SUPPLEMENTARY FIGURE LEGEND

Figure S1. Ultrastructural morphometry of AVs in WT and PS1 KO cells, Related to Figure 1. (A) AV ultrastructure in WT and PS1 KO cells grown in the presence (control) or absence (-serum) of serum. Scale bar represents 500 nm. (B) AVs were subcategorized based on morphology (AP: autophagosome - double membrane and/or undigested organelles, EAL: early autolysosome - single membrane with relatively undigested material, LAL: late autolysosome - single membrane with amorphous electron-dense material. Double arrow heads represent double membranes and single arrow heads represent single membranes. Scale bar represents 500 nm. (C) Quantitative analysis of the relative proportions of vacuoles (categorized base on B) in WT and PS1 KO cells (** for $p < 0.001$, n=20 EM images). All values are reported as mean ±S.E.M. (D) Ultrastructure of lighter and heavier AVs (AV10, AV20, respectively) and lysosomes purified from serum-starved (6 hrs) WT and PS1 KO cells. Long arrows highlight the more abundant immature AV forms in PS1 KO fractions compared to those in WT fractions. Scale bar represents 500 nm. (E) Immunofluorescence labeling using antibodies to LC3 in WT and PS1 KO cells under conditions of no treatment (Ctrl), serum starvation (w/o serum, 6 hrs), rapamycin (Rap, 10 nM; 6 hrs), rapamycin treatment followed by rapamycin removal (Rap/RC) for 6 hrs, and 3MA (10 mM; 6 hrs). Scale bar represents 10 μm.

Figure S2, Endogenous and exogenous protein degradation impairment in PS1 KO cells, Related to Figure 2. (A) Immunoblot analysis of LC3-I and –II levels in cells following leupeptin treatment. Double-immunofluorescence analysis of WT (B,C) and PS1 KO cells (D,E) with LC3 and LAMP-2 antibodies after treatment with leupeptin (0.3 mM) (C,E). Quantitative immunoblot analysis of data in (A) is presented graphically as mean ±S.E.M. for 3 different experiments. Scale bar represents 10 μm. ** for $p < 0.001$. (F) Levels of p62, an intracellular autophagy substrate, are increased in PS1KO cells. (G) WT and PS1 KO cells were preincubated with 0.5 % HRP for 30 min and chased for the indicated time periods. Internalized exogenous HRP was visualized with rodamine-conjugated anti-HRP antibody. Internalized HRP is rapidly degraded within 1 hr in WT

cells but remains for 6 hr in PS1 KO cells, indicating impaired lysosomal proteolysis in PS1 KO cells. Scale bar represents 50 μm and 10 μm (enlarged panel).

Figure S3, Cathepsin D maturation is delayed in PS1 KO cells and lysosome acidification is γ-secretase independent, Related to Figure 3. (A) Cat D immunoblots show reduced generation of the mature two-chain (31 kDa; 14 kDa) form in PS1 KO cells as in bafilomycin A1 (Baf, 100 nM) treated WT cells. (B) WT and PS1 KO cells were metabolically labeled for 30 min and chased for the indicated time periods. The generation of the mature form (31 kDa) of Cat D is impaired in PS1 KO cells. (C) Double-immunofluorescence labeling with antibodies to Cat D and CI-MPR shows strong co-localization in PS1 KO cells in contrast to untreated WT cells but similar to the pattern in WT cells treated with NH_4Cl (20 mM; 6 hrs). CI-MPR co-precipitated with anti-Cat D antibody and immunoblotted with antibodies against CI-MPR. Compared to WT cells, larger amounts of Cat D were bound to CI-MPR in PS1 KO cells and this pattern was reversed by human PS1 transfection (** $p < 0.001$, n=3). (D) MR-Cat B signal is abundantly evident in WT cells but is minimal in PS1 KO cells, similar to that in NH_4Cl treated WT cells. DIC images are depicted to aid in visualizing cells. Scale bar represents 50 μm. *In vitro* assays of Cat B (E), and L (F) enzyme activities in WT and PS1 KO cells in the absence or presence of rapamycin or NH_4Cl. (G) Nicastrin (Nct) gene knockout mouse fibroblasts were immunostained with anti-nicastrin antibody. (H) WT or Nct KO fibroblasts were incubated with 100 nM LysoTracker for 1 hr and immunolabeled with polyclonal antibody against cathepsin D. Cat D-positive compartments are LysoTracker-positive in both WT and Nct KO cells. Scale bar represents 50 μm. (I) γ-secretase inhibitor has no effect on lysosomal acidification, Cat B activity or Cat D processing. (J) WT blastocysts were incubated with 100 nM LysoTracker for 1 hr followed by L685,458 (final 10 nM, 18 hrs) treatment and immunolabeling. MR-Cat B signal is abundantly evident in γ-secretase inhibitor-treated cells but is minimal after leupeptin treatment. DIC images are depicted to aid in visualizing cells. Scale bar represents 50 μm. Quantitative analysis of data is presented as mean ±S.E.M. for 3 different experiments. ** for $p < 0.001$.

Figure S4, The hPS1 introduction restored autophagic/lysosomal system, Related to Figure 5. (A) Lysosome acidification is restored after human wild-type PS1 is stably transfected into PS KO (PS1/2 KO) cells. Scale bars represent 50 μm and 20 μm (enlarged panel). (B) PS1 introduction rescues v-ATPase V0a subunit and Cat D maturation (31 kDa form). (C) Top panel depicts western blot of PS1 and middle panel shows, in the same cell types, serum deprivation-dependent increases in proteolysis based on measurements of proteolysis in the absence of serum relative to those in the presence of serum. The increases in proteolysis at 12 hrs after serum removal were determined for WT, PS1 KO, PS KO (PS1/2 KO) and hPS1 incubated in the presence or absence of serum. Bottom panel shows the percentage of 3MA-sensitive proteolysis determined for WT, PS1 KO, PS KO and hPS1 incubated with and without 3MA (10 mM) for 12 hrs in the absence of serum (** for $p < 0.001$, n=9). (D) LC3-II Western blots and graphs depicting results of densitometric analyses of LC3-II and LC3-I immunoreactivity and expression as LC3-II/LC3-I ratios using tubulin as a loading control. Values are the mean ±S.E.M. ** for $p < 0.001$, n=3). (E) Morphometric ultrastructural analyses of electron micrographs (EM) show that engulfed materials by autophagy were sufficiently digested in serum starved conditions in WT cells compared to PS1 KO cells. Scale bar represents 500 nm.

Figure S5, Defective autophagosome accumulation and acidification in PS cKO mice, Related to Figure 6. (A) LC3 immunohistochemistry of brains of PS cKO mice show a greater level of LC3 positive staining in the PS1 altered models compared to their WT controls. Scale bar 50 and 10 μm, respectively. (B) Electron micrographs show an increase of AV and dystrophic neurite-like structures in PS cKO (arrowheads) compared to their littermate controls. Scale bars equal 500 nm. (C) DAMP, a marker which localizes to acidic compartments, was infused intraventricularly into the brains of mice, which were then analyzed by immuno-electron microscopy using DNP (arrowheads) and CatD (arrows) antibodies. (D) Graphs show quantitative results of immunogold labeling for DAMP and CatD. ** $p < 0.001$.

