Cell Reports

Supplemental Information

Presenilin 1 maintains lysosomal Ca^{2+} homeostasis by regulating vATPase-mediated lysosome acidification

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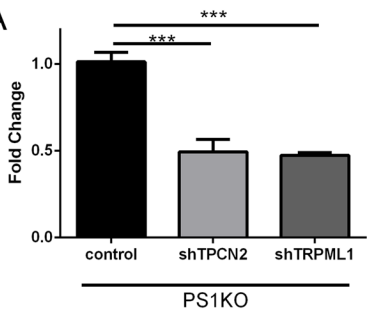

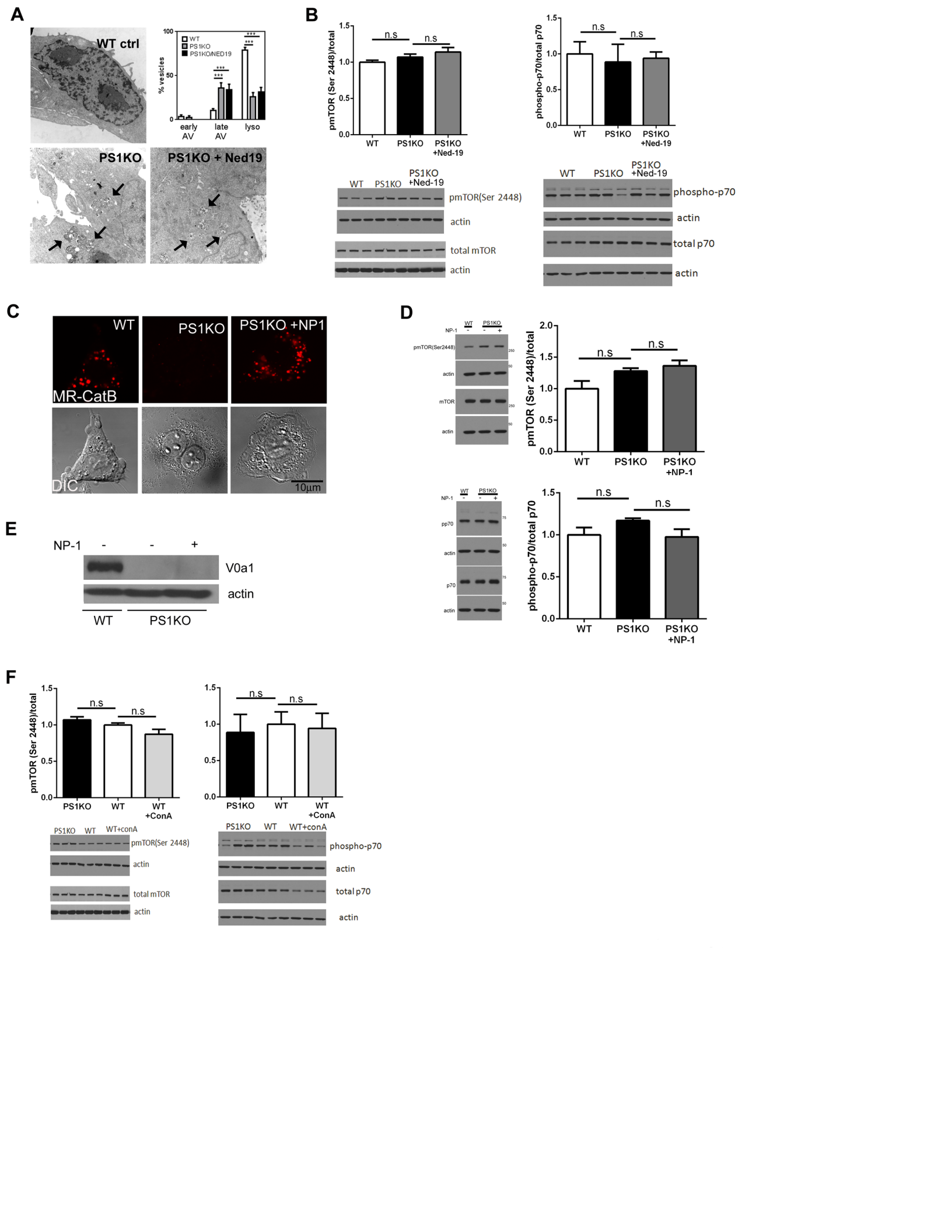
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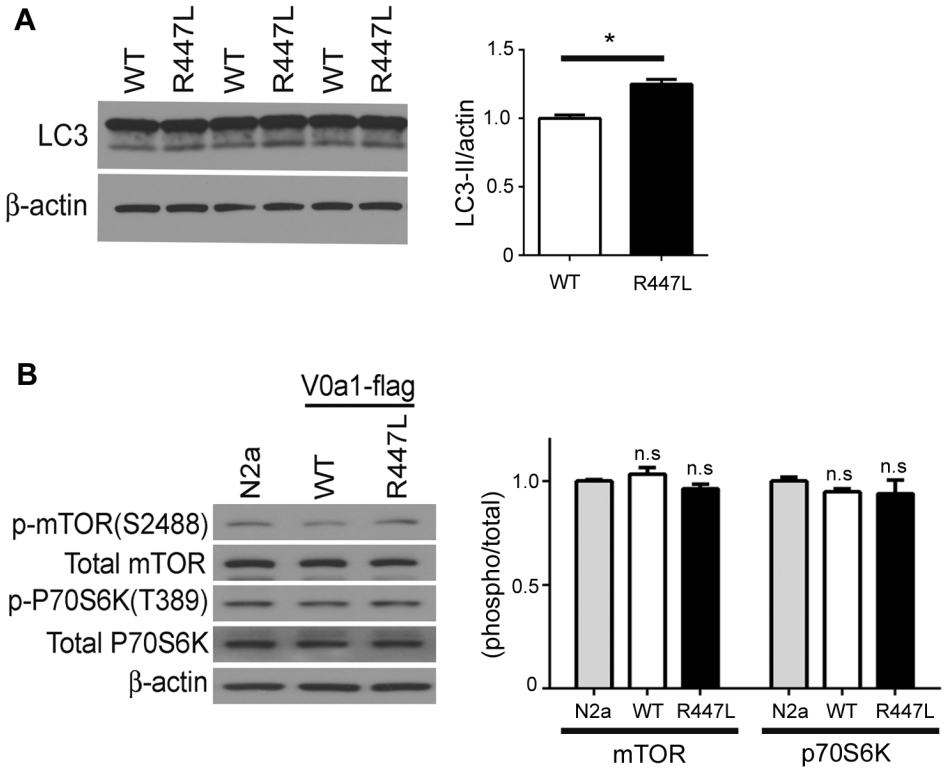
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SUPPLEMENTARY FIGURE LEGEND

