Developmental Cell, Vol. 1, 829–839, December, 2001, Copyright ©2001 by Cell Press

Myoblast Diversification and Ectodermal Signaling in *Drosophila*

Vikram Sudarsan,[1,4] Sumita Anant,[2] Preeta Guptan,[2] K. VijayRaghavan,[2] and Helen Skaer[1,3,4]
[1] Centre for Developmental Genetics
Department of Biomedical Science
Western Bank
University of Sheffield S10 2TN
United Kingdom
[2] National Centre for Biological Sciences
Tata Institute of Fundamental Research
Bangalore 560065
India

Summary

The flight muscles of *Drosophila* derive from myoblasts found on the third instar disc. We demonstrate that these myoblasts already show distinctive properties and examine how this diversity is generated. In the late larva, Vestigial and low levels of Cut are expressed in myoblasts that will contribute to the indirect flight muscles. Other myoblasts, which express high levels of Cut but no Vestigial, are required for the formation of the direct flight muscles. Vestigial and Cut expression are stabilized by a mutually repressive feedback loop. Vestigial expression begins in the embryo in a subset of adult myoblasts, and Wingless signaling is required later to maintain this expression. Thus, myoblasts are divided into identifiable populations, consistent with their allocation to different muscles, and ectodermal signals act to maintain these differences.

Introduction

Recent studies in vertebrates have identified several conserved regulatory factors whose expression patterns are suggestive of early roles in generating myoblast diversity (Jagla et al., 1995; Mennerich et al., 1998; Goulding et al., 1994). However, we know little about the mechanisms underlying the activation of these factors and their roles in generating diversity among muscles are only beginning to be tested (Birchmeier and Brohmann, 2000). Many studies, also in vertebrates, have demonstrated a key role for the ectoderm in regulating the myogenic pathway. Myoblasts respond to the action of Wnt, TGF-β, and Hedgehog (Hh) signaling pathways (reviewed in Birchmeier and Brohmann, 2000; Cossu and Borello, 1999). However, here too, we know little about the targets of these signals and the context in which they operate. For example, they could act directly on an undifferentiated pool of myoblasts to generate fibers with specific contractile properties. Alternatively, they could be required in concert with intrinsic factors that have already divided myoblasts into groups and bias the outcome of signaling so that muscle identity is specified in a combinatorial manner.

The adult flight musculature of *Drosophila* provides an amenable system in which to address these issues (Roy and VijayRaghavan, 1999). There are two muscle sets, the direct (DFM) and indirect (IFM) flight muscles, which differ from each other in their size, location, contractile properties, and innervation (Miller, 1950; Fyrberg and Beall, 1990). The adult myoblast progenitors are siblings of embryonic muscle founder cells (Carmena et al., 1995), whose identities are specified by the expression of combinations of regulatory factors (Dohrmann et al., 1990; Ruiz-Gomez et al., 1997; Baylies et al., 1998; Buff et al., 1998; Halfon et al., 2000). This provides a simple mechanism by which adult myoblasts may be specified, perhaps in a lineage-dependent manner, to contribute during metamorphosis to specific muscle groups or even to specific muscles in the adult. However, whether this is the mechanism that is actually used is not known.

The evidence to date suggests that it is not. Lawrence and Brower (1982) transplanted flight muscle myoblasts to ectopic locations and showed that these myoblasts could contribute to diverse muscles in the fly. They concluded that, in contrast to the wing disc epidermis, the adult myoblasts overlying it were still a naive population. They revealed that the identity of transplanted myoblasts was not autonomously determined but resulted from environmental cues so that they could contribute to the formation of muscle fibers according to their new location.

In trying to resolve the contradiction between this finding and the observation that intrinsic patterning of myoblasts affects at least some aspects of muscle identity (Greig and Akam, 1993; Michelson, 1994; Fernandes et al., 1994), Roy and VijayRaghavan (1997) revisited the transplantation experiments and came to the following conclusions. They agreed substantially with Lawrence and Brower (1982) that the bulk of adult flight muscle myoblasts were naive. But they also suggested that, as in the *Drosophila* embryo, adult muscles could use founder cells to seed the fusion process of myoblasts to form a mature muscle fiber. These founder cells, they argued, must have information about their identity and must be located in the correct position on the epidermis for a mature muscle fiber to form. Myoblasts, transplanted from other locations, may fuse with such founders and contribute to a normal fiber, but only as long as the developing fiber retains its original identity based on that of its "founder".

In this study we investigate the roles of intrinsic and extrinsic cues in the generation of muscle diversity. We show that Vestigial (Williams et al., 1990) and Cut (Blochlinger et al., 1988), two regulatory factors previously shown to be expressed in the adult myoblasts (Ng et al., 1996; Blochlinger et al., 1993), are expressed differentially, resulting in two populations of adult flight muscle myoblasts by the third instar. This diversity among myoblasts predicts the contribution of each population

[3] Correspondence: hs17@cam.ac.uk
[4] Present address: Department of Zoology, Downing Street, University of Cambridge CB2 3EJ, United Kingdom.

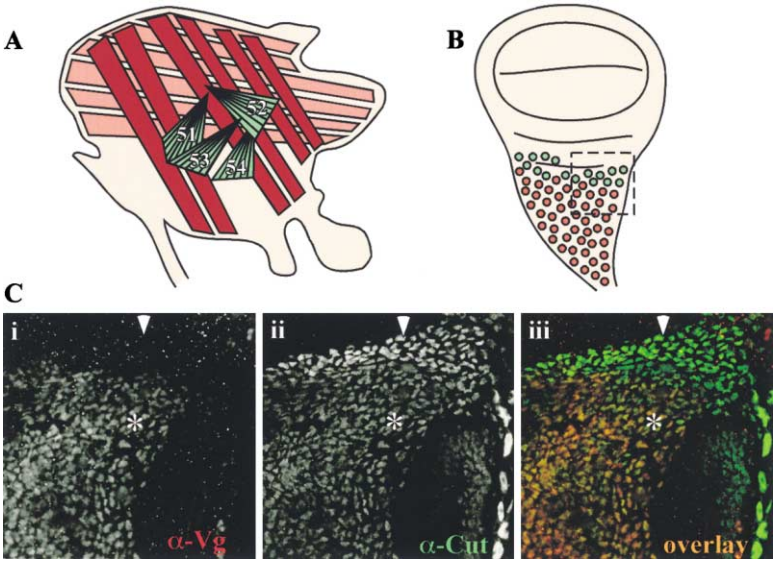

Figure 1. The Origin of the Adult Thoracic Muscles

(A) Diagram of the adult thorax to show the indirect flight muscles (IFMs in red) and direct flight muscles (DFMs in green). The DFMs consist of four muscle sets (numbered 51–54) and there are two groups of IFMs, the dorsal longitudinal muscles (DLMs, pale red) and dorsoventral muscles (DVMs, dark red). Anterior to the left.

(B) Diagram of the wing disc to show the myoblasts overlying the epithelium of the notum. Those expressing high levels of Cut are shown in green and those expressing lower levels of Cut but also expressing Vg in red. Dashed square shows the approximate region shown in (C).

(C) Confocal image of the notum of a wild-type third instar wing disc (area outlined in [B]) stained for Vg (i and red in overlay) and Cut (ii and green in overlay). Distal cells lack Vg but express high levels of Cut (white arrowheads), while proximal cells express lower levels of Cut but show staining for Vg (asterisk).

to different types of flight muscles. Vg (but low Cut)-expressing myoblasts are required for the development of the indirect flight muscles and high Cut (but not Vg)-expressing myoblasts are required for the development of the direct flight muscles. These two classes of myoblasts become distinct during embryonic myogenesis, and initially the maintenance of this diversity could well be a cell-autonomous process. However, later in development, inductive Wingless signals from ectodermal cells closely associated with myoblasts are required for the maintenance of Vg expression. Distinction between the two myoblast groups is reinforced by a mutually repressive feedback loop between Vg and Cut.

Thus, groups of myoblasts are segregated into identifiable populations early in development but require local signals from the ectoderm to maintain differences between them. We discuss the significance of this early myoblast diversification for adult flight muscle development and offer an explanation for the apparent naiveté of transplanted myoblasts.

