Mechanisms of membrane fusion: disparate players and common principles

Sascha Martens and Harvey T. McMahon

Abstract | Membrane fusion can occur between cells, between different intracellular compartments, between intracellular compartments and the plasma membrane and between lipid-bound structures such as viral particles and cellular membranes. In order for membranes to fuse they must first be brought together. The more highly curved a membrane is, the more fusogenic it becomes. We discuss how proteins, including SNAREs, synaptotagmins and viral fusion proteins, might mediate close membrane apposition and induction of membrane curvature to drive diverse fusion processes. We also highlight common principles that can be derived from the analysis of the role of these proteins.

Syncytium
A cell that contains multiple nuclei and that is formed either by cell–cell fusion or by incomplete cell division.

Hemifusion
An intermediate stage during membrane fusion that is characterized by the merger of only the contacting monolayers and not the two distal monolayers.

SNARE
(soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor). SNARE proteins are a family of membrane-tethered coiled-coil proteins that regulate fusion reactions and target specificity in vesicle trafficking. They can be divided into vesicle-associated (v)-SNAREs and target-membrane-associated (t)-SNAREs on the basis of their localization.

MRC Laboratory of Molecular Biology, Hills Road, Cambridge, CB2 0QH, UK.
e-mails:
hmm@mrc-lmb.cam.ac.uk;
martens@mrc-lmb.cam.ac.uk
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Membrane fusion is the process whereby two separate lipid bilayers merge to become one. It is essential for communication between membrane-delineated compartments in all eukaryotic cells (FIG. 1). The best-studied process involving membrane fusion is exocytosis, whereby vesicles fuse with the limiting membrane of a cell in order to release their contents (for example, hormones or neurotransmitters) into the extracellular milieu, or to deposit receptors, transporters, channels or adhesion molecules into the limiting membrane. However, large numbers of membrane-fusion events occur between intracellular compartments, and these events often involve vesicular or tubular intermediates. Fusion can either be heterotypic (when a membrane fuses with a dissimilar type of compartment; for example, synaptic vesicle exocytosis) or homotypic (when similar compartments fuse; for example, endosome–endosome fusion). More enigmatic processes involve the fusion of larger membrane-bound compartments, including whole cells in the case of syncytium formation. Furthermore, enveloped viruses gain entry into the cytosol by fusing their limiting membranes with host cell membranes (FIG. 1).

It is now believed that most, if not all, biological membrane fusion proceeds through a hemifusion intermediate[1] (FIG. 2). According to this mechanism, an intermediate stage of membrane fusion is the merger of only the closest monolayers, with full fusion resulting in complete bilayer merging. Membrane-fusion intermediates are regulated by cellular proteins that manifest their activity through the promotion of membrane–membrane proximity, by bending and remodelling membranes, or by acting upstream to regulate the lipid or protein composition of the respective lipid bilayers. In the hemifusion model the fusion pore is lipidic, but according to an alternative hypothesis — the protein-pore model — the initial fusion pore is generated and lined by transmembrane proteins rather than lipids[2]. There is good evidence that transmembrane domains of proteins are essential for efficient SNARE-dependent fusion, and replacement of these transmembrane domains with lipid anchors leads to hemifusion intermediates, whereas some viral fusion can occur in the absence of transmembrane anchors[3].

Several energy barriers have to be overcome for fusion to occur. One energetically demanding process is to bring about the close apposition of two membranes, which requires protein clearance (potentially through a process of 'sieving') and the bringing together of repulsive membrane charges (FIGS 2,3). The energy barriers related to curvature deformations during hemifusion-stalk and fusion-pore formation and expansion must also be overcome[1,4] (FIG. 2). The role of fusion proteins is to lower these barriers at the appropriate time and place to allow the regulation of the fusion process.

Numerous membrane-fusion processes have been extensively studied and many molecules that are involved in fusion have been identified (FIG. 1). From these studies it is clear that there are distinct and structurally unrelated membrane-fusion molecules. Despite this, we argue that there are general principles that operate in all fusion events.

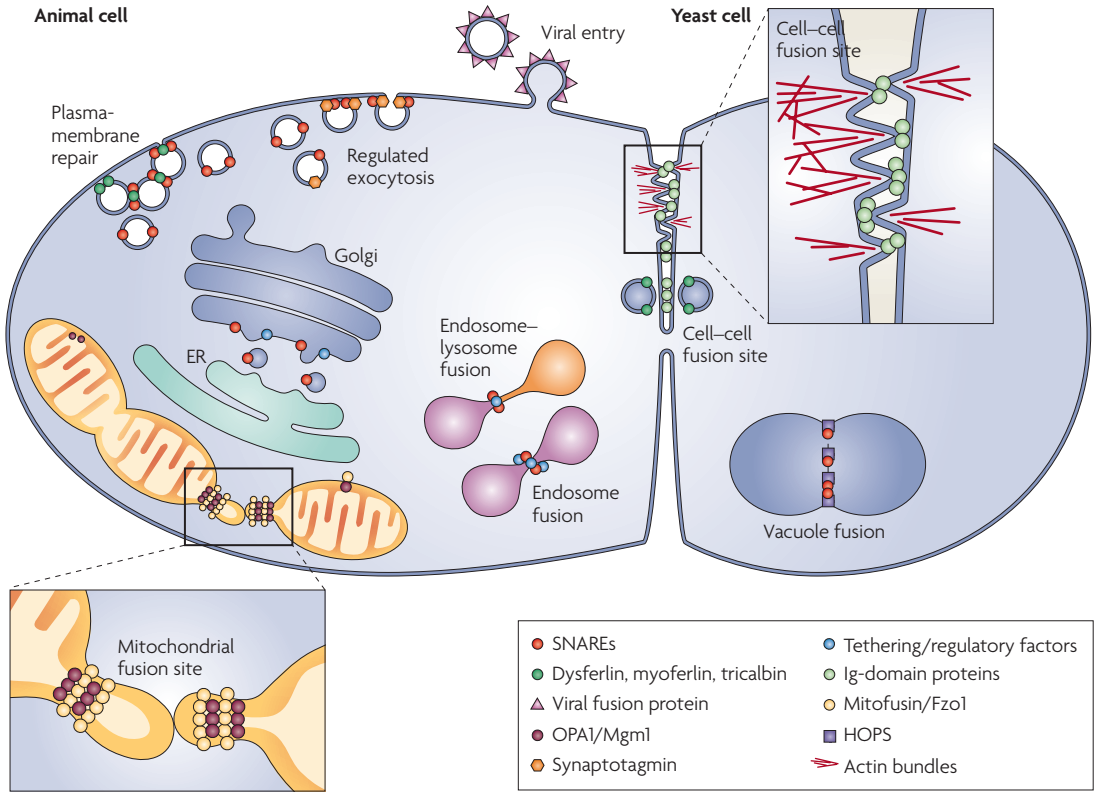


Figure 1 | **The broad spectrum of membrane-fusion events.** Membrane fusion in the secretory pathway and in the endosomal and lysosomal systems depends on SNAREs, which are assisted by tethering and regulatory factors that are generally required for efficient SNARE function. During Ca^{2+}-dependent exocytosis the SNAREs are assisted by synaptotagmins, and in endosome fusion they are assisted by the Rab5 effector EEA1. SNAREs and tethering/regulatory factors are replaced by viral fusion proteins in enveloped viruses and by immunoglobulin (Ig)-domain-containing proteins in many cell–cell fusion events. The actin cytoskeleton has also been implicated in membrane fusion and in particular in cell–cell fusion, where it might stabilize the microvilli. Furthermore, multiple-C2-domain (MC2D) proteins such as tricalbin in yeast and myoferlin in mammals have been proposed to function during plasma-membrane repair during leaky cell–cell fusion. Yeast vacuole fusion requires SNAREs and the tethering factor HOPS (homotypic fusion and vacuole protein sorting). Mitochondrial fusion is mediated by the large GTPases mitofusin and OPA1 of the dynamin superfamily. Mgm1 and Fzo1 are the yeast orthologues of OPA1 and mitofusin, respectively. Plasma-membrane repair is initiated by the influx of Ca^{2+} into the cytoplasm and is mediated by the rapid and local fusion of small vesicles with each other and with the plasma membrane. The MC2D protein dysferlin has been shown to be required for this fusion. The involvement of SNAREs in plasma-membrane repair has not been explicitly shown. ER, endoplasmic reticulum.

Membrane-fusion events generally require molecules that tether and dock membranes and bring them into close proximity, molecules that locally disturb the lipid bilayers (for example, by the induction of extreme membrane curvature) in order to reduce the energy barriers for fusion, and molecules that give directionality to the process. The driving force for membrane fusion can come from many sources — for example, from the energy that is derived from protein–lipid interactions or from protein–protein interactions — and ultimately these reactions will have been primed by ATP. Directionality might be achieved by fusion protein folding. In addition, curvature stress that promotes fusion-stalk formation will be relieved during fusion-pore opening and expansion, again giving directionality to the process from the beginning. A solid theoretical basis for a role for membrane bending in priming membrane fusion has previously been developed[1,5–8]. The different activities listed above do not have to be handled by different proteins, so the same molecules that promote hemifusion-stalk formation might promote fusion-pore expansion.

