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ERECT WING, the *Drosophila* member of a family of DNA binding proteins is required in imaginal myoblasts for flight muscle development

Susan DeSimone[1,*], Carmen Coelho[2,†], Sudipto Roy[3], K. VijayRaghavan[2,3] and Kalpana White[1]

[1]Department of Biology and Center for Complex Systems, Brandeis University, Waltham, Massachusetts 02254, USA
[2]Molecular Biology Unit, TIFR, Homi Bhabha Road, Colaba, Bombay 400005, India
[3]National Centre for Biological Sciences, TIFR Centre, PO Box 1234, IISC campus, Bangalore 560012, India

*Present address: Department of Microbiology, College of Medicine, University of Vermont, Burlington, Vermont 05405-0066, USA
†Present address: Department of Genetics, University of Cologne, 17 Gyrhofstrasse, D50923, Cologne, Germany

SUMMARY

The *erect wing* locus of the fruit fly *Drosophila melanogaster* encodes a protein, EWG, that shares extensive homology with the P3A2 DNA binding protein of sea urchin and a recently identified mammalian transcription factor. Loss-of-function *erect wing* alleles result in embryonic lethality. Viable alleles of *erect wing* cause severe abnormalities of the indirect flight muscles. We have analyzed the spatial pattern of *erect wing* expression in the developing indirect flight muscles during postembryonic development. EWG is detected, 10 hours after puparium formation, in myoblasts that will form the indirect flight muscles. The early events of muscle development are normal in *ewg* mutants. However, a few hours after the onset of *erect wing* expression in myoblasts, defects are seen in the developing indirect flight muscles which subsequently degenerate. We present results that show that the normal development of the indirect flight muscles requires *erect wing* expression in the progenitor myoblasts themselves. Finally, we examine the role of target muscles in the arborization of motor axons by studying the developing innervation to the flight muscle in *erect wing* mutants. Our study demonstrates, for the first time, a role for a regulatory gene expressed in imaginal myoblasts in *Drosophila*.

Key words: *Drosophila*, muscle, transcription factor, myoblasts, *erect wing*

INTRODUCTION

The correct patterning and differentiation of muscles require the coordinate execution of regulatory programs in several different tissues. Myoblasts must be set aside to divide and fuse to form specific muscles. There is now evidence from studies on vertebrates (reviewed by Donaghue and Sanes, 1994) and from *Drosophila* (Grieg and Akam, 1993; Michelson, 1994; Fernandes et al., 1994) that there are substantial differences amongst myoblasts, and these molecular differences may be involved in the generation of muscle diversity. In *Drosophila*, as in other insects (Williams and Caveny, 1980), muscles attach to epidermal sites; a process that involves developing muscle fibres seeking out their epidermal attachment sites in an active manner (Bate, 1990; Volk and VijayRaghavan, 1994). The nervous system also plays important roles in muscle development. The role of innervation in the expression of contractile protein isoforms has been well documented (reviewed by Donaghue and Sanes, 1994) and the nervous system has also been shown to play a determinative role in the formation of a sex- and segment-specific muscle in *Drosophila* (Lawrence and Johnston, 1986; Currie and Bate, 1995).

Myoblasts that contribute to the indirect flight muscles (IFMs) are derived from the embryonic mesoderm (Bate et al., 1991) and are attached to imaginal discs and nerves during larval development (Bate et al., 1991; Fernandes et al., 1991; Fernandes and VijayRaghavan 1993). At the onset of metamorphosis, these myoblasts migrate over the developing adult epidermis and fuse to form the adult muscles (Fernandes et al., 1991). One group of indirect flight muscles, the dorsal longitudinal muscles (DLMs) use modified larval muscles as templates for their development, while another group of very similar IFMs, the dorsoventral muscles (DVMs) appear to develop by the *de novo* fusion of myoblasts (Fernandes et al., 1991). The innervation to the IFMs develops from the modification of larval nerves (Fernandes and VijayRaghavan, 1993). Neurons that innervate larval muscles, withdraw their termini at the onset of metamorphosis, undergo specific modifications, and send out processes that grow over the developing IFMs (Fernandes and VijayRaghavan, 1993).

Flies carrying viable allelic combinations of mutations at the *erect wing* (*ewg*) locus do not have, or have greatly reduced, IFMs (Deak et. al 1982; Fleming et al., 1983) and the gene is also required in the development of the nervous system (Fleming et al., 1989; DeSimone and White, 1993). Most available mutations at the *ewg* locus cause embryonic lethality (DeSimone and White, 1993). The predominant product of the *ewg* gene is a nuclear protein, EWG, that shares extensive homology with the novel DNA binding domain of sea urchin

P3A2 DNA binding protein and a transcription regulator from mammals (DeSimone and White, 1993; Hoog et al., 1991; Calzone et al., 1991, Efiok et al., 1994; Virbasius et al., 1993). The EWG protein is specifically localized to most if not all embryonic neuronal nuclei (DeSimone and White, 1993). Expression outside the nervous system was not observed at this developmental stage. Basal level expression from an *ewg* minigene, EWG^{HS} which expresses *ewg* cDNA under the hsp-70 promoter was able to rescue both the viability and flight defects associated with *ewg* mutant alleles (DeSimone and White, 1993). Collectively these data suggest that the neural expression of *ewg* is essential for organismic viability and is required in developing and differentiated neurons.

The role of *ewg* in IFM development is especially intriguing and at least four distinct scenarios can be envisioned. Proper IFM development could require *ewg* expression in the IFMs or their precursors, in the nervous system alone (which could inductively affect IFM development), expression in both the nervous system and IFM, or expression in some other tissue. We began by investigating the postembryonic expression of EWG using antibodies that specifically recognize EWG.

We show that EWG is expressed in progenitor myoblasts of the IFMs and in the developing myofibres. The absence of this expression in pupae carrying a viable allelic combination of *ewg* mutations results in the abortion of IFM development. Ubiquitous expression of EWG in the nervous system does not rescue the IFM defects seen in *ewg* mutants. Using two different transformant constructs that express EWG, we show that the muscle phenotype very likely results from a requirement of the gene in IFM progenitor myoblasts. Finally, we show that in the absence of normal development of their target muscle, motor innervation to the developing IFMs is affected in a manner which illustrates the importance of muscles in influencing axon arborization and synapse formation.

