Supporting Information

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SI Materials and Methods

Protein Expression and Purification. Recombinant t- and v-SNARE proteins were expressed in *Escherichia coli* and purified by nickel affinity chromatography. Nonsynaptic exocytic target membrane-associated SNARE (t-SNARE) complexes were composed of an untagged syntaxin subunit and His_6-SNAP-23. The vesicle-rooted SNARE (v-SNARE) proteins were expressed in a similar way as VAMP2 (1) and had no extra residues left after the tags were proteolytically removed. Lysosomal, synaptic, and yeast exocytic SNAREs were purified as previously described (1–3). SNARE mutants were generated by site-directed mutagenesis and purified similar to WT proteins. SNAREs were stored in a buffer containing 25 mM Hepes (pH 7.4), 400 mM KCl, 1% n-octyl-β-D-glucoside, 10% (vol/vol) glycerol, and 0.5 mM Tris(2-carboxyethyl)phosphine (TCEP). The cytoplasmic domain (CD) mutants of SNAREs were purified using the same procedure as for WT proteins, except that detergents were omitted.

Recombinant untagged Munc18c protein was produced in *Sf9* insect cells using baculovirus infection. Full-length mouse Munc18c gene was subcloned into the baculovirus transfer vector pFastBac to generate a construct encoding a His_6-Munc18c fusion protein separated by a tobacco etch virus (TEV) protease cleavage site. The fusion protein was expressed in *Sf9* cells according to the manufacturer's instruction (Bac-to-Bac Baculovirus Expression System; Life Technologies). Briefly, recombinant Bacmid DNA was obtained by transforming the pFastBac-Munc18c construct into DH10Bac cells. The Bacmid DNA was transfected into *Sf9* cells grown at 27 °C in Sf900-III serum-free medium (Life Technologies). Recombinant viruses were harvested 4 d after transfection and further amplified twice to obtain viral stocks with the desired titer. At the density of 2×10^6 cells/mL, *Sf9* cells were infected with the recombinant virus at a multiplicity of infection of 1. Cells were harvested 48 h after infection, and cell pellets were stored at −80 °C. Munc18c was purified from the *Sf9* cells as previously described (4). The cells were lysed in a lysis buffer [25 mM Hepes (pH 7.4), 400 mM KCl, 10% glycerol, 20 mM imidazole, 1% Triton, 1 mM DTT, 2 mM β-mercaptoethanol, and EDTA-free protease inhibitor mixture]. The cell extract was centrifuged at 18,500 rpm (Beckman 45 Ti rotor) for 30 min at 4 °C. Munc18c protein in the cell extract was purified by nickel affinity chromatography. The His_6 tag was removed from Munc18c by TEV protease, and the protein was subsequently dialyzed overnight against a storage buffer [25 mM Hepes (pH 7.4), 150 mM KCl, 10% glycerol, and 0.5 mM TCEP]. Recombinant untagged Munc18-1 was expressed and purified from *E. coli* as we previously described (2, 5).

Liposome Coflotation Assay. Binding of soluble factors to liposomes was carried out using a coflotation assay essentially as previously described (2). Soluble factors were incubated with liposomes at 4 °C with gentle agitation. Subsequently, an equal volume of 80% Nycodenz (wt/vol) in reconstitution buffer was added and transferred to 5 mm by 41-mm centrifuge tubes. The liposomes were overlaid with 200 μL each of 35% and 30% Nycodenz, and then with 20 μL of reconstitution buffer on the top. The gradients were centrifuged for 4 h at 52,000 rpm in a Beckman SW55 rotor. Samples were collected from the 0/30% Nycodenz interface (2×20 μL) and analyzed by SDS/PAGE.

