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ladybird, a new component of the cardiogenic pathway in *Drosophila* required for diversification of heart precursors

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SUMMARY

The embryonic heart precursors of *Drosophila* are arranged in a repeated pattern of segmental units. There is growing evidence that the development of individual elements of this pattern depends on both mesoderm intrinsic patterning information and inductive signals from the ectoderm. In this study, we demonstrate that two homeobox genes, *ladybird early* and *ladybird late,* are involved in the cardiogenic pathway in *Drosophila*. Their expression is specific to a subset of cardioblast and pericardial cell precursors and is critically dependent on mesodermal *tinman* function, epidermal Wingless signaling and the coordinate action of neurogenic genes. Negative regulation by *hedgehog* is required to restrict *ladybird* expression to two out of six cardioblasts in each hemisegment. Overexpression of *ladybird* causes a hyperplasia of heart precursors and alters the identity of *even-skipped*-positive pericardial cells. Loss of *ladybird* function leads to the opposite transformation, suggesting that *ladybird* participates in the determination of heart lineages and is required to specify the identities of subpopulations of heart cells. We find that both early Wingless signaling and *ladybird*-dependent late Wingless signaling are required for proper heart formation. Thus, we propose that *ladybird* plays a dual role in cardiogenesis: (i) during the early phase, it is involved in specification of a segmental subset of heart precursors as a component of the cardiogenic *tinman*-cascade and (ii) during the late phase, it is needed for maintaining *wingless* activity and thereby sustaining the heart pattern process. These events result in a diversification of heart cell identities within each segment.

Key words: *ladybird*, cardiogenesis, heart, *Drosophila*, homeobox, *wingless*, *tinman*, *hedgehog*, *even-skipped*

INTRODUCTION

During early embryogenesis of *Drosophila*, prior to cellularization, a maternal regulatory gradient of the Dorsal gene product defines presumptive mesoderm and ectoderm territories (Maggert et al., 1995). In the ventral region, high concentrations of Dorsal induce the expression of mesoderm genes, *snail* (*sna*) (Ip et al., 1992; Maggert et al., 1995) and *twist* (*twi*) (Thisse et al., 1991) while, in the dorsal ectoderm, low Dorsal levels activate *decapentaplegic* (*dpp*) encoding a TGF-β-like signaling molecule (Huang et al., 1993; Maggert et al., 1995). At the beginning of gastrulation, cells of the mesoderm invaginate through the ventral furrow, spread dorsally to form a single layer, and subsequently split into ventral and dorsolateral lineages. This initial subdivision of the mesoderm involves inductive interactions with the dorsal ectodermal cells which express *dpp* (Staehling-Hampton et al., 1994; Frasch, 1995). Upon Dpp induction, expression of the homeobox gene *tinman* (*tin*) becomes restricted to the dorsolateral mesoderm where its activity is required for the development of visceral, cardiac and dorsal somatic lineages (Azpiazu and Frasch, 1993; Bodmer, 1993). The ventral mesoderm forms most other somatic muscle progenitors in a process involving induction by the overlying neuroectoderm (Baker and Schubiger, 1995). The first morphological event in the subdivision of the dorsal mesoderm is the formation of two layers. The outer layer, which contacts the ectoderm, gives rise to heart precursors and dorsolateral body-wall muscles, whereas the inner layer, which is derived from the *bagpipe* (*bap*)-expressing cells, contains progenitors of the visceral muscles of the midgut (Azpiazu and Frasch, 1993). Recent data indicate that the specification and subsequent differentiation of mesodermal derivatives requires additional signals from the ectoderm (Volk and Raghavan, 1994; Lawrence et al., 1995; Baylies et al., 1995; Wu et al., 1995; Park et al., 1996; Ranganayakulu et al., 1996; Azpiazu et al., 1996). The segmental origin and character of mesodermal structures, such as the heart (Azpiazu and Frasch, 1993; Lawrence et al., 1995) and the somatic muscles (Bate, 1990; Volk and Raghavan, 1994; Baylies et al., 1995), strongly suggests influences from the segmented ectoderm. Indeed, mutations of the segment polarity genes, which are involved in ectoderm patterning, also affect mesoderm segmentation (Volk and Raghavan, 1994; Baylies et al., 1995; Lawrence et al., 1995; Azpiazu et al., 1996). One member of this class of genes, *wingless* (*wg*) (Baker, 1987), encodes a secreted molecule that acts as an inductive signal for

somatic muscle precursor formation (Baylies et al., 1995; Ranganayakulu et al., 1996) and the formation of heart precursors (Lawrence et al., 1995, Wu et al., 1995; Park et al., 1996). Epidermal *wg* activity is able to rescue the heart-less wg^{-} phenotype (Lawrence et al., 1995), suggesting that the Wg signal can act across germ layers. In addition to the dorsoventral and anterior-posterior cues, the segregation of heart and somatic muscle precursors involves cell-cell interactions that are mediated by the neurogenic genes (Corbin et al., 1991; Hartenstein et al., 1992; Bate et al., 1993).

Here, we focus our analysis on the specification of *Drosophila* heart precursors and the diversification of heart cells. We show that two closely related homeobox genes, *ladybird early* (*lbe*) and *ladybird late* (*lbl*) (Jagla et al., 1993, 1994, 1997), are specifically expressed in a subpopulation of *tin*-expressing heart progenitors. Our results suggest that the *lb* genes are required to specify the identity of heart precursors.

MATERIALS AND METHODS

Fly stocks

The following fly strains were used: wg^{CX4} (Baker, 1987), wg^{IL114} (Bejsovec and Martinez-Arias, 1991), hh^{9K} (Heemskerk and DiNardo, 1991), tin^{EC40}, $tin^{Df(3R)GC14}$ (Bodmer, 1993; Azpiazu and Frasch, 1993) and a set of neurogenic mutants, N^{55e11}, bib^{ID05}, mam^{Z3}, $Df(3R)E(spl)^{Bx22}$, Dl^{RevF10} and neu^{IF65}. wg^{IL114} and hh^{9K} are temperature-sensitive alleles that behave as nulls at 29°C. To distinguish between mutant and wild-type embryos, where applicable, the balancer chromosomes were marked with a *twi-lacZ* P-element and the embryos were stained using anti-β-galactosidase antibody.

Transgenic flies

Heat-shock (Hs) stocks were used to study the effects of *lb* gene overexpression. Hs-*lbe-4* and Hs-*lbl-5* (Jagla et al., 1997) are inserted on the X chromosome. Double Hs-*lbe;lbl* transgenic flies were generated as described previously (Jagla et al., 1997). A modified deficiency stock ($Df(3R)e^{D7}$, *P(w+)tinre58/TM3ftzlacZ*; Azpiazu and Frasch, 1993), in which both *tin* and *lb* loci are missing while *tin* function is restored with a rescue construct containing genomic *tin* sequences, was used to analyse the role of *lb* in heart formation.

