

Reconstitution of leucine-mediated autophagy via the mTORC1-Barkor pathway in vitro

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Supplementation of branched chain amino acids, especially leucine, is critical to improve malnutrition by regulating protein synthesis and degradation. Emerging evidence has linked leucine deprivation induced protein breakdown to autophagy. In this study, we aimed to establish a cell-free assay recapitulating leucine-mediated autophagy in vitro and dissect its biochemical requirement. We found that in a cell-free assay, membrane association of Barkor/Atg14(L), a specific autophagosome-binding protein, is suppressed by cytosol from nutrient-rich medium and such suppression is released by nutrient deprivation. We also showed that rapamycin could efficiently reverse the suppression of nutrient rich cytosol, suggesting an essential role of mTORC1 in autophagy inhibition in this cell free system. Furthermore, we demonstrated that leucine supplementation in the cultured cells blocks Barkor puncta formation and autophagy activity. Hence, we establish a novel cell-free assay recapitulating leucine-mediated autophagy inhibition in an mTORC1-dependent manner; this assay will help us to dissect the regulation of amino acids in autophagy and related human metabolic diseases.

Introduction

Amino acids are required for activation of the mammalian target of rapamycin (mTOR) kinase, which is an atypical serine/threonine kinase that plays an indispensable role in the control of protein metabolism, cell growth, and autophagy.^{1–3} When complexed with the interacting proteins raptor (for regulatory associated protein of mTOR) and GβL (also termed mLST8) in the mammalian target of rapamycin complex 1 (mTORC1), mTOR serves as an integrator of cellular signals to control the balance between cellular anabolism and cellular catabolism.³ mTORC1 serves as an integration hub for a variety of upstream growth factor, nutrient, and stress signals and modulates a variety of anabolic (e.g., protein biosynthesis) and catabolic (e.g., autophagy) processes to adapt to the cellular environment.^{4,5} Biochemical approaches in mammalian cells showed that GTP-loaded Ras homolog enriched in brain (Rheb) stimulates mTORC1 activity both in vitro and in vivo, while GAP activity of the TSC1/TSC2 complex results in GTP hydrolysis and Rheb inhibition.^{6–9} The mTORC1 pathway is a major contributor to the anabolic response following essential amino acids (EAA) or leucine ingestion, although multiple pathways are involved.¹⁰

Cells adapt to changes in their environment by adjusting anabolic and catabolic pathways.¹¹ In times of nutrient shortage,

for example, macromolecules are degraded to produce substrates for energy production.¹ Central among the responses to nutrient deprivation is autophagy, which is characterized by formation of double-membrane vesicles (autophagosomes) that capture intracellular cargoes and deliver them to lysosomes for degradation. Autophagy plays an important role in a variety of human diseases and intracellular nutrient recycling.^{12–14} One of the most important upstream regulators of autophagy is the protein mTOR.^{2,4,15} When extracellular amino acids are limiting, autophagy recycles intracellular constituents as a way to provide an alternative source of amino acids.¹⁶ Several recent studies demonstrated that mTORC1 suppresses autophagy through regulation of the ULK1-Atg13-FIP200 complex.^{17–19}

The signaling pathway responsible for leucine-mediated autophagic response is intensively studied. It has been suggested that the class III phosphatidylinositol 3-kinase (PtdIns3K) might be a major downstream effect in amino acid deprivation-induced autophagy.¹ PtdIns3K plays a critical role in autophagy and endocytosis.^{20–25} Its specific regulation in autophagy relies on an autophagic-specific adaptor protein Barkor, also known as Atg14 or Atg14L. Barkor serves as a targeting factor for PtdIns3K recruitment to autophagosomes. Barkor is essential for autophagosome formation and its recruitment to early autophagosome structures,^{20–23} which is considered as a necessary step in

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catastrophic assembly of protein complexes on autophagosome for its nucleation, elongation and completion. Barkor directly binds to autophagic membranes through its C-terminal BATS domain and senses membrane curvature in a phosphatidylinositol 3-phosphate (PtdIns(3)P)-dependent manner.²⁶

Given the specific role of Barkor in autophagy activation, its association with autophagic membrane could serve as readout to dissect the signaling pathway regulated by leucine. To test this possibility, we established an *in vitro* cell-free assay for Barkor membrane association. Importantly, Barkor membrane association is negatively regulated by an inhibitory activity present in the nutrient-rich cell extracts, and this inhibition could be reversed by the mTORC1 inhibitor rapamycin. We further dissect the requirement of mTOR subunits in leucine-mediated autophagy inhibition. This study therefore describes the first cell-free assay recapitulating the leucine effect on autophagy activation.

