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A Tethering Complex Drives the Terminal Stage of SNARE-Dependent Membrane Fusion

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Membrane fusion in eukaryotic cells mediates the biogenesis of organelles, vesicular traffic between them, and exo- and endocytosis of important signaling molecules, such as hormones and neurotransmitters. Distinct tasks in intracellular membrane fusion have been assigned to conserved protein systems. Whereas tether proteins mediate initial recognition and attachment of membranes, SNARE protein complexes are considered as the core fusion engine. They provide mechanical energy to distort membranes and drive them through a hemifusion intermediate towards the formation of a fusion pore[1-3]. This last step is highly energy-demanding[4,5]. We combined the in vivo and in vitro fusion of yeast vacuoles with molecular simulations to show that tether proteins are critical to overcome the final energy barrier to fusion pore formation. SNAREs alone drive vacuoles only into hemifusion. Tether proteins greatly increase the volume of SNARE complexes and deform the site of hemifusion, which lowers the energy barrier for pore opening and provides driving force. Thereby, tether proteins assume a critical mechanical role in the terminal stage of membrane fusion, which is likely to be conserved at multiple steps of vesicular traffic. SNAREs and tether proteins should hence be

considered as a single, non-dissociable device to drive fusion. The core fusion machinery may then be larger and more complex than hitherto thought.

SNAREs dock apposed membranes through stepwise assembly into 4-helix bundles. They exert mechanical force through their transmembrane domains (TMDs)[1,2]. This induces fusion of the outer leaflets (hemifusion), followed by inner leaflet fusion and pore formation. Pore formation can be preceded by full zippering of the 4-helix bundle[6]. Most studies on SNARE-driven fusion focused on synaptic SNAREs, which use unique cofactors, such as synaptotagmin, to fuse highly curved neurotransmitter vesicles with exquisite speed and temporal control[7]. Most SNARE-dependent fusion reactions do not require comparably rapid and controlled fusion, the membranes to be fused are much less curved, and their SNARE density is lower. Fusion driven solely by SNAREs becomes much less effective with increasing vesicle diameter and decreasing SNARE density[8,9]. Then, multi-subunit tether complexes become important. Tether complexes facilitate membrane contact and associate with the SNARE-binding SM proteins[10-12]. They promote trans-SNARE pairing, possibly by increasing the local concentration of SNAREs and by supporting their conformational rearrangement. We investigated whether tether complexes might enhance the fusogenic potential of SNARE complexes, by increasing the force that SNAREs transmit to the bilayers, or by lowering the energy barrier for fusion pore formation.

HOPS is the tether complex for vacuole and lysosome fusion. Vacuoles from cells deleted for the Rab7-GTPase Ypt7 lack HOPS (Fig. S1a)[13]. This prevents fusion and pairing between the vacuolar SNAREs Vam3, Vti1, Vam7 and Nyv1[14]. Incubating these membranes with soluble, recombinant vacuolar Q_c-SNARE Vam7 (rVam7) allows to stimulate trans-SNARE pairing. Then, the reaction is independent of endogenous Vam7, which must otherwise be liberated by Sec18/NSF-dependent disruption of cis-SNARE complexes[15]. Fusion can hence proceed without ATP, avoiding interference by the ATP-driven chaperone NSF, which disassembles SNARE complexes unless HOPS protects them[14]. To assay trans-SNARE pairing, we separately prepared

vacuoles from *ypt7Δ* or wildtype strains expressing Nyv1-HA or Vam3-myc. We mixed them in fusion reactions with rVam7 and measured *trans*-SNARE pairing through co-immunoadsorption of Vam3-myc with Nyv1-HA. Adding rVam7 to *ypt7Δ* vacuoles induced similar trans-SNARE pairing as in wildtype vacuoles (Fig. 1a,b). We measured content mixing by transfer of a 45 kDa enzyme between the fusion partners (Fig. S2), and lipid mixing by fluorescence dequenching through dilution of rhodamine-phosphatidylethanolamine[3,16]. The trans-SNARE complexes on *ypt7Δ* vacuoles failed to induce content mixing (Fig. 1c), whereas lipid mixing was similar to wildtype (Fig. 1d,e). Thus, HOPS-free *ypt7Δ* vacuoles reached a hemifused state but failed to form a fusion pore, or to open it wide enough to let the reporter pass. Lipid mixing was sensitive to antibodies targeting the Q_a-SNARE Vam3 or the R-SNARE Nyv1, confirming that the reaction was SNARE-dependent. The *ypt7Δ* trans-SNARE complexes are probably fully zippered, because this is prerequisite for lipid mixing[17].

