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ladybird determines cell fate decisions during diversification of *Drosophila* somatic muscles

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SUMMARY

In the mesoderm of *Drosophila* embryos, a defined number of cells segregate as progenitors of individual body wall muscles. Progenitors and their progeny founder cells display lineage-specific expression of transcription factors but the mechanisms that regulate their unique identities are poorly understood. Here we show that the homeobox genes *ladybird early* and *ladybird late* are expressed in only one muscle progenitor and its progeny: the segmental border muscle founder cell and two precursors of adult muscles. The segregation of the *ladybird*-positive progenitor requires coordinate action of neurogenic genes and an interplay of inductive Hedgehog and Wingless signals from the overlying ectoderm. Unlike so far described progenitors but similar to the neuroblasts, the *ladybird*-positive progenitor undergoes morphologically asymmetric division. We demonstrate that the ectopic *ladybird* expression is sufficient to change the identity of a subset of progenitor/founder cells and to generate an altered pattern of muscle precursors. When ectopically expressed, *ladybird* transforms the identity of neighbouring, *Krüppel*-positive progenitors leading to the formation of giant segmental border muscles and supernumerary precursors of lateral adult muscles. In embryos lacking *ladybird* gene function, specification of two *ladybird*-expressing myoblast lineages is affected. The segmental border muscles do not form or have abnormal shapes and insertion sites while the number of lateral precursors of adult muscles is dramatically reduced. Altogether our results provide new insights into the genetic control of diversification of muscle precursors and indicate a further similarity between the myogenic and neurogenic pathways.

Key words: Somatic muscle, *ladybird*, Homeobox, *Drosophila*, Myogenesis

INTRODUCTION

Specification of distinctive myoblast lineages appears universal in the generation of the complex muscle pattern in both invertebrate and vertebrate embryos (Bate, 1993; Abmayr et al., 1995; Cossu et al., 1996). To date, little is known about the mechanisms that underlie the diversification of muscle precursors. Recent work in *Drosophila* has revealed some components of the genetic pathways governing early steps of myogenesis (Carmena et al., 1995; Ruiz-Gomez et al., 1997; Ruiz-Gomez and Bate, 1997; Nose et al., 1998; Carmena et al., 1998) showing that the *Drosophila* embryo is a powerful model to study the specification and patterning of muscle fibers.

Surprisingly, the mechanisms involved in the execution of early myogenic programme are similar to those of neurogenesis. In the somatic mesoderm, as in the neuroectoderm, the expression of a proneural gene *lethal of scute* (*l'sc*) delimits clusters of equivalent cells (Carmena et al., 1995). Due to lateral inhibition, involving coordinate action of neurogenic genes (Corbin et al., 1991; Bate et al., 1993), a single cell segregates from each proneural and promuscular cluster and becomes, respectively, a neuroblast or a muscle progenitor. During subsequent division, both these cell types use a common mechanism of asymmetric transmission of the Numb (Nb) protein, which seems crucial for the final diversification of both the neural and the muscular fates (Rhyu et al., 1994; Spana et al., 1995; Ruiz-Gomez and Bate, 1997, Carmena et al., 1998). Daughters of the *Drosophila* muscle progenitors, the founder cells, are thought to represent a specialized class of myoblasts able to recruit neighbouring mesodermal cells, fuse with them and produce a diversity of syncytial muscle fibers (Bate, 1990; Dohrmann et al., 1990; Abmayr et al., 1995). It has been proposed that the distinct size, shape and epidermal insertion sites of muscle fibers result from the expression, in a given founder cell, of one or a combination of putative muscle identity genes such as: *S59* (Dohrmann et al., 1990; Carmena et al., 1995), *even skipped* (*eve*) (Frasch et al., 1987; Ruiz Gomez and Bate, 1997), *vestigial* (*vg*) (Wiliams et al., 1991), *nautilus* (*nau*) (Michelson et al., 1990), *apterous* (*ap*) (Bourgouin et al., 1992), *Krüppel* (*Kr*) (Ruiz Gomez et al., 1997) and *muscle segment homeobox* (*msh*) (D'Alesio and Frasch, 1996; Nose et al., 1998). Recent studies appear to confirm this hypothesis showing that the gain or loss of function of *ap* (Bourgouin et al., 1992), *nau* (Keller et al.,

1997), *Kr* (Ruiz Gomez et al., 1997) and *msh* (Nose et al., 1998) alter the characteristics of at least a subset of muscle precursors in which they are expressed. However, low expressivity of observed phenotypes indicates that the majority of muscle fibers are specified by a combination of transcription factors rather than a single one (Ruiz Gomez et al., 1997; Nose et al., 1998). Since most putative muscle identity genes display lineage-specific expression in the differentiating central nervous system (CNS), it suggests that they may represent a further common element between the myogenic and neurogenic pathways. Indeed, the phenotypes of msh^{-} embryos (Ishiki et al., 1997; Nose et al., 1998) indicate that this gene functions to specify positional identities of both muscle and neural precursors.

In addition to the intrinsic mesodermal programme, the formation of the correct muscle pattern requires inductive signals from the epidermis. The secreted molecule encoded by the segment polarity gene *wingless* (*wg*), required for the formation of a subset of muscle founder cells (Baylies et al., 1995), is a good candidate for this function. Wg gene product can act across germ layers (Baylies et al., 1995; Jagla et al., 1997b) and influence the myogenic cascade but the mesodermal targets of Wg signaling are not yet determined.

In this study, we analyse the muscle identity functions of the *ladybird* (*lb*) genes, which belong to a distinct family encoding homeodomain-containing transcription factors (Jagla et al., 1994, 1997a,b). We show that *lb* are expressed in the promuscular cluster, progenitor, founder cell and syncytial precursor of only one larval somatic muscle, the segmental border muscle (SBM, muscle 8). We characterise the requirements for the segregation of the *lb*-positive progenitor and its particular features including the generation of two non-differentiating myoblasts, the lateral adult muscle precursors (LaPs). From the analysis of gain- and loss-of-function phenotypes, we infer that *lb* acts in concert with the myogenic pathway and is indispensable for the identity of both the SBM and the LaPs.

MATERIALS AND METHODS

Drosophila stocks

The following fly strains were used: tin^{EC40}, $tin^{Df(3R)GC14}$ (Azpiazu and Frasch, 1993; Bodmer, 1993), $Df(1)sc^{19}$ (Carmena et al., 1995), nb^{1} (Uemura et al., 1989), hh^{9K} (Heemskerk and DiNardo, 1994), wg^{IL114} (Bejsovec and Martinez-Arias, 1991) and a set of neurogenic mutants, N^{55e11}, bib^{ID05}, mam^{Z3}, $Df(3R)E(spl)^{Bx22}$, Dl^{RevF10} and neu^{IF65}. hh^{9K} and wg^{IL114} are temperature-sensitive alleles that behave as wild type at 18°C and as null at 29°C. To avoid the influence of *S59* and other genes located distally to *lbe*, as lb^{-} background, we used transheterozygous embryos, referred to as *lb-deficiency* and obtained from the cross of *Df(3R)e*-D7; *tin* rescue males with females carrying a shorter *Df(3R)e*-F1 deficiency (Azpiazu and Frasch, 1993; Jagla et al., 1997b). A Df(3R)eD7; *tin* rescue stock in which both Lbe and Lbl gene products are absent and *tin* activity is restored was kindly provided by M. Frasch. The balancer chromosomes were marked with a *twi-lacZ* or a *ftz-lacZ* P-element allowing distinction between mutant and wild-type embryos.

Ectopic expression and temperature shift

Uniform ectopic *lb* expression was induced in double transgenic *hs-lbe;hs-lbl* embryos (Jagla et al., 1997b) by the heat-shock treatment (15 minutes at 37°C in water) at the time when the muscle progenitors segregate (5 hours AEL). Alternatively, the *UAS-lbe-16-1* line, which exhibits a high level of ectopic expression and an effector line *24B-Gal4* (Brand and Perrimon, 1993), were used to express *lb* ectopically in all mesodermal cells. Temperature shifts on *hh* and *wg* thermosensitive mutant embryos were performed at 4 or at 6 hours AEL as described previously (Jagla et al., 1997b).

