Development 136, 1105-1113 (2009) doi:10.1242/dev.031567

Crossveinless and the TGFβ pathway regulate fiber number in the *Drosophila* adult jump muscle

MaryAnn S. Jaramillo, Candice V. Lovato, Erica M. Baca and Richard M. Cripps*

Skeletal muscles are readily characterized by their location within the body and by the number and composition of their constituent muscle fibers. Here, we characterize a mutation that causes a severe reduction in the number of fibers comprising the tergal depressor of the trochanter muscle (TDT, or jump muscle), which functions in the escape response of the *Drosophila* adult. The wild-type TDT comprises over 20 large muscle fibers and four small fibers. In *crossveinless* (*cv*) mutants, the number of large fibers is reduced by 50%, and the number of small fibers is also occasionally reduced. This reduction in fiber number arises from a reduction in the number of founder cells contributing to the TDT at the early pupal stage. Given the role of *cv* in TGFβ signaling, we determined whether this pathway directly impacts TDT development. Indeed, gain- and loss-of-function manipulations in the TGFβ pathway resulted in dramatic increases and decreases, respectively, in TDT fiber number. By identifying the origins of the TDT muscle, from founder cells specified in the mesothoracic leg imaginal disc, we also demonstrate that the TGFβ pathway directly impacts the specification of founder cells for the jump muscle. Our studies define a new role for the TGFβ pathway in the control of specific skeletal muscle characteristics.

Key words: *Drosophila*, TGFβ, Adult myogenesis, Crossveinless, Founder cell

INTRODUCTION

Higher animals are characterized by complex arrangements of skeletal muscles, which are used for posture and locomotion. Understanding the origin of these muscles is of crucial importance to human medicine, as a number of debilitating diseases strongly impact skeletal muscle function and maintenance (Nishino and Ozawa, 2002). It is also apparent that several muscle diseases affect some muscle groups to a greater extent than other groups; thus, understanding how individual skeletal muscles arise is a central challenge in the field.

Although there is still much to learn regarding muscle patterning in mammalian muscle specification, some significant insight has been afforded by invertebrate systems. In grasshopper embryos, Ho et al. (Ho et al., 1983) were the first to identify, among the myoblast pool, individual larger cells that appeared to seed the formation of individual muscle fibers. These 'muscle pioneers' were subsequently identified in *Drosophila* (Bate, 1990), where it was shown that single skeletal muscle fibers arise from the fusion of a muscle pioneer or 'founder' cell with a small number of fusion-competent myoblasts.

Each embryonic founder cell is also largely responsible for the acquisition of fiber-specific phenotypes, such as patterns of gene expression, innervation and muscle attachment locations. This was concluded based upon myoblast fusion mutants, where unfused founder cells still attempt to make appropriate orientations and connections (Rushton et al., 1995) (reviewed by Baylies and Michelson, 2001). Furthermore, specific muscle phenotypes arise from individual patterns of regulatory gene expression within founder cells (Crozatier and Vincent, 1999; Knirr et al., 1999; Clark et al., 2006). Thus, understanding the genetic pathways that contribute to founder cell specification will impact our understanding of muscle specification. Along these lines, signaling pathways including the Wingless pathway (Cox and Baylies, 2005) and the epidermal growth factor pathway (Buff et al., 1998) contribute to founder cell selection in the embryo. Nevertheless, there are still several parts of this specification process that have yet to be uncovered.

During *Drosophila* metamorphosis, most of the larval skeletal muscles degenerate and are replaced by new muscles arising from imaginal myoblasts (Crossley, 1978; Currie and Bate, 1991; Fernandes et al., 1991). These adult myoblasts are specified during embryogenesis and many become associated with the imaginal discs (Poodry and Schneiderman, 1970; Bate et al., 1991). Subsequently, the adult myoblasts migrate from the discs to the future locations of the muscles, and myoblasts from each disc give rise to a variety of physiologically distinct muscles (Lawrence, 1982). However, mechanisms that control the specification of many of these muscles have yet to be fully elucidated.

Does the founder cell model of muscle development also hold true for adult myogenesis in *Drosophila*? This question is important given the complex organization of the adult skeletal musculature: individual fibers can span several segments and multiple fibers are often arranged into larger muscles that are more characteristic of those found in mammals. In the adult thorax, there are two major types of muscles (reviewed by Bernstein et al., 1993): the fibrillar indirect flight muscles (IFMs) are adapted to contract at high frequency to provide the power for flight. The IFMs comprise six pairs of medial fibers termed the dorsal longitudinal muscles (DLMs), and three pairs of lateral muscles termed the dorsoventral muscles (DVMs). In addition to the fibrillar flight muscles, physiologically distinct tubular muscles are located laterally and ventrally in the thorax. Tubular muscles function in walking, jumping and angling the wings. Tubular muscles each are composed of several individual fibers grouped together. Most prominent among the tubular muscles is the tergal depressor of the trochanter (TDT, or 'jump') muscle, a large fiber found in all Diptera studied which attaches the dorsal notum to the second pair of legs. This muscle is essential for the escape response of the fly (Nachtigall and Wilson, 1967).

Department of Biology, University of New Mexico, Albuquerque, NM 87131, USA.

*Author for correspondence (e-mail: rcripps@unm.edu)

Accepted 28 January 2009

Several groups have demonstrated that the formation of some adult skeletal muscle fibers are associated with cells showing the characteristics of founders. These include the abdominal muscles, where each individual muscle is pre-figured by a cell expressing the canonical founder cell marker, *dumbfounded/kirre*, usually detected as an enhancer trap termed *duf-lacZ*, or *rp298* (Ruiz-Gomez et al., 2000). Precursors of the adult indirect flight muscles also express *duf-lacZ* (Dutta et al., 2004), and the ablation of these founder cells significantly disturbs the formation of the DVMs (Atreya and Fernandes, 2008). Interestingly, whereas founders have been observed to prefigure adult muscle development, relatively little is known of the mechanisms responsible for their specification at this stage. In fact, the process of singling out founder myoblasts, which in the embryo requires in part lateral inhibition via the Notch pathway (Carmena et al., 1995; Carmena et al., 1998), appears to occur in a Notch-independent manner in the adult (Dutta et al., 2004). Thus, understanding specification of adult muscles should provide further new insight into muscle specification mechanisms.

In this work, we have analyzed the basis of a mutation that affects the morphology of the jump muscle of the adult thorax. This mutation, which causes a reduction in TDT fiber number and defects in the morphology of the muscle, arises from mutation of the *crossveinless* (*cv*) gene, the established function of which is to modulate TGFβ signaling in the specification of wing crossveins. We demonstrate that *cv* functions in muscle development as part of the TGFβ pathway, which is activated autonomously in the adult myoblasts in order to control the number of founder cells specified for the TDT. By manipulating the TGFβ pathway, the TDT, which normally comprises 20-30 muscle fibers, can be modified to consist of as little as five fibers or as many as 50 fibers. Overall, these studies define an important function for the TGFβ pathway in adult muscle specification that might also be used in the formation of the more complex muscles found in higher animals.

MATERIALS AND METHODS

Drosophila stocks and crosses

Drosophila were grown on Carpenter's medium (Carpenter, 1950) at 25°C unless indicated. Stocks carrying cv^{1}, cv^{43}, dpp^{10638}, X chromosome deficiencies, the second chromosome deficiency covering *gbb*, and *Gal4* driver lines (unless noted) were obtained from the Bloomington Drosophila Stock Center. *UAS-Dad* (Tsuneizuni et al., 1997) and *UAS-tkv** (Adachi-Yamada et al., 1999) were from Stuart Newfeld (Arizona State University, AZ, USA); *UAS-cv* was from Larry Marsh (UC Irvine, CA, USA); *1151-Gal4* (Anant et al., 1998) was from L. S. Shashidhara (CCMB, Hyderabad, India); and *duf-lacZ* was from Upendra Nongthomba (India Institute of Science, Bangalore, India). The wild-type strain Simms-L was locally captured in Albuquerque (NM, USA) and determined to be conspecific based upon visual examination and crossing with established laboratory strains.

