Acting on identity: myoblast fusion and the formation of the syncytial muscle fiber

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Abstract

The study of *Drosophila* muscle development dates back to the middle of the last century. Since that time, *Drosophila* has proved to be an ideal system for studying muscle development, differentiation, function, and disease. As in humans, *Drosophila* muscle forms via a series of conserved steps, starting with muscle specification, myoblast fusion, attachment to tendon cells, interactions with motorneurons, and sarcomere and myofibril formation. The genes and mechanisms required for these processes share striking similarities to those found in humans. The highly tractable genetic system and imaging approaches available in *Drosophila* allow for an efficient interrogation of muscle biology and for application of what we learn to other systems. In this article, we review our current understanding of muscle development in *Drosophila*, with a focus on myoblast fusion, the process responsible for the generation of syncytial muscle cells. We also compare and contrast those genes required for fusion in *Drosophila* and vertebrates.

Keywords

Drosophila; muscle development; membrane fusion; actin cytoskeleton

Introduction

From *Drosophila* to human, skeletal muscles develop and mature through similar steps: cells of the mesoderm are first specified to be skeletal muscle cells, which then undergo cell-cell fusion to add mass to these cells. After fusion is completed, sarcomeres assemble to provide contractile force, and the muscle cell builds connections with its surrounding tissues, such as tendons and motor neurons. Many of the genes that are critical for these steps are conserved across species. This also holds true for *nautilus*, the fly ortholog of vertebrate MYOD, which is a key regulator of myogenic differentiation in *Drosophila* (Michelson et al., 1990; reviewed by (Abmayr and Keller, 1998)). As discussed below, Nautilus interacts with other

transcription and chromatin regulators, and determines muscle identity, morphology, and size (reviewed in (Dobi et al., 2015)).

During *Drosophila*'s life cycle, two periods of myoblast fusion occur to form the larval muscle system and adult muscle system, respectively (Figure 1). The larval body wall muscles form during the embryonic stage. These muscles have been intensively studied as they represent muscle at its simplest: a single muscle fiber forms a single muscle. The adult muscle system forms during the pupal stage of development. In contrast to the larva, each single adult muscle is composed of multiple muscle fibers and thus more closely resembles the muscle organization found in vertebrate skeletal muscle (reviewed by Dobi et al., 2015, Taylor, 2006). This pertains in particular to the indirect flight muscles, the largest and best-studied muscle set of the adult fly. In this review, we first give a brief overview of muscle specification (Figure 2) and then focus on a discussion of the current studies in myoblast fusion both in *Drosophila* (Figure 3, Table 1) and vertebrates (Table 2).

Preparing for fusion: specification of muscle cells

Muscle Specification in the Drosophila embryo forms the larval muscles

The body wall or skeletal muscles are derived from somatic (*Drosophila*) or paraxial (mammals) mesoderm, respectively. In *Drosophila*, the allocation of somatic mesoderm depends on the crosstalk between the mesoderm and the ectoderm (Azpiazu et al., 1996; Frasch, 1995; Staehling-Hampton et al., 1994), as well as the combination of transcription factors that are expressed in the mesoderm (reviewed in Baylies et al., 1998; Dobi et al., 2015). After the allocation of the somatic mesoderm, the bHLH protein Lethal of Scute (L'sc) is expressed in clusters of promuscle cells (Figure 2A), under the control of the Receptor Tyrosine Kinase (RTK) pathway (Carmena et al., 1995). In each cluster, a combination of the Notch and RTK-Ras signaling pathway activities restricts the expression of L'sc to one cell—the muscle progenitor cell (Figure 2B, C) (Carmena et al., 1995; 1998b; Fuerstenberg and Giniger, 1998). Each progenitor cell then undergoes an asymmetric division to form either two founder cells (FC) or a single FC and an adult muscle progenitor (AMP) (Figure 2D) (Carmena et al., 1998a; Ruiz Gomez and Bate, 1997). The cytoplasmic membrane-associated protein Numb plays a critical role in determining muscle fate. Numb antagonizes Notch-mediated lateral inhibition and allows the expression of muscle identity proteins in the muscle progenitor cells (Ruiz Gomez and Bate, 1997). Different combinations of identity transcription factors expressed in each muscle progenitor, such as Slouch, Eve, Krüppel, Apterous, and the MyoD ortholog, Nautilus, in conjunction with chromatin regulators such as Sin3a (Dobi et al., 2014) determine the different cell identities of each FC, and hence, the final muscle pattern (Figure 2E, Dobi et al., 2015). After L'sc is restricted to the progenitor cell, the remaining cells in the promuscle cell cluster adopt a fusion competent myoblast (FCM) fate. The specification of FCMs is controlled by Notch-mediated lateral inhibition as well as by the transcription factors Lame duck and Tramtrack (Boyle and Berg, 2009).

Completion of FC and FCM specification allows for fusion between the two cell types, generating syncytial muscle cells (Figures 2E and 3). Throughout the period of fusion, the transcriptional profiles in both FCs and FCMs are dynamically regulated (Bataillé et al.,

2017; Bourgouin et al., 1992; Dohrmann et al., 1990; Jagla et al., 1998). With the completion of each round of fusion, the fused FCM nuclei undergo progressive reprogramming, such that FCM-specific genes are suppressed, and the transcriptional pattern of the FC nucleus is adopted. The nucleus from the FC retains the expression of identity transcription factors after fusion. Additional experimental evidence suggests that the nucleus from the FC remains transcriptionally different from other nuclei in the same muscle fiber (Bataillé et al., 2017). Fusion events between FCs and FCMs are completed at late stage 15 (~13.5h AEL) (Beckett and Baylies, 2007; Richardson et al., 2008). Myotubes derived from myoblast fusion differentiate into fully striated, functional larval muscles (Figure 1A, C). These muscles grow without additional fusion events throughout the larval stage.

Muscle specification in the Drosophila pupa forms the adult muscles

Compared to the larval muscle, less is known about the specification process of adult *Drosophila* muscles. The prominent muscle groups in the adult *Drosophila* include the Direct Flight Muscles (DFM), the Indirect Flight Muscles (IFM), the jump muscles, the abdominal muscles, and the leg muscles. Based on morphology and gene expression, these adult somatic muscles can be divided into two types: tubular and fibrillar muscles. The *Drosophila* abdominal, jump, leg and direct flight muscles are tubular muscles with aligned myofibrils that rely on glycolytic metabolism, while the Indirect Flight Muscles (IFMs) are fibrillar muscles with distinct, unaligned myofibrils (Figure 1D) and are primarily oxidative (Peckham et al., 1990). The IFMs are composed of 7 pairs of dorsal-ventral muscles (DVMs) and 6 pairs of dorsal longitudinal muscles (DLMs) (Figure 1B). DVMs and DLMs have different orientation and attachment sites, and are generated from fusion between myoblasts and templates with different origins (reviewed in Dobi et al., 2015). The specification of DFMs requires the homeodomain transcription factors Cut and Apterous (Ghazi et al., 2000). The specification of IFMs depends on the transcriptional regulators Vestigial (Sudarsan et al., 2001) and Scalloped (Deng et al., 2009). In addition, Spalt major in the IFMs directs the fibrillar muscle fate (Schönbauer et al., 2011). Readers are directed to reviews on the specification of adult muscle for more detail (Dobi et al., 2015; Maqbool et al., 2006; Roy and Vijayraghavan, 1999).