Immunocytochemistry

The following primary antibodies were used: monoclonal anti-Lbe (1:1), rabbit anti-Lbl (1:5000), monoclonal anti-En (1:1), rabbit anti-β-galactosidase (1:8000), rabbit anti-dMEF2 provided by H. Nguyen (1:2000), rabbit anti-MHC provided by D. Kiehart (1:2000), rabbit anti-β3 tubulin, provided by A. Paululat (1:200); rabbit anti-Kr (1:2000) provided by P. Carrera and G. Vorbrüggen, rabbit anti-Twi provided by F. Perrin-Schmitt (1:1000) and rabbit anti-Human Phospho-Histone H3 (Upstate Biotechnology) (1:200). Detections of antibody stainings were performed using ABC-Elite-peroxidase or ABC-alkaline phosphatase kits (Vector Laboratories). In the case of fluorescent stainings, we have used secondary antibodies conjugated with Cy3, Cy5, Cy2 (Jackson Immuno-Research) or Oregon Green Protein (Molecular Probes). Colour reactions were developed using diaminobenzidine (for peroxidase) or NBT (for alkaline phosphatase) as substrates. Whole-mount embryos were photographed on the Axiophot microscope under Nomarski optics or scanned on the Leica confocal microscope.

RESULTS

lb expression is restricted to the segmental border muscle lineage and to the precursors of lateral adult muscles

ladybird early (*lbe*) and *ladybird late* (*lbl*) genes are located in the 93E homeobox gene cluster together with *tinman* (*tin*), *bagpipe* (*bap*) (Azpiazu and Frasch, 1993; Bodmer, 1993) and *S59* (Dohrmann et al., 1990) genes known to be involved in the mesoderm differentiation programme (Jagla et al., 1997a). We have previously shown that *lbe* and *lbl* are co-expressed in segmentally repeated populations of cardioblasts and pericardial cells and play an important role in the diversification of heart precursors (Jagla et al., 1997b). The analysis of the *lbe* and *lbl* expression patterns also showed that both genes are expressed in the same group of somatic mesodermal cells that give rise to a syncytial muscle fiber (Jagla et al., 1997a,b).

To determine the identity of *lb*-positive muscle, we used confocal microscopy of late-stage embryos stained with anti-Mef2 antibody, which labels all myoblast nuclei (Bour et al., 1995) or with anti-Myosin (Myo) (Leiss et al., 1988), which labels the muscle fiber architecture (Fig. 1). Double staining revealed that, in the stage 16 embryo, *lb* is co-expressed with *mef-2* in the 6 to 7 myoblast nuclei per hemisegment (Fig. 1C). Spatial location of these nuclei coincides with the segmental borders indicating that they belong to the SBM. This observation is fully confirmed by *lbe/myo* double staining (Fig. 1D) clearly showing that *lb* is expressed in only one muscle fiber, the SBM.

To examine the spatial relationship between the *lb*-expressing progenitor/founders and other myoblast lineages, we have used three markers: *twist* (*twi*) (Thisse et al., 1991), *Kr* (Ruiz Gomez et al., 1997) and an enhancer trap line rP298 (Nose et al., 1998) (Fig. 2). *twi* labels segregating muscle progenitors (Fig. 2A) but is no longer expressed in muscle

founder cells (Fig. 2F; see also Carmena et al., 1995). The only myoblasts with persistent *twi* expression are the non-differentiated precursors of adult muscles (Bate et al., 1991; see also Fig. 2B). The SBM progenitor, which co-expresses *twi* and *lb*, in comparison to other progenitors, shows some particularities. Unlike *S59* and *Kr* progenitors (Carmena et al., 1995), it divides giving three progeny (Fig. 2A,B,C,G,F): a *twi*-negative SBM founder cell (arrow in Fig. 2F,C,G) which recruits neighbouring myoblasts to built the syncytial SBM fiber and two adult muscle precursors with persistent *twi* expression (arrowheads in Fig. 2B,C,G). The position of the latter cells, close to the SBM, indicates that they correspond to lateral adult muscle precursors (LaPs) (Bate et al., 1991). The distinct fates of *lb*-positive SBM and LaP myoblasts are already apparent during late stage 12 (Fig. 2D,H). In embryos from the enhancer trap line rP298, known to express *lacZ* in the progenitors and founders of all larval muscle fibers (Nose et al., 1998), the SBM lineage co-expresses *lb* and *lacZ* while the LaPs do not (Fig. 2H). In contrast, neither of the *lb*-positive myoblasts express *Kr*, which labels neighbouring lateral and ventral muscle precursors (Fig. 2E,I).

lb expression is associated with all stages of SBM formation

Taking advantage of the restricted expression pattern of *lb* genes, we followed the somatic mesodermal cells that give rise to SBM. We found that *lb* activity is associated with all stages of SBM formation, namely the promuscular cluster (Fig. 3A,F), progenitor cell (Fig. 3B,G), founder cell (Fig. 3C,H), fusing myoblasts (Fig. 3D,I) and syncytial fiber (Fig. 3E,J). The SBM arises from a cluster of 6-7 mesodermal cells, each of which weakly expresses *lb* (Fig. 3A,F). During early extended germ band stage (about 5 hours AEL), *lb* expression becomes restricted to, and upregulated in, only one large cell, the SBM progenitor (Fig. 3B,G). This cell, as detected by double staining with a marker of mitosis, undergoes two divisions (Fig. 3G,H). The first division (right panel of Fig. 3G) gives rise to the SBM founder and is morphologically asymmetric (left panel of Fig. 3H; compare also with Fig. 2F) while the second, most likely symmetric, results in two LaPs

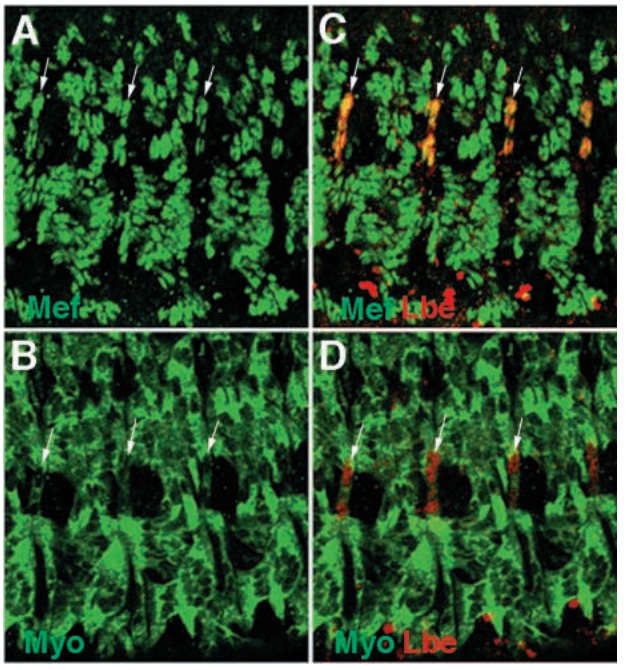

Fig. 1. *lb* expression is associated with the SBM. (A-D) Confocal scans of the ventrolateral part of stage 16 embryos double stained for Lbe and two muscle markers: Mef2 and Myosin. (A,C) Myoblast nuclei labelled using anti-Mef2 antibody (green) and (C) anti-Lbe antibody (red). SBM nuclei (arrows) that co-express Mef2 and Lbe appear yellow. (B,D) The muscle fiber architecture stained with anti-Myosin (green) and (D) myoblasts expressing *lbe* (red). The *lbe*-positive myoblast nuclei are associated with only one muscle fiber per segment, the SBM (arrows).