Results

Expression of Vestigial and Cut Reveals Two Populations of Thoracic Myoblasts that Contribute to Different Muscles

The flight muscles of the adult thorax are shown schematically in Figure 1A. The indirect flight muscles (IFMs, in red) are further divided into two classes on the basis of their location; the dorsal longitudinal muscles (DLMs, pale red) and the dorsoventral muscles (DVMs, dark red). These, together with the direct flight muscles (DFMs, in green, numbered 51–54), constitute the dorsal muscles of the adult thorax and derive from myoblasts that lie over the notal region of the developing wing disc epithelium (Figure 1B). They have been considered to be a uniform population of cells, capable of contributing to a variety of muscles (Lawrence and Brower, 1982). Consistent with this view, they uniformly express the transcription factors encoded by *twist* (*twi*) and *Dmef2* (Currie and Bate, 1991; Fernandes et al., 1991; Ranganayakulu et al., 1995; this study).

When we examined the expression of other molecular markers, we found that these myoblasts are segregated into two distinct groups well before the onset of myoblast fusion. In late third instar discs, a large group of proximally situated myoblasts expresses Vestigial (Vg), while a more distal group located around the hinge region does not express this gene (Figure 1C). These two groups of myoblasts also differ in their levels of Cut expression; Vg-expressing cells show low levels of Cut while cells not expressing Vg are marked by high levels of Cut.

We asked whether the segregation of these two groups of myoblasts, expressing different combinations of transcription factors, predicts a distinctive contribution to the development of specific adult flight muscles. We examined the development of the IFMs in adult viable alleles of *vg*. In two strong alleles, vg^{1} and $vg^{83b27\text{-}R}$, the DLMs are severely reduced and the DVMs are minimal or completely missing (10/10 examined; data shown for $vg^{83b27\text{-}R}$; Figure 2B). To assess the gain-of-function phenotype, we expressed a UAS-*vg* transgene in all wing disc-associated myoblasts using the Gal4 driver 1151 (Roy and VijayRaghavan, 1997, 1998). This leads to alterations in the development of the DFMs, so that muscles 51 and 52 are always missing (Figure 2C). In contrast, the IFMs develop normally (DLMs shown in Figure 2D; 9/10 examined). Viable hypomorphic allelic combinations of *cut* show no muscle phenotype and therefore do not allow us to readily analyze the effects of loss of function on adult muscle development. To assess the effects of *cut* gain of function, we expressed uniformly high levels in all the myoblasts, which results in the virtual complete loss of IFMs (Figure 2F), whereas the DFMs are unaffected (Figure 2E; 10/10 examined).

Thus, while the overexpression of Vg or Cut leads to the reduction or loss of one class of muscle, it is striking that the alternative class is not expanded, as would be

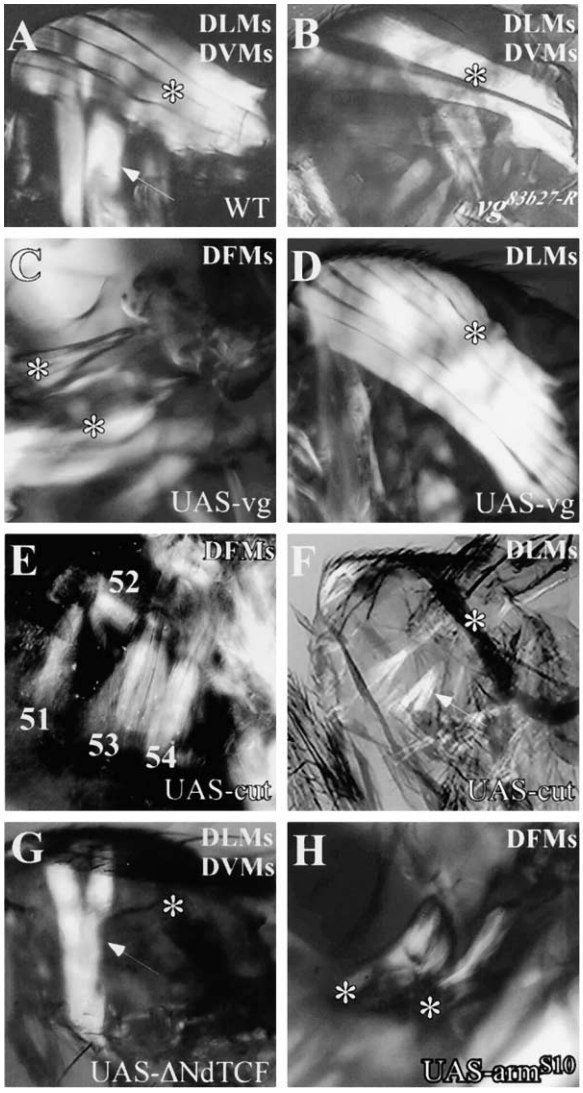

Figure 2. Loss- and Gain-of-Function Phenotypes

(A) Indirect flight muscles (IFMs) in a wild-type adult. The white asterisk indicates the DLMs and the arrow a subset of the DVMs.

(B) In a strong allele for *vg*, the DLMs are severely reduced (asterisk) and the DVMs are completely missing.

(C and D) Overexpression of *vg*. While the DFMs are variably lost (white asterisks mark two remaining DFMs in [C]), the IFMs are not affected (white asterisk marks the DLMs in [D]).

(E and F) Overexpression of *cut*. The DFMs are unaffected (labeled in [E] and arrowed in [F]), but the IFMs are lost. (Asterisk marks the site of lost DLMs in [F].)

(G) Overexpression of DNdTCF. The IFMs are strongly reduced (asterisk marks the site of DLMs; the arrow indicates the few remaining DVMs).

(H) Overexpression of activated Armadillo. The DFMs are variably affected, with a phenotype identical to the overexpression of *vg* (white asterisks indicate two remaining DFMs, compare with [C]).

expected if ectopically expressing cells switched their fate and executed the newly specified myogenic program. We therefore investigated the alternative possibility that cells forced into a new developmental program die. Pupal wing discs in which Cut had been overexpressed in all the myoblasts were stained for the general myoblast marker, Twi, and for acridine orange. Twi staining reveals an approximately 10-fold reduction in the number of myoblasts overlying the IFM larval templates (Figures 3A and 3B), and acridine orange shows a greatly increased incidence of cell death (Figure 3C and 3D). Driving maximal levels of Cut (at 29°C; see Figure 7) results in a dramatic reduction in disc-associated myoblasts even earlier, by the end of the larval period. Together these results indicate that alterations in the pattern of gene expression in myoblasts leads to cell death.

The location of the two populations of myoblasts on the notum, together with phenotypes for the gain and loss of function for *vg* and for *cut* gain of function, suggests that the IFMs derive from proximal, Vg-expressing, low Cut myoblasts and the DFMs from more distal, Vg-negative, high Cut myoblasts. Since these distinctive patterns of gene expression are observed in the third instar, before myoblast fusion, we can test this hypothesis by following the fate of Vg-expressing cells through pupal development. Indeed, we find that myoblasts expressing Vg contribute to the developing IFMs. Figure 4C shows Vg-expressing myoblasts overlying the larval templates on which the DLMs develop and in the muscles below after myoblast fusion (Figure 4C^{i}). After the larval templates have split, the DFMs have many Vg-expressing nuclei (Figure 4D). In contrast the vast majority of myoblasts that contribute to DFMs do not express Vg, either during fusion (Figure 4E) or later (Figure 4F, inset; 1 case in 20 DFMs showed fusion of two Vg-expressing myoblasts).

Together these results show that the third instar myoblasts are partitioned into two populations from which cells contribute to the IFMs or DFMs and that the larval patterns of *vg* and *cut* expression play an important role in specifying these populations.

Adult Myoblast Diversity Is Established in the Embryo, before the Adult Thoracic Epidermis Is Patterned

The notum epithelium is patterned during the second and third instars (Garcia-Garcia et al., 1999; Calleja et al., 2000) and could therefore act to pattern the myoblasts associated with it. As the myoblasts and wing disc arise as separate structures in the embryo, we analyzed Vg expression in early myoblasts to see whether they adopt different patterns of gene expression only after they associate with the disc epithelium. The progenitors of adult myoblasts are specified in the embryo and are characterized by the persistent expression of *twist* (Bate et al., 1991). Embryos stained for twi-lacZ expression, to identify adult progenitors, and with an antibody against Vg reveal a few Vg-expressing adult myoblasts lying between the leg and wing disc primordia from late stage 12 (stage 16 shown in Figure 4A). Vg expression is maintained in a subset of myoblasts during the first and second instars as they become associated with the wing disc (data not shown). By 74 hr AEL (early third instar), we see approximately six Vg-expressing adult myoblasts associated with the stalk of the wing disc (Figure 4B). Thus, the initial patterning of Vg expression in the adult myoblasts is independent of patterning of the wing disc epithelium.