Results

Reconstitution of leucine effect on Barkor membrane association in a cell-free system. Upon autophagy initiation, PtdIns3K is recruited by Barkor to the phagophore, the early autophagosome structure.²⁰⁻²³ This relocation ensures the nucleation of the initial membrane and promotes membrane extension by attracting downstream protein complexes to autophagosomes. We aim to design an *in vitro* assay utilizing the Barkor autophagic membrane association as readout to study the leucine effect on autophagy in mammalian cells.

For this purpose, the recombinant full-length Flag-tagged Barkor was purified from baculovirus-infected insect cells (Fig. 1A). Rubicon, another PtdIns3K subunit that predominantly localizes to endosomal structures,²²⁻²⁵ was also purified and used as a control in this assay. To prepare the autophagic membrane structures, we first tested a biochemical fractionation assay to isolate the autophagosome-enriched membrane. U₂OS cells were treated with either lysosomal protease inhibitors E64D and Pepstatin A to block lysosomal degradation, or MG132 to prevent proteasomal degradation. Cells were lysed by hypotonic buffer and homogenization, and separated into cytosolic (supernatant) and nuclear/organelles (pellet) fractions by centrifugation. As shown in Figure 1B, the free form of LC3 was detected in the cytosol (Fig. 1B, lane 1), and LC3-PE conjugated form was only detected in the pellet fraction of cells treated with lysosome inhibitor (Fig. 1B, lane 3). MG132 treatment led to a minor accumulation of LC3-II since proteasome inhibition could lead to moderate activation of autophagy (Fig. 1B, lane 2). This result confirms that autophagosome membranes are enriched in the pellet fraction. Hence, the pellet fraction could serve as a source for autophagosome enriched membrane in the *in vitro* assay.

We then examined the *in vitro* membrane association of Barkor and Rubicon in a co-sedimentation assay. We incubated purified recombinant Barkor and Rubicon with membrane pellets isolated from unstressed or starved HeLa cells. The association of Barkor to membrane from starved cells is stronger than that from unstressed cells (Fig. 1C, lane 3 compared with lane 1), suggesting

that Barkor membrane association is starvation induced. In contrast, endosome-localized Rubicon membrane association is not altered (Fig. 1C, lanes 2 and 4). This result suggests that Barkor membrane association *in vitro* correlates with its stress-inducible autophagosome targeting *in vivo*. In this experiment, endogenous LC3 lipidation form (LC3-II) was used as a loading control for equal amounts of autophagosomes.

We further tested whether cytosolic factors can regulate Barkor membrane association *in vitro*. In the reactions with recombinant Barkor and the membrane fractions from starved HeLa cells, S-100 (membrane-free cytosolic fraction after 100,000 g ultracentrifugation) collected from nutrient-rich [S100(+)] or nutrient-depleted [S100(-)] HeLa cells was added. Nutrient-rich S-100 could efficiently block the binding of Barkor to autophagosomes in a dose-dependent manner (Fig. 1D, lanes 4–7). The inhibitory activity was significantly reduced when S100 was prepared from starved HeLa cells (Fig. 1D, lanes 11–14). This result indicates that the inhibitory activity of S100 is robust in the cell extract from nutrient-rich HeLa cells but much weaker in the cell extract from nutrient-depleted HeLa cells.

Since inhibition of mTORC1 promotes autophagosome formation *in vivo*, we speculated the inhibitory activity in S-100 might arise from the mTORC1 signaling pathway. In the *in vitro* cell-free assay, we incubated the reaction mixture with the small-molecule mTORC1 inhibitor rapamycin. Rapamycin efficiently reversed the inhibitory effect of S-100 from unstressed HeLa cells on Barkor membrane association (Fig. 1E, lane 6 compared with lane 4). In contrast, rapamycin had little effect on S-100 from starved HeLa cells (Fig. 1E, lane 7 compared with lane 5). Hence mTORC1 is probably the major stress-adjustable inhibitor of Barkor membrane association in the unstressed S-100.

