

A negative feedback regulation of MTORC1 activity by the lysosomal Ca²⁺ channel MCOLN1 (mucolipin 1) using a CALM (calmodulin)-dependent mechanism

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ABSTRACT

Macroautophagy/autophagy is an evolutionarily conserved pathway that is required for cellular homeostasis, growth and survival. The lysosome plays an essential role in autophagy regulation. For example, the activity of MTORC1, a master regulator of autophagy, is regulated by nutrients within the lysosome. Starvation inhibits MTORC1 causing autophagy induction. Given that MTORC1 is critical for protein synthesis and cellular homeostasis, a feedback regulatory mechanism must exist to restore MTORC1 during starvation. However, the molecular mechanism underlying this feedback regulation is unclear. In this study, we report that starvation activates the lysosomal Ca²⁺ release channel MCOLN1 (mucolipin 1) by relieving MTORC1's inhibition of the channel. Activated MCOLN1 in turn facilitates MTORC1 activity that requires CALM (calmodulin). Moreover, both MCOLN1 and CALM are necessary for MTORC1 reactivation during prolonged starvation. Our data suggest that lysosomal Ca²⁺ signaling is an essential component of the canonical MTORC1-dependent autophagy pathway and MCOLN1 provides a negative feedback regulation of MTORC1 to prevent excessive loss of MTORC1 function during starvation. The feedback regulation may be important for maintaining cellular homeostasis during starvation, as well as many other stressful or disease conditions.

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INTRODUCTION

Macroautophagy (hereafter referred to as autophagy) is an evolutionarily conserved cellular process in which cells degrade their own organelles to generate energy and nutrients. During this process, targeted cytoplasmic constituents are isolated from the rest of the cell within a double-membraned compartment known as the autophagosome. The autophagosome then fuses with the lysosome to form the autolysosome where the cargos are degraded and recycled. Autophagy is not only an important housekeeping degradative process involved in cell growth, cell proliferation, tissue homeostasis and development, but also a cellular adaptation process that is essential for cell survival when nutrients or energy are limited.^{1–5}

The lysosome plays an essential role in sensing and signaling cellular nutrient status by recruiting MTORC1 (mechanistic target of rapamycin complex 1), a ubiquitous protein kinase acting as a master regulator of autophagy. Interestingly, MTORC1 is stimulated by amino acids through an inside-out mechanism in which amino acids must accumulate in the lysosomal lumen to initiate signaling.⁶ This process requires the vacuolar-type H⁺-translocating ATPase (V-ATPase), Ragulator and RAG/Rag GTPases. V-ATPase interacts with the Ragulator that anchors the RAG-MTORC1 complex to the lysosome. At rest, MTORC1 is localized on the lysosomal membrane

where it is activated by luminal amino acids. Starvation leads to MTORC1 dissociation from the lysosome and subsequent inhibition of MTORC1, triggering autophagy. Because MTORC1 is critically required for protein synthesis and cellular homeostasis, continuous loss of MTORC1 activity will damage the cell.^{7,8} Therefore, a feedback regulatory mechanism must exist to restore MTORC1 activity to survive stress conditions, in particular for prolonged starvation. However, the temporal feedback regulatory mechanism is unclear.

Intracellular Ca²⁺ signaling has recently been recognized as an important player in the regulation of MTORC1 and autophagy, although its exact role remains a matter of debate.^{9–12} Given that MTORC1 must be recruited to the surface of lysosomes to be activated,⁶ it is interesting to test whether lysosomal Ca²⁺ regulates MTORC1 and autophagy. In this study, we systematically examined the role of mammalian MCOLN1 (mucolipin 1), an important lysosomal Ca²⁺ release channel in MTORC1 regulation. We have demonstrated that MCOLN1 is essential for MTORC1 recruitment and activation in a Ca²⁺-CALM (calmodulin)-dependent manner. We have also provided direct evidence showing that starvation activates MCOLN1 through relieving the inhibition of MTORC1 on MCOLN1. Thus, we suggest that during starvation MCOLN1-CALM and MTORC1 form a feedback loop in which MTORC1 inhibition leads to MCOLN1-CALM activation, and this in

turn reactivates MTORC1. This feedback mechanism prevents MTORC1 from excess reduction of activity, a cellular adaptation mechanism that helps cells survive extreme conditions.

RESULTS

MCOLN1 promotes MTORC1 activity in lysosomes

Because Ca^{2+} is required for MTORC1 activation occurring on the lysosomal membrane^{6,10} and because MCOLN1 is an important Ca^{2+} release channel on the lysosomal membrane,^{13,14} we hypothesized that MCOLN1 regulates MTORC1 activity. To test this, MCOLN1 was upregulated by MCOLN1-GFP overexpression or mucolipin synthetic agonist 1 (ML-SA1, 15 μM).^{15,16} MTORC1 activity was assessed by measuring the phosphorylation of the substrate RPS6KB/p70-S6 kinase. To detect the potentiation effect of MCOLN1 on MTORC1, we first starved cells for 50 min to decrease MTORC1 and then reactivated MTORC1 by refeeding cells.⁶ Different starvation conditions have been used to study the MTORC1 signaling pathway, including complete nutrient deprivation, amino acid depletion, growth factor deprivation, and a milder but more physiological starvation.^{17–23} Our preliminary data suggested that MCOLN1 upregulation increased MTORC1 activity when complete nutrient deprivation (HBSS), amino acid depletion (DMEM without amino acid), or milder (HBSS or amino acid-free DMEM containing 1% fetal bovine serum [FBS]) conditions were applied (Fig. S1). To mimic physiological conditions, we adopted the milder starvation protocol (HBSS + 1% FBS).^{17–23} As shown in Fig. 1A, MTORC1 activity was increased by either MCOLN1 overexpression or ML-SA1 (15 μM) treatment in HEK293T cells, and this was inhibited by BAPTA-AM (10 μM). These data suggest that MCOLN1 regulates MTORC1 via releasing lysosomal Ca^{2+} . To consolidate our conclusion, we tested whether upregulating MCOLN1 prevented MTORC1 inactivation. For this purpose, 30 min starvation was applied to ensure the presence of substantial amounts of MTORC1 on the lysosomal membrane where it is activated by MCOLN1. Consistently, in HEK293T cells, MCOLN1 overexpression or ML-SA1 (15 μM) treatment also prohibited the loss of MTORC1 activity upon starvation (Fig. 1B). Interestingly, under normal conditions, MCOLN1 upregulation (Fig. 1C) did not affect MTORC1 activity. This is consistent with previous reports showing that activating MCOLN1 does not change MTORC1 activity.^{24,25}

Because MCOLN1 has been suggested to regulate the fusion of autophagosomes with lysosomes²⁶ and subsequent protein degradation,^{27–29} an increase in MTORC1 activity could be attributed to an elevated nutrient regeneration, which is required for the reactivation of MTORC1. To test this, we examined whether activation of MCOLN1 promotes autophagosome-lysosome fusion. Autophagosome-lysosome fusion was studied using tandem mRFP-EGFP-LC3.^{25,30,31} Because the low pH of autolysosomes quenches the fluorescent signal of EGFP but not mRFP, autophagosomes (high pH) and autolysosomes (low pH) will be labeled with yellow (i.e., mRFP⁺ EGFP⁺) and red (i.e., mRFP⁺ EGFP⁻) signals, respectively. An increase in the ratio of yellow to red puncta represents a blockade of autophagosome and lysosome fusion. We found that MCOLN1

upregulation inhibited autophagosome and lysosome fusion (Fig. S2A–S2C). This is in agreement with previous studies showing that MCOLN1 activation inhibits autophagosome-lysosome fusion.²⁵ The mechanism underlying MCOLN1 inhibiting autophagosome-lysosome fusion remains a fascinating question to be investigated. Altogether, we suggest that MCOLN1 facilitates MTORC1 activation likely through a mechanism independent of nutrient regeneration.

To further support our hypothesis, we investigated the physical association between MCOLN1 and MTORC1 using co-immunoprecipitation (co-IP) in HeLa cells transfected with MCOLN1-GFP and MTOR-FLAG. We found that MCOLN1 weakly associated with MTORC1 (Fig. 1D), and this was dramatically increased by Ca^{2+} (10 μM) application and by activating MCOLN1 with ML-SA1 (15 μM) (Fig. 1D). Unfortunately, the unavailability of MCOLN1 antibody precluded us from assessing the interaction of endogenous MCOLN1 and MTORC1. These data suggest that MCOLN1 and MTORC1 form a signaling complex on the lysosomal membrane when MCOLN1 is activated. Interestingly, the association between MCOLN1 and MTORC1 was also regulated by nutrient availability. Weak association between MCOLN1 and MTORC1³² was increased by short-term starvation (10 min) but not long-term starvation (50 min) (Fig. 1E). These data suggest that starvation may activate MCOLN1.^{33–34} to increase its association with MTORC1,³⁵ whereas long-term or strong starvation likely causes MTORC1 dissociation from the lysosome, thereby decreasing the interaction between MCOLN1 and MTORC1.

MCOLN1 is required for MTORC1 activation in lysosomes

If MCOLN1 is important for MTORC1 activation, deletion of *MCOLN1* will lead to a decrease in MTORC1 activity. Indeed, MTORC1 reactivation was compromised by *MCOLN1* ablation in human skin fibroblasts (Fig. 2A). Supporting this, starvation suppressed MTORC1 activity more easily in *MCOLN1*^{-/-} human fibroblasts (Fig. 2B). In the meantime, inhibiting MCOLN1 using ML-SII (50 μM)¹⁶ suppressed MTORC1 activity upon starvation. MTORC1 reactivation induced by nutrient refeeding was also dramatically suppressed by ML-SII in HEK293T cells (Fig. 2C). Supporting the notion that MCOLN1-mediated Ca^{2+} release is required for MTORC1 activity, we found that MTORC1 reactivation upon nutrient refeeding was suppressed by BAPTA-AM treatment (Fig. S3). In addition, in the presence of RRAGB^{GTP}, a GTP-bound mutant which partially mimics amino acid-replete conditions,^{32,36} MCOLN1 deletion or BAPTA-AM still reduced MTORC1 activity in starved cells (Fig. 2D,E). These data further suggest that MCOLN1 regulates MTORC1 activity through a mechanism independent of nutrient regeneration. In agreement with the finding showing that MCOLN1 upregulation did not alter MTORC1 activity under normal conditions (Fig. 1C),²⁴ neither MCOLN1 ablation (Fig. 2B) nor inhibition (Fig. 2F) affected MTORC1 activity under normal conditions. Note that MTORC1 activity was assessed by measuring the phosphorylation of RPS6KB and EIF4EBP1, another MTORC1 substrate (Fig. 2C). Altogether, these data suggest that MCOLN1 is required for MTORC1 activation when cells are stressed.