To test the effect of HOPS on pore opening, we accumulated *ypt7Δ* vacuoles for 60 min in the hemifused state, added purified HOPS or subcomplexes thereof (Fig. S1b), and assayed content mixing after 15 min of further incubation. HOPS is a hexameric complex with a globular SNARE-binding domain, which contains the SNARE-binding SM-protein Vps33 and its interactor Vps16 (Fig. 1f)[18]. Vps33 (79 kDa) did not stimulate fusion (Fig. 1g), whereas the bigger Vps33-Vps16 subcomplex (159 kDa) rescued fusion to 80% of an untreated wildtype control, which had been incubated under standard fusion conditions with ATP. A Vps11-Vps39 subcomplex (240 kDa), representing the opposite end of HOPS without a SNARE interaction site, had no effect (Fig. 1g). As the biggest structure, HOPS (663 kDa) rescued *ypt7Δ* fusion to wildtype level. The endosomal CORVET complex, which is of similar size as HOPS and shares Vps33 with it[11], stimulated fusion as well as HOPS (Fig. S3). The specificity of these complexes is hence restricted to their Rab-GTPase-dependent function in membrane tethering. HOPS had not enhanced trans-SNARE pairing beyond the level attained by the preincubation with rVam7 (Fig. 1a,b) and it did not induce fusion in the absence of Vam7, nor

upon pre-incubating the vacuoles with antibodies to Vam3 (Figs. 1g, S4). Thus, rescue was SNARE-dependent. These results suggest an additional role of HOPS in fusion pore opening, which is independent of its known role in facilitating SNARE pairing[12,19].

To test whether pore opening might be driven by increased SNARE complex volume, we accumulated hemifused *ypt7Δ* vacuoles and added CBP-Vps33, which does not stimulate pore opening. When we tripled the effective molecular mass of Vps33 by adding a monoclonal antibody (150 kDa) to its CBP-tag, content mixing increased 5-fold and reached >50% of the wildtype signal (Fig. 2a). Antibodies did not stimulate fusion when Vps33 had been omitted, nor when Vps33 was used with a non-cognate HA-antibody. CBP-Vps33 alone decreased content mixing of wildtype vacuoles by 60% and this inhibition could be partially overcome by adding CBP-antibodies. This can be understood if CBP-Vps33 outcompetes endogenous HOPS for SNARE binding but by itself does not add sufficient mass to stimulate fusion.

Next, we replaced HOPS by artificial SNARE-binding proteins, using solely polyclonal antibodies to SNAREs (Fig. 2b). When added from the beginning of a reaction, SNARE antibodies interfere with trans-SNARE pairing and block fusion. To circumvent this block, we first accumulated *ypt7Δ* vacuoles in hemifusion, with trans-SNARE complexes already formed. Now, anti-Nyv1 or anti-Vam3 became strong stimulators. They rescued content mixing to 50% when added individually and to 100% when added simultaneously. Wildtype vacuoles, which contain sufficient amounts of endogenous HOPS, were hardly stimulated by the antibodies. Thus, artificial SNARE ligands substitute for HOPS in fusion pore opening in vitro.

Since bivalent antibodies to SNAREs might promote fusion by clustering SNARE complexes around a fusion site, we generated monovalent F_{ab} fragments from them (Fig. S1c). These inhibited fusion and trans-SNARE pairing when added before docking (Fig. 2c,d), but they potently stimulated content mixing of hemifused vacuoles without affecting *trans*-SNARE pairing.

Individual use of F_{ab}s to either Vam3 or Nyv1 stimulated fusion weakly, probably because F_{ab}s are smaller (56 kDa) than IgGs (150 kDa) and their dimensions permit to place no more than two F_{ab}s along a SNARE domain. F_{ab}s to a single SNARE may therefore not add sufficient volume to SNARE complexes to drive pore opening. Crowding agents[20], such as Ficoll 400, had no influence, suggesting that bulky SNARE complex ligands do not stimulate fusion by molecular crowding (Figs. S5,S3). Thus, HOPS may promote fusion pore formation in vitro by increasing the volume of SNARE complexes.

