Supporting Information

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SI Materials and Methods

Cell Culture, Transfections, Immunofluorescence Microscopy, Image Analysis, and Immunoblot Analyses. Unless otherwise specified, these procedures were done as described by us recently (1). For Mn^{2+} treatment, freshly prepared $MnCl_2$ was added to the medium at the concentrations indicated in the figure legends (1). Small interfering RNA (siRNA) against SPCA1 targeted the sequence 5′-AAGGTTGCACGTTTTCAAA AA-3′ in the SPCA1 cDNA. We have previously characterized the control siRNA (2, 3). The SPCA1-replacement constructs had three silent mutations in the sequence targeted by the siRNA (1, 2). These silent mutations were introduced using the forward primer 5′-GGTGGTGGTGG GGTTAATTAAGAAGGTCGCCCG GTTTCAAAAAAT ACCTAATGG-3′. The mutated bases in the replacement construct are underlined.

Constructs. HA-tagged SPCA1-WT construct was from Rajini Rao (The Johns Hopkins Medical School, Baltimore, MD). Point mutations in SPCA1-WT were introduced using QuikChange mutagenesis kit (Strategene) and confirmed by sequencing. Substitution of the HA-tag with a single myc epitope was done using a loop-out/loop-in modification of the QuikChange protocol. GFP-tagged Rab5-WT, Rab5-S34N, Rab5-Q79L, Rab7-WT, and Rab7-T22D constructs used in this study have been described by us previously (1).

Tertiary Structure of SPCA1. Tertiary structure of SPCA1 was derived using the algorithms of the MODBASE server (http://modbase.compbio.ucsf.edu/modbase-cgi/index.cgi; ref. 4) on the basis of homology with SERCA (5).

Preparation of Golgi Membranes by Sucrose Gradient Flotation. Purification of Golgi membranes was done essentially as described previously (6, 7). HeLa cells from five 10-cm plates were harvested in cold PBS (1 mL per plate) and cells were pelleted by centrifugation at 1,040 rpm for 2 min. The pellet was transferred to 400 μL of homogenization buffer (0.25 M sucrose, 1 mM Tris-Cl; pH 7.4, 1 mM EDTA) and passed through a 25-gauge needle ~50 times. The postnuclear supernatant (PNS) was obtained by centrifugation at 2,500 rpm for 3 min. The PNS was adjusted to 1.6 M sucrose and loaded on a step gradient consisting of cushion of 2 M sucrose at the bottom (1.1 mL), overlaid sequentially with 1.1 mL of the 1.6-M load, 1.1 mL of 1.2 M sucrose, and 0.55 mL of 0.8 M sucrose. Sucrose solutions used in the gradient contained 10 mM Tris-Cl; pH 7.4, and 1 mM $MgCl_2$. The gradient was spun in a Sw50.1 rotor (Beckman Coulter) at 38,000 rpm for 4 h at 4 °C. Fractions at the 0.8 M/1.2 M sucrose interface containing the Golgi membranes were pooled, resuspended in 4 mL of 0.25 M sucrose, 10 mM Tris-Cl; pH 7.4 and 1 mM $MgCl_2$ and sedimented by spinning in the Sw50.1 rotor at 38,000 rpm for 30 min at 4 °C. The Golgi membrane pellet obtained was then resuspended at a final protein concentration of 0.1 mg/mL in 0.25 M sucrose, 10 mM Hepes; pH 6.7, 150 mM KCl, 5 mM NaN_3, 5 mM $MgCl_2$.

