Review

Tethering Membrane Fusion: Common and Different Players in Myoblasts and at the Synapse

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Abstract: Membrane fusion is essential for the communication of membrane-defined compartments, development of multicellular organisms and tissue homeostasis. Although membrane fusion has been studied extensively, still little is known about the molecular mechanisms. Especially the intercellular fusion of cells during development and tissue homeostasis is poorly understood. Somatic muscle formation in Drosophila depends on the intercellular fusion of myoblasts. In this process, myoblasts recognize each other and adhere, thereby triggering a protein machinery that leads to electron-dense plaques, vesicles and F-actin formation at apposing membranes. Two models of how local membrane stress is achieved to induce the merging of the myoblast membranes have been proposed: the electron-dense vesicles transport and release a fusogen and F-actin bends the plasma membrane. In this review, we highlight cell-adhesion molecules and intracellular proteins known to be involved in myoblast fusion. The cell-adhesion proteins also mediate the recognition and adhesion of other cell types, such as neurons that communicate with each other via special intercellular junctions, termed chemical synapses. At these synapses, neurotransmitters are released through the intracellular fusion of synaptic vesicles with the plasma membrane. As the targeting of electron-dense vesicles in myoblasts shares some similarities with the targeting of synaptic vesicle fusion, we compare molecules required for synaptic vesicle fusion to recently identified molecules involved in myoblast fusion.

Keywords: cadherins, electron-dense plaques, F-actin, FuRMA, IgSF, intercellular junctions, myogenesis, vesicle exocytosis

INTRODUCTION

Membranes define the borders of a cell and of intracellular organelles. Yet membranes must be remodelled via membrane fusion events during organogenesis or when molecules are transported between and within cells. Intercellular fusion of single cells occurs during the development of an organism to form different organs, such as placenta and muscles. Intracellular fusion of internal membranes occurs during exocytosis and protein trafficking. One common aspect of any type of membrane fusion is that the membranes first need to be brought into close contact, from the natural distance of 10–20 nm or more down to 1 nm at the site of ultimate fusion. To achieve this, integral transmembrane proteins involved in transport or cell communication must be removed from this site. Furthermore, fusion proteins are used to reduce the energy barrier for fusion by locally disrupting the membrane structure. This leads to hemifusion and stalk formation. Finally, a fusion pore opens and expands, and the content of the fused cells or organelles is mixed.

Relative to the mechanism of intracellular fusion of organelles, only rather little is known about the mechanism of intercellular fusion. Recent progress in understanding the intercellular fusion of myoblasts, which is essential for building muscles, has been achieved by using the powerful genetic analyses of Drosophila (Önel and Renkawitz-Pohl, 2009; Rochlin et al., 2010; Önel et al., 2011; Abmayr & Pavlath, 2012). Genes encoding proteins involved in myoblast fusion have been identified. Among these proteins are cell-adhesion proteins that not only mediate the recognition and adhesion of myoblasts but also play a conserved role in establishing trans-synaptic adhesions. Also identified are intracellular proteins involved in electron-dense vesicle targeting/exocytosis and actin cytoskeleton rearrangement. The vesicles might transport a fusogen that reduces the energy barrier for membrane fusion. Proteins involved in the targeting/
exocytosis of these vesicles are related to proteins sup-
porting synaptic vesicle exocytosis. Recent models also 
propose that F-actin formation leads to membrane cur-
vature and fusion pore formation. In this review, we will 
introduce the cellular and molecular biology of myoblast 
fusion in Drosophila and then highlight the function of 
conserved proteins during the formation of neuronal con-
nections and discuss synaptic vesicle exocytosis in com-
parison to electron-dense vesicle exocytosis.

CELLULAR AND MOLECULAR BIOLOGY OF 
MYOBLAST FUSION

Cellular aspects

The larval body wall musculature of Drosophila consists 
of a stereotypic pattern of 30 abdominal muscles per 
hemisegment (Bate, 1990). The muscles arise during 
embryogenesis from the somatic mesoderm. Within the 
somatic mesoderm, two different myoblast populations 
are produced: fusion-competent myoblasts (FCMs) and 
founder cells (FCs; Ruiz-Gómez, 1998; Duan et al., 
2001). Following myoblast specification, one FC fuses 
with several FCMs to build a mature myotube, which can 
contain 4–24 nuclei. The FC dictates the identity of the 
muscle, and fused FCMs adopt the transcriptional profile 
of the FC. Somatic muscle formation in Drosophila is 
completed within 5.5 h (Beckett & Baylies, 2007). The 
fusion of an individual FCM with the FC/growing myo-
tube is even faster and is completed after approximately 
12 min (Richardson et al., 2007).