We engineered an *in vivo* system for recruiting large ligands to SNAREs, using FKBP12 and FRB, two domains undergoing rapamycin-induced dimerization[21]. FKBP12 (12 kDa) was attached to the C-terminus of Vam7, which is close to the TMDs of the SNARE complex. FRB-GFP (38 kDa) was attached to Pfk1, a subunit of the octameric, soluble phosphofructokinase (850 kDa), which is unrelated to fusion (Figs. 3a,S1d,e). Rapamycin-insensitive *tor1-1* cells served as strain background to avoid side effects of rapamycin treatment through TOR signaling[22]. *tor1-1* cells have multiple vacuoles under normal growth conditions. Rapamycin recruited Pfk1-FRB-GFP to vacuoles within less than 10 min (Fig. 3b,c). These vacuoles fused, significantly reducing their number per cell. Recruitment, but not fusion, was observed, when FKBP12 was separated from Vam7 by a 35 amino acid linker (Fig. 3d,e). Vam7 lacking FKBP12 induced neither recruitment nor fusion (Fig. 3b,c). Also an FRB-GFP chimera lacking phosphofructokinase did not provoke fusion (Fig. S6).

In vivo, HOPS recruitment controls re-fusion of vacuoles following hypertonic shock[23]. Hypertonic shock fragments yeast vacuoles in <5min, Vps41 becomes phosphorylated and dissociates into the cytosol, likely together with HOPS[24]. Vacuole recovery requires Vps41 dephosphorylation, reassociation of HOPS with vacuoles and fusion. Whereas re-fusion requires >60 min in wildtype cells, rapamycin-induced recruitment of Pfk1-FRB-GFP to SNAREs provoked premature fusion of these vacuolar fragments, circumventing the physiological inactivation of HOPS (Fig. S7). Fusion was not observed in cells lacking FKBP12 on Vam7, nor when rapamycin was omitted.

We explored the influence of bulky SNARE ligands by coarse-grained molecular dynamics (Fig. 4a). HOPS features a SNARE-binding domain of approximately 12-14 nm diameter, which probably encapsulates the SNARE complex[11,18] (Fig. 1f). Binding a sphere of the size of this head region to SNAREs markedly affects the geometry of the hemifusion stalk (Fig. 4a). The stalk restrains the apposed membranes from separating, enforcing strong local curvature. We rationalized the acceleration of fusion from the apparent work (free energy) required to thin the hemifusion stalk (Fig. 4a,b and SI). Progression from hemifusion to pore opening decreases the mutual distance of the SNARE C-termini until they associate (Fig. 4a). The presence of a HOPS sphere (14 nm) halves the energetic cost of fusion pore opening (from 67 k_BT to 34 k_BT) in a tension-less membrane system with 3 SNARE complexes (Figs. 4b, S8). Pore nucleation now requires far-less thinning of the stalk. We attribute the enhanced (non-leaky) fusion pore formation to (I) a partial, relative relaxation of the HOPS-induced curvature stress (~50 k_BT; Figs. 4c,S9), and (II) a geometrical advantage because of the pre-existing curvature (Fig. S8). Smaller contributions can be made by an initial gain in SNARE pulling force (Fig. S10). Thus, HOPS stimulates pore opening by steric effects on the site of hemifusion.

We can understand numerous unexplained findings from this perspective: (1) SNARE-associated tether protein complexes (Munc13 and its associated SM protein Munc18) are essential for fusion of synaptic vesicles. While this can reflect their role in SNARE complex assembly, Munc13/18 might also drive fusion itself[25]. Accordingly, mutations that reduce Munc18 affinity for SNAREs change fusion pore dynamics[26]. (2) Also mutating the yeast Munc18 homolog Sec1, which associates with the tether complex exocyst[27], reduces exocytosis without reducing SNARE complex abundance[28,29]. (3) On vacuoles, mutations compromising Vps33 binding to vacuolar SNAREs impair content mixing more than lipid mixing and trans-SNARE pairing[16] (4) HOPS also enhances liposome fusion more than trans-SNARE pairing[30]; (5) binding to Sec17/α-SNAP and Sec18/NSF renders trans-SNARE complexes more fusogenic[17,31].

