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Segregation of myogenic lineages in *Drosophila* requires Numb

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SUMMARY

Terminal divisions of myogenic lineages in the *Drosophila* embryo generate sibling myoblasts that found larval muscles or form precursors of adult muscles. Alternative fates adopted by sibling myoblasts are associated with distinct patterns of gene expression. Genes expressed in the progenitor cell are maintained in one sibling and repressed in the other. These differences depend on an asymmetric segregation of Numb between sibling cells. In *numb* mutants, muscle fates associated with repression are duplicated and alternative muscles are lost. If *numb* is overexpressed the reverse transformation occurs. Numb acts to block Notch-mediated repression of genes expressed in muscle progenitor cells. Thus asymmetric cell divisions are essential determinants of muscle fates during myogenesis in *Drosophila*

Key words: *numb*, inscuteable, Notch, muscle pattern, myogenesis, *Drosophila*

INTRODUCTION

The mechanisms that underlie the initiation and execution of myogenic differentiation are increasingly well understood in a number of different organisms (Yun and Wold, 1996). Much less is known about the formation of specific muscles and their spatial organisation to form a pattern. The complex arrangement of muscles produced in every segment during the development of the *Drosophila* embryo (Abmayr et al., 1995; Bate, 1993) provides us with a unique opportunity to study the machinery of muscle specification and patterning. The formation of individual muscle fibres in the *Drosophila* embryo is seeded by a special class of founder myoblasts that fuse with neighbouring mesodermal cells to form the syncytial precursors of particular muscles (Bate, 1990; Dohrmann et al., 1990; Rushton et al., 1995). In this way, the elements of the muscle pattern are laid out by the generation of specific muscle-forming cells at particular locations in the somatic mesoderm.

Recent work has shown that the founder myoblasts are produced as pairs of sibling cells by the division of muscle progenitors that segregate from surrounding cells of the mesoderm (Carmena et al., 1995). Thus, during myogenesis, a well-defined lineage leads to the production of individual muscles, just as, during neurogenesis, a characteristic sequence of cell divisions generates individual neurons and their support cells (Campos-Ortega, 1993). The analogy between neurogenesis and myogenesis is reinforced by the finding that the muscle progenitors segregate from clusters of cells that express the proneural gene *lethal of scute* and that this segregation itself depends on a process of lateral inhibition mediated by the products of neurogenic genes and the activation of Notch (Bate et al., 1993; Carmena et al., 1995; Corbin et al., 1991).

Individual progenitors, founders and the muscles that they give rise to express specific transcription factors such as *Krüppel (Kr)*, *even skipped (eve)* and *S59* (Abmayr et al., 1995; Bate, 1993). In every case, the expression of these genes is lost from one of the two sibling cells that the progenitor produces. Thus a *Kr*-expressing progenitor gives rise to two founders and hence two muscles: one expresses *Kr*, the other does not (Ruiz Gómez et al., 1997). There is experimental evidence that it is the expression of these genes, or combinations of them, that endows individual muscles with their special characteristics such as size, shape, insertion sites and innervation by specific motorneurons (Ruiz Gómez et al., 1997; Keller et al., 1997). However muscle lineages in the *Drosophila* embryo not only produce the elements of the larval muscle pattern in the form of specific muscle founder cells, they also lay out the elements of the adult muscle pattern by generating the precursors of future adult muscles at specific locations in the embryonic mesoderm (Bate et al., 1991). Adult muscle precursors differ from larval muscle founders in that they maintain expression of the bHLH gene *twist* (Thisse et al., 1988) and do not fuse with neighbouring myoblasts. Instead they proliferate during larval life to produce pools of *twist*-expressing myoblasts from which the adult muscles will be formed (Currie and Bate, 1991). Despite these differences, adult muscle precursors are produced in the embryo as the sibling cells of larval muscle founders (Carmena et al., 1995). Thus terminal divisions in the myogenic lineages of the *Drosophila* embryo produce sibling cells with different fates: either the founders of two distinct larval muscles, or a larval founder cell and the precursor of an adult muscle.

The differences between these cells could be determined by extrinsic signals or the segregation of intrinsic factors during cell division. Here we report the results of an investigation of terminal divisions in the myogenic lineages of the embryo and the mechanisms that operate during cell division to generate the distinctive cell fates that underlie muscle patterning in *Drosophila*. We find that the divisions that produce sibling founders are asymmetrical and that, as in the terminal divisions

of neurogenesis, this asymmetry depends on the cytoplasmic membrane-associated protein Numb (Guo et al., 1996; Rhyu et al., 1994; Spana et al., 1995; Spana and Doe, 1996; Uemura et al., 1989). Numb is asymmetrically distributed in muscle progenitors and segregates asymmetrically between sibling cells as each progenitor divides. It is this differential distribution of Numb that underlies the segregation of cell fates that occurs at the terminal division. The characteristic feature of embryos mutant for *numb*, or embryos where the normal asymmetry of Numb distribution is disturbed, is a defective muscle pattern caused by the duplication of some fates in the myogenic lineages accompanied by a corresponding loss of others. Thus asymmetric cell divisions determine the allocation of cell fates in muscle-forming mesoderm and are an essential prerequisite for the formation of a normal muscle pattern.

MATERIALS AND METHODS

Drosophila strains and crosses

The following flies were used: *Oregon R*, a null allele of *numb*, $numb^{1}$ (Uemura et al., 1989), two null alleles of *inscuteable*, $insc^{P72}$ and $insc^{P49}$ (Kraut et al., 1996), a lethal allele of *not enough muscles*, nem^{8} (Burchard et al., 1995), a null allele of *Notch*, N^{55e11} (Lindsley and Zimm, 1992). We also used *UAS-numb* (Zhong et al., 1996) and *UAS-Nintra* (an activated form of *Notch* gift from S. Bray and J. F. de Celis), *twist-Gal4* (Baylies and Bate, 1996) and *C612b-Gal4* (with localised strong expression of GAL4 in muscles DA2 and DO2 from stage 11, generously provided by E. Dormand and A. Brand).

For the ectopic expression of *numb* females of the stock *twist*-GAL4; *twist*-GAL4 were mated to males *UAS-numb*. Localised expression of activated *Notch* was obtained by mating *C612b-GAL4* females to *UAS-Nintra* males. In both cases, crosses were made at 29°C and embryos were allowed to develop at this temperature until they reach the desired stage before fixation for immunostaining.

