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Review

Drosophila adult muscle development and regeneration

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ABSTRACT

Myogenesis is a highly orchestrated, complex developmental process by which cell lineages that are mesodermal in origin generate differentiated multinucleate muscle cells as a final product. Considerable insight into the process of myogenesis has been obtained for the embryonic development of the larval muscles of *Drosophila*. More recently, the postembryonic development of the muscles of the adult fly has become a focus of experimental investigation of myogenesis since specific flight muscles of the fly manifest remarkable similarities to vertebrate muscles in their development and organization. In this review, we catalog some of the milestones in the study of myogenesis in the large adult-specific flight muscles of *Drosophila*. The identification of mesoderm-derived muscle stem cell lineages, the characterization of the symmetric and asymmetric divisions through which they produce adult-specific myoblasts, the multifaceted processes of myoblast fusion, and the unexpected discovery of quiescent satellite cells that can be activated by injury are discussed. Moreover, the finding that all of these processes incorporate a plethora of signaling interactions with other myogenic cells and with niche-like neighboring tissue is considered. Finally, we briefly point out possible future developments in the area of *Drosophila* myogenesis that may lead to of new avenues of genetic research into the roles of muscle stem cells in development, disease and aging.

Contents

1. Introduction

The muscles of metazoans power a wide range of movements such as walking, running, climbing, and flying and, although there is considerable variation, they generally make the largest contribution to the body mass of most animals [1,2]. In developmental

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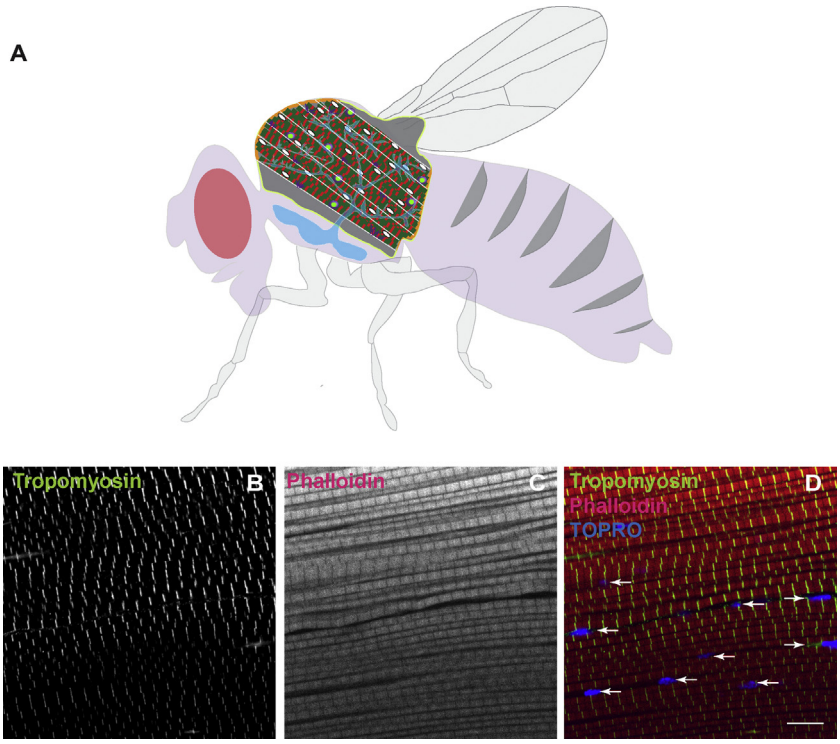

Fig. 1. A) Schematic showing adult fly thorax with six dorsal lateral muscles innervated by motor neuron (Blue). B–D) Optical section of flight muscles stained for Tropomyosin-GFP (anti-GFP, Green), phalloidin (marks F-actin, Red) and TOPRO-3 (Blue, marks all nuclei). Arrows shows position of the nuclei in the muscle syncytium.

terms, muscles derive from the mesoderm, a germ layer that together with the ectoderm and endoderm give rise to the various tissues and organs that make up the bilaterian body. In cellular terms, and contrasting with most other cell types, mature muscle cells are generally characterized by the presence of numerous nuclei. How this multinucleate syncytial organization of muscle cells originates from mononucleate precursor cells of mesodermal origin (often generically referred to as "myoblasts") during early development is a major question in muscle biology.

To address this question, developmental biologists have turned to a number of vertebrate and invertebrate model systems. Investigations carried out on one of these model systems, the fruit fly *Drosophila melanogaster*, have been useful to understanding many aspects of organismal development, especially as concerns the early stages of development, and have led to the identification and characterization of numerous conserved developmental control genes that operate in animal embryogenesis. Work focused on the development of the *Drosophila* mesoderm and its derivatives, notably the somatic, cardiac and visceral muscles, has resulted in remarkable insight into the genetic mechanisms of myogenesis. Moreover together with comparative studies on the development of vertebrate skeletal muscle, this work has uncovered conserved molecular pathways for myogenesis and identified similar cellular processes involved in transforming mononucleate myoblastic precursors into mature multinucleate muscle cells [2–4].

The indirect flight muscles (IFMs) have many developmental characteristics similar to that of vertebrate muscle. The IFMs consist of two groups of large muscles, the dorsoventral muscles (DVMs) and the dorsal longitudinal muscles (DLMs), which together power the wing stroke during flight (Fig. 1). We focus on the DLMs for much of this report. Like vertebrate muscle cells, the multifiber IFMs are formed during development by the fusion of muscle stem cell-derived myoblasts with a set of developing fibres and, once mature, manifest 'fibrillar' organization in contrast to the tubular organization of other fly muscles [5]. Many shared features of IFMs and vertebrate somatic muscle cells have made these *Drosophila* muscles excellent models for developmental genetic investigations of key aspects of myogenesis [6–10].

In this review, we consider recent findings on the cellular and molecular mechanisms involved in the development of one group of IFMs, the DLMs focusing on mesoderm-derived muscle stem cell lineages, the symmetric and asymmetric divisions through which they produce myoblasts, the multifaceted processes of myoblast fusion with template cells, and the recently discovered muscle satellite cells. In doing so, we embark on a journey from their mesodermal origins along a developmental timeline that incorporates a plethora of signaling interactions with other myogenic cells and with niche-like neighboring tissue to the ultimate formation of mature muscle cells and of quiescent satellite cells, which can be activated by injury. Finally, we will briefly point out possible future developments in the area of *Drosophila* myogenesis.

