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Visualizing new dimensions in *Drosophila* myoblast fusion

Brian Richardson^{1,2}, Karen Beckett¹, and Mary Baylies^{1,2,*}

¹ Program in Developmental Biology, Sloan Kettering Institute, New York, New York 10021

² Weill Graduate School at Cornell Medical School, New York, New York 10021

Summary

Over several years, genetic studies in the model system, *Drosophila melanogaster*, have uncovered genes that when mutated, lead to a block in myoblast fusion. Analyses of these gene products have suggested that Arp2/3-mediated regulation of the actin cytoskeleton is crucial to myoblast fusion in the fly. Recent advances in imaging in *Drosophila* embryos, both in fixed and live preparations, have led to a new appreciation of both the three-dimensional organization of the somatic mesoderm and the cell biology underlying myoblast fusion.

Introduction

Muscle fiber formation during development in both flies and mammals can be divided into two broad steps. The first step of pattern formation requires the integration of signaling and transcriptional networks to specify populations of myoblasts. The second step of morphogenesis involves the molecular and cell biological regulation of myoblast fusion and subsequent muscle guidance, attachment and innervation. In this review, we will focus on the molecular and cellular events that occur during myoblast fusion. Myoblast fusion during development is a reiterative process and is also essential to the repair of damaged muscle later during life.(1–3) Despite the centrality of the fusion process to the formation and subsequent repair of muscle, little is known about the cellular and molecular mechanisms regulating fusion. Increased understanding of this process could have therapeutic benefit in regards to muscular dystrophy and other muscle wasting diseases.

Thirty muscle fibers make up the body wall musculature in each abdominal hemisegment of the developing *Drosophila* embryo.(4) Each multinucleated fiber or muscle has a unique size, shape and orientation with respect to body axes. Each muscle is formed by the fusion of two cell types, a founder cell (FC) that seeds the specific muscle and fusion-competent myoblasts (FCMs) that provide mass. FCs are a diverse population of myoblasts: each FC has a unique identity that is characterized by the expression of transcription factors, such as Slouch (also known as S59), Even-skipped, Krüppel, Vestigial, Apterous and Nautilus.(5–7) The specific combination of transcriptional regulators expressed in each FC determines the unique morphology of the final muscle and controls the number of fusion events, muscle shape and orientation, innervation and sites of attachment to the overlying epidermis.

In contrast to FCs that control individual identities of each muscle, the FCMs are a more naïve population of myoblasts, which express the Lameduck (Lmd, also known as Myoblasts Incompetent and Gleeft) transcription factor.(8–10) Upon fusion to FCs, the nuclei of newly incorporated FCMs are reprogrammed and begin to express the transcriptional regulators of

*Correspondence to: Mary Baylies, Developmental Biology, Sloan-Kettering Institute, Box 310, 1275 York Avenue, New York 10021.

E-mail: m-baylies@ski.mskcc.org.

Both Brian Richardson and Karen Beckett contributed equally to this work.

the FC to which they have fused.(5,11–17) Although all FCMs are thought to be capable of fusing to any FC, recent studies have ascribed specific identities and revealed considerable functional diversity among this supposedly uniform and naïve population. For example, the FCM-specific gene *hibris* is expressed only in a subset of FCMs,(18) and expression of the mesodermal protein Twist is maintained in a subset of FCMs in *lmd* mutant embryos.(10) Likewise, Estrada et al. (2006) have identified several FCM-specific genes that do not require Lmd for their expression or are not expressed in all FCMs.(19) Furthermore, recent characterization of a novel GAL4 driver, which uses an FCM-specific enhancer of the *Dmef2* promoter, shows expression in only a subset of FCMs.(20) In parallel to these studies, analysis of myoblast behaviors during fusion have identified behaviors specific to subsets of the FCM population. For example, at the time of FCM specification, some FCMs only contact other FCMs, while others contact FCs or epithelial cells of the epidermis or trachea.(21) After specification, during stages 12–13 (7.5–10.5 hours AEL), small clusters of FCMs undergo cell division.(21) The significance of these extra cell divisions are not clear, but presumably are required to generate the correct number of FCMs required for fusion. Taken together, FCMs appear to contain much more information that may impact upon myoblast fusion and final muscle morphogenesis than previously anticipated. In this review, we summarize what is known about the cellular and molecular mechanisms of *Drosophila* myoblast fusion. We also discuss recent advances in the field and specifically, the timing of myoblast fusion, a novel actin structure at the site of fusion and new genes implicated in regulating the fusion process.