Golgi $^{54}Mn^{2+}$ Transport Assay. The in vitro uptake of $^{54}Mn^{2+}$ into Golgi vesicles was done as described previously (8). Pooled Golgi membranes (10 μL), corresponding to 1 μg of protein, were added to 50 μL of transport buffer [10 mM Hepes; pH 6.7, 150 mM KCl, 5 mM NaN_3, 5 mM $MgCl_2$, 25 μM carbonyl cyanide 3-chlorophenylhydrazone (CCCP), 1 mM ATP, 1.6 μCi/mL $^{54}Mn^{2+}$]. $^{54}Mn^{2+}$ was from PerkinElmer. This buffer was supplemented with 100 μM $CaCl_2$ or 100 μM $MnCl_2$ for competition with nonradioactive Ca^{2+} or Mn^{2+}, respectively. Uptake was carried out for 5 min at room temperature. The reaction was stopped by transferring the tubes to ice and adding 60 μL of stop buffer (10 mM Hepes; pH 9.0, 150 mM KCl, 10 mM EDTA). Golgi vesicles were retrieved by spinning at 15,000 rpm on a tabletop centrifuge for 10 min at 4 °C. We confirmed, by immunoblot, that we could effectively recover all of the input Golgi membranes with this spin. The supernatant was discarded and the pelleted membranes were gently washed two times in 100 μL of stop buffer and subsequently lysed in 100 μL of lysis buffer (1% Triton X-100, 0.1 N HCl in isopropanol). Radioactivity was then measured using the LS6500 liquid scintillation counter (Beckman Coulter).

Fluorescence-Activated Cell Sorting (FACS). To obtain identical numbers of cells with nearly 100% transfection of SPCA1-WT or Q747A in a transient transfection system, we cotransfected cultures with a GFP-tagged construct (Rab5-WT) and SPCA1-WT or Q747A. One day after cotransfection, identical numbers of GFP positive cells were sorted for each group using FACS (Vantage SF; Becton Dickinson). We obtained >98% cotransfection of Rab5 and the SPCA1 constructs and verified that expression of Rab5-WT-GFP did not alter the localization of SPCA1-WT or Q747A. Further, the expression levels of SPCA1-WT and Q747A were similar to each other and GPP130 was degraded in cotransfected cells expressing the Q747A mutant but not WT. FACS sorted cells were used for $^{54}Mn^{2+}$ uptake assays in permeabilized and intact cells and for Mn^{2+} toxicity experiments.

$^{54}Mn^{2+}$ Uptake in Permeabilized Cells. Uptake of $^{54}Mn^{2+}$ in permeabilized cells was done essentially as described previously (9, 10). For each assay, 8,000 cells were sorted on a coverslip placed in a well of a 24-well plate. One day after sorting, cells were permeabilized for 15 min at room temperature in 30 mM imidazole; pH 6.8, 120 mM KCl, 1 mM $MgCl_2$, 1 mM ATP, and 30 μg/mL saponin. Cells were then washed two times with PBS and incubated for 30 min in 30 mM imidazole; pH 6.8, 120 mM KCl, 1 mM $MgCl_2$, 5 mM ATP, and 0.5 μCi/mL of $^{54}Mn^{2+}$. For competition with nonradioactive Mn^{2+}, this buffer was supplemented with 100 μM $MnCl_2$. After 30 min, cells were washed two times in PBS+ 10 mM EDTA and harvested in 250 μL of lysis buffer (1% Triton X-100, 0.1 N HCl in isopropanol). Radioactivity was measured by liquid scintillation.

$^{54}Mn^{2+}$ Import/Export Assays in Intact Cells. As in the permeabilized cell assay, import and export of $^{54}Mn^{2+}$ was assayed using 8,000 FACS sorted cells. Uptake was performed at 37 °C with 0.5 μCi/mL of $^{54}Mn^{2+}$ in 500 μL of HBSS with $NaHCO_3$ without phenol red (Sigma-Aldrich) per coverslip. After uptake, cells were washed two times in PBS+ 10 mM EDTA and either directly harvested (see below) or used for the chase part of the experiment. Before the chase, cells were rapidly washed one more time with 250 μL of PBS (without EDTA) and this wash was collected and used to measure background radioactivity nonspecifically present on the coverslip. Cells were then transferred to 500 μL HBSS without $^{54}Mn^{2+}$ and incubated for 30 min. After this, the HBSS was collected and the radioactivity obtained from the last PBS wash was subtracted from that obtained in this sample and represented the total radioactivity secreted. To measure intracellular radioactivity, cells were again washed two times with PBS+ 10 mM EDTA and harvested in lysis buffer (1% Triton X-100, 0.1 N HCl in isopropanol). Competition with non-

radioactive Mn^{2+} was performed with 100 μM $MnCl_2$. Where indicated, cells were pretreated with brefeldin A (10 μg/mL, ref. 11) for 15 min before being exposed to $^{54}Mn^{2+}$.

