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# Munc18a Clusters SNARE-Bearing Liposomes Prior to Trans-SNARE Zippering

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## Abstract

Sec1-Munc18 (SM) proteins cooperate with SNAREs {SNAP [soluble NSF (N-ethylmaleimide-sensitive factor) attachment protein] receptors} to mediate membrane fusion in eukaryotic cells. Studies of Munc18a/Munc18-1/Stxbp1 in neurotransmission suggest that SM proteins accelerate fusion kinetics primarily by activating the partially zippered trans-SNARE complex. However, accumulating evidence has argued for additional roles for SM proteins in earlier steps in the fusion cascade. Here we investigate the function of Munc18a in reconstituted exocytic reactions mediated by neuronal and non-neuronal SNAREs. We show that Munc18a plays a direct role in promoting proteoliposome clustering, underlying vesicle docking during exocytosis. In the three different fusion reactions examined, Munc18a-dependent clustering requires an intact N-terminal peptide (N-peptide) motif in syntaxin that mediates the binary interaction between syntaxin and Munc18a. Importantly, clustering is preserved under inhibitory conditions that abolish both trans-SNARE complex formation and lipid mixing, indicating that Munc18a promotes membrane clustering in a step that is independent of trans-SNARE zippering and activation.

### Keywords

membrane fusion; exocytosis; SNARE; Munc18; docking; reconstitution

## Introduction

Eukaryotic membrane fusion along the secretory and endocytic pathways depends on the conserved functions of proteins in the SNARE family. Anchored to apposed membranes, cognate SNAREs form a 4-helical bundle (*trans*-SNARE complex or SNAREpin) that stresses the lipid bilayers, a key event to the fusion process (1–3). Based on whether they contribute a glutamyl (Q) or an arginyl (R) side chain at the center of the helical bundle, SNAREs can be further classified into Q- or R- subfamilies (4, 5). A typical trans-SNARE

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**Author contribution**

M.G.A and H.X. designed the experiments and wrote the manuscript. M.G.A, P.A, and H.X analyzed the data. M.G.A, P.A, B.K., and H.X performed the experiments.

**Competing Interests**

The authors declare that there are no competing interests associated with the manuscript.

complex is composed of three Q-SNAREs (Qa, Qb, and Qc) and one R-SNARE although in exocytosis, a SNAP25-like protein (e.g., SNAP23 and SNAP25) contributes two αhelices (SNARE motifs) to the 4-helical bundle. The unique pairing of Q- and R- SNAREs on different organelles is partially responsible for the specificity of membrane traffic (1).

Although trans-SNARE zippering is sufficient to drive basal levels of lipid and content mixing in various reconstituted fusion systems (6–9), additional fusion factors play important roles *in vivo* to regulate events preceding and beyond trans-SNARE complex formation (10, 11). Vesicle fusion with the plasma membrane is thought to proceed in three successive steps: 1) docking, 2) priming, and 3) merger of lipid bilayers (12–14). Microscopically, docking refers to the retention of transport vesicles to the target membrane (e.g., plasma membrane) (15). It is facilitated by membrane-tethering factors (including coiled-coil homodimers and multisubunit tethering complexes) (11), which exploit interactions with lipids and/or membrane-anchored proteins (e.g., Rab GTPase) to bring vesicles and target membranes into proximity (16). However, evidence that multisubunit tethering factors physically link two distinct membranes is still lacking, with the exception of HOPS and CORVET, which function in endo/lysosomal fusion (17). Priming renders docked vesicles competent for calcium-triggered exocytosis. Through the activities of Munc13/CAPS and other factors (12, 18–20), priming results in partially assembled trans-SNARE complexes. In the final step of exocytosis, calcium influx is thought to activate synaptotagmin, which relieves the inhibition imposed by complexin while cooperating with the trans-SNARE complex to merge the apposed lipid bilayers (21, 22).

The intricate coordination of the different steps in membrane fusion relies on members of the SM protein family (10, 23), which exploit multiple modes of interaction with the SNAREs (24–26). Four SM protein subfamilies have thus far been identified in eukaryotic cells, where they perform essential functions in exocytosis (Sec1/Munc18), endocytosis (Vps45), protein biosynthesis (Sly1), and degradation (Vps33) (27). A major role of SM protein in activating SNARE mediated fusion (after priming) was established through the elegant study of Munc18a/Munc18-1/Stxbp1 in reconstituted neural transmission (28) and was supported by the detection of direct association between SM proteins and the 4-helical SNARE bundles (28–33). While the biochemical elements responsible for such interaction have yet to be fully elucidated, it is accepted that promoting full trans-SNARE zippering is a conserved function of SM protein in membrane fusion (10, 14). Intriguingly, the recent crystallographic study of vacuolar SM protein Vps33p has revealed two distinct but parallel binding tracks for individual Qa-SNARE (Vam3p) and R-SNARE (Nyv1p) (34). The orientation and proximity of these binding tracks provide structural basis for the notion that an SM protein could catalyze the initial trans-SNARE complex assembly (priming) by binding simultaneously to Q- and R- SNAREs that are anchored in apposing membranes (11). The notion is in accordance with the observation that Munc18a-bound syntaxin1 readily forms SNARE complexes with VAMP2/synaptobrevin2 in native plasma membranes (35) and results from the reconstitution study of Munc18c in trans-SNARE zippering assays (36). Furthermore, Munc18a-dependent *de novo* SNARE assembly has lately been confirmed using single-molecular manipulations (37). In addition to priming (via partial trans-SNARE assembly) and fusion (via full trans-SNARE assembly), there is accumulating evidence that SM proteins support earlier steps in the fusion cascade. Ultra-structural studies

of the neuromuscular junctions in C. *elegans* show that the proportion of vesicles docked near neurotransmitter release sites is reduced in unc-18 mutants (38, 39). In chromaffin cells, a striking 10-fold decrease in the number of morphologically docked granules is observed in Munc18a null (40). In insulin secretion and GLUT4 translocation, Munc18 isoforms were found to promote the plasma membrane association of insulin secretory granules in pancreatic islet β cells and GLUT4 vesicles in adipocytes (41, 42). These studies highlight a positive role for Munc18 proteins in vesicle docking during exocytosis in eukaryotic cells. However, whether and how Munc18 directly connects two membranes in the docking process remains largely unknown.

In this study, we exploited reconstituted, SNARE-bearing proteoliposomes to assess Munc18a's role in vesicle docking. We focused on three sets of SNAREs that include 1) syntaxin1/SNAP25/VAMP2, 2) syntaxin3/SNAP23/VAMP2, and 3) syntaxin4/SNAP23/VAMP2. The Munc18a/syntaxin1/SNAP-25/VAMP2 tetrameric complex mediates neuronal transmission and is at least partially responsible for the exocytosis of insulin granules (43). The trimeric syntaxin4/SNAP23/VAMP2 complex is best known for catalyzing Glut4 translocation along with Munc18c in muscle cells and adipocytes (44, 45). However, Munc18a has also been detected in these cells (41) (Gene expression database Bgee) and was recently shown to promote syntaxin4/SNAP23/VAMP2-mediated lipid mixing in reconstitution (46). Considering that syntaxin4 is required for both the initial and 2$^{nd}$ phase in insulin secretion (47), whereas its "cognate" SM protein Munc18c is required only in the 2$^{nd}$ phase (48), it is conceivable that a Munc18a/syntaxin4/SNAP-23/VAMP2 complex might play an under-appreciated role in the first phase of insulin release. The physiological importance of the Munc18a/syntaxin3/SNAP23/VAMP2 complex is less clear, but Munc18a was shown to promote the lipid-mixing reaction mediated by syntaxin3, SNAP23, and VAMP2 in reconstitution (46). Interestingly, all these four components are expressed in mast cells and pancreatic islet β cells (49, 50), although it remains to be determined whether this particular SM/SNARE combination is required in any form of exocytosis in these cells. By exploiting these three sets of SNAREs in reconstitution, we report that Munc18a plays a direct role in vesicle clustering in a stage prior to trans-SNARE zippering.

