Founder myoblasts and fibre number during adult myogenesis in *Drosophila*

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Summary

We have examined the mechanisms underlying the setting of myotubes and choice of myotube number in adult *Drosophila*. We find that the pattern of adult myotubes is prefigured by a pattern of *duf-lacZ*-expressing myoblasts at appropriate locations. Selective expression of *duf-lacZ* in single myoblasts emerges from generalized, low-level expression in all adult myoblasts during the third larval instar. The number of founders, thus chosen, corresponds to the number of fibres in a muscle. In contrast to the embryo, the selection of individual adult founder cells during myogenesis does not depend on Notch-mediated lateral inhibition. Our results suggest a general mechanism by which multi-fibre muscles can be patterned.

Key words: Myogenesis, Myotube, Muscle, Founder cell

Introduction

In many organisms, muscles consist of syncitial fibres or myotubes formed by the fusion of embryonic myoblasts. The formation of syncitia increases the dimensions of muscle fibres, allowing them to operate as appropriately sized contractile elements in the locomotor machinery of the animal concerned. In the *Drosophila* embryo, the muscles of the larva form as single myotubes of different sizes that insert onto the developing epidermis. The key to understanding the specification and assembly of this complex pattern lies in the founder myoblasts that seed myotube formation. Each founder recruits and fuses with neighbouring myoblasts to form the syncitial precursor of a myotube (Bate, 1990; Bate, 1993; Abmayr et al., 1995; Rushton et al., 1995; Baylies et al., 1998; Paululat et al., 1999a). The characteristics of these myotubes are largely, if not completely, determined by transcription factors expressed in particular founder myoblasts. In adult flies and many vertebrates, additional complexity is added to the specification of muscle properties by the fact that muscles consist of many myotubes bundled together to form a contractile element. In systems such as these, the properties of myotubes must be specified, but in addition, the number of myotubes contributing to the developing muscle must be set as well. It is possible to envisage ways in which the founder-feeder model of myotube patterning could form the basis for setting the characteristics of such multi-fibre arrays.

We have examined the formation of multi-fibre muscles, i.e. muscles that function together as a contractile unit, in the adult fly (Fig. 1). In the mesothorax of the adult, the most prominent muscles are the indirect flight muscles (IFMs), whose development has been charted in some detail (Fernandes et al., 1991; Roy and VijayRaghavan, 1999). The IFMs consist of the dorsal longitudinal muscles (DLMs), an array of six large fibres, and three groups of dorsoventral muscles: DVM-I (three fibres), DVM-II (two fibres) and DVM-III (two fibres). The mesothorax contains another large muscle, namely the tergal depressor of the trochanter (TDT) or jump muscle, which consists of many fibres bundled together as a unit. The dorsal thorax also contains the direct flight muscles (DFMs), involved in changing the orientation of the wing. Each of these muscles is a multi-fibre contractile unit. The muscles in the adult abdomen are also arranged as well-defined sets of fibres, which form dorsally, laterally and ventrally in each segment (Fig. 1) (Currie and Bate, 1991).

By examining the formation of these muscles, we provide evidence that specialized founder myoblasts are a feature of adult as well as embryonic myogenesis in the fly. As adult muscles, unlike the somatic muscles of the embryo, are composed of multiple fibres, we discuss the question of how several founders are chosen for each multi-fibre contractile unit.

Materials and methods

Fly strains

To follow *duf* expression, the enhancer trap line *rp298*, which has a P element-nuclear-*lacZ* insertion in the promoter region of *duf*, was used (Ruiz-Gomez et al., 2000). The GAL4-UAS system (Brand and Perrimon, 1993) was used for directed expression of genes during adult myogenesis. The *1151-GAL4* enhancer trap strain was obtained from L. S. Shashidhara (CCMB, Hyderabad, India). This GAL4 driver is expressed in all adult myoblasts associated with the imaginal discs and nerves in the larvae. No expression is seen in the larval templates

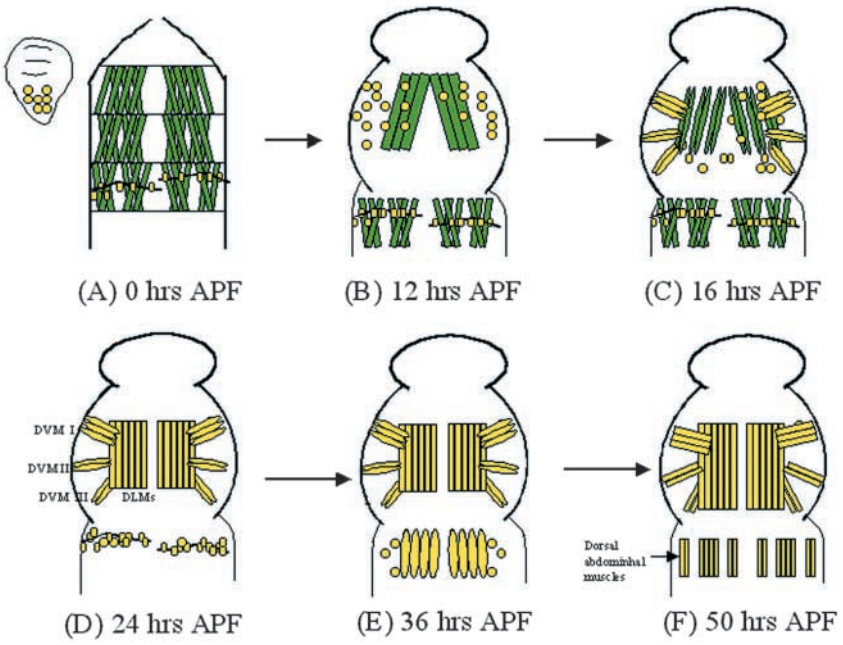

Fig. 1. Schematic representation of the development of the indirect flight muscles (IFMs) and the abdominal muscles. The IFMs are anatomically and functionally subdivided into two distinct groups: the dorsal longitudinal muscles (DLMs) and the dorsoventral muscles (DVMs). (A) The precursor myoblasts (yellow) for the IFMs are attached to the wing disc during the larval life. At the onset of metamorphosis larval muscles begin to histolyse, with the exception of three oblique muscles. (B) By 12 hours APF, histolysis is complete and the three larval templates are clearly visible. By this stage, myoblasts migrate into the muscle-forming regions. (C) The larval templates start splitting by 13 hours APF and the process is complete by 16-17 hours APF. At the same time, the DVMs form by the de novo fusion of myoblasts. (D) By 24 hours APF, the DLMs and the DVMs are complete. Muscle formation in the abdomen occurs later than in the thorax. The abdominal muscles develop from an adult myoblast pool (yellow) associated with the segmental and intersegmental nerves (A). (A-D) During early pupal stages (from 0-24 hours APF), myoblasts proliferate and migrate out along the nerves. (E) Fusion of these myoblasts begins by 28-30 hours APF. (F) The arrangement of the DLMs, the DVMs (I, II and III) and the dorsal muscles of the first abdominal segment in an adult fly: by 50 hours APF, the pattern of the adult muscles is largely complete.

(see Anant et al., 1998). *UAS-dnRac1* was obtained from the Bloomington Stock Centre. *UAS-activated Notch* (*UAS-Nintra*), *UAS-dnNotch* (Rebay et al., 1993), *UAS-MamH* and *UAS-MamN* were obtained as gifts from S. Artavanis-Tsakonas (MGH Cancer Centre, USA). The various UAS strains were put in the background of *rp298* using standard genetic techniques. For co-localization of *1151* and *duf-lacZ*, *1151-GAL4* was crossed with *rp298; UAS-nuclear localized-GFP*. Most fly cultures and crosses were grown at 25°C. The progeny of the GAL4-UAS crosses (with the exception of *1151×rp298; UAS-nls-GFP*) were grown at 25°C until early second instar stages, and then shifted to 29°C until the adult stages. Controls were grown in similar conditions.

Heat shocks

We found that the rate of development at 22°C was 0.75 times that at 25°C, and that at 31.5°C was approximately 1.3 times that at 25°C (Anant et al., 1998). Based on this, 0-hour APF pupae were grown at 22°C for 21 hours and 20 minutes (corresponding to 16 hours APF at 25°C), 24 hours (≡18 hours at 25°C), 26 hours and 40 minutes (≡20 hours APF at 25°C), 29 hours and 20 minutes (≡ 22 hours at 25°C), and then raised to 31.5°C for 6 hours, 4 hours and 30 minutes, 3 hours, and 1 hour and 30 minutes, respectively.