2. Myogenic beginnings: from mesoderm to muscle progenitors

Somatic myogenesis in *Drosophila* is a two-stage process. The muscles of the larval stage are generated during embryogenesis and are largely destroyed during pupal stages at metamorphosis. By contrast, the muscles of the adult are generated de novo during the postembryonic larval and pupal stages. Remarkably, however, the cells of the adult musculature are related to cells of the embryonically generated larval muscles as members in a common lineage that can be traced back to specified progenitor cells that arise in specific domains of the embryonic mesoderm [11].

Formation of the mesoderm begins during early embryogenesis through the process of gastrulation in which cells located ventrally, that express high nuclear levels of the maternally provided Dorsal protein, invaginate into the embryo along a ventral furrow [12]. This initial specification of mesodermal cells requires the activation by Dorsal of two zygotic genes that encode the basic helix-loop-helix transcription factor Twist, a key regulator of mesodermal tissue formation, and the transcription factor Snail. The Twist/Snail positive

cells divide, change shape, migrate dorsolaterally and form a monolayered epithelial arrangement that comes into direct contact with the overlying ectoderm during the dorsally directed invagination [13,14].

The physical proximity of the developing mesoderm to the ectoderm is a decisive step in early myogenesis. In subsequent development, signaling cross-talk between the two germ layers sets up an inductive patterning process, which together with autonomous patterning gene activity in the mesoderm itself, shapes specific cell fates in the initially "naïve" mesoderm. Numerous signaling molecules from the ectoderm are known to direct mesodermal patterning [15]. Thus, secreted Decapentaplegic is necessary for the induction of muscle cell fates in the mesoderm and secreted Wingless is a prerequisite for further patterning of these cells, resulting in the formation of somatic and cardiac muscle cell lineages [16,17]. Further autonomous patterning of the mesoderm by the segmentation gene products Even-skipped and Sloppy-paired results in a compartmentalized subdivision of the mesoderm into alternating sets of high and low Twist expression domains in a segmentally repeated manner. While the low Twist and high Even-skipped domains give rise to progenitors of the cardiac and visceral muscle lineages, the high Twist and high Sloppy-paired domains give rise to progenitors of the somatic muscle lineages [17–19]. Progenitor cell formation in the high Twist/Sloppy-paired domains occurs in local equivalence groups of cells delimited by Lethal-of-scute expression. Lateral inhibition in the equivalence group mediated by Notch signaling results in one cell in the group adopting a myogenic progenitor cell fate and the remaining cells adopting the fate of fusion-competent myoblasts [20].

2.1. Specification of embryonic muscle founder cells and adult muscle precursors

Approximately half way through embryogenesis, each of the myogenic progenitors undergoes an asymmetric cell division that produces two different daughter cells. The majority of these asymmetric cell divisions result in the generation of two muscle founder cells, which serve as seeds for embryonic muscle formation through fusion with the surrounding fusion-competent myoblasts. In this case, asymmetric division of the high Twist progenitor cell produces two low Twist founder cells. Hence, embryonic muscles made for larval life arise from fusion of low Twist mesoderm-derived founder cells with fusion competent myoblasts that have also lost Twist expression. However, in some cases the asymmetric progenitor division produces a single founder cell and an adult muscle precursor (AMP) that has the features of an adult-specific muscle stem cell and remains quiescent until larval life. While the founder cell in these cases is also a low Twist cell, the AMP continues to express high levels of the mesodermal marker Twist.

Notch/Numb interactions and asymmetric cell divisions are important factors in the generation of these two distinct progenitor sibling fates, founder cell and AMP. Asymmetric segregation to the future founder cell of Numb, a cytosolic protein that inhibits Notch signaling, tilts the cell's developmental balance towards immediate muscle generation. In contrast, the absence of Numb in the nascent AMP cell, and the resulting active Notch signaling, together with the inheritance of a high Twist state, maintains the cell's ability to avoid a commitment to immediate muscle generation since Twist and Notch working together prevent further differentiation of AMPs during embryogenesis. Hence, unlike their sibling founder cells, the AMPs remain quiescent and undifferentiated, are set aside in the embryo, and in many cases become associated with the imaginal discs by the end of embryonic development [19,21–23].

During embryogenesis, numerous myoblasts (4–25 approximately) fuse with each of the founder cells fuses with to produce the small muscles of the embryo that will operate in larval stages. Though the molecules for long range signaling to attract myoblasts to the founder cells remain unknown, numerous molecules required for cell fusion between founder cell and myoblasts have been identified in this system. Notably, the requirement of the immunoglobulin domain-containing transmembrane receptors Dumbfounded (in the founder cells) and Sticks-*n*-stones (in the fusion-competent myoblasts) for heterophylic interactions between the two cell types have been well characterized [4,24–26].

Although embryonically generated larval muscles are largely destroyed during metamorphosis, some set the stage for one aspect of adult muscle development. Thus, in the thorax, the largest muscles of the adult *Drosophila*, the DLM indirect flight muscles, are generated through the use of one set of larval muscles as a persistent scaffold-like template that is transformed into functional adult muscle by numerous swarming and fusing myoblasts derived from the high Twist AMPs. In the abdomen, the interstices of larval muscles and peripheral nerves associated with larval muscles appear to act as position-specific niches for AMPs until adult abdominal muscle development is started during the pupal stage [6,27–29].

2.2. Adult myogenesis: making muscles to power flight

The multifiber muscles of the adult fly are not only markedly larger than the muscles generated during embryogenesis, they also differ significantly in the process of their formation [21,30–33]. Adult myogenesis starts fairly early, indeed as mentioned above, it can be traced back to the embryo where AMPs are generated. These AMPs are mitotically quiescent myogenic precursors that are set aside until after larval hatching. AMPs can be identified by along the length of the embryo, and their mitotic activity during postembryonic stages leads to the formation of numerous myoblasts in all thoracic and abdominal segments. In this embryonic process, the AMPs that produce the myoblasts for muscles required for metameric structures of the body axis such as head, thorax and abdomen become situated anatomically near their future adult-specific muscle sites.

