Supplementary Information for:

Control of SNARE-driven vesicle fusion by synapsin condensates on freestanding membranes

Cha *et al.*

* Correspondence: mjshon@postech.ac.kr (MJS); seungsoo@postech.ac.kr (SSO)

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Supplementary Methods

Recombinant protein sequences

Rat syntaxin-1A for the ΔN complex (residues 191–288 lacking the H_{abc} domain; I202 and K266 were mutated to cysteine (*red*) for fluorescent labeling, and native cysteines C271 and C272 were mutated to alanine (*blue*) to prevent unwanted labeling):
MALSEIETRHSECIKLENSIRELHDMFMDMAMLVESQGEMIDRIEYNVEHAVDYVERAVSDTKKAVKY QSKARRKKCMIIIAAVILGIIIASTIGGIFG

Rat SNAP25 isoform b for the ΔN complex (residues 1–206; all native cysteines were mutated to alanine (*blue*)):
MAEDADMRNELEEMQRRADQLADESLESTRRMLQLVEESKDAGIRTLVMLDEQGEQLERIEEGMDQ INKDMKEAEKNLTDLGKFAGLAVAPANKLKSSDAYKKAWGNNQDGVVASQPARVVDEREQMAISGG FIRRVTNDARENEMDENLEQVSGIIGNLRHMALDMGNEIDTQNRQIDRIMEKADSNKTRIDEANQRAT KMLGSG

Rat VAMP2 fragment for the ΔN complex (residues 49–96 with an N-terminal His-tag):
MGSSHHHHHHSSGLEVLFQGPDNVDKVLERDQKLSELDDRADALQAGASQFETSAAKLKRKYWWK NLKMM

Rat VAMP2 (residues 1–116; A72 was mutated to cysteine (*red*) for fluorescent labeling, and a native cysteine C103 was mutated to alanine (*blue*) to prevent unwanted labeling):
MSATAATVPPAAPAGEGGPPAPPPNLTSNRRLQQTQAQVDEVVDIMRVNVDKVLERDQKLSELDDR ADALQCGASQFETSAAKLKRKYWWKNLKMMIILGVIAAIILIIIVYFST

Rat complexin-1 (residues 1–80 lacking the C-terminal domain)
GPMEFVMKQALGGATKDMGKMLGGDEEKDPDAAKKEEERQEALRQAEEERKAKYAMEAEREVMR QGIRDKYGIKKKEEREA

Rat synapsin-1 isoform a (residues 1–704 with an N-terminal His-tagged eGFP fusion (*green*)):
MGSSHHHHHHSSGLVPRGSHMASVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLT LKFICTTGKLPVPWPTLVTTLTYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRA EVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIMADKQKNGIKVNFKIRHNIEDGSVQLA DHYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNEKRDHMVLLEFVTAAGITLGMDELYKRSMNYLRR RLSDSNYMANLPNGYMTDLQRPQPPPPPPSAASPGATPGSAAASAERASTAAPVAAPAAPSPGSSG GGGFFSSLSNAVKQTTAAAAATFSEQVGGGSGGAGRGGAAARVLLVIDEPHTDWAKYFKGKKIHGEI DIKVEQAEFSDLNLVAHANGGFSVDMEVLRNGVKVVRSLKPDFVLIRQHAFSMARNGDYRSLVIGLQ YAGIPSVNSLHSVYNFCDKPWVFAQMVRLHKKLGTEEFPLIDQTFYPNHKEMLSSTTYPVVVKMGHA HSGMGKVKVDNQHDFQDIASVVALTKTYATAEPFIDAKYDVRVQKIGQNYKAYMRTSVSGNWKTNT

GSAMLEQIAMSDRYKLWVDTCSEIFGGLDICAVEALHGKDGRDHIIEVVGSSMPLIGDHQDEDKQLIV
ELVVNKMTQALPRQRDASPGRGSHSQTPSPGALPLGRQTSQQPAGPPAQQRPPPQGGPPQPGPG
PQRQGPPLQQRPPPQGQQHLSGLGPPAGSPLPQRLPSPTAAPQQSASQATPMTQGQGRQSRPVA
GGPGAPPAARPPASPSPQRQAGPPQATRQASISGPAPPKVSGASPGGQQRQGPPQKPPGPAGPIR
QASQAGPGPRTGPPTTQQPRPSGPGPAGRPTKPQLAQKPSQDVPPPIIAAAGGPPHPQLNKSQSLT
NAFNLPEPAPPRPSLSQDEVKAETIRSLRKSFASLFSD

