Supporting Information

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SI Materials and Methods

Construction of Munc18-1 and Munc18-2 KD and Expression Plasmids. For Munc18-1 knockdown (KD) in RBL-2H3 cells, parental pLKO-puro plasmid (Sigma-Aldrich) was modified: U6 promoter was replaced by H1 promoter + 19-nucleotide shRNA sequences of pSuper-Munc18-1 KD (1), creating pLKO-H1-Munc18-1-puro. The Munc18-2 KD plasmid was previously described (1). Expression plasmids were created by modifying the parental pLVX-IRES-puro (Clontech Laboratories). First, the puromycin-resistance gene was replaced by a blasticidin-resistance gene, creating pLVX-IRES-blast (2). Then, Emerald GFP (EmGFP; Clontech Laboratories) or the myc sequence were subcloned into the BamHI site, generating pLVX-EmGFP-IRES-blast and pLVX-myc-IRES-blast, respectively. cDNA of rat Munc18-1 and Munc18-2 (kind gifts from Thomas C. Südhof, Stanford University School of Medicine) were mutated to create silent nucleotide mutations (SNMs) (for Munc18-1, see ref. 1; for Munc18-2, AGGAATTACGATCGTCGAGGA, with the underline indicating SNM) within the shRNA-targeted sequences to protect the exogenously expressed gene from being degraded by anti-Munc18-1 or anti-Munc18-2 shRNA. We found that our original pCMV5-rMunc18-2 WT cDNA had two point mutations (G11V and T304R), which were corrected by site-directed mutagenesis. Point mutations (F115E, E132A, and F115E/E132A) were also made using site-directed mutagenesis. Munc18-1 insert (WT and mutants) was digested from pCMV5-rMunc18-EmGFP with EcoRI/XbaI and was subcloned into the same sites of pLVX-EmGFP-IRES-blast, pLVX-myc-IRES-blast, or pLVX-IRES-blast. Munc18-2 insert (WT and mutants) digested from pCMV5-rMunc18-2 (without stop codon) with EcoRI/XbaI was subcloned into the same site of pLVX-EmGFP-IRES-blast. Sequences of all created plasmids were verified by DNA sequencing. KD and expression plasmids were cotransfected with psPAX2 and pMD.G into human embryonic kidney (HEK)-293FT cells for generating recombinant lentivirus.

Isolation of Stable Munc18-1/2 KD and Rescued PC12 and RBL-2H3 Cells. Munc18-1/2 double KD (DKD) pheochromocytoma (PC) 12 cells were maintained in DMEM (Invitrogen) containing 5% (vol/vol) calf serum, 5% (vol/vol) horse serum (both from HyClone Laboratories), penicillin (100 U/mL)/streptomycin (0.1 mg/mL) (Sigma-Aldrich), 250 ng/mL amphotericin B (Sigma-Aldrich), puromycin (2.5 μg/mL) (Bishop), and G418 (700 μg/mL) (Bioshop). Lentiviruses expressing Munc18-1 and Munc18-2 (WT or mutants) with various tags were infected to the DKD PC12 cells and isolated by applying blasticidin (5 μg/mL) (Invivogen). WT RBL-2H3 cells were purchased from ATCC and maintained in DMEM containing 10% calf serum (HyClone Laboratories), penicillin (100 U/mL)/streptomycin (0.1 mg/mL), and 250 ng/mL amphotericin B. Lentiviruses expressing shRNA against Munc18-1 and Munc18-2 were applied to RBL-2H3 cells with polybrene (8 μg/mL) (Sigma Aldrich), and infected cells were isolated by puromycin (10 μg/mL) for Munc18-1 and G418 (700 μg/mL) for Munc18-2. After successful down-regulation was confirmed, lentiviruses expressing EmGFP-tagged rat Munc18-1 or rat Munc18-2 WT and hydrophobic pocket mutants were infected to the Munc18-1/2 DKD RBL-2H3 cells and selected with blasticidin (20 μg/mL).

