

## Patterning the dorsal longitudinal flight muscles (DLM) of *Drosophila*: insights from the ablation of larval scaffolds

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### SUMMARY

The six Dorsal Longitudinal flight Muscles (DLMs) of *Drosophila* develop from three larval muscles that persist into metamorphosis and serve as scaffolds for the formation of the adult fibers. We have examined the effect of muscle scaffold ablation on the development of DLMs during metamorphosis. Using markers that are specific to muscle and myoblasts we show that in response to the ablation, myoblasts which would normally fuse with the larval muscle, fuse with each other instead, to generate the adult fibers in the appropriate regions of the thorax. The development of these de novo DLMs is delayed and is reflected in the delayed expression of *erect wing*, a transcription factor thought to control differentiation events associated with myoblast fusion. The newly arising muscles express the appropriate adult-specific Actin isoform (88F), indicating that they have the correct muscle identity.

However, there are frequent errors in the number of muscle fibers generated. Ablation of the larval scaffolds for the DLMs has revealed an underlying potential of the DLM myoblasts to initiate de novo myogenesis in a manner that resembles the mode of formation of the Dorso-Ventral Muscles, DVMs, which are the other group of indirect flight muscles. Therefore, it appears that the use of larval scaffolds is a superimposition on a commonly used mechanism of myogenesis in *Drosophila*. Our results show that the role of the persistent larval muscles in muscle patterning involves the partitioning of DLM myoblasts, and in doing so, they regulate formation of the correct number of DLM fibers.

Key words: myogenesis, *Drosophila*, scaffolds, metamorphosis

### INTRODUCTION

The formation of a muscle involves a series of developmentally regulated events. These include the proliferation and specification of myoblasts to form distinct muscles, the regulation of muscle patterning, and the generation of muscle fiber identity. A fundamental challenge is to understand how an initial pool of myoblasts is patterned to give rise to muscles with distinct cellular and molecular identities. Two possibilities exist for generating diversity within a pool of myoblasts. Specific cells may become determined as 'founder cells' (Bate, 1990) or 'muscle pioneers' (Ho et al., 1983), with which the undetermined myoblasts fuse, to form mature muscles. Alternatively, groups of equivalent cells may be determined collectively, and then fuse with each other to give rise to distinct muscle fibers. Although both mechanisms may be used in the same animal, the use of pioneers/founders is widespread in insect muscle development (Jellies, 1990; Bate, 1993). The latter mechanism appears to be more prevalent during vertebrate myogenesis (Stockdale, 1992; Ordahl and LeDouarin, 1992), and also during much of adult myogenesis in *Drosophila* (reviewed by Crossley, 1978 and Bate, 1993).

The role of founder cells is best understood in the *Drosophila* embryo, where considerable work has been done on the molecular mechanisms of muscle patterning and the generation of cellular identity (reviewed by Bate, 1993). The

embryonic body wall contains a segmentally repeated pattern of muscle fibers with some variations in the thoracic and posterior abdominal segments (Bate, 1990, 1993). Individual muscles are identified by their size, bodywall insertion sites, innervation, as well as characteristic molecular markers (Keshishian et al., 1996). Each muscle fiber is established by a founder cell that arises from the mesodermal population, and is believed to possess the necessary information to confer muscle identity. Surrounding myoblasts fuse with the founder cell to initiate myogenesis (Bate, 1990). Evidence for founder cells was recently obtained from *myoblast city* mutants where myoblast fusion does not occur. In these mutants, founder cells are correctly specified and express many of the markers of a differentiated muscle. They are also correctly inserted into the epidermis (Rushton et al., 1995).

Cells analogous to the embryonic muscle founders have not been identified for the *Drosophila* adult musculature. The adult muscle pattern is partially specified during embryogenesis in the form of persistent *twist*-expressing cells (Bate et al., 1991). In the abdominal segments, these cells become closely associated with segmental nerves, whereas in the thorax a majority of these cells are associated with imaginal discs. The abdominal myoblasts are generated from six *twist*-expressing cells located in dorsal, lateral and ventral regions of the late embryo which multiply during larval life and early in metamorphosis. The dorsal, lateral and ventral muscle primordia

give rise to the correspondingly located adult fibers. Myogenesis takes place in close association with segmental nerves and the developing epidermis, which are thought to play a role in establishing the adult muscle pattern (Bate et al., 1991; Currie and Bate, 1991, 1995). The male specific muscle found in the fifth abdominal segment is a special case where muscle identity is determined by the innervating motoneuron(s) (Lawrence and Johnston, 1986; Currie and Bate, 1995; Taylor and Knittel, 1995).

Unlike the abdominal muscle precursors, thoracic myoblasts associate with imaginal discs that give rise to the adult epidermis. In a manner that resembles the segregation of abdominal myoblast precursors, the thoracic somatic mesoderm segregates into dorsal or ventral populations (Lawrence, 1982). In the mesothorax, myoblasts that give rise to ventral muscles are associated with the mesothoracic leg disc, while those that form the dorsal muscles are associated with the wing imaginal disc. In this paper we focus on the patterning of dorsal myoblasts associated with the wing disc. These myoblasts give rise to distinct muscle groups that express different Actin isoforms. The direct flight muscles (DFMs) express the tubular muscle Actin (79B) while the indirect flight muscles (IFMs) express the fibrillar muscle Actin (88F) (Fyrberg et al., 1983; Hiromi and Hotta, 1985; Courchesne-Smith and Tobin, 1989). A question of developmental interest is how is the myoblast pool patterned to give rise to these distinct muscle types. Moreover, the IFMs present a special case in muscle patterning. The IFMs consist of two muscle sets – the Dorsal Longitudinal Muscles (DLMs a-f) that develop using larval muscles and the Dorso-Ventral Muscles (DVMs) that develop de novo (Shatoury, 1956; Costello and Wyman, 1986; Fernandes et al., 1991). Does the identity of these muscles depend on differences in their mode of development?

