Two Pore Channel 2 (TPC2) Inhibits Autophagosomal-Lysosomal Fusion by Alkalinizing Lysosomal pH*\(^{[5]}\)

Received for publication, May 8, 2013, and in revised form, July 1, 2013 Published, JBC Papers in Press, July 8, 2013, DOI 10.1074/jbc.M113.484253

Yingying Lu\(^1\), Bai-Xia Hao\(^1\), Richard Graeff\(^3\), Connie W. M. Wong\(^6\), Wu-Tian Wu\(^6\), and Jianbo Yue\(^{1,1}\)

From the \(^5\)Department of Physiology, University of Hong Kong, Hong Kong, China, \(^6\)Department of Anatomy and State Key Laboratory of Brain and Cognitive Sciences, University of Hong Kong, Hong Kong, China, and \(^\ast\)GHM Institute of CNS Regeneration, Jinan University, Guangzhou, China

**Background:** The role and mechanism of NAADP, an endogenous Ca\(^{2+}\) mobilizing nucleotide, in autophagic regulation remain to be determined.

**Results:** Activation of NAADP/TPC2 signaling induced the accumulation of autophagosomes.

**Conclusion:** The NAADP/TPC2 signaling inhibits autophagosomal-lysosomal fusion by alkalinizing lysosomal pH.

**Significance:** Development of agonists or antagonists of NAADP should provide a novel approach to specifically manipulate autophagy.

---

**WITHDRAWN**

June 26, 2017

This article has been withdrawn by the authors. After reviewing the data, the authors found that the first author of the paper made inappropriate modifications to the background of the following figures: Figs. 2C, 2D, 3B, 3E, 4A, 4E, 5E, 6D, 8A, 8C, and supplemental Figs. S1A, S2B, and S5B. The first author admits to making the modifications. The modifications include covering cells not expressing RFP-GFP-LC3, GFP-LC3, or RFP-Lamp, incomplete cells on the edge of the images, or contaminant staining dots outside of the cells in images (Figs. 2C, 2D, 3B, 3E, 4A, 4E, 6D, 8A, 8C, and supplemental Figs. S1A and S2B). Additionally, other modifications include covering dirty dots above the bands in the Western blots (Fig. 5E and supplemental Fig. S5B) and inadvertently inserting a box in LC3 blot of Fig. 4E. Although these modifications did not change the results or interpretations of this work, these figures were not prepared according to the publication policy of the journal. Therefore, the authors wish to withdraw this article and sincerely apologize for these mistakes.

Macroautophagy (hereafter referred as autophagy) is an evolutionarily conserved catabolic degradation cellular process. Autophagy starts with sequestering of cytoplasmic material, such as misfolded proteins and/or damaged organelles, inside the phagophore followed by the elongation and closure of the autophagosome, a double-membrane vesicle. The matured autophagosome, a double-membrane vesicle. The matured autophagosome is then transported to the lysosomes where the inner membrane is degraded and the luminal contents are degraded by lysosomal enzymes for subsequent release.

* This work was supported by Research Grant Council Grants HKU 782709M, HKU 785911M, and HKU 785213M and a Special Fellow Award from the Leukemia and Lymphoma Society of America (to J. Y.).

\( ^{[5]}\) To whom correspondence should be addressed. E-mail: jyue@hku.hk.
Inhibition of Autophagy Progression by TPC2

HOPS complex (8), TECPR1 (9), histone deacetylase-6 (10), ubiquilins (11), Hrs (12), OATL1 (13), COP9 signalosome (14), presenilin 1 (15), the ESCRT III complex (16), LAMP1/2 (17), UVRAG (18), p97VCP (19), LRRK2 (20), and HSP70 (21) have been implicated in regulating this process. We have been studying the effects of lysosomal specific ion channels on autophagosomal-lysosomal fusion.

In vertebrates, three TPC2 genes, TPC1, -2, and -3, have been cloned. TPCs contain two putative pore-forming repeats, with each of the repeats containing six transmembrane domains and an intervening pore-forming loop. The transmembrane domain of TPCs is similar to that of voltage-gated Ca\(^{2+}\)/Na\(^+\) channels. Interestingly, human and rodent only encode TPC1 and TPC2. TPC2 predominantly localizes on lysosomes, whereas TPC1 is mainly on late endosomes, and TPC3 is thought to be in the recycling endosomes. Ever since their identification, TPCs have become the prime candidates for NAADP-mediated Ca\(^{2+}\) release from lysosome-related stores (22–24). NAADP is a metabolite of nicotinamide adenine dinucleotide phosphate (NADP) and is formed by a base-exchange reaction catalyzed by ADP-ribosyl cyclases, which replaces the nicotinamide moiety of NADP with nicotinic acid. NAADP mobilizes Ca\(^{2+}\) from acidic lysosome-related stores in a wide variety of cells, from plant to animal, including human (25). Ample evidence indicates that TPC2 forms NAADP-sensitive Ca\(^{2+}\)-permeable channels in many cell types. TPC2 overexpression promotes NAADP-induced Ca\(^{2+}\) release from lysosome-related stores, whereas ablating or knocking-down TPC2 abolishes NAADP-mediated smooth muscle contraction and inhibits NAADP-mediated smooth muscle contraction and Na\(^{+}\)/H\(^{+}\) antiporters. Moreover, TPC2 knock-out abolishes NAADP-mediated smooth muscle contraction and Na\(^{+}\)/H\(^{+}\) antiporters. TPC2 predominantly localizes on lysosomes, whereas TPC1 is mainly on late endosomes, and TPC3 is thought to be in the recycling endosomes. Ever since their identification, TPCs have become the prime candidates for NAADP-mediated Ca\(^{2+}\) release from lysosome-related stores (22–24). NAADP is a metabolite of nicotinamide adenine dinucleotide phosphate (NADP) and is formed by a base-exchange reaction catalyzed by ADP-ribosyl cyclases, which replaces the nicotinamide moiety of NADP with nicotinic acid. NAADP mobilizes Ca\(^{2+}\) from acidic lysosome-related stores in a wide variety of cells, from plant to animal, including human (25).

Previously, two groups found that activation of NAADP/H11001 release. Moreover, TPC2 knock-out abolishes NAADP-mediated smooth muscle contraction and Na\(^{+}\)/H\(^{+}\) antiporters. TPC2 predominantly localizes on lysosomes, whereas TPC1 is mainly on late endosomes, and TPC3 is thought to be in the recycling endosomes. Ever since their identification, TPCs have become the prime candidates for NAADP-mediated Ca\(^{2+}\) release from lysosome-related stores (22–24). NAADP is a metabolite of nicotinamide adenine dinucleotide phosphate (NADP) and is formed by a base-exchange reaction catalyzed by ADP-ribosyl cyclases, which replaces the nicotinamide moiety of NADP with nicotinic acid. NAADP mobilizes Ca\(^{2+}\) from acidic lysosome-related stores in a wide variety of cells, from plant to animal, including human (25).

