Up-regulation of lysosomal TRPML1 channels is essential for lysosomal adaptation to nutrient starvation

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Upon nutrient starvation, autophagy digests unwanted cellular components to generate catabolites that are required for housekeeping biosynthesis processes. A complete execution of autophagy demands an enhancement in lysosome function and biogenesis to match the increase in autophagosome formation. Here, we report that mucolipin-1 (also known as TRPML1 or ML1), a Ca^{2+} channel in the lysosome that regulates many aspects of lysosomal trafficking, plays a central role in this quality-control process. By using Ca^{2+} imaging and whole-lysosome patch clamping, lysosomal Ca^{2+} release and ML1 currents were detected within hours of nutrient starvation and were potently up-regulated. In contrast, lysosomal Na^{+}-selective currents were not up-regulated. Inhibition of mammalian target of rapamycin (mTOR) or activation of transcription factor EB (TFEB) mimicked a starvation effect in fed cells. The starvation effect also included an increase in lysosomal proteostasis and enhanced clearance of lysosomal storage, including cholesterol accumulation in Niemann–Pick disease type C (NPC) cells. However, this effect was not observed when ML1 was pharmacologically inhibited or genetically deleted. Furthermore, overexpression of ML1 mimicked the starvation effect. Hence, lysosomal adaptation to environmental cues such as nutrient levels requires mTOR/TFEB-dependent, lysosome-to-nucleus regulation of lysosomal ML1 channels and Ca^{2+} signaling.

lysosome | starvation | TRPML1 | TFEB | mTOR

Macroautophagy (referred to as autophagy hereafter) is a cellular adaptation process that is essential for cell survival when nutrients [e.g., amino acids (AA) and growth factors] are limited (1). During this process, protein aggregates and damaged organelles are digested to generate basic building-block catabolites that can be used for "housekeeping" biosynthesis tasks (2). In the past few decades, autophagy research has mainly focused on the mechanisms that underlie the initial phase of autophagy: autophagosome formation (3). However, the entire autophagy process requires a sufficient and sustained supply of functional lysosomes to perform autophagosome-lysosome fusion continuously (4–7). Moreover, it remains unclear how environmental cues such as nutrient availability and regulation of lysosomal function and biogenesis (particularly lysosomal adaptation) contribute to cellular homeostasis. Temporal regulation of these processes is also of interest. For example, whereas lysosome activation, manifested as increased acidification and delivery of hydrolases, may occur rapidly (within 2–3 h of starvation) during the initial phase of autophagy (6), lysosome reformation usually occurs 4–6 h after starvation (8).

Mammalian target of rapamycin complex 1 (mTORC1) is a master regulator of cell growth (9) and is localized on the lysosomal surface when free AAs are abundant via a Rag GTPase-dependent mechanism (10, 11). The activity of lysosome-localized mTORC1 is tightly controlled by Rheb GTPase, which, in turn, is regulated by growth factors in the serum (10). Thus, upon AA withdrawal and/or serum starvation, mTORC1 activity is suppressed in the lysosome (10, 11), and the activities of mTORC1 effectors are subsequently switched on or off. The effectors include S6K and 4E-BP1, which are responsible for protein synthesis; ATG13 and ULK1, which mediate autophagosomal biogenesis; and transcription factor EB (TFEB), which regulates lysosome function (10, 12–14). TFEB is a transcription factor that regulates both autophagy and lysosomal biogenesis via rapid translocation to the nucleus from the cytosol and lysosomes upon starvation (4, 12–14). Correspondingly, overexpression of TFEB has been shown to affect the expression of a unique set of genes that are related to lysosome function and autophagy (5). However, it remains to be determined what roles transcriptional and posttranscriptional regulations have in lysosome activation, consumption, and biogenesis during lysosomal adaptation to environmental changes.

Lysosomal ionic conductance regulates all aspects of lysosome function, including lysosomal degradation, catabolite export, and membrane trafficking (15). Hence, regulation of lysosomal conductance by environmental cues may serve as a primary mechanism for lysosome adaptation (16, 17). Lysosomal trafficking (including membrane fusion and fission) supplies hydrolases for lysosome activation, provides autophagic substrates for degradation (autophagosome-lysosome fusion), and generates new lysosomes from autolysosomes (lysosome reformation). A key player in lysosomal trafficking is ML1, a cation channel on the lysosomal membrane that releases Ca^{2+} from the lumen into the cytosol in

Significance

Lysosomes are the cell's degradation center. To adapt to different environmental conditions, the cell has evolved a set of delicate mechanisms to rapidly change lysosome function, which is referred to as lysosomal adaptation. Notably, lysosomal adaptation is required for cell survival under low nutrient conditions. In this study, we identified TRPML1, a lysosomal Ca^{2+}-permeant ion channel, as an essential player required for lysosomal adaptation. The activity of TRPML1 is potently (up to 10-fold) and rapidly increased upon nutrient starvation. Furthermore, pharmacological inhibition or genetic deletion of TRPML1 completely abolished the effects of starvation on boosting the degradation capability of lysosomes.

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response to trafficking cues, such as changes in levels of $PI(3,5)P_2$, a lysosome-localized phosphoinositide (7, 15, 18–21). ML1-mediated lysosomal Ca^{2+} release may regulate many aspects of lysosomal trafficking, including lysosome to trans-Golgi–network (TGN) retrograde trafficking, autophagosome-lysosome fusion, lysosome reformation, and lysosomal exocytosis (15, 19, 22, 23). Moreover, it has been demonstrated that nutrient starvation affects phosphoinositide dynamics and Ca^{2+} signaling (16, 18, 24). Therefore, the objective of this study was to investigate nutrient regulation of endogenous ML1 channels and the role of such regulation in lysosomal adaptation.

Results

Nutrient Deprivation Markedly Increases Lysosomal ML1 Currents. We measured endogenous lysosomal currents by using the whole-lysosome patch-clamp technique on enlarged vacuoles isolated from cells treated with vacuolin-1 (25–27). In most mammalian cell lines (HEK293, CHO, and Cos-1), as well as a RAW 264.7 macrophage cell line and various primary cells [mouse embryonic fibroblasts (MEFs), bone marrow-derived macrophage (BMM), neurons, and myocytes], small ML1 currents were activated by ML-SA1 (mucolipin synthetic agonist 1) or the more potent ML-SA3 (Fig. S1), which are synthetic agonists of TRPML channels (19).