Muscle patterning in the thorax is best understood for the DLMs. Prior to the onset of adult myogenesis, a wave of histolysis destroys much of the larval musculature. Among the muscles that survive are three mesothoracic muscles, which split as myoblasts fuse with them to give rise to the six DLM fibers (Fernandes et al., 1991). Recent work by Farrell et al. (1996) showed that DLMs were present in adult thoraces even when the persistent larval muscles are ablated, indicating that larval muscles are dispensable as scaffolds. In this paper we use similar experimental conditions and examine myogenesis during the pupal stages. By following DLM development under these altered conditions, we explore the nature of cues that might be involved in the initiation of myogenesis and in the patterning of DLM fibers. Our results show that when DLM myoblasts are deprived of their larval scaffolds, they can fuse de novo in a manner that resembles DVM development. De novo fusion appears to be the default mode of myogenesis, upon which the use of larval scaffolds has been superimposed. Our results also allow us to hypothesize how patterning mechanisms might act on a single pool of myoblasts to generate diversity among the muscle fibers.

## MATERIALS AND METHODS

### Laser ablations

The DLMs arise from three larval muscles, MFs 9, 10, 19'. MF9,

which is closest to the dorsal midline and gives rise to the dorsal most pair of DLMs, was ablated in third instar larvae. The larvae were anesthetized with diethyl ether for about 2-3 minutes and mounted under a coverslip in a drop of saline for live visualization. Using Nomarski optics and video enhancement (Halpern et al., 1991) MF9 was located through the cuticle of the intact larva. A microbeam pulsed laser was used to ablate the identified larval muscle (Cash et al., 1992). Following ablation, the larvae were allowed to recover in food vials. The larvae were allowed to develop to desired time points during metamorphosis. White pre-pupae were taken to be 0 hours APF (After Puparium Formation).

### Fly strains

The laser ablations were done in the following transgenic fly strains. (1) Myosin-heavy chain-lacZ (Hess et al., 1989). It is expressed in all bodywall muscles of the larva. The early stages of DLM development can be observed in this strain due to the perdurance of  $\beta$ -galactosidase from the larval stages (Fernandes et al., 1991). (2) Actin (88F)-lacZ (Hiromi et al., 1986). It is expressed only in the indirect flight muscles, and the earliest expression marks the onset of muscle differentiation. We used this line to follow myogenesis of the DLMs in ablated animals. (3)  $\beta$ -3 tubulin lacZ (Leiss et al., 1988). It is expressed in myoblasts as they begin to fuse, and subsequently in muscle fibers.

### Tissue preparation, histochemistry and immunocytochemistry

Pupae that had developed to desired time points were dissected, fixed and stained with X-gal or processed for immunocytochemistry as described by Fernandes et al. (1991). For double labeling, preparations were first stained with X-gal, washed and then processed for antibody staining. The following rabbit polyclonal antibodies were used: anti-TWIST, that marks all myoblasts, provided by Maria Leptin, and anti-ERECT WING, which marks subsets of myoblasts, provided by Kalpana White. After incubation in primary antibodies, the tissue preparations were washed in phosphate-buffered saline in 0.3% Triton X-100 (PBS-TX) and incubated with biotinylated secondary antibody (Vector Labs). Subsequently the preparations were washed in PBS-TX and incubated with a peroxidase-linked avidin/biotin complex (Vector Labs). The peroxidase label was visualised using DAB (diaminobenzidine). The tissue was then dehydrated in an alcohol series, cleared in xylene and mounted in DPX (Gurr, Poole, England).

## RESULTS

### Upon ablation of the larval scaffold, DLM fibers develop de novo

#### DLM development in normal animals

Development of the DLMs during the first 36 hours of metamorphosis has been described previously (Fernandes et al., 1991) and is summarized in Fig. 1. At the onset of metamorphosis, most of the larval bodywall muscles (Fig. 1A) are histolysed. The process is completed just as myoblasts arrive at the sites of adult myogenesis. In the mesothorax, three larval muscles, MF9, 10 and 19' survive the histolysis, and can be visualized in flies carrying a Myosin Heavy Chain-lacZ (MHC-LacZ) reporter transgene (Figs 1B, 2A). At 8 hours APF, the muscles are still larval-like in appearance (see Fig. 2A) and are birefringent. By 10 hours APF, the myofibrils disassemble, vacuoles appear in the muscle, and the muscles lose their birefringence. This process has been termed 'dedifferentiation' in other insect systems (Crossley, 1972; Williams and Caveney, 1984). By 12 hours APF, the three dedifferentiated larval

muscles elongate, and later, as myoblasts start to fuse with them, they split longitudinally (14–18 hours APF; Fig. 1C). The six DLM fibers can be first seen at 20 hours APF. As myoblast fusion continues, the muscles grow in size. Fusion is almost complete by 28 hours APF, and by 36 hours APF (Fig. 1D), the six fibers have attained one-third their final size (Robertson, 1936).

#### Time course of muscle development in the region of muscle ablation

We used a microbeam laser to ablate larval muscle fiber 9 (MF9), which gives rise to DLMs a and b, the most dorsal pair of DLMs (Fig. 1). From 8–12 hours APF there are no remnants of MF9 in the operated hemisegment or any signs of muscle regeneration ( $n=70$ ; Fig. 2B). Ablation of MF9 does not affect the other two persistent larval muscles. Thus, while three larval muscles can be seen in the control side (Fig. 2A), two muscle fibers corresponding to MFs 10 and 19' are seen in the operated hemisegment (Fig. 2B). Development of these two muscles proceeds normally. Taken together, the results indicate that MF9 ablation is specific.