Tissue preparation

White prepupae (0-hour APF) were collected on moist filter paper in a Petri dish and grown at 25°C for different intervals. For the GAL4-UAS crosses and their controls, white prepupae were collected and grown at 29°C. The pupal and larval tissues were prepared for immunohistochemistry as described previously (Fernandes et al., 1991). The pupal preparations were mounted in 70% glycerol for X-GAL stained preparations, or in Vectashield mounting medium (Vector Laboratories, Burlingame, CA) for fluorescently labeled preparations.

Immunohistochemistry

Anti-Ewg antibody raised in rabbit was used at a dilution of 1:500 (DeSimone et al., 1996). Anti-β-galactosidase monoclonal antibody and 22C10 (both from The Developmental Studies Hybridoma Bank) were used at a dilution of 1:50. Anti-Twist antibody, a gift from Siegfried Roth (University of Köln), was used at a dilution of 1:500. Vestigial antibody was a gift from Sean Carroll and was used at a dilution of 1:200. Anti-MHC antibody, a gift from Dan Kiehart, was used at a dilution of 1:500. For double-antibody stained preparations of *1151-GAL4, duf-lacZ; UASdnRac1/+* pupae (Fig. 7A-E), pupae were incubated first in anti-MHC antibody and developed in the absence of nickel sulphate (giving a light brown colour), then incubated in anti-β-galactosidase and developed in the presence of nickel sulphate (giving a black colour). For preparations stained both with X-GAL and antibody (Fig. 7F-I), pupae were first incubated overnight in X-GAL, then processed for antibody staining. DIC images were taken using a Nikon Eclipse E1000 microscope. For fluorescent images, secondary antibodies conjugated to Alexa Fluor dyes (from Molecular Probes, Eugene, OR) were used: Alexa 488 for green labeling and Alexa 568 for red labeling. Fluorescent preparations were scanned using the confocal microscope (MRC-1024, BioRad Laboratories, Hercules, CA) and analyzed using Metamorph (version 4.5, Universal Imaging).

Results

A marker for embryonic founder myoblasts identifies founders for the DVMs

We began our investigation of myotube formation in the adult by looking at a prominent set of muscles, the DVMs (Fig. 2A,B), which form de novo from groups of aggregating myoblasts in the adult thorax (Fernandes et al., 1991). We wanted to see how the three different DV muscles are formed from these myoblasts and how each muscle attains its typical fibre number. It could be that, as in the embryo, each fibre is seeded by the formation of a single, specialized founder cell. Alternatively, myoblasts might be selected in some other way; for example, as a group that would fuse to form a single

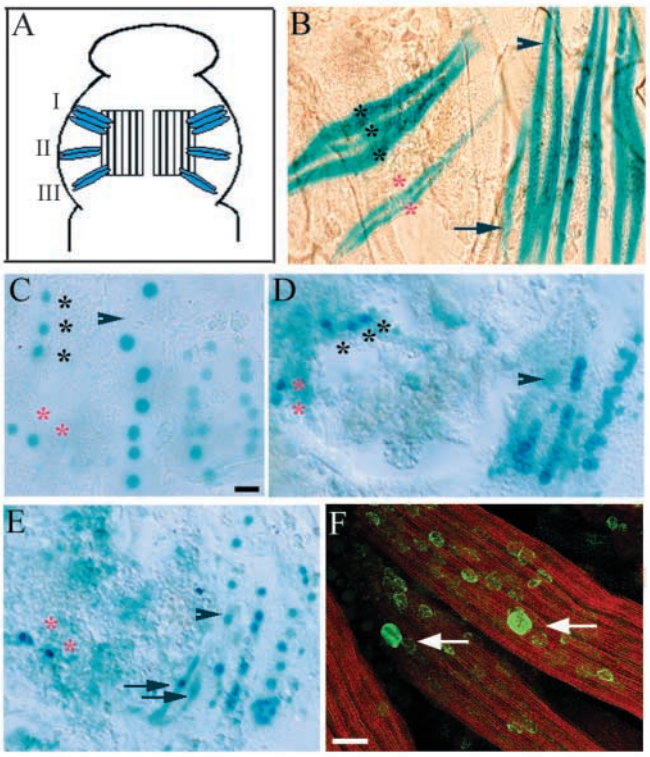

Fig. 2. Expression of *duf-lacZ* in DVMs. (A) Diagram showing the position of the three DVM bundles (blue) relative to the DLM fibres (white) in the adult fly. (B) *actin-lacZ* pupal preparation (24-hour APF) showing the DVM fibre bundles. The DVM I fibres are marked by black asterisks; fibres of DVM II are marked by red asterisks. DVM III fibres are formed beneath the DLM fibres. In this figure, one of the two DVM III fibres (black arrow) can be observed. The DLM fibre most adjacent to the DVMs is indicated by the black arrowhead. (C-E) *duf-lacZ*-expressing DVM nuclei assayed by *lacZ* staining. The nuclei corresponding to DVM I are indicated by black asterisks, to DVM II by red asterisks and to DVM III by black arrows. (C) The three *duf-lacZ*-expressing nuclei of DVM I, and the two of DVM II, at 7 hours APF. The linear arrays of nuclei, indicated by an arrowhead, are *duf-lacZ*-expressing nuclei in the adjacent larval templates and are described in more detail in Fig. 4. (D,E) Twelve-hour APF pupae showing *duf-lacZ*-expressing nuclei of DVM I and DVM II (D), and of DVM II and DVM III (E). (F) Forty-hour APF preparation of *duf-lacZ* pupa double labeled with anti-MHC (red) and anti-β-galactosidase (green) to mark the two myofibres (of DVM II) and *duf-lacZ* nuclei, respectively. One nucleus in each fibre (white arrow) expresses higher levels of *lacZ* than the rest. Anterior, top; dorsal midline, right. Scale bar: in C, 18 μm for C-E; in F, 20 μm.

myotube. To distinguish between these two alternatives, we used a P-lacZ insertion (*rp298*) in the gene *dumbfounded* (*duf*; *kirre* – FlyBase), which results in the expression of β-galactosidase in the nuclei of cells that express *duf*. *duf* itself encodes a transmembrane protein that, in the embryonic mesoderm, is exclusive to founder myoblasts and serves as an attractant for fusion competent myoblasts (Ruiz-Gomez et al., 2000). *duf-lacZ* expression is therefore a good marker for such cells, and we examined when and where *duf-lacZ* is expressed as myoblasts aggregate to form the DVMs.

At 7 hours after puparium formation (APF), at each epidermal location where a DVM fibre will develop, we see a single prominent myoblast that expresses *duf-lacZ*. Thus, we see three, two and two *duf-lacZ* expressing cells, corresponding, respectively, to the three, two and two fibres of the future DVM I, DVM II and DVM III muscles (Fig. 2C-E). These nuclei continue to express *duf-lacZ* at high levels as each of the syncitial fibres in the DVMs form (Fig. 2F). In addition, the fused adult myoblast nuclei within the syncitia show low levels of *duf-lacZ* expression (Fig. 2F). By 36 hours APF, the level of β-galactosidase in all DVM nuclei begins to fall and disappears by 70 hours APF.

These findings suggest that, just as in the embryo, the formation of myotubes in the adult may be initiated by the selection of single founder myoblasts that are identifiable by their expression of *duf-lacZ*. Other myoblasts would be recruited to these founders and fuse with them and (again, as in the embryo) these fusing cells would themselves be induced to express *duf-lacZ*, albeit at a lower level. If this view of adult fibre formation is correct, then it should be generally true for all cases in the adult where fibres form de novo from groups of aggregating myoblasts. With this in mind, we looked at the regular arrays of fibres that form dorsally and laterally in the adult abdomen. There are many such fibres laid out in a well-organized pattern and they are derived from myoblasts that, unlike the DVM cells, come not from the discs but from pools of cells associated with the abdominal nerves (Currie and Bate, 1991).