Protein expression, purification, and labeling

His-tagged, N-terminal-truncated VAMP2 and syntaxin-1A were cloned into pETduet vectors, while full-length SNAP25 and GST-tagged VAMP2 were cloned into pGEX vectors. For expression of the ΔN complex, the pETduet plasmid encoding the VAMP2 fragment and syntaxin-1A and the SNAP25 plasmid were co-transformed into *E. coli* Rosetta (DE3) pLysS cells. Cultures were grown overnight in a preculture, transferred to the main culture, and incubated at 37 °C with shaking (220 rpm) until OD600 reached 0.6, after which protein expression was induced with 1 mM IPTG for 4 h at 37 °C. Cells were harvested by centrifugation (4,000×g, 15 minutes, 4 °C) and lysed on ice by sonication in cold lysis buffer (50 mM HEPES, 500 mM NaCl, 2 mM $MgCl_2$, 0.5% Triton X-100, 1% sarcosine, 2 mM PMSF, 5 mM 2-mercaptoethanol, and 0.5 mM DNase). Clarified lysates were applied to Ni-NTA agarose (for His-tagged proteins) or glutathione agarose (for GST-tagged proteins) by gravity flow, washed extensively, and then eluted using buffers containing HEPES, NaCl, 1% n-octyl-β-D-glucoside (OG), 10% glycerol, and either 400 mM imidazole (for Ni-NTA) or 0.03 U/μL thrombin (for glutathione resin). Thrombin was deactivated with 0.3 mM PMSF after 1 h. Eluted proteins (~1 mg/mL) were fluorescently labeled overnight at 4 °C with either Alexa Fluor 647 or Cy3 maleimide (Invitrogen), ensuring a 10:1 dye-to-protein molar ratio, and free dye was removed via gel filtration. Concentration was achieved using centrifugal filters (Amicon) with a cutoff at half the target protein' size, and protein concentrations were maintained below 10 μM to prevent aggregation. Recombinant complexin-1 was purified as described previously (Shon *et al*., *Nat. Commun.* (2018)). For synapsin-1 expression, the full-length coding sequence was N-terminally fused to His-tagged eGFP in the peGFP-C1 vector, transformed into *E. coli* BL21 (DE3), and purified similarly to the SNARE proteins using Ni-NTA resin under detergent-free conditions (using buffers with Tris and TCEP instead of HEPES and 2-mercaptoethanol, and omitting detergents and glycerol).

