Coordinated development of muscles and tendons of the *Drosophila* leg

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Summary

Since Miller's morphological description, the *Drosophila* leg musculature and its formation has not been revisited. Here, using a set of GFP markers and confocal microscopy, we analyse *Drosophila* leg muscle development, and describe all the muscles and tendons present in the adult leg. Importantly, we provide for the first time evidence for tendons located internally within leg segments. By visualising muscle and tendon precursors, we demonstrate that leg muscle development is closely associated with the formation of internal tendons. In the third instars discs, in the vicinity of tendon progenitors, some Twist-positive myoblasts start to express the muscle founder cell marker *dumbfounded* (*duf*). Slightly later, in the early pupa, epithelial tendon precursors invaginate inside the developing leg segments, giving rise to the internal string-like tendons. The tendon-associated *duf-lacZ*-expressing muscle founders are distributed along the invaginating tendon precursors and then fuse with surrounding myoblasts to form syncytial myotubes. At mid-pupation, these myotubes grow towards their epithelial insertion sites, apodemes, and form links between internally located tendons and the leg epithelium. This leads to a stereotyped pattern of multifibre muscles that ensures movement of the adult leg.

Key words: Leg myogenesis, Tendons, Founder cells, *Drosophila*

Introduction

During *Drosophila* development, two waves of myogenesis are seen: the early embryonic wave, giving rise to the larval musculature; and the imaginal wave that leads to the formation of the body wall and the flight and leg muscles of the adult fly. The molecular mechanisms of larval and flight muscle development have been intensively investigated during the last decade (Bate and Rushton, 1993; Baylies et al., 1998; Baylies and Michelson, 2001; Bernard et al., 2003; Buff et al., 1998; Carmena et al., 1998a; Ghazi et al., 2000; Jagla et al., 2002; Kozopas and Nusse, 2002; Rivlin et al., 2000; Roy and VijayRaghavan, 1999; Sudarsan et al., 2001). The larval somatic muscles derive from a subset of embryonic mesodermal cells, which express a high level of *twist* (*twi*) (Bate, 1990; Bate and Rushton, 1993; Baylies et al., 1995). Individual larval muscle fibres are derived from specialised myoblasts called founder cells, each of which displays a specific and unique expression of a set of transcription factor genes known as muscle identity genes (reviewed by Frasch, 1999). Muscle founders arise from progenitor cells, which are singled out by a process of lateral inhibition mediated by the Delta (Dl)/Notch (N) signalling pathway (Brennan et al., 1999; Carmena et al., 2002; Park et al., 1998). The diversity of founders, corresponding to the diversity of muscle fibres, is generated by the asymmetric division of progenitor cells and the expression of founder-specific combinations of transcription factors (Carmena et al., 1998b; Park et al., 1998; Ruiz Gomez and Bate, 1997). Although all the information required for the specific shape, position and innervation of a given muscle is thought to reside within its founder, the formation of a fully functional syncytial muscle fibre involves fusion between the founder cells and a group of fusion-competent myoblasts (FCMs). Among the proteins implicated in myoblast fusion, the interactions of the transmembrane proteins Dumbfounded (Duf; Kirre – FlyBase) and Hibris (Hbs), which are specifically expressed in founder cells and FCMs, respectively, are essential for cell-cell contact and the initiation of fusion events (Dworak et al., 2001; Ruiz-Gomez et al., 2000). The final muscle pattern is seeded down by the establishment of contact between the growing myotubes and their tendonous epidermal insertion sites, called apodemes. Initially, this step involves the Stripe-dependent specification of apodemes (Becker et al., 1997; de la Pompa et al., 1989), followed by Epidermal Growth Factor Receptor (Egfr)-controlled dialogue between the apodemes and the myotubes (Strumpf and Volk, 1998; Vorbruggen and Jackle, 1997; Yarnitzky et al., 1997; Yarnitzky et al., 1998).

Like those in the larva, adult muscles also derive from *twi*-expressing cells. The adult abdominal body wall musculature originates from a subset of *twi*-positive cells associated with larval nerves (Currie and Bate, 1991; Dutta et al., 2004), whereas the flight and leg muscles develop from a subpopulation of *twi*-expressing cells, the so-called adepithelial cells, of wing and leg imaginal discs (Bate et al., 1991; Broadie and Bate, 1991).

Two principal types of flight muscles have been described:

the direct flight muscles (DFMs) and the indirect flight muscles (IFMs) (Fernandes et al., 1991; Miller, 1950). The DFMs are the small tubular muscles that arise from the most distal, *cut*-expressing adepithelial cells of the notum part of wing disc (Sudarsan et al., 2001). As some of these cells express the founder cell marker *duf* and the muscle identity gene *apterous* (*ap*), it has been proposed that DFMs form de novo using a myogenic pathway similar to that described for larval muscles (Ghazi et al., 2000; Kozopas and Nusse, 2002). Formation of IFMs consisting of three dorsoventral muscles (DVMs) and 6 dorsal longitudinal muscles (DLMs) involves the proximal myoblasts of the imaginal notum, which express a low level of *cut* and a high level of *vestigial* (*vg*) (Sudarsan et al., 2001), and is based on two different developmental strategies. DLMs use persistent larval muscles as a scaffold to form (Fernandes et al., 1991; Fernandes and Keshishian, 1996), whereas the DVMs form de novo.

In contrast to larval and flight muscles, practically nothing is known about the mechanism governing *Drosophila* leg myogenesis. Surprisingly, no systematic analysis of the development and morphology of appendicular *Drosophila* muscles has been performed since Miller's work, published more than 50 years ago (Miller, 1950). Here, we exploited a set of GFP-expressing *Drosophila* lines to follow the formation of appendicular muscles and tendons during larval and pupal stages. Intriguingly, the presumptive leg muscle founders segregate close to tendon precursors, and then keep contact with the invaginating internal tendons to reach the position at which the corresponding muscle fibres will develop. The confocal microscopy-based analysis reveals the multifibre, vertebrate-like organisation of *Drosophila* leg muscles, making appendicular *Drosophila* musculature an attractive model with which to study the genetic control of multifibre muscle formation.

Materials and methods

Drosophila strains

The 1151-GFP is a double transgenic strain generated from the 1151-Gal4 effector line (kindly provided by K. VijayRaghavan, Bangalore, India) and an UAS-GFPnls strain (from the Bloomington Stock Centre). The Stripe-Gal4 combined with an UAS-GFP was obtained from G. Morata (Madrid, Spain). The MHC-tauGFP strain was kindly provided by E. N. Olson and E. Chen (Dallas, USA), the RP298-lacZ/duf-lacZ line was a gift from A. Nose (Tokyo, Japan) and the odd-lacZ line was provided by C. Rauskolb (Los Angeles, USA).

To visualise muscles and tendons simultaneously, the MHC-tauGFP strain was combined with the 1151-Gal4 driver and the resulting flies crossed with the UAS-dsRED line, generously provided by S. Heuser (Meunster, Germany).

Dissections and mounting

All larvae and pupae were grown and staged at 25°C. Larvae and prepupae (up to 5 hours after pupae formation, APF) were dissected in phosphate-buffered saline (PBS), fixed for 15 minutes in 4% paraformaldehyde in PBS and stained with appropriate antibodies. Pupae older than 5 hours APF and adult flies were fixed in 4% paraformaldehyde for about 5 hours, dissected and fixed again overnight before final dissection of the developing legs. Muscle fibres from the adult legs were dissected directly in 4% paraformaldehyde, maintained for 15 minutes in the initial fixation solution and stained to visualise myoblast nuclei. All the preparations were mounted in 50% glycerol.