For Cos-1 cells grown in complete medium, only small ML-SA1–activated, inwardly rectifying, whole-endolysosome ML1 currents (I_{ML1}) were observed in most of the enlarged vacuoles (−18 ± 4 pA/pF at −120 mV, n = 14 vacuoles; 10 μM ML-SA1; Fig. 1 *A* and *E*). However, for Cos-1 cells that were serum-starved by using DMEM/F-12 medium without FBS for 4 h, up to a 10-fold increase in whole-endolysosome I_{ML1} was observed (−185 ± 32 pA/pF at −120 mV; n = 12; 10 μM ML-SA1; Fig. 1 *B* and *E*).

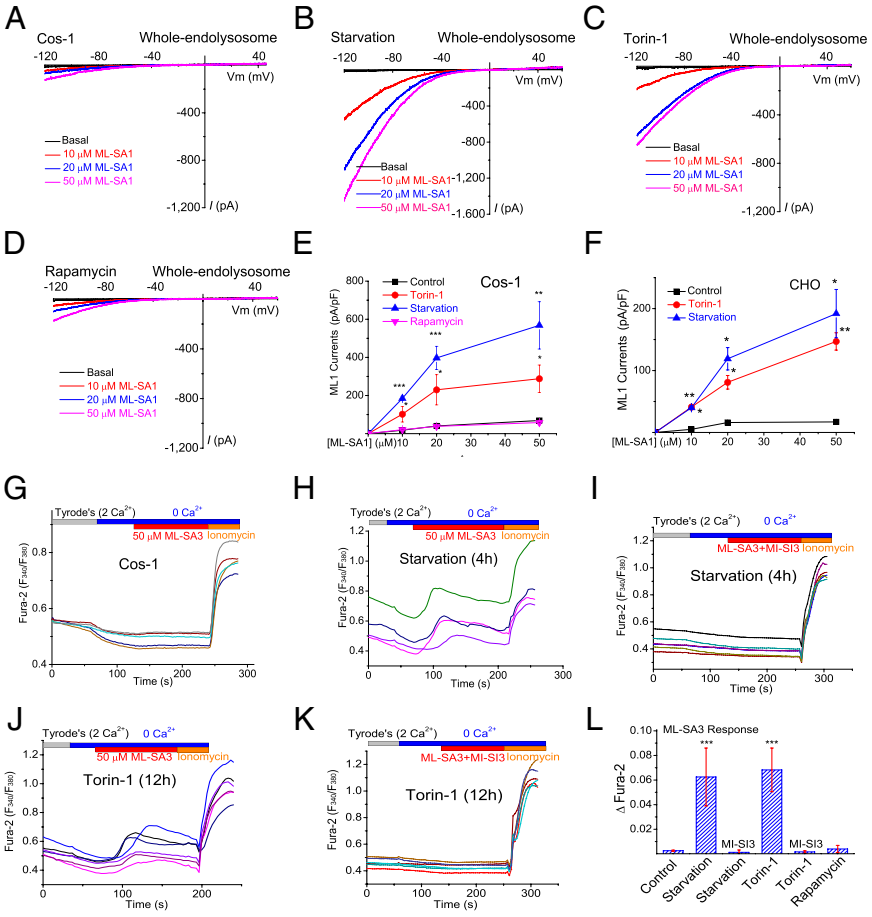

Fig. 1. Starvation and mTOR inhibition dramatically increase endogenous lysosomal ML1 currents and lysosomal Ca^{2+} release. (*A*) Representative traces of endogenous I_{ML1} for an enlarged vacuole isolated from vacuolin-1–treated Cos-1 cells grown in complete media. I_{ML1} was activated by three different concentrations of ML-SA1 (10, 20, and 50 μM) using a voltage protocol from −140 to +100 mV (only partial voltage ranges are shown). Pipette (luminal) solution was a standard external (Tyrode's) solution adjusted to pH 4.6 to mimic the acidic environment of the lysosome lumen. Bath (internal/cytoplasmic) solution was a K^+-based solution (140 mM K^+-gluconate). Note that the inward currents indicate that cations are flowing out of the endolysosome. (*B*) Whole-endolysosome I_{ML1} for Cos-1 cells after 4 h of serum starvation (DMEM/F-12 medium without FBS). (*C*) I_{ML1} for Cos-1 cells treated with Torin-1 (2 μM) for 12 h. (*D*) I_{ML1} for Cos-1 cells treated with rapamycin (2 μM) for 12 h. (*E*) Mean current densities (the current amplitude normalized to the capacitance of the vacuole) for I_{ML1} in nontreated (black), starved (blue), Torin-1–treated (red), and rapamycin-treated (pink) Cos-1 cells. (*F*) Summary of I_{ML1} for CHO cells under the different experimental conditions indicated. (*G*) ML-SA3 (50 μM) did not induce any obvious Ca^{2+} release (according to the Fura-2 ratio, F_{340}/F_{380}) in Cos-1 cells grown in complete media. Ionomycin (5 μM) was added at the conclusion of all experiments to induce a maximal intracellular release for comparison. Shown are selected traces from the same coverslip that typically contained 15–30 cells. (*H*) ML-SA3–induced Ca^{2+} release in starved Cos-1 cells. (*I*) ML-SA3–induced Ca^{2+} release in starved cells in the presence of the synthetic TRPML inhibitors, MI-SI3 (20 μM). (*J* and *K*) The effect of Torin-1 treatment (12 h) on ML-SA3–induced Ca^{2+} release. (*L*) Average ML-SA3–induced Ca^{2+} release in control, starved, Torin-1–treated, and rapamycin-treated Cos-1 cells. The results are the mean for 40–100 cells from n = 4 independent experiments. Data are presented as the mean ± SEM. Statistical comparisons were made by using variance analysis (Student's t test for *E* and *F*, and ANOVA for *L*). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Large increases in I_{ML1} were also observed when using higher concentrations of ML-SA1 (20–50 μM; Fig. 1 *A*, *B*, and *E*), and in cells that were AA-starved [AA-free DMEM/F-12 medium + 10% (vol/vol) FBS; Fig. S2*A*] or completely starved (AA-free DMEM/F-12 medium without FBS; Fig. S2*A*). However, for cells that were serum-starved for shorter durations (1–2 h), the increases in I_{ML1} were smaller (43 ± 4 pA/pF for 2 h, $n = 3$; Fig. S2 *B* and *C*). Serum starvation-induced increases in I_{ML1} were also observed in CHO cells (Fig. 1*F* and Fig. S2*D*), a RAW 264.7 macrophage cell line, and in MEFs (Fig. S2 *E*–*G*). Together, these results suggest that lysosomal ML1 channels are potently up-regulated by nutrient starvation. Because Cos-1 cells have a large cytoplasm that typically contains 200–400 lysosomes, these cells were selected for subsequent lysosomal trafficking and physiological studies.