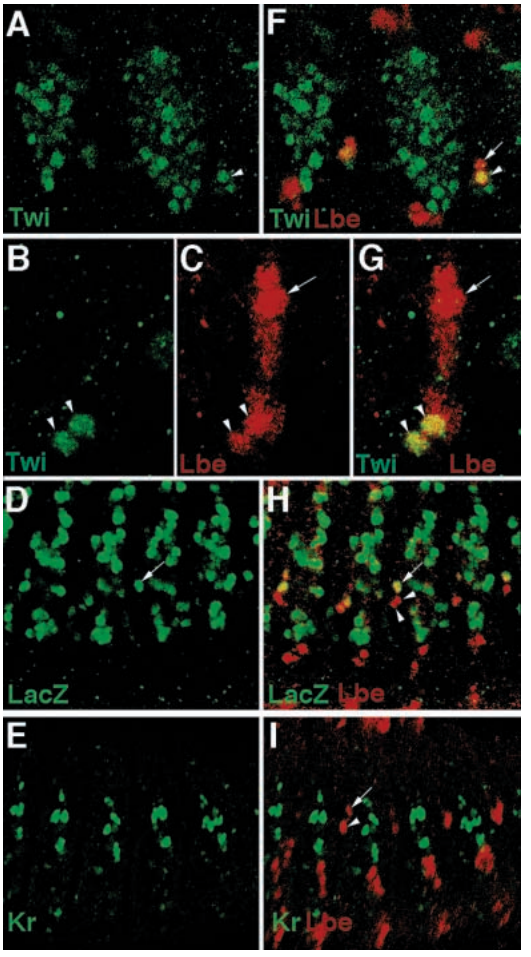

Fig. 2. *lb*-positive progenitor gives rise to the SBM founder cell and two LaPs. Confocal scans of embryos double stained for *lbe* (red) and three other markers (*twi*, *lacZ* and *Kr*) (green) expressed during early myogenesis. Myoblasts that co-express *lb* and other markers appear yellow. (A,F) At late stage 11, *lbe* is co-expressed with *twi* in only one muscle progenitor (arrowhead), which divides giving rise to the SBM founder cell (arrow). (B,C,G) At stage 14, after second division of the progenitor, two LaPs (arrowheads) co-express *twi* and *lb*. The *lb*-positive founder (arrows) migrates dorsally and fuses with neighbouring myoblasts. *lb* expression in two distinct myoblast lineages appears confirmed (D,H) by a double staining of an enhancer trap line rP298 (Nose et al., 1998) in which *lacZ* is co-expressed with *lb* in most laterally located SBM founder (arrows) but is not in two LaPs (arrowheads). (E,I) *Kr* is expressed in muscle precursors located close to the *lb*-positive SBM founder (arrow) and LaPs (arrowhead).

(right panel of Fig. 3H; see also Fig. 2B,C,G). At the end of germ band retraction (9 hours AEL), the SBM founder cell starts to migrate dorsally along the segmental border whereas the LaPs remain at their initial position (Fig. 3D,I). The migration of the SBM founder prefigures the final location of SBM syncytial fiber formed by the progressive integration of neighbouring myoblasts (Fig. 3I). At the onset of dorsal closure fusion is completed and the SBM contains 6-7 *lb*-positive nuclei (Fig. 3E,J).

Mesodermal and epidermal cues required for the segregation of SBM progenitor

The SBM progenitor corresponds to the most superficial cell from the promuscular cluster, thus suggesting a role for the overlying ectoderm during its segregation. To investigate this possibility we first determined the position of the SBM promuscular cluster with respect to the epidermal anterior and posterior compartments. As shown on Fig. 4A,F, this cluster is located ventrolaterally below the epidermal posterior compartment. After segregation, the SBM progenitor migrates to a more lateral and posterior position so that, by late stage 11 (7 hours AEL), it is detected at the segmental border (Fig. 4G,L). Since epidermal Wg and Hedgehog (Hh) signaling was shown to influence muscle formation (Baylies et al., 1995; Azpiazu et al., 1996), we analyzed the SBM-associated *lb* expression in embryos carrying *hh* and *wg* thermosensitive mutations. Temperature shifts administered during early extended germ band stage (at 4 hours AEL) revealed that the Wg and Hh signalings, mutually dependent at this time (Perrimon, 1994), are required for the promuscular *lb* activity and/or the segregation of SBM progenitors (Fig. 4B,C,F). The initial influence of these signals is no longer observed later in development (Fig. 4H,I).

In addition to signals from the epidermis, the activity of the mesodermal gene, *tin* (Azpiazu and Frasch, 1993; Bodmer, 1993), initially expressed in the whole trunk mesoderm, is involved in the early events of myogenesis. In *tin*$^{-}$ embryos, the formation of SBM promuscular clusters and segregation of *lb*-positive progenitor cells are strongly affected (compare Fig. 4D,F) leading to the absence of the majority of SBM fibers (compare Figs 3E and 4J). Since, during promuscular cluster formation (about 5 h AEL), *tin* expression becomes restricted to the dorsal mesoderm (Azpiazu and Frasch, 1993; Bodmer, 1993), its influence on ventrolaterally located SBMs is likely to be indirect and mediated via an unknown factor (see Fig. 7). The lack of neurogenic gene function, known to be involved in cell-cell interactions during lateral inhibition (Bate et al., 1993) generates an opposite phenotype. *Mastermind* (*Mam*$^{-}$) (data not shown) and *Enhancer of split* (*E(spl)*$^{-}$) embryos fail to restrict promuscular *lb* expression to only one cell (Fig. 4E) and, in consequence, display a hyperplastic *lb* pattern in later stages (Fig. 4K). In strong neurogenic mutants such as *Notch* and *Delta*, the SBM progenitors do not segregate at all or do not