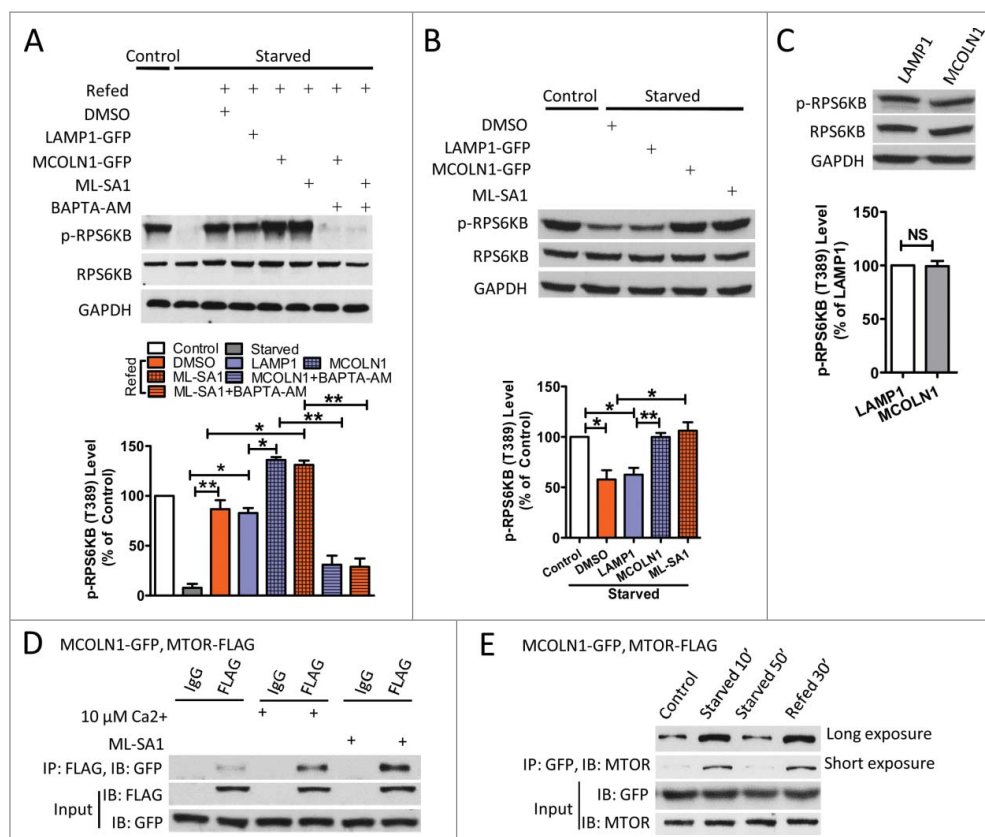


Figure 1. MCOLN1 promotes MTORC1 activation. (A) Upregulation of MCOLN1 promotes MTORC1 reactivation during refeeding, and this was inhibited by BAPTA-AM. HEK293T cells transfected with or without LAMP1-GFP, or MCOLN1-GFP were kept in normal culture medium, starved for 50 min or followed by nutrient refeeding for 15 min \pm ML-SA1 (15 μ M) and/or BAPTA-AM (10 μ M) as indicated. Cell extracts were analyzed by western blotting using anti-p-RPS6KB (T389) and anti-RPS6KB antibodies, and anti-GAPDH antibody was used as a loading control. Histograms represent the mean percentage of the ratio of p-RPS6KB:RPS6KB (mean \pm SEM, $n = 3$ independent experiments) in the indicated conditions, relative to that of the control. (B) Upregulation of MCOLN1 prohibited the loss of MTORC1 activity upon starvation. HEK293T cells transfected with or without LAMP1-GFP, or MCOLN1-GFP were starved for 30 min \pm ML-SA1 (15 μ M). Histograms represent the mean percentage of the ratio of p-RPS6KB:RPS6KB (mean \pm SEM, $n = 3$ independent experiments) in the indicated conditions, relative to that of the control. (C) MCOLN1 overexpression did not alter MTORC1 activity under normal conditions. HEK293T cells were transfected with either LAMP1-GFP or MCOLN1-GFP. MTORC1 activity was examined after the cells were cultured for 2–4 h in fresh culture medium. Histograms represent the mean percentage of the ratio of p-RPS6KB:RPS6KB (mean \pm SEM, $n = 3$ independent experiments) in the indicated conditions, relative to that of LAMP1-expressing cells. (D) Co-IP of MCOLN1 and MTORC1. HeLa cells expressing MCOLN1-GFP and MTOR-FLAG were kept in normal culture medium. Cell extracts were subject to FLAG or IgG immunoprecipitation \pm ML-SA1 (15 μ M) or 10 μ M Ca²⁺ and analyzed by western blotting for the indicated proteins. Note that culture medium was not changed before the co-IP experiments. (E) The association of MCOLN1 and MTORC1 is regulated by starvation. Short-term (10 min) starvation increased the association between MCOLN1 and MTORC1, and this was suppressed by long-term (50 min) starvation. Nutrient refeeding also increases the association between MCOLN1 and MTORC1. HEK293T cells expressing MCOLN1-GFP and MTOR-FLAG were kept in fresh culture medium, starved for 10 min and 50 min, or starved for 50 min followed by 30 min refeeding. NS, not significant; *, $P < 0.05$; **, $P < 0.01$.

MCOLN1 regulates MTORC1 depending on the culture conditions

Recently, Li et al. reported that MCOLN1 but not other lysosomal channels, such as TPCN2 and P2RX4, regulate MTORC1 under normal conditions.³⁵ However, our data suggest that MCOLN1 regulates MTORC1 only under starvation but not normal fed conditions (Fig. 1, Fig. 2).²⁴ To clarify this controversy, we repeated Li et al.'s experiment by carefully controlling the cell culture conditions. We suspected that the cells in Li et al.'s study were also under starvation conditions because Li et al. measured MTORC1 activity after 24 h culture without changing the medium.³⁵ Indeed, we found that neither MCOLN1 overexpression nor ML-SA1 affected MTORC1 activity under normal fed conditions. In contrast, both MCOLN1 overexpression and ML-SA1 increased MTORC1 activity when nonfresh medium was replaced after overnight culture (Fig. 3A,B). Consistently, MCOLN1 deletion compromised

MTORC1 activity when cells were incubated in nonfresh culture medium but not in normal fed conditions (Fig. 3C). Altogether, these data further suggest that MCOLN1 regulates MTORC1 specifically when cells encounter nutrient shortage.

MCOLN1 and Ca²⁺-dependent MTORC1 recruitment onto lysosomes

Nutrient deprivation results in the dissociation of MTORC1 from lysosomes, whereas nutrient replenishment restores lysosomal localization of MTORC1 and its activity.^{17,36-40} Given that MCOLN1 is important for MTORC1 activation, we postulated that MCOLN1 regulates MTORC1 recruitment onto lysosomes. To test this, we acutely ablated MCOLN1 in HEK293T cells using MCOLN1 shRNA-expressing lentivirus (Fig. S4), and tested whether MCOLN1 is required for

