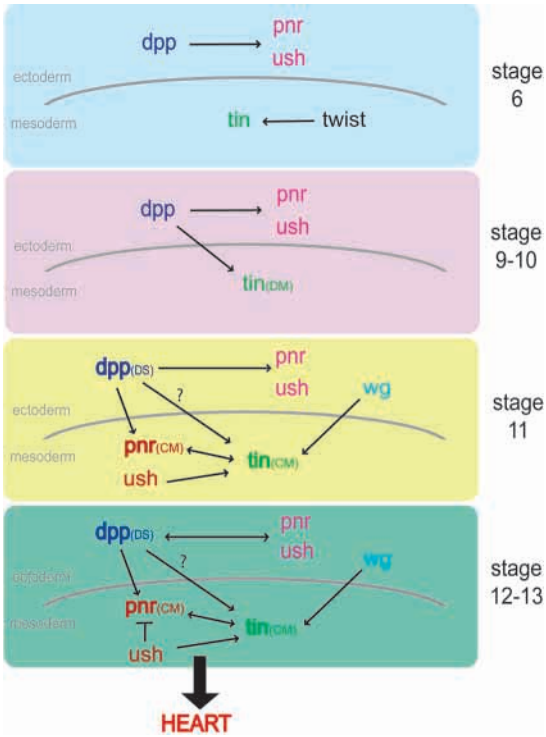


Fig. 7. The genetic network involved in *Drosophila* heart development. In the early embryo (stage 6), both *pnr* and *ush* are induced by *dpp* in the early ectoderm. Twist, a bHLH factor, induces tin expression in the early mesoderm. By stage 9/10 *tin* expression is restricted to the dorsal mesoderm (DM) via *dpp* signaling from the ectoderm. By stage 11, when *dpp* is expressed in a thinner dorsal stripe (DS), *pnr* expression is initiated in the presumptive cardiac mesoderm (CM) at the dorsal mesodermal margin, presumably by *dpp* and *wg* signaling from the ectoderm in the context of *tin* (DM). By late stage 11, we propose that *pnr* (in conjunction with ectodermal *dpp* and *wg* signaling) initiates the expression of *tin* in the cardiac mesoderm. By late stage 11 and stage 12, *ush* is needed to help maintain the expression of *tin* in the cardiac precursors. By stage 12/13, *pnr* and *ush* are also needed to maintain ectodermal *dpp* expression in the dorsal stripe and are also mediating the ectodermal signal of *dpp* in the mesoderm. In these later stages, *ush* may also be needed to limit the ability of *pnr* to activate *tin* expression in the ventrolateral mesoderm. Based on the data presented here, we propose the model that during the spatial convergence of *dpp*, *wg* and *tin* during cardiogenesis, the crucial mediator and executioner of the *dpp* signal is likely to be *pnr*.

that they both contribute to maintaining each other's expression. Both *tin* and *pnr* have been shown to be targets of Dpp signaling at stage 9/10 (Xu et al., 1998; Ashe et al., 2000). We propose that *dpp* is necessary again at stage 11 to activate and maintain *pnr* and *tin* expression in the cardiogenic region of the mesoderm (Fig. 7). First, *pnr* is activated with the help of early stage 11 *tin*, which is expressed broadly throughout the dorsal mesoderm, and *dpp*, which is expressed in a narrow dorsal ectodermal stripe. Then, at mid-stage 11, *tin* is restricted to the cardiogenic region with the help of mesodermal *pnr* as well as continuous ectodermal Dpp signaling. Once both are activated in the cardiogenic mesoderm, they are likely to contribute to the maintenance of each other's expression, probably aided again, but only moderately, by ectodermal Dpp

signaling. This interpretation is consistent with mesodermal versus ectodermal expression of dominant-negative *pnr*EnR (Fig. 4) and the *dpp* target repressor encoded by *brk* (Fig. 6). They are both equally effective in reducing cardiac-specific *tin* when expressed in the mesoderm, but ectodermal repression is more effective when dorsal-stripe *dpp* at stage 11 is also affected (as in the case of ZKr-Gal4>*UAS-brk* shown in Fig. 6G, but not with ZKr-Gal4>*UAS-pnr*EnR, data not shown).