In the case of the thoracic muscles, wing imaginal disc associated myogenic precursors generate myoblasts, which form a variety of direct and indirect flight muscles involved in flight control, while leg imaginal disc associated myogenic precursors furnish myoblasts for a diverse set of leg muscles. By contrast, the adult-specific myogenic precursors in the abdominal segments give rise to muscles that show more uniform fate. These differences may indicate that an initial common program for myoblast proliferation might be followed by a subsequent divergence in myogenesis plans to achieve segment-specific results (in this case, large muscles in the thorax and small, numerous muscle in the abdomen). Accordingly, experiments involving transplantation demonstrate the ability of adult-specific myoblasts destined to form thoracic muscle to contribute to abdominal muscle formation [34]. The underlying phenomena behind this remain unaddressed but a re-assignment of fate by local signaling and founder cells and a conserved role of fusion molecules are likely mechanisms [1,33,35].

To date, the focus of most investigations on adult myogenesis research has been on thoracic flight muscles, notably on those involved in powering the wing stroke; less is known about myogenesis of the abdominal muscles [36] or of the leg muscles [37,38] or the development of the muscles of the head. Flight muscles are categorized into the indirect flight muscles that power the wing stroke during flight and direct flight muscles that control the angle of wing movements. While far less known about the development of the direct flight muscles [35] due to their small size and the lack of markers that specifically label their developmental stages, the development of the two subsets of indirect flight muscles, the

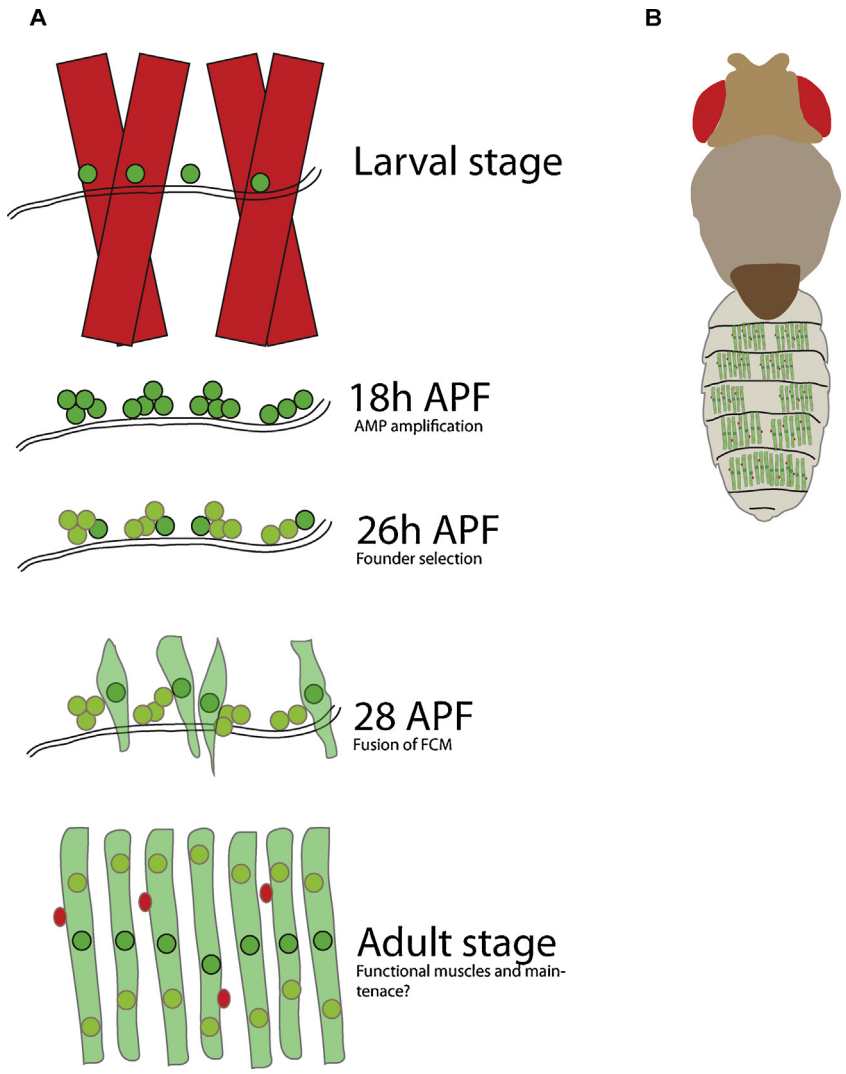

Fig. 2. Development of abdominal muscles. A-B) Schematic depiction of abdominal muscles during pupal stages. Precursors for abdominal muscles are found in the close proximity of nerves in the abdominal segment. During 18–30 APF these precursors proliferate and fuse to form abdominal muscles. Larval muscles undergo histolysis. Considering the presence of satellite cells in the flight muscles, there is the possibility of similar cells in the abdominal muscles, and these have been indicated in the adult muscle as red cells in the figure.

dorsal longitudinal muscles (DLMs) and the dorsoventral muscles (DVMs) has been described extensively [7,39,40].

Classical work has described the developmental program for the DLM indirect flight muscles. Three sets of larval muscles persist through histolysis during metamorphosis as larval templates for the adult-specific DLM muscles, namely the dorsal oblique muscles 1, 2, and 3. Although they show signs of partial degeneration, they nevertheless serve as a scaffold for fusion by migrating myoblasts. Myoblast fusion with these templates occurs at around 12–20 h APF (after puparium formation). Following the fusion process, the three muscles then split to form a total of six DLM muscle fibers per hemisegment. The final number of mature DLMs muscle fibers is dependent on the number of initial templates and on the splitting of the templates, which is directly governed by fusion of the myoblasts. Any perturbations in either of these two components severely affect DLM biogenesis [7,41]. Thus, fusion-competent adult-specific myoblasts are critical for the transformation of the persistent larval templates into the mature DLM muscles.

Myoblast fusion is also a key event in the formation of other muscles involved in flight such as the DVM indirect flight muscles and the direct flight muscles, as well as in the formation of the adult-specific muscles of the abdomen. However, in these cases, myoblast fusion is de novo and seeds muscle patterning without the use of larval templates, much like in embryonic myogenesis. Thus, the adult-specific DVMs comprising three sets of indirect flight muscles termed DVM-I (three fibers), DVM-II (two fibers) and DVM-III (two fibers) are generated by fusion of AMP-derived feeder myoblasts with Dumfounded-positive founder cells during early pupal stages; each muscle fiber is seeded by the formation of a single founder cell. After one day APF, the resulting multinuclear cells begin to differentiate into functional myotubes and express muscle specific MHC [31]. Interestingly, DVM founder cells originate from leg discs and are required to generate the correct number of fibers. Genetic ablation of these founder cells leads to supernumerary muscle phenotype arguing against their absolute requirement for muscle fiber formation [42].