Our simulations and experimental observations suggest that SNARE-ligands can drive fusion irrespective of specific molecular properties, except for their size. Therefore, any ligand that substantially increases the volume of trans-SNARE complexes close to their membrane anchors is expected to stimulate pore opening. Since SM proteins and tethering complexes are indispensable for fusion in multiple trafficking pathways[11], we propose that the driving force that they can contribute to fusion is a critical and conserved feature of their function. In physiological membranes, SNAREs and the tether/SM protein system thus act as an integrated molecular machine, in which tether/SM proteins first facilitate SNARE pairing[11] and then drive the very last step of fusion.

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Author contributions:

HJR conceived and interpreted simulation experiments and values derived from them. MDA conceived, performed and interpreted all other experiments. AM conceived the study and interpreted the results. AL and CU provided purified CORVET, HOPS and HOPS subcomplexes. All authors jointly wrote the paper.

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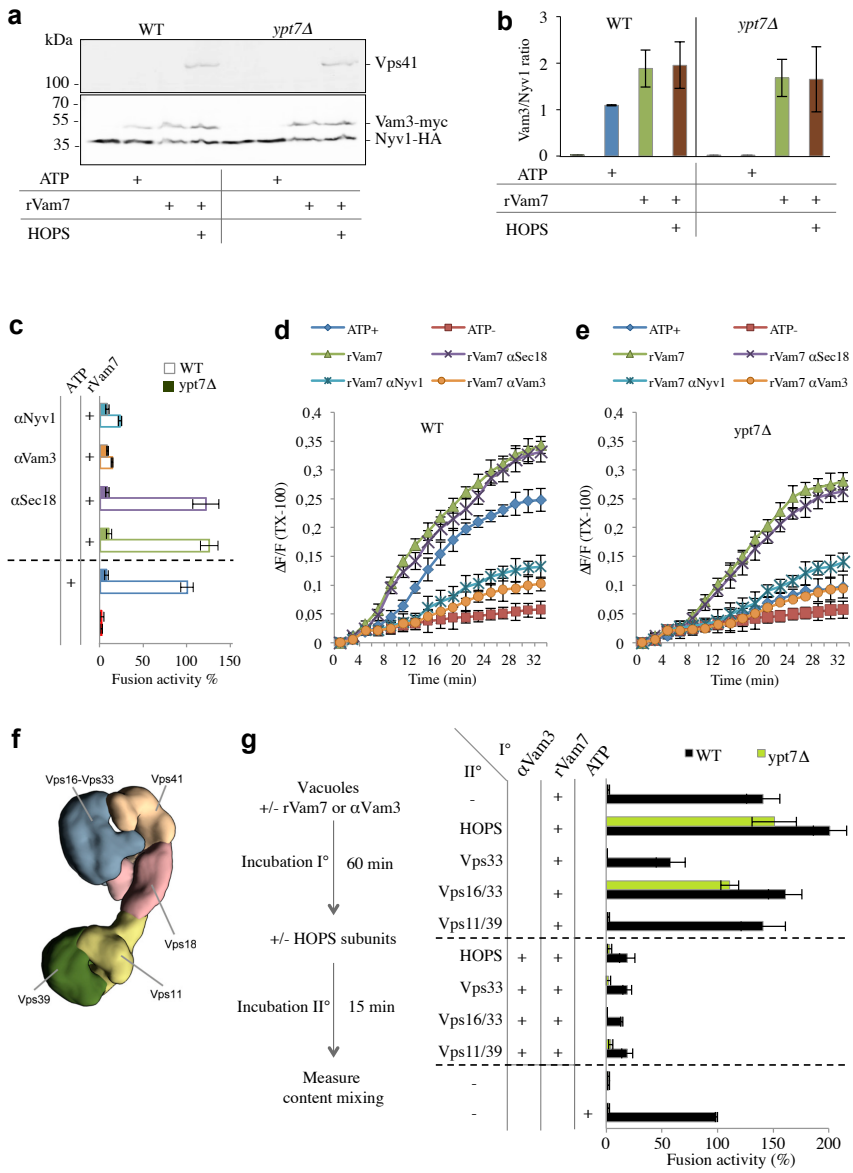