Mesodermal overexpression of *ush* and co-overexpression with *pnr* results in a decrease in the amount of cardiac-specific *tin* expression, suggesting that *ush* may not only be required along with *pnr* for heart development, but also play an inhibitory role. To test this hypothesis further, we overexpressed *pnrD4*, an allele that abolishes Ush binding to Pnr, and found not only ectopic *tin* expression at early stages of cardiogenesis, but also undiminished and even increased levels of expression at later stages. A similar phenotype was observed when both *pnrD4* and *ush* were expressed throughout the mesoderm, suggesting that *ush* plays an anti-cardiogenic role by antagonizing the activity of wild-type Pnr, but not that of PnrD4. It would be interesting to see if pan-mesodermal overexpression of wild-type *pnr* in a *ush* mutant background results in ectopic *tin* expression similar to *pnrD4*, or if a minimal amount of *ush* activity is required to maintain normal and ectopic *tin* expression even with forced *pnr* expression. Interestingly, overexpression of both *pnr* and *tin* together in the mesoderm also causes a *pnrD4*-like phenotype, as assayed with *Hand* expression, suggesting that *pnr* and *tin* collaborate during initiation and subsequent differentiation of the heart progenitors.

Although in vitro the Ush-related FOG factors are primarily known for their role as transcriptional repressors (Svensson et al., 1999; Tevosian et al., 1999), they apparently can also function as co-activators: Fog2 can synergistically activate or repress the transcriptional activity of Gata4, depending on the (cardiac) promoter and cell line used (Lu et al., 1999), and FOG-1 can cooperate with Gata1 to transactivate NF-E2, an erythroid cell-specific promoter (Tsang et al., 1998). Moreover, the ventricular hypoplasia and other heart defects observed in Fog2-deficient mice suggest a deficit rather than an excess in heart development (Tevosian et al., 2000; Svensson et al., 2000). In addition, mice with an equivalent mutation to PnrD4 knocked into the Gata4 locus, thus eliminating binding to Fog2, exhibit in many ways a similar phenotype to Fog2-deficient mice (Crispino et al., 2001). These data are consistent with the idea that Fog2 is normally involved in promoting rather than antagonizing cardiogenesis, similar to what we have found with our genetic studies during *Drosophila* heart development.

The dual role of Ush suggests that the amount of Ush may be crucial for whether it exerts its function as a an activator or repressor, perhaps by binding to different sets of co-factors in a concentration-dependent manner. Alternatively, the mode of transcriptional regulation by Ush could be stage-dependent: at stage 11, Pnr and Ush cooperate as transcriptional activators in initiating cardiac-specific *tin* expression and heart development, but later Ush becomes a repressor to limit the transcriptional activation of *tin* by Pnr

pnr and *ush* are initially broadly expressed in the dorsal ectoderm of the early embryo, but by germband retraction the ectodermal expression of *pnr* is confined to a narrow stripe of cells along the border of the amnioserosa, which overlaps with the thin dorsal *dpp* stripe (Fig. 1D). The early ectodermal expression of *ush* is restricted to the presumptive amnioserosa, and by germband extension, *ush* also overlaps with the dorsalmost region of the ectoderm (Fossett et al., 2000; Herranz and Morata, 2001). These patterns of expression suggest that *pnr* and *ush* may be acting in both germ layers. Our genetic data, including germ layer-specific expression of wild-type and dominant-negative *pnr* constructs, as well as germ layer-specific rescue experiments suggest strongly that *pnr* and *ush* function is not only needed in the mesoderm, but also in the ectoderm for heart formation (see model in Fig. 7). The ectodermal requirement for *pnr* and *ush* in heart development is probably achieved via the maintenance of *dpp* expression, as dorsal stripe *dpp* expression diminishes in *pnr* and *ush* mutants and ectodermal interference with *pnr*, *ush* and/or *dpp*-signaling function compromises the normal progression of heart development.