Histochemistry

For double-labelling experiments with antibody staining and in situ hybridization, we made the antibody staining in the presence of RNAsin and heparin followed by in situ hybridizations performed with minor modifications to the protocol of Tautz and Pfeifle (1989). Embryos were fixed following standard procedures and incubated with 3% H_2O_2 in methanol for 10 minutes followed by three washes with PBTH (filtered 1× PBS, 0.1% Tween 20 and 50 μg/ml heparin) before overnight incubation at 4°C with anti-Kr antibodies. The antibody was diluted (1:1000) in PBTH and 20 U/ml of RNAsin as well as 250 μg/ml of tRNA were added to the antibody mix. After three washes in PBTH, embryos were incubated for 30 minutes at room temperature in the biotinylated secondary antibody diluted in PBTH containing RNAsin and tRNA, followed by washes in PBTH. The embryos were then incubated in the ABC reagent (in the presence of RNAsin and tRNA) for 30 minutes and washed in PBTH before developing the colour reaction by adding DAB and H_2O_2. The reaction was stopped by adding PBTH and the embryos were then washed with PBT extensively to dilute up the heparin before proceeding to the in situ protocol. Immunological stainings of whole-mount embryos, using the Vectastain ABC Elite Kit from Vectalabs were made as described in Ruiz Gómez and Ghysen (1993). The following primary antibodies were used: anti-Numb (Rhyu et al., 1994), anti-Inscuteable (Kraut and Campos-Ortega, 1996), anti-muscle Myosin (Kiehart and Feghali, 1986), anti-Twist, anti-Krüppel (Gaul et al., 1987), anti-S59 (Dohrmann et al., 1990), anti-Eve (Patel et al., 1994), anti-Ladybird (Jagla et al., 1997) and anti-β-galactosidase (Cappel). In most cases, mutant chromosomes were balanced over chromosomes containing *lacZ* inserts and homozygous mutant embryos were identified by double staining with anti-β-gal antibodies. Stained embryos were examined and photographed using a Zeiss Axiophot microscope. Immunofluorescent stainings were performed as in Carmena et al. (1995) and fluorescent images were recorded by use of a Biorad MRC600 confocal microscope.

RESULTS

numb is expressed in muscle progenitors and asymmetrically distributed between muscle founder cells

Muscles in the *Drosophila* embryo form by a process of myoblast fusion which is initiated by the appearance of muscle founder cells in the somatic, muscle-forming mesoderm. Founders fuse with neighbouring, undifferentiated myoblasts to form the syncytial precursors of individual muscles (Bate, 1990). Each founder is one of a pair of sibling cells formed by the division of a muscle progenitor cell which segregates from a cluster of adjacent cells in the mesoderm (Carmena et al., 1995). To investigate the role of *numb* in muscle determination, we first used polyclonal antibodies against Numb to show whether the protein is expressed in the mesoderm. We found that Numb is expressed in large mesodermal cells, which, by their position, we could provisionally identify as muscle progenitors. To confirm this, we made double in situ hybridisations/antibody stainings using a cDNA probe to detect *numb* transcripts and anti-*Kr* antibody to identify the subset of muscle progenitors in which *Kr* is known to be expressed (Ruiz Gómez et al., 1997). The coexpression of *Kr*and *numb* that we observe in these experiments (Fig. 1) clearly demonstrates that *numb* is expressed in muscle progenitor cells.

To follow the distribution of Numb as progenitors divide to produce muscle founders we focussed on a subset of progenitors and founders, those that express the homeobox-containing gene *S59*. The lineages and muscles generated by these cells have already been described in detail (Carmena et al., 1995; Dohrmann et al., 1990). During normal development two ventrally located, *S59*-expressing muscle progenitor cells divide to produce three muscle founders and the precursor of an adult muscle. The more dorsal progenitor divides first giving rise to the founders of muscles VA1 and VA2 (muscle nomenclature according to Bate, 1993) followed by the more ventral progenitor which produces the VA3 founder and the ventral adult precursor (VaP). By concentrating on this well-defined group of cells, we could follow the distribution of Numb as the muscle progenitors divide. As shown in Fig. 1C, there are crescents of Numb in the progenitor cells. As the progenitors divide, Numb is included in one of the two cells formed and excluded from the other. For example, Fig. 1C shows the stage at which the more dorsal progenitor has divided, so that the cluster consists of three cells, namely the VA1 and VA2 founders and the most ventral progenitor. All three cells express *S59* (shown in red) but only two, the progenitor and one of the founders, contain Numb (green). Thus the division of a muscle progenitor produces an unequal distribution of Numb between the founders: one contains Numb, the other does not.

Asymmetric distribution of Numb in the mesoderm requires *inscuteable*

In the CNS, the asymmetric localisation of Numb depends on

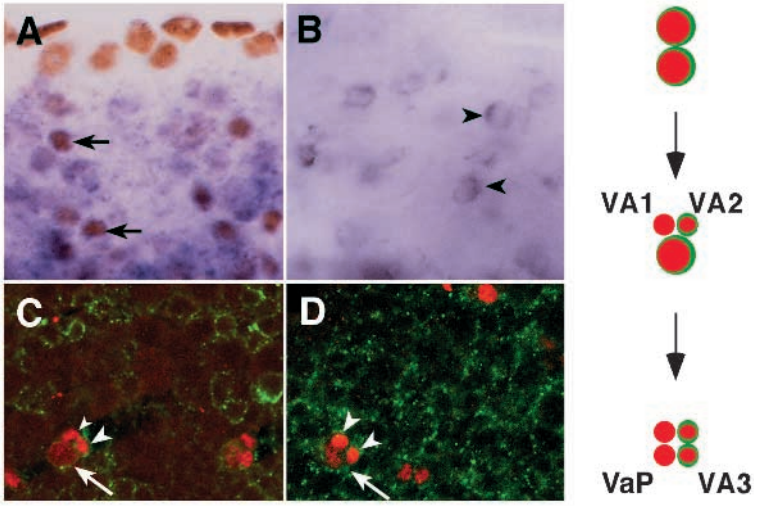

Fig. 1. Expression of *numb* and *inscuteable* in muscle progenitors and founders. (A) Expression of *numb* cDNA (purple) and Kr protein (brown) in stage 11 wild-type embryos. Some muscle progenitors that co-express *numb* and *Kr* are labelled by arrows. (B) Accumulation of Insc protein in crescents in muscle progenitors (arrowheads). (C) Expression of Numb (green) and S59 (red) proteins in stage 12 wild-type embryo. One of the two ventral *S59*-expressing progenitors has already divided to produce two founder cells. Numb is segregated to one of the founders (large arrowhead) and is absent from the other (small arrowhead). The other ventral progenitor has not divided yet and shows asymmetric distribution of Numb (arrow). (D) Expression of Numb (green) and S59 (red) proteins in stage 12 $insc^{P49}$ mutant embryos. Numb is not asymmetrically distributed in the progenitors (arrow) and segregates to both founder cells after division (arrowheads). The diagram in the right panel shows the sequential division of the ventral S59-expressing progenitors. VA1 and VA2 founders derive from the dorsalmost progenitor that divides earlier, the other progenitor generates the VA3 founder and the ventral adult precursor (VaP). Numb segregates to the VA2 and VA3 founders as deduced from the mutant phenotypes (see text). In all panels anterior is to the left and dorsal is up.