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—LC3 antibody (antibody from MBL was used in study in the embryonic stem (ES) cells, and the one from Novus was used in the study in HeLa cells; 1:50 for the immunofluorescence analysis and 1:1000 for the Western blotting analysis (WB)), p62 antibody (Novus; 1:1000 WB), cathepsin-L antibody (BD Bioscience; 1:250 WB), Lamp-1 and EGFR antibodies (Santa Cruz; 1:250 WB), Lamp-1 antibody (Cell Signaling; 1:500 immunofluorescence analysis), phospho-mTOR and mTOR antibodies (Cell Signaling; 1:500 WB), GAPDH antibody (Sigma; 1:5000 WB), and TPC2 antibody (a custom rabbit polyclonal antibody against rat TPC2; 1:500 immunofluorescence analysis and 1:1000 WB). Bafilomycin A1, rapamycin, and BAPTA-acetoxyethanol (AM) were all purchased from Sigma. NH\(_4\)Cl was purchased from Merck. Fura-2 AM, dye-quenched (DQ)-BSA-green, lysosensor Green DND-160, and LysoSensor Yellow/Blue DND-160 were purchased from Invitrogen.

Cell Culture—HeLa cells (ATCC) were maintained in DMEM (Invitrogen) supplemented with 1% fetal bovine serum (Invitrogen) and 100 units/ml penicillin/streptomycin (Invitrogen) at 5% CO\(_2\) and 37 °C. Embryonic stem cells from Prof. S. Y. Tsang (Chinese University of Hong Kong) and Prof. D. E. van der Kooy (Cambridge, MA), were normally maintained in Dulbecco’s modified Eagle’s medium plus 15% FBS (ES qualified, Cambridge Bioscience), 1% penicillin-streptomycin, 1% L-glutamine, 1000 units/ml leukemia inhibitory factor, and 0.1 mM nonyl-

Intracellular Ca\(^{2+}\) Measurement—Intracellular Ca\(^{2+}\) in HeLa cells were measured as described previously (46, 47). Briefly, cells were cultured in 24-well plates at the density of 5 \times 10\(^5\) cells/well in regular DMEM medium overnight. Before Ca\(^{2+}\) measurement, cells were incubated for 30 min in Hanks’ balanced salt solution containing 4 μM Fura-2 AM and 0.04% F127 in the

2 The abbreviations used are: TPC2, two pore channel 2; NAADP, nicotinic acid adenine dinucleotide phosphate; tLC3, GFP-LC3, RFP-GFP-LC3; BAF, bafilomycin-A1; EGFR, EGFR receptor; ES, embryonic stem; WB, Western blot; RFP, red fluorescent protein; AM, acetoxyethyl; DQ, dye-quenched.
dark at room temperature. The cells were then washed with Hanks’ balanced salt solution three times and incubated at room temperature for another 10 min. Cells were put on the stage of an Olympus epifluorescence microscope and visualized using a 20× objective. Fluorescence images were obtained by alternate excitation at 340 and 380 nm with emission set at 510 nm. Images were collected by a CCD camera every 3 s. Finally, the images were saved and analyzed by Cell R imaging software.

**Western Blot Analysis**—Western blot analysis was performed as described previously (48, 49). Briefly, cells were lysed in an ice-cold EBC lysis buffer (50 mM HEPES at pH 7.5, 0.15 M NaCl, 1 mM EDTA, 1% Nonidet P-40, 150 μM PMSF, 10 mM NaF, 10 ng/ml leupeptin, 1 mM DTT, and 1 mM sodium vanadate) and passed through a 21-gauge needle several times to disperse any large aggregates. Protein concentrations of the cell lysates were determined by Bradford protein assay. 30–100 μg of protein per lane was diluted in the standard SDS sample buffer and subjected to electrophoresis on SDS-PAGE gels. Proteins were then transferred to an Immobilon PVDF membrane (Millipore, Billerica, MA), blocked with 5% milk in TBST (20 mM Tris, 150 mM NaCl, pH 7.6), and incubated with the primary antibody overnight. After washing with TBST, the blots were probed with a secondary antibody (1:5000 dilution) for detection by chemiluminescence.

**RNA Isolation and Quantitative Real-time RT-PCR**—Total RNA was extracted from HeLa cells or mouse ES cells at various stages of neural differentiation using an RNA extraction kit (Invitrogen). The quantitative real-time RT-PCR was performed in a MiniOpticon™ Real-time PCR detection system (Bio-Rad) according to the manufacturer’s instructions. The primers for detecting tpc2 and GAPDH mRNAs are listed in supplementary Table I. GAPDH expression was normalized to GAPDH expression.

**Immunofluorescence Staining Analysis**—Immunofluorescence staining analysis was performed as described previously (50). Briefly, infected or transfected cells grown on coverslips were fixed for 20 min with 4% paraformaldehyde at room temperature and washed twice with PBS, and nuclei were labeled with DAPI to identify each individual cell. For immunostaining analysis, fixed cells were further blocked with 1% goat serum, 1% BSA, 0.1% Triton X-100 in PBS for 1 h. Thereafter, the cover glasses were incubated with primary antibodies for 2 h, washed, and incubated with a secondary antibody (Alexa Fluor® 488 or 568 goat anti-mouse or rabbit IgG, Invitrogen; 1:1000 dilution) for 1 h. Images were captured under a Zeiss LSM710 confocal microscope with a Plan-Apochromat ×63/1.4 oil differential interference contrast objective. Images were processed and analyzed with Zeiss ZEN software.

**Transmission Electron Microscopy**—Cell preparation for transmission electron microscopy was performed as described previously (51). Briefly, cells were washed in PBS and fixed in 2% formaldehyde and 2.5% glutaraldehyde for 2 h. After fixation, cells were pelleted, transferred to glass slides, and allowed to dry on a hotplate at 37 °C before embedding in 2% agarose solution. Agarose-embedded blocks were then fixed in the same fixative solution at 4 °C for 4 h. Thereafter, the blocks were washed three times with PBS and post-fixed with 1% osmium tetroxide solution in PBS at 4 °C overnight. Blocks were subsequently dehydrated in graded ethanol, embedded in pure Epon, and kept at 60 °C for 3 days. Ultrathin sections (~90 nm) were cut and stained with 8% uranyl acetate and lead citrate before observation under electron microscope.

For immunoelectron microscopy, cells were incubated with primary antibody (rabbit anti-LC3 antibody, 1:100) at 4 °C overnight. After incubation in the primary antibody, the cells were incubated in biotinylated secondary antibody (biotinylated goat anti-rabbit IgG antibody, 1:200) for 40 min at room temperature. Cells were then incubated in ABC reagents (Vectorstain ABC kit) for 60 min at room temperature, sequentially, followed by incubating in 0.05% dianaminobenzidine and 0.03% H2O2 for 3–5 min until a brown reaction product was observed. The stained cells were then processed to transmission electron microscopy study as described above.

**Neural Differentiation**—The in vitro neural differentiation of mouse ES cells was performed as described previously (52). Briefly, feeder-free ES cells were plated onto 0.1% gelatin-coated plates at a density of 0.8 × 104 cells/cm2 in N2B27 medium (1:1 mixture of DMEM/F12 and neurobasal medium supplemented with B27 plus 50 μg/ml bovine serum albumin, 100 μM β-mercaptoethanol). The medium was changed every day until day 8.

