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Cross-repressive interactions of identity genes are essential for proper specification of cardiac and muscular fates in *Drosophila*

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SUMMARY

In *Drosophila* embryos, founder cells that give rise to cardiac precursors and dorsal somatic muscles derive from dorsally located progenitors. Individual fates of founder cells are thought to be specified by combinatorial code of transcription factors encoded by identity genes. To date, a large number of identity genes have been identified; however, the mechanisms by which these genes contribute to cell fate specification remain largely unknown. We have analysed regulatory interactions of *ladybird* (*lb*), *msh* and *even skipped* (*eve*), the three identity genes specifying a subset of heart and/or dorsal muscle precursors. We show that deregulation of each of them alters the number of cells that express two other genes, thus changing the ratio between cardiac and muscular cells, and the ratio between different cell subsets within the heart and within the dorsal muscles. Specifically, we demonstrate that mutation of the muscle identity gene *msh* and misexpression of the heart identity gene *lb* lead to heart hyperplasia with similar cell fate modifications. In *msh* mutant embryos, the presumptive *msh*-muscle cells switch on *lb* or *eve* expression and are recruited to form supernumerary heart or dorsal muscle cells, thus indicating that *msh* functions as a repressor of *lb* and *eve*. Similarly, overexpression of *lb* represses endogenous *msh* and *eve* activity, hence leading to the respecification of *msh* and *eve* positive progenitors, resulting in the overproduction of a subset of heart cells. As deduced from heart and muscle phenotypes of *numb* mutant embryos, the cell fate modifications induced by gain-of-function of identity genes are not lineage restricted. Consistent with all these observations, we propose that the major role of identity genes is to maintain their restricted expression by repressing other identity genes competent to respond positively to extrinsic signals. The cross-repressive interactions of identity genes are likely to ensure their localised expression over time, thus providing an essential element in establishing cell identity.

Key words: Heart, Somatic muscles, *ladybird*, *even skipped*, *msh*, Cell fate, *Drosophila*

INTRODUCTION

The molecular pathways involved in the early events of heart and muscle formation are thought to be conserved from invertebrate to vertebrate species (for reviews, see Bodmer and Venkatesh, 1998; Jagla et al., 1998; Yun and Wold, 1996). Considerable progress in understanding how the heart and the muscle progenitors are specified has been made using *Drosophila* as a model system. However, the mechanisms that underlie commitment of cells to cardiac and muscular lineages and the requirements for generating the diversity of heart and muscle cells are not fully understood. In *Drosophila* embryos, heart tube derives from the dorsal mesoderm. It is located close to a subset of syncytial muscle fibres and composed of a small number of cell types, and is thus well suited for studying the genetic regulation of cell commitment.

To date, different intrinsic and extrinsic factors involved in progressive cell fate determination in the dorsal mesoderm of *Drosophila* have been identified (Corbin et al., 1991; Gisselbrecht et al., 1996; Shishido et al., 1997; Jagla et al., 1997; Carmena et al., 1998b; Park et al., 1998, Nose et al., 1998; Gajewski et al., 1999; Su et al., 1999; Gajewski et al., 2000; Crozatier and Vincent, 1999; Fosset et al., 2000; Ward and Skeath, 2000; Lee and Frasch, 2000, Halfon et al., 2000). The activity of the homeobox gene *tinman* (*tin*) (Azpiazu and Frasch, 1993; Bodmer, 1993) maintained by ectodermal Decapentaplegic (Dpp) signals delimits dorsal mesodermal cells (Frasch, 1995) and makes them competent to form heart, dorsal muscles and visceral mesoderm. *tin* expression in these cells is necessary but not sufficient to specify cardiac and muscular fates, as ubiquitously distributed *tin* products do not appear to cause any dorsalisation (Yin and Frasch, 1998). Segmentally repeated clusters of cells competent to become the heart and muscle progenitors form at the intersection of transversely striped *wingless* (*wg*) domain and the dorsal *dpp*/*tin* domain thus suggesting that *tin* expression is only instructive in conjunction with Wg and Dpp signalling pathways (for a review, see Frasch, 1999). In response to combined Dpp/Tin/Wg activities the mesodermal cells underlying the epidermal Dpp/Wg overlap switch on the

proneural *lethal of scute* (*l'sc*) [*l(1)sc* – FlyBase] gene encoding a bHLH protein (Carmena et al., 1998b). Subsequent Receptor Tyrosine Kinase (RTK) action subdivides this territory into small clusters of equivalent cells from which the individual progenitors segregate (Carmena et al., 1998b; Halfon et al., 2000). This step involves a process of lateral inhibition governed by the neurogenic genes, which include Notch and its ligand Delta. Progenitor cells are thought to produce a pair of sibling cells, either the founders of dorsal muscles or precursors of two types of heart cells, the cardioblasts and the pericardial cells. The distinct fates of founder cells derived from the same progenitor are determined by asymmetric segregation of the Numb protein and by lineage-specific expression of identity genes (Ruiz Gomez and Bate, 1997; Jagla et al., 1998). Recent studies have provided important insights into the mechanisms underlying specification of a subset of pericardial cells expressing the identity gene *even skipped* (*eve*) (Carmena et al., 1998a; Park et al., 1998). These cells arise from the Numb-devoid founder cell generated after asymmetric division of dorsal progenitor P2 (Ruiz Gomez and Bate, 1997; Carmena et al., 1998a). The absence of Numb makes the prospective *eve*-pericardial cells competent to respond to Notch signalling, a requirement essential for their final fate (Park et al., 1998). In addition, the intrinsic program of pericardial cell specification involves the function of the GATA factor Pannier (Pnr) (Gajewski et al., 1999) and its antagonist U-shaped (Ush) (Fosset et al., 2000).

Studies on *eve*-positive muscle and heart lineages have indicated that the closely located dorsal progenitors P2 and P15 are specified sequentially (Carmena et al., 1998a). As demonstrated by Knirr et al. (Knirr et al., 1999), for a *slouch* (*slou*)-expressing progenitor (P5/25) and adjacent *ladybird* (*lb*)-positive SBM progenitor (P8), the sequential segregation also takes place in the lateral somatic mesoderm. In this case, an ectopic expression of *lb* in P5/25 and the resulting supernumerary SBM muscles observed in *slou* mutant embryos clearly indicate that the repression of *lb* by *slou* is a prerequisite for the specification of the progenitor P5/25 (Knirr et al., 1999). Similar negative interactions between *lb* and the *muscle specific homeobox* (*msh*) gene are essential for proper fate specification of muscles derived from the *msh*-expressing progenitors P21/22, P23/24 and the *lb*-positive SBM progenitor (Jagla et al., 1999). Thus, the switches of cell fates resulting from the repression of one identity gene by an other have been previously reported in *Drosophila*; however, the mutual repression between identity genes was not considered as an integral part of intrinsic program governing cell fate specification.

In this report, we focus on the interactions of three homeobox-containing identity genes expressed in adjacent populations of heart and muscle precursors: the *lb* genes that specify a subset of heart cells (Jagla et al., 1997); the *msh* gene required for the specification of two dorsal muscles (Nose et al., 1998); and the *eve* gene, which determines two pericardial cells and a dorsal muscle (Su et al., 1999). Using misexpression experiments and confocal microscopy, we demonstrate that *msh, eve and lb* gene products act as mutual repressors. Taking in consideration cross-repressive interactions of vertebrate identity genes during specification of neural fates (Briscoe et al., 2000; Pierani et al., 2001; Moran-Rivard et al., 2001) we anticipate that mutual repression between identity genes represents an important part of intrinsic mechanisms that lead to the diversification of cell types in both invertebrate and vertebrate development.