Our results demonstrate that by using selective promoters it is possible to uncouple pleiotropic functions of a gene and create tissue-specific mutations – in the case of the present study – a ‘myoblast-specific synthetic null’ genetic condition of the *ewg* locus. To our knowledge this is the first demonstration of a function for a gene expressed in imaginal myoblasts in *Drosophila*. Our study raises several questions on the mechanism of activation of a regulatory gene in IFM progenitors and provides an important means of testing whether this could arise from ectoderm-mesoderm interactions or by other mechanisms that are intrinsic to the mesoderm.

MATERIALS AND METHODS

Immunohistochemistry

Immunohistochemistry for embryos followed procedures described by Ashburner (1989). Pupal dissections were analyzed as described earlier (Fernandes et al., 1991; Fernandes and VijayRaghavan, 1993). An EWG-specific antibody was preabsorbed on 0- to 3-hour Canton-S embryos and used at a final dilution of 1:200 to 1:500. mAb 22C10 (Fujita et al. 1982) was a gift from Seymour Benzer and was used at a dilution of 1:50. A monoclonal β-galactosidase antibody (Promega) was used at a dilution of 1:1000. The samples were processed using

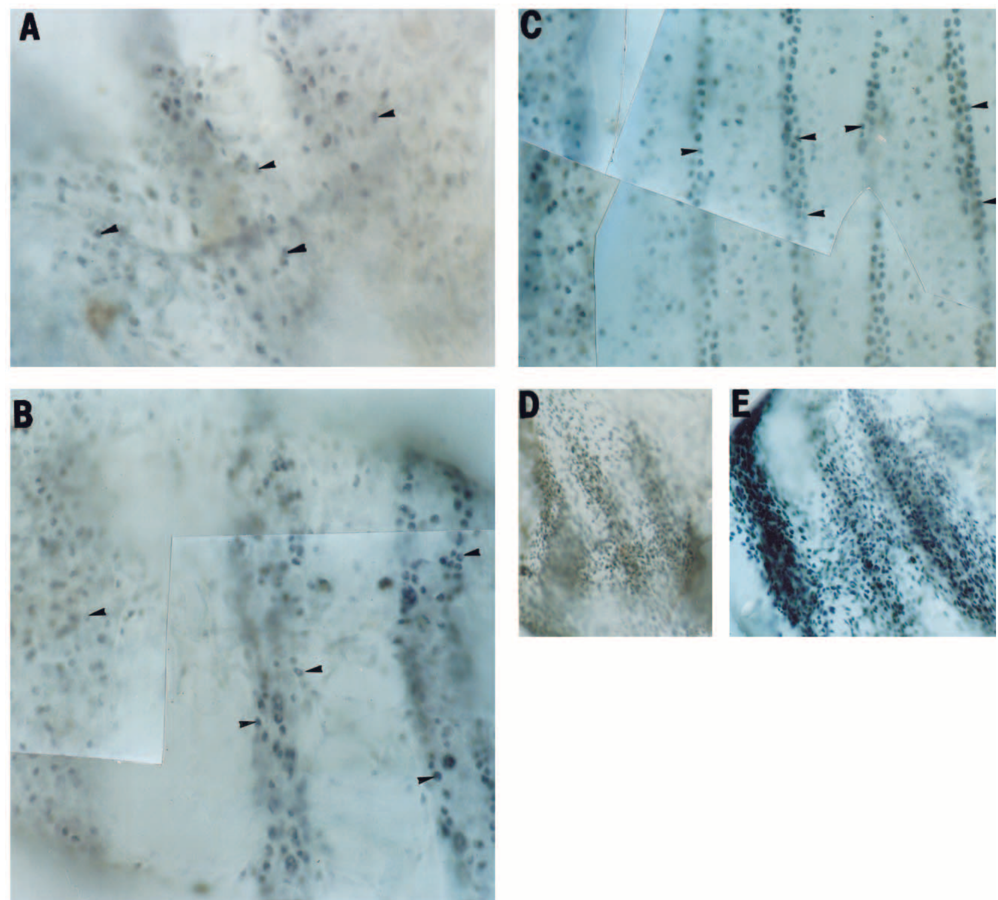

Fig. 1. EWG expression in myoblasts during the development of the DLMs. In all pictures, anterior is to the top; dorsal is to the left. The larval dorsal oblique muscle closest to the dorsal midline is muscle 9 (Fernandes and VijayRaghavan, 1993, Bate, 1993) (A) At 10 hours APF, expression is seen in a subset of myoblasts that lie above the dorsal oblique muscles. The nuclei of myoblasts are seen as dark spots of colour; some of these myoblasts are marked with arrowheads. (B) At 13 hours APF, the staining intensifies and more cells over the dorsal oblique muscles are labeled at this stage. (C) At 16 hours APF, muscle 10 and muscle 19′ (Fernandes and VijayRaghavan, 1993; Bate, 1993) have split. Arrowheads show the myoblasts aligned over the surface of these muscles. Muscle 9, closest to the left of the frame has not yet split. EWG-positive myoblasts are also seen over this muscle. D and E are 12-hour and 16-hour APF preparations labeled with TWIST-specific antibody to reveal all the myoblasts in the region. Note that the larger number of labeled cells in these preparations compared to A, B and C above.

indirect immunoperoxidase reaction or with appropriate fluorophore-conjugated secondary antibodies. Confocal microscopy analysis was done with a BioRad Model 600 confocal microscope. The confocal microscope pictures represent optical sections printed on a Sony printer.

Construction of the *EWG^NS* minigene

The *EWG^NS* mingene consists of an *ewg* cDNA, SC3 (sequence given in DeSimone and White, 1993) with 27 nt of 5′ untranslated leader and 600 nt of 3′ trailer under the control of an *elav* promoter (Yao and White, 1994) cloned into the pCaSpeR4 vector. Details of the subcloning and of P-element mediated germline transformation are available on request.

Fly Strains and methods

Canton-S was the wild-type stock used. Crosses were cultured at 25°C. All marker gene abbreviations are as in Lindsley and Zimm (1992). The reporter construct which contains the regulatory domains of the *twist* gene fused to the *lacZ* gene is referred to as *twist-lacZ* and flies carrying these constructs have been described previously (Thisse et al., 1991). The strain was generously provided by F. Perrin-Schmitt and B. Thisse of Strasbourg, France.