Figure 1: Effect of soluble Vam7 on lipid and content mixing.

a, Trans-SNARE pairing. Vacuoles were isolated from wildtype (BJ3505) or isogenic ypt7Δ cells carrying Vam3-myc or Nyv1-HA. The two vacuole populations were mixed and incubated in fusion reactions with ATP, Vam7 and HOPS as indicated. After solubilisation, proteins were pulled down with anti-HA and analyzed by SDS-PAGE and Western blotting. **b,** Signals were quantified by infrared fluorescence scanning. *Trans*-SNARE pairing is indicated by co-adsorbed Vam3-myc and quantified through the ratio Vam3-myc/Nyv1-HA. Means ± s.d. are shown. n=3. **c-e,** Hemifusion in *ypt7Δ* vacuoles: Vacuoles from wildtype and *ypt7Δ* cells were incubated in ATP-free fusion reactions with 600 nM rVam7 and 10 mg/ml BSA. Antibodies (200 nM) had been added where indicated. **c,** Content mixing was determined after 60 min, using the activation of pro-alkaline phosphatase by a maturase from the fusion partners. **d,e**, Lipid mixing was followed by dequenching of the fluorescence of Rh-phosphatidylethanolamine, which had been integrated at self-quenching concentrations into one of the fusion partners. Means ± s.d. are shown. n=3. **f,** Structure of HOPS (from[18]). **g,** Effect of HOPS subcomplexes on content mixing. Vacuoles were incubated in fusion reactions as in C for 60 min, with anti-Vam3 where indicated. Then, the samples received 400 nM purified (CBP)-tagged HOPS sub-complexes or Vps33 and were incubated for further 15 minutes before content mixing was assayed. Means ± s.d. are shown. n=3.