Figure S6, Defective autophagy in PS1-FAD human fibroblasts, Related to Figure 7. (A) LC3-II Western blot and graph depicts results of densitometric analyses of LC3-II and LC3-I immunoreactivities and expression as LC3-II/LC3-I ratios using tubulin as a loading control (** for p <0.001, n=3). All values are the mean ±S.E.M. (B) Immunostaining for LC3 of control and PS1 FAD fibroblast following incubation in the presence or absence of serum. Scale bar represents 10 μm. (C) Electron micrographs of human fibroblasts from non-affected and PS1-FAD human fibroblasts were examined following treatment in FBS (+FBS) and 12 hours in FBS-free media (-FBS). AVs with relatively undigested material and amorphous electron-dense digested materials were identified ultrastructurally in PS1 FAD fibroblasts. Scale bar represents 500 nm.** for P<0.001. All values are reported as mean ± S.E.M. of n = 30.

Figure S7, Defective acidification and lysosomal targeting of v-ATPase was impaired in PS1 FAD fibroblasts, Related to Figure 7. (A) control (7621) and PS1-FAD (6840) fibroblasts were grown in the absence of serum (6 hrs) followed by transfection of the mRFP-GFP-LC3 construct and live confocal imaging in an incubation chamber. Bottom panel shows the quantitative analysis of GFP and RFP-positive compartments. Scale bar represents 20 μm. (B) p62 immunoblot shows increased p62 protein levels in PS1-FAD fibroblasts in either the presence or absence of serum (6 hrs). Double-immunostaining shows LC3/p62 positive compartment size is increased in PS-FAD fibroblasts. * for p <0.01, ** for p <0.001. Scale bar represent 50 and 10 μm. (C) Double-immunofluorescence labeling with antibodies to v-ATPase (V0a1 subunit) and CatD showing strong co-localization in control fibroblast but significantly reduced co-localization in PS1 FAD fibroblasts. (D) Double immunostaining with antibodies to v-ATPase (V0a1 subunit) and PDI, ER marker shows strong co-localization in PS1 FAD fibroblasts but minimal co-localization in control fibroblast. All values are reported as mean ± S.E.M. for n = 30 cells. (E) v-ATPase complex formation was impaired in PS1 FAD fibroblast. Double-immunofluorescence labeling with antibodies to v-ATPase (V0a1 subunit) and v-ATPase (V1B1 subunit) shows strong co-localization in control fibroblast but significantly reduced co-localization in PS1 FAD fibroblasts. (F) Quantitative analysis of v-ATPase V0a1 subunit association with organelle markers.

Values are the mean ±S.E.M. of n = 30 cells. ** for p <0.001. (G) v-ATPase V0a1 subunit western blots and graphs depicting results of densitometric analyses of v-ATPase V0a1 immunoreactivity and expression as total amount using GAPDH as a loading control (* for p <0.05).

SUPPLEMENTARY EXPERIMENTAL PROCEDURES

Cell lines and mouse

Murine blastocysts with different presenilin (PS1) genotypes (WT, BD6; PS1 KO BD15; PS1/2 KO, BD8) previously characterized by Lai et. al. (2003) (Lai et al., 2003), were used in this study. In addition, human PS1 wt was stably transfected into the BD8 line (Laudon et al., 2004). The cells were grown in 35 mm dishes in DMEM supplemented with penicillin/streptomycin (Invitrogen), 15 % fetal bovine serum (Hyclone), and β-mercaptoethanol (Sigma). Human fibroblasts lines (see below), acquired from the Coriell Institute (Camden NJ), Karolinska Institute (Upsala, Sweden), University di Firenze (Italy) and University of Western Australia (Perth), were maintained in MEM (Invitrogen, Carlsbad CA) with 15% FBS (Hyclone, Logan, UT) at 37°C and 5% CO_2. PS1 hypomorph (Rozmahel et al., 2002) and PS cKO mice (Saura et al., 2004) were studied at 13 month and 2-3 month, respectively, together with age-matched controls. All animal experiments were performed according to "Principles of Animal Care" (NIH, 1985) and approved by the Institutional Animal Care and Use Committee at the NKI.

Compilation of Human Fibroblasts used in this study

Code	Mutation	Age	Source
PS1			
9200	M233T	43	R. Martins
9900	M233T	47	R. Martins
8170	A246E	56	Corriell Ins
6840	A246E	56	Corriell Ins
6848	A246E	56	Corriell Ins
7613	A246E	66	Corriell Ins
8711	A246E	34	Corriell Ins
4159	A246E	52	Corriell Ins
HK36	H163Y	59	R Cowburn

BS51	H163Y	44	R Cowburn
NES52	H163Y	42	R Cowburn
AK65	H163Y	30	R Cowburn
LS64	H163Y	31	R Cowburn
KR40	H163Y	55	R Cowburn
M146	M146L	53	S. Sorbi
L392	L392A	55	S. Sorbi
Control			
7621	--	57	Corriell Ins
6846	--	75	Corriell Ins
7714	--	56	Corriell Ins
5600	--	43	R. Martins
5900	--	38	R. Martins
EJ39	--	57	R. Cowburn
EB24	--	72	R. Cowburn
VS31	--	64	R. Cowburn

Antibodies and Reagents

Rabbit pAb to LC3 (1/500) (Koike et al., 2005) and LC3 (1/200, Novus) were used for cell and mouse brain, respectively, immunofluorescence studies and a polyclonal LC3 (generated in house, 1/1000) (Yu et al., 2005) was used for immunoblotting, anti-murine LAMP (LAMP-2: ABL-93, 1/200 and LAMP-1 : 1D4B, 1/5 or H4A3, 1/200) mAb was purchased from Developmental Studies Hybridoma Bank. Rabbit anti-Cathepsin D pAb (1/1000) was purchased from Scripps Laboratories for MeOH fixed cell ICC, Rabbit anti-Cathepsin D pAb (1/5000) was generated in house for 4 % PFA fixed ICC and western blot, and rabbit polyclonal antibody to Cat D (1:50, IEM) was purchased from DAKO. The Anti-PDI mouse mAb (1/5000) and Anti-GRP94 rat mAb (9G10, 1/10000) were purchased from Assay designs. Anti-Calnexin mouse mAb (1/1000) was from Affinity Bioreagent. The mouse monoclonal anti-LBPA antibody (1/2) was a generous gift from Dr. Jean Gruenberg. Anti-EEA1 mouse mAb (clone 14, 1/1000) was purchased from BD Bioscience. The mouse monoclonal anti-CI-MPR (clone 2G11, 1/500) and mouse monoclonal anti-rab7 were from Abcam. Guinea pig polyclonal anti-p62 (GP62-C, 1/2000) was from Progen Biotechni and mouse monoclonal anti-human p62 (1/1000) was from BD transduction. LysoTracker Red DND-99 (1/10000), LysoSensor yellow/blue