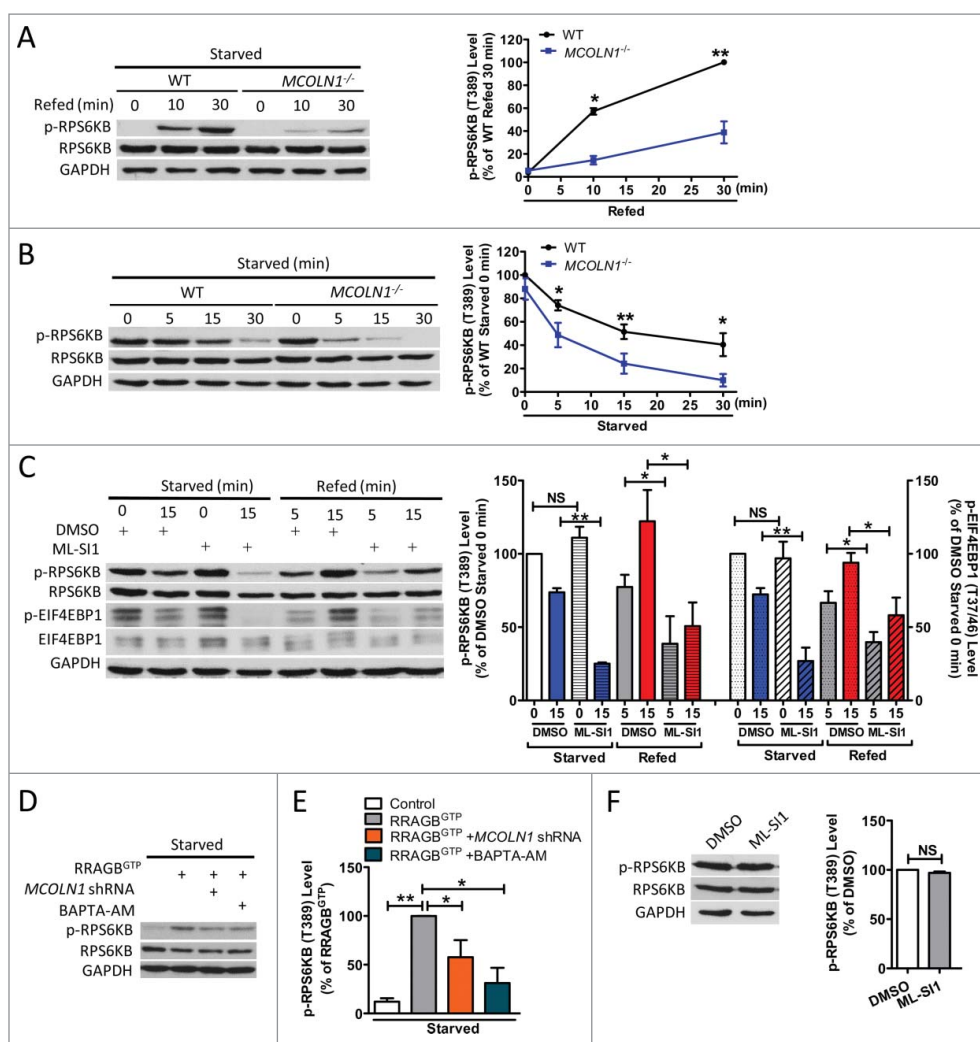


Figure 2. MCOLN1 is required for MTORC1 activation. (A) MCOLN1 ablation compromised MTORC1 reactivation during nutrient refeeding. WT and *MCOLN1*^{-/-} human skin fibroblasts were starved for 50 min or followed by nutrient refeeding for 10 or 30 min as indicated. Cell extracts were analyzed by western blotting for the indicated proteins. The graph illustrates the mean percentage of the ratio of p-RPS6KB:RPS6KB (mean \pm SEM, $n = 3$ independent experiments), relative to that in WT cells with 30 min refeeding. (B) *MCOLN1*^{-/-} cells lose MTORC1 activity more easily compared with WT cells when starved. WT and *MCOLN1*^{-/-} cells were kept in normal culture medium or starved for 5, 15 or 30 min. Cell extracts were analyzed by western blotting for the indicated proteins. The graph shows the mean percentage of the ratio of p-RPS6KB:RPS6KB (mean \pm SEM, $n = 3$ independent experiments), relative to that in fed WT cells. (C) Inhibition of MCOLN1 suppressed MTORC1 activity upon starvation and nutrient refeeding. HEK293T cells were kept in normal culture medium, starved for 15 min, or starved for 50 min followed by nutrient refeeding for 5 or 15 min \pm ML-S11 (50 μ M) as indicated. MTORC1 activity was assessed by measuring p-RPS6KB (T389) and p-EIF4EBP1 (T37/46) using western blot. The graph shows the mean percentage of the ratio of p-RPS6KB:RPS6KB (left) and p-EIF4EBP1:EIF4EBP1 (right) (mean \pm SEM, $n = 3$ independent experiments), relative to that in nontreated fed cells. (D, E) High MTORC1 activity in cells expressing constitutively active RRBAG, which partially mimics nutrient-replete conditions was suppressed by *MCOLN1* deletion and BAPTA-AM. HEK293T cells were transfected with RRBAG^{GTP} or together with scramble or *MCOLN1* shRNA; 24 h later cells were starved with amino acid-free DMEM for 30 min in the presence or absence of 10 μ M BAPTA-AM. (F) MCOLN1 inhibition did not alter MTORC1 activity under normal conditions. HEK293T cells were treated with DMSO or ML-S11 (50 μ M) for 1 h in normal medium. The graph illustrates the mean percentage of the ratio of p-RPS6KB:RPS6KB (mean \pm SEM, $n = 3$ independent experiments), relative to that in DMSO-treated cells. NS, not significant; *, $P < 0.05$; **, $P < 0.01$.

lysosomal MTOR recruitment. HEK293T cells were first starved for 60 min to completely diffuse MTOR and then refeed with nutrients for 10 min.⁶ Under starvation, MTOR normally failed to cluster onto lysosomes and instead was found in a diffuse staining pattern as shown in Fig. 4A and Fig. 4B. Interestingly, refeeding nutrients resulted in a significant increase in lysosomal MTOR puncta in control but not in *MCOLN1* knockdown cells (Fig. 4A,B). Supporting this, ML-SA1 (15 μ M) prevented starvation from dispersing MTOR in control but not *MCOLN1* knockdown cells, and this was eliminated by chelating Ca²⁺ using BAPTA-AM (10 μ M) (Fig. 4C,D). Consistently, in human fibroblasts, loss of *MCOLN1* prevented MTOR recruitment onto lysosomes (Fig. S5A,B) in response to

refeeding, and ML-SA1 (15 μ M) prevented starvation from causing dispersal of MTOR from lysosomes (Fig. S5C,D). Conversely, neither *MCOLN1* knockdown (Fig. S6A,B) nor *MCOLN1* deletion (Fig. S6C,D) affected lysosomal localization of MTOR under normal conditions. This is in agreement with the data showing that MCOLN1 was not required for MTORC1 activation under normal conditions (Fig. 1C, Fig. 2B,F). Collectively, these data suggest that MCOLN1-mediated Ca²⁺ release is specifically required for lysosomal localization of MTORC1 under stress but not normal conditions. This finding is also in agreement with the data showing that MCOLN1 was weakly associated with MTORC1 (Fig. 1D,E) and minimally active under normal fed conditions.

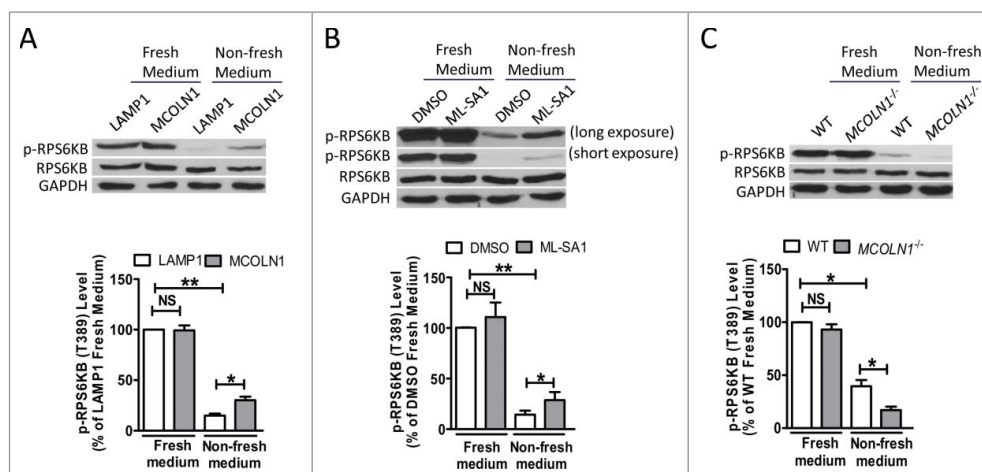


Figure 3. MTORC1 activity is regulated by culture medium conditions. (A) HEK293T cells were transfected with LAMP1-GFP or MCOLN1-GFP for 24 h, and then the culture medium was either changed to fresh complete medium for 2 h (fresh medium) or kept unchanged (nonfresh medium) before measuring MTORC1. MCOLN1 overexpression increased MTORC1 activity in cells incubated in nonfresh medium but not in fresh medium. Histograms represent the mean percentage of the ratio of p-RPS6KB:RPS6KB (mean \pm SEM, $n = 3$ independent experiments) in the indicated conditions, relative to that of fed cells expressing LAMP1. (B) Cells were cultured for 24 h, and then treated with ML-SA1 (10 μ M) for 3 h in fresh or nonfresh medium. Histograms represent the mean percentage of the ratio of p-RPS6KB:RPS6KB (mean \pm SEM, $n = 3$ independent experiments) in the indicated conditions, relative to that of fed cells treated with DMSO. (C) WT and MCOLN1^{-/-} human skin fibroblasts were cultured for 24 h, and then incubated in fresh or nonfresh medium for 2 h. Histograms represent the mean percentage of the ratio of p-RPS6KB:RPS6KB (mean \pm SEM, $n = 3$ independent experiments) in the indicated conditions, relative to that of WT fed cells. NS, not significant; *, $P < 0.05$; **, $P < 0.01$.

MCOLN1 activates MTORC1 through CALM

To understand how MCOLN1 recruits and activates MTORC1, we searched for Ca²⁺ sensor proteins that have been reported to be localized in the lysosomal membrane, including SYT7,⁴¹ PDCD6/ALG2 (programmed cell death 6),⁴² and CALM.^{43,44} We first evaluated whether these proteins are required for MTORC1 activation. We found that inhibition of CALM with W7 (3 μ M), but not expression of the dominant negative

mutant SYT7 (SYT7-DN)⁴⁵ or PDCD6 deletion⁴⁶ caused a reduction in MTORC1 activity (Fig. 5A-C and Fig. S7). We also observed that an increase in MTORC1 activity induced by MCOLN1 overexpression (Fig. 5D) or ML-SA1 (15 μ M) (Fig. 5E) was inhibited by W7 (3 μ M).

In accordance with this, MCOLN1 and CALM were strongly associated and this association was eliminated by inhibiting MCOLN1 with ML-SI1 (50 μ M) or chelating Ca²⁺ with BAPTA-AM (10 μ M) (Fig. 5F). We also detected association

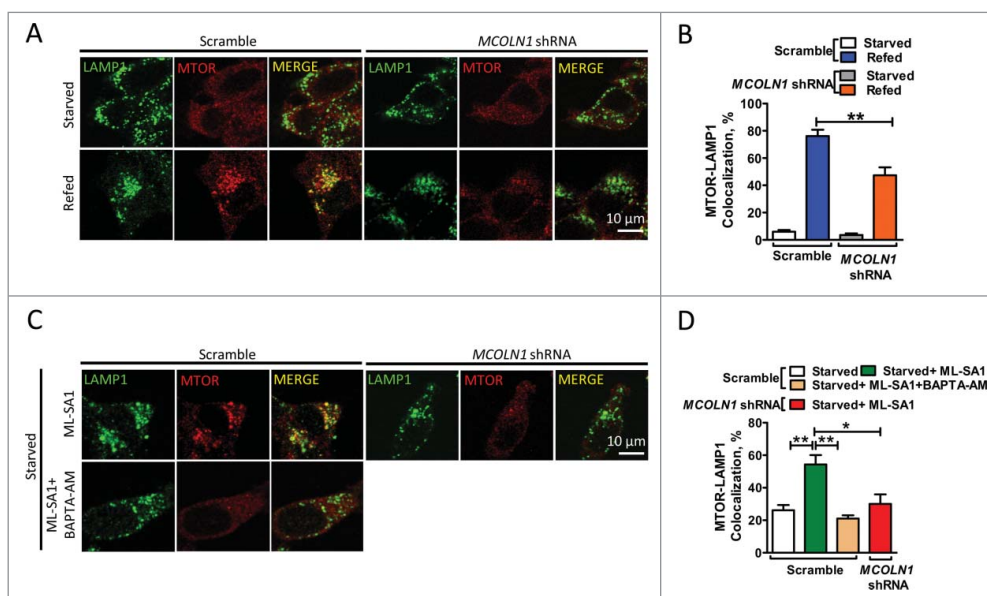


Figure 4. MCOLN1 regulates MTOR lysosome recruitment. (A) In the MCOLN1 knockdown HEK293T cell line that was generated with lentivirus carrying shRNA against MCOLN1, a decrease in MCOLN1 suppressed MTOR recruitment onto lysosomes. Scramble and MCOLN1 shRNA knockdown HEK293T cells were starved for 1 h followed by nutrient refeeding for 10 min. Cells were then fixed and immunostained with antibodies against LAMP1 and MTOR. (B) The percentage of colocalization between LAMP1 and MTOR as in (A). (C) Upregulation of MCOLN1 by ML-SA1 prohibited MTOR dissociation from lysosomes in control but not MCOLN1-deficient cells. This was blocked by BAPTA-AM. Scramble and MCOLN1 shRNA knockdown HEK293T cells were starved for 50 min in the presence of ML-SA1 (15 μ M) \pm BAPTA-AM (10 μ M) as indicated. (D) The percentage of colocalization between LAMP1 and MTOR as in (C). More than 20 cells were analyzed for each condition in 3 independent experiments. NS, not significant; **, $P < 0.01$.