The subsequent stages of myoblast fusion and muscle fiber development were studied using an Actin (88F)-lacZ reporter transgene. In this line the developing DLMs can be visualized from 14 hours APF onwards (Fernandes et al., 1991). In more than half the operated animals examined during 16–18 hours APF, small structures resembling muscle fibers are observed at multiple sites in the vicinity of the ablated MF-9 (58%; Fig. 2D). These structures express the IFM specific Actin (88F)-lacZ, are elongated in the A/P axis, and unlike the neighboring fibers, they do not span the DLM attachment sites. We have called these incipient fibers, 'muscle pre-fibers.' In the remaining animals, no pre-fibers can be detected (42%; Fig. 2C). The pre-fibers are first observed at 16 hours APF, which is about 2 hours after the lacZ reporter is turned on in the developing DLMs. Since these pre-fibers arise in the absence of a larval muscle fiber, we hypothesize that they develop by fusion of myoblasts with each other (de novo fusion). In the adjacent region, other myoblasts fuse with the remaining two persistent muscles (MFs 10 and 19') to give rise to DLMs c-f.

When examined at later times (20–24 hours APF), several distinct muscle patterns are observed in the operated hemisegments (Table 1). In a minority of animals (19%), muscle pre-fibers were not seen. In other animals (26%), pre-fibers similar to those seen during 16–18 hours APF were observed. In the remaining animals, either one (33%) or two (22%) thin muscle fibers developed (Fig. 2E). Unlike the pre-fibers, these muscle fibers are large enough to span the DLM attachment sites, but they are thinner than the normally developing DLM fibers. The de novo DLMs probably developed from pre-fibers observed during 16–18 hours APF. By 26–28 hours APF, the pre-fibers are no longer found. At this time fewer myoblasts are present than at 24 hours APF, and may not be capable of de novo myogenesis. The decline in the formation of pre-fibers is paralleled by the appearance of de novo DLM fibers, and in some cases by the elimination of pre-fibers.

By 36 hours APF muscle patterning is complete (Fernandes et al., 1991) and thereafter the muscles grow in size to fill the thorax (Reedy and Beall, 1993). The frequency at which an ablation led to the formation of de novo muscle fibers was similar to the outcome of MF9 ablation observed in adult

animals (65% found by Farrell et al., 1996 vs 68%, this paper). Our data at this stage fall into two major categories. In 32% of animals, no de novo fibers are seen (Fig. 2F), while in the remaining animals (68%), 1–2 de novo fibers are present (Fig. 2G,H). This distribution is similar to the muscle patterns seen at 26–28 hours APF, where de novo DLMs develop in roughly two-thirds of animals. The de novo fibers are usually thinner than the normally developing DLM fibers. In these animals, DLMs c-f are usually larger in diameter than their counterparts in the control hemisegments. As at 26–28 hours APF, muscle pre-fibers are never observed at 36 hours APF.

#### Distribution of myoblasts as visualized by Twist immunoreactivity

Myoblasts express the Twist protein, and can be reliably observed, using anti-Twist antibodies, during pupal development (Currie and Bate, 1991; Fernandes et al., 1991). During early metamorphosis (6–10 hours APF) myoblasts are loosely organized around the persistent larval muscles (Fernandes et al., 1991). Since development of the de novo DLMs in MF9 ablated animals is delayed, we examined whether the ablation altered myoblast distribution. Myoblasts were present in the region of the ablated fiber during 12–18 hours APF ( $n=23$ ; Fig. 3A–D). Since myoblasts had arrived at the sites of DLM myogenesis (DLM a and b) in the absence of MF9, it follows that the persistent larval muscle fiber is not required for myoblast aggregation at the appropriate thoracic sites. The dorsal extent of myoblast distribution remains unchanged when muscle pre-fibers first appear during 16–18 hours APF (Fig. 3C,D). However, from 20 hours APF onwards, the dorsal extent of myoblasts becomes restricted to the most dorsal DLM regardless of muscle pattern ( $n=14$ ; Fig. 3E,F). In the following section we follow myoblast distribution in the region of the ablated MF9. Our results indicate that DLM myoblasts unable to undergo de novo fusion are recruited into neighboring DLM fibers.

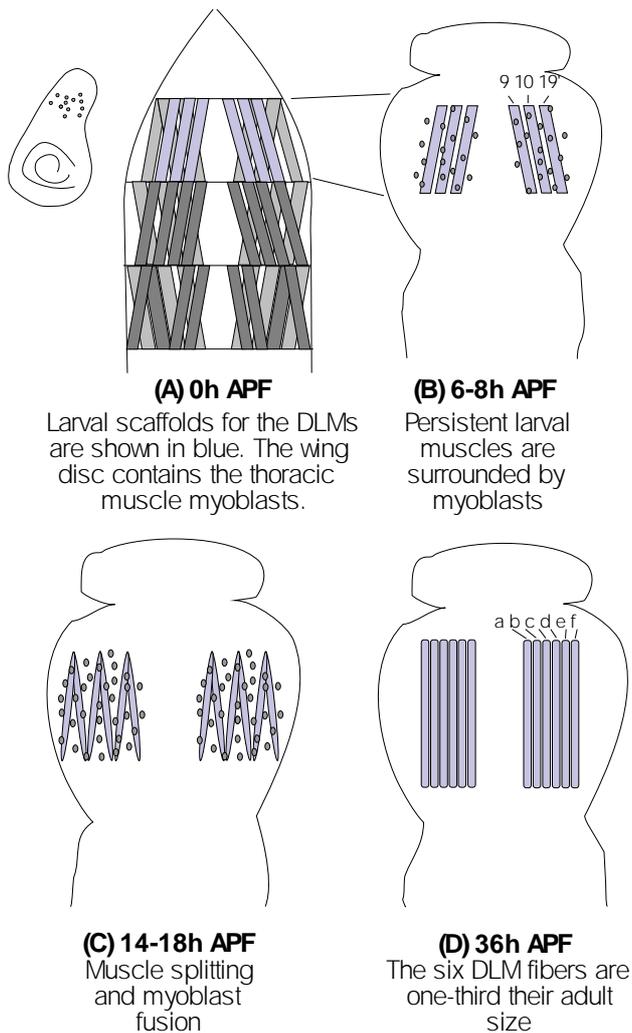
#### Fate of myoblasts as a result of MF9 ablation

##### Origins of pre-fibers

The development of muscle pre-fibers, first evident at 16 hours APF, was examined in flies bearing a  $\beta$ -3 tubulin lacZ reporter construct.  $\beta$ -3 tubulin is expressed in myoblasts prior to fusion and continues to be expressed in muscle fibers (Leiss et al., 1988; Currie and Bate, 1991). Using the reporter, we detected multiple foci of myoblast clustering in the region of ablation as early as 14 hours APF. At this time Actin-lacZ does not detect muscle pre-fibers. These myoblast clusters contained 4–6 cells (Fig. 4A). The number of such clusters is usually greater than the number of muscle pre-fibers observed later in development. It appears therefore, that myoblast fusion gives rise to small syncytia, which then coalesce to give rise to larger pre-fibers or muscle fibers that express the IFM-specific Actin (88F)-lacZ. The end-to-end alignment of myoblast nuclei in the clusters resembles the manner in which the DVMs develop (Fig. 4B). The DVMs, unlike the DLMs, normally develop de novo (Fernandes et al., 1991).