Although embryonic myoblasts are allotted to sets that express Vg and those that do not, the other transcription factor, Cut, whose level of expression also

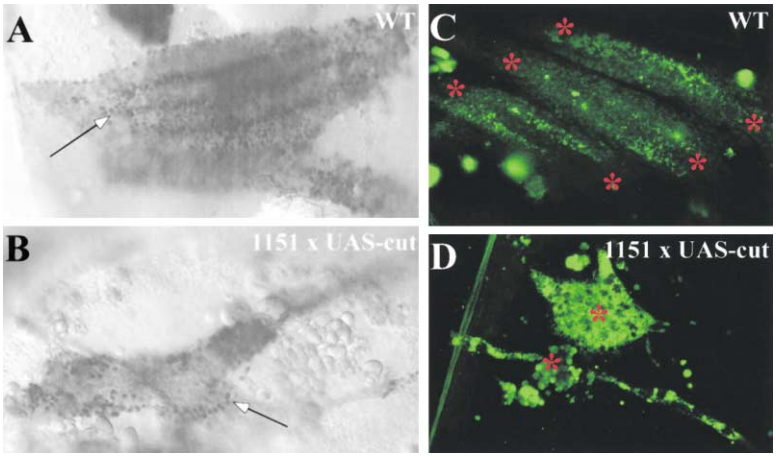

Figure 3. Overexpression of *cut* Induces Cell Death

(A and B) DLMs dissected and stained with an antibody against Twist. Compared to the wild-type (A), the number of cells associated with the larval templates (arrowed) and consequently DLM development is severely reduced when *cut* is expressed in all the myoblasts (B).

(C and D) Acridine orange staining (green) reveals that ectopic Cut results in greatly increased cell death over the developing DLMs (red asterisks; compare [D] to wild-type, [C]).

distinguishes myoblast groups, is initiated only later, in mid third instar larvae (Blochlinger et al., 1993; our own observations). During the third instar, all adult myoblasts express Cut, but they are in two distinct groups: a distal group that expresses high levels of Cut and a proximal group, also distinguished by the expression of Vg, that expresses Cut weakly (Figures 1B and 1C).

Together these data show that the adult myoblast progenitors diversify during embryogenesis before they are associated with the wing disc and suggest that these differences are maintained and elaborated after the myoblasts migrate onto the disc. Might the disc epithelium regulate these later stages of myoblast patterning?

Wg Can Cross from the Ectoderm, Late in the Third Larval Instar, to Signal to Thoracic Myoblasts

If the specification of the adult myoblasts is influenced by the disc epithelium, signals must be able to diffuse between germ layers to stimulate the myoblasts. A striking feature of the presumptive notum of the wing disc is an ectodermal stripe of *wingless* (*wg*) expression, which is initiated in the third instar (Phillips and Whittle, 1993) (Figures 5A and 5B). When conventional fixation procedures are used, Wg is detectable at the apical surface of the disc epithelium (Figure 5C), whereas the protein gradient forms on the basolateral domain, adjacent to the myoblast layer (Strigini and Cohen, 2000). To assess whether Wg secreted from epidermal cells could signal to the associated myoblasts, we expressed a truncated, nonfunctional, GPI-linked form of the Wingless receptor, DFrizzled2 (Cadigan et al., 1998), in all the adult myoblasts. We then examined whether Wg protein was detectable in the mesodermal layer. We find that Wg can cross between the germ layers and bind to the myoblasts in a graded fashion, covering a domain that is wider than the Wg stripe (Figures 5C and 5D).

Wg Functions to Elaborate and Maintain Myoblast Diversity

We asked whether myoblasts were receptive to inductive signaling from the epidermis and whether *wg* is required for the divergence of the two myoblast populations in wild-type third instar discs. Using a temperature-sensitive allele, wg^{IL114} (Nüsslein-Volhard et al., 1984), we removed Wingless function from the second instar onward. This results in a strong reduction of Vg expression in the myoblasts (Figures 6A and 6B). We tested the importance of signaling through the canonical Wg pathway by expressing a dominant-negative form of the transcriptional effector, TCF (van-de-Wetering et al., 1997; Riese et al., 1997), in all the myoblasts of the notum. We found a complete loss of Vg expression (Figure 6C), and, in the adult, the IFMs were much reduced in size (Figure 2G; 8/9 examined). In contrast, when we activated the Wg pathway by expressing arm^{S10}, a constitutively active form of Armadillo (Pai et al., 1997), throughout the notum myoblasts, we found a small expansion of Vg expression into the most distal population of myoblasts (Figure 6D). In the adult, some DFMs were lost, while those that remained were very reduced in size (Figure 2H; 10/10 examined). These results show that spatially organized Wg signaling from the ectoderm is required to maintain Vg expression in the adjoining population of myoblasts.

In order to compare the levels of Cut in wild-type and genetically manipulated cells within single populations of myoblasts, we generated mitotic clones of myoblasts carrying a mutation in *dishevelled* (*dsh*) and therefore unable to transduce Wg signaling (Geer et al., 1983; Noordermeer et al., 1994; Siegfried et al., 1994). As expected, *dsh* clones lose Vg expression, but they also show an increase in the level of Cut expression relative to neighboring wild-type myoblasts (Figures 6E and 6F). This indicates that, in addition to its role in maintaining Vg expression, Wg signaling is also required, directly or indirectly, to modulate myoblast expression of Cut. Interestingly, we find that changes in gene expression are confined to the mutant cells, indicating a cell-autonomous response; the induction of secondary signaling by Wg-activated cells is not involved.

Vg and Cut Show Mutual Repression

Since high levels of Cut are found only in the absence of Vg and Vg-expressing cells show depressed levels of Cut, we analyzed the relationship between Vg and Cut expression by inducing overexpression of each gene in all the notum myoblasts. A general expression of Vg results in uniformly low levels of Cut expression (Figures 7A and 7B), while the induction of uniformly high Cut virtually abolishes Vg expression (Figures 7C and 7D).