Below we list the alleles used in this study and their relevant properties (Fleming et al., 1983; DeSimone and White, 1993); Lindsley and Zimm (1992) nomenclature, where different, is in the parenthesis

ewg^{1}: EMS induced, viable

ewg^{l1}: EMS induced, embryonic lethal, and a protein-null (ewg^{2})

ewg^{l3}: EMS induced, embryonic lethal, and not a protein-null (ewg^{4})

ewg^{l4}: X-ray induced, stage of lethality not determined, and not a protein-null (ewg^{5})

ewg^{l5}: EMS induced, embryonic lethal, protein not determined (ewg^{10})

ewg^{l6}: EMS induced, late embryo-early larval lethal, occasional adult escapers, not a protein-null (ewg^{11})

Analysis of adult IFMs

Thoraces of 2- to 4-day old adults were fixed and processed for analysis of the IFMs as described by Restifo and White (1991).

RESULTS

EWG is expressed in the progenitors and fibres of the indirect flight muscles during pupal development.

An examination of EWG expression, using EWG-specific antibodies, during embryonic and larval development revealed no immunohistochemical signal in the mesoderm or its derivatives. However, EWG is detected in all mature embryonic and larval neurons (data not shown). EWG expression was examined in progenitors of adult IFMs in larvae and pupae. No expression is seen in the myoblasts associated with the wing or other imaginal discs during the third larval instar. Nor is EWG detected in the myoblasts associated with the nerves (Currie and Bate, 1991; Fernandes and VijayRaghavan, 1993) at this stage. After the onset of metamorphosis, myoblasts associated with the wing imaginal discs migrate over the developing adult epidermis (Fernandes et al., 1991). At this stage, and until 10 hours after pupal formation (APF), EWG is not detected in the myoblasts. At 10 hours APF, EWG protein is detected in a small population of cells in the region where the DLMs are known to develop (Fig. 1A, arrowheads). These EWG-positive cells overlie (Fig. 1A, B, C) the larval templates (Fernandes et al, 1991) that are used for the development of the DLMs. At this stage EWG expression is detected only in a small fraction of the myoblasts present over the larval templates (Fig. 1A). Two hours later, at about 13 hours APF, a more intense expression of EWG is seen over the larval templates: the staining reaction is stronger and more cells are labeled (Fig. 1B, arrowheads). At 16 hours APF the alignment of labeled cells along the surface of the larval templates is a noticeable feature (Fig. 1C, arrowheads). By 20 hours APF, the three larval templates have split and the development of the DLMs is well underway (Fernandes et al., 1991).

We analyzed the pattern of EWG expression at this and later stages in *twist-lacZ* transformants that express the reporter enzyme β-galactosidase under the control of the *twist* promoter and regulatory elements (Thisse et al, 1991). The *twist* gene is active in unfused muscle precursors and is down regulated when myoblasts fuse and differentiate (Bate, 1993). Confocal microscopy analysis of EWG and β-galactosidase double-labeled preparations at 20 hours APF reveal EWG expression in a large population of myoblasts over the developing DLM fibres as well as in almost all nuclei of the muscle syncitia (Fig. 2A). This expression pattern continues through 24 hours APF when intense expression is detected in the myoblasts as well as in the nuclei of the myofibres (data not shown). At 28 hours APF, low-level immunoreactivity is noticed in the myofibre nuclei and in the unfused myoblasts adhering to the develop-

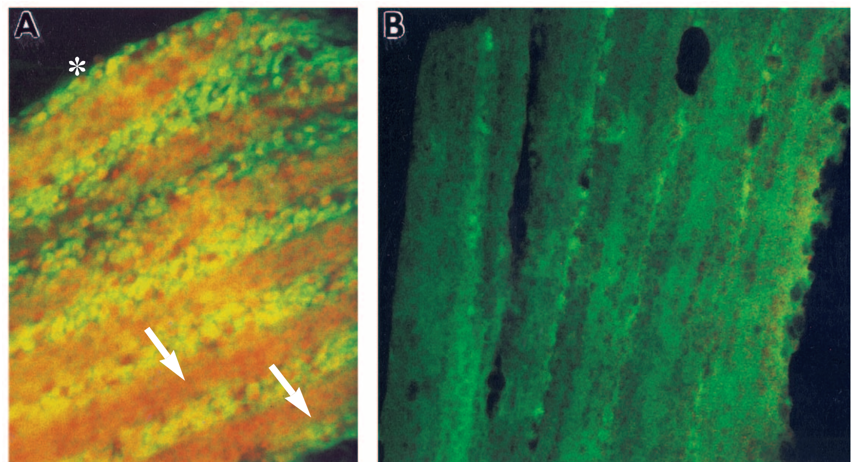

Fig. 2. Expression of EWG and reporter β-galactosidase in a *twist-lacZ* transformant animal reveals expression in both myoblasts and myofibres. Optical sections during pupal development were used to reveal nuclear-localized EWG expression (red) and cytoplasmic β-galactosidase expression (green). (A) At 20 hours APF, EWG expression is seen in a large population of myoblasts around the developing DLMs and in nuclei of the developing fibres. Top is dorsal, bottom is ventral. Unfused myoblasts are seen as green-outlined cells in between the fibres; the myoblasts surrounding the dorsal-most fibres are marked with an asterisk. This optical section passes through developing fibres and the red colour in these fibres represent nuclear EWG expression. The ventral-most fibres are marked by arrows. (B) At 28 hours APF, β-galactosidase expression (green) is seen in the fibres due to the perdurance of reporter enzyme. At this time, low levels of EWG expression is seen in the nuclei of the developing myofibres (faint red) in this optical section through the fibres.

ing muscles (Fig 2B). By 36 hours APF, fusion of myoblasts to the developing fibres is complete, as suggested by the absence of TWIST expression (Fernandes et al, 1991). At this stage residual EWG expression is detected in the myofibre nuclei of the DLMs and this completely disappears by 38 hours APF (data not shown). Thus EWG expression spans a window 10-36 hours broad during the development of the DLMs and is seen in the myoblasts as well as the nuclei of the developing myofibres. At any particular developmental stage however, expression is detected in a subset of myoblasts. Since EWG expression is detected over a period of 26 hours, and myoblast fusion is in progress in this period, it is possible that all myoblasts express EWG before fusion and that this expression continues inside the myofibre for some length of time.

EWG expression is also seen, between 12-36 hours APF, in myoblasts that will contribute to the other group of IFMs, the DVMs, and in the progenitors of the jump muscle and in their developing fibres (data not shown).