DND-160–Dextran, Bodipy-FL-Pepstain A (1 μg/ml), DAMP (30 μM stock), and mouse monoclonal antibody to DNP (1:50) were from Invitrogen. Total p70S6K (#9292, 1/1000) and phospho-p70S6K (#9206, 1/1000) were purchased from Cell Signaling. Anti-PS1 rabbit pAb (Ab14) was a generous gift from Dr. Sam Gandy, anti-PS1-NTF rabbit pAb (34-4600, 1/1000) was purchased from Zymed. Anti-PS1 loop mouse mAb (MAB5232, 1/1000) and anti-nicastrin mouse mAb (MAB5556, 1/1000) were purchased from Chemicon. β-tubulin (clone 2-28-33, 1/5000), anti-actin (clone AC-40, 1/5000), and rabbit polyclonal anti-GAPDH (1/5000) were purchased from Sigma. A rabbit pAb against mouse v-ATPase V0a1 (W249, 1/5000) and mouse pAb against human v-ATPase V0a1 (Osw2, 1/500) were generous gift from Dr. Satoshi Sato and other rabbit pAb against V0a1 subunit of the vacuolar proton pump (1/500) was purchased from Synaptic Systems. The v-ATPase V1 B1 antibody (H-180, 1/200) was purchased from Santa Cruz Biocech. Following reagents were used for autophagy modulation experiments. Rapamycin (Rapa, final 10 nM), ammonium chloride (NH_4Cl, final 20 mM), bafilomycin A1 (final 0.2 μM) and 3-methyladenine (3MA, final 10 mM) were from Sigma and leupeptin (final 0.3 mM) was from Peptide Institute Inc. The γ-secretase inhibitor (L685,458) was purchased from Sigma.

Gel electrophoresis, immunoblotting and deglycosylation

Immunoblotting was performed as previously described (Yu et al., 2005). Briefly, cells used for Western blot analysis were lysed in buffer containing 50 mM Tris (pH=7.4), 150 mM NaCl, 1mM EDTA, 1 mM EGTA, 1 % Triton X-100 and 0.5 % Tween-20 with protease and phosphatase inhibitors. Following electrophoresis on 4 -20 % gradient gel (Invitrogen), proteins were transferred onto 0.45 μm PVDF membranes (Millipore) and the membrane was incubated overnight in primary antibody then incubated with HRP conjugated secondary antibody. The blot was developed by ECL-kit (GE Healthcare). To assess v-ATPase V0a subunit glycosylation, lysate from WT and PS1KO cells were either treated for 24 hrs at 37°C with PNGase F or O-glycanase using an enzymatic deglycosylation kit according to the manufacturer's instructions (PROzyme) or with Endo H (New England Biolabs) for 24 hrs at 37°C. Cells were treated for 24 hrs at 37°C

with tunicamycin (5μg/ml). Total glycoproteins were isolated using Glycoprotein Isolation Kit, ConA according to the manufacturer's instructions (Thermo Scientific).

Subcellular fractionation

Homogenate (0.5 ml) was layered on the top layer of 10, 15, 20, 25, and 30% Optiprep (Sigma) step gradient, 2.3 ml each, into polyallomer tubes (Beckman) and centrifuged in a SW-40Ti rotor with a model L8-80M Beckman ultracentrifuge (100,000 g, 16 hours, 4°C). After centrifugation, the gradients were fractionated into 0.5 ml fractions. 40 μl of each collected fraction was mixed with an equal volume of sample buffer and then loaded onto gels. The homogenates were fractionated into cytosolic and membrane fractions by high speed centrifugation (150,000 x g, 60 min) and equal proteins was loaded on gel following 55°C for 10 min incubation with 2x urea sample buffer. All data represent an average at least three independent experiments.

Autophagic vacuole isolation

AVs were isolated by centrifugation in a discontinuous metrizamide density gradient (Marzella et al., 1982) for each cell line. Cells (5×10^8) were serum-deprived overnight to induce autophagic activity prior to AV isolation. The cells were collected, disrupted by nitrogen cavitation then homogenized in 3 volume of 0.25 M sucrose in a glass homogenizer with Teflon pestle for 10 strokes. The homogenate was filtered through double gauze and then spun at 2000 x g for 5 min to yield a supernatant and pellet of unbroken cells and nuclei. The supernatant was centrifuged at 17,000 x g for 12 min to yield a pellet and a supernatant which was spun again at 100,000 x g for 1 hr to yield a pellet containing ER and a supernatant containing cytosol. The pellet from the 17,000 x g centrifugation was resuspended in the same volume of 0.25 M sucrose and spun again at 17,000 x g for 12 min. The pellet was resuspended in 1.9 ml of 0.25 M sucrose and 2.8 ml of metrizamide (Mtz). This mixture (2.4 ml volume) was loaded on top of a 26 % (4 ml), 24 % (2 ml), 20 % (2 ml) and 15 % (2 ml) Mtz step gradient matrix. The sample on the Mtz gradient was centrifuged at 247,000 rpm for 3 hrs in an ultracentrifuge using an SW41 rotor. Each gradient interface was collected and diluted in 0.25 M sucrose. The samples were then pelleted at 24,000 x g for 10 min. Light AVs (AV10) were present in

the 15-20 % fraction, while heavy AV (AV20) was present in the 20-24 %, lysosomes were in the 24-26 % interface, and mitochondria were located in the 26% Mtz area. Fractions were pelleted and immersed in a cacodylate fixation buffer for EM analysis or analyzed directly by Western blot or enzyme assay. All data represent an average at least three independent experiments, unless otherwise indicated.

Ultrastructural and Morphometric Analyses

Following treatments, cells were prepared for EM as previously described and AVs, identified using previous morphological criteria (Yu et al., 2005), were classified and counted on electron micrographs (7900x print magnification) of 20 EM images from different lines in each experimental group.

Lysosomal pH measurement

Quantification of lysosomal pH was determined using Dextran conjugated Lysosensor Yellow/Blue DND-160 (Invitrogen). Wild Type and PS1KO blastocysts were grown in High Glucose DMEM + 15 % FBS with antibiotics to ~ 90% confluency. Cells were then trypsinized, harvested (1 X 10^6 cells/ml) and loaded with 1mg/ml of Lysosensor-dextran for 1 hour at 37°C with 5% CO_2. The cells were then washed 3X in HBSS and aliquoted at 100 μl into a black 96-well microplate. pH calibration was performed according to the protocol established by Diwu *et al* (Diwu et al., 1999). Briefly, wild type and PS1KO blastocysts were treated with 10 μM monensin and 10uM nigericin in MES buffer (5 mM NaCl, 115 mM KCL, 1.3 mM MgSO4, 25 mM MES), with the pH adjusted to a range from 3.5-7.0. The samples were read in a Wallac Victor 2 fluorimeter (Perkin Elmer) with excitation at 355 nm. The ratio of emission 440/535 nm was then calculated for each sample. The pH values were determined from the linear standard curve generated via the pH calibration samples.

In vivo vesicle acidification study

The mouse was anaesthetized with a 1 % body weight IP injection of chloral hydrate, (400 mg/kg at a concentration of 50 mg/ml, 26 g needle, and volume less than 200 ul) and allowed sufficient time to go down. Under sterile conditions, the subject was then

shaved, and cleaned at the site of the surgery, in this case the scalp. The subject was placed in position in a stereotaxic holder with drill (BenchMark). The position of the drill/burr arm was located in right ventricle (-0.22 mm from bregma; L, -1 mm; D/V, -2.5 mm), the coordinates relative to the bregma, were determined by using *The Mouse Brain* (Keith B. J. Franklin and George Paxinos, Academic Press).