In Drosophila, electron-dense structures have been 
observed at apposing membranes during myoblast 
recognition and adhesion (Doberstein et al., 1997). These 
structures are both electron-dense vesicles (Figure 1A)

Figure 1. Intermediate steps of myoblast fusion and comparison to intercellular junctions. (A, C, D and E) Electron micrographs of 
Drosophila stage 14 wild-type embryos conventionally fixed as described in Berger et al. (2008). (A) A set of electron-dense vesicles 
that align at apposing membranes of adhering myoblasts (arrowheads). (B) Serial electron micrographs from Doberstein et al. (1997; 
Figure 3) are aligned using the Velocity v5.3 software from Perkin Elmer and show a cloud of vesicles termed the prefusion complex 
(orange). (C) and (D) Electron-dense plaques between apposing myoblasts (arrows). Doberstein et al. (1997) are the first to describe 
these structures in Drosophila myoblasts and observed them in areas of plasma membrane breakdown (C and D, arrowheads). (E) 
Adherence junction between Drosophila epithelial cells with electron-dense material on both sides of the cells (arrow). Epithelial 
junctions consist of E-cadherins, which form homotypic interactions and link the cells with the actin cytoskeleton. (F) Excitatory 
synapse in the hippocampal CA1 stratum radiatum from a mouse fixed in 1% formaldehyde/1% glutaraldehyde. The cellular junction 
(arrow) between two neurons contain proteins of the IgSF family and N-cadherin.
that accumulate as a cloud (Figure 1B) and electron-dense plaques (Figure 1C). It has been suggested that the vesicles emanate from the Golgi (Kim et al., 2007), and the plaques are often found close to areas of membrane breakdown (Figure 1C and E, arrowheads; Doberstein et al., 1997). Both electron-dense structures are rare to observe in wild-type myoblasts, and their function during myoblast fusion still remains nebulous. However, the plaques show striking similarities to intercellular junctions, such as the adherence junctions in Drosophila epithelial cells (Figure 1E, arrow) and the mammalian excitatory synapse (Figure 1F, arrow).

Myoblast recognition and adhesion

Cell-adhesion molecules of the immunoglobulin super family (IgSF) in Drosophila include Dumbfounded/Kin of Irre (Duf/Kirre), Hibris (Hbs), Roughstuff/Irregular Chiasm C (Rst/IrreC), and Sticks and Stones (Sns), which are also known as the irre cell recognition module (IRM) proteins (Fischbach et al., 2009; Figure 2A; Table 1). Duf/Kirre is expressed specifically on the surface of FCs, where it attracts FCMs (Ruiz-Gómez et al., 2000) via heterophilic interaction with the FCM-specific IgSF protein Sns (Bour et al., 2000; Galletta et al., 2004). The paralog of Duf/Kirre, Rst/IrreC, is expressed on the surface of both myoblast types and mediates homophilic interactions (Galletta et al., 2004). However, Duf/Kirre can substitute for Rst/IrreC in FCs and vice versa (Strünkelberg et al., 2001). The paralog of Sns is Hbs, which, like Sns, is exclusively expressed in FCMs (Artero et al., 2001; Dworak et al., 2001) and can partially substitute for Sns (Shelton et al., 2009), but its function during fusion remains unknown.

In adhering FCs and FCMs, Duf/Kirre, Rst/IrreC and Sns are expressed in a ring-like structure at cell–cell contact points (Kesper et al., 2007; Sens et al., 2010; Önel et al., 2011). The rings expand during the fusion process, which suggests that fusion takes place within the area restricted by these IgSFs (Önel & Renkawitz-Pohl, 2009; Önel et al., 2011). Furthermore, Duf/Kirre, Hbs, Rst/IrreC and Sns are involved in the formation of a cell-communication structure that recruits further proteins, for example, Swiprosin-1 (DSwip-1; Figure 2B; Table 1) and Blown fuse (Blow; Figure 2C), to the site of cell–cell contact. The structure that forms when cells adhere has been termed fusion-restricted myogenic-adhesive structure (FuRMAS; Kesper et al., 2007). Homologs of Duf/Kirre and Sns are also important for vertebrate myoblast fusion (Srinivas et al., 2007; Sohn et al., 2009; Durcan et al., 2013), which indicates that the role of IgSF proteins in myoblast fusion is evolutionarily conserved.

Another cell-adhesion molecule that has been recently described to mediate the adhesion of FCs and FCMs during myoblast fusion is neuronal (N-)cadherin (Figure 2A; Table 1). N-cadherin is a classical member of the Ca$^{2+}$-dependent cadherin superfamily. In contrast to the IgSFs that form a ring-like structure at cell–cell contact points, N-cadherin is uniformly distributed around the plasma membrane of FCs and FCMs (Dottermusch-Heidel et al., 2012). As in mammals, the loss of N-cadherin in Drosophila does not disturb myoblast fusion (Charlton et al., 1997; Dottermusch-Heidel et al., 2012), which points towards compensatory mechanisms. In mammals, N-cadherin is replaced by other members of the classical cadherin family (Krauss, 2010). Thus, Drosophila N-cadherin could share redundant functions with another member of the cadherin family. Genetic interaction data suggest that Drosophila N-cadherin is regulated at the site of fusion by the guanine-nucleotide exchange factor Schizo/Loner (Siz), which binds to the intracellular domain of N-cadherin and activates the small Arf1-GTPase during myoblast fusion (Dottermusch-Heidel et al., 2012; Figure 2A). The cadherin extracellular region is 22 nm in length (Nagar et al., 1996), whereas the hemifusion of membranes occurs at an intermembrane distance of 1 nm (Kozlovsky et al., 2004). Hence, it seems that N-cadherin needs to be displaced from the ultimate site of fusion to reduce membrane distance. In contrast, it is believed that the IgSF molecules are redistributed from the site of fusion by the expansion of the ring structure.