In this review, we discuss SNARE-independent membrane fusion, followed by SNARE-dependent membrane fusion, concentrating on the mechanisms by which the membranes become fusogenic and the role of fusion proteins and other accessory proteins in this process.

SNARE
(soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor). SNARE proteins are a family of membrane-tethered coiled-coil proteins that regulate fusion reactions and target specificity in vesicle trafficking. They can be divided into vesicle-associated (v)-SNAREs and target-membrane-associated (t)-SNAREs on the basis of their localization.

Viral fusion

The fusion components of enveloped viral fusion have been intensively studied. Viruses bind cell-surface proteins, and subsequent membrane fusion can occur either on the plasma membrane or after internalization[9–12]. Viral-surface fusion proteins can be structurally divided into three classes. The class I fusion proteins are mainly α-helical (FIG. 4a), the class II fusion proteins are mainly composed of β-sheets, and the class III fusion proteins have a mixed secondary structure.

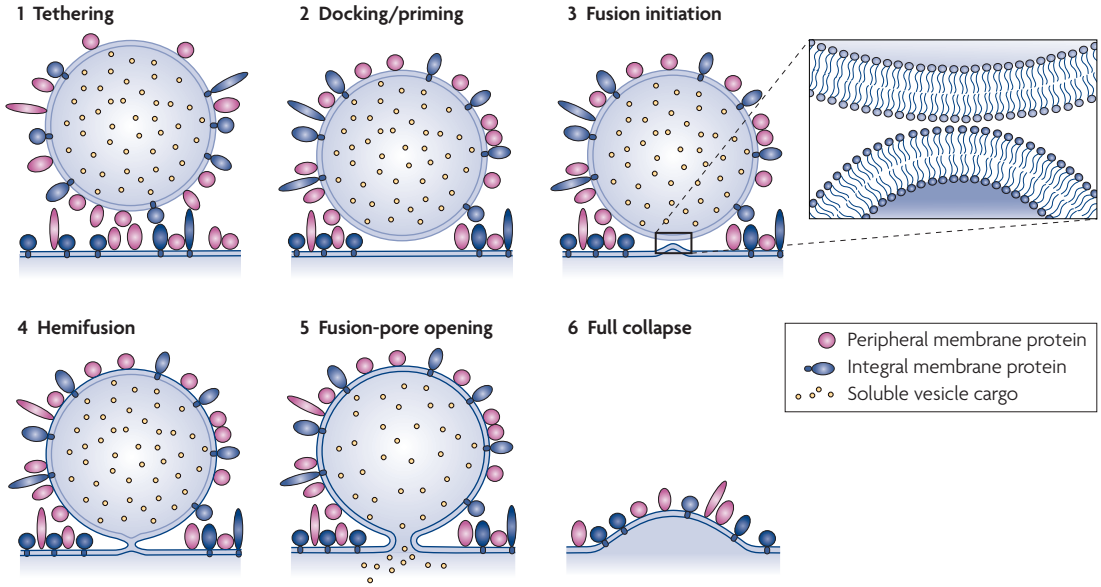


Figure 2 | **The hemifusion model for bilayer fusion.** Proposed steps in synaptic vesicle fusion with the plasma membrane. Most membrane-fusion events are likely to follow a similar sequence. Steps might not be as temporally delineated as indicated, but the stepwise depiction helps to conceptualize the process. All the steps indicated are regulated by cellular proteins. In step 1, the vesicle is transported and tethered to the appropriate membrane by specific tethering factors, which mediate the long-range recognition between the membranes. In step 2, the loosely tethered state is converted to a tightly docked state, bringing the membranes into closer proximity. In some specialized cells docking is followed by a priming step, during which the fusion machinery is assembled such that it can rapidly respond to a trigger (for example, changes in the Ca^{2+} concentration). Docking should also entail the generation of protein-denuded membranes (FIG. 3). In step 3, the high-energy barrier must be lowered to initiate membrane fusion and some membrane stress, such as curvature stress, probably facilitates the reaction. In addition, the distance between the two membranes has to be further decreased in order to bring the membranes into direct contact. Indeed, bilayers have been proposed to fuse when they are separated by ~1 nm[152]. In step 4, hemifusion occurs. Hemifusion is the defining step of this fusion model; in it apposing monolayers merge, whereas distal monolayers do not. In step 5, fusion-pore opening results from the further merger of the two distal monolayers and the release of vesicle content is initiated. In step 6, as a consequence of fusion-pore expansion, the vesicular membrane collapses into the plasma membrane and loses its identity.

Although no consensus has been reached to date, most researchers support the following model of how viral fusion is achieved (FIG. 4a). First, transmembrane viral fusion proteins are kept in an inactive state on the viral surface. Second, following exposure to an appropriate trigger (such as the interaction with a specific receptor on the cell surface and/or a pH change), the viral fusion proteins undergo dramatic conformational changes, thereby exposing fusion peptides or loops, which then insert into the target membrane. Third, either concurrently or subsequently, the fusion proteins undergo an additional conformational change that brings the transmembrane domains in the viral envelope into close proximity with the viral fusion peptides that are embedded in the target membrane. Fourth, membrane fusion occurs as a consequence of close membrane apposition, bilayer disturbance that is mediated by the transmembrane domain and bilayer disturbance that is mediated by the fusion peptide. The energy for membrane fusion is derived from the refolding of fusion proteins.

One of the best-studied viral fusion proteins is haemagglutinin, which comes from the influenza virus (FIG. 4a): this protein's homotrimeric structure has been determined in a pre-fusion state, with the three constituent fusion peptides buried within the molecule (a class I fusion protein). The structure has also been determined in a lower pH environment, whereby the fusion peptides are exposed and the trimer has undergone a dramatic change in conformation[9]. However, even in this case there are long unresolved regions between the transmembrane domains and the resolved structure at low pH, leading to uncertainty as to the precise mechanism of fusion. Although class II and class III fusion proteins are structurally different from class I fusion proteins, they probably work in a similar manner, undergoing conformational changes and exposing fusion peptides or loops[10–12].

The fusion loops or peptides of many viral fusion proteins insert partially into one monolayer (BOX1; FIG. 5), and it has been suggested that this would induce 'nipple' formation in the target membrane, bending it towards the viral envelope[11,13–16]. In support of this, small protrusions have been observed to emanate from liposomes[15] or from cells that have been incubated with activated influenza virus haemagglutinin[16]. Thus, viruses might combine two activities in their fusion proteins: close membrane proximity resulting from the conformational change that is coupled to membrane disturbance; and curvature induction that is mediated by fusion peptides and transmembrane domains.

Fusion peptide or loop
A short hydrophobic or amphiphilic peptide in a viral fusion protein that is normally only exposed during fusion and is proposed to insert into the cellular membrane.

Liposome
An artificial, bilayer-bound structure that is composed of lipids and resembles an intracellular transport vesicle.

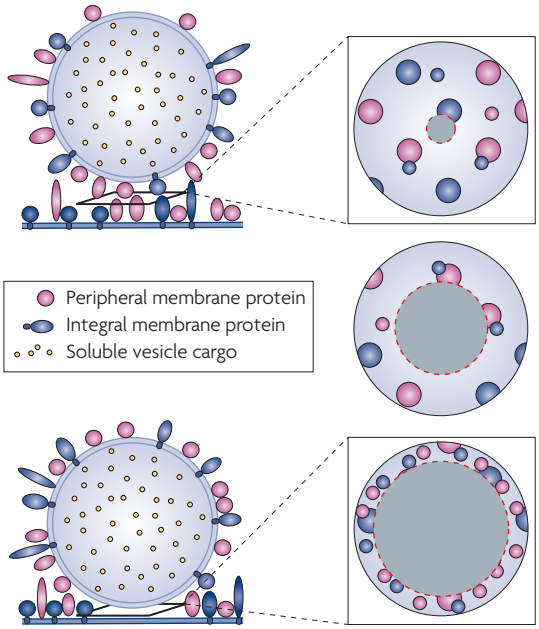


Figure 3 | **Molecular 'sieving'.** It is not known how the membrane fusion zone can become protein denuded, but we propose that it might be achieved by molecular 'sieving': the fusion machinery starts to assemble at the initial membrane contact point, but as assembly proceeds the machinery must move radially away from this point of contact. A concerted radial movement (as shown by the increase in the grey area) is probably regulated by docking and priming factors, such as MUNC13 and MUNC18, at the synapse. High membrane curvature might also contribute to this molecular sieving of bulky proteins away from the fusion zone, as many resident proteins might not tolerate the membrane distortion. The right panels are an enlargement of the fusion zones, showing the creation and enlargement of protein-free patches.

Mitochondrial fusion

Mitochondria undergo constant membrane fusion and fission events, leading to the mixing of membrane proteins and content. Fusion and fission are essential for normal mitochondrial function[17]. Inhibition of mitochondrial dynamics results in the accumulation of abnormally shaped mitochondria and reduced respiratory capacity, and deletion of various fusion components leads to embryonic lethality or neuropathy. Although several proteins are strongly implicated in this process, the molecular mechanisms are unclear.