MATERIALS AND METHODS

Drosophila strains

The following *Drosophila* strains were used: *numb2* (Uemura et al., 1989); *msh-lacZ* line rH96, the *msh* null alleles; $msh^{\Delta 68}$ and $msh^{\Delta 89}$; and the *UAS-msh-m25-m1* line (Nose et al., 1998), which exhibits high levels of ectopic *msh* expression when crossed with a GAL4 driver. For the *lb* gain-of-function experiments, we used double transgenic *Hs-lbe/Hs-lbl* flies (Jagla et al., 1997) or *UAS-lbe-16-1* strain (Jagla et al., 1997). The *lb* mutants referred to as lb^{def} have been previously described (Jagla et al., 1997). The *UAS-eve* line and the thermosensitive eve^{ts} mutants (Su et al., 1999) were kindly provided by R. Bodmer.

Ectopic expression and temperature shift

Ectopic mesodermal expression of *msh*, *lbe* and *eve* was induced using the *Gal4-UAS* system with the mesoderm-specific effector line *24B-Gal4* (Brand and Perrimon, 1993). The influence of ectopic *lb* expression on the specification of *msh*-positive muscle precursors was analysed in heat-shocked embryos derived from the cross of *Hs-lbe/Hs-lbl* males and rH96 virgin females. *Hs-lbe* and *Hs-lbl* transgenes were induced at 5 hours after egg laying (AEL) by a 20 minutes heat shock in 37°C water bath. To invalidate *eve* function during specification of muscle and heart progenitors, the eve^{ts} embryos were shifted from 18°C to 29°C at 5 hours AEL.

Immunocytochemistry and in situ hybridisation

Embryos were stained with the following primary antibodies: monoclonal anti-Lbe 1:1 (Jagla et al., 1997); rabbit anti-Kr, 1: 1000 (provided by G. Vorbrüggen); guinea pig anti-Kr, 1:500 (provided by D. Kosman); rabbit anti-β-galactosidase, 1:5000 (Cappel Laboratories); rabbit anti-Eve, 1:2000 and guinea pig anti-Eve, 1:1000 (provided by D. Kosman); rabbit anti-Odd (1:500) (provided by J. Skeath); rabbit anti-Tin, 1:800 (provided by M. Frasch); rabbit anti-dMef2, 1:1000 (provided by H. Nguyen); rabbit anti-β3-Tubulin, 1:200 (provided by R. Renkawitz-Pohl); rabbit anti-Myosin Heavy Chain, 1:2000 (provided by D. Kiehart); and monoclonal anti-Pc, 1:3 (mAb No.3, anti-pericardin antibody, provided by T. Volk). Labelled cells were detected using the ABC-Elite-peroxidase kit (Vector Laboratories) with diaminobenzidine as a substrate, or with secondary antibodies conjugated with Cy2, Cy3 or Cy5 (Jackson). In some cases, immunostaining was amplified using the Tyramide-Biotin (indirect) or Tyramide-Cy3 (direct) systems (NEN). To visualise heart and the muscle cells simultaneously, the whole-mount embryos were analysed using an OLYMPUS FV300 confocal microscope. The whole-mount in situ hybridisation experiments were performed with a digoxigenin-labelled *msh* antisense-RNA probe according to Tautz and Pfeifle (Tautz and Pfeifle, 1989). The full-length *msh* cDNA clone kindly provided by T. Isshiki was used as a template.

RESULTS

Cell types in the dorsal mesoderm and their molecular markers

Previous studies (Carmena et al., 1998b) have indicated that dorsally located heart and muscle founders derive from a precluster of *l'sc*-expressing cells determined by an interplay of epidermal Wg and Dpp signalling pathways. At the beginning of germband retraction, in each abdominal

hemisegment one can distinguish segmental heart units composed of six cardioblasts and ten pericardial cells (Ward and Skeath, 2000), as well as closely adjacent precursors of four dorsal muscles (Fig. 1A). Interestingly, the cardioblasts and the pericardial cells within the same hemisegment differ along the anteroposterior axis as pairs of cells that could be characterised by the unique combinatorial code of transcription factors (Fig. 1A). The most anterior pair of cardioblasts expresses *tin*, the adjacent pair is *tin*/*lb*-positive, while the most posterior do not express *tin* and can be detected in a *seven up-lacZ* (*svp-lacZ*) enhancer trap line (data not shown) (Ward and Skeath, 2000) as well as with the anti-Pericardin (anti-Pc) antibody (Fig. 1A,B). Among the pericardial precursors, as the most recent data indicate (Ward and Skeath, 2000), four pairs derive from the anterior compartment and one pair originates from the posterior compartment. Three out of four anterior pericardial pairs express *tin* (Fig. 1A,C). One *tin*-positive pair co-expresses *lb* and another one co-expresses *eve* (Fig. 1A,C). The *tin*-only expressing pericardial cells lie behind the cardioblasts and are often difficult to distinguish (Fig. 1C).

Two remaining *tin*-negative pairs of pericardial cells have recently been shown to express the Odd-skipped protein (Ward and Skeath, 2000) (Fig.1A). Two out of four Odd-pericardial cells are located in the posterior compartment and co-express *Svp* (Fig. 1A). Precursors of dorsal muscles DA1, DO1, DA2 and DO2 are located more lateral to the pericardial cells. Like the heart cells, they express a combination of transcription factors that allows monitoring of their identity during development (sketched in Fig. 1A). Among them, the most dorsally located DA1 and DO1 can be detected using anti-Eve and the *msh-lacZ* enhancer trap line rH96, respectively (Fig.1D) (Nose et al., 1998). In addition, in the late stage embryos, the cardioblasts and the myoblast nuclei could be visualised using anti-Mef2, while the pericardial cells could be visualised using anti-Pc antibodies (Fig. 1B). At stage 13/14 of embryogenesis, weak Mef2 staining is also detected in pericardial cells (data not shown, see also Fig. 7A). In this study, the influence of deregulated activity of heart and muscle identity genes was monitored using the cell fate markers described above.

Ectopic *lb* expression leads to heart hyperplasia concomitantly with the loss of dorsal muscles

Our previous data have shown that *lb* functions to specify the identity of two pericardial cells (*lb*-Pc) and when misexpressed, is able to change the fates of adjacent *eve*-expressing pericardial cells (*eve*-Pc) (Jagla et al., 1997) (Fig. 2C,D). The analysis of *lb* gain-of-function experiments with the use of a general heart marker *tin* has led us to the conclusion that ubiquitous mesodermal expression of *lb* results in heart hyperplasia (Jagla et al., 1997). To elucidate the origin of supernumerary heart cells in *24B-GAL4/UAS-lbe* and *Hs-lbe/Hs-lbl* embryos (see Materials and Methods), we analysed specification of the most dorsal muscle founders and formation of corresponding syncytial fibres. The DA1 founder was monitored with anti-Kr (Fig. 2A,B) and anti-Eve (Fig. 2C,D) while the specification of DO1 founder was revealed with anti-Kr (Fig. 2A,B) or with anti-*lacZ* in rH96 embryos (Fig. 2E,F). The resulting muscle fibres were detected using anti-Myosin Heavy Chain (MHC) (Fig. 2I). We found that the pattern of all founder-specific markers was significantly affected (Fig. 2B,D,F). The uniform mesodermal expression of *lbe* led to the