Signalling fusion and F-actin regulation

Recently identified players in myoblast fusion are the MARVEL domain protein Singles Bar (Sing; Estrada et al., 2007) and the EF-hand domain protein DSwip-1 (Hornbruch-Freitag et al., 2011). MARVEL (MAL and related proteins for vesicle trafficking and membrane link) domain proteins are characterized by four transmembrane helix regions. In mammals, proteins containing this domain signature are involved in membrane apposition and vesicle trafficking (Sanchez-Pulido et al., 2002). sing is transcribed in both FCs and FCMs (Estrada et al., 2007; Table 1). sing mutant embryos possess a significantly higher number of prefusion complexes. It is therefore hypothesized that Sing is involved in the targeting or in the exocytosis of the electron-dense vesicles to the plasma membrane (Estrada et al., 2007). Support for this idea comes from studies on the Ca$^{2+}$-binding protein DSwip-1. This protein was identified from a databank search for Ca$^{2+}$-binding proteins that are expressed in the somatic mesoderm. DSwip-1 contains two Ca$^{2+}$-binding EF-hand domains and one coiled-coil domain. Its mammalian homolog Swiprosin-1/EFHD2 was first discovered in human lymphocytes (Vuadens et al., 2004) and in B cells where it associates with lipid rafts (Mielenz et al., 2005). In myoblasts, DSwip-1 is only present in FCMs at cell–cell contact points during adhesion, as is Sns. DSwip-1
Figure 2. Overview of proteins involved in myoblast fusion and in mediating trans-synaptic adhesions. (A) Adhesion molecules. The IgSF proteins Duf/Kirre and Sns interact heterophilically on the surface of FCs and FCMs. Rst/IrreC interact homophilically. Whether the FCM-specific IgSF Hbs interacts heterophilically with Duf/Kirre or Rst/IrreC on the surface of FCs is not yet clear. The Ca\(^{2+}\)-dependent adhesion molecule N-cadherin is additionally involved in the recognition and adhesion of FCs and FCMs and forms homodimers. The GTPase Arf1 and Schizo/Loner (Siz) are involved in the displacement of N-cadherin, probably to reduce the distance between apposing membranes. Siz also binds to the intracellular domain of Duf/Kirre (Bulchand et al., 2010). (B) Signalling molecules. Sing, which consists of seven-transmembrane spanning helix regions, is present in FCs and FCMs and acts in FCMs together with the Ca\(^{2+}\)-binding EF-hand domain protein DSwip-1 to target or exocytose the vesicles of the prefusion complex. (C) Adaptor proteins and F-actin regulators. The multidomain protein Rols7 transfers the fusion signal from the membrane into the FC by binding to the intracellular domain of Duf/Kirre. MhcI is a downstream partner of Rols7. Furthermore, the SH2-SH3 adaptor proteins Crk and Dock transfer the fusion signal into FCs and FCMs. Crk binds to the intracellular domain of Sns. Dock binds to the intracellular domain of Duf/Kirre, Hbs and Sns. Crk and Dock link cell adhesion with Scar- and WASp-dependent Arp2/3 regulation. The regulatory Scar complex, to which Kette belongs to, regulates Arp2/3-dependent actin polymerization in FCs and FCMs. In FCMs, the Scar complex is activated by the GEF Mbc that catalyses the GDP-to-GTP exchange on the small GTPases Rac1 and Rac2 (Haralalka et al., 2011). The GEF that activates Rac1 and Rac2 in FCs is unknown. Arp2/3-dependent F-actin focus formation in FCMs is additionally controlled by the WASp/WIP complex. Competition between WASp and Blow for WIP binding leads to the formation of new actin filaments, which promote the formation of finger-like protrusions, as shown in E. (D) Model of F-actin regulation and FuRMAS expansion. One possible function of F-actin formation during myoblast fusion is possibly the expansion of the ring-like structure formed by the IgSF proteins from 1 to 5 \(\mu\)m in diameter. (E) Model of F-actin formation and membrane stress. Another possible role for F-actin could be to induce membrane stress at the FCM membrane, which subsequently induces fusion pore formation. (F) Chemical synapse. Cell-adhesion molecules of the cadherin, protocadherin, IgSF, neuroligin, neurexin and CNTNAP families connect presynaptic and postsynaptic
accumulates in sing mutant myoblasts (Hornbruch-Freitag et al., 2011). Thus, successful cell adhesion leads to the targeting or exocytosis of the prefusion complex in FCMs, which involves the Ca\(^{2+}\)-binding protein, DSwip-1, and the MARVEL domain protein, Sing (Figure 2B). A corresponding Ca\(^{2+}\)-binding protein in FCs that mediates Sing-dependent prefusion complex targeting or exocytosis has not yet been identified.