Mitochondrial homotypic fusion is unusual because it involves the fusion of both outer and inner mitochondrial membranes with cognate partners (FIG. 1). It is dependent on the dynamin superfamily GTPases OPA1 (optic atrophy protein-1) and mitofusin. Members of this family of proteins are large, self-oligomerizing GTPases that mediate membrane remodelling events such as endocytic vesicle scission[18]. It has been shown that mitofusins can tether mitochondria to each other. This tethering is mediated by the C-terminal α-helices that are provided by mitofusins in opposing mitochondria and which together form an antiparallel coiled structure. Fusion also requires the N-terminal GTPase domain of mitofusin[19]. In addition to the tethering, these proteins promote membrane tubulation[20,21], and it is likely that this is the driving force for fusion because the ends of membrane tubules are extremely fusogenic[22]. So, although they are structurally different from the viral fusion proteins, the dynamin-related molecules involved in mitochondrial fusion might provide the same functions, namely close apposition and the deformation of fusing membranes.

Cell–cell fusion

Cell–cell fusion events are essential during fertilization, development and immune responses. During fertilization the sperm fuses with the oocyte in order to release its pronucleus into the oocyte cytoplasm. Other cell–cell fusion events include the fusion of myoblasts during myotube generation, the fusion of macrophages during osteoclast and 'giant cell' formation, syncytiotrophoblast formation during placental development, and yeast-cell fusion during mating. Little is known about the molecular players that are involved in the regulation and execution of cell–cell fusion, and of what is known there is little conservation from yeast to nematodes and insects to mammals[23], suggesting that these mechanisms might have evolved independently.

The best functionally characterized cell–cell fusion molecules are the *Caenorhabditis elegans* anchor cell fusion failure-1 (AFF-1) and epithelial fusion failure-1 (EFF-1) proteins, which are required for anchor cell and epithelial cell fusion, respectively[12]. These proteins are both necessary and sufficient for cell–cell fusion and are therefore authentic fusogens[24,25]. They are needed in both of the membranes that are destined to fuse and can probably tether the membranes, but it is unclear how they mediate this fusion. Although they are conserved in nematodes, no homologues outside of the nematode lineage have been identified.

Syncytins are expressed in the placenta and function in the trophoblast cell–cell fusion that results in syncytiotrophoblast formation[26,27]. Syncytins are homologous to retroviral fusion proteins and have a potential fusion peptide. Primate and rodent lineages have probably acquired this retroviral protein independently and these proteins might function in a manner analogous to viral fusion proteins[27].

Despite the importance of sperm–egg fusion, surprisingly few candidate proteins for mediating the actual fusion event have been identified. Among the best candidates are CD9, a multiple transmembrane-domain protein on the egg surface[28–30], and IZUMO, a single transmembrane-domain protein with an extracellular immunoglobulin (Ig)-like domain on the sperm surface[31]. Interestingly, CD9 localizes to microvilli on the egg surface. Microvilli are regions of the plasma membrane with extreme curvature and will therefore be highly fusogenic[32]. Furthermore, CD9 seems to directly affect the curvature of the microvilli because CD9 deletion results in an increased microvillus diameter[32]. Thus, CD9 might be required to stabilize and/or further generate a more extreme membrane curvature that is required for the fusion step.

Dynamin superfamily
A family of GTP-binding proteins that mediate oligomerization-dependent membrane remodelling events.

Fusogen
An agent that has the ability to promote fusion between two membranes.

Syncytin
A mammalian protein that is derived from a retrovirus. Syncytins function in cell–cell fusion during trophoblast formation.

Immunoglobulin (Ig)-like domain
A common domain that is found in extracellular proteins and is composed largely of β-sheets. Ig domains are the structural unit of antibodies.

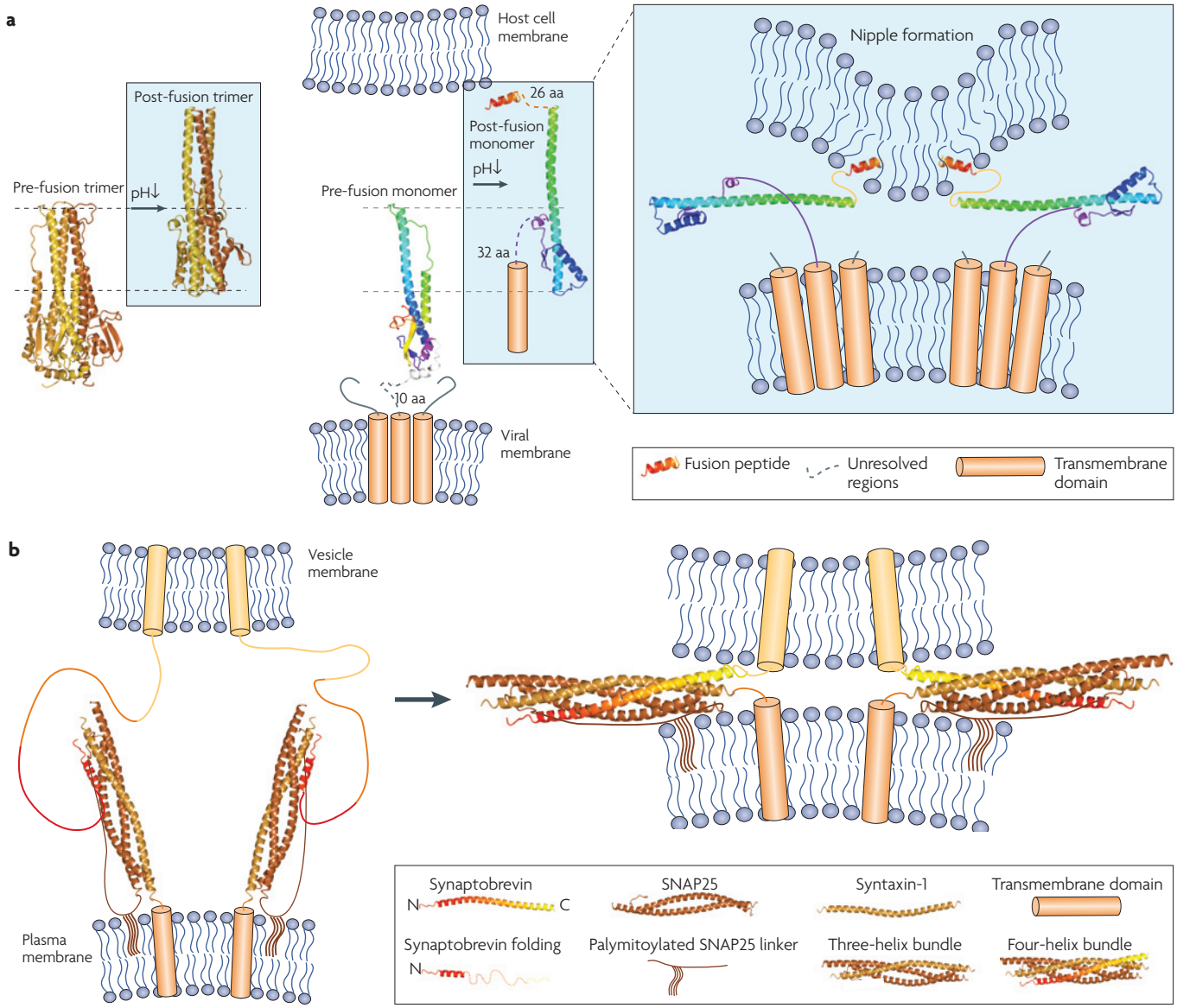


Figure 4 | **Hairpin-like structures in membrane fusion. a** | Structural changes in the influenza viral fusion protein. The fusion protein is a homotrimer and is cleaved by a host protease into the polypeptides HA1 (not shown) and HA2. HA2 has a C-terminal transmembrane domain and the N terminus will mature into the fusion peptide (Protein Data Bank code 1IBN). The pre- and post-fusion HA2 trimer structures are shown (top left). Residues 1–175 are resolved in the pre-fusion structure (PDB code 2HMG, showing chains B, D and F) and residues 40–153 are resolved in the post-fusion structure (PDB code 1HTM, showing chains B, D and F). Pre- and post-fusion structures were aligned using residues 76–105, which are part of the extended helix in both structures and constitute the region between the dotted horizontal lines. To simplify the membrane-fusion model, we use only one subunit of each component of the trimer (chain B from each structure) and have colour-coded these subunits with a gradient from red (at the N terminus, the fusion peptide) to magenta (at the C terminus). Comparisons of the coloured regions highlight the structural rearrangements that take place. The extent of unresolved N- and C-terminal residues in the post-fusion structure means that it is hypothetical how this structure is arranged with respect to the fusing membranes, but it is generally assumed that the N and C termini are both at the same end of the molecule. In this state the structure remotely resembles a hairpin, and hence the terminology 'hairpin model of membrane fusion'. In this model, a structural rearrangement from an extended to a hairpin conformation is proposed to drive the close apposition of the membranes to be fused. Fusion peptide insertion into the target membrane might generate an area of positive membrane curvature (nipple formation). **b** | SNARE-dependent membrane fusion involves the final formation of a four-helix bundle with helices contributed by three or four different SNARE proteins. Shown is the formation of the neuronal SNARE complex (PDB code 1SFC) of vesicular synaptobrevin with plasma-membrane SNAP25 and syntaxin-1. SNAP25 and syntaxin-1 can form a three-helix bundle (the intermediate structure shown on the left has not been structurally determined and so is hypothetical). This can act as the acceptor for synaptobrevin, which is unstructured before assembly and folds from the N terminus to the C terminus (see the initiation of helix assembly on the left) to form the mature SNARE complex. This folding into the fourth strand of the four-helix bundle is proposed to bring the membranes into close apposition (right). This resembles the post-fusion hairpin-like structure of the influenza virus. aa, amino acids.