## Materials and Methods

### cDNA constructs

The *E.coli* expression constructs for N-terminally MBP-tagged rat VAMP2, VAMP8, VAMP2cd, VAMP8cd, syntaxin3 (Stx3), syntaxin4 (Stx4), SNAP23, Munc18a, and for TEV were described previously (46). The cDNAs for full-length or N-terminally truncated rat syntaxin3 and syntaxin4 were inserted individually into the LIC site of pET MBP $His_6$ LIC cloning vector (gift from Scott Gradia; Addgene plasmid # 37237) to generate pET-Stx3-TCS(Tev Cleavable Site)-MBP-$His_6$, pET-Stx3ΔN-TCS-MBP-$His_6$, pET-Stx4-TCS-MBP-$His_6$, and pET-Stx4ΔN-TCS-MBP-$His_6$. Syntaxin3ΔN lacks nucleotide sequences for amino acids 2 to 27 (51), whereas syntaxin4ΔN lacks nucleotide sequences for amino acids 2 to 36 (52). pET28a-Stx1 (rat), pET28a-Stx1ΔN (rat), pET15b-SNAP23 (rat), and pET15b-SNAP25 (mouse) were kind gifts from Jingshi Shen (28, 36). Plasmid pPROEX-HTb-Munc18a (rat) is a kind gift from Axel Brünger (53).

## Recombinant proteins and peptides

Recombinant proteins including MBP-TCS-VAMP2, MBP-TCS-VAMP8, MBP-TCS-SNAP23, $His_6$-SNAP23, Stx3-TCS-MBP-$His_6$, Stx3ΔN-TCS-MBP-$His_6$, Stx4-TCS-MBP-$His_6$, Stx4ΔN-TCS-MBP-$His_6$, Stx1-$His_6$/$His_6$-SNAP25, Stx1ΔN-$His_6$/$His_6$-SNAP25, Stx4/$His_6$-SNAP23 were purified according to previously reported procedures (28, 36, 46). $His_6$-Munc18a was isolated from Rosetta2(DE3)/pPROEX-HTb-rMunc18a as described below. In brief, Rosetta2(DE3)/pPROEX-HTb-rMunc18a cells in 1 L Terrific Broth were grown to $D_{600}$= 1.2 at 37°C. Following the addition of 0.5 mM IPTG, the cultures were incubated for 4 h before cell pellets were harvested by centrifugation (5k rpm, 5 min, room temperature, GS3 rotor). The cell pellet was resuspended in 20 mL of Buffer C (50 mM HEPES-KOH, pH 7.5, 500 mM KCl, 10% glycerol, 1 mM DTT) containing 10 mM imidazole, 1 mM PMSF, 1x PIC (0.62 μg/ml leupeptin, 4 μg/ml pepstatin A, and 24.4 μg/ml pefabloc-SC). Following French Press at 900 psi, 1% TritonX-100 was added to the lysate which was then nutated for 30 min at 4°C before ultracentrifugation with a Beckman Type 70 Ti rotor (4°C, 30 min, 18,500 rpm). The supernatants were applied to 4 ml of Ni-NTA resin (Qiagen) pre-equilibrated with Buffer C, and nutated for 2 h at 4°C. Resins were then washed with 20 ml of Buffer C containing 50 mM imidazole, before the proteins were eluted in 20 ml of Buffer C containing 200 mM imidazole. $His_6$-Munc18a was dialyzed 1,000,000-fold in RB150 (20 mM HEPES-NaOH, pH 7.4, 150 mM NaCl, 10% glycerol) overnight at 4°C, concentrated using 30k MWCO Microsep™ Advanced Centrifugal Device, snap-frozen in small aliquots in liquid $N_2$, and stored at – 70°C. Protein concentration was determined by the standard Bradford assay.

Peptides corresponding to the N-terminal 10 residues (N-peptides) of the wild-type syntaxin4 (MRDRTHELRQ) and mutant syntaxin4 (MRDRTHEARQ) at 95% purity were custom made (ABI Scientific).

## Proteoliposome preparation

Unilamellar liposomes were prepared by detergent dilution and isolated on a Histodenz density gradient flotation as previously described (54). Donor proteoliposomes contain 60% POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine), 17% POPE (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine), 10% DOPS (1,2-dioleoyl-sn–glycero-3-phosphoserine), 10% cholesterol, 1.5% NBD-DHPE [N-(7-Nitrobenz-2-Oxa-1,3-Diazol-4-yl)-1,2-Dihexadecanoyl-sn-Glycero-3-Phosphoethanolamine] and 1.5% rhodamine-DHPE (Lissamine™ Rhodamine B 1,2-Dihexadecanoyl-*sn*-Glycero-3-Phosphoethanolamine), whereas acceptor proteoliposomes contain 60% POPC, 19% POPE, 10% DOPS, 10% cholesterol and 1% Dansyl DHPE [N-(5-Dimethylaminonaphthalene-1-Sulfonyl)-1,2-Dihexadecanoyl-sn-Glycero-3-Phosphoethanolamine]. NBD-DHPE and rhodamine-DHPE form a FRET pair on donor RPLs (reconstituted proteoliposomes) such that the NBD signal is quenched until after the membranes of the donor and acceptor RPLs merge (55). SNARE proteins were kept at similar densities as other reconstitution studies (28), with protein: lipid rations at or below 1: 200 for R-SNARE-bearing donor RPLs and at or below 1:500 for Q-SNARE-bearing acceptor RPLs. $His_6$-TEV was added at 60 μg/mL to remove the MBP tag from the recombinant SNAREs during proteoliposome preparation (54). Donor RPLs bear VAMP2 (N-terminal MBP tag removed) or VAMP8 (N-terminal MBP tag removed).

Acceptor RPLs bear various combinations of recombinant Qa- and Qbc- SNAREs including Stx1-$His_6$/$His_6$-SNAP25 (Figs. 1, 2 and 5), Stx1ΔN-$His_6$/$His_6$-SNAP25 (Fig. 2), Stx3/SNAP23 (C- and N- terminal MBP tags removed from Stx3 ΔN and SNAP23 respectively; Figs. 1 and 2), Stx3ΔN/SNAP23 (C- and N- terminal MBP tags removed from Stx3 and SNAP23 respectively; Fig. 2), Stx4/SNAP23 (C- and N- terminal MBP tags removed from Stx4 and SNAP23 respectively; Figs. 1, 2 and 3), Stx4ΔN/SNAP23 (C- and N- terminal MBP tags removed from Stx4ΔN and SNAP23 respectively; Figs. 2 and 3), Stx3/$His_6$-SNAP23 (C-terminal MBP tag removed from Stx3; Fig. 5), or Stx4/$His_6$-SNAP23 (tagless Stx4 was co-purified with SNAP23; Figs. 5 and 6), Stx1-$His_6$ (Fig. 4), Stx3 (C- terminal MBP tag removed; Fig. 4), Stx4 (C- terminal MBP tag removed; Fig. 4). Note that recombinant SNAP25 and SNAP23 are not lipid-modified but instead anchored to liposomes through their binary interaction with syntaxin, which has a trans-membrane domain.

After the harvest of RPLs, lipid concentrations were determined from the fluorescence of NBD-DHPE (λ ex = 460 nm, λ em = 538 nm, emission cutoff = 515 nm) for donor RPLs and dansyl-DHPE (λ ex = 336 nm, λ em = 517 nm, emission cutoff = 495 nm) for acceptor RPLs, in the presence of 100 mM β-OG (54). The size of RPLs was measured by dynamic light scattering to be around 106 nm in diameter. The copies of R- and Q- SNAREs per RPL are 270 and 96 respectively, according to the published calculation method (7). Typically, 65 - 85% of the SNAREs face outward (sensitive to trypsin digestion (56)).

## Lipid-mixing assay

Unless otherwise specified, the standard fusion reaction (20 μl) contained R-SNARE donor RPLs (50 μM lipids) and Q-SNARE acceptor RPLs (400 μM lipids) in RB150 (including 1 mM DTT). Wherever MBP-Munc18a was used, the MBP tagged was removed by premixing MBP-Munc18a with $His_6$-TEV at a molar ratio of 2:1. We routinely used Munc18a at 3.4 μM (unless otherwise specified), a concentration at which lipid-mixing rate reaches saturation (46). Following either 3 h or overnight incubation on ice, reaction mixtures were transferred to a 396 well plate and the NBD fluorescent signal, initially quenched by Rhodamine on the same donor membrane, was measured (λex = 460 nm, λem = 538 nm, emission cutoff = 515 nm) in a SpectraMAX Gemini XPS plate reader (Molecular Devices) at 37°C. The maximal, early rate of dequenching (within the first 30 min) was calculated as the increased fluorescence at any time divided by the maximal fluorescence increase in 1% Triton X-100 $[(F_t - F_0)/(F_d - F_0) \times 100]$. An increase of 1 in this parameter is defined as one unit.