DSwip-1 is not the only protein whose localization depends on successful cell adhesion. Also F-actin accumulation is dependent. The recognition and adhesion of myoblasts results in the formation of a dense and dynamic F-actin focus in FCMs and a thin F-actin sheath in FCs (Kesper et al., 2007; Richardson et al., 2007; Sens et al., 2010). The F-actin focus in FCMs co-localizes with DSwip-1, but F-actin formation does not depend on DSwip-1 (Hornbruch-Freitag et al., 2011). F-actin formation during myoblast fusion involves the evolutionarily conserved actin-related protein (Arp)2/3 complex (Figure 2C; Massarwa et al., 2007; Richardson et al., 2007; Berger et al., 2008). The activity of the Arp2/3 complex in cells is generally controlled by nucleation-promoting factors (NPFs) of the Wiskott-Aldrich Syndrome protein (WASp) family (Pollard, 2007; Takenawa & Suetsumu, 2007; Rotty et al., 2013). Both the NPF suppressor of the cyclic AMP receptor (Scar; also known as WAVE) and WASp (Massarwa et al., 2007; Schäfer et al., 2007; Richardson et al., 2007; Berger et al., 2008; Gildor et al., 2009) control Arp2/3-dependent F-actin formation during myoblast fusion (Figure 2C). Scar solely controls F-actin formation in FCs, but cooperates together with WASp in F-actin focus formation (Figure 2C; Berger et al., 2008; Sens et al., 2010; Haralalka et al., 2011). The WASp-interacting partner WIP (also known as Verprolin1 and Solitary in Drosophila) and WASp localize in FCs at cell–cell contact points (Kim et al., 2007; Massarwa et al., 2007; Schäfer et al., 2007; Berger et al., 2008). Recent studies suggest that the stability of the WIP–WASp complex is modulated by the FCM-specific protein, Blow, which competes with WASp for WIP binding (Figure 2C; Jin et al., 2011). The localization of Blow to the site of cell–cell contact is Sns-dependent (Kesper et al., 2007; Jin et al., 2011).

Scaffold and adaptor proteins bind to the intracellular domain of the cell-adhesion molecules to transfer the signal in FCs and FCMs to the actin cytoskeleton (Kim et al., 2007; Bonn et al., 2013; Kaipa et al., 2013). The multidomain protein Rol7 and the SH2-SH3 adaptor protein Dock (known as Nck in vertebrates) are able to bind to the intracellular domain of Duf/Kirre in FCs (Kreiskötber et al., 2006; Kaipa et al., 2013). In contrast, both the SH2-SH3 adaptor protein Crk and Dock bind to the intracellular domain of Sns in FCMs (Kim et al., 2007; Kaipa et al., 2013). Dock binds additionally to the intracellular domain of Hbs (Kaipa et al., 2013). To connect cell adhesion with F-actin formation, Crk interacts with Blow and WIP (Figure 2C; Kim et al., 2007; Jin et al., 2011), whereas Dock interacts with Scar, WIP and WASp (Figure 2C arrows; Kaipa et al., 2013).

At the ultrastructural level, blow mutants stop fusion after prefusion complex formation (Doberstein et al., 1997). wasp and wip mutants, in contrast, arrest fusion during membrane breakdown (Massarwa et al., 2007, Berger et al., 2008). However, Arp3 mutants stop fusion at a later state, after the formation of a fusion pore. Although a fusion pore forms in Arp3 mutants, the FCM fails to integrate into the growing myotube. Thus, Arp2/3-dependent actin polymerization seems to affect different steps of myoblast fusion.

**Possible roles for F-actin during myoblast fusion**

F-actin foci formation during myoblast fusion is highly dynamic (Richardson et al., 2007) and essential for fusion. Different F-actin regulation mutants stop fusion at different steps. So what is the function of F-actin during myoblast fusion? In wild-type myoblasts, the ring-like structure formed by Duf/Kirre, Sns and Rst/IriCe expands from 1 \(\mu\)m to 5 \(\mu\)m (Figure 2D, Kesper et al., 2007; Önel et al., 2011). This structure fails to expand in mutants defective in F-actin formation, for example, kette and blow (Kesper et al., 2007). Thus, one possible role for F-actin formation in FCs and FCMs is the expansion of the FuRMAF (Kepser et al., 2007; Figure 2D). The finding that fusion pores remain small in Arp3 mutant myoblasts indicates that F-actin is also required for the integration of the FCM into the FC/growing myotube (Berger et al., 2008). FCMs extend finger-like protrusions towards the FC/growing myotube (Figure 2E; Sens et al., 2010). blow, wip and wasp mutants fail to form these protrusions (Sens et al., 2010; Jin et al., 2011). Therefore, it has been suggested that F-actin is required to form finger-like protrusions, which induce membrane stress and lead to fusion pore formation.
Table 1. Proteins involved in myoblast fusion in *Drosophila*, in synapse formation and synaptic vesicle exocytosis.