We thank Ken Cadigan, Tom Kerppola and Jim Skeath for their helpful discussions. We also thank Manfred Frasch, Marc Haenlin, Anthea Letsou, Marek Mlodzik, Adrian Moore, Philippe Ramain, Christine Rushlow and the Bloomington Drosophila Stock Center for providing fly stocks and reagents essential for this work. S.K. is supported by a predoctoral fellowship from the American Heart Association. This work was funded by grants from the National Institutes of Health (NHLBI) and the American Heart Association to R.B.

REFERENCES

Abel, T., Michelson, A. M. and Maniatis, T. (1993). A Drosophila GATA family member that binds to Adh regulatory sequences is expressed in the developing fat body. *Development* **119**, 623-633.

Alvarez, A. D., Shi, W. S., Wilson, B. A. and Skeath, J. B. (2003). *pannier* and *pointedP2* act sequentially to regulate *Drosophila* development. *Development* **130**, 3015-3026.

Arceci, R. J., King, A. A., Simon, M. C., Orkin, S. H. and Wilson, D. B. (1993). Mouse GATA-4: a retinoic acid-inducible GATA-binding transcription factor expressed in endodermally derived tissues and heart. *Mol. Cell. Biol.* **13**, 2235-2246.

Ashe, H. L., Mannervick, M. and Levine, M. (2000). Dpp signaling thresholds in the dorsal ectoderm of the Drosophila embryo. *Development* **127**, 3305-3312.

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

Azpiazu, N., Lawrence, P. A., Vincent, J. P. and Frasch, M. (1996). Segmentation and specification of the Drosophila mesoderm. *Genes Dev.* **10**, 3183-3194.

Bhatia, S. N., Suri, V., Bundy, A. and Krauss, C. M. (1999). Prenatal detection and mapping of a distal 8p deletion associated with congenital heart disease. *Prenat. Diagn.* **19**, 863-867.

Bodmer, R., Jan, L. Y. and Jan, Y. N. (1990). A new homeobox-containing gene, msh-2, is transiently expressed early during mesoderm formation of Drosophila. *Development* **110**, 661-669.

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

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

Bodmer, R. and Venkatesh, T. V. (1998). Heart development in Drosophila and vertebrates: conservation of molecular mechanisms. *Dev. Genet.* **22**, 181-186.

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

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.

Brown, S. and Castelli-Gair Hombria, J. (2000). Drosophila grain encodes a GATA transcription factor required for cell rearrangement during morphogenesis. *Development* **127**, 4867-4876.

Byars, C. L., Bates, K. L. and Letsou, A. (1999). The dorsal-open group gene raw is required for restricted DJNK signaling during closure. *Development* **126**, 4913-4923.

Calleja, M., Herranz, H., Estella, C., Casal, J., Lawrence, P., Simpson, P. and Morata, G. (2000). Generation of medial and lateral dorsal body domains by the pannier gene of Drosophila. *Development* **127**, 3971-3980.

Chartier, A., Zaffran, S., Astier, M., Semeriva, M. and Gratecos, D. (2002). Pericardin, a Drosophila type IV collagen-like protein is involved in the morphogenesis and maintenance of the heart epithelium during dorsal ectoderm closure. *Development* **129**, 3241-3253.

Crispino, J. D., Lodish, M. B., Thurberg, B. L., Litovsky, S. H., Collins, T., Molkentin, J. D. and Orkin, S. H. (2001). Proper coronary vascular development and heart morphogenesis depend on interaction of GATA-4 with FOG cofactors. *Genes Dev.* **15**, 839-844.

Cubadda, Y., Heitzler, P., Ray, R. P., Bourouis, M., Ramain, P., Gelbart, W., Simpson, P. and Haenlin, M. (1997). U-shaped encodes a zinc finger protein that regulates the proneural genes achaete and scute during the formation of bristles in Drosophila. *Genes Dev*. **11**, 3083-3095.

