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Supplemental Information

Self-Assembly of VPS41 Promotes Sorting Required for Biogenesis of the Regulated Secretory Pathway

Cédric S. Asensio,Daniel W. Sirkis, James W. Maas, Jr., Kiyoshi Egami, Tsz-Leung To, Frances M. Brodsky, Xiaokun Shu, Yifan Cheng, and Robert H. Edwards

Inventory of Supplementary Materials:

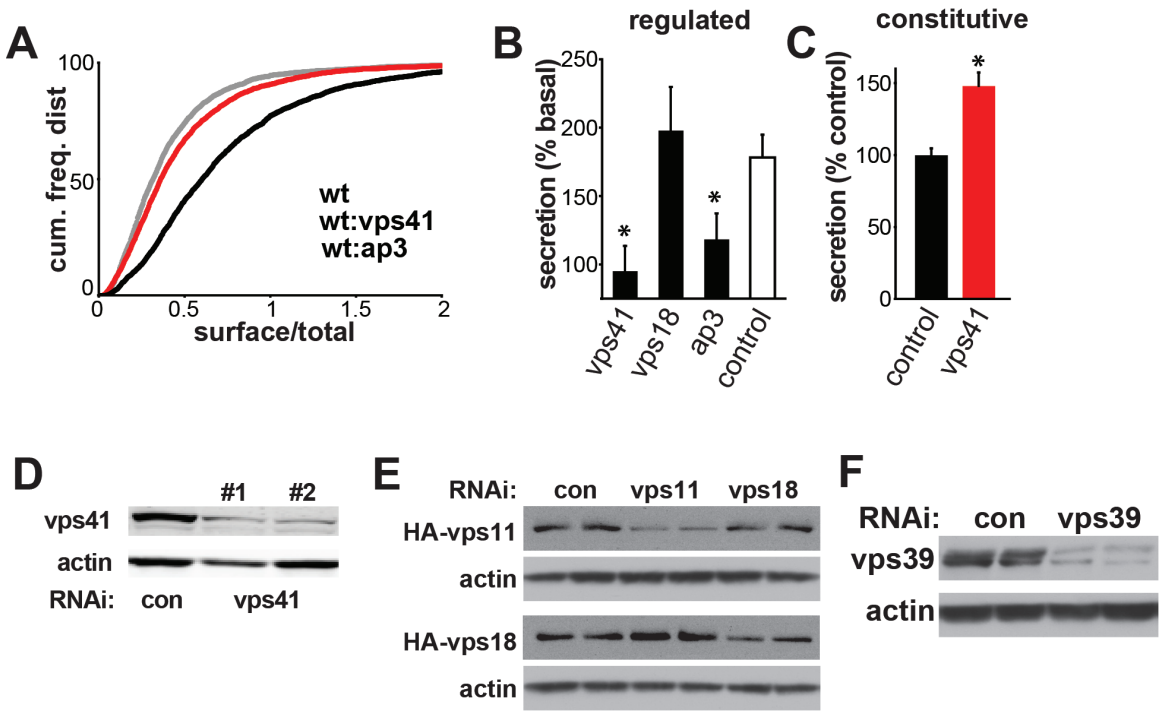


Figure S1. RNAi screen of granule group genes implicates VPS41 but not other HOPS complex subunits in the regulated secretory pathway (related to Figure 1)

(A) Drosophila S2 cells expressing GFP-HA-dVMAT were treated for 3 days with dsRNA against known granule group genes, incubated for two hours at room temperature with external HA antibody conjugated to Alexa 647, washed, and the fluorescence of individual cells determined by flow cytometry. The cumulative frequency distributions of surface (red) : total (green) dVMAT shows a small increase in surface expression for *vps41* (red). $p < 10e^{-12}$ relative to wild type control by Kolmogorov-Smirnov (B,C) S2 cells were transiently transfected with ANF-GFP and treated with dsRNA to *vps41*, *vps18* (as negative control) and *ap-3*. LPS-induced (B) and basal (C) secretion of ANF-GFP was measured using a plate reader. *, $p < 0.01$ relative to secretion from control cells (n=5-6) The bar graphs indicate mean ± s.e.m.