## Proteoliposome clustering assay

Following incubation on ice (described above), each standard reaction mixture was diluted 40 fold in ice-chilled RB150. Four micro-liters were placed on a microscope slide and covered with a 22 mm coverslip. Images were collected randomly using a Zeiss confocal fluorescence microscope. Cluster/particle sizes were measured in ImageJ (NIH) and the values were plotted on logarithmic scale against their cumulative distribution (also on logarithmic scale) using KaleidaGraph as described (57). In experiments where trans-SNARE zippering needs to be prevented, inhibitory VAMP2cd or VAMP8cd at 2 μM was premixed with Q-SNARE acceptor liposomes on ice for 1 h before donor liposomes (bearing

200 nM VAMP2) and Munc18a were added. Please note that DTT was not used in our initial experimentation (Fig. 1, S Figs. 1 and 2) because it had no impact on clustering (or lipid mixing). However, DTT at 1 mM was later included so that our subsequent reaction conditions become more comparable with other groups'.

### Trans-SNARE complex assays

Fusion reaction mixtures (20 μl) containing donor RPLs (bearing VAMP2) and acceptor RPLs (bearing $His_6$-Q-SNAREs) were incubated with or without Munc18a on ice for 3 h (Fig. 5) or overnight (Fig. 6). Three hundred eighty μL of RB150 containing 1% Triton X-100 and 20 mM imidazole was then added. Following 15 min nutation at 4°C, 350 μL of the mixture was added to the 12.5 μL of Ni-NTA agarose resin (Qiagen) pre-equilibrated with the same buffer. Following 1 h incubation at 4°C, the mixture was centrifuged at 3,000 × g for 2 min. The supernatant was discarded and the Ni-NTA resin was washed by incubation with 400 μl of RB150 containing 1% Triton X-100 and 20 mM imidazole. After centrifugation (3000 × g for 2 min at 4°C), supernatant was removed. Three additional repeats of the wash were performed, and 2x Laemmli sample buffer was then added to the nickel resin to elute $His_6$-tagged Q-SNAREs. The presence of full-length VAMP2 in the eluate was probed by Western blotting using a monoclonal anti-VAMP1/2 antibody (clone SP10; Santa Cruz), which served as an indicator for trans-SNARE formation between donor and acceptor proteoliposomes. SNAP23 and SNAP25 were detected using polyclonal anti-SNAP23 (W-17; Santa Cruz) and anti-SNAP25 (C18; Santa Cruz) antibodies respectively.

For immuno-precipitation (IP) experiments described in Fig. 6D, overnight fusion reaction (20 μL) on ice was terminated by the addition of 1 μL of anti-syntaxin4 rabbit serum (Antibodies–online; cat#: ABIN1742221), and the incubation was continued on ice for 1 h. Three hundred and eighty μL of RIPA buffer (25 mM HEPES-NaOH, pH 7.4, 150 mM NaCl, 1% NP40 alternative, 1% deoxycholate, 0.1% SDS, and 10 mM EDTA) were then added and the mixture were nutated at 4°C for 20 min before centrifugation at 16,000 × g for 5 min at 4°C. Three hundred and fifty μL of the supernatant were added to a microfuge tube containing 15 μL of protein A agarose (UBPBio) pre-equilibrated with the RIPA buffer. Following 1 h incubation at 4°C, the mixture was centrifuged at 3,000 × g for 2 min. The supernatant was discarded and the agarose resin was washed by incubation with 400 μL of RIPA buffer followed by centrifugation (3000 × g for 2 min at 4°C). After removing the supernatant, 3 additional repeats of the wash were performed, and 22.5 μL of 2x Laemmli sample buffer was then added to the agarose resin. The mixtures were boiled for 4 min and 18 μL of the eluate were used for SDS-PAGE and subsequent western blotting analysis. Goat anti-Stx4 (H-16; Santa Cruz) and mouse anti-VAMP1/2 were used as primary antibodies. SuperSignal® west femto maximum sensitivity substrate (ThermoFisher) was used to develop the western signal.

### Binding assay and densitometry

Each 30 μL reaction mixture including 2.4 μM $His_6$-Munc18a and 1.2 μM liposome-rooted syntaxin in RB150 was nutated overnight at 4°C before the addition of 150 μL of RB150, 20 μL of 200 mM imidazole, and 10 μL of Ni-NTA agarose resin (Qiagen) that had been pre-equilibrated in the binding buffer (20 mM HEPES-NaOH, pH 7.4, 150 mM NaCl, 10%

glycerol, and 20 mM imidazole). Following a 1 h incubation at 4°C, the samples were centrifuged at 1000 × g for 1 min at 4°C to pellet the resin (including associating molecules). After the supernatant was removed, the resin was washed by incubation with 200 μL of binding buffer followed by centrifugation. After two additional repeats of the wash, the resin was resuspended in 20 μL of 2x Laemmli sample buffer. Twenty μL of the eluates were subject to SDS-PAGE and Coomassie Brilliant Blue staining. For densitometry, gels were scanned and saved as grayscale 8 bits TIF files and the intensity of the protein bands was quantified in UNSCANIT Gel 5.3 (Silk Scientific) according to manufacturer's instruction.

## Results

### Munc18a-dependent clustering is R-SNARE specific

To assess if Munc18a promotes the clustering of SNARE-bearing donor and acceptor proteoliposomes, we performed the clustering reaction on ice [to prevent membrane fusion (S Fig. 1)] and before mounting for confocal microscopy, we stop further proteoliposome clustering by diluting the reaction mixture 40 fold (58–60). The size of each cluster was then captured by the CCD camera on the microscope (S Fig. 2) and measured with the ImageJ software. The cumulative size distributions were calculated to compare the clustering effects of Munc18a in various SNARE combinations (Fig. 1). We examined VAMP2-bearing and VAMP8-bearing donor liposomes in parallel because VAMP8 is largely involved in endo/lysosomal fusion and, unlike VAMP2, does not respond to Munc18a in reconstituted fusion reactions (46). When these donor proteoliposomes were incubated with SNARE-free acceptor liposomes, Munc18a had no impact on the cluster size distribution (Fig. 1A, B) at the concentration (3.4 μM) we used [we chose this concentration because higher levels of Munc18a (e.g., 6.8 μM) lead to artificial aggregation (Xu, unpublished)]. Likewise, Munc18a had no impact on the cluster size distribution when SNARE-free donor liposomes were used (S Fig. 3). In contrast, when VAMP2-bearing liposomes were incubated with acceptor liposomes bearing any of the three Q-SNARE complexes (syntaxin1/SNAP25, syntaxin3/SNAP23, or syntaxin4/SNAP23), larger clusters were detected as long as Munc18a had been added (Fig. 1C, E, G). The Munc18a effect on cluster size is specific to VAMP2 because replacing VAMP2 with VAMP8 in the donor liposome abolished the Munc18-dependent cluster size increase (Fig. 1D, F, H). This VAMP2-specific clustering by Munc18a mirrors the reported lipid-mixing results (28, 46), and suggests that unique motif(s) on VAMP2 might be responsible for direct interactions with Munc18a. Since all three Q-SNARE complexes (with different combinations of Qa- and Qbc- SNAREs) in our assay supported Munc18a-dependent clustering, we decided to investigate the underlying biochemical determinants shared by these distinct Q-SNARE complexes.

### Munc18a-dependent clustering requires N-peptide motif in syntaxin

While syntaxins vary in their domain structure, syntaxin1, 3, 4 all contain an N-peptide motif at the extreme N-terminus, which is followed by a 3-helical Habc domain, a SNARE motif, and a C-terminal trans-membrane domain (61). The binary interaction between the syntaxin1 N-peptide motif and the N-pocket in the D1 domain of Munc18a has been documented (62, 63) and shown to be critical to the fusion process (64). Direct binding

between recombinant syntaxin3 and Munc18a has also been reported (65) although the involvement of syntaxin3 N-peptide motif in the interaction is not clear. Meanwhile, Munc18a was found to bind the synthetic N-peptide of syntaxin4 with a similar affinity for the syntaxin1 N-peptide (66). To investigate the potential role of the N-peptide motif in Munc18a-dependent vesicle clustering, we prepared acceptor proteoliposomes with wild-type syntaxins or syntaxins lacking the N- peptide motif along with Qbc-SNAREs (SNAP23 or SNAP25). Clustering assays indicate that the N-peptide motif of syntaxin1, 3, or 4 is critical for Munc18a-dependent clustering (Fig. 2A). To provide quantitative evaluation of the data, the percentile of clusters larger than 5 pixels or 10 pixels from multiple independent experiments were tabulated (S Fig. 4). It is evident that Munc18a is only able to promote the formation of larger clusters when the N-peptide motif is still intact. In parallel, lipid-mixing assays show that the N-peptide motif is also indispensable for Munc18a-dependent acceleration of the fusion reaction (Fig. 2B). Importantly, removing the N-peptide motif does not affect Munc18a-independent lipid mixing (Fig. 2B), suggesting that the N-terminal deletion mutation does not cause overall conformation change.