Immunostaining and 3D modelling

The following primary antibodies were used: rabbit anti-Twi, dilution 1/2500 (from F. Perrin-Schmit, Strasbourg, France); rabbit anti-Stripe, dilution 1/200 (from T. Volk, Reohovot, Israel); rabbit anti-Histone H3 Phosporylated (H3P), dilution 1/200 (Upstate); monoclonal anti-Wingless (Wg) (DHSB); monoclonal anti-Dl, dilution1/100 (DHSB); monoclonal anti-LacZ, dilution 1/500 (DHSB). Anti-rabbit and anti-mouse secondary antibodies (Jackson) conjugated to CY3 or CY5 fluorochromes were used (dilution 1/300) to reveal the staining. Nuclei of dissected muscle fibres were stained using propidium iodide (Molecular Probes). All the preparations were analysed on an Olympus Fluoview FV300 confocal microscope. 3D modelling was performed using the Imaris™ Bitplane software.

Results

Spatial distribution of myoblasts and tendon precursors during larval and early pupal stages

To follow spatial positioning of myoblasts in leg discs during larval and prepupal stages, we generated a compound 1151-GFP transgenic line carrying an adepithelial cell-specific 1151-GAL4 driver (Anant et al., 1998) and one copy of UAS-GFP transgene. As determined by co-staining with anti-Twi antibody (Fig. 1A), GFP expression is specific to imaginal myoblasts and can be detected in a few adepithelial cells starting from the early second larval instar. Interestingly, at this time, the majority of leg myoblasts (except the most proximal stalk myoblasts) undergo proliferation (Fig. 1B) (Cohen, 1993). As judged from the markedly increased number of adepithelial cells in the leg discs from the third compared with the second larval instar (Fig. 1A,C), the myoblasts undergo several rounds of mitosis. They stop proliferating in the middle of the third instar, at the time when proliferation of the epithelial leg disc cells can still be detected (Fig. 1D). We estimate that, after the larval wave of proliferation, each third instar leg disc contains about 500 1151-GFP-positive myoblasts. In addition, at the beginning of pupal stages, a discrete subset of 1151-GFP cells that do not co-express Twi (Fig. 1E,F) and that are *stripe* (*sr*)-positive (Fig. 1G,H) can be detected. This observation indicates that, from the beginning of pupation, 1151-GAL4 drives expression not only in appedicular myoblasts but also in invaginating epithelial precursors of internal leg tendons.

To characterise adepithelial cell positions during third instar and early pupal stages, we analysed larval and prepupal leg discs co-labelled with anti-Twi and anti-Wg, or anti-Twi and anti-Dl antibodies (Fig. 2). Wg is expressed in the anteroventral part of the leg disc, allowing the dorsoventral position to be determined (Brook and Cohen, 1996; Wilder and Perrimon, 1995), whereas the Notch ligand Dl, expressed in the segmental joints, marks the proximodistal subdivision of the leg disc (de Celis et al., 1998; Mishra et al., 2001; Rauskolb, 2001; Rauskolb and Irvine, 1999). In the third instar, myoblasts are evenly distributed on the leg disc epithelium (Fig. 2A,E), suggesting that their final deployment is independent of the dorsoventral cues. By contrast, as revealed by double Twi/Dl staining (Fig. 2C,G), the arrangement of myoblasts is influenced by the segmental subdivision of the leg disc. This is particularly obvious in the proximal part of the disc where myoblasts display circular deployment (Fig. 2C,G). In addition, in prepupal leg discs (Fig. 2D,H), Twi-positive cells are not detected within the tarsal segments, a feature that is

consistent with the observation that the tarsus of the adult leg is devoid of muscles (Miller, 1950) (this study). As Twi-positive cells are detected in the tarsus of the third instar leg discs (Fig. 1C, Fig. 2E,G), we conclude that in pupal stages Twi expression is not maintained in distally located myoblasts.

In parallel, we set out to identify appendicular tendon precursors. As in embryos (Volk, 1999) and wings (Fernandes et al., 1996; Ghazi et al., 2000; Ghazi et al., 2003) progenitors of muscle attachment sites express the transcription factor Sr, we analysed *sr* expression pattern in the leg discs. To determine the positions of tendon precursors with respect to the leg axes and in relation to myoblasts, *sr* expression was analysed in imaginal discs co-stained for Twi and Wg, or for Twi and Dl (Fig. 2E-H). In the third larval instar, five domains of *sr* expression (labelled a-e; Fig. 2A,C) are visible in the leg disc epithelium. Two of them, b and c, lie within the ventral Wg domain (Fig. 2A), whereas the most prominent one, a, is located opposite, in the dorsal, Dpp-dependent side (Fig. 2A). Two other *sr* patches, d and e, occupy the most distal and proximal positions, respectively. Interestingly, during the eversion, the distal patch, d (Fig. 2B,D,F,H), forms a long central structure extending from the tip of the tarsus to the femur, and corresponds to the previously described precursor of the long tarsus tendon (Miller, 1950) (see also Figs 4, 5). Our observations indicate that the long tendon forms by the progressive invagination of *sr*-expressing epithelial cells from the d domain. As judged from the co-staining with anti-Dl antibody (Fig. 2D,H), *sr* patches are differentially distributed along the proximodistal axis. The a and b *sr* domains correspond to tendon precursors in the femur segment, c in the tibia and e, most probably, in the coxa. At the onset of eversion two supplementary *sr* expression domains can be detected (Fig. 2D,H), one, f, in the dorsal part of the coxa and the second, g, in the dorsal tibia. At 0 hours APF, patches of *sr*-expressing epithelial cells become elongated, indicating that like the most distal domain, d, they undergo invagination. Overall, these observations reveal that the appendicular tendon precursors are specified largely before the formation of leg muscle fibres. Particularly striking is the prominent early *sr* expression in the a domain located in the dorsal region of the presumptive femur (Fig. 2A,C,E,G). Immediately adjacent to this domain, we observe an accumulation of Twi-expressing cells (Fig. 2E,G).

Importantly, our observations indicate that, because of the unique dorsoventral and proximodistal position, each of the *sr*-positive tendon precursors detected in the prepupal leg discs can be correlated with the resulting internal tendon, and with the associated adult muscle (see Table 1).