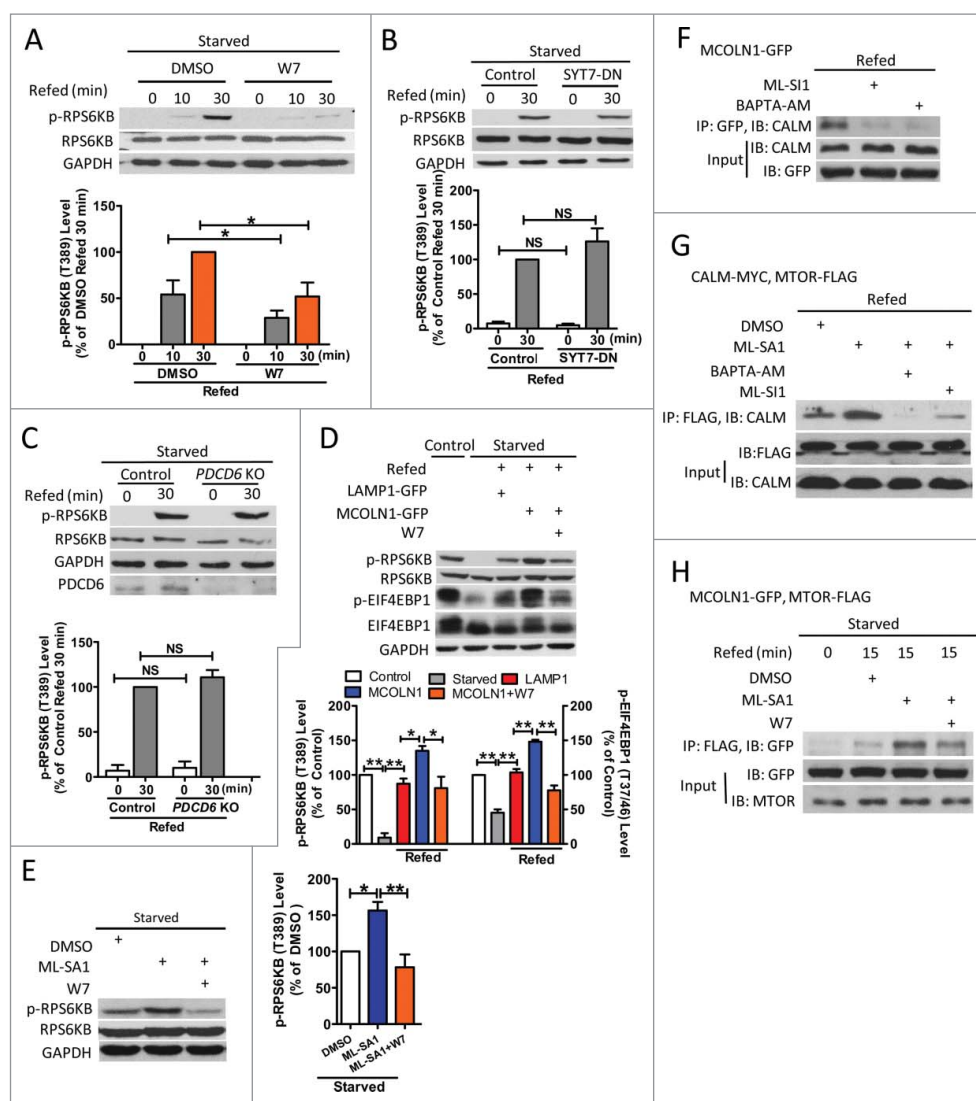


Figure 5. MCOLN1 activates MTORC1 through CALM. (A) W7 prevented MTORC1 reactivation during refeeding. HEK293T cells were starved for 50 min or followed by nutrient refeeding for 10 or 30 min \pm W7 (3 μ M) as indicated. Cell extracts were analyzed by western blotting using anti-p-RPS6KB (T389) and anti-RPS6KB antibodies, and anti-GAPDH antibody was used as a loading control. Histograms represent the mean percentage of the ratio of p-RPS6KB:RPS6KB (mean \pm SEM, $n = 3$ independent experiments) in the indicated conditions, relative to that of cells with 30 min refeeding. (B) SYT7-DN had no effect on MTORC1 reactivation during refeeding. HEK293T cells transfected with or without a dominant negative form of SYT7 (SYT-DN) were starved for 50 min or followed by nutrient refeeding for 30 min. Histograms represent the mean percentage of the ratio of p-RPS6KB:RPS6KB (mean \pm SEM, $n = 3$ independent experiments) in the indicated conditions, relative to that of cells with 30 min refeeding. (C) *PDCD6* knockout did not influence MTORC1 reactivation during refeeding. Control HEK293T cells or cells with *PDCD6* knockout were starved for 50 min or followed by nutrient refeeding for 30 min. Histograms represent the mean percentage of the ratio of p-RPS6KB/RPS6KB (mean \pm SEM, $n = 3$ independent experiments) in the indicated conditions, relative to that of cells with 30 min refeeding. (D) The increased MTORC1 activity induced by MCOLN1 overexpression was inhibited by W7. HEK293T cells transfected with or without MCOLN1-GFP were kept in normal culture medium, starved for 50 min or followed by nutrient refeeding for 30 min \pm W7 (3 μ M) as indicated. MTORC1 activity was assessed by measuring RPS6KB (T389) and p-EIF4EBP1 (T37/46) using western blot. Histograms represent the mean percentage of the ratio of p-RPS6KB:RPS6KB (left) and p-EIF4EBP1:EIF4EBP1 (right) (mean \pm SEM, $n = 3$ independent experiments) in the indicated conditions, relative to the control. (E) W7 inhibited the ML-SA1-induced increase of MTORC1 activity during starvation. HEK293T cells were starved for 15 min \pm ML-SA1 (15 μ M) with or without W7 (3 μ M) as indicated, and then lysed and subjected to immunoblotting. Histograms represent the mean percentage of the ratio of p-RPS6KB:RPS6KB (mean \pm SEM, $n = 3$ independent experiments) in the indicated conditions, relative to that of cells without treatment. (F) Co-IP of MCOLN1 and CALM. The interaction between MCOLN1 and CALM was inhibited by ML-SI1 or BAPTA-AM. HEK293T cells expressing MCOLN1-GFP were starved for 50 min followed by nutrient refeeding for 30 min \pm ML-SI1 (50 μ M) or BAPTA-AM (10 μ M) as indicated. Cell extracts were subject to GFP immunoprecipitation and analyzed by western blotting for the indicated proteins. (G) Co-IP of CALM and MTORC1. The interaction between CALM and MTORC1 during refeeding was enhanced by activating MCOLN1 with ML-SA1, and this was eliminated by BAPTA-AM or ML-SI1. HEK293T cells expressing CALM-MYC-HIS and MTOR-FLAG were starved for 50 min followed by nutrient refeeding for 15 min \pm ML-SA1 (15 μ M), BAPTA-AM (10 μ M) or ML-SI1 (50 μ M) as indicated. Cell extracts were subject to FLAG immunoprecipitation and analyzed by western blotting for the indicated proteins. (H) Co-IP of MCOLN1 and MTORC1. During nutrient refeeding, the ML-SA1-induced increase in the interaction between MCOLN1 and MTORC1 was inhibited by W7. HEK293T cells expressing MCOLN1-GFP and MTOR-FLAG were starved for 50 min, or followed by nutrient refeeding for 15 min \pm ML-SA1 (15 μ M) with or without W7 (3 μ M) as indicated. Cell extracts were subject to FLAG immunoprecipitation and analyzed by western blotting for the indicated proteins. NS, not significant; *, $P < 0.05$; **, $P < 0.01$.

between MTORC1 and CALM, and this was increased by activating MCOLN1 with ML-SA1 (15 μ M), but suppressed by chelating Ca^{2+} using BAPTA-AM (10 μ M) or inhibiting MCOLN1 with ML-SI1 (50 μ M) (Fig. 5G). In addition, the

interaction between MCOLN1 and MTORC1 was increased by ML-SA1 (15 μ M) and this was inhibited by W7 (3 μ M) (Fig. 5H). Altogether, these data suggest that MCOLN1, CALM and MTORC1 form a macromolecular complex on the

lysosomal membrane where MCOLN1 releases lysosome Ca^{2+} to activate CALM and subsequently regulate MTORC1 activity.

CALM is required for MTORC1 recruitment

Data presented above suggest that CALM may also regulate MTORC1 recruitment. In agreement with this, inhibiting CALM with W7 (3 μM) suppressed lysosomal MTOR recruitment in HEK293T cells (Fig. 6A). Consistently, in the presence of W7, ML-SA1 (15 μM) failed to prohibit starvation from inducing dissociation of MTOR from lysosomes (Fig. 6B). These data suggest that CALM may function downstream of MCOLN1 to regulate MTORC1 recruitment.