C2 domain
A domain found in many intracellular proteins that mediate Ca^{2+}-dependent protein–protein and protein–membrane interactions.

AAA-ATPases
(ATPases associated with diverse cellular activities). Enzymes that translate the chemical energy that is stored in ATP into a mechanical force.

NSF
(*N*-ethylmaleimide-sensitive fusion protein). An AAA-ATPase that uses ATP hydrolysis to disassemble the SNARE complex.

Aliphatic chain
A backbone of carbon atoms that lack aromatic groups. In cellular membranes, the aliphatic hydrocarbon chains of phospholipids and sphingolipids form the hydrophobic core of the membrane.

Intriguingly, in addition to IZUMO, several molecules with Ig-like domains were shown to be required for cell–cell fusion events. Macrophage fusion receptor (MFR) and the leukocyte surface antigen CD47 are transmembrane-domain proteins with Ig-like domains and have been implicated in homotypic macrophage fusion[33,34]. The *Drosophila melanogaster* proteins DUF, RST and SNS that are involved in myoblast fusion each contain multiple extracellular Ig-like domains, which are required for cell–cell tethering[35–37]. Whether the Ig-like domains have a role downstream of cell–cell tethering is unknown, but their widespread occurrence in cell–cell fusion events suggests that they have a general role during membrane fusion. Notably, Ig-like domains are structurally related to C2 domains, which are found in synaptotagmins (FIG. 6), proteins that are central to intracellular membrane-fusion events (see below)[38]. By analogy to synaptotagmins, it is therefore possible that some of the Ig-like domains that are involved in fusion bind and insert into the target membrane, thereby inducing membrane curvature and thus facilitating membrane fusion. The actin cytoskeleton has also been strongly implicated in myoblast fusion in *D. melanogaster*[23]. It is therefore conceivable that the Ig-like-domain-containing proteins tether the two cells that are destined to fuse with their extracellular domains and mediate actin polymerization and reorganization with their intracellular domains, leading to highly curved plasma-membrane protrusions that bring the two plasma membranes against each other (FIG. 1, insert).

SNARE-dependent membrane fusion

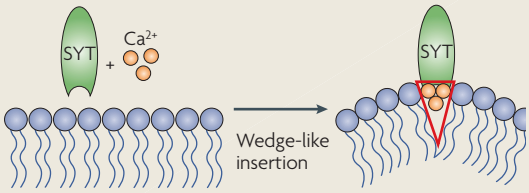
SNARE superfamily proteins are essential for many intracellular membrane-fusion events[39]. SNARE motifs are the regions in each SNARE protein that contribute to the formation of a highly stable four-helix bundle, called the SNARE complex (FIG. 4). Each SNARE motif contributes one helix to this four-helix bundle and the helices are all aligned in parallel[40,41]. The folding of this bundle is thought to drive the fusion reaction.

From extensive research over the past 20 years, the following consensus on SNARE function has emerged. At least one SNARE protein that contains a transmembrane domain must be present in each of the membranes that are destined to fuse (although this has recently been challenged[42]). The SNARE complex forms first in a *trans* configuration, in which the SNAREs that are involved in the formation of the complex are localized in different membranes (FIG. 4b). The subsequent formation of the complete SNARE complex temporally coincides with membrane fusion, which results in the formation of a *cis* complex in which all of the contributing SNARE proteins are localized to the same membrane. This *cis* complex is then disassembled by the action of αSNAP (an adaptor protein) and the AAA-ATPase NSF, freeing the SNAREs for the next round of fusion[39]. It is notable that SNAREs never function alone, and in every fusion event that has been analysed in detail, other molecules are required for efficient membrane fusion. Intriguingly, most of these molecules bind to both membranes and to the SNAREs. Next, we discuss vacuole, endosome and synaptic vesicle fusion events in more detail.

Yeast vacuole fusion. In yeast, the functional equivalent of the mammalian lysosome is the vacuole, and yeast vacuoles can undergo homotypic fusion[43] (FIG. 1). Homotypic yeast vacuole fusion is required for the reassembly of larger vacuoles, as occurs during the budding of haploid cells and in zygotes[44]. Yeast vacuole fusion depends on the SNAREs Vam3, Vam7, Vti1 and Nyv1 (REFS 45,46).

Whether yeast SNARE proteins are sufficient for physiological fusion is still under debate. It has recently been shown that the yeast vacuolar SNARE proteins

Box 1 | **Membrane-curvature induction caused by shallow insertions**



The region of a monolayer that is occupied by the glycerol backbones of lipids has maximal rigidity and therefore works as a fulcrum for monolayer bending. So, proteins that insert only partly into one monolayer, to expand the region occupied by glycerol backbones, work as wedges in the membrane (see figure). Given that voids cannot exist in the membrane, the aliphatic chains of neighbouring lipids respond by tilting. This results in the local bending of the monolayer towards the insertion and, owing to bilayer coupling, the bending of the whole bilayer. Several molecules have been proposed to bend the bilayer in this manner. Among them are the C2 domains of synaptotagmins (SYTs) and synaptotagmin-like proteins[22], epsin1 (REF. 150) and SAR1 (REF. 151). In the case of synaptotagmin-1, the double C2-domain module is required for curvature induction. Although curvature induction is a feature that is shared by other double C2 domains it is unclear whether it applies to all C2 domains.

It is thought that curvature induction by shallow insertions involves the coordinated insertion of multiple molecules that might be held in close proximity by a scaffold. In the case of epsin this scaffold is the clathrin cage and in the case of SAR1 it is probably the coatomer protein coat. For synaptotagmin it is likely that the interaction with SNARE proteins leads to a larger fusion complex. Furthermore, vesicle-localized synaptotagmin molecules will cluster at the area of vesicle–plasma-membrane contact, and the presence of two linked C2 domains per synaptotagmin molecule increases the avidity of the module for the target membrane, allowing them to work cooperatively. Other factors might also determine the extent to which the membrane can respond to shallow and coordinated insertions, namely the membrane tension, the lipid composition and the presence of transmembrane proteins, which will also be major contributors to bilayer coupling. So, highly curved membranes can be stable if their spontaneous curvature is close to the geometrical one (this is how lysolipids stabilize curved 'nipples' and prevent their transformation into stalks).

