Patterning Muscles Using Organizers: Larval Muscle Templates and Adult Myoblasts Actively Interact to Pattern the Dorsal Longitudinal Flight Muscles of *Drosophila*

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Abstract. Pattern formation in muscle development is often mediated by special cells called muscle organizers. During metamorphosis in *Drosophila*, a set of larval muscles function as organizers and provide scaffolding for the development of the dorsal longitudinal flight muscles. These organizers undergo defined morphological changes and dramatically split into templates as adult fibers differentiate during pupation. We have investigated the cellular mechanisms involved in the use of larval fibers as templates. Using molecular markers that label myoblasts and the larval muscles themselves, we show that splitting of the larval muscles is concomitant with invasion by imaginal myoblasts and the onset of differentiation. We show that the Erect wing protein, an early marker of muscle differentiation, is not only expressed in myoblasts just before and after fusion, but also in remnant larval nuclei during muscle differentiation. We also show that interaction between imaginal myoblasts and larval muscles is necessary for transformation of the larval fibers. In the absence of imaginal myoblasts, the earliest steps in metamorphosis, such as the escape of larval muscles from histolysis and changes in their innervation, are normal. However, subsequent events, such as the splitting of these muscles, fail to progress. Finally, we show that in a mutant combination, null for Erect wing function in the mesoderm, the splitting of the larval muscles is aborted. These studies provide a genetic and molecular handle for the understanding of mechanisms underlying the use of muscle organizers in muscle patterning. Since the use of such organizers is a common theme in myogenesis in several organisms, it is likely that many of the processes that we describe are conserved.

Many major themes of developmental biology are illustrated by the formation of skeletal muscles. Myogenesis involves a precisely choreographed sequence of cell lineage–dependent specification, cell proliferation, migration, cell–cell interactions, and differentiation. Multinucleated muscle fibers arise from the fusion and differentiation of mononucleated progenitors, myoblasts. Muscle fibers differ from each other in their position, innervation, patterns of gene expression, and physiological properties. Myoblasts must be able to seek out epidermal sites of muscle formation, fuse to form properly oriented fibers, and get appropriately innervated to give rise to the precise pattern that is observed in the mature animal. One way to understand how such diversity is generated and how muscle pattern is organized is to systematically screen for mutations that affect different developmental stages of particular muscle fibers and then analyze what roles these genes play during the normal development of these muscles.

A system ideally suited for such analyses are the indirect flight muscles (IFMs)[1] of the fruit fly *Drosophila melanogaster*. The two groups of the IFMs, the dorsal longitudinal muscles (DLMs) and the dorso-ventral muscles (DVMs) have distinct developmental histories: the DLMs develop using persistent larval muscles as scaffolds (Tiegs, 1955; Shatoury, 1956; Shafiq, 1963; for review see Crossley, 1978), whereas the DVMs are constructed de novo by the fusion of imaginal myoblasts (Fernandes et al., 1991). The availability of several reporter genes and antibody probes that label to reveal different stages of development of these muscles (Fernandes et al., 1991; Barthmaier and Fyrberg, 1995), their innervation (Fernandes and VijayRaghavan,

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1. *Abbreviations used in this paper*: APF, after puparium formation; DLM, dorsal longitudinal muscle; DVM, dorso-ventral muscle; *ewg,* erect wing; IFM, indirect flight muscle; *MHC*, myosin heavy chain; UAS, upstream activating sequence.

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1993), and attachment (Fernandes et al., 1996), thus provide important tools for a molecular and genetic analysis of the mechanism of their development (Fernandes et al., 1994; DeSimone et al., 1996; Roy and VijayRaghavan, 1997; Roy et al., 1997; Sandstrom et al., 1997).

The progenitors of the IFMs are associated with the wing imaginal disc during larval life and, during early pupation, these myoblasts divide and spread over the developing dorsal mesothorax at the sites of muscle formation (Bate et al., 1991; Fernandes and VijayRaghavan, 1993). At the onset of pupation, while all other larval muscle fibers in the thoracic segments undergo histolysis, three muscles, dorsal oblique 1, 2, and 3 persist and split into six templates that serve as scaffolds for DLM development (for larval muscle nomenclature see review by Bate, 1993). Further fusion of myoblasts with these templates results in the elaboration of the final DLM pattern of six fibers (Fernandes et al., 1991). These early events in the development of the DLMs are summarized diagrammatically in Fig. 1. The transformation of larval muscles into DLM templates is best visualized by staining for β-galactosidase activity that perdures after larval expression of the enzyme from the myosin heavy chain (*MHC*) promoter has ceased (Fernandes et al., 1991). During early pupation, at ~10–12 h after puparium formation (APF), the persistent larval muscles appear vacuolated (see Fig. 2 *a*). These vacuoles are probably the surface blebbing described in early studies of the transformation of these muscles, and of similar muscles in other dipterans (for review see Crossley, 1978). Subsequently, at ~15 h APF, each muscle fiber splits along its longitudinal axis, the dorsal-most muscle splitting last, resulting in the formation of six templates (see Fig. 2 *b*). At the time these changes take place in the larval muscles, metamorphic changes occur in the motor nerves that innervate these muscles. The motor innervation of the persistent larval muscles that form part of the intersegmental nerve fiber withdraw their neuromuscular synaptic contacts and, as the muscles split and myoblasts fuse to form the DLM fibers, new axonal branches develop to innervate them (see Fig. 1; also see Fernandes and VijayRaghavan, 1993). An analogous series of events that delineate the attachment of the DLM fibers to the epidermis with particular reference to the *stripe* gene has been described (Fig 1; also see Fernandes et al., 1996) and a detailed and critical electron and light microscopic analysis of this process is also available (Reedy and Beall, 1993*b*).

The role of the persistent larval muscles in the development of the DLMs has been addressed systematically only recently. Laser ablation experiments have suggested that although DLM development can proceed apparently normally in their absence, the larval muscles are required for regulating the numbers of DLM fibers formed, and in this sense are important in determining certain aspects of pattern in the developing DLMs (Farrell et al., 1996; Fernandes and Keshishian, 1996). The molecular mechanisms involved in the splitting of the larval muscles into templates for DLM development are unknown. From the sequence of events and interactions of various tissues alluded to above, at least three mechanisms, not mutually exclusive, appear plausible: (*a*) the splitting process is an autonomous property of the persistent larval muscles, additionally involving its interaction with the epidermis and perhaps even the nervous system, but not the imaginal myoblasts themselves; (*b*) inductive instructions from the metamorphosing motor nerves mediate splitting and are essential for the process to proceed. The close apposition of myoblasts and nerves, both in larval life as evidenced by nerve-associated myoblasts in the thorax and during pupal development (Fernandes and VijayRaghavan, 1993) further strengthen this possibility; and (*c*) interactions with imaginal myoblasts are essential, and somehow engender the transformation of these muscles.