Preparation of samples for microscopy

Samples were prepared for paraffin sectioning as described by Lyons et al. (Lyons et al., 1990) and modified by Cripps et al. (Cripps et al., 1998). Sections were cut at 8-12 μm, and stained with Hematoxylin and Eosin (Sigma) for evaluation of TDT structure. Stained slides were dehydrated through 100% ethanol, soaked in xylene, and mounted in Cytoseal-XYL (VWR Scientific Products). TDT fibers were counted from both sides of the thorax and treated as independent samples. Averages for each genotype were calculated and comparisons were performed using Student's *t*-test at Graphpad.com.

Cryosections were prepared by embedding adult flies in OCT medium followed by freezing. Sections were cut at 15 μm at –18°C and air dried. Next, samples were fixed for 5 minutes at room temperature with 1.9% v/v formaldehyde in 1×PBS, washed and used for antibody staining as described in the following section.

For pupal dissections, newly pupariated animals were marked, and aged for the appropriate time until harvesting and dissection. All pupal samples were dissected in a Sylgard-coated petri dish (Dow Corning) and pinned open. After dissection, samples were fixed for 30 minutes on ice with 5% formaldehyde in 1×PBS, washed in PBTx [1×PBS, 0.2% v/v Triton-X100, 0.2% w/v Blocking Agent (Roche)], and then subjected to blocking and antibody incubations (see below).

For documentation of adult wings, wings were removed from adult flies and stored in 70% (v/v) ethanol overnight, then transferred twice to 100% ethanol. Wings were next soaked in 100% xylene, and mounted using Cytoseal-XYL (VWR Scientific Products) for photography.

Immunostaining and in situ hybridization

Fixed and washed samples were subjected to immunostaining essentially as described by Patel (Patel, 1994) and modified by Molina and Cripps (Molina and Cripps, 2001). Primary antibodies used were: anti-βPS-integrin 1:10 (Brower et al., 1984) (University of Iowa Developmental Studies Hybridoma Bank, IA); anti-Z(210) 1:100 (Vigoreaux et al., 1991) (kindly supplied by Jim Vigoreaux, University of Vermont, VT, USA); anti-MEF2 1:2000 (Lilly et al., 1995) (kindly supplied by Bruce Paterson, NIH); rabbit anti-β-galactosidase 1:1000 (AbCam); and mouse anti-β-galactosidase (Promega). For immunofluorescence, secondary antibodies were Alexa conjugated (Molecular Probes), and mixed with Alexa-488 phalloidin at 1:500 (Molecular Probes) and DAPI used at 2 μg/ml (Sigma). For immunohistochemistry, secondary antibodies and detection were carried out using the Vectastain Elite staining kit and diaminobenzidine (DAB) substrate according to the manufacturer's recommendations.

In situ hybridization was carried out using digoxigenin-labeled probes (Roche) according to the method of O'Neill and Bier (O'Neill and Bier, 1994). Probes for *cv* were generated from the plasmid pOT2/SD27025 (Drosophila Genomics Resource Center, Indiana University, IN, USA): sense probes were synthesized from plasmid cut with *Xho*I using T7 RNA polymerase; antisense probes were generated from plasmid cut with *Eco*RI using SP6 RNA polymerase.

Images were collected using an Olympus BX-51 stereomicroscope using either DIC or fluorescence optics. Digitally captured images were assembled into figures using Adobe Photoshop.

Jump testing

Jump tests were performed essentially as described by Cripps et al. (Cripps et al., 1994). Briefly, flies lacking wings and aged 2-3 days after eclosion were induced to jump from a platform elevated 10 cm above a piece of white paper. The landing location was marked and the lateral distance from the edge of the platform to the landing point was measured in mm. Average distances were calculated for more than 20 individuals for each genotype and compared using Student's *t*-test.

RESULTS

Variation in TDT fiber number in *Drosophila* strains

The jump muscle of the *Drosophila* thorax comprises large and small cells, organized into a rosette. There are generally 26-28 large cells and four small cells (O'Donnell et al., 1989; Peckham et al., 1990). The cells are visualized in paraffin sections cut horizontal to the axis of the muscle (Fig. 1A), and also are outlined in anti-β-PS integrin stains of horizontal cryosections (Fig. 1B). Large and small cells can additionally be distinguished based upon the presence (large cells) or relative absence (small cells) of the Z(210) Z-disc-associated protein (Fig. 1C) (Vigoreaux et al., 1991).

Control animals in some of our publications have shown fewer fibers than the canonical 26-28 (Baker et al., 2005), prompting us to evaluate TDT fiber number in a range of strains. The number of small cells only occasionally deviated from four (a range of three to five); however, there was significant variation in the number of large cells (summarized in Table 1). Some strains showed the published 26-28 large fibers (Fig. 1A), other lines showed as few as 18 large

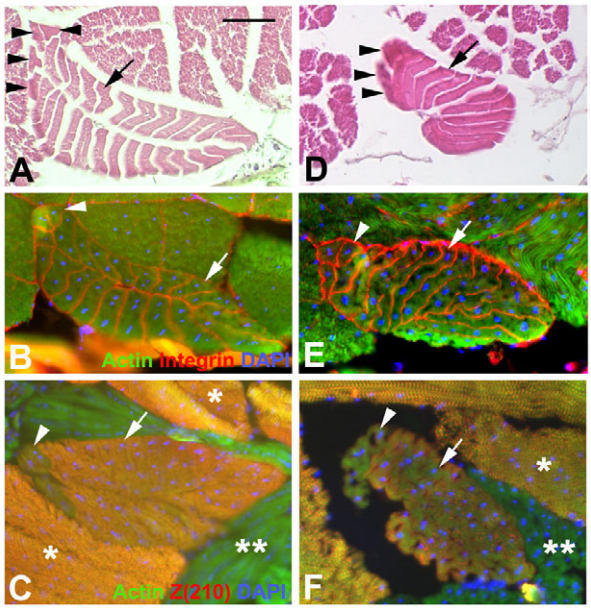

Fig. 1. Characterization and analysis of TDT fiber number in laboratory strains. (**A**) In Oregon R wild type, the jump muscle comprises four small cells (arrowheads) and ~26 large cells (arrow), as visualized in paraffin wax-embedded sections stained with Hematoxylin and Eosin. (**B**) In *y w*, this number is reduced to four small cells and ~19 large cells, visualized here using: anti-β-integrin (red), which outlines individual fibers; phalloidin (green), to visualize F-actin; and DAPI (blue), to visualize nuclei. (**C**) The TDT can also be identified based upon its accumulation of the Z(210) Z-disc associated protein (red). This protein is detected at high levels in the indirect flight muscles (single asterisks) and in the large cells of the TDT (arrow), and at very low levels in the small cells of the TDT (arrowhead); however, it is not detected in other tubular muscles of the thorax (double asterisk). (**D-F**) Comparable images of the TDT in *y cv*^{1} *v f car* mutants. The number of large fibers is always reduced and the number of small cells is occasionally reduced. Frequently, the fibers fail to organize properly into the rosette characteristic of the wild-type TDT. Scale bar: 50 μm.

fibers (Fig. 1B). These data include a locally caught strain named Simms-L, which showed an average of 22 large cells. Nevertheless, in all but one of these strains, the shape and arrangement of the fibers was maintained. Given the importance of this muscle to the escape response of the fly, we sought to investigate the genetic basis of this variation in more detail.

One strain tested carried the X-chromosome linked visible mutations *yellow*, *crossveinless*, *vermillion*, *forked*, *carnation* (*y cv*^{1} *v f car*). For this strain, the number of TDT large fibers was severely reduced, to an average of 13 (Table 1; Fig. 1D,E). Although both large and small cells were detectable (Fig. 1F), the overall morphology of the muscle was usually abnormal, with the cells failing to form a ring of fibers. We also observed an overall increase in the size of the individual fibers. We attribute the latter observation to a common size of myoblast pool being divided into fewer distinct fibers in the mutants. This would cause more myoblasts to contribute to each fiber and might result in an increase in fiber volume. The model that fiber size corresponds to the number of contributing myoblasts has already been proposed for the indirect flight muscles (Farrell et al., 1996). Given the large reduction in fiber number in the *y cv*^{1} *v f car* strain, we defined the cause of this phenotype.