Supplementary Figures

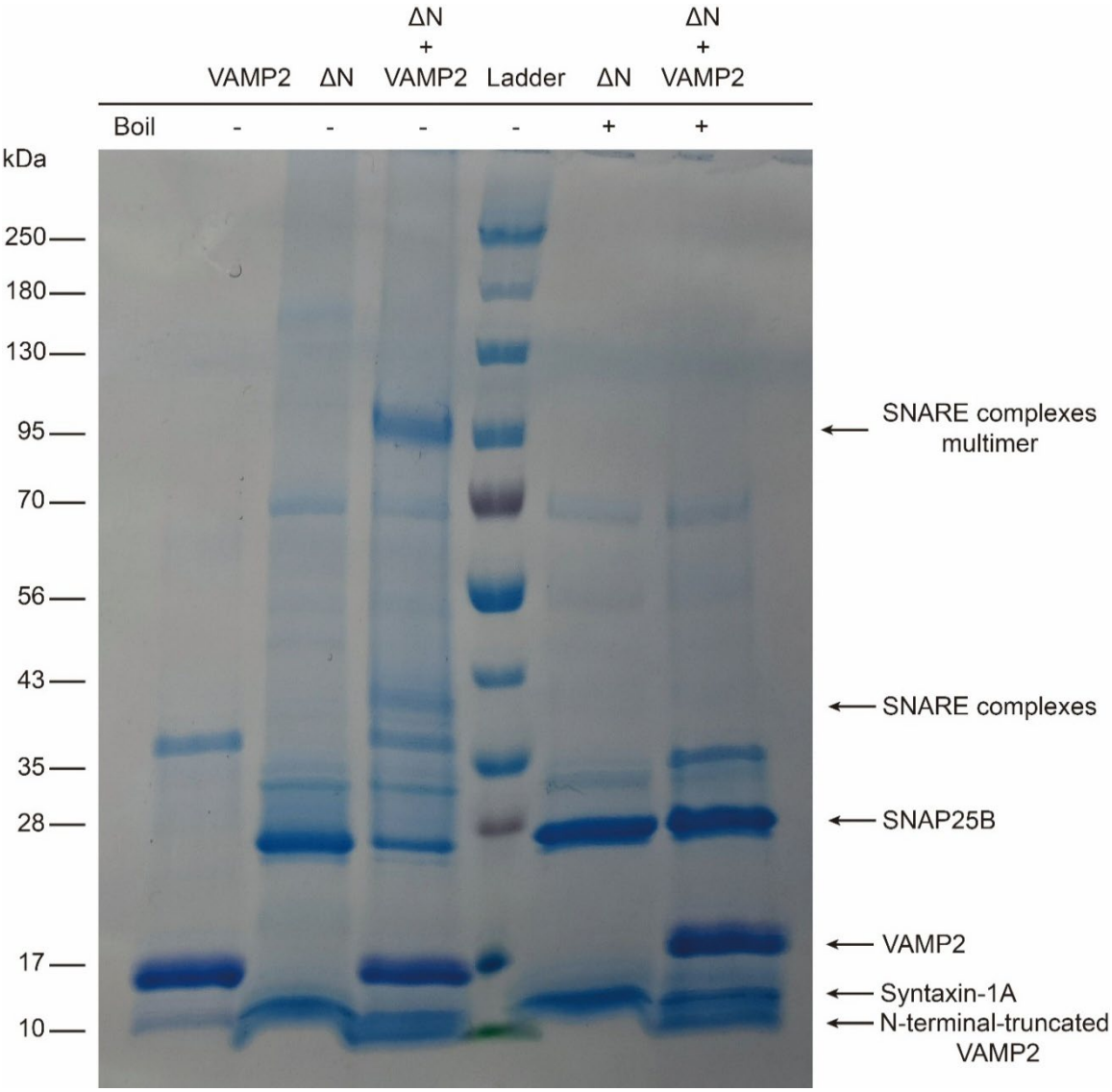

Figure S1. Purification of VAMP2, ΔN complexes, and SNARE complexes

SDS-PAGE gel showing purified VAMP2, ΔN complexes, and SNARE complexes formed by mixing 150 pM VAMP2 with ΔN complexes. Bands from boiled samples treated with 10 mM DTT demonstrate that monomeric and multimeric SNARE complexes can be disassembled.

