# A cascade of multiple proteins and lipids catalyzes membrane fusion

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**ABSTRACT** Recent studies suggest revisions to the SNARE paradigm of membrane fusion. Membrane tethers and/or SNAREs recruit proteins of the Sec 1/Munc18 family to catalyze SNARE assembly into *trans*-complexes. SNARE-domain zippering draws the bilayers into immediate apposition and provides a platform to position fusion triggers such as Sec 17/α-SNAP and/or synaptotagmin, which insert their apolar "wedge" domains into the bilayers, initiating the lipid rearrangements of fusion.

Intracellular membrane fusion is essential for cell growth, hormone secretion, and neurotransmission. Fusion occurs by mechanisms that are conserved from yeast to humans and among most organelles. Intracellular membrane fusion normally requires Rab-family GTPases (Grosshans *et al.*, 2006); tethering proteins, which bind selectively to GTP-bound Rabs and are termed "effectors" (Baker and Hughson, 2016); SNARE proteins (soluble NSF [*N*-ethylmaleimide–sensitive factor] attachment protein receptor), which bind to each other to form SNARE complexes (Jahn and Scheller, 2006); Sec 1/Munc18 (SM) family proteins, which orchestrate SNARE complex assembly (Rizo and Südhof, 2012; Baker *et al.*, 2015); the ATPase Sec 18/NSF, which disassembles SNARE complexes (Zhao *et al.*, 2015); and the SNARE- and Sec 18/NSF-binding protein Sec 17/SNAP (soluble NSF attachment protein). SNAREs are membrane proteins that have canonical heptad repeat domains (SNARE motifs) with a central argininyl or glutaminyl residue and are thus termed R- or Q-SNAREs, respectively (Fasshauer *et al.*, 1998). There are three families of Q-SNAREs: Qa, Qb, and Qc. SNARE complexes consist of four-helix bundles with the composition RQaQbQc and are termed *cis* when each SNARE is anchored to the same membrane and *trans* when individual SNAREs are anchored to apposed bilayers prior to fusion. Pioneering studies suggested a paradigm (Table 1, left) in which SNAREs associate spontaneously in-*trans*, zipper their SNARE domains, and thereby stress and deform the bilayers via their C-terminal *trans*-membrane anchors, driving lipid rearrangements that lead to membrane fusion (Hanson *et al.*, 1997; Weber *et al.*, 1998). In this model, the other proteins required for fusion act only by regulating *trans*-SNARE complex levels. Here, we compare recent studies of the machineries controlling yeast vacuolar fusion (Figure 1A) and neurotransmitter release (Figure 1B) and propose a substantially revised membrane fusion paradigm (Table 1).

## FUSION ASSAYS

Understanding fusion rests on the quality and specificity of fusion assays. Fusion studies are guided by assays that measure lipid mixing (Struck *et al.*, 1981; Weber *et al.*, 1998) and content mixing (Smolarsky *et al.*, 1977; Wilschut *et al.*, 1979; Nickel *et al.*, 1999; Zucchi and Zick, 2011). Pioneering studies used R-SNARE proteoliposomes bearing two fluorescent lipids, one quenching the other via fluorescence resonance energy transfer (FRET). Upon fusion with nonfluorescent Q-SNARE proteoliposomes, fluorescence dequenching is seen, ascribed to lipid mixing and dilution of the fluorophores. Either neuronal SNAREs (Weber *et al.*, 1998) or yeast vacuolar SNAREs (Fukuda *et al.*, 2000) support fluorescence dequenching in this assay. However, the fluorescence dequenching signal, although reliant on *trans*-SNARE complex assembly, does not entirely arise from membrane fusion or even hemifusion because the dequenching signal is dramatically different when the two fluorescence lipids are on the vacuolar R-SNARE proteoliposomes or the vacuolar Q-SNARE proteoliposomes (Zick and Wickner, 2014), whereas fusion or hemifusion should yield equivalent dequenching signals independent of which proteoliposome bears the fluorescent lipids. Fluorescent lipid dequenching can also result from lipid transfer between the proteoliposomes induced by the SNAREs, perhaps promoted by their highly charged juxtamembrane regions, or from lysis followed by membrane reannealing.