Up-Regulation of Lysosomal ML1 Channels by Pharmacological Inhibition of mTOR. mTORC1 is the primary nutrient sensor in the lysosome (12–14). The activity of mTORC1 can be measured by detecting the level of phosphorylated S6K kinase (p-S6K). Following nutrient starvation of Cos-1 cells, p-S6K became undetectable (Fig. S3*A*). To investigate whether mTORC1 is involved in ML1 up-regulation, two selective mTOR inhibitors were used to treat cells. Whereas rapamycin is a partial, allosteric inhibitor of mTOR, Torin-1 is a full, catalytic inhibitor that completely suppresses the functions of mTORC1 (6, 28, 29). Following a 4-h and 12-h treatment with each of these mTOR inhibitors, whole-endolysosome I_{ML1} was found to markedly increase (by Torin-1; Fig. 1 *C* and *E*), or remain unchanged (by rapamycin; Fig. 1 *D* and *E*), respectively. In contrast, both Torin-1 and rapamycin completely suppressed levels of p-S6K (Fig. S3*A*) and strongly induced autophagosome formation (30). Thus, starvation-induced ML1 up-regulation may occur via a mechanism distinct from the initiation of autophagy, but likely through the processes downstream of mTORC1 inhibition.

Elevated ML1-Mediated Lysosomal Ca^{2+} Release in Starved Cells. Next, ML1-mediated lysosomal Ca^{2+} release in intact cells was measured by using Fura-2 Ca^{2+} imaging. Lysosomal Ca^{2+} release was induced in a zero Ca^{2+} (free $[Ca^{2+}] < 10$ nM) external

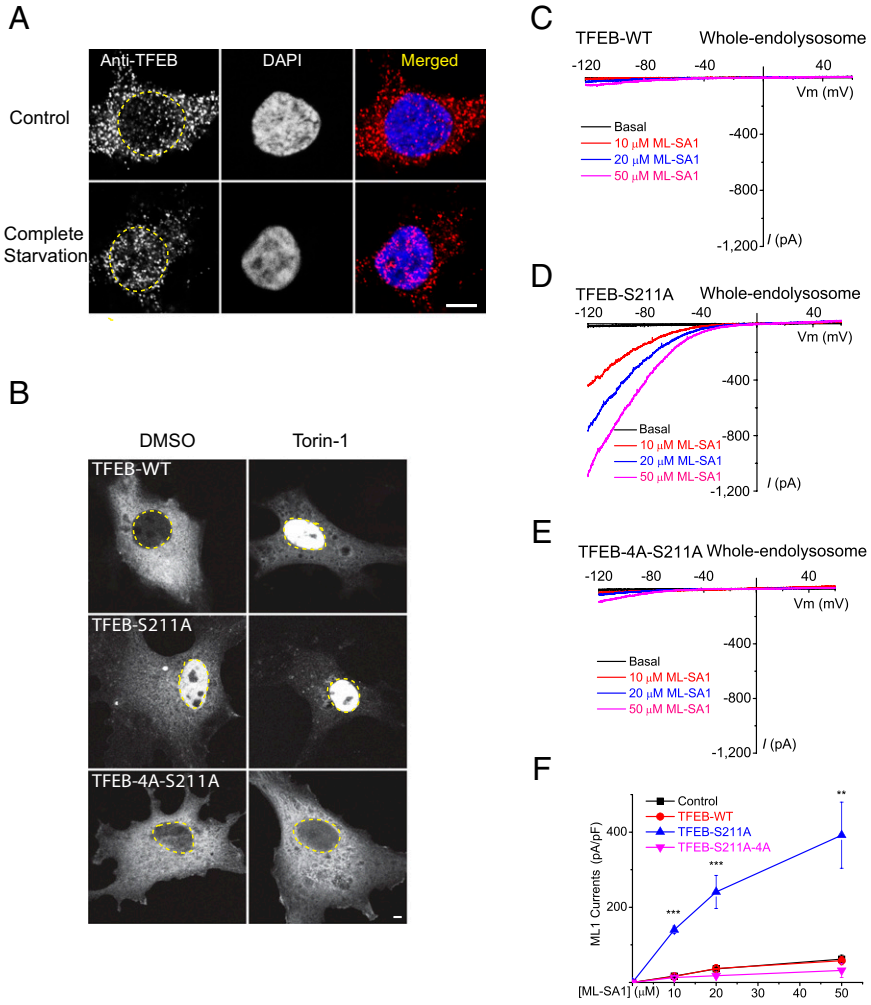

Fig. 2. Activating mutation of TFEB is sufficient to up-regulate lysosomal ML1 currents. (*A*) Complete starvation (AA free + serum free) promoted endogenous TFEB nuclear translocation in HeLa cells. Endogenous TFEB was recognized by an anti-human TFEB antibody. Nuclei were stained with DAPI and indicted by yellow dotted lines. (*B*) The effects of Torin-1 treatment (4 h) on the subcellular localization of overexpressed TFEB-mCherry, TFEB-S211A-mCherry, and TFEB-4A-S211A-mCherry. (Scale bars: 5 μm.) (*C*–*E*) Representative traces of whole-endolysosome I_{ML1} for Cos-1 cells transfected with TFEB-WT (*C*), TFEB-S211A (*D*), and TFEB-4A-S211A (*E*). (*F*) Mean current densities for I_{ML1} in nontransfected (black; $n = 14$ vacuoles), TFEB-WT (red; $n = 4$), TFEB-S211A (blue; $n = 13$), and TFEB-S211A-4A (pink; $n = 3$)-transfected Cos-1 cells. Data are presented as the mean ± SEM. Statistical comparisons were made by using variance analysis (*t* test). $^{**}P < 0.01$; $^{***}P < 0.001$.