inscuteable (*insc*; Kraut et al., 1996). *insc* is known to be expressed in single cells in the embryonic mesoderm (Kraut and Campos-Ortega, 1996) and, using an antibody to Inscuteable, we find that it too is expressed in crescents in muscle progenitor cells (Fig. 1B). In the absence of *insc*, *numb* is still expressed in the mesoderm, but the protein is no longer localised asymmetrically as crescents in the progenitor cells. Similarly, when a progenitor divides, in the absence of *insc*, Numb is equally partitioned between the two founder cells which it gives rise to (Fig. 1D).

numb is required to confer distinct fates on the daughter cells of muscle progenitors

There are severe defects in the muscle pattern formed by embryos carrying null mutations in *numb* (Uemura et al., 1989). To analyse the requirement for *numb* in the myogenic lineages, we made a detailed study of these defective patterns. We find that the defects are caused by the loss of some muscles and the transformation of others. There are clear cases where muscles are duplicated while neighbouring muscles are missing. These duplications/losses include (a) VA1 (duplicated) and VA2 (missing) (see Fig. 2G,H) (b) DO5 (duplicated) and LL1 (missing) and (c) LT3 (duplicated), LT4 (missing) (see Fig. 2D,E). One way of interpreting these muscle transformations is that they result from changes in the fate of sibling founder cells caused by the absence of Numb. Specifically, if the absence of Numb leads to a failure in the normal distinction between two sibling founder cells, then both cells should now form the same muscle.

To test this idea, we looked in detail at the fates of cells in the ventral *S59*-expressing clusters where lineages and fates have already been described. As outlined above, the more dorsal progenitor produces the VA1 and VA2 founders, whereas the ventral progenitor produces VaP and the VA3 founder. In $numb^{1}$ embryos, VA1 and VaP are duplicated whereas VA2 and VA3 are missing (Fig. 2G,J). Thus one of the two cell types produced by each progenitor is duplicated with the concomitant loss of the other. It is likely that all adult precursors are paired with larval founder cells as alternative fates. Each of the six persistent *twist*-expressing precursors in an abdominal hemisegment (Bate et al., 1991) is duplicated in $numb^{1}$ embryos (Fig. 2J). In the case of the lateral adult precursors, this duplication is associated with a loss of the segment border muscle (SBM); in the case of the three dorsal precursors, it is associated with the loss of dorsal muscles (Fig. 2A).

The implication of these findings is that, in muscle lineages, as in neural lineages, Numb acts to switch sibling cells between alternative fates and that, in *numb* mutant embryos, both cells adopt the fate characteristic of the cell from which Numb is normally absent. If this is so, then expression of Numb in both cells should reverse the phenotype of $numb^{1}$ by switching both cells into the fate normally adopted by the sibling to which Numb is normally segregated. We tested this in two ways: first, we used the Gal4 system to misexpress *numb* throughout the mesoderm from early stages onwards; second, we analysed the phenotype of *insc* mutant embryos in which (see above) Numb is equally partitioned between sibling muscle founder cells.

Ectopic expression of *numb* leads to loss of adult muscle precursors and duplication of larval muscles

We used *twi*-Gal4 and a UAS-*numb* construct (Brand and Perrimon, 1993; Zhong et al., 1996) to induce generalised expression of Numb throughout the mesoderm from early stages of embryogenesis onwards. Under these conditions, muscles are duplicated or transformed and adult muscle precursors are lost (Fig. 2). These changes are the reverse of those that we see with loss of *numb*. For example, VA2 is duplicated at the expense of VA1 (Fig. 2I); DO5 is transformed to LL1 and LT3 is transformed to LT4 (Fig. 2F). VaP is missing and VA3 is duplicated (Fig. 2L,I). Lateral and dorsal adult precursors are also missing (Fig. 2L) and the SBM (not shown) and dorsal muscles are duplicated (Fig. 2C). Despite these aberrations in the larval muscle pattern, some embryos develop to form adult flies. These adults are flightless and do not jump and they lack ventral abdominal muscles that would normally be generated from VaP (Fig. 3).

The transformations of muscles are corroborated by corresponding changes in the patterns of gene expression in muscle