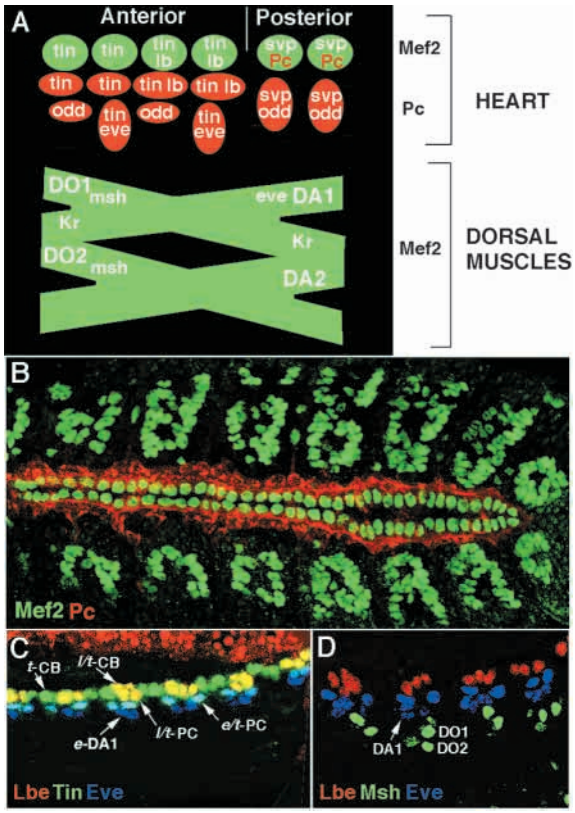

Fig. 1. Molecular markers of cardiac and dorsal muscle cells. (A) A scheme summarising expression of the so far defined markers of cardiac and muscular fates (Jagla et al., 1997; Ruiz Gomez et al., 1997; Nose et al., 1998; Su et al., 1999; Ward and Skeath, 2000; Gajewski et al., 2000) and representing one hemisegment of a developing *Drosophila* embryo. Notice that cardiac precursors diversify as pairs of cells. Individual pairs of heart cells and individual muscle precursors express a unique combination of molecular markers. (B) Dorsal view of stage 15 wild-type embryo, showing the cardioblasts and dorsal muscles stained with anti-Mef2 (green) and the pericardial cells visualised by anti-Pericardin (red). (C) Lateral view of stage 13 wild-type embryo triple-stained for *tin* (green), *lbe* (red) and *eve* (blue) demonstrates how the different subsets of cardiac cells can be identified. (D) The *RH96 msh-lacZ* embryo triple stained for *lbe* (red), *msh/lacZ* (green) and *eve* (blue) shows that *lbe*, *msh* and *eve* are expressed in adjacent non-overlapping populations of dorsal mesodermal cells. Abbreviations: DO1,dorsal oblique muscle 1; DO2, dorsal oblique muscle 2; DA1, dorsal acute muscle; DA2, dorsal acute muscle 2; t-CB, tin-positive cardioblasts; l/t-CB, lb/tin-positive cardioblasts; l/t-PC, lb/tin-positive-pericardial cells; e/t-PC, eve/tin-positive pericardial cells.

60% loss of DA1 founders and 40% loss of DO1 founders (Table 1; Fig. 2B). Affected Kr expression (Fig. 2B) was not linked to selective *Kr* repression, as the reduced number of DA1 and DO1 muscle precursors was also detected by anti-Eve (Fig. 2D) and anti-Msh (Fig. 2F) antibodies.

This specific loss of identity of most dorsally located muscle founders most probably leads to morphological defects observed in dorsal somatic musculature of late stage embryos overexpressing *lb* genes (Fig. 2I). As revealed by the anti-MHC antibody (Fig.2I), the gaps in somatic musculature are associated with the significantly enlarged heart area, suggesting that the supernumerary heart cells are generated by a shift in

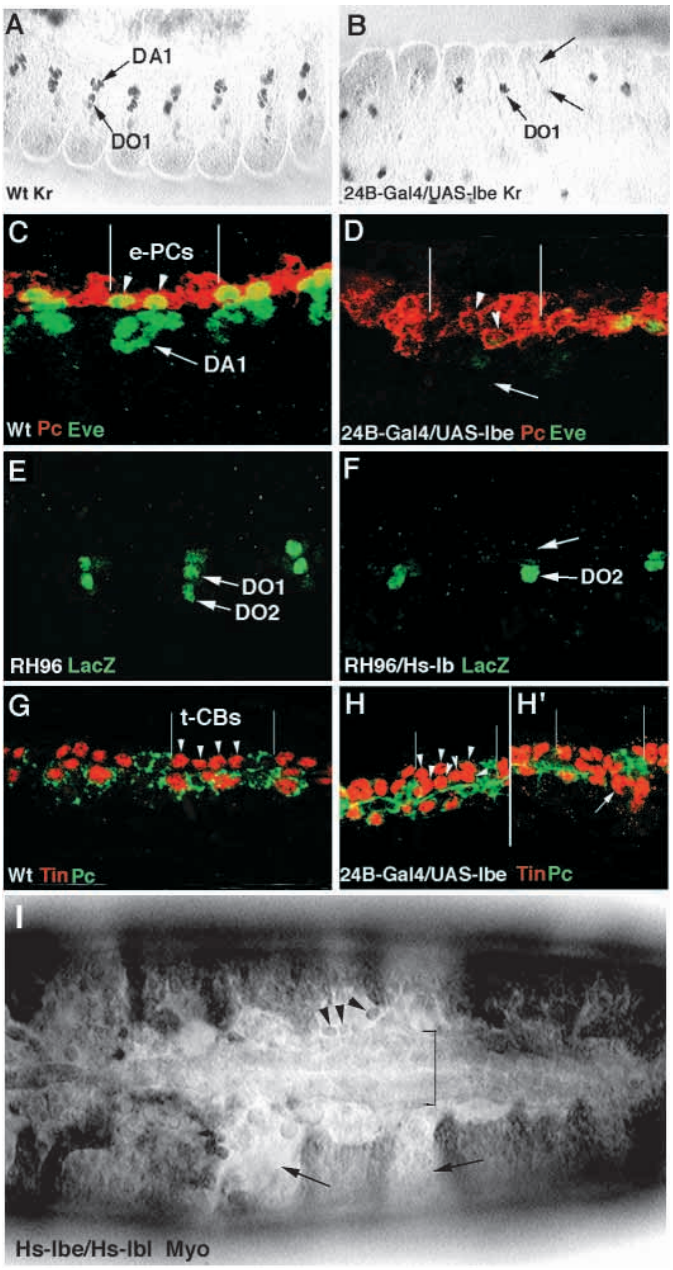

Fig. 2. Ectopic *lb* represses endogenous *eve* and *msh*, leading to the modification of cardiac and muscular fates. (A,E) Dorsolateral views of stage 13 and (C,G) of stage 14 embryos, showing wild-type expression of cardiac and dorsal muscle markers. (B,D,F,H,H′) Comparable views of embryos overexpressing *lbe* under control of (B,D,H,H′) *24B-Gal4* or (F) *heat-shock* promoter. Ectopic *lb* leads to the loss of *Kr*-labelled DA1 and DO1 founders (arrows in B), *eve*-labelled pericardial cells (e-PCs, arrowheads in D) and DA1 muscle (arrow in D), as well as *lacZ*-positive DO1 founder in *msh-lacZ* embryos, RH96 (arrow in F). Concomitant with this reduced expression of muscle and pericardial markers, a uniform mesodermal expression of *lb* provokes heart hyperplasia manifested by supernumerary *tin*-positive cardioblasts (arrowheads in H) or *tin*-positive pericardial cells (arrow in H′). (I) Morphological defects in muscle and heart formation induced by ectopic *lb* and visualised by the anti-MHC staining. The gaps in dorsal musculature (arrows) and non-fused myoblasts (arrowheads) are associated with the enlarged heart area (bracket). Abbreviations: DO1,dorsal oblique muscle 1; DO2, dorsal oblique muscle 2; DA1, dorsal acute muscle; DA2, dorsal acute muscle 2; t-CB, tin-positive cardioblasts; l/t-CB, lb/tin-positive cardioblasts; l/t-PC, lb/tin-positive-pericardial cells; e/t-PC, eve/tin-positive pericardial cells.

