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Towards a molecular pathway for myoblast fusion in *Drosophila*

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Intercellular fusion among myoblasts is required for the generation of multinucleated muscle fibers during skeletal muscle development. Recent studies in *Drosophila* have shed light on the molecular mechanisms that underlie this process, and a signaling pathway that relays fusion signals from the cell membrane to the cytoskeleton has emerged. In this article, we review these recent advances and discuss how *Drosophila* offers a powerful model system to study myoblast fusion *in vivo*.

Membrane fusion is one of the most fundamental processes in life. Cell–cell fusion is the most poorly understood of the three types of membrane-fusion events (intracellular fusion of organelles; virus–cell fusion and cell–cell fusion). Cell–cell fusion is crucial for the development of multicellular organisms and is required for processes as diverse as fertilization, the formation of bone and placenta, and myogenesis [1,2]. Despite the diversity of the cell types that undergo fusion, the cellular events that are involved in this process – cell recognition, adhesion and membrane merger – are common to all of these cell types, which suggests that shared molecular mechanisms might be used.

Myoblast fusion, by which mononucleated myoblasts fuse to form multinucleated muscle fibers, is an essential early step during skeletal muscle differentiation. Most studies of myoblast fusion during the past three decades have been carried out in mammalian cell-culture systems in which myoblast fusion can be synchronized [3,4]. These *in vitro* studies have implicated several classes of protein in myoblast fusion, including cell-adhesion molecules, metalloproteases, calmodulin, protein kinases and phospholipases [4,5]. However, it remains to be determined whether these proteins are involved in myoblast fusion *in vivo* (for a review of recent advances regarding the genes that regulate mammalian myoblast fusion, see Ref. [6]).

Considering the limitations of *in vitro* studies, an *in vivo* system is desirable for investigating the molecular mechanisms that underlie myoblast fusion. The fruit fly *Drosophila* provides an ideal paradigm for such a purpose. The somatic musculature (or larval body-wall muscle) of *Drosophila* is functionally equivalent to vertebrate skeletal muscle. As in vertebrates, myoblast fusion is an indispensable step during *Drosophila* myogenesis. Furthermore, the distinctive cellular changes during the fusion process, including myoblast recognition, adhesion, alignment and membrane coalescence, are morphologically similar between *Drosophila* and vertebrates [3,4,7]. Thus, it is conceivable that the genes that are involved in myoblast fusion in *Drosophila*, or a portion of them at least, have evolutionarily conserved roles in vertebrate myogenesis. Despite the similarities between fly and vertebrates, the *Drosophila* musculature is much less complex (at most, 30 myoblasts per fiber, compared with thousands of myoblasts per fiber in vertebrates) and its development takes less time (hours, compared with days and weeks in vertebrates) [8]. These features, together with the powerful molecular and genetic tools that are available, make *Drosophila* a tractable system to unravel the molecular mechanisms that control myoblast fusion *in vivo*. In this article, we discuss the basic developmental and cell biology of myoblast fusion in *Drosophila* and highlight recent advances in the molecular and genetic investigations of this process.

The developmental biology of myoblast fusion

Primary and secondary myotubes in vertebrates

Vertebrate skeletal muscles originate from the embryonic mesoderm. Skeletal muscle cells, or myoblasts, are derived from epithelial somites and are specified by the sequential actions of the paired-box transcription factor Pax-3 and the myogenic basic helix–loop–helix (bHLH) transcription factors MyoD and Myf5 [9]. The withdrawal of proliferating myoblasts from the cell cycle in response to extracellular cues is accompanied by the fusion of myoblasts to form multinucleated myotubes. The early wave of myoblast fusion produces primary myotubes that function as scaffolds for the later waves of fusion that lead to the formation of secondary and tertiary myotubes. During the final wave of embryonic myogenesis, a pool of 'muscle satellite cells' is formed. Some satellite cells remain quiescent for a period of time, after which they proliferate, differentiate and fuse with existing muscle fibers during exercise and injury, and in degenerative muscle diseases [10,11].

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Muscle founder cells and fusion-competent cells in Drosophila

Based on their different behaviors during fusion, two myoblast cell types have been revealed by studies of *Drosophila* myogenesis: muscle founder cells and fusion-competent cells. Muscle founder cells function as 'attrac-attractants' for the surrounding fusion-competent cells and they prefigure many properties of future muscle fibers, including position, orientation, size, attachment sites and patterns of nerve innervation [8]. Muscle founder cells are further divided into different subsets by the expression of different 'selector' transcription factors such as Nautilus, Krüppel, S59, Apterous, Vestigial, Even skipped and Ladybird [12,13]. The neighboring fusion-competent cells fuse with founder cells and, thereafter, adopt the same selector-gene expression profile. Initially, a founder cell fuses with one or two competent cells to form binucleated or trinucleated muscle precursors [14]. Additional rounds of fusion between these precursors and fusion-competent cells result in the formation of multinucleated myotubes [14]. Thus, myoblast fusion in *Drosophila* occurs in two step-wise phases. Recent *in vitro* studies of mammalian myoblast fusion have also revealed two phases of fusion: first, the fusion between a subset of myoblasts to form nascent myotubes and, second, additional rounds of fusion between myoblasts and nascent myotubes [6]. However, it is not clear whether the two-phase fusion process occurs *in vivo* and whether a founder-cell population exists during the first phase of mammalian myoblast fusion.

Muscle founder cells and fusion-competent cells are specified by a hierarchy of transcription factors during *Drosophila* myogenesis [5,12,13,15] (Figure 1). During early embryogenesis, the bHLH transcription factor Twist (Twi) is required to specify the embryonic mesoderm. After gastrulation, the mesoderm is subdivided into regions of alternating high and low Twi expression. The domains with high levels of Twi expression contain clusters of cells that express another gene, *lethal of scute*, that encodes a bHLH transcription factor. These clusters of cells form the so-called myogenic equivalence groups. One muscle progenitor cell from each myogenic equivalence group is then specified by a Notch- and Delta-mediated lateral inhibition process. This single cell undergoes one round of asymmetric cell division to generate either two muscle founder cells or one founder cell and one adult muscle precursor. The remaining cells of the myogenic equivalence group differentiate as fusion-competent cells. This later stage of myogenic differentiation also seems to be controlled by additional transcription factors. For example, *lame duck* (*lmd*) [also called *myoblast incompetent* (*minc*) and *gleeful* (*glee*)] encodes a Gli family transcription factor that is required for the differentiation of fusion-competent cells [16–18]. In *lmd*/*minc*-mutant embryos, there is an absence of fusion-competent cells, whereas founder cells are properly specified. Interestingly, one of the downstream target genes of *lmd*/*minc*/*glee* is *Dmef*2, which encodes a MADS-box transcription factor that is required for the differentiation of all the somatic, cardiac and visceral muscle lineages. At present, it is not clear whether other transcription factors are required for the differentiation of all muscle founder cells, as Lmd/Minc/Glee is in fusion-competent cells.

