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Membrane Fusion Intermediates via Directional and Full Assembly of the SNARE Complex

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Cellular membrane fusion is thought to proceed through intermediates including docking of apposed lipid bilayers, merging of proximal leaflets to form a hemifusion diaphragm, and fusion pore opening. A membrane-bridging four-helix complex of soluble *N*-ethylmaleimide–sensitive factor attachment protein receptors (SNAREs) mediates fusion. However, how assembly of the SNARE complex generates docking and other fusion intermediates is unknown. Using a cell-free reaction we identified intermediates visually and then arrested the SNARE fusion machinery when fusion was about to begin. Partial and directional assembly of SNAREs tightly docked bilayers, but efficient fusion and an extended form of hemifusion required assembly beyond the core complex to the membrane-connecting linkers. We propose that straining of lipids at the edges of an extended docking zone initiates fusion.

Synaptic vesicle fusion is mediated by the vesicular SNARE synaptobrevin 2 (syb) and the plasma membrane SNAREs SNAP-25A (SN25) and syntaxin 1A (syx). Binding of syb to a 1:1 syx:SN25 acceptor complex forms a SNARE complex consisting of a four-helix bundle held together by 16 layers (numbered -7 to +8) of interacting amino acids (*1*). The SNARE complex alone is sufficient for mediating fusion in vitro (*2*), the prevalent model being that directional N-to-C terminal zippering of a trans SNARE complex pulls opposing membranes together. Once contact has been established, fusion is thought to proceed via a symmetrical stalk intermediate that expands to form a hemifusion diaphragm (*3*) (Fig. 1A). An expanding pore formed within the diaphragm then completes fusion. Some studies have reported the involvement of some of these intermediates (*4*) while others have attempted to arrest them by disrupting SNARE assembly (*5*); however, key questions remain unanswered, including what is the nature of the relevant docking interaction that leads to fusion and, just as important, how does the assembly of the SNARE complex give rise to docking and other intermediates that occur thereafter.

To address these questions, we pursued a minimalist approach in which docking and fusion is recapitulated with purified SNAREs reconstituted in liposomes. Previously, a transient docking state was inferred when using liposomes containing a syx:SN25 acceptor complex that is stabilized by a C-terminal syb fragment (ΔN syb 49-96, hereby denoted ΔN syb) (Fig. 1B) (*6*). This "ΔN complex" contains a free binding site for syb at the N terminus, accelerating syb trans binding and preventing dead end 2:1 syx:SN25 complexes (*7*). Docking of large SNARE-liposomes (radii ~40-100 nm, Fig. 1C) had a longer lifetime than smaller ones (radii ~15-25 nm), a finding we attribute to reduced curvature stress (*8*, *9*) of the large liposomes which approaches that of giant liposomes (Fig. 1C inset). We confirmed this behavior in fluorescence resonance energy transfer (FRET)-based lipid-mixing assays. Here, a pronounced lag phase was observed with large liposomes, suggesting lower curvature delays the initiation of fusion once contact of membranes has been established (Fig. 1D).

To monitor trans SNARE complex binding and ΔN syb displacement the experiments were repeated with fluorophore-labeled versions of full-length syb (syb^{28A488}) and ΔN syb ($\Delta N\ syb\ 49\text{-}96^{79A488}$). Accordingly, SNARE complex zippering began without delay at the very N terminus (Fig. 1E) but ΔN syb displacement slowed down further zippering toward the C-terminal end (fig. S2). However, this delay originated only from the displacement of ΔN syb's first N-terminal layers whereas the remaining C-terminal half of ΔN syb dissociated rapidly thereafter, allowing zippering to proceed (fig. S3). Based on these findings, we reasoned that the delay between trans SNARE binding and the initiation of lipid-mixing, caused first by ΔN syb displacement and then more prominently by the low membrane curvature stress of the large liposomes used here (Fig. 1D), opens a time window to identify intermediate states of the membranes on their way to fusion by EM (EM).