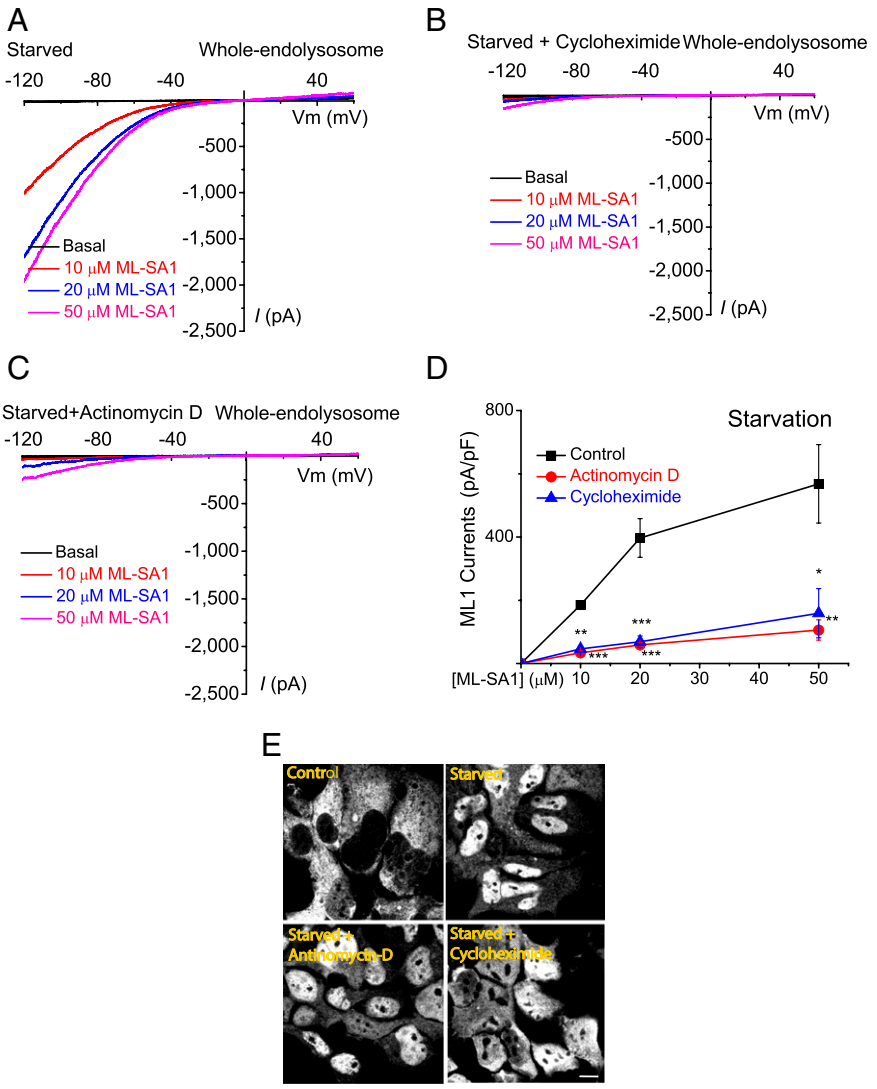

Fig. 3. ML1 up-regulation by starvation via transcription- and translation-dependent mechanisms. (*A*) Representative traces of whole-endolysosome I_{ML1} for starved cells. (*B* and *C*) The effects of cycloheximide (2 μg/mL; *B*) and actinomycin D (10 μg/mL; *C*) on I_{ML1} for starved Cos-1 cells. (*D*) Mean densities for I_{ML1} in starved cells treated with cycloheximide (blue; $n = 3$) and actinomycin D (red; $n = 3$). (*E*) The effects of actinomycin D or cycloheximide on TFEB localization in HEK293 cells stably expressing TFEB-mCherry. (Scale bar: 5 μm.) Data are presented as the mean ± SEM. Statistical comparisons were made by using variance analysis (*t* test). $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

solution by using ML-SA compounds as reported (19). No measurable Ca^{2+} release was detected in Cos-1 cells treated with up to 50 μM ML-SA1 or ML-SA3 (Fig. S1). These results are consistent with the small amplitude of I_{ML1} that were recorded for these cells (Fig. 1 *G* and *L*). In contrast, for Cos-1 cells that were serum-starved for 4 h, ML-SA3 induced an obvious Ca^{2+} response (Fig. 1 *H* and *L*), whereas smaller responses were induced by ML-SA1 (Fig. S3*B*). ML-SA3 was thus chosen for Ca^{2+} imaging studies. ML-SA3–induced Ca^{2+} responses were completely abolished in the presence of the TRPML-specific synthetic inhibitor, ML-SI3 (23) (Fig. 1 *I* and *L*), thereby supporting the specificity of the responses observed. Moreover, large ML-SA3 responses were observed in Cos-1 cells that were treated with Torin-1, and not in Cos-1 cells treated with rapamycin (4–12 h; Fig. 1 *J*–*L*). Collectively, these results are in general agreement with the electrophysiological analyses of vacuolin-enlarged vacuoles and demonstrate that nutrient deprivation dramatically increases ML1-mediated lysosomal Ca^{2+} release in intact Cos-1 cells.

Activating Mutation of TFEB Is Sufficient to Cause ML1 Up-Regulation. Of the three major mTOR effectors, TFEB is sensitive to Torin-1 and not sensitive to rapamycin (12–14). Whereas most mTORC1 substrates are inhibited by both rapamycin and Torin-1, TFEB nuclear translocation is triggered only after mTORC1 is completely inhibited (14). In HeLa cells, complete starvation resulted in a rapid translocation of endogenous TFEB from the cytoplasm to nucleus (Fig. 2*A*). Similarly, Cos-1 cells transfected with wild-type (WT) TFEB-mCherry and treated with Torin-1 exhibited a marked increase in the nuclear localization of TFEB (Fig. 2*B*). In contrast, constitutively active TFEB-S211A-mCherry (12) was associated with predominant nuclear localization of TFEB in the presence or absence of starvation or Torin-1 (Fig. 2*B*). Correspondingly, increased expression of endogenous Lamp1 was observed (Fig. S3 *C* and *D*). However, when mutations in TFEB included the removal of four charged residues within the putative nuclear localization motif (Arg^{245}-Arg^{248} to Ala^{245}-Ala^{248}; TFEB-S211A) (13), nuclear localization was completely abolished (Fig. 2*B*).

Endogenous whole-endolysosome I_{ML1} dramatically increased in cells transfected with TFEB-S211A (Fig. 2 *D* and *F*), but not in cells transfected with WT TFEB (Fig. 2 *C* and *F*) or TFEB-4A-S211A (Fig. 2 *E* and *F*). In addition, ML1-mediated lysosomal Ca^{2+} release increased in cells transfected with TFEB-S211A and not in cells transfected with WT TFEB or TFEB-4A-S211A (Fig. S3*E*). Overexpression of TFEB-WT in HeLa cells was reported to increase *ML1* mRNA levels (4). However, TFEB activity may not increase significantly in healthy cells, because WT TFEB mostly exhibited cytoplasmic localization in complete media (Fig. 2*B*). Nevertheless, when TFEB nuclear translocation was induced in TFEB-WT–transfected Cos-1 cells treated with Torin-1 for 4 h, we observed a synergistic increase in I_{ML1} with a trend toward statistical significance (Fig. 2*C* and Fig. S3 *F* and *G*). In combination, these results suggest that activation of TFEB underlies the effect of starvation on ML1.