Table 1. Variation in the number of large TDT fibers over different laboratory stocks

Genotype	*n**	Average number of fibers	s.e.m.†
Oregon-R	20	26.90	0.50
Simms-L	7	22.14	0.70
y w	8	19.87	1.04
w^{1118}	8	22.63	0.71
cv-2^{1}	14	19.07	0.46
det^{1}	8	29.13	1.25
duf-lacZ	7	23.29	0.64
TM3 / svp^{1}	12	26.00	0.30
y cv^{1} *v f car*	26	13.00	0.37

*Number of muscles counted.
†Standard error of the mean.

Identification of *cv* as the gene responsible for TDT defects

Using standard genetic crosses and analyzing TDT fiber number in individuals, the reduction in fiber number in the X-chromosome stock arose from a recessive mutation that mapped to the marked X chromosome (data not shown).

To localize the mutation, we crossed *y cv*^{1} *v f car* with a wild-type strain to generate *y cv*^{1} *v f car* /+ + + + + females, which were then backcrossed to wild-type males. Male recombinant offspring from this cross were collected, genotyped using the visual markers, and individually assessed for TDT fiber number. The results are shown in Table 2. In all cases the TDT fiber phenotype segregated according to the allele of *cv* that was present: *cv*$^{+}$ recombinants showed a normal fiber number, whereas *cv*^{1} recombinants showed defective TDTs. These results placed the TDT mutation in the proximity of *cv*.

To localize the mutation more precisely, we analyzed the phenotype of females heterozygous for the *cv*^{1} allele on one X chromosome and a deficiency on the other X. Combination of *cv*^{1} with *Df (1) N73* (deficient for 5C2 to 5D5-6) showed a normal TDT phenotype (Fig. 2A), whereas *cv*^{1} heterozygous with *Df (1) C149* (deficient for 5A8-9 to 5C5-6) uncovered the TDT phenotype (Fig. 2B). Similarly, we found that duplication of this segment of the X chromosome could rescue the TDT phenotype: in *cv*^{1} */ Dp(1;Y) dx*$^{+}$*1* males (where a wild-type X chromosome segment comprising 5A8-9 to 6D8 is translocated to the Y), the TDT phenotype was normal (Fig. 2C). These data placed the TDT mutation in the genomic region 5A8-9 to 5C2 of the X chromosome.

Given the close linkage of the TDT mutation to the *cv*^{1} allele, and given that *cv* resides at 5A13 of the X chromosome, we next tested whether mutation of *cv* might be responsible for the TDT phenotype by analyzing *cv*^{43}, an allele which is null for *cv* gene function, and which has a genetic background distinct to that of *cv*^{1} (Vilmos et al., 2005). *cv*^{43} homozygotes also showed a severe TDT phenotype (Fig. 2D), and *cv*^{1}/*cv*^{43} female heterozygotes had defective TDT structure (Fig. 2E). These studies demonstrated that mutation of *cv*, in addition to affecting the crossveins of the adult wing (Bridges, 1920), affects the normal development of the adult jump muscle.

We also determined whether genetic rescue of the *cv* mutation would rescue the jump muscle phenotype. We used the Gal4-UAS system (Brand and Perrimon, 1993) to determine whether Gal4-driven expression of *cv* was sufficient to rescue the mutant phenotype. We also followed the crossveinless phenotype in the wing to control for our manipulations; wild type and mutant are indicated in Fig. 3A,B.

Table 2. Mapping the TDT fiber number phenotype on the X chromosome

Genotype/Y	1*	2	3	4	5	6
y cv v f car	16	16	14	15	13	
y cv v f +	NT†					
y cv v + +	12	12	12	13	14	12
y cv + + +	15	15	18			
y + + + +	25	24	24	26	23	26
+ + + + +	24	23	24	25	26	23
+ + + + car	23	23	24			
+ + + f car	25	25	25	25	25	
+ + v f car	24	21	22	22	21	
+ cv v f car	14	12				

*1-6 represent individuals from each genotype that were assayed for TDT fiber number. The number in the column represents the average number of large TDT fibers per TDT for each individual.
†NT, not tested, as no recombinants were recovered.

When we generated a homozygous stock of the genotype w^{1118} cv^{43}*; UAS-cv*, this stock showed some rescue of the crossvein phenotype in the wings (Fig. 3C, left panel). Upon sectioning these adults, the TDT phenotype was also rescued (Fig. 3C, right panel). We attribute this finding to the *UAS-cv* transgene used being slightly leaky, such that in the homozygous condition it generates sufficient Cv protein to rescue the two phenotypes. By contrast, when we studied w^{1118} cv^{43}*; UAS-cv/+* (i.e. mutants carrying just one copy of the *UAS-cv* transgene), adults showed the mutant crossvein and TDT phenotypes (Fig. 3D), indicating that two copies of the UAS construct were required for rescue.

Rescue of the crossvein and TDT phenotypes confirmed that loss of *cv* function was responsible for the TDT phenotype. We extended the rescue experiment by crossing w^{1118} cv^{43}*; UAS-cv* homozygous females to males carrying either the ectodermal driver *patched-Gal4* (Wilder and Perrimon, 1995), or the mesodermal driver *24B-Gal4* (Brand and Perrimon, 1993). In male offspring for both of these cases (offspring of the genotype w^{1118} cv^{43}*/Y; UAS-cv/ptc-Gal4* or w^{1118} cv^{43}*/Y; UAS-cv/+; 24B-Gal4/+*), significant rescue was observed for both the crossvein and the TDT (Fig. 3E,F).

The observation that either a mesodermal or an ectodermal driver could rescue the wing and muscle phenotypes of cv^{43} can be explained by the demonstration that Cv is a secreted protein (Shimmi et al., 2005; Vilmos et al., 2005), and might diffuse from a source to the target tissue. It is interesting to note that *24B-Gal4* can direct sufficient Cv synthesis that the wing vein phenotypes can be rescued. In this instance, it is possible either that *24B-Gal4* is expressed at some levels outside of the mesoderm, or that the production of Cv in this background sufficiently stabilizes the ligand to which it binds in order to allow signaling over large distances.

To determine whether TDT defects are a common phenotype among mutants showing wing crossvein alterations, we studied the fiber number in mutants for *crossveinless-2* (*cv-2*) and *detached* (*det*). In both of these cases, no obvious fiber defect was observed (Table 1), indicating that *cv* must play either a unique or a more crucial function in muscle development than other members of this mutant class.

Effects of reduced fiber number upon TDT function

We next determined whether reductions in the number of TDT fibers affected TDT function. This muscle is solely responsible for the jump response in flies (Nachtigall and Wilson, 1967; Elliot et al., 2007), thus we carried out jump tests of wild-type, mutant and rescued flies. For w^{1118} controls, the jumping distance was 48 ±2 mm, whereas w^{1118} cv^{43} males jumped 38±3 mm. This difference was significant using the Student's *t*-test (P=0.02). When we analyzed the jumping ability of w^{1118} cv^{43}*; UAS-cv* rescued males flies (the same genotype used in Fig. 3C), the jumping distance was rescued to 50±3 mm, essentially indistinguishable from wild type. These experiments revealed that the TDT defects in *cv* mutants result in subtle, albeit significant defects in jump muscle performance. Clearly, reduction in TDT fiber number causes severe abnormalities in both muscle morphology and muscle performance.