Liposome Docking Assay. The t-SNARE liposomes were prepared in a similar way as the liposome fusion assays except that 2% of biotin-conjugated 1,2-Dioleoyl-sn-Glycero-3-Phosphoethanolamine (DOPE) lipid was included. The biotin-labeled t-SNARE liposomes were incubated with avidin-conjugated agarose beads at room temperature for 1 h. The bead-bound t-SNARE liposomes were then used to pull down rhodamine-labeled v-SNARE liposomes, which were identical to the v-SNARE liposomes used in lipid-mixing assays. The pull-down reactions were performed in the liposome reconstitution buffer at 4 °C in the presence or absence of 5 μM Munc18c. After washing three times with the reconstitution buffer, CHAPSO (SoltecVentures) was added to the final concentration of 1% to solubilize the bead-bound liposomes. The avidin beads were removed by centrifugation at 4,000 rpm (Eppendorf 5417R microcentrifuge) for 2 min. Rhodamine fluorescence in the supernatant was measured in a BioTek microplate reader. In negative control reactions, t-SNARE liposomes were substituted with protein-free liposomes.

***Trans*-SNARE Formation Assay.** Reconstituted t- and v-SNARE liposomes were incubated at 4 °C for the indicated periods in the presence or absence of 5 μM Munc18c before a 10-fold excess amount of inhibitory VAMP2 CD was added. The liposomes were solubilized with 1% CHAPS (Anagrade), and the t-SNAREs were precipitated using nickel beads (through binding to His_6/SNAP-23). The presence of full-length VAMP2 in the precipitates was probed by Western blotting using a monoclonal anti-VAMP2 antibody (clone Cl69.1; Synaptic Systems), which was used as an indicator for *trans*-SNARE formation between liposomes. Syntaxin-4 was detected using a polyclonal anti–syntaxin-4 antibody (Sigma).

Cryo-EM. Reconstituted v- and t-liposomes were mixed at equal concentrations (final lipid concentration = 2.5 mM) in the presence of reconstitution buffer, VAMP2 CD (20 μM), or Munc18c (5 μM). After 1 h of incubation at 4 °C, the samples were diluted threefold with the reconstitution buffer. Five microliters of each sample was then applied to perforated carbon-coated grids (Quantifoil R2/4, Electron Microscopy Sciences) that had been glow discharged in the presence of amylamine to reduce the tendency of the liposomes to stick to the carbon support film. Samples were quick-frozen into vitreous ice by plunging into liquid ethane. Images were recorded on a Tecnai TF20 electron microscope operating in a low-dose mode. Both freezing and imaging were carried out at the Boulder Laboratory for 3D Electron Microscopy. Typical imaging conditions included a magnification of 50,000× and 3- to 5-μm defocus. Images were collected on a CCD camera and processed using ImageJ (National Institutes of Health). Liposomes were counted if they were within the imaging field and not in contact with the carbon support (because liposomes had a tendency to accumulate on the carbon). We counted 14–23 fields for each sample. Liposomes were measured for diameter and cluster size. Clustering was scored whenever two liposomes were in contact or within two bilayer diameters (~10 nm) of contact.

Isothermal Titration Calorimetry Measurements. Isothermal titration calorimetry (ITC) measurements were performed at 25 °C using a VP-ITC instrument (Microcal). Munc18c and SNARE complexes were dialyzed overnight in a binding buffer [25 mM Hepes (pH 7.4), 150 mM KCl, 10% glycerol, and 0.5 mM TCEP]. Munc18c protein (4 μM) was loaded into the sample cell of the VP-ITC instrument, followed by the injection of SNAREs (73 μM) into the sample cell. After polynomial baseline corrections to remove the slight drifts in initial data points, the data were

fitted with a nonlinear least squares routine using Microcal Origin software.

Munc18c Overexpression and Glucose Uptake in 3T3-L1 Adipocytes. 3T3-L1 fibroblasts (American Type Culture Collection) were cultured and differentiated to adipocytes using an established protocol (6). 3T3-L1 adipocytes were either not transduced (no virus) or transduced with increasing concentrations (×1 and ×2) of V5-Munc18c lentivirus. At 5 d posttransduction, Munc18c expression levels were determined by immunoblotting whole-cell lysates with Munc18c-specific or V5 epitope antibodies. To measure glucose uptake, cells were serum-starved and either untreated or treated with insulin (1 or 100 nM) for 20 min. Uptake of ^{3}H-2-deoxyglucose into the cells was measured as previously described (7).