##### Myoblasts in the region of MF9 are recruited into neighboring DLM fibers

We observed that between 20–24 hours APF, myoblasts do not extend beyond the dorsal most DLM fiber. We also observed



**Fig. 1.** Overview of DLM development. (A) Of the 21 bodywall muscle fibers in the second thoracic segment of the third instar larva (Bate 1993), only three muscles (shown in blue) persist into metamorphosis. These serve as scaffolds for Dorsal Longitudinal Muscle (DLM) development. Myoblasts are associated with the epithelium of the wing imaginal disc. (B) Early in pupal development, a wave of histolysis destroys most of the larval thoracic bodywall musculature. In the mesothorax, the three persistent larval muscles are surrounded by myoblasts. (C) As myoblasts begin fusing, the larval muscles, begin to split, and (D) give rise to the six DLM fibers (a-f). The muscles subsequently grow to fill the thoracic space.

that the DLM fibers adjacent to the de novo fibers increase in size relative to their counterparts in the control hemisegments. This raises the possibility that myoblasts which would have fused into the larval muscle, were instead fusing into neighboring fibers. Using the  $\beta$ -3 tubulin lacZ transformant, we observed that distinct nuclear rows are present in the developing DLMs. At the beginning of the splitting process (14-16 hours APF), each developing fiber is characterized by the presence of 1-2 rows of myoblast nuclei that have fused into the larval muscle ( $n=16$ ; Fig. 4C). When the DLM fibers are formed at 20 hours APF, they typically have about 3 rows of nuclei, and by 24 hours APF, 4 distinct rows are visible ( $n=26$ ;

Fig. 4E). In operated hemisegments, the two DLM fibers that arise from MF10 (adjacent to MF9) have the same number of rows of nuclei in both the experimental and in the control hemisegment during 14-18 hours APF ( $n=16$ ; Fig. 4C,D). However, during 20-24 hours APF the DLMs generated from MF10 have more nuclear rows in the operated side (5-6) than the control (4), ( $n=13$ ; Fig. 4E,F). Taken together with the fact that the *de novo* fibers are thinner, it follows that myoblasts from the ablated region may be recruited into the neighboring DLMs. The increase in number of nuclear rows is most pronounced in DLMs c and d, which are closest to the region of MF-9 (Fig. 4F). These results imply that the persistent larval muscles partition the initial pool of myoblasts, which is manifested in the ordered number of nuclear rows seen in the developing DLMs.

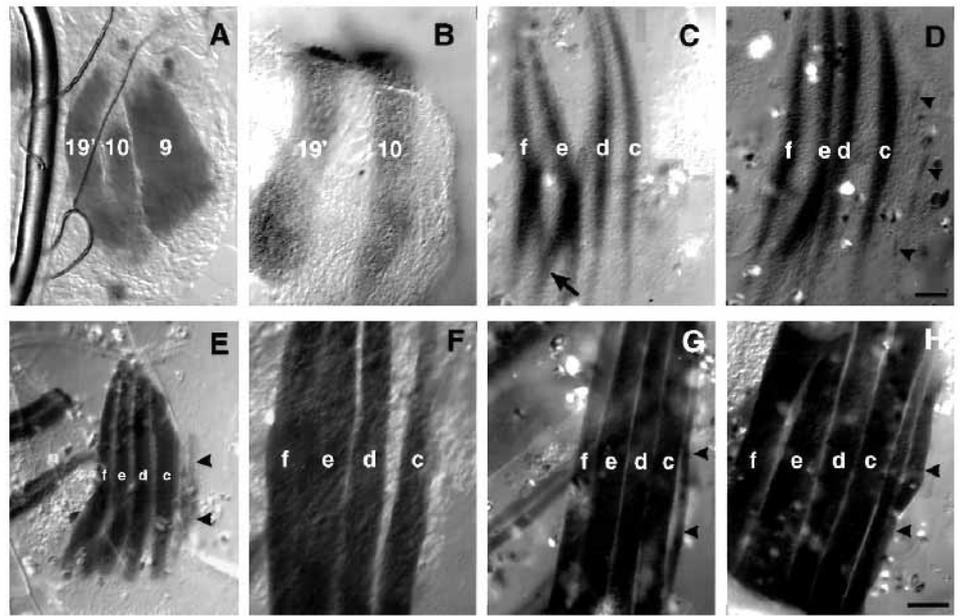
### ***erect wing* expression in DLM myoblasts is altered as a result of MF9 ablation**

Erect Wing (EWG) is a transcription factor expressed by IFM myoblasts, and is required for the formation of these muscles (DeSimone and White, 1993; DeSimone et al., 1996). Using immunolabelling, EWG is first detected at 10 hours APF in a subset of myoblasts closely apposed to the larval muscles (DeSimone et al., 1996; Fig. 5A). Since fusion of myoblasts with the larval scaffold is preceded by the expression of *ewg* in the myoblasts, we examined whether the expression was altered in operated hemisegments. Following MF9 ablation, myoblasts in the region of the ablated fiber do not express *ewg* (Fig. 5B) in contrast to those on the surface of the adjacent MFs 10 and 19'. Thus the onset of *ewg* expression appears to be a local effect involving an interaction between the larval muscle and the myoblasts. At 16 hours APF, as the larval muscles are splitting, myoblasts continue to express *ewg*, whereas in the region of ablation, *ewg* is not expressed (Fig. 5C,D). At this stage, two rows of *ewg*-expressing nuclei are seen within each developing DLM fibre. This arrangement of nuclei was also detected using the  $\beta$ -3 tubulin lacZ transformant line (see above; Fig. 4). As pre-fibers appear, we notice that *ewg* is expressed in myoblasts that are in the immediate vicinity of the de novo fibers (data not shown). We hypothesize that initial fusion gives rise to a pre-fiber, which then recruits surrounding myoblasts for de novo fusion, causing them to express *ewg*.