Formation of the adult-specific abdominal muscles is also de novo and involves AMPs that are arranged in a repeated pattern in abdominal segments A1-A8 in contact with larval nerves and larval muscles [22,43]. Following proliferation of the precursors in each abdominal segment, a subset of founder cells is selected from the precursor pool and the remaining cells differentiate into fusion-competent myoblasts that then fuse with the founder cell to generate the multinucleate functional muscle fibers (Fig. 2). Interestingly, and in contrast to embryonic myogenesis, selection of the founder cells for adult-specific abdominal myogenesis does

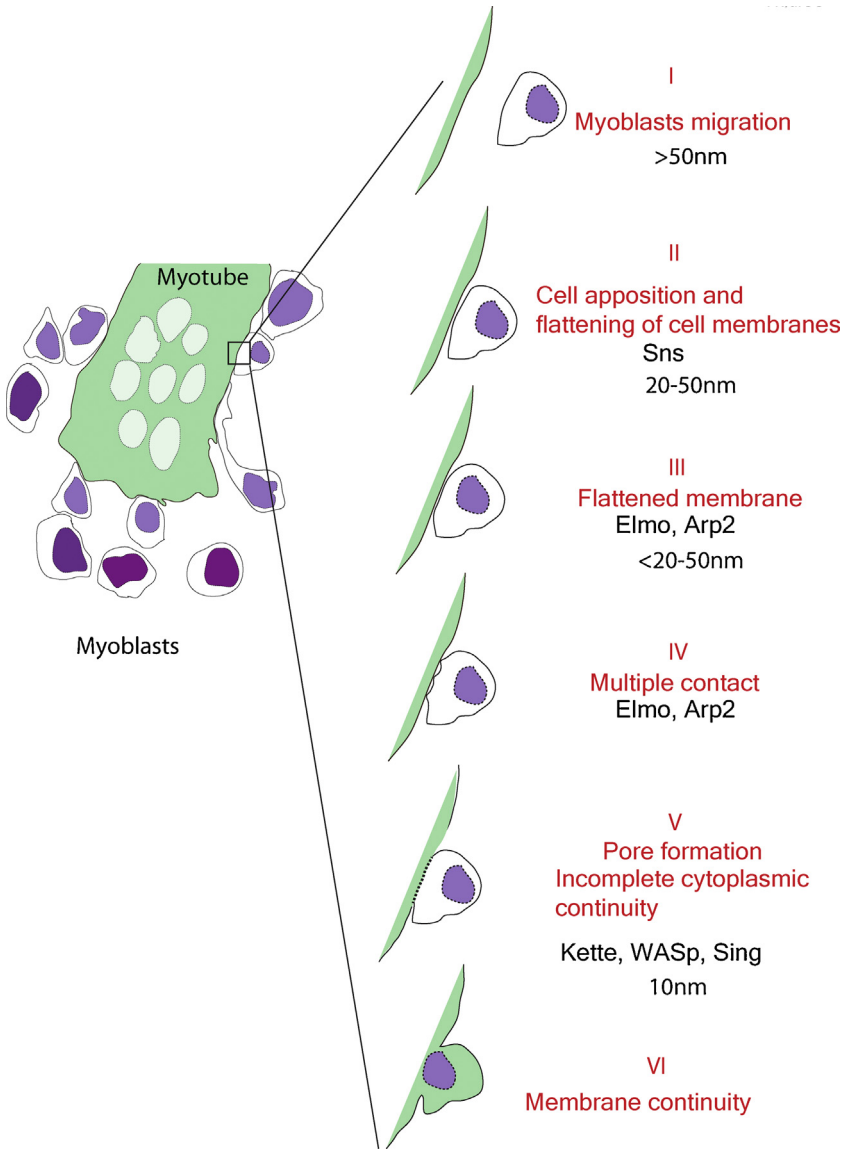

Fig. 3. Stages of myoblast fusion. I. migrating myoblasts are more than 50 nm distant from the myotube. II. Successful adhesion upon Duf-Sns interaction brings myoblasts 20–50 nm proximity. III. Myoblast flatten on to myotube in an Arp2/3 and ELMO dependent manner to form long surface contact. IV. Branched actin polymerizing machinery and Singles bar mediate multiple cell–cell contacts that serve as origin for nascent fusion pore formation. V. multiple fusion pores form at the contact surface to bridge the cytoplasmic continuity. VI. Membrane remnants are removed to unite myoblast into myotube. The genes involved in the various stages of the fusion are mentioned in black letters. Similarly, the distances between fusing myoblast membrane and myotube membrane from electron microscopic studies are mentioned in black letters [27].

not involve Notch mediated lateral inhibition. Rather founder cell selection and maturation is mediated by the FGF receptor Heartless and involves an interplay of the positive FGF signaling regulator Heartbroken and the negative FGF signaling regulator Sprouty [36]. The autonomous identity of the mesoderm is important for choosing muscle founder cells in the correct segmental pattern. The authors show this by removal of the function of *Antennapedia*, the Hox gene expressed in the mesoderm of the third thoracic segment. This results in the transformation of founder cells to a second-thoracic pattern [44].

3. AMPs and imaginal discs: Epithelial signaling to adult-specific muscle stem cells

As mentioned above, the development of the DLM indirect flight muscles has become of particular interest. They are the largest muscles of *Drosophila*, require fusion of hundreds of myoblasts over a short developmental period, and resemble vertebrate skeletal muscles in their structure [45]. A key early event in the adult-specific development of the DLM flight muscles is the close association of mesoderm-derived AMPs and ectoderm-derived wing discs. Thus, following their generation through asymmetric division of embryonic muscle progenitors, the Twist-positive AMPs are found in close apposition to neighboring imaginal discs [1,19,33].

Recent work on the development of the DLM flight muscles has revealed new insights into the cellular and molecular myogenic interactions between AMPs and wing imaginal discs that result in the generation of fusion competent myoblasts [10]. In the mesothoracic (T2) segment, AMPs acting as adult-specific muscle stem cells sequentially manifest two different modes of proliferative activity during larval stages that result in the generation of pools of post-mitotic myoblasts for DLM formation. During both proliferation modes, the AMPs, which retain a high Twist status, remain closely apposed to the epithelial surface of the notum-part of the wing imaginal disc. During early larval stages (24–48 h AEL, after egg laying), these embryonically generated muscle stem cells manifest

a first symmetric mode of division that serves to expand the AMP pool. Activation of Notch signaling in the AMPs by its ligand Serrate located in disc epithelium orchestrates this first proliferative phase.