TGFβ signaling controls TDT fiber number

Recently, *cv* was shown to be a component of the TGFβ signaling pathway, facilitating Dpp signaling in the formation of crossveins (Shimmi et al., 2005; Vilmos et al., 2005). To determine whether the *cv* muscle phenotype arises from disruptions in TGFβ signaling, we tested the effects upon TDT development of activation and repression of the TGFβ pathway.

To achieve this, we firstly crossed the adult myoblast driver *1151-Gal4* to a line carrying *UAS-tkv**, an autonomous constitutive activator of the TGFβ pathway. We analyzed TDT structure in adult offspring (termed *1151>UAS-tkv**) using both paraffin sections and immunostained cryosections. Compared with wild type (Fig. 4A-C), the mutant offspring showed a striking increase in the number of

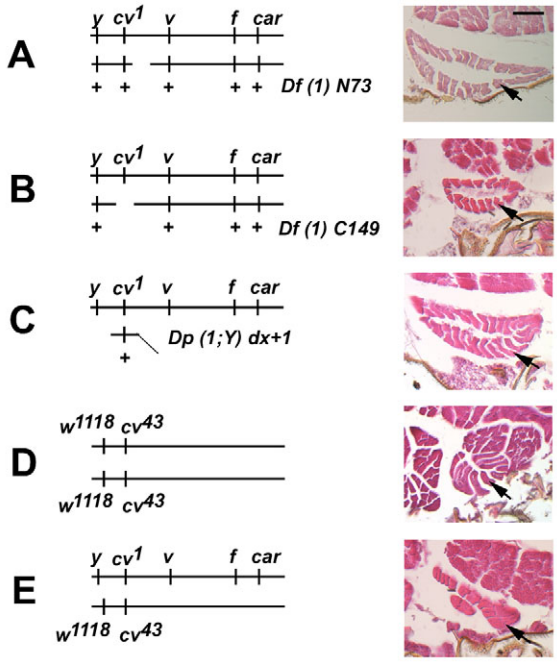

Fig. 2. Aneuploid mapping of the TDT fiber phenotype on the X chromosome. (**A**,**B**) In females, a deficiency adjacent to the *cv* gene did not uncover the defect in fiber number when heterozygous with *y* cv^1 *v f car* (A; there are 27 large fibers in this example), but a deficiency that also uncovered *cv* did uncover the TDT fiber defect (B; there are only 14 large fibers in this example). (**C**) Furthermore, a duplication for the *cv* region rescued the *y* cv^1 *v f car* TDT defect in otherwise hemizygous males (21 large fibers). (**D**,**E**) To determine whether the fiber defect arose from mutation of *cv*, we tested a different *cv* allele, cv^{43}. (D) Females homozygous for cv^{43} showed severe TDT defects (D; nine large fibers), as did cv^1/cv^{43} trans-heterozygotes (E; approximately nine large fibers). Arrows indicate TDT. Scale bar: 50 μm.

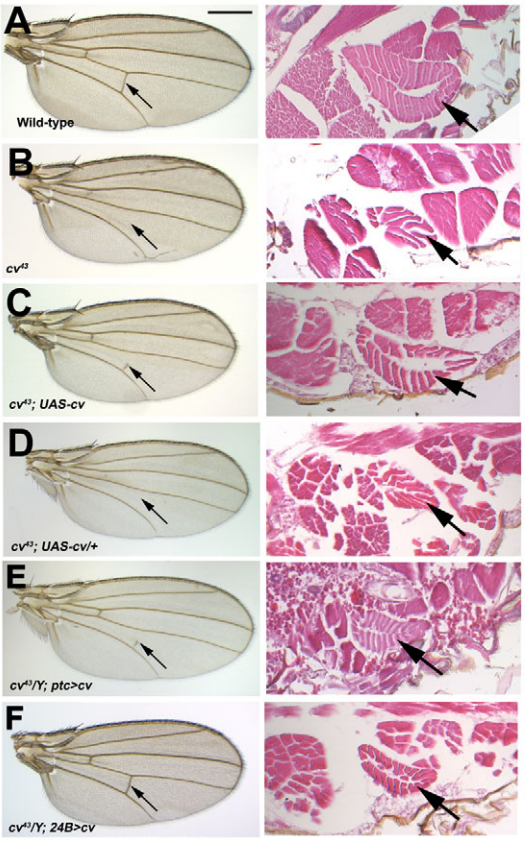

Fig. 3. Rescue of the posterior crossvein and TDT phenotypes of *cv* mutants. (**A**) Wild type. The posterior crossvein is present in the wing (arrow in left panel) and the TDT is normal (arrow, right panel). (**B**) In cv^{43} mutants, the posterior crossvein is absent (arrow in left panel) and the TDT phenotype is apparent (arrow in right panel. (**C**,**D**) In cv^{43}; *UAS-cv* homozygotes, there is partial rescue of the posterior crossvein (C; arrow in left panel), and essentially complete rescue of the TDT (arrow in right panel). This effect probably arises from slight leakiness of the *UAS-cv* transgene, as one copy of the *UAS-cv* is not sufficient to rescue either phenotype (D). (**E**,**F**) Both the wing and the TDT phenotypes can be rescued using drivers that are predominantly ectodermal (*ptc-Gal4*; E) or mesodermal (*24B-Gal4*, F) (arrows). Scale bar: 400 μm for wing images; 50 μm for paraffin sections.

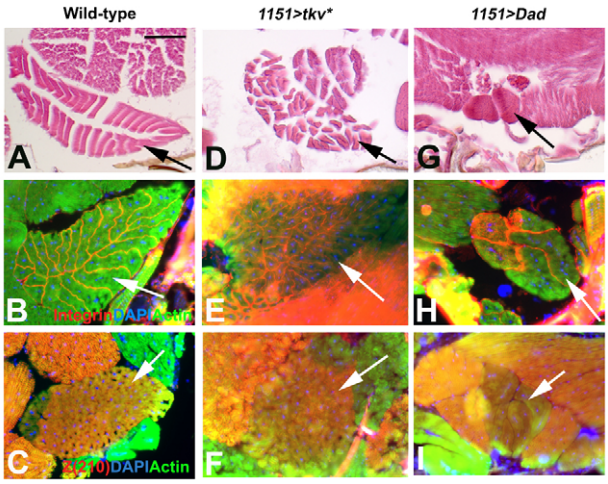

Fig. 4. The TGFβ pathway controls TDT fiber number. TDT fiber number and identity were assayed in both paraffin (A,D,G) and frozen sections (B,C,E,F,H,I) of wild type and mutant combinations. Stains are as in Fig. 1. (**A-C**) Wild-type, showing the numbers of large TDT cells (arrows). (**D-F**) In *1151-Gal4/+; UAS-tkv*/+* adults, the number of TDT fibers (arrows) was increased significantly. This could be observed in paraffin sections (D), as well as in the cryosections, where the β-integrin stain outlined a very large number of individual fibers (E). The identity of this muscle as the TDT was confirmed by anti-Z(210) reactivity (F). (**G-I**) In *1151-Gal4/+; UAS-Dad/+* adults, the number of TDT fibers was drastically reduced (arrows), although the identity of the fibers was retained, visualized using anti-Z(210) reactivity (I). Scale bar: 50 μm.

TDT fibers, although each individual fiber was somewhat smaller than in wild-type muscles (Fig. 4D,E). We consistently observed over 50 fibers per TDT, approximately twice the normal number. By immunostaining we observed that most of the fibers showed accumulation of Z(210), a characteristic of the large TDT cells (Fig. 4F). Thus, fiber number, but not fiber fate, was altered in these mutants.

We next analyzed the effects of repressing the TGFβ pathway upon TDT formation, by creating flies of the genotype *1151>Dad*, where *Dad* encodes an intracellular repressor of the TGFβ pathway. Here, there was a severe reduction in the total number of TDT fibers to approximately six (Fig. 4G-I).

The opposing effects upon TDT fiber number of activating or repressing the TGFβ pathway provided strong evidence for the involvement of this pathway in TDT development. Moreover, as we used a Gal4 driver line active in the adult myoblasts, we also conclude that the TGFβ pathway is activated in the myoblasts themselves, rather than in an adjacent cell population.