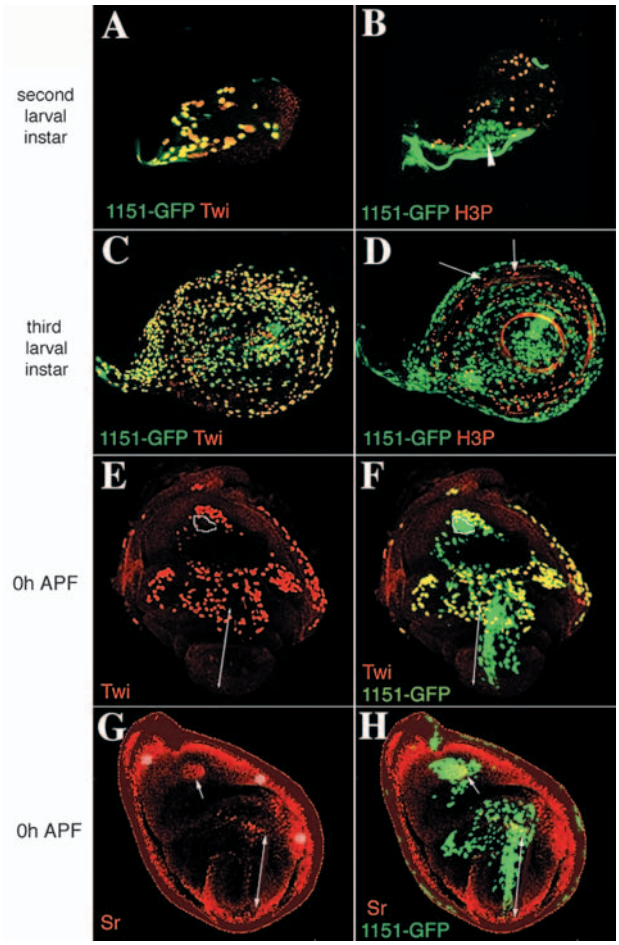

Fig. 1. Appendicular myoblasts in second and third instar larvae. (A,B) Leg discs from a second larval instar. (A) All the Twi-positive myoblasts co-express the 1151-GFP marker. (B) All second instar appendicular myoblasts, except those located in the stalk (arrowhead), express a mitosis marker (red), indicating that they are proliferating. (C,D) Leg discs from late third instar larvae. (C) Several hundreds of appendicular myoblasts co-expressing 1151-GFP and Twi are detected. (D) In the third larval instar, the majority of leg disc myoblasts do not display mitotic activity. Proliferating epithelial cells are indicated by arrows. (E,F) A prepupal leg disc stained for 1151-GFP and Twi, showing a subset of non-mesodermal (Twi-negative) 1151-GFP cells. These cells are seen within the outlined area and along the double-headed arrow. (G,H) Non-mesodermal 1151-GFP cells co-express Sr, indicating that they correspond to tendon precursors. Presumptive tarsal (double-headed arrow) and femur tendon precursors (arrow) are indicated. Asterisks in G point to the background staining resulting from the weak signal to background ratio seen when using the available anti-Sr antibody.

duf-lacZ-expressing myoblasts are associated with tendon precursors

To characterise the possible link between tendon and muscle development, we set out to analyse the distribution of presumptive appendicular muscle founder cells. For this purpose, we analysed leg discs derived from larvae and pupae carrying one copy of a founder-specific *duf-lacZ* marker, known as RP298 (Nose et al., 1998), and one copy of a compound transgene, Stripe-GAL4/UAS-GFP. Our analysis (Fig. 3A-F) reveals that the Twi-positive myoblasts lying close to the *sr* a domain correspond to the presumptive *duf-lacZ*-expressing muscle founder cells. In the middle of third larval instar, about thirty myoblasts organised around the a tendon domain co-express Twi and the reporter gene *duf-lacZ* (Fig. 3A,D). Interestingly, at the beginning of pupation (Fig. 3B,E), the a domain epithelial cells, together with the adjacent *duf-lacZ*-expressing myoblasts, start to invaginate inside the leg disc. This invagination leads to the spatial rearrangement of *sr*-expressing tendon precursors (Fig. 3C,F), which form a long internal string-like domain. As another type of epithelial invagination that leads to the formation of segmental leg joints is regulated by Notch signaling and its direct target *odd skipped* (*odd*) (Bishop et al., 1999; Mirth and Akam, 2002; Hao et al.,

Table 1. Muscles and tendons of the *Drosophila* leg

Muscle name	Associated tendon*	Number of muscle fibres†
Coxa		
Trochanter depressor muscle, trdm	Trochanter depressor tendon, trdt (e or f)	8-10
Trochanter levator muscle, trlm	Trochanter levator tendon, trlt (e or f)	8-10
Trochanter reductor muscle trrm	Trochanter reductor tendon, trrt	3
Trochanter		
Femur depressor muscle, fedm	Femur depressor tendon, fedt	7-9
Femur reductor muscle, ferm	Femur reductor tendon, fert	4-5
Femur		
Tibia depressor muscle, tidm	Tibia depressor tendon, tidt (b)	12-15
Tibia levator muscle, tilm	Tibia levator tendon, tilt (a)	10-12
Long tendon muscle 2, ltm2	Long tendon, lt (d)	3
Tibia reductor muscle, tirm	Tibia reductor tendon, tirt	8-10
Tibia		
Tarsus depressor muscle, tadm	Tarsus depressor muscle, tadt (c)	20-24 (first leg) 28-33 (second and third leg)‡
Tarsus levator muscle, talm	Tarsus levator muscle, talt (g)	8-10
Long tendon muscle 1, ltm1	Long tendon, lt (d)	6-7
Tarsus reductor muscle 1, tarm1		4-5
Tarsus reductor muscle 2, tarm2		3

*Corresponding larval/pupal tendon precursors shown in Figs 2 and 4 and labeled a-g are shown in parentheses.

†Number of muscle fibres composing appendicular muscles in the adult fly defined by examining leg muscles from the five adult MHC-tauGFP females and males. Minor variations in the number of muscle fibres were observed in nearly all muscles (except for trrm, ltm2 and tarm2). These variations are independent of sex and leg pair.

‡The tadm muscle of the first leg is composed of a markedly lower number of muscle fibres than its counterparts in the second and the third leg pair. The differences in the number of tadm muscle fibres are independent of sex.

2003), we tested the expression of *odd* with respect to that of *sr*. Interestingly, in the prepupal leg disc, the *odd* and *sr* expression domains are largely overlapping (Fig. 3H-J), which suggests that similar mechanisms may control the invagination of segmental joints and internal tendons. As schematised in Fig. 3G-G‴, the invaginating tendons are covered by the associated *duf-lacZ* cells. Importantly, at the beginning of pupation, the number of *duf-lacZ*-positive cells associated with the invaginating *sr* a domain increases to about 50 (Fig. 3B,E). As the number of muscle fibres that will form in this area is less than 50 (see Table 1), we hypothesise that only some of the myoblasts that initially express *duf-lacZ* differentiate into founder cells. This is in agreement with the recent observation of Dutta et al. (Dutta et al., 2004), who report that founder cells of the adult *Drosophila* muscles characterised by high *duf-lacZ* expression arise from a pool of myoblasts with a low *duf-lacZ* expression level.

In addition to the a domain, at the beginning of pupation the first *duf-lacZ*-expressing cells also segregate more distally. They lie close to another patch of *sr*-positive tendon precursors (Fig. 3E,F), which most likely correspond to the tibia c domain (see also Fig. 2D,H). This observation suggests that appendicular muscles and tendons develop in a coordinated manner in all leg segments.