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4. Latham CF, et al. (2006) Molecular dissection of the Munc18c/syntaxin4 interaction: Implications for regulation of membrane trafficking. *Traffic* 7(10):1408–1419.
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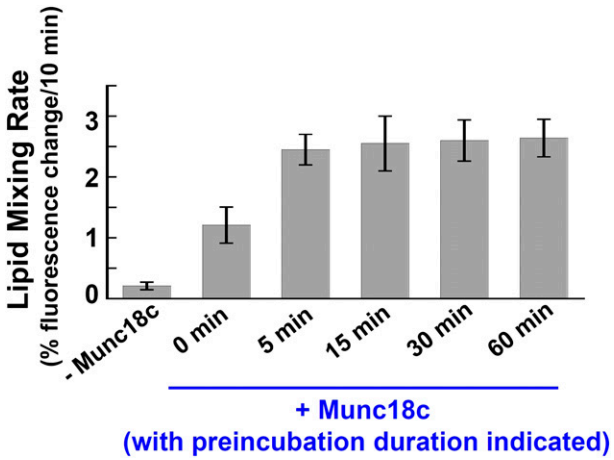

Fig. S1. Effect of 4 °C preincubation on the stimulatory activity of Munc18c in fusion. Munc18c was preincubated with v- and t-SNARE liposomes at 4 °C for the indicated periods before the temperature was elevated to 37 °C to initiate fusion. The fusion reactions were measured by a FRET-based lipid-mixing assay. Each reaction contained 5 μM t-SNAREs and 1.5 μM v-SNARE. Munc18c was added to a final concentration of 5 μM. Data are presented as the percentage of fluorescence change per 10 min. Error bars indicate SD.