(D-F) Confirmation of HOPS subunit knock-down in mammalian cells. (D) COS-7 cells were transiently cotransfected with VPS41-myc and either control siRNA or two siRNAs to VPS41. Immunoblotting the extracts for myc shows a substantial reduction in VPS41 with the two siRNAs to VPS41 relative to control. (E) COS-7 cells were transiently cotransfected with VPS11 or VPS18 siRNAs as well as either HA-VPS11 or HA-VPS18. Immunoblotting the extracts for HA shows a substantial and specific reduction relative to cells cotransfected with control siRNAs. (F) PC12 cells were transfected with VPS39 siRNA and immunoblotting of the extracts for VPS39 shows a strong reduction relative to control cells.

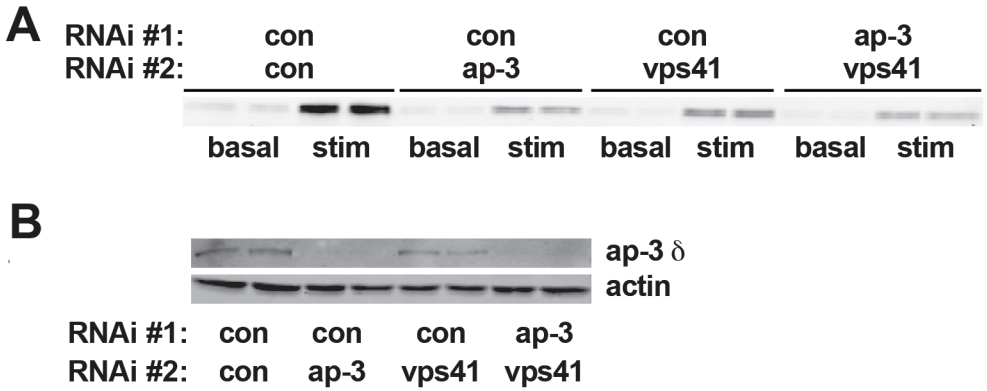
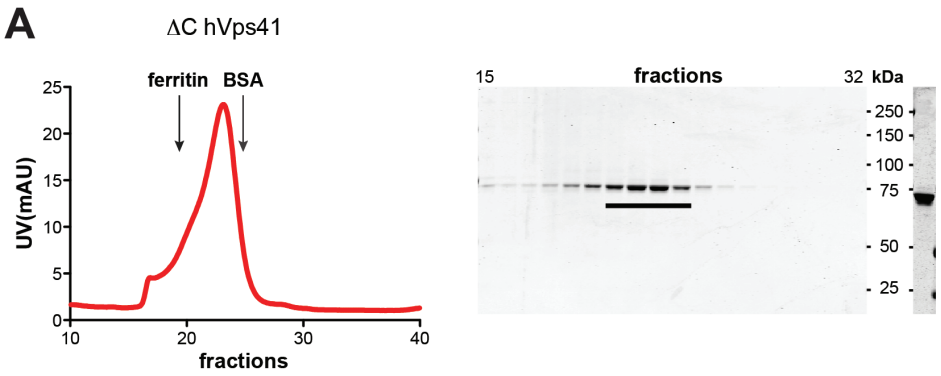
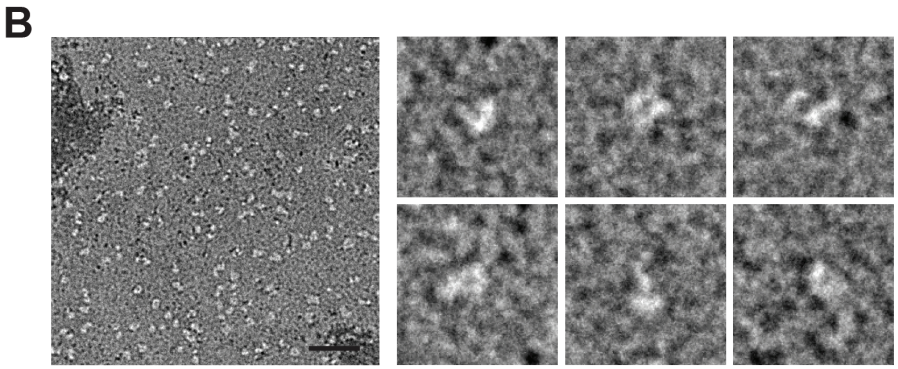


Figure S2. Functional interaction of VPS41 and AP-3 (related to Figure 3)

PC12 cells were transiently transfected in duplicate with control, VPS41 or AP-3 siRNA (each at 50 nM), and the secretion of SgII measured by immunoblotting as in Figure 1. (A) AP-3 and VPS41 RNAi each impair regulated release of SgII, but do not have an additive effect. (B) The efficiency of AP-3 knockdown is not affected by VPS41 RNAi.