The formation of the syncitial muscles in the abdomen begins at about 28 hours APF (Currie and Bate, 1991). We looked at a stage prior to this to see whether single *duf-lacZ*-expressing cells appear before fibres form. Once again, we observed a striking correspondence between forming fibres and *duf-lacZ* expression in the abdomen, with every fibre preceded by a single *duf-lacZ*-expressing nucleus at the appropriate position. At 24 hours APF, we observe an array of *duf-lacZ*-expressing cells in each of the dorsal hemisegments. One of the hemisegments (A4) is shown in Fig. 3A. The monoclonal antibody 22C10 also labels these cells (Fig. 3B,C). By 28 hours APF, these cells are in positions where the future muscle fibres will form (Fig. 3D). By 50 hours APF, when the formation of the syncitial fibres is largely complete, we observed one nucleus in each fibre that expresses *duf-lacZ* at higher levels than the rest (Fig. 3E). We observe a similar pattern of *duf-lacZ* expression in the developing lateral muscles. At positions where the future lateral muscles will form there are single *duf-lacZ*-expressing cells that are also 22C10 positive (Fig. 3F). These mononucleate cells develop into multinucleate fibres (see Fig. 3G,H), which each contain several *duf-lacZ*-expressing nuclei.

duf-lacZ is expressed as DLMs form on larval templates

On the face of it, the development of the DLMs follows a different scheme. Here, the muscles assemble on a set of pre-existing templates provided by a small set of persistent larval fibres, the three larval oblique muscles (LOMs) (shown in Fig. 4B). Subsequently, the three templates split to form the six fibres of the DLMs (Fig. 1, Fig. 4C) (Fernandes et al., 1991). Despite this novel form of myogenesis, it has been suggested that the larval fibres serve a founder-like function in organizing the development of the DLMs (Farrell et al., 1996), and it is

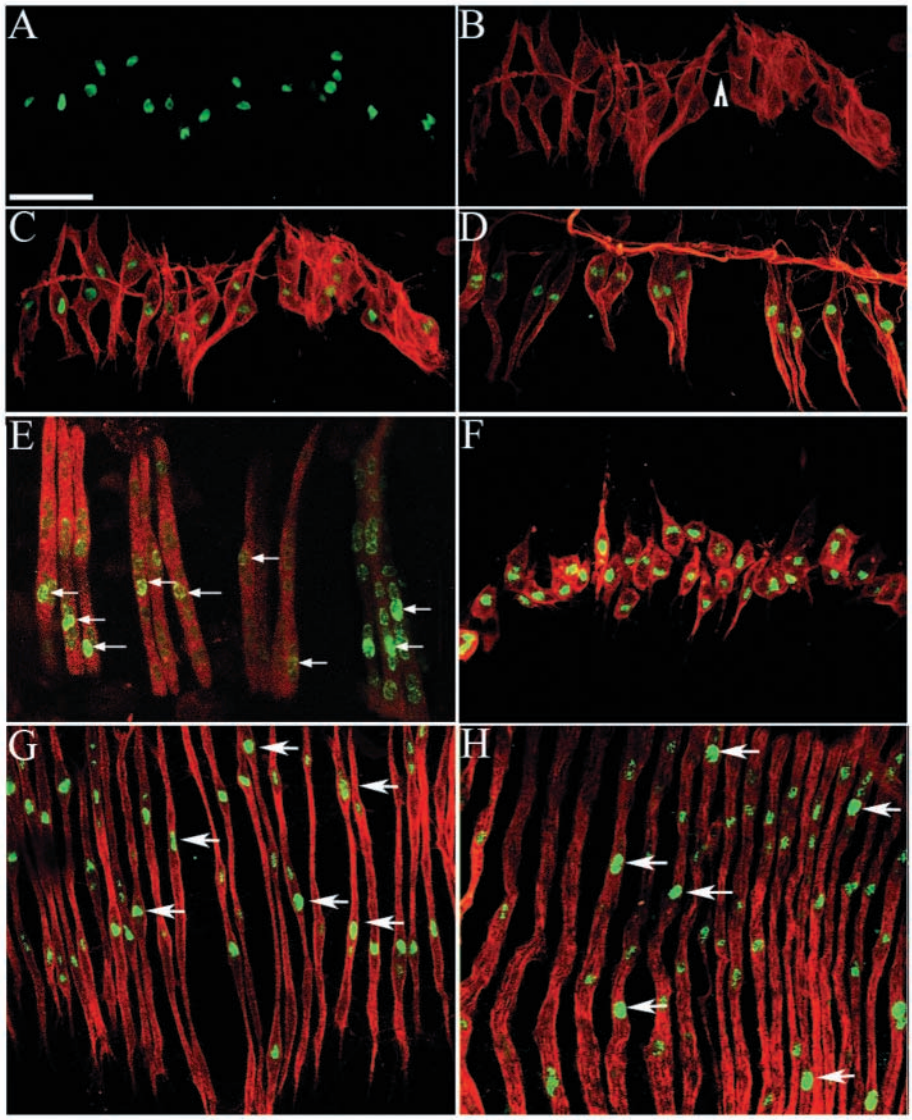

Fig. 3. Expression of *duf-lacZ* in abdominal muscles. (A-E) *duf-lacZ* expression in developing dorsal muscles of the abdomen. (A-C) One hemisegment of a 24-hour APF *duf-lacZ* pupa double labeled with anti-β-galactosidase (green) and 22C10 (red). (A) Single cells with *duf-lacZ* expression in their nuclei. (B) The same preparation as shown in A showing the *duf-lacZ*-expressing cells stained by 22C10. The nerve to which the myoblasts remain attached initially is also labeled by 22C10 and is indicated by the white arrowhead. (C) Merged image of A and B. (D) A 28-hour APF *duf-lacZ* pupa similarly double labeled as in C, showing the *duf-lacZ*-expressing cells stretching out at positions where future muscle fibres will form. (E) A 50-hour APF *duf-lacZ* pupa double labeled with anti-MHC (red) and anti-β-galactosidase (green), showing a subset of dorsal muscles. By this stage, formation of the multi-nucleate fibres is largely complete. Each fibre has one prominent *duf-lacZ*-expressing nucleus (white arrows), presumably belonging to the single cell that preceded the multi-nucleate fibre. The remaining nuclei express *duf-lacZ* at a lower level. (F-H) *duf-lacZ* expression in developing lateral muscles of the abdomen. (F,G) *duf-lacZ* pupa double labeled with anti-β-galactosidase (green) and 22C10 (red). (F) Twenty-four hours APF. Single cells expressing *duf-lacZ* are present in the region where the future lateral muscles will form. These cells, like the cells in the dorsal region, are labeled by 22C10. (G) The developing lateral myotubes at 36 hours APF. The fibres, labeled by 22C10, are now multi-nucleate. (H) Lateral muscles at 41 hours APF, labeled by anti-β-galactosidase (green) and anti-MHC (red). In both G and H, each fibre contains a single nucleus with high levels of *duf-lacZ* expression (white arrows), against a backdrop of fainter expression in the other nuclei. For some fibres in H, nuclei with higher *duf-lacZ* expression are not present in the field of view. In A-E, anterior is to the top, dorsal midline to the right; in F-H, anterior is to the right, dorsal midline to the bottom. Scale bar: 50 μm.

certainly true that the adult myoblasts aggregate on the larval fibres and fuse with them to form syncitial myotubes. With this in mind, we decided to look at the expression of *duf-lacZ* during DLM development. We found that *duf-lacZ* is indeed expressed in the founder analogues, the larval templates of the adult muscles. *duf-lacZ* expression begins in the three larval templates at 6.5 hours APF (Fig. 4D). All of the nuclei in each of the three templates appear to express *duf-lacZ*. The expression of *duf-lacZ* continues as the templates split and form the six fibres of the DLMs (Fig. 4E-G). By 36 hours APF, the intensity of β-galactosidase staining starts to decrease, and by 70 hours APF the staining has disappeared. The *duf-lacZ*-expressing nuclei in the templates could be larval nuclei or the nuclei of adult myoblasts that have already fused with the templates by 6.5 hours APF. Larval nuclei are large and easily distinguishable from the smaller nuclei of the adult myoblasts (Crossley, 1978). The transcription factor Erect wing (Ewg) (DeSimone et al., 1996) is expressed in the nuclei of the larval muscles and this expression persists as the larval muscles are transformed to templates. Ewg is also expressed in adult myoblasts as they migrate over the templates to fuse with them (Roy and VijayRaghavan, 1998). These two subsets of nuclei, both expressing Ewg, can be easily distinguished on the basis of their size (as can also be seen in Fig. 4I). A double label using antibodies against β-galactosidase and Ewg reveals that *duf-lacZ*-expressing nuclei co-localise with Ewg-expressing nuclei, which by their size are larval in origin (Fig. 4H-J).

duf-lacZ expression precedes adult myogenesis and is initially seen in many myoblasts

If *duf-lacZ* expressing founder myoblasts are, as in the embryo, a decisive factor in controlling the spatial pattern of adult myogenesis, then we need to understand how this pattern of expression develops and is controlled. We first looked at the