DLM defects are observed in *ewg* mutants after the period where protein expression is detected in adult myoblasts

We chose to study IFM development in the *ewg*^{1}/*ewg*^{l1} pupae as this genotype exhibits the most severe IFM defects: either a complete loss or a severe reduction in the width of the affected muscles. These phenotypes may result from any one or a combination of the following scenarios: there may be an inadequate number of myoblasts present on the imaginal discs; there may be an adequate number present on the disc but an inadequate number reaching their target sites; or many or most myoblasts may fail to fuse to form muscles. In pupae of the genotype *ewg*^{1}/*ewg*^{l1}, at 12 hours APF, the stage when EWG protein is first detected in wild-type myoblasts, TWIST-expressing cells are seen in a pattern that cannot be distinguished from the wild type (data not shown). Thus, the early events in IFM formation are not detectably affected in *ewg* mutants. However, when *ewg*^{1}/*ewg*^{l1} pupae were examined, EWG protein was not detected in myoblasts. The absence of detectable EWG signal suggests that the *ewg*^{1} allele is very likely close to a protein null in myoblasts.

To examine IFM development at later stages, *ewg*^{1}/*ewg*^{l1} animals were dissected during pupal development and labeled with a monoclonal antibody mAb 22C10 (Fujita et al., 1982) that labels neurons and can also be used to view developing muscles (Fernandes and VijayRaghavan, 1993). Preparations aged between 14 and 16 hours APF displayed normally developing muscle syncitia when compared to wild-type preparations (data not shown; see also Fernandes et al., 1991 for wild-type development). The first signs of defects in IFM development are observed at about 18 hours APF when degeneration of the ventral-most DLMs (Fig. 3A, arrow) are seen. A little later, at 20 hours APF, the DLMs have completely degenerated (Fig. 3B). Significantly, no apparent defects are observed in the DVMs at this stage, while adults with *ewg* mutations do show variable phenotypes in this group of muscles. The DVM defects must therefore manifest at later stages in development. The stage at which the mutant phenotype is first observed (Fig. 3A) is after the process of splitting of the larval templates has taken place in the wild type (Fig. 3C). However, while the development of the wild-type DLMs proceeds and results in the formation of six innervated units (Fig. 3D), the mutant muscles usually degenerate (Fig. 3B).

Neural-restricted expression of EWG provides the vital ewg function

Analysis of an *ewg* minigene *EWG*^{HS1} (the SC3 *ewg* cDNA under the control of the hsp-70 promoter; DeSimone and White, 1993), had previously shown that the basal expression of this minigene could rescue the embryonic lethality associated with loss of *ewg* function (DeSimone and White, 1993). However, these analyses did not address the question of tissue-specific *ewg* requirements. In particular, we wanted to examine if nervous system-specific expression was sufficient to provide the *ewg*-associated vital function, and if this expression could either rescue or ameliorate the muscle phenotype.

Our strategy to achieve neural-restricted *ewg* expression consisted of fusing *ewg* cDNA sequence to a neural-specific promoter. We chose the promoter of the *Drosophila elav* gene for several reasons. In contrast to *ewg,* the *elav* gene is expressed exclusively in neurons throughout development. The overall postembryonic and embryonic expression patterns of *ewg* and *elav* in the nervous system are strikingly similar (Robinow and White, 1991; DeSimone and White, 1993; DeSimone, 1992). Although no direct comparisons have been made, our impression, based on when the transcripts are first seen in the embryo and the first discernible immunoreactive signal seen in the eye disc, is that *elav* expression precedes *ewg* expression by about one half hour in both the embryo and the eye disc. Thus, the expression of *ewg* under the control of *elav* promoter would ensure the availability of EWG in maturing neurons at the normal onset of *ewg* expression. Finally, a 3.5 kb fragment from the *elav* locus has been shown to confer neuronal-specific expression on a heterologous reporter gene (Yao and White, 1994).

To construct the neural-specific transgene *EWG*^{NS}, SC3 cDNA sequence was fused directly to the *elav* promoter with only 27 nt of *ewg* untranslated leader included. This was done to minimize the chance of including possible *ewg* enhancer elements. In all, seven different transformant lines were isolated, of these, four autosomal inserts were further analyzed. Immunohistochemistry of *ewg*^{l1};*EWG*^{NS4} embryos using EWG-specific antibodies revealed EWG presence in the predicted pattern. EWG was detected in the nuclei of central nervous system (CNS) and peripheral nervous system (PNS) neurons similar to the pattern detected in wild-type embryos (Fig. 4A as compared to Fig. 5 in DeSimone and White, 1993). A closer examination of EWG expression in the PNS of *ewg*^{l1};*EWG*^{NS4} further demonstrated that the transgene restored EWG expression in all PNS neurons: Fig. 4B shows a preparation highlighting the dorsal cluster of PNS neurons in the third thoracic segment (T3) and the first (A1) and second (A2) abdominal segments. Characteristic differences in the pattern of PNS neurons in these segments are known and EWG-specific antibodies label these neurons to clearly reveal these differences (Fig. 4B). Similarly, a CNS preparation from an embryo of the genotype *ewg*^{l1};*EWG*^{NS4}, shows EWG in all neuronal nuclei (Fig. 4C). Expression of the *EWG*^{NS} transgene was not observed outside the nervous system in embryonic, larval or pupal stages. In addition, no expression of EWG outside of the nervous system was observed in *ewg* null mutants that carry the *EWG*^{NS} transgene (data not shown).

Four independent EWG^{NS} transgene inserts were tested for rescue of the lethality associated with ewg^{l1}, ewg^{l3}, ewg^{l4}, ewg^{l5} and ewg^{l6}. Previous analyses have shown that ewg^{l1}, ewg^{l3}, ewg^{l4}, ewg^{l5} cause embryonic lethality, whereas ewg^{l6} has no specific lethal stage and a few adult escapers are observed (Fleming et al., 1983). Immunoblot analysis of mutant embryos has shown that ewg^{l1} is a protein null, whereas ewg^{l3} and ewg^{l4} show an immunoreactive protein band at the expected position (DeSimone and White, 1993). Each transgene insert was able to provide vital *ewg* function as judged by survival of adults hemizygous for all the lethal alleles tested that carry one copy of the transgene (data not shown). Since ewg^{l1} appears to be a protein null (DeSimone and White, 1993), the rescue of this allele should serve as an accurate reflection of the vital function provided by the transgene. Rescued males carrying an *ewg* mutant allele on the X chromosome and one copy of an autosomal transgene insert were used for further analysis.