To mimic the leucine inhibition on autophagy, we supplemented starved S-100 with different concentration of leucine in the cell-free assay. In the presence of S-100 from starved HeLa cells, Barkor membrane association activity was robust, which could be efficiently blocked by leucine addition (Fig. 1E, lanes 8 and 9). The inhibitory effect is also mediated through mTORC1, since rapamycin could reverse the leucine inhibition (Fig. 1E, lanes 10 and 11). Without S-100, leucine supplementation alone failed to inhibit Barkor membrane association (Fig. 1E, lanes 12 and 13). Not all amino acids have shown a strong inhibition on mTOR signaling pathway and autophagy. Leucine is the most robust one, whereas cysteine barely has any effect. We further investigated if cysteine could inhibit Barkor membrane association as efficient as leucine *in vitro*. At the same concentration that leucine inhibits Barkor membrane association (Fig. 1F, lanes 3 and 4), cysteine had no inhibitory effect (Fig. 1F, lanes 5 and 6), indicating that the inhibition is leucine-specific. These results therefore recapitulate the *in vivo* requirement of leucine inhibition in a cell-free system, and delineate a cellular pathway from mTORC1 to Barkor for signal transduction.

Since the membrane fraction might also contain membranes other than autophagic membranes, we examined the nonautophagic membrane binding of Barkor. In Atg5 knockout (KO) MEFs, no autophagosome is formed.^{27,28} Barkor association with the membrane fraction from Atg5 KO cells is much less than that

mTOR is the major inhibitor present in cytosolic extract for blocking Barkor membrane association.

Leucine deprivation induces autophagy in HEK 293T cells. To determine whether leucine deprivation induces autophagy, we generated a HEK 293T cell line stably expressing EGFP-LC3. LC3 serves as an immunohistological marker for the autophagosome.^{27,28} EGFP-LC3 expressing cells were cultured in the medium without leucine for different time periods. Initially, EGFP-LC3 displayed mostly diffused cytoplasmic staining indicating a very low level of basic autophagy (Fig. 2A). Upon leucine deprivation, the average number of LC3 puncta per cell representing the magnitude of *in vivo* autophagy activity was significantly augmented in a time-dependent manner, peaking at 4 h upon leucine deprivation (Fig. 2A and B). Consistently, the conversion of LC3-I to LC3-II was also increased (Fig. 2C and D). In addition, the cellular level of p62, an autophagy substrate,^{27,28} was reduced upon leucine deprivation (Fig. 2C and E). In summary, leucine deprivation elicits substantial autophagy activation in HEK 293T cells.

Leucine supplementation suppresses autophagy induced by leucine deprivation. We further tested if leucine supplementation inhibits autophagy. We used a human osteosarcoma U₂OS cell line stably expressing Myc-LC3 to perform a leucine-deprivation and re-supplementation assay. Consistent with the previous observations in HEK293T cells, leucine deprivation also dramatically increased the number of autophagic vacuoles decorated by LC3 in U₂OS cells (Fig. 3A and B). Re-supplementation with different concentration of leucine repressed autophagy activity in a dose-dependent manner (Fig. 3A and B).

To validate the autophagy inhibition by leucine supplementation, we examined the LC3-II and p62 level in U₂OS cells deprived and re-supplemented with leucine. As shown in Figure 3C, leucine deprivation significantly increased the level of LC3-II, which was accompanied with p62 reduction (Fig. 3C).

Consistently, 10 mM leucine supplementation restored the autophagy activity to basal levels (Fig. 3C). As expected, rapamycin potently reversed the leucine supplementation effect, proving that rapamycin-sensitive mTORC1 activation is required for leucine-mediated autophagy inhibition (Fig. 3C).

To ensure that mTOR activity is essential for leucine inhibition, we examine the phosphorylation of S6K, one of the mTOR physiological substrates.²⁹ As expected, S6K phosphorylation was dramatically suppressed by leucine deprivation and restored by leucine replenishment (Fig. 3D).

Leucine supplementation can suppress Barkor autophagosome targeting *in vivo*. Since Barkor autophagosome targeting is an essential step in autophagosome formation,²⁶ we investigated if leucine deprivation and supplementation affect this step. We examined the subcellular localization of Barkor in the absence or presence of leucine. The percentage of U₂OS cells with Barkor puncta and puncta per cell were dramatically increased after 6 h incubation with leucine-free medium (Fig. 4). As a positive control, the mTORC1 inhibitor rapamycin also stimulated the Barkor puncta formation. Leucine supplementation (3 mM) dramatically decreased Barkor puncta (Fig. 4). These data indicate that Barkor autophagosome targeting is induced by leucine deprivation and suppressed by leucine supplementation *in vivo*.