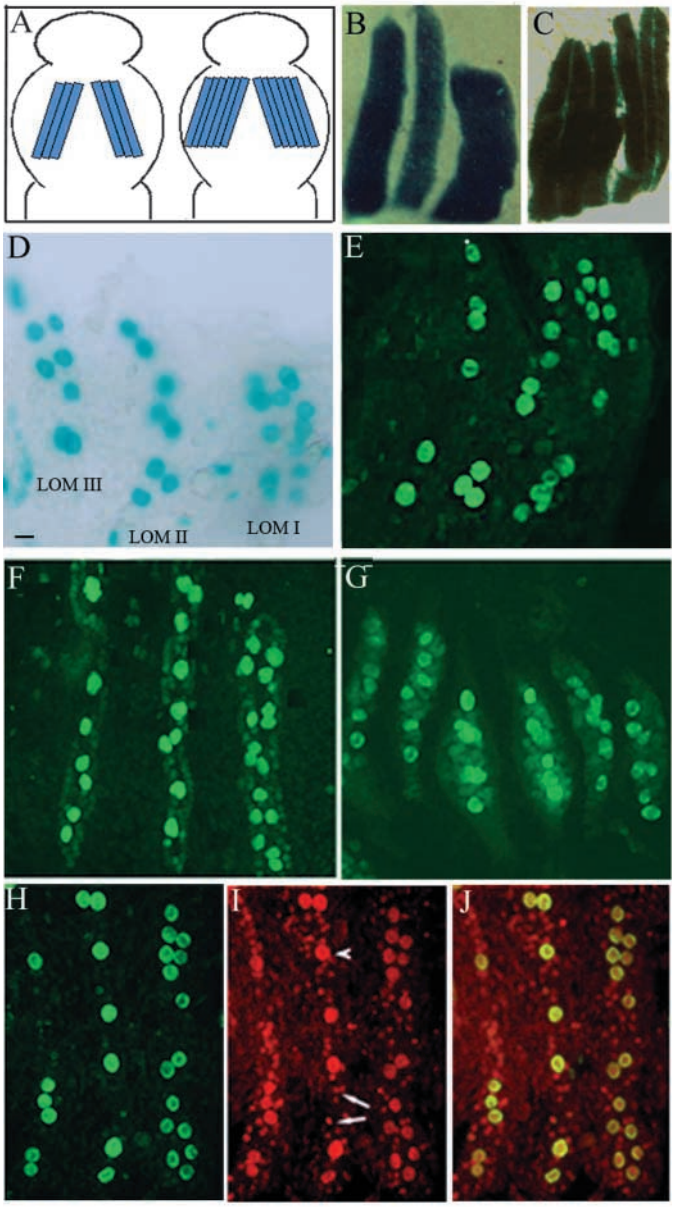

Fig. 4. *duf-lacZ* expression in developing DLMs. (A) The position of the larval templates and the six DLM fibres with respect to the fly notum. (B) The three larval templates at 12 hours APF; (C) the six DLM fibres at 26 hours APF, as revealed by histochemical staining in *Mhc-lacZ* pupae. (D-G) *duf-lacZ* expression followed by assaying for β-galactosidase activity (D) and anti-β-galactosidase labeling (E-G). (D) A 6.5-hour APF *duf-lacZ* pupal preparation showing β-galactosidase histochemical activity in the three larval templates. The three templates have been labeled as LOM I, II and III. Each template has 8-13 *duf-lacZ*-expressing nuclei aligned along their length. Some nuclei in LOM I and LOM II are out of focus. (E-G) *duf-lacZ* expression at 12 hours APF (E), 14 hours APF (F) and 24 hours APF (G), when the DLMs are in the six fibre stage. (H-J) Confocal images of a 12-hour APF *duf-lacZ* pupa stained with anti-β-galactosidase (green) and anti-Ewg (red) antibodies. (H) The three larval templates expressing *duf-lacZ*. (I) Ewg expression in larval nuclei, and in the nuclei of incoming adult myoblasts. White arrowhead points to a larval nucleus expressing Ewg in the template fibre; arrows indicate the adult nuclei. Note the difference in size between the nuclei of the two different origins. (J) Merged image showing the co-localization of β-galactosidase (green) with Ewg (red), expressed in the larval nuclei. Anterior is to the top; dorsal midline to the right. Scale bar 20 μm for D-J.

emergence of *duf-lacZ* expression in the myoblasts of the abdomen. The abdominal muscles develop from pools of muscle-forming cells that are associated with the nerves that innervate the larval muscle field (Bate et al., 1991; Currie and Bate, 1991). These cells in turn are derived from single adult muscle progenitors that arise in the embryo as the siblings of embryonic muscle founder cells (Ruiz-Gomez and Bate, 1997). Unlike their embryonic founder cell siblings, the adult precursors maintain *twist* expression and proliferate during larval life to form pools of nerve-associated, *twist*-expressing myoblasts. The adult precursors do not express *duf-lacZ* in the embryo (Ruiz-Gomez et al., 2000). These myoblasts then migrate, aggregate and fuse to form the muscles of the adult abdomen (Fig. 1). When we looked at the expression of *duf-lacZ* in these precursor cell populations in late third instar larvae, we found that all the cells express *duf-lacZ* at a low level (Fig. 5A,B). This expression persists during early pupal stages (13-14 hours APF; Fig. 5C) but, by 16 hours APF, levels of β-galactosidase in the myoblasts have declined and, at 20 hours APF (Fig. 5D), only a subset of Twist-positive myoblasts remains β-galactosidase positive. By 24 hours APF, however, there is a clear upregulation of *duf-lacZ* expression in particular nuclei (Fig. 5E), and by 28 hours APF, these *duf-lacZ*-expressing cells are positioned at the sites where individual muscle fibres will now form (Fig. 5F). These are the same nuclei that are present in 22C10-stained cells, as shown previously in Fig. 3D, and that continue to express high levels of *duf-lacZ* within the fully formed fibres (Fig. 3E). We find a similar sequence of *duf-lacZ* expression in the lateral clusters of myoblasts (data not shown). Expression does not persist into the adult and begins to diminish by 70 hours APF.

We find a similar sequence of expression in the myoblasts of the wing disc that give rise to the DLMs and DVMs (Lawrence, 1982). In late third instar larvae, *duf-lacZ* expression can be detected at low levels in all of these cells (Fig. 6A-C). To follow this pattern of expression in the pupa, we double-labeled *duf-lacZ* pupae with markers for adult myoblasts, using either *UAS-GFP* driven by *1151-GAL4*, which is expressed in all adult myoblasts (Anant et al., 1998), or antibodies to the transcription factor Vestigial (Vg), which is expressed in most of the myoblasts on the wing disc (Sudarsan et al., 2001). These stainings reveal that the generalized low level of *duf-lacZ* expression had disappeared by 12 hours APF and had been replaced by expression in specific cells. For example, Fig. 6D shows 1151-driven GFP expression in myoblasts of DVM II. *duf-lacZ* expression is seen in a single nucleus in each of the two fibres of DVM II (Fig. 6E). Fig. 6F, a merged image, shows *duf-lacZ*-expressing nuclei co-localizing with GFP expression. Double labeling with antibodies against Vg and β-galactosidase again shows co-expression in specific nuclei (Fig. 6G-I). In a similar fashion, *duf-lacZ* expression is downregulated in myoblasts that will fuse with the larval templates: Fig. 4H,I show the absence of *duf-lacZ* in the myoblasts that have migrated over the larval templates. We conclude that, as in the abdomen, the