IFM defects are not rescued in *ewg* mutations carrying the EWG^{NS} transgene

We examined the rescued $ewg^{lx}/Y\ EWG^{NS}$ flies for the erect wing phenotype (*x* stands for different lethal alleles). A large proportion of $ewg^{l1}/Y;\ EWG^{NS}$ and $ewg^{l3}/Y;\ EWG^{NS}$ surviving adults showed erect wing posture similar to that observed for ewg^{1} mutant animals. The percentage of erect wing males were within ranges that have been observed for ewg^{1}/Y animals (Fleming 1987). Erect wing posture was not observed in flies of genotype $ewg^{l2}/Y;\ EWG^{NS}$, $ewg^{l4}/Y;\ EWG^{NS}$, $ewg^{l5}/Y;\ EWG^{NS}$ and $ewg^{l6}/Y;\ EWG^{NS}$. The $ewg^{l1}$and ewg^{l3} rescued males were further analyzed for IFM morphology (Materials and Methods). The results of this analysis showed that all $ewg^{l1};EWG^{NS}$ and $ewg^{l3};EWG^{NS}$ males, whether with erect or non-erect wings exhibited muscle defects that always included complete absence of DLMs with varying degree of DVM defects (Fig. 5).

To substantiate further the hypothesis that EWG is required in myoblasts for IFM development, and that this requirement is independent of the requirement or expression of EWG in the nervous system, flies with additional doses of the neural-specific transgenes were analyzed. Flies of the genotype, $ewg^{l1}/Y;\ EWG^{NS3}/EWG^{NS3}$ and $ewg^{l1}/Y;\ EWG^{NS4}/EWG^{NS4}$ and $ewg^{l1}/Y;\ EWG^{NS3}/EWG^{NS3};\ EWG^{NS4}/EWG^{NS4}$ are viable but exhibit IFM defects indistinguishable from those of the flies that carry a single dose of the neural-specific transgene. Protein analysis of the flies with increased doses of the neural-specific transgene demonstrate an increased amount of EWG protein as examined on western blots (data not shown).

The above results show that expression of *ewg* in the nervous system rescues the embryonic lethality but not adult muscle defects. When flies of the genotype EWG^{HS1}/CyO (DeSimone and White, 1993) are crossed to ewg^{l1} homozygous females rescued by the EWG^{NS} minigene, half the male progeny carry the EWG^{HS1} minigene that drives an *ewg* cDNA under the control of a heat shock promoter. These flies, when grown at 25°C can fly and, upon dissection, are seen to have all the DVMs and three DLMs (Fig. 6C). Thus, while high level of EWG in the nervous system restores viability but does not rescue the IFM defects, expression from a heat shock promoter, even in the absence of heat-shock, restores IFMs to a significant extent.

The dissection of *ewg* requirement in the nervous system and in the IFMs allows the study of the role of target muscles in axonal branching

The expression of EWG in pupal myoblasts is at a time when extensive restructuring of larval motoneurons to innervate adult muscle is taking place (Fernandes et al., 1993). How much of this restructuring of the nervous system, is a cell-autonomous property of the motoneurons and what aspects of the observed changes are directed by the target muscles?

Recognition of molecular cues on the target muscles by their growth cones have been shown to be important in the embryonic neuromuscular development (Cash et al., 1992; Fernandes and Keshishian, 1995). Similar mechanisms have been suggested in the development of innervation to the IFMs (Fernandes and VijayRaghavan, 1993; Fernandes et. al., 1994). Mutations at the *ewg* locus provided us with an opportunity to look at neuromuscular specificity during the development of the IFMs.

Since ewg^{l1}; Tf(3) EWG^{NS4} animals lack DLMs we decided to investigate the fate of their innervation during the early development and subsequent degeneration of their targets. The early events of synapse withdrawal and neurite outgrowth in these animals are not any different from that in the wild type (C. C., unpublished observations). In these animals however, the larval muscles fail to split and subsequently degenerate (S. R., K.W. and K. V. R., unpublished data).

Fig. 6A shows the pattern of DLMs and their innervation at 24 hours APF in a wild-type animal. We have chosen to compare the development of two DLMs, each marked by a ‘star’ in Fig. 6A. These are muscles 45c and 45d in the nomenclature of Miller (Miller, 1950; Bate, 1993). The branching of the innervation over these muscles at 24 hours APF is shown by arrowheads (Fig. 6A). Muscles 45c and 45d are each innervated by a separate motoneuron whose cell body is located ipsilateral to the muscle. Each motoneuron branches over the muscle sending one process towards the anterior (top right of Fig. 6A) and another process towards the posterior of the fibre (bottom left of Fig. 6A). The anterior branch of motoneurons over muscles 45c and 45d is shown in detail in the inset (Fig. 6A). In animals of the genotype ewg^{l1}; Tf(3) EWG^{NS4}, the muscle marked with a ‘star’ (Fig. 6B) is the ‘un-split’ muscle 45c and 45d prior to degeneration; the inset shows the innervation over the region corresponding to that shown for the wild type in Fig. 6A. It can be seen that only one branch is present over the ‘un-split’ muscle (arrowhead, Fig. 6B). This suggests that a muscle of this genotype, which will not develop further and will soon degenerate, shows characteristics of only one of either muscle 45c or 45d as inferred by the pattern of innervation. IFM development in animals of the genotype ewg^{l1}; Tf(3) EWG^{NS4} is partially rescued by the presence of an EWG^{HS} transgene even in the absence of heat-shock. Such animals develop to adulthood with three ‘un-split’ DLMs (Fig. 6C). However, the innervation over the 45c/45d fused muscle (Fig. 6C) is a composite of that normally seen separately over 45c and 45d (Fig. 6A). We also investigated the fate of the DLM innervation upon target degeneration. Fig. 6D shows a 48 hours APF preparation of an animal of the genotype ewg^{l1}; Tf(3) EWG^{NS4}. The remnant DVMs sometimes present in these animals often show grossly abnormal innervation (Fig. 6D). In other similar preparations, nerve trunks are seen over the