Muscle Specification in vertebrates

Similar to *Drosophila*, the specification of muscle progenitor cells in vertebrates also requires a combination of signals from adjacent tissues (reviewed in Munsterberg et al., 1995; Bryson-Richardson and Currie, 2008). Extracellular signals induce the expression of Paired Box (Pax) 3 and Pax7 in the somites (Duprez et al., 1998). Pax genes have dual roles: they enhance the proliferation of muscle precursor cells (Relaix et al., 2005), and they activate the expression of MyoD and Myf5, two members of the myogenic regulatory factor (MRF) family (Maroto et al., 1997) (Sato et al., 2010), reviewed in (Bryson-Richardson and Currie, 2008). MRFs are a family of four transcription factors: MyoD, Myf5, myogenin, and MRF4. MRFs activate the expression of genes required for muscle differentiation. MyoD and Myf5 are the first MRFs expressed in muscles and function redundantly to trigger myoblast specification (Rudnicki et al., 1993). Myogenin functions after muscle progenitor formation and regulates terminal differentiation of committed myoblasts (Hasty et al., 1993). Similar to Myogenin, MRF4 also regulates terminal differentiation of myoblasts and

functions upstream of MyoD to direct the specification of myogenic cells (Kassar-Duchossoy et al., 2004). In addition to MRFs, other transcription factors are required for muscle differentiation, such as the Myocyte enhancer factor 2 (Mef2), which plays a conserved role in muscle cell differentiation from *Drosophila* to human (Dodou et al., 2003; Lilly et al., 1994; Molkentin et al., 1995). After the specification of myogenic cells, two waves of myogenesis take place to form the final vertebrate muscle pattern. The first wave generates primary myofibers. The primary fibers function similarly to FCs in *Drosophila*, and determine the shape and identity of muscles (slow or fast). The second wave generates secondary myofibers that align alongside the primary myofibers and adds mass to the muscles (Harris et al., 1989). The formation of both types of myofibers requires fusion of myoblasts. *In vitro* experiments suggest that the fusion process occurs in two periods: the first involves the fusion of individual myoblasts to form the nascent myotubes; the second period involves fusion between the myotube and additional myoblasts (reviewed in Rochlin et al., 2010).

Making Larval muscle: Myoblast Fusion in the *Drosophila* Embryo

Recognition and Adhesion

Myoblast fusion begins with cell-cell recognition and adhesion (Figure 3A, Table 1). In *Drosophila*, recognition and adhesion between the FC and FCMs are mediated by cell-specific immunoglobulin super family (IgSF) members. The IgSF members expressed in the FC are Dumbfounded (Duf) and Roughest (Rst) (Ruiz Gomez et al., 2000). Duf and Rst are transmembrane proteins that function redundantly to mediate recognition and adhesion (Strünkelnberg et al., 2001). In the FCMs, Sticks and Stones (Sns) and Hibris (Hbs) are the counterparts of Duf and Rst (Artero et al., 2001; Bour et al., 2000). Hbs can partially compensate for Sns function, but its activity is less efficient than Sns (Shelton et al., 2009). Expression of Rst is also detected in a subset of FCMs; however, its function in the FCM is unclear (Strünkelnberg et al., 2001). The interaction between Duf-Sns and Rst-Hbs is required for cell recognition and adhesion. Ectopic expression of Duf induces myoblast aggregation and adhesion, but is insufficient to trigger fusion (Ruiz Gomez et al., 2000). The interaction between Duf/Rst-Sns/Hbs is also required in other developmental events such axon guidance and eye development (Bao et al., 2010; Ramos et al., 1993), and this "cassette" of transmembrane proteins is referred to as an "adhesion module" in these contexts. If the FC/myotube and FCMs are not in direct contact before adhesion, cell migration is required to bring the two types of cells into close proximity (Richardson et al., 2007; Rochlin et al., 2010). An intriguing recent development in the understanding of fusion-related adhesion is the identification of N-cadherin as a contributor to myoblast fusion in the *Drosophila* embryo. N-cadherin is a member of the cadherin family of adhesion proteins. N-cadherin is present, but not restricted at the fusion site (Dottermusch-Heidel et al., 2012). One possible function of N-cadherin is to mediate cell adhesion through the formation of cellular junction-like structures at the fusion site (Hamp et al., 2016). For membrane fusion to proceed, however, N-cadherin needs to be removed from the fusion site (Hamp et al., 2016). Kette and Schizo/Loner, upstream regulators of Arp2/3-mediated actin rearrangements (see Actin rearrangement section below), are reported to facilitate removal of N-cadherin (Hamp et al., 2016, Dottermusch-Heidel et al., 2012, Onel et al., 2014).

Notably, N-cadherin loss of function does not result in fusion defects in the *Drosophila* embryo (Charlton et al., 1997; Dottermusch-Heidel et al., 2012), suggesting potential redundant functions with other members of the cadherin family.

Actin rearrangements at the fusion site

After the cells adhere to one another, signals from these transmembrane recognition and adhesion molecules cause the actin cytoskeleton in both the FC and FCM to undergo remodeling, resulting in a filamentous-actin (F-actin)-based focus structure in the FCM and a thin sheath of F-actin on the opposing inner membrane in the FC (Figure 2B). The actin focus in the FCM mediates the formation of invasive finger-like membrane protrusions (Sens et al., 2010). This actin structure in the FCM is thought to provide the necessary force to push the lipid bilayers of the cells into close proximity. In response to the mechanical forces generated by the invasive protrusions from the FCM, non-muscle Myosin II (MyoII) is recruited to the fusion site in the FC/myotube. The accumulation of MyoII generates cortical tension, facilitates cell membrane juxtaposition, and promotes fusion pore formation (see below) (Kim et al., 2015).