Figure S1: Verification of gene knockdown, related to Figure 1. (A) PS1KO cells were transfected with either GFP-shTPCN2 or GFP-shTRPML1 for 72 hours and the fold change in mRNA expression assessed by quantitative PCR. There was significant reduction in levels of mRNA for either TPCN2 or TRMPL1. Graph is plotted ±SEM for an average of 3 experiments. *** denotes $p<0.001$.

Figure S2: Additional experiments demonstrating lack of rescue with Ned-19, related to Figure 2; Treatment with NP-1 does not affect levels of V0a1 subunit, related to Figure 2; Treatment with Ned-19, NP-1, or ConA does not alter induction of autophagy, related to Figure 2, 3, and 4. (A) Percentages of AVs or lysosomes remains unchanged in Ned-19 treated (24 hrs, 0.5μM) PS1KO blastocysts. (B) Levels of either phospho-mTOR or phospho-p70S6K are unchanged with Ned-19 (24hrs, 0.5μM) treatment. (C) Levels of MR-CatB are elevated with NP-1 (24hr, 1mg/mL) treatment. (D) Levels of either phospho-mTOR or phospho-p70S6K remained unchanged with NP-1 (24hrs, 1mg/mL) treatment. (E) Levels of V0a1 are unchanged with NP-1 (24hr, 1mg/mL) treatment. (F) Levels of phospho-mTOR and phospho-p70S6K are unchanged with ConA (50nM, 24hrs) treatment in WT cells.