A
ΔC hVps41
ferritin
BSA
UV(mAU)
fractions
15
fractions
32 kDa
250
150
100
75
50
25


B

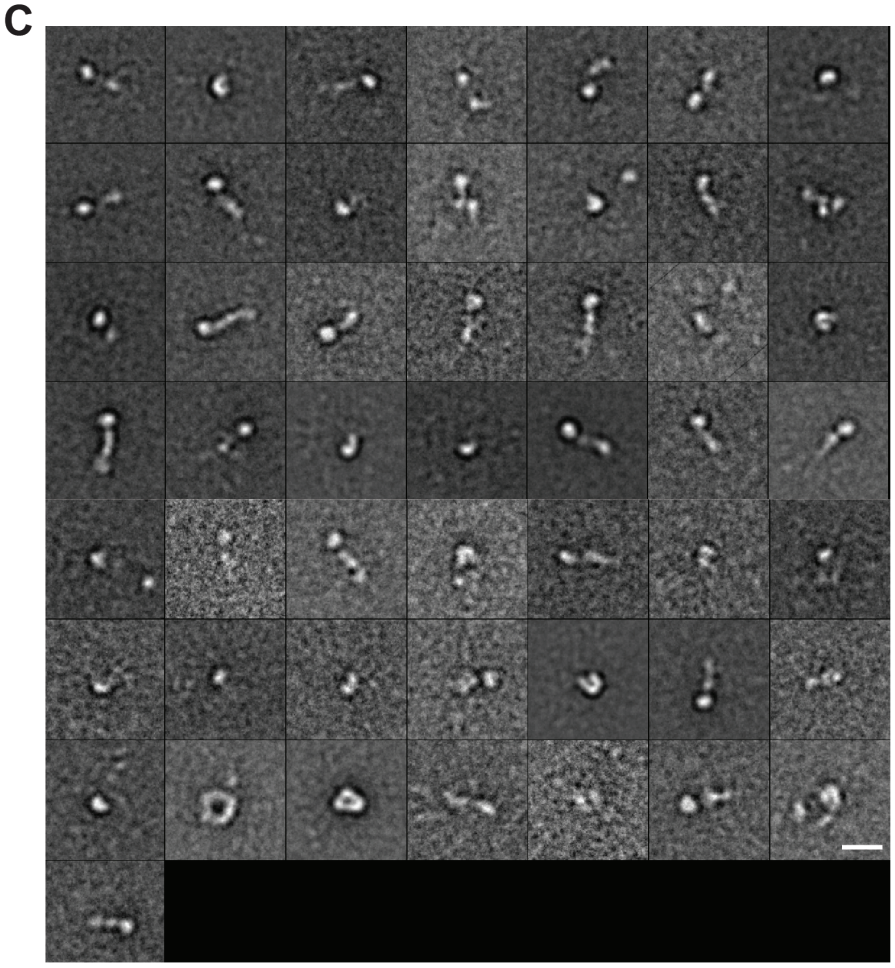

C

Figure S3. Purification of human VPS41 lacking the C-terminus and VPS41 class averages (related to Figure 5)

(A) hVPS41 containing residues 1 to 594 (DC) was purified from Sf9 cells by metal affinity chromatography followed by gel filtration (Superdex 200) in 10 mM Tris, pH 7.9. UV absorbance was used to monitor the outflow from size exclusion chromatography (left). The fractions were then separated by electrophoresis and stained using Coomassie Blue (right). Fractions containing monomeric hVPS41 were pooled, concentrated and separated again by electrophoresis (extreme right). (B) Negative stain EM of recombinant DC hVPS41 shows single particles (left panel). Panels on the right show single particles at higher magnification. Size bar, 50 nm. (C) Unassembled, recombinant hVPS41 was negative stained and imaged by EM. Class averages were generated from the single particle data set. Size bar, 20 nm.