Viability Assays. The methylthiazolylphenyl-tetrazolium bromide (MTT; EMD Chemicals) assay was performed using 20,000 FACS sorted cells as described by us previously (1). Propidium iodide was from Sigma-Aldrich and was used as described by the manufacturer. Briefly, cells were cotransfected with SPCA1-WT or Q747A and a transfection marker (mCherry or GFP-tagged Rab5-WT). One day after transfection, cultures were either exposed to 1 mM Mn^{2+} or left untreated. Sixteen hours after exposure to Mn^{2+}, cells were washed two times in binding buffer (10 mM Hepes; pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 1.8 mM $CaCl_2$) and then incubated in 1% propidium iodide in binding buffer for 15 min at room temperature. At the end of the incubation period, cells were again washed in binding buffer and immediately mounted for microscopy. Images were collected as described previously (12).

VSVG-GFP Trafficking Assays. Trafficking of VSVG-GFP was analyzed as described earlier (2, 3). Cells were transfected with VSVG-GFP alone or in combination with SPCA1-Q747A for 24 h and then shifted to 40 °C for 16 h. Cultures were then shifted to 32 °C for 20, 40, and 60 min. At the end of the time course, cultures were incubated with an antibody against the ectodomain of VSVG (2, 3) on ice for 30 min. Subsequently, cultures were fixed, stained, and imaged.

Antibodies and Chemicals. Polyclonal (pAb) and monoclonal (mAb) antibodies against GPP130, pAb against giantin and GP73, and mAb against myc and VSVG have been described by us (1, 3, 11, 12). Monoclonal antibodies against HA epitope and tubulin were from Sigma-Aldrich and that against LAMP-2 was from Abcam. All chemicals were from Sigma-Aldrich unless otherwise specified.

Statistical Analyses. Comparison between two groups was performed using two-tailed Student's *t* test assuming equal variances. Multiple group comparison at the same time was done using single-factor analysis of variance (ANOVA) with the Tukey–Kramer post hoc test (NCSS 2007 software). Asterisks in the bar graph indicates $P < 0.05$.

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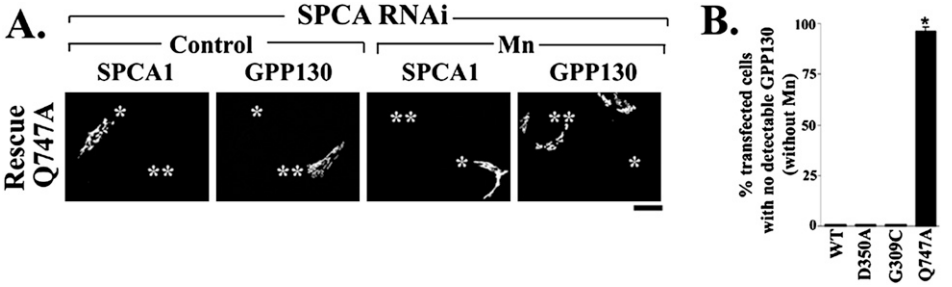

Fig. S1. (*A*) HeLa cells treated with anti-SPCA1 siRNA for 48 h were transfected with RNA-resistant HA-tagged SPCA1-Q747A and, after 24 h, were exposed to 500 μM of Mn^{2+} for 4 h and stained using anti-HA (to detect SPCA1) and anti-GPP130. GPP130 was degraded in cells expressing SPCA1-Q747A after knockdown of endogenous SPCA1 without the addition of Mn^{2+} (single asterisk). Cells not expressing the rescue construct did not exhibit a loss of GPP130 even after Mn^{2+} treatment thus verifying knockdown efficiency (double asterisk). (Scale bar, 10 μm.) (*B*) Quantitation of percentage of transfected cells that did not have detectable GPP130 after SPCA1 knockdown and without treatment with Mn^{2+} (mean ± SE; n = 100 cells per experiment from three independent experiments; $P < 0.05$).