A major regulator of the actin focus during myoblast fusion is Arp2/3. Arp2/3 is a multi-subunit protein complex that binds to actin filaments and polymerizes new actin filaments at a 70-degree angle from the pre-existing actin filament. Arp2/3 is activated by the nucleation-promoting factors (NPFs), Wiskott-Aldrich Syndrome protein (WASp) and SCAR (also known as WAVE). Signal transduction from the membrane to these actin cytoskeleton regulators is partially mediated by the activity of Crk, as well as Dreadlock (Dock), the *Drosophila* homolog of Nck. Both Dock and Crk are adapter proteins with *src* homology 2 (SH2)/SH3 domains. Dock can interact with all four IgSF transmembrane proteins that have SH2 or SH3 domains, but it only genetically interacts with Duf, Sns, and Hbs (Kaipa et al., 2013). In addition, rescue experiments using a series of *sns* deletions and site-directed mutations suggest that Sns functions through interacting with a complex array of proteins (Kocherlakota et al., 2008). In the FCMs, the Dock SH3 domain biochemically and genetically interacts with WASp, as well as the WASp regulator, Verprolin 1 (Vrp1, also known as Solitary (Sltr) and D-Wip) (Kaipa et al., 2013). In both the FC and FCMs, Dock biochemically and genetically interacts with the Arp2/3 NPF SCAR. Through a similar mechanism as Dock, Crk biochemically interacts with Sns. In a FCM, Crk physically interacts with the WASp regulatory proteins Vrp1 and Blow (Kim et al., 2007). Thus, the adaptor proteins Dock and Crk function as links that relay signals from the membrane to the actin cytoskeleton.

In the FC, another adaptor protein that links activity at the membrane to the actin cytoskeleton is Rolling pebbles (Rols, also known as Antisocial (Ants)/Rols). Rols is recruited to the fusion site by Duf. In a positive feedback loop, Rols replenishes Duf at the membrane, and thus enables additional rounds of fusion (Menon et al., 2005). Rols interacts with two proteins that regulate the actin cytoskeleton organization: Myoblast city (Mbc) (Chen and Olson, 2001; Rau et al., 2001) and D-titin (Menon and Chia, 2001). Mbc is the *Drosophila* homolog of the mammalian protein DOCK1, which mediates actin rearrangements by acting as a guanine nucleotide exchange factor (GEF) for the small

GTPase Rac. However, recent data suggest that the downstream target of Rols is not Mbc: rescue experiments suggest that the activity of Mbc is not required in the FC for the initial fusion event (Haralalka et al., 2011). Another actin cytoskeletal protein that can bind to Rols is D-titin (Menon and Chia, 2001). D-titin is recruited to the fusion site by Rols and mediates myoblast fusion in a mechanism that remains unclear. D-titin is also involved in maintaining myotube morphology (Menon and Chia, 2001; Zhang et al., 2000).

While Mbc appears not to be required by the FCs initially, Schizo/Loner, another GEF that regulates actin rearrangements, is required in FCs to mediate fusion. Schizo/Loner is recruited to the fusion site by Duf and Rst (Bulchand et al., 2010). Domain analysis suggests that the function of Schizo/Loner is to recruit and activate the small GTPase dARF6 at the fusion site. Schizo/Loner and dARF6 control the membrane localization of Rac (Chen et al., 2003). However, *arf6* maternal/zygotic null mutants do not have a myoblast fusion defect (Dyer et al., 2007), and the fusion phenotype in a dominant negative *dARF6* background is not as severe as that in the *schizo/loner* mutant. Together these data suggest that the downstream target of Schizo/Loner is another GTPase or that there is redundancy with other dARF family members such as dARF1 (Chen et al., 2003).

Once activated, the small GTPase Rac regulates the function of SCAR and is critically important in both FCs and FCMs to mediate fusion. The activity of SCAR is controlled by the pentameric WRC complex (the WAVE/SCAR regulatory complex). The WRC is composed of Kette/Nap1, Sra1, Abi, Hspc300, and SCAR itself (Eden et al., 2002). Rac activity is essential for the localization of the WRC to the fusion site, as well as activation of SCAR via the release of the SCAR VCA domain from an inhibited state (Chen et al., 2010; Gildor et al., 2009). As part of the WRC, Kette is also essential for myoblast fusion as it controls the localization and stability of SCAR (Richardson et al., 2007). It has not been reported whether loss of function in the other WRC components, Sra1, Abi and Hspc300, lead to a fusion phenotype. This may be due to a lack of appropriate mutant alleles, as well as a maternal supply of protein/mRNA in the embryo that masks the role of these proteins during the time period in which fusion takes place. Nevertheless, overexpression of WRC components in muscle cells results in fusion block and suggests a potential role of other WRC components during fusion (Deng et al., 2016).

In the FC, SCAR activates its downstream target Arp2/3, and rearranges the actin cytoskeleton into a thin sheath along the fusion site (Sens et al., 2010). In the FCM, SCAR activity is also regulated by Rac. However, Rac localization and activation is regulated through Mbc (Haralalka et al., 2011). Mbc is recruited to the fusion site by the phospholipid PI(4,5)P2 (PIP2). When PIP2 activity is sequestered via overexpression of the pleckstrin homology domain of phospholipase C-gamma (PH$^{plc\gamma}$), Mbc is concentrated in the cytoplasm instead of at the fusion site, leading to mislocalization of active Rac (Bothe et al., 2014). Mbc interacts with ELMO using its SH3 domain and forms the Mbc/ELMO complex. The GEF activity of Mbc/ELMO is tightly regulated during myoblast fusion, and it controls the level of active Rac. Both overexpression and loss of function of Mbc/ELMO cause defects in myoblast fusion, which are reminiscent of constitutively active Rac and Rac mutants, respectively (Geisbrecht et al., 2008). When activated by Mbc/ELMO, Rac, in turn, binds to the WRC and regulates Arp2/3 activity at the fusion site.

In addition to SCAR, the other NPF, WASp, also regulates Arp2/3 at the fusion site. Unlike SCAR, which functions both in FCs and FCMs, WASp activity is only required in the FCM (Jin et al., 2011). The activity of WASp is regulated by Blown Fuse (Blow) and Vrp1. The WASp-homology-1 (WH1) domain in WASp can bind to the WASp-binding domain (WBD) in Vrp1 (Ramesh and Geha, 2009). The interaction between WASp and Vrp1 is required to stabilize and localize WASp at the fusion site (Jin et al., 2011). This interaction is disrupted when Blow competes with WASp for Vrp1 WBD binding (Jin et al., 2011). Thus, after cell recognition and adhesion, a signal is transduced from the membrane via the adaptor protein Crk, which recruits Blow and Vrp1 to the fusion site. At the fusion site, the WASp-Vrp1 complex promotes actin polymerization by regulating Arp2/3 activity. Blow suppresses WASp activity by competing with WASp for Vrp1 binding and by dissociating the WASp-Vrp1 complex. Since the binding affinity between Blow-Vrp1 is lower than WASp-Vrp1, dissociated WASp interacts with Vrp1 again and promotes another round of branched actin polymerization (Jin et al., 2011). It has been reported that in FCs, the function of WASp and SCAR is redundant, as overexpression of WASp in the FCs can partially restore fusion in *kette* mutant background (Hamp et al., 2016). FCM-specific reduction of the WASp level in a *kette* mutant background rescues fusion defects. It has been proposed that a particular ratio of active WASp and SCAR needs to be maintained for fusion to occur (Hamp et al., 2016).