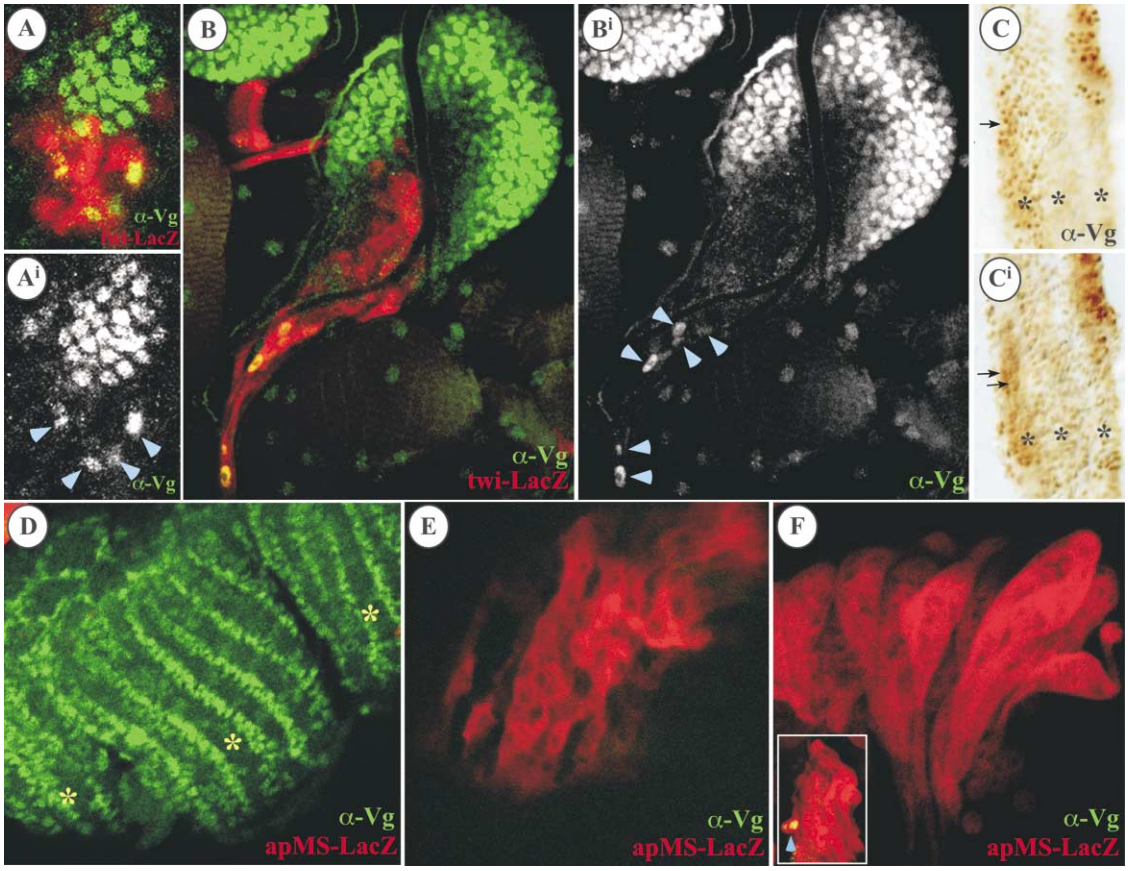

Figure 4. Vg Expression during the Development of Adult Flight Muscles

(A) Mesothoracic segment of a stage 16 embryo labeled with antibodies against β-Gal to identify adult myoblasts (twi-LacZ, red) and Vg (green). A subset of adult myoblasts lying between the leg and wing disc primordia express Vg ([A^i], arrowheads). Later in development these myoblasts will be distributed to the wing and leg discs. n.b., the large group of cells staining strongly for Vg is in the presumptive wing blade.

(B) Early third instar disc (74 ± 2 hr) stained for twi-LacZ (red) and Vg (green). Vg-expressing myoblasts can be seen associated with the stalk of the disc (blue arrowheads in [B^i]). The presumptive wing blade ectoderm expresses Vg strongly.

(C) The larval templates on which the DLMs form dissected from a pupa (20–22 hr APF), showing Vg expression in myoblasts (arrow in [C]) overlying the template (asterisks in [C]) and in myotube nuclei (arrows in [C^i]) as fusion with the templates (asterisks) progresses (lower plane of focus shown in [C^i]).

(D) The DLMs (30 hr APF), after the templates have split and muscles have formed, stained for Vg (green) and ap-MS-LacZ (red, to mark the DFMs). Continued expression of Vg is seen in rows of nuclei within each fiber (asterisks indicate three out of the six fibers). No expression of ap-MS-LacZ is seen in the DLMs.

(E) DFM myoblasts (20–22 hr APF), stained as in [D]. A cluster of myoblasts in the process of forming a DFM fiber expresses the DFM marker, ap-MS-LacZ, and shows no expression of Vg.

(F) A DFM (30–32 hr APF), stained as in [D], showing continued expression of ap-MS-LacZ and no expression of Vg. Inset shows the single example out of twenty, in which Vg-expressing nuclei (blue arrow) were seen in a DFM.

These results reveal a negative regulatory loop between these two gene products, which from the third instar will act to maintain the distinction between the two groups of notum myoblasts, namely, those expressing Vg, that will contribute to the IFMs, and those expressing Cut at high levels that contribute to the DFMs.

Discussion

To make a mature muscle, myoblasts must migrate to specific ectodermal sites, exit from the cell cycle, fuse to produce syncytial fibers, and differentiate to express muscle-specific proteins. In addition to these general requirements, myoblasts acquire specific characteristics enabling them to form fibers with distinctive structural, contractile, and synaptic properties. Our results from this study of adult flight muscle development in *Drosophila* provide evidence for early diversification of myoblasts, the maintenance of this diversity by Wg signaling, and a bias in the contribution of these different myoblasts to distinct adult muscle types.

Diverse Myoblast Populations Are Required for Flight Muscle Development

Much of our understanding of the mechanisms underlying specification of muscle identity has come from the *Drosophila* embryo (reviewed in Baylies et al., 1998). Muscle cell fate is determined by the expression of a combination of transcription factors in a single founder cell for each muscle (Dohrmann et al., 1990; Bourgouin et al., 1992; Ruiz-Gomez et al., 1997). The founder cell is

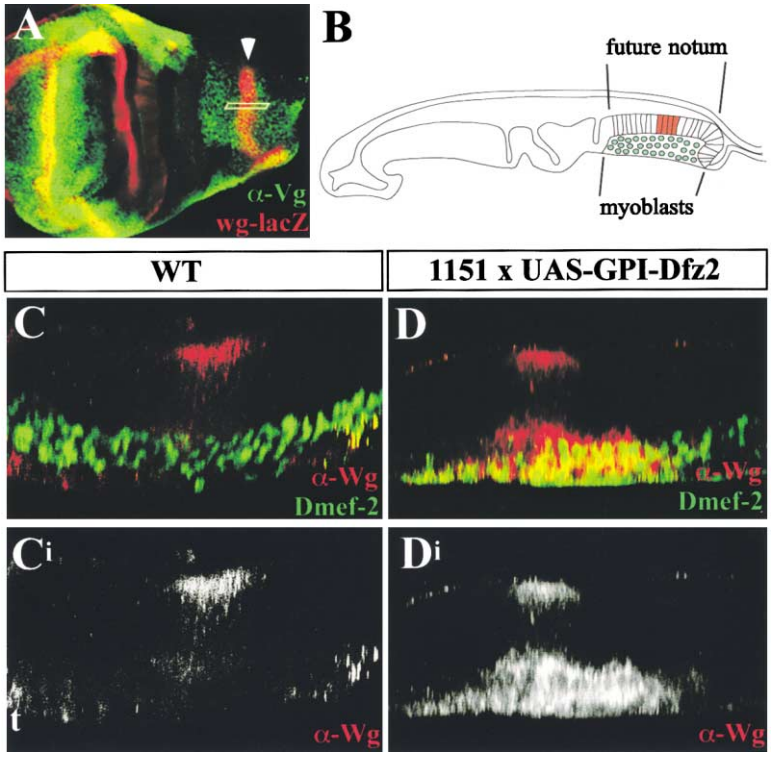

Figure 5. The Expression of Wg in the Notal Region of the Third Instar Wing Disc

(A) Wing disc stained for Vg (green) and β-gal (red) in a wg-LacZ line. The reporter is expressed in a longitudinal stripe in the epidermis of the notum (arrowhead).

(B) Cross-sectional (X-Z) diagram of the wing disc to show the relative position of the epidermis, expressing Wg (red), and the myoblasts (green).

(C and D) Confocal X-Z section of the region boxed in (A) stained for the myoblast marker, Dmef2 (green), and Wg (red). In the wild-type (C), Wg is seen only in the epidermis ([C^i], t: tracheal staining), while, after the overexpression of Dfz2 in the myoblasts, Wg can be seen in the myoblast domain extending many cell diameters beyond the epidermal stripe (D).

chosen from a domain of equivalent cells by Notch-mediated lateral inhibition, and the equivalence domain is itself specified by a combination of inductive instructions from the ectoderm (Carmena et al., 1998a). The founder cell not only specifies the identity of the syncytium, but gives a polarity to the fusion process (Bour et al., 2000; Ruiz-Gomez et al., 2000). Thus, these two important aspects of myogenesis are directed primarily by properties of a single cell. How general is this mechanism?

We have shown that adult myoblasts, as early as during embryogenesis, fall into two classes based on the expression of Vg and that each population later acquires properties that favor its contribution to the formation of specific muscles. We have followed Vg-expressing myoblasts during pupal development and show that they contribute to the IFMs (Figures 4C and 4D). We also show that the myoblasts contributing to the DFMs in the main do not express Vg (Figures 4E and 4F); we have only rarely seen a Vg-expressing myoblast fused with a DFM. Moreover, Vg-negative high Cut-expressing myoblasts are seen around the hinge region on the imaginal disc epidermis, close to the site of the future DFMs.

