Supplementary Materials and Methods

Antibodies

To generate polyclonal rabbit VAC14 antibody, full length human *VAC14* cDNA was cloned into pMALc2H$_{10}$T (Kristelly et al, 2004). Recombinant MBP-10x His tagged VAC14 protein was purified from E. coli (Rosetta) using Ni-NTA agarose (Qiagen, 30210). The MBP-10x His tag was cleaved with AcTEV proteases (Invitrogen, 12575-015) and the tag removed via Ni-NTA agarose. 0.1% Triton X-100 was added to stabilize VAC14. Purified protein was injected into a rabbit. Serum was affinity purified by enrichment of the IgG fractions with protein A Sepharose-4B (Sigma, P9424). Nonspecific IgGs were depleted on a total *Vac14*$^{-/-}$ column made from Actigel (Sterogene Bioseparations Inc., 2731) conjugated with a total protein extract from *Vac14*$^{-/-}$ fibroblasts. VAC14 specific IgGs were purified on an affinity column with VAC14 protein conjugated to Actigel. Antibodies were eluted with a decreasing pH step gradient: 0.1 M citrate (pH5.0, 4.5, 4.0, 3.5, 3.0) and 0.2 M glycine (pH 2.8). Eluted fractions were analyzed by western blot and pooled. The best fractions were eluted at pH 3.0.

Chicken anti-EEA1 was a gift from Dr. Silvia Corvera (University of Massachusetts Medical School, Worcester, MA). Commercial antibodies: GAPDH antibody (Ambion, AM4300); surface GluA2 antibody (Chemicon, MAB397); C-terminal GluA2 antibody (Rabbit pAb, AB1768, Chemicon); LAMP-1 antibody (University of Iowa Hybridoma Bank, 1D4B); LAMP-2 antibody (University of Iowa Hybridoma Bank, ABL-93); LBPA antibody (Echelon Biosciences, Z-SLBPA); MAP2 antibodies (Santa Cruz, sc-32791, Millipore, AB5622, Sigma-Aldrich, M4403); PSD95 antibody (Thermoscientific, MA1-045); synapsin antibody (Synaptic Systems,

106004), synaptobrevin antibody (Calbiochem, NB07); synaptotagmin antibody (Calbiochem, 573824); TAU-1 antibody (Chemicon, MAB3420); vGlut1 antibody (Millipore, AB5905). All secondary antibodies are from Molecular Probes (Invitrogen, Carlsbad, CA).

Cell culture

Hippocampal neurons were cultured from E14-18 embryos from crosses of *Vac14*$^{+/-}$ heterozygous mice (C57BL/6J × 129/Ola mixed background). Culture medium included neurobasal medium (Invitrogen, 10888022), 1x B27 supplement (Invitrogen, 17504-044), 2 mM L-glutamine (Invitrogen, 25030-081) and 1x antibiotics/antimycotics (Invitrogen, 15240-062). Hippocampi were dissected and digested in 0.25% trypsin (Invitrogen, 25200) for 15 minutes; the reaction was stopped with 0.5 mg/ml soybean trypsin inhibitor (Invitrogen, 17075-029) in PBS. Tissues were triturated in culture medium ~10-15 times. Cells were counted and plated on poly-D-lysine (Sigma P0899) coated coverslips or Mattek dishes (Mattek Corporation, P35G-0-14-C). For experiments requiring distinct single cells, 5, 000 – 20, 000 cells were seeded per 12 mm coverslip (Fisher Scientific, 12-545-82). For electrophysiology experiments, 65 000 cells were seeded per Mattek dish. Media were changed every two days until day 10.

Mouse primary fibroblasts were cultured from P0 pups as described (Zhang et al, 2007).

Immunofluorescence microscopy

A large fraction of VAC14 protein is cytosolic in mammalian cells. To localize membrane bound VAC14, mouse fibroblasts were treated with 0.05% saponin for 1 minute before fixation. Localization of proteins in neurons was performed without this step. Unless noted elsewhere,

cells were fixed in 4% paraformaldehyde for 15 minutes. Cells were blocked in filtered 2% goat serum/2% donkey serum/5% BSA/PBS/0.1% saponin for 1 hour, incubated with primary and then secondary antibodies for 1 hour each and mounted with Prolong Gold Antifade reagent (Invitrogen, P36930). *Vac14*$^{-/-}$ fibroblasts or neurons were used as negative controls in all immunofluorescence experiments using anti-VAC14 antibody. Images were taken on an inverted Olympus FV1000 laser scanning confocal microscope or a Zeiss 510 laser scanning confocal microscope.

For PSD95 experiments, neurons were fixed with 2% paraformaldehyde/2% sucrose for 15 minutes and permeabilized with 0.1% Triton X-100 for 10 minutes.

To label surface GluA2, mouse GluA2 antibody (IgG2a) was incubated with live neurons for 15 minutes. Neurons were washed with cold PBS containing 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$, fixed in 4% paraformaldehyde/4% sucrose, blocked with 2% BSA for 20 minutes and incubated with fluorescent antibody (Goat α M-488). Quantitation of GluA2 puncta was done in IMAGE J. Dendrites were straightened and intensity of individual puncta analyzed using modified IMAGEJ particle analysis macros. All intensities are normalized to the mean of wild-type. To label total GluA2 and Map2, following surface GluA2 labeling, neurons were permeabilized with 0.1% TritonX-100 and incubated with rabbit GluA2 and MAP2 (IgG1, Sigma-Aldrich) antibodies for 1 hr at room temperature, and then incubated with fluorescent secondary antibodies (Goat α IgG2a-488, Goat α IgG_1-555, Goat α Rabbit-647).

To label lysosomes, mouse primary fibroblasts were incubated with 1 mg/ml Texas red Dextran (Invitrogen D1864, Mw 70, 000) for 1 hour and chased in normal medium for 24 hours before fixation.

