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Diversification of muscle types in *Drosophila* embryos

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Abstract

Drosophila embryonic somatic muscles represent a simple and tractable model system to study the gene regulatory networks that control diversification of cell types. Somatic myogenesis in *Drosophila* is initiated by intrinsic action of the mesodermal master gene *twist*, which activates a cascade of transcriptional outputs including myogenic differentiation factor Mef2, which triggers all aspects of the myogenic differentiation program. In parallel, the expression of a combinatorial code of identity transcription factors (iTFs) defines discrete particular features of each muscle fiber, such as number of fusion events, and specific attachment to tendon cells or innervation, thus ensuring diversification of muscle types. Here, we take the example of a subset of lateral transverse (LT) muscles and discuss how the iTF code and downstream effector genes progressively define individual LT properties such as fusion program, attachment and innervation. We discuss new challenges in the field including the contribution of posttranscriptional and epitranscriptomic regulation of gene expression in the diversification of cell types.

Introduction

Understanding the gene regulatory pathways that control progressive diversification of cell fates is of major interest not only to fill existing gaps in our fundamental knowledge but also to cue dynamically developing regenerative medicine. Differentiated cells can be reprogrammed to the ground state of induced pluripotent stem cells (iPSCs), which then can engage in specific differentiation programs and produce different cell types. Defining gene pathways that operate in development to form particular cell subsets is thus of great importance, with potential applications for cell replacements in pathological conditions.

The muscle network in *Drosophila* embryos, composed of 30 muscle fibers per abdominal hemisegment, offers a tractable system for studying cell diversification. The embryonic *Drosophila* muscles develop and undergo differentiation triggered by a generic myogenic pathway involving intrinsic and extrinsic cues. However, each of the 30 muscle fibers displays a specific size, orientation, number of nuclei, attachment and innervation. How these

features are acquired at the muscle-specific level remains elusive, although muscle founder cells (FCs), from which muscle fibers originate, are thought to carry instructive information required for individual muscle identity. This information is provided by the combinatorial code of identity transcription factors (iTFs) that are activated in subsets of FCs to regulate expression of downstream targets necessary for the acquisition of individual muscle properties.

Here we discuss the development of *Drosophila* embryonic muscles and the acquisition of their diversity, with particular emphasis on the lateral transverse (LT) muscles, where the expression of a combinatorial code of iTFs and their targets have become better understood in recent years.

Extrinsic and intrinsic cues that promote muscle development

Muscles originate from the mesoderm, which at early stages is composed of pluripotent cells. The transcription factor (TF) *twist*, activated by the dorsal-ventral embryonic axis determinant Dorsal, is expressed in the presumptive mesoderm [1][2]. *Twist* acts as a selector gene that is both necessary and sufficient to initiate mesoderm development [3]. Following gastrulation, mesodermal cells proliferate, migrate dorsally, and subdivide into different tissue primordia, including the heart, visceral muscle, and somatic muscle. At this stage, an interplay of conserved extrinsic Wnt/Wg, BMP/Dpp, Notch and RTK signals is required to modulate *twist* expression and to switch on the expression of another selector gene, *tinman (tin).* High Twist activity is essential for the specification of somatic body wall muscle precursors, while Tin drives the cardiac muscle developmental program [4][5][6] and, by activating *bagpipe (bap)*, also that of visceral muscles [7]. Many important downstream regulators of somatic muscle development including Mef2 and miR-1 were found to be direct Twi targets [8][9]. Interestingly, the same extrinsic regulatory inputs (Wg, Dpp, RTKs), supplemented by the intrinsic Twi and Tin action, activate the cell fate-specifying factors within the cardiac and somatic mesoderm [10][11]. For example, the regulatory requirements for *even-skipped (eve),* encoding the TF that acts to specify the identity of a dorsally located DA1 muscle founder cell (FC) and a pericardial FC have been described in detail [5]. This led to the identification of the *eve* enhancer carrying binding sites for the effectors of Wg, Dpp and RTK signaling pathways and for tissue-specific factors Twi and Tin. All these factors are required for the initiation of *eve* expression in dorsal mesodermal cells, whereas repression by other identity TFs Ladybird (Lb) and Msh ensures restriction of *eve* expression to the DA1 muscle founder and two pericardial cells [12].