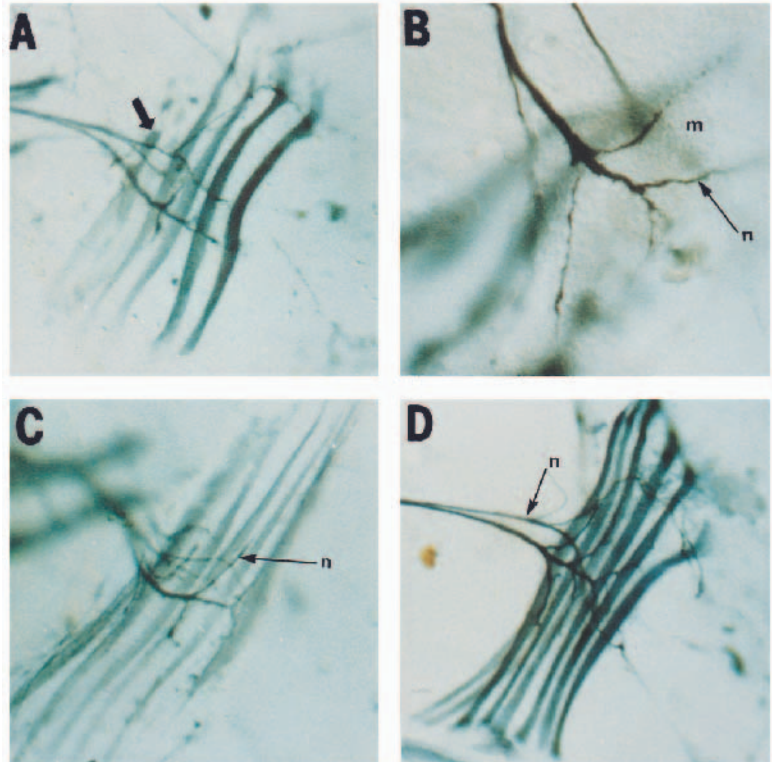

Fig 3. Developmental aberrations in the DLMs due to loss of *ewg* function in ewg^1/ewg^{l1} animals. In all pictures, dorsal midline is on the right; top is anterior. Preparations are stained with the monoclonal antibody mAb 22C10 that labels both the muscles and the nerves. (A) An 18 hours APF preparation showing fragmentation of the two ventral most DLM fibres (arrow). (B) At 20 hours APF, all DLMs have completely degenerated (m marks the position where the muscles would have normally been present and n marks the persisting innervation). (C,D) The developing DLMs in wild-type CS strain at 16 hours and 20 hours APF respectively. The innervation is marked with an 'n'.

epidermis. The implications of these results are discussed in the next section.

DISCUSSION

Myoblast expression of ewg is required for IFM development

The *ewg* gene provides an essential function in embryogenesis and in the development of IFMs. A central question regarding the role of *ewg* in the IFM development is whether the gene is required in the nervous system, in the myoblasts, or in both tissues for proper IFM formation. To address these issues we have developed an *ewg* transgene, EWG^{NS}, that does not express in myoblasts, but expresses in a pan-neural fashion and thus provides the neural function associated with this locus. Flies carrying this transgene in the *ewg* null allele background are 'myoblast-specific null' for the *ewg* gene product, but have full neural function. Such genetically engineered 'myoblast-specific null' flies were studied to analyze the role of myoblast expression of *ewg* locus.

In general, analysis of loss of function mutations at later stages of development or in a specific tissue, for genes like *ewg*, requires the use of genetically mosaic animals or conditional mutations so that the early requirement for viability can be satisfied. These methods are not without limitations. With mosaic technologies, it is not always possible to mark all tissues satisfactorily; moreover, each mosaic animal is unique, so the analysis is complicated. In the case of conditional mutants, although viability issues may be overcome, true null conditions are hard to achieve and tissue-specific null conditions are again not readily possible.

EWG is present in the myoblasts that will form the IFMs and the nuclei of the developing myofibres. EWG is not detected in the myoblasts prior to metamorphosis, is first seen at 10 hours APF and is not detected in adult myofibres. This suggests that the IFM defect associated with ewg^1/ewg^{l1} might

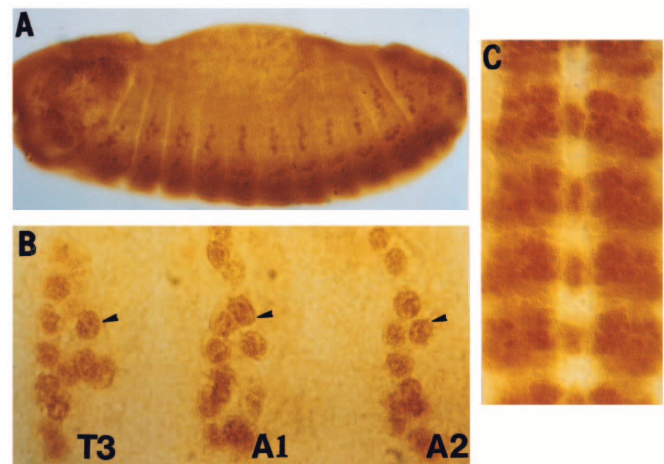

Fig 4. EWG expression in ewg^{l1}; EWG^{NS4} embryos is restricted to the nervous system. Embryos from a stock of ewg^{l1}; EWG^{NS4} were prepared for immunohistochemistry as described in Materials and methods. In A and B anterior is left and ventral is down; in C anterior is top. (A) CNS and PNS expression in stage 14 embryo. The brain and ventral nerve cord are slightly out of focus. (B) High magnification of the dorsal cluster of PNS neurons in T3, A1 and A2 segments. Arrowheads point to cells in the plane of focus and show nuclear localization of EWG. (C) Ventral view of the nerve cord showing labeling in CNS neurons.