Mouse was injected with DAMP (20 µl of 30 mM stock solution prepared in PBS) by intra-ventricular method with 1.5 µl/min speed. After 4hrs, animals were anesthetized and perfused with a fixative containing 0.1 % glutaraldehyde and 4 % paraformaldehyde in sodium cacodylate buffer (Electron Microscopy Sciences). Brains were dissected and immersed in the same fixative for 4 hrs and then 40 µm sagital sections were made using a vibratome. The sections were processed routinely for electron microscopy and embedded in LR white. Ultrathin sections were mounted on nickel grids. Sections were treated with 4% NRS for 2hrs at room temperature. For DAMP, the sections were incubated with an antibody to DNP (Invitrogen 1:50) and CatD (1:50, DAKO) overnight. Grids were subsequently washed with PBS and incubated for 2 hrs in room temperature with secondary antibody coupled with 10 nm gold. Sections were washed with PBS and were stained briefly with uranyl acetate and lead citrate. Sections were examined and photographed with a Philips CM10 electron microscope.

Intracellular Protein Degradation Measurements

Total protein degradation in cultured cells was measured by pulse-chase experiments (Auteri et al., 1983). Confluent cells were labeled with [^{3}H]-leucine (2µCi/ml) for 48 hrs at 37°C in order to preferentially label long-lived proteins. Following labeling, cells were extensively washed and maintained in complete medium (DMEM + 10 % fetal bovine serum), under which conditions autophagy is suppressed, or in serum-deprived medium, where autophagy is induced. Under both conditions, after washing the cells, the medium was supplemented with unlabeled 2.8 mM leucine to prevent [^{3}H]-leucine reincorporation into newly synthesized proteins. Aliquots of the medium taken at different time-points were precipitated with 10 % TCA, filtered using a 0.22 µm pore membrane and radioactivity in the flow-through was measured. Proteolysis is expressed as the percentage of the initial acid-precipitable radioactivity (protein) transformed to

acid-soluble radioactivity (amino acids and small peptides) over time. To inhibit autophagy in this system, 20 mM NH_4Cl or 10 mM 3MA was added immediately after the labeling period and maintained at that concentration throughout the chase. NH_4Cl blocks lysosomal degradation, so this procedure permits one to estimate the relative contributions of lysosomal and non-lysosomal pathways to overall protein degradation. 3MA blocks formation and fusion of autophagosomes to lysosomes and is used to block macroautophagic contributions to proteolysis. Degradation rates of short-half life proteins were determined by the same procedure but after a labeling period of 30 min at 37 °C. Nearly all turnover of short-lived proteins is due to proteasomal activity. Protein synthesis was determined as the incorporation of [^{3}H]leucine into acid-insoluble material in the presence of an excess (2.8 mM) unlabeled leucine in the medium. Under these conditions incorporation of radioactivity into protein accurately reflects rates of proteins synthesis and minimizes differences due to alteration of amino acid transport and/or intracellular amino acid pool sizes.

Confocal laser scanning microscopy

Immunocytochemistry was performed as previously described (Yu et al., 2005). Secondary antibodies used were from Invitrogen: goat anti-mouse/rabbit/rat Alexa Fluor 488 and goat anti-mouse/rabbit/rat Alexa Fluor 568. Cells were imaged using a plan-Apochromat 40x or 100x/1.4 oil DIC objective lens on the laser scanning confocal microscope, LSM 510 META, with LSM software v3.5 (Carl Zeiss MicroImaging Inc). Images were analyses using ImageJ program (NIH). Organelles with low internal pH were labeled by LysoTracker DND-99 dye (Invitrogen) at the final 100 nM for 30 min. Following 4 % PFA fixation, cells were further count stained with Cat D, LMAP1, or LC3 antibody for 4 hrs then visualized with Alexa Fluor 488 conjugated secondary antibody. Active Cat D was labeled by adding Bodipy-FL-pepstatin A directly to the medium at a final concentration of 1 μg/ml for 1 hr. Following 4 % PFA fixation, cells were counter stained with Cat D antibody for 4 hrs and visualized with Alexa Fluor 568 conjugated secondary antibody. For assessing Cat B activation, MagicRed–Cathepsin B (Immunochemistry Technologies) for active Cat B was added to the cells at the concentration suggested by company (1:260). Cells were incubated for 30 min with MR-

Cathepsin B prior to mounting them under the confocal microscope. To block v-ATPase proton pump activity, ammonium chloride (NH_4Cl) was added directly to the medium at a final concentration of 20 mM for 6 hrs prior to LysoTracker addition.

Co-immunoprecipitation

Wild type mouse blastocysts cells were lysed with lysis butter (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 100 mM EDTA, and protease inhibitor cocktail) with 1 % digitonin. Immunoprecipitations of complex proteins with anti-PS1 pAb, anti-V-ATPase V0a1 subunit pAb, anti-Sec61α, or anti-STT3B were performed using the Seize Primary immunoprecipitation kit (Pierce Biotechnology) (Hiesinger et al., 2005).

Degradation of HRP internalized by fluid phase endocytosis.

HRP degradation was evaluated in cells that were incubated for 30 min at 37 °C with medium containing 5 mg/ml horseradish peroxidase (HRP) (HRP type II; Sigma) and washed three times with phosphate-buffered saline then chased 37 °C for 0, 1, and 6 hrs with fresh media. Following fixation, the cells were immunolabeled with rodamine-conjugated anti-HRP antibody (Jackson lab).

Enzymatic assays

Cathepsin B and L activity were assayed as described previously (Nakanishi et al., 1994) and Cathepsin D activity was assayed using [^{14}C]methemoglobin as previously described (Dottavio-Martin and Ravel, 1978). A 50 μl aliquot of enzyme was incubated at 37 °C with 100 μl of 0.4 M acetate buffer (pH 3.2) containing 10 mg/ml [^{14}C]methemoglobin. Cathepsin D activity was expressed in terms of methemoglobin degrading activity; one unit of enzyme activity is defined as the capacity to degrade one nanomol of methemoglobin per minute.

Cathepsin D metabolic labeling

Cells were grown in a 3.5 cm dish to 5 x 10^5 cells/dish, after which they were metabolically labeled for 30 minutes with 100 mCi/ml [35S]methionine/cysteine (EXPRE35S35S Protein Labeling Mix, NEG0702 14MC: Perkin Elmer) and chased with

normal medium for specific periods. The labeled cells were lysed and subsequently processed for immunoprecipitation with anti-rat cathepsin D antibody and protein G-agarose bead (Santa Cruz Biocech). The immunoprecipitates were analyzed by 10 % SDS-PAGE.

Transfection of plasmid

Fibroblast were transfected with mRFP-GFP-LC3 (Kimura et al., 2007) from Dr. Tamotsu Yoshimori (Osaka University) using Lipofectamine 2000 (Invitrogen) according to manufacture's instruction. Following transfection fibroblasts were incubated for 24 hrs and before imaging incubated for another 6 hrs with serum or without serum medium.