Heat-shock treatment and temperature shift

The transgenic embryos were aged at 25°C and heat-shock treated, at different times of development (indicated in the text), for 15 minutes at 37°C in water. The temperature-sensitive *wg* and *hh* mutants were aged at 18°C and then raised to the non-permissive temperature (29°C) at 4, 5.5, 6.5, 8 or 9 hours AEL. The rate of development at 18°C is 0.5 times lower than at 25°C and 1.4 times faster at 29°C (Wu et al., 1995).

Immunocytochemistry

Detections of the antibody stainings were performed using ABC-Elite-peroxidase or ABC-alkaline phosphatase kits (Vector Laboratories). Antibody dilutions were as follows: monoclonal anti-Lbe (1:1), rabbit anti-Lbl (1:5000), rabbit anti-Eve (1:5000), guinea pig anti-Eve (provided by Dave Kosman) (1:500), rabbit anti-β-galactosidase (1:8000), rabbit anti-Tin (1:2000), rabbit anti-Wg (provided by Roel Nusse) (1:1000) and rabbit anti-MEF2 (provided by Hanh Nguyen) (1:1000). Colour reactions were developed using diaminobenzidine (for peroxidase) or NBT (for alkaline phosphatase) as substrates. Fluorescent microscopy with secondary antibodies conjugated with FITC, Texas Red or Cy5 (1:200; Jackson Immuno Research) was used to determine the position of Lb-positive cells with respect to Eve, MEF2 or Tin-positive heart precursors. The double or triple stainings were analysed using a Leica confocal microscope TCS 4D with 40× or 100× objectives.

RESULTS

lb expression defines a subset of cardioblast and pericardial cell progenitors located below anterior ectodermal compartments

lbe and *lbl* were previously shown to be expressed in the same heart progenitors, but the exact identity of these cells was not known (Jagla et al., 1997). Therefore, we examined the spatial relationship between heart progenitors expressing *lb*, *tin*, *even-skipped* (*eve*) or *mef-2* using confocal microscopy (Frasch et al., 1987; Bodmer, 1993; Azpiazu and Frasch, 1993; Bour et al., 1995; Lilly et al., 1995). As shown in Fig. 1A, at early stage 12, *lb* is expressed in clusters of about four cells per hemisegment in the developing heart region. These cells represent a segmental subset of *tin*-expressing heart progenitors, which form a continuous row at the dorsal crest of the mesoderm at this stage (Fig. 1G). *eve* expression begins at a slightly earlier time than *lb* in similar clusters of cells. It appears that two cells from each segmental *eve* cluster develop into a particular type of pericardial cells, termed *e*-PCs. Double stainings for *lb* and *eve* expression demonstrate that the *e*-PC progenitors are distinct from the *lb*-expressing heart progenitors and located posteriorly adjacent to them in each segment (Fig. 1B). Similar stainings of embryos at later stages show that the *lb*-expressing cells give rise to a subpopulation of cardioblasts (CBs) and a second type of pericardial cells, termed *l*-PCs. As shown in Fig. 1C-F, cell rearrangements during stage 12, which involve a 90°, clockwise rotation of the heart progenitor clusters within each segment, place the *lb*-expressing cells at the dorsal side and move the *eve*-expressing cells ventrally to them. This morphogenetic process results in a dorsal row of cardioblasts and ventrolaterally adjacent rows of pericardial cells on either side of the embryo. At stage 14, generally four out of six cardioblasts per hemisegment express both *tin* and *mef-2* (Fig. 1H-J). Double stainings with Lb antibodies show that the two anterior *tin*- and *mef-2*-expressing cardioblasts in each hemisegment co-express *lb* (Fig. 1H-J). In addition, *tin* and *lb* are co-expressed in the *l*-PCs, which are located ventrally below the cardioblasts (Fig. 1H). However, *lb* is not expressed in the *e*-PCs, which are found in more lateral positions at this stage (Fig. 1J). These results indicate a diversification among cardioblasts of each segment, as well as among the pericardial cells, that is already apparent during stage 11.

Because of the important role of *wg* in heart development, we compared its domains of expression with the locations of *lb*- and *eve*-expressing heart progenitors. As shown in Fig. 2, in embryos before and during germ-band retraction, both *lb*- and *eve*-labeled heart-progenitors are localized in the mesodermal areas below each ectodermal *wg* stripe. This arrangement is compatible with a role of *wg* in the specification and/or maintenance of the developmental fates of these cells.

tin and *wg* are required for *lb* expression in the heart

Since heart development requires *tin* function (Azpiazu and Frasch, 1993; Bodmer, 1993) and *lb*-positive heart progenitors emerge from *tin*-expressing dorsal mesodermal cells, we tested for *lb* activity in *tin* mutants. We find that segregation of both