Once the differentiation of the somatic muscle compartment begins, Twi expression is reduced and maintained only in adult muscle precursors (AMPs), the muscle stem cells. AMPs

arise via asymmetric cell divisions of a subset of somatic muscle progenitor cells [13][14]. Each embryonic abdominal hemisegment features six AMPs with persistent Twi [15]. AMPs also express two repressors of myogenic differentiation, *holes-in-muscles (Him)[16]* and *zinc finger homeobox 1 (Zfh1)* [17] both activated by the Notch pathway, thus highlighting the crucial and conserved role of Notch in muscle cell stemness.

The finding that a tissue domain is determined by the coordinated action of extrinsic signaling cues and intrinsic tissue-specific selector genes (*twi*, *tin*) inspired a CodeFinder approach [18]. This approach was used to find a large number of regulatory modules regulated by the interplay of the signals described above and muscle- or heart-specific factors. The CodeFinder and a few other approaches [19][20][21][22] provided the large datasets and qualitative modeling describing the activity of genes during the determination of muscular and cardiac cell fates. Likewise, transcriptional profiling and ChIP assays have identified a broad set of genes and their regulatory motifs regulated by the key evolutionarily conserved muscle differentiation factor Mef2 [23][24].

Mef2 is required for the terminal differentiation of skeletal, visceral, and cardiac musculature [25][26][27]. By regulating transcriptional activity of a large set of genes, Mef2 directs practically all somatic muscle differentiation events, including fusion, attachment, and innervation, but also the capacity of muscle to contract [23][24]. For example, the identification of *lame duck* (*lmd), hibris (hbr)*, and *N-cadherin* (*N-cad)* as direct Mef2 targets is consistent with the involvement of Mef2 in myoblast fusion processes, which are affected in *Mef2* mutant embryos [25][26]. In the same line of evidence, the presence of Mef2-dependent cis-regulatory modules (CRMs) close to *Netrin B (NetB)*, which is involved in the attraction of motoneurons, suggests that the Mef2-dependent formation of presynaptic active zones [28] may involve NetB. Also, the established involvement in cell adhesion and muscle attachment of *inflated (if)* and *delilah (dei)* as direct Mef2 targets, supports the role of Mef2 in the essential muscle differentiation step, the attachment to tendon cells. Finally, Mef2-binding CRMs are present in the vicinity of *sallimus* (*sls), tropomyosin 1 (Tm1), 87E actin (Act87E)*, and *pod-1 coronin (pod1)* genes encoding muscle structural proteins and sarcomeric components, but also upstream of genes encoding proteins involved in ion transport, channel activity, and metabolism (e.g., *slowpoke* (*slo), inositol 1,4,5,-triphosphate receptor (Itp-r83A), Na+/H+ hydrogen exchanger 2 (NheII)*, and *mitochondrial aconitase* (*Acon*)). The regulation of this class of genes may reflect a role for Mef2 in muscle contraction and the transmission of neural stimulation [23].

The myogenic differentiation factor Mef2 triggers all aspects of the muscle differentiation program. The extrinsic inputs are necessary to subdivide progenitors into heart, visceral and somatic fields that switch expression of specific selector genes and the

downstream network of cell identity transcription factors that trigger cell fate diversification programs.