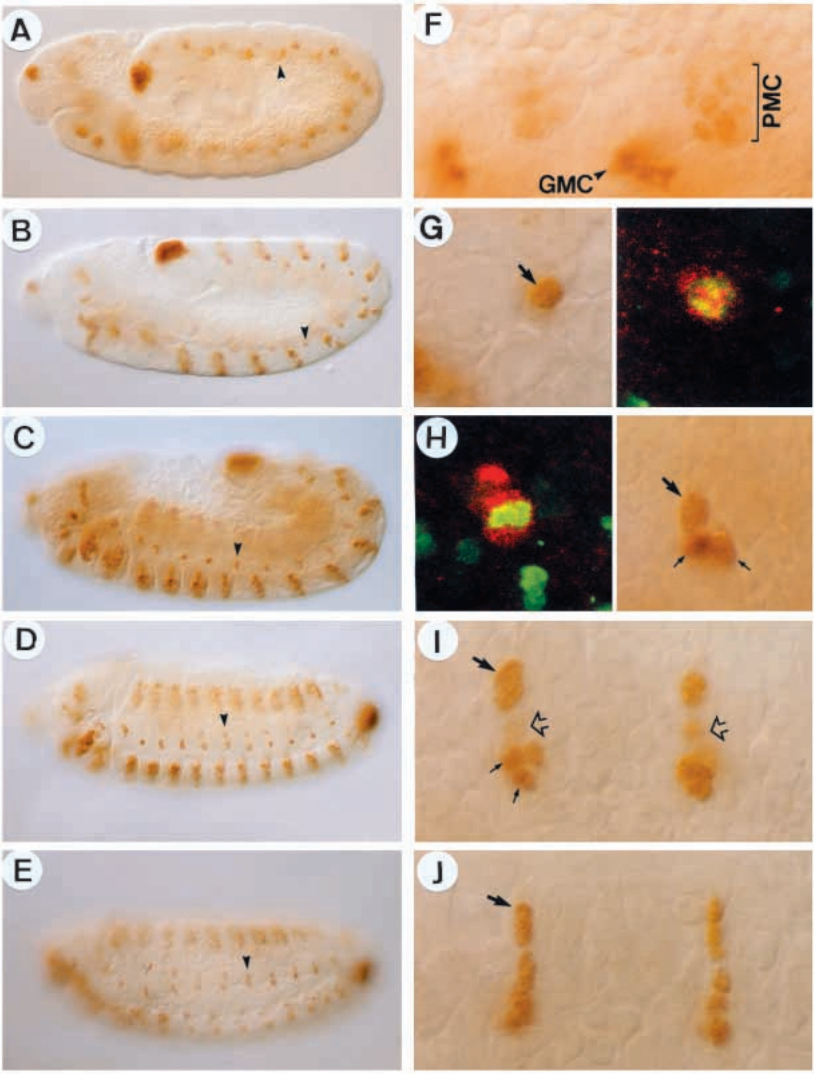

Fig. 3. SBM fiber formation. (A-J) Immunostainings of embryos with anti-Lbe antibody. (F-J) Higher magnification of *lb*-positive myoblasts indicated on A-E by arrowheads. (A,F) SBM promuscular clusters. (B,G) SBM progenitor (arrow in left G subpanel). (Right G subpanel) Confocal view of the early phase of the first asymmetric division of the progenitor, double stained for Lbe (red) and for Phospho-Histone H3 (green), which labels condensed chromatin in dividing cells. Areas with the overlapping red and green staining appear yellow. (C,H) Divisions of progenitor. (Left H subpanel) Confocal view stained as for the right G subpanel showing the nucleus of SBM progenitor at the beginning of its second division (with the green/yellow chromatin) and its first progeny, the SBM founder (red). (Right H subpanel) The progeny of progenitor after its second division: the SBM founder cell (arrow) and two LaPs (small arrows). (D,I) Fusion of the SBM founder cell with its neighbouring myoblasts (open arrows). Note that after the second asymmetric division the SBM founder migrate dorsally (compare H and I) while the LaPs remain at their original position (small arrows). (E,J) Newly formed syncytial fibers. Developmental stages of the embryos: (A,F) stage 10, (B,G) early stage 11, (C,H) early stage 12, (D,I) stage 13, (E,J) stage 15. In all panels anterior is to the left and dorsal is up. Abbreviations: GMC, ganglion mother cell; PMC, promuscular cluster.

divide properly (data not shown). In contrast, the loss of function of a proneural gene, *lethal of scute* (*l'sc*), which is specifically expressed in promuscular clusters and segregating muscle progenitors (Carmena et al., 1995), has no significant influence on SBM formation (data not shown). Altogether our analysis of how the segregation of SBM progenitor is regulated indicates that cell commitment in the somatic mesoderm is conferred by a coordinate input of mesodermal and epidermal cues.

lb defines cell fates of myoblasts forming SBM and LaPs

To investigate the role of *lb* activity in the specification of SBM and LaP myoblast lineages, we examined the pattern of larval and adult muscle precursors in embryos ectopically expressing *lb* and in embryos lacking *lb* activity (see Materials and Methods). The comparison of SBM formation in the wild-type (Fig. 5A), the *hs-lb* (Fig. 5B) and *UAS-lb* embryos (Fig. 5H) revealed that, in about 70% of hemisegments, the ectopic *lb* expression leads to the formation of enlarged (Fig. 5B,H) or duplicated (Fig. 5H) SBMs. Similarly, in *24B-Gal4/UAS-lbe* embryos, the number of LaPs with persistent *twi* expression is significantly increased (Table 1; compare Fig. 5J and K. The overproduction of SBM (Fig. 5B,H) and LaPs (Fig. 5K) is frequently accompanied by the loss of some neighbouring lateral muscle fibers (compare Fig. 5G,H) suggesting that the ectopic expression of *lb* may change the identity of a subset of early

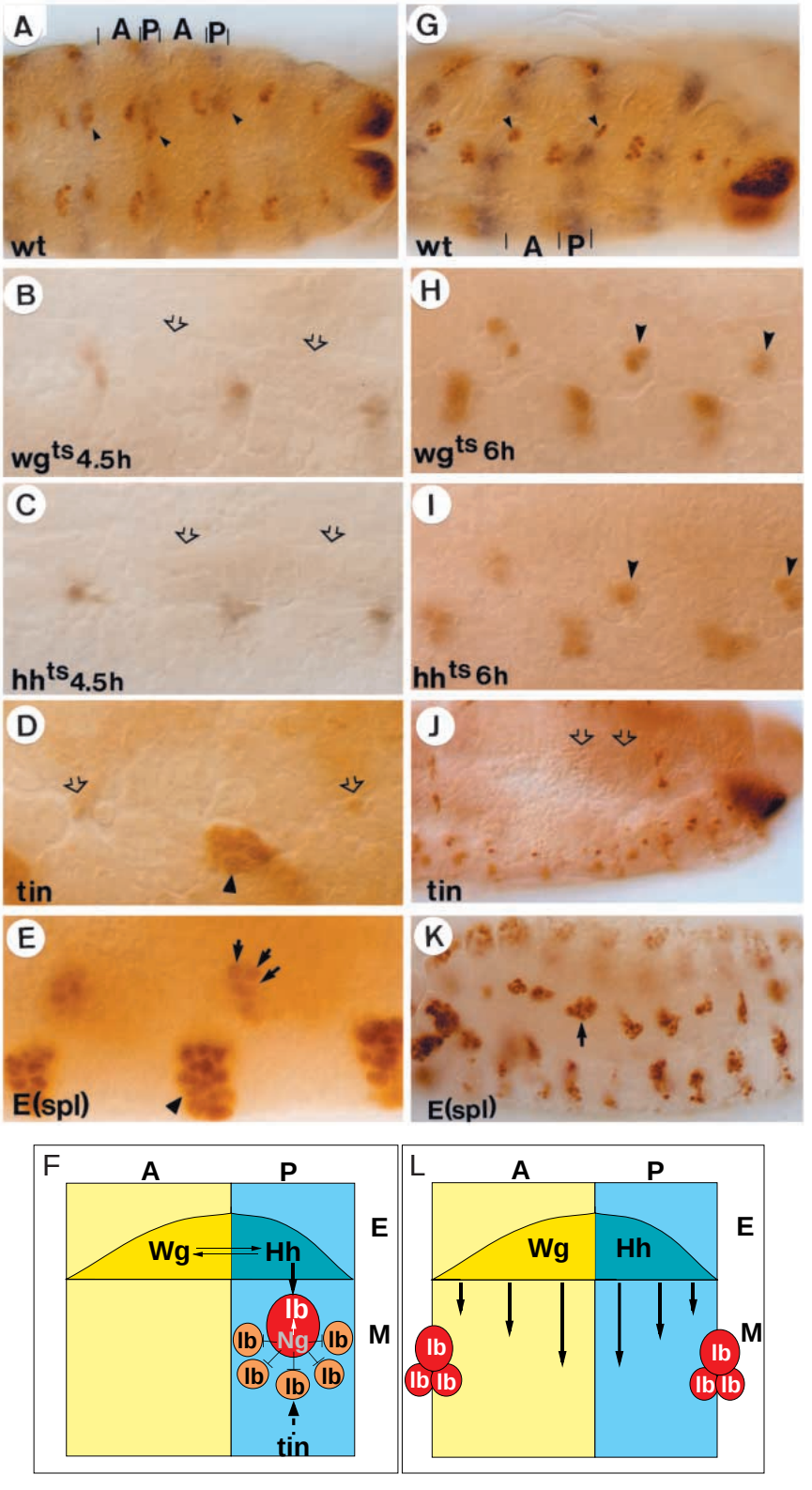

Fig. 4. Mesodermal and epidermal factors contributing to the specification of SBM progenitor. (A,G) Embryos double stained with anti-Lbl antibody (orange/brown) and with anti-Engrailed antibody (blue), which labels the epidermal posterior (P) compartment. Arrowheads point to *lb*-positive promuscular clusters. (B) *lb*-positive cells are absent (open arrows) in wg^{ts} and (C) hh^{ts} embryos shifted to the non-permissive temperature at 4.5 h AEL. (H,I) The temperature shift at 6 hours AEL has no effect on *lb* expression in SBM precursors (arrowheads). (D,J) In tin^{-} embryos, the SBM promuscular clusters and the majority of SBM fibers are absent (open arrows). The arrowhead points to the *lb*-expressing neural cells. (E) In *E(spl)* mutants, multiple cells (arrows), instead of one progenitor, segregate from the *lb*-positive promuscular clusters. Arrowhead points to supernumerary *lb*-expressing neural cells. (K) In the same mutants, at later stages, the hyperplastic muscular *lb* pattern is visible (arrow). (F) Schematic representation of SBM progenitor segregation (large red cell) from lb-positive promuscular cluster, involving indirect *tin* action (dotted arrow), epidermal Hh signaling and lateral inhibition conferred by the neurogenic genes (Ng). (L) A scheme showing the location of *lb*-positive cells at the beginning of germ band retraction with the respect to epidermal domains from which Wg and Hh signals penetrate the mesoderm (arrows). Developmental stages: (A-E) late stage 10, (G-I) late stage 11, (J) stage 15, (K) stage 14. (A) Ventral, (G) ventrolateral and (B-E and H-K) lateral views of embryos with anterior to the left. Abbreviations: E, epidermis; M, mesoderm.