β-Hexosaminidase Release Assays from RBL-2H3 cells. RBL-2H3 cells were plated in 24-well plates; 2–3 d after plating, the cells that were to be stimulated by the IgE-dependent method were sensitized with 0.01 μg/mL dinitrophenyl (DNP)-IgE *Spe7* antibody (Sigma Aldrich) overnight. Medium was changed for cells that were to be stimulated by ionomycin dependent method. The next day, the cells were washed twice with physiological saline solution (PSS) containing 145 mM NaCl, 5.6 mM KCl, 2.2 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5.6 mM glucose, 15 mM Hepes, and 0.1% BSA at pH 7.4. Degranulation was stimulated by adding 500 μL PSS plus the following to the respective cells: 50 ng/mL DNP-human serum albumin (HSA) (Sigma-Aldrich), 0.5 μM ionomycin (Sigma-Aldrich), or 2.5 μM ionomycin. After 1 h of incubation at 37°C, samples were collected and centrifuged at 4°C for 3 min. Supernatant was transferred to a new set of tubes for measuring the released β-hexosaminidase. Cells were solubilized in 500 μL 0.5% Triton X-100, and samples were collected for measuring the remaining β-hexosaminidase. Then, 200 μL 1 mM p-nitrophenyl *N*-acetyl-β-D-glucosaminide (Sigma-Aldrich) was added and incubated at 37°C for 1 h. The reaction was quenched by adding 500 μL 0.05 μM sodium carbonate buffer, and absorbance at 405 nm was measured by spectrophotometer. The optical density $(OD)_{405}$ of supernatant/OD_{405} of total × 100% was calculated to be the percentage of β-hexosaminidase release.

[^{3}H]-Noradrenaline Release Assays from PC12 cells. PC12 cells were plated in 24-well plates. Three to 4 d after plating, the cells were labeled with 0.5 μCi [^{3}H]-Noradrenaline (NA) in the presence of 0.5 mM ascorbic acid for 12–16 h. The labeled PC12 cells were incubated with the fresh complete DMEM for 1–5 h to remove unincorporated [^{3}H] NA. The cells were washed once with PSS, and NA secretion was stimulated with 200 μL PSS or high K^+-PSS (containing 81 mM NaCl and 70 mM KCl). Secretion was terminated after a 15-min (high K^+) incubation at 37°C by chilling to 0°C, and samples were centrifuged at 4°C for 3 min. Supernatants were removed and the pellets were solubilized in 0.1% Triton X-100 for liquid scintillation counting.

Protein Interaction Experiments Between Recombinant Syntaxin-11 and Munc18-2. Mouse syntaxin-11 cDNA was subcloned into pGex-KG, using EcoRI/XbaI sites. pGex-KG-syntaxin-11 along with empty vector, were transformed into BL21 (Codon+) cells, and the bacteria were grown at 37°C until confluent. Recombinant protein expression was induced by adding 50 μM isopropyl-β-D-thiogalactopyranoside at 30°C for 3 h. Cells were lysed using 0.1% Triton X-100 and sonications in PBS containing 1 mM EDTA and protease inhibitors (10 μg/mL leupeptin and 10 μg/mL aprotinin). Supernatant portions were then mixed with glutathione agarose beads (Pierce Biotechnology). The next day, the mixtures were washed extensively with PBS containing 20% sucrose, PBS containing 0.1% Triton X-100, and PBS, in that order. Samples were saved for binding with Munc18-2.

EmGFP, WT Munc18-2-EmGFP, and hydrophobic pocket mutants were transfected into HEK-293FT cells. After 3 d, the cells were lysed with potassium glutamate buffer containing 20 mM Hepes at pH 7.2, 120 mM KGlu, 20 mM potassium acetate, 2 mM EGTA, and 0.1% Triton X-100 as a detergent. After centrifugation, KGlu buffer plus detergent containing solubilized lysates were mixed with either 10 μg GST or GST-syntaxin-11. The next day, the mixtures were washed extensively with KGlu buffer containing 0.1% Triton X-100. The samples were then dried and 2× SDS/PAGE sample buffer was added and boiled. The supernatant was subjected to SDS/PAGE followed by Coomassie Blue staining and immunoblots with mouse monoclonal anti-EmGFP antibody.