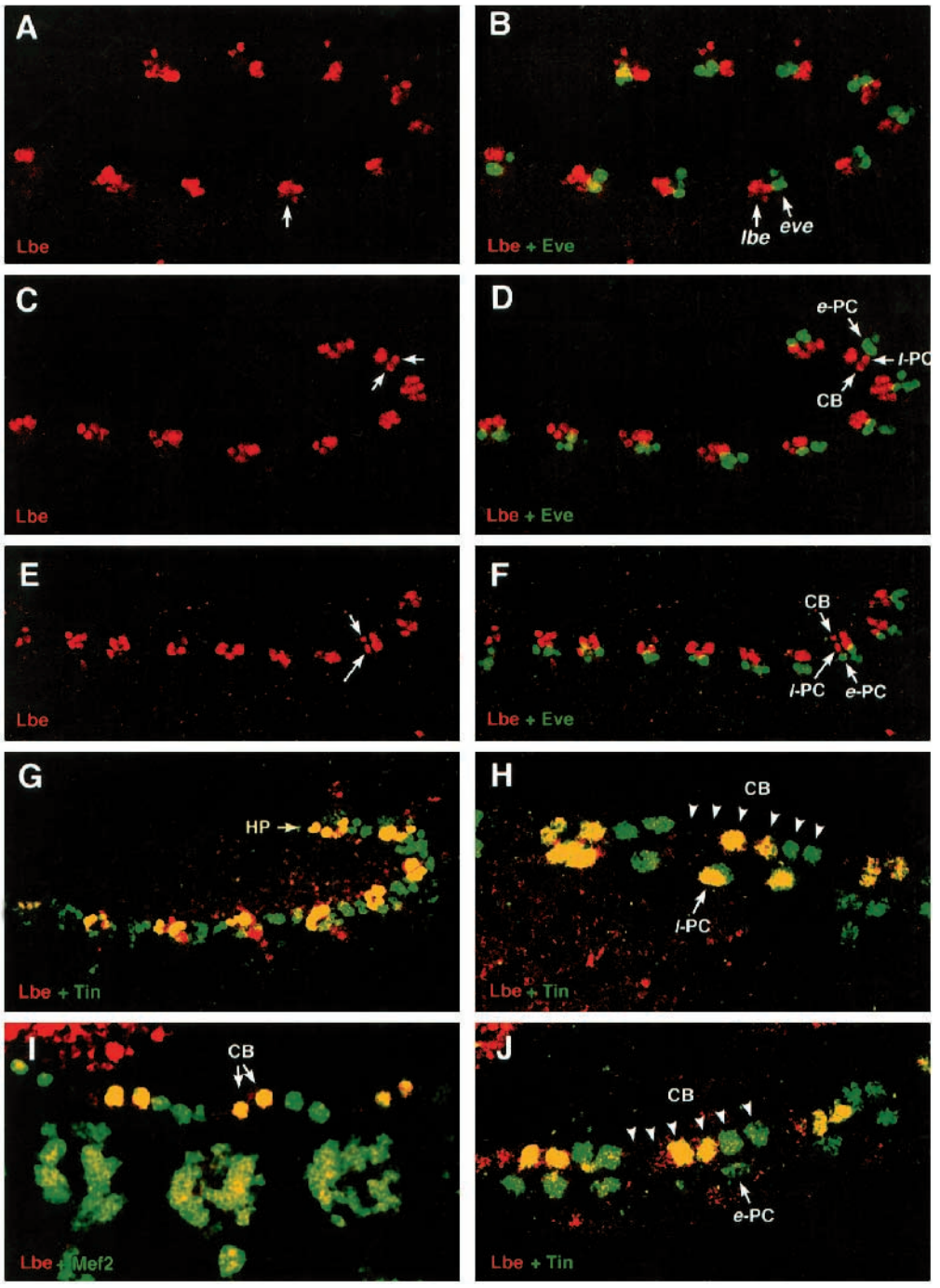

Fig. 1. *lb* is expressed in specific subtypes of cardioblasts and pericardial cells. Shown are confocal scans of embryos from different stages stained with anti-Lbe antibodies (red signals) and with antibodies against other heart markers (green signals). Cells that co-express *lb* with other markers appear yellow. Note that only scans through mesodermal cells were used, while those showing epidermal *lb*-expression were omitted. Dorsal is up and anterior is to the left. (A,B) Early stage 12 embryo (7.5 hours AEL) stained for Lbe (red) and Eve (green). The *eve*-expressing cells are located just posteriorly to the *lbe*-expressing clusters in each segment (the yellow signals seen in some areas resulted from the merging of different optical sections containing green and red nuclei). (C,D) Late stage 12 embryo (9 hours AEL) stained as in A,B. *eve*-expressing pericardial cells are in the process of moving towards the ventral side of the *lb*-expressing heart progenitors. (E,F) Stage 13 embryo (9.5 hours AEL) stained as in A,B. *lb*-expressing heart progenitors form a dorsal row and *eve*-expressing pericardial cells a ventrally adjacent row of developing heart cells. (G) Stage 12 embryo stained for Lbe (red) and Tin (green). The *lb*-expressing cells form a segmental subset of *tin*-expressing heart progenitors (HP). (H) Stage 14 embryo (11 hours AEL) stained for Lb and Tin as in G. Lateral view at high magnification showing a row of cardioblasts and *l*-PCs before dorsal closure. Four out of six cardioblasts per hemisegment express *tin* and the two anterior ones express *lb*. The *l*-PCs are located ventrally to the cardioblasts. (I) Dorsolateral view of a stage 14 embryo, stained for Lbe and MEF-2. The anterior two of the four MEF-2-stained cardioblasts express *lb* (deeper scans of *l*-PCs were omitted). (J) Dorsal view of stage 14 embryo, stained for Lbe and Tin as in G. *e*-PCs, which at this stage are located laterally to the cardioblast row, lack *lb* expression. Abbreviations: CB, cardioblasts; *e*-PC, *eve*-expressing pericardial cells; *l*-PC, *lb*-expressing pericardial cells.

cardioblasts and pericardial cells, as monitored by *lb* expression, does not occur in tin^{-} embryos (compare Fig. 3A and Fig. 3C) and *lb*-heart cells are missing after germ-band retraction (compare Fig. 3B and Fig. 3D). To investigate the influence of Wg and Hh signaling on *lb*-positive cardiac cells, we shifted wg^{IL114} and hh^{9K} thermosensitive mutant embryos at 4 or 6 hours of development to non-permissive temperatures. The influence of *wg* (Jagla et al., 1997) and *hh* (our unpublished observation) on *lb* expression in the dorsal epidermis allowed us to identify the homozygous mutant embryos. In wg^{IL114} embryos lacking either early (from 4 hours AEL) or late (from 6 hours AEL) *wg* function, *lb* expression in heart precursors is absent (Fig. 3E,F), indicating that initiation of *lb* activity requires both *tin* and *wg* action and is maintained by Wg signaling from the dorsal epidermis. In contrast, loss of early *hh* function does not prevent *lb* expression (Fig. 3G), while, in the absence of late Hh signaling, *lb* expression is expanded (Fig. 3H).