Figure S3: Additional experiments demonstrating phenotype of V0a1^{R447L}-flag cell lines, related to Figure 6. (A) Immunoblot analysis of LC3-I and LC3-II levels in V0a1-flag and V0a1^{R447L}-flag cells. Results were plotted as ratios normalized to V0a1-flag. (B) Immunoblot analysis of phospho- mTOR and P70S6K levels in V0a1-flag and V0a1^{R447L} mutant cells. Results were plotted as ratios normalized to native N2a cells. * denotes $p<0.05$, ** denotes $p<0.001$, *** denotes $p<0.0001$. Error bars represents standard error of the mean (±S.E.M). β-actin blot provided as a loading control.

SUPPLEMENTARY EXPERIMENTAL PROCEDURES

Cell lines and constructs

Murine blastocysts with different PS1 genotypes (WT, BD6; PS1KO, BD15) were grown in DMEM (Invitrogen, 11995-073) supplemented with penicillin/streptomycin (Invitrogen), Glutamax, 15 % fetal bovine serum (Hyclone), NEAA (Invitrogen) and β-mercaptoethanol (Sigma). Human and MLIV fibroblasts were purchased from Coriell Cell Repository and have numbers GM005399 and GM002527 respectively and maintained in 10% FBS in DMEM with 5% CO_2.Murine neuroblastoma (N2A) cells were maintained in DMEM with penicillin/streptomycin and 10% FBS at 37°C and 5% CO_2. Mouse PS1 (MSS208049) and scramble (12935-200) siRNA were purchased from Invitrogen and mouse vATPase V0a1 siRNA (11975) from Thermo Scientific. Cells were transfected using Lipofectamine RNAiMAX (Invitrogen). Primary cortical neuronal culture were derived from E15 stage pups from PS1 (+/-) crosses and cultured on poly-D-lysine (100 mg/ml, Sigma) coated plates with Neurobasal medium containing B27, 0.5 mM Glutamax and 50 units/ml Penicillin/Streptomycin.

Murine neuroblastoma cells (N2a) were maintained in DMEM (Life technologies, 11995-073)/OPTI-MEM Life technologies, 51985091) with10% FBS (GE Healthcare, SH30070.01), and penicillin/streptomycin (Life technologies, 15140-122) at37°C and5% CO_2. A musculus v-ATPase V0a1-Flag construct (EX-Mn20338-M13) was purchased from GeneCopoeia. A V0a1 R447L-flag mutant DNA construct was generated using a QuikChange II XL site-directed mutagenesis kit (Agilent technologies, #200521) according to manufacturer's instructions with 5`-GCATGGTGTTCAGCGGCCTATACATTATTCTTCTGATG-3` and 5`-CATCAGAAGAATAATGTATAGGCCGCTGAACACCATGC-3` primer set. WT and R447L mutant constructs were transfected using LipoJet (SignaGen, Sl100468) according to

manufacturer's instructions. Selection for cells containing the required construct was performed in normal growth medium with G418 (Sigma, 500μg/ml) for 14 days. After establishing the stable cell line, cells were maintained with G418 (300 μg/ml) containing medium.

shRNA mediated gene knockdown

Either GFP-shTPCN2 or GFP-shTRPML1 (Qiagen) were transfected with Lipofectamine 2000 (Invitrogen) for 72 hours and calcium measurements for cells containing the GFP signal were calculated for either cytosolic or lysosomal calcium. Knockdown efficiency was quantified by qPCR using primers for either TPCN2 or TRPML1 compared to GAPDH (Applied Biosystems,).