Table 1. Expressivity of loss and gain of function *lb* phenotypes monitored by counting *twi*-expressing lateral (LaP) and ventral (VaP) adult precursors

Genotype	*wt*	*lb-def*	*UAS-lb*
LaP	2	0.53	8.44
VaP	1	1.54	0.09

LaP and VaP cells were counted in 100 abdominal hemisegments of stage 15 embryos stained with anti-Twi antibody. The number of cells per hemisegment is presented.

myoblasts (progenitors/founder cells) and modify the muscle pattern. Indeed, in *hs-lb* (data not shown) and *24B-Gal4/UAS-lbe* embryos, the number of *Kr*-expressing muscle precursors just adjacent to the SBM (see Fig. 2I) is dramatically reduced (compare Fig. 5D and E) indicating *lb*-induced transformation of myoblast identities.

The lb^{-} embryos display opposite muscle phenotypes. In 57% of hemisegments, we note the absence of muscle fibers at the SBM positions (Fig. 5C) and instead, within segmental borders or around the normal SBM location, we detect some unfused myoblasts (Fig. 5C,I). In the remaining 43% of hemisegments, the muscle fibers lying within or close to the segmental borders display abnormal shapes and insertion sites clearly distinct from those of SBM (Fig. 5I). Similarly, the specification of LaPs is affected in lb^{-} embryos. In 55% of hemisegments, they are absent (Fig. 5L) and, in 38% of hemisegments, we observe only one LaP (not shown) instead of two (Fig. 5J). Surprisingly, alteration of *lb* activity also affects *lb*-negative precursors of ventral adult muscles (VaP)

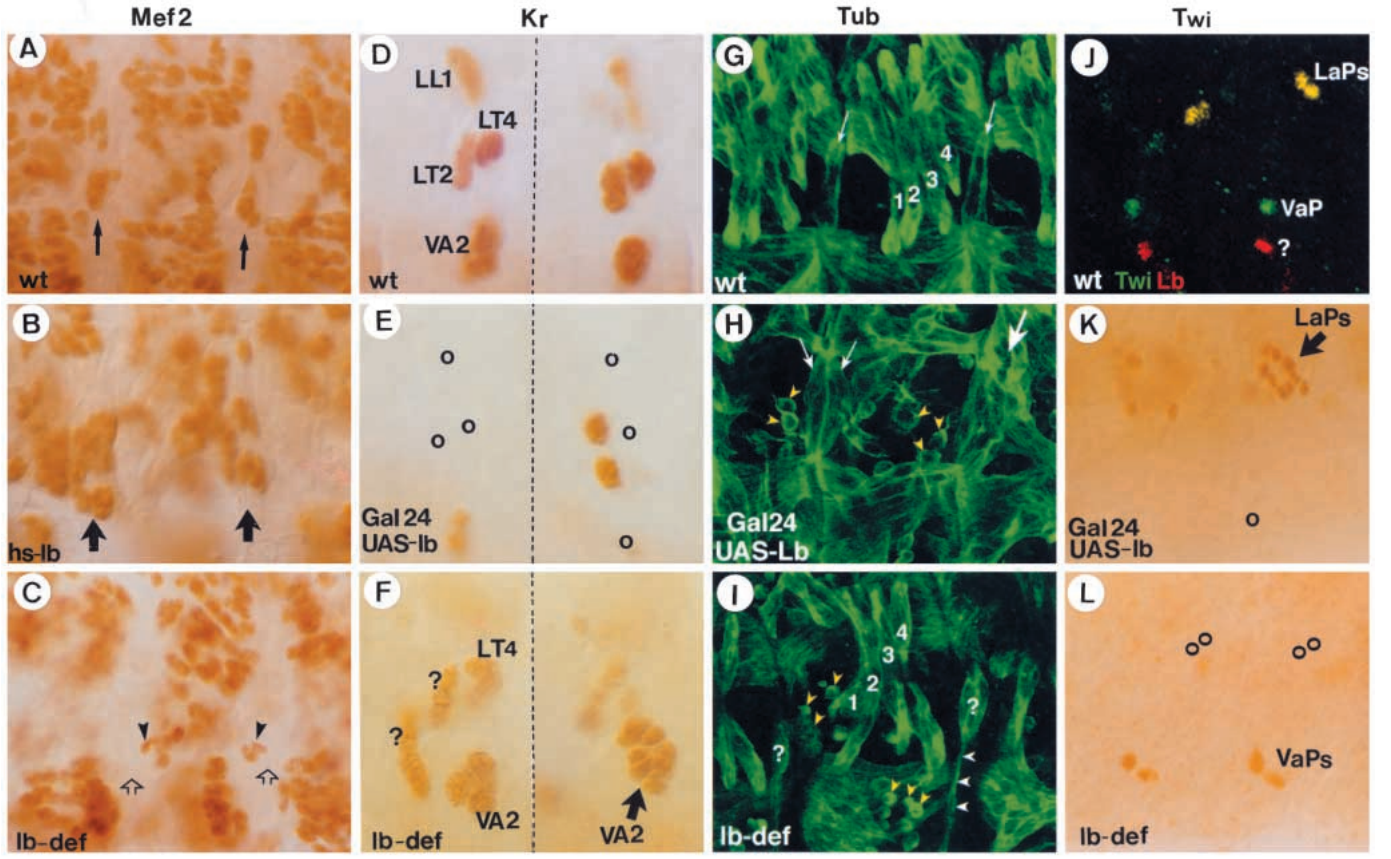

Fig. 5. *lb* determines the identity of both the SBM and LaPs. (A-C) Muscle pattern of the lateral part of abdominal segments immunostained with anti-Mef2. (A) Arrows point to SBMs of a wild-type embryo. (B) In *hs-lb* embryos, supernumerary myoblasts fuse to form enlarged SBMs (large arrows). (C) In embryos lacking *lb* activity, the SBMs are absent (open arrows). Arrowheads indicate some myoblasts lying near segmental borders. (D-F) Patterns of *Kr*-positive muscle precursors. (G-I) Confocal scans of muscle fiber architecture labeled with anti-β3-Tub. (J-L) Patterns of lateral (LaP) and ventral (VaP) precursors of adult muscles: (J) double stained for *twi* (green) and *lbe* (red) or (K,L) detected with anti-Twi antibody. Note that, in J, the *lb*-positive SBM cells are not shown. Dotted lines (D-F) indicate the segmental borders. (D,G,J) Wild-type, (E,H,K) *24B-Gal4;UAS-lbe* and (F,I,L) *lb*-deficiency embryos. Ectopic *lb* expression in all mesodermal cells (E) changes the identity of the majority of *Kr*-positive muscle precursors (open circles) and (H) leads to the formation of duplicated (two arrows) or enlarged (large arrow) SBMs as well as to (K) the hypertrophic pattern of the LaPs (large arrow) and absence of the VaP (open circle). Yellow arrowheads in H point to unfused myoblasts lying within usual emplacement of lateral transverse muscles. In *lb*-deficiency embryos the lack of *lb* activity (F) leads to the formation of supernumerary (?) or enlarged (large arrow) *Kr*-expressing muscle precursors as well as to (L) the absence of LaPs (open circles) and duplication of VaP. (I) In *lb*-def embryos, the presence of unfused myoblasts around the normal SBM location (yellow arrowheads) and/or the formation of muscle fibers which lay within segmental borders but have abnormal shape (?) and insertion sites. This latter is illustrated by a muscle fiber (?) with a long philopodium (white arrowheads), most likely unable to recognise epidermal insertion site specific for the SBM. In all panels, anterior is to the left and dorsal is up. Abbreviations: 1,2,3,4, lateral transverse muscles LT1, 2, 3 and 4; LL1, lateral longitudinal muscle 1; VA2, ventral acute muscle 2.