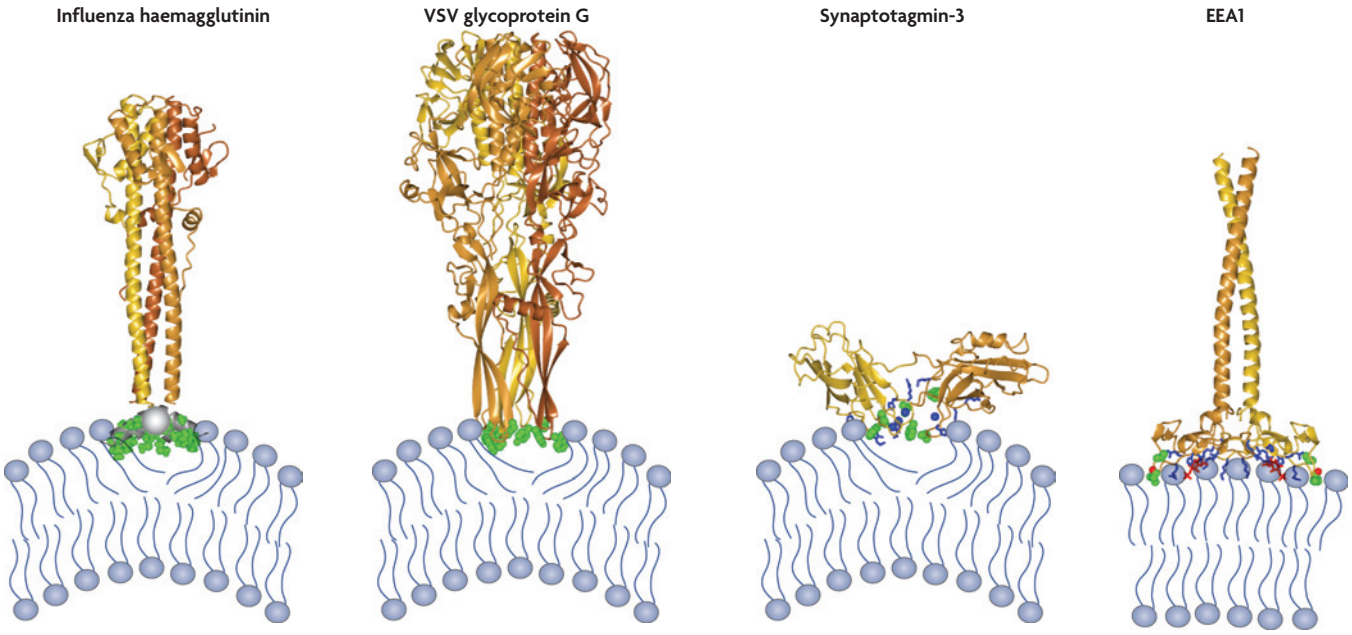


Figure 5 | **Membrane insertions and bending by membrane-fusion molecules.** Several molecules that are involved in membrane fusion have shallow insertions into one monolayer of the membrane and have been shown to induce, or are likely to induce, membrane curvature (BOX 1). The low-pH-induced form of the homotrimeric influenza protein haemagglutinin (Protein Data Bank code 1HTM) exposes an N-terminal fusion peptide that is proposed to insert in a shallow manner into the host cell membrane. Two fusion peptides of the homotrimer are shown as structural elements surrounded by hydrophobic residues (shown in green). Similar to haemagglutinin, the low-pH form of glycoprotein G of vesicular stomatitis virus (VSV; PDB code 2CMZ) inserts hydrophobic loops into the outer monolayer of the host cell membrane. This is very similar to the Ca^{2+}-induced insertion of hydrophobic loops into the plasma membrane shown here for the C2A and C2B domains of synaptotagmin-3 (PDB code 1DQV). Early endosomal antigen-1 (EEA1; PDB code 1JOC) binds to phosphatidylinositol-3-phosphate (PtdIns3P)-containing regions of membrane and inserts hydrophobic loops into it. This might induce membrane curvature and therefore assist the SNARE-dependent membrane fusion of endosomes. Positively charged residues are shown in blue and PtdIns3P head groups are shown in red.

require active Rab GTPases and Rab effectors such as HOPS (homotypic fusion and vacuole protein sorting) to function efficiently[47]. Overexpression of SNAREs bypasses the requirement for Rab GTPases and effectors but results in vacuole lysis and 'leaky' fusion, indicating that SNAREs are not sufficient for non-leaky fusion *in vivo*[47]. In addition, overexpression of SNAREs does not increase the total amount of vacuole fusion in an *in vitro* assay[47], which suggests that SNAREs need active help from accessory molecules such as the HOPS complex[42]. HOPS is a multi-subunit protein complex that is required for vacuole tethering. Purified HOPS interacts with vacuolar SNAREs and phosphoinositides[48], and HOPS is therefore ideally positioned to have an active role during the fusion process. It will be interesting to see whether HOPS also has a direct effect on membrane curvature.

Endosomal fusion. Early endosomes fuse homotypically as well as heterotypically with late endosomes or lysosomes, in order to allow the efficient recycling and degradation of their contents. As for vacuole fusion in yeast, SNAREs are essential for endosome fusion. Among the SNAREs that have been identified are syntaxin-6, syntaxin-13, VTI1A and VAMP4 (REF. 49). Although these SNAREs have been shown to be able to promote liposome fusion *in vitro*, they are not sufficient for membrane fusion *in vivo*. Analogous to the requirement of HOPS for efficient vacuole fusion in yeast, the tethering factor and Rab5 effector EEA1 is essential for homotypic endosome fusion *in vivo*. As with the HOPS complex, EEA1 directly interacts with SNAREs and lipids[50–52]. Furthermore, residues of the FYVE domain of EEA1 insert into the membrane following phosphatidylinositol-3-phosphate binding[53] (FIG. 5). This membrane insertion might induce curvature stress and thereby induce endosomal fusion. Many tethering factors bind to SNAREs and membranes and might therefore have a more direct role during fusion than the mere tethering and targeting activity that was previously assumed[54].

Synaptic vesicle fusion

The fusion of synaptic vesicles and dense-core granules with the plasma membrane is essential for neurotransmitter and hormone release and therefore for efficient intercellular communication. Synaptic vesicle exocytosis is specialized in a way that allows neurons to communicate in a fast and controlled manner. These fusion events are tightly coupled to the influx of extracellular Ca^{2+} into the cell and are remarkably fast, with synaptic vesicle fusion occurring within less than 1 ms after Ca^{2+} influx. Dense-core granule fusion is approximately tenfold slower than synaptic vesicle fusion. The ability to stimulate synaptic and dense-core granule fusion by Ca^{2+} elevation has given us remarkable insights into the regulation and kinetics of fusion.

Rab GTPase
A small GTP-binding protein that regulates membrane traffic by interacting with effector proteins.

Endosomes
Various intracellular compartments that are the central sorting stations for molecules that are either derived mainly from the plasma membrane or taken up from the extracellular medium.

FYVE domain
A protein domain that is named after the first four proteins in which it was found (Fab1, YOTB/ZK632.12, Vac1 and EEA1) and that binds to the membrane lipid phosphatidylinositol-3-phosphate.

Dense-core granules
Vesicles that are 200–300 nm in diameter and are seen as electron dense by electron microscopy. In some cells they undergo Ca^{2+}-dependent exocytosis.

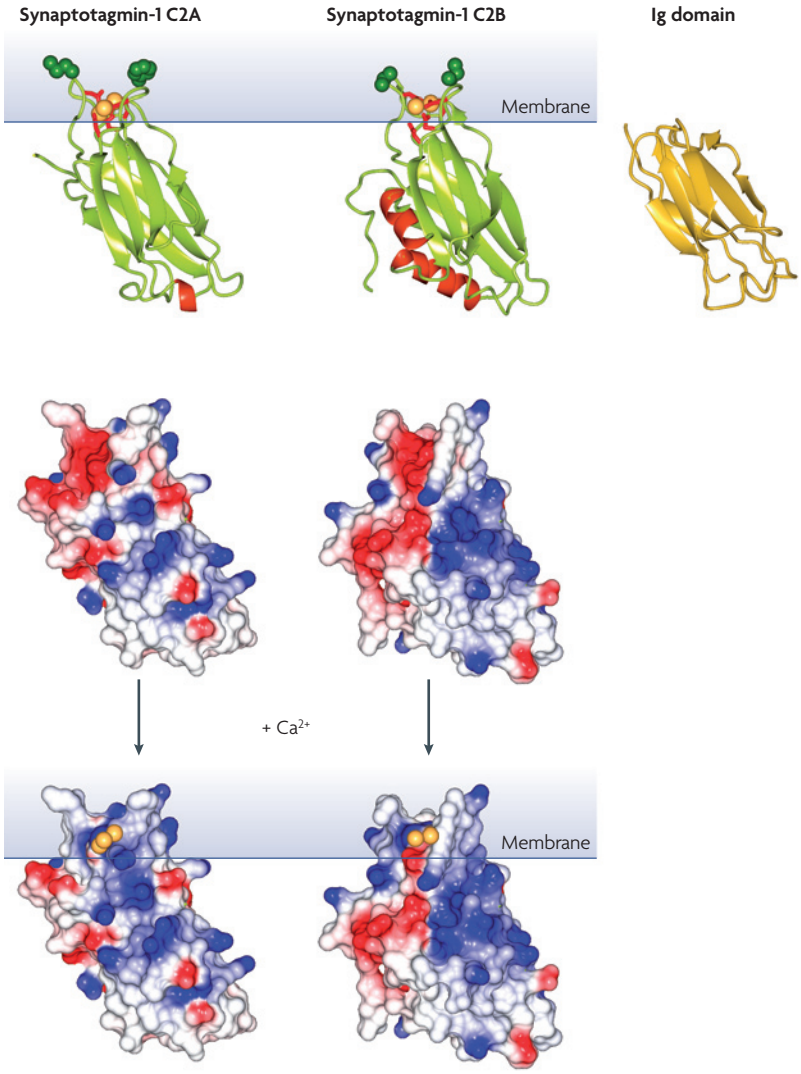


Figure 6 | **Ca^{2+}-dependent membrane interaction of C2 domains.** Ca^{2+} (yellow spheres) is coordinated by five Asp residues (red residues) on loops at the end of the C2 domains of synaptotagmin C2A (Protein Data Bank code 1BYN) and C2B (PDB code 1UOW). This neutralizes the negative surface electrostatic potential of this region (compare the bottom panels) and allows the C2 domains to interact with membranes. From membrane insertion measurements we know that the tips of the loops (including the indicated hydrophobic residues in dark green) are buried in the membrane. The C2 domain and the immunoglobin (Ig) domain have similar folds, both being composed of β-sheets connected by loops. The variable domain of the heavy chain of the monoclonal antibody MCPC603 (PDB code 1MCP) is shown. Ig domains are often found in proteins that are involved in cell–cell fusion, and given the structural similarity to the C2 domains it is possible that at least some of these domains have an active role during membrane fusion by causing the induction of membrane curvature.