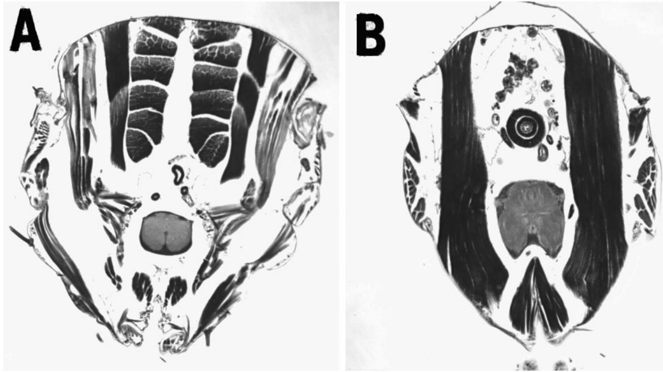

Fig 5. Vital function is restored but IFM abnormalities remain in 'myoblast-null' mutants of *ewg*. Photomicrographs of frontal sections through the thorax of male adults. (A) Wild type; (B) $ewg^{l3}/Y;\ EWG^{NS4}/+$. Note the six DLM fibres, in each hemi-thorax, readily seen in the wild type, and the empty space in between the DVM fibres (long strip of black in each hemi-thorax) in the mutant. The picture of the mutant is representative of the phenotype seen in all 'myoblast null' synthetic mutants of the *ewg* locus, irrespective of the dosage of EWG^{NS} genes.

be due to the reduced levels or absence of EWG in myoblasts (the low expression seen in myofibres could be due to the perdurance of the protein in nuclei upon fusion or could reflect a requirement for EWG in the fibre also). The phenotype observed in ewg^{l1}; EWG^{NS} flies is consistent with this notion. The differences between the phenotypes of ewg^{l1}/ewg flies and ewg^{l1}; EWG^{NS} flies are most likely due to residual EWG activity in the ewg^{l1}/ewg flies. Since ewg^{l1} shows all the characteristics of a 'null' allele, we denote animals of the genotype, ewg^{l1}; EWG^{NS}, in which the lethality has been rescued by expression of the NS4 transgene in the nervous system, as 'myoblast-null' alleles. The patterns of expression of *β3 tubulin* (Kimble et al., 1989), *twist*, *actin 88F*, and *myosin* (Fernandes et al., 1991) have also been described for the developing IFMs. From these studies and the data presented here we conclude that *ewg* expression follows TWIST, is roughly coincident with *β3 tubulin* and precedes *actin* and *myosin* expression in the developing fibre.

Adult flies of the genotype ewg^{1}/ewg^{l1} and those of the genotype $ewg^{l1};\ EWG^{NS}$ both show a variable IFM phenotype with some DVMs often present. A possible scenario to explain the variable expression of the muscle phenotype is that EWG

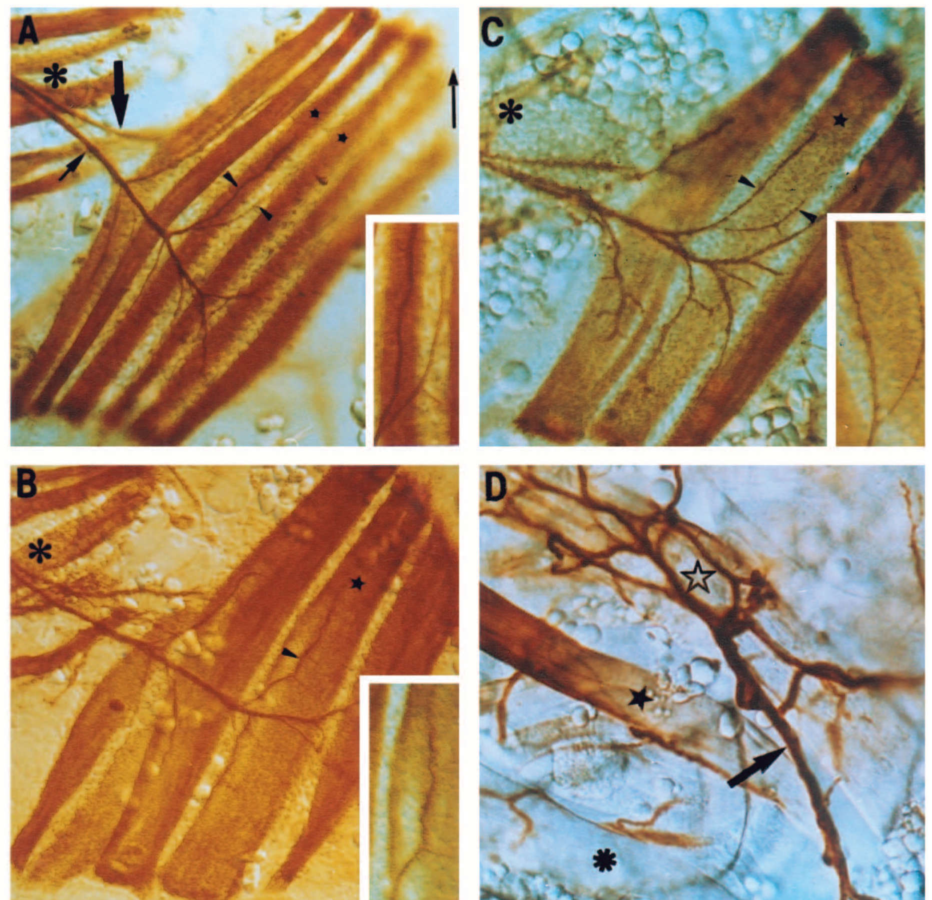

Fig 6. Muscle phenotypes affect the pattern of axonal branching. A comparison of the patterning of motor neurons to the DLMs in wild type, in $ewg^{l1};\ EWG^{NS4}$ /+ animals and in $ewg^{l1};EWG^{NS4}$ /+ animals with muscles partially rescued by a leaky EWG^{HS1} transgene. Preparations are labeled with the mAb 22C10. In all pictures, anterior is to the top and dorsal midline to the right, indicated by a thin vertical arrow in A. The asterisk marks the region of the developing DVMs in A, B and C. (A) Wild-type innervation to the DLMs in a 24 hours APF preparation. The small arrow marks the branch of the nerve trunk containing the motor axons that innervate the DLMs. Note the branches to the individual muscle fibres. The large arrow marks the branch of the nerve that contains neurons from sensory structures on the epidermis. The arrowheads mark the anterior branches of the axons that innervate the muscles marked with a 'star' The arborization of the two axons are shown in detail in the inset. (B) Innervation in animals of the genotype $ewg^{l1};\ EWG^{NS4}$ /+ (the 'myoblast-null') at 24 hours APF. Three 'un-split' DLM units are seen prior to degeneration. The muscle marked with a 'star' is located at a position where the two muscles similarly marked in A would have normally developed. The arrowhead shows axonal branching over the muscle and this arborization is shown in detail in the inset. Note that only one anterior process is seen over this muscle. (C) A leaky EWG^{HS1} transgene in the $ewg^{l1};\ EWG^{NS4}$ /+ (the 'myoblast-null') background partially rescues the DLM phenotype. However, only three differentiated DLMs are seen in the adults that emerge. These DLMs are shown at 24 hours APF and the muscle marked with a 'star' would normally have split to form the muscles marked similarly in A above. The innervation over this muscle, shown by arrowheads, is very similar to that seen over the two fibres in A. Two anterior axon branches are seen and are shown in detail in the inset. (D) At 48 hours APF the DLMs have degenerated in a 'myoblast-null' *ewg* mutant animal. The black star and the asterisk denote the jump muscle and a DVM, respectively. The arrow shows a nerve trunk twisted over one of the remnant DVMs, marked with an open star.