Cellular aspects of myoblast fusion

Like other types of cell–cell fusion events, myoblast fusion is a multistep process. The initial steps of cell recognition and adhesion can be observed readily at the light-microscopy level. In *Drosophila*, for example, fusion-competent cells are seen to extend membrane protrusions (filopodia) towards founder cells and the tips of the filopodia are observed to be attached to the founder-cell membrane [19]. The electron microscopy (EM) studies of *Drosophila* myoblast fusion that were carried out by Doberstein *et al.* are particularly informative with respect to the subcellular changes that follow the initial recognition and adhesion of myoblasts [7] (Figure 2). The authors observed paired vesicles (called prefusion complexes) that had electron-dense margins at the sites of cell–cell contact. These vesicles line up with each other across the apposed membranes of two adhering myoblasts. The prefusion complex then resolves into electron-dense plaques between apposed myoblasts while the two cells become elongated and align themselves along their long axes. Subsequently, cytoplasmic continuity forms through multiple small zones (fusion pores) between the apposed plasma membranes, followed by vesiculation of the residual membranes. Eventually, these events lead to the formation of multinucleated myotubes.

These detailed cell biology studies of myoblast fusion have raised many questions regarding the mechanisms that underlie this process. How do fusion-competent cells sense the signal from founder cells for fusion? What mediates the attraction and adhesion between the two cell populations? How are fusion signals transduced to the cytoskeleton to affect its rearrangement, which is a prerequisite for cell alignment and fusion? What are the components of the prefusion complex? What mediates the breakdown of the plasma membrane and how do fusion pores form? A genetic approach to address these fundamental questions is to isolate mutations that cause specific defects in myoblast fusion. The identification and the functional characterization of the corresponding genes are beginning to reveal a signaling cascade that transduces the fusion signal from the cell surface to changes in the cytoskeleton during *Drosophila* myoblast fusion. These recent advances are discussed later.

The molecular biology of myoblast fusion

Myoblast recognition and adhesion: the transmembrane receptors

The first step during myoblast fusion is the recognition between muscle founder cells and fusion-competent cells. This seems to be mediated by cell-type-specific transmembrane receptors (Figure 3 and Table 1). In founder cells, two immunoglobulin (Ig)-domain-containing cell-adhesion molecules – Dumbfounded (Duf) [also called Kin of Irregular chiasm C (Kirre)] and Roughest (Rst) [also called Irregular chiasm C (IrreC)] – function redundantly to attract fusion-competent cells [20,21]. The deletion of both *duf* and *rst* causes a complete block of fusion, whereas the overexpression of either gene can attract fusion-competent