We found many docked liposomes during the lag phase by negative stain (Fig. 2A) and cryo-EM (Fig. 2, B-D) and distinguished docked membranes with both minimal (Fig. 2B) and extended docking zones (Fig. 2, C and D). Because none of these interactions implicate any lipid-mixing and occur during the lipid-mixing lag phase where SNARE assembly takes place, we hypothesized that these docked states are generated by partial complex zippering on their way to fusion. Surprisingly, we also identified a small number of liposomes containing only one bilayer in between the two lumina (Fig. 2E), the ultrastructural signature of an extended hemifusion diaphragm (Fig. 2F). Hemifusion (like docking) was SNARE-dependent, an important distinction given that even misfolded proteins can mediate hemifusion (*10*) (fig. S4). However, we were unable to distinguish small differences in the counting of docking and hemifusion by EM, preventing us from making definite conclusions about the reaction sequence.

To clarify this question, we resorted to alternative biophysical assays and used a combination of fluorescence cross-correlation spectroscopy (FCCS) and FRET to follow the evolution of docking (*6*). FCCS-FRET revealed an initial and instantaneous accumulation of docking (without

fusion) followed by a plateau (Fig. 2G), suggesting that all docked states observed by EM are transient intermediates and are part of the same pathway. This finding appeared initially to contradict the observation that many docked liposomes were still seen by EM after lipid-mixing was completed (fig. S4), but we note that FCCS-FRET does not detect docking between liposomes that have already docked and fused previously. We therefore postulate that docked liposomes at the end of the reaction have already fused at least once but contain insufficient SNAREs for additional cycles of fusion. Although one SNARE complex is sufficient for fusing small liposomes (*11*), it appears that more complexes are required for fusing larger ones (see further below).

To monitor the evolution of hemifusion, we measured lipid-mixing in the inner leaflet of a merging bilayer (*12*, *13*) and compared it to the expected value when full-fusion (i.e., no hemifusion) conditions are assumed (Fig. 2H and S5). Inner leaflet lipid-mixing begins to diverge after ~3-5 min from the expected value (Fig. 2H), meaning that hemifusion begins to develop mainly after the lag phase and that it arises sequentially from docked intermediates identified by EM. Surprisingly, inner leaflet lipid-mixing remained below the expected value throughout the rest of the reaction, indicating that hemifusion accumulates and becomes kinetically trapped, in agreement with the appearance of hemifused liposomes with extended diaphragms seen at the end of the reaction by EM (fig. S4).

To pinpoint the regions of the SNARE complex responsible for tight docking, extended hemifusion and fusion, we sought to arrest intermediates by perturbing SNARE assembly at the transition between the four-helix bundle and the membrane-connecting linker, a region with a critical role in fusion (*5*, *14*, *15*). To this end, we made a single deletion at position 84 of syb (syb Δ84) that disrupts the last +8 layer of the bundle, (inset of Fig. 4C). Syb Δ84 was unable to fuse large liposomes, although it partially induced lipid-mixing on small ones, a result consistent with our earlier supposition that large liposomes require more energy (and therefore more SNARE complexes) to fuse (Fig. 3A). We confirmed that syb Δ84 displaced the ΔN syb fragment from the ΔN complex (fig. S6), suggesting it could assemble into SNARE complexes and dock liposomes but not fuse them. This was confirmed by FCCS-FRET (Fig. 3B) and EM where almost all liposomes were seen arrested at the tightly docked state with extremely rare sightings of hemifusion (Fig. 3, C and D).

Several key conclusions can be derived from this analysis: 1) tight docking is a result of partial complex assembly which does not exceed layer +7, validating our hypothesis that tightly docked liposomes are a result of partial zippering; 2) assembly beyond the +7 layer of the SNARE complex is essential for generating extended hemifusion and fusion; 3) in line with directional N-to-C terminal assembly (*16*), the tight bilayer arrangement with extended contact zones represents an intermediate state that was stalled on its way to fusion. Further supporting this conclusion, lipid-mixing by syb Δ84 can be partially restored by increasing curvature, suggesting that large liposomes would otherwise fuse were it not for its higher energy barrier; 4) because bilayers are tightly held together with no resolvable space in between, complexes are arrested in trans and are probably distantly distributed along the vertex ring as described in yeast vacuoles (*17*); and 5) lipids at the edges of the extended docking zone are highly strained where splaying of lipid tails or stalks are likely to initiate fusion (*3*, *18*).