In this study we show that the transformation of residual larval muscles during DLM development is mediated by the activity of imaginal myoblasts and is not an autonomous property of the larval muscles themselves. We show that the Erect wing (Ewg) protein is expressed in larval muscle nuclei remaining in the developing adult fiber. This, and the absence of splitting in an *ewg* mutant combination, suggests that myoblasts and templates interact actively to pattern the DLM fibers. Our results throw light on the cellular processes involved in the early morphogenetic events of a set of adult muscles in *Drosophila* that develop using muscle organizers, and sets the stage for the identification and analysis of other genes that regulate such processes.

Materials and Methods

Fly Strains

The Canton S strain was used as the wild-type strain in all control experiments unless otherwise mentioned. The *MHC-lacZ* transgenic strain contains the regulatory regions of the *Drosophila* muscle *MHC* gene fused to the *lacZ* reporter and is expressed in all embryonic, larval, and adult muscle fibers (Hess et al., 1989; Fernandes et al., 1991). The *actin (88F)-lacZ* strain has an in-frame gene fusion of the IFM-specific *actin (88F)* gene to the *lacZ* gene (Hiromi et al., 1986; Fernandes et al., 1991). The *twist-lacZ* transformant strain carries a transgene that consists of the regulatory domains of the *twist* gene fused to the *lacZ* gene (Thisse et al., 1991; DeSimone et al., 1996).

The X chromosome *1151-GAL4* enhancer trap strain was obtained from L.S. Shashidhara (Centre for Cellular and Molecular Biology, Hyderabad, India) and its expression pattern was examined by using the reporter strain upstream activating sequence *UAS-lacZ* (Brand and Perrimon, 1993). We have found that this strain can drive β-galactosidase expression from this reporter in the adult muscle precursors associated with the imaginal discs and nerves in the larvae, and in almost all the developing and differentiated adult muscle fibers (Roy and VijayRaghavan, 1997; Anant et al., 1998).

The *UAS-p^{21}* strain was kindly provided by I. Hariharan (Massachusetts General Hospital Cancer Centre, Charlestown, MA). This strain carries a transgene insert on the X chromosome that consists of a cDNA of the human cyclin dependent kinase inhibitor *p^{21}* gene (Harper et al., 1993) placed under the control of the GAL4 protein-responsive UAS.

Two different strains containing a null allele of *ewg* (*ewg^{l1}*)were used: *ewg^{l1}, y, cho, sn*; *NS^4* and *ewg^{l1}, y, w, sn; NS^4*. *NS^4* represents a transgene that consists of an *ewg* cDNA under the control of the regulatory regions of the *Drosophila* neuron-specific gene *embryonic lethal abnormal visual system* (DeSimone et al., 1996). This transgene provides *ewg* vital function that is required in neurons for embryonic viability, and since its expression is restricted only to neurons, the presence of this transgene in the background of the *ewg^{l1}* null allele effectively produces an *ewg* null condition in the mesoderm.

Antibody Labeling and β-Galactosidase Histochemistry

Pupal and larval tissues were prepared for immunohistochemistry as described previously (Fernandes et al., 1991). Anti-Ewg antibody raised in rabbit was used at a dilution of 1:500 (DeSimone et al., 1996), an anti–β-galactosidase monoclonal antibody (Promega Corp., Madison, WI) was used at

a dilution of 1:250, an anti-Twist antibody raised in rabbit (gift of S. Roth, Max Planck Institute, Tübingen, Germany) was used at a dilution of 1:5,000 and an antihorseradish peroxidase antibody raised in rabbit (Sigma Chemical Co., St. Louis, MO), was used at a dilution of 1:4,000. The labeling reactions were developed using either the Vectastain ABC immunoperoxidase kit (Vector Labs, Inc., Burlingame, CA) according to the manufacturer's instructions, or using appropriate fluorophore-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Histochemical staining for β-galactosidase expression was done according to standard procedures (Fernandes et al., 1991).

Microscopy

After antibody labeling and β-galactosidase histochemistry, tissue preparations were mounted in 75% glycerol and examined using a Leitz Aristoplan microscope (Wetzlar, Germany) equipped with Nomarski (differential interference contrast) optics or laser scanning confocal microscopy (MRC-600; Bio-Rad Laboratories, Hercules, CA) using a Zeiss Axiophot microscope (Carl Zeiss, Inc., Thornwood, NY).

Results

Postfusion Columnar Organization of Ewg-expressing Myoblast Nuclei Marks the Onset of DLM Differentiation

Our earlier studies have shown that the *ewg* gene is required for IFM development (DeSimone et al., 1996). These experiments also showed that *ewg* expression can be detected in the imaginal myoblasts as early as 10 h APF when these cells are swarming over the persistent larval muscles. We therefore used antibodies to the Ewg protein to label myoblasts and antibodies to β-galactosidase to label the persistent larval muscles in pupae of flies expressing the reporter enzyme from the *MHC* promoter to examine the process of splitting more closely. Optical sections of double-labeled pupal preparations with antibodies to Ewg and β-galactosidase at 10–12 h APF revealed Ewg expression in the nuclei of the imaginal myoblasts that have fused with the larval muscles (Fig. 3 *a*). Similar labeling experiments at a later developmental stage (15–16 h APF) showed Ewg-expressing myoblast nuclei and associated cytoplasm organizing themselves into two neat longitudinal columns in each larval muscle (Fig. 3 *b*). These longitudinal columns of imaginal myoblast nuclei with the associated cytoplasm actually represent the splitting larval muscles that are observed on histochemical staining for β-galactosidase in the *MHC-lacZ* transformant strain (Fig. 2 *b*; also see Fernandes et al., 1991).

Ewg and Actin (88F)-lacZ Labeling Reveal That the Columnar Organization of Nuclei Are Early Differentiating DLMF Fibers

The splitting of the persistent larval muscles can also be revealed using histochemical staining for β-galactosidase expression from the actin (88F) promoter that is first expressed at ~14–15 h APF in the splitting larval muscles (Fig. 3 *c*; Fernandes et al., 1991). This actin gene is predominantly expressed in the IFMs, and the initiation of the actin (88F)-lacZ reporter activity is an indication of the onset of flight muscle differentiation programme (Hiromi et al., 1986; Fernandes et al., 1991). Double-labeling experiments with antibodies specific to Ewg and β-galactosidase on 14–15 h APF pupae carrying the actin (88F)-lacZ transgene revealed that the longitudinal columns that form by the aggregation of Ewg-expressing nuclei actually represent nascent myofibers that have begun to differentiate and express the actin (88F) gene (Fig. 3 *d*). This analysis of the behavior of Ewg expressing myoblasts during early stages of DLM development suggests that the process of larval muscle splitting involves myoblast fusion with the persistent larval muscles, and the organization of their nuclei into longitudinal columns with the associated cytoplasm, followed by the expression of structural components of the myofiber.