Specification of TDT muscle fibers

To define the cellular basis for the TDT defects, we studied the early development and specification of the TDT in *duf-lacZ* at late larval and early pupal time points. By correlating this expression with markers of the muscle lineage, we defined crucial events in TDT formation, which could be compared with *cv* mutants. First, the TDT is pre-figured by the specification of founder cells at the proximal region of the T2 leg imaginal disc. The founders were somewhat difficult to discern during the late larval stage (Fig. 5A), either because they were just being specified, or because the *duf-lacZ* reporter was only just becoming activated. In the early pupal stage [2 hours after puparium formation (APF)] 12-15 *duf-lacZ*-expressing founder cells were observed in this region of the imaginal disc (Fig. 5B), and by 8 hours APF had increased to an average number of 21.5±1.3 founder cells (Fig. 5C).

As pupal development proceeded, this group of founder cells migrated laterally and dorsally, among a large number of MEF2-positive myoblasts. By 16 hours APF, the TDT founders had increased in number to 25.4±1.5, similar to the final number of large plus small fibers in the mature TDT of *duf-lacZ* adults (Fig. 5D; Table 1). Later than 16 hours APF there was no significant increase in the number of founder cells for the TDT, and instead the formation of linear fibers began to take place.

These observations first suggested that the fibers of the TDT each develop according to the founder cell model. Second, the specification of TDT founders takes place over several hours at the end of the larval stage and early during pupal development. These findings are consistent with the observations of Rivlin et al. (Rivlin et al., 2000) who elegantly followed the appearance and migration of 'muscle pioneers' for the TDT, which are probably coincident with the founder cells. In addition, similar data are

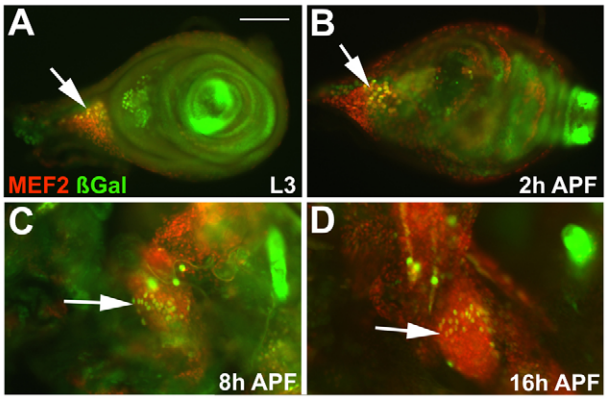

Fig. 5. Specification of founder cells during development of the TDT. In order to follow TDT specification, we studied *duf-lacZ* expression in wild type during late larval to early pupal development. Founders for the TDT can be first observed in the T2 leg imaginal disc at the late third instar (L3) larval stage (**A**), identified as being positive for both β-Gal (green) and MEF2 (red). At this stage, the number of specified founders is ~12. During early pupal development, these cells spread laterally and dorsally across the developing ectoderm. Two hours after puparium formation (APF), there were 12-15 founders (**B**), and this number increased to approximately 21 founder cells by 8 hours APF in wild-type (**C**). (**D**) By 16 hours APF, the number of founders in wild type was roughly 25, comparable with the final number of TDT fibers. Arrows indicate TDT founder cells. Scale bar: 50 μm.

presented by Atreya and Fernandes (Atreya and Fernandes, 2008), although their study did not focus specifically upon TDT development.

We studied next TDT development in *cv* mutants by creating a *duf-lacZ* cv^{43} recombinant chromosome. We followed founder cell number in *duf-lacZ* controls and *duf-lacZ* cv^{43} mutants during formation of the muscles (16 hours APF images are shown in Fig. 6A,C), and we studied the appearance of muscle fibers at 24 hour APF (Fig. 6B,D). Owing to the relatively large size of the muscles at this stage, the images presented do not show all of the founders cells or muscle fibers forming the muscles under study. Representative focal planes are shown for each sample.

Interestingly, we observed that the initial specification of TDT founders was normal in the *cv* mutants. By 8 hours APF, the number of founder cells in the mutants was 20.2±0.8, which was not significantly different from wild type at this timepoint. By 16 hours APF, however, there were slightly fewer TDT founder cells observed in the cv^{43} mutants, averaging 18.3±0.5 founder cells (Fig. 6C). This number was significantly less than wild-type at 16 hours APF ($P<0.01$, Student's *t*-test), and identified this timepoint as the first at which defects became apparent in the *cv* mutants. This value of ~18 founder cells is slightly larger than the number of large plus small fibers in the *cv* mutants, and it is possible that there is additional loss of founder cells prior to the initiation of fiber formation.

At 24 hours APF, the formation of individual fibers could be easily discerned in both wild-type (Fig. 6B) and cv^{43} mutants (Fig. 6D). However, the number of fibers was clearly reduced in the mutants compared with controls, and the mutant fibers were also disorganized. Taken together, these studies indicated that in the *cv* mutants the process of founder cell specification was stalled, and that the reduction in founder cell numbers was the direct cause of the fiber number defect.

We next determined whether founder cell number and the formation of initial TDT fibers was altered when we interfered with TGFβ signaling via expression in the myoblasts of activated *tkv* or of *Dad*. Consistent with our observations of fiber number in the adults, we saw striking effects upon founder cell and fiber number in young mutant pupae. For expression of activated *tkv*, the number of founder cells was clearly increased at 16 hours APF. This is apparent in comparing Fig. 6E with 6A. The increase in founders was also reflected by an increase in the total number of fibers that were initially specified (Fig. 6F).

When we expressed *Dad* in the adult myoblasts, the opposite effect was observed: there were significantly fewer founder cells (only two or three can be discerned in Fig. 6G), although we also noted that there was an overall reduction in the total number of myoblasts, as determined by anti-MEF2 staining. At 24 hours APF, TDT fibers were difficult to identify as they were so few in number, and also showed severe hypoplasty (Fig. 6H).

Taken together, the studies shown in Fig. 6 define for the first time a crucial role for TGFβ signaling in the specification of TDT founder cells. These results will be further evaluated in the Discussion.

TGFβ ligands controlling TDT development

We next sought to identify the TGFβ ligand responsible for controlling TDT fiber number. Ligand encoded by the *dpp* gene is expressed at high levels in the imaginal discs and during pupal development (Masucci et al., 1990) and expression of *lacZ* from a *dpp* enhancer trap lies close to the TDT founder cells throughout early pupal development (data not shown). Furthermore, ligand encoded by *glass bottom boat* (*gbb*) is also expressed broadly in the imaginal discs (Khalsa et al., 1998). Thus, we focused upon these genes as potential contributors to TDT founder cell specification.

To determine whether Dpp or Gbb might be important for TDT founder specification, we tested whether double-heterozygotes for a ligand-encoding gene and for *cv* mutant alleles showed significantly fewer TDT fibers than did single heterozygotes for either gene mutation. The results of this analysis are presented in Fig. 7A,B. For Dpp, we observed, first, that the number of fibers in the dpp^{10638}/+ heterozygotes was reduced slightly compared with that of cv^{43}/+, suggesting that haploinsufficiency for *dpp* might be an important factor in muscle specification. More importantly, the combination of both *cv* and *dpp* mutant alleles resulted in a fiber count further reduced relative to either single heterozygotes alone, and which was significant based upon Student's *t*-test (Fig. 7A). These studies identified Dpp as at least one of the TGFβ ligands that impacts TDT fiber number during pupal muscle development.

For Gbb, we also investigated whether haploinsufficiency would exacerbate the TDT phenotype in a cv^{43}/+ background. In this instance, we observed a more striking effect upon fiber number in the double heterozygotes (Fig. 7B). The double mutants displayed a highly significant reduction in fiber number when compared with controls. These data suggest that both Dpp and Gbb ligands might play important roles in specification of TDT muscle fibers.