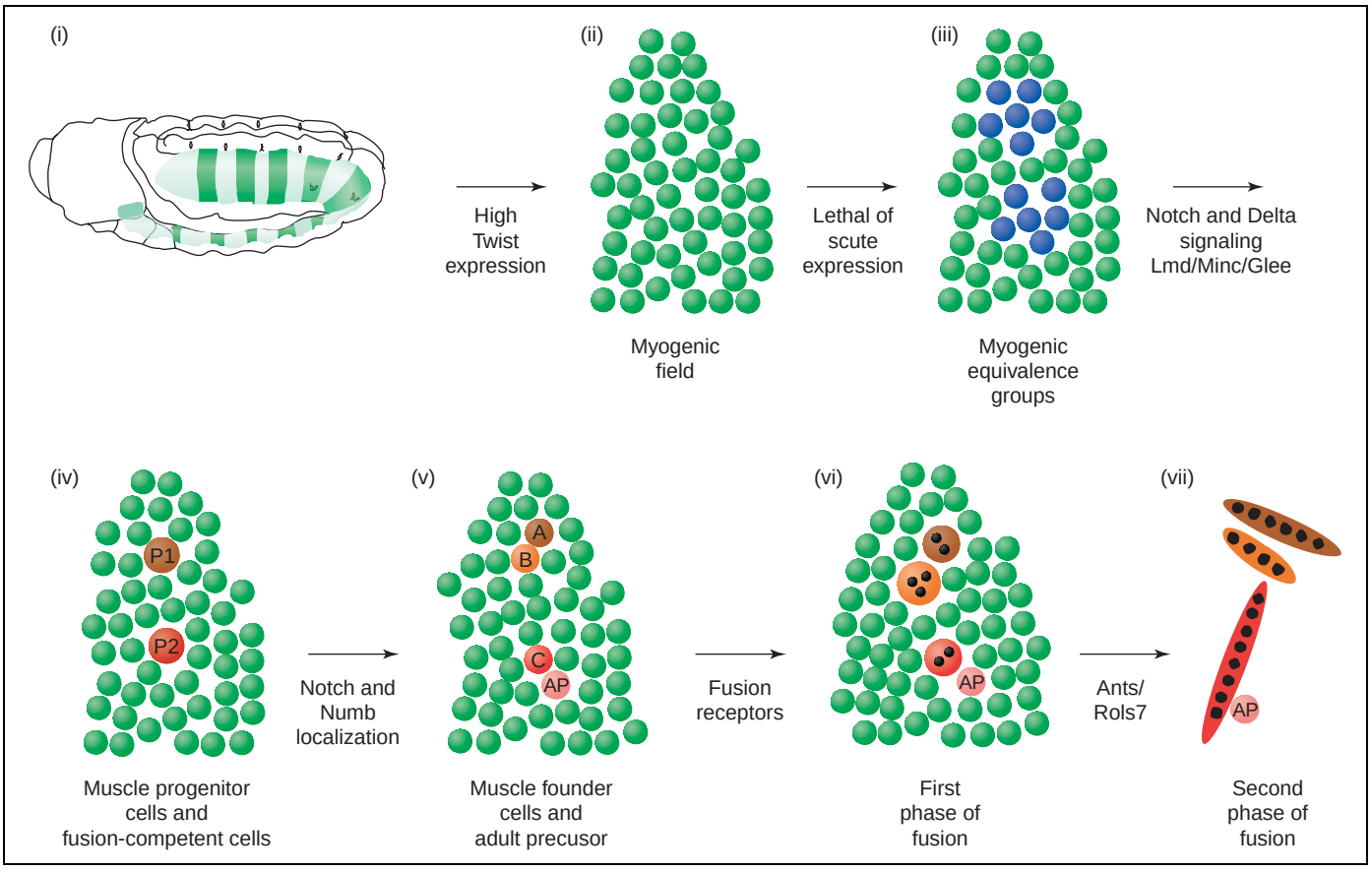

Figure 1. Overview of *Drosophila* muscle development. **(i)** A stage-11 embryo showing alternating levels of Twist (Twi) expression. Cells that express high levels of Twi (dark green) acquire a myogenic fate **(ii)**. **(iii)** Clusters of cells (myogenic equivalence groups; blue) within the myogenic field express Lethal of scute. **(iv)** A muscle progenitor cell (P1 or P2) is singled out from each equivalence group by a lateral inhibition process that is mediated by Notch and Delta signaling. The remaining cells in the equivalence group are specified to become fusion-competent cells by a process that requires the transcription factor Lmd/Minc/Glee. **(v)** Each progenitor cell undergoes asymmetric cell division to produce either two founder cells (A and B) or one founder cell (C) and one adult muscle precursor (AP). Each founder cell expresses a specific muscle-identity gene that is also known as a selector gene. **(vi)** Founder cells attract surrounding fusion-competent cells to fuse with them. This is mediated by specific 'fusion receptors' and downstream signaling components. The first phase of fusion yields binucleated or trinucleated muscle precursors. A fusion-competent cell expresses the same selector gene after fusing with a founder cell. **(vii)** Muscle precursors continue to attract additional fusion-competent cells in the second phase of fusion, which requires the function of Antisocial (Ants) [also called Rolling pebbles (Rols7)] and leads to the formation of multinucleated myotubes. Modified, with permission, from Ref. [13].

myoblasts to the ectopic sites of expression. In fusion-competent cells, Sticks and stones (Sns), which is also an Ig-domain-containing cell-adhesion molecule, is required for fusion because the loss of *sns* results in a lack of fusion [22]. Another fusion-competent cell-specific cell-adhesion molecule is the paralog of Sns Hibris (Hbs) [23,24]. Hbs is not essential for myoblast fusion but it seems to inhibit Sns function. The overexpression of *hbs* blocks myoblast fusion, whereas the loss of *hbs* causes only minor fusion defects.

The careful examination of the cellular behavior of fusion-competent cells in *duf rst* double-mutant or *sns* single-mutant embryos revealed that these myoblasts do extend filopodia, albeit with random orientations [5,20]. The failure of these filopodia to attach to founder cells is consistent with the hypothesis that Duf, Rst and Sns are required for the initial recognition and adhesion between the two cell populations. In addition, there is evidence that Duf and Sns might interact directly with each other to mediate cell adhesion because cultured *Drosophila* cells (S2 cells) that express Duf can aggregate with Sns-expressing cells [15,23].

It remains to be determined how fusion-competent cells are attracted to the founder cells initially. One possibility is that fusion-competent cells randomly extend filopodia to locate the founder cells. Alternatively, fusion-competent cells might sense a kind of concentration gradient from the founder cells and extend filopodia specifically in that direction. It is also unclear how the sites of fusion are selected. For example, the transmembrane protein Duf might be localized to predetermined sites in founder cells by intrinsic cues. Alternatively, extrinsic contacts made by the filopodia from fusion-competent cells could have a role in determining Duf localization in founder cells. Detailed studies of receptor localization during the fusion process will provide clues to the answers to these questions.

Signal transduction: from membrane to cytoskeleton

Two events occur after a fusion-competent cell makes contact with a founder cell. First, the fusion-competent cell moves towards the founder cell. Second, the fusion-competent cell aligns with the founder cell, thus juxtaposing the two cell membranes. These cellular events require changes in the actin cytoskeleton. Thus, rearrangement of the actin cytoskeleton in both founder cells and fusion-competent cells is a prerequisite for myoblast fusion. How is the fusion signal transduced to the cytoskeleton to effect the rearrangement of the cytoskeleton? The recent