Spatial arrangements of FCs and FCMs influence myoblast fusion

Studies of muscle morphogenesis using light microscopy have primarily focused on the final muscle pattern, using antibodies against Myosin heavy chain or Beta-tubulin. While this approach has been useful for identifying fusion mutants with drastically disrupted muscle patterns, these markers do not label myoblasts throughout the fusion process and are therefore inadequate for studying the cellular aspects of fusion. The availability of reagents that specifically label FCs, FCMs and the cell cortices has allowed the identification and tracking of myoblasts throughout the fusion process, as well as investigation of how the three-dimensional arrangement of myoblasts contributes to the fusion process. These advances have revealed that the spatial arrangements of FCs and FCMs are complex, with the relationships between these cell types changing during the period of fusion.(21)

At stage 12 (7.5–9.5 hours AEL), when myoblast fusion has just begun, the mesoderm appears folded upon itself ventrally and consists of multiple layers of FCs and FCMs. The FCs concurrently form the most external and internal cells with the FCMs in between. Initial fusion events appear to occur between an FC and its nearest FCM neighbor. By stage 13 (9.5–10.5 hours AEL), the ventral FCs and FCMs rearrange their positions, resulting in a multi-layered mesoderm with FCs in the external layers and FCMs distributed throughout both external and internal layers. By stage 14 (10.5–11.5 hours AEL), the growing myotubes are arranged in the external layers, prefiguring the final muscle pattern. The majority of remaining FCMs are located internally at this time. Further fusion appears to require recruitment and migration of FCMs from these internal layers. Hence, the arrangement of myoblasts over time appears critical for myoblast fusion.

FCs form at stereotypical positions with respect to the AP and DV axes of the embryo and determine where the final muscles will form (Fig. 1A).(4,22) While the position of many FCs using expression of known FC identity genes had been previously characterized,(11–16,23–26) a recent three-dimensional map has demonstrated the relative position of all FCs within a single mesodermal hemisegment as fusion begins.(21) This approach has shown that FCs form characteristic groupings (dorsal, dorsal–lateral, lateral and ventral) that are maintained throughout fusion and correspond to the final muscle pattern (Fig. 1A). However, within these

groups, there is often variation in the precise position of FCs with respect to one another. Regardless of this variation, muscles still form in the correct positions indicating that movement of FCs or muscle guidance must compensate for this subtle variation. This map can now be used to identify the specific FCs in which mesodermal gene products are expressed. For example, using this approach we have determined that the transcriptional regulator, Twist, is expressed in the FCs for the DO1, DO3, LT2, LT4 and VA1-3 muscles at stage 13 (Fig. 1B).

Timing of myoblast fusion in *Drosophila*

Myoblast fusion is a reiterative process that occurs from stage 12 to 15 (7.5–13 hours AEL). (4,21) The size of each muscle is determined by the number of fusion events; some muscles are generated from two fusion events whereas other fuse 25 times.(4) While there is some variation in the final size of individual muscles, a characteristic mean number of nuclei has been determined in several muscles (Table 1).(21) FCs do not all begin to fuse at the same time. It is likely that the timing of their specification impacts the time at which they start to fuse. Bate proposed that fusion begins in the ventral mesoderm based on analysis of when bi- and trinucleate myotubes become apparent.(4,22) Our subsequent analysis using FC identity gene expression suggests that this may not be true as the dorsal DA1 and DO2 muscles begin to fuse at stage 12, prior to the ventral VT1 and VA2 muscles that begin to fuse at stage 13. (21) The exact time when each muscle stops fusing is not known, although all fusion is completed by the end of stage 15. Prior observations suggested that since a muscle stops fusing once it reaches its final size, smaller muscles will stop fusing before larger muscles.(22) However, recent analysis of the small VT1 muscle (2–4 nuclei at stage 15) shows that fusion events occur throughout stages 12 to 15, suggesting that this may not be the case.(21)

Analysis of the fusion profile of individual muscles has shown that fusion occurs in two temporal phases.(21) During the first 3 hours of fusion (stage 12–13, 7.5–10.5 hours AEL) 9–27% of fusion events occur, while the remaining 73–91% of fusion events occur in the final 2.5 hours of the process (stage 14–15, 10.5–13 hours AEL). One explanation for these results is that individual fusion events happen faster later in fusion. However, live imaging studies have suggested the timing of individual fusion events does not change over time,(23) suggesting instead that the frequency with which fusion events occur must increase later during the fusion process.

