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Membrane lysis during biological membrane fusion: collateral damage by misregulated fusion machines

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In the canonical model of membrane fusion, the integrity of the fusing membranes is never compromised, preserving the identity of fusing compartments. However, recent molecular simulations provided evidence for a pathway to fusion in which holes in the membrane evolve into a fusion pore. Additionally, two biological membrane fusion models—yeast cell mating and in vitro vacuole fusion—have shown that modifying the composition or altering the relative expression levels of membrane fusion complexes can result in membrane lysis. The convergence of these findings showing membrane integrity loss during biological membrane fusion suggests new mechanistic models for membrane fusion and the role of membrane fusion complexes.

Introduction

Membrane fusion is a ubiquitous process in biology that allows for delivery, mixing, and sorting of soluble and membrane integrated macromolecules across membrane barriers. Despite enormous diversity of fusion reactions, the job description components catalyzing membrane fusion remain simple: tether, destabilize, and fuse membranes without allowing contents leakage across the bilayer (Jahn et al., 2003; Sollner, 2004; Wickner and Schekman, 2008). In the prevailing model of membrane fusion, the catalyst that drives the coalescence of juxtaposed bilayers, termed a fusase, initiates the formation of a hemifusion stalk, a nonbilayer intermediate that joins the apposed leaflets of the fusing membranes (Fig. 1, stage 2) (Chernomordik and Kozlov, 2008). Axial expansion of the stalk leads to a single bilayer consisting of the other two leaflets—termed a hemifusion diaphragm—that separates the two compartments (stage 3). Rupture of the hemifusion diaphragm results in a fusion pore (stage 4). At no point in this process are the contents of the two fusing membrane exposed to the environment between the membranes; thus, compartmental identity is preserved. This characteristic of the fusion process is considered vital to biological membrane fusion because leakiness in the fusion pathway could have disastrous consequences for the cell. Depending on their longevity and degree of occlusion, uncontained membrane holes would allow the dissipation of ion gradients, the escape of potentially harmful hydrolases from intracellular compartments, and cell lysis if plasma membranes were compromised during cell–cell or cell–virus fusion events. Thus, it comes as a surprise that recent work has shown that vacuole fusion and yeast mating are prone to lysis when the balance of fusion players is altered (Jin et al., 2004; Aguilar et al., 2007; Starai et al., 2007), and some reports suggest that viral fusases may also cause membrane holes (Shangguan et al., 1996; Blumenthal and Morris, 1999; Frolov et al., 2003). Here we review those perturbations that cause fusases to make holes instead of nonleaky fusion pores and discuss how fusase organization and hypothetical fidelity factors could promote formation of fusion pores over membrane lysis.

Lysis during biological membrane fusion

SNARE-driven vacuole lysis. Analogous to lysosomes, yeast vacuoles are an acidified compartment specialized for protein and membrane degradation. These large (0.5–1 µm in diameter) organelles undergo fusion and fission and are maintained at 1–5 vacuoles per cell (Wang et al., 2002). The SNARE-dependent fusion of yeast vacuoles has been extensively studied in vitro. Before fusion, Rab-dependent docking results in expansive membrane contact, termed boundary membrane, between neighboring vacuoles. The ring-shaped vertex microdomain at the edges of this boundary domain accumulates many fusion-relevant proteins, including the Rab GTPase Ypt7p, the HOPS Rab effector complex, and the vacuolar SNAREs (Wang et al., 2002). Fusion initiates around the vertex ring, resulting in fused vacuoles with the boundary membranes released into the lumenal space.

Wickner and colleagues created a strain of yeast with GFP in the vacuole lumen (Starai et al., 2007). By monitoring the release of lumenal GFP in the in vitro vacuole fusion assay, they were able to assess vacuole lysis during the fusion reaction. With the physiological ratio of Rab, effector complex, and SNAREs, they observed a low background of vacuole lysis (which was likely a result of handling the purified vacuoles). Surprisingly,

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Abbreviation used in this paper: HA, hemagglutinin.

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when the SNARE Vam7p was added in excess, which results in increased trans-SNARE complex formation, vacuole lysis increased (Vam7p has a PX domain for membrane association, but no transmembrane anchor). The Vam7p-induced lysis was concentration dependent and required full-length Vam7p capable of SNARE pairing. Similarly, vacuoloes isolated from strains overexpressing all four vacuolar SNARE proteins were also prone to lysis. Vacuole lysis was blocked by antibodies that inhibit cis-SNARE disassembly, vacuole docking, and trans-SNARE pairing. Furthermore, vacuole lysis and vacuole fusion followed identical kinetics.