Requirement of mTOR adaptors in leucine-mediated autophagy inhibition. The rapamycin-sensitive mTOR activity is regulated by several adaptors, including Rheb, Raptor and GβL, through distinct mechanisms. To determine the roles of these adaptors in leucine regulation, we depleted the individual adaptors in HEK 293T cells by RNA interference (Fig. 5). mTOR activity was compromised but still detectable in the unstressed cells depleted with Rheb, Raptor and GβL (Fig. 5 and data not show). The small GTPase Rheb is the key downstream effector of TCS1/2 in controlling mTORC1 activity, and Raptor is an essential scaffold for the mTOR kinase activity *in vivo*.²⁹ In Rheb

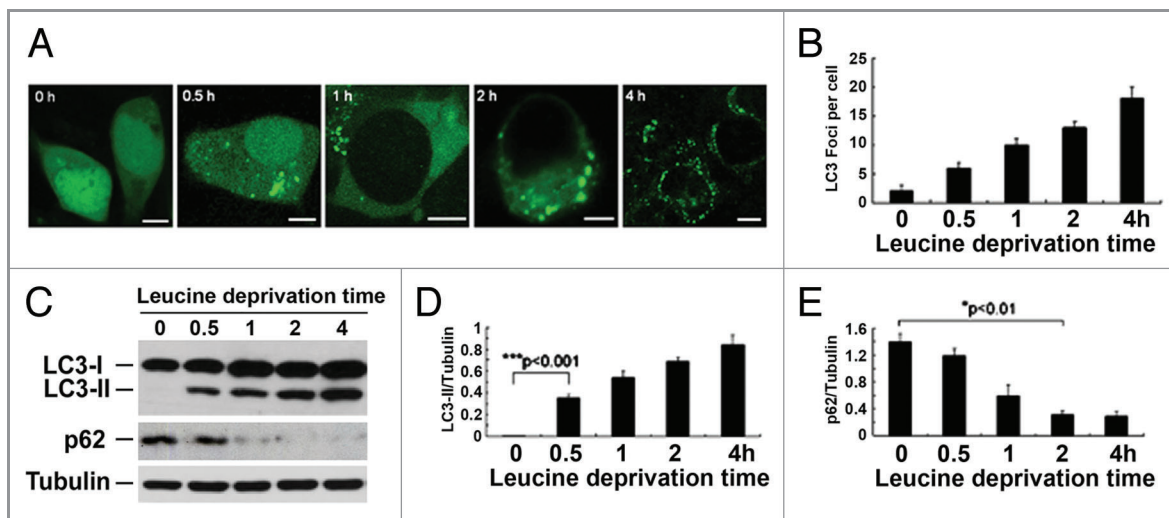


Figure 2. Leucine deprivation activates autophagy. (A) Fluorescent EGFP-LC3 detected in transfected HEK 293T cells upon incubation in the leucine-free medium for 0 h, 0.5 h, 1 h, 2 h and 4 h, respectively. (B) Quantification of EGFP-LC3 dots per cell as described in (A). (C) Western blot of LC3, p62 and tubulin in HEK 293T cells treated by the leucine-free medium as indicated. (D and E) Quantification of LC3-II/tubulin and p62/tubulin as described in (C). Data are means \pm SEM for at least three different experiments.