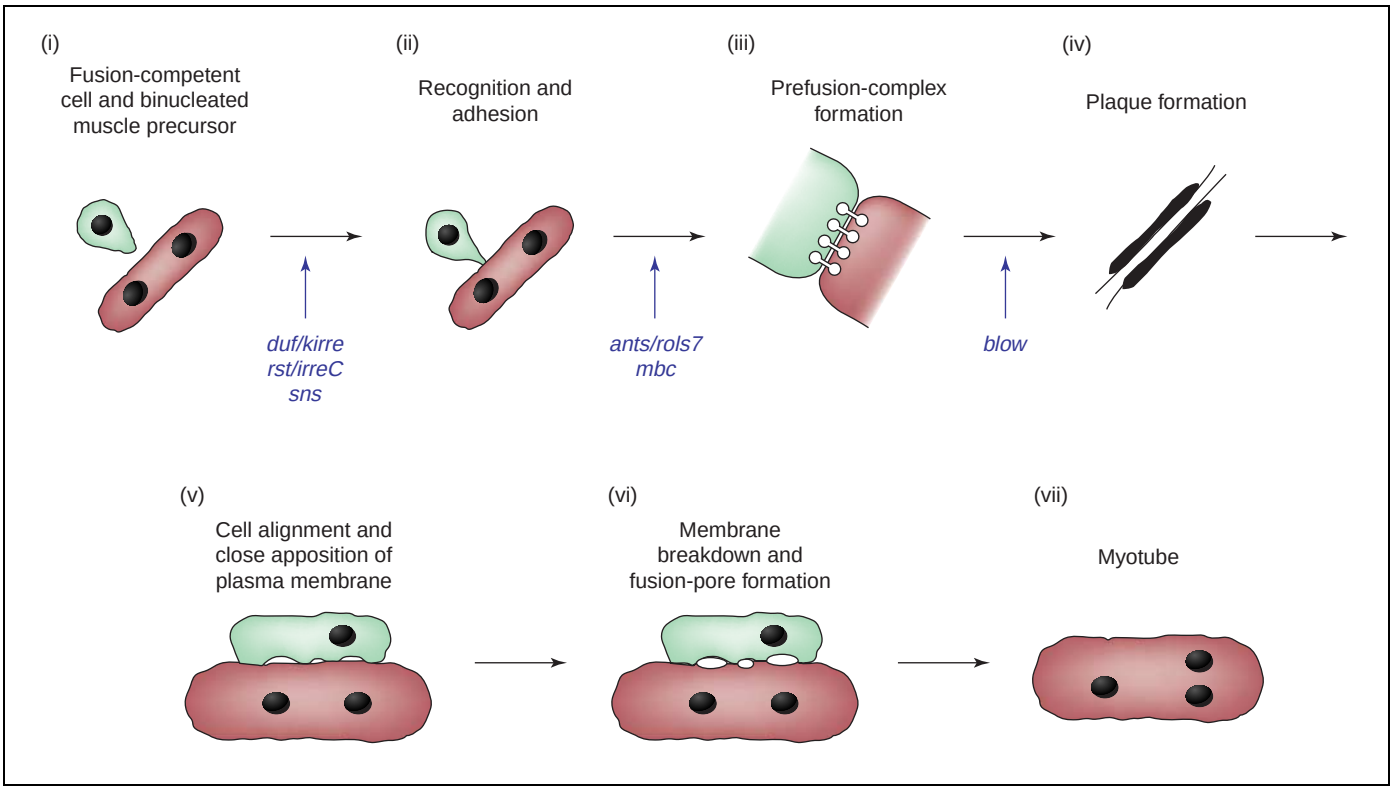

Figure 2. Myoblast fusion is a multistep process. **(i)** A fusion-competent cell (green) extends filopodia towards a binucleated muscle precursor (brown). **(ii)** The fusion-competent cell recognizes and attaches to the muscle precursor. **(iii)** Paired vesicles with electron-dense margins (prefusion complexes) form along the apposed membranes. **(iv)** An electron-dense plaque forms along the membranes. **(v)** The cells align along their entire long axes. **(vi)** The apposed membranes break down, accompanied by the formation of fusion pores. **(vii)** A multinucleated myotube is formed. The genes illustrated in blue have been shown to function at different stages of myoblast fusion. Modified, with permission, from Ref. [7]. © (1997) Rockefeller University Press.

identification of Antisocial (Ants) [also called Rolling pebbles (Rols7)], which is an adaptor protein that links the fusion receptor to components of the cytoskeleton, and Loner, which is a regulator of the ADP-ribosylation factor (ARF)6 small GTPase, has provided insights into the signaling mechanisms that relay the fusion signal from the fusion receptor to the cytoskeleton in founder cells (Figure 3 and Table 1).

Ants/Rols7: an adaptor protein that links fusion receptors to the cytoskeleton in founder cells

The identification of the founder-cell-specific adaptor protein Ants/Rols7 has facilitated the understanding of signal transduction in founder cells [25–27]. The gene *ants/rols7* encodes a protein that has multiple potential protein–protein-interaction motifs, including nine ankyrin repeats, three tetratricopeptide repeats and a

Table 1. Proteins involved in *Drosophila* myoblast fusion[a]

Protein	Localization	Structure	Vertebrate homolog	Refs
Transmembrane receptors				
Dumbfounded (or Kin of irregular chiasm C)	FC	Ig domains;TM; APD; PDZ-binding motif	DM-GRASP/BEN/SC1	[20]
Roughest (or Irregular chiasm C)	FC, FCC	Ig domains; TM; APD; PDZ binding motif	DM-GRASP/BEN/SC1	[21]
Sticks and stones	FCC	Ig domains; fibronectin type-III domain; TM; target sites for kinases	nephrin	[22]
Hibris	FCC	Ig domains; fibronectin type-III domain; TM; target sites for kinases	nephrin	[23,24]
Intracellular proteins				
Antisocial (or Rolling pebbles)	FC	Lipolytic-enzyme signature sequence; ATP- and GTP-binding site; ankyrin repeats; TPR; coiled-coil domain	mants	[25–27]
Loner	FC	IQ motif; Sec7 domain; PH domain; coiled-coil domain	ARF–GEP_{100}	[30]
Myoblast city	FC, FCC	SH3 domain; Crk binding sites; Docker domain	Dock180	[31,32]
DCrk	Mesoderm	SH2 and SH3 domains	CRK-II and CRKL	[40]
Drac1 and Drac2	Mesoderm	GTPase	Rac	[39]
dARF6	Ubiquitous	GTPase	ARF6	[30]
D-Titin	FC, FCC	Ig domains; fibronectin type-III domains; PEVK domain	Titin	[28,29]
Paramyosin	Mesoderm	Coiled-coil domains	Unknown	[45]
Blown fuse	FC, FCC	PH domain	Unknown	[7]

[a]Abbreviations: APD, autophosphorylation domain; ARF, ADP-ribosylation factor; FC, founder cell; FCC, fusion-competent cell; GEP, guanine-nucleotide-exchange protein; Ig, immunoglobulin; PH, pleckstrin homology; SH, Src homology; TM, transmembrane domain; TPR, tetratricopeptide repeat.