Dai, Y. S., Cserjesi, P., Markham, B. E. and Molkentin, J. D. (2002). The transcription factors GATA4 and dHAND physically interact to synergistically activate cardiac gene expression through a p300-dependent mechanism. *J. Biol. Chem.* **277**, 24390-24398.

Durocher, D., Charron, F., Warren, R., Schwartz, R. J. and Nemer, M. (1997). The cardiac transcription factors Nkx2-5 and GATA-4 are mutual cofactors. *EMBO J*. **16**, 5687-5696.

Evans, T. and Felsenfeld. G. (1989). The erythroid-specific transcription factor Eryf1: a new finger protein. *Cell* **58**, 877-885.

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

Frasch, M., Hoey, T., Rushlow, C., Doyle, H. and Levine, M. (1987). Characterization and localization of the even-skipped protein of Drosophila. *EMBO J.* **6**, 749-759.

Fossett, N., Zhang, Q., Gajewski, K., Choi, C., Kim, Y. and Schulz, R. A. (2000). The multitype zinc-finger protein U-shaped functions in heart cell specification in the Drosophila embryo. *Proc. Natl. Acad. Sci. USA* **97**, 7348-7353.

Fossett, N., Tevosian, S. G., Gajewski, K., Zhang, Q., Orkin, S. H. and Schulz, R. A. (2001). The Friend of GATA proteins U-shaped, FOG-1, and FOG-2 function as negative regulators of blood, heart, and eye development in Drosophila. *Proc. Natl. Acad. Sci. USA* **98**, 7342-7347.

Fu, Y., Yan, W., Mohun, T. J. and Evans, S. M. (1998). Vertebrate tinman homologues XNkx2-3 and XNkx2-5 are required for heart formation in a functionally redundant manner. *Development* **124**, 4439-4449.

Gajewski, K., Fossett, N., Molkentin, J. D. and Schulz, R. A. (1999). The zinc finger proteins Pannier and GATA4 function as cardiogenic factors in Drosophila. *Development* **126**, 5679-5688.

Gajewski, K., Zhang, Q., Choi, C. Y., Fossett, N., Dang, A., Kim, Y. H., Kim, Y. and Schulz, R. A. (2001). Pannier is a Transcriptional Target and Partner of Tinman during Drosophila Cardiogenesis. *Dev. Biol.* **233**, 425-436.

Gisselbrecht, S., Skeath, J. B., Doe, C. Q. and Michelson, A. M. (1996). *heartless* encodes a fibroblast growth factor receptor (DFR1/DFGF-R2) involved in the directional migration of early mesodermal cells in the Drosophila embryo. *Genes Dev.* **10**, 3003-3017.

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

Grepin, C., Dagnino, L., Robitaille, L., Haberstroh, L., Antakly, T. and Nemer, M. (1994). A hormone-encoding gene identifies a pathway for cardiac but not skeletal muscle gene transcription. *Mol. Cell. Biol.* **14**, 3115-3129.

Haenlin, M., Cubadda, Y., Blondeau, F., Heitzler, P., Lutz, Y., Simpson, P. and Ramain, P. (1997). Transcriptional activity of Pannier is regulated negatively by heterodimerization of the GATA DNA-binding domain with a cofactor encoded by the u-shaped gene in Drosophila. *Genes Dev.* **11**, 3096-3108.

Han, Z., Fujioka, M., Su, M., Liu, M., Jaynes, J. B. and Bodmer, R. (2002). Transcriptional integration of competence modulated by mutual repression generates cell-type specificity within the cardiogenic mesoderm. *Dev Biol.* **252**, 225-240.

Han, Z. and Bodmer, R. (2003). Myogenic cells fates are antagonized by Notch only in asymmetric lineages of the Drosophila heart, with or without cell division. *Development* **130**, 3039-3051.