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SUPPLEMENTARY FIGURE LEGEND

Figure S1. Ultrastructural morphometry of AVs in WT and PS1 KO cells, Related to Figure 1. (A) AV ultrastructure in WT and PS1 KO cells grown in the presence (control) or absence (-serum) of serum. Scale bar represents 500 nm. (B) AVs were subcategorized based on morphology (AP: autophagosome - double membrane and/or undigested organelles, EAL: early autolysosome - single membrane with relatively undigested material, LAL: late autolysosome - single membrane with amorphous electron-dense material. Double arrow heads represent double membranes and single arrow heads represent single membranes. Scale bar represents 500 nm. (C) Quantitative analysis of the relative proportions of vacuoles (categorized base on B) in WT and PS1 KO cells (** for $p < 0.001$, n=20 EM images). All values are reported as mean ±S.E.M. (D) Ultrastructure of lighter and heavier AVs (AV10, AV20, respectively) and lysosomes purified from serum-starved (6 hrs) WT and PS1 KO cells. Long arrows highlight the more abundant immature AV forms in PS1 KO fractions compared to those in WT fractions. Scale bar represents 500 nm. (E) Immunofluorescence labeling using antibodies to LC3 in WT and PS1 KO cells under conditions of no treatment (Ctrl), serum starvation (w/o serum, 6 hrs), rapamycin (Rap, 10 nM; 6 hrs), rapamycin treatment followed by rapamycin removal (Rap/RC) for 6 hrs, and 3MA (10 mM; 6 hrs). Scale bar represents 10 μm.

Figure S2, Endogenous and exogenous protein degradation impairment in PS1 KO cells, Related to Figure 2. (A) Immunoblot analysis of LC3-I and –II levels in cells following leupeptin treatment. Double-immunofluorescence analysis of WT (B,C) and PS1 KO cells (D,E) with LC3 and LAMP-2 antibodies after treatment with leupeptin (0.3 mM) (C,E). Quantitative immunoblot analysis of data in (A) is presented graphically as mean ±S.E.M. for 3 different experiments. Scale bar represents 10 μm. ** for $p < 0.001$. (F) Levels of p62, an intracellular autophagy substrate, are increased in PS1KO cells. (G) WT and PS1 KO cells were preincubated with 0.5 % HRP for 30 min and chased for the indicated time periods. Internalized exogenous HRP was visualized with rodamine-conjugated anti-HRP antibody. Internalized HRP is rapidly degraded within 1 hr in WT

cells but remains for 6 hr in PS1 KO cells, indicating impaired lysosomal proteolysis in PS1 KO cells. Scale bar represents 50 μm and 10 μm (enlarged panel).

Figure S3, Cathepsin D maturation is delayed in PS1 KO cells and lysosome acidification is γ-secretase independent, Related to Figure 3. (A) Cat D immunoblots show reduced generation of the mature two-chain (31 kDa; 14 kDa) form in PS1 KO cells as in bafilomycin A1 (Baf, 100 nM) treated WT cells. (B) WT and PS1 KO cells were metabolically labeled for 30 min and chased for the indicated time periods. The generation of the mature form (31 kDa) of Cat D is impaired in PS1 KO cells. (C) Double-immunofluorescence labeling with antibodies to Cat D and CI-MPR shows strong co-localization in PS1 KO cells in contrast to untreated WT cells but similar to the pattern in WT cells treated with NH_4Cl (20 mM; 6 hrs). CI-MPR co-precipitated with anti-Cat D antibody and immunoblotted with antibodies against CI-MPR. Compared to WT cells, larger amounts of Cat D were bound to CI-MPR in PS1 KO cells and this pattern was reversed by human PS1 transfection (** $p < 0.001$, n=3). (D) MR-Cat B signal is abundantly evident in WT cells but is minimal in PS1 KO cells, similar to that in NH_4Cl treated WT cells. DIC images are depicted to aid in visualizing cells. Scale bar represents 50 μm. *In vitro* assays of Cat B (E), and L (F) enzyme activities in WT and PS1 KO cells in the absence or presence of rapamycin or NH_4Cl. (G) Nicastrin (Nct) gene knockout mouse fibroblasts were immunostained with anti-nicastrin antibody. (H) WT or Nct KO fibroblasts were incubated with 100 nM LysoTracker for 1 hr and immunolabeled with polyclonal antibody against cathepsin D. Cat D-positive compartments are LysoTracker-positive in both WT and Nct KO cells. Scale bar represents 50 μm. (I) γ-secretase inhibitor has no effect on lysosomal acidification, Cat B activity or Cat D processing. (J) WT blastocysts were incubated with 100 nM LysoTracker for 1 hr followed by L685,458 (final 10 nM, 18 hrs) treatment and immunolabeling. MR-Cat B signal is abundantly evident in γ-secretase inhibitor-treated cells but is minimal after leupeptin treatment. DIC images are depicted to aid in visualizing cells. Scale bar represents 50 μm. Quantitative analysis of data is presented as mean ±S.E.M. for 3 different experiments. ** for $p < 0.001$.

Figure S4, The hPS1 introduction restored autophagic/lysosomal system, Related to Figure 5. (A) Lysosome acidification is restored after human wild-type PS1 is stably transfected into PS KO (PS1/2 KO) cells. Scale bars represent 50 μm and 20 μm (enlarged panel). (B) PS1 introduction rescues v-ATPase V0a subunit and Cat D maturation (31 kDa form). (C) Top panel depicts western blot of PS1 and middle panel shows, in the same cell types, serum deprivation-dependent increases in proteolysis based on measurements of proteolysis in the absence of serum relative to those in the presence of serum. The increases in proteolysis at 12 hrs after serum removal were determined for WT, PS1 KO, PS KO (PS1/2 KO) and hPS1 incubated in the presence or absence of serum. Bottom panel shows the percentage of 3MA-sensitive proteolysis determined for WT, PS1 KO, PS KO and hPS1 incubated with and without 3MA (10 mM) for 12 hrs in the absence of serum (** for $p < 0.001$, n=9). (D) LC3-II Western blots and graphs depicting results of densitometric analyses of LC3-II and LC3-I immunoreactivity and expression as LC3-II/LC3-I ratios using tubulin as a loading control. Values are the mean ±S.E.M. ** for $p < 0.001$, n=3). (E) Morphometric ultrastructural analyses of electron micrographs (EM) show that engulfed materials by autophagy were sufficiently digested in serum starved conditions in WT cells compared to PS1 KO cells. Scale bar represents 500 nm.

Figure S5, Defective autophagosome accumulation and acidification in PS cKO mice, Related to Figure 6. (A) LC3 immunohistochemistry of brains of PS cKO mice show a greater level of LC3 positive staining in the PS1 altered models compared to their WT controls. Scale bar 50 and 10 μm, respectively. (B) Electron micrographs show an increase of AV and dystrophic neurite-like structures in PS cKO (arrowheads) compared to their littermate controls. Scale bars equal 500 nm. (C) DAMP, a marker which localizes to acidic compartments, was infused intraventricularly into the brains of mice, which were then analyzed by immuno-electron microscopy using DNP (arrowheads) and CatD (arrows) antibodies. (D) Graphs show quantitative results of immunogold labeling for DAMP and CatD. ** $p < 0.001$.