Western blot analysis

Tissues or brain regions were dissected from P0 pups and homogenized in RIPA buffer (50 mM Tris, pH 7.4; 150 mM NaCl; 1 mM EDTA; 1% deoxycholic acid; 1% NP-40; 0.1% SDS; 10 mM NaF; 1 mM Na_3VO4; 1x protease inhibitor cocktail (Sigma P8215)). Protein concentrations were determined by BCA assay (Thermoscientific 23228). Equal amount of total protein (15 μg) were separated by SDS-PAGE, transferred to nitrocellulose membrane and blotted with anti-VAC14 (1:2000) or anti-GAPDH (1: 300, 000). Quantitation was done with IMAGE J's Gel Analyzer function. All lanes are normalized to the first lane.

Transfection

Neurons were transfected with 0.5-1 μg *Citrine* or *VAC14-Citrine* plasmid (Zhang et al, 2007) with the CalPhos Transfection kit (CloneTech; Mountain View, CA). Experiments were performed 24-48 hrs after transfection.

Electron microscopy

Brain tissue from P0 pups was fixed in 2.5 % glutaraldehyde in 0.1 M Sorensen's buffer for one hour and post-fixed for one hour in 1% osmium tetroxide in the same buffer. Samples were rinsed in double distilled water to remove phosphate and then en bloc stained with aqueous 3% uranyl acetate for one hour. Samples were dehydrated in ascending concentrations of ethanol,

treated with propylene oxide, and embedded in Epon epoxy resin. Semi-thin sections were stained with toluidine blue for tissue identification. Ultra-thin sections (70 nm) of the mid and hind brain were post stained with uranyl acetate and lead citrate. Samples were examined with a Philips CM100 electron microscope at 60 KV, images were captured with a Hamamatsu ORCA-HR digital camera system, and AMT software (Advanced Microscopy Techniques Corp., Danvers, MA).

Image Quantitation

For quantification of VAC14 colocalization with endocytic and autophagic markers, the Puncta Analyzer plugin (gift from Dr. Cagla Eroglu) (Ippolito & Eroglu, 2010) for ImageJ was used. This plugin identifies and counts the numbers of green (VAC14) and red (EEA1, LAMP1, LBPA or LC3) puncta at user-defined thresholds and calculates the number of colocalizing puncta. It also labels colocalized puncta after the analysis so that the result can be validated. Before processing, the nonspecific nuclear staining in the VAC14 channel, if present, was removed. Rolling ball radius method was used to subtract the background. The Puncta Analyzer plug-in is optimized for dual-labeling. Thus, in the VAC14/EEA1/LAMP1 triple label experiments, calculations were performed as follows. First, the number of VAC14 that colocalized with EEA1 (N_{EEA1}) or LAMP1($N_{LAMP1)}$ were measured. Then, the thresholded LAMP1 and EEA1 channels were combined into a single channel and then the number of VAC14 puncta that colocalized with either EEA1 or LAMP1 (N_e) was determined. The number of VAC14 puncta that colocalized with neither marker (N_n) was calculated by: N_n = total VAC14 – N_e. The number of VAC14 puncta that colocalized with both LAMP1 and EEA1 (N_b) was calculated by: Nb = $N_{EEA1} + N_{LAMP1} - N_e$. The number of VAC14 puncta that colocalized with only EEA1 was

calculated by: $N_{EEA1}' = N_{EEA1} - N_b$. The number of VAC14 puncta that colocalized with only LAMP1 was calculated by: $N_{LAMP1}' = N_{LAMP1} - N_b$.

To quantify surface GluA2 puncta with ImageJ, a user written macro was used. Dendrites were straightened with ImageJ's Straighten plugin. Straightened dendrite images were made binary with user-defined threshold and then treated with the watershed filter. Puncta were identified by IMAGEJ's "analyze particles..." command. The integrated intensity values were calculated from the original pixels that lie within each punctate region. This macro is available upon request.

To quantify total internalized GluA2, regions of interest (soma or dendrites) were measured directly with ImageJ. Soma were selected freehand. Apical dendrites were straightened and a fixed length (35 μm) was used. Area and integrated intensity were measured with IMAGEJ. Intensity values were normalized to the area.

Supplementary Legend

Figure S1. VAC14 is widely distributed in all tissues and brain regions tested.

(A) Polyclonal rabbit anti-VAC14 antibody specifically recognizes VAC14 on western blots. Brain extracts from P0 wild-type and *Vac14*$^{-/-}$ pups were homogenized and detected by western blot analysis using anti-VAC14.

(B) VAC14 is expressed in all tissues tested. Brain, heart, lung, liver and kidney were dissected from P0 pups. Western blots were probed with affinity purified rabbit anti-VAC14 antibody.

(C) VAC14 is found in all tested regions of the brain.

(B-C) *Vac14*$^{-/-}$ littermates indicate the absence of VAC14 in all *Vac14*$^{-/-}$ tissues. Blots shown are representative of 3 independent experiments. VAC14 expression levels were quantified using the ratio of VAC14/GAPDH band densities and normalized to the first lane. Error bars, STD.

Figure S2. Vacuoles in cultured *Vac14*$^{-/-}$ hippocampal neurons.

(A) *Vac14*$^{-/-}$ neurons form vacuoles as early as 1 DIV. Starting from 12 DIV, vacuoles were frequently observed in both the soma and the neurites (arrows).

(B) Wild-type and *Vac14*$^{-/-}$ neurons were fixed and labeled with LAMP1 or EEA1. Vacuoles in *Vac14*$^{-/-}$ neurons (arrows) are positive for LAMP1 (late endosomal and lysosomal marker) but not EEA1 (early endosomal marker).

(C) Vacuolation in neurons is not rescued by suppressing neuronal activity. Hippocampal neurons from wild-type or *Vac14*$^{-/-}$ embryos were treated with 2 μM TTX, 20 μM APV and 40 μM CNQX starting from 3 DIV. Media were changed every other day in both drug treated and control dishes. Neurons were imaged at 18 DIV. (A-C) Bar = 10 μm.