Cellular activities required for fusion

The ability to clearly identify and distinguish FCs/myotubes and FCMs has identified novel cell behaviors during fusion (Fig. 2).(21,23) For example, cell-type-specific labeling allowed the identification of a large group of Lmd-positive FCMs that are located underneath growing myotubes as fusion begins. Fixed and live imaging utilizing labeled actin revealed that these FCMs extend filopodia and lamellopodia, consistent with migratory behavior. These FCMs may migrate large distances in multiple directions and/or through several cell layers.

Similar labeling experiments in both fixed and live preparations allowed identification of an actin structure at the site of myoblast adhesion that occurs just prior to fusion.(23) These studies have revealed that F-actin accumulates into a focus structure (also known as a fusion restricted myogenic adhesive structure, or FURMAS) at the site of myoblast adhesion (Fig. 2a).(23,24) Actin foci have an average area of $1.9 \mu\text{m}^2$, although larger and smaller foci are observed. (23) This most likely reflects their dynamic nature as they form and dissolve. Many, but not all, proteins implicated in myoblast fusion localize to the actin focus, suggesting that this is the site of their activity during fusion. Crucially, live imaging that specifically follows the actin cytoskeleton using an actin–GFP fusion protein has revealed that this accumulation of actin cytoskeletal components marks the actual site of subsequent myoblast fusion.(23) All fusion events are preceded by the formation and dissolution of this structure and data indicate that the

cell membranes are intact when an actin focus is present. This actin focus, however, is dynamic, reflecting that *Drosophila* myoblast fusion is a relatively rapid process, with the average fusion event being approximately 12 minutes from appearance of the focus to its dissolution and incorporation of a new nucleus into the myotube. Consistent with new data obtained from individual muscle fusion profiles determined from fixed preparations, live imaging also indicated that the number of fusion events changes over time, with relatively few fusion events taking place early in the process (stage 12–13) and the majority of fusion taking place later (stage 14–15). The function of the actin focus during fusion, however, remains an open question. Actin polymerization may provide a force to push the membranes together.⁽²⁵⁾ Alternatively, actin may play a role in vesicle trafficking to or from this site of fusion.⁽²⁶⁾ Further study into the cytoskeletal dynamics and connections to membrane changes are required.

While advances in light microscopy have revealed several new aspects of myoblast behavior at the cellular and subcellular level, pioneering work by Doberstein et al. used transmission electron microscopy (TEM) to examine myoblast fusion at the ultrastructural level.⁽²⁷⁾ Through this approach, they identified several different stages of fusion and ordered them into a sequential process based on the prevalence of a structure at a particular developmental stage. This model proposed that following adhesion between an FC/myotube and an FCM, clusters of electron-dense vesicles, referred to as “prefusion complexes”, form and align along the neighboring membranes of the attached cells. Next, electron-dense plaques form, presumably from the contents of the prefusion complexes. Finally the cells align with apposed plasma membranes, at which point fusion pores open in the vicinity of the plaques, and the cells undergo cytoplasmic mixing and ultimately, fusion to become one cell (Fig. 2b).

Despite the insights to the fusion process provided by ultrastructural studies, certain observations raise questions concerning the proposed sequential order in which the steps of fusion take place. First, fusion is not a synchronized process with all myoblasts starting and stopping the fusion cycle at the same time. Hence, many fusion events at different steps in the fusion cycle are captured by a particular section prepared for TEM. Why one step would be favored as occurring before another is unclear. Second, fusion was recently found to be a relatively rapid process, with an average fusion event taking only 12 minutes.⁽²³⁾ Hence, all of the events captured by TEM would have to occur extremely rapidly. Taken together, these observations make the relationship between the steps of the fusion process extremely difficult to ascertain. Therefore, it remains a possibility that electron-dense plaques form before prefusion complexes, for example. Alternatively, all myoblasts may not go through all of the steps of fusion identified by TEM. A major challenge is to link what is seen by TEM to that seen by light microscopy. For example, can live markers be developed to label and monitor the electron-dense vesicles? What are the contents of these vesicles? What is the connection between the events described for actin focus/dissolution and the prefusion steps? Further development of methodologies to connect these two views of the subcellular events underlying fusion is necessary.