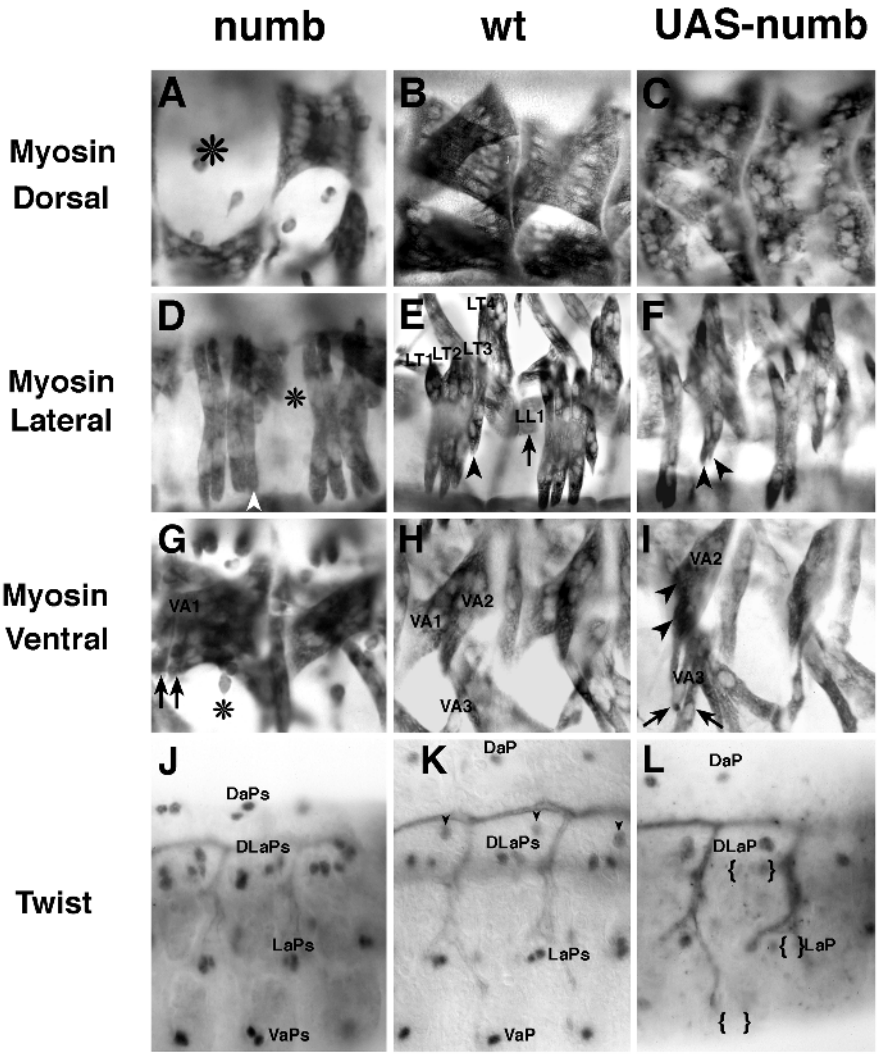

Fig. 2. Mesodermal phenotypes produced by loss and gain of function of *numb.* Left column shows *numb*^{1} mutant embryos (numb), central column wild type (wt) and right column embryos with ectopic expression of *numb* in the mesoderm (UAS-numb, see Materials and Methods). (A-I) Embryos are stained with anti-Myosin to show dorsal (A-C), lateral (D-F) and ventral (G-I) muscle subsets in two consecutive abdominal segments. (J-L) Embryos are stained with anti-Twist to show adult muscle precursors. In the absence of Numb, changes in the muscle pattern consist of absences (represented by asterisks) of the dorsal set of muscles (A), muscle LL1 (D, compare with arrow in E) and muscle VA3 (G, see H for comparison) or muscle transformations as those of muscle LT4 towards LT3 (more dorsal attachment typical of LT4 converted to ventral attachment typical of LT3: white arrowhead in D, compare with arrowhead in E) and VA2 to VA1 (arrows in G, compare H). Muscle absences are associated with ectopic adult precursor cells which are shown in J (duplication of anti-Twist-stained nuclei compare J with K). When *numb* is ectopically expressed in the mesoderm the opposite transformations are induced. Ectopic muscles develop in the dorsal region (C), the number of adult precursors is reduced (brackets for DLaP in L), LT3 is transformed into LT4 (arrowheads in F), VA1 is transformed into VA2 (arrowheads in I) and an ectopic VA3 develops instead of the ventral adult precursor (arrows in I and bracket in L). In all panels, anterior is to the left and dorsal is up. Muscle nomenclature as in Bate (1993). Arrowheads in K indicate positions of alary muscle nuclei that also express *twist* (Bate et al., 1991).

founder cells and the muscle precursors that they give rise to. During normal myogenesis, there is a characteristic sequence and pattern to the expression of genes such as *Kr*, *S59* and *eve*, each of which marks a subset of the developing muscles. Initially, each is expressed in particular muscle progenitors and then in the two sibling founder cells that each progenitor produces. Expression then disappears from one founder cell but is maintained in the other, with the result that the members of each muscle pair now differ in the expression of one of these genes. In *numb*^{1} embryos, the expression of *Kr* (Fig. 4D), *S59* (not shown) and *eve* (Fig. 4A) is initiated normally but is lost from both founder cells after they are formed. Thus, in *numb*^{1} embryos, there are no muscles that express *Kr*, *eve* or *S59*. In contrast, when numb is ectopically expressed throughout the mesoderm, *Kr*, *S59* and *eve* expression are maintained in both founders and in the muscle precursors that they give rise to. In these embryos *Kr*, *S59* and *eve*-expressing muscles are duplicated (Fig. 4C,F).

Taken together, these results reveal a requirement for *numb* in the mesoderm in making the choice between alternative fates in the somatic muscle lineages. In each case, presence or absence of Numb acts as a switch between alternative muscle fates, which mirrors the unequal distribution of Numb between founder cells that we observe in normal embryos. Because of the changes in gene expression and muscle pattern that we observe, we can confirm the previous designations of VA1 and VA2, VA3 and VaP, as being derived from pairs of sibling cells and identify likely additional siblings as follows: LT1 and LT2; LT3 and LT4; LL1 and DO5; SBM and lateral adult precursor; and, tentatively, DA1 and the most dorsal adult precursor.

Lack of function of *inscuteable* produces phenotypes in the mesoderm similar to ectopic *numb*

The development of the normal muscle pattern requires an asymmetric distribution of Numb between sibling muscle founder cells. This requirement is reflected in the characteristic muscle phenotypes that we have described for loss of *numb* and constitutive *numb* expression throughout the mesoderm. For this reason, mutations that interfere with the normal segregation of Numb between founder cells should also produce muscle patterning defects. *insc* is required for asymmetric distribution of Numb in the CNS (Kraut et al., 1996) and we have shown that it is also required in the mesoderm. *insc* mutant embryos have a defective muscle pattern (Kraut and Campos-Ortega, 1996), although these defects have not been described

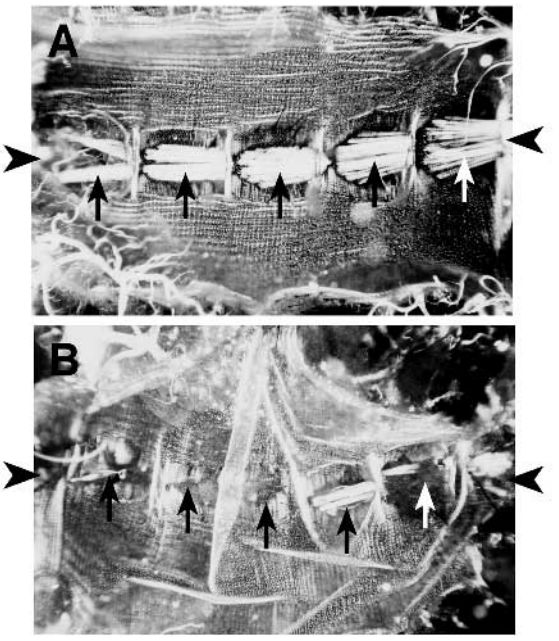

Fig. 3. Loss of adult muscles produced by ectopic expression of *numb*. Ventral view of adult abdomens (female) opened dorsally and dissected flat to reveal ventral muscles (arrows) under polarised light. (A) Wild type and (B) UAS-*numb*. Ventral muscles are missing in UAS-*numb* flies. In both panels, anterior is to the left and arrowheads point to the ventral midline.