**LysoSensor Green DND-189 Inhibition of Autophagy Progression by TPC2**

The pH calibration curve was generated as described previously (54). Briefly, cells were trypsinized and labeled with 2 μM LysoSensor Green DND-189 in prewarmed regular medium for 20 min at 37 °C. Then the cells were washed twice with PBS and immediately analyzed by flow cytometry (collecting FL1 fluorescence, and 10,000 cells were collected for each sample) or in a microplate reader (excitation/emission = 485/530 nm) in triplicate. Quantification of lysosomal pH was performed using a ratio-metric lysosomal pH dye LysoSensor Yellow/Blue DND-160. The pH calibration curve was generated as described previously (55). Briefly, cells were trypsinized and labeled with 2 μM LysoSensor Yellow/Blue DND-160 for 30 min at 37 °C in regular medium, and excessive dye was washed out using PBS. The labeled cells were treated for 10 min with 10 μM monensin and 10 μM nigericin in 25 mM MES calibration buffer, pH 3.5–6.0, containing 5 mM NaCl, 115 mM KCl, and 1.2 mM MgSO4. Quantitative comparisons were performed in a 96-well plate, and the fluorescence was measured with a microplate reader at 37 °C. Light emitted at 440 and 535 nm in response to excitation at 340 and 380 nm was measured, respectively. The ratio of light emitted with 340- and 380-nm excitation was plotted against the pH values in MES buffer, and the pH calibration curve for the fluorescence probe was generated from the plot using Microsoft Excel.

**NH4Cl Chase Assay**—HeLa cells were seeded in regular DMEM medium overnight and then incubated with 100 μM NH4Cl in regular DMEM medium without sodium bicarbonate for 20 min at 37 °C. Thereafter, NH4Cl was removed, and the cells were washed with regular medium without sodium bicarbonate continuously and thoroughly. Samples were collected at
Inhibition of Autophagy Progression by TPC2

the indicated time and finally analyzed by Western blotting analyses or for pH measurement in a microplate reader.

Statistical Analysis—All data were presented as the means ± S.E. Student’s t test was performed to determine the differences among grouped data. * denotes statistically significant with p < 0.05.

RESULTS

Reconstitution of NAADP Competence in HeLa Cells by TPC2 Overexpression—HeLa cell line is an autophagy-competent cell line (supplemental Fig. S1, A and B). Yet HeLa cells are not responsive to NAADP-induced Ca$$^{2+}$$ changes (Fig. 1A), which might be due to the low expression level of endogenous TPC2 (supplemental Fig. S1C). We thus first constructed a stable rat TPC2-overexpressing HeLa cell line by lentiviral infection. As shown in Fig. 1B, TPC2 was overexpressed in the lysosome as indicated by its co-localization with the lysosomal marker Lamp1 in a subset of cellular puncta (the TPC2-positive vesicles were actually enlarged in some cells as compared with the control). Likewise, the HeLa cells expressing a rat TPC2 249L/P mutant, in which a highly conserved leucine residue within a putative pore region of rat TPC2 was mutated to proline (34), were constructed by lentiviral infection (Fig. 1B). A cell permeant NAADP analog, NAADP-AM (55), induced Ca$$^{2+}$$ release only in wild type TPC2-overexpressing HeLa cells and not in TPC2 249L/P mutant-expressing cells (Fig. 1A) and displayed a characteristic bell-shaped dose-response curve due to the activation of its receptor by low concentrations of NAADP and its desensitization by high concentrations of NAADP (56). A CA$$^{2+}$$-induced Ca$$^{2+}$$ release was markedly inhibited by preincubation with Ned-19 (56), an NAADP antagonist, indicating that HeLa cells can be reconstituted to be NAADP competent via TPC2 overexpression.

Inhibition of Autophagic Flux by TPC2—Remarkably, under the electron microscopy (EM), typical autophagic vacuoles were observed in TPC2-overexpressing HeLa cells maintained in normal (nutrient-rich) culture conditions but not in control cells (Fig. 2A). Similar to previous reports (43), lipidated LC3-II, a reliable autophagosome marker, was markedly increased in TPC2-overexpressing cells compared with that in TPC2 mutant expressing or control HeLa cells (Fig. 2B). Interestingly, p62, an autophagic substrate (57), was also accumulated in TPC2-overexpressing cells compared with that in control HeLa cells, suggesting that TPC2 overexpression results in a defect of autophagic degradation instead of simply promoting autophagy (Fig. 2B). Consistently, endogenous LC3-II puncta (Fig. 2C) or GFP-LC3 puncta (Fig. 2D and supplemental Fig. S1A) were greatly increased in TPC2-overexpressing cells and did not co-localize with Lamp1, which was similar to the cells treated with bafilomycin (BAF), an inhibitor of the vacuolar proton pump that blocks the fusion of autophagosomes with lysosomes (58). These data suggest that these LC3-II puncta present in TPC2-overexpressing cells are autophagosomes, not autolysosomes.

Because it is possible that the accumulation of LC3-II in TPC2-overexpressing cells is partly due to the increased early autophagic activity, we briefly (1 h) treated control or TPC2-overexpressing HeLa cells with BAF in normal or serum-free medium. We reasoned that BAF treatment could further induce the accumulation of LC3-II and p62 if TPC2, like starvation, promotes early autophagic progression. However, BAF treatment (1 h) only induced the accumulation of LC3-II and p62 in control cells, not in TPC2-overexpressing cells, regardless of whether cells were in normal or starvation states (Fig. 3A and supplemental Fig. S1B). This suggests that TPC2 is not involved in early autophagy induction. Also notably, starvation not only induced the accumulation of LC3-II but also decreased p62 in both control and TPC2-overexpressing cells (comparing lanes 5 and 6 in Fig. 3A), suggesting that TPC2 overexpression only inhibits but does not abolish the fusion of autophagosomes with lysosomes.

After the initiation of the phagophores, autophagosomes undergo a stepwise maturation process from early to late autophagosomes, which ultimately fuse with lysosomes to form autolysosomes. Any missteps at the maturation process could lead to the accumulation of autophagosomes (1). Therefore, we next assessed whether TPC2 signaling compromises early autophagosome maturation. To do so, GFP-LC3 or GFP-LC3/TPC2-expressing cells were transfected with RFP-ATG5. Upon starvation (nutrient-rich), ATG5 was diffused in both control and TPC2-overexpressing cells (suggesting that TPC2 does not influence ATG5 localization, on the other hand, overexpression of ATG5, some of which localize with LC3 in both cell types (Fig. 3B). These data suggest that TPC2 signaling does not inhibit the early autophagosome (59) maturation.

Interestingly, we tested whether the accumulated autophagosomes in TPC2-overexpressing HeLa cells are the result of basal cellular autophagy activity. To do so, we knocked-down ATG5, which is required for the elongation and closure of autophagosomes in both control and TPC2-overexpressing HeLa cells (supplemental Fig. S2A). As expected, not only did TPC2 overexpression in ATG5-knockdown cells fail to induce autophagosome(s) accumulation (Fig. 3C and supplemental Fig. S2B), but also knockdown of ATG5 in TPC2-overexpressing cells markedly decreased the accumulated LC3-II and p62 (Fig. 3D). These results place TPC2 downstream of ATG5 in autophagy regulation and again suggest that TPC2 overexpression does not completely block the fusion of autophagosomes with lysosomes.