To complement these data, we studied the pupal expression pattern of *cv* by in situ hybridization. We generated *cv* sense and antisense probes, and validated their functionality in embryos. As reported by Vilmos et al. (Vilmos et al., 2005), we observed specific expression of *cv* surrounding the embryonic tracheal pits (Fig. 7C), whereas no signal was obtained from a sense control probe (Fig. 7D).

In 16 hour APF pupal samples, we also observed expression of *cv* in the forming cuticle, close to where the TDT myoblasts had migrated (Fig. 7E). Although the signal was relatively weak, it was

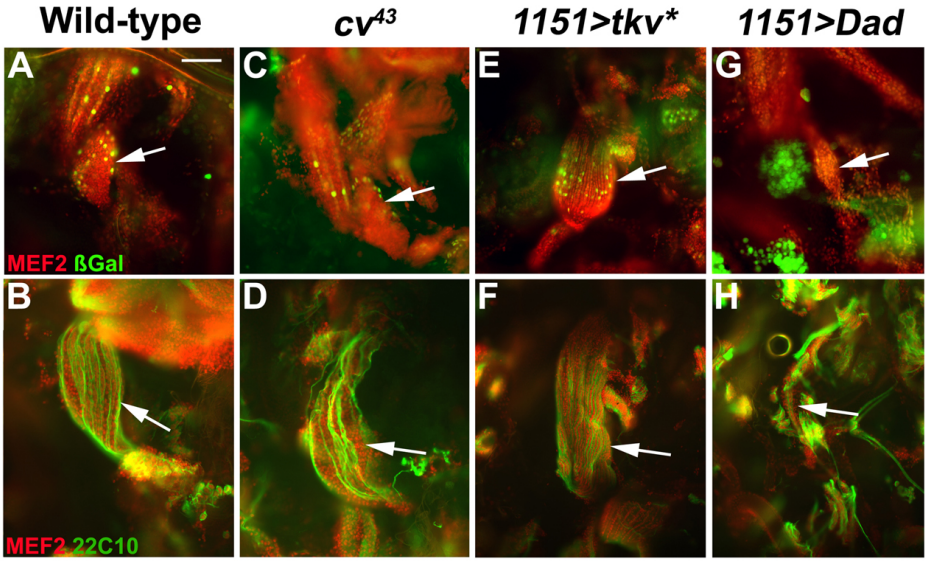

Fig. 6. The TGFβ pathway regulates TDT founder cell specification. (A-H) Control and TGFβ pathway mutants were analyzed for TDT founder cell specification 16 hours APF using a *duf-lacZ* marker (A,C,E,G) and for TDT fiber formation 24 hours APF (B,D,F,H). Arrows indicate the developing TDT. Owing to the thickness of the samples at this stage, not all founder cells nor all fibers are apparent in a single focal plane; thus, representative planes are shown. In *duf-lacZ* controls, founder cells were apparent at the location of the developing TDT (**A**), and these subsequently gave rise to clearly defined fibers by 24 hours APF (**B**). In cv^{43} mutants, founder cells were still present (**C**), although significantly reduced in number compared with wild type (see text for details). The fibers that were subsequently formed (**D**) were fewer in number than in wild type and somewhat disorganized. Upon activation of the TGFβ pathway (*1151>tkv**), large numbers of founder cells were observed at 16 hours APF (**E**), and a concomitant increase in the number of nascent fibers was observed at 24 hours APF (**F**). Upon repression of the TGFβ pathway (*1151>Dad*), there were very few founder cells observed at 16 hours APF (**G**). In addition, based upon the accumulation of MEF2 at this stage, there were significantly fewer myoblasts present to form the TDT. By 24 hours APF in these mutants, the TDT was barely visible and only comprised a handful of fibers (**H**). Scale bar: 50 μm.

reproducibly greater than in sense probe control preparations that were stained in parallel (Fig. 7F). We are currently generating promoter-*lacZ* gene fusions of *cv*, in order to expand upon these data. These findings will be presented elsewhere.

Taken together, identification of the most likely TGFβ ligands that contribute to TDT founder cell specification, and presentation of the pupal *cv* expression pattern at 16 hours APF further strengthen our model for the specification of TDT founder cells via the TGFβ pathway.

DISCUSSION

The specification and action of founder cells is crucial to normal muscle development in *Drosophila*, where founder cells impart unique phenotypes for each muscle. Here, we have shown that the founder cell model also holds true for the adult tubular muscles such as the TDT, or jump, muscle. More importantly, we demonstrate that the specification of TDT founder cells arises from activation of the TGFβ pathway, a pathway that has not been previously implicated in controlling founder cell number at the adult stage of development. Our data demonstrate that it is most probably the activation of the TGFβ pathway within myoblasts that impacts founder cell specification, as in our overexpression experiments we used a *Gal4* driver which is active in the adult myoblasts.

The TGFβ pathway has roles in a range of developmental processes, and the addition of adult muscle development here extends a detailed list (reviewed by Kollias and McDermott, 2008). In mammals, TGFβ can affect muscle development in several ways, including inhibition of differentiation (e.g. Yanagisawa et al., 2001) and inhibition of muscle regeneration in vivo (Cohn et al., 2007). Interestingly, the TGFβ molecule myostatin acts in mammalian muscle development to modulate the number of muscle fibers: in myostatin mutants in a variety of mammalian models, there is a profound increase in both muscle fiber number and muscle mass (reviewed by Kollias and McDermott, 2008). Despite these similarities, there is not yet sufficient evidence to suggest that Cv-mediated modulation of the Dpp pathway plays a similar role in *Drosophila* to that played by myostatin in mammals. This is at least partly because the effects of *cv* mutants that we observe here are restricted to a small subset of the adult musculature. A *Drosophila* gene named *Myoglianin* and encoding a molecule with strong sequence similarity to myostatin has been described, although no mutant alleles have been characterized (Lo and Frasch, 1999).

Our genetic interaction data suggest roles for both Dpp and Gbb in TDT fiber specification. Each of these ligands function in wing vein development (e.g. Khalsa et al., 1998; Ralston and Blair, 2005); thus, a combinatorial role for them in founder cell specification would not be unprecedented. We also note that the *Drosophila* genome encodes a number of additional TGFβ-related molecules (O'Connor et al., 2006), and such molecules, in addition to Dpp and Gbb, might contribute to TDT founder cell specification.

Although manipulation of the TGFβ pathway showed clear effects upon the numbers of TDT founder cells, we also note that inhibition of the pathway, via *UAS-Dad*, caused a decrease in the number of total myoblasts as visualized by MEF2 staining. This observation suggests that, in addition to founder cell specification, the TGFβ pathway in adult myoblasts impacts either myoblast proliferation or myoblast survival. This observation is consistent with our finding that, in *cv* mutants, the number of founder cells reduces slightly as pupal development proceeds.