Diversification of muscle cell types

Identity code: combinatorial expression of muscle identity transcription factors

Identity genes code for transcription factors (iTFs) expressed in subsets of muscle progenitors and/or FCs, and for which loss-of-function mutations display defects in the muscle subset where they are normally expressed. Twenty iTFs have been identified so far during embryonic myogenesis (19 having known vertebrate orthologs [29]) and examinations of mutant embryos have highlighted their functions in muscle morphogenesis.

iTFs regulate, in a muscle type-specific manner, the number of fusion events, growth, guidance to attachment sites, and innervation. These mechanisms are modulated by the partial overlapping expression patterns of iTFs leading to muscle-specific combinations of transcription regulators. This combinatorial code of iTFs is thought to lead to a diversity in morphologies and properties of muscles by providing precise expression levels of "executing" genes.

Although most of the iTFs have non-regionalized expression patterns along the dorso-ventral axis, a few, such as Lateral muscles scarcer (Lms) in the lateral region (LT1-4), are restricted to subdomains [30]. Furthermore, a few iTFs are specific to only one muscle, such as Ladybird (Lb) present only in the segmental bordure muscle (SBM)[31].

Some iTFs are expressed by progenitors and their expression is maintained in sibling founders and muscles (*lb, apterous* (*ap*)), while others are differentially regulated in sibling founders leading to their loss from one of them and the corresponding muscles (*Kr, mid*; [32][33]).

Studies based on loss of function of iTFs have highlighted some common characteristics, among which is their ability to mediate cross repressive interactions responsible for the downregulation of iTFs of other muscles [34][12]. For example, members of the iroquois complex Araucan (Ara) and Caupolican (Caup) are required for the specification of LT fate [33]. LT muscles comprise four muscles and express five additional iTFs (Msh, Kr, Ap, Lms and Mid; Figure 1). *Ara/caup* are expressed in LTs but also with *slouch (slou)* in DT1 and *lb* in SBM and can act as repressors or activators depending on the level of activity of Ras/MAPK pathways. In LTs, where the Ras/MAPK pathway is inactive, they act as repressors of two other iTFs, *slou* and *vg*, preventing muscle fate conflict. These results suggest that at least in the LT muscles, not only the combinatorial code of iTFs but also the signaling inputs required for their activity will determine a specific muscle identity. Further studies will probably discover other identity genes and modulatory pathways as there are a number of muscles whose specific identity mechanisms we do not know. In addition, only a few studies have so far been conducted to identify direct target genes of iTFs, and none have yet been successfully

performed to better understand the temporal overall combinatorial integration of several iTF inputs.

The identity code is transmitted to all the nuclei in the syncytium through the process of myoblast fusion. Each FCM nucleus entering a founder cell protoplasm will undergo a progressive phase of transcriptional reprogramming to acquire the corresponding muscle identity [35][36][37]. The molecular mechanisms behind this process are still unknown but it is highly likely that future studies based on single nucleus genomic analysis will provide some answers.

Muscle type specific fusion program

Once muscle founders are specified, they undergo a complex process of fusion with a pool of FCMs present in their vicinity, which will determine the number of nuclei present in each muscle fiber. Several sequential cellular events are required to bring the two cell membranes into close proximity. These steps involve cell recognition and adhesion, actin cytoskeleton rearrangements, fusogenic protrusion, lipid mixing, and fusion pore formation, leading to the integration of the FCM content in the growing myotube [38]. The fusion process occurs between stages 12 and 15 (10-13h AEL) but the duration and rate of fusion events appear to vary between muscle types, suggesting a muscle type-specific fusion program.

Recognition of FCs and FCMs is mediated by transmembrane proteins belonging to the immunoglobulin superfamily. Dumbfounded (Duf, also called Kirre), is present on the surface of the FC, while Sticks-and-stones (Sns) is located on the FCM [39][40][41][42][43].

The duration and level of Duf/Kirre on the FC membrane along with other fusion proteins, appears to be a rate-limiting factor in myoblast fusion during embryonic myogenesis.