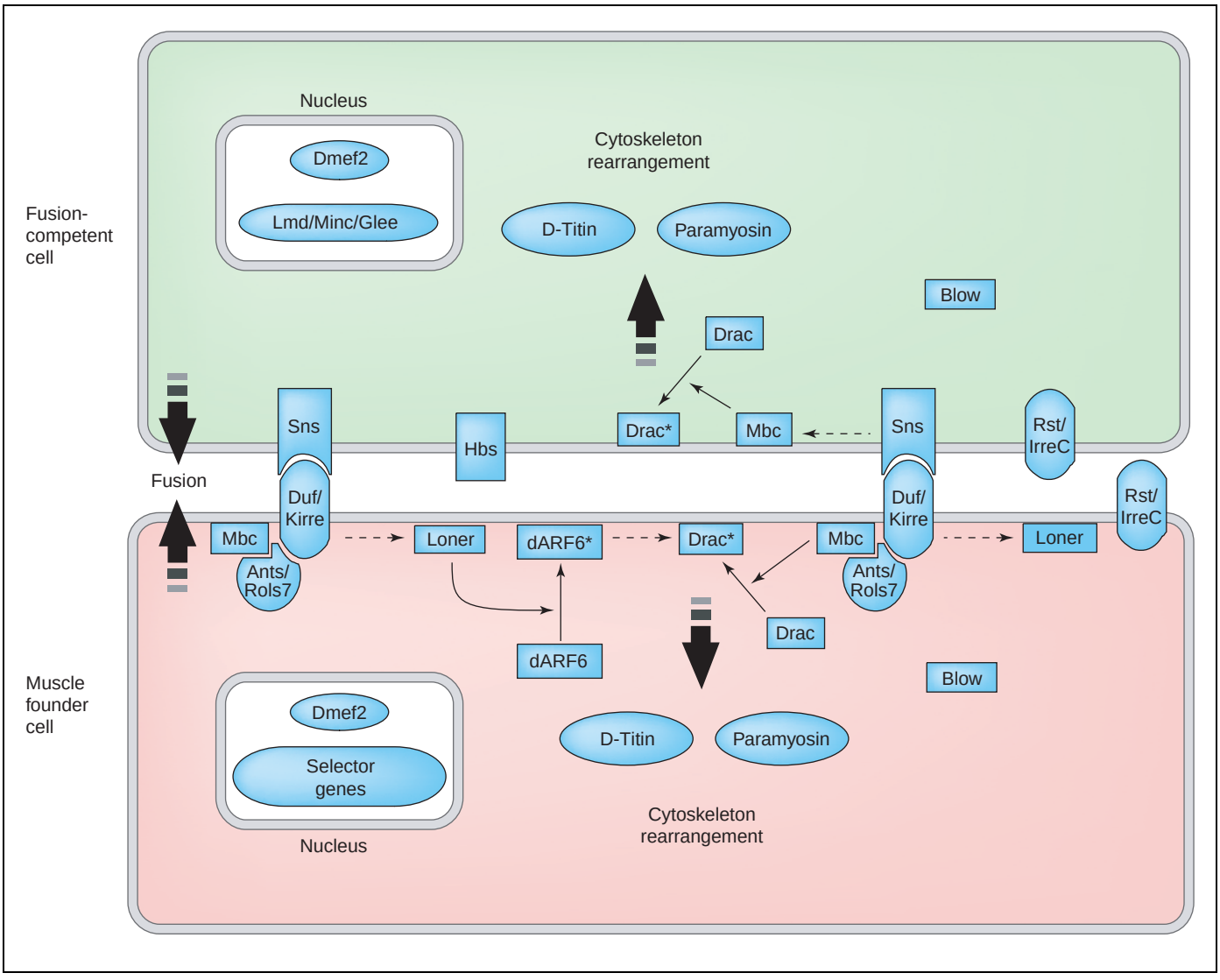

Figure 3. A model of myoblast fusion in *Drosophila*. A muscle founder cell (pink) functions as an 'attractant' for a fusion-competent cell (green). The identity of a muscle founder cell is specified by selector genes (in the nucleus). Fusion-competent cells are specified by the transcription factor Lmd/Minc/Glee. Dmef2 is required for the muscle differentiation of both founder and fusion-competent cells. The transmembrane receptors Dumbfounded (Duf) [also called Kin of Irregular chiasm C (Kirre)] and Roughest (Rst) [also called Irregular chiasm C (IrreC)] are expressed and required in the founder cell (Rst/IrreC is also present in the fusion-competent cell), whereas two other receptors, Sticks and stones (Sns) and Hibris (Hbs), are expressed specifically in the fusion-competent cell. Duf/Kirre and Sns might interact with each other to mediate cell adhesion. Duf/Kirre might also interact with Hbs, and Rst/IrreC might interact with itself and Sns (not shown). In the muscle founder cell, Antisocial (Ants) [also called Rolling pebbles (Rols7)] functions as an adaptor protein that interacts with both Duf/Kirre and Myoblast city (Mbc) to assist the transduction of fusion signals. Mbc is an unconventional guanine-nucleotide-exchange factor (GEF) that activates the small GTPase Drac (active form denoted by *). Duf/Kirre also independently recruits Loner, which is an ADP-ribosylation factor (ARF)6 GEF, to sites of fusion. Loner activates *Drosophila* ARF6 (dARF6; activated form denoted by *) and the Loner–dARF6 module is required for the proper subcellular localization of Drac. In turn, Drac regulates actin-cytoskeleton rearrangements. The downstream effectors of Drac might include the structural proteins D-Titin and Paramyosin. Blow is a pleckstrin homology (PH)-domain-containing cytoplasmic protein that has an unknown function at present. Unbroken arrows indicate direct interactions and the conversion of Drac and dARF6 from inactivated to activated states. Broken arrows indicate indirect interactions that are probably mediated by additional proteins. Relatively little is known about the signaling components in the fusion-competent cell. It is not clear whether this cell type has an adaptor protein such as Ants/Rols7 and whether there is a module that is similar to Loner and ARF6. Modified, with permission, from Ref. [30].

coiled-coil domain. It also contains a RING finger and a lipolytic-enzyme signature sequence. Mutations in *ants/rols7* block the fusion process after the initial step of myoblast recognition and adhesion. Occasionally, fusion proceeds to a binucleated or trinucleated stage, which suggests that *ants/rols7* is essential for the second phase of fusion but might have a redundant role or no function during the first phase [26,27]. *ants/rols7* is expressed specifically in founder cells at the time of fusion and, strikingly, the protein is localized to discrete subcellular foci [25,26]. These discrete foci correspond to subcellular sites of fusion, as revealed by their colocalization with the structural protein D-Titin that localizes to the sites of myoblast contact [26,28,29]. Interestingly, the specific subcellular localization of Ants/Rols7 depends on the founder-cell-specific transmembrane receptors Duf and Rst. Ants/Rols7 is distributed throughout the cytoplasm in *duf rst* double-mutant embryos, whereas it is localized to specific subcellular foci in wild-type embryos [25,26]. Consistent with Duf being required for the subcellular localization of Ants/Rols7, Duf can associate physically with Ants/Rols7 and recruit it from the cytoplasm to the membrane-contact regions between aggregating S2 cells [25,30].

Further insights into the function of Ants/Rols7 have come from physical interactions detected between Ants

and Myoblast city (Mbc) [25], which is another essential component of the myoblast-fusion process [31,32]. *Drosophila* Mbc belongs to the CDM family of proteins that also includes *Caenorhabditis elegans* Ced-5, and mammalian Dock180 and Dock2 [33]. CDM proteins in *C. elegans* and mammalian cells are involved in an evolutionarily conserved signaling pathway (Ced-2, Ced-12, Ced-5 and Ced-10 in *C. elegans* and CrkII, ELMO, Dock180 and Rac in mammals) that modulates the small GTPase Rac, which is a crucial regulator of cytoskeletal dynamics [34–36]. This pathway mediates cytoskeletal rearrangements during the phagocytosis of apoptotic cells and during cell movements [37]. It has been suggested that Dock180 forms an unconventional two-part guanine-nucleotide-exchange factor (GEF) for Rac with the ELMO protein [38]. It is conceivable that *Drosophila* Mbc also regulates the activity of the small GTPase Drac during myoblast fusion, although the signaling mechanisms of Mbc are understood less well. Consistent with this hypothesis, Drac1 and Drac2 are required for myoblast fusion in *Drosophila* [39]. The physical interactions between Ants and Mbc and between Ants and Duf suggest that Ants could function as an intermediary protein that relays the fusion signal from the cell-surface receptor Duf to the cytoskeleton through the regulation of Mbc and Drac activity [25]. It remains to be determined whether Ants/Rols7 regulates the GEF activity or the subcellular localization of Mbc. Furthermore, it will be interesting to investigate whether the homologs of CrkII and ELMO are involved in myoblast fusion in *Drosophila* [40].