### **DISCUSSION**

At the onset of metamorphosis, three larval mesothoracic muscle fibers survive the wave of histolysis that destroys most of the larval bodywall musculature. These muscle fibers (MFs 9, 10 and 19') serve as scaffolds for the development of the six DLM fibers (DLMs a-f). When one or more of the larval scaffolds is ablated, DLM fibers are seen in the adult, suggesting that the larval fibers are not obligate for DLM development (Farrell et al., 1996). We show that in the absence of a larval scaffold, DLM myoblasts fuse with each other to generate adult fibers. The de novo fibers express the appropriate flight muscle-specific Actin (88F), although they are developmentally delayed in comparison to the normally developing DLMs. Adjacent to the de novo DLMs, DLMs c-f develop normally by the fusion of myoblasts into the two

**Fig. 2.** Upon ablation of MF9, DLMs can develop de novo. The muscles in A and B are visualized using the MHC-lacZ, while in the rest of the panels (C-G), the Actin (88F)-lacZ has been used. (A) 8 hours APF (control). The three persistent larval muscles, 9, 10 and 19'. (B) 12 hours APF (ablated). The clean ablation of MF9 in the larva is evident. Elongation of MFs 10 and 19' takes place on schedule in the operated hemisegment. (C) 18 hours APF (ablated). No muscles develop in the region of ablation during 14-18 hours APF (41%). Only 4 developing DLMs (c-f) can be seen. DVM III is indicated by the arrow. (D) 18 hours APF (ablated). In a majority of animals Actin-lacZ-positive pre-fibers (arrowheads) are seen (58%). Unstained myoblasts can also be seen surrounding the muscles. (E) 24 hours APF (ablated): arrowheads indicate the de novo developing muscle fibers. DLMs c-f and the DVMs develop normally. (F-H) De novo DLM fibers seen at 36 hours APF (ablated): none (F), one (G), or two (H). Fibers that develop in the region of ablation are smaller in size than the normally developing fibers. The DLMs are one-third their adult length. Dorsal midline is to the right. Bar, 50  $\mu$ m.

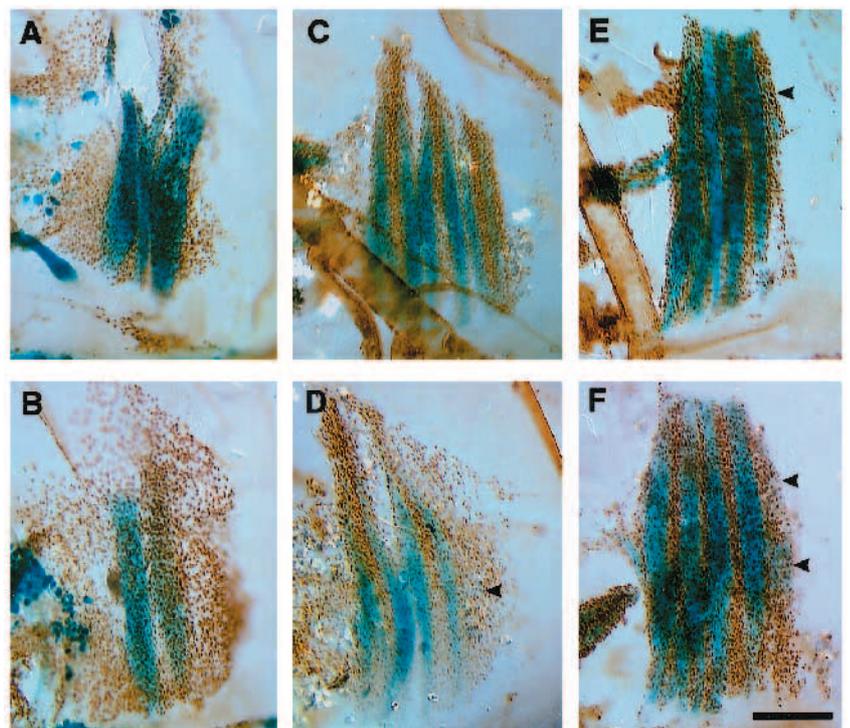


unablated larval muscle fibers (MFs 10 and 19'), showing that the two modes of myogenesis can occur side by side. Development of the de novo DLMs in the context of myogenesis of the dorsal mesothoracic muscles is shown schematically in Fig. 6.

The de novo myogenesis of DLM fibers is a departure from the normal course of events that take place during the formation of the DLMs, and raises several questions about the

process of myogenesis. (A) How do myoblasts become appor- tioned to distinct muscle forming sites? (B) How is a choice made between de novo fusion and fusion with a larval scaffold? (C) How is fusion initiated in the absence of a larval scaffold? (D) How are muscles patterned with or without the aid of larval muscles to generate the appropriate muscle identity? These aspects of myogenesis will be discussed in the following sections.

**Fig. 3.** Myoblast distribution remains unchanged in operated animals until 18 hours APF but changes by 24 hours APF. (A) 12 hours APF (control): muscles revealed using the MHC-lacZ transformant. The elongated fibers are surrounded by myoblasts detected with anti-Twist antibodies. (B) 12 hours APF (ablated): Myoblast distribution is not visibly altered as a result of the ablation. (C) 18 hours APF, (control): the developing DLM fibers are surrounded by myoblasts. The muscles are revealed using an Actin-lacZ transformant line. (D) 18 hours APF (ablated): myoblasts are present in the region of MF9. Pre-fibers stain faint blue (arrowhead) (E,F) 24 hours APF, (ablated). Myoblasts are not present beyond the de novo developing fibers (indicated by arrowheads). (E) prefiber (F) Two de novo fibers Bar=50  $\mu$ m.