Vacuole lysis by high SNARE activity compliments earlier observations regarding SNARE-containing liposome integrity after reconstitution of neuronal SNAREs (Dennison et al., 2006). Vesicles containing syntaxin at a high protein/lipid ratio exhibited increased contents leakage. Together, these studies suggest that although SNAREs are the minimal bilayer destabilization machinery, other factors assist in converting membrane destabilization to membrane fusion. An exciting explanation for SNARE-dependent vacuole lysis is that trans-SNARE pairs are balanced with regulatory proteins that govern membrane integrity during membrane fusion (Sudhof, 2007). These regulating factors are not capable of handling the many trans-SNARE complexes formed when SNAREs are overexpressed, and vacuole lysis results.

Lysis of yeast mating pairs. Lysis is also observed during cell fusion of mating yeast. Fusion of haploid cells of opposite mating type yields diploid zygotes (White and Rose, 2001; Chen et al., 2007). The mating reaction begins with pheromone sensing, which results in cell cycle arrest, polarized growth toward a mating partner ("shmooing"), and induction of a mating-specific transcriptional program. When a polarized shmoo meets a mating partner, their cell walls are woven together and a small channel at the center of the mating pair is cleared, such that the plasma membranes may come into contact (Gammie et al., 1998). Membrane fusion rapidly ensues, and further cell wall remodeling and fusion pore expansion allow for widening of the mating pair neck to allow for nuclear congression and fusion ("karyogamy").

Efficient membrane fusion requires the mating-specific, multipass membrane proteins Prm1p and Fig1p (Heiman and Walter, 2000; Aguilar et al., 2007). Prm1p localizes to the cell surface and is enriched at sites of contact between cells of a mating pair. Its activity is required in only one partner. When *PRM1* is deleted in both **a** and α cells, only 40% of mating pairs correctly complete membrane merger and cell fusion. Of the remaining mating pairs, most arrest at the step of membrane fusion. Cell wall removal continues such that large areas of membrane are in direct apposition, with only 8 nm separating the outer leaflets of the facing plasma membranes. Due to the absence of cell wall at the interface to separate the mating partners, the opposed membranes grow and retract such that the cytoplasm of one partner invades the space of the other, forming a membrane-contained structure ("cytoplasmic bubbles"). These cytoplasmic bubble structures are stable; they can grow and retract dramatically without losing integrity and allowing mixing between the distinct cytoplasms. The prezygotes remain arrested for up to 2 h, by which point individual cells resume the cell cycle and begin budding, or repolarize in an attempt to mate with another nearby cell. In addition to fusion failure and extension of cytoplasmic bubbles, a large fraction of *prm1* × *prm1* mating pairs undergo simultaneous cell lysis (Jin et al., 2004). Like Prm1p, Fig1p is highly enriched at the site of cell fusion (Aguilar et al., 2007). *fig1* × *fig1* mating pairs exhibit many of the same membrane fusion defects as *prm1* × *prm1* mating pairs, including cytoplasmic bubbles and simultaneous cell lysis. However, the *fig1* defects are milder and less penetrant compared with *prm1* mutants and the majority of *fig1* × *fig1* mating pairs are able to complete fusion.

The simplest explanation for the lysis and membrane fusion defects of *prm1* and *fig1* mutants is that both are caused by misregulation of the cell membrane fusase. The apposed, unfused cell membranes observed in mating pairs lacking Prm1p and Fig1p provide evidence that the cell fusase is not functioning properly. The concomitant cell lysis defect suggests that the fusase is active but misregulated, generating holes in the cell membranes instead of merging them. Two characteristics of the cell lysis suggest the phenomenon is catalyzed by the cell fusion machinery: the requirement of membrane contact and the timing of the two events. Lysis requires membrane contact, as would activation of the cell–cell fusase. Consistent with this view, deletion of *FUS1* and *FUS2*, which results in arrest of mating pairs at the upstream step of cell wall removal, suppresses the *prm1* lysis phenotype (Jin et al., 2004). Also, by analyzing many fusion events in a population using time-lapse microscopy, it became evident that lysis events initiate with the same timing as opening of fusion pores in successful mating pairs (Aguilar et al., 2007). Finally, concomitant with mating pair lysis, a small amount of cytoplasmic mixing is observed, consistent with fusion pores opening simultaneously with the appearance of membrane holes that result in mating pair lysis (Aguilar et al., 2007).