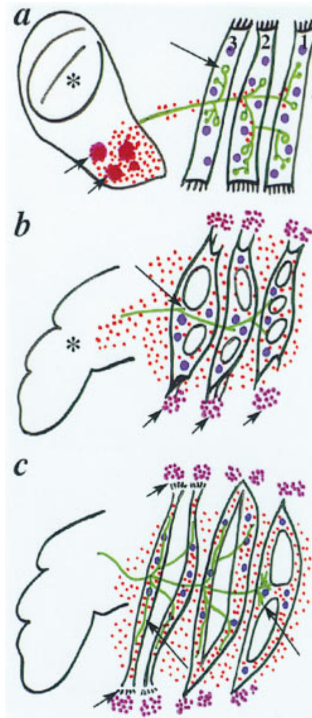

Figure 1. Diagrammatic representation of the early developmental events in the patterning of the DLMs. (*a*) During larval life, myoblasts (*red dots*) that give rise to the DLMs and other dorsal mesothoracic muscles remain sequestered in the wing disc (*asterisk*) and associated with motor nerves (*green*) innervating the larval muscles, dorsal oblique *1*, *2*, and *3*. The attachment sites for the IFMs are prefigured on the notal epithelium of the wing disc by expression of stripe in groups of cells (*short arrows*). *Blue*, larval muscle nuclei; *long arrow*, a synaptic bouton on the larval muscle. *Top*, anterior; *vertical green arrow*, dorsal midline. Orientation of the subsequent panels is similar. (*b*) During early pupal development (6–10 h APF), the myoblasts migrate out from the everting wing disc (*asterisk*) and swarm over the three remnant larval fibers, which unlike other larval muscles, escape histolysis. The larval muscles appear vacuolated, and there is a regression of the synaptic terminals of the motor neurons (*long arrow*). The stripe expressing epidermal cells (*purple spots*) that will become the attachment sites for the DLMs position themselves adjacent to the larval muscles (*short arrows*, posterior attachment sites). (*c*) By 16 h APF, the larval fibers have begun to split longitudinally to form the templates for DLM development, and the motor neurons send out fresh arborizations over the splitting muscles (*long arrows*). Filopodial extensions from the ends of the templates anchor them to the attachment sites on the epidermis (*short arrows*). Fusion of imaginal myoblasts with the templates results in further elaboration of DLM pattern.

Larval Muscle Nuclei Do Not Degenerate and Express Ewg during Metamorphosis

Interestingly, Ewg expression is seen in the nuclei of the larval muscles themselves during early development of the DLMs (refer to Fig. 3 *a*). The polyploid larval muscle nuclei are larger than imaginal myoblast nuclei and can be easily distinguished from the latter (Crossley, 1978). During development of the DLMs, the larval muscle nuclei align themselves with the columns of imaginal nuclei in the cytoplasm of the larval muscles as they split into two longi-

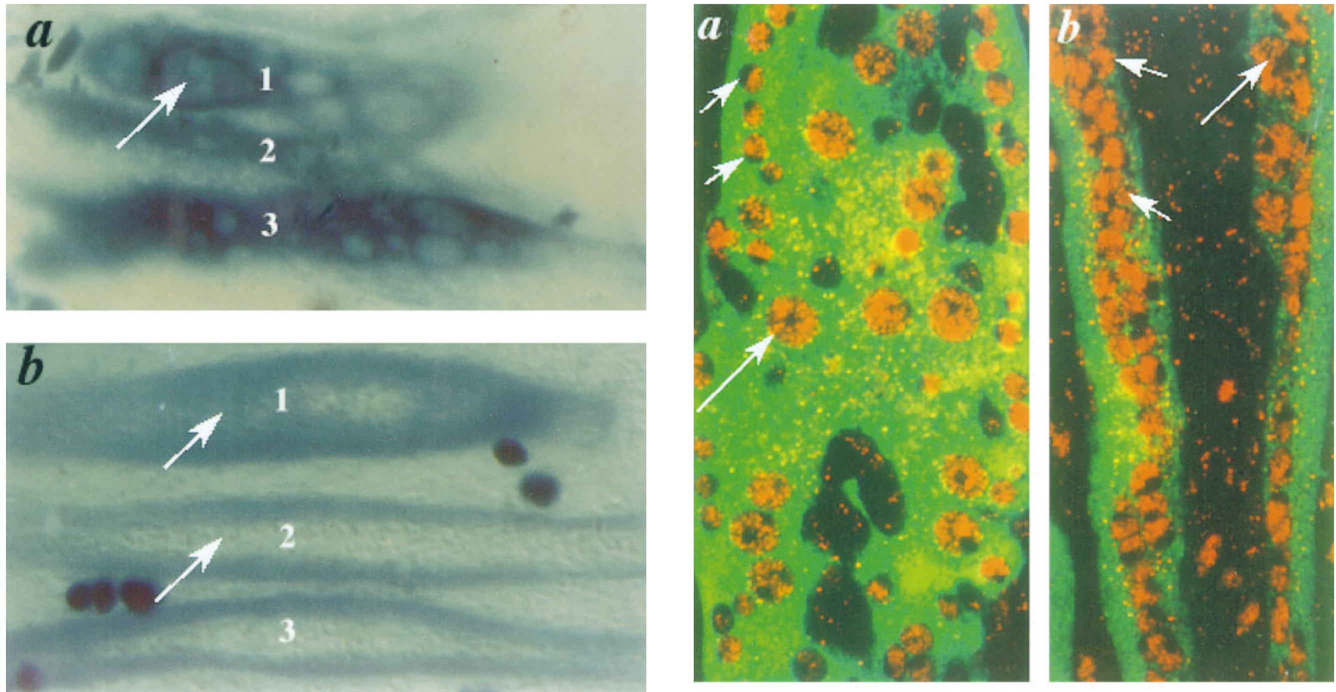

Figure 2. Transformation of the persistent larval muscles during early development of the dorsal longitudinal muscles. (*a*) A 12-h APF pupal preparation showing characteristic morphological changes in the larval muscles (*1*, *2*, and *3*) just before splitting as visualized by staining for β-galactosidase expression from the MHC promoter in the MHC-lacZ transformant strain. Note the presence of vacuole-like structures in these muscles (*arrow*). These could actually be surface blebs that have been described in early light microscopic observations (for review see Crossley, 1978). (*b*) At 15 h APF, the process of splitting is underway. The ventral fibers (*2* and *3*) have split and the dorsal-most fiber (*1*) is in the process of splitting. *Arrows*, spaces between the split muscles. In both panels only one hemisegment is shown. *Top*, dorsal midline; *left*, anterior.

tudinal strands enveloping the nuclei (refer to Fig. 3, *b* and *d*). At the very least, these results identify larval nuclei as present during adult muscle development and show that the remnant larval muscles are not mere bags of membrane. In addition, they suggest that these nuclei may not merely have the ability to localize Ewg protein from fusing myoblasts, but perhaps themselves are transcriptionally active. These possibilities are discussed later (see Discussion).