Table 1. Modifications of the number of cardiac and muscle precursors induced by ectopic expression of *lb*, *msh* and *eve*

	Heart precursors			Muscle precursors			
	Lb			Msh DO1			
	Tin CB	CB/PC	Eve PC	Eve DA1	DO2	Kr DA1	Kr DO1
Wt	4	4	2	1	2	1	1
UAS-lb	7.5	-	0.3	0.4	0.6*	0.5	0.4
UAS-msh	1.2	0.3	0.4	0.1	-	-	1.8
UAS-eve	1.6	0.1	-	-	0.8	1.5	0.3

Number of cells represents an average from the 50 hemisegments. Cells were counted in abdominal segments A2 to A7. The 24B-GAL4 driver was used to overexpress *lb*, *msh* and *eve* genes in the mesoderm.
*Embryos from the cross of *rh96 msh-lacZ* strain with *Hs-lbe/lbl* strain.
–, not determined.

fate of muscle founders, rather than by aberrant proliferation of cardiac precursors. Interestingly, the majority of cardiac cells induced by ectopic *lb* expression corresponds to the *tin*-positive cardioblasts (Fig. 2H). On average, mesodermal ectopic expression of *lb* results in the generation of 7.5 *tin*-labelled cardioblasts per hemisegment instead of 4 (Table 1). In addition, the accumulation of supernumerary *tin*-positive pericardial cells is seen in about 20% of hemisegments (Fig. 2H′). These pericardial cells are distinct from *eve*-Pc, as ectopic *lb* suppresses *eve*-positive fates (Fig. 2D). Altogether, these observations suggest that *lb*, when ectopically expressed, is able to repress *msh* and *eve*, leading to the transformation of dorsally located muscle founders into the *tin*-positive heart cells.

Ectopic *msh* suppresses cardiac fates

One possible explanation of the exclusive non-overlapping expression of Lb, Msh and Eve proteins in small subsets of adjacent dorsally located mesodermal cells (see Fig. 1D) is that these genes are able to repress each other. To test this possibility, we induced ubiquitous mesodermal expression of *msh*. Previous work (Nose et al., 1998) has indicated that *msh* is required for the specification of two dorsal muscles and is not expressed in the heart. Indeed, as shown in Fig. 1D, *msh* is not expressed in the heart field. However, our data clearly demonstrate the ectopic *msh* leads to alterations in both dorsal muscle and heart cell populations (Fig. 3). The number of heart cells is dramatically reduced (Fig. 3). The majority of pericardial cells and about 70% of *tin*-expressing cardioblasts are lacking (Table 1; compare Fig. 3A with 3B). Among suppressed cardiac cell fates are those specified by the *lb* and *eve* gene activities (Fig. 3C-F; see also Table 1).

In embryos that overexpress *msh*, we observe less than 10% of *lb*-positive and about 30% of *eve*-positive heart cells when compared with the wild type (Table 1). The inhibitory influence of ectopic *msh* on a large spectrum of cardiac fates is particularly obvious once the pericardial cells and the cardioblasts are simultaneously visualised (Fig. 3G-J).

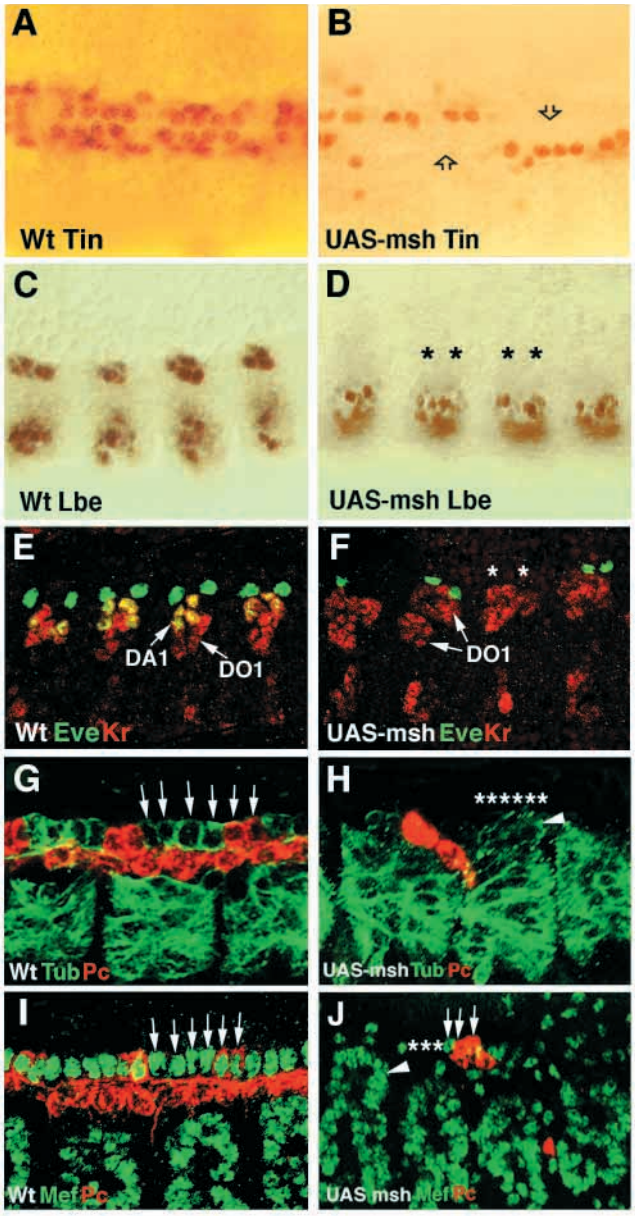

Fig. 3. Gain of *msh* function affects specification of a large subset of cardiac cells. Nomarski (A-D) and confocal (E-J) micrographs of the dorsal regions of embryos stained with anti-Tin (A,B), anti-Lbe (C,D) or double-stained with anti-Eve/anti-Kr (E,F), anti-Pericardin/anti-β3-Tubulin (G,H) and with anti-Pericardin/anti-Mef2 (I,J). Wild-type (A,C,E,G,I) and transgenic embryos (B,D,F,H,J) ectopically expressing *msh* under control of 24B-GAL4 driver. Note that the mesodermal gain of *msh* function leads to a significant reduction of *tin*-, *lbe*- and *eve*-positive heart cells (open arrows in B and asterisks in D,F) and to the switch from DA1 to DO1 identity (compare E with F). Simultaneous visualisation of heart and dorsal muscles (G-J) clearly demonstrates that ectopically expressed *msh* leads to the reduction of either the pericardial cells (compare red staining in G,I with that H,J) and the cardioblasts (compare arrows in G,I with asterisks in H,J). Both specification of anterior Tub-positive and posterior Pc-positive cardioblasts are affected (compare G with H). Loss of cardiac cells is accompanied by expansion of dorsal muscles (arrowheads).

Surprisingly, *msh* is able to affect specification of both the anterior (β3-Tub-labelled) and the posterior (Pc-labelled) cardioblasts (compare Fig. 3G with 3H). Moreover, the major part of Pc-positive pericardial cells is missing (Fig. 3H,J). As shown by double Kr/Eve staining (Fig. 3E,F), *msh* is also able to change the fates of dorsal muscles. Specifically, in *UAS-msh* embryos, the majority of *eve*-positive DA1 muscle precursors are substituted by supernumerary DO1 muscles. Thus, the loss of cardiac and particular muscle cells is accompanied by overproduction of other dorsal muscle lineages (Fig. 3H,J), suggesting that *msh* may change the identity of dorsal mesodermal cells and recruit them to the alternative myogenic pathways.

Cell fate switches in embryos lacking functional Msh

To test whether *msh* determines cell fates by repressing a set of identity genes, we analysed specification of heart and dorsal muscle cells in *msh* null mutant embryos. Loss of *msh* function leads to the generation of supernumerary *tin*- (compare Fig. 4A with 4B) and *mef*-positive (compare Fig. 4C with 4D) heart cells. Interestingly, the *msh*-induced heart hyperplasia seems to result from the generation of two distinct types of additional cardiac cells. The first category corresponds to the supernumerary cardioblasts (arrows in Fig. 4B,D), while the second type of cells (arrowheads in Fig. 4B,D) is located more laterally than the regular cardioblasts. Based on identical position of supernumerary *odd*-pericardial cells observed in *msh* mutant embryos (arrowheads in Fig. 4F), we propose that the loss of *msh* function leads to the specification of enlarged number of both the cardioblasts and the pericardial cells. The increased number of heart cells in *msh* mutants may indicate that in the wild-type embryos, *msh* represses the activity of genes specifying cardiac fates. In order to check this possibility, we traced the development of DO1 and DO2 muscles in embryos lacking *msh* function. As indicated in Fig. 4G (arrows), at least some of the presumptive *msh*-positive founders (labelled by the *lacZ* in null $msh^{\Delta 89}$ strain) (Nose et al., 1998) switch on *lb* expression. As *lb* is never co-expressed with *msh* in wild-type embryos, we conclude that *msh* functions to repress *lb* activity.