Cell Preparation for Confocal Immunofluorescence Microscopy. Sterilized circular glass coverslips (0.25 mm in width, 1.8 cm in diameter) were placed in 2.2-cm wells within 12-well cell culture plates. The coverslips were then coated for 1 h with poly-D-lysine (0.1 mg/mL) at room temperature. RBL-2H3 cells were allowed to adhere to the coverslips for 3 d. Then the cells were washed with PBS and fixed for 15 min with PBS containing 4% paraformaldehyde (PFA). PFA was then removed from each well and the cells rinsed three times (10 min each time) with 1 mL PBS per well. The fixed cells were then permeabilized with PBS containing 0.2% Triton X-100 and 0.3% BSA for 5 min, followed by three washes with PBS. Nonspecific sites were blocked for 1 h at room temperature in PBS containing 0.3% BSA. For the primary antibody against syntaxin-11, we used either GST or GST-syntaxin-11 absorbed antibody to ensure specificity. Briefly, rabbit polyclonal anti-syntaxin-11 antibody was absorbed overnight with 20 μg GST or GST-syntaxin-11 in PBS + 0.3% BSA, such that antibody concentration is 1:500. The supernatant was then applied to the permeabilized cells as the primary antibody (1:1,000) for 1 h. After three washes in blocking buffer, Alexa488-conjugated goat anti-rabbit antibodies (diluted 1:1,000; Invitrogen) were applied for 1 h. The samples were washed again three times in blocking buffer, and 300 nM DAPI was applied for 30 min. The slides were mounted in Fluoromount-G reagent (Southern Biotechnology). Immunofluorescence staining was recorded with a laser confocal scanning microscope (LSM510; Carl Zeiss) with an oil immersion objective lens (63×).

Construction of Syntaxin-11 KD Plasmids and Isolation of Stable Syntaxin-11 KD RBL-2H3 cells. To the KD rat syntaxin-11 gene, we targeted the 21-nucleotide sequence of CGGTGCAGTACAAGAAGAAGA in rat syntaxin-11. CTCGAG was used as a linker sequence. These oligonucleotides containing sense and antisense of the target sequences were annealed, phosphorylated, and ligated to AgeI/EcoRI sites of pLKO-puro (Sigma-Aldrich) generating the syntaxin-11 KD plasmid (pLKO-syntaxin-11-4). Inserted sequences were verified by DNA sequencing. This KD plasmid was co-transfected with psPAX2 and pMD.G into HEK-293FT cells for generating recombinant lentivirus.

WT RBL-2H3 cells were purchased from ATCC and maintained in DMEM containing 10% calf serum (HyClone Laboratories), penicillin (100 U/mL)/streptomycin (0.1 mg/mL), and 250 ng/mL amphotericin B. Lentiviruses expressing shRNA against rat syntaxin-11 were applied to RBL-2H3 cells with polybrene (8 μg/mL) (Sigma Aldrich), and infected cells were isolated by puromycin (10 μg/mL).

1. Han L, et al. (2009) Rescue of Munc18-1 and -2 double knockdown reveals the essential functions of interaction between Munc18 and closed syntaxin in PC12 cells. *Mol Biol Cell* 20(23):4962–4975.

2. Han GA, et al. (2011) Munc18-1 domain-1 controls vesicle docking and secretion by interacting with syntaxin-1 and chaperoning it to the plasma membrane. *Mol Biol Cell* 22(21):4134–4149.