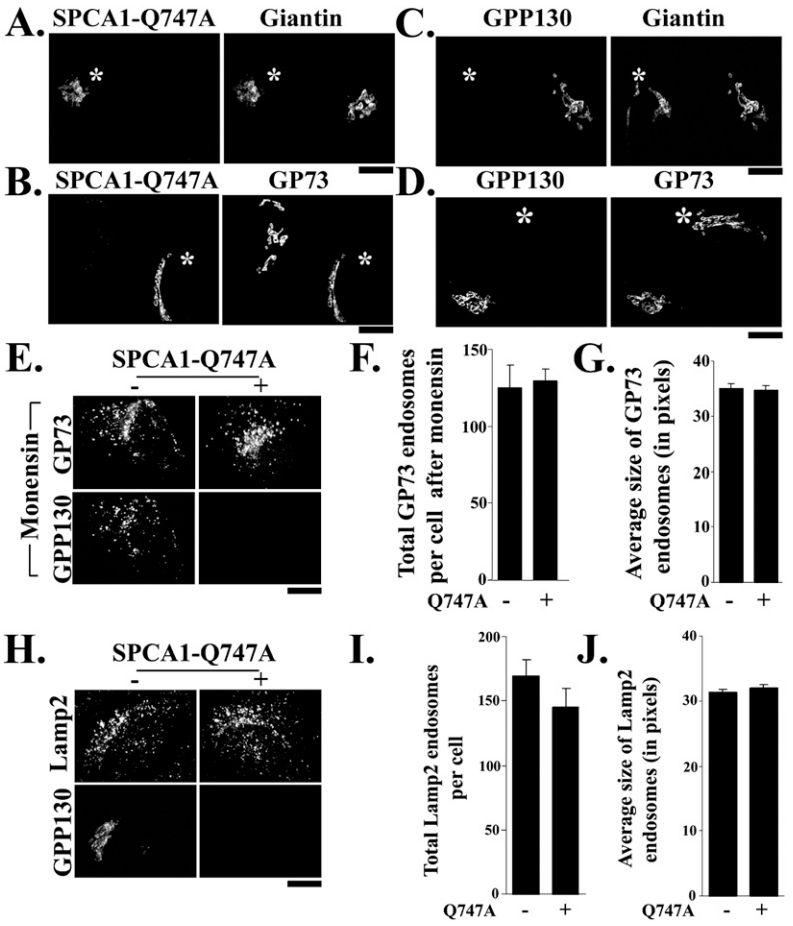

Fig. S2. (*A*–*D*) HeLa cells were transfected with HA-SPCA1-Q747A and imaged 24 h later to detect HA and giantin or GP73 in *A* and *B*, respectively, or GPP130 and giantin, or GP73 in *C* and *D*. Single asterisk denotes Q747A transfected cells. (Scale bar, 10 μm.) (*E*) HeLa cells were transfected with SPCA-Q747A for 24 h. Controls were untransfected. Cultures were then exposed to 10 μM of monensin for 30 min, fixed, and stained to detect GPP130 and GP73. (Scale bar, 10 μm.) (*F* and *G*) Quantitation of average number of GP73 endosomes per cell and average size of each GP73 endosome in cells expressing SPCA1-Q747A and controls was obtained using the Analyze Particle function of ImageJ (mean ± SE; $n = 10$ cells per condition; $P > 0.05$. The experiment was independently replicated three times). (*H*) HeLa cells were transfected with SPCA1-Q747A for 24 h. Controls were untransfected. Cultures were fixed and stained to detect LAMP-2 and GPP130. (Scale bar, 10 μm.) (*I* and *J*) Quantitation of LAMP-2 endosomes was done as described for *F* and *G* above (mean ± SE; $n = 10$ cells per condition; $P > 0.05$. The experiment was independently replicated three times).