Figure S6, Defective autophagy in PS1-FAD human fibroblasts, Related to Figure 7. (A) LC3-II Western blot and graph depicts results of densitometric analyses of LC3-II and LC3-I immunoreactivities and expression as LC3-II/LC3-I ratios using tubulin as a loading control (** for p <0.001, n=3). All values are the mean ±S.E.M. (B) Immunostaining for LC3 of control and PS1 FAD fibroblast following incubation in the presence or absence of serum. Scale bar represents 10 μm. (C) Electron micrographs of human fibroblasts from non-affected and PS1-FAD human fibroblasts were examined following treatment in FBS (+FBS) and 12 hours in FBS-free media (-FBS). AVs with relatively undigested material and amorphous electron-dense digested materials were identified ultrastructurally in PS1 FAD fibroblasts. Scale bar represents 500 nm.** for P<0.001. All values are reported as mean ± S.E.M. of n = 30.

Figure S7, Defective acidification and lysosomal targeting of v-ATPase was impaired in PS1 FAD fibroblasts, Related to Figure 7. (A) control (7621) and PS1-FAD (6840) fibroblasts were grown in the absence of serum (6 hrs) followed by transfection of the mRFP-GFP-LC3 construct and live confocal imaging in an incubation chamber. Bottom panel shows the quantitative analysis of GFP and RFP-positive compartments. Scale bar represents 20 μm. (B) p62 immunoblot shows increased p62 protein levels in PS1-FAD fibroblasts in either the presence or absence of serum (6 hrs). Double-immunostaining shows LC3/p62 positive compartment size is increased in PS-FAD fibroblasts. * for p <0.01, ** for p <0.001. Scale bar represent 50 and 10 μm. (C) Double-immunofluorescence labeling with antibodies to v-ATPase (V0a1 subunit) and CatD showing strong co-localization in control fibroblast but significantly reduced co-localization in PS1 FAD fibroblasts. (D) Double immunostaining with antibodies to v-ATPase (V0a1 subunit) and PDI, ER marker shows strong co-localization in PS1 FAD fibroblasts but minimal co-localization in control fibroblast. All values are reported as mean ± S.E.M. for n = 30 cells. (E) v-ATPase complex formation was impaired in PS1 FAD fibroblast. Double-immunofluorescence labeling with antibodies to v-ATPase (V0a1 subunit) and v-ATPase (V1B1 subunit) shows strong co-localization in control fibroblast but significantly reduced co-localization in PS1 FAD fibroblasts. (F) Quantitative analysis of v-ATPase V0a1 subunit association with organelle markers.

Values are the mean ±S.E.M. of n = 30 cells. ** for p <0.001. (G) v-ATPase V0a1 subunit western blots and graphs depicting results of densitometric analyses of v-ATPase V0a1 immunoreactivity and expression as total amount using GAPDH as a loading control (* for p <0.05).

SUPPLEMENTARY EXPERIMENTAL PROCEDURES

Cell lines and mouse

Murine blastocysts with different presenilin (PS1) genotypes (WT, BD6; PS1 KO BD15; PS1/2 KO, BD8) previously characterized by Lai et. al. (2003) (Lai et al., 2003), were used in this study. In addition, human PS1 wt was stably transfected into the BD8 line (Laudon et al., 2004). The cells were grown in 35 mm dishes in DMEM supplemented with penicillin/streptomycin (Invitrogen), 15 % fetal bovine serum (Hyclone), and β-mercaptoethanol (Sigma). Human fibroblasts lines (see below), acquired from the Coriell Institute (Camden NJ), Karolinska Institute (Upsala, Sweden), University di Firenze (Italy) and University of Western Australia (Perth), were maintained in MEM (Invitrogen, Carlsbad CA) with 15% FBS (Hyclone, Logan, UT) at 37°C and 5% CO_2. PS1 hypomorph (Rozmahel et al., 2002) and PS cKO mice (Saura et al., 2004) were studied at 13 month and 2-3 month, respectively, together with age-matched controls. All animal experiments were performed according to "Principles of Animal Care" (NIH, 1985) and approved by the Institutional Animal Care and Use Committee at the NKI.

Compilation of Human Fibroblasts used in this study

Code	Mutation	Age	Source
PS1			
9200	M233T	43	R. Martins
9900	M233T	47	R. Martins
8170	A246E	56	Corriell Ins
6840	A246E	56	Corriell Ins
6848	A246E	56	Corriell Ins
7613	A246E	66	Corriell Ins
8711	A246E	34	Corriell Ins
4159	A246E	52	Corriell Ins
HK36	H163Y	59	R Cowburn

BS51	H163Y	44	R Cowburn
NES52	H163Y	42	R Cowburn
AK65	H163Y	30	R Cowburn
LS64	H163Y	31	R Cowburn
KR40	H163Y	55	R Cowburn
M146	M146L	53	S. Sorbi
L392	L392A	55	S. Sorbi
Control			
7621	--	57	Corriell Ins
6846	--	75	Corriell Ins
7714	--	56	Corriell Ins
5600	--	43	R. Martins
5900	--	38	R. Martins
EJ39	--	57	R. Cowburn
EB24	--	72	R. Cowburn
VS31	--	64	R. Cowburn

Antibodies and Reagents

Rabbit pAb to LC3 (1/500) (Koike et al., 2005) and LC3 (1/200, Novus) were used for cell and mouse brain, respectively, immunofluorescence studies and a polyclonal LC3 (generated in house, 1/1000) (Yu et al., 2005) was used for immunoblotting, anti-murine LAMP (LAMP-2: ABL-93, 1/200 and LAMP-1 : 1D4B, 1/5 or H4A3, 1/200) mAb was purchased from Developmental Studies Hybridoma Bank. Rabbit anti-Cathepsin D pAb (1/1000) was purchased from Scripps Laboratories for MeOH fixed cell ICC, Rabbit anti-Cathepsin D pAb (1/5000) was generated in house for 4 % PFA fixed ICC and western blot, and rabbit polyclonal antibody to Cat D (1:50, IEM) was purchased from DAKO. The Anti-PDI mouse mAb (1/5000) and Anti-GRP94 rat mAb (9G10, 1/10000) were purchased from Assay designs. Anti-Calnexin mouse mAb (1/1000) was from Affinity Bioreagent. The mouse monoclonal anti-LBPA antibody (1/2) was a generous gift from Dr. Jean Gruenberg. Anti-EEA1 mouse mAb (clone 14, 1/1000) was purchased from BD Bioscience. The mouse monoclonal anti-CI-MPR (clone 2G11, 1/500) and mouse monoclonal anti-rab7 were from Abcam. Guinea pig polyclonal anti-p62 (GP62-C, 1/2000) was from Progen Biotechni and mouse monoclonal anti-human p62 (1/1000) was from BD transduction. LysoTracker Red DND-99 (1/10000), LysoSensor yellow/blue