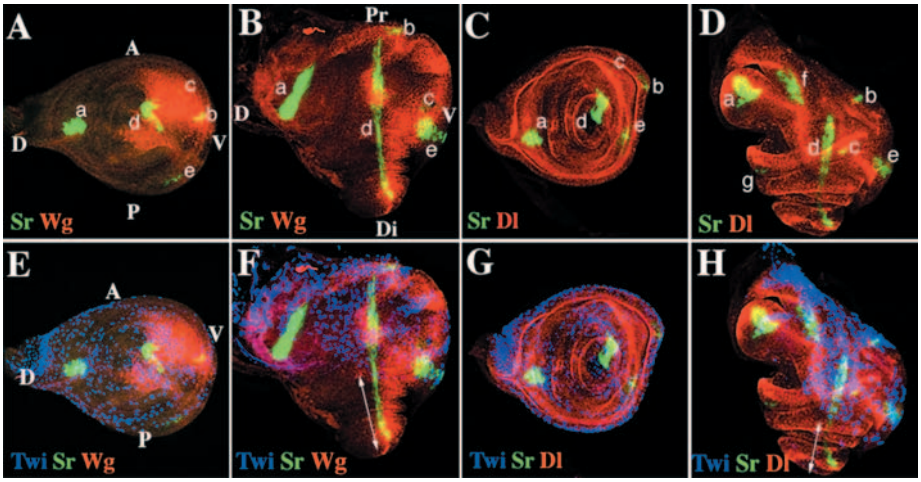

Fig. 2. Spatial distribution of appendicular myoblasts and tendon precursors during larval and early pupal stages. (A-D) Tendon precursor position revealed by Stripe-Gal4/UAS-GFP with respect to the Wg-positive anteroventral domain (A,B) and the Dl-labelled proximodistal axis (C,D). (A,C) In the third instar leg disc, five GFP patches are observed. They are designated a in dorsal, b and c in ventral, e in posteroventral, and d in the distal part of the leg epithelium. (B,D) At 0 hours APF, tendon precursors invaginate and become extended. The proximodistal positions of tendon precursors are as follows: the a and b patches lie within the femur, c lies within the tibia and d crosses the tarsus, tibia and femur. At the onset of eversion (D) two supplementary tendon precursors, e in the proximal region (coxa, trochanter) and g in the dorsal tibia, are detected. (E-H) Merged images showing myoblast (blue) and tendon precursor (green) organisation in relation to the dorsoventral and proximodistal axes. Note that at 0 hours APF (F,H), myoblasts are excluded from the most distal part of the disc (double-headed arrows). Disc orientation is noted by: A, anterior; P, posterior; V, ventral; D, dorsal; Pr, proximal; and Di, distal.

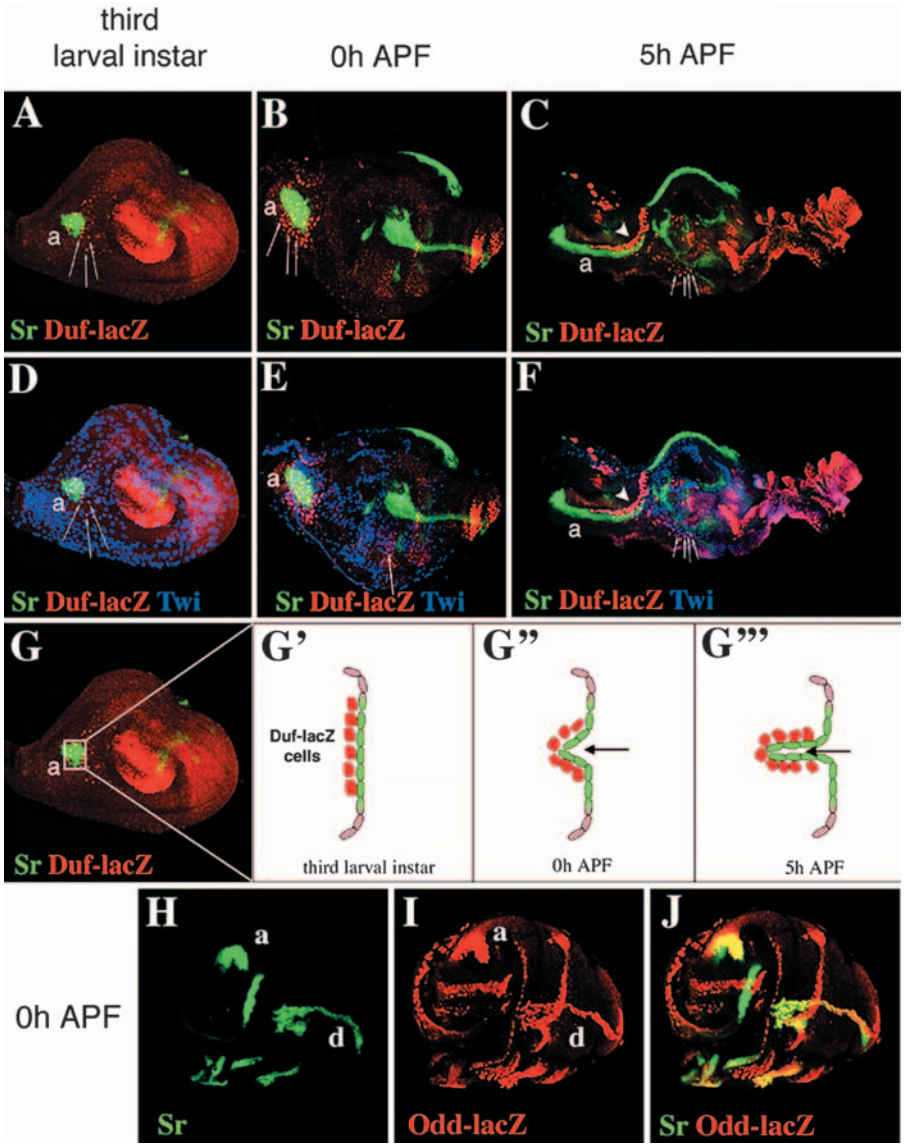

Fig. 3. Appendicular *duf-lacZ*-expressing cells are associated with tendon precursors. (A-C) *duf-lacZ*-expressing cells visualised in leg discs dissected from *duf-lacZ*; Stripe-Gal4/UAS-GFP (A) larvae, (B) prepupae and (C) pupae. (A) About 50 *duf-lacZ*-labelled cells (arrows) are detected in the dorsal femur close to the *sr*-expressing a domain. Notice that the *duf-lacZ* transgene is also expressed in the epithelium of the most distal leg part. (D-F) Merged Twi/*duf-lacZ* views confirm the presence of presumptive founder myoblasts around the a tendon domain. (B,E) At the prepupal stage (0 hours APF), the number of *duf-lacZ*-expressing cells around the a domain increases (arrows, compare A with B). Some of them lose *twi* expression. At 0 hours APF, *duf-lacZ*-positive cells also appear in other regions (dotted arrow in E). (F) At 5 hours APF, the *sr*-expressing a domain extends to form a long structure in the femur. The associated *duf-lacZ* cells follow the extending tendon precursor (arrowheads in C and F). Few myoblasts co-expressing *duf-lacZ* and Twi are visible near the g domain in the tibia (arrows in F). (G-G‴) A model schematising the invagination of the tendon precursors during the leg disc eversion, showing the associated *duf-lacZ*-expressing cells. (H-J) Prepupal leg disc double-stained for Stripe-GFP (H) and Odd-lacZ (I; merged image is shown in J), showing co-expression of *odd* with *sr* in invaginating tendon precursors.

Major events of appendicular myogenesis during pupal metamorphosis

Starting from mid-pupation secretion of the cuticle makes pupal legs inaccessible for antibody staining and thus refractory to the analysis of muscle and tendon development by immunostaining-based methods. To overcome this technical problem, we took advantage of two GFP-expressing *Drosophila* lines (1151-GFP and Stripe-GFP) that enabled us to visualise appendicular muscles and tendons during metamorphosis (Fig. 4). Importantly, this analysis confirms the presence of internal tendons in all leg segments (Fig. 4, see also Fig. S1 in supplementary material). At 8 hours APF, string-like tendon precursors are detected in the Stripe-GFP and the 1151-GFP-expressing leg discs (Fig. 4A,B). Analysis of the proximodistal and dorsoventral positions of the detected tendons (Figs 3, 4) shows from which previously described *sr*-expressing domain (Fig. 2) they arise (to better understand the morphological changes that occur during leg disc eversion refer to the scheme presented in Fig. S1 in supplementary material). In the femur, the a and b domains give rise to the dorsal and ventral tendons, respectively (compare Fig. 2H with Fig. S1 and Fig. 4A). Similarly, the dorsal and the ventral tibia tendons arise from the c and g domains, respectively. In addition to these tendons, the long tendon originating from the d domain can be detected in the tarsus, tibia and femur (Fig. 4A-D and Fig. S1 in supplementary material). Thus, unlike the embryonic muscle attachment sites (Volk, 1999), the *sr*-expressing progenitors of appendicular tendons give rise to internal string-like tendons. As revealed by the analysis of everting leg discs (data not shown), *sr* expression diminishes and is no longer detected in the long tendon starting from 25 hours APF. By contrast, all the internal tendons, including the long tendon, are detected in the 1151-GFP pupae (Fig. 4D and data not shown).