Nutrient-Sensitive Regulation of ML1 Depends on Gene Expression and Protein Synthesis. ML1 up-regulation may result from a posttranslational modification or an increase in mRNA/protein expression. Based on the observation that starvation-induced increases in I_{ML1} occurred at a similar degree for all of the ML-SA1 concentrations tested, an increase in agonist potency is unlikely to account for the up-regulation. Instead, it is hypothesized that increased expression, or lysosomal targeting, of ML1 proteins plays a key role, due to the fact that Torin-1 treatment and starvation only modestly increased *ML1* mRNA levels (<twofold; Fig. S4). Notably, when transcription or protein synthesis was blocked by using antinomycin D (31) or cycloheximide (32), respectively, starvation-induced I_{ML1} increases were almost abolished (Fig. 3 *A*–*D*). In contrast, treatment with antinomycin D did not affect TFEB nuclear translocation, whereas treatment with cycloheximide partially blocked translocation (Fig. 3*E*). Lysosome-resident membrane proteins are reported to have an extremely slow turnover rate with $t_{1/2} > 3$ d (33), suggesting that degradation of TRPML1 proteins is likely negligible over the time course of starvation. Because increases in transcription and translation of ML1 proteins and transcripts cannot fully account for the large increase in currents, it is possible that unidentified posttranslational modification mechanisms may increase agonist efficacy. Taken together, these results suggest that starvation-induced ML1 up-regulation involves the synthesis of ML1 proteins or auxiliary proteins that modulate TRPML1 channel function.

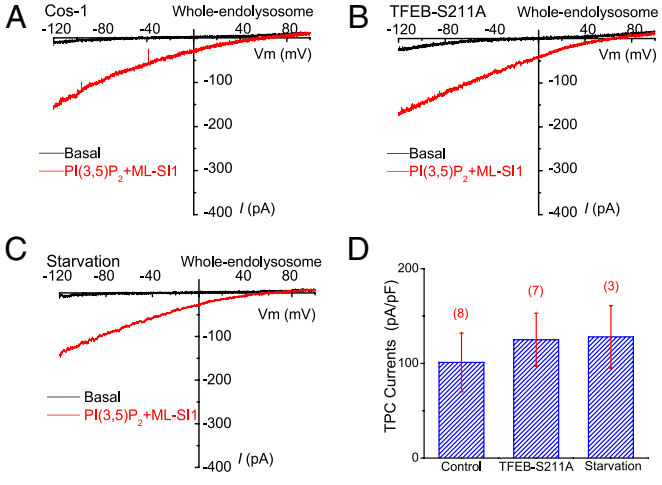

Fig. 4. Starvation or TFEB activation does not increase the endogenous TPC currents in the lysosome. (*A*) Representative traces of endogenous whole-endolysosome, Na^+-selective TPC currents (I_{TPC}) activated by $PI(3,5)P_2$ (100 nM) in the presence of the TRPML inhibitor, ML-SI1 (10 μM). (*B*) Whole-endolysosome I_{TPC} for a TFEB-S211A–transfected Cos-1 cell. (*C*) Whole-endolysosome I_{TPC} for a serum-starved Cos-1 cell. (*D*) Mean current densities for I_{TPC} of control, TFEB-S211A–transfected, and starved Cos-1 cells. The number of cells being recorded was indicated in parentheses. Data are presented as the mean ± SEM. Statistical comparisons were made by using variance analysis (*t* test).

Lysosomal Na^+-Selective Currents Are Not Affected by Nutrient Starvation. Two-pore (TPC) Na^+-selective channels have recently been proposed to be components of nutrient-sensing machinery in the cell (34). Both ML1 and TPC channels are activated by $PI(3,5)P_2$ (26, 27). Upon $PI(3,5)P_2$ activation, TPC currents were isolated by using MI-SI1 (Fig. S1*A*) to block ML1 currents (23). Compared with control Cos-1 cells (Fig. 4 *A* and *D*), neither TFEB-S211A (Fig. 4 *B* and *D*) nor starvation (Fig. 4 *C* and *D*) increased whole-endolysosome TPC currents. Hence, starvation may only selectively up-regulate certain lysosomal channels.

Regulation of TFEB Activation by $PI(3,5)P_2$. Nutrient deprivation inactivates Rag GTPases, which may mediate the recruitment of mTORC1 and TFEB to lysosomes (35). Nutrient deprivation also results in a rapid decrease in lysosomal $PI(3,5)P_2$ levels, which have been reported to affect mTOR localization and activity (24, 36, 37). The role of $PI(3,5)P_2$ in TFEB activation was investigated by using two different inhibitors of the $PI(3,5)P_2$-synthesizing enzyme, PIKfyve: YM201636 (38) and Apilimod (39). HEK293 cells that stably expressed TFEB were treated with YM201636 or Apilimod, and TFEB nuclear translocation was observed in both experiments. Moreover, the extent of translocation in each case was comparable to that observed with Torin-1 treatment (Fig. 5 *A* and *B*). Correspondingly, the treatment of Cos-1 cells with Apilimod, but not vacuolin-1, increased ML1-mediated lysosomal Ca^{2+} release (Fig. 5*D* and Fig. S5 *A* and *B*). Previously, lysosome inhibitors have been found to induce TFEB nuclear translocation by reducing mTOR activity (40). However, treatment with Apilimod did not cause an obvious inhibition of lysosomes or lysosomal membrane damage (Fig. S5*C*). In addition, p-S6K levels were only slightly reduced following YM201636 treatment and remained unchanged following Apilimod treatment (Fig. 5*C*). Similar results were observed for p-4E-BP1, another mTORC1 effector. In sharp contrast, rapamycin, which was unable to induce TFEB nuclear translocation (Fig. 5*A*), completely suppressed the level of p-S6K (Fig. S3*A*). Because $PI(3,5)P_2$ levels are potently reduced by both compounds (38, 39), these results suggest that regulation of TFEB nuclear translocation during starvation may use a $PI(3,5)P_2$-dependent mechanism that is independent of mTOR. Because $PI(3,5)P_2$ is an endogenous agonist of ML1 (26), starvation-induced ML1 up-regulation may occur as the result of a compensatory mechanism caused by a reduction in the levels of $PI(3,5)P_2$.