MTORC1-dependent MCOLN1 activation by starvation

Recent studies have suggested that nutrient deprivation increases MCOLN1 expression via TFEB (transcription factor EB) that is activated by MCOLN1-mediated Ca^{2+} release.^{47,48} However, the molecular mechanism underlying the initial MCOLN1 activation upon starvation is unknown, especially considering that transient starvation has been shown to activate MCOLN1-mediated Ca^{2+} release.⁴⁷ Because MCOLN1 is suppressed by MTORC1 via direct phosphorylation,³³ we tested whether nutrient starvation inhibited MTORC1 to release the

suppression of MCOLN1 by MTORC1. RRAG are widely known to regulate MTORC1 signaling.³⁶ Expression of RRAG^{GTP} displayed a high level of MTORC1 activity in starved cells, whereas expression of RRAG^{GDP}, a GDP-bound RRAG mutant, suppressed MTORC1 activity in refed cells following starvation (Fig. S8).^{32,36} Therefore, HEK293T cells expressing genetically encoded calcium indicator for optical imaging (GECO)-MCOLN1 together with RRAG^{GDP} or RRAG^{GTP} (RRAG^{WT} as control) were employed to identify the influence of MTORC1 on MCOLN1. MCOLN1-mediated lysosomal Ca^{2+} release was monitored by measuring ML-SA1 (10 μM)-mediated GECO-MCOLN1 response as previously reported.⁴⁵ As expected, nutrient starvation induced an increase in GECO-MCOLN1 response, which was reversed by nutrient refeeding (Fig. 7A,B). Expression of RRAG^{GTP} decreased GECO-MCOLN1 response in starved cells, whereas expression of RRAG^{GDP} increased GECO-MCOLN1 response in refed cells (Fig. 7E). In agreement with this, pretreatment with AZD8055 (1 μM , 30 min), a potent MTOR inhibitor,⁴⁹ enhanced the ML-SA1-evoked GECO-MCOLN1 response (Fig. S9). Notably, neither treatments affect the GECO-MCOLN1 response to glycyl-phenylalanine 2-naphthylamide (GPN, 200 μM), a substrate of the lysosomal exopeptidase CTSC (cathepsin C) that induces lysosome osmolysis to dump all Ca^{2+} out, or ionomycin (2 μM , with 2 mM Ca^{2+} in the bath),

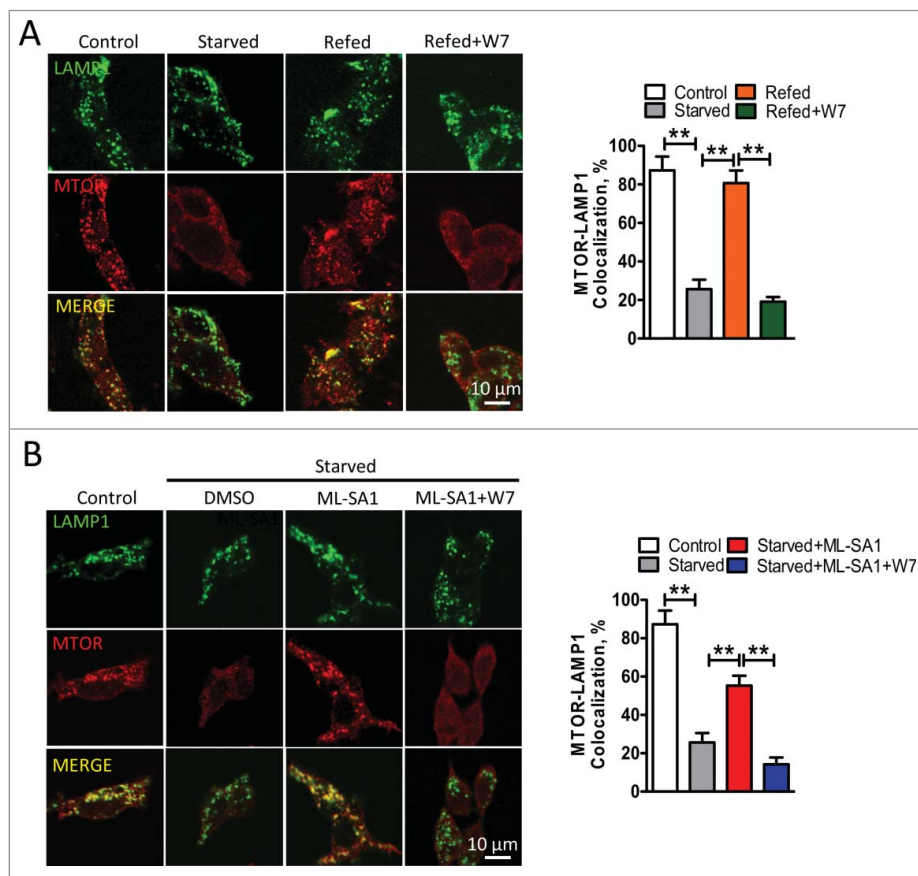


Figure 6. MCOLN1 regulates MTOR recruitment onto lysosomes through CALM. (A) Inhibiting CALM by W7 prohibited MTOR recruitment induced by nutrient refeeding. HEK293T cells were starved for 50 min, followed by nutrient refeeding for 10 or 15 min \pm W7 (3 μM) as indicated. The data was expressed as a percentage of colocalization between LAMP1 and MTOR. (B) W7 eliminated the effect of ML-SA1 on preventing MTOR dissociation from lysosomes. HEK293T cells were kept in normal culture medium, or starved for 50 min \pm W7 (3 μM) and/or ML-SA1 (15 μM) as indicated. The data are expressed as a percentage of colocalization between LAMP1 and MTOR. More than 20 cells were analyzed for each condition in 3 independent experiments. **, $P < 0.01$.