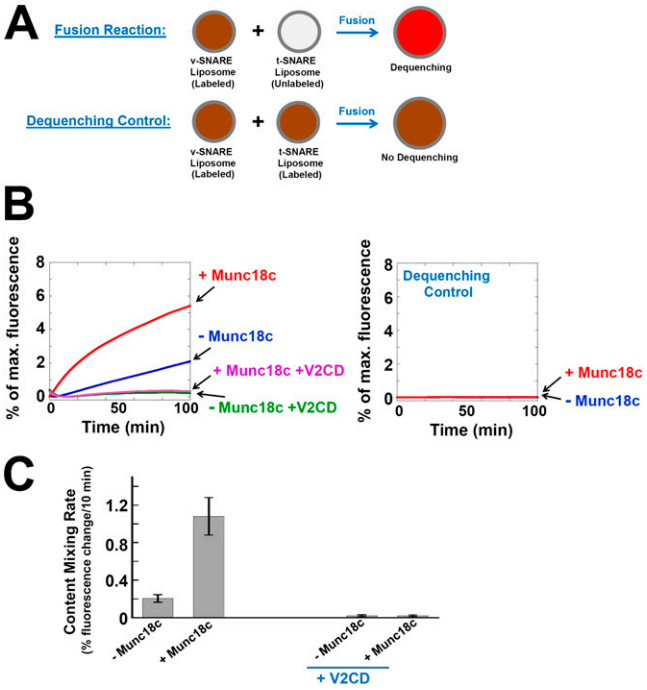

Fig. S2. Munc18c promotes the content mixing of SNARE-mediated membrane fusion. (*A*) Diagram of the liposome–liposome content-mixing assay. The soluble dye sulforhodamine B (50 mM) was encapsulated in the v-SNARE liposomes, in which its fluorescence was inhibited by self-quenching. Fusion of the v-SNARE liposomes with unlabeled t-SNARE liposomes led to the dilution and dequenching of sulforhodamine B. In dequenching controls, the sulforhodamine B dye was included in both v- and t-SNARE liposomes. If content leakage did not occur, no sulforhodamine B dequenching would be observed. (*B*) Content mixing of the reconstituted fusion reactions. The v-SNARE liposomes were directed to fuse with unlabeled t-SNARE liposomes in the absence or presence of 5 μM Munc18c. Each fusion reaction contained 5 μM t-SNAREs and 1.5 μM v-SNARE. Data are presented as the fluorescence increase over time. In negative controls, 20 μM VAMP2 CD was added to the fusion reactions. In dequenching controls, increases in sulforhodamine B fluorescence were not observed, indicating that no detectable content leakage occurred during the fusion reactions. max., maximum. (*C*) Initial content-mixing rates of the fusion reactions shown in *B*. Data are presented as the percentage of fluorescence change per 10 min. Error bars indicate SD.

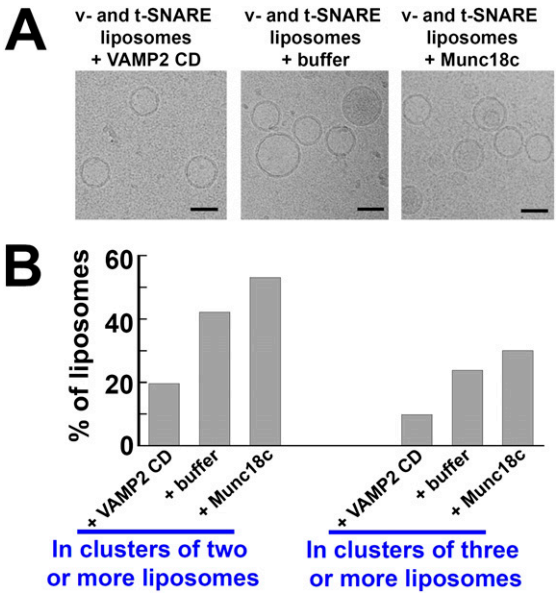

Fig. S3. Cryo-EM analysis of liposome docking. (*A*) Reconstituted t- and v-liposomes were mixed at equal concentrations (final lipid concentration = 2.5 mM) in the presence of reconstitution buffer, VAMP2 CD (20 μM), or Munc18c (5 μM). After 1 h of incubation at 4 °C, the samples were visualized on a Tecnai TF20 electron microscope. (Scale bar: 50 nm.) (*B*) Quantification of liposome–liposome docking. Liposomes were counted if they were within the imaging field and not in contact with the carbon support (because liposomes in all conditions had a tendency to accumulate on the carbon). We counted 14–23 fields for each sample. Liposomes were measured for diameter and cluster size. Clustering was scored whenever two liposomes were in contact or within two bilayer diameters (~10 nm) of contact.

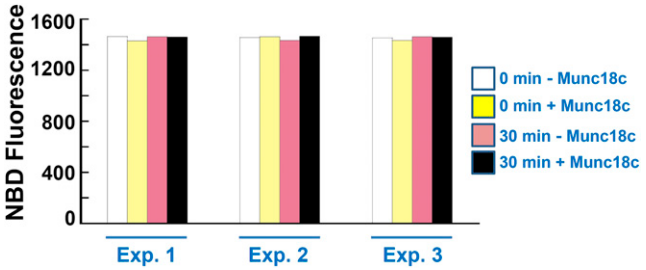

Fig. S4. Lipid mixing did not occur in the *trans*-SNARE assembly assays. On the completion of the *trans*-SNARE assembly assays (Fig. 3 *C* and *D*), the *N*-(7-nitro-2,1,3-benzoxadiazole-4-yl) (NBD) emissions of the samples were measured in a similar way as in liposome lipid-mixing reactions. The NBD fluorescence did not change after the incubation, indicating that lipid mixing did not occur during the *trans*-SNARE assembly reactions. Data of three independent experiments are shown in experiments (Exp.) 1–3.