At the onset of the last larval stage (72 h AEL), the AMPs transit to an asymmetric, stem cell mode of cell division in which they self-renew and at the same time generate a differentiated daughter cell, a postmitotic myoblast. Concurrently, the epithelium of the notum region of the wing disc, to which the AMPs are closely apposed, undergoes several changes in signaling, notably an activation of the Wnt pathway due to a marked increase in the expression of the ligand Wingless at this time point (72 h AEL). Activation of this pathway is both sufficient and necessary to initiate the asymmetric division of AMPs in the third instar stage. Wingless signaling from the disc induces expression of Numb in the AMP, which is asymmetrically distributed to only one of the two daughter cells that result from the ensuing asymmetric division. In the daughter cell that receives Numb, Notch signaling is inhibited and as a result this cell exits the cell cycle and differentiates as a postmitotic myoblast. In contrast, the daughter cell that does not receive Numb retains high levels of Notch signaling activity, maintains its muscle stem cell fate and continues to divide in the asymmetrical mode to form more postmitotic myoblasts resulting in a large increase in the myoblast pool available for further DLM muscle differentiation (Fig. 3).

3.1. The wing imaginal disc as a novel dynamic stem cell niche

The proximity of AMPs acting as muscle stem cells to the wing disc epithelium acting as a stem cell niche is essential for both symmetrical and asymmetrical AMP division modes. Thus, the molecular crosstalk between mesoderm and ectoderm which started early from the embryonic stages continues throughout postembryonic development and is critical for adult-specific myogenic proliferation. Indeed, since discrete groups of cells in the disc epithelium are fated to become future muscle attachment sites ("tendon cells", see below), the continuous close "ad-epithelial" contact between AMPs and disc epithelium may presage possible coordination between muscles and tendons even before metamorphosis brings the two lineages together [37,46].

Given that the disc epithelium is involved in numerous other developmental signaling pathways such as those involving Decapentaplegic and Hedgehog, it may also be involved in imparting regionalized identity to AMP muscle stem cells or their progeny and hence contribute to differential myoblast function or muscle type. For example, the diverging development of direct flight muscles versus indirect flight muscles has been shown to depend on the differential expression of the Cut and Vestigial transcription factors. Wingless signaling from the disc notum is crucial for this in that it segregates the Twist positive precursor pool into a high Vestigal, low Cut sub-pool which contributes to indirect flight muscles and a high Cut, low Vestigial sub-pool which contributes to direct flight muscles [33].

In view of the multiple key roles of the wing disc epithelium in signaling pathways required for muscle stem cell expansion, proliferation, and maintenance, we consider this tissue to be a new dynamic-transient niche for muscle stem cells in *Drosophila*. This muscle stem cell niche is comparable in functional respects to the well-known tissue niches that regulate stem cell dynamics and homeostasis in other contexts such as germline, intestine, skin and blood [47,48]. However it differs from these in its dynamic-transient nature. The wing disc epithelium constantly and dramatically changes its dimensions due to the marked growth that occurs during larval stages. Moreover, it manifests a marked change in the spatial pattern of the various signaling molecules that it expresses during development. Finally, after metamorphosis it is transformed into the dorsal structures of the adult mesothorax and is from then on no longer available as a niche for tissue stem cells. The novel features of this tissue stem cell-niche architecture pose challenging questions for future studies of muscle stem cell biology in *Drosophila* [49,10].

Table 1
The list of Gal4 drivers, lacZ lines and other fly lines useful for muscle developmental studies.

Gal4 driver/ Enhancer elements	Tissue expression	Reference
Ap Gal4	Niche-wing epithelium	[51]
Mef2 Gal4	Stem and postmitotic myoblasts	[52]
Pnr Gal4	Niche-wing epithelium	[53]
Notch Gal4	Stem cells	[54]
MHC Gal4	Mature muscle	[55]
Duf Gal4	Founder cells	[56]
Sns Gal4	Postmitotic/fusion competent myoblast	[57]
Delta GFP	Muscle fibres adult stage	[58]
NRE GFP	Insect satellite cells	[59]
Neur-LacZ	Mature muscle	[60]
Sns-lacZ	Postmitotic/fusion competent myoblast	[57]
Duf-lacZ	Founder cells	[61]
Zfh-1 Gal4	Insect satellite cells	[62]
Act88F gal4	Mature DLMs	[63]
Stripe gal4	Tendon cells	[35]
P103.3 gal4	Motor neuron	[64]

3.2. Myoblast-myotube fusion: swarming of myoblasts and a coalition of membranes

Following the generation of a large pool of adult-specific IFM myoblasts, the myoblasts contributing to the DLMs congregate around the larval template muscles. These fusion competent myoblasts, which initially maintain a naive state, through the expression of Notch and Twist, then express *Sns* (*Sticks and stones*) and *Wip* (*WASP interacting protein*) and eventually fusion with the larval muscle templates to generate functional indirect flight muscle. Fusion of myoblasts starts the terminal differentiation of myoblasts. Mef2 is highly expressed in myoblasts prior to fusion and also after fusion. Before fusion, the Him gene is known to antagonise Mef2, thus controlling premature myosin expression. Notch along with Him gene is involved in keeping Mef2 from acting early during development, inhibiting premature differentiation of myoblasts and besides this, Notch directly known to control Him gene levels [50].

Myoblast fusion is a multi-step process involving migration and adhesion to the fusion partner followed by membrane contacts and fusion pore formation. While most of the previous work on myoblast fusion has focused on embryonic stages, the availability of targeted genetic access through specific Gal4 driver lines together with development of florescent markers and RNAi based gene knock-down techniques has brought adult flight muscles into the limelight. A list of some of the genetic driver lines currently used to study adult flight muscle development is given in Table 1.

In the past few years, a number of genetic studies using electron microscopy and fluorescence microscopy have advanced our understanding of the multiple stages of myoblast fusion in adult *Drosophila* myogenesis [9,26]. Notably, visualization and analysis of the numerous myoblasts undergoing fusion with larval templates at around 20 h APF, the peak of fusion, has provided an excellent opportunity to capture multiple myoblasts at various stages of the fusion process. In the following, we describe these stages by focusing primarily on recent advances in understanding DLM indirect flight muscle fusion, compare it to the embryonic myoblast fusion, and add a note on conserved molecular players or the processes

where appropriate. A simplified summary scheme of some of these developmental events is shown in Fig. 3.