Molecular activities required for fusion

Genetic studies have identified a number of gene products required for the fusion process (Table 2).^(2,28,29) Mutation of these gene products leads to a myoblast fusion defect, which is indicated by thin “mini-muscles” formed by FCs attempting to achieve normal muscle morphology, surrounded by free FCMs that have been unable to fuse. These gene products fall into several classes based on their predicted functions and localization. Four transmembrane proteins containing immunoglobulin domains function in the recognition and adhesion of myoblasts. Dumbfounded/Kirre (Duf) and Roughest/Irre function in the FC,^(30,31) while Sticks and Stones (Sns) and Hibris (Hbs) function in the FCM.^(18,32,33) Duf and Rst directly

bind with *Sns*, and these proteins seem to be the main mediators of myoblast adhesion. *Duf* and *Rst* perform redundant functions and removal of both genes leads to a complete fusion defect.(31) Removal of *sns* also has been reported to cause a complete fusion defect,(32) although recent data suggest that single fusion events do occur in this mutant.(34) *hbs*, however, is more complicated: removal and overexpression both cause mild fusion phenotypes.(18,33) *hbs* and *sns* genetically interact, and it has been proposed both that *Hbs* serves as a negative regulator of *Sns*,(18) and that it acts redundantly with *Sns*.(34) Clearly further analysis is required to clear up this discrepancy.

Downstream of adhesion in the FC is Rolling Pebbles/Antisocial (*Rols*), an adapter protein with two proposed functions.(35–37) First, *Rols* is thought to physically link *Duf* to components and regulators of the cytoskeleton, namely *Titin* and *Myoblast City* (*Mbc*), the *Drosophila* Dock180 homolog.(35,36) Second, *Rols* is thought to play a role in replenishing *Duf* at FC membranes following each FC–FCM fusion cycle, thereby promoting the next round of fusion.(34) Removal of *rols* leads to a severe, but incomplete, fusion defect, with one or two fusion events occurring per FC.(21,34,36,37)

Genetic analysis has highlighted the critical role of the actin cytoskeleton and its regulators to the fusion process. *Mbc* and its homolog Dock180 play an established role in regulating the small GTPase *Rac*,(38–41) which, among its many proposed functions, is an important regulator of the actin cytoskeleton. Importantly, *Rac* signaling activates SCAR/WAVE by regulating a multi-protein complex consisting of *Kette*/*Nap1*, *Sra-1*/*CYFIP*/*Pir121*, and *HSPC300* bound to SCAR/WAVE.(42,43) This complex appears important in stabilizing and localizing SCAR/WAVE during fusion.(23) SCAR/WAVE belongs to the WASP family of proteins, which are capable of binding actin monomers and activating *Arp2/3*, an actin-related protein complex that promotes the polymerization and branching of actin.(44–46) Consistent with a role for this pathway in myoblast fusion, mutations in *Rac*, *kette* and *SCAR* lead to strong fusion defects.(21,23,47–49) Zygotic *Arp3* mutants have a mild fusion defect, although maternal contribution of this gene product has, thus far, prevented a complete loss-of-function analysis.(23) The WASP protein itself presents an alternative mechanism of activating *Arp2/3*.(44,46) Mutations in WASP and its regulator Wasp-interacting protein/Solitary (*D-WIP*/*Sltr*) lead to strong fusion defects, highlighting the critical nature of the *Arp2/3* pathway to the fusion process.(25,26,50)

Loner/*Schizo* is also critically required for fusion, as mutation in this gene leads to a fusion block.(21,51) *Loner* is a GEF that regulates the GTPase, *Arf6*. *Arf6* has several characterized roles, including regulation of membrane trafficking and regulation of *Rac*.(52–54) Consistent with these functions acting in myoblast fusion, *Rac1* is not normally localized in *loner* mutants.(51)

Two additional fusion proteins have less clear roles. *blownfuse* (*blow*) mutants have a complete fusion block.(21,27) *Blow* is a PH domain-containing protein, suggesting that lipid binding and/or membrane localization are important to its function.(2) The only other clue to the function of *blow* is a genetic interaction with *kette*.(49) *singles bar* (*sing*) mutants exhibit a severe fusion block, with one fusion event happening per FC. *Sing* contains a MARVEL domain, which is known to be important for membrane apposition events such as tight junction formation and vesicular transport.(55)