Figure S3. *Vac14*$^{-/-}$ neurons form vacuoles in culture, yet arborization is similar to wild-type neurons. (A) Examples of Stage I-V hippocampal neurons. Hippocampal neurons start out as fibroblast-like lamellipodia (Stage I). Then several short neurites emerge (Stage II). One extends further than the others and commits to the fate of an axon (Stage III). The other neurites (dendrites) extend further and form complicated networks (Stage IV and V). Bar = 10 μm. (B) *Vac14*$^{-/-}$ neurons undergo normal arborization. Cultured wild-type and *Vac14*$^{-/-}$ neurons were plated at a density that allowed visualization of single neurons (5, 000 to 10, 000 per 1.91 cm^2), and randomly selected neurons were imaged every twenty-four hours by phase contrast microscope. The numbers of neurons at different stages were counted and percentage calculated. N = 50-80 neurons for each day. Similar results were obtained in three independent experiments.

Figure S4. Polyclonal rabbit anti-VAC14 antibody specifically recognizes VAC14 in fixed cells.

(A) Immunofluorescence with anti-VAC14. Puncta in the cytoplasm were observed in wild-type but not *Vac14*$^{-/-}$ cells. Nonspecific nuclear staining was frequently present in cells cultured from both wild-type and *Vac14*$^{-/-}$ mutant mice. Bottom panels, DIC images.

(B, C, D, E) *Vac14*$^{-/-}$ controls for Figure 2 B, C, D, F, respectively. Bar = 10 μm.

(F-G) Quantitation of VAC14 colocalization with endocytic and autophagic markers.

(F) Percentages of VAC14 puncta that colocalized with EEA1, LAMP1, neither marker or both markers were quantified from triple labeling of VAC14/EEA1/LAMP1 immunofluorescence (N = 11 cells). Error, STD.

(G) Percentages of VAC14 puncta that colocalized with LBPA, Dextran or LC3 were quantified from double labeling of VAC14 and the marker of interest (N= 7 for LBPA, 16 for Dextran, 15 for LC3). Error bars, STD.

Figure S5. VAC14 distribution in the endomembrane system.

Diagram of proposed PI(3,5)P_2 distribution in the endomembrane system. Based on the localization of endogenous VAC14, PI(3,5)P_2 localizes on early endosomes, late endosomes, lysosomes, and possibly autophagosomes. CCV, clathrin coated vesicles; EE, early endosomes; LE, late endosomes, RE, recycling endosomes; AP, autophagosomes; AL, autolysosomes; TGN, trans-Golgi network.

Figure S6. VAC14 partially colocalizes with EEA1 or LAMP2 in the soma.

Wild-type and *Vac14*$^{-/-}$ neurons were double labeled with rabbit anti-VAC14 and chicken anti-EEA1 (A) or rat anti-LAMP2 (B). Arrows; colocalization between VAC14 and EEA1 or LAMP2. Bar = 5 μm.

Figure S7. Polyclonal rabbit anti-VAC14 antibody specifically recognizes VAC14 in fixed neurons. (A-B) *Vac14*$^{-/-}$ controls for Figure 2 A-B respectively.

(C) Quantitation of VAC14 puncta colocalization with endocytic markers in neurites. Percentages of VAC14 puncta that colocalized with EEA1 and LAMP1were quantified from triple labeling of VAC14/EEA1/LAMP1 immunofluorescence (N = 12 neurites). Error, STD.

(D-E) *Vac14*$^{-/-}$ controls for Figure 2 C-D, respectively.

(A) Bar = 10 μm. (B, D, E) Bar = 5 μm.

Figure S8. Total expression of GluA2 is similar.

Cultured hippocampal neurons at 14 DIV were collected, lysed and total protein separated by SDS-PAGE. To confirm linearity of the assay, a series of dilutions of WT animal B was run on the same gel. Membranes were probed with anti-GluA2 and anti-tubulin antibodies.

Figure S9. No defects were observed in the entry of internalized GluA2 into the degradation pathway in *Vac14*$^{-/-}$ neurons. Hippocampal neurons from wild-type or *Vac14*$^{-/-}$ embryos were treated with the lysosomal inhibitor leupeptin then live labeled with GluA2 antibodies. Endocytosis was stimulated with 50 μM NMDA for 10 minutes. Surface bound GluA2 antibodies were removed with an acid wash. Neurons were then fixed and labeled with LAMP1 antibodies. The percentage of internalized GluA2 puncta that colocalized with LAMP1 was calculated from a fixed length of apical dendrites (the first 35 μm dendrites from soma) (N = 57 for wild-type and 61 for *Vac14*$^{-/-}$. $p = 0.63$. t-test). Bars, 5 μm.

Supplementary Reference

Ippolito DM, Eroglu C (2010) Quantifying synapses: an immunocytochemistry-based assay to quantify synapse number. *Journal of Visualized Experiments: JoVE*

Kristelly R, Gao G, Tesmer JJG (2004) Structural Determinants of RhoA Binding and Nucleotide Exchange in Leukemia-associated Rho Guanine-Nucleotide Exchange Factor. *Journal of Biological Chemistry* **279:** 47352-47362

Zhang Y, Zolov SN, Chow CY, Slutsky SG, Richardson SC, Piper RC, Yang B, Nau JJ, Westrick RJ, Morrison SJ, Meisler MH, Weisman LS (2007) Loss of Vac14, a regulator of the signaling lipid phosphatidylinositol 3,5-bisphosphate, results in neurodegeneration in mice. *Proceedings of the National Academy of Sciences of the United States of America* **104:** 17518-17523