We took advantage of this "docking mutant" and tested if the Ca^{2+} sensor synaptotagmin (syt), which might stabilize fusion intermediates by bending bilayers (*19*), can assist SNAREs in the conversion from docking to hemifusion/fusion, but found it could restore neither, consistent with the view that syt operates upstream of complex assembly (*20*) (fig. S7). We therefore turned to our mutational analysis by exploring single and double deletions further downstream of syb in the linker region (inset of Fig. 4C), ensuring complete assembly of the core four-helix bundle (see fig. S8 for characterization of syb linker mutants). Fusion and extended hemifusion was restored for all three mutants tested, albeit with lower efficiency than wild-type syb (Fig. 4 and fig. S8C), confirming that hemifusion is preceded by tight docking and showing that efficient fusion requires zippering of the linker in agreement with the crystal structure of the full SNARE complex (*21*). Additionally, the hemifusion/fusion ratio was unaffected (Fig. 4B), indicating no involvement of the linkers in the transition from hemifusion to fusion, although this may be the role of the transmembrane domain (TMD) (*22*, *23*).

Based on our findings, we can assign approximate regions responsible for the generation of docking, extended hemifusion and fusion by directional and full assembly of the SNARE complex (Fig. 4C). Our conclusion that membrane merging begins after assembly of the core complex contrasts to a recent study that used myricetin to stall a partially zippered complex and arrest a topologically-undefined hemifusion state (*24*). However, myricetin also binds to acyl chains (*25*) and may increase membrane fusogenicity or leakiness. Our finding that extended hemifusion accumulates suggests it is a kinetically trapped intermediate resulting from a high-energy barrier for pore opening, a state that has been observed in cortical granules (*26*). Nevertheless, additional factors, for instance specific lipid requirements, may lower that barrier and make the extended hemifusion conformation a viable intermediate as suggested by studies of giant liposomes and simulations (*27*, *28*), although it is still not clear whether a localized rather than an extended form of hemifusion may also be a biologically relevant intermediate (see fig. S9 for a discussion).

Our findings question the routinely made assumption that fusion must start from a symmetrical toroidal stalk. Instead, the SNARE fusion machinery seems to work by pulling the membranes as tightly as possible which strains the edges of an extended docking zone. Such a mechanism shares some similarities with what appears to occur in vacuoles (*17*) and observed with greater detail in simulations (*27*, *29*, *30*). Our work now suggests that the tight pulling mechanism is a conserved feature of SNARE-mediated fusion.

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Supplementary Text
Figs. S1 to S9
References (*31*–*53*)

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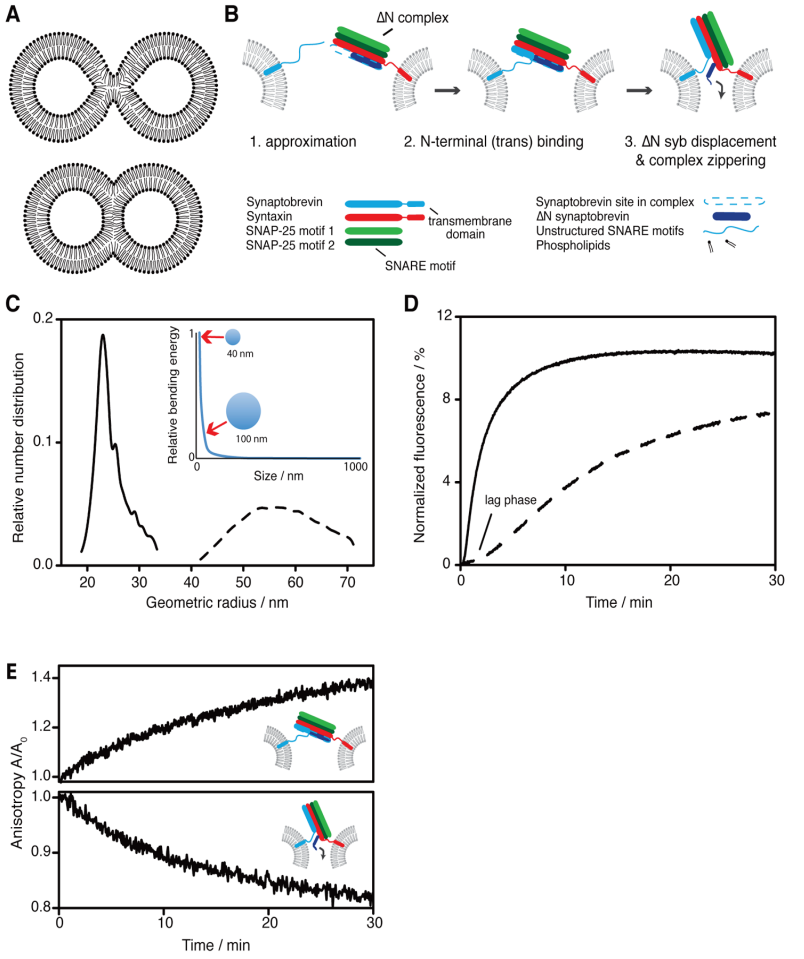