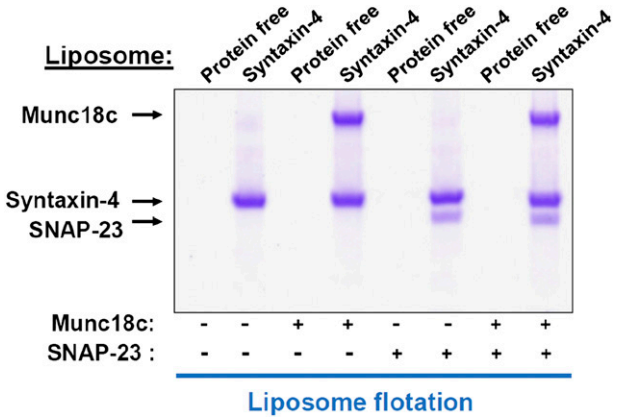

Fig. S5. Munc18c does not inhibit t-SNARE complex assembly. A Coomassie blue-stained gel shows that Munc18c did not inhibit t-SNARE assembly in the liposome coflotation assay. Syntaxin-4 liposomes were incubated with 5 μM Munc18c or buffer at 4 °C for 1 h. Subsequently, 5 μM SNAP-23 was added. After 2 h of incubation at 4 °C, the samples were floated up on a Nycodenz gradient. Input materials for these coflotation experiments are shown in Fig. 6*B*.

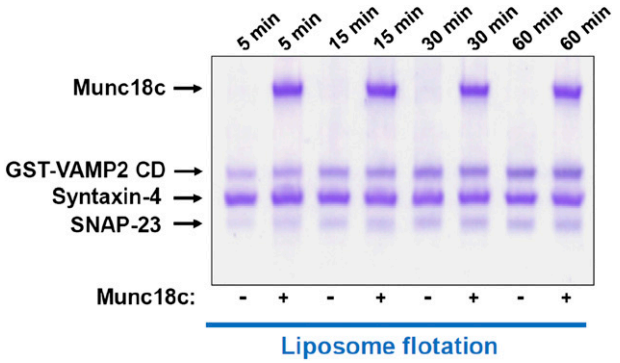

Fig. S6. Munc18c does not inhibit the assembly of the ternary SNARE complex. A Coomassie blue-stained gel shows the time course of SNARE complex assembly in the liposome coflotation assay. Syntaxin-4 liposomes were incubated with 5 μM Munc18c or buffer at 4 °C for 1 h. Subsequently, SNAP-23 and VAMP2 CD (aa 1–95) were added. After incubation at 4 °C for the indicated periods, the samples were floated up on a Nycodenz gradient. To visualize VAMP2 CD better by Coomassie blue staining, a GST tag was included at its N terminus. Input materials for these coflotation experiments are shown in Fig. 6*B*.