Figure S1

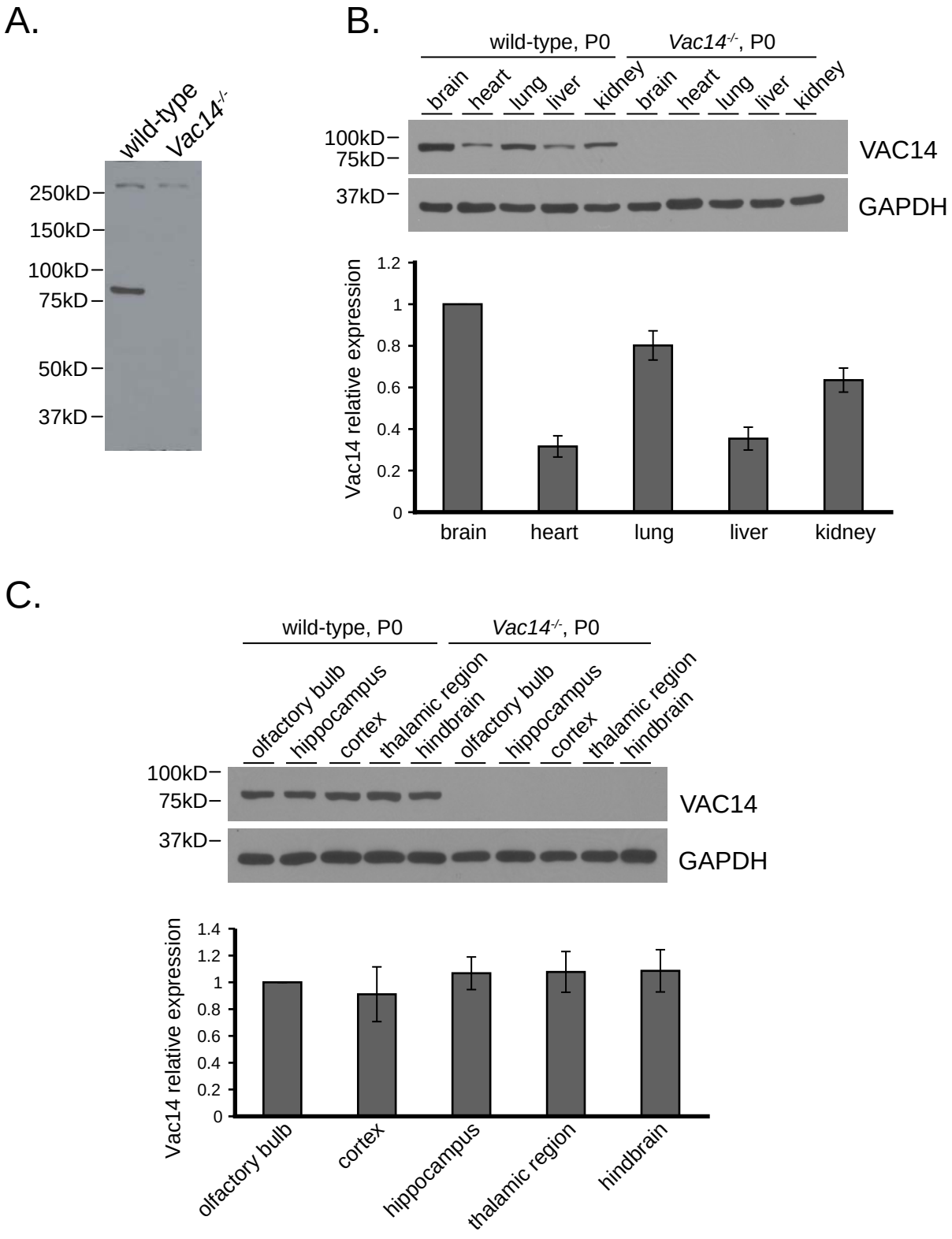

A.
wild-type
Vac14-/-
250kD
150kD
100kD
75kD
50kD
37kD
B.
wild-type, P0
Vac14-/-, P0
brain
heart
lung
liver
kidney
100kD
75kD
37kD
VAC14
GAPDH
Vac14 relative expression
1.2
1
0.8
0.6
0.4
0.2
0
C.
wild-type, P0
Vac14-/-, P0
olfactory bulb
hippocampus
cortex
thalamic region
hindbrain
100kD
75kD
37kD
VAC14
GAPDH
Vac14 relative expression
1.4
1.2
1
0.8
0.6
0.4
0.2
0