Loner: a guanine-nucleotide-exchange factor that regulates the ARF6 small GTPase during myoblast fusion

The recent characterization of the fusion-defective mutant *loner* has provided a new element to the understanding of the signaling cascade that regulates cytoskeletal rearrangement during *Drosophila* myoblast fusion (Figure 3 and Table 1). The *loner* gene encodes a putative GEF that contains a Sec7 domain and an adjacent pleckstrin homology (PH) domain [30]. The Sec7 domain is found in GEFs for the ARF family of small GTPases [41], whereas PH domains have been implicated in binding to phospholipids in the plasma membrane [42]. Rescue experiments have demonstrated that both of these domains are essential for the function of Loner *in vivo* [30]. Loner is expressed in founder cells, in which it is localized in discrete subcellular foci (as is the case for Ants/Rols7). However, Loner is colocalized only partially with Ants, which suggests that only a portion of the Loner protein is localized to the sites of fusion. The transmembrane receptor Duf is required for the proper subcellular localization of Loner in founder cells, which is also the case for Ants. Furthermore, Duf can recruit Loner from the cytoplasm to the membrane-contact regions between aggregating S2 cells. However, the subcellular localization of Loner is not dependent on that of Ants and vice versa. Thus, it seems that Ants and Loner are recruited independently to sites of fusion by the transmembrane receptor Duf [30].

How does Loner mediate myoblast fusion? The presence of a Sec7 domain suggests that Loner might function as a GEF for the ARF family of small GTPases. *In vitro*, the purified Sec7 domain of Loner displays specific GEF activity towards *Drosophila* ARF6 (dARF6), which suggests that dARF6 might be a physiological target of Loner [30]. Consistent with this hypothesis, the overexpression of a dominant negative form of dARF6 in founder cells blocks myoblast fusion [30]. Together, these observations reveal a novel Loner–dARF6-mediated signaling module that has an essential role in myoblast fusion. However, loss-of-function mutations of *dARF6* will, ultimately, be required to strengthen this conclusion.

The relationships between the small GTPases dARF6 and Drac1

The identification of dARF6 and Drac1 as essential components of myoblast fusion raises important questions regarding the relationships between these two small GTPases during the fusion process. Studies in cultured mammalian cells have implicated ARF6 in membrane trafficking and actin-cytoskeleton rearrangements, which are two processes that have potential relevance to myoblast fusion [43]. In particular, there is evidence that ARF6 regulates cytoskeletal rearrangement by controlling the subcellular localization of Rac1 [44]. In *Drosophila* muscle founder cells, the Loner–dARF6 module seems to control the subcellular localization of Drac1. In *loner*-mutant embryos, Drac1 is distributed throughout the cytoplasm rather than being concentrated to the sites of fusion, as is seen in wild-type embryos [30]. Thus, similar to what occurs in mammalian cells, the Loner–dARF6 module could signal to the actin cytoskeleton through the regulation of Drac1 (Figure 3 and Table 1). However, considering the widespread roles for ARF6 in diverse processes, such as its regulation of the enzymes that are responsible for lipid modification and its involvement in regulated secretion events, it remains to be determined whether these other functions of ARF6 also contribute to myoblast fusion.

The downstream effectors of Drac

Considering the pivotal role of Drac in *Drosophila* myoblast fusion, it would be interesting to determine the downstream effectors of Drac during actin-cytoskeleton rearrangement. The characterization of the structural proteins D-Titin and Paramyosin in muscle development might help to do this. D-Titin and Paramyosin were identified initially as sarcomeric proteins. However, recent studies have revealed unexpected functions for them during myoblast fusion [28,45]. Both proteins are present at myoblast-contact sites during fusion and are important, although not essential, for the fusion process [28,29,45]. In addition, the proper localization of D-Titin is dependent on Ants/Rols7 (the adaptor protein that is associated with the putative Drac1 GEF Mbc) [27]. These studies, together with the interactions between D-Titin and the actin cytoskeleton and between Paramyosin and the actin cytoskeleton, have led to suggestions that the two structural proteins have a role in the organization of the actin-cytoskeleton elements that are required for fusion [28,29,45] and that they might be among the many downstream effectors of Drac (Figure 3 and Table 1).

Questions outstanding

Studies of *Drosophila* myoblast fusion are beginning to reveal a signaling pathway in muscle founder cells that transduces signals from fusion receptors into changes in the cytoskeleton. Meanwhile, these studies raise new questions for future investigations, as highlighted next.

Identification of components of a 'fusion complex'

The presence of multiple potential protein–protein-interaction motifs in Ants/Rols7, combined with the observation that Duf recruits both Ants/Rols7 and Loner to sites of fusion, suggests that Duf and Ants/Rols7 might function within a scaffold to anchor multiple proteins to the sites of fusion, where a multiprotein 'fusion complex' mediates the cellular changes that accompany myoblast fusion. The identification of additional components of this fusion complex, through both genetic and biochemical approaches, is likely to provide important insights into myoblast fusion. It will also be important to examine the subcellular localization of the fusion complex at the EM level to determine how the fusion complex relates to the distinct ultrastructural entities that have been observed during myoblast fusion, such as paired vesicles and plaques.

How do juxtaposed membranes fuse with each other?

Cytoskeletal rearrangement is a prerequisite for the membrane merger of two apposing cells. It is required for the two membranes to align effectively so that their lipid bilayers are closely juxtaposed for fusion to proceed. Little is known about the actual fusion process. For example, it is unclear how the two membranes are destabilized, how fusion pores form and which molecules are involved in these events. During virus–cell fusion, a hydrophobic peptide in the fusogenic viral glycoprotein mediates the juxtaposition and fusion of two membranes [2,46], although no fusogen-like sequences have been identified in the known proteins that are involved in myoblast fusion. However, the founder-cell adaptor protein Ants/Rols7 contains a lipolytic-enzyme signature sequence that is often present in lipases that are involved in the modification of the lipid bilayer [47]. An isoform of Ants/Rols7 that lacks the N-terminal region that includes the lipolytic-enzyme signature sequence can no longer rescue the myoblast-fusion phenotype in *ants*/*rols7*-mutant embryos [26]. It will be interesting to determine the specific contribution of this lipolytic-enzyme signature sequence to membrane dynamics during myoblast fusion.