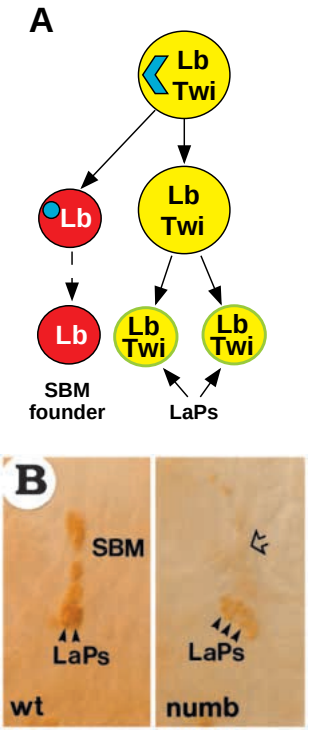

Fig. 6. Divisions of *lb*-positive progenitor. (A) The *lb*-positive progenitor divides two times. First, a morphologically asymmetric division leads to the transmission of Nb protein (blue chevron) to the SBM founder. The second division of a progenitor devoid of Numb gives rise to two *twi* and *lb*-positive LaPs. (B) The *lb* staining of wild-type (left, subpanel) and nb^- embryo (right, subpanel) shows the formation of a supernumerary LaP and absence of SBM (open arrow) in nb^- background. In these embryos, all progeny of *lb*-positive progenitors are devoid of Nb and become LaPs.

(see Table 1), which are duplicated in *lb*-deficient embryos (Fig. 5L) and absent when *lb* is ectopically expressed (Fig. 5K). Thus, we speculate that this feature is linked with the *lb* expression in a mesodermal cell (?) lying close to the VaP (Fig. 5J) and reflect the functions of *lb* in cell fate specification. Altogether these findings imply that *lb* is required for the acquisition of specific muscle properties and that the presence or absence of Lb proteins is sufficient to switch a subset of muscle founders between alternative fates.

DISCUSSION

Myogenesis in *Drosophila* leads to the formation of a segmentally repeated pattern of 30 different body wall muscles. Compared with vertebrate myogenesis, it appears much less complex, nevertheless an obvious similarity can be observed: (i) in both invertebrate and vertebrate embryos, somatic muscle primordia are specified as a population of multipotent mesodermal cells, respectively, within segmental units or somites, (ii) these cells become committed to a muscle fate under the influence of evolutionarily conserved intrinsic MyoD-like and MADS-box regulators and extrinsic inductive Wg/Wnts signals (for review see Yun and Wold, 1996) and (iii) in both systems, the Notch/Delta cell-cell signaling pathway mediates lateral inhibition involved in the segregation of muscle progenitors (Corbin et al., 1991; Bate et al., 1993; Lindsell et al., 1995). Extensive conservation of these early steps of the myogenic cascade makes *Drosophila* a model of choice to address one of the most important and as yet unanswered questions: how distinct characteristics of otherwise similar muscles are determined? Here we investigate the genetic pathways that lead to the diversification of *Drosophila* larval muscles showing that the identity of one of them is specified by the homeobox genes *lbe* and *lbl*.

Regulation of *lb* activity during myogenesis

During myogenesis, the *lb* genes display a highly restricted expression pattern suggesting that they require a precise regulatory apparatus. Indeed, both mesodermal information and inductive signals from the epidermis are involved in the proper regulation of *lb* activity in myoblast lineages leading to the formation of SBM and LaPs. Initially, *tin* function is required for activation of promuscular *lb* expression. However, this *tin* influence is likely to be indirect and mediated via an unknown factor, since *tin* expression at this time (5 hours AEL) becomes restricted to the dorsal mesoderm (Azpiazu and Frasch, 1993; Bodmer, 1993) while the *lb*-positive promuscular cells are located ventrolaterally. The indirect activation of *lb* by *tin* seems confirmed by the presence of about 20% of *lb*-expressing SBM fibers in tin^- embryos (see Fig. 4I). Interestingly, *tin*-dependent *lb* activation does not occur, in embryos lacking early *wg* or *hh* functions, providing further evidence for the role of epidermal signalings in the early events of myogenesis (see also Baker and Schubiger, 1995; Baylies et al., 1995, Ranganayakulu et al., 1996). Our analysis of mutants of neurogenic genes also confirm that the cell-cell interactions that lead to lateral inhibition are indispensable for the segregation of a proper number of SBM muscle progenitors (see also Corbin et al., 1991; Bate et al., 1995).

Particular features of *lb*-positive progenitor and derived myoblast lineages

In contrast to *S59* (Dohrmann et al., 1990; Carmena et al., 1995), *Kr* (Ruiz Gomez et al., 1997), *msh* (Nose et al., 1998) and other muscle identity genes, *lb* expression is restricted to only one muscle progenitor and its progeny. Recently, Ruiz Gomez and Bate (1997) and Carmena et al. (1998) have shown that the Nb protein is asymmetrically located in the progenitors and during their division transmitted to only one of the two daughter cells. As indicated by the loss of *Kr*-, *S59*- and *eve*-expressing muscles and the overproduction of adult precursors in *nb* mutant embryos (Ruiz Gomez and Bate, 1997), *nb* may determine the different fates of the sibling myoblasts.

After division of the progenitors expressing *Kr*, *S59* and *eve*, the activity of these genes disappears from founders devoid of Nb before they start to fuse with neighbouring myoblasts and is maintained in muscle precursors that arise from Nb-containing founders (Ruiz Gomez and Bate, 1997). The *lb*-positive progenitor displays distinct features. After its division, *lb* expression is maintained in both the SBM and LaP lineages although, as indicated by the nb^- phenotype (lack of SBM; see Fig. 6B), the Nb protein is rather transmitted to the SBM founder than to the LaPs. These data indicate that *lb* expression may play an important role in the determination of LaP identity and is maintained in these myoblasts in a *nb*-independent manner.