| Drosophila | Cell type | Protein class | Drosophila | C. elegans | Mammals | Cell type | Protein class |
|------------|-----------|---------------|------------|------------|---------|-----------|---------------|
| Du\(\text{f/Kirre}\)\(^1\) | FC, FCM | IgSF | Duf/Kirre\(^1\) | Rst/IrrC\(^3\) | Rst/IrrC\(^3\) | Presynaptic? | IgSF |
| Rst/IrrC\(^3\) | FC, FCM | IgSF | Rst/IrrC\(^3\) | Postsynaptic | Postsynaptic | Postsynaptic | IgSF |
| Sns\(^5\) | FC | IgSF | Hbs\(^4\) | Postsynaptic | Postsynaptic | Postsynaptic | IgSF |
| Hbs\(^6\) | FC | IgSF | N-cadherin\(^9\) | Postsynaptic | Atypical | Cadherin (7TM) |
| N-cadherin\(^9\) | FC, FCM | Cadherin | N-cadherin\(^9\) | Fmi-1\(^13\) | Postsynaptic |

Tethering factors: Myoblast fusion

| Drosophila | Cell type | Protein class | Drosophila | C. elegans | Mammals | Cell type | Protein class |
|------------|-----------|---------------|------------|------------|---------|-----------|---------------|
| None | | | | | | | |

MARVEL domain proteins:

| Drosophila | Cell type | Drosophila | C. elegans | Mammals | Expression |
|------------|-----------|------------|------------|---------|------------|
| Sing\(^18\) | FC, FCM | Synaptogyrin\(^19\) | Unc-11\(^20\) | Synaptogyrin\(^21\) | Abundant expression on the vesicle membrane |

Intracellular Ca\(^{2+}\) binding proteins:

| Drosophila | Cell Type | Drosophila | C. elegans | Mammals |
|------------|-----------|------------|------------|---------|
| DSwip-1\(^25\) | | Unc13\(^14\) | Unc-13\(^15\) | Munc13-1,2,3\(^16\) |

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\(^1\)Ruiz-Gómez et al. (2000), Strünkelnberg et al. (2001).
\(^2\)Komori et al. (2008).
\(^3\)Strünkelnberg et al. (2001).
\(^4\)Sugie et al. (2010).
\(^5\)Bour et al. (2000).
\(^6\)Artero et al. (2001), Dworak et al. (2001).
\(^7\)Shen and Bargmann (2003).
\(^8\)Shen et al. (2004).
\(^9\)Dottermusch-Heidel et al. (2012).
\(^10\)Prakash et al. (2005).
\(^11\)Fannon and Colman (1996), Uchida et al. (1996).
\(^12\)Bao et al. (2007).
\(^13\)Najarro et al. (2012).
\(^14\)Aravamudan et al. (1999).
\(^15\)Maruyama and Brenner (1991).
\(^16\)Brose et al. (1995).
\(^17\)Takamori et al. (2006).
\(^18\)Estrada et al. (2007).
\(^19\)Stenius et al. (1995).
\(^20\)Nonet et al. (1999).
\(^21\)Stevens et al. (2012).
\(^22\)Nonet et al. (1993).
CONSERVED PLAYERS INVOLVED IN SYNAPSE FORMATION AND MAINTENANCE

To form functional networks, neurons are interconnected via chemical or electrical synapses. In chemical synapses, action potentials arriving at presynaptic terminals induce neurotransmitter release via Ca$^{2+}$-dependent synaptic vesicle exocytosis (Figure 2F). The released neurotransmitters diffuse through the synaptic cleft and bind to receptors on the postsynaptic membrane, thereby depolarizing the postsynaptic membrane potential and activating or modulating signal transduction pathways. A prerequisite for a functional chemical synapse is the establishment and maintenance of trans-synaptic adhesions. This involves the function of cell-adhesion molecules that link presynaptic and postsynaptic structures. A number of cell-adhesion molecules have been implicated in synapse maturation and maintenance including neurexins/neuroligins; leucine-rich repeat proteins, such as leucine-rich repeat transmembrane neuronal proteins, netrin-G ligands and signalling lymphocyte activation molecules; tyrosine kinase receptor proteins, such as EphrinB and their ligands; and integrins (Figure 2F). As these protein classes have been reviewed recently in detail (Togashi et al., 2009; Missler et al., 2012; Thalhammer & Cingolani, 2013) and are not involved in myoblast fusion, we focus here only on the cell-adhesion molecules cadherins and Ig-domain proteins.

The most ancient Ig-domain proteins that constitute trans-synaptic adhesions in mammals and invertebrates are the evolutionarily conserved Nephrin and Nephrin proteins (Giagtzoglou et al., 2009; Fischbach et al., 2009). In Caenorhabditis elegans, synapse formation of the hermaphrodite-specific motor neuron (HSNL) is controlled by the Neph1 homolog SYG-1 and the Nephrin homolog SYG-2 (Shen & Bargmann, 2003; Shen et al., 2004; Table 1). SYG-2 is expressed on vulva epithelial cells, where it interacts with SYG-1 on the HSNL neuron. SYG-2 thereby recruits SYG-1 to the site, where the presynapse is formed.