To understand the mechanisms underlying the fusion block observed in these mutants, several approaches have been taken. Firstly, actin foci have been analyzed for the fusion mutants, which sort out into three distinct classes (Table 2).(23) In class one, no or fewer actin foci are detected: *sns* mutants and *duf/rst*-deficient embryos have no actin foci, while *rols* mutants have a drastically reduced number of actin foci. In class two mutants, normal-sized foci are detected,

but with increased number, presumably due to the block in fusion. The *loner* mutation falls into this class. The third class of mutations is typified by more and larger foci. Members of this class include *mbc*, *blow*, *kette*, *Rac* and *Scar*. The actin focus phenotype of *D-WIP/sltr* mutants remains controversial. Focus size appears wild type,(23) placing *D-WIP/sltr* into the second class of fusion mutants. Kim et al. (2007) report that *sltr*-deficient FCMs do not form actin foci, as judged by overlap with the FC-specific protein Rols7.(26) However, in our hands using the same technique, actin foci are detected in both FCs and FCMs in *sltr* mutants (B.E.R. and M.K.B., unpublished observations). Clear labeling of cell membranes will be necessary to resolve this issue. Finally, the actin focus phenotype of *sing* mutants remains to be tested, while the actin focus phenotype of *Arp2/3* mutants awaits stronger loss-of-function reagents.

Secondly, some fusion mutants have been analyzed by TEM. *mbc* mutants almost completely lack prefusion complexes, suggesting that Mbc is required before, during or immediately following myoblast recognition/adhesion.(27) *blow* mutants contain normal numbers of prefusion complexes, but lack electron-dense plaques, consistent with a role for Blow in their formation.(27) Larger electron-dense plaques are found in *kette* mutants compared to wild type, indicating that Kette may be responsible for their breakdown.(49) *sing* mutants contain elevated numbers of prefusion complexes compared to wild-type, suggesting that Sing is necessary for fusion to progress past this step.(55) The TEM phenotype of *D-WIP/sltr* mutants remains controversial. Kim et al. (2007) have reported that *D-WIP/sltr* mutants appear to have a defect in vesicle targeting, as paired vesicle complexes are seen not only at the site of FC/FCM adhesion, but also between FCMs in these mutants.(26) However, Massarwa et al. (2007) found that *D-WIP/sltr* mutants progress to the final stage of fusion, with fusion pores formed between adherent cells.(25) Further studies are required to resolve these conflicting observations.

Given these data sets, it is tempting to equate the actin foci with the electron-dense material (prefusion complex and plaques) seen by TEM. However, there is a strong argument against this conclusion. We observe enlarged actin foci in a number of fusion mutants, including *kette*, *mbc* and *blow*.(23) Whereas all show enlarged foci, TEM analysis reveals distinct phenotypes; *mbc* mutants are deficient in recognition/adhesion, *blow* mutants lack electron-dense plaques, and *kette* mutants show a block at plaque formation.(27,49) Therefore, it is possible that all steps revealed by TEM occur at the time in which an actin focus is present. Recent TEM work suggests that actin is important for the targeting of vesicles to plasma membrane at the site of myoblast adhesion.(26) How the diffuse actin observed in these studies relates to the dynamic but concentrated accumulation of F-actin into foci at the plasma membrane as seen by live and fixed imaging remains in question.

Models of myoblast fusion

The two-step model of myoblast fusion proposes that, in the first step, a bi- or trinucleate precursor cell is formed and then, in the second step, all subsequent fusion events occur until the muscle reaches its final size.(37,49) This model also suggests that distinct gene products and subcellular events, as analyzed by TEM, are required for each step of the fusion process. The idea of a precursor cell is based on Bate's initial description of *Drosophila* myoblast fusion. (4) In the absence of cell markers, the first visible difference between FCs/myotubes and FCMs was the appearance of bi- and trinucleate cells that he termed precursor cells. However, no functional importance was ascribed to these cells other than the fact that they were formed from FCs that had begun to fuse. However, due to the identification of mutants that showed either no (e.g. *mbc*) or limited (e.g. *kette*, *rols*) fusion by the end of muscle development, it was proposed that the formation of a precursor cell is an essential and distinct step during myoblast fusion.(37,49)