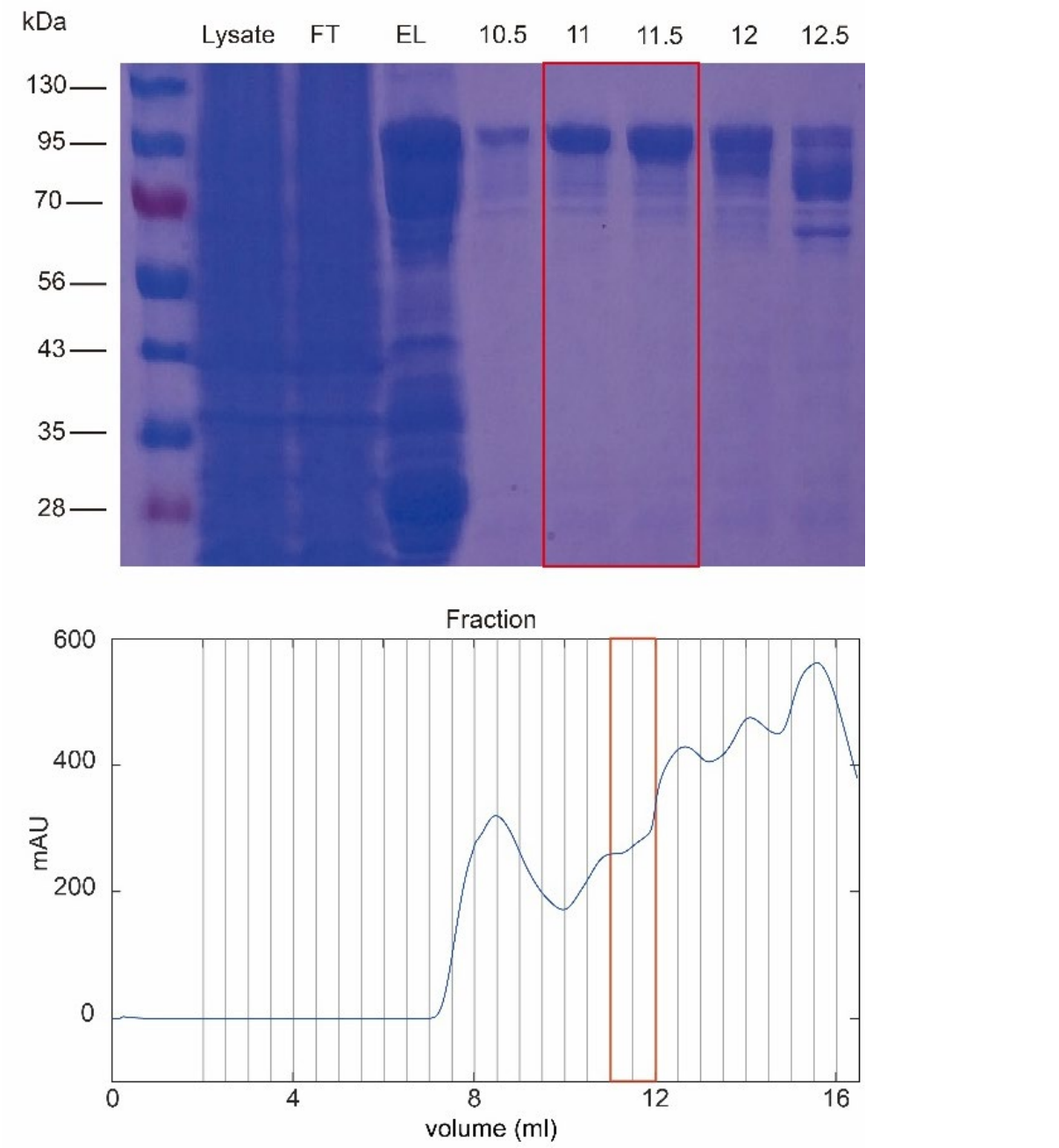

Figure S2. Purification of eGFP-tagged synapsin-1

SDS-PAGE analysis showing the lysate, flow-through (FT), eluate (EL), and the selected elution fractions (*red box*) for purified eGFP–synapsin-1 following size-exclusion chromatography. Below the gel is the corresponding UV absorbance chromatogram at 280 nm.

Supplementary Video Legends

Video S1. Formation of freestanding lipid bilayers over an electron microscopy grid
Time-lapse video capturing the real-time formation of lipid bilayers as a transmission electron microscopy (TEM) grid, initially floated on a lipid-oil mixture (90 mol% DOPC and 10 mol% DOPS), was submerged into an aqueous buffer. Images were acquired under white-light illumination.

Video S2. Asymmetric quenching of NBD fluorescence in a freestanding lipid bilayer
NBD-labeled PE lipids (1 mol%) were locally quenched by sodium dithionite delivered via a micropipette. The freestanding bilayer was imaged under 488 nm illumination (0.5 s exposure per frame) with reduced laser power to minimize photobleaching. A control video segment at the end shows abrupt membrane rupture and complete loss of fluorescence.

Video S3. Comparison of ΔN complex diffusion on freestanding and supported lipid bilayers
A video sequence showing two experimental conditions: first, freely diffusing Alexa 647-labeled ΔN complexes embedded in a freestanding lipid bilayer; and second, the same proteins embedded in a supported lipid bilayer modified with 0.5 mol% PEG5000-ceramide to enhance protein mobility, exhibiting limited mobility. Images were acquired under 633 nm laser illumination with a 30 ms exposure per frame.

Video S4. Probing the orientation of ΔN complexes within a freestanding lipid bilayer
In the first part with the upright configuration (see Fig. 2J in the main text), a glass probe with a rounded tip contacted the top side of the membrane, excluding Alexa 647-labeled ΔN complexes from the contact region as the probe was moved around, thus reducing local fluorescence. In the second, flipped configuration (see Fig. 2K in the main text), the same probe contact did not displace ΔN complexes, indicating that they now resided on the bottom side of the bilayer. These results demonstrate that ΔN complexes were preferentially embedded from the side on which the protein solution was applied. Images were acquired under 633 nm laser illumination with a 30 ms exposure per frame.

Video S5. Comparison of VAMP2 diffusion in the absence and presence of complexin
In the first part, rapidly diffusing Cy3-labeled VAMP2 was observed in a freestanding bilayer after fusion of VAMP2 vesicles with ΔN-containing membranes. In the second part, with 1 μM complexin present, many VAMP2 vesicles remained intact and diffused more slowly,

consistent with complexin's clamping effect and arrest of fusion. Images were acquired under 532 nm laser illumination with a 30 ms exposure per frame. Video playback is slowed to 1/2 of the original speed.

Video S6. Effect of synapsin on VAMP2 vesicle binding to ΔN complexes

Video showing clustered Cy3-labeled VAMP2 vesicles (*green*) colocalized with Alexa Fluor 647-labeled ΔN complexes (*red*) on FLBs, with and without 10 μM synapsin. 3% PEG was used to promote synapsin condensation. Images were acquired using a dual-view setup under 532 nm and 633 nm illumination, with a 30 ms exposure per frame. Video playback is slowed to 1/4 of the original speed.

Video S7. VAMP2 vesicles in the presence of synapsin before and after condensate dispersal

Video showing 1 mol% DiD-labeled VAMP2 vesicles (*red*) in the presence of 10 μM eGFP-tagged synapsin (*green*) with 3% PEG, before and after introducing 5% 1,6-hexanediol for condensate dispersal. Images were acquired using a dual-viewer setup under 488 nm and 633 nm laser illumination. The exposure time per frame was 0.03 s. Video playback is slowed to 1/4 of the original speed.