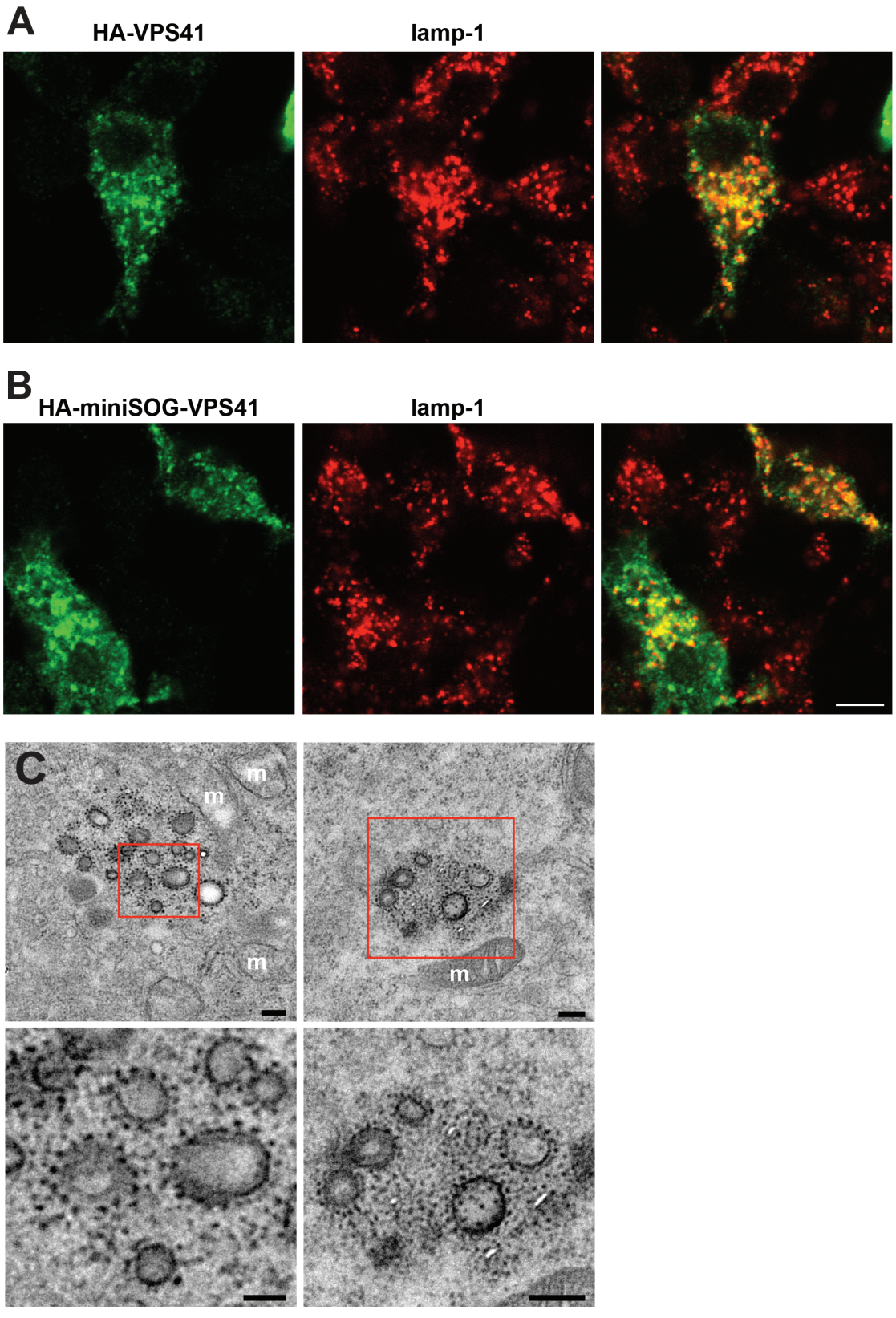

A
HA-VPS41
lamp-1
B
HA-miniSOG-VPS41
lamp-1
C
m
m
m
m

Figure S4. The miniSOG tag does not affect VPS41 localization by confocal microscopy but produces clustered membrane vesicles surrounded by a periodic array of electron-dense puncta (related to Figure 7)