| Current paradigm | New paradigm |
|---|---|
| SNAREs self-assemble | SNARE assembly catalyzed by SM proteins |
| The force of zippering extends via SNARE *trans*-membrane anchors to bend bilayers for fusion | *Trans*-membrane anchors are replaceable by prenyl anchors |
| Passive lipid bilayers | Specific lipid properties can be crucial for fusion[a]: |
| |     Small–head group, non–bilayer-prone lipids |
| |     Acidic lipids and phosphoinositides bind peripheral membrane proteins |
| |     Fluidity of fatty acyl chains |
| |     Lipids control SNARE enrichment at fusion microdomain |
| Other proteins needed for fusion just act by regulating the amount of *trans*-SNARE complex | To fuse, *trans*-SNARE linked membranes need: |
| |     HOPS or, for neurons, Munc18-1 and Munc13 |
| |     Small–head group lipids |
| |     Sec 17/SNAPs and/or, for neurons, synaptotagmin-1/$Ca^{2+}$ |

[a]Some of these concepts were proposed in the 1980s and 1990s but were largely omitted in the model postulating that SNAREs alone execute membrane fusion.

**TABLE 1:** A new paradigm for membrane fusion.

Indeed, SNAREs will cause extensive lysis at unphysiologically high levels (Chen *et al.*, 2006; Dennison *et al.*, 2006; Starai *et al.*, 2007; Zucchi and Zick, 2011; Zick and Wickner, 2016). The second, more reliable assay of fusion monitors the mixing of the lumenal contents of proteoliposomes, for instance, from the development of FRET between lumenal soluble fluorophores (Zucchi and Zick, 2011). To avoid spurious signal from lysis, this assay is performed with an excess of an external unlabeled blocking compound. In some studies, fluorescent lipid dequenching and protected lumenal content mixing were measured simultaneously with the same proteoliposomes. These assays showed that unphysiologically high levels of vacuolar SNAREs alone, which support lipid fluorescence dequenching, yield little mixing of protected lumenal contents (Zick and Wickner, 2014) and thus reflect processes other than fusion.

The high levels of SNAREs that support some slow proteoliposome fusion while triggering substantial membrane permeability and even lysis (Zucchi and Zick, 2011) also bypass the physiological requirements for other proteins and lipids. In reconstitutions with lower, more physiological levels of yeast vacuolar SNAREs, fusion requires 1) Rab and Rab-effector mediated tethering (Zick and Wickner, 2016); 2) SM protein–mediated SNARE complex assembly (Baker *et al.*, 2015); 3) specific lipid head groups, for example, acidic lipids (Orr *et al.*, 2015), phosphoinositides (Cheever *et al.*, 2001; Stroupe *et al.*, 2006; Mima and Wickner, 2008; Karunakaran and Wickner, 2013), and small, nonbilayer prone lipids (Zick *et al.*, 2015a); and 4) fluid fatty acyl chains (Zick and Wickner, 2016). These functions are integrated by the hexameric <u>ho</u>motypic fusion and vacuole <u>p</u>rotein <u>sor</u>ting (HOPS) complex, which has the vacuolar SM protein as a subunit and two other subunits that directly bind the Rab Ypt7 for membrane tethering (Hickey and Wickner, 2010; Ostrowicz *et al.*, 2010).