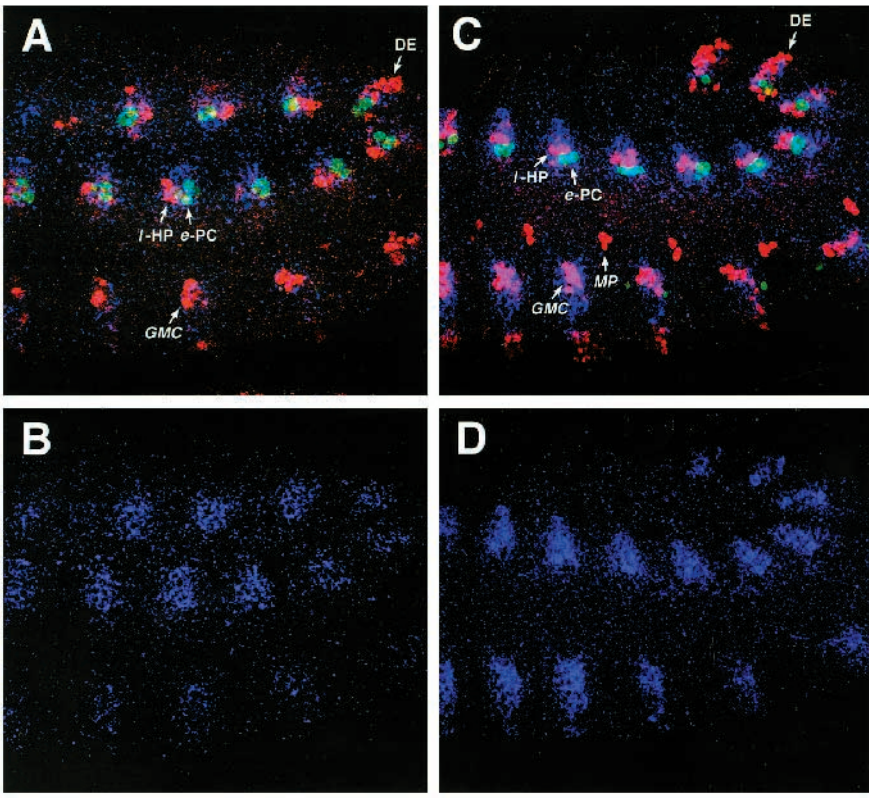

Fig. 2. *lb* and *eve*-expressing heart progenitors are located below dorsal ectodermal *wg* stripes. Confocal scans of ectodermal Wg stainings (blue) were merged with scans of mesodermal Lbe (red) and Eve (green) signals. To allow visualization of heart progenitors, scans showing Lbe-stained epidermal cells were largely omitted. (A) Early stage 12 embryo stained for Lbe (red), Eve (green) and Wg (blue). (B) Same embryo as in A showing ectodermal Wg staining. (C) Late stage 12 embryo stained as in A. (D) Same embryo as in C showing ectodermal Wg staining. Abbreviations: DE, dorsal ectoderm; *e*-PC, *eve*-expressing pericardial cells; *l*-HP, *lb*-expressing heart progenitors. GMC, ganglion mother cells; MP, muscle precursors.

Thus, epidermal Wg and Hh signaling have opposing influences on *lb* activity in heart progenitors. Wg signal is required to induce *lb* in anteriorly located cardiac cells, while the Hh signal in the cells underlying posterior epidermal compartments acts to inhibit *lb* expression (Fig. 3I).

lb-dependent late *wg* activity is required for proper heart patterning

Our previous analysis of the epidermal *lb* function revealed that late *wg* activity in dorsal and caudal epidermis is maintained by a *wg-lb* regulatory feedback loop (Jagla et al., 1997). Since *lb*-dependent dorsal epidermal domains of *wg* expression overlie the heart mesoderm (see Fig. 2), the Wg signal sent by epidermal cells may continue to reach the cardiac cells during this period. The time window during which *wg* can influence heart development was determined with the thermosensitive wg^{IL114} allele and three different antibodies as markers, anti-Eve (Fig. 4A-C), anti-Tin (Fig. 4D-F) and anti-MEF2 (Fig. 6G-I). The loss of early *wg* activity, starting from 3.5-4.5 hours of development (Fig. 6B,E,H), abolishes dorsal mesoderm differentiation and determination of *eve*-, *tin*- and *mef2*-positive cardiac lineages, suggesting that the Wg signal is required for specifying both pericardial cells and cardioblasts. Later, during germ-band retraction, when cardiac lineages have been defined, *wg* activity continues to play a role in heart development (Fig. 4C,F,I). wg^{IL114} embryos shifted to the non-permissive temperature at 7.5 hours AEL have a reduced number of *eve*-positive pericardial cells (Fig. 4C), *mef2*-cardioblasts (Fig. 4I) and disrupted heart pattern as revealed by Tin staining (Fig. 4F). There is a correlation between the severity of the heart defects and the start of the temperature shift, such that earlier loss of *wg* function causes stronger defects in heart formation (data not shown). Since late *wg* activity in the dorsal epidermis is maintained by *lb* (Jagla et al., 1997), we conclude that the formation of a normal heart pattern requires the *wg-lb* regulatory loop.

Neurogenic mutations affect *lb* expression in the heart

Previous data have shown that, similar to the neural cells, the neurogenic genes are required for the segregation of a proper number of somatic muscle founder cells and heart progenitors (Corbin et al., 1991; Hartenstein et al., 1992; Bate et al., 1993). The analysis of *lb* expression patterns revealed that *lb*-expressing heart progenitors contribute to the increased number of cardiac precursors in neurogenic mutants (e.g., *N, Dl, E(spl), mam* – Fig. 5) *(bib, neu* – data not shown). Thus, in extended-germ-band embryos, the expansion of *lb* expression in the CNS is accompanied by the formation of enlarged clusters of *lb*-positive heart cells (Fig. 5A,C,E). Supernumerary *lb*-heart precursors appear both in embryos displaying weak (Fig. 5A) and severe (Fig. 5E) neurogenic phenotypes. At this early stage, the (abnormal) segregation of cardiac cells does not seem to be disturbed by the neurogenic phenotypes in the dorsal epidermis (these embryos lack for instance *lb* expression in dorsal epidermal cells – Fig. 5E). As the germ band retracts, the degree of disruption of the heart pattern in different mutant backgrounds correlates with the degree of CNS expansion. Thus, *E(spl)* or *mam* embryos with moderate neurogenic phenotypes show heart hyperplasia accompanied by ectopic *lb* expression (Fig. 5B,D) while, in *Dl* (Fig. 5F), *N* and *neu* (data not shown) mutants, dorsal vessel morphogenesis is strongly disrupted and the expanded *lb* expression in the heart decays in late stage embryos. This late loss of *lb* is most likely due to

the progressive disruption of the dorsal epidermis and the loss of epidermal Wg signaling that is required for *lb* expression.

Overexpression of *lb* influences heart development

The modulation of the activity of genes involved in heart formation such as *tin*, *wg* and *hh* leads to a deficit or hyperplasia of heart precursors (Wu et al., 1995; Park et al., 1996). Since *lb* is specifically expressed in a subset of heart progenitors, we wondered whether increased levels of *lb* gene products may influence specification of the heart lineages. To address this question, we used transgenic flies carrying *lbe*, *lbl* or both *lb* cDNAs driven by a heat-shock promoter (Jagla et al., 1997) and antibodies labeling cardioblasts (anti-MEF2), pericardial cells (anti-Eve) or both types of heart precursors (anti-Tin). Overexpression of *lbe* or *lbl* at 5-6 hours AEL, during the segregation of cardioblasts and pericardial cells, leads to a significant hyperplasia of heart progenitors, as monitored by *tin* expression (compare panels A, E and B, F in Fig. 6). Surprisingly, the number of *eve*-expressing pericardial cells is strongly reduced (compare panels C, E and D, F in Fig. 6). The analysis of embryos in which ubiquitous expression of both *lb* transgenes was maintained between 5 and 9 hours AEL confirms that ectopic *lb* activity can recruit additional cardiac cells. After germ-band retraction, we observe expanded Tin domains in the majority of these embryos (compare panels G, H and M, N in Fig. 6). (Fig. 6I, J), as well as supernumerary *mef-2*-cardioblasts (compare panels K, L and M, N in Fig. 6). Since, in the same embryos, *eve*-pericardial cells are missing, this raises the possibility that *lb* determines the identity of a distinct type of pericardial cells and that misexpression of *lb* in *e-PCs* leads to their transformation into *l*-PCs. To further investigate this eventuality, we analysed *eve* expression in $Df(3R)e^{D7}$, *tin* rescue embryos in which both *lbe* and *lbl* genes are deleted, while *tin* function is restored by a P-insertion (Azpiazu and Frasch, 1993). We find that, in embryos lacking *lb*, *eve* expression is expanded (Fig. 7). At stage 12, instead of 2-3 cells per hemisegment, the *eve* clusters contain 4-5 heart precursors (Fig. 7A). After germ-band retraction, additional *eve*-cells contribute to the formation of the heart and are found at positions where *lb*-cells are normally located (Fig. 7B). Since heart hyperplasia was not observed in $Df(3R)e^{D7}$, *tin* rescue embryos (Azpiazu and Frasch, 1993 and our unpublished observations), these supernumerary *eve*-cells result most likely from the transformation of *l*-PCs into *e*-PCs. To exclude that this is due to the absence of genes located distally to *lb*, the $Df(3R)e^{D7}$, *tin* rescue flies were crossed with a stock carrying a shorter deficiency, $Df(3R)e^{F1}$ (Jagla et al., 1997). The analysis of transheterozygous embryos revealed the expansion of *e*-PCs