Antibodies and Reagents

Anti-LC3 rabbit pAb (Novus 100-2220, 1/1000) and anti-calnexin mouse mAb (NB300-518, 1/1000) were purchased from Novus.Anti-PS1 loop mouse mAb (MAB5232, 1/1000) and anti-nicastrin mouse mAb (MAB5556, 1/1000) were purchased from Millipore. Rabbit polyclonal anti-GAPDH (1/5000) anti-beta actin mouse mAb (A4700, 1/10,000) were purchased from Santa Cruz. pAb against V0a1 subunit (sysy, 1/1000) was purchased from Synaptic Systems. Anti-V1B2 (ab73404, 1/2000) and V1E1 (ab111733, 1/2000) were purchased from Abcam. Rabbit anti-Cathepsin DpAb (1/5000) was generated in house. Anti-murine LAMP2 (ABL97, 1/200) mAb was purchased from Developmental Studies Hybridoma Bank. Anti-PDI antibody (SPA891, 1/5000) was purchased from Stressgen. Anti-Flag antibody (2368, 1/1000), P70S6K (9202, 1/1000), pP70S6K (9205, 1/1000), p-mTOR (2971, 1/1000) and mTOR (2972, 1/1000) were purchased from Cell Signaling. Ribophorin-I (AB38451, 1/1000) was from Abcam. Anti-Nicastrin mAb antibody (MAB5556, 1/1000) was from Millipore. LysoTracker RedDND-99 (L7528), Bodipy-FL-pepstatin A (P12271) and LysoSensor yellow/blue-dextran (L22460) were from Life technologies. Anti-APP antibody (C1/6.1, 1/1000) was produced in-

house (Jiang et al., 2010). Magic-Red Cathepsin B detection kit was from Immunochemistry Technologies, LLC.Anti-EEA1 mouse mAb (clone 14, 1/1000) was purchased from BD Bioscience. Guinea pig polyclonal anti-p62 (GP62-C, 1/2000) was from Progen Biotechnik. Anti-Calnexin mouse mAb (1/1000) was from Affinity Bioreagent. Cycloheximide (C7698), MG-132 (M7449) and G418 (A1720) were from Sigma. Cyto-ID autophagy detection kit (ENZ-51031-K200) was from Enzo Life sciences.

Gel electrophoresis and immunoblotting

Cells used for western blot analysis were lysed in buffer containing 50 mM Tris (pH7.4), 150 mM NaCl, 1mM EDTA, 1 mM EGTA, 1% Triton X-100 and 0.5 %Tween-20 with protease and phosphatase inhibitors (Roche). Samples were mixed with 2x urea sample buffer and incubated 15 min at 55°C for vATPase and PS1 electrophoresis, otherwise samples mixed with 2x SDS sample buffer and incubated 5 min at 100°C followed by electrophoresis. The blot was developed using an ECL-kit (Invitrogen).

Confocal laser scanning microscopy

Immunocytochemistry was performed as previously described (Yu et al., 2005). Organelles with low internal pH were labeled by LysoTracker DND-99 at a final concentration of 100 nM for 1 hr. For assessing *in vivo* CatB activation, cells were incubated with MagicRed–CatB for 30 min, and then imaged. 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 washes with PBS, fresh media was added for live imaging using a plan-Apochromat 40x or 100x/1.4 oil DIC objective lens on a LSM510 META laser scanning confocal microscope, with images acquired using LSM software 3.5 (Carl Zeiss MicroImaging Inc). Images were analyzed using the Image J program (NIH).

Deglycosylation and cycloheximide treatment

To assess flag-tagged V0a1 mutant glycosylation, samples were treated for 24 hrs at 37 C with PNGase F using an enzymatic deglycosylation kit according to the manufacturer's instruction (PROzyme). To assess the stability of the V0a1 mutant, samples were treated with CHX (100 μg/ml) for 0, 2, 4, 8, 24 hrs with or without pre-treatment with MG-132 (1 μM/ml, 24 hrs) and then analyzed by SDS-PAGE.

Subcellular Fractionation

Cell homogenate (0.5ml) was added onto the top layer of a 10, 15, 20, 25, and 35% Optiprep (Sigma) step gradient containing 2.3 ml of each percentage in polyallomer tubes (Beckman). After centrifugation in a SW-40Ti rotor within a model L8-80M Beckman ultracentrifuge (1000,000 x g, 16 hrs, 4 C), the gradients were fractionated into 0.5 ml fractions and a 40 μl aliquot of each fraction was loaded onto the SDS-PAGE gel.