Fig. 1. (**A**) A symmetrical toroidal stalk (top) and an expanded hemifusion diaphragm (bottom) are thought to be intermediates in the SNARE-mediated fusion pathway. (**B**) The ΔN complex is necessary for promoting fast N-terminal binding for trans SNARE complex formation. (**C**) Size distributions of small (solid) and large (dashed) liposomes reconstituted with syb determined by light scattering (see fig. S1 for characterization of the reconstitution). Inset: schematic showing liposome size dependence (for a single component system) of the elastic bending energy of a lipid in the outer monolayer (*8*). (**D**) Large liposomes (dash) exhibit a prolonged lag phase in lipid-mixing compared to small liposomes (solid) at the same SNARE density. (**E**) Fluorescence anisotropy of large liposomes containing syb ^{28A488} (top panel) and ΔN syb 49-96^{79A488} complex (bottom panel) were added to large liposomes containing their respective non-labeled SNARE binding partners, revealing that formation of trans SNARE complexes and ΔN syb displacement begin without delay. Note that anisotropy likely decreases when ΔN syb displacement has already initiated without the fragment being fully removed.

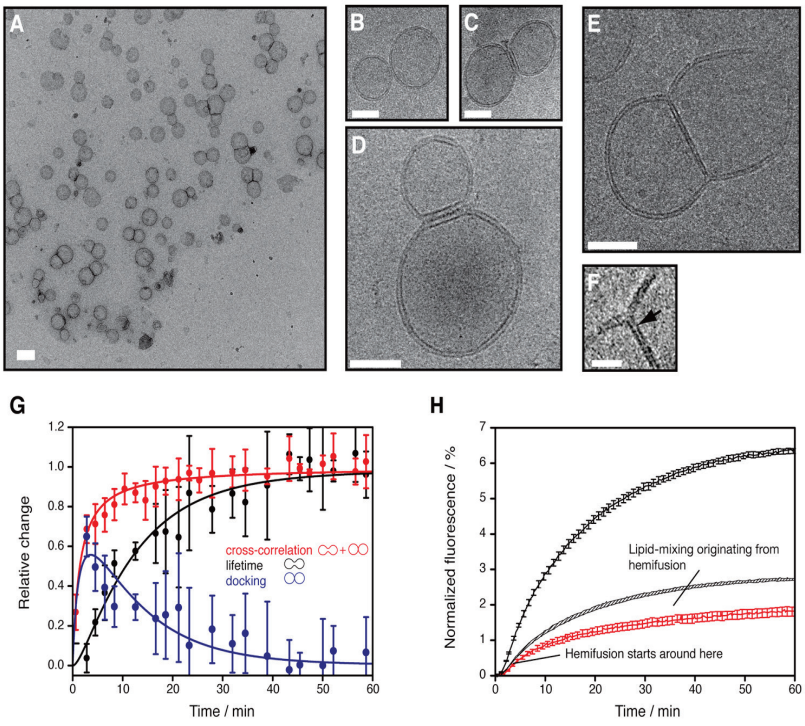

Fig. 2. Ultrastructural and biophysical identification of docking and hemifusion. (**A**) Negative stain EM depicting liposomes engaged in docking taken at ~3 min after mixing. Bar = 200 nm. Docked liposomes with minimal (**B**) and extensive (**C-D**) contact zones were observed by cryo-EM ~1-2 min after mixing. (**E-F**) Hemifused liposomes were identified by an extended diaphragm consisting of a single bilayer (arrow). Bar = 50 nm except in (F) where bar = 20 nm. (**G**) Discrimination of docking and fusion by FCCS-FRET. Cross-correlation between labeled liposomes (reflecting both fused and docked liposomes, red) were subtracted from changes in fluorescence lifetime (reflecting fused liposomes, black) to reveal the evolution of docked liposomes (blue). Data are mean +/− SD ($N \geq 5$). (**H**) Total (black) and inner leaflet (red) lipid-mixing measured and compared to the expected inner leaflet lipid-mixing (cross-shaded region). Hemifusion begins to form after ~3-4 min and accumulates thereafter. Bars represent 95% confidence intervals ($N \geq 3$).