Although Munc18a is known to interact with syntaxin1 and syntaxin3 (65, 67), a binary interaction between syntaxin4 and Munc18a had thus far escaped detection. In fact, immunoprecipitation experiments and direct binding assays all appear to have excluded Munc18a as a likely partner for syntaxin4 (41, 65), despite the fact that Munc18a shows (slightly) stronger affinity for the N-peptide of syntaxin4 than that of syntaxin1 (66). Since detergent lysates or soluble domains of syntaxin4 were used in the majority of the binding studies, which might introduce unexpected artifacts (25), we decided to use full-length syntaxin4 anchored in lipid bilayers as an alternative platform in our binding assay. We incubated $His_6$-tagged Munc18a with liposomes bearing either syntaxin3 or syntaxin4 overnight at 4°C. We then added Ni-NTA agarose to the mixtures. After further incubation at 4°C for 1h, we used a low-speed spin to bring down $His_6$-Munc18a and associating molecules. SDS-PAGE followed by Coomassie blue staining shows that syntaxin4 is pulled-down by $His_6$-Munc18a at a level comparable to syntaxin3 (Fig. 2C, compare lanes 3 and 5 in the top panel), a previously established binding partner for Munc18a (41, 65). Importantly, incubation of Ni-NTA agarose with proteoliposomes in the absence of $His_6$-Munc18a did not bring down any syntaxin (lane 1), suggesting that centrifugal force did not cause the liposomes to pellet and that there is no unspecific interaction between syntaxin and the Ni-NTA resins under our experimental conditions. The truncated syntaxin3 (syntaxin3ΔN) associates with Munc18a to a lesser extent (lane 4), at around 27% of wild-type level (Fig. 2D), whereas even lesser syntaxin4ΔN was found to co-precipitate with Munc18a (Fig. 2C and D). Taken together, the N-peptide motifs in syntaxin3 and syntaxin4 are important for their binary interaction with Munc18a.

At least two models could explain the requirement of the N-peptide motif in Munc18a-mediated clustering: i) the N-peptide motif of syntaxin directly connects Munc18a to Q-SNARE bearing membranes; ii) the N-peptide motif allosterically activates Munc18a and can act as an isolated unit. To distinguish the two, we exploited synthetic N-peptide of syntaxin4 that was tested initially in reactions containing VAMP2-bearing donor liposomes and syntaxin4ΔN/SNAP23-bearing acceptor liposomes. At the range of concentrations (from 11 μM to 300 μM) tested, the synthetic N-peptide (pre-incubated with Munc18a) did not

demonstrate any stimulatory impact on lipid mixing or vesicle clustering (Fig. 3A and S Fig. 5, lane 7), suggesting the N-peptide could not function as an isolated unit in the fusion reaction. This is not due to the low affinity between the N-peptide and Munc18a, because when added to reactions containing VAMP2-bearing donor liposomes and syntaxin4/SNAP23-bearing acceptor liposomes, the wild-type N-peptide (Stx4-$N^{wt}$) significantly inhibited Munc18a-mediated lipid mixing and vesicle clustering (Fig. 3B and S Fig. 5, lane 3). Importantly, the mutant N-peptide (Stx4-$N^{L8A}$), which is structurally incompatible with the N-pocket on Munc18a (63), did not exhibit any inhibitory effect on reactions containing liposomes that bear wild-type SNAREs (Fig. 3B, lane 6; S Fig. 5, lane 4), suggesting that the added Stx4-$N^{wt}$ can specifically and successfully compete against the membrane-bound full-length syntaxin4 for Munc18a at the concentrations we used. We therefore favor the notion that the N-peptide motif of sytaxin4 bridges Q-SNARE-bearing membranes and Munc18a to facilitate membrane clustering. We cannot conclude the same for syntaxin3 however, because under the same conditions tested above, the synthetic N-peptide of syntaxin3 did not show any specific impact on lipid mixing or proteoliposome clustering (data not shown).

## Differential requirement for SNAP-25-like proteins in membrane clustering

Since syntaxin pairs with SNAP25-like proteins on the same membrane during membrane fusion, we went on to determine if SNAP-25-like proteins contribute in anyway to the clustering reaction. We prepared accepter liposome with syntaxins alone, and incubated them with VAMP2-bearing donor liposomes, with or without Munc18a. While Munc18a-dependent clustering was still observed when syntaxin1-bearing acceptor liposomes were used, it was largely abolished in reactions containing syntaxin3-bearing or syntaxin4-bearing acceptor liposomes (Fig. 4 and S Fig. 6). Whether SNAP23 contributes to clustering via physical association with Munc18a or via modulating syntaxin conformation (i.e., by keeping syntaxin in the open state) warrants future investigation. Unfortunately, recombinant syntaxin1ΔN by itself was difficult to purify from bacterial lysates (Xu, unpublished), which prevented us from assessing the role of N-peptide motif in SNAP25-independent clustering.

## Munc18a-dependent clustering is independent of trans-SNARE zippering

Because proteoliposome clustering could lead to trans-SNARE assembly in reconstituted fusion reactions, it remains a possibility that Munc18a binds and stabilizes a partially-assembled trans-SNARE complex on ice, and in doing so, drive a reversible clustering reaction in the direction of stabilized (larger) clusters. To establish that Munc18a indeed has a direct role in clustering, it would be crucial to uncouple clustering from trans-SNARE complex formation. We therefore asked if keeping the reaction on ice [which permits clustering (Fig. 1)] would be sufficient to prevent trans-SNARE zippering, following a straightforward strategy established by Shen and colleagues in the study of Munc18c (36). To this end, we incubated SNARE-bearing acceptor and donor liposomes either with or without Munc18a for 3h on ice, after which 1% Triton X-100 was used to dissolve the membranes. $His_6$-tagged Q-SNAREs were then pulled down from the detergent lysates by Ni-NTA agarose resin (Fig. 5A). The eluates were analyzed via western blotting for R-SNARE VAMP2, as an indicator for trans-SNARE zippering (36). As it turned out, VAMP2 was found in association with syntaxin4/SNAP23 when Munc18a was added in the beginning of the 3h incubation (Fig. 5B), suggesting that even on ice, Munc18a was able to

promote limited trans-SNARE pairing. This is not due to new SNARE complex formation in detergent lysates because introducing Munc18a immediately after Triton X-100 addition did not result in detectable VAMP2 in immunoblotting (lane 1). Nor is it due to post-fusion cis-SNARE complex formation because incubation of proteoliposomes on ice prevents lipid mixing (S Fig. 1). In contrast, little or no VAMP2 was found in association with syntaxin3/SNAP23 and syntaxin1/SNAP25, regardless of the presence of Munc18a. Therefore, it seems that different sets of SNAREs vary in their capability to form trans-SNARE complexes in the presence of Munc18a, and that reducing the reaction temperature to 0°C effectively blocked the formation of the neuronal trans-SNARE complex.