Enzyme activity for Cathepsins

To assess CatB activation *in situ* within cells, MagicRed– Cathepsin B (Immunochemistry Technologies) at the concentration suggested by the company (1:260) and cells were incubated for 30min. To assess levels of CatD activation, Bodipy-FL-pepstatin A (Life technologies) was added to the cells in a final concentration of 1 μg/ml for 60 min. After washing the cells with PBS, new medium was added for live imaging by confocal microscopy.

Lysosomal pH measurement

Procedures were performed as previously described (Wolfe et al., 2013). Following the addition of 2ul of 0.05mg/ml LysoSensor Yellow/Blue-dextran treatments, cells were incubated for 24 hours. The samples were then read in a Wallac Victor 2 fluorimeter (Perkin Elmer) with excitation at 355 nm. The ratio of emission 440 nm/535 nm was then calculated for each sample. The pH values were determined from the standard curve generated via pH calibration samples.

Ultrastructural analyses

Procedures were performed as previously described (Lee et al., 2010). Following treatments, cells were prepared for EM as previously described and AVs, identified using previous morphological criteria. Sections were examined and photographed with a Philips CM10 electron microscope.

Lysosomal Isolation

Cells were incubated in growth medium containing 1mM HEPES, pH 7.2 and 10% Dextran conjugated magnetite (Liquid Research LLC) for 24 hrs, then chased in normal growth media for 24 hrs. Cells were washed in PBS then harvested in 4ml of ice cold Buffer A (15 mM KCL, 1.5mM MgAc, 1mM DTT, 1mM HEPES, 1X Protease Inhibitor Cocktail (Sigma). Cells were then homogenized with 40 strokes of a tight fitting pestle in a Dounce homogenizer then passed through a 22G needle 5 times. After homogenization, 500ul of ice cold Buffer B (220mM HEPES, 375mM KCL, 22.5mM MgAc, 1mM DTT, DNase I) was added and samples were then centrifuged at 750 x g for 10 mins. The supernatant was then decanted over a QuadroMAC LS column that had previously been equilibrated with 0.5% BSA in PBS. The pellet was subjected to re-addition of 4ml cold Buffer A, 500µl cold Buffer B and then resuspended and recentrifuged. This second supernatant was also passed over the column and allowed to flow through via gravity. DNase I (10µl/ml in PBS) was added and the column was then incubated for 10 min and then washed with 1ml cold PBS. Lysosomes were eluted by removing the column from the magnetic assembly, adding 500µl of PBS and forced through the column using a plunger.

The vATPase activity assay

Lysosome-enriched fractions (32.5 μg total protein) were mixed with 3 vol of Buffer A (100 mM MES–Tris buffer, 80 mM KCl, 6 mM $MgCl_2$, 150 mM NaCl, pH 7.0) and incubated at 37°C for 5 min. After incubation, the reaction was started by addition of 2 mM ATP and incubated 20 min at 37°C. The ATPase reaction was stopped after 25 min by the addition of 2 ml of molybdate solution (2% v/v H_2SO_4, 0.5% w/v ammonium molybdate and 0.5% w/v SDS) and 0.2 ml of ascorbic acid (2% w/v), then developed for 5 min at room temperature. Control samples were measured in the presence of the vATPase inhibitor ConA (μM) and the experimental values were subtracted accordingly. Absorbance was measured at 750 nm and solutions of KH_2PO_4were used to generate a standard curve.

Proton Translocation assay

Proton transport activity into the lumen of isolated lysosomes was measured by fluorescence quenching of 9-amino-6-chloro-2-methoxyacridine (ACMA) in the presence or absence of 1 μM concanamycin A. Lysosomes (25 μg) were added to a cuvette containing 2 ml of reaction buffer [10 mM BisTrisPropene (BTP)-MES, pH 7, 25 mM KCl, 2 mM MgSO4, 10% glycerol and 2 μM ACMA]. The reaction was started by the addition of 1 mM ATP in BTP, pH 7.5, a measurement (ex412/em480)taken every 5 seconds for 600 seconds on a SpectraMax M5 multimode reader(Molecular Devices).