PC12 cells transfected with HA-VPS41 (A) or HA-miniSOG-VPS41 (B) were stained with a mouse monoclonal antibody to HA and a rabbit polyclonal antibody to lamp-1, followed by an anti-mouse antibody conjugated to Alexa Fluor 488 and an anti-rabbit antibody conjugated to Cy3, and visualized by confocal microscopy. Representative micrographs show that fusion to miniSOG does not obviously perturb the localization of VPS41, which remains punctate and colocalized with lamp-1. Scale bar, 5 μm. (C) PC12 cells were transfected with VPS41 siRNA and RNAi-resistant miniSOG-VPS41, fixed, incubated with DAB as described in the legend to Figure 7 and processed for EM. Approximately one quarter of cells transfected with miniSOG-VPS41 (but not miniSOG alone) showed clustered membrane vesicles decorated with a regular array of electron-dense puncta. Scale bars indicate 200 nm. (m: mitochondria).

Table S1. AP-3-associated genes tested for defects in the regulated secretory pathway (related to Figure 1)

Complex	CG	Name	p-value #1	p-value #2
BLOC-1				
	6856	dtnbp1	0.006	0.01
	14145	bloc1s2	1	0.725
	14149	cappuccino	1	0.999
	30077	bloc1s1	0.778	1
	34131	muted	0.835	1
BLOC-2				
	9770	hps5	0.06	1
	14562	hps3	0.594	0.06
BLOC-3				
	4966	hps4	0.512	0.365
	12855	hps1	0.999	0.967
HOPS				
	3093	deep orange (vps18)	1	0.178
	7146	vps39	0.657	0.493
	8454	vps16A	0.265	0.329
	12230	carnation (vps33A)	1	1
	18028	light (vps41)	10E-12	10E-12
	32350	vps11	0.942	0.812
none				
	8024	lightoid (rab32/rab38)	0.875	0.741

dsRNA to the genes listed above were tested specifically for the ability to increase cell surface expression of wild type GFP-HA-dVMAT in S2 cells (Asensio et al., 2010). Of these genes, only *vps*41 increased cell surface dVMAT.

Supplementary Experimental Procedures

Cell culture

Drosophila S2 cells were maintained in Schneider medium (Invitrogen, CA) supplemented with 10% heat-inactivated fetal bovine serum at 27° C in a humidified incubator. Transfection of S2 cells was performed with Fugene HD (Roche) according to the manufacturer's instructions. Sf9 cells were maintained in Sf900 II medium supplemented with 2% heat-inactivated fetal bovine serum, and grown in suspension at 27° C in a humidified incubator. PC12 cells were maintained in DME-H21 medium supplemented with 10% horse serum and 5% calf serum (Cosmic) at 37° C. COS7 cells were maintained in DME-H21 medium supplemented with 10% fetal bovine serum at 37° C. PC12 and COS7 cells were transfected with 50 nM siRNA with or without 200-500 ng cDNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Molecular biology

The rat vps41 sequence was amplified from PC12 cell cDNA by PCR with the following primers to introduce an Nhe I restriction site at the 5' end and a myc epitope at the 3' end: 5'-GATGCTAGCGCCATGGCGGAAGCAGAGGAGCA-3' (rat vps41 sense); 5'-ATCCCATGGTTACAGGTCCTCCTCGCTGATCAGCTTCTGCTCTCCGCCCTTCTTC ATCTCCAGGATGGC-3' (rat vps41 antisense). The rat vps11 sequence was amplified from PC12 cell cDNA by PCR using the following primers to introduce a Not I restriction site and HA epitope at the 5' end and an Xho I restriction site at the 3' end: 5'-ATCGCGGCCGCCACCATGGAGTACCCATACGACGTACCAGATTACGCTGGCGG CAGCGAAGGCCAGATCTGGTTCTTGC-3' (rat Vps11 sense); 5'-GATCTCGAG