The extent of *prm1* × *prm1* mating pair lysis was greatly increased in the absence of extracellular Ca^{2+}, jumping from 20% to 50% of the mating pairs (Aguilar et al., 2007). The increase in mating pair lysis in the absence of extracellular Ca^{2+} is balanced by a similar decrease in mating pair fusion, again suggesting the engagement of the fusion machine can have two possible outcomes: productive fusion or lysis. Conversely, *prm1* × *prm1* mating pair lysis can be suppressed by high concentrations of Ca^{2+}. Calcium may play a direct role in the fusion step by interacting with lipid head groups of the opposed bilayers or the proteins that comprise the fusion machinery (Papahadjopoulos et al., 1990). However, wild-type mating pairs do not require calcium to avoid extensive lysis. Alternatively, Ca^{2+} could prevent mating pair lysis by initiating a wound repair process to fix membrane defects initiated by the fusase. In cell culture wound-healing models, membrane holes are repaired by fusion of lysosomal membrane delivery via a Ca^{2+}-dependent mechanism that involves the membrane protein synaptotagmin VII (Reddy et al., 2001). Synaptotagmin VII can sense changes in intracellular calcium levels and influence membrane fusion events via calcium and phospholipid binding C2 domains (Rizo and Sudhof, 1998). In yeast, the tricalbin family of proteins has been identified as potential synaptotagmin homologues based

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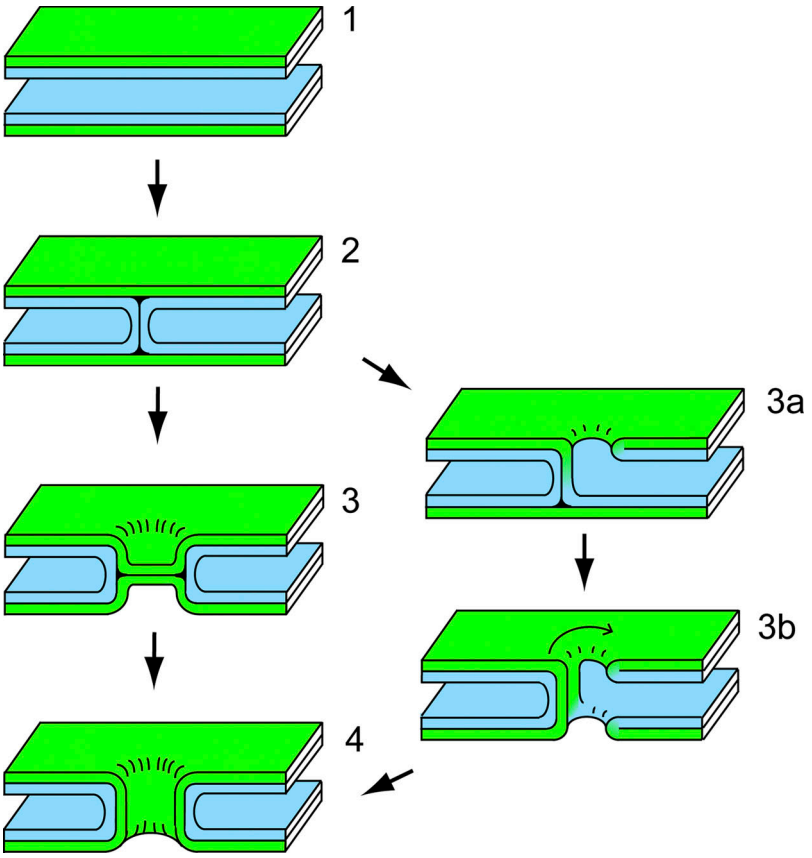