ML1 Is Required for the Clearance of Cholesterol Accumulation from Lysosomes in Niemann–Pick Disease Type C Cells. Lysosomal Ca^{2+} may regulate cellular clearance and cholesterol export in Niemann–Pick disease type C (NPC) cells (19). To investigate whether ML1 up-regulation by nutrient deprivation reduces cholesterol accumulation in NPC cells, Filipin staining was used to evaluate free cholesterol levels (19). Both starvation conditions and Torin-1 treatment dramatically reduced cholesterol accumulation in NPC1 CHO cells (Fig. 6 *A* and *B*), NPC1 knockout (KO) macrophages (Fig. 6*C* and Fig. S6*A*), NPC-like macrophages, and CHO cells that were pharmacologically induced by U18666A, a blocker of cholesterol transport (19) (Fig. 6 *D* and *E* and Fig. S6 *B* and *E*). ML1 overexpression also had a comparable clearance effect on U18666A-treated primary mouse macrophage (Fig. 6 *G* and *H*). An increase in ML1 channel activity following treatment with ML-SA1 had a subtle

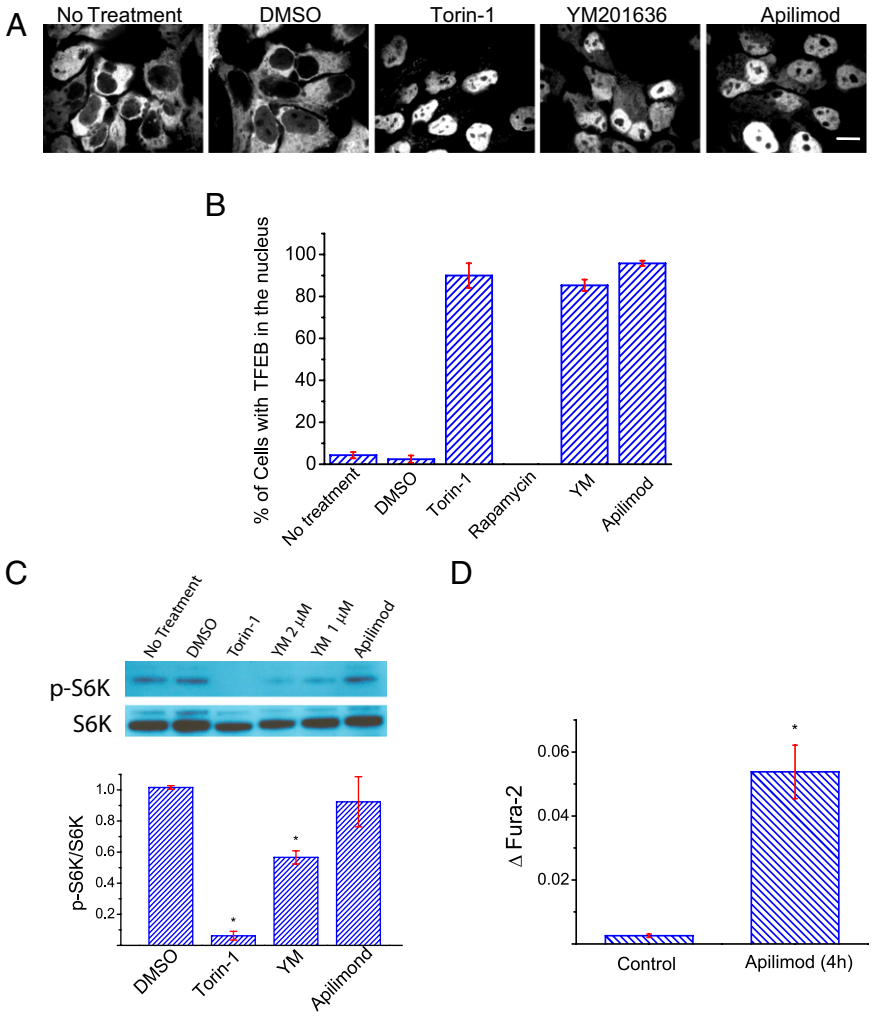

Fig. 5. A decrease in PI(3,5)P_2 levels promotes TFEB nuclear localization. (*A*) Confocal imaging of TFEB localization in cells treated with DMSO, Torin-1 (1 μM), YM201636 (YM, 1 μM), or Apilimod (1 μM). (Scale bars: 5 μm.) (*B*) Quantitation of data from *A* ($n = 6$). Nuclear localization was determined by using an arbitrary criterion of the fluorescent intensity of TFEB-mCherry in the nucleus being >150% of the cytoplasmic signal. (*C*) Western blot analysis of phosphorylated S6K (p-S6K) levels following treatment with DMSO, Torin-1, YM, and Apilimod-treated cells. Ratios of p-S6K to total S6K under different experimental conditions are shown in *C, Lower* ($n = 4$). (*D*) The average ML1-mediated lysosomal Ca^{2+} release in Apilimod-treated Cos-1 cells. The results were averaged from 40 to 100 cells from $n = 3$ independent experiments (Fig. S5 *A* and *B*). Data are presented as the mean ± SEM. Statistical comparisons were made by using variance analysis (*t* test). *$P < 0.05$.

effect on cholesterol levels in Torin-1–treated NPC cells and starved NPC cells (Fig. 6 *A*–*C*). However, a larger effect was observed for U18666A-treated RAW macrophage cells overexpressing ML1 (Fig. S6 *C* and *D*). These results suggest that ML1 up-regulation may play a critical role in starvation-induced cellular clearance.

In the presence of MI-SI3, cholesterol accumulation in NPC cells was not reduced by starvation or Torin-1 treatment (Fig. 6 *A*–*E*). Similarly, starvation or Torin-1 treatment did not reduce cholesterol accumulation in ML1 KO macrophage treated with U18666A (Fig. 6*F* and Fig. S6*F*). Thus, the channel activity of ML1 is absolutely required for starvation- or Torin-1–induced reduction in cholesterol accumulation in NPC cells.

ML1 Is Required for Starvation-Induced Enhancement of Lysosomal Proteolytic Function. To further investigate the role of ML1 in cellular adaptation, lysosomal proteolytic activity was measured by using an assay that yields red fluorescence according to the proteolytic degradation of DQ-red-BSA (41). Consistent with previous studies (41, 42), DQ-BSA degradation was enhanced one- to twofold following the complete starvation of Cos-1 cells (Fig. 7 *A* and *B*). In blind experiments, the starvation effect was completely abolished by MI-SI3, whereas ML-SA1 treatment led to a small increase in proteolytic activity (Fig. 7 *A* and *B*). Therefore, ML1 may have a general role in regulating the adaptation responses of a cell to changes in nutrient availability.