Figure S2

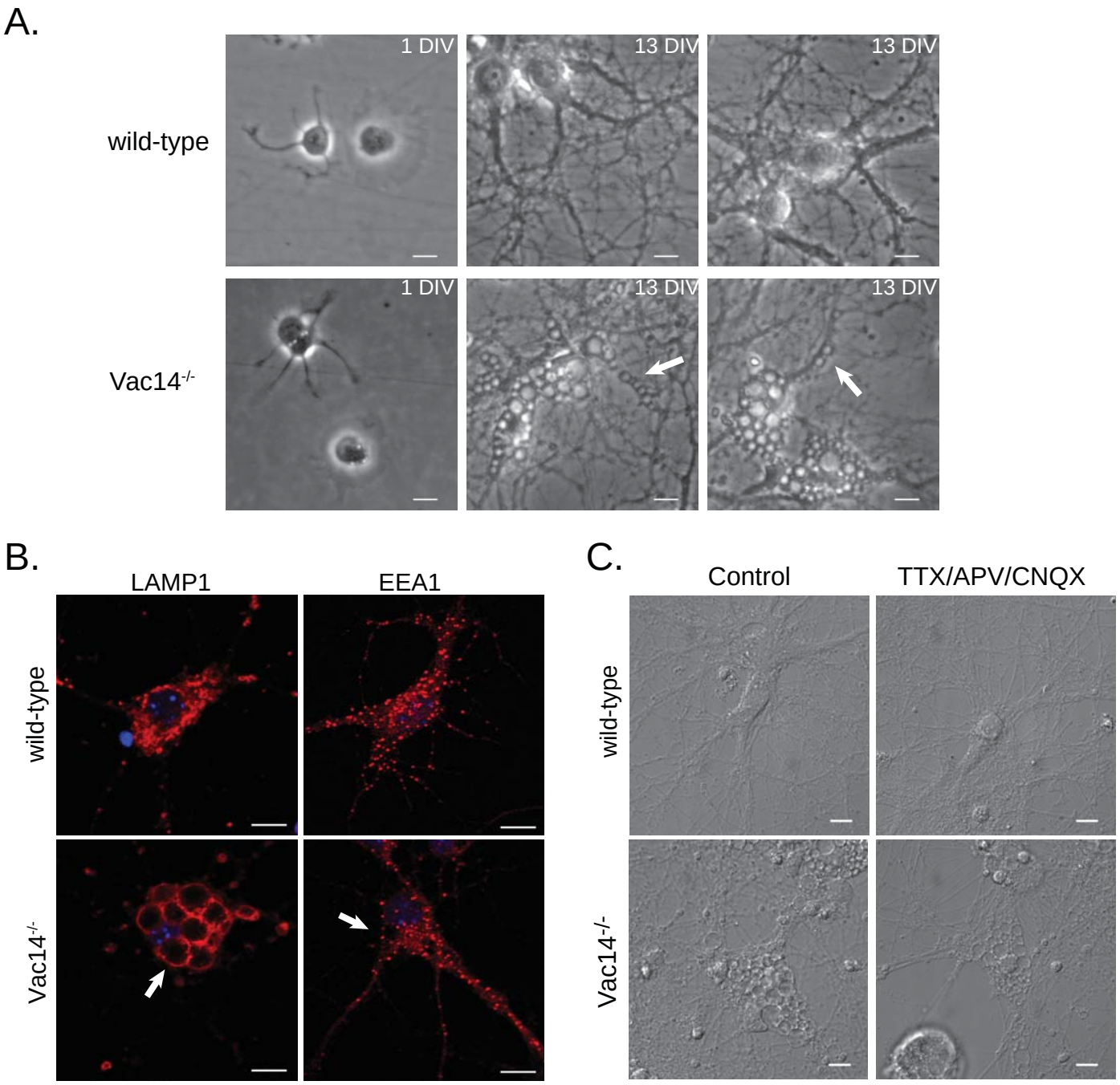
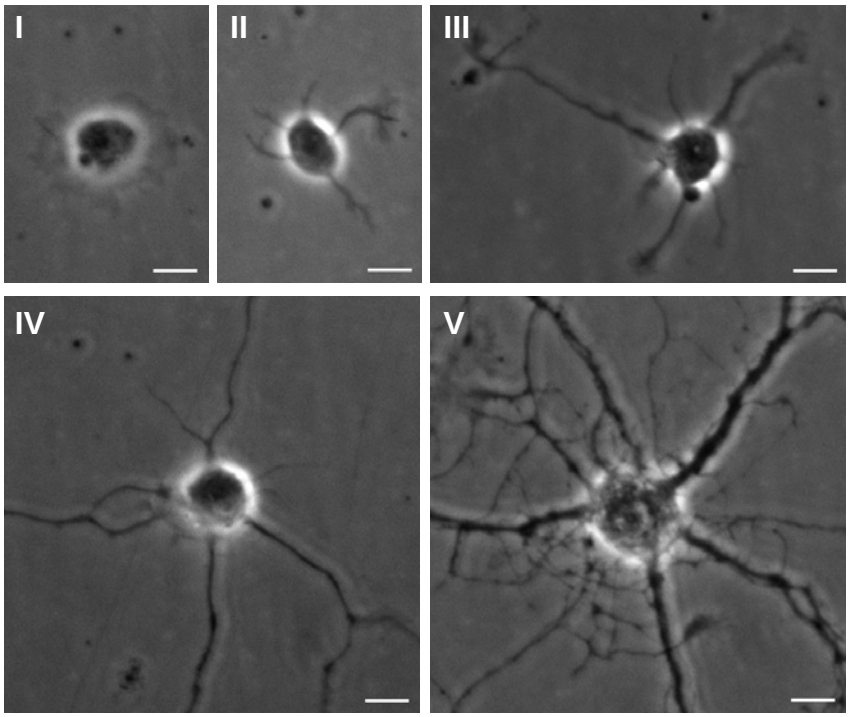

A.
wild-type
Vac14-/-
1 DIV
13 DIV
13 DIV
1 DIV
13 DIV
13 DIV
B.
LAMP1
EEA1
wild-type
Vac14-/-
C.
Control
TTX/APV/CNQX
wild-type
Vac14-/-

Figure S3

A.


I
II
III
IV
V

B.

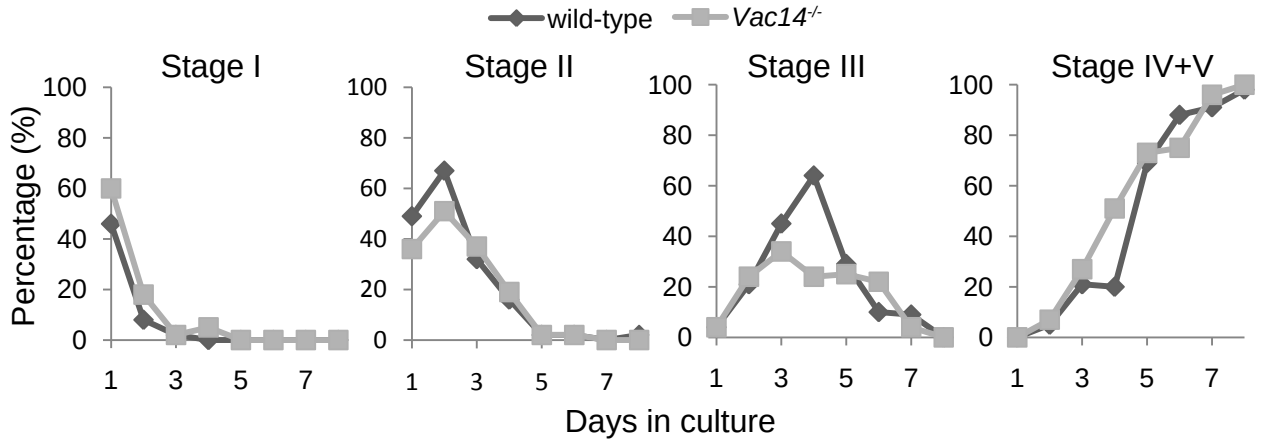

wild-type
Vac14-/-
Stage I
Stage II
Stage III
Stage IV+V
Percentage (%)
100
80
60
40
20
0
1
3
5
7
Days in culture