While *duf* is expressed in all FCs, its transcription appears to be controlled in a complex way where spatiotemporal activation / repression appears to be regulated by specific enhancers in different types of muscles [44]. This suggests that the transcriptional control of a key player in myoblast fusion may be the source of muscle diversity.

At present, only few studies have been able to find links between the iTFs code and downstream target genes controlling fusion events. It has been shown that the non-muscle type restricted expression of three genes, *Mp20*, *Pax* and *mspo* is tightly controlled by iTFs, including the iTFs Lb, Slou, and Eve, leading to a varying number of fusion events proportional to the level of their combinatorial expression [34][45] (Figure 1). *Mp20* and *Pax* encode cytoskeletal proteins modulating actin organization and promote fusion, while Mspo, an ECM component participating in cell-matrix maintenance, seems to repress it. The mechanism involving these genes is still unclear but could be linked to the stabilization of FC-FCM interaction required for the progression of the fusion process. More recently, using a muscle subset-specific translatomic approach (TRAP) [46], we identified a novel effector gene, called

gelsolin (gel), expressed under the control of iTFs Ap and Lms in LT muscles [47]. *gel* is activated at the onset of muscle fusion and participates in fusion arrest specifically in LT muscles (Figure 1). In *gel* mutant embryos, though at low frequency, LTs exhibit a greater number of nuclei leading in some cases to muscle fiber splitting. The opposite effect (increase in unfused myoblasts) is observed in the gain of gel function experiment using an FC-specific driver.