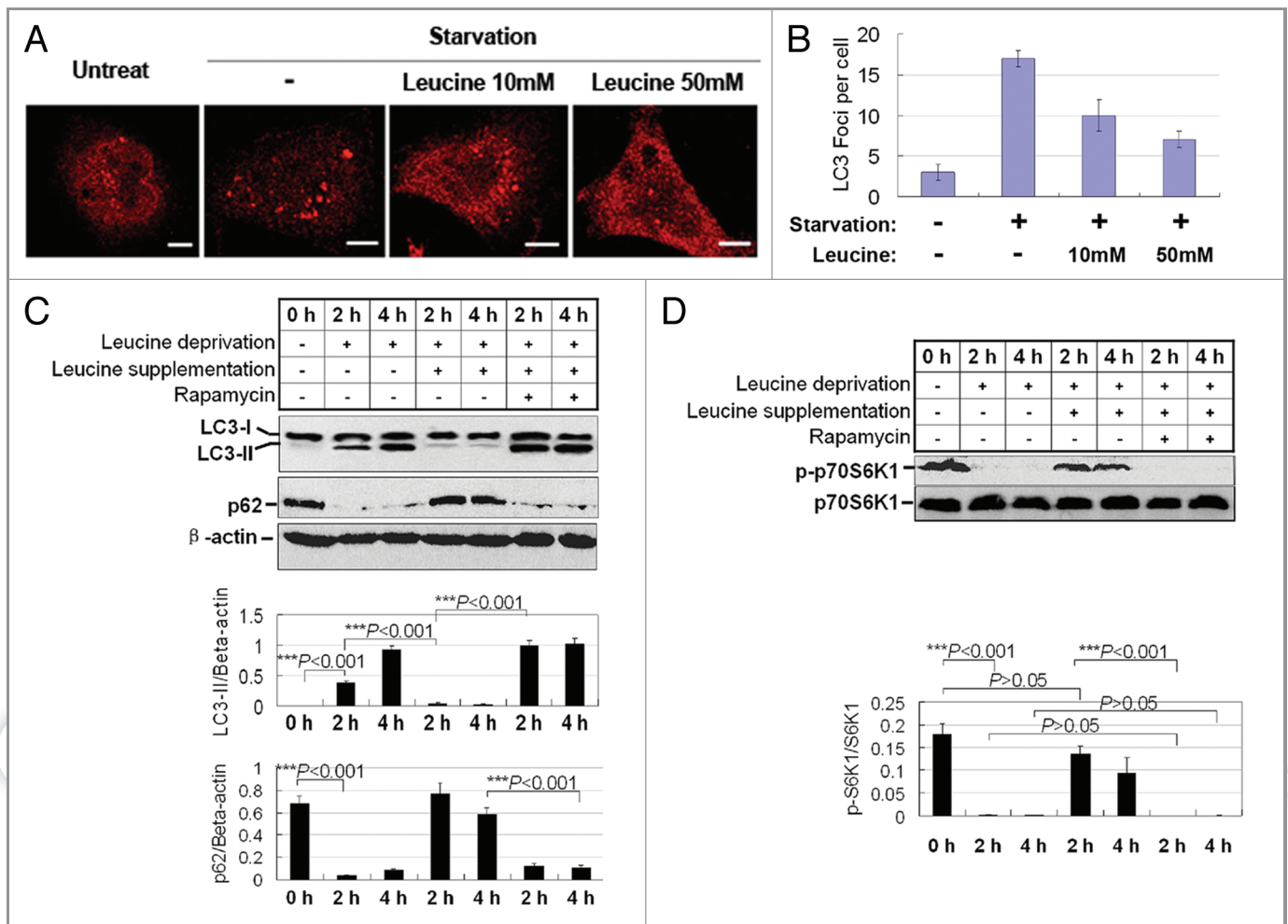


Figure 3. Leucine supplementation inhibits autophagy via the mTORC1 pathway. (A) Leucine supplementation blocks autophagosome formation. U₂OS cells were treated with leucine-free medium alone or different doses of leucine (10 mM and 50 mM). Myc-LC3 was detected by anti-Myc antibody. (B) Autophagosomes marked by Myc-LC3 described in (A) were counted under a fluorescence microscope (Data are means ± SEM of 20 cells). (C) Leucine supplementation inhibits autophagy. Leucine was added to the leucine-free medium with or without rapamycin (2 μM) for 2 h and 4 h in HEK 293T cells. LC3, p62 and β-actin (used as loading control) protein levels were assessed in HEK 293T cells by western blot (top). Quantification of LC3-II/tubulin and p62/tubulin is shown as the relative expression levels (bottom). Data are means ± SEM for at least three different experiments. (D). Leucine supplementation inhibits mTOR activity. Cells with the same treatments as described in (C) are probed with phosphorylated p70S6K (p-p70S6K), p70S6K, and β-actin (top), and the quantification of p-p70S6K/ p70S6K is plotted (bottom).

(Fig. 5A) or Raptor depleted cells (Fig. 5D), autophagy activity, presented as LC3-II conversion and p62 degradation, were strongly stimulated by leucine deprivation, but leucine supplementation failed to inhibit autophagy activity (Fig. 5B, C, E and F). Interestingly, depletion of another positive regulator of the mTOR signaling complex, GβL,³⁰ still respond to leucine deprivation and supplementation (Fig. 5G–I). In the cells treated with a control siRNA, autophagy and mTOR activity is reversibly controlled by leucine deprivation and supplementation (Fig. 5J–L).