in detail. In addition, it has recently been shown that *insc* is allelic to the known muscle patterning gene, *not enough muscles* (*nem*) (Burchard et al., 1995, Kirr et al., 1997). We reexamined the muscle pattern in *insc*/*nem* mutant embryos with the expectation that the phenotype would resemble that of constitutive *numb* expression in the mesoderm, because *numb* will be equally segregated between sibling muscle founder cells in such embryos. In the event, we find that there are muscle transformations and ectopic muscles in the pattern, which resemble those that result from uniform expression of Numb throughout the mesoderm although the effects are less pronounced (Fig. 5). This weaker effect may be due to equal partitioning of Numb between both siblings at a lower level than that achieved by Gal4-induced overexpression. Ectopic muscles are formed dorsally (Fig. 5A) and this is associated with a loss of *twi*-expressing adult muscle precursors (Fig. 5C,D). SBM is duplicated (Fig. 5E) and lateral adult precursors are missing (Fig. 5C,D). LT3 is transformed towards LT4 (Fig. 5E) and these changes are accompanied by corresponding alterations in gene expression. For example, the homeobox gene *ladybird*, normally expressed in SBM (Jagla et al., 1997), is also expressed in the ectopic SBM (Fig. 5B,F) and *Kr* expression is maintained in all four LT muscles instead of just LT2 and LT4 (Fig. 5E inset). Thus the requirement for Insc for the asymmetric distribution of Numb between sibling muscle founders and the muscle phenotypes that result from a failure in this segregation, confirm the role of Numb in the assignment of founder cells to different developmental pathways.

Numb antagonises Notch in determining cell fates in the mesoderm

One proposal for the action of Numb in determining differences in cell fate is that it operates by interfering with the activation of Notch (Campos-Ortega, 1996; Guo et al., 1996; Spana and Doe, 1996). In this view, alternative cell fates are set by an interaction between an intrinsic factor (Numb), segregated by cell division, and extrinsic signals operating through the activation of the Notch signalling pathway. Sibling cells are consigned to different fates by implementation (absence of Numb) or nonimplementation (presence of Numb) of Notch-dependent developmental pathways. This would suggest that, as in neurogenesis, there are two requirements for Notch during myogenesis: first in a process of lateral inhibition leading to a segregation of progenitor cells and then in switching progeny cells between alternative fates. However, analysing the role of

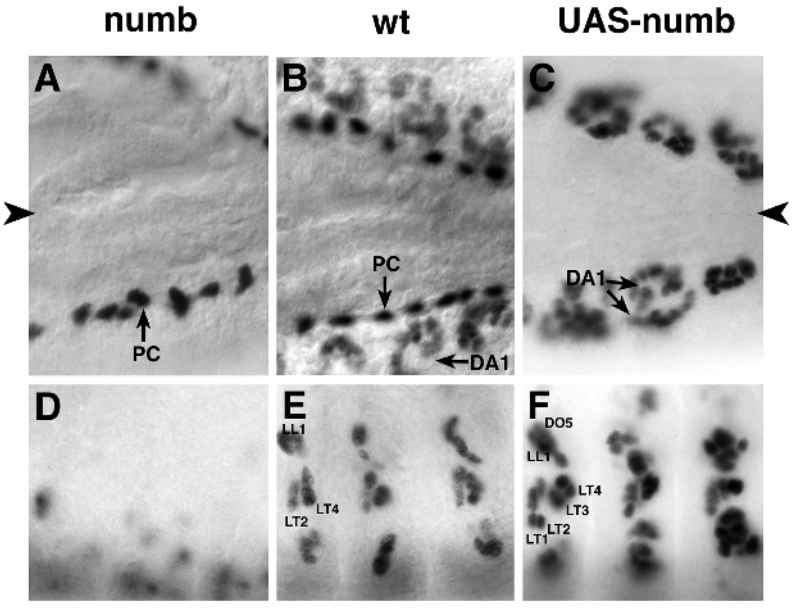

Fig. 4. Changes in the expression of muscle marker genes produced by loss of *numb* and ectopic *numb*. (A-C) Dorsal view of stage 15 *numb*^{1} (A), wild-type (B) and *UAS-numb* (C) embryos stained with anti-Eve. In wild-type embryos, *eve* is expressed in the dorsal muscle DA1 and in a subset of pericardial cells (PC in B). *numb*^{1} embryos lack DA1 (A) whereas in *UAS-numb* embryos DA1 is duplicated as reflected by ectopic Eve (C, compare Fig. 2C). The excess of PC cells in *numb*^{1} (A) and their absence in *UAS-numb* (C) embryos is accompanied by a reciprocal change in the number of cardial cells (not shown). (D-F) Lateral view of stage 14 *numb*^{1} (D), wild-type (E) and *UAS-numb* (F) embryos stained with anti-Kr. In normal development Kr is only maintained in one of the two founders derived from *Kr*-expressing progenitors. Thus, in the lateral region it is maintained in LT2, LT4 and LL1 and lost from LT1, LT3 and DO5 (E). In the absence of *numb*, Kr is lost from all precursors (D, ventral expression is CNS), whereas when *numb* is ectopically expressed Kr is maintained in both precursors arising from the sibling founders (F).

Notch in determining the alternative fates of muscle-forming cells is complicated by the fact that the segregation of muscle progenitor cells depends on the earlier process of lateral inhibition that is mediated by the activation of Notch (Bate et al., 1993; Carmena et al., 1995; Corbin et al., 1991). In *N* mutant embryos, there is widespread disruption of the muscle pattern, both because of an overproduction of muscle progenitor cells and lack of myoblast fusion and also because the epidermis on which the pattern forms is deranged (Bate et al., 1993).

Despite this requirement for *N* during progenitor segregation and the disruption of the muscle pattern in *N* mutant embryos, such embryos can still be used to analyse the effects of loss of *N* on the allocation of cell fates in sibling cells of the myogenic lineages. We do this by assaying the expression of genes that are normally expressed by one founder but not both, and by assaying persistent *twist* expression as a marker for adult precursor cells. In N^{55e11} embryos, there is a large increase in the number of myoblasts expressing muscle founder markers such as *Kr* (Fig. 6A) and *S59* (Bate et al., 1993), whereas persistent *twi* expression characteristic of adult muscle precursors is lost (Fig. 6B).