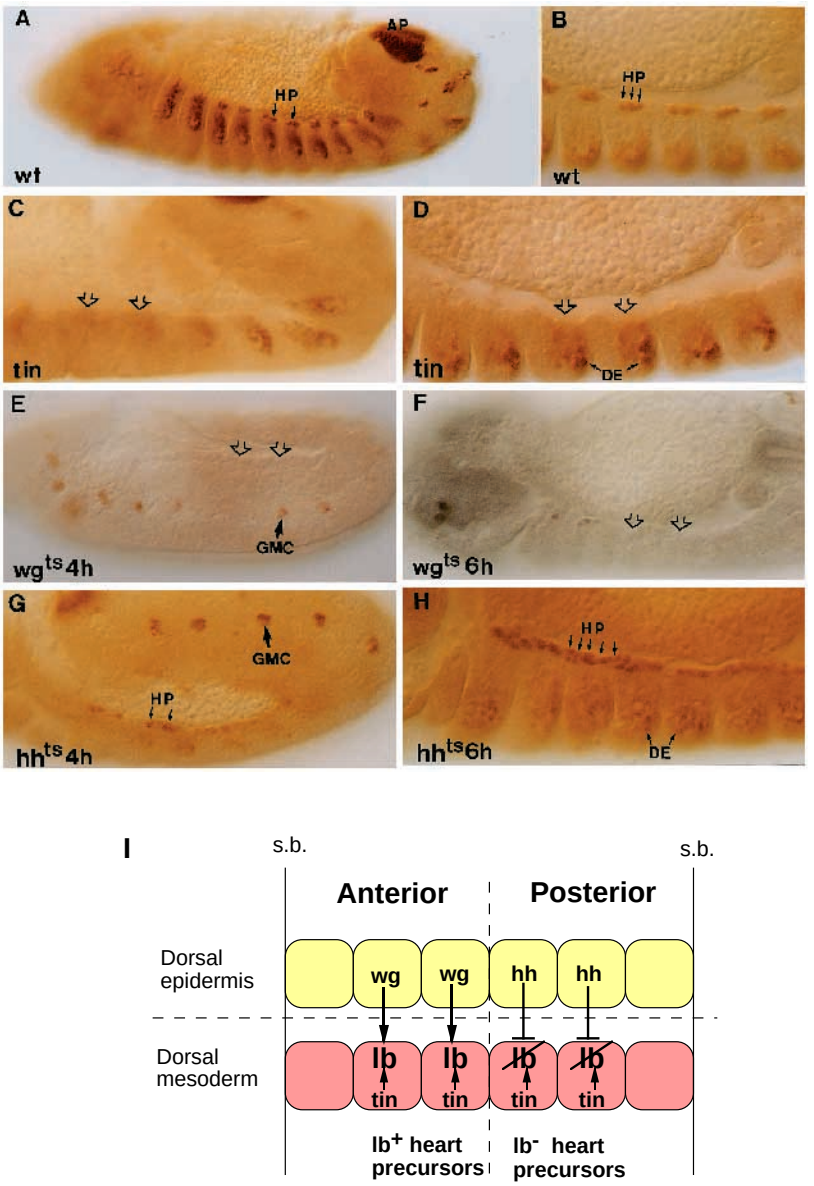

Fig. 3. *lb* expression in the heart depends on *tin* activity and Wg signaling. (A,B) Distribution of Lbe gene products in wild-type, (C,D) tin^{EC40}, (E,F) thermosensitive (wg^{ts}) wg^{IL114} and (G,H) thermosensitive (hh^{ts}) hh^{9K} embryos, visualized by immunostaining with anti-Lbe antibody. *lb* expression in segmentally repeated clusters of heart precursors (arrows in A and B) does not appear during germ-band retraction in *tin* mutants (C) and is completely missing (open arrows) in shortened tin^{-} embryos (D). Loss of heart-associated *lb* activity (open arrows) could be induced in thermosensitive *wg* embryos by a temperature shift at 4 hours AEL (E) or at 6 hours AEL (F). In contrast, *lb* expression is ectopically expanded in embryos lacking late Hh signaling after (6 hours AEL) (H). Loss of early *hh* function (from 4 to 5 hours AEL) leads to the partial inhibition of epidermal Wg signaling (Perrimon, 1994) and, as a consequence, slightly reduces *lb* expression in the heart (G). (I) A scheme of regulation of *lb* expression in the heart precursors of a stage 11 embryo, indicating the dependence of *lb* activity on both mesodermal *tin* function and epidermal Wg signaling. *lb* expression is restricted to the heart cells underlying *wg* domains (mesodermal A domains) and inhibited in the P domains (for nomenclature see Azpiazu et al., 1996) by Hh signals. Developmental stages of the embryos: (E,G) stage 11, (A,C) stage 12, (B,D,F,H) stage 13. Abbreviations: AP, anal plate; DE, dorsal epidermis; GMC, ganglion mother cells; HP, heart precursors. All whole-mounts are oriented with the anterior to the left and photographed under Nomarski optics.