Larval Muscles Do Not Split in Absence of Imaginal Myoblasts

Imaginal myoblasts, specified in the embryo, divide actively during larval life to produce large clusters of cells

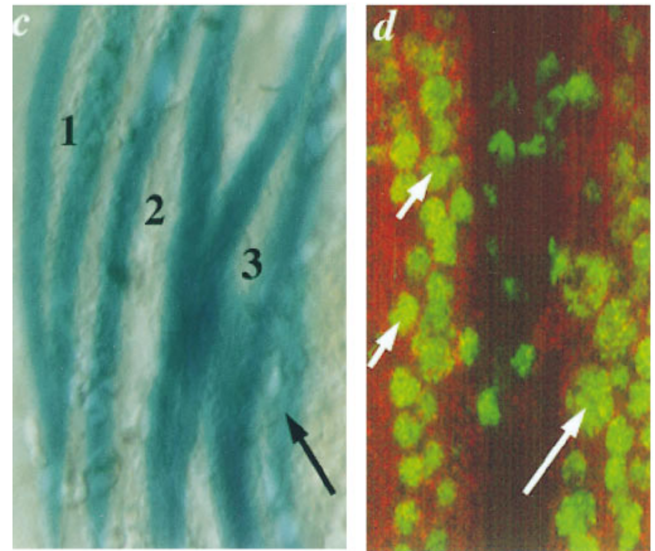

Figure 3. Ewg-expressing myoblasts fuse with the remnant larval muscles and split them into longitudinal columns that form nascent myofibers and express muscle differentiation genes. (*a*) An optical section of a 12- h APF preparation showing Ewg expression in the nuclei of imaginal myoblasts that have fused with a persistent larval muscle (*red*, *short arrows*, *small nuclei*). Ewg expression is also observed in the nuclei of the larval muscle (*red*, *long arrow*, *large nucleus*). *Green labeling*, cytoplasmic β-galactosidase expression from the *MHC* promoter in the larval muscle. (*b*) An optical section of a 15-h APF preparation showing the organization of the Ewg expressing nuclei (*red*, *short arrows*) into two longitudinal columns in the larval muscle. *Long arrow*, larval nucleus. The cytoplasm of the larval muscle (*green*, labeled by β-galactosidase expression from the *MHC* promoter) partitions into longitudinal strands enveloping the nuclei. Compare this figure with the splitting larval muscles as revealed by histochemical staining for β-galactosidase expression from the *MHC* promoter at 15 h APF in Fig. 2 *b*. Note that the split muscle strands in Fig. 2 *b* correspond to the longitudinal columns of nuclei and associated cytoplasm in 3 *b*. In *a* and *b*, only the dorsal most larval muscle (dorsal oblique *1*) is shown. (*c*) A 16-h APF pupal preparation showing larval muscle splitting by histochemical staining for β-galactosidase expression from the *actin (88F)* promoter. The split muscles are numbered. The arrow indicates the position of a developing DVM. (*d*) Double labeling for Ewg and β-galactosidase expression in the *actin (88F)-lacZ* transgenic strain showing β-galactosidase expression (*red*) in the cytoplasm associated with the longitudinal columns of Ewg expressing nuclei (*green*). *Short arrows*, imaginal myoblast nuclei; *long arrow*, persistent larval nucleus. *Top*, anterior; *left*, dorsal midline.

that remain associated with the imaginal discs and motor nerves innervating larval muscles (Bate et al., 1991; Fernandes and VijayRaghavan, 1993). During pupal development, these cells exit from cell cycle and fuse to form adult muscles. To investigate the role of imaginal myoblasts in the splitting of the larval muscles into DLM templates, we selectively eliminated these cells during larval development by inhibiting their proliferation. To do this, we ectopically expressed a cell cycle inhibitor protein, the human cyclin dependent kinase inhibitor, p^{21} (Harper et al., 1993), in proliferating imaginal myoblasts during larval life. A previous study has shown that p^{21}, when ectopically expressed in dividing precursor cells in the eye imaginal disc of *Drosophila,* can strongly inhibit their proliferation (de-Nooij and Hariharan, 1995). We have used the GAL4-UAS system (Brand and Perrimon, 1993) to target p^{21} expression to imaginal myoblasts during early larval development using an enhancer-trap GAL4 strain *1151* (refer to Materials and Methods and the references therein on the characterization of the domains of expression of *1151*) and a *UAS-p^{21}* transgene. The effect of p^{21} misexpression on the proliferation of myoblasts associated with wing imaginal discs was assayed using the *twist-lacZ* reporter gene and also with antibodies raised against the Twist protein. Ectopic expression of p^{21} in the wing disc–associated myoblasts strongly inhibited their proliferation, and very few cells on the wing discs of late third instar larvae labeled with the above markers were detected (Fig. 4, *a–d*). Similar results were obtained when these discs were labeled with antibodies against the Cut protein that is expressed in the disc-associated adult myoblasts in a pattern similar to Twist (Blochlinger et al., 1993; data not shown). These observations demonstrate the efficacy of ectopic expression of p^{21} in arresting division and thereby eliminating the bulk of imaginal myoblasts.

We next examined the effects of myoblast depletion on transformation of the persistent larval muscles into DLM templates. First, the larval muscles escape histolysis as in the normal development, indicating that this process is not mediated by myoblasts. This is pertinent because the *1151* driver is also expressed in nerve-associated myoblasts, although not in larval muscles themselves (Anant et al., 1998). Examination of *1151/UAS-p^{21}* pupae at ~16 h APF revealed that, in the absence of myoblasts, the splitting process was inhibited (Fig. 5 *a*). Using antibodies against the Ewg protein few, if any, myoblasts were found to be associated with these muscles (Fig. 5, *b* and *c*). At a slightly later time in development, the muscles degenerated (data not shown). This result suggests that the transformation of the larval muscles into templates for DLM development is mediated by interactions with imaginal myoblasts and is not an autonomous property of the larval muscles themselves. It also emphasizes the fact that the predominant function of the persistent larval muscles could be to func-