**Table 1. De novo muscle fiber formation at the site of ablation\***

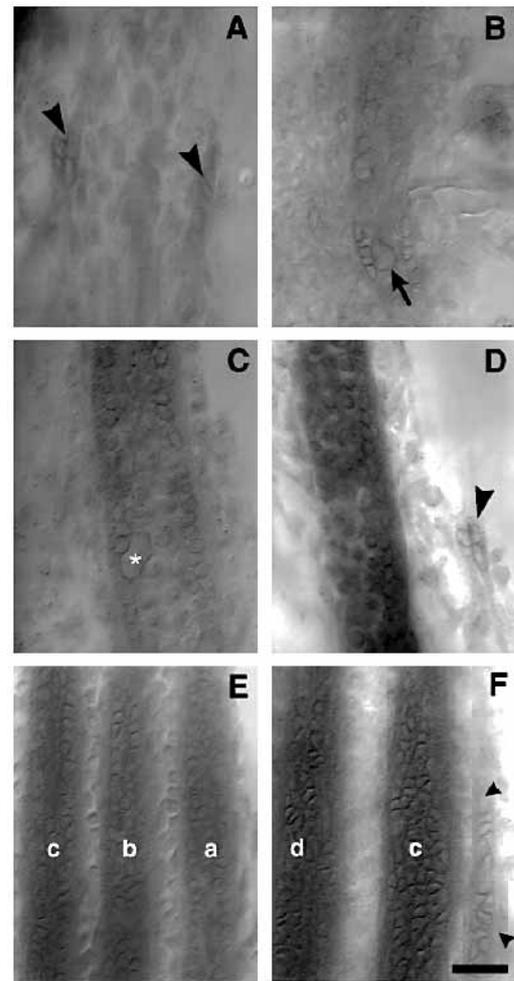
Age (hours)	No fibres (%)	Multiple pre-fibres† (%)	1 fibre (%)	2 fibres (%)	<i>n</i>
14-18	41	58	0	0	31
20-24	19	26	33	22	27
26-28	26	0	48	26	19
34-36	32	0	40	28	25

\*In all categories 4 fibers developed from the non-ablated regions.  
†Short incipient fibers expressing Act88F lacZ (see Fig. 2).

### Arrival and early patterning of myoblasts at the sites of myogenesis

One of the first steps in the formation of a muscle is the arrival of mesodermal cells at the sites of myogenesis. In the *Drosophila* embryo, it is the process of gastrulation that brings the Twist-expressing mesoderm to lie above the epidermis and the CNS. Subsequently, muscle founders arise in close association with these two tissues (Bate, 1990; Baylies et al., 1995). During adult muscle development, abdominal muscle myoblasts migrate along segmental nerves to arrive at their sites of myogenesis (Currie and Bate, 1991). In the thorax, myoblasts are also found along nerves (Fernandes and VijayRaghavan, 1993). However, the bulk of myoblasts are disc-associated and are found in the thoracic cavity soon after the discs evaginate (Fernandes et al., 1991). When the larval muscle is removed by ablation (this study), or when the muscle is genetically removed as in the duplicated thorax of *Bithorax* mutants (Egger et al., 1990; Schneiderman et al., 1993; Fernandes et al., 1994), myoblasts are still capable of aggregating in the region of DLM development. Thus, for DLM development, it appears that myoblasts arrive at the sites of myogenesis independent of any influence from the larval muscles. The possibility of residual extracellular matrix from the ablated larval fibers influencing myoblast aggregation was ruled out by performing muscle ablations in first instar larvae (Farrell et al., 1996), when the muscles are about one tenth the size that they are in the third instars (Keshishian et al., 1993). The adult muscle patterns obtained as a result were essentially the same as those obtained when ablations were carried out in third instar larvae.

The wing myoblasts are specified as a dorsal lineage as early as the first larval instar (Lawrence, 1982), and give rise to the IFMs (DLMs, DVMs) and the DFMs (direct flight muscles) during metamorphosis. Whether the segregation of these myoblast groups takes place prior to, or soon after disc evagination remains unknown. For mammalian muscle it has been proposed that generation of distinct muscle lineages can occur in several ways, including segregation of myoblasts, selective migration of groups of myoblasts or through selective proliferation (Stockdale, 1992). It is likely that in *Drosophila*, interactions with the epidermis bring about all of the above, and in case of the wing disc, such interactions could be responsible for segregation of the associated myoblasts into distinct primordia that give rise to the various muscles in the dorsal thorax. The IFMs and the DFMs each express a characteristic Actin isoform (Courchesne-Smith and Tobin, 1989; Hiromi and Hotta, 1985; Fyrberg et al., 1983). The commitment to express distinct Actin isoforms appears to be associated with the distinctive muscle morphology of the two muscle types –