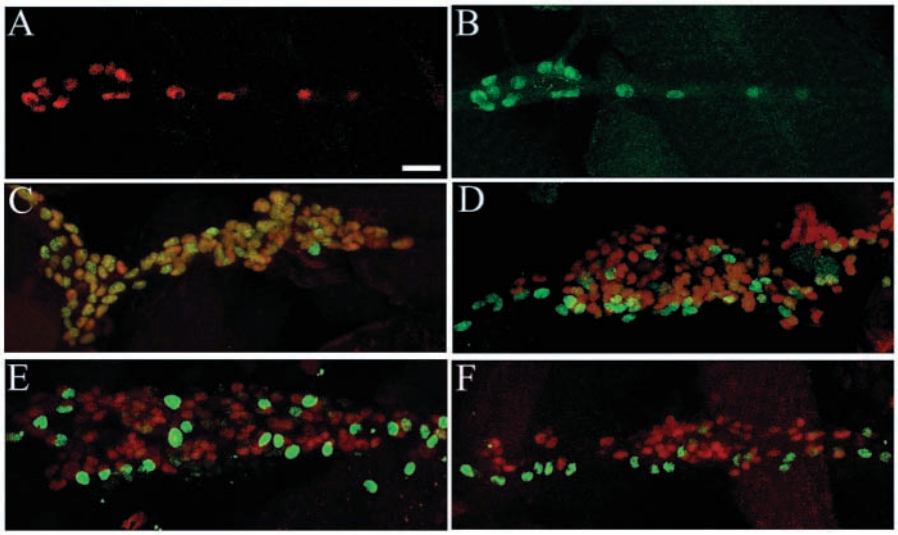

Fig. 5. Expression of *duf-lacZ* during abdominal myogenesis. Preparations of *duf-lacZ* pupae double labeled with anti-β-galactosidase (green) and anti-Twist (red). Panels A and B are of the same preparations shown separately in the red and green channels. (C-F) Merged images. (A) Twist-expressing nerve-associated myoblasts in the dorsal hemisegment of A3 in a late third instar larva. (B) *duf-lacZ* expression in the same cells as in A. (C) A 13-hour APF pupa, again showing co-localization of *duf-lacZ* with Twist in almost all nerve-associated myoblasts. (D) By 20 hours APF, *lacZ* expression has faded from many of the myoblasts, and only a subset of myoblasts shows co-localization. (E) At 24 hours APF, the number of Twist and *duf-lacZ* co-localizing myoblasts is further reduced. By this stage, individual myoblasts expressing high levels of *duf-lacZ* are seen. (F) Twenty-eight hours APF. Single *duf-lacZ*-expressing myoblasts are observed at positions corresponding to future fibre forming sites. In all figures anterior is to the top, dorsal to the right. Scale bar: 20 μm.

duf-lacZ-expressing founders of the thoracic muscles other than the DLMs are derived from the pool of adult myoblasts, all of which initially express *duf-lacZ* at low levels.

duf-lacZ-expressing myoblasts form fibres at appropriate positions when myoblast fusion is defective

The decisive function of muscle founder cells in myotube formation is revealed in the embryo by their unique capacity to form muscles in mutants where myoblast fusion is compromised. Thus in embryos that are defective in the machinery of myoblast fusion, founder myoblasts differentiate to form thin mononucleate fibres at appropriate positions and with molecular characteristics similar to those of normal wild-type muscles (Rushton et al., 1995). Other myoblasts aggregate on the differentiating founders but cannot fuse with them and do not succeed in differentiating to form muscle fibres themselves. These observations show that, in the embryo, myoblasts are of two kinds: founders, which are competent to complete myogenesis in the absence of fusion, and feeders, which can only contribute to myogenesis after fusion with a founder.

To show whether there is a similar division of myoblasts during adult myogenesis, we generated a fusion-defective phenotype during adult myogenesis, by overexpressing a dominant-negative form of the protein Rac1, a member of the small GTPase superfamily involved in the process of fusion (Paululat et al., 1999b). Overexpression of the dominant-negative Rac1 (Rac1N17) in the embryonic mesoderm severely delays the fusion process and results in abnormal fusion in the later stages

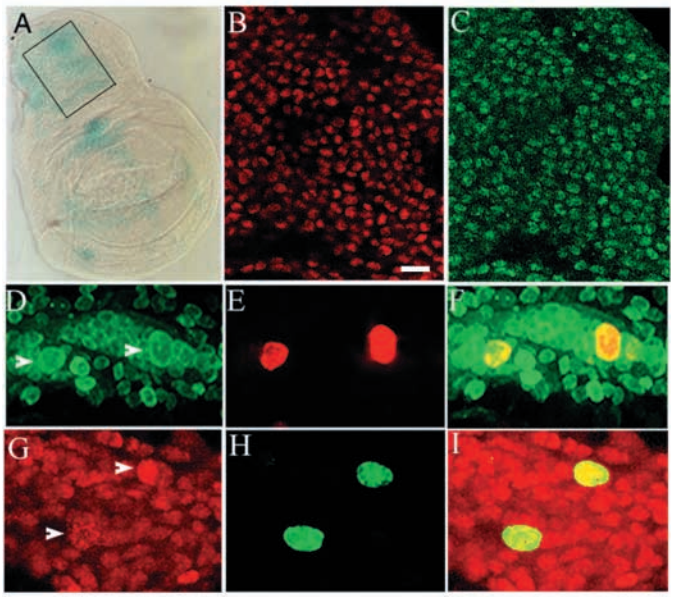

Fig. 6. Expression of *duf-lacZ* in the adult thoracic myoblast pool. (A-C) Expression of *duf-lacZ* in the wing disc-associated myoblasts. (A) Faint *duf-lacZ* expression in the myoblasts that adhere to the notal region of the wing disc. The region outlined by the black box is magnified in B and C. (B,C) Twist-expressing myoblasts (red; B) that also express *duf-lacZ* (green; C). (D-F) Co-localization of *duf*-lacZ with 1151Gal4-expressing myoblasts. (D) Confocal image of a 12-hour APF hemithorax showing the 1151-GAL4 expressing myoblasts of the developing DVM II fibres. 1151Gal4-driven expression is revealed by UAS-nls-GFP, with *duf-lacZ* in the background. Each arrowhead indicates a nucleus, which is larger in size than the other nuclei within the syncitium. (E) The *duf-lacZ*-expressing myoblasts of DVM II, assayed by anti-β-galactosidase labeling. These two nuclei are the same as those indicated by arrowheads (D). (F) Merged image showing the co-localization of *duf-lacZ* (red) with 1151GAL4 (green). (G-I) Co-localization of *duf-lacZ* with Vg. (G) Confocal image showing Vg-expressing myoblasts that have fused to, or have swarmed over, the DVM II syncitium. Vg expression is observed by anti-Vg labeling on a 12-hour APF *duf-lacZ* pupa. Amidst the cluster of myoblasts, arrowheads indicate the myoblasts that also express *duf-lacZ*. (H) Anti-β-galactosidase labeling reveals the *duf-lacZ*-expressing myoblasts of DVM II in the same preparation. (I) Merged image showing the co-localization of Vg with *duf-lacZ*. Scale bar: 50 μm for B,C; 30 μm for D-I.