To eliminate the complication from trans-SNARE complex formation between non-neuronal SNAREs (e.g., syntaxin4/SNAP23/VAMP2) in our clustering assay, we pre-incubated syntaxin4/SNAP23-bearing acceptor liposomes with the cytoplasmic/soluble domain of VAMP2 (VAMP2cd) or VAMP8 (VAMP8cd), both syntaxin4/SNAP23 cognates (46). These soluble domains would occupy the binding sites on syntaxin4/SNAP23, preventing the Q-SNARE subcomplex from further interaction with the membrane anchored, full-length VAMP2 (at a tenth of the soluble VAMPs in quantity). Donor liposomes bearing VAMP2 were then added together with Munc18a (Fig. 6A). As expected, the pre-incubation of soluble VAMPs abolished Munc18a-dependent trans-SNARE complex assembly, judging by the pull-down assay (Fig. 6C) that was performed in mild, nonionic detergent (1% Triton X-100), or the co-IP assay (Fig. 6D) that was performed in harsher, ionic detergents (RIPA buffer). Although the pre-incubation of soluble VAMPs abolished lipid mixing (Fig. 6B), it did not inhibit proteoliposome clustering (Fig. 6E, S Fig. 7). Importantly, the inhibitory VAMPs themselves (without Munc18a) did not promote membrane clustering (S Fig. 7, compare lanes 5 and 6 to 1). Therefore, the Munc18a-dependent clustering we observed between VAMP2-bearing donor liposomes and syntaxin4/SNAP23-bearing acceptor liposomes takes place independent of trans-SNARE zippering. Similarly, the inhibitory VAMPs permitted Munc18a-dependent clustering of liposomes bearing the other two sets of SNAREs while blocking Munc18a-mediated lipid mixing (data not shown).

## Discussion

Reconstitution studies pioneered by Rothman and colleagues have been instrumental in unraveling the mechanisms of Munc18 proteins in membrane fusion. Using SNARE-bearing SUVs, Shen et al showed a decade ago that Munc18a acts on the pre-assembled neuronal trans-SNARE complex (28), establishing Munc18a as an integral component of the SNARE machinery for exocytosis. Evidence soon emerged that Munc18a could also scaffold trans-SNARE assembly (68) and promote SNARE-dependent adhesion of LUVs to GUVs (69). These studies provided important, early clues that Munc18a may coordinate multiple steps in the fusion cascade. However, to delineate the precise role of Munc18a in each of these steps requires methods to uncouple fusion subreactions that are otherwise interconnected. In this report, we dissected Munc18a function in a reconstituted assay that resolves membrane clustering (docking) and trans-SNARE zippering (priming). We now present three lines of evidence indicating that Munc18a mediates membrane clustering independently of trans-SNARE zippering. First, ice incubation permits neuronal-SNARE-dependent clustering while preventing neuronal trans-SNARE complex formation (Figs. 1 and 5). Second,

syntaxin1-bearing liposomes and VAMP2-bearing liposomes can be clustered by Munc18a in the absence of SNAP25 (Fig. 4), a Qbc-SNARE essential to neuronal exocytosis and the formation of the neuronal SNARE complex (70, 71). Third, soluble VAMPs abolish non-neuronal trans-SNARE zippering and lipid mixing, without interfering with membrane clustering (Fig. 6). We propose that Munc18a-mediated clustering underlies vesicle docking that is observed in both neuronal and non-neuronal exocytosis (38–42, 72). However, the clustering effect may be unique to Munc18a because we have evidence that Munc18c could stimulate some of the same lipid-mixing reactions without promoting vesicle clustering (Voth and Xu, unpublished).

Under our experimental conditions, the clustering activity of Munc18a requires the N-peptide motif in both neuronal and non-neuronal syntaxins (Fig. 2). Since the measured *K*d of Munc18a and N-peptide interaction is at or above 31 uM (66), a lot higher than the concentration of syntaxin in reconstitution or *in vivo*, it is likely that Munc18a exploits additional binding sites in syntaxin or Qbc-SNAREs to mediate clustering. In agreement to this, Munc18a has been found to simultaneously bind the N-peptide motif and the rest of the cytoplasmic domain of syntaxin1 in either closed or open conformation (73, 74). Additionally, there is evidence for direct interaction between Munc18a and SNAP25 (75). Concerning R-SNARE on the other hand, Munc18a-dependent clustering was abolished when VAMP2 was replaced with VAMP8 (Fig. 1), suggesting that unique motifs in R-SNARE VAMP2 (28) might be required in Munc18a-mediated vesicle clustering/docking.

The absence of syntaxin N-peptide motif negatively affects both the proteoliposome clustering and lipid mixing (Fig. 2A and B), indicating that either the N-peptide/Munc18a interaction is required in both events, or exclusively in the early event (i.e., clustering) that is mechanically coupled to the late event (i.e., lipid mixing). The latter scenario is consistent with a previous study of syntaxin1-mediated neurotransmission, which shows N-peptide function is limited to a stage prior to trans-SNARE complex formation (64). Based on our findings, we propose that in addition to recruiting Munc18a to the site of fusion (64), the N-peptide/Munc18a interaction is required in vesicle docking. How Munc18a transitions from clustering/docking SNARE-bearing membranes to subsequent steps in the fusion cascade will require further investigation.

All the syntaxins used in this study can adopt a closed conformation (42, 51, 76–78), when the inhibitory Habc domain folds back to bind the SNARE domain, preventing these syntaxins from interaction with their cognate Qbc- and R- SNAREs. Whether Munc18a-mediated clustering requires the opening of the syntaxin inhibitory domain is not clear. However, considering that in Figure 6, the tagless syntaxin4 and $His_6$-SNAP23 were co-purified (via the $His_6$ tag on SNAP23) and co-inserted into the lipid bilayers (via the trans-membrane domain of syntaxin4) in a 1:1 ratio, it is rational to assume that all the Stx4/$His_6$-SNAP23-bearing liposomes start with their Habc domain in the open position, and that this open conformation is compatible with Munc18a in clustering (Fig. 6E). Another interesting observation is that the membrane-anchored full-length syntaxin1 by itself exists in both open and closed states (79). It is tempting to speculate that a greater proportion of syntaxin1 might exist in the open state than that of sytnaxin3 and syntaxin4, enabling syntaxin1 to facilitate Munc18a-dependent clustering without the SNAP25 (Fig. 4).

What would be the functional relationship between Munc18a-mediated clustering and exocyst-mediated membrane tethering during exocytosis? Like other multisubunit tethering factors, exocyst is thought to promote the initial contact between a vesicle and the target membrane, via interactions with lipids and Rab/Rho family GTPases (16, 61, 80). However, exocyst-dependent membrane tethering has not been directly demonstrated in reconstitution (17). Based on the observations that i) the exocyst interacts functionally and physically with Sec1p (Munc18 ortholog in yeast) (81, 82) and ii) the number of docked vesicles are significantly reduced in Munc18a-deficient cells (39–41), we propose that exocyst-mediated tethering needs to be stabilized by Sec1p/Munc18a, which initiates the physical connection/association between R- and Q-SNAREs. Although SM proteins are known to catalyze the assembly of trans-SNARE complex (34, 83), our study indicates that Munc18a-mediated clustering occurs before and independently of trans-SNARE zippering. Since these two activities of Munc18a may involve distinct but overlapping binding sites in SNAREs, identification of point mutations that specifically abolish either activity will be of great interest in future studies.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Appendix. Supplementary data

Supplementary data to this article is going to be online.

## Abbreviations

| | |
|---|---|
| **DTT** | dithiothreitol |
| **FRET** | fluorescence resonance energy transfer |
| **GUV** | giant unilamellar liposome vesicle |
| **LUV** | large unilamellar liposome vesicle |
| **MBP** | maltose binding protein |
| **Munc18** | mammalian homolog of uncoornidated-18 |
| **RPL** | reconstituted proteoliposome |
| **SM** | Sec1p-Munc18 |

| | |
|---|---|
| **SNARE** | soluble N-ethylmaleimide-sensitive factor attachment protein receptors |
| **SUV** | small unilamellar liposome vesicles |
| **VAMP** | vesicle associated membrane protein |