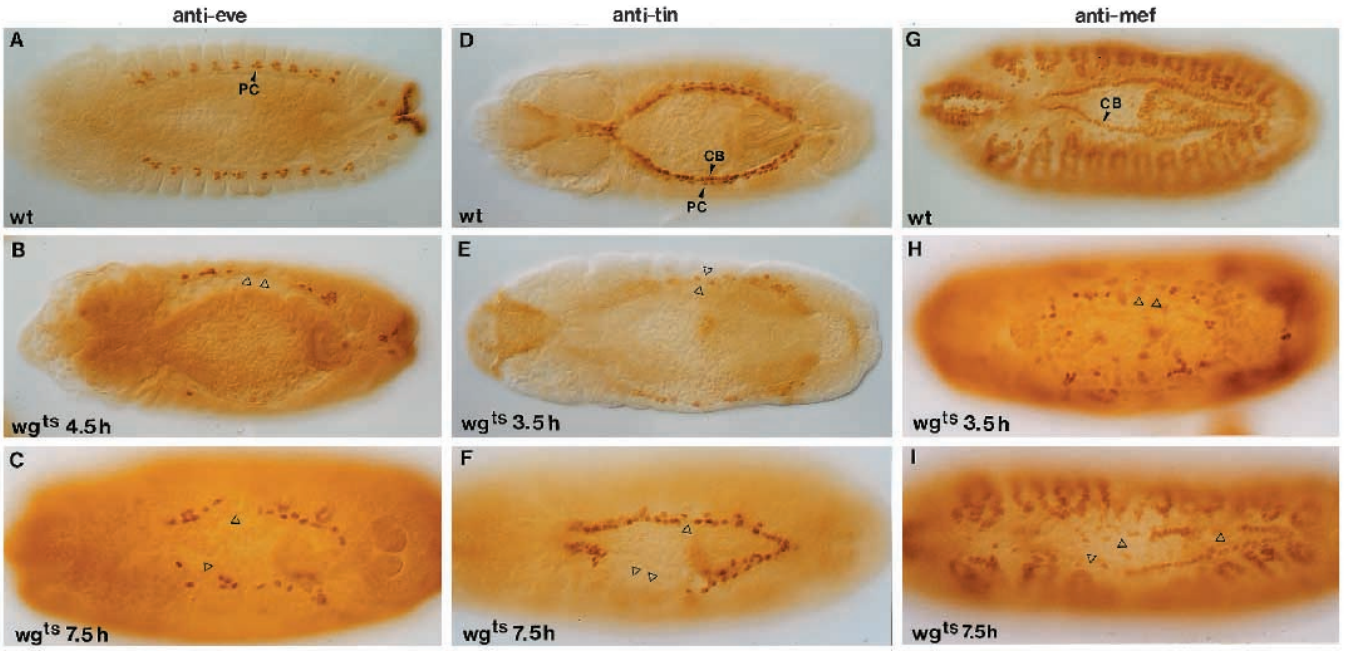

Fig. 4. *lb*-dependent late Wg signaling is required for proper heart patterning. (A,D,G) Wild-type and (B,C,E,F,H,I) thermosensitive wg^{ts} mutants shifted to nonpermissive temperature (E,H) at 3.5, (B) 4.5 or (C,F,I) 7.5 hours AEL. Pericardial cells are detected with anti-Eve antibody (A-C), cardioblasts with anti-MEF2 antibody (G-I) and the general heart pattern is revealed by anti-Tin staining (D-F). Loss of early *wg* function abolishes specification of all cardiac lineages (see open arrowheads in B, E and H). Late, *lb*-dependent Wg signaling (Jagla et al., 1997) is required for normal heart pattern formation (see lacking heart components indicated by open arrowheads in C, F and I). Developmental stages of the embryos: (A,B,E,H) late stage 12, (D) stage 13, (G,C,F,I) stage 14. Abbreviations are as in Fig. 3. All whole-mounts show dorsal views oriented as in Fig. 3.

similar to that presented in Fig. 7 (data not shown). Taken together, these data indicate that *lb* cooperates with *tin* to determine a subset of *tin*-positive cardiac precursors, thus providing spatial information along the anterior/posterior axis during differentiation of the heart mesoderm.

DISCUSSION

In both vertebrates and invertebrates, the heart originates from bilateral mesodermal primordia and there is increasing evidence suggesting that molecular control mechanisms in the specification of cardiac lineages have been conserved during evolution (for review see Bodmer, 1995 ; Olson and Srivastava, 1996). However, the subsequent development of the heart differs between vertebrates and invertebrates. Cells in the linear heart tube of vertebrates assume different characteristics along the anterior/posterior axis, an important prerequisite for later morphogenesis that includes looping and chamber formation. In contrast, the morphology of the insect heart appears rather uniform along the anterior/posterior axis and the heart remains as a linear tube. Here, we show that the heart precursors in *Drosophila* assume different identities along the anterior/posterior axis within each segment. The *lb* genes, which encode homeodomain-containing transcription factors, are expressed in a specific subset of cardioblasts, as well as in a particular type of pericardial cells within each segment, and play a role in diversification of heart

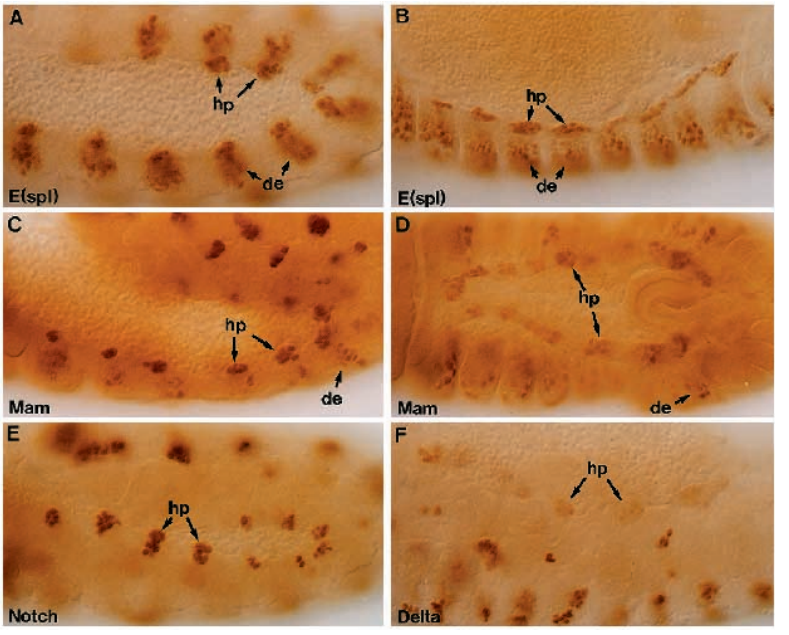

Fig. 5. Neurogenic mutations influence the segregation of *lb*-heart cells and affect dorsal vessel formation. (A,C,E) *lb*-positive cardiac cells were detected in germ-band-extended and (B,D,F) -shortened neurogenic mutant embryos using anti-Lbe antibody. (A) Enlarged clusters of heart precursors (hp) expressing *lb* segregate in embryos lacking *E(spl)*, (C) *mam* and (E) *N* gene functions. Note that the supernumerary *lb*-heart cells segregate even if *lb* expression in the dorsal epidermis (de) disappears (compare A and E). (B) After germ-band retraction ectopic *lb* expression could be detected in the heart of *E(spl)* and (D) *mam* embryos but (F) is reduced to background levels in *Dl* embryos, which display a severe neurogenic phenotype. (A,C,E,F) Lateral views and (B,D) dorsolateral views of whole-mount embryos oriented and photographed as in Fig. 3.

precursors. Like other *Drosophila* genes involved in important developmental decisions, *lb* genes have been evolutionarily conserved and have homologs in mouse and humans (Jagla et al., 1995).