Importantly, the 1151-GFP line also allows the monitoring of the position of myoblasts with respect to the developing internal tendons. At 8 hours APF, most myoblasts are dispersed within the everting leg segments, and only some of them (most likely those corresponding to the founder cells) are closely associated with extending tendons (Fig. 4A). The myoblast distribution changes during the next few hours so that, at 20 hours APF (Fig. 4C), nearly all the 1151-GFP-positive myoblasts are aligned around the internal tendons. We estimate that the non-associated myoblasts seen within the tibia and femur represent no more than 5% of the total number of 1151-GFP-positive adepithelial cells. At the beginning of pupation,

some dispersed 1151-positive cells are also present in the tarsus (Fig. 4A). They most probably correspond to myoblasts that have stopped expressing Twi (Figs 2, 3).

At the time the tarsal 1151-GFP-expressing cells disappear (at about 20 hours APF), 1151-GFP-expressing cells in other leg segments start to form prefusion complexes (Fig. 4C,F). Thus at 25 hours APF, we can discern the first precursors of syncytial muscle fibres, composed of 5 to 10 myoblast nuclei (Fig. 4D,G), which indicates that fusion processes are initiated between 20 and 25 hours APF. Newly formed muscle fibres are tightly arranged around the internal tendons and are not yet attached to the epithelium (Fig. 4G). Interestingly, at the same time we observe that the number of dispersed 1151-GFP-positive cells increases (Fig. 4D), suggesting that they have proliferated to generate a new pool of myoblasts. In the next 10 hours, these myoblasts most probably ensure the second wave of fusion, giving rise to the multinucleated muscle fibres (Fig. 4E,H). The newly formed myotubes appear to be associated on their distal sides with the internal tendons. The establishment of the contact between the proximal extremity of a myotube and the corresponding apodeme takes place between 40 and 55 hours APF (Fig. 4G,I,J). As in embryos, precursors of apodemes in the leg epithelium express *sr* (Fig. 4I,J), suggesting that a similar genetic pathway controls the differentiation of larval and adult muscle attachment sites. In parallel to the establishment of epithelial insertion, appendicular myotubes enter the phase of terminal myogenic differentiation marked by the expression of myofibrillar proteins, here evidenced by the

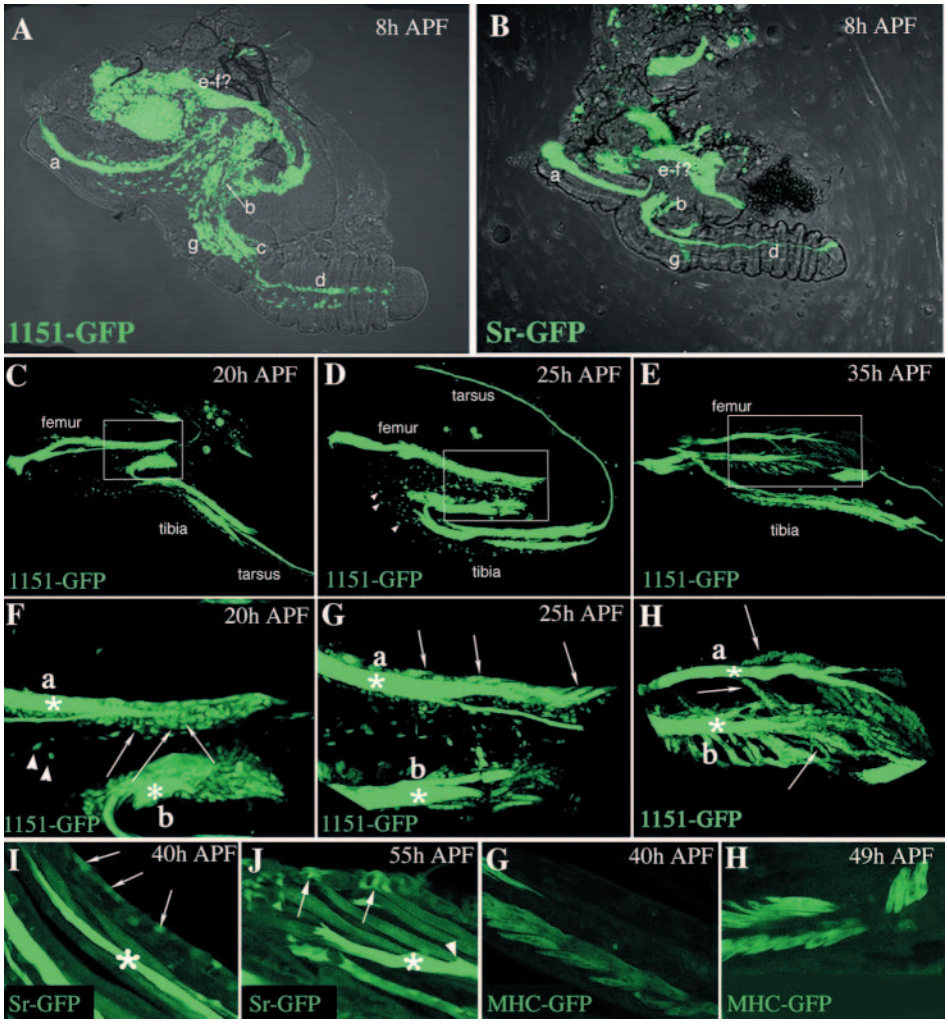

Fig. 4. Development of internal tendons and late steps of appendicular myogenesis. Tendons are visualised in the leg discs of (A) 1151-Gal4/UAS-GFP and (B) Stripe-Gal4/UAS-GFP pupae at 8 hours APF. Tendon precursors are annotated as in Fig. 2. Two tendon precursors, a and b, are located within the femur, two, g and c, are located in the tibia, and two others, e and f, in the proximal part of the leg. At this stage we are unable to define precisely the identity of proximal tendon precursors annotated e and f. (A) In addition to tendon precursors, 1151-Gal4/UAS-GFP also marks myoblasts, showing that they are deployed in all leg segments. A few individual 1151-GFP-positive cells, corresponding most probably to myoblasts, are detected at 8 hours APF in the tarsus. These cells do not express *twi* (see Fig. 7) and are no longer detected in later stages of development (see D). (C-E) General views and (F-H) enlarged views of dissected femur, tibia and tarsus from 1151-Gal4/UAS-GFP pupae at three different times during metamorphosis. In enlarged views asterisks mark the tendons. (C) General organisation of tendons and myoblasts at 20 hours APF. (F) Enlarged view of the region indicated in C, showing a proximal part of the femur a and b tendons. Myoblasts (arrows) lie tightly around the tendons. Note that only very rare myoblasts (arrowheads in F) appear not to be associated with tendons. (D) At 25 hours APF, the number of non-associated myoblasts increases (arrowheads). (G) Enlarged area corresponding to that shown in D. The arrangement of myoblasts associated with tendons has changed so that they form syncytial muscle fibre precursors composed of 5 to 10 nuclei (arrows). (E) At 35 hours APF, the precursors of the muscle fibres elongate. Myoblasts not associated with tendons are no longer detected. (H) Enlarged view of the region indicated in E, showing an increased number of myoblast nuclei in the muscle fibres (arrows). (I,J) Proximal part of the ventral tibia dissected from the Stripe-Gal4/UAS-GFP pupae, showing differentiation of the epithelial muscle attachment sites, the apodemes (arrows). Asterisks indicate the ventral internal tibia tendon, previously annotated c. Muscle fibres are visualised by their autofluorescence. (I) At 40 hours APF, Stripe-GFP expression appears in the nuclei of some epithelial cells (arrows). At this time, muscle fibres seem not to be attached to the epithelium. (J) At 55 hours APF, the Stripe-GFP cells are easy to detect. They have delaminated from the leg epithelium to form muscle attachment sites called apodemes (arrows). Muscle fibres attach to the apodemes. Note that a morphologically distinct area forms at the junction between the muscle fibres and the internal tendon (arrowhead). (G,H) Ventral tibia regions from MHC-tauGFP pupae, showing Myosin Heavy Chain (MHC-tauGFP) expression in differentiating myotubes. (G) At 40 hours APF, low levels MHC-tauGFP expression can be detected in appendicular tibia fibres. (H) About 10 hours later, MHC-tauGFP levels increase.