Since we have shown that adult precursors are formed as siblings of larval muscle founders (Carmena et al., 1995), Notch activation appears to be required for the acquisition of one of two alternative fates by sibling muscle-forming cells. This includes the distinction between adult muscle precursor and larval muscle founder: in this case, the decision is mediated by the activation of Notch in the adult precursors. This accords with our previous demonstration that ectopic Numb blocks formation of adult precursors.

If Notch activation is required for the allocation of cells to the adult precursor fate, then expressing an activated form of Notch during late phases of muscle determination should increase the number of adult precursors at the expense of larval muscles. We performed this experiment using a Gal4 line that is locally expressed in the progenitors of dorsal muscles (C612b; not shown) to promote expression of a constitutively active Notch construct (see Materials and Methods). In such embryos, there is a duplication of persistent *twi*-expressing cells dorsally (Fig. 6D) and this is associated with a loss of dorsal muscles (Fig. 6C). In this case, the phenotype corresponds to that of *numb* mutant embryos and suggests that the loss of dorsal muscles is not caused by a failure in the segregation of dorsal progenitors but by the assignment of both daughter cells to the adult precursor fate through the activation of Notch.

DISCUSSION

So far as is known, the larval muscles formed during embryogenesis in *Drosophila* are, biochemically and physiologically, a uniform set of contractile elements (Bate, 1993; Bernstein et al., 1993). However, each muscle is a unique component of the machinery with which the larva moves. In this sense, there are functionally important distinctions between the muscles that depend on their different positions, their anchorage sites in the epidermis, their size and, most importantly, their specific connections with innervating motorneurons. Thus the general pathway of myogenesis is subject to local controls that lead to the formation of individual muscles with specific characteristics.

The issue of how muscles acquire their distinctive properties in the context of a general pathway of myogenic differentiation is common to all organisms in which the function of the neuromuscular system depends on the formation of a particu-

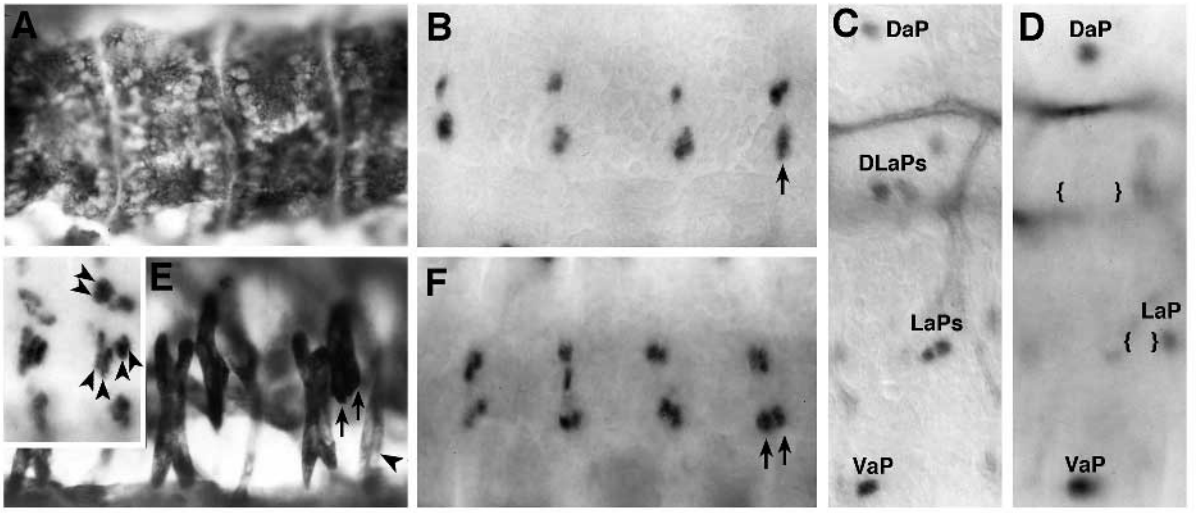

Fig. 5. Mesodermal phenotypes in *not enough muscles* (*nem*) mutant embryos. Lack of function of *insc/nem* induces transformations similar to those seen when *numb* is ectopically expressed in the mesoderm. (A) Dorsal view of nem^{8} embryo stained with anti-Myosin to show supernumerary muscles that develop at the expense of adult precursors. Concomitant loss of dorsolateral adult precursors (DLaPs) shown by top bracket in anti-Twist staining pattern for nem^{8} embryo (D, compare wild-type Twist pattern in C). Similarly, anti-Myosin staining of lateral region of nem^{8} embryo (E) shows duplication of SBM (arrowhead in E) at expense of lateral adult precursors (LaPs) as shown in D (compare C). Note also that in these embryos, muscle LT3 is transformed to LT4 (arrows in E, compare Fig. 2E,F). Muscle transformations are accompanied by changes in gene expression. This is shown for *Kr* expression in lateral muscles in a nem^{8} embryo in the inset in E (compare Fig. 4E,F) and for *ladybird* (*lb*) expression in SBM in a nem^{8} embryo in F. Arrows in F indicate *lb* expression in the duplicated SBM (compare wild-type expression of *lb* in B, arrowed). Anterior is to the left and dorsal is up.

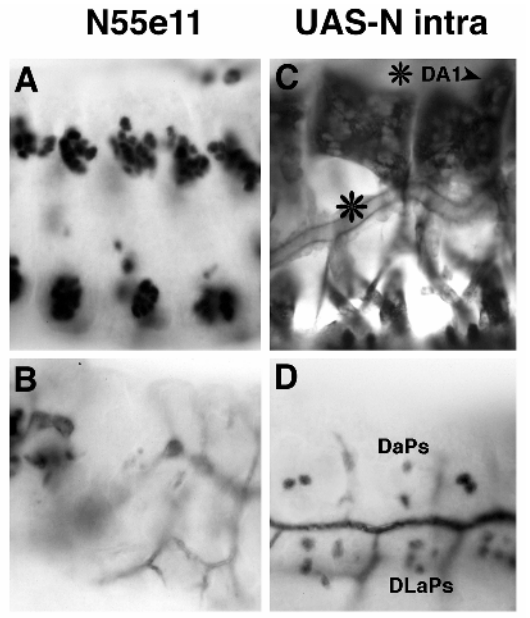

Fig. 6. Requirement for *Notch* during the acquisition of alternative fates by sibling progeny of muscle progenitors. (A) Dorsolateral view of a late stage 12 N^{55e11} embryo stained with anti-Kr showing enlarged clusters of *Kr*-expressing founders. (B) Dorsolateral region of a stage 15 N^{55e11} embryo stained with anti-Twi showing absence of persistent *twi*-expressing cells. (C,D) Dorsolateral views of embryos stained with anti-Myosin (C) or anti-Twist (D) in which an activated form of Notch has been expressed in dorsal progenitors using a mesodermal GAL-4 line (see Materials and Methods). When activated Notch is locally provided in muscle progenitors there is a reduction in the number of dorsal muscles (asterisks in C) and an increase in the number of adult precursors (D, compare to Fig. 2K), indicating that Notch activation can switch muscle founder cells towards their sibling fate, in this case adult precursors. In all panels, anterior is to the left and dorsal is up.