Discussion

By directly patch clamping lysosomal membranes, we demonstrated that the current density of lysosomal ML1 channels is selectively and dramatically up-regulated within hours of nutrient starvation. This up-regulation was also mimicked with pharmacological inhibition by mTORC1, with reduced levels of PI(3,5)P_2, and with TFEB activation (and nuclear translocation). Hence, nutrient-sensitive regulation of ML1 channels may link lysosome function with nutrient availability via mTORC1, PI(3,5)P_2, and TFEB. Although our study has focused on the effect of mTORC1 inhibition on up-regulation of TRPML1, it has recently been demonstrated that the *Drosophila* homolog of TRPML1, TRPML, regulates the activity of TORC1 in vivo (43). Hence, TRPML1 and TORC1 may constitute a feedback loop to regulate amino acid homeostasis in vivo. Although TFEB

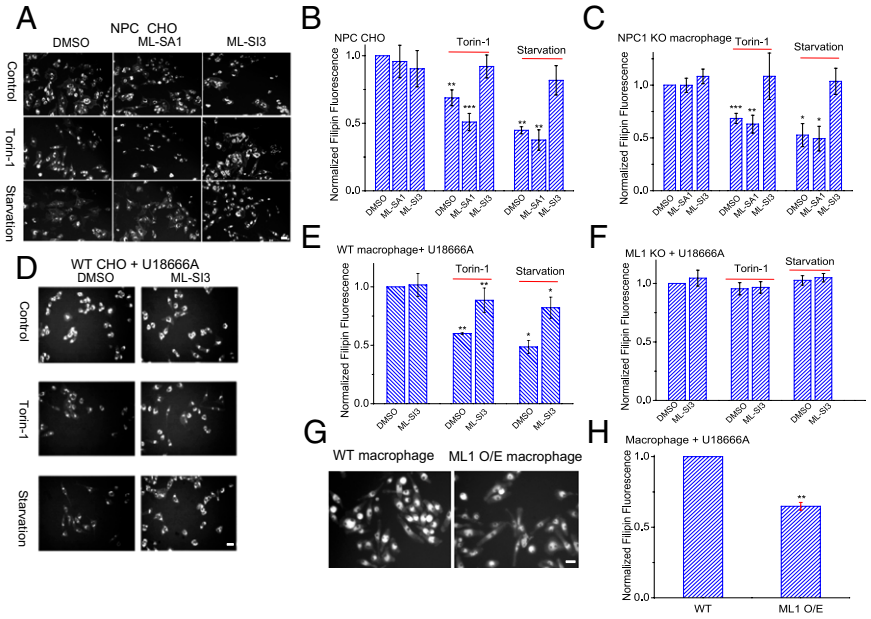

Fig. 6. Reduction of lysosome storage by starvation or mTOR inhibition requires ML1. (*A*) The effects of starvation (4 h), Torin-1 treatment (12 h), and TRPML synthetic modulator treatment (20 μM ML-SA1 or 20 μM ML-SI3) on cholesterol accumulation in NPC CHO cells (49). Free cholesterol was detected by using Filipin staining. (*B* and *C*) Normalized cholesterol levels in NPC CHO cells (*B*; $n = 5$) and NPC1$^{-/-}$ primary macrophage (*C*; $n = 3$) upon starvation or mTOR inhibition in the presence of ML-SA1 and ML-SI3 as indicated. (*D*) Torin1 treatment or starvation reduced cholesterol accumulation in NPC-like CHO cells that were pharmacologically induced by U18666A (2 μg/mL, 16 h). This reduction was abolished in the presence of MI-SI3. (*E* and *F*) Torin-1 treatment or starvation reduced cholesterol levels in WT macrophage (*E*; $n = 3$), but not ML1$^{-/-}$ macrophage (*F*; $n = 3$) treated with U18666A. (*G* and *H*) Stable overexpression (O/E) of ML1 reduced the cholesterol accumulation in U18666A-treated WT macrophage ($n = 3$). Data are presented as the mean ± SEM. The results were averaged from at least three independent experiments, each with 100–300 cells. Statistical comparisons were made by using ANOVA. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. (Scale bars: 100 μm.)

activation is known to trigger the expression of many lysosomal genes required for lysosome biogenesis (4, 5), the large increase in current density for ML1, and not for lysosomal TPC Na^+ channels, suggests that ML1 up-regulation plays an active role in lysosomal adaptation (Fig. 7*C*). Thus, ML1 up-regulation may represent one of the key functional changes that occur in a lysosome, and the up-regulation of ML1 may be required for lysosomal adaptation. Consistent with this hypothesis, ML1 was found to be required for starvation-induced enhancement of lysosomal proteolytic activity and cholesterol export. It is possible that starvation-induced enhancement of ML1-mediated lysosomal Ca^{2+} release may also facilitate lysosomal trafficking (19) for the following reasons. Given the timeframe for autophagosome-lysosome fusion (0.5–4 h after starvation) (8), ML1 up-regulation may promote Ca^{2+}-dependent fusion of autophagosomes and lysosomes (Fig. 7*C*) for the autophagy process (15, 44). ML1 channels may also be directly sensitized via a post-translational mechanism, thereby increasing lysosomal activity and proteolytic function. Further studies are needed to confirm and elucidate these mechanisms. Second, during the next phase of lysosomal adaptation (2–6 h after starvation), ML1 up-regulation may promote lysosomal reformation and biogenesis (Fig. 7*C*) (45, 46) required for sustained autophagy (8). However, the direct evidence to support the role of ML1 in autophagosome-lysosome fusion and lysosomal reformation is still lacking. Therefore, during prolonged starvation, an increase in lysosomal reformation and biogenesis could also indirectly increase autophagosome-lysosome fusion to boost lysosome function.

What is the significance of starvation-induced ML1 up-regulation given that starvation also reduces the level of $PI(3,5)P_2$, which is the only known endogenous agonist of ML1 (26)? The level of $PI(3,5)P_2$ is reduced upon starvation, but rapidly re-elevated upon readdition of AAs or growth factors (8, 24, 37). Although starvation initially (<0.5 h after starvation) reduces global $PI(3,5)P_2$ levels, thereby causing TFEB activation and subsequent ML1 up-regulation, upon prolonged starvation (>2–4 h), the efflux of lysosomal AAs that are produced during the course of lysosomal degradation may readily trigger newly synthesis (i.e., resynthesis) of $PI(3,5)P_2$, thereby causing mTOR reactivation and TFEB inactivation (8, 16, 24). In addition, $PI(3,5)P_2$ levels may also increase transiently and locally in lysosomes, presumably in a nutrient-independent manner, to regulate lysosomal membrane trafficking (36). Hence, $PI(3,5)P_2$ is involved in nutrient regulation of lysosomal functions in at least two distinct steps.