Discussion

In this study, we demonstrate that leucine regulates Barkor membrane association, an essential event in autophagosome biogenesis. Recently, Barkor/Atg14(L) was identified as an autophagy-specific subunit of the class III PI3-kinase complex

in mammalian cells by four research groups including us.^{20–23} In this study, we demonstrate that Barkor membrane association is regulated by an inhibitory activity in the unstressed cytosol but not in the starved cytosol. Since this activity could be reversed by rapamycin, mTOR is very likely the responsible inhibitor. In the starved cells with low mTOR activity, leucine supplementation could stimulate mTOR activity and inhibit Barkor membrane association in the cell free system. This data therefore provide the direct evidence that leucine controls autophagy through a mTOR-Barkor pathway. Reconstitution of leucine effect on autophagy in the cell-free system provides an effective system to further dissect the signaling pathway regulated by leucine.

Although the mTOR activity is an essential part of the leucine-mediated signaling pathway, interestingly, we have observed different response to nutrients in the cells depleted with different mTOR adaptors. Leucine deprivation in the Rheb or Raptor

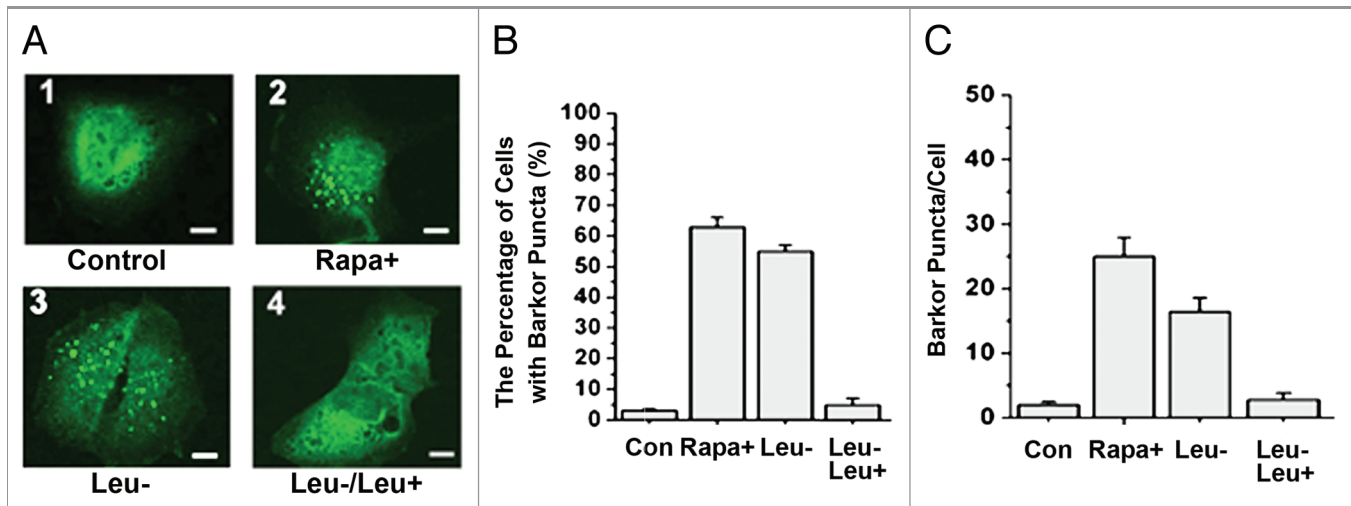


Figure 4. Leucine regulates Barkor puncta formation in vivo. (A) For these treatments, U₂OS cells were transfected with pCDNA5-GFP-Barkor. Twenty-four hour later, cells were subjected to nutrient-rich medium for 6 h [Control, (1)], nutrient-rich + 250 nM rapamycin for 6 h (2), leucine-free medium for 6 h (3), leucine-free medium + 3 mM leucine for 6 h (4), and then were fixed using 3.5% paraformaldehyde and observed by confocal microscope. (B) Quantification of the percentage of cells with Barkor puncta (%) in cells described in (A). (C) Quantification of Barkor puncta per cell in cells described in (A).

deficient cells strongly induces autophagy and mTOR inactivation that cannot be reversed by leucine supplementation, whereas in GβL depleted cell both autophagy and mTOR activity is sensitive to leucine deprivation and supplementation. It is possible that the residual GβL might still sustain some function, despite that GβL protein level is significantly reduced in siRNA treated cells. Nevertheless, this observation is coincident with the nutrient-sensitive association of Raptor-mTOR and nutrient-insensitive interaction of GβL-mTOR.³⁰ It is also likely that GβL has Rheb and Raptor-independent functions. Knockout of GβL/mLST8 is accompanied by a selective loss of mTORC2 function, whereas mTORC1 function appears to be preserved.³¹ In addition to these mTORC1 adaptors, it will be interesting to explore the contribution of Ulk1 and Atg13 in our cell-free assays, considering the substantial functional and physical interaction among these molecules. The detailed mechanism will be further explored in the future study. It is probable that unknown mechanism(s) is also involved in this regulation.