Corresponding with these observations, manipulation of Vg and Cut results in differential defects in IFM and DFM development. Hypomorphic *vg* mutations affect the IFMs but not the DFMs, while the overexpression of Vg in all adult myoblasts perturbs the DFMs but not the IFMs. Analysis of myoblast number and acridine orange staining for dying cells shows that forcing the expression of high levels of Cut in all myoblasts leads to cell death, resulting in disruption of IFM but not DFM development. This suggests that defects in muscle development following alterations in the expression of Vg or Cut are largely due to cell death rather than an alteration in the specificity of muscle development. Myoblasts with altered patterns of gene expression may well die when they find themselves in an inhospitable environment (for example, lacking appropriate fusion partners or attachment sites). Together these data show that in normal development, Vg-expressing myoblasts contribute to the IFMs and that other myoblasts on the wing disc contribute to the DFMs. It is not clear to us how exclusive these contributions might be, but, in any case, we expect that the multinucleate nature of muscles will be tolerant to a degree of sloppiness in this process and may permit some mixing of myoblasts.

Our results could be interpreted to mean that adult flight muscle identity is specified in a population of myoblasts rather than in a single cell as in the embryo. But this does not exclude the presence of founder cells in adult myogenesis, which not only direct the fusion process but confer information about the size, orientation, muscle insertion sites, and innervation of the muscle being made. In addition to these characteristics, IFMs and DFMs exhibit specific contractile properties conferred by the expression and organization of their contractile proteins (reviewed in Fyrberg and Beall, 1990). Myoblast diversity could play a role in establishing these characteristics. Similar mechanisms may operate in the embryo, giving properties to populations of fusion-competent myoblasts, in addition to those conferred by founder cells, to specify muscle identity (Martin et al., 2001).

Adult Myoblast Diversity Is Established in the Embryo

We have identified Vg as a factor in the early diversification of myoblasts. Vg is expressed on the third larval instar wing imaginal disc in many, but not all, myoblasts. The origins of these molecular differences among myoblasts can be traced back to the embryo; as early as

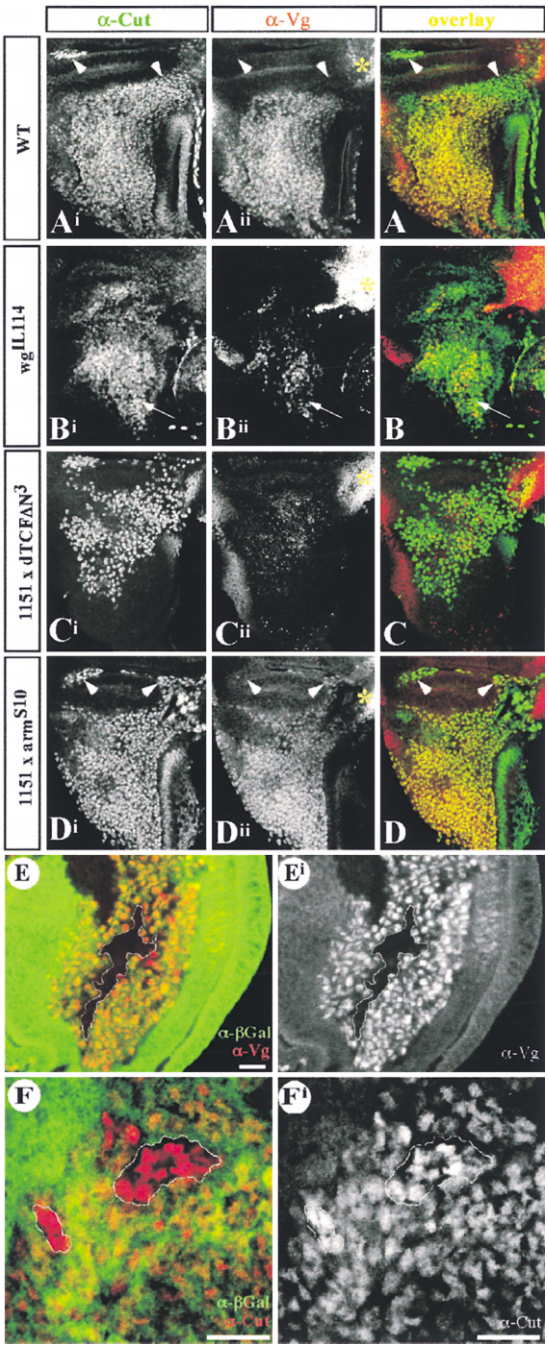

Figure 6. The Role of the Wg Pathway in Myoblast *vg* Expression

(A) Expression of Cut (A^i) and Vg (A^{ii}) in a wild-type third instar wing disc. Arrowheads, distal-most myoblasts; yellow asterisk in (A^{ii}), epidermal Vg expression.

(B) Third instar disc from a wg^{IL114} line shifted to the restrictive temperature at 72 hr after egg laying. Vg is dramatically reduced in the proximal myoblasts (arrows in [B^{ii}] and [B]).

(C) Disc in which a dominant-negative TCF has been expressed in the myoblasts. Myoblast Vg expression is lost (C^{ii}). Cut staining indicates that there are fewer, larger myoblasts in this condition (compare [C^i] with [A^i]).

(D) Disc in which an activated *armadillo* transgene has been expressed in the myoblasts. Vg is ectopically expressed in some of the distal myoblasts ([D^{ii}], right arrowhead) but not in others ([D^{ii}], left arrowhead). Proximal myoblast expression of Vg is unaffected.

(E and F) Expression of Vg and Cut in *dishevelled* mutant clones. Clones of *dsh* mutant myoblasts (marked by the absence of arm-LacZ, green) in the proximal, Vg-expressing domain fail to express Vg (red in [E] and [E^i]) but show elevated levels of Cut compared with wild-type myoblasts ([F^i] and red in [F]).

stage 12, adult myoblasts have already diversified into Vg-expressing and nonexpressing subtypes.

Adult thoracic myoblasts, already subdivided into these two classes, are associated with ectodermal cells of the wing imaginal disc in the late embryo. However, the patterning of the wing epithelium itself is a gradual process that continues during larval life. The expression of Wg in the presumptive notum begins only in the early third instar larva (Phillips and Whittle, 1993). Thus, the initial diversification of myoblasts occurs before notum Wg expression begins.

Each abdominal muscle fiber in the adult develops from the progeny of a single cell set aside in the embryo (Broadie and Bate, 1991). These adult progenitors arise from the asymmetric division of specific precursor cells in the embryo (Ruiz-Gomez and Bate, 1997; Baylies et al., 1998; Carmena et al., 1998b). The identity of muscle founders and adult progenitors results from the asymmetric distribution of Numb, which biases Notch-mediated signaling, so that the cell inheriting Numb becomes the founder of a larval muscle, while the daughter that retains Notch activity becomes an adult muscle progenitor (Ruiz-Gomez and Bate, 1997; Carmena et al., 1998b). Misexpressing *numb* in all myoblasts can therefore markedly reduce the segregation of adult progenitors. When these animals develop to the pupal stage, the abdominal musculature is greatly reduced, and adults that emerge are often flightless (Ruiz-Gomez and Bate, 1997). Our observations (data not shown) indicate that this results from a severe reduction in both IFMs and DFMs.

These results indicate that adult flight muscle myoblasts originate from the siblings of larval founder cells in the embryo, raising the possibility that common mechanisms operate in these lineages to establish larval founder cell identities and the diversification of adult progenitors.

Adult Myoblast Diversity Is Maintained by Wg Signaling

While differences among myoblasts are established early, inductive signals are required to maintain this diversity later during larval life. We have shown that Wg signaling from the presumptive notum is required for continued Vg expression in the underlying myoblasts.

Wg signaling acts in two ways. First, it has an effect on the proliferation of Vg-expressing cells. When Wg signaling is activated, the number of Vg-expressing cells increases, and, conversely, when it is removed, the number of cells in the region decreases, quite apart from the loss of Vg expression (Figure 6C). The second effect, maintenance of Vg expression, is a cell-autonomous response of each myoblast to Wg signaling. This is demonstrated in clones of cells that remove *dsh* function, in which Vg expression is lost from every mutant cell, showing that neighboring Vg-expressing cells cannot rescue defects in Wg signaling. This result and the presence of Wg response elements in *vg* regulatory sequences (Kim et al., 1996) are consistent with a direct role for Wg signaling in the maintenance of myoblast Vg expression.