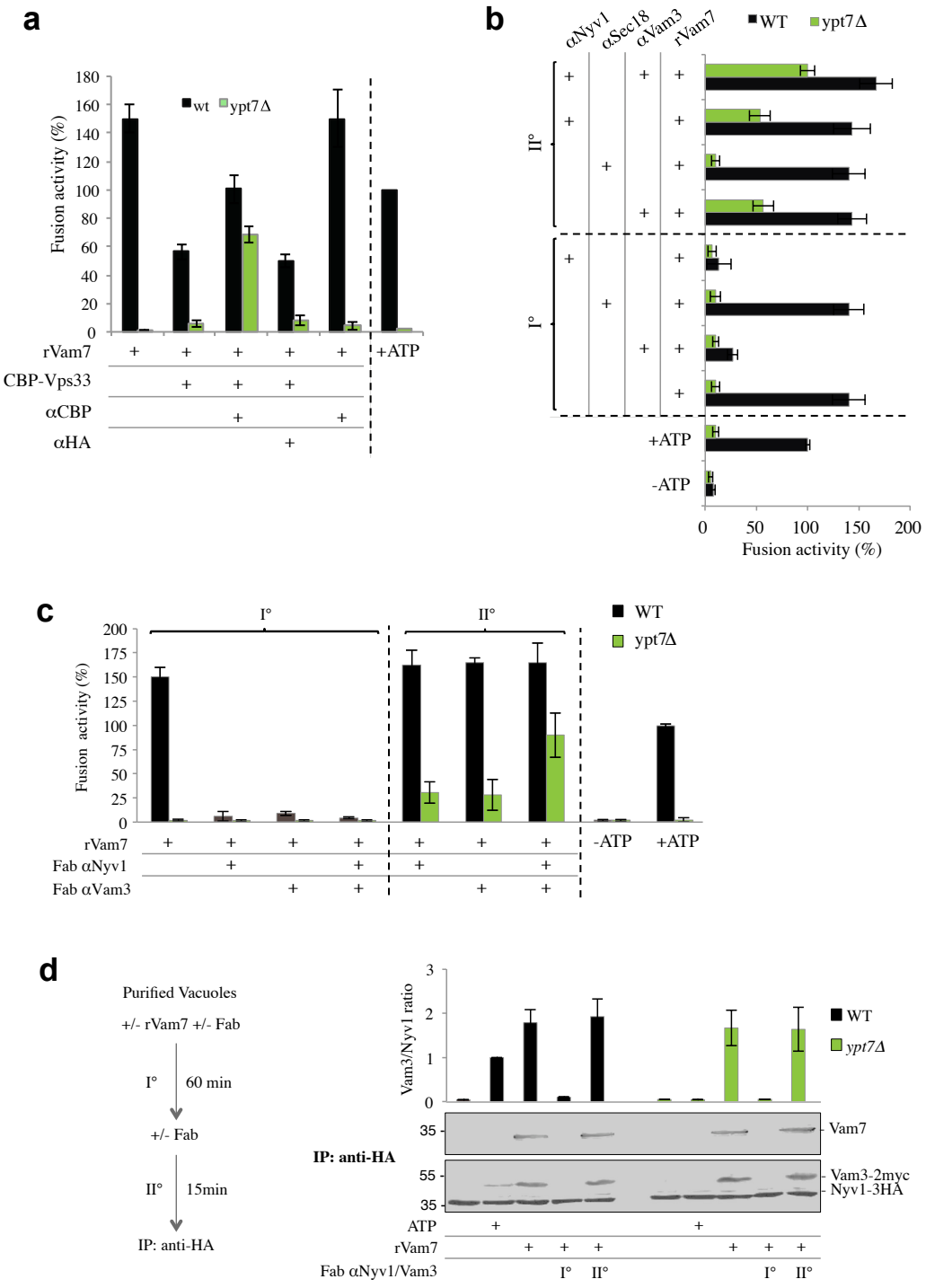

Figure 2: Fusion pore opening driven by ligands increasing SNARE complex size in vitro. **a,** Vps33. Fusion reactions with wildtype and *ypt7Δ* vacuoles were started as in Fig. 1g. After the first 60 min incubation period, samples received recombinant CBP-Vps33 (400nM), antibodies to CBP or HA (200 nM), or buffer only. After further 15 min, content mixing was assayed. Means ± s.d. are shown. n=3. **b,** Antibodies. Two-stage fusion reactions were performed as in a, but only with rVam7. Antibodies (200 nM) against Vam3, Nyv1 or Sec18/NSF were added either during the first 60 min incubation (I°) or during the second incubation (II°) of 15 min. Means ± s.d. are shown. n=3. **c,** F_{ab} fragments. Experiment as in b, but with F_{ab} fragments instead of antibodies. n=3. **d,** *Trans*-SNARE pairing was assayed using tagged strains as in Fig. 1a. Reactions with staged addition of rVam7 and F_{ab}s were run as in c. Means ± s.d. are shown. n=3.