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

Heikinheimo, M., Scandrett, J. M. and Wilson, D. B. (1994). Localization of transcription factor GATA-4 to regions of the mouse embryo involved in cardiac development. *Dev. Biol.* **164**, 361-373.

Heitzler, P., Haenlin, M., Ramain, P., Calleja, M. and Simpson, P. (1996). A genetic analysis of pannier, a gene necessary for viability of dorsal tissues and bristle positioning in Drosophila. *Genetics* **143**, 1271-1286.

Herranz, H. and Morata, G. (2001). The functions of pannier during Drosophila embryogenesis. *Development* **128**, 4837-4846.

Ip, H. S., Wilson, D. B., Heikinheimo, M., Tang, Z., Ting, C. N., Simon, M. C., Leiden, J. M. and Parmacek, M. S. (1994). The GATA-4 transcription factor transactivates the cardiac muscle-specific troponin C promoter-enhancer in nonmuscle cells. *Mol. Cell. Biol.* **14**, 7517-7526.

Jagla, K., Frasch, M., Jagla, T., Dretzen, G., Bellard, R. and Bellard, M. (1997). Ladybird, a new component of the cardiogenic pathway in Drosohila required for diversification of heart precursors. *Development* **124**, 3471-3479.

Jaynes, J. B. and O'Farrel, P. H. (1991). Active repression of transcription by the engrailed homeodomain protein. *EMBO J.* **10**, 1427-1433.

Jazwinska, A., Rushlow, C. and Roth, S. (1999). The role of brinker in mediating the graded response to Dpp in early Drosophila embryos. *Development* **126**, 3323-3334.

Jiang, Y. and Evans, T. (1996). The Xenopus GATA-4/5/6 genes are associated with cardiac specification and can regulate cardiac-specific transcription during embryogenesis. *Dev. Biol.* **174**, 258-270.

Kelley, C., Blumberg, H., Zon, L. I. and Evans, T. (1993). GATA-4 is a novel transcription factor expressed in endocardium of the developing heart. *Development* **118**, 817-827.

Kirkpatrick, H., Johnson, K. and Laughon, A. (2001). Repression of dpp targets by binding of brinker to mad sites. *J. Biol. Chem.* **276**, 18216-18222.

Knirr, S., Azpiazu, N. and Frasch, M. (1999). The role of the NK-homeobox gene slouch (S59) in somatic muscle patterning. *Development* **126**, 4525-4535.

Kolsch, V. and Paululat, A. (2002) The highly conserved cardiogenic bHLH factor Hand is specifically expressed in circular visceral muscle progenitor cells and in all cell types of the dorsal vessel during Drosophila embryogenesis. *Dev. Genes Evol.* **212**, 473-485.

Kuo, C. T., Morrisey, E. E., Anandappa, R., Sigrist, K., Lu, M. M., Parmacek, M. S., Soudais, C. and Leiden, J. M. (1997). GATA4 transcription factor is required for ventral morphogenesis and heart tube formation. *Genes Dev.* **11**, 1048-1060.

Laverriere, A. C., MacNeill, C., Mueller, C., Poelmann, R. E., Burch, J. B. and Evans, T. (1994). GATA-4/5/6, a subfamily of three transcription factors transcribed in developing heart and gut. *J. Biol. Chem.* **269**, 23177-23184.

Lee, H. H. and Frasch, M. (2000). Wingless effects mesoderm patterning and ectoderm segmentation events via induction of its downstream target *sloppy paired*. *Development* **127**, 5497-5508.

Lee, Y., Shioi, T., Kasahara, H., Jobe, S. M., Wiese, R. J., Markham, B. E. and Izumo, S. (1998). The cardiac tissue-restricted homeobox protein Csx/Nkx2.5 physically associates with the zinc finger protein GATA4 and cooperatively activates atrial natriuretic factor gene expression. *Mol. Cell. Biol.* **18**, 3120-3129.