Signal transduction in fusion-competent cells

Little is known about how fusion signals are transduced in fusion-competent cells. The cytoplasmic region of the transmembrane receptor Sns, which is specific to fusion-competent cells, contains proline-rich sequences, potential phosphorylation sites for various kinases and stretches of evolutionarily conserved sequences that have unknown physiological functions [22]. Mbc, which regulates the cytoskeleton in founder cells, is also present in fusion-competent cells and might provide a similar function by regulating Drac and cytoskeletal rearrangements during fusion [32]. It will be interesting to determine whether *Drosophila* homologs of CrkII and ELMO, in addition to Mbc and Drac, are required in fusion-competent cells. It will also be interesting to find out whether there is an adaptor protein in fusion-competent cells that is equivalent to Ants/Rols7 in founder cells and that links the Sns receptor to the cytoskeleton. Ongoing genetic screens in *Drosophila* might identify these and other potential components of fusion-competent cells and shed light on the signal-transduction pathway that is employed in this cell type.

Drosophila *myoblast fusion: relevance to mammalian myogenesis*

Given the conserved cellular events that are involved in *Drosophila* myoblast fusion and mammalian myogenesis, it is conceivable that the genes that are required for *Drosophila* myoblast fusion might have conserved roles in mammalian myogenesis. Curiously, the mammalian homologs of the Ig-domain-containing transmembrane receptors Duf, Rst, Sns and Hbs are not expressed in the developing mesoderm. In fact, the mouse homolog of *duf* and *rst* (*SC-1*) is expressed predominantly in the nervous system [48]. In addition, the mouse homolog of Sns and Hbs (nephrin) has been implicated in kidney development [49]. Thus, it seems that the initial recognition and adhesion between myoblasts during vertebrate myoblast fusion might use a different set of transmembrane receptors. This might reflect the differences in the molecular events that lead to the specification of myoblasts in flies and vertebrates. However, preliminary studies suggest that the intracellular components of the myoblast-fusion network might be conserved between *Drosophila* and vertebrates after a fusion signal has triggered the recognition and adhesion of myoblasts. One of the mouse orthologs of *ants*, *mants1*, is expressed in a variety of mesodermal tissues, including somites, limb buds and body-wall muscles [25]. The transient expression of *mants1* coincides with muscle differentiation, which suggests that it might have a role in muscle differentiation and myoblast fusion. The Loner–ARF6 module might also have a role in mammalian myogenesis because a dominant negative form of ARF6 blocks MyoD-induced myotube formation in a cell-culture model [30]. Future experiments involving knockout or transgenic mice should address definitively whether these fusion genes have conserved roles in mammalian myoblast fusion.

Myoblast fusion and muscle disease

Most studies of human muscle disease have focused on genes such as dystrophin that affect the sarcolemma [47]. Because embryonic myogenesis requires myoblast fusion to occur, complete loss-of-function mutations in fusion genes are likely to cause embryonic lethality. However, hypomorphic alleles of these genes might result in congenital or postnatal muscle diseases. In fact, both centronuclear myopathy and myotonic dystrophy are characterized by minute myofibers, which suggests that myoblast fusion might be defective in these muscle diseases [50,51]. In addition to its role during myogenesis, myoblast fusion is also required for muscle growth and repair during exercise and muscle injury. For example,

satellite cells can proliferate and fuse with existing myotubes during exercise or they can fuse with injured muscle fibers to repair lesions. It is conceivable that similar molecular mechanisms might be involved in adult satellite-cell fusion and in myoblast fusion during embryogenesis. Therefore, certain types of adult myopathies might be associated with defects in the genes that are required for myoblast fusion during myogenesis. The elucidation of the molecular and cellular mechanisms of myoblast fusion might provide insights into this intriguing cell biology phenomenon and lead to an understanding of and, ultimately, therapeutic interventions in human muscle diseases.

Concluding remarks

Recent studies in the fruit fly *Drosophila* have provided novel insights into the molecular mechanisms that control myoblast fusion during myogenesis. However, it is likely that only the tip of the iceberg has been uncovered so far. Future studies that combine genetics with biochemical, cell biology and genomic approaches will, undoubtedly, provide this area of investigation with more exciting discoveries. In addition, the combination of insights from studies in both *Drosophila* and vertebrates will facilitate our understanding of this fascinating biological process.