The Drosophila orthologs of Neph1 and Nephrin are Duf/Kirre and Rst/IrreC, and Sns and Hbs, respectively. Hbs and Rst/IrreC are also essential for the communication between retinal axons and their postsynaptic partners in eye development. During the development of the visual centre in Drosophila, presynaptic photoreceptor cells (R cells) extend their axons to the lamina layer. The R axons express the segment polarity gene hedgehog, which induces the differentiation of lamina precursor cells into postsynaptic lamina precursor cells (pLPCs). As a consequence, pLPCs express Hbs, which interacts with Rst/IrreC and enables pLPCs to associate with R axon bundles (Sugie et al., 2010; Table 1).

IRMs have also been identified in vertebrates. In mouse embryos, the Duf/Kirre homolog Neph2/Kirrel3 is expressed in the central nervous system and in the dorsal root ganglia of the peripheral nervous system (Komori et al., 2008; Völker et al., 2012). Furthermore, Neph1 and Neph2/Kirrel3 are expressed in the nervous system of mouse and chick embryos (Völker et al., 2012). However, Neph3/Kirrel2 is transcribed in the cerebellar anlage and the spinal cord of mice embryos. Furthermore, Neph2/Kirrel3 possibly plays a role in axonal pathfinding, cell recognition and synapse formation between dorsal root ganglia neurons and their target cells (Komori et al., 2008; Table 1).

Another conserved protein involved in trans-synaptic adhesion is N-cadherin (Table 1). It controls synaptic morphology and function (Hansen et al., 2008; Sheng et al., 2013). Furthermore, N-cadherin acts as a trans-synaptic sensor for synaptic activity, which promotes N-cadherin dimer formation (Tanaka et al., 2000). Immunoelectron microscopy studies have shown that N-cadherin localizes to sites of synaptic vesicle exocytosis, which suggests a role in vesicle trafficking (Uchida et al., 1996). Indeed, presynaptic N-cadherin has been implicated in the regulation of synaptic vesicle recruitment and recycling (Stan et al., 2010). In unstimulated neurons, N-cadherin is distributed uniformly along the synaptic cleft, and evidence suggests that upon synaptic stimulation, N-cadherin redistributes at the synapse (Yam et al., 2013). However, during KCl-induced depolarization, less N-cadherin is found in the region where synaptic vesicle exocytosis takes place, and a protein redistributes to the periphery of the synaptic cleft. After KCl-induced depolarization, N-cadherin redistributes again more uniformly. Thus, the localization of N-cadherin is directly correlated to synaptic activity. These findings possibly indicate that N-cadherin–free regions must be created at the region to which the synaptic vesicle fuses.

CONSERVED PRINCIPLES AND PLAYERS IN SYNAPTIC VESICLE FUSION

During exocytosis, filled vesicles fuse with the plasma membrane to release their contents into the extracellular space. This intracellular membrane fusion has been studied extensively during synaptic vesicle exocytosis (McMahon et al., 2010; Jahn & Fasshauer, 2012; Wu et al., 2013). The release of neurotransmitters from synaptic vesicles follows a core principle of intracellular fusion that involves tethering, docking and priming of vesicles (James & Martin, 2013). These events lead to the zipper ing of soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) that catalyse the fusion process and possess fusogenic characteristics. Fusogens are proteins that drive membrane fusion and destabilize the lipid bilayers by inducing local membrane stress (Oren-Suissa & Podbilewicz, 2007). One of the main criteria for
a fusogen is that it should be expressed at the ultimate site and time of fusion. Furthermore, it should be sufficient to fuse cells that normally do not fuse. In an elegant study, Hu et al. (2003) demonstrated that expression of the neuronal vesicle (v-)SNARE Synaptobrevin (Figure 3A) and the neuronal target-membrane (t-)SNAREs Syntaxin-1 and SNAP-25 (Figure 3A) on the cell surface is able to induce the heterologous fusion of Chinese hamster ovary (CHO) and fibroblast cells.

During vesicle fusion, the N-terminal regions of the SNARE proteins, where the SNARE motif is localized, assemble into a trans-SNARE complex (Südhof & Rizo, 2011). Through SNARE assembly, the distance of the apposing membranes is reduced to 3–4 nm, and membranes are destabilized (Chen et al., 2006). However, the time needed for trans-SNARE complex assembly \textit{in vitro} is much lower than that \textit{in vivo}, which suggests the involvement of assisting proteins (Fasshauer et al., 2002). A set of proteins that regulate SNARE-mediated membrane fusion has been identified and some of them are discussed next.