In *msh* mutants, *lb* is de-repressed in the presumptive *msh*-positive muscle founder cells, which leads to their recruitment into cardiogenic pathway. However, only some *msh*-devoid cells are able to adopt cardiac fates (see irregular heart hyperplasia in Fig. 4B,D), indicating that msh^{-} cells may be alternatively recruited to other differentiation pathways. Indeed, in msh^{-} background, some of the *msh*-positive myoblasts (most probably fusion-competent cells that form DO1 muscle) are recruited to built enlarged *eve*-labelled DA1 muscle precursors (Fig. 4H). These myoblasts, once incorporated into *eve*-positive DA1 syncytium, lose their *lacZ* expression progressively (Fig. 4H), indicating that *eve* has a capacity to repress *msh*. Most likely, for this reason, in $msh^{\Delta 89}$ mutants we detect only few *lacZ*-expressing cells that contribute to DA1 muscles even if these muscle fibres are significantly enlarged (Fig. 4H). This observation is consistent with statistically rare incidence of *lb* and *lacZ* co-expression in $msh^{\Delta 89}$ embryos and with comparatively low *lacZ* expression in the presumptive *msh*-positive cells recruited to the cardiogenic pathway (arrow in Fig. 4H). The detection of only few cells co-

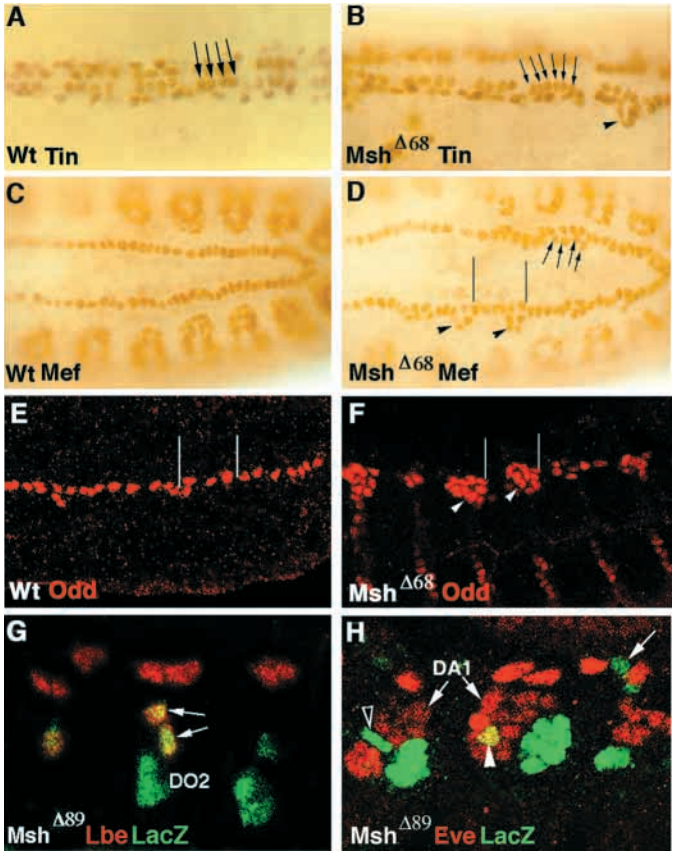

Fig. 4. Cell fate transformations in *msh* mutant embryos. Dorsal region of (A,C,E) *Oregon R*, (B,D,F) $msh^{\Delta 68}$ and (G,H) $msh^{\Delta 89}$ embryos are shown. Both *msh* alleles are null. In $msh^{\Delta 89}$ embryos, the presumptive *msh*-positive myoblasts can be monitored by *lacZ* expression (Nose et al., 1998). As revealed by Tin (A,B) and Mef2 (C,D) staining, loss of *msh* function leads to the formation of supernumerary cardioblasts (arrows in B,D). Abnormal *tin* and *mef* expression is also seen in cells located more laterally (arrowheads in B,D). As shown by Odd staining (E,F) these cells correspond to supernumerary *odd*-expressing pericardial cells (arrowheads in F). In comparison with the wild-type situation (see Fig. 1D), the loss of *msh* function leads to the activation of *lbe* (arrows in G) in some of the presumptive *msh*-positive myoblasts. These cells (as shown in G) can be detected at the late stage 12, thus suggesting that they correspond to the founders or to the first attracted fusion-competent cells. They most probably contribute to the enlarged heart (B,D). In addition, the msh^{-} myoblasts (presumably fusion competent cells) may also adopt an alternative myogenic fate and contribute to enlarged *eve*-positive DA1 muscles (white arrowhead in H). Open arrowhead indicates a not yet integrated *lacZ*-positive myoblast close to the *eve*-positive DA1 syncytium. Arrow in H points at a *lacZ*-positive cell located at abnormal position, it is now in the area of the cardiac cells. Note that the *msh*, *eve* and *lb* expression domains are never overlapping in wild-type embryos (see Fig. 1D). For the abbreviations, refer to the legend to Fig. 1.

expressing *lacZ* and *lb*, or *lacZ* and *eve* in germband retracted $msh^{\Delta 89}$ embryos (Fig. 4G,H), associated with relatively important perdurance of β-gal (Halfon et al., 2000), suggests also that the repressive action of *lb* and *eve* on *msh* appears early in development (most probably at the progenitor stage).

Different regulative behaviours of cells overexpressing *eve*

Adjacent heart and muscle cells achieve their rigidly determined states as they await metamorphic cues provided by secreted EGF and FGF signals (Buff et al., 1998), and by localised activation of transcription factors, such as Lb and Msh. Our data indicate that *lb* prevents presumptive cardiac cells from assuming a muscle-specific identity by repressing the muscle fate inducing gene *msh*. Similarly, *msh* is able to repress *lb* and to promote specification of dorsal muscle instead of cardiac fates. In contrast to *msh* and *lb*, *eve* is expressed in both cardiac (pericardial cells) and muscular (DA1) precursors, and is involved in cell fate specification processes in these two territories (Su et al., 1999). The requirement for *eve* and its mode of action can be demonstrated by misexpressing the gene outside of its endogenous expression pattern. We used 24B-GAL4 line to drive *eve* expression ubiquitously in differentiating mesoderm and have found that a significant number of cells from the dorsal mesoderm assume altered identities. In particular, ectopic *eve* represses *lb* and leads to the loss of *lb*-expressing heart cells (Fig. 5A,B; Table 1). Surprisingly, not only the *lb*-positive pericardial cells but also the cardioblasts are missing. This effect appears similar to the loss of cardiac cells in UAS-msh embryos (Fig. 3), except that in embryos that misexpress *eve*, a subset of *tin*-labelled cardiac cells is still specified or even overproduced (see arrowheads in Fig. 5B).

As revealed by Mef2 staining (Fig. 5D), only some of the remaining heart cells correspond to the cardioblasts, indicating that *eve* promotes preferentially a particular type of pericardial fate (most probably *eve*-Pc). This assertion is also supported by the negative influence of ectopic *eve* on another subset of pericardial cells expressing *odd* (Fig. 5E,F). Concomitant with the loss of the majority of cardiac cell types, ubiquitous mesodermal expression of *eve* leads to the formation of supernumerary dorsal muscles (Fig. 5D, Table 1). As shown by Mef2 (Fig. 5D) and double Kr/Odd staining (Fig. 5F), the supernumerary dorsal muscles appear preferentially in segments with the reduced number of heart cells, thus suggesting that *eve* is also able to convert cardiac into muscular fates. The additional Kr-positive muscles (Fig. 5F) cannot however correspond to DO1 fibres, as in situ hybridisation experiments with *msh* probe (Fig. 5G,H) clearly show that the ectopic *eve* represses *msh*, leading to the loss of DO1 and DO2 identities. Taken together, these observations demonstrate different regulative behaviour of cells that overexpress *eve* and reflect a binary decision they may take to achieve their identity.