p62, as a cargo receptor for some ubiquitinated proteins, is first incorporated into autophagosomes before being targeted to lysosomes for degradation (59). Therefore, it is also necessary to clarify whether accumulated p62 in TPC2-overexpressing cells is due to the defects in cargo incorporation into autophagosomes. As shown in Fig. 3E, under normal culture conditions, p62 puncta were already markedly accumulated in TPC2-overexpressing cells compared with control cells, and most of them were co-localized with LC3-II. Moreover, similar to control cells, starvation further induced LC3-II puncta and p62 puncta in TPC2-overexpressing cells (Fig. 3E). This indicates that TPC2 does not prevent p62 incorporation into autophagosomes and suggests that accumulated p62 in TPC2-overexpressing cells is due to the defects in later stages of autophagy.
Inhibition of the Fusion between Autophagosomes and Autolysosomes by NAADP/TPC2 Signaling in HeLa Cells

To further differentiate autophagosomes and autolysosomes during autophagy, we infected the control or TPC2-overexpressing HeLa cells with lentiviruses carrying expression cassettes that encode RFP-GFP-LC3 (tflC3), in which RFP and GFP are tan-

**FIGURE 1.** Reconstitution of NAADP-competent HeLa cells by overexpressing TPC2. A, NAADP-AM (100 nm) only induced Ca\(^{2+}\) release in wild type, but not in an inactive mutant, TPC2-overexpressing HeLa cells. B, shown is localization of Lamp1-RFP, TPC2, and TPC2 249L/G in HeLa cells. A rabbit polyclonal TPC2 antibody was used to detect overexpressing TPC2. Scale bar = 5 μm. C, NAADP-AM induced Ca\(^{2+}\) release in TPC2-overexpressing HeLa cells, which was markedly inhibited by Ned-19 (10 μM). Note the bell-shaped concentration dependence curve for NAADP-AM. Quantification of the [Ca\(^{2+}\)]\(_i\) peak induced by drug treatment in A and C is expressed as the mean ± S.E., n = 30–40 cells. The asterisk indicates the results of t test analysis; p < 0.05.
dem-tagged to the N terminus of LC3 (60). Before fusion with lysosomes, the LC3-II-positive autophagosomes are shown by both GFP and RFP signals as yellow puncta, and after fusion, autolysosomes are shown by only RFP signals as red only puncta because GFP loses its fluorescence in acidic pH. As expected, yellow puncta (autophagosomes) were markedly increased with few red puncta signals (autolysosomes) in wild type, not mutant, TPC2-overexpressing cells, which was similar to the effects of BAF in control cells. In contrast, starvation dramatically increased both yellow and red puncta in control cells (Fig. 4A and B). We further performed immunoelectron microscopy analyses to distinguish autophagosomes from autolysosomes in control and TPC2-overexpressing HeLa cells (Fig. 4C). The cells were incubated with anti-LC3 antibody followed by diaminobenzidine staining and were subsequently subjected to EM analyses. Two types of autophagic structures were quantified as the mean ± S.E., n = 80 cells. The asterisks indicate the results of t test analysis; p < 0.05.

FIGURE 2. TPC2 overexpression induced a defect in autophagic degradation in HeLa cells. A, large numbers of autophagic vacuoles were observed in TPC2-overexpressing cells, but not in control cells, under electron microscope. B, wild type, not an inactive mutant, TPC2 overexpression induced the accumulation of both LC3-II and p62 in control and TPC2-overexpressing HeLa cells. C, wild type, not an inactive mutant, TPC2 overexpression markedly induced endogenous LC3-II puncta (green) in HeLa cells, which were not colocalized with RFP-Lamp1. Scale bar = 5 μm. Quantification of LC3 puncta is expressed as the mean ± S.E., n = 80 cells. The asterisks indicate the results of t test analysis; p < 0.05. D, starvation induced LC3 puncta, some of which were colocalized with LAMP1 puncta, whereas the LC3 puncta induced by bafilomycin or TPC2 expression failed to co-localize with LAMP1 in RFP-LAMP1/GFP-LC3 HeLa cells. Scale bar = 10 μm.
Inhibition of Autophagy Progression by TPC2

FIGURE 4. TPC2 signaling inhibited the progression of autophagy in HeLa cells. A. HeLa cells were transfected with a RFP-GFP tandem fluorescent-tagged LC3 (tfLC3) plasmid. TPC2 overexpression induced yellow puncta with few red-only puncta signals in cells. In contrast, starvation dramatically increased both yellow and red puncta in control cells. Scale bar = 10 μm. B. Quantitation of % of yellow puncta or red only puncta per cell of A are expressed as the mean ± S.E., n = 80 cells. The asterisks indicate the results of t test analysis; p < 0.05. C. Control and TPC2-overexpressing cells were processed for immunoelectron microscopy analyses. C1, shown are representative autophagosomal structures, whose surfaces are LC3-positive, in TPC2-overexpressing HeLa cells; C2, shown are representative autolysosomal structures, whose inside are LC3-positive, in TPC2-overexpressing HeLa cells. Quantitation of autophagosomes and autolysosomes per cell are expressed as the mean ± S.E., n = 40 cells (bottom panel). ns, not significant. D, NAADP-AM (100 nM) increased the accumulation of both LC3-II and p62 only in TPC2-overexpressing, not the control, HeLa cells. E, Ned-19 (10 μM) decreased the levels of both LC3-II and p62 in TPC2-overexpressing, but not the control, HeLa cells.

autophagosomes whose surfaces are LC3-positive (Fig. 4C1) and autolysosomes whose inside are LC3-positive (Fig. 4C2). Consistently, the numbers of autophagosomes in TPC2-overexpressing cells were significantly more than those in control cells, whereas autolysosomes were hard to find in both control and TPC2-overexpressing cells (the bottom panel of Fig.
Inhibition of Autophagy Progression by Endogenous TPC2 Signaling in Mouse Embryonic Stem Cells—Upon withdrawal of self-renewal stimuli, mouse ES cells can spontaneously and robustly differentiate into neural progenitors in adherent monolayer culture (61). Interestingly, autophagy was markedly induced during neural differentiation of mouse ES cells (Fig. 5A and supplemental Fig. S4A). TPC2 is detectable in mouse ES cells (Fig. S5A and supplemental and quantitative RT-PCR analyses showed that the level of TPC2 mRNA was dynamically regulated during neural differentiation of mouse ES cells (supplemental Fig. S5B). We used lentivirus-mediated short hairpin (sh)RNA cDNA to stably knockdown the endogenous TPC2 and overexpress TPC2 (Fig. 5B and Supplemental Fig. S5B).

The control scramble-shRNA or TPC2-overexpressing cells were cultured to differentiate into neural lineages after the monolayer culture protocol. Similar to HeLa cells, the LC3-II levels of both LC3-II and p62 only in TPC2-overexpressing HeLa cells. Not surprisingly, in TPC2 knockdown cells during neural differentiation, more yellow LC3 puncta (autophagosomes) existed in TPC2-overexpressing cells, whereas more red-only LC3 puncta (autolysosomes) appeared in TPC2 knockdown cells (Fig. 5F), indicating that autophagosomal-lysosomal fusion is indeed facilitated by TPC2 knockdown but inhibited by TPC2 overexpression. Clearly, these data further demonstrate that the endogenous TPC2 signaling inhibits the fusion between autophagosomes and autolysosomes as well. In addition, we found that TPC2 knockdown facilitated mouse ES cells to differentiate into neural progenitors, whereas TPC2 overexpression inhibited mouse ES cells differentiated into neural progenitors (61).
ATPase and that it has been previously shown that NAADP increases the pH of acidic Ca\(^{2+}\) stores in sea urchin egg (67, 68), we speculated that TPC2 overexpression might increase lysosomal pH as well. We first applied LysoSensor Green DND-189 (pK\(_a\) = 5.2) to qualitatively measure lysosomal pH (53). LysoSensor Green DND-189 permeates cell membranes and accumulates in acidic intracellular organelles, and its fluorescence increases or decreases in acidic or alkaline environments, respectively. As shown in Fig. 7A, supplemental Fig. S5, C and D, TPC2 overexpression indeed raised lysosomal pH in HeLa cells, which was similar to that by 1 h of BAF treatment but was less than that by 3 h of BAF treatment. This also explains why only 3 h, not 1 h, of BAF treatment further induced the accumulation of LC3-II and p62 in TPC2-overexpressing HeLa cells (supplemental Fig. S5, A and B). We further quantified lysosomal pH by dual-emission ratio imaging of LysoSensor Yellow/Blue DND-160-stained cells (supplemental Fig. S5E) and found that lysosomal pH in TPC2-overexpressing HeLa cells was increased.