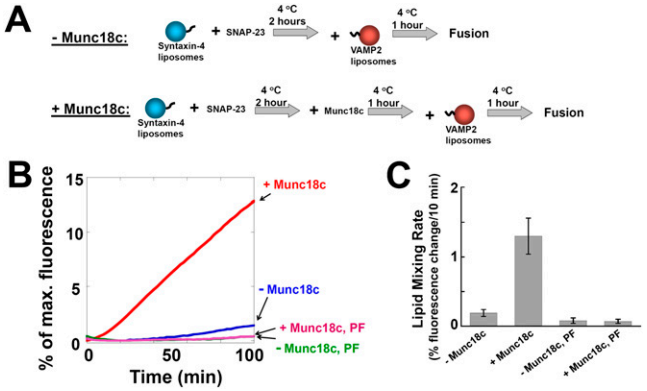

Fig. S7. Munc18c stimulates the fusion reaction when added after SNAP-23. (*A*) Diagram illustrating the experimental procedures for the reconstituted fusion reactions. (*B*) Syntaxin-4 liposomes were incubated with 5 μM SNAP-23 at 4 °C for 2 h before 5 μM Munc18c or buffer was added. After 1 h at 4 °C, VAMP2 liposomes were introduced. After another hour of incubation at 4 °C, the temperature was raised to 37 °C to initiate fusion. The fusion reactions were measured by a FRET-based lipid-mixing assay. In the negative control experiments, t-SNARE liposomes were substituted with protein-free (PF) liposomes. Each fusion reaction contained 5 μM t-SNAREs and 1.5 μM v-SNARE. (*C*) Initial lipid-mixing rates of the reconstituted fusion reactions shown in *B*. Error bars indicate SD.

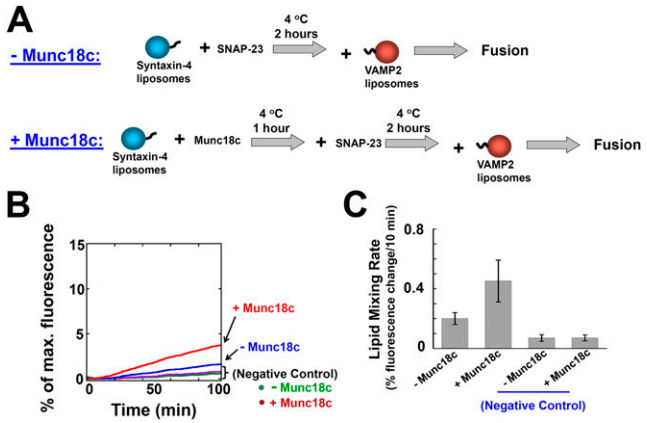

Fig. S8. Munc18c does not inhibit the SNARE-dependent fusion reaction. (*A*) Diagram illustrating the experimental procedures of the reconstituted fusion reactions. (*B*) Munc18c binding to syntaxin-4 did not inhibit the SNARE-dependent fusion reaction. Syntaxin-4 liposomes were incubated with or without Munc18c at 4 °C for 1 h before SNAP-23 was added. After another 2 h of incubation at 4 °C, VAMP2 liposomes were introduced and the samples were immediately incubated at 37 °C to initiate fusion. In negative control experiments, t-SNARE liposomes were substituted with protein-free liposomes. Each reaction contained 5 μM t-SNAREs and 1.5 μM v-SNARE. The final concentrations of Munc18c and SNAP-23 were at 5 μM. (*C*) Initial lipid-mixing rates of the reconstituted fusion reactions shown in *B*. Error bars indicate SD.