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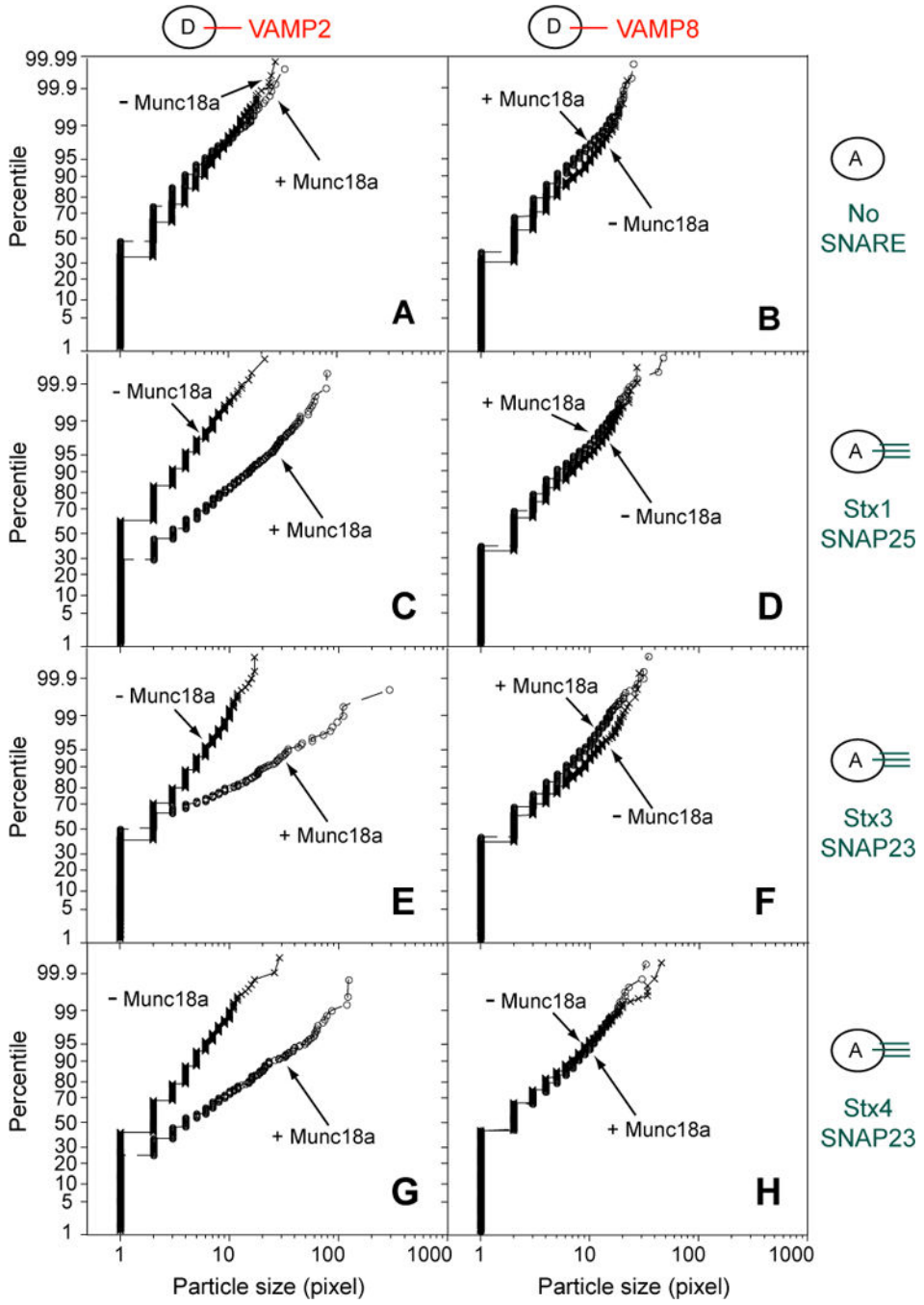


**Figure 1. Munc18a selectively promotes the clustering of SNARE-bearing liposomes**

Donor and acceptor proteoliposomes as specified were mixed in a 1:8 molar ratio, with either Munc18a or Munc18a buffer. Following overnight incubation on ice, diluted samples were subject to confocal fluorescence microscopy. Cluster/particle sizes in four randomly collected images were measured and their cumulative distribution is presented on the vertical axis. The Wilcoxon test indicates that the particle size distributions for the "– Munc18a" condition and the "+ Munc18" condition in C), E), and G) are significantly different ($P < 0.001$). This experiment is a representative of 3 repeats.

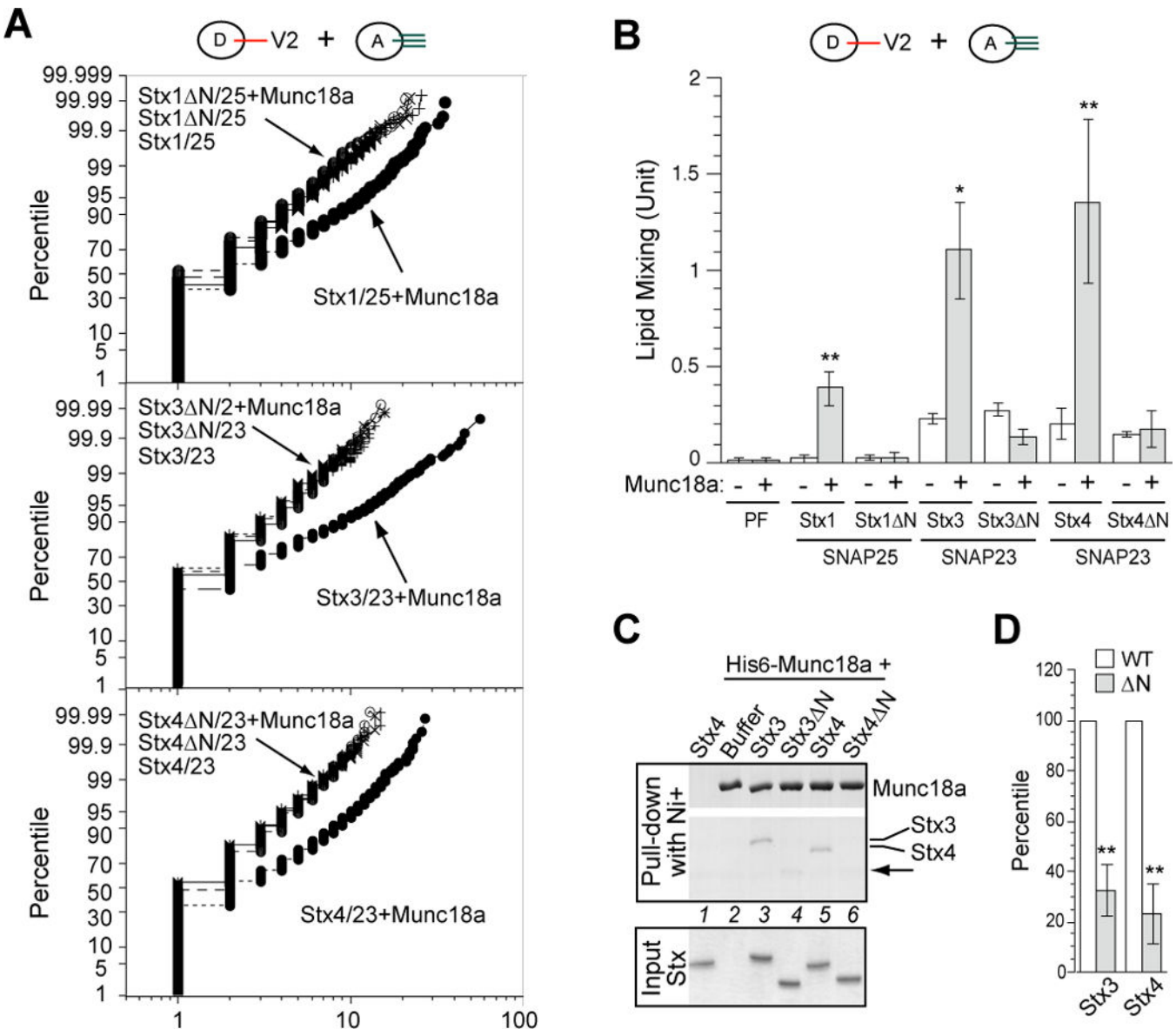


**Figure 2. N-peptide motifs are required in Munc18a-dependent clustering and lipid-mixing acceleration**

VAMP2-bearing donor liposomes were incubated 3 h on ice with acceptor proteoliposomes as specified, with or without Munc18a. Each sample was then aliquoted for clustering and lipid-mixing assays. **A)** Cumulative distribution plots show the results of the clustering assay. **B)** Following the lipid-mixing assay, the mean values of the maximal lipid-mixing rates are presented and error bars indicate SD (n=4). Student's t-test was used to assess the significance of the difference between the "– Munc18a" results and the "+ Munc18" results. * indicates $P < 0.05$ whereas ** indicates $P < 0.01$. Note that the extent of Munc18a-dependent acceleration of lipid mixing does not necessarily correlate linearly with the extent of change in clustering, which implies either the insufficient resolution of the clustering assay or possibly different modes of action exploited by Munc18a in clustering and lipid mixing respectively**. C)** To determine the biochemical interaction between Munc18a and Q-

SNAREs, $His_6$-Munc18a was incubated with specified proteoliposomes at 4°C overnight before Ni-NTA agarose was added to bring down $His_6$-Munc18a and associating molecules. Eluates were subject to SDS-PAGE followed by Coomassie Brilliant Blue staining. The top and bottom panels are from two separate gels. **D)** Densitometry was performed and the intensity of the syntaxin bands in the pull-down was normalized for the input. The values of the wild-type syntaxin3 and syntaxin4 were set as 100% respectively and then used to calculate the relative levels of mutant syntaxins in the pull-down. Student's t-test was used to assess the significance of the difference between the "WT" results and the "ΔN" results. ** indicates $P < 0.01$. Error bars represent SD (n=3).