**FIGURE 5.** TPC2 signaling inhibited the progression of autophagy during neural differentiation of mouse ES cells. A, in vitro neural differentiation of mouse ES cells initiated by monolayer adherent culture markedly induced LC3-II in D3 mouse ES cells. B, TPC2 knockdown by shRNA in ES cells was determined by TPC2 immunoblot analyses. C, TPC2 is expressed in lysosome-related organelles, as it is co-localized with LysoTracker (red). Scale bar = 50 μm. D, TPC2 overexpression induced accumulation of LC3-II in ES cells during neural differentiation. Treatment with bafilomycin (100 nm) only induced the accumulation of LC3-II in control cells but not in TPC2-overexpressing cells. E, TPC2 knockdown decreased the accumulation of LC3-II in ES cells during neural differentiation. Treatment with bafilomycin (100 nm) induced the accumulation of LC3-II in both control and TPC2 knockdown cells. F, mouse D3 ES cells were infected with RFP-GFP tandem fluorescent-tagged LC3 (tlLC3) lentiviruses. TPC2 overexpression induced yellow puncta with few red only puncta signals, whereas TPC2 knockdown increased the red-only puncta in ES cells after 4 days of neural differentiation. Quantification of at least 50 cells from three independent experiments is expressed as the mean ± S.E. and shown in the right columns. The asterisks indicate the results of t test analysis; p < 0.05. Scale bar = 10 μm.
from pH 4.9 in control cells to pH 5.2 (Fig. 7B). Similar results were observed in mouse ES cells (Fig. 7, C and D). In addition, NAADP-AM transiently raised lysosomal pH only in TPC2-overexpressing HeLa cells (supplemental Fig. S5F), which correlated with its effects on LC3-II accumulation (Fig. 3D). Next, we assessed whether reacidifying lysosomes in TPC2-overexpressing HeLa cells could relieve the fusion blockage of autophagosomes and lysosomes. An established ammonium chloride (NH₄Cl) pulse-wash technique was adopted to artificially acidify lysosomal pH (69). To do so, cells were initially incubated with NH₄Cl. The accumulation of ammonia in lysosomes resulted in an increase of lysosomal pH, which was indicated by a drop of LysoSensor Green DND-189 fluorescence in NH₄Cl pulsing cells compared with that in cells without NH₄Cl pulsing (time 0 in Fig. 7E). When NH₄Cl was removed from the medium by thorough washing, the efflux of ammonia caused the pH in the lysosomes to drop rapidly and transiently reach a value lower than that before the ammonium chloride was added, which was indicated by the increases of LysoSensor Green DND-189 fluorescence in NH₄Cl-pulsing cells after washing (Fig. 7E). The transient reacidified lysosomal pH produced after washing out ammonium chloride, mitigated the fusion blockage in TPC2-overexpressing cells (Fig. 7F). Taken together, these data clearly demonstrate that TPC2 signaling suppresses autophagosomal-lysosomal fusion by alkalizing the lysosomal pH.

TPC2 on Rab7 Recruitment to Autophagosomes—Rab7, a small GTPase, is required for autophagosome-lysosome fusion (6). We, thus, assessed the effects of TPC2 overexpression on the localization of Rab7 by transfecting RFP-Rab7 into GFP-LC3 or GFP-LC3/TPC2-overexpressing HeLa cells. We found that starvation induced the co-localization of Rab7 with both GFP-LC3 and Lamp1 in control cells (Fig. 8A). On the other hand, RFP-Rab7 puncta were colocalized with Lamp1, but few of them were colocalized with LC3-GFP in TPC2-overexpressing cells (Fig. 8B). Next, we assessed whether transient reacidified lysosomal pH by NH₄Cl pulse-wash in TPC2-overexpressing HeLa cells restores the association of Rab7 with autophagosomes. As shown in Fig. 8C, in the majority (2/3) of TPC2-overexpressing HeLa cells, reacidified lysosomal pH (10 min after NH₄Cl washing) markedly decreased LC3 puncta, which is consistent with the fact that re-acidification decreased LC3-II levels in TPC2-overexpressing cells as shown in Fig. 7F. Interestingly, Rab7 failed to co-localize with the remaining LC3 puncta in these cells. We speculate that lysosomal reacidification in the majority of TPC2-overexpressing cells quickly relieves the fusion blockage of autophagosomes and lysosomes to form autolysosomes, thereby leading to the degradation of LC3-II and the quenching of GFP fluorescence. The remaining LC3 puncta in these cells likely represent the immature autophagosomes induced by the repetitive post NH₄Cl washing. However, around 1/3 of TPC2-overexpressing cells still contained large numbers of LC3 puncta after removing NH₄Cl from the medium, and Rab7 puncta were indeed co-localized with LC3-II in these cells (Fig. 8D). We reason that lysosomal reacidification in these cells might be slow and just start to
recruit Rab7 to the autophagosomes but not yet induce the fusion of autophagosomes with lysosomes. Taken together, these results suggest that TPC2 signaling prevents the recruitment of Rab7 to autophagosomes by alkalizing lysosomal pH to inhibit autophagosomal-lysosomal fusion.

TPC2 on General Endosomal-Lysosomal Degradation—Because the increase of lysosomal pH normally compromises the lysosomal activity, we assessed whether TPC2 signaling simply disrupts the general lysosomal functions to inhibit autophagy maturation. The processing of cathepsin L from the precursor form to its mature form has been commonly used as a marker for lysosomal activity, yet we found that the processing of cathepsin L was only slightly compromised in TPC2-overexpressing HeLa cells compared with that in control cells (Fig. 9A), which might be due to the fact that TPC2 overexpression just marginally increased lysosomal pH (Fig. 7B). Next, an epi-

![Figure 7](image.png)