Mesodermal and epidermal regulation of *lb* activity during cardiogenesis

Double-staining experiments with different markers revealed that *lb*-expressing cardiac cells correspond to a subset of cardioblasts and pericardial cells. At the beginning of the extended-germ-band stage, these cells segregate from the dorsal mesodermal cells in a process that depends on mesodermal *tin* function. Co-localisation with the epidermal *wg* domains, detected by confocal microscopy, shows that they are positioned below the anterior compartments. In addition to *tin* activity, the formation of *lb*-heart cells requires Wg signaling, which is consistent with the findings of Wu et al. (1995). Using a thermosensitive *wg* allele, we show that *lb* expression in the heart depends on Wg that is secreted from the dorsal epidermis. Since *lb* expression is limited to the heart cells that directly contact overlying *wg* domains, it seems that only high Wg concentrations are able to induce and maintain *lb* activity. In contrast, cardiac cells located below posterior compartments are under the influence of Hh signals which suppresses *lb*. This opposing action of Wg and Hh on mesodermal *lb* expression is reminiscent of the regulation of *bap* activity during visceral mesoderm development at earlier stages (Azpiazu et al., 1996). These results indicate that *wg* and *hh*, in addition to their earlier requirements for the formation of all heart progenitors, play important roles in the diversification of heart cells within each segment.

Our analysis of *lb* expression provides further evidence that

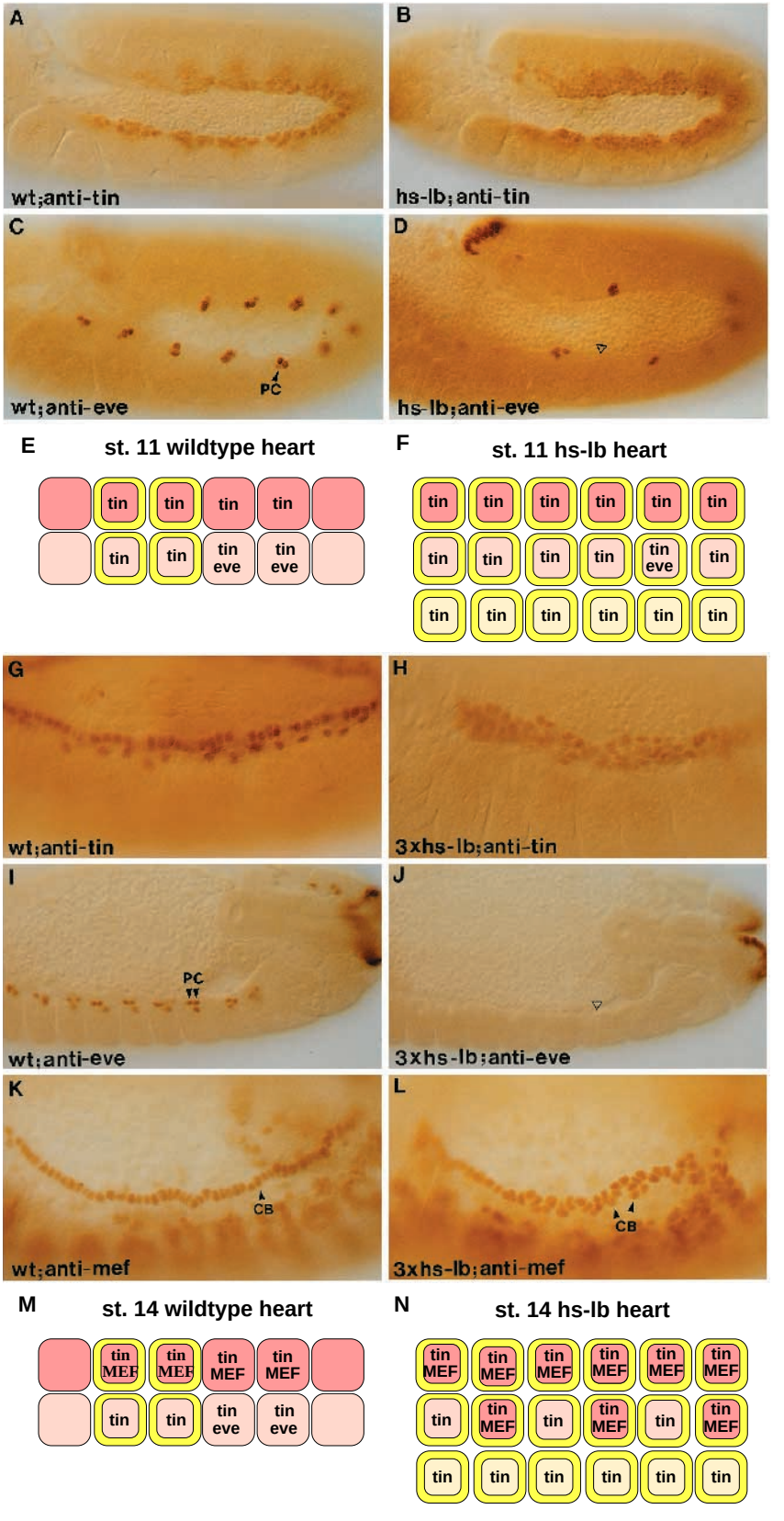

Fig. 6. Ubiquitous expression of *lb* leads to heart hyperplasia and transformation of cell identities. (A,C, E,G,I,K,M) Cardiac lineages in wild-type, (B,D,F) Hs-*lbe* and (H,J,L,N) Hs-*lbe;*Hs-*lbl* transgenic embryos were detected by anti-Tin (A,B,G,H), anti-Eve (B,D,I,J) and anti-MEF-2 (K,L) antibodies. (B,F) Ubiquitous *lbe* expression induced at 5 hours of development by a 15 minute heat shock leads to hyperplasia of *tin*-positive heart precursors and (D,F) to the reduction of the number of *eve*-pericardial cells. (H,N) Continuous expression of both *lb* transgenes between 5 and 9 hours AEL maintains ectopic *tin* activity, (L,N) recruits supernumerary *mef-2*-positive cardioblasts and (J,N) leads to the inhibition of *eve* expression (open arrowhead) in pericardial cells. (E,F and M,N) Summaries of heart-associated gene expression in (E,M) wild-type and (F,N) Hs-*lb* embryos observed (E,F) before and (M,N) after germ-band retraction. The upper row of cells represents the cardioblasts and the lower one corresponds to the pericardial cells. Heart precursors that express *lb* are labeled in yellow. Developmental stages of the embryos: (A-D) stage 11, (I,J) early stage 13, (K,L) stage 13, (G,H) stage 14. Abbreviations: CB, cardioblasts; PC, pericardial cells. All whole mounts show lateral or dorsolateral views oriented as in Fig. 3.