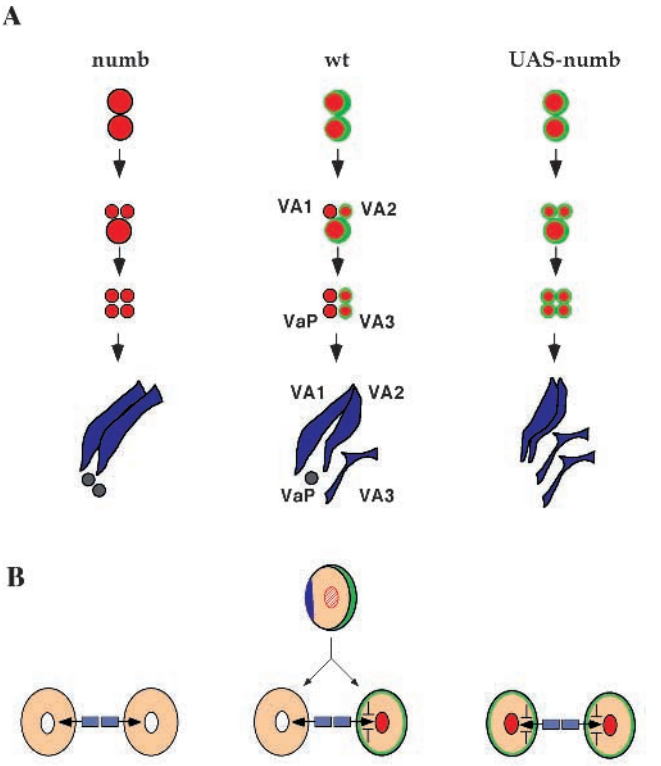

Fig. 7. Diagrams illustrating effects of loss and gain of *numb* expression on the segregation of lineages in the mesoderm. (A) Development of VA1-3 and VaP in wild-type, $numb^{1}$ and UAS-*numb* embryos. During normal development, the two ventral S59-expressing muscle progenitors divide sequentially to give rise to four cells, the founders for muscles VA1-3 and the ventral adult precursor (VaP). Numb is asymmetrically distributed in both progenitors and the generation of the four distinct cells requires the segregation of Numb to one sibling cell. Thus, after division of the dorsalmost progenitor, Numb is segregated to the VA2 founder and excluded from VA1. Similarly the division of the second progenitor generates a VA3 founder containing Numb and VaP that lacks Numb. In *numb* mutant embryos, each sibling adopts the fate of the cell that does not receive Numb in the wild type, so producing duplicate VA1s and VaPs. When Numb is provided ectopically in the mesoderm, the opposite fates are adopted by the two sibling cells of each pair, giving rise to two VA2 and two VA3. The presence of Numb is indicated by the green outline in progenitors and founder cells. (B) Implementation of alternative fates in the sibling cells produced by muscle progenitors. Numb and Inscuteable are asymmetrically distributed in muscle progenitors (Numb, green and Insc blue sector). Progenitors differ in the combination of marker genes that they express: e.g. DA1 progenitor co-expresses *eve* and *Kr*, VA2 progenitor *S59* and *Kr*, indicated by crosshatched red nucleus. Segregation of Numb to one of the founders after division ensures that the two sibling cells will adopt different fates. Implementation of the Notch pathway in the sibling cell that does not receive Numb results in the repression of genes such as *S59*, *Kr* and *eve* (empty nuclei) and allows the maintenance of *twi* expression in the adult precursor cells. In the sibling cell receiving Numb, the Notch signalling pathway is blocked and the expression of progenitor genes such as *S59*, *Kr* and *eve* is maintained (red nuclei). In the absence of Numb, activation of Notch results in repression of *S59*, *Kr* and *eve* in both sibling cells, whereas ectopic activation of Numb in both sister cells interferes with the activation of Notch and produces two cells that adopt the alternative fate.

lar pattern of muscles, each with special mechanical functions and an appropriate type of innervation. In *Drosophila*, and it is likely, in vertebrate embryos too, segmental differerences between muscles are regulated autonomously by patterns of homeotic gene expression in the mesoderm (Greig and Akam, 1993; Michelson, 1994). However, the formation of thirty different muscle fibres within a segment requires a different kind of control. Recent work has shown that the key to understanding this control is the fact that the formation of multinucleate muscle fibres is seeded by single myoblasts known as founder cells (Rushton et al., 1995).

Individual founder myoblasts express transcription factors such as *Kr* and *S59* that are characteristic of particular muscle subsets and, in the absence of myoblast fusion, are capable of differentiating to form mononucleate muscle fibres with appropriate positions, attachments and innervation (Rushton et al., 1995). Neighbouring myoblasts do not complete myogenesis in the absence of fusion but remain rounded and relatively undifferentiated (Rushton et al., 1995). In normal development the founders fuse with their neighbours and recruit them to their distinctive pattern of gene expression, so forming the syncytial precursors of individual muscles (Dohrmann et al., 1990). Because founder myoblasts are uniquely competent to complete myogenesis, they gate the process of muscle formation and the

expression of genes such as *Kr* and *S59* in these cells leads to the development of muscles with distinct, different characteristics (Ruiz Gómez et al., 1997). Thus, a group of fusing myoblasts acquires the distinctive properties of an individual muscle as a result of the prior specification of a unique founder cell at a particular location in the somatic mesoderm.