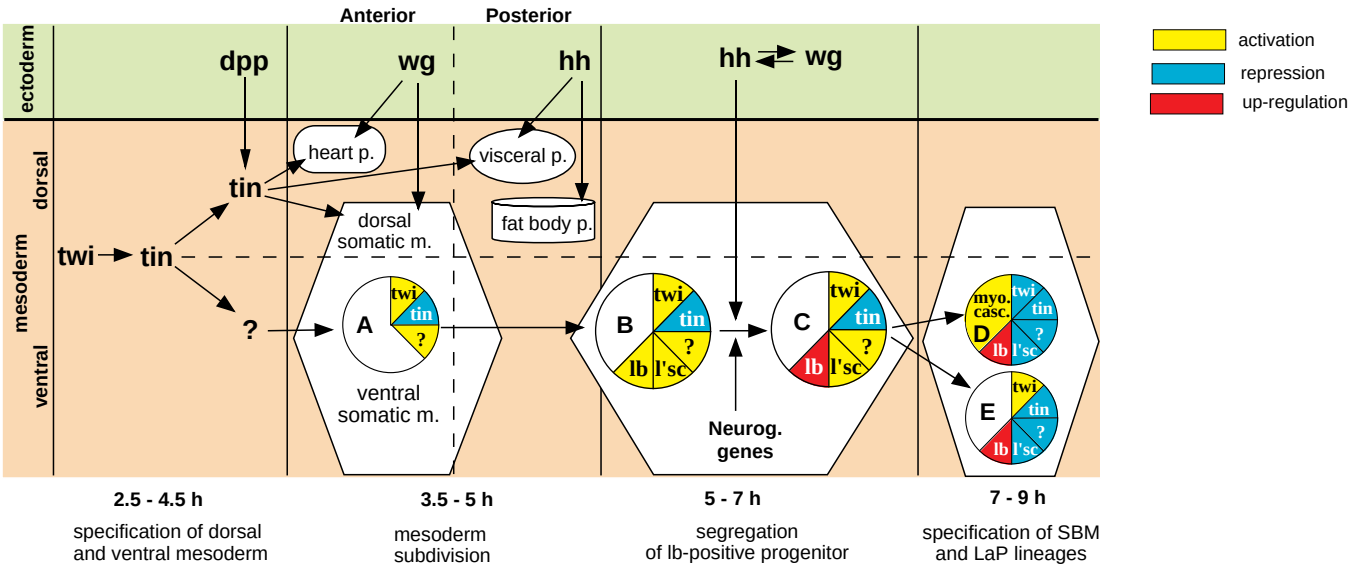

Fig. 7. Schematic representation of genetic events leading to the specification of SBM and LaP myoblast lineages. During gastrulation, mesodermal cells express *twi*, which activates *tin* in the region of the trunk. In the response to ectodermal Dpp signaling (Frasch, 1995), *tin* expression becomes restricted to the dorsal mesoderm suggesting that an unknown factor (?) specifies ventrally located mesodermal cells. Epidermal Wg and Hh signalings provide positional information leading to the mesoderm segmentation. Dorsal mesoderm located under the anterior, *wg*-expressing epidermal compartment gives rise to the heart and to the dorsal somatic muscles while the posteriorly located dorsal mesodermal cells (under the Hh influence) develop on visceral and fat body primordia (Azpiazu et al., 1996). In parallel, the ventral somatic muscles arise from the anteriorly located ventral mesodermal cells. Genetic events leading to the specification of two ventral somatic lineages: SBM founder and LaPs are illustrated in a series of circles, labelled A-E showing the activation or repression of gene activity. Multipotent ventral somatic mesodermal cells (A) differentiate to *l'sc*-expressing cells organized in promuscular clusters. In the case of the SBM promuscular cluster, these cells (B) start to express *lb.* Under the influence of epidermal signals and cell-cell signaling mediated by the neurogenic genes, the *lb* expression in the SBM progenitor (C) is upregulated. The SBM founder cell (D) continues to express *lb* and switches off *twi* activity while the LaPs (E) maintain both *lb* and *twi* expression. Since *twi* expression does not prevent differentiation of larval muscles (Baylies and Bate, 1996), its inactivation in the SBM founder, most likely, has no influence on the activation of the myogenic regulatory cascade. In contrast, according to Anant et al., 1998, persistent *twi* expression may keep, during embryonic and larval life, a nondifferentiated fate of adult muscle precursors.

The observation that the *lb* progenitor, unlike *S59* and *Kr* progenitors (Carmena et al., 1995; Ruiz Gomez et al., 1997), produces three progeny (one SBM founder and two LaPs) instead of two, suggests a distinct pathway for the division. Indeed, as indicated by confocal microscopy (see Fig. 3G,H), the *lb*-positive progenitor divides two times. Surprisingly, its first division is morphologically asymmetric giving rise to a smaller SBM founder cell (see Figs 3H, 6). This finding indicates that the muscle progenitors can produce two daughter cells of the same size, like neural midline precursors (Carmena et al., 1995), and also divide like neuroblasts to generate smaller progeny. The second division of the *lb*-positive progenitor produces two LaPs, which do not contribute to the larval muscles and, most likely due to the persistent *twi* expression, remain nondifferentiated throughout embryogenesis. As was previously shown by Bate (1991), the LaPs proliferate during larval life and give rise to the majority of abdominal adult lateral muscles. Unexpectedly, the second myoblast lineage specified by *lb* expression, the SBM myoblasts, also contributes to the adult body wall muscles. During pupation, unlike other larval somatic muscles, the SBM fibers resist on histolysis and serve as a template for the formation of adult muscles (Bate et al., 1991). The role of *lb* in this 'long life' phenomenon remains to be elucidated.

Interestingly, *lb* genes are also expressed in a group of promuscular cells from which the *lb*-positive progenitor segregates. This early *lb* activity overlaps that of *l'sc* expressed in all promuscular clusters (Carmena et al., 1995) suggesting that *lb* may exert its own promuscular function and/or cooperate with *l'sc* in the specification of the SBM promuscular cluster. The unaffected development of SBM in *l'sc*$^{-}$ embryos (data not shown) seems to confirm this possibility.

lb as a component of myogenic pathway

According to our observations, cell commitment in the somatic mesoderm involves three steps: (i) formation of promuscular clusters; (ii) segregation of progenitors and (iii) specification of founder cells or adult muscle precursors. Since, *lb* expression is associated with all these steps of SBM and LaPs commitment, it strongly suggests that *lb* genes may determine the identity of these two myoblast lineages. Indeed, in the embryos lacking *lb* function, the SBM and LaP formation is disturbed and we detect near segmental borders unfused or supernumerary *Kr*-expressing myoblasts. Thus, we conclude that, in the absence of *lb* activity, the prospective SBM myoblasts behave as noncommitted cells unable to form syncytial fibers or are recruited to built enlarged neighbouring muscles. Opposed muscle transformations occur in the embryos with ectopic *lb* expression. In the majority of segments, *Kr*-expressing progenitor/founder cells from the lateral domain are redefined, become *lb*-positive and contribute to the formation of giant SBMs or supernumerary LaPs. These

data clearly show that *lb* functions to specify the cell fate of myoblasts in which it is expressed. Thus, we infer that *lb* is sufficient to determine the identity of the SBM founder and required, together with *twi*, to define distinct features of LaPs.

Taking into consideration the requirements for the specification of *lb*-expressing myoblast lineages, we propose a model of cell commitment where the sequential activation or repression of defined, or as yet unknown, genes allows a pluripotent mesodermal cell to become the SBM founder or a LaP (Fig. 7). During gastrulation, the mesodermal cells of the trunk, from which body wall muscles arise, in addition to *twi*, express *tin*. The *tin* activity becomes restricted to the dorsal mesoderm, thus suggesting that another gene specifies the fate of ventrally located cells. The dorsal mesoderm, under the influence of epidermal signals, subdivides into the cardiac, visceral and fat body primordia while the ventral mesoderm mainly gives rise to the somatic musculature. Most likely, due to the action of *l'sc* (Carmena et al., 1995), a small portion of dorsal and the majority of ventral mesodermal cells become committed to promuscular clusters from which the progenitors are selected. The segregation of muscle progenitors requires a coordinate action of neurogenic genes as well as epidermal Wg and Hh signalings. The progenitors and, subsequently, their progeny are committed to different myoblast lineages by a network of identity genes such as *S59*, *Kr*, *msh* and *lb.* In the case of the *lb*-expressing progenitor, two myoblast lineages are produced: the *twi*-negative SBM founder and two LaPs with persistent *twi* activity. Similar to the co-expression of *lb* and *twi* in LaPs, several other myoblast lineages have been shown to express more than one identity gene. For example, in the DA1 founder, *Kr* is co-expressed with *eve* and in VA2 with *S59* (Ruiz Gomez et al., 1997), thus suggesting that the combined activation of identity genes may play an important role in the acquisition of individual muscle properties.

In this work, we describe the myogenic functions of *lb* homeobox genes showing that altered *lb* expression leads to the transformation of myoblast identities. Since *lb* provides positional information to a subpopulation of cardiac cells (Jagla et al., 1997b), we conclude that *lb* exerts similar functions during muscle and heart formation. The role of *lb* genes in the determination of identity is reminiscent of that of homeotic genes but exerted at the 'micro' levels where a cell lineage not a body part is concerned.

The finding that the *Drosophila lb* determines cell fate decisions during diversification of somatic muscles suggests a similar role for the vertebrate *lb* homologs (Jagla et al., 1995, Dietrich et al., 1998) thus allowing the investigation of diversification of vertebrate skeletal muscles.