3.3. Myoblast migration: a largely unexplored territory

During embryonic myogenesis, fusion competent myoblasts migrate over short distances to fuse with founder cells. As previously mentioned, the immunoglobulin superfamily adhesion molecules, Dumbfounded and Sticks-*n*-stones are thought to be key elements in this short distance myoblast migration. Dumbfounded, which is expressed exclusively in founder cells, attracts Sticks-*n*-stones on FCMs (Fusion competent myoblasts) to mediate migration. Ectopic expression of Dumbfounded is sufficient to alter the migratory behavior of embryonic fusion competent myoblasts [61].

In contrast, DLM myoblasts must migrate over relatively long distances. The wing imaginal disc associated muscle stem cells (the AMPs discussed above) generate a semi-differentiated population of myoblasts. After the wing disc eversion, these *twist* expressing myoblasts migrate towards the larval templates by associating with segmental nerves in the T2 segment [65]. Unlike in the embryo, migration of adult-specific myoblasts appears to require long range signals as they utilize the parallelly metamorphosing nerve tracks through muscle-nerve interaction [6,65]. However, the long-range signals that involve muscle-nerve interactions are poorly understood, In addition, filopodia emanating from template myotubes and the adhesion molecules are shown to be crucial for local migration of myoblast after they reach the vicinity of myotubes (see below) [9,26,66].

Cytoskeletal remodeling has been shown to play an important role in embryonic myoblast migration. RNAi mediated knockdown of Kette, a subunit of the SCAR/WAVE pathway of the Arp2/3 NPF system, resulted in the failure of myoblasts to acquire the stereotypical teardrop shape characteristic of migrating cells. Whereas SCAR localized to the polarized part of the cell in wild-type myoblasts, in Kette knockdown myoblasts SCAR is distributed throughout the cytoplasm [67]. In adult-specific DLM myoblasts, intracellular mechanisms such as cytoskeletal remodeling and cell shape changes have yet to be studied.

3.4. Adhesion: filopodia in long and close range membrane apposition

As in other forms of cellular fusion, adhesion of the membranes of the fusing partners is an early critical step during myoblast fusion. While earlier studies in *Drosophila*, like in other experimental systems, were focused on understanding fusion pore formation and membrane merger, not much was known initially about the cellular mechanisms that mediate adhesion. The molecular machinery of adhesion is now well established in embryonic myoblast fusion [61] and the same molecular machinery appears to be conserved in DLM myoblast fusion.

Two important observations have been reported in recent DLM myoblast fusion studies. First, an ultrastructural study of DLM myoblast fusion has uncovered a role for adhesion in bringing fusing cells into close contact with one another [26]. Second, filopodia have been shown to emanate from the larval muscle template (myotube) surface, contact the fusion competent myoblasts, and facilitate the adhesion process through Dumbfounded/Sticks-*n*-stones interactions [66]. Thus, simultaneous knockdown of Sticks-*n*-stones and Hibris perturbed the adhesion process, and myoblasts were more than 50 nm farther apart from myotubes as compared to 20–30 nm in their wild type counterparts. Interestingly, knockdown of elements of the molecular machinery that generates filopodia (Enabled and IRSP53) resulted in a phenotype similar to that of the Sticks-*n*-stones/Hibris knockdown. This observation is further strengthened by localization of Sticks-*n*-stones to the contact sites between filopodia and myoblasts [66].

Together, these ultrastructural, immunohistochemical and genetic studies strongly suggest that interactions between myotube filopodia and myoblasts initiate an adhesion process that brings myoblasts 20–30 nm close to the myotube membrane. It might be tempting to speculate that these interactions between myotube filopodia and fusion competent myoblasts could also be responsible for initiating the terminal differentiation of the myoblasts that form DLMs. However, as shown by [66], the fate of myoblasts is not dependent on filopodia, leaving the cellular and molecular mechanisms responsible for the myoblast fate change elusive.

3.5. Membrane contacts and fusion pore formation

Work on embryonic myoblast fusion has shown that successful myoblast-founder cell adhesion leads to activation of downstream molecules that initiate Arp2/3 dependent polymerization of branched actin in the two fusing cells and notably in the formation of a prominent actin focus in the myoblast. This phenomenon has also been shown to occur in adult-specific DLM myoblast fusion. However, the ultrastructural features of the actin foci have been under debate. In the embryo, the actin focus is thought to correspond to actin-based invasive podosomes that extend from the myoblast surface into the founder cell [45,68]. In contrast, in adult-specific DLM myoblast fusion, such invasive podosomes are less frequent although the actin foci do form [26,28]. In DLM myoblast fusion, following their adhesion, myoblasts move closer to their template myotube (10–20 nm) and membranes of the myoblasts make several distinct physical contacts along the length of the myotube membrane. Since ultrastructural analyses indicate that fusion pores between the two cells also form at multiple sites and that cell–cell contacts and fusion pores coexist, it is in principle possible that the cell–cell contacts act as the origins of the fusion pores.

Genetic experiments suggest that the branched actin polymerization machinery (Wasp, SCAR, Arp2/3, ELMO) and a MARVEL domain containing transmembrane protein (Singles bar) are essential for the formation of cell–cell contacts and eventual fusion [26]. Moreover, despite the apparent difference in the mode of the fusion process during embryonic and adult myogenesis, these elements of the branched actin polymerization machinery as well as the same membrane adhesion proteins (Sticks-*n*-stones, Hibris, Dumbfounded and, Roughest) are utilized in both cases. Nevertheless, the molecular nature of the cell–cell contacts in terms of fusion machinery localization has not been adequately addressed. While a previous study has shown that localization of Dumbfounded on the myotube is nonuniform [9], it is not clear if the initiation of polymerization of branched actin at the cell-contacts takes place at the sites of Dumbfounded and Sticks-*n*-stones localization. To address this and other possibilities, future experiments on protein localization focused on the cell–cell contacts and nascent fusion pores using immunogold labeling, correlative light and electron microscopy, and super resolution technology will be important.