Recent studies have indicated that in addition to the branched actin network generated by Arp2/3, the linear actin filament nucleated by Diaphanous (Dia) also plays a role during myoblast fusion (Deng et al., 2015; Haralalka et al., 2014). Dia is a member of the formin family of actin polymerization proteins. Dia is enriched at the actin focus during the formation of *Drosophila* somatic musculature and dorsal pharyngeal musculature. Dia loss of function blocks myoblast fusion (Deng et al., 2015). Interestingly, overexpression of active Dia also leads to fusion defects (Deng et al., 2015; Haralalka et al., 2014), suggesting that the activity of Dia is highly regulated. Notably, in addition to Dia, the WASp family member WHAMY can also promote the assembly of linear actin filament. It has been reported that WHAMY synergizes with WASp during myoblast fusion (Brinkmann et al., 2016). Altogether, these data suggest that a balance between linear and branched actin filament is required at the fusion site for fusion to occur.

Fusion pore formation

A critical step in cell fusion is the formation of fusion pores that allow for cytoplasmic continuity between the fusing cells (Figure 3C). Studies from virus-cell fusion and *C. elegans* epithelial cell fusion have identified fusogens that mediate fusion pore formation (Mohler et al., 2002; reviewed in Sapir et al., 2008). A fusogen is a transmembrane protein that localizes to the fusion site and its presence is essential and sufficient to trigger membrane fusion (Sapir et al., 2008). Despite the identification of many gene products that are required for myoblast fusion in both *Drosophila* and vertebrates, no fusogen has been identified in these systems to date. Therefore, the mechanism that brings the membranes in close proximity to allow pore formation during myoblast membrane fusion remains a critical, open question.

It has been proposed that the actin cytoskeleton plays a crucial role in forcing the juxtaposition of the FC/FCM cell membranes, leading to the formation and expansion of fusion pores (reviewed by (Onel and Renkawitz-Pohl, 2009; Schejter, 2016; Schulman et al., 2015). During *Drosophila* embryonic myoblast fusion, the F-actin focus in the FCM forms podosome-like structures at the fusion site, which provide an invasive force on the apposing FC and pushes the cell membranes of the FCM and FC into closer proximity. The F-actin focus later evolves into a single-channel fusion pore on the membrane of the fusing cells (Sens et al., 2010). Studies aimed at investigating the function of actin regulators during pore formation have been performed. In these studies, the exchange of fluorescent proteins between the FCM and the FC were measured as a reporter of cytoplasmic continuity in various fusion mutant backgrounds. From these experiments, it has been proposed that SCAR-regulated actin remodeling is required for the initiation of fusion pore formation (Gildor et al., 2009). The function of WASp and its regulators is required for the formation of invasive structures (Jin et al., 2011; Kim et al., 2007; Sens et al., 2010). Therefore it is believed that WASp-regulated actin remodeling is required for pore formation (Jin et al., 2011; Kim et al., 2007; Sens et al., 2010) and expansion (Gildor et al., 2009). Notably, the actin focus resolves prior to fusion, suggesting that actin and other proteins located at the fusion site need to be removed, allowing fusion to occur. Thus far, the removal of proteins associated with the actin cytoskeleton at the fusion site has not been well studied. One potential protein involved in this process is Schizo/Loner, which has been reported to remove N-cadherin from the fusion site to permit fusion (Dottermusch-Heidel et al., 2012). In addition, the transmembrane protein *singles bar* (*sing*) is required during myoblast fusion. *sing* mutant embryos display a cell fusion block with accumulation of vesicles at the fusion site, suggesting a potential role of Sing in vesicle trafficking during fusion (Estrada et al., 2007).

Other studies suggest alternative models of fusion pore formation (Dhanyasi et al., 2015; Doberstein et al., 1997; Onel and Renkawitz-Pohl, 2009). Electron microscopy images have shown the presence of prefusion complexes at the fusion site. These prefusion complexes contain electron-dense, paired vesicles at the apposed plasma membranes. It has been suggested that these paired vesicles resolve into membrane plaques. Subsequently, the juxtaposed plasma membranes vesiculate and form sacs that enclose the extracellular space. Multiple fusion pores are observed in these situations (Doberstein et al., 1997). Similar membrane structures and multiple fusion pores at the fusion site have also been reported during myoblast fusion which leads to the formation of the *Drosophila* adult flight muscle (Dhanyasi et al., 2015). These data emphasize the requirement for the delivery and the clearing of vesicles at the fusion site as critical for membrane breakdown (Doberstein et al., 1997). These data also suggest that instead of one fusion pore, multiple pores are formed to facilitate fusion. It is still unclear whether these differences of fusion pore number arise from different fixation methods, context or other variables (reviewed in (Schejter, 2016)).

Making Adult Muscle: Myoblast Fusion in the *Drosophila* Pupa

The *Drosophila* adult muscles form during metamorphosis in the pupal stages. During the first few hours of metamorphosis, most of the *Drosophila* larval body wall muscles are destroyed through histolysis, but a few muscles, such as the Dorsal Oblique (DO1-3)

muscles, escape degradation and serve as templates for some adult muscles, particularly the Dorsal Longitudinal Flight muscles (Fernandes et al., 1991). The growth of adult muscles is achieved through hundreds of cell-cell fusion events, in which a pool of myoblasts fuse with either template myotubes or muscle precursor cells (Fernandes et al., 1991).

The pathways that regulate myoblast fusion to form the *Drosophila* adult muscles are not fully understood. Based on our current knowledge, the mechanisms that regulate myoblast fusion during adult myogenesis are similar to those that regulate embryonic myogenesis. Fusion occurs between the adult muscle progenitors (AMPs) and myoblasts that migrate from the imaginal discs to form the adult abdominal muscles, the leg muscles, and the DVMs. The DLMs are formed through fusion between myoblasts and the muscle templates that are preserved during histolysis (Fernandes et al., 1991). The AMPs associated with the wing imaginal disc go through two phases of amplification during adult muscle development. The initial phase is a symmetrical division which is regulated by Notch signaling and leads to increased AMP numbers. The second phase is an asymmetrical division that is regulated by Notch and Wnt signaling and results in AMP self-renewal and the generation of myoblast progeny required for the later fusion events (Gunage et al., 2014). It has been proposed that during muscle injury, the AMPs could function similarly to satellite cells, whose progeny fuse with myotubes to repair muscles (Gunage et al., 2014). A recent study has shown that dIlp6 signals from a muscle niche can activate dormant AMPs through the Insulin-Notch-dMyc cascade (Aradhya et al., 2015).