**FIGURE 7.** TPC2 signaling increased lysosomal pH to suppress autophagy progression. A, TPC2 overexpression or BAF treatment induced an increase of lysosomal pH in HeLa cells as determined by FACS analyses of LysoSensor DND-189-stained cells. B, quantification of lysosomal pH in control and TPC2-overexpressing HeLa cells as determined by dual-emission ratio imaging of LysoSensor Yellow/Blue DND-160-stained cells. C and D, TPC2 overexpression or BAF treatment induced an increase of lysosomal pH in D3 ES cells as determined by FACS analyses (C) and microplate reader measurement (D) of LysoSensor DND-189-stained cells. E, washing out NH4Cl in both control and TPC2-overexpressing HeLa cells after pulsing with NH4Cl (100 μM) for 20 min transiently reacidified lysosomal pH. The data are expressed as the mean ± S.D.; n = 4. F, washing out NH4Cl decreased the accumulation of LC3-II and p62 in TPC2-overexpressing cells after been pulsing with NH4Cl (100 μM) for 20 min. The first lanes show samples without NH4Cl pulsing. The graph in B and D represents data from three independent experiments, and the data are expressed as the mean ± S.D.; n = 3. The asterisks indicate the results of t test analysis; p < 0.05.
dermal growth factor receptor (EGFR) degradation assay was performed to examine whether the TPC2 signaling also affects the general endosomal-lysosomal pathway. In this assay both control and TPC2-overexpressing HeLa cells were treated with EGF. In principle, after EGF binds to its receptors (EGFR), the receptor complex undergoes endocytosis and is targeted to lysosomes where it is ultimately degraded. As shown in Fig. 9B and supplemental Fig. S6A, BAF inhibited EGF-triggered EGFR degradation in a dose-dependent manner, whereas TPC2 overexpression had little effect on it, which was similar to that in FIGURE 8.

TPC2 prevents the recruitment of Rab-7 to autophagosomes in HeLa cells. A, starvation (60 min) induced RFP-Rab7 puncta, which were colocalized with GFP-LC3 puncta (left panel) but not with GFP-LC3 puncta in TPC2-overexpressing cells pulsed with NH4Cl for 10 min (right panel). B, TPC2-overexpressing cells still contained large number of LC3 puncta, which were co-localized with RFP-Rab7. Scale bar = 10 μm.

FIGURE 9. TPC2 did not inhibit general endosomal-lysosomal degradation in HeLa cells. A, shown is processing of cathepsin L from the precursor form to its mature form in control or TPC2-overexpressing HeLa cells treated with or without bafilomycin for 1 or 3 h. B, TPC2 overexpression failed to change EGF-induced EGFR degradation, whereas BAF (100 nM) completely abolished it in HeLa cells. C, TPC2 overexpression did not inhibit the degradation of DQ-BSA-green in HeLa cells, whereas BAF (100 nM) markedly inhibited it.
control cells. Finally, a DQ-BSA-green degradation assay was applied to measure the general endosomal-lysosomal degradation. DQ-BSA-green is a BSA labeled with a self-quenching fluorescent dye. After DQ-BSA-green is delivered to lysosomes via endocytosis, it is hydrolyzed into single dye-labeled peptides by lysosomal proteases, thereby relieving self-quenching, and the fluorescence can subsequently be monitored by flow cytometry. As shown in Fig. 9C and supplemental Fig. S6B, TPC2 overexpression also had little effect on BSA degradation, whereas BAF markedly inhibited it. Collectively, these results document that TPC2 signaling does not inhibit the general endosomal-lysosomal degradation.

DISCUSSION

Here we provide definitive evidence that NAADP/TPC2 signaling induced the accumulation of matured autophagosomes by inhibiting autophagosome-lysosomal fusion (Figs. 2–5). Perturbing TPC2/NAADP signaling by either TPC2 knockdown or the addition of an NAADP antagonist, on the other hand, facilitated the progression of autophagy by promoting the formation of autolysosomes (Figs. 3–5). Instead of affecting or functioning downstream of mTOR (Fig. 6, A and B), TPC2 signaling actually alkalinized lysosomal pH to suppress autophagosome-lysosomal fusion (Fig. 7), likely by preventing the recruitment of Rab-7 to autophagosomes (Fig. 8). Yet TPC2 signaling had little effect on general endosomal-lysosomal degradation (Fig. 9).

The acidic milieu in lysosomes is generated by the V-ATPase, a multisubunit protein complex that pumps protons into the lysosomal lumen against an electrochemical gradient at the expense of ATP hydrolysis (70). The influx of protons into lysosomal lumen results in lower ATP levels and dissociation of mTOR from lysosomes (66). However, here we found that overexpression of a wild type, not a pore-mutant, TPC2 in cells maintained in nutrient-rich medium already alkalinized lysosomal pH and suppressed autophagosome-lysosome fusion (Figs. 2–5). Regarding how NAADP/TPC2-triggered Ca\(^{2+}\) release raises lysosomal pH, we speculate that either a putative Ca\(^{2+}/H^+\) exchanger (75) or an unidentified lysosomal Ca\(^{2+}\)-ATPase (76), similar to SERCA, or the coupling of several lysosomal protonation counter-transporters, such as sequential action of Ca\(^{2+}/Na^+\) exchangers and Na\(^+/H^+\) exchangers, can be activated to refill the lysosomal Ca\(^{2+}\) pools at the expense of proton efflux upon Ca\(^{2+}\) release from lysosomes via TPC2. Obviously, deciphering how Ca\(^{2+}\) enters lysosomes is the key to resolving this mystery. However, other than the V-ATPase, ion channels or transporters responsible for the formation of the unique ionic environment, e.g. Na\(^+\), Cl\(^-\), K\(^+\), and Ca\(^{2+}\), inside lysosomes remain unknown (75). A future lysosomal proteomics study should help in identifying the respective transporters.

Here we found that the HeLa cell line, an NAADP-incompetent line, can be reconstituted to be NAADP-competent by overexpressing a wild type, but not a pore-mutant, TPC2 (Fig. 1, A and C), and treatment of TPC2-overexpressing cells with Ned-19, a NAADP antagonist, markedly inhibited NAADP-induced Ca\(^{2+}\) release (Fig. 4A, C). Thus, these data provide unambiguous evidence that NAADP can activate TPC2 for Ca\(^{2+}\) release and, thereby, finding that TPC2 is an NAADP effector. We also found that TPC2 overexpression in the absence of endogenous NAADP treatment, in cell lines such as HeLa (Fig. 7, A–D) and HEK293 (Fig. 9), although the effects of TPC2 overexpression might be due to endogenous NAADP, NAADP/TPC2 signaling on Ca\(^{2+}\) release (Fig. 1, A and C) and autophagy maturation (Fig. 4D and supplemental Fig. S3A) in TPC2-overexpressing cells were transient. It has recently been revealed that NAADP does not directly bind to TPC2, arguing that NAADP might first bind to accessory proteins within the TPC2 complex and subsequently activate TPC2 (40–42). Therefore, the effects of TPC2 overexpression in HeLa or mouse ES cells might be caused by accessory proteins in the TPC2 complex, and NAADP, induced by extracellular stimuli or cellular stress (25, 77), could bind to these accessory proteins to further activate TPC2. However, at this stage we still could not exclude the possibility that the effects of TPC2 overexpression are due to endogenous NAADP, as the transient effects of NAADP-AM might be due to its instability in aqueous solution (55).