The presynaptic response to an action potential can be divided into two major phases. Immediately (<1 ms) after depolarization of the synapse a burst of small synaptic vesicle exocytosis is observed; this is referred to as synchronous release. The synchronous release phase is followed by isolated exocytic events, which are collectively termed asynchronous release[55]. Both synchronous and asynchronous release are strictly Ca^{2+} dependent but are at least partly dependent on different molecular machineries[56,57]. Spontaneous release of single vesicles that is uncoupled from synaptic depolarization also occurs sporadically.

Detailed analysis of exocytosis has given us an insight into which molecules are involved in the actual fusion step and which molecules are involved in upstream events[55,58,59]. According to studies of large dense-core granule exocytosis (and therefore, by implication, of synaptic vesicle fusion), the only molecules that are directly involved in the fusion step are synaptotagmins and the SNAREs, and manipulation of either has a direct effect on the kinetics of fusion[60]. Other accessory molecules, such as complexin, mammalian homologue of *C. elegans* UNC-13 (MUNC13), MUNC18 and CAPS (calcium-dependent secretion activator), seem to have upstream involvement, because manipulation has an effect on the amount of exocytosis but not on the kinetics. There are many possible roles of the accessory molecules upstream of fusion, including targeting, priming and membrane-denuding (FIGS 2, 3).

Neuronal SNAREs. At the synapse, the SNAREs that are involved in synaptic vesicle fusion are synaptobrevin and the two plasma-membrane SNAREs SNAP25 and syntaxin-1 (REF. 39) (FIG. 4b). The targeted deletion of each SNARE protein results in an almost complete abolition of exocytosis. The residual release seen in synaptobrevin$^{-/-}$ and *Snap25*$^{-/-}$ mice is likely to be attributable to compensation by other isoforms[61,62]. Furthermore, it was discovered in the early 1990s that the neuronal SNAREs are the targets for several neurotoxins, which proteolytically cleave individual SNAREs and thereby inhibit exocytosis[63–65]. Moreover, the neuronal SNAREs mediate membrane fusion when they are reconstituted into artificial liposomes[66,67].

These findings leave little doubt that the SNAREs are central players during synaptic vesicle fusion and have led to the now widely accepted model in which the SNAREs (that is, the vesicular synaptobrevin and the plasma-membrane SNAP25 and syntaxin-1) mediate fusion by forming a tight complex, at the centre of which lies a four-helix bundle (FIG. 4; note the similarity between the helical bundles of the SNARE complex and viral fusion proteins). Formation of the SNARE complex brings the vesicular and plasma membranes into close proximity (a gap of ~3–4 nm would accommodate the SNARE complex). In addition, it is proposed that part of the energy that is released following SNARE-complex formation is transduced through the transmembrane domains into the lipid bilayers, resulting in membrane destabilization. This close proximity of the membranes and the lipid-bilayer destabilization would then result in fusion. Indeed, although it is hard to determine accurately, the energy that is released by SNARE-complex formation seems to be sufficient to overcome the energy barrier for membrane fusion[68]. According to this model, other molecules, such as MUNC18 and MUNC13, the synaptotagmins and the complexins, have essential roles in the temporal and spatial regulation of the SNAREs but would have no direct role during the actual fusion process.

However, although there is no doubt that the SNARE complex brings the two membranes that are destined to fuse into close apposition, it is unclear how much of, or how, the energy that is released during complex formation

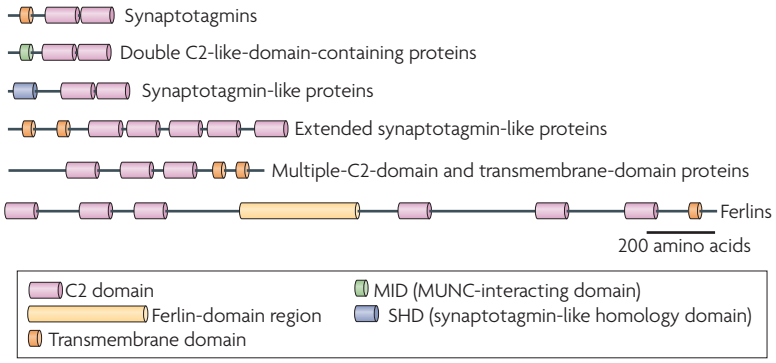


Figure 7 | **Multiple-C2-domain-containing proteins.** The domain structures of selected multiple-C2-domain-containing proteins (MC2Ds) that are implicated in vesicle fusion. Synaptotagmins (SYTs; of which there are 17 including SYT1 (accession number NP_005630)) are characterized by their double C2 domains and an N-terminal transmembrane domain. Double C2-like-domain-containing proteins (DOCs; of which there are three: DOC2A/α (accession number AAH41769), DOC2B/β and DOC2γ have a double C2 module preceded by a MUNC-interacting domain (MID) which binds to MUNC13. Rabphilin 3A (exophilin 1) is closely related to the DOCs but has an N-terminal Rab-interacting domain and no MID domain. Synaptotagmin-like proteins (SLPs; of which there are five: SLP1 (accession number NP_116261), SLP2 (exophilin4), SLP3, SLP4 (granuphilin-A) and SLP5) have a similar domain structure to DOCs but have a Rab GTPase-binding domain at the N-terminus, known as a synaptotagmin-like homology domain. The extended synaptotagmin-like proteins (E-SYTs; of which there are three: E-SYT1 (accession number NM_015292), E-SYT2 and E-SYT3) contain up to five C2 domains and two N-terminal transmembrane domains. The MC2D and transmembrane-domain proteins (MCTPs; of which there are three: MCTP1 (accession number Q6DN14), MCTP2 and MCTP3) have a variable N-terminal sequence, three C2 domains and two C-terminal transmembrane domains. Little is known about the functions of E-SYTs and MCTPs. The ferlins (of which there are four: dysferlin (accession number NP_003485), myoferlin, otoferlin and ferl1) have up to six C2 domains, a C-terminal transmembrane domain and central ferlin homology domains.

is transduced into the membrane. It has been shown that SNARE-mediated fusion of artificial liposomes *in vitro* requires non-physiological, high SNARE densities, and this fusion is accompanied by the induction of liposome leakage[69,70]. At lower SNARE densities, the neuronal SNAREs were shown to require either curvature stress or destabilizing agents, such as PEG, to mediate efficient fusion. This is reminiscent of yeast vacuole fusion, in which the overexpression of SNAREs abrogates the need for Rab GTPase and the tether factor, and in which the resulting fusion is accompanied by significant lysis[47].

In addition, to ensure that the energy that is released during SNARE-complex formation is directly transmitted into the membrane, it has been suggested that the linker between the SNARE motif and the transmembrane domain must be rigid. One might assume that mutations of this linker would radically interfere with fusion. However, this is evidently not the case as the insertion of two Pro or Gly residues into the linker of synaptobrevin had little effect on fusion *in vitro* and *in vivo*[71,72]. *In vitro* experiments with mutated syntaxin-1, which was designed to conformationally uncouple the SNARE domain from the transmembrane domain, also showed surprisingly minor effects on fusion[72]. Furthermore, the exchange of the entire transmembrane domain for long, bilayer-spanning lipids was tolerated, at least *in vitro*[73]. In summary, the current evidence suggests that the SNAREs bring the two membranes into close proximity, but it is unclear to what extent they have a direct membrane-destabilizing role. So, other molecules probably function alongside the SNAREs by helping to trigger the actual fusion step *in vivo*.

Another important parameter is the state of the SNAREs before the fusion event. Two extreme scenarios are conceivable. In the first scenario, the SNAREs are completely disassembled before fusion. Ca^{2+} influx then somehow triggers the complete zippering of the SNARE complex. In this way, the energy that is released during complex formation would be freed concurrently with the membrane-fusion process and would therefore be available for fusion. In the second scenario, the SNARE complex is already completely formed before the Ca^{2+} trigger. In this case, the energy for the subsequent fusion stages would have to be provided by other molecules. The available data point to an intermediate between these two extremes. Recent work in chromaffin cells suggests that the SNARE complex is already partially assembled before the Ca^{2+} trigger and that the C-terminal part of the complex assembles during fusion[74]. How a partially assembled SNARE complex can be stabilized is unclear, although complexins have been proposed to provide this function[75–77].

The SNARE complex does not act alone. Molecules have been identified that are required for Ca^{2+}-dependent exocytosis at the synapse, the most prominent of which are MUNC18, MUNC13, complexin and synaptotagmin. Compelling evidence suggests that synaptotagmin-1 is the Ca^{2+} sensor for membrane fusion. Synaptotagmin-1 function is intimately linked to the SNARE complex, and so far this is the only molecule besides the SNAREs that has been shown to have a direct effect on the kinetics of exocytosis[78].