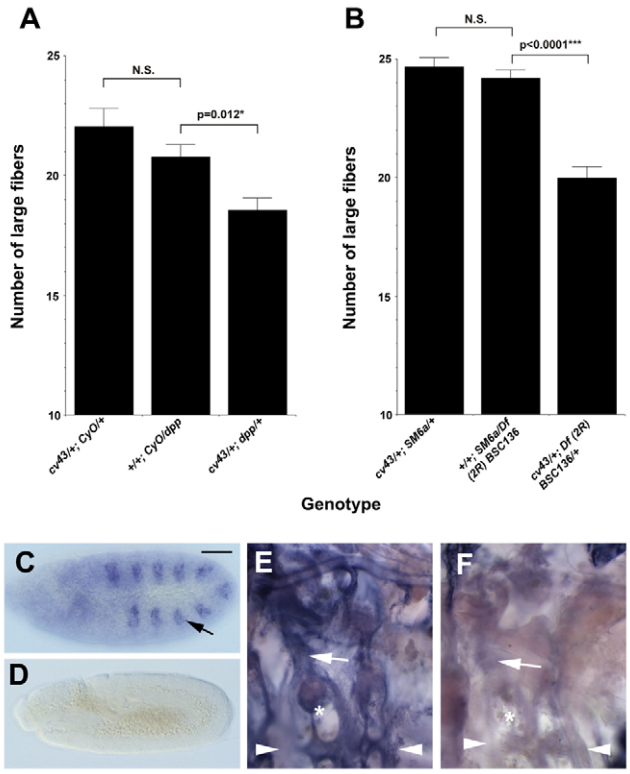

Fig. 7. A role for Dpp and Gbb in TDT fiber number specification. (**A**,**B**) Combination of cv^{43} with mutants or deficiencies for *dpp* (A) or *gbb* (B) resulted in significant reductions in TDT fiber number, observed in double-heterozygotes compared with any single heterozygotes. (C-F) Evaluation of *cv* expression during development via in situ hybridization. (**C**,**D**) In stage 11 embryos, a *cv* antisense probe revealed significant expression (arrow) around the tracheal pits (C) (Vilmos et al., 2005), whereas a control sense probe showed no specific signal (D). Embryos are oriented with anterior towards the left and dorsal side uppermost. (**E**) In 16-hour APF pupae, the antisense probe showed significant hybridization to a region of the cuticle (arrow) located just lateral to the lumen of the developing second leg (marked with an asterisk). (**F**) In control pupae, the sense probe did not show hybridization to this region. The ventral midline is indicated by paired arrowheads in E,F, and anterior is towards the left. Scale bar: 100 μm for C,D; 25 μm for E,F.

Specification of TDT founder cell number appears to be subject to significant variability in *Drosophila*. This observation contrasts sharply with many of the other muscles of the animal, which show relatively invariant fiber numbers. These include the skeletal body wall muscles of the larva (Bate, 1993), and the indirect flight muscles of the adult (Cripps and Olson, 1998; Farrell et al., 1996). Perhaps the variability in TDT fiber number is a reflection of the multi-step TGFβ pathway responsible for its specification, where variation in one or a few of the signaling components required for founder cell specification will ultimately impact the number of founder cells specified. Similar to the studies presented here, a genetic approach should be able to identify additional genes whose products function in this founder specification pathway and are responsible for the strain-specific differences in TDT fiber number that we have characterized. The identified genes might encode novel new members of the TGFβ signaling pathway active in myoblasts, or might identify mild alleles of known pathway members.

Variability in the organization of skeletal musculature is also documented in higher animals. In humans, the palmaris longus muscle of the forearm is commonly used as a source for tendon transplants. However, this muscle is absent, either unilaterally or bilaterally, in up to 15% of humans, depending strongly upon the ethnicity of the population being tested (Thompson et al., 1921; Thompson et al., 2001; Sebastin et al., 2005). This variability also has a strong heritable component (Thompson et al., 1921), and there is also significant variation in the size of this muscle (Sebastin et al., 2005). Similarly, the plantaris major also shows variable loss in humans (Vanderhooft, 1996). Our studies, which define how muscle fibers in the adult can be specified, point to potential mechanisms that might contribute to this variability in higher animals.

We are very grateful to the individuals who provided crucial reagents for this work: Larry Marsh, Stuart Newfeld, Upendra Nongthomba, L. S. Shashidhara and Jim Vigoreaux. We also thank Aaron Johnson, Stuart Newfeld and John Sparrow for valuable discussions during this work. We thank Anton Bryanstev for assistance with cryosections. The project was funded by grant R01 GM61738 from the NIH to R.M.C. We acknowledge technical support from the Department of Biology's Molecular Biology Facility, supported by NIH grant number 1P20RR18754 from the Institute Development Award (IDeA) Program of the National Center for Research Resources. Deposited in PMC for release after 12 months.

References

Adachi-Yamada, T., Nakamura, M., Irie, K., Tomoyasu, Y., Sano, Y., Mori, E., Goto, S., Ueno, N., Nishida, Y. and Matsumoto, K. (1999). p38 mitogen-activated protein kinase can be involved in transforming growth factor beta superfamily signal transduction in Drosophila wing morphogenesis. *Mol. Cell. Biol.* **19**, 2322-2329.

Anant, S., Roy, S. and VijayRaghavan, K. (1998). Twist and Notch negatively regulate adult muscle differentiation in Drosophila. *Development* **125**, 1361-1369.

Atreya, K. B. and Fernandes, J. J. (2008). Founder cells regulate founder number but not fiber formation during adult myogenesis in Drosophila. *Dev. Biol.* **321**, 123-140.

Baker, P. W., Kelly Tanaka, K. K., Klitgord, N. and Cripps, R. M. (2005). Adult myogenesis in *Drosophila melanogaster* can proceed independently of Myocyte enhancer factor-2. *Genetics* **170**,1747-1759.

Bate, M. (1990). The embryonic development of larval muscles in Drosophila. *Development* **110**, 791-804.

Bate, M. (1993). The mesoderm and its derivatives. In *The Development of Drosophila melanogaster*, Vol. 2 (ed. M. Bate and A. Martinez Arias), pp. 1013-1090. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

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. and Michelson, A. M. (2001). Invertebrate myogenesis: looking back to the future of muscle development. *Curr. Opin. Genet. Dev.* **11**, 431-439.

Bernstein, S. I., O'Donnell, P. T. and Cripps, R. M. (1993). Molecular genetic analysis of muscle development, structure and function in Drosophila. *Int. Rev. Cytol.* **43**, 63-152.

Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-415.

Bridges, C. B. (1920). The mutant crossveinless in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. USA* **6**, 660-663.

Brower, D. L., Wilcox, M., Piovant, M., Smith, R. J. and Reger, L. A. (1984). Related cell-surface antigens expressed with positional specificity in Drosophila imaginal discs. *Proc. Natl. Acad. Sci. USA* **81**, 7485-7489.

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

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., Murugasu-Oei, B., Menon, D., Jimenez, F. and Chia, A. (1998). inscuteable and numb mediate asymmetric muscle progenitor cell divisions during Drosophila myogenesis. *Genes Dev.* **12**, 304-315.

Carpenter, J. M. (1950). A new semisynthetic food medium for Drosophila. *Drosoph. Inf. Serv.* **24**, 96-97.

Clark, I. B. N., Boyd, J., Hamilton, G., Finnegan, D. J. and Jarman, A. P. (2006). D-six4 plays a key role in patterning cell identities deriving from the Drosophila mesoderm. *Dev. Biol.* **294**, 220-231.