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REFERENCES

Abmayr, S. M., Erickson, M. S. and Bour, B. A. (1995). Embryonic development of the larval body wall musculature of *Drosophila melanogaster. Trends Genet.* **11**, 153-159.

Anant, S., Roy, S. and VijayRaghavan, K. (1998). Twist and Notch negatively regulate adult muscle differentiation in *Drosophila. Development* **125**, 1361-1369.

Azpiazu, N. and Frasch, M. (1993). *tinman* and *bagpipe*: two homeo box 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.

Baker, R. and Schubiger, G. (1995). Ectoderm induces muscle specific gene expression in *Drosophila* embryos. *Development* **121**, 1387-1398.

Bate, M. (1990). The embryonic development of larval muscles in *Drosophila. Development* **110**, 791-804.

Bate, M., Rushton, E. and Currie, D. A. (1991). Cells with persistent *twist* expression are the embryonic precursors of adult muscles in *Drosophila*. *Development* **113**, 79-89.

Bate, M., Rushton, E. and Frasch, M. (1993). A dual requirement for neurogenic genes during *Drosophila* myogenesis. *Development* **1993 Supplement**, 149-161.

Bate, M. (1993). The mesoderm and its derivatives. In *The development of Drosophila melanogaster*, **vol. 2**, (ed. M. Bate and A. Martinez- Arias). pp. 1013-1090. Cold Spring Harbor, New York: CSH Laboratory Press.

Baylies, M. K., Martinez-Arias, A. and Bate, M. (1995). *wingless* is required for the formation of a subset of muscle founder cells during *Drosophila* embryogenesis. *Development* **121**, 3829-3837.

Bejsovec, A. and Martinez-Arias, A. (1991). Roles of *wingless* in paterning the larval epidermis in *Drosophila. Development* **113**, 471-485.

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

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

Bourgouin, C., Lundgren, S. E. and Thomas, J. B. (1992). *apterous* is a *Drosophila* LIM domain gene required for the development of a subset of embryonic muscles. *Neuron* **9**, 549-561.

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.

Carmena, A., Bate, M. and Jimenez, F. (1995). *lethal of scute*, a proneural gene, participates in the specification of muscle progenitors during *Drosophila* embryogenesis. *Genes Dev.* **9**, 2373-2383.

Carmena, A., Murugasu-Oei, B., Menon, D., Jimenez, F. and Chia, W. (1998). *inscuteable* and *numb* mediate asymmetric muscle progenitor cell division during *Drosophila* myogenesis. *Genes Dev.* **12**, 304-315.

Corbin, V., Michelson, A. M., Abmayr, S. M., Neel, V., Alcamo, E., Maniatis, T. and Young, M. W. (1991). A role for the *Drosophila* neurogenic genes in mesoderm differentiation. *Cell* **67**, 311-323.

Cossu, G., Tajbakhsh, S. and Buckingham, M. (1996). How is myogenesis initiated in the embryo. *Trends Gene Therapy* **12**, 218-223.

D'Alessio, M. and Frasch, M. (1996). *msh* may play a conserved role in dorsoventral patterning of the neuroectoderm and mesoderm. *Mech. Dev.* **58**, 217-231.

Dietrich, S., Schubert, R. F., Healy, C., Sharpe, P. T. and Lumsden, A. (1998). Specification of the hypaxial musculature. *Development* **in press**.

Dohrmann, C., Azpiazu, N. and Frasch, M. (1990). A new *Drosophila* homeobox gene is expressed in mesodermal precursor cells of distinct muscles during embryogenesis. *Genes Dev.* **4**, 2098-2111.

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

Heemskerk, J. and DiNardo, S. (1994). *Drosophila* hedgehog acts as a morphogen in cellular patterning. *Cell* **76**, 449-460.

Isshiki, T., Takeichi, M. and Nose, A. (1997). The role of *msh* homeobox gene during *Drosophila* neurogenesis: implication for the dorsoventral specification of the neuroectoderm. *Development* **124**, 3099-3109.

Jagla, K., Stanceva, I., Dretzen, G., Bellard, F and Bellard, M. (1994). A distinct class of homeodomain proteins is encoded by two sequentialy expressed *Drosophila* genes from the 93E/D cluster. *Nucl. Acids Res.* **22**, 1202-1207.

Jagla, K., Dollé, P., Mattei, M.-G., Jagla, T., Schuhbaur, B., Dretzen, G., Bellard, F. and Bellard, M. (1995). Mouse *Lbx1* and human *LBX1* define

a novel mammalian homeobox gene family related to the *Drosophila ladybird* genes. *Mech. Dev.* **53**, 345-356.

Jagla, K., Jagla, T., Heitzler, P., Dretzen, G., Bellard, F. and Bellard, M. (1997a). *ladybird*, a tandem of homeobox genes that maintain late *wingless* expression in dorsal and terminal epidermis of the *Drosophila* embryo. *Development* **124**, 91-100.

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

Keller, C. A., Erickson, M. S. and Abmayr, S. M. (1997). Misexpression of *nautilus* induces myogenesis in cardioblasts and alters the pattern of somatic muscle fibers. *Dev. Biol.* **181**, 197-212.

Leiss, D., Hinz, U., Gasch, A., Mertz, R. and Renkawitz-Pohl, R. (1988). *β3 tubulin* expression characterises the differentiating mesodermal germ layer during *Drosophila* embryogenesis. *Development* **104**, 525-531.

Lindsell, C. E., Schwaber, C. J., Boulter, J. and Weinmaster, G. (1995). Jagged- a mammalian ligand that activates *notch1*. *Cell* **80**, 909-917.

Michelson, A. M., Abmayr, S. M., Bate, M., Martinez-Arias, A. and Maniatis, T. (1990). Expression of *Myo-D* family member prefigures muscle pattern in *Drosophila* embryos.*Genes Dev.* **4**, 2086-2097.

Nose, A., Isshiki, T. and Takeichi, M. (1998). Regional specification of muscle progenitors in *Drosophila*: the role of the *msh* homeobox gene. *Development* **125**, 215-223.

Perrimon, N. (1994). The genetic basis of the patterned baldness in *Drosophila. Cell* **76**, 781-784.

Ranganayakulu, G., Schultz, R. A. and Olson, E. N. (1996). Wingless signaling induces *nautilus* expression in the ventral mesoderm of the *Drosophila* embryo. *Dev. Biol.* **176**, 143-148.

Rhyu, M., Jan, L. and Jan, Y. N. (1994). Asymmetric distribution of numb protein during division of the sensory organ precursor confers distinct fates to daughter cells. *Cell* **76**, 477-491.

Ruiz Gomez, M., Romani, S., Hartmann, C., Jäckle, H. and Bate, M. (1997). Specific muscle identities are regulated by *Krüppel* during *Drosophila* embryogenesis. *Development* **124**, 3407-3414.

Ruiz Gomez, M. and Bate, M. (1997). Segregation of myogenic lineages in *Drosophila* requires Numb. *Development* **124**, 4857-4866.

Spana, E., Kopczynski, C., Goodman, C. and Doe, C. (1995). Asymmetric localisation of numb autonomously determines sibling neuron identities in the *Drosophila* CNS. *Development* **121**, 3187-3195.

Thisse, C., Perrin-Schmitt, F., Stoetzel, C. and Thisse, B. (1991). Sequence-specific transactivation of the Drosophila twist gene by the dorsal gene product. *Cell* **65**, 1191-1201.

Uemura, T., Shepherd, S., Ackerman, L., Jan, L. Y. and Jan, Y. N. (1989). *numb*, a gene required in determination of cell fate during sensory organ formation in *Drosophila* embryos. *Cell* **58**, 349-360.

Wiliams, J. A., Bell, J. B. and Carroll, S. B. (1991). Control of *Drosophila* wing and haltere development by the nuclear *vestigial* gene product. *Genes Dev.* **5**, 2481-2495.

Yun, K. and Wold, B. (1996). Skeletal muscle determination and differentiation: story of a core regulatory network and its context. *Curr. Opin. Cell Biol.* **8**, 877-889.