In summary, our data show that leucine inhibits autophagy via mTOR-Barkor signaling pathway. Investigating how autophagosome formation is initiated or inhibited, and proceeded remains difficult. The findings presented here provide an insight into the role of leucine in regulating mammalian autophagic activity and will serve as a resource for further mechanistic analysis of intracellular amino acids recycling and metabolism so critical for protein homeostasis.

Materials and Methods

Reagents and Antibodies. Rapamycin (R8781) and leucine (L8912) were purchased from Sigma Aldrich. Rabbit anti-Phospho-S6K1 (Thr³⁸⁹) (9234), rabbit anti-S6K1 (2708), rabbit anti-Rheb (4935), rabbit anti-Raptor (2280), rabbit anti-GβL (3274), rabbit anti-Flag (2044), rabbit anti-p62 (5114) antibodies,

rabbit anti-β-tubulin (5346), mouse monoclonal anti-β-actin antibodies (3700) and mTOR (2983) were obtained from Cell Signaling Technology. Rabbit anti-LC3 (L7543) was obtained from Sigma Aldrich.

Cell culture, cell transfection and cell lysate preparation. HEK 293T and U₂OS cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma Aldrich, D9443) containing 10% fetal bovine serum (Hyclone, 30396) and 1% penicillin-streptomycin (Invitrogen, 15070) in 5% CO₂ at 37°C. For leucine-deprivation assay, HEK 293T and U₂OS cells were cultured in DMEM-low glucose without L-leucine medium (MP Biomedicals, 1642149) supplemented with 10% dialysis FBS (Sigma, 12105C). Cell transfection was performed with Lipofectin 2000 (Invitrogen, 11668) according to the protocol provided by the manufacturer. Whole-cell lysates (WCLs) used for immunoblotting of different cell lines were prepared in tandem affinity purification buffer [20 mM Tris·HCl (pH 7.5), 150 mM NaCl, 0.5% Nonidet P-40, 1 mM NaF, 1 mM Na₃VO₄, 1 mM EDTA, protease inhibitor mixture (Roche, 04693124001)].²¹

Immunofluorescence staining. Cells were transfected with different plasmids. Twelve hours after transfection, cells were trypsinized and transferred to 6-well dishes with coverslips. Twenty-four hours later, cells grown on coverslips were fixed with 4% paraformaldehyde solution in PBS at room temperature for 20 min. After permeabilization with PBS buffer containing 0.1% Triton X-100 at room temperature for 20 min, cells were incubated with primary antibodies (anti-Myc or anti-GFP) at 37°C for 2 h. After washing with PBS buffer containing 0.1% Triton X-100, cells were incubated with rhodamine red-conjugated secondary antibodies at 37°C for 2 h. Slides were examined by using a laser scanning confocal microscope (Zeiss LSM 510 META UV/Vis).²¹

Vector-based small interference RNA expression. Construction of the vector PGCsilencer H1/Neo/GFP/RNAi (control-shRNA, Rheb-shRNA, Raptor-shRNA and GβL-shRNA)