With chemically defined proteoliposomes bearing the vacuolar R-SNARE or Q-SNAREs, substantial *trans*-SNARE complex forms without HOPS (Zick and Wickner, 2014), and any attendant very slow fusion is abolished by Sec 17/Sec 18/ATP (Mima *et al.*, 2008; Xu *et al.*, 2010; Zick and Wickner, 2014). Because these SNARE disassembly factors are always present in the cytosol, how are *trans*-SNARE complexes protected from Sec 17/Sec 18/ATP action? Studies of the fusion of isolated yeast vacuoles show that HOPS inhibits *trans*-SNARE complex disassembly by Sec 17/Sec 18 (Xu *et al.*, 2010). The fusion-productive pathway requires that SNARE complexes assemble via SM-protein association. HOPS fulfills the SM function because the vacuolar SM protein Vps33 is a HOPS subunit, catalyzing the assembly of SNARE complexes (Baker *et al.*, 2015; Orr *et al.*, 2017). Under these conditions, there is minimal lysis (Zick and Wickner, 2016). HOPS-dependent fusion is actually stimulated by Sec 17/Sec 18, likely because Sec 17 binds to SNAREs and enhances fusion through its apolar N-terminal loop (Zick *et al.*, 2015b), and Sec 17/Sec 18 may disassemble unproductive complexes that hinder functional *trans*-SNARE complex formation.

Synaptic vesicle fusion has common features with the yeast vacuolar fusion but also special features that fulfill the exquisite regulatory requirements of neurotransmission. The most overt difference is that release is acutely triggered by $Ca^{2+}$ via the $Ca^{2+}$ sensor synaptotagmin-1 (Fernandez-Chacon *et al.*, 2001). Like vacuolar fusion, synaptic exocytosis also requires SNAREs, but in this system, the Qb and Qc SNARE motifs are in a single protein (SNAP-25; no relation to SNAPs). Moreover, the Qa SNARE (syntaxin-1) adopts a self-inhibited "closed" conformation in which the N-terminal $H_{abc}$ domain is bound to the SNARE motif (Dulubova *et al.*, 1999; Misura *et al.*, 2000), a feature not observed in the yeast vacuolar homologue Vam3 (Dulubova *et al.*, 2001). Closed syntaxin-1 binds tightly to the SM protein Munc18-1 (Dulubova *et al.*, 1999), which, together with Munc13s, orchestrates SNARE complex assembly in a manner that is resistant to NSF and α-SNAP (Ma *et al.*, 2013). Munc13s play a crucial function in neurotransmitter release through their MUN domain (Basu *et al.*, 2005), which helps to open syntaxin-1 (Richmond *et al.* 2001; Ma *et al.