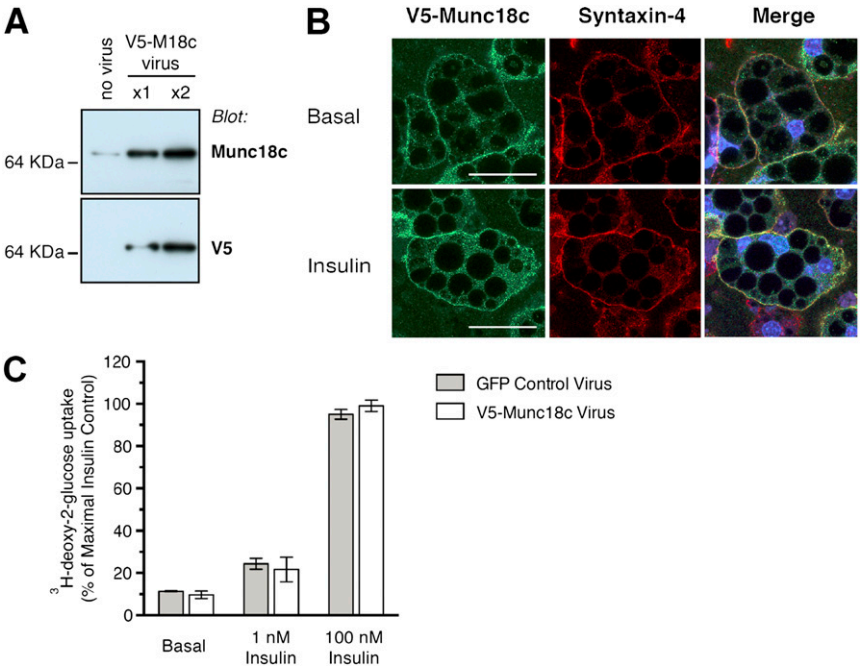

Fig. S9. Munc18c overexpression has no effect on insulin-stimulated glucose uptake in 3T3-L1 adipocytes. (*A*) Lentiviral expression of V5-Munc18c in 3T3-L1 adipocytes. 3T3-L1 adipocytes were either not transduced (no virus) or transduced with increasing concentrations (×1 and ×2) of V5-Munc18c lentivirus. At 5 d posttransduction, Munc18c expression levels were determined by immunoblotting whole-cell lysates using Munc18c-specific (*Upper*) or V5 epitope (*Lower*) antibodies. Molecular weight markers are shown. (*B*) V5-Munc18c colocalized with endogenous syntaxin-4 at the plasma membrane in the absence and presence of insulin stimulation. V5-Munc18c lentivirus-expressing 3T3-L1 adipocytes were serum starved and then incubated in the absence (Basal) or presence of 100 nM insulin (Insulin) for 20 min. Cells were fixed and labeled for Munc18c (using mouse anti-V5 antibodies) and syntaxin-4 (using rabbit anti–syntaxin-4 antibodies). Representative confocal microscopy images are shown. DAPI was used to stain nuclei (shown in blue). (Scale bars: 30 μm.) (*C*) Overexpression of V5-Munc18c had no effect on insulin-stimulated glucose transport in 3T3-L1 adipocytes. 3T3-L1 adipocytes were transduced with GFP expressing control virus or V5-Munc18c virus. At 5 d posttransduction, 100% of adipocytes were expressing V5-Munc18c (determined by immunofluorescence microscopy). The cells were serum-starved and either untreated (Basal) or treated with insulin (1 nM or 100 nM) for 20 min. Uptake of ^{3}H-2-deoxy-glucose into the cells was measured as we previously described (7). Data are presented as a percentage of the maximal insulin GFP control and represent two independent experiments. Error bars indicate SD.

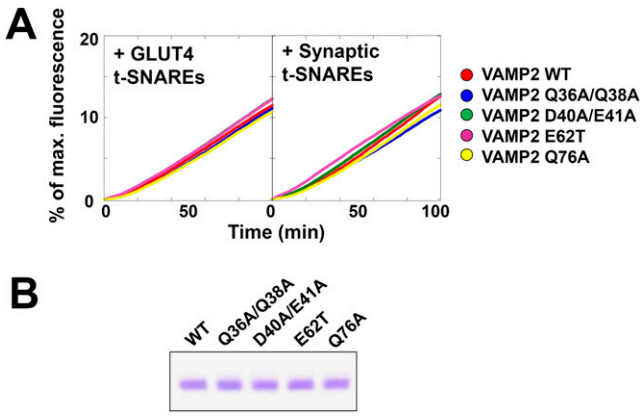

Fig. S10. Effects of VAMP2 mutations on the basal SNARE-mediated membrane fusion. (*A*) WT or mutant VAMP2 liposomes were directed to fuse with liposomes reconstituted with either synaptic t-SNAREs (syntaxin-1/SNAP-25) or GLUT4 exocytic t-SNAREs (syntaxin-4/SNAP-23). The fusion reactions were measured by a FRET-based lipid-mixing assay. Representative lipid-mixing reactions are shown. To compare the basal fusion rates better, the surface densities of VAMP2 on these liposomes were about threefold higher than those on regular liposomes. (*B*) Coomassie blue-stained gel showing that WT and mutant VAMP2 proteins were reconstituted into liposomes at comparable surface densities.