Currently, the nature of fusion pore formation remains one of the major unresolved dilemmas in *Drosophila* myogenesis. Ultrastructural studies of embryonic myoblast fusion provide contradicting results. An early study, using traditional chemical fixation method to preserve tissue integrity, reported that multiple pores mediate myoblast fusion [69]. A later study, using more advanced cryofixation method to preserve ultrastructure, claimed that myoblast membranes form actin-based invasive podosomes that invade the myotube. According to this study, a single fusion pore forms at the tip of the podosome whose expansion causes the union of both the cells [68]. A number of other electron microscopy studies report

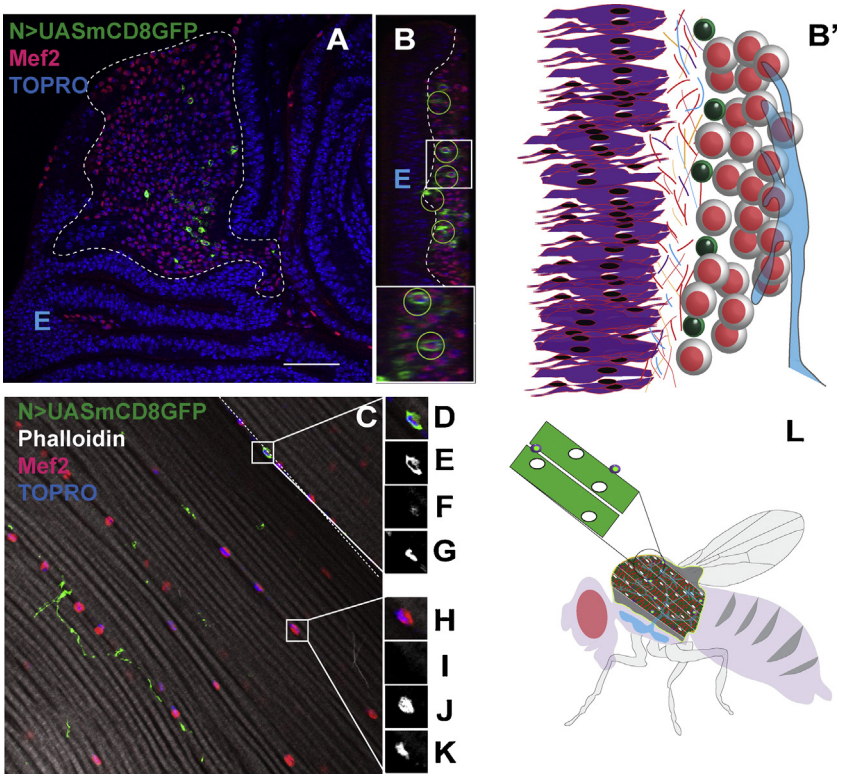

Fig. 4. (A B, B') A third instar wing imaginal disc from Notch Gal > UASmCD8:GFP larvae. Muscle stem cells are labeled with membrane GFP (Anti-GFP, green) and are co-stained with Mef2 (Anti-Mef2, red) and TOPRO (blue) labeling all the nuclei. The orthogonal section of disc shows muscle stem cells marked with green circle. Muscle stem cells are in close proximity to the wing disc epithelium (denoted by E) marked by white dotted line in (B). Inset in B shows zoomed in view of the section marked by white square depicting two GFP positive cells co-immunolabeled with Mef2. n = 6 B') Schematic of B showing stem cells (Green) and postmitotic myoblasts (red) on disc epithelium (purple cells). The branched blue structure represents trachea. (C-L) Single optical section of flight muscles. Flight muscles labeled with membrane-tethered GFP (green, anti-GFP immuno labeling), TOPRO-3 (blue) and Mef2 (anti-Mef2, red). Dotted lines mark muscle fiber boundaries. (D-G) GFP-labeled cell represent a satellite cell (Notch gal4 > UAS mCD8:GFP) in the adult stage. D is the merge showing GFP labeled cell (green, anti-GFP immunolabeling) (E) with low levels of Mef2 staining (anti-Mef2, red) (F) and TOPRO (blue) marks the nuclei (G) (H-K) Single Mef2 positive muscle nuclei within Phalloidin labeled muscle cell (nuclei with high levels of Mef2). H is the merge showing GFP negative cell (green, anti-GFP immunolabeling) (I) with high levels of Mef2 staining (anti-Mef2, red) (J) and TOPRO (blue) marks the nuclei (K) n = 7. n = number of animals processed for a given experiment. Scale bar 50 μm. (L) Schematic depiction of C showing cross section of flight muscles with satellite cell adjacent to muscle fiber.

that multiple fusion pores occur during embryonic myoblast fusion [70–72]. The most recent study of DLM myoblast fusion, which utilized a hybrid protocol of chemical and cryofixation methods, has shown that fusion pores formed at multiple sites establish cytoplasmic continuity [26]. Clearly, more ultra-structural studies in *Drosophila* and other systems are needed to resolve the problem of fusion pore formation.

3.6. Muscle attachment: stripe expression in myotendinous junction formation

In order to differentiate fully into functional muscles, the multinuclear myofibers must attach to tendon cells and form a muscle attachment structure referred to as a myotendinous junction. In *Drosophila*, the adult-specific flight muscle fibers first stably attach to tendon cells and only then assemble their contractile intracellular myofibrils [73]. The epidermal cells that serve as attachment sites for the DLMs are located in the notal region of the wing disc epithelium directly adjacent to the site of adult-specific myoblast proliferation. These prospective muscle attachment cells and their location in the disc epidermis are specified by expression of the *stripe* gene [27,41]. (The actual physical attachment between the epidermal tendon cell and muscle cells at the myotendinous junction is mediated by interactions between shared and cell type-specific integrin adhesion molecules during pupal stages.) Interestingly, expression of Stripe is seen on the wing disc as early as the third larval instar. The reason for this relatively early specification of cells that will form future insertion points for adult muscles is not clear, but the fact that myoblasts remain in close association with these cells during their residence on the prospective notum suggests the possibility that instructive pattering information could be exchanged between the myoblasts and the Stripe-expressing prospective tendon cells.

While molecules such as Stripe, Talin and PS Integrins have been identified as important players in myotendinous junction formation, the ensemble of molecular mechanisms involved in formation and maintenance of the attachment between muscle fibers and tendon cells remains poorly understood. To address this lack of information, a genetic screen using *stripe*-Gal4 targeted RNAi gene knockdown has been carried out [74]. This screen revealed numerous candidate genes and subsequent developmental and phenotypic analysis of these candidates led to the identification of 19 novel molecules that act in the myotendinous system including molecules involved in cell adhesion, transcriptional activity, protein folding, intracellular transport and enzymatic function. Among these, the endoplasmatic reticulum-to-Golgi transport protein Tango1 was shown to be essential for proper development of tendon precursors and myotendinous junction formation.