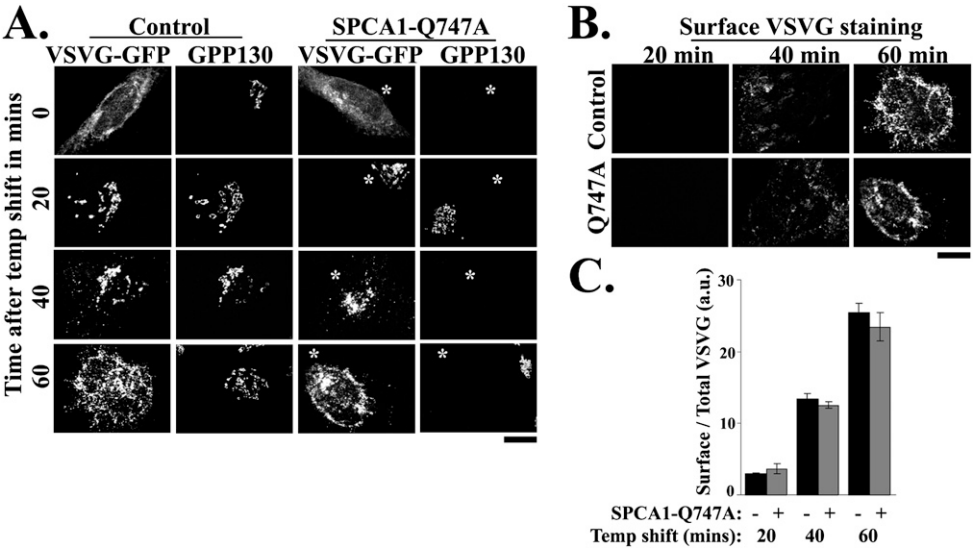

Fig. S3. (*A*) The VSVG-GFP transport assay was performed as described in *Materials and Methods*. Cells were stained and imaged to detect GFP, GPP130, and VSVG. *A* shows only the GFP and GPP130 channels for each time point depicted. Asterisks denote SPCA1-Q747A transfected cells. (Scale bar, 10 μm.) (*B*) Surface VSVG staining for the 20-, 40-, and 60-min time points depicted in *A*. (Scale bar, 10 μm.) (*C*) Quantitation of the ratio of the average surface to total VSVG staining per cell (mean ± SE; $n = 10$ cells per condition per time point; $P > 0.05$ for the difference in surface/total VSVG ratio between control and SPCA1-Q747A at each time point. The experiment was replicated three times, independently).