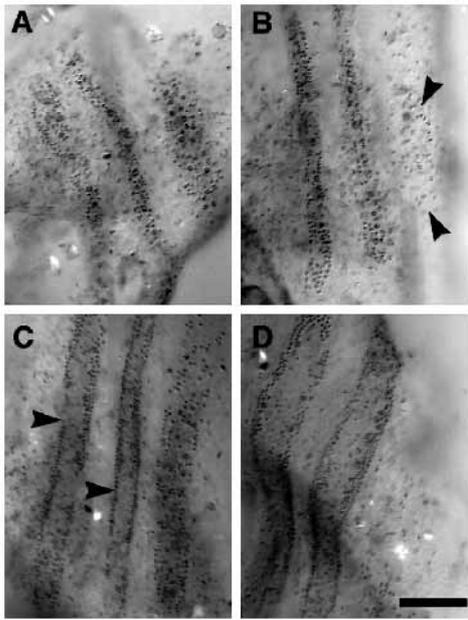


**Fig. 4.**  $\beta$ -3 tubulin distribution reveals that the formation of muscle pre-fibers is initiated by end to end alignment of myoblasts. (A) Clusters of aligned myoblasts in the region of ablation are first observed using the  $\beta$ -3 tubulin-lacZ transformant. Two clusters of 4-6 myoblasts are indicated by arrowheads. (B) Similar alignments of myoblasts can be seen during DVM development (arrow). At 12 hours APF, nuclei align at random sites along the length of the primordium. (C) 14 hour control: muscle 10 has a single row of myoblast nuclei on either side of the splitting muscle fiber. The asterisk marks a persistent larval nucleus. (D) 14 hours APF (ablated): muscle 19 has a row of nuclei on either side of the splitting fiber. A cluster of 4 myoblasts is seen in the region of the ablated MF9. (E) 24 hours APF (control): DLMs a, b and c. Each of them has about 3-4 rows of nuclei. (F) 24 hours APF (ablated): arrowheads indicate a developing musclepre-fiber. DLMs c and d have many more rows of nuclei (4-5) than their counterparts in the control hemisegment. Bar, 10  $\mu$ m.

the IFMs have a fibrillar type arrangement, while the DFMs and most other thoracic muscles are tubular (Crossley, 1978). This commitment may be intrinsic to the myoblasts, since the de novo DLM fibers express the appropriate Actin isoform despite a change in the mode of myogenesis.

### Initiation of fusion

Having arrived at the sites of myogenesis, the next task faced by the myoblasts is that of fusion. One possible scenario is that,



**Fig. 5.** The persistent larval muscles induce Erect Wing expression in myoblasts. (A) 12 hours APF, (control): a subset of myoblasts, that are present in close proximity to the muscle fiber express EWG prior to fusion with the larval muscle. (B) 12 hours APF (ablated): myoblasts in the region of the ablated muscle (arrowheads) do not express EWG. (C) 16 hours APF: after fusion, EWG-expressing myoblast nuclei are arranged in rows in the splitting muscles (arrowheads). The dorsal-most muscle (MF9) has not begun splitting. (D) 16 hours APF (ablated): in the region of ablation no EWG-expressing myoblasts are present. Bar, 50  $\mu$ m.

as in the *Drosophila* embryo and in the grasshopper (Ball et al., 1985; Xie et al., 1992; Steffens et al., 1995), there are founder cells which are endowed with the information that is necessary for establishing a muscle (Bate, 1990; Rushton et al., 1996). Thus far, we have not observed single specialized cells akin to the embryonic founders during pupal myogenesis.

Our results suggest that the persistent larval muscle fibers may function like the developing embryonic muscle fibers or the grasshopper pioneers in recruiting myoblasts for fusion. These events probably involve a communication between the pioneers/founders and the myoblasts that fuse with them. In case of the DLMs, myoblasts are seen around the persistent larval muscles soon after disc evagination. At this time the muscles are larval-like in appearance. As the larval fibers begin dedifferentiating, myoblasts become segregated to the three larval muscles (Fernandes et al., 1991), and fusion is initiated soon after (DeSimone et al., 1996). It is conceivable that an interaction between myoblasts and the larval muscles provides the cues for initiating fusion. Some evidence for such an interaction comes from the effect of muscle ablation on *ewg* expression. *ewg* is first expressed in a subset of DLM myoblasts prior to fusion, and is thought to regulate differentiation events downstream of fusion (DeSimone et al., 1996). The *ewg* expressing myoblasts are normally in close contact with the persistent larval muscles and when MF9 is ablated, *ewg* is not expressed by the DLM myoblasts present in the region. It is very likely that due to the absence of a larval muscle, the myoblasts no longer receive a signal to fuse and

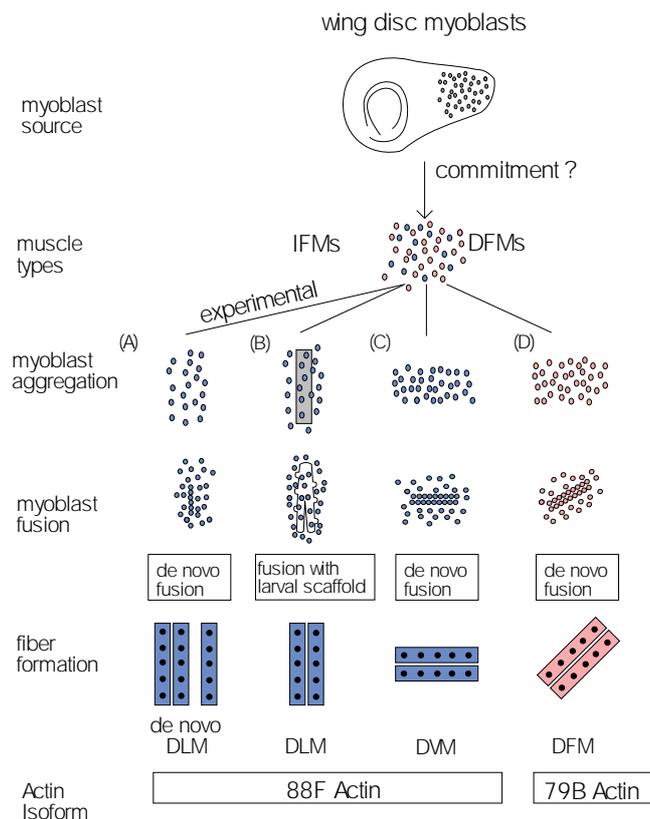
therefore do not express *ewg*. However, *ewg* expression turns on when a de novo fiber begins developing, indicating that cues for the initiation of myogenesis are now available to myoblasts. Another molecule that is expressed in myoblasts at the time of fusion is the PS2 integrin (Fernandes et al., 1996). However, the expression is transient and possibly occurs in those myoblasts that first fuse with the larval scaffold. In cultured vertebrate myoblasts, integrins are expressed prior to fusion, and are thought to be involved in signaling events leading to the differentiation of myoblasts (Menko and Boettiger, 1987).