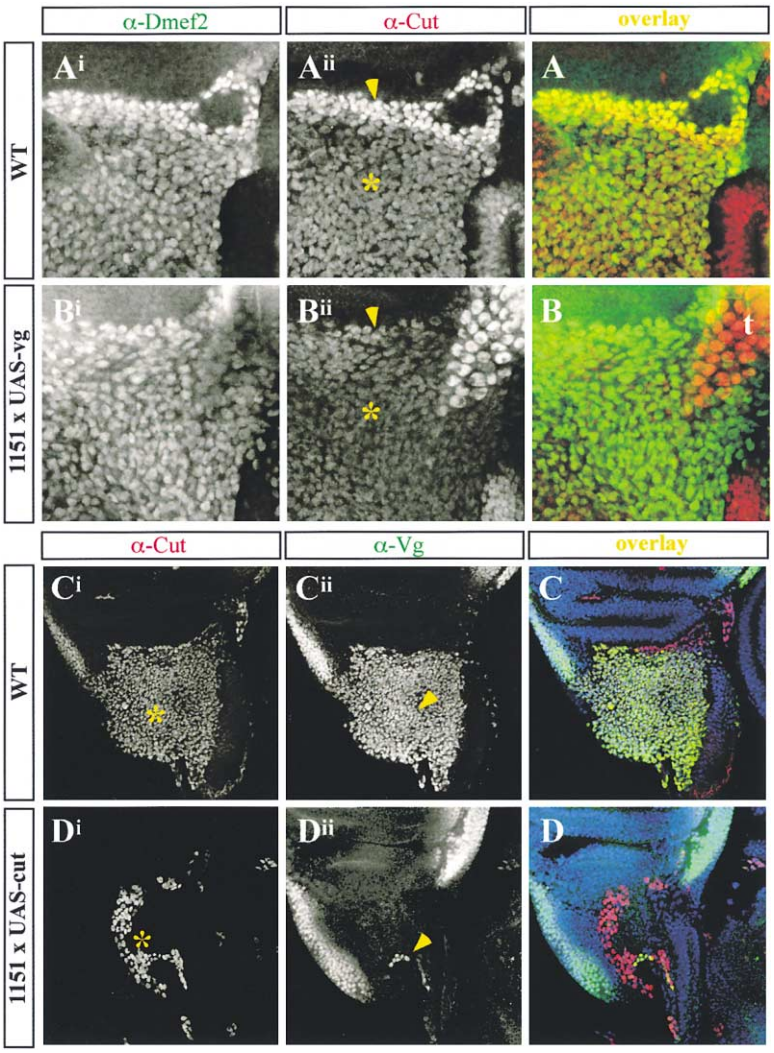

Figure 7. Overexpression of *vg* and *cut* in the Notum-Associated Myoblasts of Third Instar Discs

(A and B) Dmef2 expression (green) marks all myoblasts. Discs stained for Cut (red). In wild-type discs (A), Cut is expressed at higher levels in the distal myoblasts (arrowhead in [A^{ii}]) compared with the proximal cells (asterisk). Ectopic expression of *vg* in these cells reduces Cut expression, leading to uniform levels of Cut throughout the myoblasts ([B], t: tracheal cells). n.b., the apparent increase in staining for Dmef2 results from bleed through from the FITC-Cut channel and does not reflect an increase in Dmef2 expression.

(C and D) Discs stained for Vg (green). Increasing the levels of *cut* expression in the proximal myoblasts results in strong reduction of Vg (arrowheads in [C^{ii}] and [D^{ii}]). As shown in Figure 3, overexpression of *cut* also results in extensive cell death accounting for the overall reduction in myoblast number (asterisks in [C^{i}] and [D^{i}]). Cut (red); DNA (blue).

We have shown that the capacity of myoblasts to initiate *vg* expression in response to Wg signaling is limited. The effects of expressing an activated Arm in all myoblasts are not uniform. Vg expression is induced in some but not all distal myoblasts within the high Cut-expressing domain. However, the consequences for adult flight muscle development are identical to the phenotype we observe when we express *vg* in all adult myoblasts. The IFMs are unaffected while the DFMs are severely reduced but not completely lost. These observations suggest that there is a subset of high Cut-expressing myoblasts that neither responds to the activation of Wg signaling nor changes its behavior when forced to express *vg*.

We have shown that Wg is required from the adjacent ectoderm, not for the initiation of Vg expression, but for its maintenance. This has consequences for the interpretation of experiments in which myoblasts were transplanted (Lawrence and Brower, 1982). In this situation, the environmental cues that reinforce the distinctive character of myoblasts are altered so that they could be reprogrammed, becoming competent to contribute to muscles in their new location.

A Feedback Loop Acts to Reinforce Myoblast Diversity

Manipulation of the levels of *cut* and *vg* expression point to mutual negative regulatory feedback between these genes. One model for the mechanism underlying this loop is shown schematically in Figure 8. In this scheme, Wg signaling acts to maintain *vg* expression. Expression of Vg results in the repression of high *cut* expression, although a direct input of Wg signaling is not ruled out. In contrast, cells that express high levels of Cut repress *vg*.

Vg has been shown to have an obligate requirement for the *scalloped* (*sd*) gene product as a cofactor. Wing blade development depends on a regulatory network activated by Wg signaling and involving genes such as *cut*, *vg*, and *sd* (Couso et al., 1994; reviewed in Cohen, 1996 and Bray, 1999). The Vg and Cut interactions we observe suggest that a similar regulatory network could act during adult myoblast proliferation, and, consistent with this, *sd* is expressed during adult flight muscle development (Anand et al., 1990; Campbell et al., 1992). Other elements participating in this regulatory network must be *apterous* (Ghazi et al., 2000) for the DFMs and *erect wing* for the IFMs (DeSimone et al., 1996; Roy

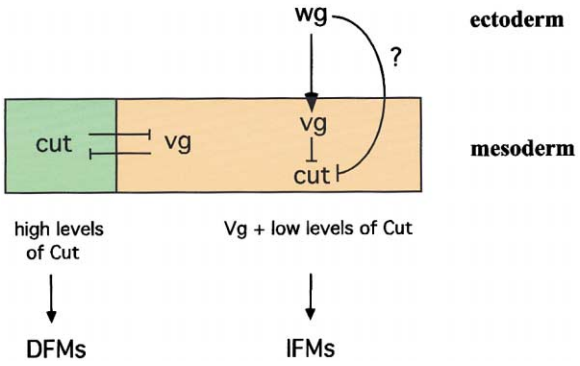

Figure 8. Model for the Regulation of *cut* and *vg* Expression in the Notum Myoblasts

Wg signaling from the epidermis of the presumptive notum acts to maintain Vg expression in adjacent myoblasts. Mutual repression between Cut and Vg reinforces the effects of Wg to establish the identity of distinct myoblast populations that were initially set aside during embryogenesis. Resulting high-Vg/low-Cut myoblasts contribute to the indirect flight muscles (IFMs), while high-Cut/Vg-negative cells contribute to the direct flight muscles (IFMs).

and VijayRaghavan, 1999), genes whose expression in specific muscle sets begins later in pupal development.

Myoblast Diversification: A More General Mechanism?

We have described a mechanisms for subdividing myoblasts and provide evidence that this process is important for generating muscles of different kinds in the adult. Flight muscles are large, are made up of multiple fibers, and develop by the fusion of populations of myoblasts that migrate over large distances in close contact with the epidermis—features shared by developing vertebrate muscles. The combination of intrinsic and extrinsic cues, such as we have described for adult flight muscle myoblasts, might also act to regulate vertebrate myoblast characteristics.

In the mouse, limb muscle precursors are marked out by the expression of a transcription factor, Ladybird (Lbx1), which is required for migration of these cells into the developing limb bud (Schafer and Braun, 1999). In the zebrafish slow and fast muscle subtypes are specified by signaling through the Hedgehog pathway (Blagden et al., 1997). These studies show the importance of both cell-autonomous and cell-nonautonomous cues for vertebrate muscle diversification, but less is known about the relationship between these regulatory factors. Given the conservation of such molecular networks and the similarity of many cellular features of flight muscle development and vertebrate myogenesis, the fly offers the opportunity to unravel further details of the mechanisms that are required to generate complex patterns of large muscles.