Synaptotagmin-1. Synaptotagmin-1 belongs to a large protein family and is localized to synaptic vesicles by a single N-terminal transmembrane domain (FIG. 7). It was first isolated as a conserved antigen with an apparent molecular weight of 65 kDa and named p65 accordingly[79]. It was then discovered that p65 contains two cytoplasmic C2 domains[80] and so the protein was subsequently renamed synaptotagmin[81]. C2 domains are independently folded, evolutionarily conserved domains that are composed of β-sheets connected by loops[82]. They show structural similarity to Ig domains[38] (FIG. 6). Many C2 domains bind Ca^{2+} and are found in proteins that are involved in signalling and membrane trafficking. It is clear that most C2-domain-containing proteins that are involved in signalling carry only one copy of the domain, whereas molecules that are involved in membrane trafficking and fusion tend to carry multiple C2 domains (MC2Ds).

In synaptotagmin-1, Ca^{2+} binding is mediated by pockets that are made from the loops at one end of the C2 domains. These pockets are lined by Asp residues[83,84], and Ca^{2+} binding results in an electrostatic switch that changes the net charge in this region from negative to positive and thereby enables the C2 domain to interact with and partially insert into the negatively charged membranes[85,86] (FIG. 6). Some other C2 domains

show no membrane binding or can bind to membranes in a Ca^{2+}-independent manner. C2 domains can also engage in protein–protein interactions. Furthermore, synaptotagmin-1 C2 domains bind SNAREs in a Ca^{2+}-regulated manner[75].

Targeted deletion of synaptotagmin-1 in mice results in the loss of synchronous, but not asynchronous, neurotransmitter release[57]. In synapses in which synaptotagmin-1 is not expressed, synaptotagmin-2 and synaptotagmin-9 are probably the functional substitutes[87]. Other Ca^{2+}-binding synaptotagmins cannot functionally replace synaptotagmin-1 in cortical neurons, suggesting that these proteins have a role in non-synchronous release or in membrane-fusion events elsewhere[87]. In auditory synapses, the MC2D protein otoferlin substitutes for synaptotagmin-1 (see below)[88]. The C2 domains of synaptotagmin-1 have been extensively studied, and Ca^{2+}-dependent and Ca^{2+}-independent interactions with neuronal SNAREs and phospholipids have been reported. The C2A domain shows Ca^{2+}-dependent interactions with syntaxin-1 (REF. 89), SNAP25 (REF. 90) and negatively charged phospholipids[91]. The C2B domain shows Ca^{2+}-independent interactions with the SNAP25–syntaxin-1–t-SNARE (target-membrane-associated SNARE) complex[92], with phosphatidylinositol-4,5-bisphosphate and with phosphatidylinositol-3,4,5-trisphosphate[93,94] and, as with the C2A domain, shows Ca^{2+}-dependent interactions with negatively charged phospholipids[95].

A truncated protein that contains both C2 domains (C2AB) shows weak Ca^{2+}-independent SNARE-complex binding, which is enhanced in the presence of Ca^{2+} (REF. 75). Synergistic membrane binding of the C2AB domain has been shown, and the combined domain inserts deeper into the membrane than the isolated C2A and C2B domains[85,86,96,97]. In addition, C2AB domains, but not the isolated domains, induce positive membrane curvature *in vitro*[22]. The Ca^{2+}-dependent SNARE- and membrane-binding activities of synaptotagmin-1 are both essential for triggering exocytosis[89,98–103].

Several models have been put forward to explain how synaptotagmin-1 triggers the rapid exocytosis of synaptic vesicles and dense-core granules. The first model proposes that synaptotagmin-1 directly affects SNAP25 and syntaxin-1, resulting in t-SNARE-complex formation, which in turn provides a high-affinity receptor for synaptobrevin. It was further proposed that Ca^{2+}-dependent phosphatidylserine binding by synaptotagmin-1 is important for its action on the t-SNAREs[104]. The second model proposes that synaptotagmin-1 displaces complexin from a partially assembled SNARE complex. According to this model, complexin functions as a fusion clamp, preventing the complete zippering of the SNARE complex. Complexin displacement by Ca^{2+}-bound synaptotagmin-1 thus enables the SNARE complex to form fully, thereby inducing membrane fusion. According to the first model, the SNARE complex is formed after the Ca^{2+} trigger, whereas the second model assumes that the SNARE complex is at least partially formed before the Ca^{2+} trigger[75–77]. A third variation proposes that the C2B domain of synaptotagmin-1 simultaneously binds to the vesicular and plasma membranes following Ca^{2+} binding, thus inducing very close membrane apposition. Furthermore, the C2B domain was suggested to bend the membrane, owing to its positive electrostatic potential, and thereby promote hemifusion-stalk formation[105,106].

We have recently put forward a fourth model for synaptotagmin-1 function[22]. Our model is based on the observation that Ca^{2+}-dependent membrane binding induces extreme curvature in the target membrane, which in the case of synaptic vesicle fusion will be the plasma membrane. Curvature induction is required for the stimulation of SNARE-dependent membrane fusion in an *in vitro* assay. Membrane-curvature induction by synaptotagmin-1 has two major effects: first, it buckles the plasma membrane towards the vesicle, thereby reducing the distance between the membranes; second, it puts the lipids in the end cap of the buckle under curvature stress, and thereby reduces the energy barrier for the subsequent lipid rearrangements that occur during hemifusion and fusion-pore opening[22]. Ca^{2+}-dependent SNARE binding would be required to target the membrane buckle. Our model is not incompatible with Ca^{2+}-dependent complexin displacement. According to our model, synaptotagmin-1 has a direct fusogenic role rather than a regulatory role and so would be classified as a fusion protein. This fusogenic role, which is mediated by Ca^{2+}-dependent membrane-curvature induction, would explain why Ca^{2+} is the final trigger for exocytosis and why synaptotagmin manipulation affects the kinetics of exocytosis in chromaffin cells[78].

Multiple-C2-domain proteins in membrane fusion

Synaptotagmin-related molecules, which are defined as proteins that contain two or more linked C2 domains, belong to a larger protein superfamily of MC2Ds. Many of these proteins have been implicated in membrane-fusion processes and many fusion events might therefore be initiated by the induction of membrane curvature by these proteins.

The synaptotagmin family. The human genome encodes 17 synaptotagmins[107] (FIG. 7) and the expression of most synaptotagmins is highest in neuronal tissues, although some synaptotagmins show a widespread tissue distribution[103,108]. Synaptotagmin-2 and probably synaptotagmin-9 are functionally equivalent to synaptotagmin-1, although small but significant differences exist[87,109,110]. Based on the C2-domain sequence we predict that both synaptotagmin-2 and synaptotagmin-9 can induce membrane curvature.

Synaptotagmin-7 shows a broad tissue distribution and Ca^{2+}-dependent membrane binding[111] and is likely to induce membrane curvature. Synaptotagmin-7 has been implicated in a number of Ca^{2+}-dependent membrane-fusion events, including lysosomal exocytosis during plasma-membrane resealing after injury[112] and insulin secretion from pancreatic β-cells[113]. Synaptotagmin-7 has also been implicated in exocytosis of dense-core granules in PC12 cells[114] (but see REF. 110). However, synaptotagmin-$7^{-/-}$ mice are viable and show no neurological phenotype[115].

Synaptotagmin-3 is less well-studied. It binds to membranes in a Ca^{2+}-dependent manner and induces membrane curvature[22]. It was the first synaptotagmin for which a crystal structure of the double C2-domain fragment was obtained[116]. The biological function of synaptotagmin-3 is unclear: it localizes to the plasma membrane in neurons but is also implicated in intracellular trafficking in the endosomal compartment in T cells and mast cells[117,118].

Mammalian synaptotagmin-4 does not show Ca^{2+}-dependent membrane binding[119]. Synaptotagmin-$4^{-/-}$ mice are viable and show only a subtle neurological phenotype, and synaptotagmin-4 has been suggested to be redundant with its close relative synaptotagmin-11 (about which little is known)[120]. It was recently shown that synaptotagmin-4 might function in a membrane-fusion step that is required for the maturation of dense-core granules in PC12 cells. This activity was dependent on Ca^{2+}-independent binding of syntaxin-6 (REF. 121).

Synaptotagmin-5 binds to membranes in Ca^{2+}-dependent manner, induces membrane curvature and is proposed to be involved in insulin exocytosis[22,122]. Synaptotagmin-6 binds to membranes in a Ca^{2+}-dependent manner, and we predict that it can also induce membrane curvature. Synaptotagmin-6 was proposed to be required for exocytosis of vesicles during the acrosome reaction in sperm[123]. Synaptotagmin-12 was shown to increase the incidence of spontaneous neurotransmitter release events, although the mechanistic basis for this is enigmatic[124]. It will be interesting to learn which of the membrane-fusion steps involve the other synaptotagmins. It seems likely that at least the synaptotagmins that bind membranes will have direct fusogenic roles.