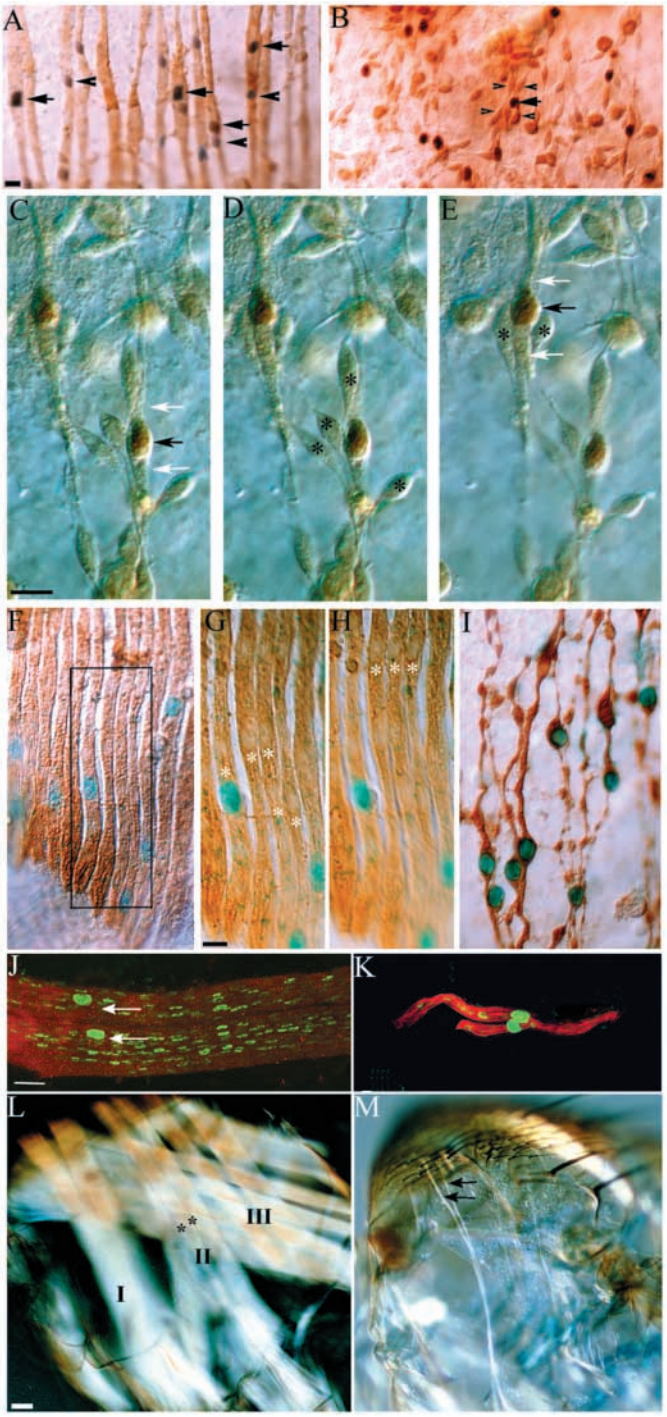

Fig. 7. Effects of dominant-negative Rac1 expression in adult myoblasts. (A) Fibres of wild-type lateral muscles expressing myosin (brown) at 31 hours APF (29°C). Each fibre has a brightly stained *duf-lacZ* nucleus (black arrow). In some fibres, this nucleus is not present within the field of view. Other nuclei in the fibres express *duf-lacZ* at lower levels and are seen in a few fibres (black arrowheads). (B) *1151Gal4*/*UAS-Rac1N17*-misexpression pupa of the same stage showing the absence of syncitial fibres. The putative founder cells (one of them indicated by the black arrow) are correctly specified and express *duf-lacZ* and myosin. Unfused myoblasts also express myosin and, in some cases, can be seen clustered around *duf-lacZ*-expressing founders (arrowhead). (C-E) Two lateral founders, of the same stage as shown in in B, at a higher magnification. The founder cells extending processes in an orientation similar to that of the wild-type lateral fibres. The nucleus of each cell expressing *duf-lacZ* is indicated by a black arrow. (C) Extended processes of one of the founder cells are in focus (indicated by white arrows). (D) Same preparation as in C, shown at a different plane of focus. Black asterisks mark some of the unfused myoblasts surrounding the founder cell. (E) Processes of the second founder cell are in focus in this image (white arrows). Unfused myoblasts are again seen clustered around this founder. (F-I) X-Gal and anti-MHC-stained lateral muscle fibres at 42 hours APF (in 29°C) in wild-type (F,G,H) and *1151Gal4*/*UAS-Rac1N17*-misexpression (I) pupae. (F) Lateral muscles showing one *duf-lacZ*-expressing nucleus in each fibre. This nucleus corresponds to the high *duf-lacZ*-expressing founder nucleus observed in the fluorescent images in Fig. 3G and H. X-Gal staining cannot detect the low β-galactosidase activity in the remaining nuclei of the syncitium. These nuclei can be detected at a higher magnification as shown in G and H. (G,H) Magnified views of the region outlined by the box in F, at different planes of focus showing the presence of multiple nuclei in each fibre. Each white asterisk is placed below a nucleus in the plane of focus. (I) In the absence of normal fusion, thin mononucleate lateral fibres span the region. (J,K) Fluorescent preparations at 36 hours APF (at 29°C). (J) Wild-type DVM II expressing myosin (red) and *duf-lacZ* (green). White arrows indicate the nucleus in each syncitium that expresses high levels of *duf-lacZ*. (K) Developing fibres of DVM II in a pupa with Rac1N17 misexpression in adult myoblasts. The DV muscle fibres are reduced in size but the pattern of one high *duf-lacZ*-expressing nucleus per fibre remains unaffected. (L) Wild-type pattern of DLMs, and DVMs I, II and III, in one hemisegment of an adult CS fly (grown at 29°C). Black asterisks indicate the DVM II fibres. (M) Muscles in one thoracic hemisegment of a fly after Rac1N17 misexpression in adult myoblasts. In focus are the two fibres of DVM II (black arrows). Fibre size is severely reduced but fibre number remains unchanged. In A-I, anterior is to the right, dorsal midline to the bottom; in J-K, anterior is to the top, dorsal to the right; in L-M, anterior is to the left, dorsal to the top. Scale bars: in A, 4 μm for A,B,F,I; in C, 4 μm for C,D,E; in G, 4 μm for G,H; in J, 20 μm for J,K; in L, 40 μm for L,M.

(Luo et al., 1994). We found that overexpression of Rac1N17 in the adult myoblast pool severely reduces myoblast fusion, the effect being most dramatic in the lateral muscles of the abdomen and, to a lesser extent, in the thoracic muscles. Nevertheless, *duf-lacZ*-expressing myoblasts are present in the correct number at the correct positions. Preparations of wild-type and *1151GAL4/UAS-Rac1N17* pupae are shown (Fig. 7A,B). In the absence of fusion, each putative founder cell begins to express myosin at the appropriate stage, elongates and differentiates into a thin myotube. Two such lateral founders (in different planes of focus) that have begun to extend processes are shown in Fig. 7C-E. Myoblasts that have failed to fuse cluster around the *duf-lacZ*-expressing cells and, as in the embryo, express myosin, but do not differentiate further (Fig. 7D,E). Wild-type lateral fibres with the founder nucleus in each fibre expressing *duf-lacZ* are shown for comparison (Fig. 7F). X-Gal staining does not detect the low *duf-lacZ* expression in the remaining nuclei of these fibres. But that these fibres are multinucleate is evident when observed at a higher magnification (Fig. 7G,H). In the absence of fusion, the founders eventually develop into mononucleate, myosin-expressing fibres (Fig. 7I), like the mononucleate muscles observed in the embryos of fusion mutants.