Experimental Procedures

Drosophila Stocks and Genetics

Oregon R or Canton S flies were used as wild-type. vg^{1} *and* $vg^{83b27\text{-}R}$ are viable recessive alleles of *vestigial* (Williams et al., 1990). For misexpression of UAS-transgenes in the wing disc-associated adult myoblasts, we used a 1151-Gal4; twi-lacZ stock (Roy and VijayRaghavan, 1997, 1998). 1151-Gal4 is expressed in the adult myoblasts and fat body from the first instar. UAS-*vg* was obtained from S. Carroll. UAS-*cut* was obtained from K. Blochlinger (Blochlinger et al., 1991). UAS-GPI-*Dfz2* was obtained from S. Cohen. wg^{IL114} is a temperature-sensitive allele. wg TS experiments were carried out essentially according to Couso et al. (1994), except that wg^{IL114} homozygous discs were identified by the lack of Cut expression in the wing margin. Eggs were collected for 2 hr and allowed to develop at 18°C for 160 hr (equivalent to 80 hr at 25°C) and subsequently grown at the restrictive temperature of 25°C for a further 40 hr, after which larvae were dissected and fixed. We chose only animals that showed morphological characteristics of third instar larvae and discs that lacked Cut in the margin but expressed Cut in the sensory neurons (Couso et al., 1994) as a measure of age. For all other timed dissections, eggs were collected for 4 hr and aged appropriately at 25°C.

Generation of *dsh* Mutant Clones

yw, dsh^{v26} *FRT18/FM7* virgins were crossed to *w, arm-lacZ FRT18* ; hs-flp^{38} males (both stocks obtained from D. Strutt). Flies from the cross were transferred to bottles on day 1, eggs collected overnight and clones were induced by heat shocking at 37°C for 2 hr both on days 3 and 4. *dsh* mutant clones were identified by lack of β-Gal staining, and myoblasts were identified by the expression of Cut or Vg depending on the experiment.

Immunohistochemistry

Dissected larvae were fixed for 15 min in 4% paraformaldehyde in cold PBS. Immunostaining was done according to Ghazi et al. (2000). Monoclonal antibodies against Wg and Ct were obtained from the Developmental Studies Hybridoma Bank. The antibody against Wg was preabsorbed on young embryos at 1:10 dilution and used at 1:1000 final dilution. The antibody against Cut was used at 1:100 dilution. Rabbit antibodies against Vg were kindly provided by S. Carroll and used at 1:500 dilution. Rabbit antibodies against Dmef2 were kindly provided by B. Paterson and used at 1:500 dilution. Monoclonal antibodies against β-Gal (Promega) were used at 1:1000 dilution. For fluorescent detection, FITC-, Cy3-, or Cy5-conjugated secondary antibodies were used. For some experiments, we performed an extra amplification step by using streptavidin-conjugated Cy3. All fluorophores were obtained from Jackson Laboratories and confocal microscopy performed on a Leica TCS NT confocal system. Images were assembled using Photoshop and Canvas software.

Adult Muscle Preparations

Adult thoracices were prepared by freezing flies (spread on a slide and stuck down on a drop of 70% glycerol) in liquid nitrogen. Flies were then cut sagitally (along the center for IFMs and more laterally for the DFMs), dehydrated through an ethanol series, mounted in DPX, and viewed under polarized light. Pupal dissections were performed according to Fernandes et al. (1991).

Acknowledgments

We thank Karen Blochlinger, Steve Cohen, Sean Carroll, Juan Pablo Couso, Bruce Paterson, and David Strutt for stocks and reagents and the Developmental Studies Hybridoma Bank for antibodies. We are grateful to Barry Denholm, Peter Lawrence, Veronica Rodrigues, Sudipto Roy, and David Strutt for helpful discussion and Michael Bate for many illuminating discussions and helpful comments on the manuscript. This work is funded by an INLAKS Foundation scholarship to V.S., the Wellcome Trust (H.S.), a British Council-ISTR grant (K.V. and H.S.) and a Department of Biotechnology grant to K.V.

Received July 9, 2001; revised September 21, 2001.

References

Anand, A., Fernandes, J., Arunan, M.C., Bhosekar, S., Chopra, A., Dedhia, N., Sequiera, K., Hasan, G., Palazzolo, M.J., Vijay Raghavan, K., and Rodrigues, V. (1990). *Drosophila* enhancer-trap transposants—gene expression in chemosensory and motor pathways and identification of mutants affected in smell and taste ability. J. Genet. *69*, 151–168.

Bate, M., Rushton, E., and Currie, D.A. (1991). Cells with persistent

twist expression are the embryonic precursors of adult muscles in *Drosophila*. Development *113*, 79–89.

Baylies, M.K., Bate, M., and Ruiz-Gomez, M. (1998). Myogenesis: a view from *Drosophila*. Cell *93*, 921–927.

Birchmeier, C., and Brohmann, H. (2000). Genes that control the development of migrating muscle precursor cells. Curr. Opin. Cell Biol. *12*, 725–730.

Blagden, C.S., Currie, P.D., Ingham, P.W., and Hughes, S.M. (1997). Notochord induction of zebrafish slow muscle mediated by Sonic hedgehog. Genes Dev. *11*, 2163–2175.

Blochlinger, K., Bodmer, R., Jack, J., Jan, L.Y., and Jan, Y.N. (1988). Primary structure and expression of a product from *cut*, a locus involved in specifying sensory organ identity in *Drosophila*. Nature *333*, 629–635.

Blochlinger, K., Jan, L.Y., and Jan, Y.N. (1991). Transformation of sensory organ identity by ectopic expression of Cut in *Drosophila*. Genes Dev. *5*, 1124–1135.

Blochlinger, K., Jan, L.Y., and Jan, Y.N. (1993). Postembryonic patterns of expression of *cut*, a locus regulating sensory organ identity in *Drosophila*. Development *117*, 441–450.

Bour, B.A., Chakravarti, M., West, J.M., and Abmayr, S.M. (2000). *Drosophila* SNS, a member of the immunoglobulin superfamily that is essential for myoblast fusion. Genes Dev. *14*, 1498–1511.

Bourgouin, C., Lundgren, S.E., and Thomas, J.B. (1992). *apterous* is a *Drosophila* LIM domain gene required for the development of a subset of embryonic muscles. Neuron *9*, 549–561.

Bray, S. (1999). *Drosophila* development: Scalloped and Vestigial take wing. Curr. Biol. *9*, R245–R247.

Broadie, K.S., and Bate, M. (1991). The development of adult muscles in *Drosophila*: ablation of identified muscle precursor cells. Development *113*, 103–118.

Buff, E., Carmena, A., Gisselbrecht, S., Jimenez, F., and Michelson, A.M. (1998). Signalling by the *Drosophila* epidermal growth factor receptor is required for the specification and diversification of embryonic muscle progenitors. Development *125*, 2075–2086.

Cadigan, K.M., Fish, M.P., Rulifson, E.J., and Nusse, R. (1998). Wingless repression of *Drosophila frizzled 2* expression shapes the Wingless morphogen gradient in the wing. Cell *93*, 767–777.

Calleja, M., Herranz, H., Estella, C., Casal, J., Lawrence, P., Simpson, P., and Morata, G. (2000). Generation of medial and lateral dorsal body domains by the *pannier* gene of *Drosophila*. Development *127*, 3971–3980.

Campbell, S., Inamdar, M., Rodrigues, V., Raghavan, V., Palazzolo, M., and Chovnick, A. (1992). The *scalloped* gene encodes a novel, evolutionarily conserved transcription factor required for sensory organ differentiation in *Drosophila*. Genes Dev. *6*, 367–379.

Carmena, A., Bate, M., and Jimenez, F. (1995). *lethal of scute*, a proneural gene, participates in the specification of muscle progenitors during *Drosophila* embryogenesis. Genes Dev. *9*, 2373–2383.

Carmena, A., Gisselbrecht, S., Harrison, J., Jimenez, F., and Michelson, A.M. (1998a). Combinatorial signaling codes for the progressive determination of cell fates in the *Drosophila* embryonic mesoderm. Genes Dev. *12*, 3910–3922.

Carmena, A., Bate, M., and Jimenez, F. (1998b). *inscuteable* and *numb* mediate asymmetric muscle progenitor cell divisions during *Drosophila* myogenesis. Genes Dev. *12*, 304–315.

Cohen, S.M. (1996). Controlling growth of the wing: *vestigial* integrates signals from the compartment boundaries. BioEssays *18*, 855–858.