We now know that there are very close similarities between the process of specifying single cells in the mesoderm during myogenesis and the specification of cells in the ectoderm during neurogenesis (Carmena et al., 1995). Founder cells are generated as pairs from cells known as muscle progenitors that are in turn selected from clusters of cells in the somatic mesoderm. These cell clusters express the proneural gene *lethal of scute* and progenitors are selected from them by a process of lateral inhibition depending on the activation of the Notch signalling pathway (Carmena et al., 1995). By analogy with the proneural clusters of the PNS (Ghysen and Dambly-Chaudière, 1988), it is likely that these 'promuscle' clusters are the endpoint for a process of integrating positional signals that leads to the formation of a uniquely specified progenitor at that location in the mesoderm. This process of specification will dictate the characteristics of muscle founder cells and muscles formed at this position, just as positional differences between neurons are likely to depend on the sense organ precursor from which they are derived (Merritt and Whitington, 1995).

However, each progenitor gives rise to two distinct muscle founder cells (Carmena et al., 1995). Muscles that are formed from sibling founders have shared properties (for example, similar orientations, innervation by related motorneurons) and these are reflected in the common expression of genes such as *apterous* (Bourgouin et al., 1992) and *connectin* (Nose et al., 1992). However, differences between sibling founders and the muscles that they form appear to depend on the differential maintenance of patterns of gene expression that are initiated in the parent progenitor cell. In every case, we find that genes such as *Kr*, *S59* and *eve* are expressed in the progenitor but that expression is maintained in only one of the two sibling founder cells that the progenitor gives rise to and these distinctive patterns of gene expression are correlated with differences between the muscles that are formed. We have also shown (Carmena et al., 1995) that, in particular instances, larval founder cells are paired with the precursors of adult muscles rather than with sibling muscle founder cells. In all these instances, the formation of the adult precursor is associated with repression of progenitor cell marker genes and the maintained expression of *twist*.

The view of the myogenic lineages that emerges from these studies is that uniquely specified progenitor cells give rise to two different cells each of which initiates the formation of an individual muscle. The two cells represent two alternative states: one in which genes expressed in the progenitor are maintained in the founder and the alternative where the expression of these genes is repressed. Where the sibling cells are founders, recent work shows that maintaining or losing the expression of a progenitor gene, such as *Kr*, can be sufficient to switch the two muscles between the alternative fates (Ruiz Gómez et al., 1997). However, such transformations are not always complete, implying that the misexpression of a single transcription factor may be insufficient to respecify muscle fates. A striking feature of the work that we report here is that loss or gain of Numb can provoke complete transformations in the muscle pairs that we have studied, indicating that *numb* is a pivotal element in determining patterns of gene expression and cell fates in the myogenic lineages. Nonetheless the expressivity of the changes that we observe is incomplete and we have quantified this by assaying persistent *twist*-expressing cells in the different mutant backgrounds that we have used (Table 1).

Table 1. Expressivity of phenotypes monitored by counting persistent *twist*-expressing cells in *numb^{1}*, *UAS-numb* (constitutive mesodermal expression of *numb*) and *UAS-N^{intra}* (activated Notch expressed in dorsal muscle progenitors) embryos

Genotype	Hemiseg.	VaP	LaP	DLaP	DaP
numb^{1}	30	60/(30)	95/(60)	91/(60)	59/(30)
UAS-numb	18	5/(18)	31/(36)	15/(36)	14/(18)
UAS-N^{intra}	39	N.C.	N.C.	131/(78)	46/(39)

Cells were counted in numbers of hemisegments (Abdominal 3,4 and 5) as indicated. VaP, LaP, DLaP and DaP: Ventral, Lateral, Dorso-Lateral and Dorsal adult Precursors respectively. N.C. (Not Counted). Numbers in brackets are for wild type (Bate et al., 1991).

The differential distribution of Numb between the founders shows that the division of the progenitor is intrinsically asymmetrical. As in the neural lineages, the asymmetry of Numb distribution is itself dependent on the presence of Insc in the progenitor cells (Kraut et al., 1996; this paper). In the absence of Numb, expression of progenitor cell marker genes is lost and alternative muscle fates including *twist*-expressing adult precursors are duplicated (Fig. 7A). If Numb is overexpressed, i.e. present in both founders, the number of cells maintaining marker gene expression is doubled and the alternative fates, including the adult precursors, are lost (Fig. 7A). Thus Numb is an essential part of a mechanism that blocks the repression of marker gene expression in one of a pair of sibling muscle-forming cells. The resemblance of the *insc* phenotype to that of ectopic *numb* expression confirms that *numb* function depends on a differential distribution of Numb between the two sibling cells.

As in neural lineages, the phenotype of *numb* overexpression in the mesoderm resembles that of *N* loss of function, namely an overexpression of progenitor cell marker genes and a concomitant loss of adult muscle precursors. There is a corresponding match between loss of Numb and ectopic expression of a constitutively active form of Notch. This strongly suggests that the differential distribution of Numb between the two daughter cells allows the activation of the *N* signalling pathway to be blocked in the sibling cell to which Numb is segregated, presumably by way of a physical interaction between Numb and the cytoplasmic domain of Notch (Guo et al., 1996; Zhong et al., 1996) (Fig. 7B). Thus the bifurcation of the myogenic lineage depends on an interaction between external signals operating through Notch and an intrinsic factor, Numb, that is differentially distributed at the progenitor cell division (Fig. 7B). However, our preliminary evidence from an analysis of double mutants for *N* and *numb* suggests that *numb* function may not be exclusively through blocking Notch activation.

The significance of the resemblance between muscle and neural lineages should not be overemphasised. The key feature of myogenesis that makes it analogous to neurogenesis is the

requirement for a pattern of single specified cells which seed the muscle pattern by forming at particular locations in the mesoderm. This spaced pattern clearly resembles a spaced pattern of any other cell type including neurons and it is known that proneural and neurogenic genes, as well as in all likelihood *numb* and *insc*, function in a number of different tissues and lineages (Hoch et al., 1994). However, the fact that muscle formation in *Drosophila* is preceded by an essential phase of myoblast diversification may be of wider relevance for our understanding of myogenesis and its control. In vertebrate embryos, a number of regulatory factors contribute to the formation of different kinds of muscle fibres but there is good evidence that intrinsic differences between embryonic myoblasts determine the formation of different classes of primary myotubes (Donoghue and Sanes, 1994). In zebrafish embryos, founder populations of myoblasts for particular muscles can be distinguished by the expression of the lineage marker Engrailed (Hatta et al., 1990) or by the early expression of MyoD (Schilling and Kimmel, 1997). How the mesoderm is partitioned to produce these early distinctions between myoblasts is far from clear. In this sense, the local controls of myogenesis that we describe in *Drosophila*, where asymmetric cell divisions lead to a diversification of founder myoblasts, may have special relevance for our understanding of the way in which muscle-forming mesoderm is subdivided to produce distinct populations of embryonic myoblasts.

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