Synaptogyrin and Synaptophysin are abundantly expressed on synaptic vesicles and belong to the MARVEL domain family (Rehm et al., 1986; Stenius et al., 1995; Table 1). Both proteins have been implicated in playing a role in the biogenesis of synaptic vesicles (Thiele et al., 2000; Stevens et al., 2012), and it has been suggested that Synaptophysin promotes the formation of highly curved membranes (Thiele et al., 2000). Furthermore, Synaptophysin is able to bind to the v-SNARE Synaptobrevin. However, not much is known about the function of either protein during synaptic vesicle release and recycling. The genetic analysis of Synaptogyrin and Synaptophysin in mammals is complicated as different homologs exist that possibly behave in a compensatory manner (Janz et al., 1999; Spiwoks-Becker et al., 2001). \textit{Drosophila} lacks Synaptophysin, and produces Synaptogyrin (Table 1). \textit{Drosophila} Synaptogyrin regulates neurotransmitter release and short-term synaptic plasticity (Stevens et al., 2012). Besides Synaptogyrin, the \textit{Drosophila} genome encodes two other MARVEL-domain–containing proteins: the Sing protein relevant for myoblast fusion and CMTM4 (Stevens et al., 2012).

Tethering factors mediate the initial interaction between an intracellular vesicle and its target membrane (Barlowe, 1997; Cao et al., 1998). These factors are either large coiled-coil proteins or multi-subunit tethering complexes. The complexes have a homologous tertiary structure, termed complex associated with tethering containing helical rods (CATCHR; Yu et al., 2010). Munc13 and Munc18 are regulatory proteins that prepare the SNAREs for complex assembly (Figure 3A). Munc13 shows structural similarities to the CATCHR region of Sec6, a subunit of the exocyst tethering complex (Li et al., 2011). Munc18 conducts Syntaxin-1 to the target membrane and stabilizes a closed form of Sytaxin-1 (Hata et al., 1993; Misura et al.,

![Figure 3](image.png) **Figure 3.** Steps in synaptic vesicle exocytosis. Ca$^{2+}$ influx activates the assembly of the SNARE complex and is required during various steps of vesicle fusion. (A) The vesicle is tethered to the plasma membrane. This involves Munc13 of the CATCHR family and the SM protein Munc18. (B) The zipping of the neuronal SNARES Syntaxin, SNAP-25 and Synaptobrevin brings the apposing membranes into proximity at 3–4 nm. (C) Fusion pore initiation involves the function of the Ca$^{2+}$–dependent C2 domain protein Synaptotagmin. After a fusion pore has formed, the content of the vesicle is released into the synaptic cleft.
SNAREs and Synaptotagmin-1 are specialized proteins that induce membrane curvature during vesicle fusion (Martens et al., 2007; Marsden et al., 2011) and thereby lead to the opening of a fusion pore (Figure 3C). Synaptotagmin-1 is located to synaptic vesicles and contains two cytoplasmic C2 domains. The C2A domain shows interactions with t-SNAREs in a Ca\(^{2+}\)-dependent manner (Chapman et al., 1995; Gerona et al., 2000), but the C2B domain shows interactions with t-SNAREs in a Ca\(^{2+}\)-dependent manner (Rickman & Davletov, 2003). Different models have been proposed as to how Synaptotagmin initiates fusion pore formation. One model also suggests that Synaptotagmin-1 serves as a Ca\(^{2+}\)-dependent fusogen (Martens & McMahon, 2008).

**Do SNARE-like fusogens exist in myoblast fusion or are they involved in the exocytosis of a myoblast-specific fusogen?**

SNAREs and Synaptotagmin-1 are specialized proteins that induce membrane curvature during vesicle fusion (Martens et al., 2007; Marsden et al., 2011) and partially fulfill the criteria of a fusogen. A comparable fusogen in Drosophila myoblast fusion has not yet been identified. However, the Ig domains of the IRM proteins that are involved in myoblast adhesion are structurally related to the C2 domains of Synaptotagmin-1 (Martens & McMahon, 2008). Heterologous expression of Duf/Kirre and Sns, or Rst/IrreC and Sns in Drosophila non-muscle S2 or in murine C2C12 cells cannot induce cell–cell fusion (Galletta et al., 2004; Kesper et al., 2007). Thus, although these proteins are expressed at the ultimate site and time of fusion, they are not sufficient to fuse cells that normally do not fuse.

In pioneering work on the ultrastructural analysis of Drosophila myoblast fusion, Doberstein et al. (1997) proposed several possible functions of the electron-dense structures present at apposing membranes. One possibility was that the electron-dense vesicles fuse to the plasma membrane and generate the electron-dense plaques (Figure 4A). However, prefusion complexes are seen more often than electron-dense plaques, and sometimes electron-dense plaques and vesicles are observed together (Figure 2D). Another possibility is that the electron-dense vesicles deliver fusion-relevant components or even a fusogen to the apposing membranes. The identification of *blow* and *sing* mutants and their ultrastructural phenotype substantiate the important role of electron-dense vesicles during fusion. Furthermore, *Sing*, like Synaptogyrin, contains a MARVEL domain and possibly mediates electron-dense vesicle exocytosis. As *Sing* and the Ca\(^{2+}\)-binding protein DSwip-1 seem to act in concert, the exocytosis of these vesicles could be triggered by Ca\(^{2+}\) influx. However, the measurement of Ca\(^{2+}\) influx during fusion is difficult, because myoblast fusion occurs asynchronously and several FCMs can adhere and fuse to an FC/growing myotube at the same time.