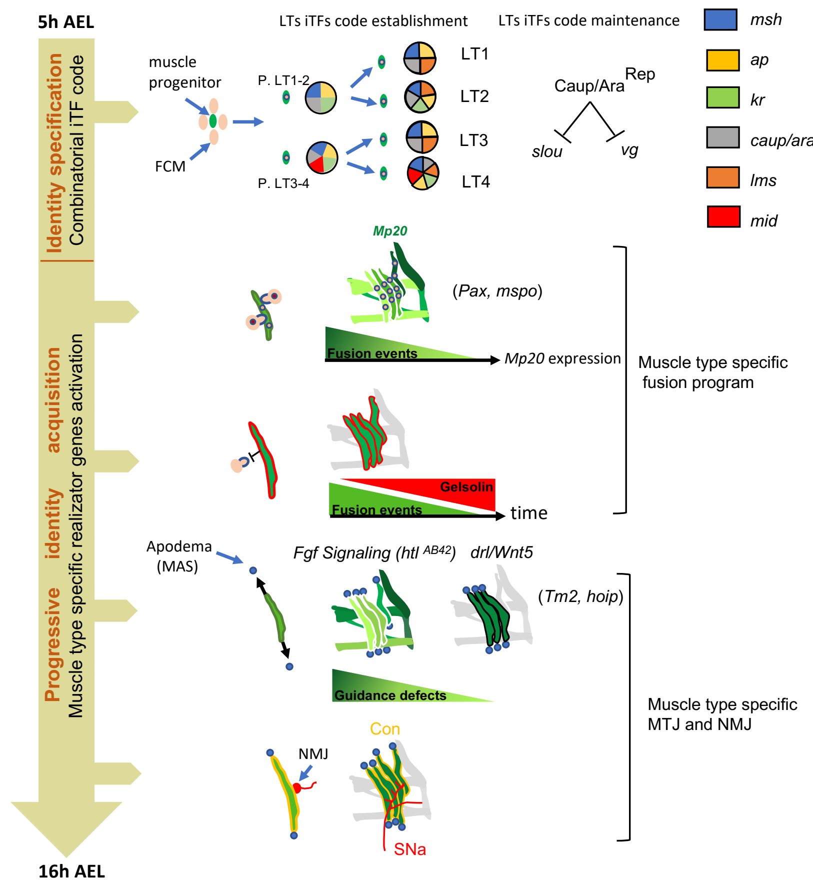

Figure 1. Scheme depicting different steps in specification and acquisition of muscle identity during the development of *Drosophila* LT muscles. The scheme encompasses a developmental time window between 5 and 16 h after egg laying (AEL). During the identity specification phase, LT muscle progenitors (P^{LT1-2} and P^{LT3-4}) segregate from the clusters of equivalent cells and then divide, giving rise to four LT founders, each of which is characterized by a distinct combinatorial expression code of six iTFs (*msh, ap, Kr, caup/ara, lms, mid*). LT iTFs (Caup/Ara repressor form) retain the identity code by repressing other iTFs genes essential for other muscle subtypes (*slou, vg*). Next, during the acquisition of specific LT muscles identities, several downstream effector genes are activated to set up LT-specific fusion programs (*Mp20, Pax, mspo, gel*) but also at later developmental stages LT-specific attachment (*FGF signaling, drl/Wnt5, hoip, Tm2*) and innervation (*Con*). FCM: Fusion Competent Myoblast, MAS: Muscle Attachment Site, NMJ: Neuro-Muscular Junction, SNa: Segmental Nerve a.

The fusion modulator gene *gel* becomes the first one identified with restricted muscle expression and is therefore one of the few known muscle identity executor genes. This protein

possesses severing activity allowing actin depolymerization, but its precise function in the fusion arrest is still elusive. Several members of this actin depolymerization/polymerization family are involved in different steps of muscle building, and partial redundancy can occur, making functional assessment difficult [48][49]. However, future studies will help gain a better understanding of the involvement of the actin cytoskeleton regulation in muscle development and disease, which is central to many processes and appears to be a major driver of muscle cell identity.

In summary, these studies highlight two main modes of regulation of the genes executing muscle identity with (i) fusion genes having broad expression in all FCs but with complex modulation of the level of expression specific to each muscle, probably mediated by the combinatorial code of iTFs, and (ii) fusion genes having a specific muscle subset expression and playing a safeguard role for the permanent arrest of fusion events.

Muscle guidance and myotendinous junction

Concomitantly with the fusion process, myotubes are directed to specific muscle attachment sites to associate with tendon cells through the process of myotube guidance. Some evidence has shown that iTFs and syncytial nuclei identity reprogramming are required for the correct achievement of this process [36].

Following a step of migration to sites of myogenesis, fusing FCs polarize and mature into nascent myotubes, which extend two leading edges in opposite directions. During this step, the extracellular environment will participate in the navigation of filopodia towards specific muscle attachment sites.

Relatively little is known about the molecular pathways that direct this extremely complex and precise process.

Studies have shown the requirement of the Slit-Robo or Kon-Grip signaling pathways, but in both cases, their disruption affects the attachment of only a subset of muscles in each segment [50][51]. More recently, muscle cell fluorescent sorting combined with RNA-seq experiments identified FGF signaling as an essential component of the myotube guidance pathway to modulate the actin cytoskeleton during muscle morphogenesis [52]. The authors showed in this study that the FGF ligands Pyramus (Pyr) and Thisbe (Ths) are necessary for myotube guidance and appear to act as paracrine signals from the ectoderm toward the developing myotube. The myotube expressing *htl* will then restrict the Rho / Rac activity and thus regulate the actin cytoskeleton. Again, in this example, a wide range of defects are observed in the *htl* mutant based on muscle identity with the LO1 or DT1 muscles showing a high frequency of guidance / attachment defects, while the LT muscle morphogenesis does not appear to be heavily dependent on the FGF pathway (Figure 1).