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References

1 Blumenthal, R. *et al*. (2003) Membrane fusion. *Chem. Rev.* 103, 53–69
2 Hernandez, L.D. *et al*. (1996) Virus–cell and cell–cell fusion. *Annu. Rev. Cell Dev. Biol.* 12, 627–661
3 Wakelam, M.J. (1985) The fusion of myoblasts. *Biochem. J.* 228, 1–12
4 Knudsen, K.A. (1992) Fusion of myoblasts. In *Membrane Fusion* (Wilschut, J. and Hoekstra, D. eds), pp. 601–626, Marcel Decker
5 Abmayr, S.M. *et al*. (2003) Cell and molecular biology of myoblast fusion. *Int. Rev. Cytol.* 225, 33–89
6 Horsley, V. and Pavlath, G.K. (2004) Forming a multinucleated cell: molecules that regulate myoblast fusion. *Cells Tissues Organs* 176, 67–78
7 Doberstein, S.K. *et al*. (1997) Genetic analysis of myoblast fusion: *blown fuse* is required for progression beyond the prefusion complex. *J. Cell Biol.* 136, 1249–1261
8 Bate, M. (1993) The mesoderm and its derivatives. In *The Development of* Drosophila melanogaster (Bate, M. and Martinez Ariazs, A. eds), pp. 1013–1090, Cold Spring Harbor Laboratory Press
9 Buckingham, M. (2001) Skeletal muscle formation in vertebrates. *Curr. Opin. Genet. Dev.* 11, 440–448
10 Bischoff, R. (1994) The satellite cell and muscle regeneration. In *Myogenesis* (Engel, A.G. and Franszini-Armstrong, C. eds), pp. 97–118, McGraw-Hill
11 Seale, P. *et al*. (2001) The potential of muscle stem cells. *Dev. Cell* 1, 333–342
12 Frasch, M. (1999) Controls in patterning and diversification of somatic muscles during *Drosophila* embryogenesis. *Curr. Opin. Genet. Dev.* 9, 522–529
13 Baylies, M.K. *et al*. (1998) Myogenesis: a view from *Drosophila*. *Cell* 93, 921–927
14 Bate, M. (1990) The embryonic development of larval muscles in *Drosophila*. *Development* 110, 791–804
15 Dworak, H.A. and Sink, H. (2002) Myoblast fusion in *Drosophila*. *Bioessays* 24, 591–601
16 Duan, H. *et al*. (2001) *Drosophila* Lame duck, a novel member of the Gli superfamily, acts as a key regulator of myogenesis by controlling fusion-competent myoblast development. *Development* 128, 4489–4500
17 Furlong, E.E. *et al*. (2001) Patterns of gene expression during *Drosophila* mesoderm development. *Science* 293, 1629–1633
18 Ruiz-Gomez, M. *et al*. (2002) *myoblasts incompetent* encodes a zinc finger transcription factor required to specify fusion-competent myoblasts in *Drosophila*. *Development* 129, 133–141
19 Paululat, A. *et al*. (1999) Essential genes for myoblast fusion in *Drosophila* embryogenesis. *Mech. Dev.* 83, 17–26
20 Ruiz-Gomez, M. *et al*. (2000) *Drosophila* dumbfounded: a myoblast attractant essential for fusion. *Cell* 102, 189–198
21 Strunkelnberg, M. *et al*. (2001) *rst* and its paralogue *kirre* act redundantly during embryonic muscle development in *Drosophila*. *Development* 128, 4229–4239
22 Bour, B.A. *et al*. (2000) *Drosophila* SNS, a member of the immunoglobulin superfamily that is essential for myoblast fusion. *Genes Dev.* 14, 1498–1511
23 Dworak, H.A. *et al*. (2001) Characterization of *Drosophila hibris*, a gene related to human nephrin. *Development* 128, 4265–4276
24 Artero, R.D. *et al*. (2001) The immunoglobulin-like protein Hibris functions as a dose-dependent regulator of myoblast fusion and is differentially controlled by Ras and Notch signaling. *Development* 128, 4251–4264
25 Chen, E.H. and Olson, E.N. (2001) Antisocial, an intracellular adaptor protein, is required for myoblast fusion in *Drosophila*. *Dev. Cell* 1, 705–715
26 Menon, S.D. and Chia, W. (2001) *Drosophila* rolling pebbles: a multidomain protein required for myoblast fusion that recruits D-Titin in response to the myoblast attractant Dumbfounded. *Dev. Cell* 1, 691–703
27 Rau, A. *et al*. (2001) *rolling pebbles* (*rols*) is required in *Drosophila* muscle precursors for recruitment of myoblasts for fusion. *Development* 128, 5061–5073
28 Zhang, Y. *et al*. (2000) *Drosophila* D-titin is required for myoblast fusion and skeletal muscle striation. *J. Cell Sci.* 113, 3103–3115
29 Machado, C. and Andrew, D.J. (2000) D-Titin: a giant protein with dual roles in chromosomes and muscles. *J. Cell Biol.* 151, 639–652
30 Chen, E.H. *et al*. (2003) Control of myoblast fusion by a guanine nucleotide exchange factor, Loner, and its effector ARF6. *Cell* 114, 751–762
31 Rushton, E. *et al*. (1995) Mutations in a novel gene, *myoblast city*, provide evidence in support of the founder cell hypothesis for *Drosophila* muscle development. *Development* 121, 1979–1988
32 Erickson, M.R. *et al*. (1997) *Drosophila myoblast city* encodes a conserved protein that is essential for myoblast fusion, dorsal closure, and cytoskeletal organization. *J. Cell Biol.* 138, 589–603
33 Nolan, K.M. *et al*. (1998) Myoblast city, the *Drosophila* homolog of DOCK180/CED-5, is required in a Rac signaling pathway utilized for multiple developmental processes. *Genes Dev.* 12, 3337–3342
34 Hall, A. (1998) Rho GTPases and the actin cytoskeleton. *Science* 279, 509–514
35 Van Aelst, L. and D'Souza-Schorey, C. (1997) Rho GTPases and signaling networks. *Genes Dev.* 11, 2295–2322
36 Ridley, A.J. (2001) Rho family proteins: coordinating cell responses. *Trends Cell Biol.* 11, 471–477
37 Grimsley, C. and Ravichandran, K.S. (2003) Cues for apoptotic cell engulfment: eat-me, don't eat-me and come-get-me signals. *Trends Cell Biol.* 13, 648–656
38 Brugnera, E. *et al*. (2002) Unconventional Rac–GEF activity is mediated through the Dock180–ELMO complex. *Nat. Cell Biol.* 4, 574–582
39 Hakeda-Suzuki, S. *et al*. (2002) Rac function and regulation during *Drosophila* development. *Nature* 416, 438–442
40 Galletta, B.J. *et al*. (1999) Identification of a *Drosophila* homologue to vertebrate Crk by interaction with MBC. *Gene* 228, 243–252
41 Donaldson, J.G. and Jackson, C.L. (2000) Regulators and effectors of the ARF GTPases. *Curr. Opin. Cell Biol.* 12, 475–482
42 Chardin, P. *et al*. (1996) A human exchange factor for ARF contains Sec7- and pleckstrin-homology domains. *Nature* 384, 481–484
43 Chavrier, P. and Goud, B. (1999) The role of ARF and Rab GTPases in membrane transport. *Curr. Opin. Cell Biol.* 11, 466–475

44 Radhakrishna, H. *et al*. (1999) ARF6 requirement for Rac ruffling suggests a role for membrane trafficking in cortical actin rearrangements. *J. Cell Sci.* 112, 855–866
45 Liu, H. *et al*. (2003) *Drosophila* paramyosin is important for myoblast fusion and essential for myofibril formation. *J. Cell Biol.* 160, 899–908
46 Jahn, R. *et al*. (2003) Membrane fusion. *Cell* 112, 519–533
47 Upton, C. and Buckley, J.T. (1995) A new family of lipolytic enzymes? *Trends Biochem. Sci.* 20, 178–179
48 Fournier-Thibault, C. *et al*. (1999) BEN/SC1/DM-GRASP expression during neuromuscular development: a cell adhesion molecule regulated by innervation. *J. Neurosci.* 19, 1382–1392
49 Lenkkeri, U. *et al*. (1999) Structure of the gene for congenital nephrotic syndrome of the finnish type (NPHS1) and characterization of mutations. *Am. J. Hum. Genet.* 64, 51–61
50 Farkas-Bargeton, E. *et al*. (1988) Immaturity of muscle fibers in the congenital form of myotonic dystrophy: its consequences and its origin. *J. Neurol. Sci.* 83, 145–159
51 Wockel, L. *et al*. (1998) Abundant minute myotubes in a patient who later developed centronuclear myopathy. *Acta Neuropathol. (Berl.)* 95, 547–551