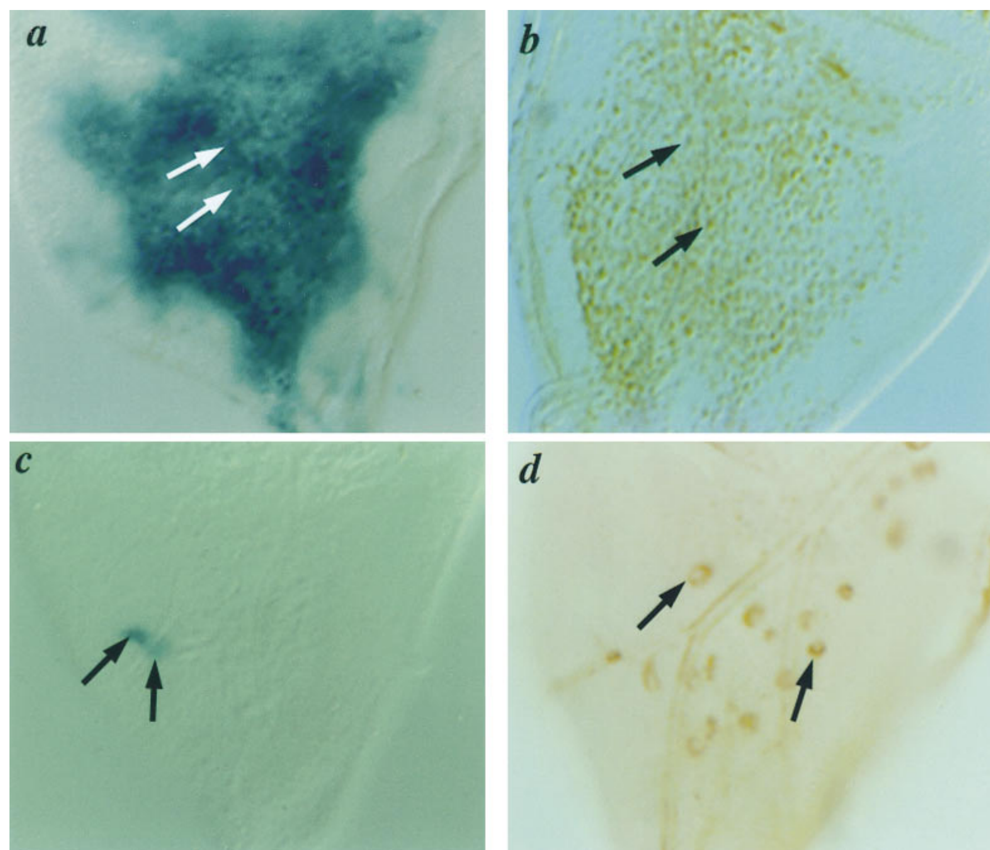

Figure 4. Ectopic expression of the cyclin dependent kinase inhibitor p^{21} inhibits adult myoblast divisions on the wing disc. (*a* and *b*) Notal region of a wild-type wing imaginal disc showing the large population of imaginal myoblasts as revealed by staining for *twist-lacZ* expression (*a*) and for expression of the Twist protein (*b*) (*arrows*). These cells will give rise to the DLMs and other muscles in the mesothorax. (*c* and *d*) Misexpression of p^{21} using *UAS-p^{21}* transgene and a GAL4 line that specifically expresses in the wing-disc associated myoblasts strongly inhibits their divisions. Such wing discs, when stained for *twist-lacZ* expression (*c*), or when labeled with anti-Twist antibodies (*d*), reveal the presence of very few adult myoblasts (*arrows*). Note that some of the myoblast nuclei in *1151/UAS-p^{21}* discs appear larger compared to those of wild-type myoblasts and this could possibly be due to DNA replication without concomitant cell division on ectopic p^{21} expression in these cells.

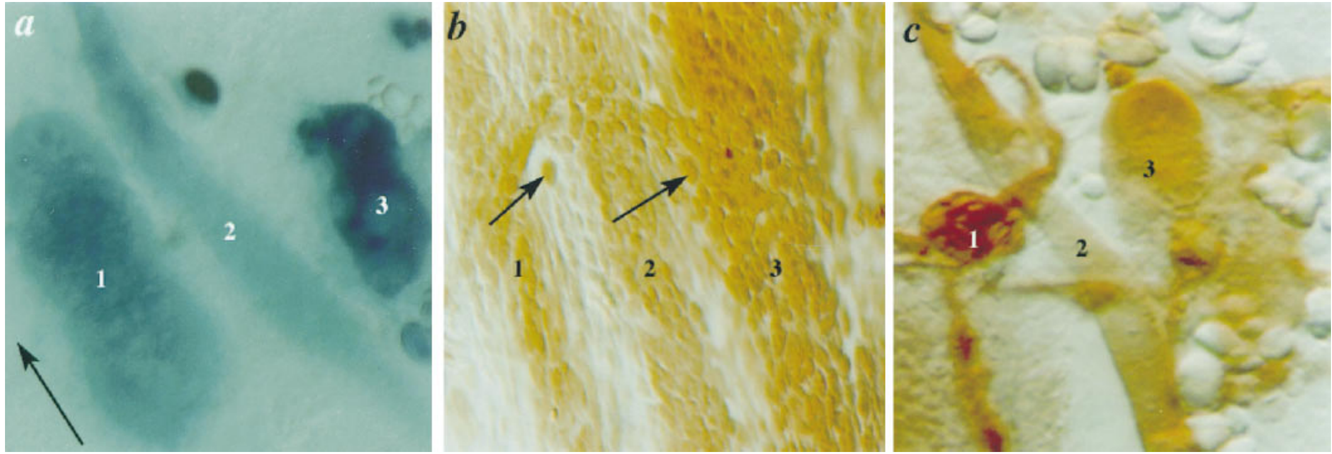

Figure 5. In the absence of imaginal myoblasts, the larval muscles do not split during pupation. (*a*) The larval muscles at 15 h APF visualized by staining for β-galactosidase expression from the MHC promoter in the absence of imaginal myoblasts from the wing disc. Note the absence of splitting in these muscles. The muscles are numbered. Note that muscle *3* is in the process of degeneration. Compare with the wild-type preparation in Fig 2 *b*. *Arrow*, dorsal midline; *left corner*, anterior. (*b*) Ewg expression in imaginal myoblasts (*arrows*) in a wild-type pupal preparation at 14 h APF over the larval muscles (*1*, *2*, and *3*). Note the presence of large numbers of myoblasts over the larval muscles, revealed by Ewg immunoreactivity. (*c*) A 14-h APF 1151/UAS-p^{21} pupal preparation stained with anti-EWG antibodies as in *b*, showing almost complete absence of imaginal myoblasts. Note that in the absence of these myoblasts the degenerating larval muscles are readily visible (*1*, *2*, and *3*). Orientations of *b* and *c* are as in *a*.

tion as positional cues for the imaginal myoblasts for DLM development, and they are, by themselves, incapable of developing into even rudimentary adult muscles although they have remnant larval nuclei and are innervated.