Among the proteins interacting with F-actin, the sarcomeric protein Tropomyosin-2 (Tm2) has been identified as playing a non-canonical function in the guidance of myotubes [53]. The *Tm2* mRNAs are targeted by the RNA binding protein Hoi-polloi (Hoip), which stimulates *Tm2* mRNA translation and myotube guidance. This is particularly the case in the LT1-3 and LL1 muscles where the overexpression of *Tm2* in *hoip* mutant embryos rescues the elongation phenotype. Overexpressing a mutant version of *Tm2* lacking the actin interaction domain was sufficient to affect elongation of myotubes in these muscles. In a similar manner as for muscle fusion, myotube guidance is also dependent on muscle type-specific gene expression. This is particularly the case in the LT muscles, where the transmembrane receptor tyrosine kinase, a Ryk family member, Derailed (Drl) regulates elongation of LT1-3 muscles to their attachment sites [54] (Figure 1). In *drl* mutant embryos myotubes bypass their appropriate attachment sites and often the new attachment sites do not express tendon cell master regulator *stripe*. Interestingly, this LT overgrowth occurs only on the ventral side, indicating that guidance mechanisms also differ at the two poles of the growing muscle. To control the guidance of the LT muscles, it is likely that the Drl receptor interacts with the secreted protein Wnt5, and together they would be needed to stop muscle extension at the correct position [55]. While *drl* is only required in the LTs muscles, *Wnt5* gain of function in either tendon cells or muscles can rescue LT muscle attachment defects observed in *Wnt5* mutant embryos.
Once the myotubes reach the tendon cells, both cell types secrete proteins from the extracellular matrix (ECM) to form the myotendinous junction (MTJ). Tendon cells deposit Laminin (Lan) and Thrombospondin (Tsp) and also secrete Slowdown (Slown), which temporarily sequesters Tsp until its partners, muscle integrins (αPS2/βPS), are present. This helps prevent immature MTJ formation [56][57][58]. The formation of integrin dimers and their binding to ECM components allows the anchoring of muscles and tendons [59][60].
Altogether, these studies highlight that muscle guidance requires the complex integration of signaling pathways, broadly expressed actin/ECM regulators, and muscle type-specific genes responsible for more subtle phenotypes. Further studies will be needed to understand this process mechanistically and how it is specifically driven in each muscle. For example, synchronized analysis at single cell/nuclei resolution in myotube and tendon cells should provide insight into the activation of specific gene expression in these tissues and into the downstream impact of the activation of signaling pathways.

Muscle-specific innervation by motoneurons

Once the muscles are attached to the tendon cells, they will be innervated to respond to central nervous system signals. How the motoneurons find and recognize their correct muscles has been a model system for studies over the past decades in many organisms, but our knowledge is still relatively limited. Motoneurons, like muscle founders, express a

combinatorial code of transcription factors providing the information to make stereotyped pathway choices as they extend toward the regions where their appropriate target muscles arise. Motoneurons extend growth cones out toward their target muscles in six major peripheral nerve branches: the intersegmental nerves (ISN, ISNb and ISNd) two branches of the segmental nerve (SNa, c) and the transversal nerve (TN) [61][62]. Dorsal muscles are innervated via the ISN, while lateral and ventral muscles are innervated via the ISNb and d and the SNc. In this process, homophilic cell adhesion molecules (Capricious (Caps), Fasciclin-3 (Fas3) or Connectin (Con)) present in both the muscle and their motoneurons seem to play important roles [63][64][65]. One of the best examples is the ability of Con, a leucin-rich repeat protein, to participate in the recognition of LT muscles by the motoneuron growth cones of the SNa (Figure 1). However, muscle innervation is also dependent on the attractive/repulsive signaling such as NetB in the dorsal muscles respectively through Frazzled or UNC-5, which are differentially expressed in motor neurons.

In a recent study, an unexpected role of adult muscle precursors (AMPs), *Drosophila* muscle stem cells, was discovered in guiding motor neurons to their muscle target [66]. AMPs not only have the ability to attract the navigating ISN and SNa nerve branches but are also necessary for the innervation of muscles in the lateral domain. Lateral AMPs, which continue to express the iTFs of the progenitor from which they derive, express an immunoglobulin superfamily coding gene *sidestep* in a precise time window and could attract the SNa expressing *beat-1a*. This interaction between muscle stem cells and innervation would be of interest to explore in the context of muscle regeneration where satellite cells are mobilized after injury, together with the process leading to the re-innervation of muscle fibers.