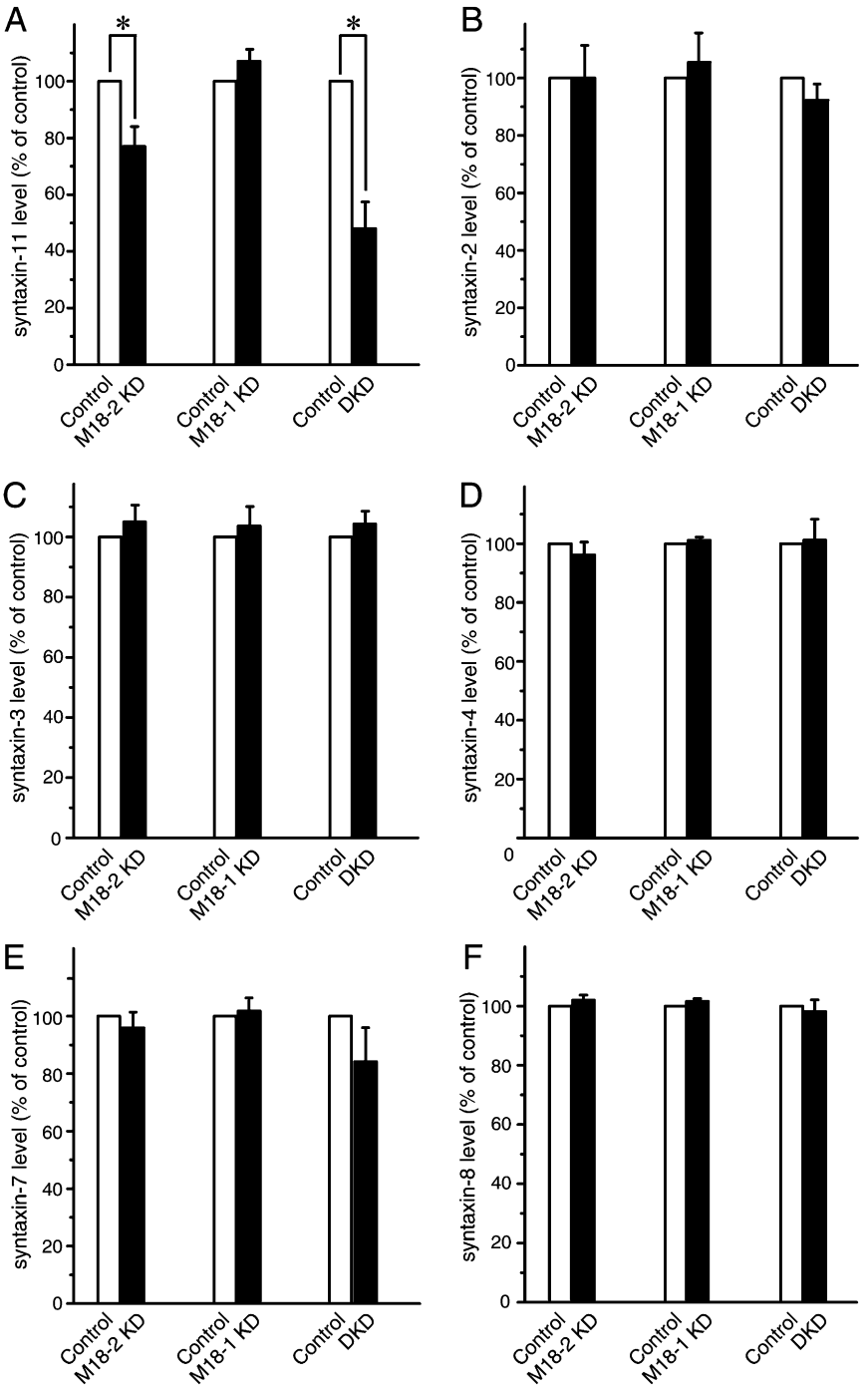

Fig. S1. Quantification of various syntaxin isoforms reveals a strong down-regulation of syntaxin-11 and a mild decrease in syntaxin-7 level in Munc18-1/2 double knockdown RBL-2H3 cells. (*A–F*) Images of these proteins on the film were quantified for densitometry and normalized to the control using ImageJ (National Institutes of Health). Error bars, SEM (n = 3–8). The statistical significance of the differences in syntaxin-11 level between control, Munc18-2 knockdown, and Munc18-1/2 double knockdown is indicated. *$P < 0.05$ (Student t test).