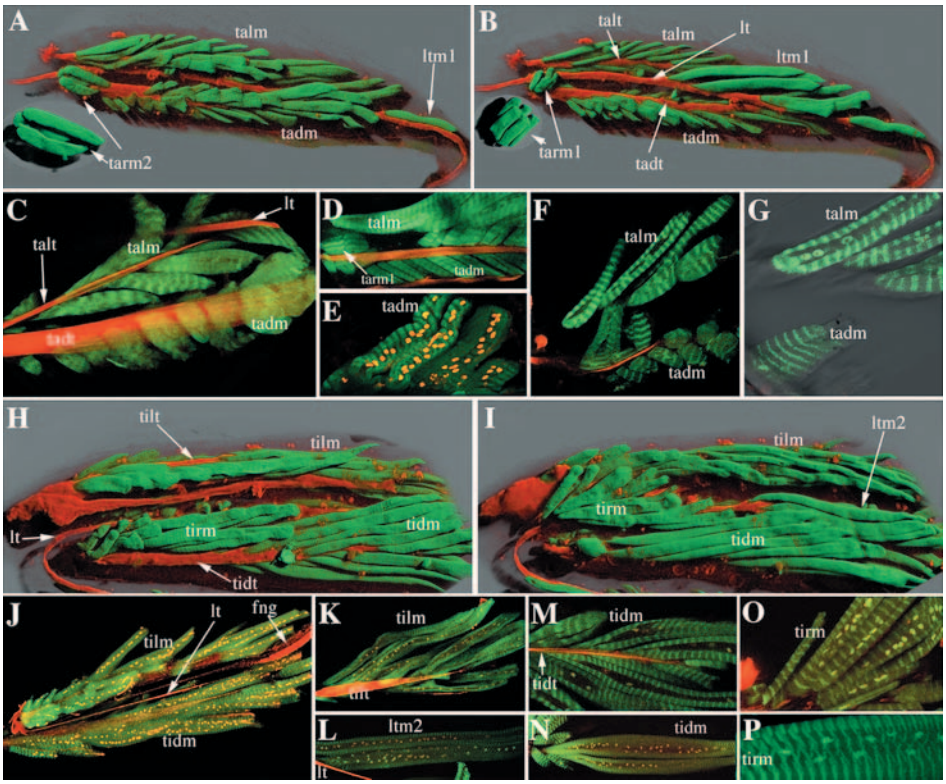

Fig. 5. Organisation of appendicular muscles and tendons. (A,B,H,I) Computer assisted 3D reconstruction of muscles and tendons from 55-hour APF whole-mount legs expressing muscle-specific MHC-tauGFP (green) and tendon-specific 1151-driven DsRED (red). A,B and H,I show muscles and tendons of the tibia and femur, respectively. (C,E-G) Dissected adult tibia muscles from MHC-tauGFP legs. (D) A view of the tibia muscles from a whole-mounted MHC-tauGFP adult leg. In C, D and F, tendons (red) are revealed by their strong autofluorescence. (E) MHC-tauGFP depressor muscle fibres stained with propidium iodide (PI) to show the nuclei (red) within the syncytial myotubes. (F,G) A higher accumulation of MHC-tauGFP is seen in the tarsus levator (talm) compared with in the tarsus depressor (tadm) muscle. (J-P) Dissected adult femur muscles expressing MHC-tauGFP. (J) General view of muscles (green) and tendons (red) of the femur dissected from MHC-tauGFP/1151-DsRED leg stained with PI to reveal the nuclei (yellow). A neural ganglion of the femur (fng) associated with the long tendon (lt) is indicated. (K) Tibia levator muscle (tilm). (L) Long tendon (lt)-associated muscle (ltm2). (M) Proximal and (N) distal part of the tibia depressor muscle (tidm). Note that the tibia levator tendon (tilt) shown in K is much larger than the depressor tendon (tidt) shown in M. The opposite is true (C) for the tarsus levator (talt) and depressor (tadt) tendons. (O,P) Tibia reductor muscle fibres (tirm). The nuclei in K, L and N are stained with PI and appear yellow. (M) Note the alignment of sarcomeres in neighbouring tidm fibres. (P) An enlarged view of a tibia depressor fibre (tidm), showing two rows of peripherally located nuclei. All muscle and tendon names and corresponding abbreviations are defined in Table 1.

progressively stronger fluorescence of muscle fibres from MHC-tauGFP pupae (Fig. 4G,H).

Muscles and tendons of the adult leg

To identify individual muscles and tendons and assign their position, we analysed a double-fluorescent *Drosophila* line that expresses 1151-driven DsRED in the tendons and MHC-tauGFP in the muscles. Our observations indicate that a robust muscle morphogenesis takes place between 40 and 50 hours APF (Fig. 4 and data not shown), establishing a final pattern of muscles and tendons as early as 55 hours APF. As the tendon-specific 1151-DsRED expression is stronger in the pupae than in the adult, we decided to use 55-hour APF pupae to revisit the appendicular musculature. The general muscle organisation is independent of sex and leg pair (data not shown). except for the jump muscle, which is specific to the second leg pair (Miller, 1950).

Two major multifibre muscle types are present in the tibia, femur and coxa (Fig. 5A,B,H,I and Fig. S2A-C in supplementary material). These are the levator and the depressor muscles. The levator muscles, designated talm (tarsus levator muscle), tilm (tibia levator muscle) and trlm (trochanter levator muscle) (Figs 5, 6 and Fig. S2 in supplementary material), are located dorsally. They are organised around the levator tendons, designated talt, tilt and trlt, respectively (Figs 5, 6 and Fig. S2 in supplementary material). The ventral sides of the tibia, femur and coxa harbour the depressor muscles, tadm, tidm and trdm, organised around the corresponding tadt, tidt and trdt depressor tendons (see Table 1 for abbreviations). As these tendons have not been described previously (Fig. 6C), the morphology of the levator and the depressor muscles described here differs from that described by Miller (Miller, 1950). The depressors and the levators in the same leg segment display distinct features. This results primarily from the different lengths and diameters of the internal tendons to which the depressor and levator muscle fibres are attached (Fig. 6A,B). In tibia and coxa segments, the diameter of the depressor tendons is greater than that of the levator tendons, whereas the comparatively thicker levator tendon is present in the femur (schematised in Fig. 6B). Moreover, depressor tendons are generally longer than levator tendons, and this is particularly notable in the tibia. As a consequence of the marked differences in tendon morphology, the number of muscle fibres that build tadm is higher than of talm (Table 1). In contrast to the tibia, the number of muscle fibres constituting depressors and levators in femur and coxa segments is more balanced (Table 1). Taken together, we interpret the described differences in depressor and levator morphology as reflecting the potential efforts to which these muscles are dedicated. Our GFP-based simultaneous detection of muscles and tendons also identified two muscles in the trochanter (Fig. 6 and Fig. S2A-D in supplementary material). These are the femur depressor (fedm) and the femur reductor (ferm) muscles, making the trochanter the only leg segment with different muscle organisation.