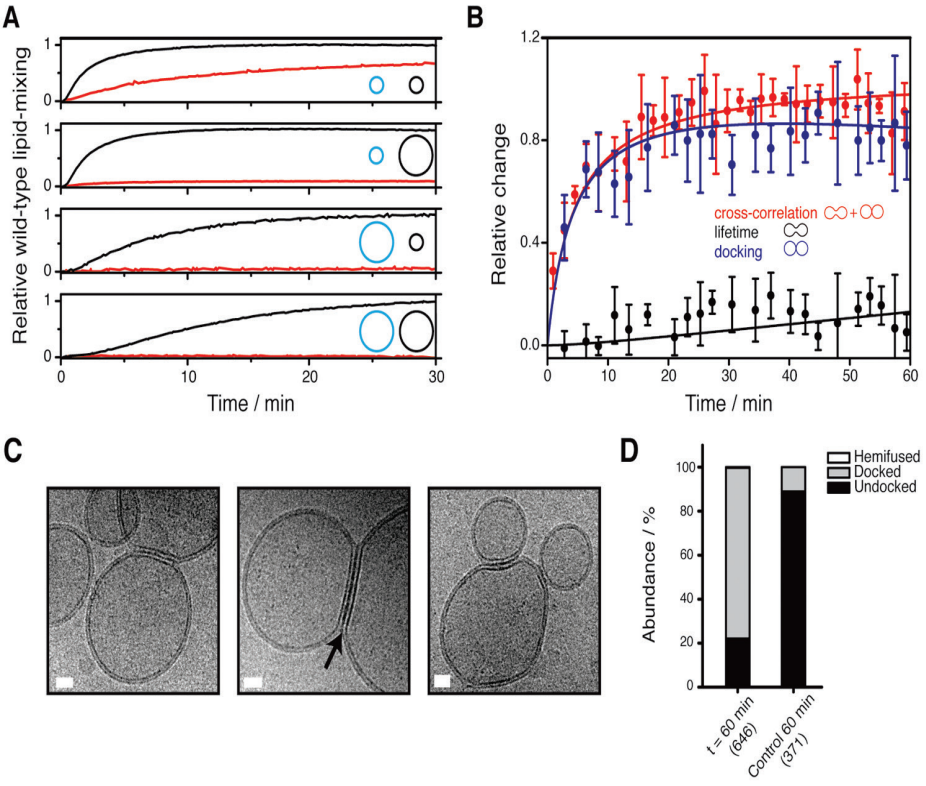

Fig. 3. Disruption of the C-terminal +8 layer of the SNARE complex arrests a tightly docked intermediate. (**A**) Comparative lipid-mixing of wild-type syb (black) and syb Δ84 (red) according to the depicted liposome size combinations (location of ΔN complex is indicated in light blue), showing curvature affects the ability of syb Δ84 to mediate fusion. Traces were normalized to wild-type syb which was set arbitrarily to 1. (**B**) FCCS-FRET analysis of large syb Δ84 liposomes showing accumulation of docking. Data are mean +/− SD ($N \geq 5$). (**C**) Examples of cryo-EM images of syb Δ84 and ΔN complex large liposomes depicting the arrest at the tightly docked state. The edges of an extended docking zone results in straining of lipids (arrow). Bar = 20 nm. (**D**) Counting of docking of syb Δ84 and ΔN complex liposomes observed by cryo-EM after 1 hour, confirming the vast majority of liposomes were arrested in the tightly docked state. Control was performed in the presence of excess soluble syb 1-96 showing that docking was SNARE-dependent.