Larval Muscles Do Not Split in an Ewg Mesoderm-null Mutant

The accessibility, with antibody probes and reporter genes, of cellular events involved in the early stages of DLM patterning using larval organizers led us to investigate the possible genetic basis of this elaborate process. The close correspondence of the spatio-temporal expression of *ewg* and the transformation events in the larval muscles prompted us to investigate the role of this gene in larval muscle splitting. *ewg* has a sustained and ubiquitous expression in all postmitotic neurons in the embryo, and this expression is essential for embryonic viability (DeSimone and White, 1993; DeSimone et al., 1996). To examine the role of *ewg* in flight muscle development, we had constructed a transgenic strain (ewg^{l1}*, y, w, sn;* NS^4; refer to Materials and Methods) that carries an *ewg* transgene consisting of an *ewg* cDNA under the control of the neural-specific regulatory elements of the *Drosophila embryonic lethal abnormal visual system* gene in the null background of the ewg^{l1} allele (DeSimone et al., 1996). This neural-specific transgene provides essential *ewg* function required for viability, but since these flies completely lack *ewg* expression in the mesoderm they are mesoderm-null, and they lack most of the IFMs. We examined the early events in larval muscle transformation in this *ewg* mesoderm-null background to elucidate the role of this gene in this process. Examination of the larval muscles in 14–16 h APF pupae of *ewg* mutant flies show that although myoblasts are present, migrate normally, and get distributed over the larval muscles, the splitting process is aborted (Fig. 6 *a*). Optical sections of the unsplit muscles revealed that fusion of imaginal myoblasts with the larval muscles was not affected in this *ewg* mutant background (Fig. 6 *b*). However, the progressive alignment of the fused myoblast nuclei into longitudinal columns was not observed in this situation. The unsplit muscles initiate differentiation as revealed by the normal onset of *actin (88F)-lacZ* expression, but by 26 h APF, begin to fragment and degenerate (data not shown). Although this mutant phenotype in the developing DLMs clearly justifies the role of *ewg* in mediating the splitting process, the situation is complicated by our observation that this phenotype is sensitive to genetic background. Thus, although strains that are ewg^{l1}*, y, w, sn;* NS^4 exhibit this phenotype consistently, strains carrying the same *ewg* allele in a different genetic background, ewg^{l1}*, y, cho, sn;* NS^4, however are not affected in the splitting process. Further, animals carrying a viable allele of *ewg*, ewg^1, which exhibits no Ewg immunoreactivity in the mesodermal cells, are also not affected in the splitting process (Coelho, 1994). The developing DLMs nevertheless degenerate at similar stages in all these strains.

Given that there is a genetic background-dependent variation of the effect of removal of *ewg* function during IFM development, and given the complex nature of products from the locus (Fleming et al., 1993), the most parsimonious conclusion, consistent with the pattern of expression of the gene during IFM development, is that *ewg* removal can, at least in one genetic background, affect splitting of the larval muscles. We discuss this in relation to IFM differentiation below (see Discussion).

Early Events of Nervous System Metamorphosis Are Normal in the Absence of Imaginal Myoblasts

Unlike the *Drosophila* embryo, where muscles are patterned independently of the developing innervation, muscle development in the imago presents a contrasting situation where myogenesis and development of the innervation

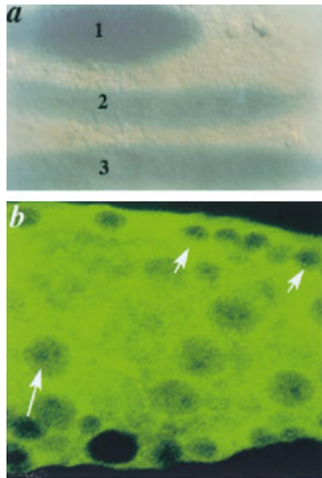

Figure 6. Remnant larval muscles do not split in the absence of ewg function in imaginal myoblasts. (*a*) A 16-h APF pupal preparation of an *ewg*^{l1}*, y, w, sn; NS*^{4} fly stained for β-galactosidase activity from the *MHC* promoter in the larval muscles showing absence of splitting (the muscles are marked *1*, *2*, and *3*). Compare with a similar stage wild-type preparation in Fig. 2 *b*. (*b*) Optical section through an unsplit larval muscle in an *ewg* mutant pupa showing invasion of the larval fiber by imaginal myoblasts. The large nuclei (*large arrow*) are the nuclei of the larval muscles themselves, whereas the more numerous and smaller nuclei (*small arrows*) are those of imaginal myoblasts that have fused with the larval muscle. Compare with Fig. 3 *b*. *Green labeling*, β-galactosidase expression from the *MHC* promoter in the larval muscle. Note the complete absence of Ewg immunoreactivity in the larval muscle and imaginal myoblast nuclei in these animals. In both panels, *left*, anterior; *top*, dorsal midline.

proceed in synchronicity (Broadie and Bate, 1993; Fernandes and VijayRaghavan, 1993). This process has been best investigated for the developing DLMs, and it begins with the withdrawal of synaptic contacts of the nerves from the persistent larval muscle targets, followed by the elaboration of new arborizations over these muscles as these muscles split and develop into templates for DLM development (refer to Fig. 1). It has been suggested that cues emanating from the metamorphosing innervation could play an important role in the splitting of the larval muscles into DLM templates (Fernandes et al., 1991; Fernandes and VijayRaghavan, 1993). This proposition is based on the following observations: new arborizations from the motor nerves are observed at a time when the larval muscles are splitting, and these could, by some unknown mechanism, instruct the larval muscles to split. Moreover, during larval life, apart from imaginal myoblasts on the wing imaginal discs, a subset of imaginal myoblasts remain associated with the larval nerves (Bate et al., 1991; Fernandes and VijayRaghavan, 1993). These myoblasts could derive essential patterning signals from the motor nerves and somehow be involved in the transformation of the remnant larval muscles. If neural signals are important for larval muscle transformation, then selective ablation of these neurons during imaginal development should affect DLM development. However, laser ablation of the flight muscle motor nerve does not seem to affect the normal development of the DLMs (Fernandes and Keshishian, 1998). This would suggest that innervation does not play a crucial role in the development and patterning of the DLMs.

Given this scenario, we decided to examine whether metamorphic changes in the larval motor nerves that occur during pupal development could proceed in the absence of imaginal myoblasts in *1151/UAS-p*^{21} animals, or whether cues from these myoblasts dictate patterning events in the developing innervation. We therefore examined the motor nerves of *1151/UAS-p*^{21} pupae at 14 h APF for withdrawal of their larval neuromuscular contacts and for the development of neurite outgrowths over the unsplit larval muscles using antihorseradish peroxidase immunohistochemistry that fortuitously labels all neurons and their arborizations in *Drosophila* (Jan and Jan, 1982). We found that even in the absence of imaginal myoblasts in *1151/UAS-p*^{21} animals, the motor nerves were able to withdraw their synaptic contacts from the larval muscles on schedule, and were also able to send out neurite processes over the degenerating unsplit muscles (Fig. 7, *a–c*). These observations demonstrate that much of the early metamorphic changes of the flight muscle innervation such as withdrawal of synaptic terminals from their target larval muscles and elaboration of neurites over the developing DLMs are autonomous properties of the nerves, most likely induced by hormonal changes during early pupation and are not dictated by cues from the developing DLMs, a situation similar to hormonally mediated reprogramming of motor neurons in the tobacco moth, *Manduca sexta* (Truman and Reiss, 1995). However, the evolution of the final innervation pattern is likely to be dictated by cues from the developing DLM fibers (Fernandes et al., 1994; DeSimone et al., 1996).

Discussion

In this study, we have focused on the cellular mechanisms of pattern formation in the developing DLMs using larval muscle fibers as scaffolds. The use of larval muscles for the development of the DLMs has long been recognized, not only in *Drosophila* and other related dipterans, but in other insects as well (for review see Crossley, 1978; also see Smit and Velzing, 1986; Cifuentes-Diaz, 1989). With reporter genes that allowed the visualization of the early events during flight muscle development in *Drosophila*, Fernandes et al. (1991) first documented the transformation of the larval muscles into templates for the development of the DLMs. However, the mechanisms by which the larval muscles are modified into templates have always remained an enigmatic problem. Early observations (for review see Crossley, 1978), coupled with more recent electron microscopic studies (Reedy and Beall, 1993*a*) have revealed that imaginal myoblasts invade the larval muscles during early pupal development. In this study, we were able to demonstrate that the transformation of residual larval fibers into templates for DLM development is dependent on the invasion and fusion of the imaginal myoblasts with these fibers. In addition, we identify a role for the *ewg* gene in this process.