Perspectives

Several new research paths need to be taken or further pursued to gain more knowledge of the mechanisms of embryonic muscle diversification. One of them calls on the study of metabolism regulation in different muscle types and particularly its impact on gene expression. Zinc ion plays essential roles in protein stability and function for Zn finger transcription factors, proteins bearing RING fingers and LIM domains, and zinc regulatory proteins such as metallothionein and matrix metalloproteinases [67]. In addition, during embryonic myogenesis, the zinc transporter Foi is necessary for the normal development of somatic and visceral muscles [68]. In Foi mutant embryos, iTFs expression is maintained, showing a normal segregation of FCs, but the muscle pattern is strongly affected. Interestingly, some muscles are absent or strongly morphologically affected (LTs), while others are practically normal (SBM). These results suggest a specific muscular need for zinc ions for the formation of muscle fibers and demonstrate a new modulating role in muscle diversification awaiting further exploration.

Another interesting area of investigation lies in the study of muscle type-specific alternative splicing and post-transcriptional regulation. So far, most studies have focused on transcriptional regulation or on the functional analysis at the level of a gene. However, in adult muscle fibers, one of the most important drivers of diversity between fibrillar and tubular fibers is the sarcomeric protein isoform content [69][70]. During embryonic myogenesis, the impact of these processes is largely unknown. Comparison of nascent transcription with ribosomal footprint or TRAP-seq experiments will certainly fill this gap in the near future and identify muscle type-specific RNA binding proteins together with isoforms of transcripts present in a muscle cell at a given time. Finally, a growing field in the regulation of cell fate choices links RNA modifications, called the epitranscriptome, to the regulation of several molecular mechanisms, including transcription, splicing, RNA export, mRNA stability or translation [71][72][73][74][75][76][77]. Identifying the impact of these modifications on muscle diversification and their potential involvement in muscle disease presents a completely new challenge.

From a larger perspective, an important question is to which extent the molecular and cellular mechanisms of *Drosophila* muscle diversification could help in the understanding how muscle diversity in vertebrates is acquired. We anticipate that a global view on transcriptional signatures underlying specification of different muscle types in vertebrates will arise from the single cell RNA sequencing analyses of the developing somites [78], the source of skeletal muscle progenitor cells. However, few studies already suggest that the orthologs of *Drosophila* iTFs play similar muscle identity functions during vertebrate myogenesis. For example, the Mid ortholog Tbx1 is critical for pharyngeal arches-derived muscles development [79] whereas Pitx2, a counterpart of the *Drosophila* iTF Ptx1 specifies extra-ocular muscle founder cells [79][80]. Also differential requirement for two Myogenic Regulatory Factors (MRFs) Myf5 and MyoD underlies specification of cranial and limb muscle fiber populations in zebrafish [81] in a way similar to iTFs-based specification of muscle types in *Drosophila*. Moreover, the myoblast fusion, which plays a key role in setting muscle diversity, displays several similarities between fruit fly and vertebrates. In *Drosophila* it involves cellular asymmetry with “receiving” founder cells expressing membrane-associated molecule Dumbfounded (Duf) and “attacking” fusion competent myoblasts expressing Stick and Stones (Sns) that interacts with Duf [38] . The view that a similar asymmetry, although with different molecular actors (Myomaker and Myomixer), exists during myoblast fusion in vertebrates is supported by the molecular asymmetry of myoblast fusion observed recently in the regenerating *mdx* muscles [82] but also by the formation of finger-like actin-based protrusions at the fusion sites in mouse muscle [83].

Thus, we foresee that *Drosophila* model will continue to inform about the logic of complex processes driving diversification of muscle types.

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