The IFMs are composed of two muscle groups, the DLMs and the DVMs, which differ in their mode of fusion. While the DLMs develop using larval scaffolds, the DVMs develop de novo (Costello and Wyman, 1986; Fernandes et al., 1991). Given the similarity between the initiation of DVM and de novo DLM myogenesis, our results show that the mechanism of de novo fusion is common to both the DLM and the DVM myoblasts. Furthermore, for the DLMs, the mechanism of fusion with a larval scaffold appears to be a superimposition. We do not know if the nature of the signal that initiates myogenesis in the DVMs and the de novo DLMs is similar. A likely candidate is the epidermis. For the DLMs, as a result of larval muscle ablation, underlying interactions with the epidermis may become prominent and direct de novo fusion. Alternatively, the process of de novo fusion may simply be due to a mesoderm autonomous event.

#### Role of the larval muscle in DLM development

In general, annelid and insect muscles are patterned through the use of muscle organizers (Jellies, 1990). These are distinct mesodermal cells that serve as scaffolds or targets for myoblast fusions, determine orientation of the muscle fibers and in some cases delineate sites of attachment. To what extent do the persistent larval muscles function as classical muscle organizers? In the leech, muscle organizers are obligate muscle scaffolds, since their ablation does not allow myogenesis to take place (Jellies, 1990). A similar result was obtained for a coxal muscle in grasshopper (Ball et al., 1985). The larval scaffolds differ in this respect. In their absence, DLM development can still occur (Farrell et al., 1996; this paper). Unlike the embryonic founders which impart information about the identity of the muscle fiber, the larval muscles are not required for establishing DLM identity. The de novo fibers appear to have the appropriate identity with respect to expression of the correct Actin isoform, insertion at appropriate sites in the epidermis and innervation by the correct motor neuron (Fernandes and Keshishian, 1995).

Our results indicate that the persistent larval muscles play an important role in controlling the number of DLM fibers. One of the many features that distinguish the DLMs from the DVMs is that they always have a constant number of fibers, while in the DVMs, variability in fiber number is seen (de la Pompa et al., 1989). However, variations in adult DLM fiber number are seen when two or more larval fibers are ablated (Farrell et al., 1996). When all three larval scaffolds are ablated, 2-12 adult DLM fibers are seen. By following myogenesis in ablated animals during pupal development we show that the control of fiber number is most likely to be achieved by controlling the partitioning of myoblasts between the larval scaffolds. The adult abdominal muscles, which like the DVMs, develop de novo, also have variable numbers of fibers (Broadie and Bate, 1991; Taylor and Knittel, 1995). In contrast, the



**Fig. 6.** Formation of the de novo DLMs in relation to myogenesis of the wing disc myoblasts. Formation of (A) de novo DLMs, (B) DLMs, (C) DVMs, (D) DFM.

embryo and the larva have an invariant muscle fiber pattern, where each fiber arises from a specific founder cell (Bate, 1990; Bate, 1993). For both the DLMs and the embryonic musculature, there is a precise pattern of motoneuron connectivity (Ikeda and Koenig, 1988; Keshishian et al., 1996). The invariant muscle fiber number in both these systems likely plays an important function in motor control.

An important aspect of muscle pattern is correct orientation and the ability to find appropriate insertion sites. The de novo DLM fibers are always aligned in the correct A/P axis, showing that information about muscle fiber orientation can come from sources other than the larval muscle. The possibility of adjacent unablated muscle fibers influencing orientation of the fibers is unlikely because, in animals where all three larval scaffolds were ablated, the adult fibers were present in the correct orientation (Farrell et al., 1996). In the beetle *Tenebrio*, it has been shown that the epidermis provides cues for muscle orientation (Williams and Caveney, 1980). In those experiments, when the epidermis is surgically rotated, muscles appropriately changed their orientation. Finally, we observe that not all the muscle pre-fibers give rise to mature de novo DLMs (Table 1). One of the likely explanations is that the developing syncytium is eliminated since it cannot find the appropriate insertion sites. This elimination takes place around the time that the DLMs undergo a transient change in the morphology of muscle attachments (Table 1) and muscle size (Shatoury, 1956; Fernandes et al., 1991). It is possible that the persistent larval muscles play a role in enabling the developing DLM

fibers to make appropriate contacts with the epidermal cells that serve as insertion sites.

The use of larval muscle fibers as scaffolds is a common strategy for the generation of DLMs in many insects. However, there are variations to this common theme. In the butterfly *Pieris*, myoblasts begin invading the larval precursors of the DLMs as early as the last larval instar (Cifuentes-Diaz, 1989). In the blowflies *Calliphora* (Perez, 1910) and *Lucilia* (Peristianis and Gregory, 1971), the DLMs develop in a manner similar to *Drosophila*. On the other hand, there are some insects in which DLMs are generated de novo. For example, in a primitive Dipteran, *Simulium*, strands of indirect flight muscle myoblasts proliferate during metamorphosis (Hinton, 1959) and give rise to the adult muscles. In the wax moth, *Galleria*, the DLMs also develop without the use of larval scaffolds (Sahota and Beckel, 1967). In this case, pockets of myoblasts are seen in the early pupa that rapidly proliferate to give rise to the DLMs as well as the DVMs. The significance of the use of larval scaffolds in some insects and the lack of them in others is not clear. Given the short time period of pupation in *Drosophila* (4 days compared to considerably longer in other insects), and given that the DLMs are one of the effectors of the escape response, we propose that the larval scaffolds are necessary to ensure efficient partitioning of myoblasts thus controlling generation of the correct fiber number.

In conclusion, we show that though the larval muscle is not required for myogenesis to take place, it does play a role in the correct spatiotemporal initiation of DLM myoblast fusion, and in the partitioning of myoblasts to individual fibers. Our studies also suggest that de novo myogenesis is a common mechanism of muscle formation in the adult. Since the DLM myoblasts are capable of fusing de novo, we believe that larval scaffolds are superimposed on a common mode of de novo fusion, and are a mechanism by which DLM and DVM myoblasts may become distinct.

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