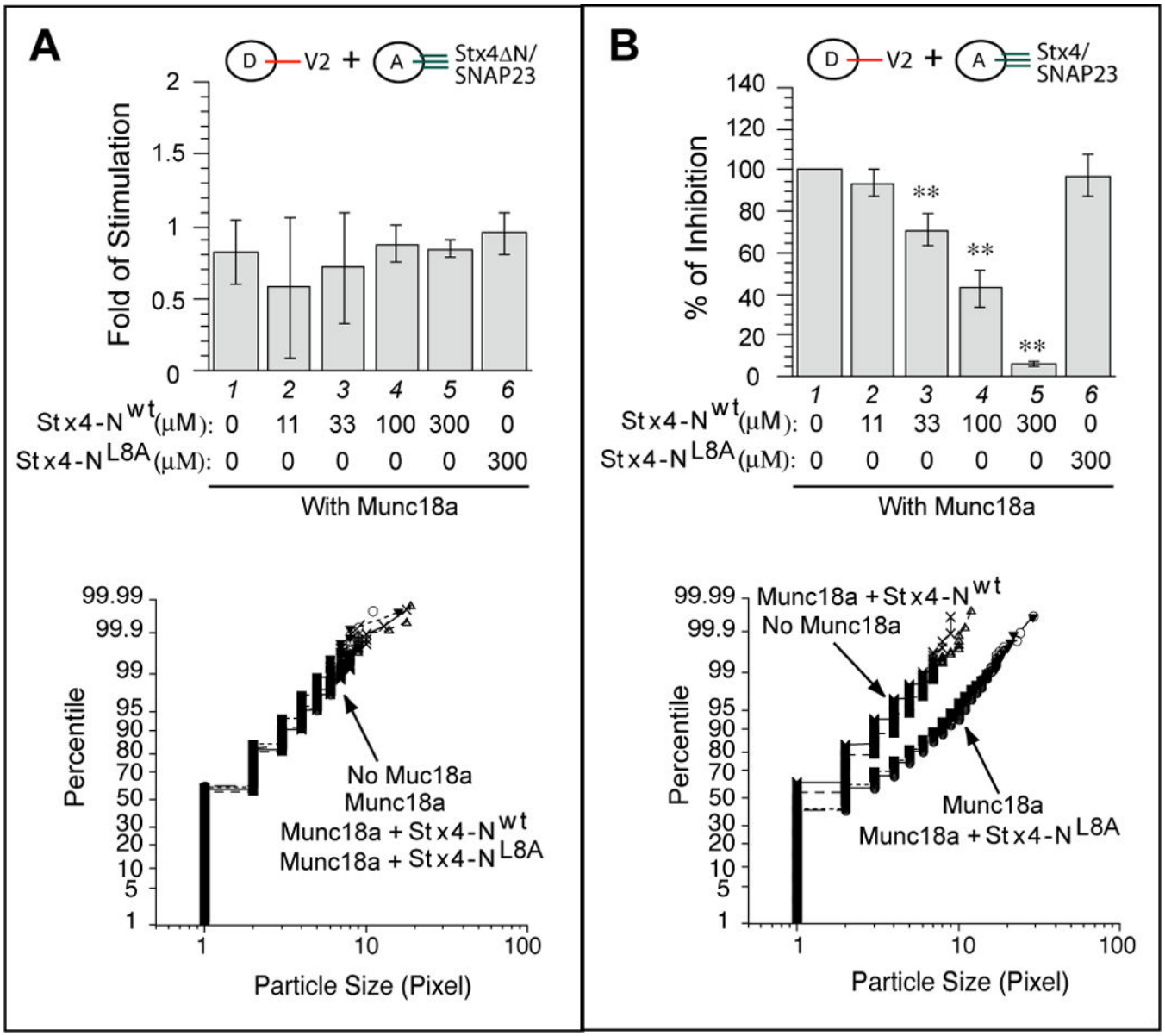


**Figure 3. The impact of synthetic N-peptides on Munc18a's function**

Donor and acceptor proteoliposomes as indicated were incubated 3 h on ice alone or with Munc18a (2μM) that had been preincubated overnight with specified amounts of Stx4-$N^{wt}$, Stx4-$N^{L8A}$, or control buffer. The reaction mixtures were then divided for lipid-mixing assay and clustering assay respectively. **A)** To assess the potential of N-peptide to stimulate Munc18a function in reactions containing VAMP2-bearing donor liposomes and syntaxin4ΔN/SNAP23-bearing acceptor liposomes, the ratio of the maximal lipid-mixing rate of each Munc18a-containing reaction over that of the SNARE-only reaction was calculated and shown as fold of stimulation. **B)** To assess the potential of N-peptide to inhibit Munc18a activity in reactions containing VAMP2-bearing donor liposomes and syntaxin4/SNAP23-bearing acceptor liposomes, the maximal lipid-mixing rate of the peptide-free reaction (but with Munc18a) was set at 100% and then used to calculate the

relative values of the other reactions. Student's t-test was used in statistical analysis. ** indicates $P < 0.01$. Error bars indicate SD (n=4). Cumulative distribution plots in A) and B) highlight the impact of the wild-type and mutant N-peptides on membrane clustering.

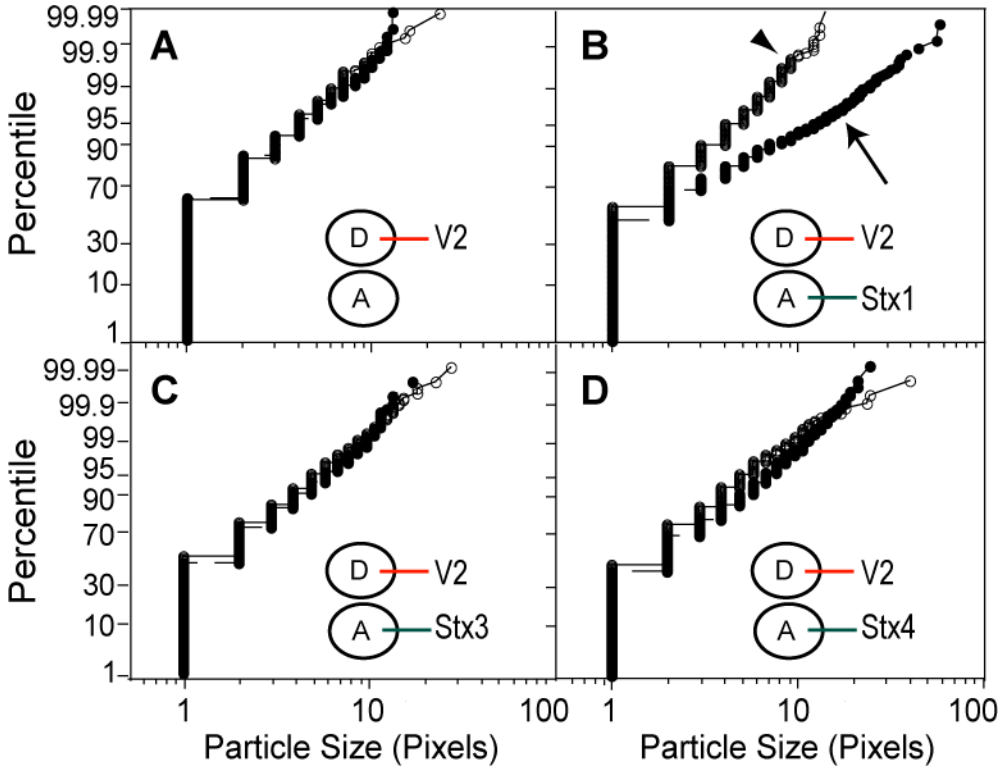


**Figure 4. Requirement for Qbc-SNAREs in Munc18a-dependent clustering**

VAMP2-bearing donor liposomes and various acceptor liposomes as specified were incubated on ice for 3 h, with or without Munc18a. The samples were examined with confocal microscopy and cluster/particle sizes were determined by ImageJ. The cumulative distribution plot of a typical experiment (out of 7) is presented.