Re-examination and quantification of the fusion profile of individual muscles in fusion mutants provides evidence that is not fully consistent with the two-step model.(21) First, this analysis demonstrates that no fusion mutant shows absolutely no fusion events. Even *mbc* mutant embryos that show the strongest fusion block display occasional fusion events.(21,56) Second, the rare fusion events that do occur in the fusion mutants analyzed can occur at any stage of myoblast fusion, not just during early fusion.(21) Third, in those fusion mutants in which two to three fusion events per FC frequently occur (*kette*, *rols* and *Rac*), there are also cases of FCs in which no fusion happens, demonstrating that these gene products can be required for both initial or later fusion events, depending on the FC.(21) We propose that the limited fusion events that can occur in the many of the fusion mutants are due to inefficiency because of residual gene product activity. It has not been demonstrated that maternal contribution does not partially mask the zygotic function of the genes. For example, *mbc*, *Rac*, *kette* and *loner* all have RNA expression in the early embryo that could be maternally loaded and could therefore affect the final phenotype.(41,47,51,57) Inefficient fusion could thus explain the differences in final nuclei number observed between *kette*, *mbc*, *loner* and *Rac* mutants and the intermediate fusion blocks often observed with rescue experiments.(21,49) *rols*, in contrast, is clearly not maternally loaded.(35–37) The phenotype of *rols* mutants appears to be explained by its role in replenishment of Duf at FC membranes, which may not be required for all fusion events.(34)

These data in combination with the two temporal phases of fusion described above have led us to propose a new model to describe the process of myoblast fusion at the tissue level (Fig. 3).(21) We propose that, in the first phase of fusion (stage 12–13, 7.5–10.5 hours AEL), rare and limited fusion events occur between FCs and adjacent, external FCMs. In the second phase of fusion (stage 14–15, 10.5–13 hours AEL), frequent fusion events occur as FCMs move externally to fuse with growing myotubes. This model proposes that all gene products and subcellular behaviors (e.g. actin foci, prefusion complexes, fusion plaques) previously identified are required for both phases of myoblast fusion. An interesting question highlighted by this model is how the transition between the two phases occurs. We propose that this transition between infrequent and frequent fusion events is due to a limiting factor and/or FCM migration. There may be limiting amounts of a fusion protein during the first phase of fusion that becomes more abundant at the transition point. It is possible that Duf may be such a limiting factor as levels of cell-surface Duf expression are known to be tightly regulated.(34) The timing at which this transition point occurs also corresponds to the time at which FCMs appear to separate from one another and become migratory. It is possible that limited fusion can occur before this point in time due to the small number of FCM in direct contact with FCs. Once FCMs can migrate to their targets, fusion becomes more frequent.

One issue not addressed by this new model concerns the first fusion event. Blow and D-Wip/Sltr are initially expressed only in FCMs;(26,58) after the first fusion event, these proteins are located in both cell types. How or if this initial asymmetry in protein localization affects this first fusion event is not known, but this must be considered during mutant analysis.

Conclusions

The ability to visualize and measure cellular dynamics during *Drosophila* myoblast fusion has brought a new dimension to the study of cell–cell fusion. As genetic screens reveal new genes to incorporate into our models of fusion, the knowledge of the site of fusion and a particular actin structure at that site provides new assays to measure the role of specific genes in this process. Further assays, such as those to measure FCM migration, as well as new reagents to probe the cellular changes required for fusion, such as vesicle and membrane markers, will continue to evolve our knowledge of this process. It remains a significant challenge to connect what is seen at the light level to that detected by TEM. The function of the structures seen by

light and TEM, such as the actin focus and the electron-dense plaques, to the fusion process still is unclear. Likewise the membrane changes that lead to two cells becoming one are still unknown. Initial studies suggest that similar genes and structures are required for myoblast fusion in mammals. The actin cytoskeleton appears important to the fusion process in many systems. Whether the myoblast fusion module will be conserved across all cell–cell fusion events remains to be tested.

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Abbreviations

DO	dorsal oblique
DA	dorsal acute
DT	dorsal transverse
VT	ventral transverse
VA	ventral acute
VL	ventral longitudinal
FC	founder cell
FCM	fusion competent myoblast
TEM	transmission electron microscopy
AEL	after egg lay
AP	anterior posterior
DV	dorsal ventral
GFP	green fluorescent protein
GTPase	

guanine triphosphatase

GEF

guanine nucleotide exchange factor

PH

pleckstrin homology

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Figure 1.