DND-160–Dextran, Bodipy-FL-Pepstain A (1 μg/ml), DAMP (30 μM stock), and mouse monoclonal antibody to DNP (1:50) were from Invitrogen. Total p70S6K (#9292, 1/1000) and phospho-p70S6K (#9206, 1/1000) were purchased from Cell Signaling. Anti-PS1 rabbit pAb (Ab14) was a generous gift from Dr. Sam Gandy, anti-PS1-NTF rabbit pAb (34-4600, 1/1000) was purchased from Zymed. Anti-PS1 loop mouse mAb (MAB5232, 1/1000) and anti-nicastrin mouse mAb (MAB5556, 1/1000) were purchased from Chemicon. β-tubulin (clone 2-28-33, 1/5000), anti-actin (clone AC-40, 1/5000), and rabbit polyclonal anti-GAPDH (1/5000) were purchased from Sigma. A rabbit pAb against mouse v-ATPase V0a1 (W249, 1/5000) and mouse pAb against human v-ATPase V0a1 (Osw2, 1/500) were generous gift from Dr. Satoshi Sato and other rabbit pAb against V0a1 subunit of the vacuolar proton pump (1/500) was purchased from Synaptic Systems. The v-ATPase V1 B1 antibody (H-180, 1/200) was purchased from Santa Cruz Biocech. Following reagents were used for autophagy modulation experiments. Rapamycin (Rapa, final 10 nM), ammonium chloride (NH_4Cl, final 20 mM), bafilomycin A1 (final 0.2 μM) and 3-methyladenine (3MA, final 10 mM) were from Sigma and leupeptin (final 0.3 mM) was from Peptide Institute Inc. The γ-secretase inhibitor (L685,458) was purchased from Sigma.

Gel electrophoresis, immunoblotting and deglycosylation

Immunoblotting was performed as previously described (Yu et al., 2005). Briefly, cells used for Western blot analysis were lysed in buffer containing 50 mM Tris (pH=7.4), 150 mM NaCl, 1mM EDTA, 1 mM EGTA, 1 % Triton X-100 and 0.5 % Tween-20 with protease and phosphatase inhibitors. Following electrophoresis on 4 -20 % gradient gel (Invitrogen), proteins were transferred onto 0.45 μm PVDF membranes (Millipore) and the membrane was incubated overnight in primary antibody then incubated with HRP conjugated secondary antibody. The blot was developed by ECL-kit (GE Healthcare). To assess v-ATPase V0a subunit glycosylation, lysate from WT and PS1KO cells were either treated for 24 hrs at 37°C with PNGase F or O-glycanase using an enzymatic deglycosylation kit according to the manufacturer's instructions (PROzyme) or with Endo H (New England Biolabs) for 24 hrs at 37°C. Cells were treated for 24 hrs at 37°C

with tunicamycin (5μg/ml). Total glycoproteins were isolated using Glycoprotein Isolation Kit, ConA according to the manufacturer's instructions (Thermo Scientific).

Subcellular fractionation

Homogenate (0.5 ml) was layered on the top layer of 10, 15, 20, 25, and 30% Optiprep (Sigma) step gradient, 2.3 ml each, into polyallomer tubes (Beckman) and centrifuged in a SW-40Ti rotor with a model L8-80M Beckman ultracentrifuge (100,000 g, 16 hours, 4°C). After centrifugation, the gradients were fractionated into 0.5 ml fractions. 40 μl of each collected fraction was mixed with an equal volume of sample buffer and then loaded onto gels. The homogenates were fractionated into cytosolic and membrane fractions by high speed centrifugation (150,000 x g, 60 min) and equal proteins was loaded on gel following 55°C for 10 min incubation with 2x urea sample buffer. All data represent an average at least three independent experiments.

Autophagic vacuole isolation

AVs were isolated by centrifugation in a discontinuous metrizamide density gradient (Marzella et al., 1982) for each cell line. Cells (5×10^8) were serum-deprived overnight to induce autophagic activity prior to AV isolation. The cells were collected, disrupted by nitrogen cavitation then homogenized in 3 volume of 0.25 M sucrose in a glass homogenizer with Teflon pestle for 10 strokes. The homogenate was filtered through double gauze and then spun at 2000 x g for 5 min to yield a supernatant and pellet of unbroken cells and nuclei. The supernatant was centrifuged at 17,000 x g for 12 min to yield a pellet and a supernatant which was spun again at 100,000 x g for 1 hr to yield a pellet containing ER and a supernatant containing cytosol. The pellet from the 17,000 x g centrifugation was resuspended in the same volume of 0.25 M sucrose and spun again at 17,000 x g for 12 min. The pellet was resuspended in 1.9 ml of 0.25 M sucrose and 2.8 ml of metrizamide (Mtz). This mixture (2.4 ml volume) was loaded on top of a 26 % (4 ml), 24 % (2 ml), 20 % (2 ml) and 15 % (2 ml) Mtz step gradient matrix. The sample on the Mtz gradient was centrifuged at 247,000 rpm for 3 hrs in an ultracentrifuge using an SW41 rotor. Each gradient interface was collected and diluted in 0.25 M sucrose. The samples were then pelleted at 24,000 x g for 10 min. Light AVs (AV10) were present in

the 15-20 % fraction, while heavy AV (AV20) was present in the 20-24 %, lysosomes were in the 24-26 % interface, and mitochondria were located in the 26% Mtz area. Fractions were pelleted and immersed in a cacodylate fixation buffer for EM analysis or analyzed directly by Western blot or enzyme assay. All data represent an average at least three independent experiments, unless otherwise indicated.

Ultrastructural and Morphometric Analyses

Following treatments, cells were prepared for EM as previously described and AVs, identified using previous morphological criteria (Yu et al., 2005), were classified and counted on electron micrographs (7900x print magnification) of 20 EM images from different lines in each experimental group.

Lysosomal pH measurement

Quantification of lysosomal pH was determined using Dextran conjugated Lysosensor Yellow/Blue DND-160 (Invitrogen). Wild Type and PS1KO blastocysts were grown in High Glucose DMEM + 15 % FBS with antibiotics to ~ 90% confluency. Cells were then trypsinized, harvested (1 X 10^6 cells/ml) and loaded with 1mg/ml of Lysosensor-dextran for 1 hour at 37°C with 5% CO_2. The cells were then washed 3X in HBSS and aliquoted at 100 μl into a black 96-well microplate. pH calibration was performed according to the protocol established by Diwu *et al* (Diwu et al., 1999). Briefly, wild type and PS1KO blastocysts were treated with 10 μM monensin and 10uM nigericin in MES buffer (5 mM NaCl, 115 mM KCL, 1.3 mM MgSO4, 25 mM MES), with the pH adjusted to a range from 3.5-7.0. The samples were read in a Wallac Victor 2 fluorimeter (Perkin Elmer) with excitation at 355 nm. The ratio of emission 440/535 nm was then calculated for each sample. The pH values were determined from the linear standard curve generated via the pH calibration samples.

In vivo vesicle acidification study

The mouse was anaesthetized with a 1 % body weight IP injection of chloral hydrate, (400 mg/kg at a concentration of 50 mg/ml, 26 g needle, and volume less than 200 ul) and allowed sufficient time to go down. Under sterile conditions, the subject was then

shaved, and cleaned at the site of the surgery, in this case the scalp. The subject was placed in position in a stereotaxic holder with drill (BenchMark). The position of the drill/burr arm was located in right ventricle (-0.22 mm from bregma; L, -1 mm; D/V, -2.5 mm), the coordinates relative to the bregma, were determined by using *The Mouse Brain* (Keith B. J. Franklin and George Paxinos, Academic Press).