Figure S4

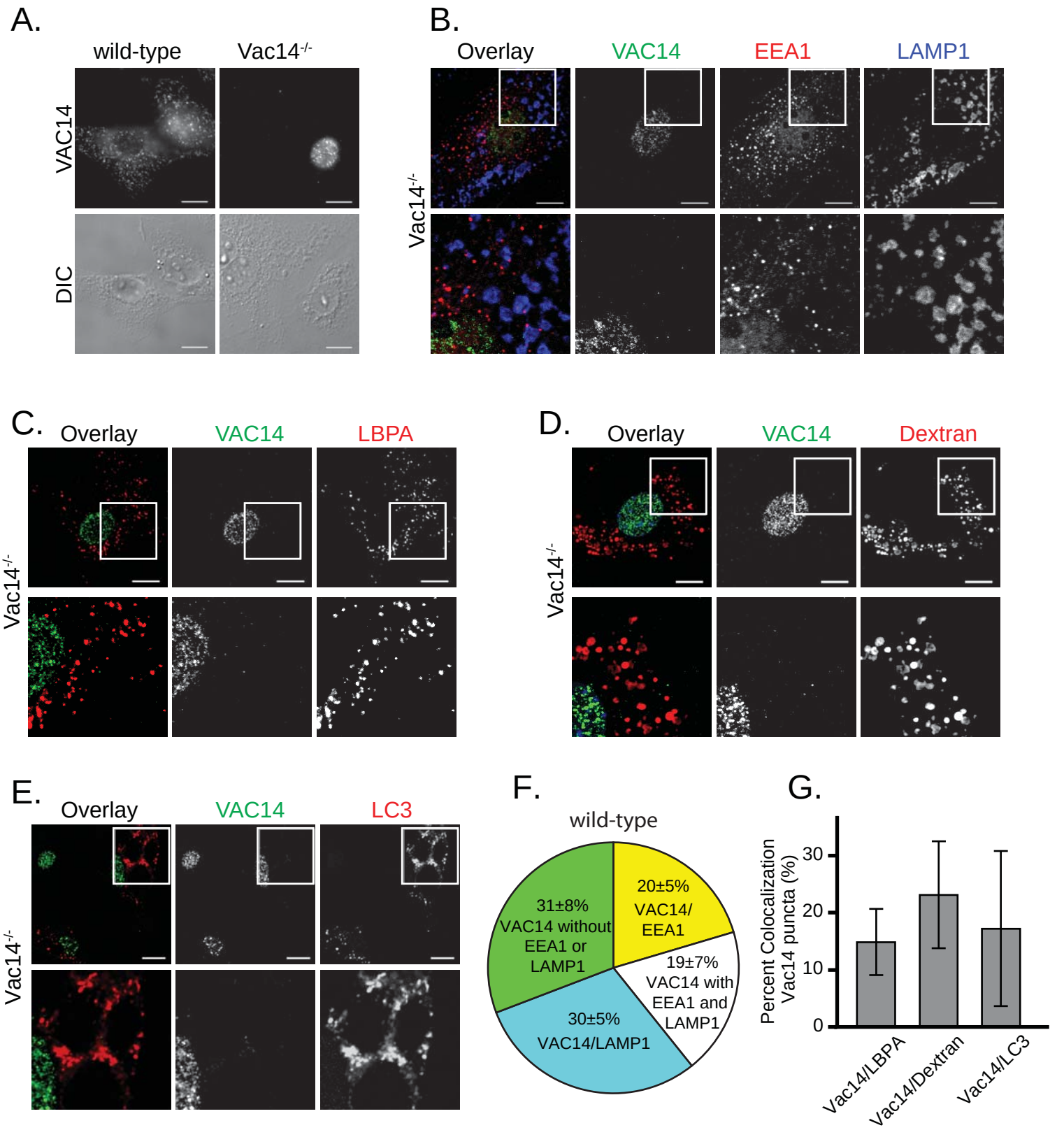

A.
wild-type
Vac14-/-
VAC14
DIC
B.
Overlay
VAC14
EEA1
LAMP1
Vac14-/-
C.
Overlay
VAC14
LBPA
Vac14-/-
D.
Overlay
VAC14
Dextran
Vac14-/-
E.
Overlay
VAC14
LC3
Vac14-/-
F.
wild-type
20±5%
VAC14/
EEA1
19±7%
VAC14 with
EEA1 and
LAMP1
30±5%
VAC14/LAMP1
31±8%
VAC14 without
EEA1 or
LAMP1
G.
Percent Colocalization
Vac14 puncta (%)
0
10
20
30
Vac14/LBPA
Vac14/Dextran
Vac14/LC3