Similar to embryonic myoblast fusion, the transmembrane proteins Duf, Rst, Sns, and Hbs are required to mediate cell recognition and adhesion (Gildor et al., 2012). After adhesion, the fusing myoblast flattens on the myotube, such that the cell membranes are in tight apposition (Dhanyasi et al., 2015). Actin accumulates primarily at the myoblast side of the fusion site, after receiving signals from the cell membrane, and forms a focus-like structure. As with embryonic myogenesis, actin rearrangements during adult myogenesis require the function of Arp2/3, as well as the actin nucleation-promoting factors (NPFs) WASp and SCAR. Loss of function studies revealed that WASp is required for fusion pore initiation. (Mukherjee et al., 2011). Similar to embryonic myoblast fusion, the MARVEL domain protein Sing is expressed in adult muscles, and its activity is required for adult myoblast fusion (Brunetti et al., 2015).

Despite these similarities, however, there are differences between *Drosophila* embryonic and adult myogenesis. In embryos, FCMs and FCs are positioned relatively close to one another prior to fusion. The FCMs in contact with FCs/myotubes are responsible for the early fusion events. The FCMs that are located more internally migrate prior to making contact and fusing with the FCs/myotubes (Beckett and Baylies, 2007; Richardson et al., 2008). During adult myogenesis, myoblasts often need to migrate long distances from the imaginal discs to the muscle template/myotubes. More importantly, during migration, these myoblasts maintain a semi-differentiated state via the Notch signaling pathway. Each myoblast expresses the Notch ligand Delta and represses the differentiation of its neighboring cells. Notch signaling decays when myoblasts reach the vicinity of the myotubes, and this allows for the terminal differentiation of the myoblasts and the expression of FCM markers such as Sns (Gildor et al., 2012).

During adult muscle formation, the adhesion between myoblasts and myotubes also requires filopodia that emanate from the myotube surface. The filopodia are actin-based structures that bring the myoblasts and myotubes into close proximity. The formation and maintenance of filopodia requires the activity of Enabled and IRSp53 (Segal et al., 2016). The requirement for these two proteins during embryonic myoblast fusion has not been reported. In addition, reducing WASp and SCAR activity simultaneously in embryos abolishes actin focus formation (Bothe et al., 2014). However, double knockdown of WASp and SCAR during adult muscle formation results in enlarged actin foci, suggesting novel roles of the NPFs during *Drosophila* adult myoblast fusion (Mukherjee et al., 2011).

Making muscle in Vertebrates: Myoblast Fusion

Compared to *Drosophila*, the vertebrate muscle is a more complex system, both in terms of its structure and the regulatory mechanisms required during development. Over the past few decades, researchers have identified numerous genes that are required for myoblast fusion in vertebrates (reviewed in Rochlin et al., 2010; Demonbreun et al., 2015). Nevertheless, how these different proteins coordinate fusion remains unclear. The morphological steps that are required for myoblast fusion and many of the genes necessary for the process are conserved between *Drosophila* and vertebrates. Therefore, we review recent updates in vertebrate fusion-related genes based on their localization and which steps they are involved in during fusion (Table 2).

Similar to the process of *Drosophila* myoblast fusion, the myotube-myoblast fusion during muscle development in vertebrates is also asymmetric and requires recognition molecules located on the cell membrane. One pair of membrane proteins required for fusion is Integrin vLA-4 and its receptor VCAM-1. Integrin vLA-4 is expressed in myotubes and VCAM-1 in myoblasts. Interactions between vLA-4 and VCAM-1 are required for the alignment of the secondary myofiber with the primary myofiber, as well as for the second phase of muscle cell fusion (Rosen et al., 1992). In addition to vLA-4 and VCAM-1, the transcription factor NFAT2C also mediates cell recognition by regulating the expression of IL-4. IL-4 is a cytokine that functions as a secreted myoblast recruitment factor (Horsley et al., 2003). Another pair of membrane proteins required for fusion is the immunoglobin superfamily proteins Jamb and Jamc. In zebrafish, Jamb and Jamc are expressed in fast-muscle myoblasts. The physical interaction between Jamb and Jamc is required for myoblast fusion during the formation of fast-twitch muscles. Loss of function of either protein severely impairs myoblast fusion (Powell and Wright, 2011; Sedwick, 2011). In addition, it has been shown that Nephrin, an ortholog of the *Drosophila* transmembrane protein Sns, localizes on myoblasts and mediates cell recognition (Sohn et al., 2009). In zebrafish, Kirrel, an ortholog of Nephrin, is localized to membranes of myoblasts and is required for fusion (Srinivas et al., 2007). After recognition, adhesion molecules such as M-cadherin (Hollnagel et al., 2002), N-cadherin (Radice et al., 1997), and integrin family members (Schwander et al., 2003) mediate the adhesion and alignment of the myoblasts. It has been reported that the G-protein coupled receptors BAI1 and BAI3 also play a role during fusion, through signal transduction from the membrane to the cytoskeleton via the Dock1/Rac1 pathway (Hamoud et al., 2014; Hochreiter-Hufford et al., 2013).

Unlike in *Drosophila,* an actin focus structure has not been reported during vertebrate myoblast fusion. Nevertheless, actin cytoskeletal rearrangements play a critical role in vertebrate muscle formation. Similar to *Drosophila mbc* mutants*, Dock1*-null mice embryos exhibit severely impaired myoblast fusion and skeletal muscle content is reduced (Laurin et al., 2008). Dock1 is the mammalian ortholog of Mbc, and it functions as a GEF protein that activates Rac GTPase. In a conditional *Rac1* knockout mouse model, the recruitment of Arp2/3 and F-actin to the cell-contact site is reduced, resulting in impaired myoblast migration and fusion (Vasyutina et al., 2009). Experiments using C2C12 mouse myoblast cell culture suggest that Nap1, the mammalian ortholog of Kette and the downstream target of Rac1, is also required for regulating WAVE dependent actin remodeling during myoblast fusion (Nowak et al., 2009). Moreover, a recent study reported the requirement of the non-muscle myosin IIA (NM-MHC-IIA) during myoblast fusion. NM-MHC-IIA is involved in the formation of the cortical actin wall in the FC/myotube in the rat L6 myoblast cell culture, providing structural and mechanical support to facilitate membrane alignment and fusion pore formation (Duan and Gallagher, 2009). Together, these data suggest a conserved role of actin during muscle development.