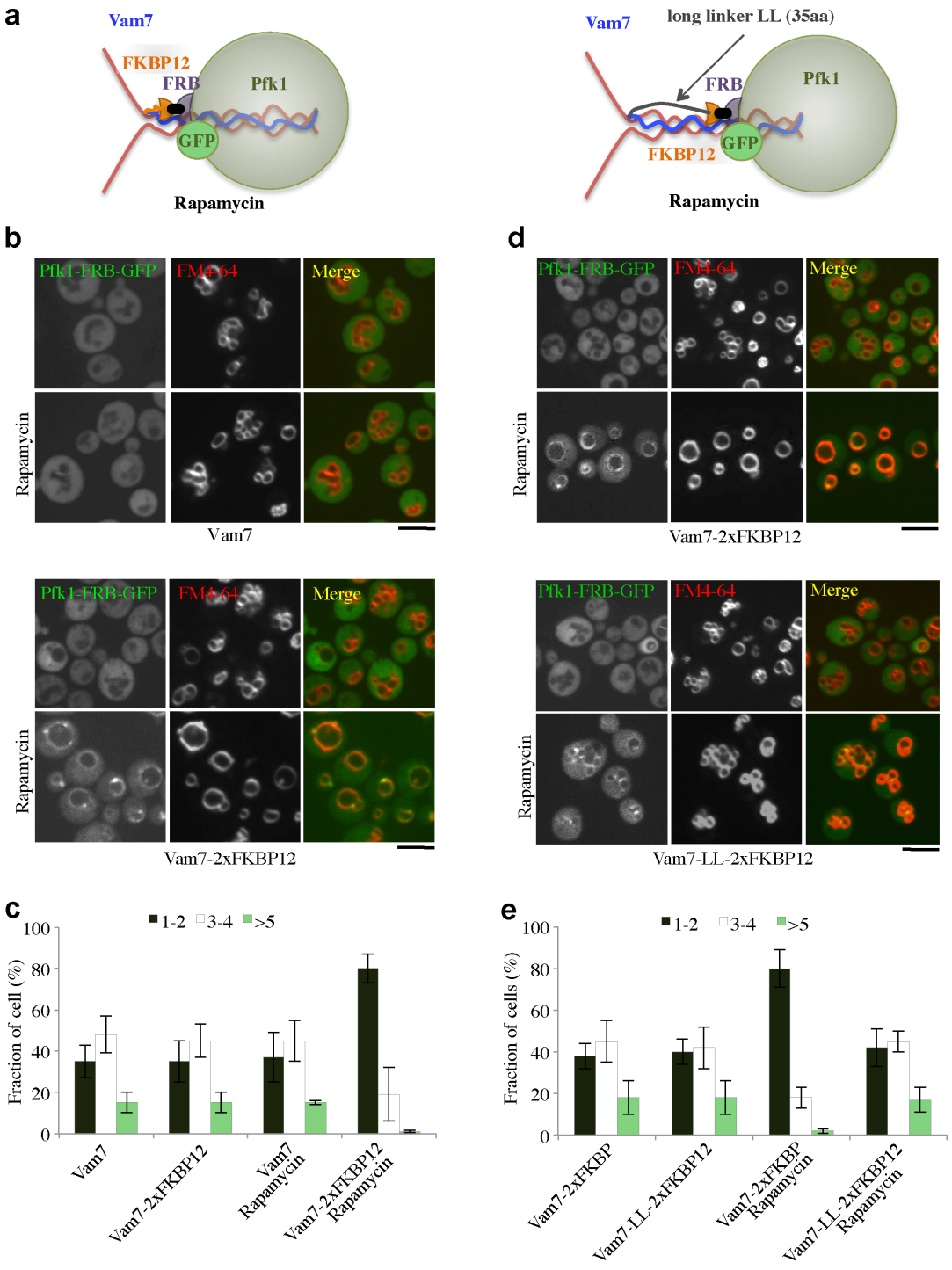

Figure 3: Effect of SNARE complex enlargement on vacuole fusion in vivo.
a, Schematic view of rapamycin-induced FKBP12/FRB-tagged Pfk1-recruitment to the SNARE complex without and with a long linker between Vam7 and FKBP12. **b, d,** In vivo vacuole morphology. Logarithmically growing cells, carrying Pfk1-FRB-GFP and (**b**) Vam7 or Vam7-2xFKBP12, or (**d**) Vam7-LL-2xFKBP12 with a 35 amino acid linker, were stained with the vacuole tracer FM4-64. Cells were incubated with 10 µM rapamycin for 10 min where indicated and analyzed by spinning disc microscopy. Scale bar: 5 µm. **c, e,** Quantification of b and d, respectively. The cells were grouped into three categories according to the number of vacuoles visible per cells. 100 cells were analyzed per sample. Means and s.d. are shown. n=3.