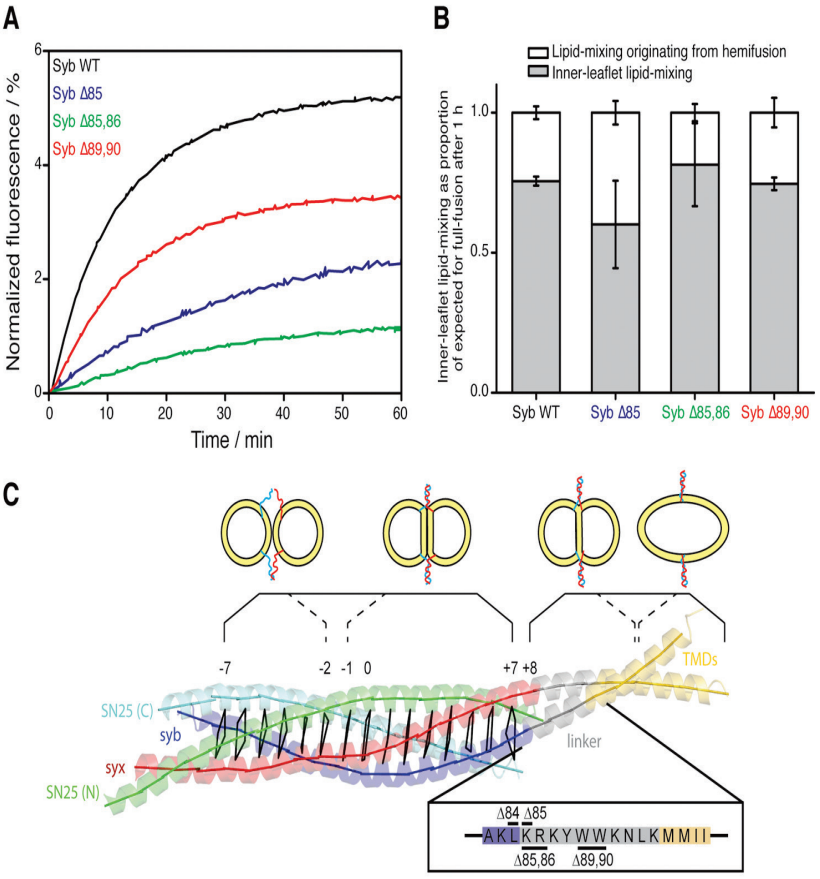

Fig. 4. Full assembly of the core SNARE complex restores hemifusion and the linkers are needed for efficiently completing fusion. (**A**) Comparative lipid-mixing of linker deletion mutants depicting a decrease in fusion in large liposomes. (**B**) Inner leaflet lipid-mixing as a proportion of expected assuming full-fusion conditions for wild-type syb and linker deletion mutants. Values were taken 1 hour after mixing and bars represent 95% confidence intervals ($N \geq 3$). (**C**) Ribbon structure (*21*) of the fully assembled SNARE complex showing the interacting layers (black lines, +8 layer indicated) of the four-helix bundle, the linker and TMD and the proposed regions which give rise to docking, extended hemifusion and fusion. Dashed lines indicate borders of regions that are based on other studies (*22*, *23*) or on biochemical characterization. Inset: deleted amino acids used for mutation analysis.