It has been a major challenge to identify muscle-specific genes that ensure that fusion occurs only between myoblasts rather than other cell types. The first such gene found in vertebrates is Myomaker/Tmem8c. Myomaker is a transmembrane protein that is specifically expressed in the muscles during muscle development and regeneration (Landemaine et al., 2014; Luo et al., 2015; Millay et al., 2013; 2014; Zhang and Roy, 2017). In humans, reduced Myomaker function is linked to Carey-Fineman-Ziter syndrome (Di Gioia et al., 2017), a congenital myopathy. Myomaker is required for fusion and is localized to the adhesion site. Expressing Myomaker in fibroblasts is sufficient to induce fusion between fibroblasts and myoblasts, but it is not sufficient to induce fibroblast-fibroblast fusion (Millay et al., 2013). These reports suggest that additional proteins on the myoblasts are required to interact with Myomaker to induce fusion.

Recently, Gm7325, the potential counterpart of Myomaker, has been identified by three different labs, and was given the name Myomixer (Bi et al., 2017), Myomerger (Quinn et al., 2017) and Minion (Zhang et al., 2017). Gm7325 is an 84 amino acid microprotein with a similar expression pattern to Myomaker. The function of Gm7325 is required for Myomaker-induced fusion, as simultaneous expression of Myomaker and Gm7325 induces fusion between non-muscle cells (Bi et al., 2017; Quinn et al., 2017; Zhang et al., 2017). The mechanism by which Myomaker and Gm7325 regulates fusion is not well understood. A physical interaction between Myomaker and Gm7325 has been reported (Bi et al., 2017), but remains controversial (Quinn et al., 2017; Zhang and Roy, 2017). In Gm7325- and Myomaker-deficient myoblasts, cell fusion is blocked at different fusion stages. Based on whether the cell membranes align in the deficient myoblasts, it has been proposed that Myomaker is necessary for cell recognition, while Gm7325 is required for downstream events such as actin cytoskeleton remodeling (Zhang et al., 2017).

Cell fusion in vertebrates is required not only during early embryonic myogenesis, but also during muscle regeneration after damage. As with *Drosophila* adult muscle progenitors (AMPs), a pool of myoblasts remains undifferentiated during vertebrate myogenesis. These

myoblasts are satellite cells that associate with myofibers. Satellite cells contribute to post-natal muscle growth, as well as to muscle repair. The specification of satellite cells is critically dependent on Pax7, as no satellite cells are found in *Pax7* mutant mice (Seale et al., 2000). With intact muscles, satellite cells are mitotically quiescent and express Pax7 and Myf5, but not Myogenin or MyoD (reviewed in (Yin et al., 2013)). Upon muscle injury, myofiber necrosis triggers inflammatory responses, which induce satellite cells to proliferate (Tidball, 1995). After proliferation, the majority of satellite cells differentiate and start to express MyoD and myogenin (Rantanen et al., 1995). These cells later fuse with damaged myofibers or fuse with each other to generate new muscle fibers (Grounds and Yablonka-Reuveni, 1993). Thus far, the process of satellite cell fusion is mechanistically similar to myoblast fusion during primary and secondary myofiber formation.

Conclusion

Myoblast fusion in organisms ranging from the fly to human occurs through conserved steps: cell recognition, membrane apposition, actin rearrangement, pore formation, and cytoplasmic continuity. The investigation of myoblast fusion in *Drosophila* continues to provide valuable insights to vertebrate muscle development. However, there are many questions that remain unanswered: How do muscles control the number of fusion events during development and during repair? How do the nuclei added to a myotube adopt the program of the growing myotube? How are movement and transcriptional diversity among the added myonuclei controlled? And when considering the mechanisms of fusion, how are the ratios of linear and branched actin filaments controlled at the actin focus? What are the actin depolymerization factors that resolve the actin focus after fusion pore formation? What causes pore formation between the engaged cells? The knowledge gained from answering these questions in model systems such as *Drosophila* will illuminate how the key steps of fusion are controlled and how the syncytial muscle cell develops and is maintained.

Acknowledgments

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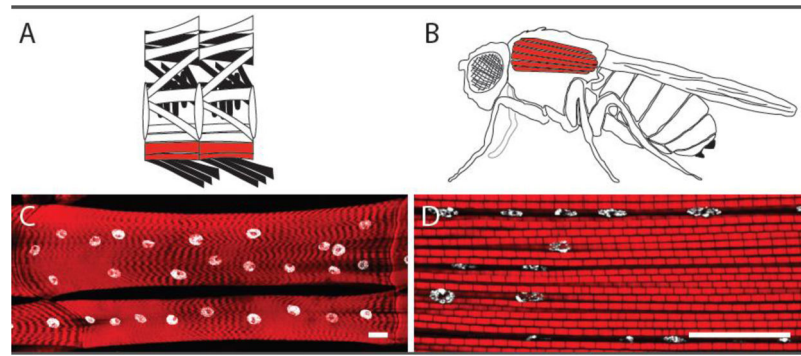
Figure 1. Larval and adult *Drosophila* muscles