Figure S5

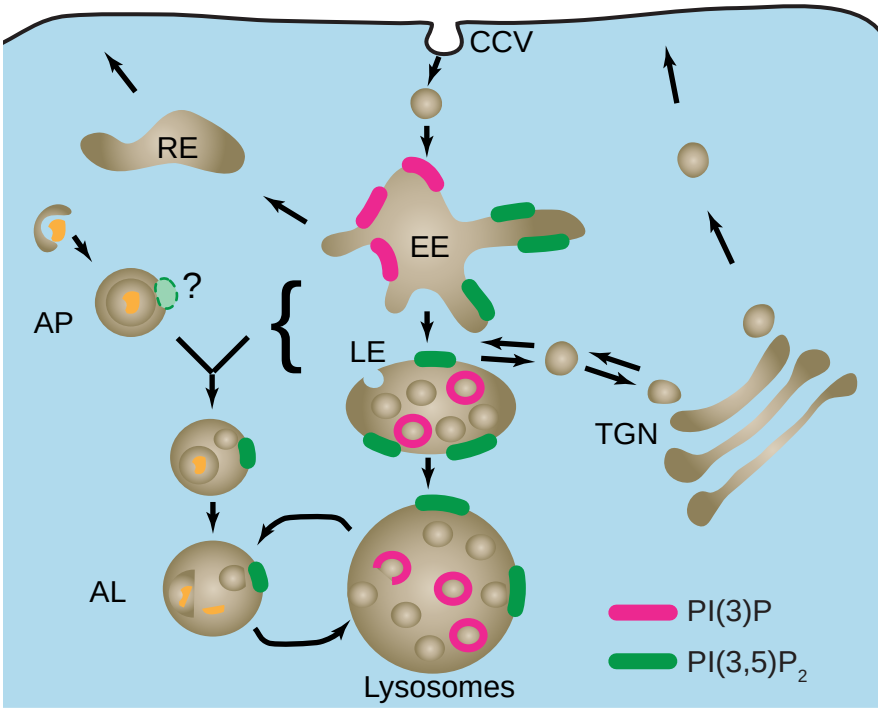


Figure S6

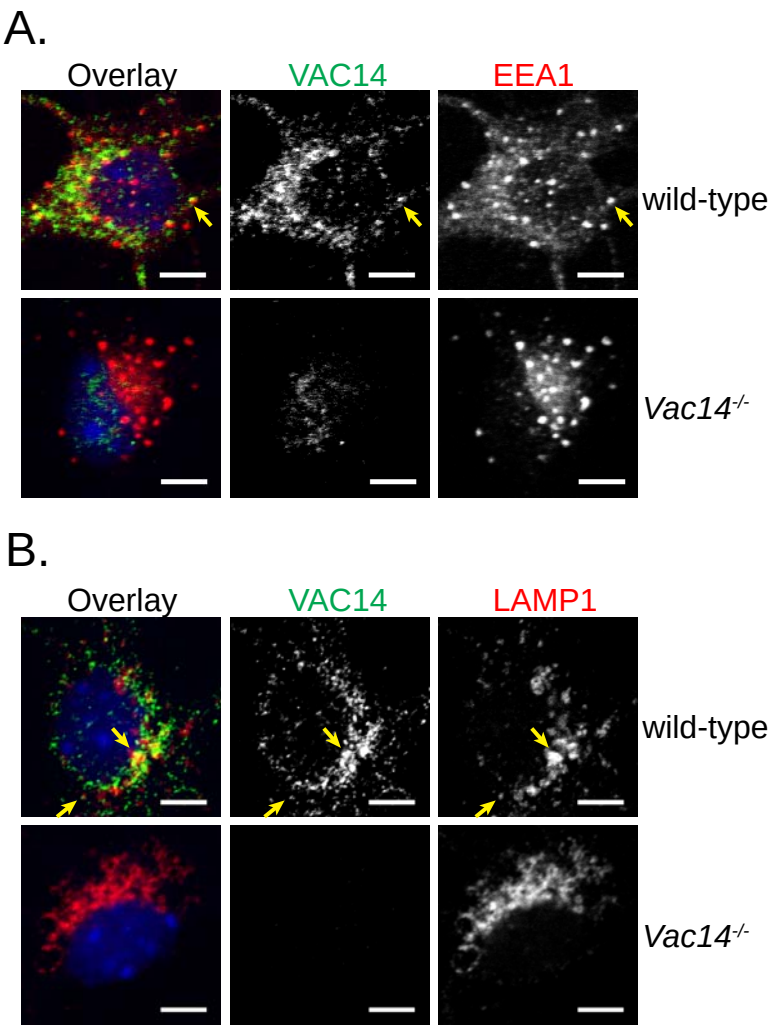

A.
Overlay
VAC14
EEA1
wild-type
Vac14-/-
B.
Overlay
VAC14
LAMP1
wild-type
Vac14-/-

Figure S7

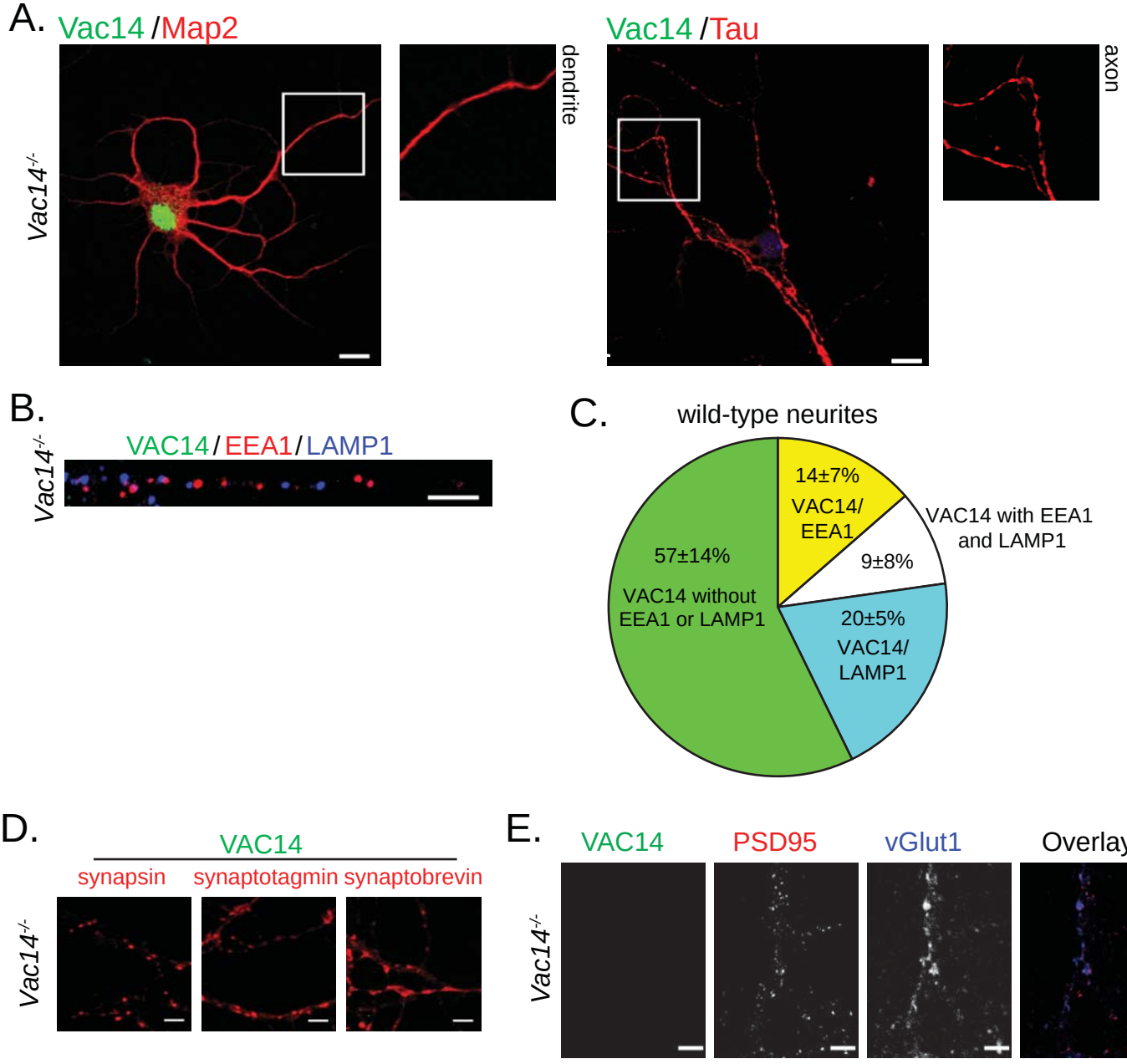

A.
Vac14 /Map2
Vac14 /Tau
dendrite
axon
Vac14-/-
B.
VAC14 / EEA1 / LAMP1
Vac14-/-
C.
wild-type neurites
14±7%
VAC14/
EEA1
VAC14 with EEA1
and LAMP1
9±8%
57±14%