DOC2 proteins. The double C2-like domain-containing (DOC2) proteins are highly similar to the synaptotagmins, given that they contain two C-terminal C2 domains (FIG. 7). Three DOC2 proteins, DOC2A/α (accession number AAH41769), DOC2B/β (NP_003576) and DOC2γ (EAW74657), are encoded in humans. Unlike the synaptotagmins, the DOC2 proteins do not have an N-terminal transmembrane domain, but they do share a conserved N-terminal domain, which has been reported to bind MUNC13 (REFS 125,126). In addition, an interaction with MUNC18 has been reported[127]. It has been shown that only the C2A, but not the C2B, domain of DOC2B/β binds to phospholipids in a Ca^{2+}-dependent manner[128]. However, the C2A and C2B domains have only been studied as glutathione-*S*-transferase fusion proteins and thus the C2 domains await a more detailed analysis. From its sequence it is not apparent why the C2B domain does not show Ca^{2+}-dependent membrane binding. DOC2A/α expression is restricted to the brain, whereas DOC2B/β shows a more widespread expression pattern and in the brain does not overlap with DOC2A/α[127].

Doc2a$^{-/-}$ mice have mild defects in synaptic transmission during sustained stimulation[129]. Overexpression of DOC2A/α has been shown to enhance stimulated secretion in PC12 cells[130], whereas DOC2B/β has been shown to promote insulin secretion in MIN6 cells[131]. The precise step at which DOC2A/α and DOC2B/β function has not yet been elucidated. DOC2A/α and DOC2B/β translocate from the cytosol to the plasma membrane in a Ca^{2+}-dependent manner[132,133]. Translocation occurs at submicromolar Ca^{2+} concentrations, which suggests that *in vivo* the two proteins bind Ca^{2+} with significantly higher affinity than does synaptotagmin-1. This feature would fit with the hypothesis of a high-affinity Ca^{2+} sensor for asynchronous membrane fusion, and awaits further study.

Synaptotagmin-like proteins. In mammals there are five synaptotagmin-like proteins (SLPs), which are defined by a conserved synaptotagmin-like homology domain (SHD) at the N terminus (FIG. 7) that binds to Rab GTPases[134]. SLP1–5 contain two tandem C2 domains at their C termini that are homologous to the C2A and C2B domains of the synaptotagmins. The current literature on SLPs is confusing, partly because different names are used to describe the same gene product. SLP1 is also referred to as JFC1, SLP2 is referred to as exophilin-4 and SLP4 is referred to as granuphilin A. We refer to the following sequences: SLP1 (accession number NP_116261), SLP2 (NP_996810), SLP3 (NP_001009991), SLP4 (NP_542775) and SLP5 (NP_620135).

Several studies implicate the SLP proteins in the regulation of membrane trafficking events in professional secreting cells. However, there is no consensus regarding the function of the individual SLP proteins, and both positive and negative effects on exocytosis have been described. SLP1 has been reported to positively effect PSAP (prostate-specific acid phosphatase) secretion from LNCaP cells[135]. SLP1 and SLP2 have been implicated in secretion from cytotoxic T cells[136]. SLP2 (the SLP2-a isoform) has been shown to positively affect basal mucus secretion in gastric cells, although the reported effects were very small[137]. SLP2 is further implicated in targeting Rab27a-coated melanosomes to the plasma membrane in melanocytes[138]. This effect was attributed to its N-terminal Rab27a-binding activity and to phospholipid binding by its C2A domain. Similarly, SLP2 has been proposed to dock glucagon-containing granules to the plasma membrane in pancreatic α-cells. Glucagon granule docking has further been suggested to be negatively regulated by Ca^{2+} owing to a negative effect of Ca^{2+} on phospholipid binding by the C2A domain[139]. Following overexpression of *Slp4*, more insulin granules are found to be associated with the plasma membrane in MIN6 cells, an effect that is attributed to SLP4's interaction with syntaxin-1 (REF. 140). Overexpression of SLP4 also reduced the amount of insulin that is secreted after stimulation, whereas basal secretion levels were slightly enhanced[141]. In *Slp4*$^{-/-}$ cells, stimulated insulin secretion is augmented, although fewer granules are found docked at the plasma membrane[142]. The apparent inhibitory effect of SLP4 on stimulated insulin secretion might be mediated by its binding to syntaxin-1 and, by implication, by stabilization of the closed conformation of syntaxin-1 (REF. 141). However, the interpretation of the *Slp4*$^{-/-}$ results is complicated, because the levels of syntaxin-1 and MUNC18 proteins are also reduced in these cells[142].

Ferlins. The ferlin protein family has four members in humans (FIG. 7). These proteins are large and contain at least four C2 domains and a C-terminal transmembrane domain. Strong evidence suggests that the ferlins are directly involved in the regulation and triggering of Ca^{2+}-dependent membrane-fusion events. Otoferlin was shown to be required for synaptic vesicle release in auditory synapses, where it was proposed to functionally replace synaptotagmin-1 (REF. 88). Significantly, otoferlin was shown to bind the SNARE proteins syntaxin-1 and SNAP25 (REF. 88). From sequence alignments we predict that at least some of the C2 domains of otoferlin show Ca^{2+}-dependent membrane binding and insertion and are therefore likely to induce membrane curvature.

Dysferlin is another prominent member of the ferlin family. Dysferlin is required for Ca^{2+}-dependent plasma-membrane repair in muscle cells[143]. Plasma-membrane injuries are repaired by rapid, Ca^{2+}-dependent membrane-fusion events at and around the site of injury[144]. Mutations in dysferlin result in muscle degeneration, highlighting its importance in fusion during plasma-membrane repair[145,146]. The first C2 domain of both dysferlin and myoferlin has been shown to bind phospholipids in a Ca^{2+}-dependent manner[147,148]. Myoferlin is required for productive cell–cell fusion during myoblast formation[148], but its precise role is unclear. Interestingly, the yeast MC2D tricalbin-3 has been implicated in cell–cell fusion during mating and was suggested to prevent lysis during this process by causing Ca^{2+}-dependent plasma-membrane repair[149].

These results suggest that the ferlins have roles that are analogous to that of synaptotagmin-1 during synaptic vesicle release. In addition, the occurrence of MC2Ds separated by long linkers in the ferlins suggests a function for the ferlins in clustering membranes. This would be particularly useful during plasma-membrane repair, in which massive vesicle fusion is required at the site of injury.

Concluding remarks

Membrane-fusion events are mediated by a bewildering number of unrelated molecules. However, we propose that most fusion events require the coordination of two activities and that both of these are essential for efficient fusion. First, membranes have to be brought into close proximity and, second, bilayers that are destined to fuse must be destabilized. We propose that both can be achieved by the local induction of curvature, which brings the bilayers into close apposition and induces high local membrane stress, which can be relieved by fusion. Highly curved membranes, such as those of small vesicles and the end caps of ER tubules, will also not fuse unless they come into close enough proximity, are under stress and (in the case of the end-caps) are protein-free. The viral fusion proteins might combine the induction of close membrane proximity (mediated by the refolding of the protein) and the induction of high curvature (mediated by the shallow insertion of the fusion peptide into the target membrane). These two activities are not necessarily provided by only one polypeptide. For example, at the synapse synaptotagmin acts alongside the SNAREs to trigger fusion, whereas Rab GTPase effectors such as HOPS might assist SNAREs in other fusion events. Future studies will need to unravel how membrane proximity and curvature induction are coordinated *in vivo*.

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DATABASES
Protein Data Bank: http://www.pdb.org/pdb/home/home.do
1BYN | 1DQV | 1HTM | 1IBN | 1JOC | 1MCP | 1SFC | 1UOW | 2CMZ | 2HMG |
FirstGlance in Jmol (3D structures): http://molvis.sdsc.edu/fgij/index.htm
1BYN | 1DQV | 1HTM | 1IBN | 1JOC | 1MCP | 1SFC | 1UOW | 2CMZ | 2HMG |
UniProtKB: http://ca.expasy.org/sprot
αSNAP | AFF-1 | CAPS | CD9 | CD47 | complexin | DOC2A/α | DOC2B/β | DOC2γ | DUF | dysferlin | EEA1 | EFF-1 | MFR | IZUMO | mitofusin | MUNC13 | myoferlin | Nyv1 | OPA1 | otoferlin | Rab27a | Rab5 | RST | SLP1 | SLP2 | SLP4 | SNAP25 | SNS | synaptobrevin | synaptotagmin-1 | synaptotagmin-2 | synaptotagmin-3 | synaptotagmin-4 | synaptotagmin-5 | synaptotagmin-6 | synaptotagmin-7 | synaptotagmin-9 | synaptotagmin-11 | synaptotagmin-12 | syntaxin-1 | syntaxin-6 | SYT1 | tricalbin-3 | Vam3 | Vam7 | VAMP4 | Vti1 | VTI1A

FURTHER INFORMATION
Harvey T. McMahon's homepage: http://www.endocytosis.org
Leiden muscular dystrophy pages: dysferlin: http://www.dmd.nl/dysf_home.html
SNPs3D of ferlins: http://www.snps3d.org/search/?q=fer-1
Structural Classification of Proteins (SCOP):
Synaptotagmin-like: http://scop.mrc-lmb.cam.ac.uk/scop/data/scop.b.c.bd.b.c.html l
Synaptotagmin home page: http://www.endocytosis.org/Synaptotagmin/

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