Drosophila founder cell (FC) and body wall muscle maps. **A:** The FC map shows the relative position of all 30 FCs prior to myoblast fusion based on three-dimensional reconstructions of single mesodermal hemisegments (left panel). The final positions of the muscles seeded by these FCs are shown in the right panel. FCs are located in four groups: dorsal (red), dorsal-lateral (yellow), lateral (green) and ventral (blue). FCs identified using the expression of FC identity markers are outlined in black, while those identified based on position are outlined in grey. DO, dorsal oblique; DA, dorsal acute; DT, dorsal transverse, LL, lateral longitudinal; LT, lateral transverse; LO, lateral oblique; SBM, segment border muscle; VT, ventral transverse; VO, ventral oblique; VA, ventral acute; VL, ventral longitudinal. **B:** The

mesodermal transcription factor, Twist, is expressed in a subset of FCs prior to fusion. The left panel shows a three-dimensional reconstruction of a single mesodermal hemisegment at stage 13 and shows the colocalization of Twist (red) with the FC marker rp298–lacZ (green) in the FCs that will form the VA1–VA3, LT2, LT4, DO3 and DO1 muscles. A schematic of these data is shown in the panel on the right.

A Light Microscopy

B Electron Microscopy

Figure 2.

Cellular behaviors observed during *Drosophila* myoblast fusion. **A:** Behaviors observed by fluorescent light microscopy.(4,5,21,23,24,32) *Searching:* Founder cells (FCs) extend several actin-based protrusions in all directions prior to fusing. Fusion-competent myoblasts (FCMs), however, appear to extend only one actin based protrusion directionally towards an FC or myotube. *Migration:* FCMs assume a teardrop shape as they migrate towards an FC or myotube. An F-actin-based protrusion (red lines) extends in the direction of migration. This protrusion is broader than that observed during searching behavior. *Actin focus:* A spherical F-actin structure (red circle) forms at the site of adhesion between an FC or myotube and an FCM. The focus marks the subsequent site of myoblast fusion and colocalizes with several important fusion regulators. *Membrane breakdown and cell mixing:* Following dissolution of the actin focus, the membrane between the cells breaks down, allowing cytoplasmic and nuclear transfer and the formation of a cell with an additional nucleus. The newly fused nucleus from the FCM becomes transcriptionally “entrained” to the identity of the FC/myotube. **B:** Behaviors observed by transmission electron microscopy.(27) Box represents close up on the site of cell–cell adhesion, prior to fusion. *Prefusion complex* Electron-dense vesicles symmetrically appose across the membranes of the two cells. *Electron-dense plaques:* Electron-dense material presumably forms from the contents of the prefusion complex, and is also symmetrically distributed across the membranes of the two cells. *Fusion pores:* Gaps form as the membranes vesiculate, allowing cytoplasmic transfer and cell mixing.

Figure 3.

Model of *Drosophila* myoblast fusion. Myoblast fusion occurs between stages 12 and 15 (7.5–13 hours AEL) in two phases. At early stage 12, prior to myoblast fusion, mesodermal cells are arranged in multiple layers. The FCs (red and blue) are located externally, while the FCMs (green) are located both externally and internally. During the first phase of fusion (stage 12–13, 7.5–10.5 hours AEL), infrequent fusion events occur between the FCs and the external FCMs. The majority of fusion events occur in the second phase of fusion (stage 14–15, 10.5–13 hours AEL) as the internal FCMs dissociate from one another and migrate externally.

Table 1

Mean number of nuclei per muscle at stage 15

Muscle	Mean number of nuclei [*]	Range
DA1	9 ^a , 10 ^b	6–16 ^a
DO2	11 ^a	8–17 ^a
DT1	6 ^a	4–8 ^a
VT1	3 ^a , 4 ^c	2–4 ^a , 3–5 ^c
VA2	9 ^a	6–13 ^a
VA3	4 ^c	3–4 ^c
VL4	9 ^c	7–11 ^c

^{*} Rounded to the nearest whole number.

^a From Beckett and Baylies (2007).

^b From Menon et al. (2005).

^c From Bate (1990).