Cossu, G., and Borello, U. (1999). Wnt signaling and the activation of myogenesis in mammals. EMBO J. *18*, 6867–6872.

Couso, J.P., Bishop, S.A., and Martinez-Arias, A. (1994). The *wingless* signalling pathway and the patterning of the wing margin in *Drosophila*. Development *120*, 621–636.

Currie, D.A., and Bate, M. (1991). The development of adult abdominal muscles in *Drosophila*: myoblasts express *twist* and are associated with nerves. Development *113*, 91–102.

DeSimone, S., Coelho, C., Roy, S., VijayRaghavan, K., and White, K. (1996). ERECT WING, the *Drosophila* member of a family of DNA binding proteins is required in imaginal myoblasts for flight muscle development. Development *122*, 31–39.

Dohrmann, C., Azpiazu, N., and Frasch, M. (1990). A new *Drosophila* homeo box gene is expressed in mesodermal precursor cells of distinct muscles during embryogenesis. Genes Dev. *4*, 2098–2111.

Fernandes, J., Bate, M., and Vijayraghavan, K. (1991). Development of the indirect flight muscles of *Drosophila*. Development *113*, 67–77.

Fernandes, J., Celniker, S.E., Lewis, E.B., and VijayRaghavan, K. (1994). Muscle development in the four-winged *Drosophila* and the role of the *Ultrabithorax* gene. Curr. Biol. *4*, 957–964.

Fyrberg, E.A., and Beall, C. (1990). Genetic approaches to myofibril form and function in *Drosophila*. Trends Genet. *6*, 126–131.

Garcia-Garcia, M.J., Ramain, P., Simpson, P., and Modolell, J. (1999). Different contributions of *pannier* and *wingless* to the patterning of the dorsal mesothorax of *Drosophila*. Development *126*, 3523–3532.

Geer, B.W., Lischwe, T.D., and Murphy, K.G. (1983). Male fertility in *Drosophila melanogaster*: genetics of the *vermilion* region. J. Exp. Zool. *225*, 107–118.

Ghazi, A., Anant, S., and VijayRaghavan, K. (2000). Apterous mediates development of direct flight muscles autonomously and indirect flight muscles through epidermal cues. Development *127*, 5309–5318.

Goulding, M., Lumsden, A., and Paquette, A.J. (1994). Regulation of Pax-3 expression in the dermomyotome and its role in muscle development. Development *120*, 957–971.

Greig, S., and Akam, M. (1993). Homeotic genes autonomously specify one aspect of pattern in the *Drosophila* mesoderm. Nature *362*, 630–632.

Halfon, M.S., Carmena, A., Gisselbrecht, S., Sackerson, C.M., Jimenez, F., Baylies, M.K., and Michelson, A.M. (2000). Ras pathway specificity is determined by the integration of multiple signal-activated and tissue-restricted transcription factors. Cell *103*, 63–74.

Jagla, K., Dolle, P., Mattei, M.G., Jagla, T., Schuhbaur, B., Dretzen, G., Bellard, F., and Bellard, M. (1995). Mouse *Lbx1* and human *LBX1* define a novel mammalian homeobox gene family related to the *Drosophila ladybird* genes. Mech. Dev. *53*, 345–356.

Kim, J., Sebring, A., Esch, J.J., Kraus, M.E., Vorwerk, K., Magee, J., and Carroll, S.B. (1996). Integration of positional signals and regulation of wing formation and identity by *Drosophila vestigial* gene. Nature *382*, 133–138.

Lawrence, P.A., and Brower, D.L. (1982). Myoblasts from *Drosophila* wing discs can contribute to developing muscles throughout the fly. Nature *295*, 55–57.

Martin, B.S., Ruiz-Gomez, M., Landgraf, M., and Bate, M.A. (2001). A distinct set of founders and fusion-competent myoblasts make visceral muscles in the *Drosophila* embryo. Development *128*, 3331–3338.

Mennerich, D., Schafer, K., and Braun, T. (1998). Pax-3 is necessary but not sufficient for *lbx1* expression in myogenic precursor cells of the limb. Mech. Dev. *73*, 147–158.

Michelson, A.M. (1994). Muscle pattern diversification in *Drosophila* is determined by the autonomous function of homeotic genes in the embryonic mesoderm. Development *120*, 755–768.

Miller, A. (1950). The Internal Anatomy and Histology of the Imago of *Drosophila melanogaster*, M. Demerec, ed. (New York: Cold Spring Harbor Laboratory Press).

Ng, M., Diaz-Benjumea, F.J., Vincent, J.P., Wu, J., and Cohen, S.M. (1996). Specification of the wing by localized expression of Wingless protein. Nature *381*, 316–318.

Noordermeer, J., Klingensmith, J., Perrimon, N., and Nusse, R. (1994). *dishevelled* and *armadillo* act in the *wingless* signalling pathway in *Drosophila*. Nature *367*, 80–83.

Nüsslein-Volhard, C., Wieschaus, E., and Kluding, H. (1984). Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster*. Roux Arch. Dev. Biol. *193*, 267–282.

Pai, L.M., Orsulic, S., Bejsovec, A., and Peifer, M. (1997). Negative

regulation of Armadillo, a Wingless effector in *Drosophila*. Development *124*, 2255–2266.

Phillips, R.G., and Whittle, J.R. (1993). *wingless* expression mediates determination of peripheral nervous system elements in late stages of *Drosophila* wing disc development. Development *118*, 427–438.

Ranganayakulu, G., Zhao, B., Dokidis, A., Molkentin, J.D., Olson, E.N., and Schulz, R.A. (1995). A series of mutations in the D-MEF2 transcription factor reveal multiple functions in larval and adult myogenesis in *Drosophila*. Dev. Biol. *171*, 169–181.

Riese, J., Yu, X., Munnerlyn, A., Eresh, S., Hsu, S.C., Grosschedl, R., and Bienz, M. (1997). LEF-1, a nuclear factor coordinating signaling inputs from *wingless* and *decapentaplegic*. Cell *88*, 777–787.

Roy, S., and VijayRaghavan, K. (1997). Homeotic genes and the regulation of myoblast migration, fusion, and fibre-specific gene expression during adult myogenesis in *Drosophila*. Development *124*, 3333–3341.

Roy, S., and VijayRaghavan, K. (1998). Patterning muscles using organizers: larval muscle templates and adult myoblasts actively interact to pattern the dorsal longitudinal flight muscles of *Drosophila*. J. Cell Biol. *141*, 1135–1145.

Roy, S., and VijayRaghavan, K. (1999). Muscle pattern diversification in *Drosophila*: the story of imaginal myogenesis. Bioessays *21*, 486–498.

Ruiz-Gomez, M., and Bate, M. (1997). Segregation of myogenic lineages in *Drosophila* requires *numb*. Development *124*, 4857–4866.

Ruiz-Gomez, M., Coutts, N., Price, A., Taylor, M.V., and Bate, M. (2000). *Drosophila dumbfounded*: a myoblast attractant essential for fusion. Cell *102*, 189–198.

Ruiz-Gomez, M., Romani, S., Hartmann, C., Jäckle, H., and Bate, M. (1997). Specific muscle identities are regulated by Krüppel during *Drosophila* embryogenesis. Development *124*, 3407–3414.

Schafer, K., and Braun, T. (1999). Early specification of limb muscle precursor cells by the homeobox gene *Lbx1h*. Nat. Genet. *23*, 213–216.

Siegfried, E., Wilder, E.L., and Perrimon, N. (1994). Components of *wingless* signalling in *Drosophila*. Nature *367*, 76–80.

Strigini, M., and Cohen, S.M. (2000). Wingless gradient formation in the *Drosophila* wing. Curr. Biol. *10*, 293–300.

van-de-Wetering, M., Cavallo, R., Dooijes, D., van-Beest, M., van-Es, J., Loureiro, J., Ypma, A., Hursh, D., Jones, T., Bejsovec, A., et al. (1997). Armadillo coactivates transcription driven by the product of the *Drosophila* segment polarity gene *dTCF*. Cell *88*, 789–799.

Williams, J.A., Atkin, A.L., and Bell, J.B. (1990). The functional organization of the *vestigial* locus in *Drosophila melanogaster*. Mol. Gen. Genet. *221*, 8–16.