Lysosomal Ca^{2+} Measurements

Cells were plated on glass bottom dishes the night before treatment. For baseline calcium measurements, cells were incubated with 25mg/mL rhod-dextran for 12 hrs before imaging. Cells were pretreated with Ned-19 (Enzo) for 24 hrs prior to the addition of 25mg/ml rhod-dextran. Cells were pretreated with ConA (Sigma) for 4 hrs before the addition of rhod-dextran.

Cells were incubated in rhod-dextran for a minimum of 12 hrs. After incubation, cells were chased for 30 mins in complete media before it was exchanged for calcium-free HBSS (Invitrogen) and cells were imaged. The mean intensity of the rhod signal of single cells (30 to 40 per experiment) was analyzed using ImageJ (NIH). For all cytosolic measurements, cells were incubated with either 5μM Oregon-Green 488 Bapta-1 AM (Life Technologies) or 2μM Fura-2 AM (Life Technologies) for 1 hour and then chased with complete medium for 30 minutes. Cells were washed with HBSS (Invitrogen) and imaged and analyzed using ImageJ (NIH).

Lysosomal Ca^{2+} release measurements

Release of lysosomal Ca^{2+} was measured using methods adapted from those previously described (Lloyd-Evans et al., 2008). Cells were plated on μ-Slide 8 well imaging dishes (ibidi), left to adhere overnight and then treated with 2μg/ml U18666a for 24 hrs as appropriate. Post incubation, cells were loaded with either 5μM Fluo3-AM or 5μM Fura 2-AM (StratechScientific LTD) in DMEM with 1% BSA and 0.0025% Pluronic acid F127 for 1 hr at 37°C then washed, left for 10 minutes to allow deesterification of the calcium dye, and imaged in 1x HBSS with 1mM HEPES, 1mM $MgCl_2$ and 1mM $CaCl_2$. Ca^{2+} free HBSS was used for all human fibroblast experiments. Intracellular Ca^{2+} responses were recorded using a Zeiss Colibri LED microscope system with an AxiocamMrm CCD camera and Zeiss Axiovision software version 4.7 with the additional physiology module for live cell Ca^{2+} imaging, after addition of 1μM thapsigargin (Sigma), 5μM ionomycin (Calbiochem), 300μM GPN (Sigma), 100nM NAADP-AM (synthesized according to (Parkesh et al., 2008) or as required for the experiment). Cytoplasmic regions of interest (ROIs) were drawn and analyzed per field of view per experiment. 20μM ML-SA1 (EMD) was used for blastocyst experiments and 50μM ML-SA1 was used for human fibroblast experiments. PS1KO cells were treated with 5μg/mL anti-TRPML1 (Sigma) for 16

hours prior to ML-SA1 treatment. PS1KO cells were treated with 10μM YM201636 (Invitrogen) for 1 hour prior to experiments.

Acidic nanoparticle treatment

Poly (DL-lactide-co-glycolide) (PLGA) ResomerH RG 502 H was purchased from Boerhinger Ingelheim Inc., VA and prepared as previously described (Baltazar et al., 2012). Nanoparticles used in this study were prepared at 1mg/ml in complete culture medium after sonication for a minimum of 3x5min; solutions were vortexed in between sonication periods and after the final sonication step. Immediately following sonication, nanoparticle solutions were passed through a 0.8μm filter to remove clumped particles, and then applied directly to cells for the treatment time indicated. All nanoparticle formulations were made up for same-day use only.

Analytical Procedures

Quantitative colocalization analysis was performed using ImageJ software (NIH Image) with colocalization analysis plugins (Wright Cell Imaging facility). The value shown represents Pearson's coefficient. Statistical analysis was calculated by two-tailed paired Student's t-test using GraphPadInStat (GraphPad Software Inc.) unless otherwise indicated. All experiments were performed in triplicate unless otherwise indicated. Error bars represent standard error of the mean (±S.E.M).

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