Mouse was injected with DAMP (20 µl of 30 mM stock solution prepared in PBS) by intra-ventricular method with 1.5 µl/min speed. After 4hrs, animals were anesthetized and perfused with a fixative containing 0.1 % glutaraldehyde and 4 % paraformaldehyde in sodium cacodylate buffer (Electron Microscopy Sciences). Brains were dissected and immersed in the same fixative for 4 hrs and then 40 µm sagital sections were made using a vibratome. The sections were processed routinely for electron microscopy and embedded in LR white. Ultrathin sections were mounted on nickel grids. Sections were treated with 4% NRS for 2hrs at room temperature. For DAMP, the sections were incubated with an antibody to DNP (Invitrogen 1:50) and CatD (1:50, DAKO) overnight. Grids were subsequently washed with PBS and incubated for 2 hrs in room temperature with secondary antibody coupled with 10 nm gold. Sections were washed with PBS and were stained briefly with uranyl acetate and lead citrate. Sections were examined and photographed with a Philips CM10 electron microscope.

Intracellular Protein Degradation Measurements

Total protein degradation in cultured cells was measured by pulse-chase experiments (Auteri et al., 1983). Confluent cells were labeled with [^{3}H]-leucine (2µCi/ml) for 48 hrs at 37°C in order to preferentially label long-lived proteins. Following labeling, cells were extensively washed and maintained in complete medium (DMEM + 10 % fetal bovine serum), under which conditions autophagy is suppressed, or in serum-deprived medium, where autophagy is induced. Under both conditions, after washing the cells, the medium was supplemented with unlabeled 2.8 mM leucine to prevent [^{3}H]-leucine reincorporation into newly synthesized proteins. Aliquots of the medium taken at different time-points were precipitated with 10 % TCA, filtered using a 0.22 µm pore membrane and radioactivity in the flow-through was measured. Proteolysis is expressed as the percentage of the initial acid-precipitable radioactivity (protein) transformed to

acid-soluble radioactivity (amino acids and small peptides) over time. To inhibit autophagy in this system, 20 mM NH_4Cl or 10 mM 3MA was added immediately after the labeling period and maintained at that concentration throughout the chase. NH_4Cl blocks lysosomal degradation, so this procedure permits one to estimate the relative contributions of lysosomal and non-lysosomal pathways to overall protein degradation. 3MA blocks formation and fusion of autophagosomes to lysosomes and is used to block macroautophagic contributions to proteolysis. Degradation rates of short-half life proteins were determined by the same procedure but after a labeling period of 30 min at 37 °C. Nearly all turnover of short-lived proteins is due to proteasomal activity. Protein synthesis was determined as the incorporation of [^{3}H]leucine into acid-insoluble material in the presence of an excess (2.8 mM) unlabeled leucine in the medium. Under these conditions incorporation of radioactivity into protein accurately reflects rates of proteins synthesis and minimizes differences due to alteration of amino acid transport and/or intracellular amino acid pool sizes.

Confocal laser scanning microscopy

Immunocytochemistry was performed as previously described (Yu et al., 2005). Secondary antibodies used were from Invitrogen: goat anti-mouse/rabbit/rat Alexa Fluor 488 and goat anti-mouse/rabbit/rat Alexa Fluor 568. Cells were imaged using a plan-Apochromat 40x or 100x/1.4 oil DIC objective lens on the laser scanning confocal microscope, LSM 510 META, with LSM software v3.5 (Carl Zeiss MicroImaging Inc). Images were analyses using ImageJ program (NIH). Organelles with low internal pH were labeled by LysoTracker DND-99 dye (Invitrogen) at the final 100 nM for 30 min. Following 4 % PFA fixation, cells were further count stained with Cat D, LMAP1, or LC3 antibody for 4 hrs then visualized with Alexa Fluor 488 conjugated secondary antibody. Active Cat D was labeled by adding Bodipy-FL-pepstatin A directly to the medium at a final concentration of 1 μg/ml for 1 hr. Following 4 % PFA fixation, cells were counter stained with Cat D antibody for 4 hrs and visualized with Alexa Fluor 568 conjugated secondary antibody. For assessing Cat B activation, MagicRed–Cathepsin B (Immunochemistry Technologies) for active Cat B was added to the cells at the concentration suggested by company (1:260). Cells were incubated for 30 min with MR-

Cathepsin B prior to mounting them under the confocal microscope. To block v-ATPase proton pump activity, ammonium chloride (NH_4Cl) was added directly to the medium at a final concentration of 20 mM for 6 hrs prior to LysoTracker addition.

Co-immunoprecipitation

Wild type mouse blastocysts cells were lysed with lysis butter (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 100 mM EDTA, and protease inhibitor cocktail) with 1 % digitonin. Immunoprecipitations of complex proteins with anti-PS1 pAb, anti-V-ATPase V0a1 subunit pAb, anti-Sec61α, or anti-STT3B were performed using the Seize Primary immunoprecipitation kit (Pierce Biotechnology) (Hiesinger et al., 2005).

Degradation of HRP internalized by fluid phase endocytosis.

HRP degradation was evaluated in cells that were incubated for 30 min at 37 °C with medium containing 5 mg/ml horseradish peroxidase (HRP) (HRP type II; Sigma) and washed three times with phosphate-buffered saline then chased 37 °C for 0, 1, and 6 hrs with fresh media. Following fixation, the cells were immunolabeled with rodamine-conjugated anti-HRP antibody (Jackson lab).

Enzymatic assays

Cathepsin B and L activity were assayed as described previously (Nakanishi et al., 1994) and Cathepsin D activity was assayed using [^{14}C]methemoglobin as previously described (Dottavio-Martin and Ravel, 1978). A 50 μl aliquot of enzyme was incubated at 37 °C with 100 μl of 0.4 M acetate buffer (pH 3.2) containing 10 mg/ml [^{14}C]methemoglobin. Cathepsin D activity was expressed in terms of methemoglobin degrading activity; one unit of enzyme activity is defined as the capacity to degrade one nanomol of methemoglobin per minute.

Cathepsin D metabolic labeling

Cells were grown in a 3.5 cm dish to 5 x 10^5 cells/dish, after which they were metabolically labeled for 30 minutes with 100 mCi/ml [35S]methionine/cysteine (EXPRE35S35S Protein Labeling Mix, NEG0702 14MC: Perkin Elmer) and chased with

normal medium for specific periods. The labeled cells were lysed and subsequently processed for immunoprecipitation with anti-rat cathepsin D antibody and protein G-agarose bead (Santa Cruz Biocech). The immunoprecipitates were analyzed by 10 % SDS-PAGE.

Transfection of plasmid

Fibroblast were transfected with mRFP-GFP-LC3 (Kimura et al., 2007) from Dr. Tamotsu Yoshimori (Osaka University) using Lipofectamine 2000 (Invitrogen) according to manufacture's instruction. Following transfection fibroblasts were incubated for 24 hrs and before imaging incubated for another 6 hrs with serum or without serum medium.

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