*, 2011) and is homologous to tethering factors from diverse membrane compartments (Pei *et al.*, 2009). In addition, Munc13s have multiple regulatory domains that mediate presynaptic plasticity for information processing in the brain, in part through interactions with other very large regulatory proteins such as the Rab3 effector RIM (Rizo and Südhof, 2012). The Munc13 $C_1$, $C_2B$, and $C_2C$ domains are adjacent to the MUN domain and likely help to bridge the vesicle and plasma membranes (Liu *et al.*, 2016), whereas the N-terminal $C_2A$ domain of Munc13-1 may also control vesicle tethering by forming a tripartite complex with RIMs and Rab3s (Dulubova *et al.*, 2005). This suggests that Munc18-1 and Munc13s perform similar roles in tethering and SNARE complex assembly as Vps33 and the other five subunits of the vacuolar HOPS complex, but formation of the closed syntaxin-1–Munc18-1 complex and the involvement of
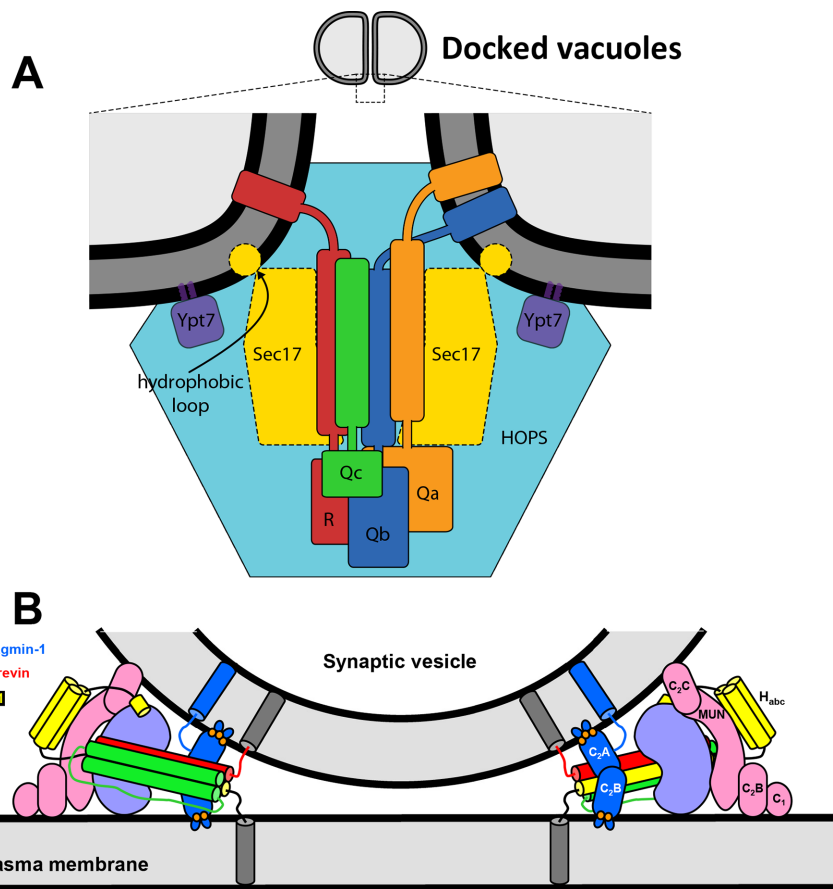
**FIGURE 1:** The machineries of yeast vacuole and human neuronal membrane fusion. (A) Fusion at the bent membrane microdomain relies on Rab-bound HOPS, locally enriched fusogenic lipids (Fratti *et al.*, 2004), and *trans*-paired SNAREs and can be triggered by membrane insertion of a hydrophobic N-terminal "wedge" domain of SNARE-bound Sec 17 (Zhao *et al.*, 2015; Zick *et al.*, 2015b). Reproduced with permission from Zick *et al.* (2015b). (B) Potential arrangement of selected components of the neurotransmitter release machinery as they are about to promote synaptic vesicle fusion. In the model, the SNARE complex formed by syntaxin-1 (yellow), SNAP-25 (green), and synaptobrevin (red) is assembled and bound to Munc18-1 (purple), Munc13-1 (pink), and synaptotagmin-1 (blue). Only the conserved C-terminal region of Munc13-1 is shown, with interactions involving its $C_1$, $C_2B$, and $C_2C$ domains helping to bridge the synaptic vesicle and plasma membranes. The $C_2$ domains of synaptotagmin-1 are shown with three ($C_2A$) and two ($C_2B$) bound $Ca^{2+}$ ions (orange) and insertion of the $Ca^{2+}$-binding loops into the two membranes. The relative orientation of the synaptotagmin-1 $C_2$ domains, Munc18-1, and Munc13-1 with respect to the SNAREs is completely speculative. Many other components of the release machinery are not shown but could also help to bridge the two membranes (e.g., Rab3s and RIMs) or induce fusion through interactions with membranes (e.g., SNAPs and complexins).