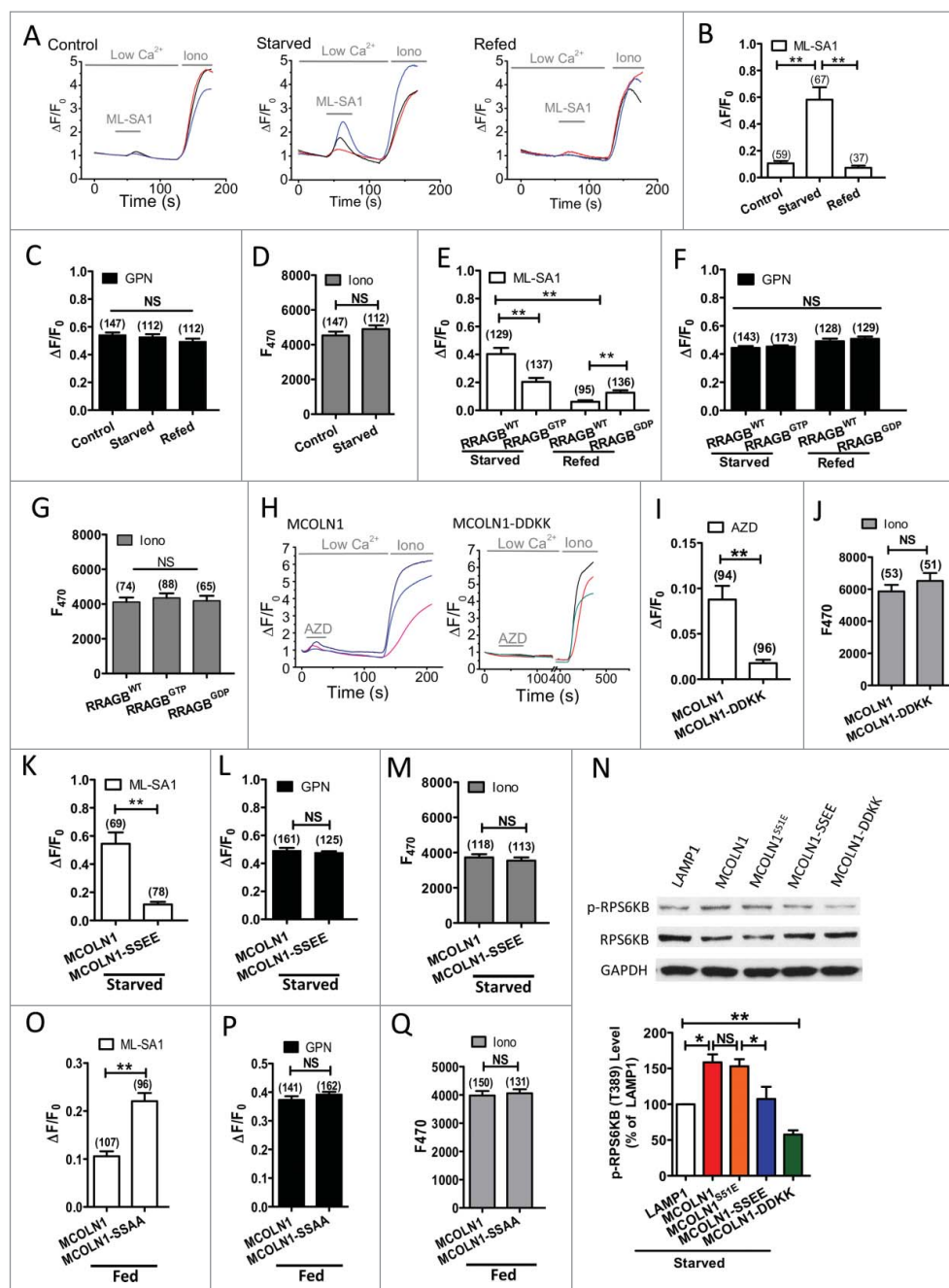


Figure 7. MTORC1-dependent regulation of MCOLN1-mediated lysosomal Ca^{2+} release. (A) Starvation increased MCOLN1 activity as indicated by elevated GECO-MCOLN1 responses to ML-SA1 in HEK293T cells expressing GECO-MCOLN1. Cells were kept in normal culture medium, starved for 50 min or followed by nutrient refeeding for 15 min prior to the measurement. (B) Summary of ML-SA1-induced GECO-MCOLN1 responses as in (A). (C, D) Starvation or refeeding did not affect GECO-MCOLN1 response to GPN (200 μM) (C) or ionomycin (Iono, 2 μM) (D). (E) RRAG^{GTP} decreased GECO-MCOLN1 responses in starved cells, whereas RRAG^{GDP} increased GECO-MCOLN1 responses in refeeding cells. HEK293T cells expressing GECO-MCOLN1 and RRAG^{WT} together with RRAG^{GTP} or RRAG^{GDP} were subjected to starvation, or nutrient refeeding (DMEM + 10% FBS, 15 min). (F, G) GECO-MCOLN1 response to GPN (200 μM) (F) and Ionomycin (2 μM) (G) was comparable to conditions in (E). (H–J) Inhibition of MTOR with AZD8055 (1 μM) induced GECO responses in HEK293T cells expressing GECO-MCOLN1 but not GECO-MCOLN1-DDKK, a nonconducting mutant of MCOLN1. GECO signals were measured in the absence of external Ca^{2+} . GECO responses to ionomycin (2 μM , with 2 mM Ca^{2+} in the bath) was used to compare the expression levels of GECO-MCOLN1 and GECO-MCOLN1-DDKK. (K) S571,576E phosphomimetic mutation of GECO-MCOLN1 (GECO-MCOLN1-SSEE) decreased ML-SA1-induced GECO responses upon starvation compared to GECO-MCOLN1. HEK293T cells expressing GECO-MCOLN1 and GECO-MCOLN1-SSEE, respectively, were subjected to starvation (50 min) prior to the measurement. (L, M) GECO-MCOLN1 response to GPN (200 μM) (L) or ionomycin (2 μM) (M) was comparable in conditions as in (H). (N) WT MCOLN1 but not MCOLN1-SSEE and MCOLN1-DDKK increased MTORC1 activity. HEK293T cells expressing LAMP1-GFP, MCOLN1-EGFP, MCOLN1^{S51E}-EGFP, MCOLN1-SSEE-EGFP and MCOLN1-DDKK-GFP, respectively, were starved for 30 min. MCOLN1-DDKK-GFP (MCOLN1^{D471,472K}-GFP) is a MCOLN1 nonconducting pore.⁴⁵ MCOLN1^{S51E}-EGFP is a control phosphomimetic mutant that is not related to MTORC1 phosphorylation.³³ Cell extracts were analyzed by western blotting using anti-p-RPS6KB (T389) and anti-RPS6KB antibodies, and anti-GAPDH antibody was used as a loading control. Histograms represent the mean percentage of the ratio of p-RPS6KB:RPS6KB (mean \pm SEM, $n = 3$ independent experiments) in the indicated conditions, relative to that of cells expressing LAMP1. (O–Q) Summary of ML-SA1-induced GECO responses in HEK293T cells expressing GECO-MCOLN1 and GECO-MCOLN1-SSAA under normal fed conditions. MCOLN1-SSAA displayed a higher activity in normal fed conditions compared to MCOLN1. However, MCOLN1-SSAA did not have an effect on lysosomal Ca^{2+} content. These findings suggest that the lysosome must have a mechanism to maintain its Ca^{2+} homeostasis. In this case, although MCOLN1-SSAA increases Ca^{2+} release, a compensational mechanism exists to increase Ca^{2+} uptake that depends on the endoplasmic reticulum Ca^{2+} release.^{70–73} NS, not significant; *, $P < 0.05$; **, $P < 0.01$.

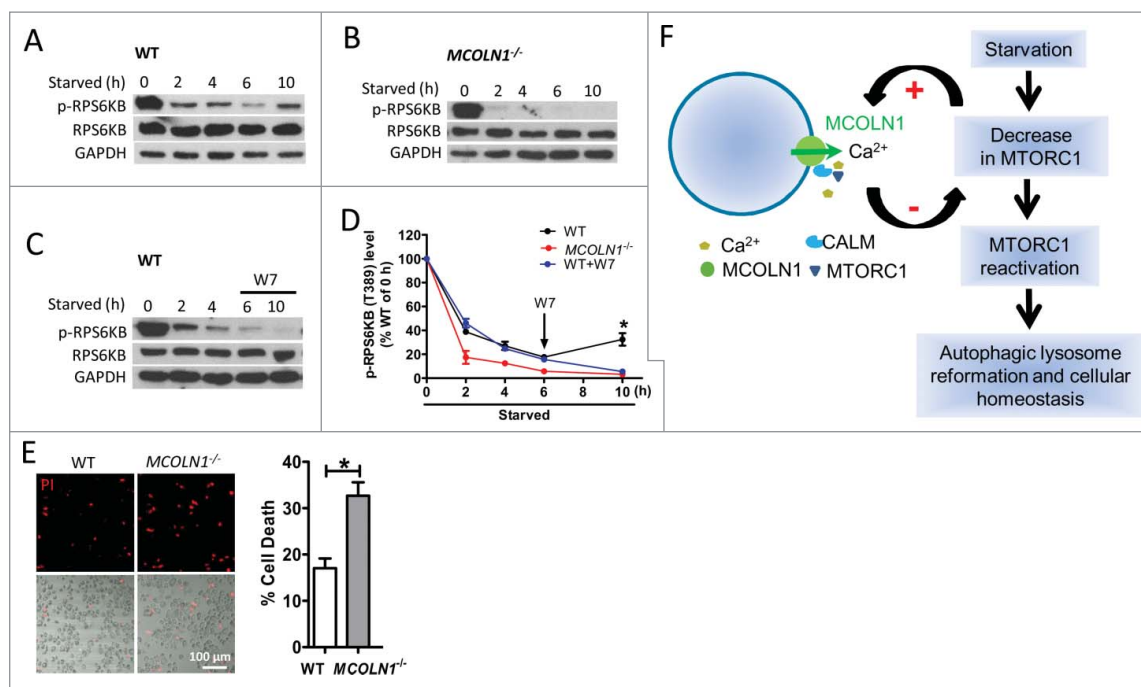


Figure 8. MCOLN1 is required for MTORC1 reactivation during prolonged starvation. (A) MTORC1 reactivation by prolonged starvation. WT human fibroblasts were starved in DMEM medium in the absence of FBS for 2, 4, 6, and 10 h.⁵¹ Cell extracts were analyzed by western blotting using anti-p-RPS6KB (T389) and anti-RPS6KB antibodies, and anti-GAPDH antibody was used as a loading control. (B) Loss of MTORC1 reactivation in *MCOLN1*^{-/-} human fibroblasts. (C) W7 inhibited MTORC1 reactivation in human fibroblasts. W7 (3 μ M) was added in the DMEM medium during the 6 to 10 h of starvation. (D) Quantification of MTORC1 activity by assessing p-RPS6KB using western blot. Histograms represent the mean percentage of the ratio of p-RPS6KB:RPS6KB (mean \pm SEM, $n = 3$ independent experiments) in the indicated conditions, relative to that of fed WT cells. (E) HBSS starvation for 48 h significantly decreased cell viability in *MCOLN1*^{-/-} human skin fibroblasts compared to WT fibroblasts. Cell death was revealed by propidium iodide (PI) staining (mean \pm SEM, $n = 3$ independent experiments). (F) Illustration of a feedback regulation between MTORC1 and MCOLN1 during starvation. Starvation caused a decrease in MTORC1 activity, leading to an increase in MCOLN1-mediated Ca^{2+} release. Ca^{2+} binds to CALM, promoting lysosomal MTORC1 recruitment and reactivation. This feedback regulatory mechanism is essential for autophagic lysosome reformation and cellular homeostasis during starvation. *: $P < 0.05$.

suggesting that they do not affect lysosomal Ca^{2+} content or MCOLN1 expression (Fig. 7F,G).⁴⁵ To directly test whether MTORC1 inhibition activates MCOLN1, GECO-MCOLN1 signals were monitored upon acute AZD8055 application. As shown in Fig. 7H-J, AZD8055 treatment induced ‘transient’ MCOLN1 activation. This is consistent with a previous report that starvation activates MCOLN1.⁴⁷ Altogether, we suggest that starvation continuously activates MCOLN1 to retain MTORC1 on the lysosome and to maintain MTORC1 activity.

Starvation changes cytoplasmic pH,¹⁷ which may affect the GECO-MCOLN1 signals.⁵⁰ To exclude this possibility, we transfected HEK293T cells with G-GECO vectors and compared GECO fluorescence intensity between control and starvation groups. We found that GECO signals were not changed by our starvation conditions (Fig. S10). These data suggest that the ML-SA1 induced GECO-MCOLN1 response was specifically caused by MCOLN1 activation but not other nonspecific effects. It has been reported that MTORC1 phosphorylates human MCOLN1 on Ser572 and Ser576 leading to a decrease in MCOLN1 activity (corresponding to mouse MCOLN1 Ser571 and Ser576).³³ To test if starvation upregulates MCOLN1 via suppressing MTORC1-mediated MCOLN1 inhibition, the lysosomal Ca^{2+} release from the S571,576E phosphomimetic mutation of mouse MCOLN1 was examined. Upon starvation, cells expressing GECO-MCOLN1^{S571,576E} (GECO-MCOLN1-SSEE) exhibited a significant decrease in ML-SA1-mediated Ca^{2+} release compared to cells expressing GECO-MCOLN1 (Fig. 7K), whereas lysosomal Ca^{2+} content

(Fig. 7L) or MCOLN1 expression (Fig. 7M) was not altered. Consistently, an increase in MTORC1 activity induced by MCOLN1 overexpression was diminished by overexpressing MCOLN1-SSEE, but not MCOLN1^{S51E}, a control mutant that does not respond to MTORC1 (Fig. 7N).³³ Additionally, the nonphosphorylatable GECO-MCOLN1^{S571,576A} (GECO-MCOLN1-SSAA) mutation exhibited an increased GECO response compared with GECO-MCOLN1 in normal fed conditions (Fig. 7O-Q). Altogether, these data provide direct evidence that nutrient starvation upregulates MCOLN1-mediated Ca^{2+} release by relieving MTORC1’s inhibition of the channel.