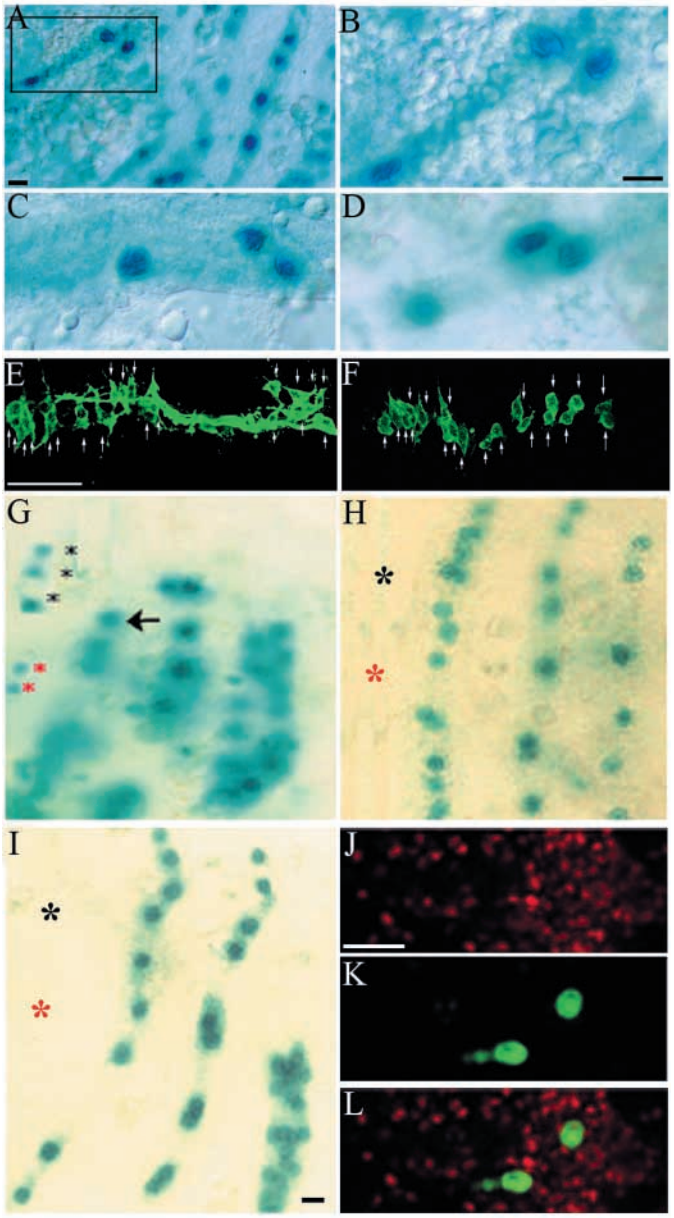

Fig. 8. Notch signalling and founder cell numbers. (A) Wild-type preparation of 24-hour APF *duf-lacZ* pupa (grown at 29°C). The box outlines *duf-lacZ*-expressing founders of DVM I fibres. (B) The *duf-lacZ* founders of DVM I (present within the outlined region in A) shown at a higher magnification. (C,D) *duf-lacZ*-expressing DVM I founders in animals expressing dnNotch (C) and MamN (D). The number of founders is unchanged. The relative orientation of the three *duf-lacZ*-expressing cells with respect to each other varies slightly in B, C and D. Such variation is also observed in wild-type preparations. (E,F) 22C10 staining in the dorsal A4 hemisegment of a N^{ts1}pupa grown entirely at permissive temperatures (E) and of a heat-pulsed N^{ts1} pupa (F), grown for 24 hours at 22°C and then pulsed for 4 hours and 36 minutes at non-permissive temperature (see Materials and methods for details). The small arrows indicate the 22C10-stained cells, whose number (20 in this case) remains unchanged despite reduced Notch function in N^{ts1} pupae. (G) A 12-hour APF preparation from a *duf-lacZ*-expressing pupa (grown at 29°C), showing the wild-type pattern of founders in DVMs and DLMs. The three black asterisks indicate the three DVM I founders, while the red asterisks show the two founders corresponding to DVM II. The arrow indicates *duf-lacZ* nuclei belonging to the larval template adjacent to the DVMs. (H) *duf-lacZ* expression in a pupa (similar age to that in G) in which Nintra has been expressed in all myoblasts. DVM founders are missing in these pupae. Bold asterisks mark the region where the founders are expected. (I) *duf-lacZ* expression in a *1151Gal4*/*UAS-Twi* pupa. Constitutive expression of Twist in the myoblasts results in an absence of DVM founders. (J-L) Complementary expression pattern of *duf-lacZ* and Twist in the myoblasts. (J) Confocal section of Twist-expressing myoblasts in the presumptive region of formation of DVM II. (K) Same image as that in J showing *duf-lacZ*-expressing founders of DVM II. (L) Merged image showing the absence of Twist immunoreactivity in the *duf-lacZ*-expressing nuclei. Anterior is to the top, dorsal midline to the right. Scale bars: in A, 9 μm; in B, 9 μm for B-D: in E, 25 μm for E,F; in I, 20 μm for G-I; in J, 15 μm for J-L.

The putative founders of the DVMs in the thorax of *1151GAL4/UAS-Rac1N17* pupae are also present in a wild-type pattern and initiate fibre formation, as shown in Fig. 7K. Some fusion does occur, but to a lesser extent than normal. The DVM II fibres shown in Fig. 7K are not mononucleate but have fewer nuclei than wild-type fibres of the same stage (Fig. 7J). These fibres ultimately give rise to muscles, albeit thin, at the correct position and with the correct number of fibres (Fig. 7M). These results suggest that where myoblast fusion is prevented during adult myogenesis, a population of *duf-lacZ*-expressing myoblasts segregates normally, as in the embryo, and that, like the founders in the embryo, these cells uniquely have the capacity to complete differentiation to form muscles. They also demonstrate that, as in the embryo, by the onset of fibre formation adult myoblasts are of two classes: fusion-competent cells that do not express *duf-lacZ* and founders that do express *duf-lacZ*. It is these latter cells that have the capacity to complete myogenic differentiation even when fusion is blocked or reduced.

Selection of *duf-lacZ*-expressing founders is not mediated by lateral inhibition during adult myogenesis

In the *Drosophila* embryo, the diversification of muscle forming mesoderm into founders and fusion-competent cells occurs through a process of lateral inhibition mediated by Notch (Corbin et al., 1991; Carmena et al., 1995; Carmena et al., 1998). As we have now shown that single *duf-lacZ*-expressing cells are selected and appear to act as founder myoblasts during adult myogenesis, it is important to show whether, as in the embryo, a Notch-dependent lateral inhibition pathway mediates this selection process. To test whether Notch has a function in selecting specific myoblasts for *duf-lacZ* expression, we used a dominant-negative and a constitutively active form of Notch. We reasoned that if lateral inhibition were involved, then overexpression of a dominant-negative form of Notch (dnNotch) in adult myoblasts would lead to an increase in the number of *duf-lacZ*-expressing founders, whereas overexpression of the active form (Nintra) should suppress *duf-lacZ* expression altogether.

In fact, the results of these experiments appeared to be contradictory: thus, expression of *UAS-dnNotch* caused no change in the number of DVM founders (Fig. 8C) and flies of the genotype *1151GAL4, UAS-dnNotch* had the correct number of DLM and DVM fibres (data not shown). We verified this conclusion by reducing Notch function in two additional ways.

We reduced function in the Notch signalling pathway in myoblasts by overexpressing truncated forms of the protein Mastermind (Mam), an essential component of the Notch signalling pathway. Mam interacts with the intracellular domain of Notch and with Suppressor of Hairless, and forms a transcriptional activation complex (Wu et al., 2000; Kitagawa et al., 2001). Two truncated versions of Mam, MamH and MamN, when overexpressed by the GAL4-UAS system behave as dominant-negative proteins and elicit Notch loss-of-function phenotypes (Helms et al., 1999). Overexpression of either *UAS-MamN* (Fig. 8D) or *UAS-MamH* (data not shown) in myoblasts using *1151-GAL4* had no effect on the number of DVM founders. We further examined the role of *Notch* by using a conditional allele, N^{ts1} (Shellenbarger and Mohler, 1975). Because of the close proximity of the *duf* and *Notch* loci, we could not generate *duf-lacZ*, N^{ts} recombinants and hence used 22C10 as the marker for founder cells in the abdomen. The earliest time at which myoblasts expressing high levels of *duf-lacZ* are also labeled with 22C10 is at 24 hours APF (Fig. 3A-C). We removed Notch function for different periods (2 hours, 4 hours, 6 hours and 8 hours) before this stage by raising N^{ts} animals to the non-permissive temperature and looked at the number of 22C10-stained cells associated with the abdominal nerves. The numbers of 22C10-expressing cells in the dorsal (Fig. 8F) or lateral segments (data not shown) of the abdomen were examined and shown to be unaffected in these experiments. We know that all three approaches – using the dominant-negative *Notch* or *mastermind* constructs, and using N^{ts} animals – are effective, as they all can reduce the levels of Twist expression in adult myoblasts (data not shown), a known consequence of *Notch* reduction in adult myoblasts (Anant et al., 1998).

Taken together, these results suggest that Notch is not required for the selection of *duf-lacZ*-expressing myoblasts. However, in the converse experiment, expression of Nintra in the myoblasts does suppress the formation of founders, as we would expect for a selection mechanism based on lateral inhibition (Fig. 8H). How can these apparently contradictory findings be reconciled? Earlier studies from our laboratory showed that Notch acts as a positive regulator of Twist in the myoblast population (Anant et al., 1998). Thus, Nintra expression in adult myoblasts leads to maintained expression of Twist in these cells and to a failure of muscle differentiation. If the absence of founders that we observe is a consequence of this sustained expression of Twist in the myoblasts, then we would expect that simply expressing Twist constitutively in the myoblasts would mimic the activated-Notch phenotype. Using *1151-GAL4* to drive Twist expression in the adult myoblasts, we found that at 12 hours APF there were no DVM founders (Fig. 8I), suggesting that a decline in Twist expression, which is antagonized by the action of Nintra, is a requirement for elevated *duf-lacZ* expression. Indeed, founders are the first cells in the myoblast pool to show declining levels of Twist expression, with the result that the *duf-lacZ*-expressing founders and Twist-expressing myoblasts are mutually exclusive cell populations (Fig. 8J-L).

Discussion

The spatial organization of myotube formation

A central issue for our understanding of myogenesis is the organization of muscle differentiation to produce a muscle pattern. In different organisms, the fusion of groups of myoblasts to form syncitial myotubes is clearly regulated in such a way that, at appropriate positions, a set number of fibres form, resulting in a well-defined pattern of muscles on which subsequent movement depends. In the *Drosophila* embryo, this patterning process largely depends on the controlled segregation of a specialized class of myoblasts, the founders, each of which seeds the formation of a myotube. However, each of the larval muscles that form in the embryo consists of a single myotube, and it could be argued that the founder myoblasts are a special feature of a system that generates thirty different myotubes from the mesoderm of a single hemisegment. In the adult fly, however, the situation is more akin to that found in many vertebrates and other organisms, with each muscle consisting of multiple myotubes that associate together to form a larger contractile element. The question we have addressed here is a general one for all such systems, namely how the number and location of the forming myotubes is controlled in such a multi-fibre system.

An essential feature of embryonic myogenesis in the fly is the division of muscle-forming mesoderm into myoblasts of two kinds: founders and fusion-competent cells (Baylies et al., 1998). During embryogenesis, fusion is an asymmetric process in which founders and fusion-competent cells fuse with each other, but neither class can fuse with itself. This ensures that, wherever a founder segregates, it acts as a seed for the formation of a single myotube. During adult myogenesis, groups of myoblasts aggregate to form muscles consisting of multiple myotubes: our question is therefore, how does a fixed number of myotubes arise from this aggregate? Is it by the formation of an appropriate number of founder myoblasts and, if so, how is this controlled? Or is it by some totally different process that might be of general relevance for the formation of such multi-fibre aggregates?