Eve and Lb act as repressors during specification of cardiac and muscular fates

In order to provide direct demonstration that Eve and Lb behave as repressors, we have analysed the influence of loss of function of both genes on cell fate specification in the dorsal mesoderm of the *Drosophila* embryos. The previously described thermosensitive eve^{ts} mutants (Su et al., 1999) and *tin* rescue construct-carrying lb^{def} mutants (Jagla et al., 1997) were used in this study. In eve^{ts} embryos, non-functional Eve protein is still synthesised, which makes it possible to follow the *eve*-positive cells. As shown in Fig. 6A (compare with wild type shown in Fig. 7C) loss of *eve* function at 5 hours of

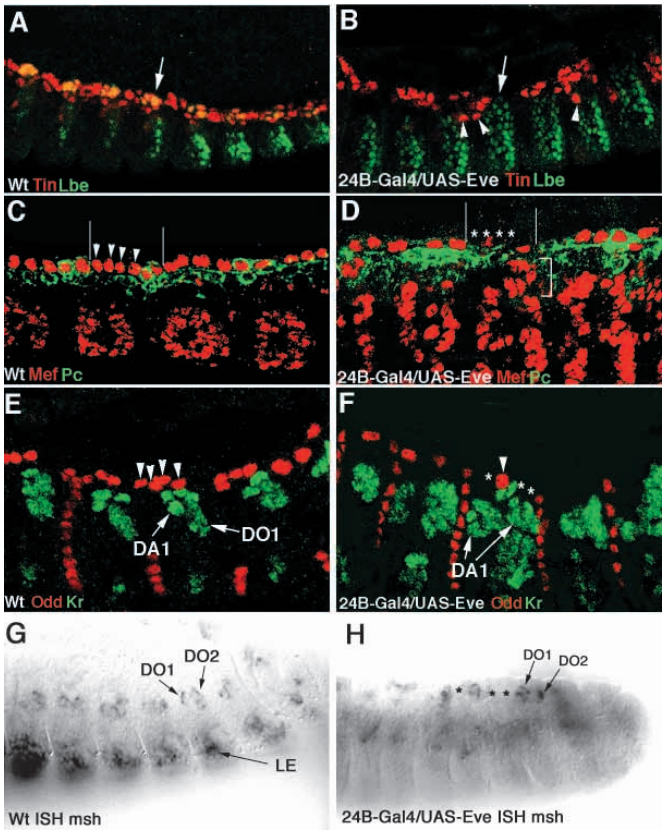

Fig. 5. Cardiac and muscular phenotypes of embryos overexpressing *eve*. Confocal (A-F) and Nomarski (G,H) micrographs of the dorsal regions of wild-type (A,C,E,G) and transgenic embryos (B,D,F,H) expressing *eve* under control of 24B-GAL4 driver. The late stage 12 (A,G), stage 13 (B,H) and stage 14 embryos (C-F) are shown. Ectopic *eve* suppresses endogenous *lb* expression and leads to the loss of *lb/tin*-positive cardiac cells (arrow in A,B). Note also an overproduction of a subset of *tin*-positive heart cells lying more laterally (arrowheads in B). (C,D) Double Mef/Pc staining demonstrates that the gain of *eve* function results in a significant reduction in the number of cardioblasts (asterisks in D), concomitant with an increased number of Mef2-positive cells at the position of dorsal muscles (bracket in D). The Pc staining appears slightly enlarged (D). As revealed by double Kr/Odd staining (E,F), ectopic *eve* leads to the formation of supernumerary DA1 muscles (F) in segments that lack Odd pericardial cells (asterisks in F). Simultaneously, in situ hybridisation with a *msh* probe (G,H) shows that *eve* is able to repress the *msh*-expressing DO1 and DO2 fates (asterisks in H). Abbreviations: DO1,dorsal oblique muscle 1; DO2, dorsal oblique muscle 2; DA1, dorsal acute muscle; DA2, dorsal acute muscle 2.

development (see Materials and Methods) leads to de-repression of *lb* in the *eve*-positive mesodermal cells. Consequently, activated *lb* represses *eve* so that, in the germband retracted embryos, expression of mutated Eve protein becomes progressively lower. We observe supernumerary *lb*-positive cells (arrowheads in Fig. 6A) in segments in which *eve* is completely repressed. Consistent with the model of mutual repression between *lb* and *eve*, the loss of *lb* function (Fig. 6B) leads to the specification of supernumerary *eve*-expressing cells. As demonstrated by double Kr/Eve staining (arrowheads in Fig. 6B), the additional

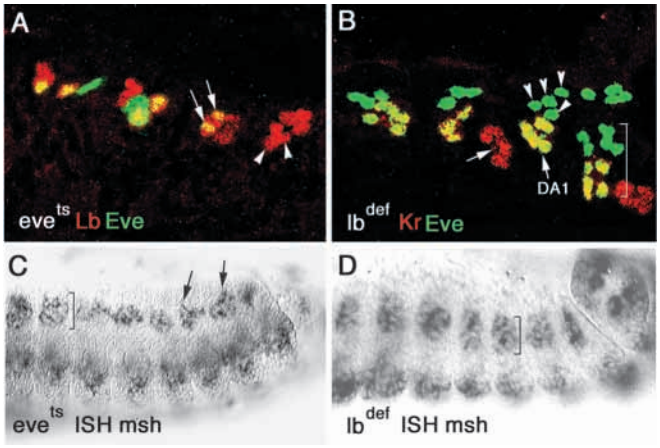

Fig. 6. Cell fate modifications in *eve* and *lb* mutant embryos. Confocal (A,B) and Nomarski (C,D) micrographs representing the dorsolateral views of late stage 12 (A,C,D) and late stage 13 (B) embryos. (A,C) Temperature shift-induced loss of *eve* function leads to de-repression of *lb* in presumptive *eve*-positive mesodermal cells (arrows in A) and to enlarged pattern of *msh* transcripts (bracket and arrows in C) in dorsal muscle area. For wild-type *msh* expression in dorsal mesoderm revealed by in situ hybridisation, refer to Fig. 5G. (B) Double Kr/Eve immunostaining showing that loss of *lb* function leads to the generation of supernumerary *eve* pericardial cells (arrowheads) and *eve*-positive dorsal muscles (bracket). Notice that we interpret the *eve*-only expressing cells within the bracket as dorsal muscles because of their lateral position. (D) Loss of *lb* function leads also to enlarged *msh* expression within the dorsal domain (bracket), thus suggesting that Lb acts as transcriptional repressor of *msh*.

eve-positive cells (compare with the wild type; Fig. 3E) generally adopt a pericardial identity and only some of them contribute to enlarged dorsal muscles (bracket in Fig. 6B). The repressive action of *eve* and *lb* during specification of cardiac and muscular fates is also confirmed by the analysis of *msh* expression (Fig. 6C,D). Compared with the wild type (Fig. 5G), in *eve*$^{-}$ (Fig. 6C) and *lb*$^{-}$ embryos (Fig. 6D), the *msh* transcripts are detected in much broader area, thus indicating ectopic activation of the gene.