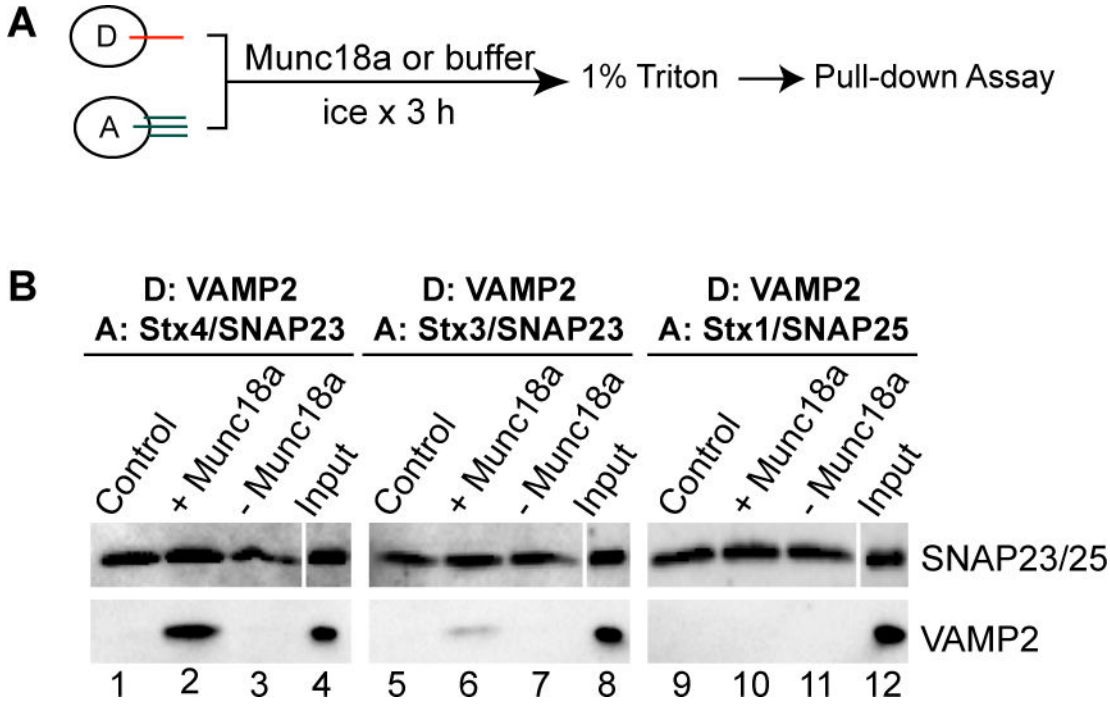


**Figure 5. Munc18a-dependent trans-SNARE assembly on ice**

**A)** Illustration of the experimental procedure. **B)** In Triton X-100 lysates, $His_6$-tagged Q-SNAREs were precipitated by Ni-NTA resin and subjected to SDS-PAGE and immunoblotting, using antibodies specific to SNAP23 (lanes 1 to 8; top panel) or SNAP25 (lanes 9–12, top panel; 50% input is shown to gauge the relative efficiency of each pull-down experiment. The empty space between lanes 3 and 4, 7 and 8, 11 and 12 specifies two regions of the same blot from one gel. VAMP2 co-precipitated with $His_6$-tagged Q-SNAREs was probed by monoclonal antibody (lanes 1 to 12; bottom panel; 5% of the Input is shown). To control for Munc18a-dependent SNARE complex formation in detergent lysates, acceptor and donor liposomes received Munc18a immediately after detergent addition in lanes 1, 5, and 9. This experiment is a representative of 3 independent repeats.

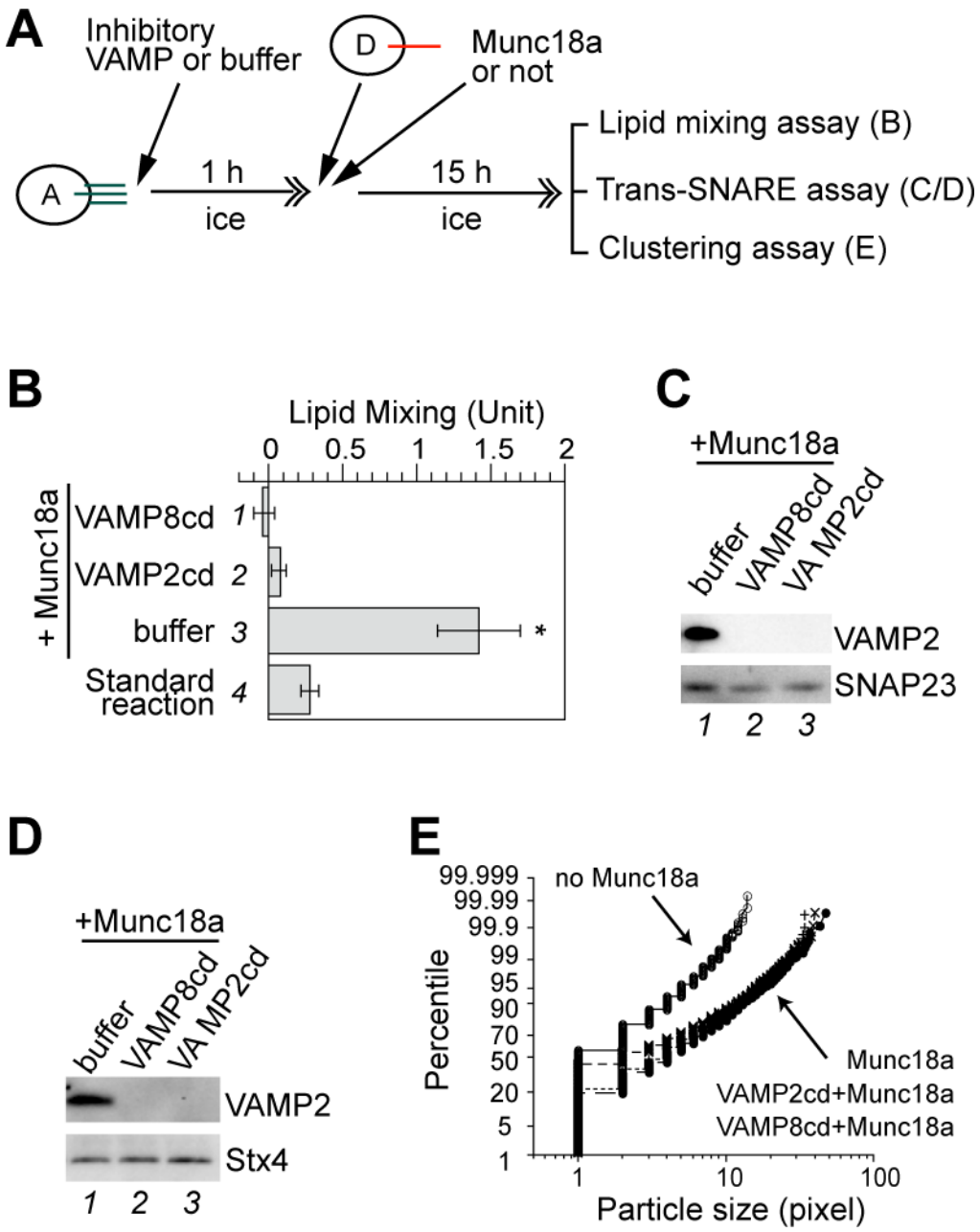


**Figure 6. Munc18a promotes proteoliposome clustering in a step prior to trans-SNARE zippering**

**A)** Incubation procedure. Syntaxin4/$His_6$-SNAP23-bearing acceptor liposomes were incubated with either the soluble, inhibitory VAMPs (10-fold molar excess in comparison to the membrane-bound VAMP2 added next) or buffer for 1 h on ice before receiving the VAMP2-bearing donor liposomes and Munc18a. Following another 15 h incubation on ice, samples were aliquoted and subject to **B)** lipid-mixing assay, **C)** pull-down assays using Ni-NTA resin, **D)** co-immunoprecipitation using immobilized anti-Stx4 antibody, and **E)** clustering assay. Student's t-test was used to assess the statistical difference between the results in lane 3 and the rest in B). * indicates $P < 0.05$. Error bars indicate SD (n=3).