The FuRMAS that forms at cell–cell contact points is reminiscent of other cell communication structures, such as invadopodia and the immunological synapse (Önel & Renkawitz-Pohl, 2009; Sens et al., 2010). It has been proposed that the formation of protrusion-like structures in FCMs induces fusion pore formation (Sens et al., 2010).

Recent evidence pointing towards the existence of a fusogen during myoblast fusion comes from studies of mammalian myoblast fusion. The newly identified transmembrane protein Myomaker is essential for myoblast fusion in mice, and forced expression of Myomaker in fibroblasts promotes the fusion of these cells (Millay et al., 2013). Consequently, the hunt for a fusogen involved in Drosophila myoblast fusion continues.

**FUTURE DIRECTIONS**

Cell–cell fusion is fundamental for animal development. In mammals, only gametes, trophoblasts, osteoclasts, macrophages and myoblasts are able to fuse. In all cell types, transmembrane proteins bring the cells that are designated to fuse into contact, and most of the transmembrane proteins identified contain Ig domains. But IgSF proteins on their own are not able to induce local membrane stress, a prerequisite to destabilize the lipid bilayers of the plasma membranes. The fusogens *Syncytin-1* and *Syncytin-2* involved in trophoblast fusion, which is essential for placenta formation, are retroviral and have been adopted during evolution (Blond et al., 2000; Mi et al., 2000). One hypothesis is that viral-like or SNARE-like fusogens are involved in mediating cell–cell and myoblast fusion, respectively (Oren-Suissa & Podbilewicz, 2007). Another proposal is that F-actin formation in Drosophila triggers membrane fusion by inducing actin-propelled invasive membrane protrusions (Sens et al., 2010). However, the identification of Myomaker as a possible fusogen in mouse myoblasts has placed doubt on this proposal. Nevertheless, the existence of a fusogen does not exclude that F-actin formation is a driving force in myoblast fusion and assists the process (Shilagardi et al., 2013).

Studying the ultrastructural features of F-actin formation has lost sight of other features that accompany myoblast fusion, for example, electron-dense vesicles and plaques. We believe that integrating these structures into current models of myoblast fusion will increase our understanding. We suggest that the electron-dense plaques represent cellular junctions that contain the IgSF proteins and N-cadherin (Figure 4B, step 1). The displacement of N-cadherin from the ultimate site of fusion possibly...
Figures 4. Models for the function of electron-dense vesicle and plaque formation. (A) Based on ultrastructural analyses of various myoblast fusion mutants, Doberstein et al. (1997) suggested that the electron-dense vesicles of the prefusion complex fuse to the plasma membranes and generate electron-dense plaques. They hypothesized that the plaques are an intermediate between fusion of the vesicles with the plasma membranes and the formation of pores between the apposed plasma membranes. (B) The striking similarity of electron-dense plaques to Cadherin-containing cellular junctions suggests that the plaques represent a cell-adhesion structure (step 1). Interestingly, the distance of the apposing plasma membranes within the plaques differs from the plasma membrane distance where membrane breakdown starts (see Figure 1C). This could be due to the displacement of N-cadherin from the ultimate site of fusion (step 2). The IgSF proteins keep the membranes in proximity (at least at 3–4 nm; step 3). The role of electron-dense vesicles during myoblast fusion is still hypothetical. Studies by Kim et al. (2007) suggest that they arise from the Golgi (step 4). Some myoblast fusion mutants stop the process after prefusion complex accumulation, which indicates that the prefusion complex plays an essential role during myoblast fusion, possibly in delivering a fusogen to the ultimate site of fusion, as suggested by Doberstein et al. (1997).

brings the membranes into proximity (step 2). The signalling cascade triggered by cell-adhesion molecules leads to the release of the electron-dense vesicle from the Golgi to the ultimate site of fusion (step 3), which is now free of N-cadherin. This could explain why fusion pore formation is observed next to the electron-dense plaques.

Future challenges will be: (i) to determine which factors lead to electron-dense vesicle release and whether this process is Ca$^{2+}$-dependent, and (ii) to determine how the electron-dense vesicles are targeted to the plasma membrane and whether F-actin (Kim et al., 2007) and tethering factors known from SNARE-mediated vesicle
fusion are involved. The exocyst multi-subunit tethering complex has been shown to tether post-Golgi secretory vesicles for SNARE-induced membrane fusion. However, protein interactions of the exocyst have demonstrated that it also plays a pivotal role, for example, in invadopodia formation (Sakurai-Yageta et al., 2008). Nevertheless, also proteins containing a CATCHR region, for example, Munc13, could be involved in the targeting of the vesicles. Another challenge will be (iii) to determine whether the vesicles are exocytosed and what they contain.

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