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

Lin, W. H., Huang, L. H., Yeh, J. Y., Hoheisel, J., Lehrach, H., Sun, Y. H. and Tsai, S. F. (1995). Expression of a Drosophila GATA transcription factor in multiple tissues in the developing embryos. Identification of homozygous lethal mutants with P-element insertion at the promoter region. *J. Biol. Chem*. **270**, 25150-25158.

Lo, P. C., Skeath, J. B., Gajewski, K., Schulz, R. A. and Frasch, M. (2002). Homeotic genes autonomously specify the anteroposterior subdivision of the Drosophila dorsal vessel into aorta and heart. *Dev. Biol.* **251**, 307-319.

Lockwood, W. and Bodmer, R. (2002). Patterns of wingless, decapentaplegic and tinman positions the Drosophila heart. *Mech. Dev.* **114**, 13-26.

Lu, J. R., McKinsey, T. A., Xu, H., Wang, D. Z., Richardson, J. A. and Olsen, E. N. (1999). FOG-2, a heart- and brain-enriched cofactor for GATA transcription factors. *Mol. Cell. Biol.* **19**, 4495-4502.

Marvin, M. J., di Rocco, G., Gardiner, A., Bush, S. M. and Lassar, A. B.

(2001). Inhibition of Wnt activity induces heart formation from posterior mesoderm. *Genes Dev.* **15**, 316-347.

Mlodzik, M., Hiromi, Y., Weber, U., Goodman, C. S. and Rubin, G. M. (1990). The Drosophila seven-up gene, a member of the steroid receptor gene superfamily, controls photoreceptor cell fates. *Cell* **60**, 211-224.

Molkentin, J. D., Lin, Q., Duncan, S. A. and Olsen, E. N. (1997). Requirement of the transcription factor GATA4 for heart tube formation and ventral morphogenesis. *Genes Dev*. **11**, 1061-1072.

Molkentin, J. D. (2000). The zinc finger-containing transcription factors GATA-4,-5,-6. *J. Biol. Chem.* **275**, 38949-38952.

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

Morrisey, E. E., Ip, H. S., Lu, M. M. and Parmacek, M. S. (1996). GATA-6: a zinc finger transcription factor that is expressed in multiple cell lineages derived from lateral mesoderm. *Dev. Biol.* **177**, 309-322.

Murphy, A. M., Thompson, W. R., Peng, L. F. and Jones, L., 2nd (1997). Regulation of the rat cardiac troponin I gene by the transcription factor GATA-4. *Biochem. J.* **322**, 393-401.

Orkin, S. H. (1998). Embryonic stem cells and transgenic mice in the study of hematopoiesis. *Int. J. Dev. Biol.* **42**, 827-934.

Padgett, R. W., St. Johnston, R. D. and Gelbart, W. M. (1987). A transcript from a Drosophila pattern gene predicts a protein homologous to the transforming growth factor-beta family. *Nature* **325**, 81-84.

Pandur, P., Lasche, M., Eisenberg, L. M. and Kuhl, M. (2002). Wnt-11 activation of a non-canonical Wnt signaling pathway is required for cardiogenesis. *Nature* **481**, 636-641.

Park, M., Wu, X., Golden, K., Axelrod, J. and Bodmer, R. (1996). The Wingless signaling pathway is directly involved in Drosophila development. *Dev. Biol.* **177**, 104-116.

Park, M., Yaich, L. E. and Bodmer, R. (1998). Mesodermal cell fate decisions in Drosophila are under the control of the lineage genes numb, Notch, and sanpodo. *Mech. Dev.* **75**, 117-126.

Pehlivan, T., Pober, B. R., Brueckner, M., Garrett, S., Slaugh, R., van Rheeden, R., Wilson, D. B., Watson, M. S. and Hing, A. V. (1999). GATA4 haploinsufficiency in patients with interstitial deletion of chromosome region 8p23.1 and congenital heart disease. *Am. J. Med. Genet.* **83**, 201-206.