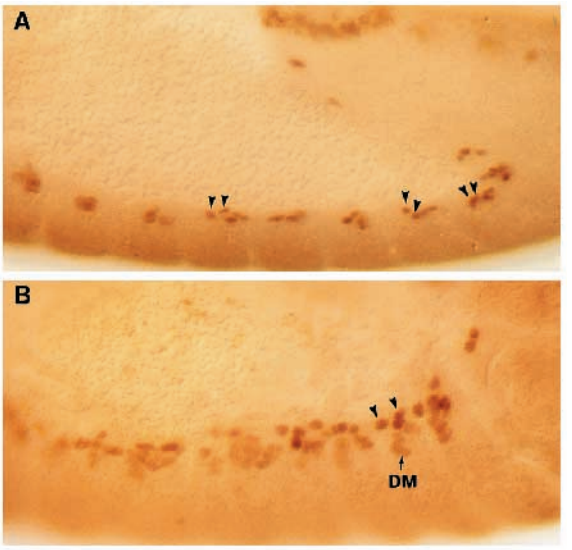

Fig. 7. Loss of *lb* function changes the identity of *l*-PCs. (A) Stage 12 and (B) stage 14 *Df(3R)e*-D7, *tin* rescue embryos stained with anti-Eve antibody. Note that, in stage 12 embryos lacking *lb* (A), the *eve*-clusters, which normally contain 2-3 cells per hemisegment (see Fig. 6C), are enlarged to 4-5 cells. After germ-band retraction (B), an average of two supernumerary heart cells continue to express *eve*. These cells (arrowheads) are located at positions that correspond to the normal location of the *l*-PCs. Abbreviations: DM, dorsal muscle 1.

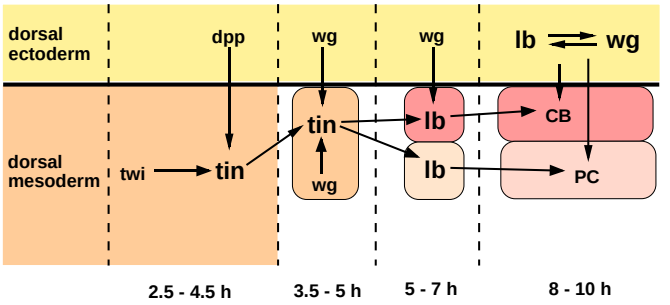

Fig. 8. Schematic representation of the role of *lb* in the cardiogenic pathway of *Drosophila*. The heart develops from the dorsal mesoderm and is specified by *tin* activity. *tin* expression initiated by *twi* is dorsally restricted by inductive Dpp signals from the overlying ectoderm and maintained by Wg signaling. In the extended-germ-band stage embryo (5-7 hours AEL), the coordinate action of mesodermal *tin* and epidermal Wg is required to induce *lb* expression in a defined subset of heart precursors. *lb*-expressing cardiac cells are located below anterior ectodermal compartments and *lb* activity determines positional identities of these cells. Later during germ-band retraction (8-10 hours AEL), ectodermal *lb* maintains late Wg signaling, which is required for proper heart patterning.

the formation of a normal heart requires neurogenic gene function. Hyperplasia of *lb*-cardiac precursors in any of the mutants for neurogenic genes indicates that the previously observed increase of heart cell number, as monitored with enhancer trap markers (Hartenstein et al., 1992), does not occur at the expense of *lb*-expressing cells. It is possible that both *e*-PCs and *l*-PCs fail to segregate in neurogenic mutants, thus giving rise to supernumerary *lb*-expressing cardioblasts.

Dual role of *lb* in *Drosophila* heart formation

The pattern of *lb* expression in progenitors of cardioblasts and pericardial cells suggests a role for the Lb gene products in the specification and diversification of cardiac lineages. To investigate this hypothesis, we have used transgenic embryos expressing *lbe*, *lbl* or both *lb* genes ubiquitously. Overexpression of *lb* during segregation of the cardiac lineages leads to a hyperplasia of heart precursors. This may indicate that *lb* can activate *tin* and perhaps other early heart determination genes to recruit ectopic mesodermal cells into the cardiogenic pathway. Since *lb* expression itself depends on *tin* function and is associated with a subpopulation of *tin*-positive heart cells, this activity of ectopic *lb* may reflect an auto- and crossregulatory loop between *tin* and *lb* to maintain their mutual expression in heart progenitors during normal development. Moreover, ubiquitous *lb* expression leads to a loss of *eve*-positive pericardial cells suggesting that ectopically expressed *lb* can change the identity of heart cells. Since, in embryos lacking *lb* function, *eve* expression is expanded, presumably because of a transformation of *l*-PCs into *e*-PCs, we conclude that *lb* is required for the diversification of cell fates within the heart. In addition, late *wg* activity in the dorsal epidermis, which depends on ectodermally expressed *lb* (Jagla et al., 1997), plays a role in maintaining *lb* expression in heart progenitors and in heart pattern formation. Together, our results provide new insights into *Drosophila* cardiogenesis, showing that *lb* genes exert at least two functions. First, autonomous, during the segregation of the heart precursors, where *lb* genes act to specify a subset of cardioblasts and pericardial cells, and second, nonautonomous, in heart pattern formation by maintaining late Wg signaling from epidermal cells (Fig. 8).

The 93E homeobox gene cluster as a control unit of mesoderm differentiation

As we have shown previously (Jagla et al., 1994, 1997), *lb* homeobox genes reside in the 93E region of the third chromosome, distally to *tin* and *bap* (Azpiazu and Frasch, 1993) and proximally to *S59* and *C15* (Dohrmann et al., 1990; P. Andermann, M. Frasch and E. Weinberg, unpublished data). All these genes code for homeodomain-containing transcription factors that are involved in various aspects of mesoderm differentiation. *tin* and *bap*, located in the most proximal part of the cluster, determine the formation of the heart and the visceral mesoderm primordia (Azpiazu and Frasch, 1993; Bodmer, 1993) whereas the distally located *S59* and *C15* homeobox genes appear to play roles in founder cell specification of body wall and alary muscles (Dohrmann et al., 1990; P. Andermann, M. Frasch and E. Weinberg, unpublished data). *lb* genes occupy a central position in the cluster and are expressed in a subset of *tin*-heart cells, corresponding to anterior cardioblasts and pericardial cell precursors in each segment. In addition, *lb* expression is specific to the segmental border muscle founder cells (Jagla et al., 1997, and data not shown). Interestingly, mesodermal *bap*, *S59*, *C15* and *lb* expression all require *tin* function, suggesting that these 93E homeobox genes act in the *tin*-dependent cascade of genetic interactions. Since the members of the 93E cluster are evolutionarily conserved (for review see Harvey, 1996; P. Andermann, M. Frasch and E. Weinberg, unpublished data), it would be interesting to determine whether the homologs are also involved in a similar pathway in vertebrates.

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