Table 2
Gene products required for *Drosophila* myoblast fusion

Gene Product	Expression	Localization	Domains	Proposed Function	Actin focus class*	References
Duf (Kirre)	FC	Membrane	Ig, TM, AP, PDZBD	Cell adhesion, attraction	1	Ruiz-Gomez et al. (2000); Strunkelberg et al. (2001)
Rst (Irre)	FC/FCM	Membrane	Ig, TM, AP, PDZBD	Cell adhesion, attraction	1	Strunkelberg et al. (2001)
Sns	FCM	Membrane	Ig, TM, FN3, PDZBD	Cell adhesion	1	Bour et al. (2000)
Hbs	FCM	Membrane	Ig, TM, FN3, PDZBD	Cell adhesion	Unknown	Artero et al. (2001); Dworak et al. (2001)
Rols (Ants)	FC	Membrane Cytoplasm	LM, RING, PL, AR, TPR, CC	Adapter, signaling	1	Menon and Chia (2001); Chen and Olson (2001); Rau et al. (2001)
D-Titin	FC/FCM	Cytoskeleton	Ig, FN3, PEVK	Cytoskeleton component	Unknown	Zhang et al. (2000); Machado et al. (2000)
Mbc	FC/FCM	Cytoplasm Cytoskeleton	SH3, DHR (GEF), CB	Cytoskeleton (Rac activation)	3	Rushton et al. (1995); Erickson et al. (1997)
Loner (Siz)	FC/FCM**	Cytoplasm	CC, Sec7 (GEF), IQ, PH	Cytoskeleton (Arp6/Rac activation)	2	Chen et al. (2003); Richardson et al. (2007)
Kette (Hem)	FC/FCM	Cytoplasm Cytoskeleton	Unknown	Cytoskeleton (Scar regulation)	3	Schroter et al. (2004)
Blow	FC/FCM***	Cytoplasm	PH	Unknown	3	Doberstein et al. (1997); Schroter et al. (2004)
Rac1	FC/FCM	Cytoplasm	GTPase	Cytoskeleton (Scar complex regulation)	3 (with Rac2)	Luo et al. (1994); Hakeda-Suzuki et al. (2002); Chen et al. (2003)
Rac2	Unknown	Unknown	GTPase	Cytoskeleton (Scar complex regulation)	3 (with Rac1)	Hakeda-Suzuki et al. (2002)

Gene Product	Expression	Localization	Domains	Proposed Function	Actin focus class [*]	References	
Scar	FC/FCM	Cytoplasm	Cytoskeleton	SHD, PR, VCA	Cytoskeleton (Arp2/3 activation)	3	Richardson et al. (2007)
Arp2/3	Unknown	Unknown	AR, WDR, ATPase	Cytoskeleton (Actin polymerization)	Unknown	Richardson et al. (2007)	
Sltr/D-WIP (Vrp1)	FC/FCM ^{***}	Cytoplasm	ProfBD, WH2, PR, W _{sp} BD	Cytoskeleton (WASP regulation)	2	Massarwa et al. (2007); Kim et al. (2007)	
WASP	Unknown	Cytoplasm	WH1, PIP2BD, GBD, PR, VCA	Cytoskeleton (Arp2/3 activation)	Unknown	Schafer et al. (2007)	
Sing	FC/FCM	Membrane (predicted)	MARVAL, TM	Vesicle Fusion	Unknown	Estrada et al. (2007)	

^{*} Actin foci classes from Richardson et al. (2007): 1 = no/reduced number of foci; 2 = increased foci number, wild-type size; 3 = increased foci number, increased size.

^{**} Originally reported as FC-specific, but more recently shown in both cell types.

^{***} Evidence that expression is first solely in FCMs, and then begins in myotubes following the first fusion event.

Abbreviations: Ig = Immunoglobulin, TM = Transmembrane, AP = Autophosphorylation, PDZBD = PDZ Binding Domain, FN3 = Fibronectin3, LM = Lipolytic Motif, RING = RING finger, PL = P-loop, AR = Ankyrin Repeats, TPR = Tetratricopeptide Repeats, CC = coiled-coiled, SH3 = Src Homology 3, DHR = Dock Homology Region, GEF = Guanine Nucleotide Exchange Factor, CB = Crk Binding, PH = Pleckstrin Homology, SHD = Scar Homology Domain, PR = Proline Repeats, VCA = WH2, Central and Acidic, AR = Actin Related, WDR = WD Repeat, ProfBD = Profilin Binding Domain, WH2 = WASP Homology 2, W_{sp}BD = Wasp Binding Domain, WH1 = WASP Homology 1, PIP2BD = PIP2 Binding Domain, GBD = GTPase Binding Domain.