content mixing at more physiological densities of neuronal SNAREs (Lai *et al.*, 2013). In fact, neuronal SNARE complexes can dock proteoliposomes for long times without fusion (Kyoung *et al.*, 2011). Synaptotagmin-1 dramatically stimulates neuronal SNARE-dependent lipid mixing (Tucker *et al.*, 2004; Lee *et al.*, 2010) and content mixing (Kyoung *et al.*, 2011; Lai *et al.*, 2013) in a $Ca^{2+}$-dependent manner. Such fusion is abolished by NSF and α-SNAP unless Munc18-1 and Munc13-1 are present to protect against SNARE disassembly (Ma *et al.*, 2013). In reconstitutions including the neuronal SNAREs, synaptotagmin-1, Munc18-1, Munc13-1, NSF, and α-SNAP, proteoliposome docking and lipid mixing occur in the absence of $Ca^{2+}$ but with little content mixing, which is rapidly triggered upon $Ca^{2+}$ addition (Liu *et al.*, 2016). These findings suggest that, as in the vacuolar system, SNARE complex formation is not sufficient for fusion and that efficient fusion requires additional factors.

## MULTIPLE SNARE COMPLEX FUNCTIONS

Although *trans*-SNARE complexes do not suffice for rapid fusion, they are essential to the process. How do they function? Early work suggested that the SNARE *trans*-membrane anchors might exert force to distort the bilayer as they joined the SNARE domains in a long, continuous α-helix (McNew *et al.*, 1999). However, recent studies showed that the SNARE *trans*-membrane anchor domains can be functionally replaced by prenyl anchors for neuronal (Zhou *et al.*, 2013) or yeast vacuolar (Jun *et al.*, 2007) membrane fusion and that the endoplasmic reticulum–to-Golgi SNARE transmembrane anchors are required more for SNARE trafficking than for fusion (Chen *et al.*, 2016). Once the SNAREs are fully zippered, there is likely no additional force exerted on the apposed bilayers when the anchors are lipidic. We suggest that SNARE complex zippering

Munc13s (instead of those five subunits of HOPS) enable multiple modes of regulation of neurotransmitter release, some of which may tether the synaptic vesicle and plasma membranes. For perhaps this reason, synaptic release appears to be much less dependent on Rabs than other types of membrane fusion (e.g., vacuolar fusion). Rab3s do perform a regulatory role in neurotransmitter release (Schluter *et al.*, 2006), as do many other proteins, including complexins and CAPS (Südhof, 2013; Rizo and Xu, 2015).

Reconstitution experiments with the fluorescent lipid dequenching assay suggested that the neuronal SNAREs alone can induce fusion, but the high protein densities required for SNARE activity in these assays compromise membrane integrity (Nickel *et al.*, 1999; Dennison *et al.*, 2006; Zucchi and Zick, 2011). Content mixing using very high neuronal SNARE densities was reported (Nickel *et al.*, 1999), but single-vesicle assays revealed almost no

fulfills three other functions: bringing the polar head groups of the apposed membrane bilayers into immediate apposition, serving as a scaffold to bind fusion triggers (see later discussion), and assembling a fusion-prone microdomain with a lipid phase that is enriched in lipids such as sterol, diacyl glycerol, and phosphatidylethanolamine (Fratti *et al.*, 2004; Zick *et al.*, 2015a), which can readily rearrange into nonbilayer phases that lead to fusion.

We propose a new fusion paradigm (Table 1) in which full *trans*-SNARE complex assembly is not the last discernible step that leads to fusion. SNAREs regulate the local enrichment of fusogenic lipids (Fratti *et al.*, 2004), and SNARE-bound proteins can directly insert into the bilayers, triggering lipid rearrangements. Once SNARE complexes have assembled, vacuolar fusion still needs small–head group lipids (Zick *et al.*, 2015a) and HOPS (Zick and Wickner, 2014). Moreover, vacuolar *trans*-SNARE complexes bind Sec 17, allowing a

membrane-proximal Sec 17 N-domain apolar loop (Winter *et al.*, 2009) to insert into the bilayer to enhance fusion (Zick *et al.*, 2015b). Although it is unclear how much each of these factors contributes to lowering the energy barrier for the radical rearrangements of adjacent bilayers that constitute fusion, pioneering studies by Schwartz and Merz (2009) showed that Sec 17, through its apolar N-domain loop, restored fusion to docked vacuoles that were incapable of completing SNARE zippering due to C-terminal truncation of the Qc-SNARE motif. Because the membrane insertion of the Sec 17 apolar loop can replace C-terminal SNARE zippering, it might provide comparable energy for fusion.