To obtain a precise description of the entire appendicular

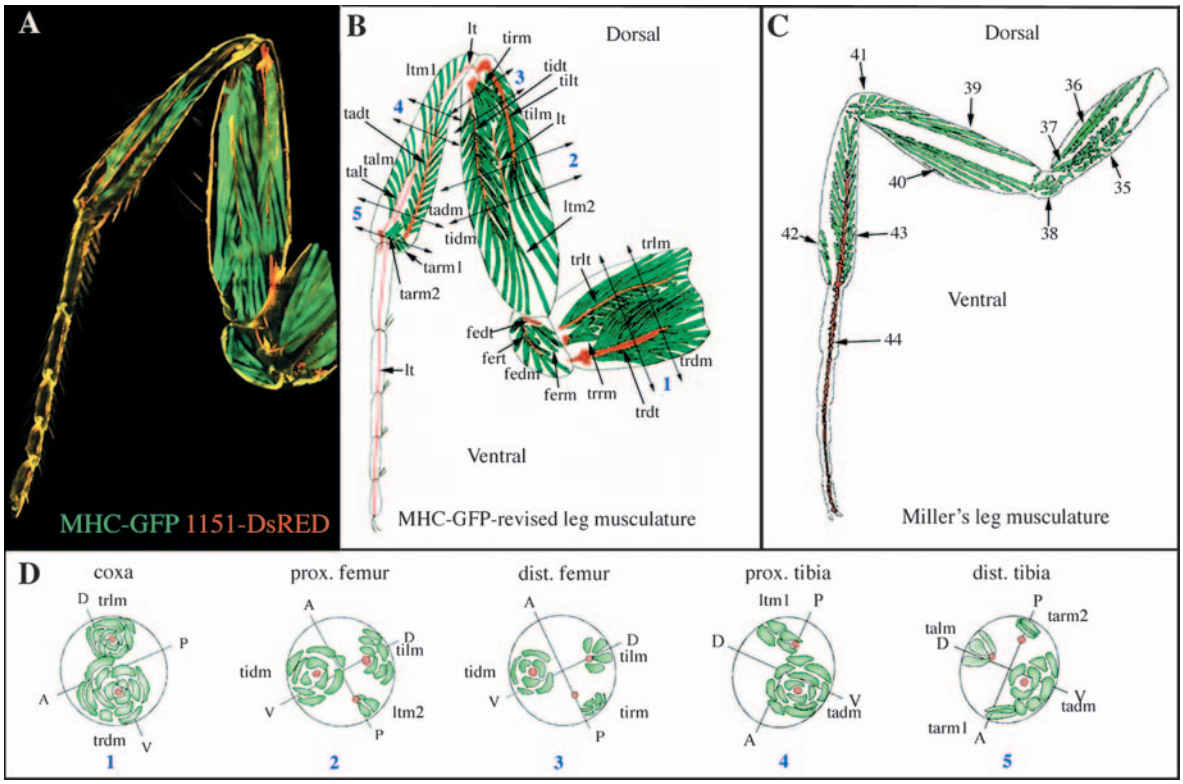

Fig. 6. Comparison of appendicular muscles revealed by MHC-tauGFP staining with the leg musculature described by Miller. (A) Adult leg muscles (green) and tendons (red) visualised by MHC-tauGFP and 1151-GAL4/UAS-DsRED staining, respectively. (B) Schematic representation of appendicular muscles (green) and tendons (red). Regions located between double-headed arrows, labelled 1-5, are represented in the cross-sections shown in D. (C) Adult *Drosophila* leg muscles identified by Miller and labelled according to Miller's description (Miller, 1950). Tarsal segments are devoid of muscles and harbour only the long tendon, 44. A comparison of B and C reveals the presence of previously undescribed muscles and tendons within the leg. These are the tarsus reductor muscles tarm1 and tarm2, the long tendon muscles ltm1 and ltm2, and a femur depressor muscle, fedm, as well as internal depressor and levator tendons in all leg segments. This implies that the levator and the depressor muscle fibres attach to the internal tendons and display an organisation that is different from that proposed by Miller. (D) Schematic representation of cross-sections through coxa (1), femur (2,3) and tibia (4,5) in the regions indicated in B. The cross-sections show the locations of muscles and tendons with respect to the dorsoventral and anteroposterior axes. Note that all of the levator muscles are located dorsally, and the depressors ventrally. All names and abbreviations of muscle and tendons are defined in Table 1.

musculature, we determined the average number of muscle fibres constituting all the individual leg muscles (Table 1). Our observations indicate that minor variations in the number of muscle fibres may exist in wild-type legs, but the only major variation is observed in the tarsus depressor muscle, in the first compared with the second and third leg pairs. In all, 14 multifibre muscles composed of about 140 fibres are present within the tibia, femur, trochanter and coxa (Table 1), and no muscles form in the tarsus.

Finally, we propose a new nomenclature for the appendicular muscles and tendons (Table 1, Fig. 6) with respect to the dorsoventral, anteroposterior and proximodistal axes of the leg. The presence, previously identified by Miller (Miller, 1950), of nine appendicular muscles labelled 35 to 44 (Fig. 6C), including depressors and levators, is confirmed (Fig. 6A,B). Our analysis also identifies five leg muscles that were not described by Miller (compare Fig. 6B with 6C, see Table 1). These are two tarsus reductor muscles, tarm1 and tarm2, the femur depressor muscle, fedm, and two long tendon muscles, one in the tibia and one in the femur, which we designate ltm1 and ltm2, respectively (Fig. 5A,B,H,I, Fig. 6B,D, Table 1). Tarm1 and tarm2 are the only leg muscles that are not associated with the internal tendons. Tarm1 is composed of five, and tarm2 of three, short aligned fibres attached on both sides to the epithelial apodemes (Fig. 5A,B). Tarm1 is located on the posterior, and tarm2 on the anterior, side of the tibia/tarsus junction (Fig. 5A,B, Fig. 6D). Fibres forming the long tendon muscles are attached on the posterior sides of the tibia and femur (Fig. 5B,I, Fig. 6D).

For a better characterisation of muscle fibres constituting appendicular musculature, we dissected individual muscles from different leg segments of the adult MHC-tauGFP flies (Fig. 5C-G,J-P, Fig. S2D-I in supplementary material). The analysis of dissected adult leg muscles confirms the general muscle and tendon organisation from the 55-hour APF pupae (compare Fig. 5A with 5C,D). Dissection revealed the striated character of syncytial muscle fibres. Depending on muscle type, appendicular fibres were found to contain 10 to 25 nuclei (Fig. 5E,L,O,P, Fig. S2D-F) arranged in two rows (Fig. 5P, Fig. S2E). Interestingly, within the tibia and femur, the levator muscles display comparatively stronger MHC-tauGFP expression than the depressors (Fig. 5F,G,J), whereas the opposite is observed in coxa (Fig. S2I in supplementary material). Moreover our analysis by transmitted light and

electron microscopy revealed a classic sarcomeric organisation of muscle fibres with a canonical succession of Z, I, H and M bands (Fig. S3A-C in supplementary material). Interestingly, when comparing the depressor and the levator muscle ultrastructure, marked differences in sarcomere size and the number of mitochondria were found (see Fig. S3 for more details).