The events in the transformation of the larval muscle organizers can be examined in a step-wise manner. Their escape from histolysis must be a consequence of autonomous properties of the muscles themselves. Transforming the segmental identity of the nervous system and the epidermis, but not the mesoderm, of the third thoracic segment towards the second, does not result in the formation of larval templates, in the transformed segment (Fernandes et al., 1994). The mechanism of splitting of the muscles themselves can most simply be explained as a consequence of a precisely choreographed set of events that occur dur-

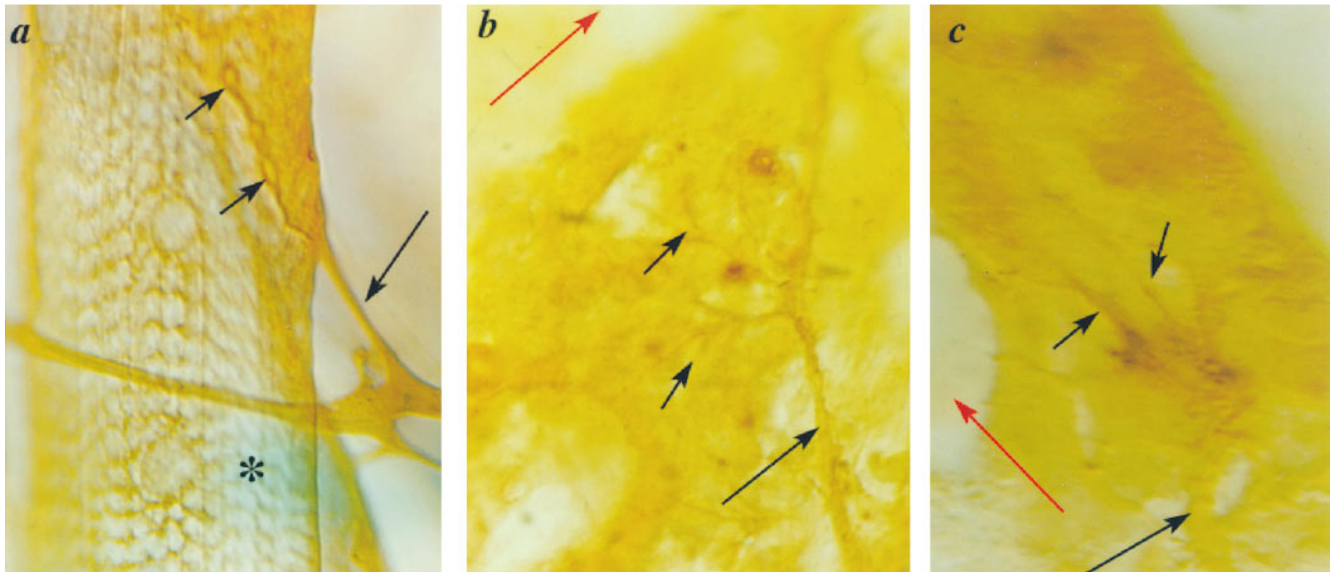

Figure 7. Metamorphic changes in the innervation to the persistent larval muscles during early pupal development can occur in the absence of imaginal myoblasts. *(a)* Neuromuscular junction in a third instar larva, showing the characteristic presence of synaptic boutons, revealed by antihorseradish peroxidase antibody staining (*short arrows*). *Asterisk*, dorsal oblique muscle *1*; *long arrow*, a branch of the intersegmental nerve. (*b*) A similarly stained wild-type 14-h APF pupal preparation showing neurite outgrowths from the ends of the motor nerve over one of the persistent larval muscles (*short arrows*). *Long arrow*, a branch of the motor nerve. Note that the synaptic boutons, characteristic of the larval neuromuscular junction in *a*, have been completely withdrawn. (For further details of bouton withdrawal and neurite outgrowth see Fig. 2 in Fernandes and VijayRaghavan, 1993). (*c*) A similarly stained preparation of a 14-h APF *1151/UAS-p*^{21} pupa showing neurite arborizations (*short arrows*) over one larval muscle in the absence of imaginal myoblasts. *Long arrow*, a branch of the motor nerve. Compare with *b*. In *a*: *top*, anterior; *left*, dorsal midline. In *b*: *right corner*, anterior. In *c*: *left corner*, anterior; *red arrows*, dorsal midline.

ing onset of differentiation. The end requirements are six DLM fibers, innervated correctly and attached to specific sites, and whose appropriate formation requires myoblasts, templates, epidermal cues, and the nervous system. Thus, at the onset of metamorphosis, Twist-expressing myoblasts swarm over the templates and Ewg expression is seen in myoblasts just before fusion (Fernandes et al., 1991; DeSimone et al., 1996). Fusion of myoblasts and the concomitant onset of differentiation, in addition to active processes autonomous to the templates must be involved in the recognition of attachment sites on the epidermis. It is this precise juxtaposition of developing muscle fibers and their epidermal attachment sites that has been well documented (Fernandes et al., 1996) and is essential for the generation of the six DLM fibers seen in the adult. In the absence of the templates (Farrell et al., 1996; Fernandes and Keshishian, 1996) two effects are noteworthy. First, muscle development is greatly delayed. Ewg expression is delayed in the myoblasts in the region where the templates are absent, suggesting that the proper onset of Ewg expression is mediated by interactions of the myoblasts with the larval muscles. Thus, templates provide both spatial and temporal cues. Second, delayed muscle development is aberrant, and the six DLM units are not correctly generated. These results, along with our observations on the expression pattern of Ewg (Fig. 3) suggest that, in normal development, the onset of muscle differentiation mediated by Ewg, together with the definition of a correct subset of *stripe*-expressing attachment cells for the muscles in the epidermis (Fernandes et al., 1996) are essential events in DLM patterning. Once distinct pairs of muscle–epidermal attachment positions have been precisely defined for each fiber, subsequent splitting may be a consequence of the forces resulting from fiber differentiation. These forces must also be specifically regulated during early myogenesis and cannot be merely the contractile activity of the muscle in response to neuronal input as neuromuscular activity is first detected late in pupal development (Salkoff, 1985).