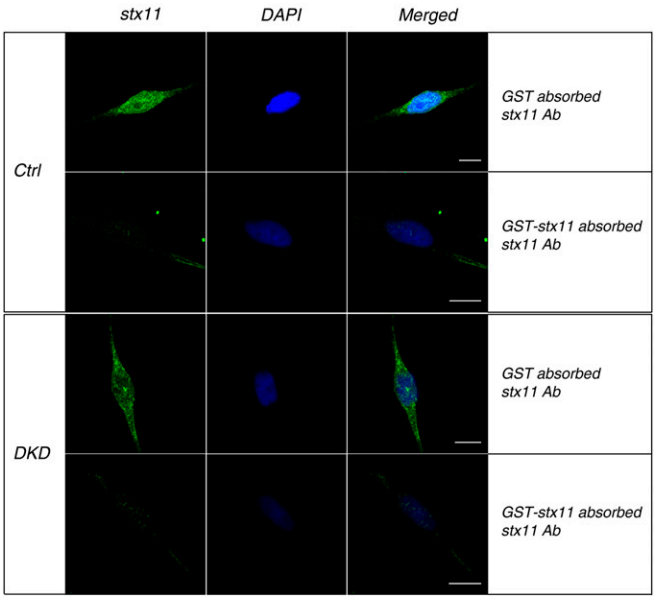

Fig. S2. Confocal immunofluorescent microscopy images ensuring specificity of the antibody as well as unaffected subcellular localization of syntaxin-11 on Munc18-1/2 double knockdown. Control and stable Munc18-1/2 double knockdown RBL-2H3 were permeabilized and stained with either GST or GST-syntaxin-11 absorbed anti-syntaxin-11 antibody followed by Alexa488-conjugated goat anti-rabbit antibody and DAPI. *Left*, syntaxin-11 staining; *Center*, DAPI; *Right*, merged pictures. (Scale bar, 10 μm.)

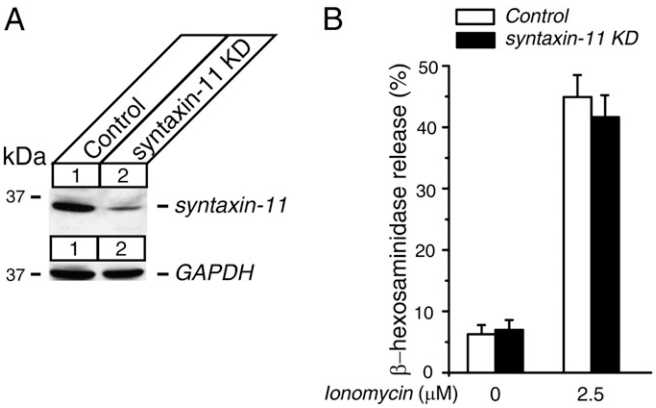

Fig. S3. Strong and stable knockdown of syntaxin-11 in RBL-2H3 cells does not show defects in β-hexosaminidase release. (*A*) Stable syntaxin-11 knockdown RBL-2H3 cells were generated by lentivirus-mediated shRNA. Twenty micrograms of cell homogenates were analyzed by SDS/PAGE and immunoblotting, using the antibodies indicated. The signals were detected with enhanced chemiluminescence detection system. The numbers on the left indicate the position of a molecular weight marker. (*B*) β-hexosaminidase release was stimulated by applying 2.5 μM ionomycin for 1 h from control, as well as syntaxin-11 knockdown cells. Error bars, SEM ($n = 6$).