Figure 1. **Models for lipid rearrangements leading to the formation of a fusion pore.** The left pathway depicts the classical model for membrane fusion via rupture of a hemifusion diaphragm. Membranes are brought into close apposition (1), the two cis leaflets (blue) fuse to form a hemifusion stalk (2), the stalk expands forming a hemifusion diaphragm in which trans leaflets (green) are in contact (3), and rupture of the hemifusion diaphragm results in a fusion pore (4). In contrast to the classical model for membrane fusion, an alternative pathway, via intermediates drawn on the right, does not always maintain compartmental identity. Formation of a hemifusion stalk results in the nucleation of holes adjacent to the stalk (3a and 3b), which encircles the holes to form a fusion pore.

on their structural similarities (transmembrane anchors coupled to multiple C2 domains) and role in membrane traffic. The C-terminal C2 domains of two tricalbin members, Tcb1p and Tcb3p, exhibit Ca^{2+}-stimulated membrane binding (Schulz and Creutz, 2004). Intriguingly, deletion of *TCB3*, but not of *TCB1* or *TCB2*, increased *prm1* × *prm1* mating pair lysis to 50% of mating pairs even in the presence of extracellular Ca^{2+} (Aguilar et al., 2007). Thus, wound repair processes may mask the true lytic extent of mating in the absence of Prm1p.

Viral fusase-induced lysis. Enveloped viruses must fuse with host cells to transfer their genomes. These fusion events are catalyzed by virally encoded transmembrane proteins. A few studies have found that viral fusases create membrane holes concurrent with fusion pore opening.

The influenza fusase hemagglutinin (HA) fusion molecule has been studied in many heterologous contexts. During HA-mediated virus–liposome fusion, membrane holes were generated with identical kinetics to lipid mixing, as monitored by the release of large dextran molecules (Shangguan et al., 1996). Similarly, video microscopy revealed content leakage after hemifusion diaphragm formation during fusion of HA-expressing fibroblasts with erythrocytes (Blumenthal and Morris, 1999). Finally, conductance measurements during HA-mediated cell–cell fusion showed that membrane permeability increased during early stages of fusion (Frolov et al., 2003). This permability decreased as fusion pores opened, suggesting that membrane leakiness results from membrane rearrangements during pore formation. Thus, it appears that the membrane-destabilizing properties of viral fusases can result in membrane lysis, particularly when they are manipulated to fuse membranes different from the viral envelope and physiologically relevant target cells.

In vivo, HIV infection of lymphocytes can result in cell lysis. Cell culture models of this phenomenon showed that cell lysis requires coexpression of the HIV-1 fusase, gp41, and its receptor CD4 (Cao et al., 1996). Curiously, however, the kinetics of lysis initiation are very slow: cells lyse days after maximal gp41 expression is achieved. Using various inhibitors it was demonstrated that the lethal fusase–receptor interaction occurs intracellularly (Madani et al., 2007). In contrast to the fusase-catalyzed cell membrane breaches discussed above, further work is required to elucidate how gp41 activity results in cell lysis.

Fusion machines and the pathway of membrane fusion

In the prevailing model, membrane fusion does not risk the integrity of compartmental identity (Fig. 1, left pathway). Yet, as described above, leakiness in fusion has been observed in three separate classes of membrane fusion when the balance of fusion players or identity of fusing membranes is altered. These findings raise two important questions: Where in the pathway of membrane fusion is lysis initiated, and how is the fusion machinery designed to prevent this outcome?

Mechanism of biological membrane fusion. The pathway to membrane fusion must include nonbilayer intermediates; generating or resolving these intermediates may be the step where the above lysis examples diverge. Recently, a new model for membrane fusion has been proposed in which compartmental identity is temporarily lost (Muller et al., 2003).

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Simulations of membrane fusion using coarse-grained lattice models predicted that the stalk intermediate promotes the formation of adjacent holes in the bilayers (Fig. 1, stage 3a and 3b). These holes are then surrounded by the stalk to form a fusion pore (3b). This pathway is less energetically costly than the traditional, nonleaky hemifusion hypothesis (Katsov et al., 2006). Lysis could emerge from this pathway if these membrane holes expand before the stalk can encircle them to form the fusion pore. A similar leaky structure would be created if, instead of bilayer rupture within the hemifusion diaphragm, a hole opens in one of the two bilayers adjacent to the hemifusion diaphragm.

Alternatively, lysis may occur before formation of the hemifusion stalk as a consequence of trying to transition to the nonbilayer intermediate. Strongly bending membranes may be a strategy for destabilizing bilayers such that they will form a stalk intermediate (Kozlov and Chernomordik, 1998). This could be a risky endeavor—generation of unstable, highly curved membranes could result in membrane rupture.