Live imaging of myotendious junction formation in pupal stages revealed further insight into the dynamics of tendon cell and developing DLM interactions [73,74]. Given the experimental accessibility of the DLM myotendinous junction during develop-

ment and in the adult, the genome-wide availability of tagged proteins in the *Drosophila* model [75] should allow an in depth analysis of molecular mechanisms involved in the interactions between muscles and tendons. This is likely to provide more general insight into the cell–cell interactions involved in the development of this and other myotendinous systems.

4. Muscle satellite cells: Novel adult muscle stem cells in *Drosophila*

Once the myotendinous junction is formed, the appropriate motoneuronal innervation is established, and the intracellular contractile myofibrils are assembled, the multinucleate flight muscle is mature, fully differentiated, and ready to play its role in flight behavior. Remarkably, however, not all of the cells associated with the mature DLM muscle have undergone terminal differentiation in the adult fly. Recent advances in the field of *Drosophila* muscle biology have led to the discovery of satellite cells, adult-specific muscle stem cells associated with the mature DLMs as small unfused cells located at the surface of the muscle fibers (Fig. 4C–L).

These satellite cells, lineal descendants of the AMPs that generate the adult-specific myoblasts during development, are normally quiescent. However, following muscle injury they undergo a Notch-Delta signaling-dependent proliferation process by which they generate fusion competent myoblast progeny that can fuse with the injured fiber in a manner that is thought to contribute to muscle repair [76]. Thus, in flies as in vertebrates, the muscle stem cell lineage that generates the adult-specific muscles during normal development is also available for adult myoblast production in muscle tissue in response to damage [77].

In contrast to the DLM muscle fiber cells, the satellite cells as tissue stem cells escape from terminal differentiation and retain their “stemness” in the uninjured adult for an indefinite period. An important role in maintaining the stem cell status of the satellite cells appears to be played by the zinc finger homeodomain transcription factor Zfh1, which has been shown to counteract the myogenic differentiation program [78]. During larval stages, Zfh1 is expressed in all of the AMP lineal cells on the notum of the wing imaginal disc, both precursors and myoblasts, and prevents them from acquiring fusion competence. Lineage tracing experiments using the G-trace method show that these adult AMPs are the descendants of larval muscle progenitors that maintained their identity through the expression of Notch and Zfh-1 and hence remain unfused. In contrast, in the adult, Zfh1 expression is limited to the pool of unfused satellite cells and is never seen in the intact muscle DLM fibers [76]. Indeed, expression of Zfh1 is a highly specific marker for satellite cells in the adult. Two, long and short, isoforms of Zfh-1 are expressed in AMP lineages. Interestingly, while the majority of the myoblasts express an isoform of Zfh1 that is subject to *miR-8* microRNA mediated downregulation that allows their differentiation into myocytes in early pupal stages, the satellite cells express a different RNA isoform of Zfh1 that cannot be targeted by *miR-8* and is, hence, maintained as a “stemness” factor in these adult muscle stem cells [79].

In contrast, muscle progenitors at the larval stages express low levels of mir-8 and the short isoform of Zfh-1, which lacks a binding site for mir-8, thereby maintaining high levels of the short isoform and maintaining stem cell status. Earlier study on IFM myoblast fusion suggested the role of Notch in the regulation of myoblast differentiation markers such as sns and wip. Downregulation of Notch through RNAi knockdown released the suppression on Sns and all myoblasts express Sns. Alternatively, constitutive expression of intracellular domain of notch (NICD) completely wiped off Sns expression [9]. Together these data suggest a sequence of signaling steps that Notch maintains Zfh-1 levels to maintain the stem cell identity and high levels of mir-8 down regulates Zfh-1 promoting them to the terminal differentiation. But the connection between mir-8 and terminal differentiation markers such as sns and wip is not yet known.

5. Discussion and concluding remarks

Earlier work on myogenesis in *Drosophila* focused primarily on the development of the larval musculature during embryogenesis. These genetic studies were highly successful in identifying the developmental stages and the controlling genes through which derivatives of the mesoderm are transformed into functional muscle cells of larval stages [11,25]. Moreover, the findings obtained in the *Drosophila* model system were useful in identifying comparable developmental mechanisms and genetic control elements that operate in vertebrate myogenesis. More recently, the availability of sophisticated genetic and clonal methods for targeted expression and manipulation of genes during postembryonic stages and in the adult, have made it possible to investigate the development of the adult musculature in the fly. Notably, investigations of myogenesis in the large multifiber, multinucleate DLM indirect flight muscle, which has the most similarities in developmental origin and structural organization to vertebrate somatic muscle, have generated a wealth of novel data on muscle development.

This work has resulted in new insight into the fields of muscle stem cells, stem cell niches, myogenic proliferation, cell–cell fusion and adult tissue stem cells, many of which are likely to be significant for unraveling the genes and developmental genetic pathways involved muscle development, maintenance and repair in vertebrates as well [79,76,80]. Clearly there is still much to be learned about adult muscle development in *Drosophila*. How do myoblasts recognize and migrate to the appropriate future site of fusion, what controls their transformation into myocytes, what controls the intricate processes of fusion pore formation with their templates, what are the inputs of motor neurons to muscle formation and how do hormones influence adult myogenesis? These and other questions must await further investigation.

Currently, one of the most promising and exciting avenues for future research has been opened up by the recent discovery of adult muscle satellite cells in *Drosophila*. In vertebrates, muscle satellite cells are known to play key roles in mediating the regenerative responses to injury and degenerative disease and are also involved in adult muscle growth [47]. The remarkable similarities in the structural and functional features of vertebrate satellite cells and the satellite cells in *Drosophila* are likely to provide a solid basis for future genetic investigations of the mechanisms involved in muscle damage, repair, and even disease-based degeneration using the wealth of classical and molecular genetic tools available in powerful genetic model system of the fly. Last but not least, given the increasing evidence for the age-related decline in satellite cell number and function in humans (e.g. [81], investigations on the satellite cells of aging flies may be useful for obtaining a more in-depth understanding of the causes of muscle wasting phenotypes observed in the aging population.

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