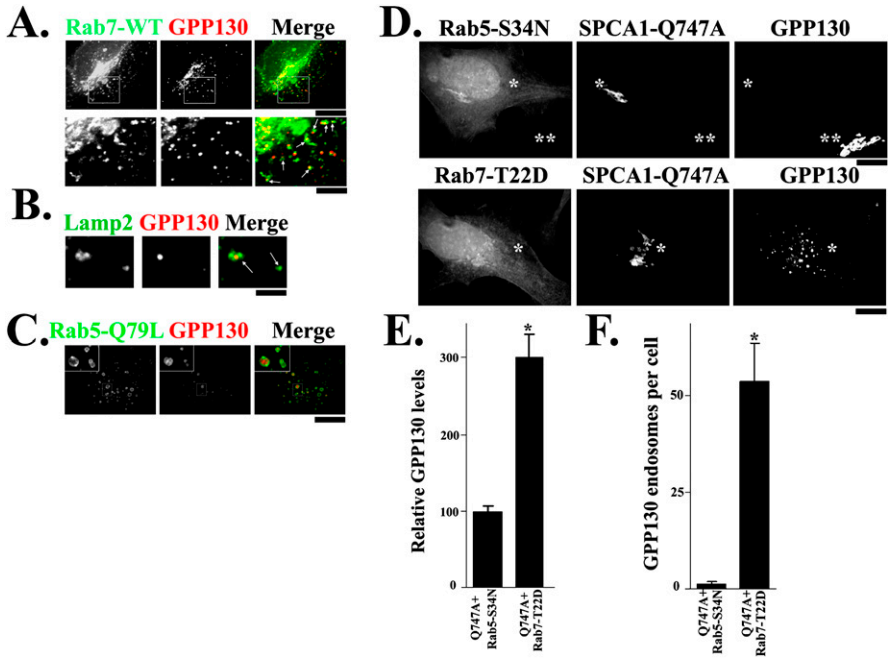

Fig. S4. (*A*) Cells were cotransfected with Rab7-WT-GFP and HA-SPCA1-Q747A for 24 h and imaged to detect GPP130 and GFP. Cotransfection frequency was >98%. GPP130 was detectable in peripheral punctae only in GFP positive cells. (Scale bar in main panel, 10 μm; scale bar for inset, 2 μm.) (*B*) Cells were transfected with SPCA1-Q747A and 24 h later imaged to detect LAMP-2 and GPP130. (Scale bar, 2 μm.) (*C*) Cells were cotransfected with Rab5-Q79L and SPCA1-Q747A for 24 and imaged to detect GPP130 and GFP. Note that transfection of Rab5-Q79L alone does not target GPP130 to Rab5-MVBs (1). (Scale bar, 10 μm; inset, 2.5×). (*D*) Cells were cotransfected with HA-SPCA1-Q747A and either Rab5-S34N or Rab7-T22D and imaged 24 h later to detect GFP, HA, and GPP130. Cotransfection was >98%. GPP130 was not detectable in cotransfected cells expressing Rab5-S34N (single asterisk), whereas it remained Golgi localized in untransfected cells in the same culture (double asterisk). Cotransfected cells expressing Rab7-T22D (single asterisk) exhibited large cytoplasmic punctae positive for GPP130. Transfection of the dominant negative Rab constructs by themselves do not alter the localization of GPP130 (1). (Scale bar, 10 μm.) (*E*) Quantitation of mean GPP130 levels per cell after transfection of Rab5-S34N or Rab7-T22D and SPCA1-Q747A from *D* above. Mean GPP130 level in Rab5-S34N cells is normalized to 100 (mean ± SE; $n = 10$ cells from one experiment per group, the experiment was independently replicated three times; $P < 0.05$). (*F*) Quantitation of total GPP130 particles per cell in cotransfected cells from *D* above (mean ± SE, $n = 15$ cells from one experiment per group, the experiment was replicated three times independently, $P < 0.05$). Particles were counted using National Institutes of Health Image J as described previously (1).

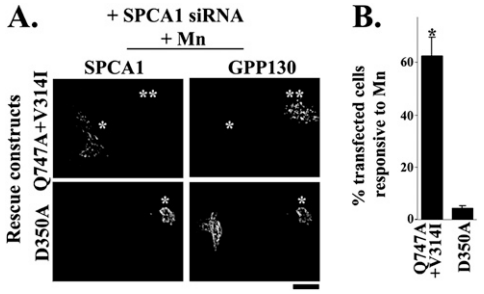

Fig. S5. (*A*) Cells treated with anti-SPCA1 siRNA for 48 h were transfected with RNA-resistant HA-tagged SPCA1-Q747A+V314I or SPCA1-D350A. After 24 h, they were exposed to 500 μM of Mn^{2+} for 4 h and imaged to detect SPCA1 using an anti-HA antibody and GPP130. GPP130 was degraded in cells expressing SPCA1-Q747A+V314I but not SPCA1-D350A (single asterisk). Cells not expressing the rescue construct did not exhibit a GPP130 Mn^{2+} response (double asterisk). (Scale bar, 10 μm.) (*B*) Quantitation of percentage of transfected cells that did not have detectable GPP130 after SPCA1 knockdown and treatment with Mn^{2+} (mean ± SE; n = 50 cells per experiment from three independent experiments; $P < 0.05$).