In an earlier study, we had reported the expression pattern of Ewg during flight muscle development and had described the phenotype of the developing IFMs in a viable combination of *ewg* alleles (DeSimone et al., 1996). We had also reported in the same study that in a synthetic genetic condition that completely eliminates *ewg* function from the mesodermal cells (mesoderm-specific null), the DLMs not only degenerate like in the viable allelic combination, but before degeneration they develop as three unsplit fibers. We had studied the effects of this altered DLM pattern on the pattern of motor nerve innervation. In this study, we have extended our previous observations on Ewg expression and have used it as a convenient marker to chart the cellular events during early stages of larval muscle transformation by the imaginal myoblasts. In addition, we have studied the development of the DLMs at the cellular level in the mesoderm-specific null genetic condition and have shown a possible connection between the pattern of Ewg expression and its mutant phenotype in the DLMs in this genetic background. Consistent with an active role that we propose for the templates is the observation of Ewg expression in the nuclei of the larval muscles

that indicate that these muscles could possibly be transcriptionally active. The presence of Ewg in larval nuclei could be a consequence of remnant expression from the larval stage, where we see low-level but consistent expression in somatic muscles (Roy, 1997). Alternatively, it is possible that the presence of Ewg immunoreactivity in these larval nuclei during DLM formation is due to the translocation of Ewg protein synthesized by imaginal myoblasts after they have fused with the larval muscles or the imaginal myoblast nuclei induce Ewg expression in the nuclei of the larval muscles on fusion. To resolve some aspects of this issue, we have looked for Ewg expression in the nuclei of the unsplit larval muscles in *1151/UAS-p*^{21} animals. We find that the nuclei of the unsplit muscles in these animals do exhibit Ewg immunoreactivity (data not shown). One possibility is that this could be due to transcription from the nuclei of the few imaginal myoblasts that consistently remain in these animals and fuse with the larval muscles, followed by nuclear translocation of Ewg translated in the muscle cytoplasm into nuclei of the larval muscles. The other possibility is that the Ewg protein detected in larval nuclei is a consequence of these nuclei being transcriptionally active. Thus, whereas the source of Ewg immunoreactivity in the larval muscle nuclei during DLM development remains unresolved, the presence of Ewg expression in these nuclei allowed us to clearly demonstrate that these nuclei actually get incorporated into the developing DLM fibers. In addition, the ability to maintain nuclear Ewg expression suggests that these nuclei have at least some kind of biological activity and are not mere "ghosts". This observation is particularly important because electron microscopic studies of the metamorphosing larval muscles and of homologous larval muscles in *Calliphora* have shown that subcellular components such as sarcomeric organization is lost, and there is disassembly of the contractile apparatus (Crossley, 1972; Reedy and Beall, 1993*a*).

The onset of differentiation of the mature fiber after fusion of myoblasts is an essential requirement for the splitting of the larval muscles. This is demonstrated by *ewg* mutant phenotypes. In an *ewg*–mesoderm-null combination (Fig. 6 *a*) these muscles fail to split. The pattern of Ewg expression during DLM development (DeSimone et al., 1996; this study) is consistent with this phenotype. Ewg expression is not observed in the mature DLMs, and this gene may therefore be a regulator of early events in muscle differentiation. The identification of target genes of Ewg may help in deciphering the molecular events in the process of splitting. However, the muscle phenotype in *ewg* mutants appears to be sensitive to the genetic background as seen in the situation where the same mesoderm-null combination can allow splitting of the larval muscles. Given the strong correlation between the expression pattern of Ewg in muscle development and the nonsplitting phenotype observed in one genetic combination, we interpret our results in the following manner. We propose that Ewg expression is required for the early steps in fiber differentiation, and splitting of the DLM templates is one of the earliest steps in this process. However, we suggest that other genes also play a similar spatial and temporal role in DLM development and in corresponding early steps of DVM development. This is reflected by the variability in the *ewg* mutant phenotype. This variation is actually most striking in the DVMs where there is a substantial variation in the expressivity of the phenotype (Fleming et al., 1983, 1989; de la Pompa et al., 1989). We have used this variability to our advantage by screening for supressors of the *ewg* DLM phenotype and, consistent with the above finding of sensitivity to genetic background, several modifiers with poor penetrance of the supressor phenotype have been isolated (Sunanda, M.S., unpublished data).

The importance of the developing innervation in patterning imaginal muscles in the fly is emphasized by the fact that the development of a sex-specific muscle, the muscle of Lawrence, in the fifth abdominal segment of male flies is critically dependent on the segmental identity and sex of the innervating motor neuron and not on the genetic identity of the myoblasts themselves nor that of the epidermis to which it attaches (Lawrence and Johnston, 1986). Specific ablation of the innervation to the muscle of Lawrence has been shown to completely arrest the development of this muscle in male flies (Currie and Bate, 1995). A similar consequence could be envisioned for the DLMs. In this case however, the motor nerves themselves appear not to be directly involved in the early patterning events of these muscle fibers, since ablation of these nerves during late larval development does not affect aspects of DLM patterning during pupation (Fernandes and Keshishian, 1998). Furthermore, early events in the transformation of the nervous system like the withdrawal of synaptic contacts from the larval muscles and elaboration of primary neurite branches are also autonomous properties and can take place in the absence of templates (Fernandes and Keshishian, 1998), or as we have shown in this study (Fig. 7*c*), in the absence of imaginal myoblasts.

The widespread occurrence of instances where muscle patterning is nucleated by muscle organizers suggests that these cells do play important functions in muscle patterning and have been evolutionarily selected for (for review see Jellies, 1990). For instance, special cells called comb cells or C cells organize the intricate pattern of muscle fibers on the body wall of the leech (Jellies and Kristan, 1988). In the grasshopper, each muscle is prefigured by a single large mesodermal cell, the muscle pioneer, that recognizes attachment sites and provides cues to the developing motor neuron to establish the correct pattern of synaptic contacts (Ho et al., 1983). Other mesodermal cells fuse with the pioneer and give rise to the mature muscle. The developing fiber often splits up into a set of muscle fascicles in a manner very reminiscent of the splitting process of the larval muscles during DLM development in *Drosophila.* In the *Drosophila* embryo, muscle development is nucleated by special myoblasts called founder cells (Bate, 1990; Rushton et al., 1995). Myoblasts can fuse to founder cells but not to each other and form pioneer fibers that configure muscle pattern. Neighboring fibers with identical intrinsic properties and pattern can nevertheless attach to distinct adjacent epidermal sites, exemplifying the importance of context in fiber formation (Ruiz-Gómez and Bate, 1997). One unifying function of organizers in myogenesis could be to provide myoblasts and developing motor neurons with spatial cues so that the muscle's fibers are produced and innervated with a high level of precision. They could act as sites of myoblast aggregation and func-

tion to partition them into groups that give rise to individual muscle fascicles. Occurrence of muscle organizers has not been definitively proven in vertebrates, but at least in the zebrafish embryo, there is a group of early maturing muscle pioneers that develop along the trunk, in close apposition with the notochord (Halpern et al., 1993). Recent studies on the mechanisms of specification of these cells tend to suggest that they play an important function in somite patterning (Currie and Ingham, 1996; van Eeden et al., 1996). It will be interesting to evaluate the mechanisms by which muscle organizers help to organize muscle patterns in these diverse organisms. It is possible that muscle development in these organisms involve cellular processes that are similar to the ones that we have found to operate during DLM development in *Drosophila.*

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