protein acts in concert with several other regulatory proteins such that its absence would not invariably result in the derailment of IFM development. That the *ewg* mutant phenotype is stronger in DLMs than in the DVMs and not apparent in other muscles where the gene is expressed, such as the jump muscle and in the dilator of the pharynx (S. D, unpublished), would suggest that the requirement for *ewg* is highest in the DLM, lower in the DVM and lowest in the jump muscle and the dilator of the pharynx. Consistent with this interpretation is the observation that *ewg* 'myoblast-null' adults (ewg^{l1}; EWG^{NS4}) are unable to jump; and that this phenotype can be rescued by the leaky expression of the EWG^{HS} transgene (S. R. unpublished observations).

In contrast to the EWG^{NS} minigene analyzed in this study, the EWG^{HS} minigene provides full *ewg* function for viability and IFM development. We consider that the expression pattern is the causal factor in these differences as both transgenes contain the same open reading frame. Since high levels of expression of EWG in the nervous system does not rescue the muscle phenotype, and EWG is not detected in the epidermis but is detected in myoblasts, we must conclude that it is the expression of EWG in developing IFM progenitors that rescues the muscle phenotype.

The role of muscles in patterning innervation.

Whereas imaginal muscles are derived from myoblasts set aside during embryogenesis, the adult motor innervation is derived from the modification of mature larval neurons. The pupal development of DLM innervation (Fernandes and VijayRaghavan, 1993) takes place, substantially, concurrently with the development of the muscles themselves. In wild-type animals four of the six DLMs are innervated by one motor neuron each while two DLMs, the dorsal-most two units, are innervated by a single neuron whose cell body is located contra-lateral to the muscles (Ikeda et al., 1980). Axons that innervate the DLMs show characteristic branching patterns over each fibre. What are the mechanisms that operate to pattern adult motor innervation over the DLMs?

In animals that are 'myoblast-null' for *ewg* function, the DLMs fail to develop. Some myoblasts appear to fuse to the remnant larval muscles that serve as templates for the DLMs, but the developing adult muscles soon degenerate without the splitting of the templates (S. R., K. W. and K. V. R. unpublished data). These 'un-split' templates are innervated, however the innervation shows a branching pattern characteristic of one of the two axons that normally innervate the two DLMs that will arise from the splitting of each template (Fig. 6B). When the EWG^{HS} minigene is introduced into strains that carry 'myoblast-null' *ewg* mutations, partial restoration of muscles is seen in animals that are grown at 25°C. This partial rescue results in animals with three fused DLMs instead of the normal six. The innervation to each of the three 'unsplit' muscles, which do not degenerate, now resembles a composite of that normally seen over the two muscles that will form in the wild type. Thus, in animals that are 'myoblast-null' for *ewg* function, the cues for the branching of part of the innervation are present whereas the cues for the branching of other aspects are absent. Upon expression of EWG in the developing muscles (note that in all cases, high level of EWG expression in the nervous system is present from the *elav* promoter) the axons branch in a manner similar to the wild type, suggesting that at least some cues for axon branching must come from the target muscles (Fig. 6C). While an analysis of individual neurons and their projections is needed to reach conclusions on the properties of each motoneuron, it is evident that this system provides a convenient graded method of studying the temporal and spatial effects of changes in the developing muscle upon its innervation. The abnormal innervation seen over remnant DVMs in *ewg* mutant animals (Fig. 6D) can be caused by at least two different mechanisms. After the developing DLMs degenerate their innervation could meander, 'searching' for muscles with recognizable cues in a manner that has been reported in the embryo when normal target muscles are removed (Cash et al., 1992). These neurons could then innervate the remnant DVMs. Another possibility is that the abnormal innervation seen here and in Fig. 6B are trivial consequences of muscle degeneration on their innervation. Further experiments are needed to distinguish between these possibilities.

Requirement of neural ewg expression

The neural component of *ewg* function is both necessary and sufficient for organismal viability as the neural-specific EWG^{NS} transgenes rescue ewg^{-} lethality. The differences in the phenotypes of the rescued adults ($ewg^{lx}/Y;EWG^{NS}$) must reflect the underlying molecular differences among the different *ewg* alleles. Since ewg^{l1} is a protein null, the only *ewg* function in ewg^{l1}/Y; EWG^{NS} is from the transgene, indicating that the nervous system-specific expression is not sufficient for muscle development. Data presented here suggest that ewg^{l3} encodes a functionally null protein. However, ewg^{l4}/Y; EWG^{NS}, ewg^{l5}/Y EWG^{NS} and ewg^{l6}/Y; EWG^{NS} flies have normal wing posture and are capable of flight. This suggests that although these *ewg* alleles cause lethality, they encode proteins that are inadequate in vital function but are functional for IFM development. Indeed, in the case of ewg^{l6}/Y; for example, the few viable animals that emerge manifest no muscle defects (Fleming, 1983). Further, these lethal alleles do not show a muscle defect in *trans* with an ewg^{1} allele, indicating that they do not affect the function in myoblasts. Because a single minigene EWG^{HS1}, can provide full rescue we believe that a single polypeptide is functional in both the nervous system and the muscle, or if two distinct isoforms exist, they are generated from the same open reading frame. Therefore the differences in functionality of the alleles could result from quantitative or qualitative differences in protein expression, or from amino acid substitutions in key functional domains of the molecule.

In conclusion, our study demonstrates a function for a regulatory gene expressed in imaginal myoblasts and in developing adult fibres. While there are several regulatory genes that have been identified that are expressed in developing myoblasts in *Drosophila*, their functional roles have largely been inferred from their amino acid sequence and their similarity to mammalian factors (Michelson et al., 1990; Patterson et al., 1991). Recent studies have demonstrated a role for the *mef-1* gene, expressed in embryonic myoblasts, in muscle development (Bour et al, 1995; Lilly et al, 1995). Our study has dissected the functional requirement for EWG in nervous system and adult flight muscle and provides a basis for the further understanding of the processes that operate in specifying adult muscle pattern.

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