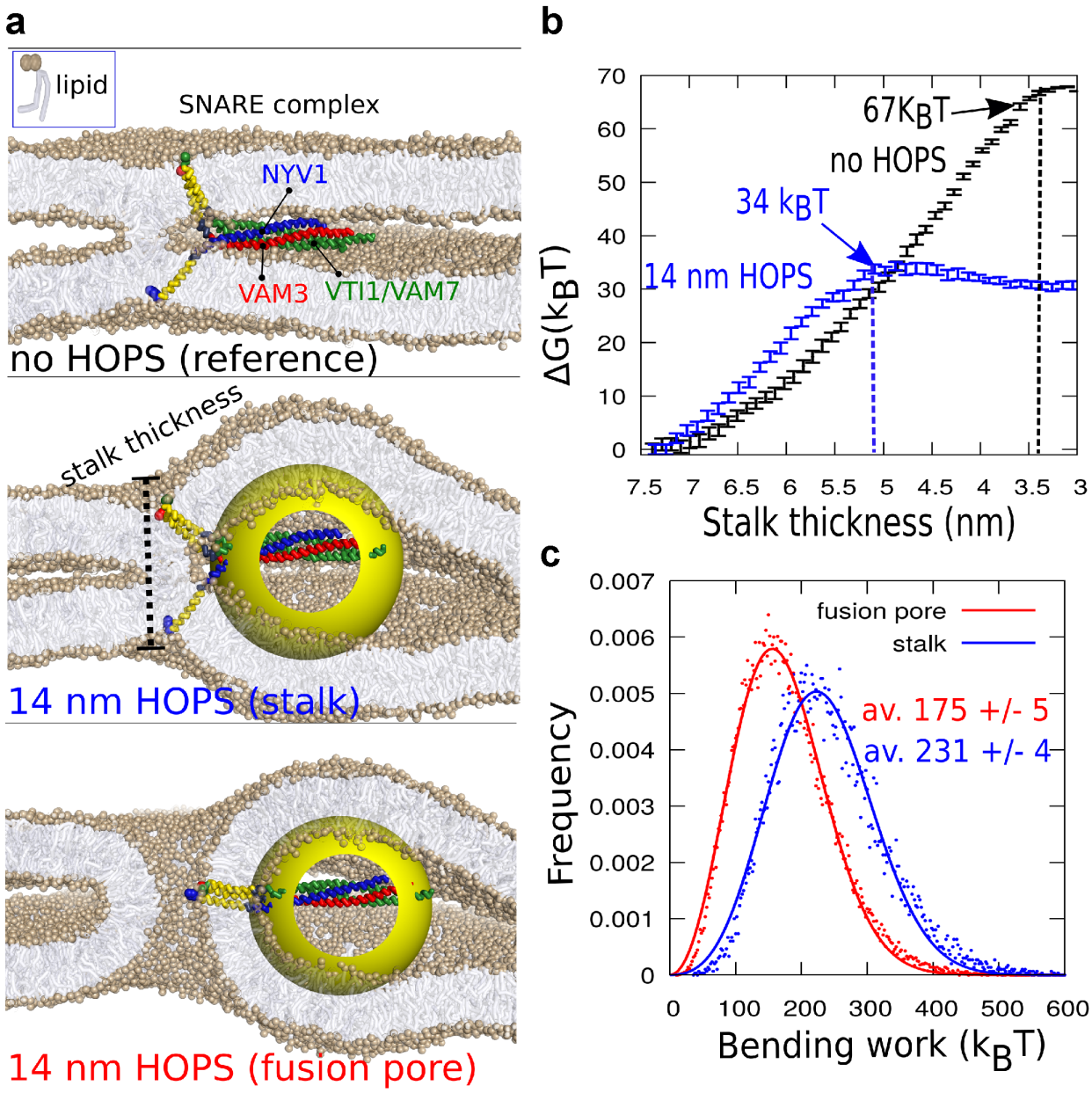

Figure 4: Molecular dynamics simulations on the influence of steric constraints at the (hemi-)fusion site.

a, Simulation setup: Two hemifused membranes in the presence of the SNARE complex that is either free or bound to a sphere equivalent to the size of the SNARE-binding domain of HOPS (14 nm). The size of 'HOPS' and positioning of the SNARE complex are inspired by EM and crystal structures[9,11,18]. To enhance clarity, only one SNARE complex is illustrated. **b,** The free-energy of fusion pore formation is derived by measuring the work (free energy) required to thin the stalk until fusion pore nucleation occurs[32] (see Fig. S8). Arrows and dotted lines indicate the point of pore nucleation. **c,** Work distribution reflecting the equilibrium work that HOPS must perform to bend the membrane in the presence of a stalk (average work: 231+/- 4 k_BT) or of a fusion pore (average work: 175 +/- 5 k_BT), respectively. A reduction of ~50 k_BT indicates that fusion pore formation is associated with release of local membrane stress.