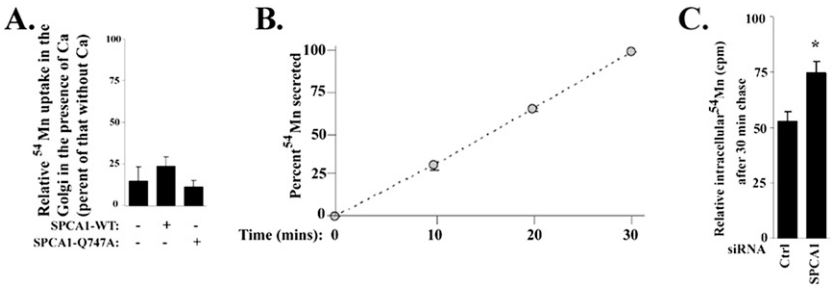

Fig. S6. (*A*) Uptake of $^{54}Mn^{2+}$ by isolated Golgi vesicles was performed as described in *Materials and Methods*. To inhibit the activity of SERCA, 10 μM of thapsigargin was included in the transport buffer (13) and uptake was performed in the presence and absence of excess cold Ca^{2+}. Uptake in the absence of Ca^{2+} was normalized to 100 separately for untransfected, WT, or Q747A and uptake in the presence of Ca^{2+} is expressed relative to that for each condition (mean ± SE; n = 3 transport assay; $P > 0.05$, for the difference in relative uptake between untransfected, WT and Q747A-expressing cells after cold Ca^{2+}). (*B*) HeLa cells were loaded with $^{54}Mn^{2+}$ for 30 min, washed, and chased. The supernatant was collected every 10 min. Total $^{54}Mn^{2+}$ counts released after 30 min was normalized to 100 (mean ± SE; n = 3; $P < 0.05$). (*C*) Control or anti-SPCA1 siRNA treated cells were loaded with $^{54}Mn^{2+}$ for 30 min, washed, and chased for 30 min. Intracellular radioactivity after a 30-min loading was normalized to 100 for both groups. Radioactivity retained after the 30-min chase is expressed relative to that (mean ± SE, n = 3, $P < 0.05$ for the difference in intracellular radioactivity retained). The percentage of retention of intracellular Mn^{2+} in cultures treated with anti-SPCA1 siRNA is ~75%, and not closer to 100%, which can be attributed to incomplete knockdown of SPCA1.

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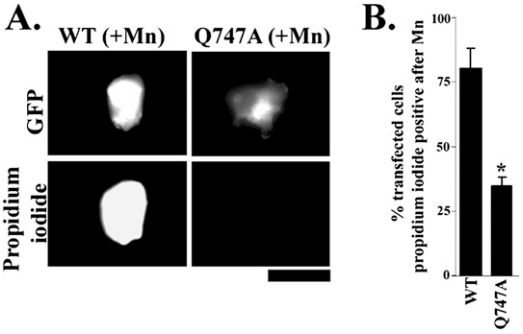

Fig. S7. (*A*) PC-12 cells were cotransfected with GFP and SPCA1-WT or Q747A using the Neon transfection system of Invitrogen for 24 h. Cells were then exposed to 1 mM of Mn^{2+} for 16 h. Controls were untreated. Cell viability was assessed using propidium iodide staining. (Scale bar, 10 μm.) (*B*) Quantitation of percentage of transfected cells positive for propidium iodide from *A* above (mean ± SE, n = 25 cells from three independent experiments per group, $P < 0.05$).