MCOLN1 is essential for MTORC1 reactivation during prolonged starvation

Following starvation-induced autophagy, autolysosomes degrade their contents to regenerate nutrients. Although MTORC1 signaling is inhibited during initiation of autophagy, it is reactivated by degradation products, such as amino acids, during prolonged starvation, which triggers the termination of autophagy and the formation of nascent lysosomes from autophagic membranes through an evolutionarily conserved cellular process called ALR (autophagic lysosome reformation).⁵¹ ALR is critical for maintaining lysosome homeostasis.^{51,52} Given that MCOLN1 is important for MTORC1 activation, we aimed to test whether MCOLN1 plays an essential role in MTORC1 reactivation during ALR. For this purpose, a standard mild starvation protocol for ALR assessment was adopted. We found

that prolonged starvation reactivated MTORC1 in WT (Fig. 8A,D) but not *MCOLN1*^{-/-} human skin fibroblasts (Fig. 8B,D). In agreement with the notion that CALM is downstream of MCOLN1, W7 (3 μ M) inhibited MTORC1 reactivation during prolonged starvation (Fig. 8C,D).

Starvation is an important evolutionarily conserved cellular process for maintaining lysosome homeostasis.^{51,52} Excessive starvation will cause autophagosome accumulation and cell death.^{1,3,7,31} In line with the role of MCOLN1 in MTORC1 activation, loss of MCOLN1 also causes accumulation of autophagosomes²⁷⁻²⁹ and cell death (Fig. 8E) during starvation.^{28,53,54} Therefore, we think that impaired MTORC1 reactivation during starvation may contribute to the pathology of MLIV (Fig. 8).²⁷ Broadly, the MCOLN1-MTORC1 feedback loop may be important for many other LSDs⁵¹ and common neurodegenerative diseases.⁵⁵⁻⁵⁷

DISCUSSION

The kinase MTORC1 is a key regulator of autophagy induction in response to nutrient shortage. Its activation suppresses autophagy, whereas its inhibition promotes autophagy. Because MTORC1 is also an important regulator of protein synthesis, cell growth and cellular homeostasis, continuous loss of MTORC1 activity during nutrient and energy deprivation leads to cell death.^{7,8} Thus, a feedback modulating mechanism must exist to maintain MTORC1 activity during starvation, in particular for prolonged starvation that induces ALR, an important cellular adaptation mechanism critical for lysosome homeostasis and cell survival.^{51,52} However, the molecular mechanism underlying the feedback regulation of MTORC1 is unclear. In this study, we have shown that upregulating MCOLN1 promotes, whereas downregulating MCOLN1 suppresses, lysosomal MTORC1 recruitment and MTORC1 activity during starvation. We have also shown that MCOLN1 regulates MTORC1 through activating CALM, and both MCOLN1 and CALM are required for MTORC1 reactivation during prolonged starvation. In addition, we provide direct evidence that starvation activates MCOLN1 by relieving MTORC1's inhibitory effect on the channel. Thus, MCOLN1 and MTORC1 form a negative feedback loop to maintain sustained MTORC1 activity during nutrient deprivation. MTORC1 inhibition due to starvation activates MCOLN1, which in turn promotes MTORC1 activity through Ca²⁺-mediated activation of CALM (Fig. 8F). This is also supported by a recent publication showing that MCOLN1 but not other lysosomal channels, such as TPCN2 and P2RX4, regulates MTORC1 when cells were cultured for 24 h without changing medium,³⁵ which leads to nutrient shortage (Fig. 3).

If this is true, why did MCOLN1 activate MTORC1 during the 15-min refeeding? We think that the negative feedback loop between MTORC1 and MCOLN1 is still functional during short-term refeeding because MTORC1 activity gradually recovers (Fig. 2A,C). Altogether, our data suggest that under normal conditions when MCOLN1 is inhibited by MTORC1 (Fig. 7 and Fig. S9),³³ MCOLN1 is dispensable for lysosomal MTORC1 recruitment and MTORC1 activity (Fig. 1C,2B,F, Fig. 8 and Fig. S6), whereas under stressful conditions, the

reduction in MTORC1 activity activates MCOLN1-CALM, leading to MTORC1 restoration.

MCOLN1 regulating MTORC1 could also be attributed to nutrient regeneration during starvation because MCOLN1 has been suggested to regulate the fusion of autophagosomes with lysosomes²⁶ and subsequent protein degradation.²⁷⁻²⁹ However, we (Fig. S2A-C) and others²⁵ suggested that upregulation of MCOLN1 did not increase autophagosome and lysosome fusion. We and others³⁵ also found that knocking down MCOLN1 attenuated the activation of MTORC1 by overexpression of constitutively active RRAGB (Fig. 2D,E), which partially mimics amino acid-replete conditions (Fig. S8).^{32,36} We thus suggest that MCOLN1 regulating MTORC1 activity is likely through a direct stimulation of MTORC1 by Ca²⁺-CALM but not nutrient regeneration. This is also in agreement with the finding that Ca²⁺ and CALM activate the kinase activity of the isolated MTORC1 complex in vitro.³⁵ However, we cannot exclude the possibility that during prolonged starvation MCOLN1 may modulate MTORC1 activity through promoting nutrient regeneration.²⁷⁻²⁹

In contrast, previous work has suggested a positive feedback loop formed by MCOLN1 and TFEB during starvation,^{47,48} i.e. starvation activates MCOLN1 leading to TFEB activation through PPP3/calcineurin (protein phosphatase 3), which in turn increases the expression of MCOLN1. Because TFEB is the master transcriptional regulator of lysosomal biogenesis and autophagy,^{58,59} regulation of its activity would be important for efficient autophagy induction and sustained autophagic flux. Notably, activation of this transcriptional pathway is independent of MTORC1.^{47,48} Previous studies have also suggested that starvation causes perinuclear clustering of lysosomes.^{17,24} In this case, activated MCOLN1 interacts with the PDCD6-DCTN/dynactin-dynein complex to drive lysosomes toward the perinuclear area, and then fuse with autophagosomes.¹⁷ Therefore, MCOLN1 is implicated in cellular adaptation to starvation using 3 different mechanisms, i.e. a negative feedback post-translational pathway involving MCOLN1-CALM-MTORC1-MCOLN1, a positive feedback transcriptional pathway involving MCOLN1-PPP3-TFEB-MCOLN1, and a lysosome positioning machinery involved MCOLN1-PDCD6-DCTN/dynactin-dynein. Future studies are needed to elucidate how these pathways work cooperatively to maintain cellular hemostasis in response to nutrient starvation and other types of cellular stresses.

Intracellular Ca²⁺ signaling is implicated in autophagy regulation.^{12,60} In parallel to the endoplasmic reticulum (ER) and the mitochondrion, the lysosome has also been recognized as an intracellular Ca²⁺ store.^{61,62} However, the role of lysosomal Ca²⁺ in MTORC1 has been neglected although MTORC1 needs to be recruited to and activated on the surface of lysosomes.⁶ Although emerging evidence suggests that the lysosomal Ca²⁺ release channel MCOLN1 is involved in autophagy, the specific role of MCOLN1 in MTORC1²⁴⁻²⁶ is controversial. Wong et al.²⁶ suggest that *Drosophila* MCOLN1 is required for MTORC1 activation. Later studies suggest that activating MCOLN1 does not change MTORC1 activity.^{24,25} Most recently, Li et al.³⁵ suggest that MCOLN1 plays an important role in regulating MTORC1. We have now resolved this controversy by clarifying that MCOLN1 regulation of MTORC1

occurs specifically under stressful conditions. Because of the similarities between *Drosophila* and mammalian MCOLNs, we think that MCOLN1 regulating MTORC1 signaling is evolutionarily conserved.

In summary, we have further demonstrated that MCOLN1-mediated lysosomal Ca^{2+} signaling as an essential component in the canonical MTORC1 pathway. MCOLN1 and MTORC1 form a negative feedback loop to maintain cellular hemostasis during starvation. This feedback regulation of MTORC1 may provide an essential adaptation process that aids cellular survival under extreme conditions.^{7,8} MTORC1 and autophagy are associated with many physiological and pathological processes such as sustained exercise, development, differentiation, LSDs, neurodegenerative diseases, cancer, inflammatory, infectious and autoimmune conditions.^{1-5,63} By promoting MTORC1 reactivation and maintaining cellular homeostasis, the MCOLN1-MTORC1 feedback regulatory mechanism may play an important role in these conditions. In particular, defective ALR blocks the termination of autophagy,^{51,52} thereby leading to impaired autophagic flux^{56,57,64} and cell death (Fig. 8E).^{51,55-57,65} Upregulating the MCOLN1-CALM-MTORC1 pathway may represent a potential therapeutic strategy for LSD patients and classical neurodegenerative diseases that are characterized by aberrant ALR.^{51,55}

Materials and methods

Cell culture

HEK293T cells (ATCC, CRL-3216TM) and HeLa cells (ATCC, CRL-3216TM) were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific, 11995065) supplemented with 10% FBS (Thermo Fisher Scientific, 12483020). Human skin *MCOLN1*^{-/-} fibroblasts (Coriell Institute for Medical Research, GM02629) from MLIV patients and WT nondisease fibroblasts (Coriell Institute for Medical Research, GM00969) were maintained in DMEM supplemented with 15% non-heat-inactivated FBS. HEK293T cells were transiently transfected using Lipofectamine 3000 (Thermo Fisher Scientific, L3000015) according to the manufacturer's manual with the indicated plasmids for further experiments. For taking images, cells were seeded on 0.01% poly-L-lysine (Sigma-Aldrich, P4832)-coated glass coverslips and cultured for 24 h before further experiments. In this study, a mild starvation condition HBSS (Thermo Fisher Scientific, 14170112) + 1% FBS¹⁷⁻²³ was adopted to purposely mimic the physiological condition unless specifically indicated, and DMEM + 10% FBS was used as refeeding.

Antibodies and reagents

The following primary antibodies were used for immunofluorescence staining and western blotting: anti-LAMP1 (Developmental Studies Hybridoma Bank, H4A3); anti-CALM/calmodulin (Abcam, ab45689); anti-FLAG (Thermo Fisher Scientific, MA1-91878); anti-GFP (GF28R; Thermo Fisher Scientific, MA5-15256); anti-MTOR (7C10; Cell Signaling Technology, 2983); anti-p-RPS6KB (T389; Cell Signaling

Technology, 9206); anti-RPS6KB (Cell Signaling Technology, 9202); anti-p-EIF4EBP1 (T37/46; Cell Signaling Technology, 2855); anti-EIF4EBP1 (Cell Signaling Technology, 9644); anti-PDCD6/ALG2 (Thermo Fisher Scientific, PIPA 527639); and anti-GAPDH (H-12; Santa Cruz Biotechnology, SC-166574). Secondary antibodies include goat-anti-mouse IgG-HRP (Santa Cruz Biotechnology, sc-2005); goat-anti-rabbit IgG-HRP (Thermo Fisher Scientific, PI31460); Alexa Fluor 488 goat anti-mouse antibodies (Thermo Fisher Scientific, A-11001); and Alexa Fluor 594 goat anti-rabbit antibodies (Thermo Fisher Scientific, A-11012). Chemical compounds: ML-SA1 (Tocris Bioscience, 4746); BAPTA-AM (Tocris Bioscience, 2787/25); GPN (Santa Cruz Biotechnology, sc-252858); ionomycin (Cayman Chemical, 11932-1); ML-SII (Enzo Life Sciences Inc, BML-CR112-0010); W-7 (Sigma-Aldrich, A3281); AZD8055 (Sigma-Aldrich, A-2345).