Ponzielli, R., Astier, M., Chartier, A., Gallet, A., Therond, P. and Semeriva, M. (2002). Heart tube patterning in Drosophila requires integration of axial and segmental information provided by the Bithorax Complex genes and hedgehog signaling. *Development* **129**, 4509-4521.

Ramain, P., Heitzler, P., Haenlin, M. and Simpson, P. (1993). pannier, a negative regulator of achaete and scute in Drosophila, encodes a zinc finger protein with homology to the vertebrate transcription factor GATA-1. *Development* **119**, 1277-1291.

Ranganayakulu, G., Elliott, D. A., Harvey, R. P. and Olsen, E. N. (1998). Divergent roles for NK-2 class homeobox genes in cardiogenesis in flies and mice. *Development* **113,** 35-54.

Rehorn, K. P., Thelen, H., Michelson, A. M. and Reuter, R. (1996). A molecular aspect of hematopoiesis and endoderm development common to vertebrates and Drosophila. *Development* **122**, 4023-4031.

Riechmann, V., Irion, U., Wilson, R., Grosskortenhaus, R. and Leptin, M. (1997). Control of cell fates and segmentation in the Drosophila mesoderm. *Development* **124**, 2915-2922.

Riechmann, V., Rehorn, K. P., Reuter, R. and Leptin, M. (1998). The genetic control of the distinction between fat body and gonadal mesoderm in Drosophila. *Development* **125**, 713-723.

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

Rushlow, C., Colosimo, P. F., Lin, M. C., Xu, M. and Kirov, N. (2001). Transcriptional regulation of the Drosophila gene zen by competing Smad and Brinker inputs. *Genes Dev*. **15**, 340-351.

Saller, E. and Bienz, M. (2001). Direct competition between Brinker and Drosophila Mad in Dpp target gene transcription. *EMBO J.* **2**, 298-305.

Sam, S., Leise, W. and Hoshizaki, D. K. (1996). The serpent gene is necessary for progression through the early stages of fat-body development. *Mech. Dev*. **60**, 197-205.

Sato, M. and Saigo, K. (2000). Involvement of pannier and u-shaped in regulation of Decapentaplegic-dependent wingless expression in developing Drosophila notum. *Mech. Dev.* **93**, 127-138.

Schneider, V. A. and Mercola, M. (2001). Wnt antagonism initiates cardiogenesis in Xenopus laevis. *Genes Dev.* **15**, 304-315.

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.

Sepulveda, J. L., Belaguli, N., Nigam, V., Chen, C., Nemer, M. and Schwartz, R. J. (1998). GATA-4 and Nkx-2.5 coactivate Nkx-2 DNA binding targets: role for regulating early cardiac gene expression. *Mol. Cell. Biol.* **18**, 3405-3415.

Sepulveda, J. L., Vlahopoulos, S., Iyer, D., Belaguli, N. and Schwartz, R. J. (2002). Combinatorial expression of GATA4, Nkx2-5, and serum response factor directs early cardiac gene activity. *J. Biol. Chem.* **277**, 25775-25782.

Sivasankaran, R., Vigano, M. A., Muller, B., Affolter, M. and Basler, K. (2000). Direct transcriptional control of the Dpp target omb by the DNA binding protein Brinker. *EMBO J*. **19**, 6162-6172.

Smith, S. T. and Jaynes, J. B. (1996). A conserved region of engrailed, shared among all en-, gsc-, Nk1-, Nk2- and msh-class homeoproteins, mediates active transcriptional repression in vivo. *Development* **122**, 3141-3150.

Svensson, E. C., Huggins, G. S., Dardik, F. B., Polk, C. E. and Leiden, J. M. (1999). A functionally conserved N-terminal domain of the Friend of GATA-2 (FOG-2) protein represses GATA-4-Dependent transcription. *J. Biol. Chem.* **275**, 20762-20769.