Our results show that in the adult fly multi-fibre arrays arise by the choice of an appropriate number of founder cells, identifiable in the adult by the elevated expression of *duf-lacZ*.

A marker for embryonic founders is expressed during adult myogenesis

An earlier study of myogenesis in the adult thorax suggested on morphological grounds that cells equivalent to founder myoblasts in the *Drosophila* embryo were involved in the formation of at least three muscles: the tergo-trochanteral (jump) muscle, and DVMs I and II (Rivlin et al., 2000). In addition Kozopas and Nusse showed *duf-lacZ* expression in one kind of adult muscle, the developing direct flight muscles (DFMs), but did not examine the function of these cells nor address the mechanisms by which they are selected (Kozopas and Nusse, 2002). We decided to extend these studies by looking, during adult development, at the expression of a marker for embryonic founders, *duf-lacZ*. By early pupal stages we were clearly able to identify selective expression of *duf-lacZ* in both the thoracic and abdominal myoblasts. Furthermore, in both cases the pattern of expression mirrored the pattern of emerging fibres. Thus, where two or three fibres form, as in the DVMs, we saw two or three *duf-lacZ*-expressing cells amongst the aggregating myoblasts; where an array of fibres form, as in the lateral and dorsal musculature of the abdomen, we saw an array of single cells expressing the

marker, prior to the formation of syncitial myotubes. We also saw that these *duf-lacZ*-expressing cells were able to seed individual fibres in the absence of fusion, or where fusion was severely reduced. These findings emphasize not only the presence of a specialized class of myoblasts amongst the adult muscle-forming cells, but suggest that these cells, one per fibre, are the founder myoblasts. In addition, because of the known function of Duf as an attractant for myoblasts (Ruiz-Gomez et al., 2000), it seems likely that the same patterns of asymmetric gene expression that characterize founders and fusion-competent cells prior to fusion in the embryo are recapitulated in the adult muscle-forming population as syncitial myotubes are formed.

We were surprised to find that *duf-lacZ* is also expressed in what have previously been thought of as analogues of founders, namely the three persistent larval muscles that act as templates in each hemithorax, organizing the development of the large DLMs from the swarms of adult myoblasts that aggregate about them as metamorphosis begins. We assume that *duf* expression in these cells serves to attract the adult myoblasts to the templates, with which they then fuse to form the six fibres of the adult DLMs.

The onset of *duf-lacZ* expression in adult myoblasts

At the end of the third larval instar, *duf-lacZ* is detected at a low level in most, if not all, adult myoblasts. This expression disappears and is replaced by selective expression at a much higher level in the cells that we identified as founder myoblasts, as we have described and discussed above. We suspect that this initially uniform expression at a low level may reflect the origins of the adult myoblasts from lineages that generate muscle founder cells in the embryo. In those cases that have been studied in detail, it has been shown that the pools of myoblasts from which adult myotubes will form, arise from a small number of adult muscle precursor (AP) cells in the embryo. In the case of the ventral abdominal muscle of the adult, for example, it can be shown that, in each hemisegment, the pool of myoblasts that will generate the several myotubes that make up this muscle are all derived from a single ventral AP cell in the embryo (Ruiz-Gomez and Bate, 1997) (reviewed by Baylies et al., 1998). This cell in turn is the sibling of the founder myoblast that seeds the formation of larval muscle VA3. This lineage is typical of the many muscle lineages that generate muscle-forming cells in the embryo: the terminal division in each lineage generates either two sibling founder myoblasts (e.g. VA1 and VA2), or a founder and an adult precursor (VA3 and VAP). While the founders manifest their muscle forming potential in the embryo, express *duf-lacZ* and seed myotubes, the muscle-forming potential of the AP cells is suppressed. These cells proliferate in the larva, and first differentiate during metamorphosis as adult muscle formation begins in the pupa. Thus the adult myoblasts are clonal descendants of single cells that are themselves the products of founder myoblast-generating lineages in the embryo. We suggest that in the hormonal environment of the third larval instar, aspects of the founder lineage of these clones begin to be expressed. However, uniform expression of *duf-lacZ* in a population of myoblasts has no apparent functional sense. Therefore, the uniform pattern of expression (perhaps reflecting the developmental history of the cells concerned) must be replaced by local upregulation in a few cells that will act as founders and downregulation in other myoblasts that will now respond to the localized Duf signal. This is the sequence that we observe in both the abdomen and the thorax, and we suggest that it is the control of this process that is decisive for the formation of the correct pattern and number of myotubes.

Regulation of *duf-lacZ* expression in the adult myoblasts: the role of Notch-mediated lateral inhibition

Our experiments indicate that a lateral inhibition mechanism such as that which leads to the segregation of muscle progenitor cells in the muscle forming mesoderm of the embryo is not responsible for the later segregation of *duf-lacZ*-expressing cells from among the adult myoblasts. For these experiments, we focused our attention on the de novo segregation of the single cells that appear to seed the formation of the DVM fibres. We found that if Notch signalling is removed or blocked by the expression of dominant-negative constructs in the adult myoblasts, there was no effect on the segregation of an appropriate number of cells expressing *duf-lacZ* at the sites where the DVM or abdominal fibres are formed. This is in stark contrast to the effects of reduced levels of Notch in the embryo, which lead to an overproduction of founder cells expressing *duf-lacZ*. However, the DVM founder myoblasts are lost when Notch is constitutively active in the adult myoblasts and this resembles effects seen in the embryo when Notch is constitutively expressed. We suspect that this loss of adult founders reflects an indirect effect of Notch, which, when activated, maintains *twist* expression in the myoblasts concerned. We find that persistent expression of *twist* alone blocks the appearance of founder myoblasts in the adult. Our observations lead us to conclude that lateral inhibition mediated by Notch is unlikely to be the mechanism underlying the segregation of the adult founder myoblasts.

How is *duf-lacZ* expression regulated?

As lateral inhibition does not appear to select cells from the muscle-forming population for *duf-lacZ* expression, we consider two other putative sources of muscle patterning cues: the epidermal sites at which individual muscle fibres will attach and the nerve fibres that will innervate them. In the thorax, *duf-lacZ* expression in the larval templates for the DLMs is first seen at the time *stripe*-expressing adult epidermal cells (Lee et al., 1995; Fernandes et al., 1996) are juxtaposed adjacent to the templates (A. Ghazi, unpublished). Preliminary results indicate that reduction of the number of *stripe*-expressing cells results in a reduction of *duf-lacZ* nuclei in the LOMs, and increasing *stripe* expression increases *duf-lacZ* expression (A. Ghazi, unpublished). The large number of *stripe*-expressing cells that attach to each thoracic fibre make the decisive experiments (complete removal of *stripe*-expressing tendon cell precursors and misexpression of *stripe* in a large ectopic domain) difficult to perform. The role of tendon cells, if any, in founder selection or *duf* expression may have to wait for other approaches that shed light on the signalling pathways involved.

Innervation might also play an important role in fibre formation through the mediation of *duf* expression. Laser ablation experiments have shown that DLMs can be formed even if the normal larval templates have been ablated. However, if the larval templates and the innervation are both removed then the DLMs fail to form (Fernandes and

Keshishian, 1998). This suggests that, where muscles form de novo, innervation is an essential ingredient for the initiation of fibre formation and may therefore play a role in the selection of *duf-lacZ*-expressing founder myoblasts from the adult myoblast population. This view is reinforced by the finding that, when there is no innervation, the DVMs, which normally form de novo, do not develop at all (Fernandes and Keshishian, 1998). It is well known that innervation is essential for the formation of the male specific muscle (MSM) in the abdomen (Lawrence and Johnston, 1986). There is a close association between nerve fibre branches and forming muscle fibres in the abdomen, and the MSM is itself a local aggregation of such muscle fibres. Now that we have a marker that identifies the earliest stages of fibre formation, we plan further experiments to investigate the part played by innervation and attachment in selecting the cells that seed fibre formation.

The broad conclusion is that an external cue from the region where the muscle is destined to form is likely to set the number of contributing myotubes [one external cue, Wingless, is required for maintenance of identity of groups of myoblasts (Sudarsan et al., 2001), but we have not yet established what external cues act to select individual myoblasts from this pool]. In *Drosophila*, this process seems to be mediated by the selection of *duf*-expressing founder cells, each of which seeds the formation of a fibre. The number and pattern of fibres could then be set by the strength and distribution of the founder-inducing cue. We can envisage a similar process operating in vertebrate myogenesis as myoblasts aggregate and fuse to form a pattern of primary myotubes. Whether here too the patterning of fibres depends on the induction of founder or seed myoblasts at sites of muscle formation is an important question that remains to be resolved.

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