Discussion

Previous reports (Miller, 1950; Trimarchi and Schneiderman, 1993) have suggested a particularly complex organisation of the *Drosophila* leg muscles, but no systematic studies have been conducted to describe the morphology of the appendicular *Drosophila* muscles and the main steps of their formation in detail. Here, we provide a set of data that fills this gap. Importantly, we find that in contrast to other hitherto described *Drosophila* muscles, the leg muscles are organised around the internal tendons and, in this feature, resemble multifibre vertebrate muscles.

Appendicular myogenesis versus larval and flight muscle formation

A common feature of all *Drosophila* muscles is that they arise from *twi*-expressing non-differentiated cells. Leg muscles originate from a restricted subpopulation of such cells (5-10 myoblasts) associated with the embryonic leg disc primordia. These cells start to proliferate in the second instar larvae to form a population of about 500 myoblasts that are randomly deployed on the disc epithelium and also are known as adepithelial cells (Bate et al., 1991; Broadie and Bate, 1991). Unlike the embryonic promuscular cells, they do not seem to be organised into clusters of cells from which progenitors of individual muscles segregate (Carmena et al., 1995), but rather to follow the segmental subdivision of the leg disc within the proximodistal axis. This leads to the early loss of *twi* expression in adepithelial cells from the tarsal segments. The main feature of all *Drosophila* muscles that form de novo, including the larval body wall and the adult direct flight muscles, is that they develop from the specialised myoblasts named muscle founder cells (Bate, 1990; Baylies et al., 1998; Kozopas and Nusse, 2002; Dutta et al., 2004). The leg muscles belong to this category of muscle, and our study shows that their formation is preceded by the specification of cells expressing the muscle founder marker *duf-lacZ*. How the *duf-lacZ*-expressing cells segregate from the population of adepithelial cells and how they become muscle founders remains unclear, but their association with *sr*-positive tendon progenitors suggests that interactions between these two cell types may promote their differentiation.

Interestingly, in third instar leg discs, *duf-lacZ* cells segregate in around only one out of five *sr*-expressing epithelial domains. This domain, termed the a domain, is located in the dorsal Dpp-dependent portion of the disc, suggesting that Dpp signalling may be involved in eliciting this group of presumptive founders. Similar to the leg tendon precursors described here, *sr*-expressing domains have been reported in the notum of the third instar wing discs (Fernandes et al., 1996; Ghazi et al., 2000; Ghazi et al., 2003). These *sr*-positive domains have been reported to be involved in flight muscle patterning (Ghazi et al., 2003).

In spite of all the similarities, marked differences in appendicular versus flight and larval body wall musculatures exist that can be explained by the specific properties of leg tendons. As demonstrated by our analyses of Stripe-GFP-expressing leg discs, at the end of third instar, concomitant with disc evagination, the epithelial domains of tendon progenitors start to invaginate inside the disc. This leads to the formation of internal tendons that have not been described in other body parts of the adult fly. Importantly, the presumptive founder cells associated with the invaginating tendon precursors are vectored and deployed throughout the proximodistal axis of the leg segments. Such a system provides an effective way to generate multifibre muscles in an invertebrate leg devoid of internal skeleton.

The mechanisms governing the formation of internal tendons remain to be elucidated; however, the co-expression of *sr* with *odd* in invaginating tendon precursors suggests a potential involvement of Notch. *odd* was previously described as an important element of the Notch-dependent cascade that controls the invagination of segmental joints (Bischop et al., 1999; Hao et al., 2003). Thus, it is possible that a similar set of genes controls the different epithelial invagination events that occur in the developing leg.

Using transgenic lines that express GFP in tendon precursors (Stripe-GFP), in myoblasts and in tendons (1151-GFP), and in developing myotubes (MHC-tauGFP), we were able, for the first time, to monitor appendicular myogenesis during pupa metamorphosis. At 20 hours APF, a large number of myoblasts are associated with the internal tendons, suggesting that the founder cells that are initially linked to tendons have attracted fusion-competent myoblasts to form prefusion complexes. Five hours later we can discern muscle precursors composed of 5 to 10 nuclei, indicating that the first wave of fusion takes place between 20 and 25 hours APF. Shortly after, at 35 hours APF, the second fusion wave occurs, giving rise to the multinucleated myotubes that are attached on one side to the internal tendons. The timing of the observed fusion events is comparable to that reported previously for the de novo forming DFMs (Ghazi et al., 2000). The next myogenic steps, including myotube growth, recognition of cognate *sr*-expressing epithelial attachment sites and induction of expression of myofibrillar proteins, are similar to the previously described events that lead to the formation of the flight and body wall muscles (Becker et al., 1997; Frommer et al., 1996; Vorbruggen and Jackle, 1997; Yarnitzky et al., 1997). The most important, unique, feature of leg muscle fibres that makes them different from other *Drosophila* muscles is their association with the internal tendons.

General organisation and nomenclature of the *Drosophila* leg muscles

The appendicular muscle pattern revealed by our study, with two principal muscles (levator and depressor) in each leg segment, resembles that described by Miller (Miller, 1950). However, the organisation of the muscle fibres composing levators and depressors is different, as they are attached to internal tendons that have not been described previously. The only tendon reported by Miller was the long tendon of the tarsus. Our analysis shows that this tendon extends to the femur and harbours two previously undescribed muscles, which we designate ltm1 and ltm2.

Overall, the computer-assisted reconstruction of the leg musculature enabled us to identify all the appendicular muscles and tendons, to define their anteroposterior, dorsoventral and proximodistal positions, and to determine the number of muscle fibres that compose the individual muscles. As this is the first reported systematic analysis of the *Drosophila* leg musculature, we propose designations and their corresponding abbreviations (see Table 1) for all the identified muscles and tendons. In general, the proposed designations reflect the muscle and tendon functions. For example, muscles located in the femur that ensure movements of the adjacent tibia are named tibia levator (tilm) and tibia depressor (tidm) muscle. This nomenclature is largely based on that of Miller (Miller, 1950).

Our observations also indicate that the general pattern of appendicular muscles is invariant in males and females. However, muscle fibres that contribute to depressors and levators display distinct characteristics, suggesting differences in the genetic programme that ensures their specification. Most specifically, they differ at the ultrastructural level, displaying variations in sarcomere size and number of mitochondria. As determined by the analyses of dissected appendicular muscles, the number of nuclei that contribute to the mature fibres differs in the different types of muscle, but is relatively invariant when the same muscles from two different legs are compared. This suggests a precise control mechanism that sets up the complex events of appendicular myogenesis in *Drosophila*.

The association of muscle and tendon precursors in the imaginal leg discs of *Drosophila* reported here resembles the temporally and spatially linked development of avian tendons and muscles described in the chick hind limb (Kardon, 1998). In addition, as demonstrated recently (Brent et al., 2003), the specification of tendon progenitors in vertebrate embryos takes place very early in development, in a compartment immediately adjacent to the myotome. Thus it seems that conserved mechanisms may control the co-ordinated development of muscles and tendons in both the *Drosophila* leg and vertebrate embryos. An attractive possibility is that the muscle and tendon progenitors mutually promote each other's specification. The existence of such a mechanism could be easily tested in the future using *Drosophila* as a model system.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/131/24/6041/DC1

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