Two recent papers found that TPC2 forms a Na\(^+\) channel in lysosomes that can be potentiated by phosphatidylinositol 3,5-diophosphate, an endolysosome-specific phosphoinositide, and inhibited by ATP (66, 78). It was proposed that high intracellular ATP levels enable mTOR to phosphorylate TPC2, thereby keeping it at a closed state. On the other hand, cell starvation results in lower ATP levels and dissociation of mTOR from TPC2, thus activating TPC2 to allow Na\(^+\) release from lysosomes (66). However, here we found that overexpression of a wild type, not a pore-mutant, TPC2 in cells maintained in nutrient-rich medium already alkalinized lysosomal pH and suppressed autophagosome-lysosome fusion (Figs. 2–5), suggesting that TPC2 in high ATP levels is already open. Starvation promoted autophagy, e.g. LC3-II induction and p62 degradation, in both control and TPC2-overexpressing cells (Figs. 3A and 6A), arguing that nutrient deprivation does not further...
activate TPC2 to inhibit autophagy progression. Thus these data suggested that TPC2 suppresses autophagosome-lysosome fusion independent of mTOR or ATP levels. However, the role of intracellular phosphatidylinositol 3,5-disphosphate or Na\(^+\) release from lysosomes on TPC2-mediated autophagy inhibition remains to be determined.

Also notably, these two papers found that NAADP cannot activate TPC2 for Ca\(^{2+}\) release from lysosomes mainly by performing a patch clamp study on enlarged endolysosomes treated with vacuolin-1 (66, 78), which is contrary to some of results presented in this study and a body of work published previously (26–31). Further investigations are needed to resolve the controversy, and a recent study suggests that a GFP tagged on the N terminus of TCP1 disrupts NAADP action (79).

Interestingly, mutation of TPC2, M484L, or G734E has been associated with blond versus brown hair by genome-wide association studies for pigmentation (80). The melanosome, a lysosome-related organelle, is the site of synthesis and transport of melanin pigments, thereby providing tissues with color and photoprotection. In addition, autophagy and autophagy regulators have been shown to play roles in both the biogenesis of melanosomes and melanosome destruction (81). Therefore, it is of interest to assess whether and how the two identified TPC2 mutants affect melanosome biogenesis or destruction via autophagy related processes. Without doubt, TPC2 signalling could play roles in other autophagic-related cellular processes. Given that autophagy plays important roles in a wide variety of cellular processes and dysfunctional autophagy is associated with many human diseases, it is of interest to pursue TPC2 signaling to identify autophagic pathway. Development of novel pharmaceutical regulators to provide alternative pharmacological treatment of autophagy-related human diseases, for example, Niemann-Pick disease, a human lysosomal storage disorder, has been associated with abnormal lysosomal Ca\(^{2+}\) homeostasis, and its cellular phenotype is similar to that in TPC2-overexpressing cells (Fig. 2A) (29, 82). Whether TPC2-mediated autophagic regulation contributes to this disorder is, therefore, of great interest to pursue.

Acknowledgments—We thank Grant Churchill for providing several batches of NAADP-AM and Ned-19, Connie Lam and Yong-Juan Zhao in Prof. Hon-Chung Lee’s laboratory for sharing reagents, and King-Ho Cheung, Hon-Chung Lee, and members of the Yue laboratory for advice on the manuscript. Confocal imaging and FACS analyses were performed in Faculty of Medicine Core Facility at the University of Hong Kong.

REFERENCES

1. Yang, Z., and Klionsky, D. J. (2010) Eaten alive. A history of macroautophagy. *Nat. Cell Biol.* 12, 814–822
2. Mairi, M. C., Zalckvar, E., Kimchi, A., and Kroemer, G. (2007) Self-eating and self-killing. Cross-talk between autophagy and apoptosis. *Nat. Rev. Mol. Cell Biol.* 8, 741–752
3. Cárdenas, C., and Foskett, J. K. (2012) Mitochondrial Ca\(^{2+}\) signals in autophagy. *Cell Calcium* 52, 45–51
4. Lorin, S., Pierron, G., Ryan, K. M., Codogno, P., and Djavaheri-Mergny, M. (2010) Evidence for the interplay between INK and p53-DRAM signalling pathways in the regulation of autophagy. *Autophagy* 6, 153–154
5. Klionsky, D. J., Elazar, Z., Seglen, P. O., and Rabinstein, D. C. (2008) Does bafilomycin A1 block the fusion of autophagosomes with lysosomes? *Autophagy* 4, 849–950
6. Iäger, S., Bucci, C., Tanida, I., Ueno, T., Kominami, E., Safﬁg, P., and Eskelinen, E. L. (2004) Role for Rab7 in maturation of late autophagic vacuoles. *J. Cell Sci.* 117, 4837–4848
7. Nair, U., Iotwani, A., Geng, J., Gammoh, N., Richerson, D., Yen, W. L., Griffith, J., Nag, S., Wang, K., Moss, T., Baba, M., McNew, J. A., Jiang, X., Regini, F., Melia, T. J., and Klionsky, D. J. (2011) SNARE proteins are required for macroautophagy. *Cell* 146, 290–302
8. Nair, D. P., Brett, C. L., and Merz, A. J. (2009) Vps-C complexes. Gatekeepers of endolysosomal trafﬁc. *Curr. Opin. Cell Biol.* 21, 543–551
9. Chen, D., Fan, W., Lu, Y., Ding, X., Chen, S., and Zhong, Q. (2012) A mammalian autophagosome maturation mechanism mediated by TECPR1 and the Atg12-Atg5 conjugate. *Mol. Cell* 45, 629–641
10. Su, M., Shi, J. J., Yang, Y. P., Li, J., Zhang, Y. L., Chen, J., Hu, L. F., and Liu, C. C. (2011) HDAC6 regulates aggresome-autophagosome degradation pathway of α-synuclein in response to MPP+–induced stress. *J. Neurochem.* 117, 112–120
11. N’Diaye, E. N., Debnath, J., and Brown, E. J. (2009) Ubiquilins accelerate autophagosome maturation and promote cell survival during nutrient starvation. *Autophagy* 5, 535–575
12. Tamai, K., Tanaka, F., Tada, Y., Yamamoto, A., Nakagawa, I., Yoshimori, T., Ueno, Y., Terasaki, T., and Sugamura, K. (2007) Role of Hrs in maturation and function of mammalian cells. *Biochem. Biophys. Res. Commun.* 358, 26–31
13. Shinoda, K., Sugiyama, T., Waguiri, S., and Fukuda, M. (2011) Presenilin-dependent Rab33B-GAP, regulates autophagy. *Nature* 492, 839–853
14. Zhao, Z., Li, L., and Wang, X. (2011) COP9 signalling regulates autophagosome maturation. *Circulation* 124, 2117–2128
15. Uijtewaal, P. J., Ploegh, H. L., and Nijman, K. D. (2010) Presenilins mediate efficient proteosome-lysosome system. *Autophagy* 7, 664–665
16. Kolesnick, R., Signeur, A., Ahmad, S. T., Young, S. G., and Gao, F. B. (2007) The JNK–FAS–fAS FF-1 dysfunction causes autophagosome accumulation and neurodegeneration. *Curr. Biol.* 17, 1561–1567
17. Eskelinen, E. L. (2006) Roles of LAMP-1 and LAMP-2 in lysosome biogenesis. *Mol. Aspects Med.* 27, 495–502
18. Liang, C., Lee, J. S., Inn, K. S., Gack, M. U., Li, Q., Roberts, E. A., Vergne, I., Deretic, V., Feng, P., Akazawa, C., and Jung, J. U. (2008) Beclin1-binding UVRAG targets the class C Vps complex to coordinate autophagosome maturation and endocytic trafficking. *Nat. Cell Biol.* 10, 776–787
19. Ju, J. S., and Weihl, C. C. (2010) p97/VCP at the intersection of the autophagy and the ubiquitin proteasome system. *Autophagy* 6, 283–285
20. Gómez-Suaga, P., Churchill, G. C., Patel, S., and Hilfiker, S. (2012) A link between LRRK2, autophagy and NAADP-mediated endolysosomal calcium signalling. *Biochem. Soc Trans.* 40, 1140–1146
21. Leu, J. I., Pimkina, J., Frank, A., Murphy, M. E., and George, D. L. (2009) A small molecule inhibitor of inducible heat shock protein 70. *Mol. Cell* 36, 15–27
22. Galiane, A. (2011) NAADP receptors. *Cold Spring Harb. Perspect Biol.* 3, a004036
23. Hooper, R., and Patel, S. (2012) NAADP on target. *Adv. Exp. Med. Biol.* 740, 325–347
24. Zhu, M. X., Ma, J., Parrington, J., Calcara, P. J., Galiane, A., and Evans, A. M. (2010) Calcium signaling via two-pore channels. Local or global, that is the question. *Am. J. Physiol. Cell Physiol.* 298, C430–C441
25. Lee, H. C. (2012) Cyclic ADP-ribos and nicotinic acid adenine dinucleotide phosphate (NAADP) as messengers for calcium mobilization. *J. Biol. Chem.* 287, 31633–31640
26. Calcara, P. J., Ruas, M., Pan, Z., Cheng, X., Arredouani, A., Hao, X., Tang, J., Rietdorf, K., Teboul, L., Chubang, K. T., Lin, P., Xiao, R., Wang, C., Zhu, Y., Lin, Y., Wyatt, C. N., Parrington, J., Ma, J., Evans, A. M., Galiane, A., and Zhu, M. X. (2009) NAADP mobilizes calcium from acidic organelles through two-pore channels. *Nature* 459, 596–600
27. Pitt, S. J., Funnell, T. M., Sitasesan, M., Venturi, E., Rietdorf, K., Ruas, M., Ganesan, A., Gosain, R., Churchill, G. C., Zhu, M. X., Parrington, J.,
64. Korolchuk, V. I., Saiki, S., Lichtenberg, M., Siddiqi, F. H., Roberts, E. A., Imarisio, S., Jahreiss, L., Sarkar, S., Futter, M., Menzies, F. M., O’Kane, C. J., Deretic, V., and Rubinsztein, D. C. (2011) Lysosomal positioning coordinates cellular nutrient responses. Nat. Cell Biol. 13, 453–460