ML1 has been recently implicated in regulating lysosomal cholesterol export in NPC cells (19). However, although starvation and mTOR inhibition were sufficient to reduce cholesterol accumulation in NPC cells in the present study, such effects were not seen when ML1 was genetically deleted or pharmacologically inhibited. Conversely, overexpression of ML1 was found to mimic the effect of starvation on reducing cholesterol accumulation. Proteolytic analyses also support the observation that nutrient starvation stimulates lysosomal activity, and ML1 channel activity is required for nutrient-sensitive regulation of proteostasis. Collectively, ML1 may play an essential role in lysosomal adaptation during normal physiology and disease. Hence, up-regulation of ML1 expression may provide an opportunity to protect NPC and other lysosomal storage diseases (LSDs). There are potent mTOR inhibitors that are undergoing clinical trials (47), although there is a potential for cell growth to be negatively affected in these trials (10). Correspondingly, drugs such as Apilimod that potently increase TFEB activity without inhibiting mTOR are more promising. However, Apilimod may block lysosome biogenesis and Apilimod-induced vacuole formation may be harmful to cells (39). Further research is needed to identify new reagents that can specifically activate TFEB and induce ML1 expression without affecting other cellular processes. However, the capacity for ML-SA compounds to potently up-regulate ML1 channel activity, in combination with strategies

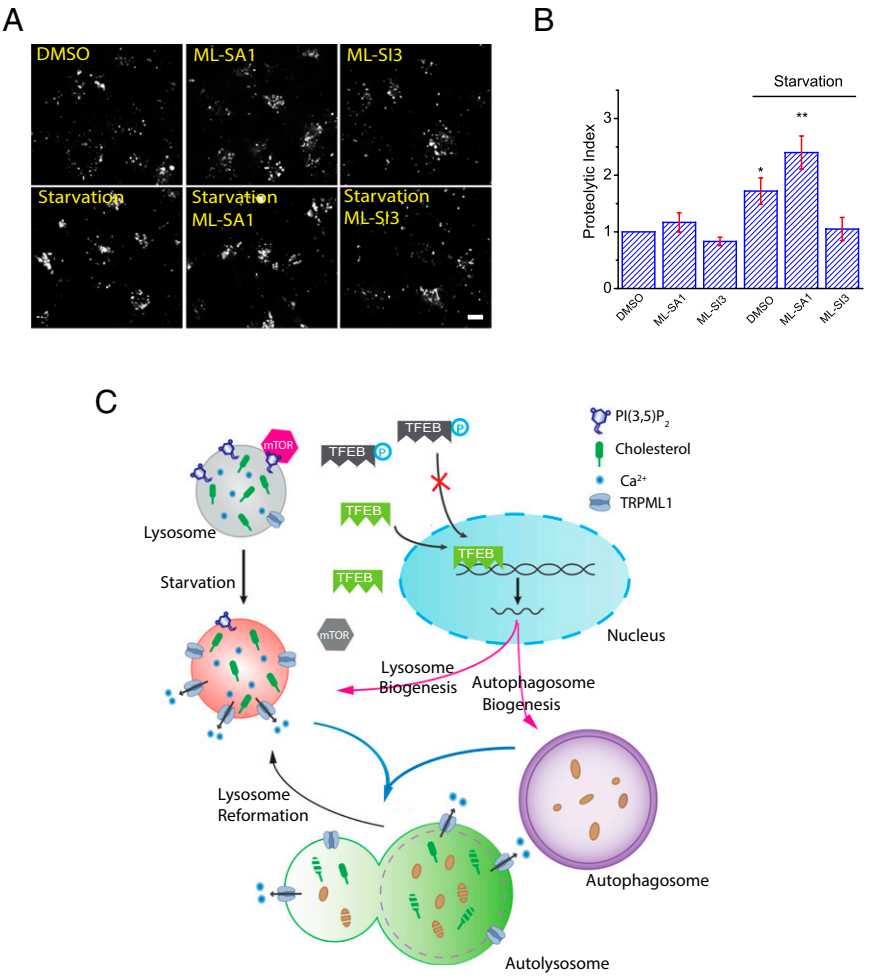

Fig. 7. ML1 regulates starvation-induced lysosomal proteolysis. (*A*) Confocal imaging of DQ-red-BSA Cos-1 cells grown in complete media versus AA-free + serum-free media (complete starvation) in the presence of ML-SA1 (20 μM) or MI-SI3 (20 μM). (Scale bar: 5 μm.) (*B*) Normalized proteolytic index values for completely starved Cos-1 cells treated with ML-SA1 or MI-SI3. Data are presented as the mean ± SEM. Statistical comparisons were made by using ANOVA ($n = 7$). *$P < 0.05$; **$P < 0.01$. (*C*) A working model to illustrate the role of ML1 up-regulation in starvation-induced enhancement of lysosomal function. Briefly, nutrient deprivation results in an inhibition of mTOR kinase activity and a decrease in lysosomal $PI(3,5)P_2$ levels, both of which promote TFEB nuclear translocation. Activated TFEB induces the expression of genes needed for autophagosome biogenesis and lysosome biogenesis. Through transcriptional, translational, and posttranslational mechanisms, lysosomal ML1 channel activity is dramatically up-regulated to increase lysosomal Ca^{2+} release, thereby promoting both autophagosome-lysosome fusion and lysosome reformation from autolysosomes. Note that upon completion of degradation during prolonged starvation, the efflux of lysosomal AAs may cause mTOR reactivation and $PI(3,5)P_2$ resynthesis (8, 16, 24). In addition, $PI(3,5)P_2$ levels may also increase transiently and locally in lysosomes, presumably in a nutrient-independent manner, to regulate lysosomal membrane trafficking (36). Thus, under these conditions, $PI(3,5)P_2$ elevation may increase the activity of lysosomal ML1 channels to promote lysosomal biogenesis and trafficking. Subsequently, autophagic lysosomal degradation is enhanced.

to up-regulate the TFEB-ML1 pathway, may represent a treatment strategy applicable to both LSDs and metabolic diseases.

Materials and Methods

Endolysosomal electrophysiology was performed in isolated endolysosomes by using a modified patch-clamp method. Cells were treated with vacuolin-1, a lipid-soluble polycyclic triazine that can selectively increase the size of endosomes and lysosomes. For filipin staining, cells were fixed and then stained with 0.05 mg/mL filipin in PBS supplemented with 10% FBS. Images were taken by using a fluorescence microscope with a UV filter. See *SI Materials and Methods* for details of experimental procedures.

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