Cohn, R. D., van Erp, C., Habashi, J. P., Soleimani, A. A., Kleion, E. C., Lisi, M. T., Gamradt, M., ap Rhys, C. M., Holm, T. M., Loeys, B. L. et al. (2007). Angiotensin II type 1 receptor blockade attenuates TGF-beta-induced failure of muscle regeneration in multiple myopathic states. *Nat. Med.* **13**, 204-210.
Cox, V. T. and Baylies, M. K. (2005). Specification of individual Slouch muscle progenitors in Drosophila requires sequential Wingless signaling. *Development* **132**, 713-724.
Cripps, R. M. and Olson, E. N. (1998). Twist is required for muscle template splitting during adult Drosophila myogenesis. *Dev. Biol.* **203**, 105-116.
Cripps, R. M., Becker, K. D., Mardahl, M., Kronert, W. A., Hodges, D. and Bernstein, S. I. (1994). Transformation of *Drosophila melanogaster* with the wild-type myosin heavy-chain gene: rescue of mutant phenotypes and analysis of defects caused by overexpression. *J. Cell Biol.* **126**, 689-699.
Cripps, R. M., Black, B. L., Zhao, B., Lien, C. L., Schulz, R. A. and Olson, E. N. (1998). The myogenic regulatory gene *Mef2* is a direct target for transcriptional activation by Twist during Drosophila myogenesis. *Genes Dev.* **12**, 422-434.
Crossley, A. C. (1978). The morphology and development of the Drosophila muscular system. In *The Genetics and Biology of Drosophila*, vol. 2b (ed. M. Ashburner and T. R. F. Wright), pp. 499-560. London: Academic Press.
Crozatier, M. and Vincent, A. (1999). Requirement for the Drosophila COE transcription factor Collier in formation of an embryonic muscle: transcriptional response to Notch signaling. *Development* **126**, 1495-1504.
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.
Dutta, D., Anant, S., Ruiz-Gomez, M., Bate, M. and VijayRaghavan, K. (2004). Founder myoblasts and fibre number during adult myogenesis in Drosophila. *Development* **131**, 3761-3772.
Elliott, C. J. H., Brunger, H. L., Stark, M. and Sparrow, J. C. (2007). Direct measurement of the performance of the Drosophila jump muscle in whole flies. *Fly* **1**, 68-74.
Farrell, E. R., Fernandes, J. and Keshishian, H. (1996). Muscle organizers in Drosophila: the role of persistent larval fibers in adult flight muscle development. *Dev. Biol.* **176**, 220-229.
Fernandes, J., Bate, M. and Vijayraghavan, K. (1991). Development of the indirect flight muscles of Drosophila. *Development* **113**, 67-77.
Ho, R., Ball, E. and Goodman, C. (1983). Muscle pioneers: large mesodermal cells that erect a scaffold for developing muscles and motorneurones in grasshopper embryos. *Nature* **301**, 66-69.
Khalsa, O., Yoon, J. W., Torres-Schumann, S. and Wharton, K. A. (1998). TGF-β/BMP superfamily members, Gbb-60A and Dpp, cooperate to provide pattern information and establish cell identity in the Drosophila wing. *Development* **125**, 2723-2734.
Kollias, H. D. and McDermott, J. C. (2008). Transforming growth factor-β and myostatin signaling in skeletal muscle. *J. Appl. Physiol.* **104**, 579-587.
Knirr, S., Azpiazu, N. and Frasch, M. (1999). The role of the NK-homeobox gene *slouch* (*S59*) in somatic muscle patterning. *Development* **126**, 4525-4535.
Lawrence, P. A. (1982). Cell lineage of the thoracic muscles of Drosophila. *Cell* **29**, 493-503.
Lilly, B., Zhao, B., Ranganayakulu, G., Paterson, B. M., Schulz, R. A. and Olson, E. N. (1995). Requirement of MADS domain transcription factor D-MEF2 for muscle formation in Drosophila. *Science* **267**, 688-693.
Lo, P. C. H. and Frasch, M. (1999). Sequence and expression of *myoglianin*, a novel Drosophila gene of the TGF-β family. *Mech. Dev.* **86**, 171-175.
Lyons, G. E., Schiaffino, S., Barton, P., Sassoon, D. and Buckingham, M. (1990). Developmental regulation of myosin gene expression in mouse cardiac muscle. *J. Cell Biol.* **111**, 2427-2436.
Masucci, J. D., Miletnberger, R. J. and Hoffman, F. M. (1990). Pattern-specific expression of the Drosophila decapentaplegic gene in the imaginal disks is regulated by 3′ cis-regulatory elements. *Genes Dev.* **4**, 2011-2023.
Molina, M. R. and Cripps, R. M. (2001). Ostia, the inflow tracts of the Drosophila heart, arise from a genetically distinct subset of cardial cells. *Mech. Dev.* **109**, 51-59.
Nachtigall, W. and Wilson, D. M. (1967). Neuro-muscular control of Dipteran flight. *J. Exp. Biol.* **47**, 77-97.
Nishino, I. and Ozawa, E. (2002). Muscular dystrophies. *Curr. Opin. Neurol.* **15**, 539-544.
O'Connor, M. B., Umulis, D., Othmer, H. G. and Blair, S. S. (2006). BMP morphogen gradients in the Drosophila embryo and pupal wing. *Development* **133**, 183-193.
O'Donnell, P. T., Collier, V. L., Mogami, K. and Bernstein, S. I. (1989). Ultrastructural and molecular analyses of homozygous-viable *Drosophila melanogaster* muscle mutants indicate there is a complex pattern of myosin heavy-chain isoform distribution. *Genes Dev.* **3**, 1233-1246.
O'Neill, J. W. and Bier, E. (1994). Double-label in situ hybridization using biotin and digoxigenin-tagged RNA probes. *Biotechniques* **17**, 870, 874-875.
Patel, N. H. (1994). Imaging neuronal subsets and other cell types in whole-mount Drosophila embryos and larvae using antibody probes. *Methods Cell. Biol.* **44**, 445-487.
Peckham, M., Molloy, J. E., Sparrow, J. C. and White, D. C. S. (1990). Physiological properties of the dorsal longitudinal flight muscle and the tergal depressor of the trochanter muscle of *Drosophila melanogaster*. *J. Muscle Res. Cell Motil.* **11**, 203-215.
Poodry, C. A. and Schneiderman, H. A. (1970). The ultrastructure of the developing leg of *Drosophila melanogaster*. *Rouxs Arch. Dev. Biol.* **166**, 1-44.
Ralston, A. and Blair, S. S. (2005). Long-range Dpp signaling is regulated to restrict BMP signaling to a crossvein competent zone. *Dev. Biol.* **280**, 187-200.
Rivlin, P. K., Schneiderman, A. M. and Booker, R. (2000). Imaginal pioneers prefigure the formation of adult thoracic muscles in *Drosophila melanogaster*. *Dev. Biol.* **222**, 450-459.
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.
Rushton, E., Drysdale, R., Abmayr, S. M., Michelson, A. M. and Bate, M. (1995). Mutations in a novel gene, *myoblast city*, provide evidence in support of the founder cell hypothesis for Drosophila muscle development. *Development* **124**, 3407-3414.
Sebastin, S. J., Puhaindran, M. E., Lim, A. Y. T., Lim, I. J. and Bee, W. H. (2005). The prevalence of absence of the Palmaris longis: a study in a Chinese population and a review of the literature. *J. Hand Surg.* **30B**, 525-527.
Shimmi, O., Ralston, A., Blair, S. S. and O'Connor, M. B. (2005). The crossveinless gene encodes a new member of the Twisted gastrulation family of BMP-binding proteins which, with Short gastrulation, promotes BMP signaling in the crossveins of the Drosophila wing. *Dev. Biol.* **282**, 70-83.
Thompson, J. W., McBatts, J. and Danforth, C. H. (1921). Hereditary and racial variations in the musculus Palmaris longus. *Am. J. Phys. Anthropol.* **4**, 205-220.
Thompson, N. W., Mockford, B. J. and Cran, G. W. (2001). Absence of the palmaris longus muscle: a population study. *Ulster Med. J.* **70**, 22-24.
Tsuneizuni, K., Nakayama, T., Kamoshida, Y., Kornberg, T., Christain, J. and Tabata, T. (1997). *Daughters against Dpp* modulates *dpp* organizing activity in Drosophila wing development. *Nature* **389**, 627-630.
Vanderhooft, E. (1996). The frequency of and relationship between the palmaris longus and plantaris tendons. *Am. J. Orthop.* **25**, 38-41.
Vigoreaux, J. O., Saide, J. D. and Pardue, M. L. (1991). Structurally different Drosophila striated muscles utilize distinct variants of Z-band-associated proteins. *J. Muscle Res. Cell Motil.* **12**, 340-354.
Vilmos, P., Sousa-Neves, R., Locacsovich, T. and Marsh, J. L. (2005). *crossveinless* defines a new family of Twisted-gastrulation-like modulators of bone morphogenetic protein signaling. *EMBO Rep.* **6**, 262-267.
Wilder, E. L. and Perrimon, N. (1995). Dual functions of wingless in the leg imaginal disc. *Development* **121**, 477-488.
Yanagisawa, M., Nakashima, K., Takeda, K., Ochiai, W., Takizawa, T., Ueno, M., Takizawa, M., Shibuya, H. and Taga, T. (2001). Inhibition of BMP2-induced, TAK1 kinase mediated neurite outgrowth by Smad6 and Smad7. *Genes Cells* **6**, 1091-1099.