A) Schematic representation of the larval muscles. The Ventral Longitudinal (VL) muscles 3 (top) and 4 (bottom) are highlighted in red. **B)** Schematic representation of the adult muscles. The Dorsal Longitudinal Muscles (DLMs) that compose the major portion of the Indirect Flight Muscles are highlighted in red. **C)** Representative image of the 3rd instar larval VL muscles 3 and 4. **D)** Representative image of the DLMs where it is possible to observe multiple myofibrils and nuclei. **C** and **D)** Actin labeling in sarcomere, red; Nuclei, white. Scale bars, 25μm.
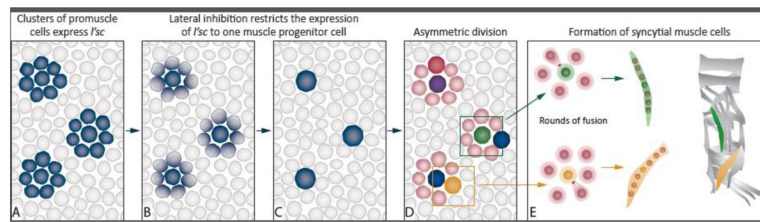
Figure 2. Specification, Fusion and Differentiation of Muscle Cells
A) Expression of *l'sc* occurs in clusters of mesodermal cells with competency to become muscle progenitor cells. **B** and **C)** The activity of the Notch and RTK-Ras pathways restricts the expression of *l'sc* to a single muscle progenitor cell of a particular identity. **D)** The remaining cells from the initial cluster of promuscle cells become FCMs (pink). The muscle progenitor cells undergo asymmetric division to form either two founder cells (red and purple) or one founder cell (green or yellow) and an adult muscle progenitor (blue). **E)** Founder cells shown on 2D express different identity genes and undergo several rounds of fusion (actin foci, red) to form syncytial muscle cells with different characteristics. The green founder cell expresses *apterous* and forms muscle LT1 and the yellow founder cell expresses *nautilus* to form muscle VA1 (modified from (Dobi et al., 2015)). The myotubes mature by attaching to tendon cells and developing sarcomeres (not shown). The final position of these muscles is highlighted in the hemisegment schematic on the right.
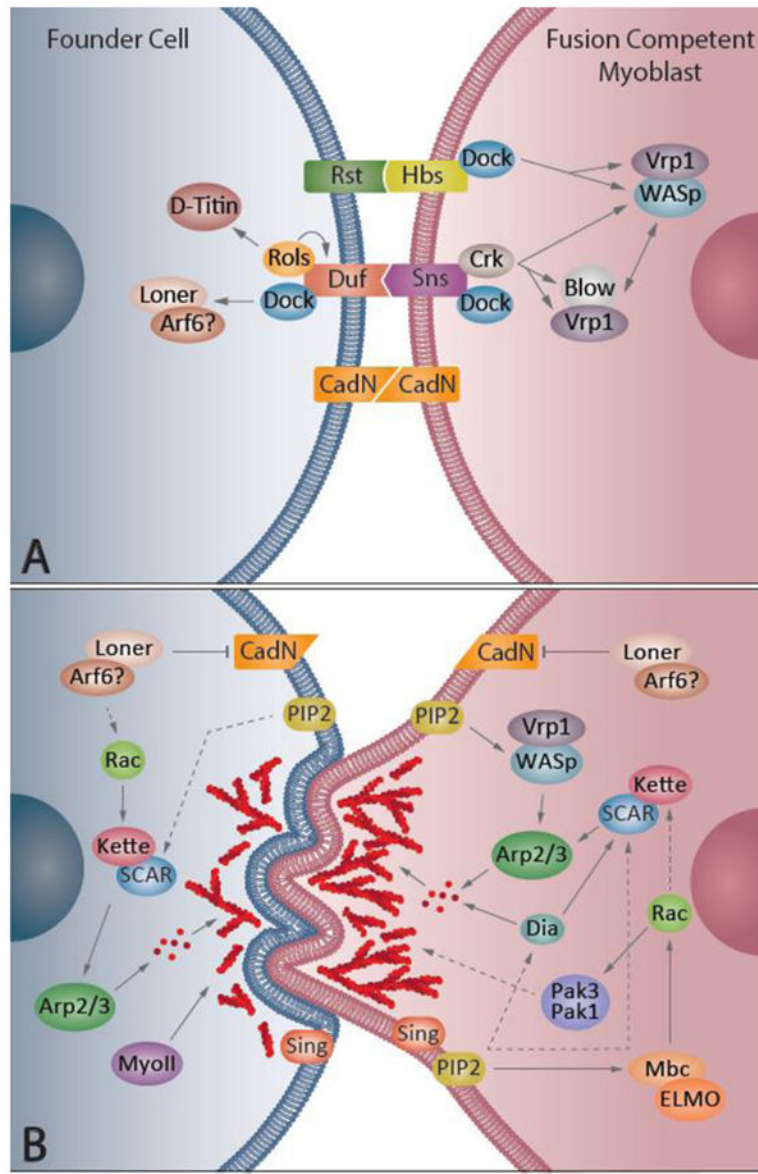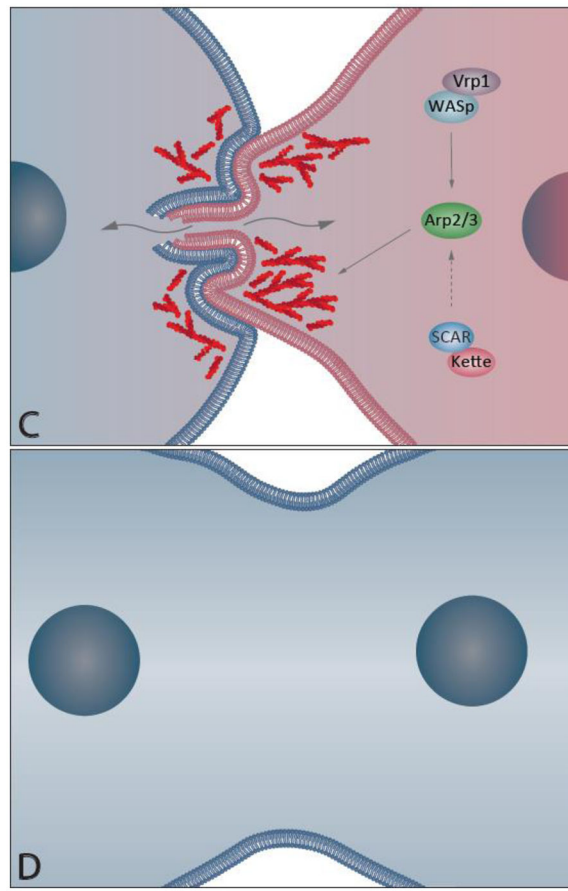
Figure 3. Schematic representation of myoblast fusion

In the *Drosophila* embryo, fusion of an individual founder cell (FC) with multiple fusion competent myoblasts (FCMs) gives rise to a single multinucleated muscle cell; the number of FCM fusion events determines the number of nuclei in the mature muscle cell. **A) Recognition and Adhesion:** FC and FCM express different membrane proteins that allow the two cells to recognize and adhere to each other. When in contact, each cell initiates the fusion process by recruiting several proteins to the fusion site. **B) Actin Focus Formation:** Through the activity of the fusion machinery on both the FC and the FCM, actin (red) monomers are assembled into filaments and form a dense F-actin focus on the FCM side and a thin layer on the FC side. The FCM actin focus invades the FC with multiple finger-like protrusions. N-cadherin is removed from the membrane to allow for the next steps of fusion. **C) Pore Formation.** The formation of a fusion pore in the membrane occurs, allowing for the exchange of cytoplasm between both cells. The repression of the FCM transcriptional profile begins (FCM pink nucleus turns blue). **D) Post Fusion.** After one fusion event, the resulting cell has one additional nucleus that has the transcriptional profile of the FC (blue nuclei). At this moment, the cell either prepares for another round of fusion or stops fusing with FCMs.