65. Yu, L., McPhee, C. K., Zheng, L., Mardones, G. A., Rong, Y., Peng, J., Mi, N., Zhao, Y., Liu, Z., Wan, F., Hailey, D. W., Oorschot, V., Klumperman, J., Baehrecke, E. H., and Lenardo, M. J. (2010) Termination of autophagy and reformation of lysosomes regulated by mTOR. Nature 465, 942–946

66. Cang, C., Zhou, Y., Navarro, B., Seo, Y. J., Aranda, K., Shi, L., Battaglia-Hsu, S., Nissim, L., Clapham, D. E., and Ren, D. (2013) mTOR regulates lysosomal ATP-sensitive two-pore Na+/H+ channels to adapt to metabolic state. Cell 152, 778–790

67. Morgan, A. J., and Galione, A. (2007) NAADP induces pH changes in the lumen of acidic Ca2+/H+ stores. Biochem. J. 402, 301–310

68. Morgan, A. J., and Galione, A. (2007) Fertilization and nicotinic acid adenine dinucleotide phosphate induce pH changes in acidic Ca2+/H+ stores in sea urchin eggs. J. Biol. Chem. 282, 37730–37737

69. Ohkuma, S., and Poole, B. (1978) Fluorescence probe measurement of the intralysosomal pH in living cells and the perturbation of pH by various agents. Proc. Natl. Acad. Sci. U.S.A. 75, 3327–3331

70. Mindell, J. A. (2012) Lysosomal acidification mechanisms. Annu Rev. Physiol. 74, 69–86

71. DiCiccio, J. E., and Steinberg, B. E. (2011) Lysosomal pH and analysis of the counter ion pathways that support acidification. J. Gen. Physiol. 137, 385–390

72. Graves, A. R., Curran, P. K., Smith, C. L., and Mindell, J. A. (2008) The Cl−/H+ antiporter CIC-7 is the primary chloride permeation pathway in lysosomes. Nature 453, 788–792

73. Deriy, L. V., Gomez, E. A., Zhang, G., Beacham, D. W., Hopson, J., Gallan, A. J., Shevchenko, P. D., Bindokas, V. P., and Nelson, D. J. (2009) Disease-causing mutations in the cystic fibrosis transmembrane conductance regulator determine the functional responses to phosphates. J. Biol. Chem. 284, 35926–35938

74. Christensen, K. A., Myers, J. T., and Swanson, J. A. (2002) pH-dependent regulation of lysosomal calcium in macrophages. J. Cell Sci. 115, 599–607

75. Morgan, A. J., Platt, F. M., Lloyd-Evans, E., and Galione, A. (2011) Molecular mechanisms of endolysosomal Ca2+ signalling in health and disease. Biochem. J. 439, 349–374

76. Patel, S., and Docampo, R. (2010) Acidic calcium stores open for business. Expanding the potential for intracellular Ca2+ signaling. Trends Cell Biol. 20, 277–286

77. Guse, A. H. (2009) Second messenger signaling. Multiple receptors for NAADP. Curr. Biol. 19, R521–R523

78. Wang, X., Zhang, X., Dong, X. P., Samie, M., Li, X., Cheng, X., Goschka, A., Shen, D., Zhou, Y., Harlow, J., Zhu, M. X., Clapham, D. E., Ren, D., and Xu, H. (2012) TPC proteins are phosphoinositide-activated sodium-selective ion channels in endosomes and lysosomes. Cell 151, 372–383

79. Churamani, D., Hooper, R., Rahman, T., Brailiou, E., and Patel, S. (2013) The N-terminal region of two-pore channel 1 regulates trafficking and activation by NAADP. Biochem. J. 453, 147–151

80. Sulem, P., Gudbjartsson, D. F., Stacey, S. N., Helgason, A., Rafnar, T., Jakobsdottir, M., Steinberg, S., Gudjonsson, S. A., Palsson, A., Thorleifsson, G., Pálsson, S., Sigurgeirsson, B., Thorisdottir, K., Ragnarsson, R., Benediktsdottir, K. R., Aben, K. K., Vermeulen, S. H., Goldstein, A. M., Tucker, M. A., Kiviat, N. B., Olafsson, J. H., Gulcher, J., Kong, A., Thorsteinsdottir, U., and Jonsson, K. (2008) Two newly identified genetic determinants of pigmentation in Europeans. Nat. Genet. 40, 835–839

81. Ho, H., and Ganesan, A. K. (2011) The pleiotropic roles of autophagy regulators in melanogenesis. Pigment Cell Melanoma Res. 24, 595–604

82. Lloyd-Evans, E., Morgan, A. J., He, X., Smith, D. A., Elliot-Smith, E., Sillicon, A. H., Churchill, G. C., Schuchman, E. H., Galione, A., and Platt, F. M. (2008) The cystic fibrosis transmembrane conductance regulator determine the functional responses to phosphates. Nat Med. 14, 1247–1255