Assembling a fusion machine. Viral fusases and SNAREs are sufficient to fuse lipid bilayers and biological membranes, yet this feat is not achieved by a single HA trimer or trans-SNARE pair. Instead, these proteins are assembled into a greater fusion machine, consisting of multiple core fusases (i.e., HA, gp41, a trans-SNARE pair) and, in most cases, regulatory proteins (i.e., HOPS, synaptotagmin, complexin) (Tang et al., 2006). Additionally, lipids act as regulators and facilitators of membrane fusion, recruiting fusase subunits and allowing highly curved membrane intermediates (Fratti et al., 2004; Chernomordik and Kozlov, 2008). Core fusase regulators have been described to govern specificity and timing of the fusion event. Might others ensure membrane integrity during lipid rearrangement? The characteristics of this fusion machine realize the fusogenic, and may limit the lytic potential of core fusases.

Both viral and intracellular fusion use the concerted action of multiple fusases to achieve the energy required for membrane fusion. Kinetic analysis of fusion by cells expressing HA with different surface densities estimated a minimum of three HA trimers mediate membrane fusion (Danieli et al., 1996), and modeling has suggested that the concerted action of at least eight HA trimers, including two in the activated state, are required to open a fusion pore (Bentz, 2000). In addition to recruiting multiple fusases, the geometry of their association is likely important for efficacy of the fusion machine. The geometry of the HA fusion machine is thought to be circular and to surround the hemifusion stalk and nascent fusion pore (Chernomordik et al., 1998). Multiple trans-SNARE pairs are required to achieve fusion, and atomic force microscopy showed that SNAREs also associate in a ring-like fashion (Hua and Scheller, 2001; Cho et al., 2002; Hofmann et al., 2006). If a fusion machine were haphazardly assembled, the membrane-destabilizing activities of the core fusases may result in membrane lysis instead of a fusion pore. The geometrical information behind HA oligomerization is likely inherent in the molecule, but this may not be the case for SNAREs or the as-yet unidentified yeast fusase, allowing for the possibility of fusase organization by an independent protein factor. At a gross localization level, both the Rab Ypt7p and regulatory lipids are required for the enrichement of SNAREs at the vertex ring of contacting vacuoles (Wang et al., 2003; Fratti et al., 2004). Furthermore, the Ypt7p effector HOPS directly interacts with the SNARE Vam7p, stimulates trans-SNARE complex formation, and proofreads trans-SNARE pairs (Stroupe et al., 2006; Collins and Wickner, 2007; Starai et al., 2008). Despite the intimate relationship between HOPS and vacuolar SNAREs, HOPS does not limit vacuole lysis driven by high