Svensson, E. C., Huggins, G. S., Lin, H., Clendenin, C., Jiang, F., Tufts, R., Dardik, F. B. and Leiden, J. M. (2000). A syndrome of tricuspid atresia in mice with a targeted mutation of the gene encoding Fog-2. *Nat. Genet.* **25**, 353-356.

Tevosian, S. G., Deconinck, A. E., Cantor, A. B., Rieff, H. I., Fujiwara, Y., Corfas, G. and Orkin, S. H. (1999). FOG-2: A novel GATA-family cofactor related to multitype zinc-finger proteins Friend of GATA-1 and U-shaped. *Proc. Natl. Acad. Sci. USA* **96**, 950-955.

Tevosian, S. G., Deconinck, A. E., Tanaka, M., Schinke, M., Litovsky, S. H., Izumo, S., Fujiwara, Y. and Orkin, S. H. (2000). FOG-2, a cofactor for GATA transcription factors, is essential for heart morphogenesis and development of coronary vessels from epicardium. *Cell* **101**, 729-739.

Tolkunova, E. N., Fujioka, M., Kobayashi, M., Deka, D. and Jaynes, J. B. (1998). Two distinct types of repression domain in engrailed: one interacts with the groucho corepressor and is preferentially active on integrated target genes. *Mol. Cell. Biol.* **18**, 2804-2814.

Tomoyasu, Y., Ueno, N. and Nakamura, M. (2000). The Decapentaplegic morphogen gradient regulates the notal wingless expression through induction of pannier and u-shaped in Drosophila. *Mech. Dev.* **96**, 37-49.

Tsai, S. F., Martin, D. I., Zon, L. I., D'Andrea, A. D., Wong, G. G. and Orkin, S. H. (1989). Cloning of cDNA for the major DNA-binding protein of the erythroid lineage through expression in mammalian cells. *Nature* **339**, 446-451.

Tsang, A. P., Fujiwara, Y., Hom, D. B. and Orkin, S. H. (1998). Failure of megakaryopoiesis and arrested erythropoiesis in mice lacking the GATA-1 transcriptional cofactor FOG. *Genes Dev.* **12**, 1176-1188.

Venkatesh, T. V., Park, M., Ocorr, K., Nemaceck, J., Golden, K., Wemple, M. and Bodmer, R. (2000). Cardiac enhancer activity of the homeobox gene tinman depends on CREB consensus binding sites in Drosophila. *Genesis* **26**, 55-66.

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

Winick, J., Abel, T., Leonard, M. W., Michelson, A. M., Chardon, I. L., Holmgren, R. A., Maniatis, T. and Engel, J. D. (1993). A GATA family transcription factor is expressed along the embryonic dorsoventral axis in Drosophila melanogaster. *Development* **199**, 1055-1065.

Wodarz, A., Hinz, U., Engelbert, M. and Knust, E. (1995). Expression of crumbs confers apical character on plasma membrane domains of ectodermal epithelia of Drosophila. *Cell* **82**, 67-76.

Wu, X., Golden, K. and Bodmer, R. (1995). Heart development in Drosophila requires the segment polarity gene *wingless*. *Dev. Biol.* **169**, 619-628.

Xu, X, Yin, Z., Hudson, J. B., Ferguson, E. L. and Frasch, M. (1998). Smad proteins act in combination with synergistic and antagonistic regulators to target Dpp responses to the Drosophila mesoderm. *Genes Dev.* **12**, 2354-2370.

Yamamoto, M., Ko, L. J., Leonard, M. W., Beug, H., Orkin, S. H. and Engel, J. D. (1990). Activity and tissue-specific expression of the transcription factor NF-E1 multigene family. *Genes Dev.* **4**, 1650-1652.

Yarnitzky, T. and Volk, T. (1995). Laminin is required for heart, somatic muscles, and gut development in the Drosophila embryo. *Dev. Biol.* **169**, 609-618.

Zhang, H., Levine, M. and Ashe, M. L. (2001). Brinker is a sequence-specific transcriptional repressor in the Drosophila embryo. *Genes Dev.* **15**, 261-266.