TTACGTTCCCCTCCTGGAGTGC-3’ (rat Vps11 antisense). The rat vps18 sequence was amplified from a cDNA clone (ATCC IMAGE ID: 7936869) by PCR using the following primers to introduce a Not I restriction site and HA epitope at the 5’ end and an Xho I restriction site at the 3’ end: 5’- ATCGCGGCCGCCACC ATGGAGTACCCATACGACGTACCAGATTACGCTGGCGGCAGCGCGTCCATACT GGATGAGTATGAGG-3’ (rat vps18 sense); 5’-GATCTCGAG CTACAGCCAGCTGAGGTGTTCCTC -3’ (rat vps18 antisense). The rat vps41 N-terminal deletion mutant (amino acids 162-853) was generated by PCR using the following primers to introduce an NheI restriction site and HA epitope tag at the 5’ end, and a blunt end at the 3’ end: 5’-GATAGCTAGCCACCATGGAGTACCCATACGACGTACCAGATTACGCTGGCAAG TCTTCTGTCCTGCACGAAGG-3’ (sense); 5’-CTAACCGGTCTACTTCTTCATCTCCAGGATGGCA-3’ (antisense). The rat vps41 C-terminal deletion mutant (amino acids 1-595) was generated by PCR using the following primers to introduce an Nhe I restriction site at the 5’ end, and an HA epitope tag at the 3’ end: 5’-GATAGCTAGCCACCATGGCGGAAGCAGAGGAGC-3’ (sense); 5’-CTAAGCGTAATCTGGTACGTCGTATGGGTACTCATGCTGCAGCTCTGGCCTG-3’ (antisense). The siRNA-resistant rat vps41 sequence was generated by overlap extension PCR using the following primers: 5’-ATCATTTTGGCAAAGAATTCGC-3' (sense #1); 5’-GTTCATCCAAGTTCTTTCAAATAGCAG-3' (antisense #1); 5'-CTGCTATTTGAAAGAACTTGGATGAAC-3' (sense #2); 5'-GTGGTTTCAGAACGGGGTCC-3' (antisense #2). GFP-/HA-dVMAT and ANF-GFP

were previously described (Asensio et al., 2010). For expression in rat PC12 cells, all cDNAs were subcloned into pCAAGS.

Human full length and C-terminal deletion mutant vps41 sequence were amplified by PCR using the following primers to introduce a Nco I restriction site at the 5' end and a Not I restriction site at the 3' end, respectively: 5'-CATGCCATGGCGGAAGCAGAGGAGCAGG-3' (full length sense); 5'-GCGATGCGGCCGCCTATTTTTTCATCTCCAAAATTG-3' (full length antisense); 5'-CATGCCATGGCGGAAGCAGAGGAGCAGG-3' (C-terminal deletion sense); 5'-GCGATGCGGCCGCCTACTGTAGCTCTGGTCTGTCTTCCA-3' (C-terminal deletion antisense). The PCR products were digested and subcloned into pFastBac HTA (Invitrogen) for baculovirus generation.

Antibodies

The HA.ll mouse monoclonal antibody was obtained from Covance, SgII rabbit polyclonal antibody from Meridian Life Science, actin mouse monoclonal antibody from Millipore, Zenon Alexa 647 labeling kit from Invitrogen, myc 9E10 mouse monoclonal antibody from Babco, myc rabbit polyclonal antibody from Cell Signaling, synaptophysin (p38) monoclonal antibody from Sigma-Aldrich and synaptotagmin 1 (syt1) rabbit polyclonal antibody from Synaptic Systems. The rabbit polyclonal antibody to VPS39 was a generous gift of R. Piper (University of Iowa).

siRNAs sequences

siRNAs to rat AP-3δ1 (5'-UUCUUGGUCAUGAUCCAUGTG-3' antisense and 5'-CAUGGAUCAUGACCAAGAATT-3' sense), and to rat VPS41 (5'-AUAGGUAUACAAUUCUGCCAG -3' #1 antisense and 5'-

GGCAGAAUUGUAUACCUAUTT-3' #1 sense, and 5'-UGUUCAUCCACGUCCGUUCAA-3' #2 antisense and 5'-GTTCGGACGUGGAUGAACATT-3' #2 sense) were obtained from Ambion. SMARTpool siRNAs from Dharmacon were obtained for Vps11 (5'-AGAGUAACAUGAAACGCUA-3'; 5'-CGUCCUAUGUGAUCCGUAA-3'; 5'-GGAGAUAUUACCCGGGACA-3', 5'-GCUUAGACACAUCGAGAAC-3'), and for Vps18 (5'-GCUCAAGAUCGCUCGGCAU-3'; 5'-CUGAAUCUCUACCGGGAUA-3'; 5'-GAAUGUGCUUGGCGAGACU-3'; 5'-CGGGAGUGCUUCCGUACUU-3').