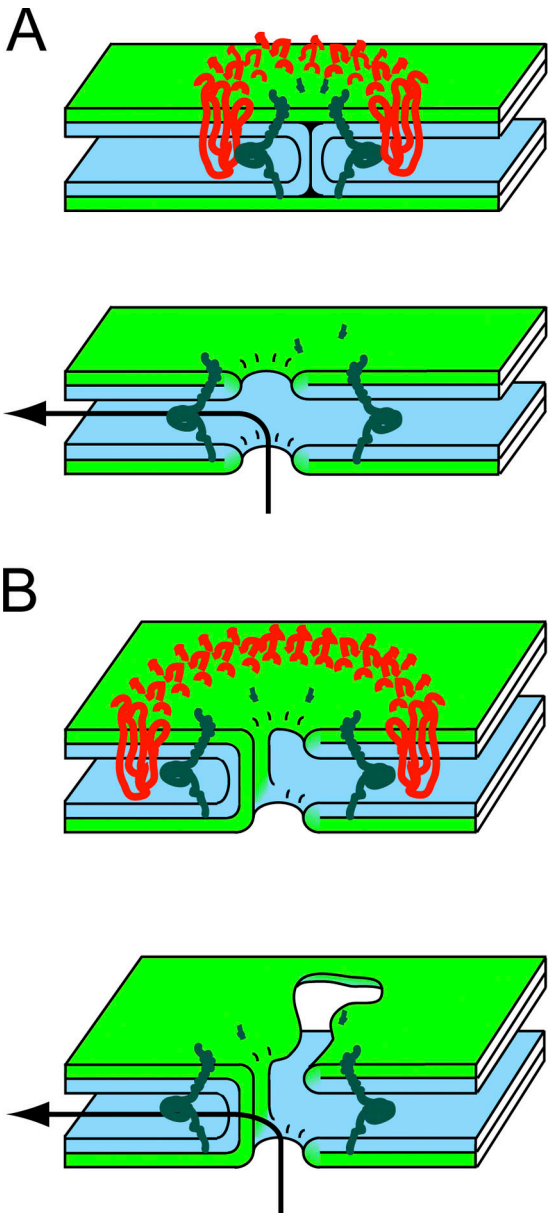

Figure 2. **Models for regulation of fusion integrity by nonfusase factors.** Fusase molecules are drawn in green, integrity promoting factors in red. (A) Regulation of lytic potential by organizing fusase molecules. Architectural factors recruit core fusases into a ring-shaped fusion machine, which guides the membrane-destabilizing activity of the core fusases toward fusion pore formation (top). In the absence of these factors, core fusase activity is not geometrically coordinated, resulting in membrane rupture (bottom). (B) Restriction of membrane hole expansion by a ring of membrane proteins. If the pathway to membrane fusion were inherently leaky (see Fig. 1), the risks of membrane hole expansion may be mitigated by protein factors surrounding the nascent fusion pore (top). In their absence, hole expansion may proceed and result in loss of compartmental integrity (bottom).

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SNARE concentrations (Starai et al., 2007, 2008). Nonetheless, the concept of fusion facilitators arranging a greater complex of trans-SNARE pairs remains enticing. Imagining cooperative SNARE function during neurotransmitter release, an organizing architectural factor was invoked that functioned to arrange multiple trans-SNARE pairs into a ring-like fusion machine (Rizo et al., 2006). Prm1p could regulate cell fusion in an analogous manner during yeast mating by interacting with and orienting core fusase molecules (Fig. 2 A, top). In the absence of Prm1p, a decreased ability to assemble active fusion machines results in apposed but unfused membranes. Incorrectly assembled fusion machines may destabilize membranes, but not in a productive stalk-promoting manner, resulting in cell lysis (Fig. 2 A, bottom).

Alternatively, instead of regulating protein fusases, integrity-promoting accessory factors could control lipid diffusion to control dangerous fusion intermediates such as the hypothetical membrane holes described above (Fig. 2 B). HA-mediated cell–cell fusion has been arrested in a state of hemifusion without lipid mixing; clustered HA trimers are believed to cause this restriction (Chernomordik et al., 1998). Modeled on these observations, Prm1 may act by preserving the lipidic environment set up by the core fusase or by stopping expansion of membrane holes (Shangguan et al., 1996; Jin et al., 2004). Consistent with this corral-like structural role, Prm1 forms covalent homodimers, but it is not known if these dimers further oligomerize (unpublished data).

Conclusion

Convergence of molecular simulations and experimental data suggests that lysis is not simply an irrelevant experimental artifact of membrane fusion assays. Accordingly, we must revisit the classical model of membrane fusion. The experimentally verified stalk structure is not in question, but different rearrangements that risk loss of compartmental identity could occur before fusion pore formation. Specific factors might be involved containing or avoiding these risks, and identifying such factors would be an extremely valuable advance in our understanding of how the activity of fusases is controlled to fuse membranes with high fidelity. Finding proteins that can suppress vacuole lysis without lowering SNARE activity could help establish such late stage regulation. Moving from the other direction, the identification of proteins that interact with Prm1p may yield a fusase responsible for cell fusion. If the predictions outlined here are correct, removing this fusase should eliminate both fusion and lysis outcomes of *prm1* × *prm1* mating pairs. When available, comparing the mechanism by which the cell–cell fusase merges membranes to the mechanisms described for viral and intracellular fusion will describe the breadth of strategies for joining membranes of different character and in different contexts. Finally, our understanding of the diversity of fusion machines will greatly benefit with the characterization of the reovirus FAST proteins, a new class of fusion proteins that mediate cell fusion (Salsman et al., 2005; Top et al., 2005). Given their small size (14 kD) and simple domain structure, answering questions about the arrangement and stoichiometry of the FAST proteins at the cell surface and the lipid rearrangements they catalyze promises new insight into the control of biological membrane fusion.

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