Molecular biology

GECO-MCOLN1, LAMP1-GFP, MCOLN1-GFP and MCOLN1-DDKK-GFP, CALM-MYC-HIS, MTOR-FLAG (FG4R), SYT7-DN, MCOLN1-HA were home-made or employed as described previously.^{46,66} The RRAGB^{WT} (19313, David Sabatini), RRAGB^{GDP} (T54L, 19314, David Sabatini), RRAGB^{GTP} (Q99L, 19315, David Sabatini), psPAX2 (12260, Didier Trono), pMD2.G (12259, Didier Trono) and mRFP-EGFP-LC3 (21074, Tamotsu Yoshimori) clones were purchased from Addgene. Knockout of *PDCD6* using the CRISPR-Cas9 system was carried out as previously reported.⁴⁶ Specific and efficient RNAi-mediated knockdown of endogenous human *MCOLN1* expression in HEK293T cells was performed as previously described.^{15,67,68} Oligonucleotide 5'-GCTACCTGACCTTCTCCACA-3'¹⁵ was cloned into the shRNA expression vector pLKO.1 (Addgene, 8453; deposited by Dr. Bob Weinberg). GECO-MCOLN1^{S571,576E} mutant and GECO-MCOLN1^{S571,576A} mutant were constructed using a site-directed mutagenesis kit (Thermo Fisher Scientific, A14604).

Immunoprecipitation and western blot

Cell lysates were incubated with 80 μl 50% protein A/G-agarose beads (Santa Cruz Biotechnology, sc-2003) in phosphate-buffered saline (PBS; 144.0 mg/L KH_2PO_4 , 9000.0 mg/L NaCl and 795.0 mg/L $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$) for 15 min at 4°C to reduce background proteins that nonspecifically bound to the beads. After centrifugation at 12,000 \times g for 15 min to remove the beads, aliquots of cell lysates were incubated with the desired antibodies or control IgG at 4°C overnight in a final volume of 1 ml RIPA-PBS buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM Na_2 EDTA, 1 mM EGTA, 1% NP-40 [Sigma-Aldrich, 74385], 1% sodium deoxycholate [Sigma-Aldrich, S1287], 2.5 mM sodium pyrophosphate [Sigma-Aldrich, 221368], 1 mM b-glycerophosphate [Cayman Chemical, 14405], 1 mM Na_3VO_4 [Sigma-Aldrich, 450243], 1 $\mu\text{g}/\text{ml}$ leupeptin [Sigma-Aldrich, L2884]) with constant rocking. After antibody incubation, protein A/G-agarose beads were added and the samples were incubated at 4°C for 4 h, followed by centrifugation at 300 g for 10 min at 4°C. The beads were then

washed 3 times with pre-cooled RIPA buffer without proteinase inhibitors and each time centrifuged at 300 g for 10 min at 4°C. Immune complexes were resolved by SDS-PAGE and subjected to immunoblotting. Proteins were analyzed by standard western blot analysis methods. Proteins were resolved on 10% SDS-PAGE and transferred onto PVDF membranes (Bio-Rad, 164-0177). Nonspecific binding was blocked using 5% skim milk in TBS-T (0.1% Tween-20 [Fermentas, BP337500] in 2420.0 mg/L Tris-HCl, 8000.0 mg/L NaCl, pH 7.4) for 2 h at room temperature. Membranes were then incubated with specific primary antibody solution at 4°C overnight with gentle constant shaking. After 3 thorough TBS-T washes, the membranes were incubated with corresponding HRP-conjugated secondary antibody at room temperature for 1 h. Immunoreactive bands were visualized using Clear ECL (Bio-Rad, 170-5060) and autoradiography.

Lentivirus production and cell transduction

HEK293T cells were seeded in 6-cm dishes. When 50–70% confluent, the cells were cotransfected with 4 µg lentiviral vector pLKO.1 (Addgene, 8453), 3 µg psPAX2 (Addgene, 12260) and 1 µg pMD2.G (Addgene, 12259) using Lipofectamine 3000 according to the manufacturer's instructions. The supernatants were harvested at 24, 48 and 72 h, centrifuged at 300 g for 10 min, and then passed through 0.45-µm filters. The concentrated virus was stored at –80°C.

HEK293T cells were seeded in 10-cm culture dishes. When cells reached 40% confluence, lentiviral conditional medium was added. After 48 h, cells were treated with 1 µg/ml puromycin (Thermo Fisher Scientific, A11138-03) for 3 d to select transduced cells, and then *MCOLN1* shRNA expression was induced by 100 ng/ml doxycycline (Sigma-Aldrich, D9891) for at least 48 h.

Real-time qPCR

HEK293T cells were transduced with lentivirus carrying scramble shRNA or *MCOLN1* shRNA. After total RNA was extracted, cDNA was generated with an iScriptTM Advanced cDNA Synthesis Kit (Bio-Rad, 1725037), and real-time qPCR was carried out using GoTaq[®] qPCR Master Mix (Promega Corporation, A6001). Real-time PCR primers were from Thermo Fisher Scientific, the primer sequence (5'-3') is as follows: human *MCOLN1* (F-CGGATGACACCTTCGCAGCCTAC, R-CGGATGACACCTTCGCAGCCTA C); *GAPDH* (F-GAAGGTGAAGGTCGGAGT C, R-GAAGATGGTGGATGGATTTC). *GAPDH* was used as a control.

Confocal microscopy

Confocal fluorescence images were taken using an inverted Zeiss LSM510 Axiovert200M confocal microscope with a 40 × or 63 × oil-immersion objective. Sequential excitation wavelengths at 488 nm and 543 nm were provided by argon and helium-neon gas lasers, respectively. Emission filters BP500-550 and LP560 were used for collecting green and red images in channels 1 and 2, respectively. After sequential excitation, green and red fluorescent images of the same cell were saved

with ZEN2012 software. Images were analyzed by Zeiss software. Intensity of fluorescence was analyzed using ImageJ. The image size was set at 1,024 × 1,024 pixels.

Immunocytochemistry

Cells grown on coverslips were washed with PBS twice and fixed in 4% paraformaldehyde (Sigma-Aldrich, P6148) in PBS for 15 min at room temperature. Fixed cells were permeabilized with 0.1% Triton X-100 (Sigma-Aldrich, T8787) in PBS for 5 min and then blocked with 3% bovine serum albumin (Thermo Fisher Scientific, 3600500) in PBS for 60 min at room temperature. After 3 PBS washes, cells were incubated with primary antibodies at 4°C overnight. After 3 more PBS washes, cells were incubated with fluorescence-conjugated secondary antibodies for 45 min at room temperature in the dark. Images were acquired using a confocal microscope (LSM510, Zeiss; USA) with a 63X oil-immersion objective lens and captured using ZEN2012 software.

GECO Ca²⁺ imaging

HEK293T cells transfected with the indicated constructs were trypsinized and plated onto glass coverslips for 4–6 h prior to experiments. The fluorescence intensity at 470 nm (F_{470}) was monitored using the EasyRatioPro system. Lysosomal Ca²⁺ release was measured under a 'low' external Ca²⁺ solution, which contained 145 mM NaCl, 5 mM KCl, 3 mM MgCl₂, 10 mM glucose, 1 mM EGTA, 20 mM HEPES, pH 7.4). Ca²⁺ concentration in the nominally free Ca²⁺ solution is estimated to be 1–10 µM. With 1 mM EGTA, the free Ca²⁺ concentration is estimated to be < 10 nM based on the Maxchelator software (<http://maxchelator.stanford.edu/>). For measuring control or refed, but not starved or AZD8055-treated cells, 1 × amino acid mix was added to external solutions during the measuring.

Analysis of cell death

Cell death was analyzed by measuring the permeability of the plasma membrane to propidium iodide (PI; Sigma-Aldrich, P4170). After the indicated treatment, cells were collected and suspended in PBS. Cells were then stained with 2 µg/ml PI for 5–10 min at room temperature, and a 10-µl suspension was applied onto a microscope slide, covered with a cover slide and viewed immediately under a fluorescence microscope.

Data analysis

Quantification of western blot and confocal image was carried out using ImageJ software. For the analysis of colocalization between MTOR and LAMP1, a constant threshold was applied to all images, followed by application of the colocalization plug-in of ImageJ software. All channels were then projected (max) and quantified using Analyze particle plugin (particles 5 pixels and above were included).⁶⁹ Due to the high density of lysosomes in human fibroblasts, we used particles' area instead of particles' number to calculate the colocalization. The data were expressed as the percentage of MTOR area that colocalized with LAMP1 area. Data are presented as mean ± SEM

from at least 3 independent experiments. Statistical comparisons were made using analysis of variance (ANOVA) and Student *t* test. *P* values of < 0.05 were considered statistically significant. *, *P* < 0.05; **, *P* < 0.01.

Abbreviations

ALR	autophagic lysosome reformation
CALM	calmodulin
co-IP	co-immunoprecipitation
ER	endoplasmic reticulum
GPN	glycyl-phenylalanine 2-naphthylamide
LAMP1	lysosomal associated membrane protein 1
LSD	lysosomal storage disease
MTORC1	mechanistic target of rapamycin complex 1
PDCD6/ALG2	programmed cell death 6
PI	propidium iodide
PPP3/calcineurin	protein phosphatase 3
SYT7	synaptotagmin 7
SYT7-DN	dominant negative mutant SYT7
TFEB	transcription factor EB
V-ATPase	vacuolar-type H ⁺ -translocating ATPase
WT	wild type

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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