VAC14 without
EEA1 or LAMP1
20±5%
VAC14/
LAMP1
D.
VAC14
synapsin
synaptotagmin
synaptobrevin
Vac14-/-
E.
VAC14
PSD95
vGlut1
Overlay
Vac14-/-

Figure S8

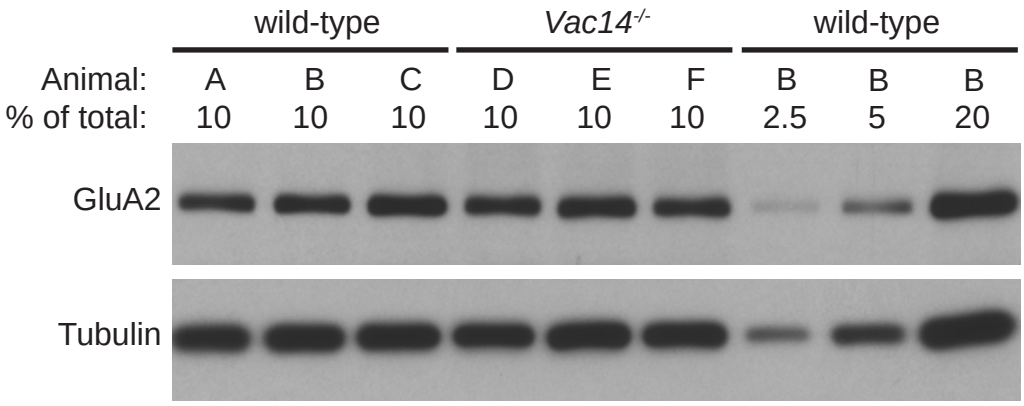
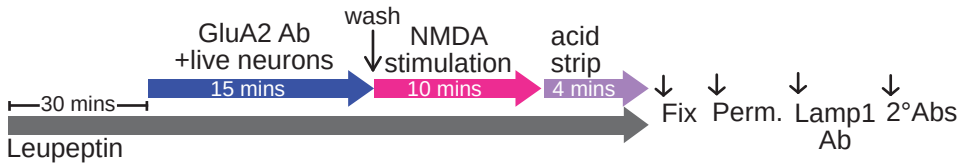
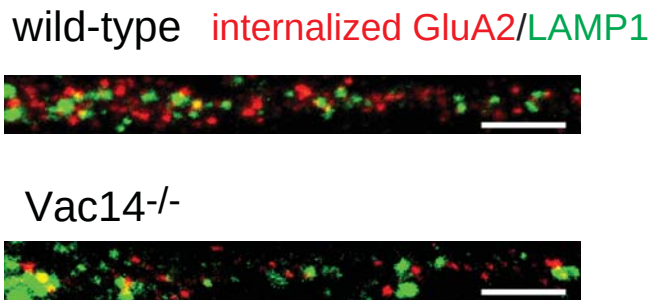

wild-type
Vac14-/-
wild-type
Animal: A B C D E F B B B
% of total: 10 10 10 10 10 10 2.5 5 20
GluA2
Tubulin

Figure S9

A.



B.



C.