Cell fate modifications in embryos misexpressing *lb*, *msh* and *eve* are not lineage restricted

Cell fate respecification observed after ectopic expression of *lb*, *msh* and *eve* is restricted to a defined subset of cardiac and/or muscular cell lineages. To learn more about the mechanisms of identity gene action, we asked whether the modifications of cell fates induced by misexpression of *lb* concerned cells other than siblings of founders in which the *lb* gene is normally expressed. As previously shown (Jagla et al., 1997) (Fig. 1), the *lb* genes are expressed in two cardioblasts and two pericardial cells per hemisegment, and when ectopically expressed they lead to the overproduction of *tin*-CB concomitantly with the loss of *eve*-PC and dorsal muscles (see Fig. 2). One way to check whether the affected cells arise from the same progenitors as the *lb*-expressing cells is to compare the influence of *numb* on their specification. It has been previously shown (Carmena et al., 1998a) that eve-PC and eve-DA1 founders derive from the asymmetrically dividing progenitors P2 and P15. According to the recent

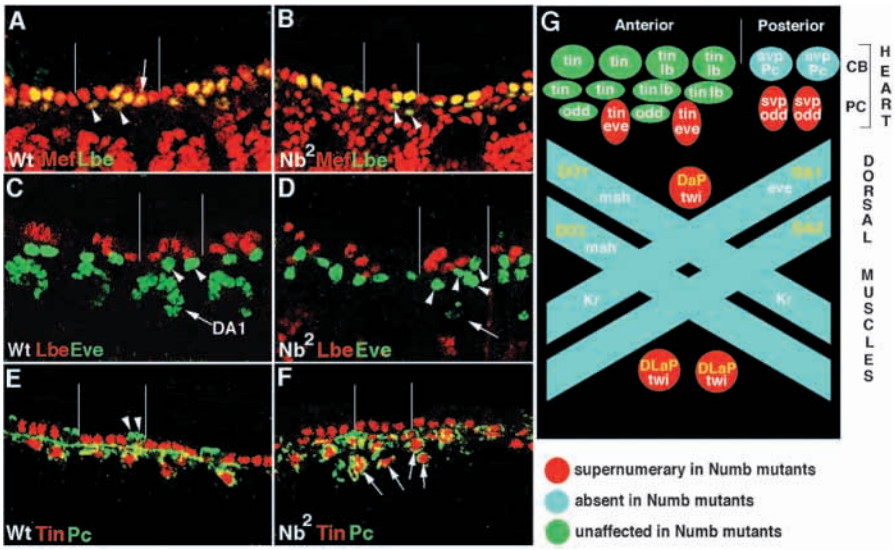

Fig. 7. The heart comprises cells that arise from symmetrically and asymmetrically dividing progenitors. Dorsolateral views of stage 14 wild-type (A,C,E) and *numb*^{2} mutant embryos (B,D,F). As demonstrated by double Mef/Lbe staining (A,B), loss of *numb* function has no influence on the number of anterior *lb*-positive cardioblasts. By contrast, a third transiently *lb*-positive cardioblast located more posteriorly (arrow in A) is absent in *numb* mutants (B). The number of *lb*-positive pericardial cells is not affected by *numb* mutation (arrowheads in A and B), thus indicating that the anterior *lb*-positive cardiac progenitors divide symmetrically. This is fully confirmed by double Lbe/Eve staining (C,D) showing that the loss of DA1 (arrow), and duplication of *eve* pericardial cells (arrowheads) in *numb* mutants coincide with an unchanged profile of *lbe* expression. The symmetric versus asymmetric divisions of progenitors that give rise, respectively, to anterior and posterior cardioblasts are visualised by double Tin/Pc staining (E,F). Arrowheads indicate posterior Pc-positive cardioblasts that are absent in F, while arrows in F indicate supernumerary pericardial cells. (G) A summary of the so far defined influence of *numb* on cardiac and dorsal muscle precursors. The scheme refers to previously published data of Ruiz Gomez and Bate (Ruiz Gomez and Bate, 1997), Carmena et al. (Carmena et al., 1998) and Ward and Skeath (Ward and Skeath, 2000).

lineage tracing (Ward and Skeath, 2000), individual muscle and heart cells arise from progenitors that undergo either the asymmetric or symmetric divisions. We demonstrate that the number of the *lb*-positive cardioblasts and the *lb*-positive pericardial cells (arrowheads in Fig. 7A,B) is not affected in *numb* mutant embryos (Fig. 7) suggesting that they arise from the progenitors dividing symmetrically. Thus, the *lb*-positive and *eve*-positive dorsal mesodermal cells cannot be generated from the common progenitor cells, revealing that the ectopic *lb* influences the fates of cells that are not lineage linked. In addition, with the use of tyramide-enhancement system (NEN) employed to strengthen the *lb* staining ,we show that *lb* is also expressed at a weaker level in a third cardioblast (arrow in Fig. 7A) immediately posterior to the highly expressing pair. In contrast to the anterior *lb*-cardioblasts, this cardioblast is absent in *numb* mutants (see Fig. 7B), indicating that it originates from the asymmetrically dividing progenitor. The assumption that the anterior cardioblasts are generated by symmetric division, while the posterior pair by the asymmetric division is fully confirmed by double Tin/Pc staining (Fig. 7E,F). Taking into consideration these data and the so far described mesodermal phenotypes of *numb* mutants (Ruiz Gomez and Bate, 1997; Carmena et al., 1998a; Ward and Skeath, 2000), we propose a scheme (Fig. 7G) that summarises the influence of *numb* on cell fates in dorsal mesoderm. Together, our results support the lineage tracing described by Ward and Skeath (Ward and Skeath, 2000) and reveal that when *lb* is ectopically expressed, it can alter the identity of cells arising from the adjacent *lb*-negative progenitors.

DISCUSSION

How an initially pluripotent cell acquires its final fate remains one of the most intriguing unresolved issues. The analyses of genes that govern heart and muscle development in *Drosophila* have provided important insights into cell fate specification processes (for reviews, see Baylies et al., 1998; Wilson, 1999; Frasch, 1999; Paululat et al., 1999). It has become clear that the acquisition of cell identity requires a coordinated integration of extrinsic signals such as Wg, Hh and Dpp (Carmena et al., 1998b; Xu et al., 1998; Lee and Frasch, 2000, Halfon et al., 2000), Notch-dependent cell-cell signalling (Park et al., 1998; Crozatier and Vincent, 1999), and the intrinsic information provided by the localised expression of transcription factors encoded by the so-called identity genes (Ruiz Gomez and Bate, 1997; Jagla et al., 1998; Nose et al., 1998; Knirr et al., 1999; Crozatier and Vincent, 1999; Capovilla et al., 2001). According to the commonly accepted hypothesis (Bate, 1990; Dohrmann et al., 1990), the individual muscles and heart cells are specified at the founder cell stage. The most recent data (Ruiz Gomez and Bate, 1997; Jagla et al., 1997; Su et al., 1999: Knirr et al., 1999) indicate that the diversification of mesodermal cell types is governed by combinatorial code of identity gene expression. We have focused on intrinsic program that leads to the specification of a subset of cardiac and muscular cells located in the dorsal mesoderm of *Drosophila* embryos. Our data reveal cell fate plasticity in this region and suggest the instructive role of identity gene expression not only at the level of the founder cell, but also at the progenitor cell level. Gain- and loss-of-function phenotypes of *lb*, *msh* and *eve* clearly indicate that the cross-repressive interactions of these genes are essential for the proper cell fate acquisition.