Table 1

List of gene products required in *Drosophila* myoblast fusion

Protein	Localization	Function	Reference(s)
Arp2/3	Cytoplasm	Actin polymerization	(Richardson et al., 2007)
Blown Fuse (Blow)	Cytoplasm	Regulation of WASp activity	(Doberstein et al., 1997), (Jin et al., 2011)
Crk	Cytoplasm	Adaptor protein	(Balagopalan et al., 2006; Galletta et al., 1999)
Diaphanous (Dia)	Cytoplasm	Actin polymerization	(Deng et al., 2015)
Dumbfounded (Duf)	FC - Membrane	Recognition and Adhesion	(Ruiz Gomez et al., 2000)
Hibris (Hbs)	FCM - Membrane	Recognition and Adhesion	(Artero et al., 2001)
Kette	Cytoplasm	SCAR activation	(Richardson et al., 2007)
Schizo/Loner	Cytoplasm	Rac localization and N-cadherin removal	(Chen et al., 2003), (Dottermusch-Heidel et al., 2012)
Myoblast City (Mbc)	Cytoplasm	Regulation of Rac activity	(Erickson et al., 1997)
Myosin II (Myo II)	FC - Cytoplasm	Cortical tension generation on the FC/myotube	(Kim et al., 2015)
N-cadherin	Membrane	Adhesion	(Dottermusch-Heidel et al., 2012)
PI(4,5)P2	Membrane	Actin cytoskeleton remodeling	(Bothe et al., 2014)
Rac	Cytoplasm	SCAR activation	(Hakeda-Suzuki et al., 2002)
Rolling pebbles (Rols)	Cytoplasm	Adaptor protein involved with Duf recycling	(Chen and Olson, 2001), (Rau et al., 2001)
Rst	FC - Membrane	Recognition and Adhesion	(Strünkelnberg et al., 2001)
SCAR	Cytoplasm	Arp2/3 activation	(Richardson et al., 2007)
Singles Bar (Sing)	Membrane	Vesicle trafficking	{Estrada:2007hg}
Sticks and Stones (Sns)	FCM - Membrane	Recognition and Adhesion	(Bour et al., 2000)
Verprolin1 (Vrp1)	Cytoplasm	Regulation WASp activity	(Massarwa et al., 2007), (Jin et al., 2011)
WASp	Cytoplasm	Arp2/3 activation	(Schäfer et al., 2007)
WHAMY	Cytoplasm	Actin polymerization	(Brinkmann et al., 2016)

Table 2

List of gene products required for vertebrate myoblast fusion

Protein	Location and function	Animal model	Reference(s)
Adam12	Membrane Mediates myoblast adhesion Interaction with the actin-binding protein α-actinin-2 promotes myoblast fusion	Mouse	(Galliano et al., 2000; Zolkiewska, 1999)
BAI1	Membrane Mediates recognition of phosphatidylserine on apoptotic cells Regulates myoblast fusion through Dock1/Rac1 pathway	Mouse	(Hochreiter- Hufford et al., 2013)
BAI3	Membrane Interacts with the scaffold protein ELMO to regulate myoblast fusion, possibly through the ELMO/DOCK/Rac1 pathway	Chicken	(Hamoud et al., 2014)
Caveolin-3	Membrane Involved in myoblast fusion (unclear role) Contradictory fusion phenotypes in different experimental models	Mouse	(Galbiati et al., 1999; Volonte et al., 2003)
Cdc42	Cytoplasm Required for actin rearrangement at the fusion site	Mouse	(Vasyutina et al., 2009)
CKIP-1	Cytoplasm Regulates myoblast fusion by interacting with both phosphoinositides on the membrane and Arp2/3 subunit	Mouse Zebrafish	(Baas et al., 2012)
Crk Crkl	Cytoplasm Adaptor proteins Crk/Crkl genetically interact with DOCK1/DOCK5 to regulate myoblast fusion	Zebrafish	(Moore et al., 2007)
DOCK1	Cytoplasm Required for myoblast fusion by activating Rac1	Zebrafish Mouse	(Laurin et al., 2008; Moore et al., 2007)
DOCK5	Cytoplasm Functions redundantly with DOCK1 Also involved in post-fusion myofiber development	Zebrafish Mouse	(Laurin et al., 2008; Moore et al., 2007)
EHD2	Membrane and cytoplasm Regulates myoblast fusion by mediating endocytic recycling of membrane proteins, such as Myoferlin	Mouse	(Doherty et al., 2008)
Gm7325 Myomixer Minion Myomerger	Membrane Interacts with Myomaker to induce fusion Functions in cell recognition (proposed)	Mouse	(Bi et al., 2017; Quinn et al., 2017; Zhang et al., 2017)
JAM-B JAM-C	Membrane Both physically interact to mediate fusion Facilitate myocytes recognition and adhesion (proposed)	Zebrafish	(Powell and Wright, 2011)
Kirrel	Membrane Required for fast muscle precursor fusion	Zebrafish	(Srinivas et al., 2007)
M-cadherin	Membrane Mediates myoblast adhesion Promotes fusion by activating the Rac1 pathway	Mouse	(Charrasse et al., 2007; 2006)
MOR23	Membrane Regulates cell adhesion during myoblast fusion	Mouse	(Griffin et al., 2009)
Myoferlin	Membrane Regulates myoblast fusion through unclear mechanism, potentially by acting as a scaffold protein that links fusion proteins with the membrane	Mouse	(Doherty et al., 2005)
Myomaker	Membrane Muscle-specific protein that induces fusion during muscle development and repair Mediate cell recognition (proposed)	Mouse Zebrafish Chicken	(Landemaine et al., 2014; Luo et al., 2015; Millay et al., 2013; Zhang and Roy, 2017)
N-WASp	Cytoplasm Required in both fusing partners	Mouse	(Gruenbaum-Cohen et al., 2012)

Protein	Location and function	Animal model	Reference(s)
Rac1	Cytoplasm Required for proper recruiting of Arp2/3 to the contact site Regulates actin remodeling during fusion Unlike in *Drosophila*, constitutively active Rac1 in zebrafish leads to myoblasts hyperfusion	Mouse Zebrafish Chicken	(Sieiro et al., 2017; Srinivas et al., 2007; Vasyutina et al., 2009)
Stab2	Membrane Involved in the phosphatidylserine-dependent fusion of myoblasts during muscle development and regeneration	Mouse	(Park et al., 2016)
Talin 1/2	Cytoplasm Regulates myoblast fusion, potentially by mediating the interaction between integrins and cytoskeleton	Mouse	(Conti et al., 2009)
VLA4 VCAM1	Membrane VLA-4 and its counter receptor VCAM-1 are involved in secondary myogenesis	Mouse	(Rosen et al., 1992)
β-1 integrin	Membrane Involved with membrane breakdown during myoblast fusion	Mouse	(Schwander et al., 2003)