Electron microscopy

PC12 cells were plated onto aclar film discs (Pella) coated with poly-L-lysine, transfected twice with siRNA (50 nM), washed with cmf-PBS 2 days after the second transfection and fixed with 2.5% glutaraldehyde in cmf-PBS. The discs were washed three times with 0.1 M sodium cacodylate buffer (pH 7.2) and postfixed for 30 min on ice with 1% osmium tetroxide in cacodylate buffer containing 1.6% potassium ferricyanide. The discs were then washed three times with cacodylate buffer, three times with water, incubated with 0.5% uranyl acetate for 30 min (in the dark), and washed again with water. The samples were dehydrated in a graded series of ethanols while progressively lowering the temperature from 4° C to -40° C, then embedded in Epon resin. After peeling off the aclar, sections were cut and then viewed in a FEI Tecnai 12 electron microscope (Phillips) at 120 kV, capturing images with a digital camera at 6800 magnification, and analyzing them with ImageJ. Morphologically identifiable LDCVs were counted for each cell sectioned, and the cytoplasmic area determined by subtracting the area of the nucleus from the whole cell. Density was calculated as the number of LDCVs per cell section

divided by the cytoplasmic area. LDCV and core diameters were determined as the shortest distance between points on opposite sides.

For the photo-oxidation experiments, PC12 cells were plated as described above, transfected with VPS41 siRNA (50 nM) one day after plating, and transfected again two days later with siRNA and siRNA-resistant miniSOG-VPS41 or cytosolic miniSOG as control. Three days after transfection, cells were fixed by adding an amount of 4% glutaraldehyde in 0.1 M sodium cadodylate, pH 7.4 equal in volume to the culture medium, incubated for 30 min, washed in 0.1 M cacodylate buffer and non-specific staining blocked by incubation in 10 mM KCN, 10 mM aminotriazole, 50 mM glycine and 0.1 M cacodylate buffer. The photo-oxidation, post-fixation and embedding procedures were carried out as previously described (Shu et al., 2011), with the samples illuminated by a blue LED (450 ± 20 nm) at 200 mW/cm2 for 60-90 min. The final sections were imaged as above.

Neuronal culture and live imaging

Dissociated primary cultures of hippocampal neurons were prepared from postnatal rats and transfected as previously described (Onoa et al., 2010) with plasmids encoding NPY-pHluorin, mCherry-Synaptophysin and the pJHUB vector encoding either control or VPS41 shRNA (5'-GGATGAAAGCGGAGAACATATTTCAAGAGAATATGTTCTCCGCTTTCATCC-3') along with blue fluorescent protein (Tracy et al., 2011). For live-cell imaging, cultures at 14 days *in vitro* (DIV) were mounted in a laminar flow perfusion chamber on an inverted Nikon TE300 fluorescence microscope, stimulated with field electrodes and the images collected at 10 Hz under epifluorescence illumination with a xenon lamp,

distinct filter sets for pHluorin, mCherry, and BFP, a 63X 1.2 N.A. water objective, and QuantEM 512SC CCD camera. Neurons were incubated at room temperature (24° C) in Tyrode's solution containing glutamate receptor antagonists 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μM) and D,L-2-amino-5-phosphonovaleric acid (APV, 50 μM) (Onoa et al., 2010), and imaged for 60 seconds under basal conditions, followed by 45 seconds of stimulation at 30 Hz. Action potentials were generated by passing 1 ms bipolar current pulses through platinum-iridium electrodes to yield fields of 5-10 V/cm. After stimulation, Tyrode's solution containing NH_4Cl (in mM: 69 NaCl, 2.5 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 50 NH_4Cl, 30 glucose, 25 HEPES, pH 7.4) was used to reveal the total fluorescence of pHluorin-tagged protein. NPY-pHluorin events, detected as an abrupt increase in fluorescence, were scored manually in axons identified based on the presence of synaptophysin-mCherry$^+$ puncta. The movies were analyzed using MetaMorph.