The identity gene dependent switches between cardiac and muscular fates

We have previously observed the ability of ectopically expressed *lb* to inhibit *eve* in founder of the DA1 muscle (Jagla et al., 1997). This effect may be due to either a specific inhibition of *eve* by *lb* or a more general regulatory mechanism

of fate specification. Data presented here favour the latter possibility, showing that the gain of *lb* function affects expression of several identity genes and consequently influences fates of cells in which these genes are expressed. Specifically, embryos that ectopically express *lb* have an increased number of *tin*-positive heart cells with a concomitant reduction of dorsal muscles. To demonstrate that the supernumerary cardiac cells result from cell fate switches, rather than from additional proliferation, we used $msh^{\Delta 89}$ mutants (Nose et al., 1998) displaying heart hyperplasia similar to that observed in embryos overexpressing *lb*. In this particular *msh* mutant, the presumptive *msh*-positive muscle cells monitored by *lacZ* start to express cardiac markers. This suggests that switches from muscular to cardiac fates contribute to heart hyperplasia induced by deregulation of identity genes. Interestingly, the ectopic expression of *lb* and *msh* leads to reciprocal phenotypes, and indicates that the identity genes specifically expressed in the heart promote dorsal mesodermal cells to enter the cardiogenic pathway, while the muscle identity genes promote the myogenic pathway. However, more detailed analysis shows that ectopic *lb* promotes only specific cardiac fates and ectopic *msh* only specific muscle identities, thus indicating that the identity genes instruct dorsal mesodermal cells to adopt the specific cardiac or muscular fates, rather than make a choice between cardiac and muscular development. This property is particularly well illustrated by the phenotypes generated by the ectopic *eve*, which is involved in the specification of a subset of heart and dorsal muscle cells and when ectopically expressed promotes specification of supernumerary cells of both types. Moreover, deregulated heart and dorsal muscle identity genes affect preferentially fates of mesodermal cells located in dorsal but not in ventral regions (T. J. and K. J., unpublished), thus suggesting that the identity gene action is instructive only in a permissive context. This observation is in complete agreement with the model of competence domain proposed by Carmena et al. (Carmena et al., 1998b). According to this concept, the high level of Wg and Dpp signals present in the anterodorsal region (under the intersection of Wg and Dpp epidermal domains) provides a major cue that direct mesodermal cells into cardiac or dorsal muscle development. In relation to this model, our data design a new regulatory mechanism that provides a paradigm of how the intrinsic transcription factors and extrinsic signalling molecules converge to specify cell fates.

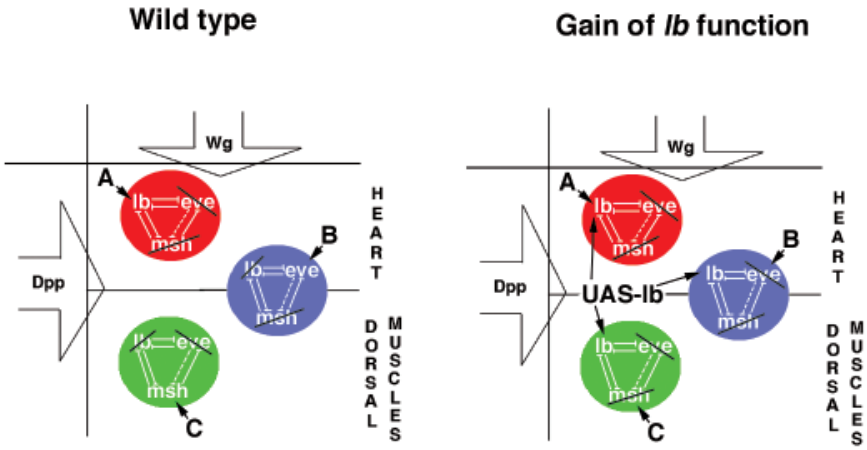

Fig. 8. Governing cell fate diversity by cross-repressive interactions of identity genes. In the dorsal mesoderm of the *Drosophila* embryos, the extrinsic Wg and Dpp signals create a permissive context for specification of cardiac and dorsal muscle cells in a large region named competence domain (Carmena et al., 1998). This domain is subdivided into clusters of equivalent cells (promuscular clusters) by combinatorial code of RTK signalling pathways depicted here symbolically as A-C. As previously shown for *eve*-positive progenitors P2 and P15 (Carmena et al., 1998), transient localised expression of a combination of RTKs is able to activate precise expression of identity genes in well-defined promuscular clusters and resulting progenitors. We propose that in the next step, the identity genes activated in adjacent populations of progenitors, owing to their repressive activities, will prevent expression of other identity genes that are potentially able to be activated by Wg and Dpp signals in the same progenitors. These cross-repressive interactions maintain the restricted expression of identity genes (left). The mutual repression at the progenitor levels provides also a comprehensive way to explain large-scale fate modifications induced by gain of function of identity genes (right).

Cell fate modifications with the respect of lineage tracing

The 24B-GAL4 line (Brand and Perrimon, 1993) used in our misexpression experiments allows the pan-mesodermal expression of identity genes to be driven from the progenitor stage onwards. This implies that the cell fate modifications we see may result either from identity gene action at the progenitor or at the subsequent founder cell stage. Endogenous identity gene expression is often detected in progenitor cells (Carmena et al., 1998a; Jagla et al., 1998; Knirr et al., 1999), even if cell fate specification is assumed after progenitor division at the founder stage. This raises a possibility that this early identity gene expression provides instructive information to the progenitor cells and contributes to progressive cell fate specification. An argument in favour of such a mechanism is our observation that the cell fate modifications induced by gain of function of each of the tested identity genes are not lineage restricted. For example in the case of *lb* overexpression, the affected cell lineages arise from at least three distinct progenitors (P2, P15 and P_{DO1}) (Carmena et al., 1998a; Nose et al., 1998). As indicated by our analysis of *numb* mutants, none of these asymmetrically dividing progenitors gives rise to *lb*-positive heart cells, which arise from progenitors that divide symmetrically.

The fine cell fate tuning by cross-repressive interactions of identity genes

Our findings suggest cross-repressive interactions that occur between transcription factors that specify adjacent and non-overlapping populations of muscle and heart cells. Most likely, in normal development, these interactions have a functional relevance once the progenitor cells segregate, and then continue to play an important role in the next step of cell fate diversification, namely in founder cells. The gain- and loss-of-function experiments we present indicate that the identity

genes may function as repressors starting from the progenitor stage onwards. However, the earliest activation of inappropriate identity gene as a result of the loss of function of repressor (in *msh*$^{\Delta 89}$ embryos) was documented in founder cells.

In the model we propose (Fig. 8), cross-repressive interactions allow the refinement of the potentially imprecise pattern of identity gene expression induced by the interplay of Wg and Dpp signalling pathways. As elegantly demonstrated by Carmena et al. (Carmena et al., 1998b) and Halfon et al. (Halfon et al., 2000), Wg and Dpp create a permissive context for the development of cardiac and dorsal muscle precursors. In such a context, the transcription factors that specify these two types of cells (e.g. *lb*, *eve* and *msh*) are expected to be activated in all dorsal mesodermal cells. The local restriction of identity gene expression is, however, provided by a combinatorial signalling code mediated by two receptor tyrosine kinases, the *Drosophila* epidermal growth factor receptor and the Heartless (Htl) fibroblast growth factor receptor (Carmena et al., 1998b). In the model proposed by Carmena et al., transient localised activity of these two mesodermal signalling pathways is thought to subdivide the large competence domain into small clusters of equivalent cells from which individual progenitors segregate. Depending on the combination of RTKs activities, the individual identity genes are activated only in a defined equivalence group and in the resulting progenitor (Carmena et al., 1998b). Our study defines an additional step to the aforementioned model. We propose that the major role of identity genes is to maintain their restricted expression in progenitors and subsequently in founder cells by repressing other identity genes competent to respond positively to Wg and Dpp signals. These cross-repressive interactions are likely to ensure constant localised identity gene expression over time, thus providing a crucial element in establishing cell identity.

It is noteworthy that the principles of the mutual repression model outlined here resemble those governing neural identity in the ventral neural tube of chicken and mouse embryos (Briscoe et al., 2000; Pierani et al., 2001; Moran-Rivard et al., 2001). In both systems, negative regulation between the transcription factors establishes sharp boundaries of the identity gene expression and controls later aspects of cell pattern. Consistent with this interspecies homology, the cross-repression mechanism may be universally used in different biological systems as a crucial component of intrinsic control of diversification.

The cross-repression mechanism also provides an effective way for establishing discrete domains of identity gene expression; however, several questions remain to be addressed:

(1) Does the repressive influence observed between identity genes result from direct interactions

(2) How do the different RTK signalling pathways subdividing the competence domain communicate with the identity gene action

(3) What is the role of asymmetric versus symmetric division in determining cell fates in the dorsal mesoderm.

One possible way of addressing these issues, and an important challenge for future studies, will be to determine the integration of regulatory cues at molecular level by functional analysis of enhancers that drive restricted expression of identity genes.

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