Reconstitutions of synaptic vesicle fusion indicate that synaptotagmin-1, Munc18-1, and Munc13-1 contribute to make fusion highly efficient in a $Ca^{2+}$-dependent manner, and such $Ca^{2+}$ dependence also requires NSF and α-SNAP (Liu *et al.*, 2016). The participation of the α-SNAP apolar loop in fusion has not been studied, but it is clear that $Ca^{2+}$-dependent interactions of the $Ca^{2+}$-binding loops of the synaptotagmin-1 $C_2$ domains with phospholipids are critical to triggering neurotransmitter release (Fernandez-Chacon *et al.*, 2001; Rhee *et al.*, 2005). These loops contain hydrophobic residues that insert into lipid bilayers and basic residues that bind to negatively charged lipid head groups (Chapman and Davis, 1998; Zhang *et al.*, 1998), which may facilitate membrane fusion by inducing membrane curvature (Arac *et al.*, 2006; Martens *et al.*, 2007) or favoring nonbilayer intermediates. Recent structural studies of synaptotagmin-1–SNARE complexes (Brewer *et al.*, 2015; Zhou *et al.*, 2015) yielded distinct orientations of the synaptotagmin-1 $C_2B$ domain that do not appear to support this model. However, these structures were elucidated in the absence of membranes, and downstream rearrangements may allow synaptotagmin-1 to promote fusion through insertion of its $Ca^{2+}$-binding loops into the bilayers. Other proteins, such as Munc18-1, Munc13-1, and complexin, might also stimulate fusion through direct interactions with membranes (Seiler *et al.*, 2009; Xu *et al*, 2011; Liu *et al.*, 2016). Diacyglycerol and phosphatidylinositol 4,5-bisphosphate ($PIP_2$) also facilitate fusion (Liu *et al.*, 2016), but the roles of lipids have not been extensively studied in this system.

Further studies will be required to test all of these ideas and characterize the macromolecular complexes that induce membrane fusion. Docked vacuoles are known to contain small fusion microdomains that are enriched in the components that drive membrane fusion, including Ypt7, the SNAREs, the tethering/SM protein complex HOPS, and key lipids such as ergosterol, diacylglycerol, and phosphatidylinositol 3-phosphate (Figure 1A; Wang *et al.*, 2002; Fratti *et al.*, 2004). Strikingly, the SNAREs and these key lipids are interdependent for their enrichments in this microdomain, in that SNARE ligands block lipid enrichment and lipid ligands or lipases block SNARE enrichments (Fratti *et al.*, 2004). At presynaptic plasma membranes, active zones concentrate the components that regulate and trigger neurotransmitter release (Südhof, 2012). The insolubility of these massive protein networks has hindered quantitative characterization of their protein and lipid composition, but there is evidence for synergistic formation of clusters enriched in $PIP_2$ and syntaxin-1 that may aid in vesicle docking via interactions with synaptotagmin-1 (Honigmann *et al.*, 2013). With continued advances in cryo–electron microscopy, it may be feasible to characterize the architecture of membrane fusion machines and understand their mechanisms of action in the near future.

## NEW PARADIGM

The lipid bilayers of biomembranes are covered with proteins, blocking bilayer apposition when membranes collide (Table 1).

Activated, organelle-specific Rabs bind tethering effectors, which cause stable membrane associations. After tethering, multiple proteins and lipids interact to form membrane fusion microdomains apposed in-*trans*. Among the effectors, or proteins that bind to effectors, are Sec 1-Munc18 family proteins, which catalyze SNARE complex assembly in-*trans*. SNARE zippering brings the membranes into such close apposition that the bilayers come into direct contact. Additional fusion proteins are recruited to the *trans*-SNARE framework. These proteins include Sec 17/SNAP proteins, which bind to the *trans* 4-SNARE motif bundles, inserting their apolar N-domain loops into the bilayer to help trigger lipid rearrangements. At the synapse, several factors (synaptotagmin, complexin, and perhaps Munc13) block fusion until a $Ca^{2+}$ pulse triggers membrane insertion of the $Ca^{2+}$-binding loops of the synaptotagmin-1 $C_2$ domains. After fusion, Sec 17 and Sec 18 act with the energy of ATP hydrolysis to disassemble the resulting *cis*-SNARE complexes and free the SNAREs to participate in additional rounds of *trans*-pairing.

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