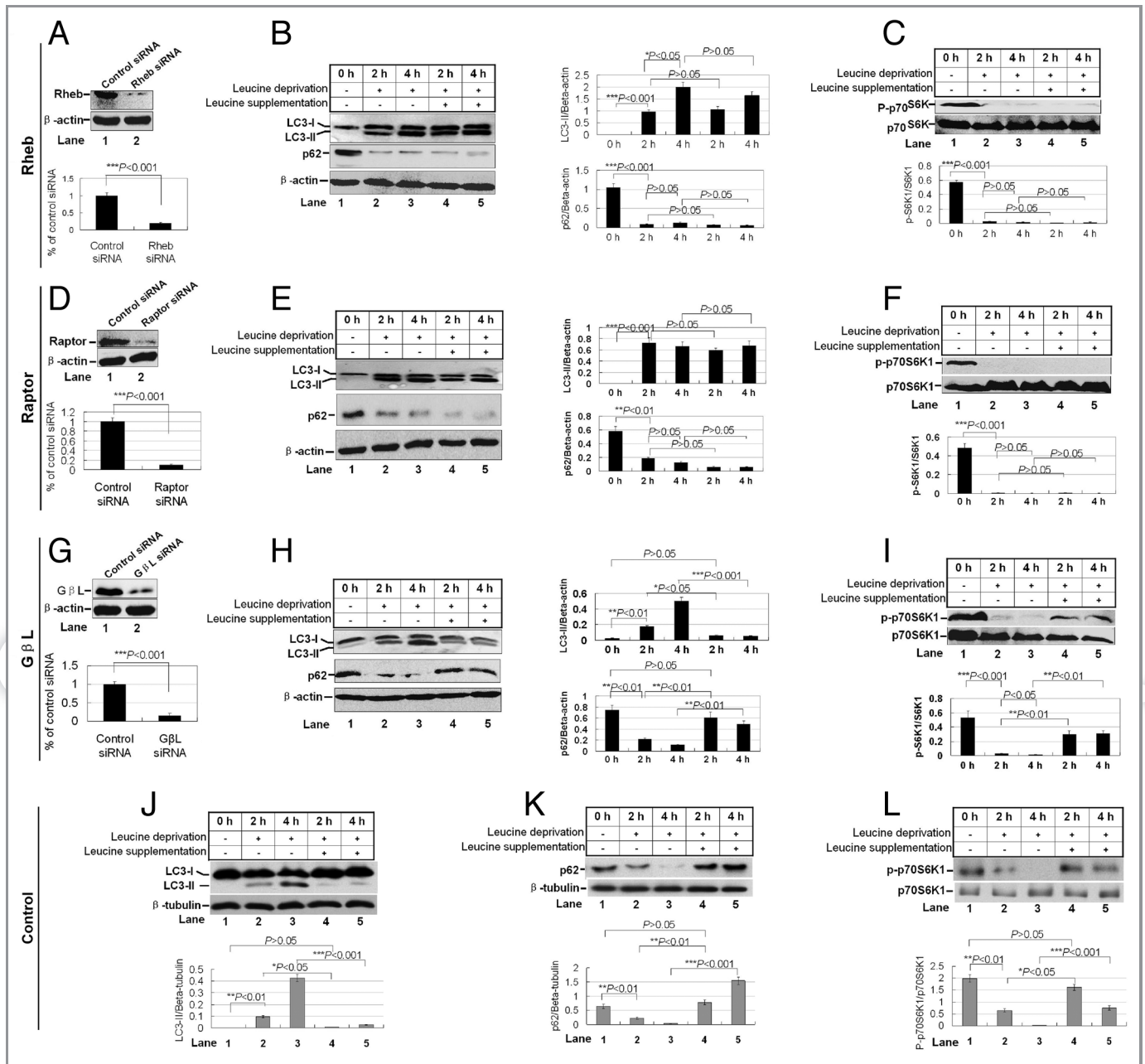


Figure 5. The contribution of mTORC1 adaptors in leucine-mediated inhibition of autophagy and mTOR activity. HEK 293T cells were transduced with pGCsilencer™ RNAi vectors expressing shRNA against Rheb. (A, D and G) western blotting for Rheb, Raptor and G β L expression (top) and quantification of these proteins compared with β -actin (bottom). (B, E and H) Leucine deprivation and supplementation on autophagy. HEK 293T cells were incubated in leucine-free medium in the absence or presence of 30 mM leucine for 2 h and 4 h. Cell lysates were analyzed by western blotting for the indicated antibodies (left), and the quantification of LC3-II/ β -actin and p62/ β -actin as the relative expression levels (right). Data are means \pm SEM for at least three different experiments. (C, F and I) Leucine deprivation and supplementation on mTOR activity. The same cell lysates as described in (B, E and H) were probed with p-p70S6K and p70S6K antibodies (top) and quantified (bottom). (J, K and L). A control siRNA was used in the cells with leucine deprivation and supplementation. The protein levels of LC3-II, p62 and p-p70S6K were detected (top) and quantified (bottom).

was fulfilled by Shanghai GeneChem Co., Ltd. The shRNA sequences were designed with “siRNA Target Finder” provided by Ambion. The shRNA coding sequence for human Rheb knockdown is 5'-UCAGUGUAGUUUGUUGUUAA-3', for raptor is 5'-GGACAACGGCCACAAGUACTT-3', and for G β L is 5'-AGCACATCCGCATGTATGATCTC-3'.

Western blotting and treatments. Rapamycin and leucine were added in fresh media as indicated in text or figure legends. Treatments were terminated by rapid removal of medium with cells on ice, followed by cell lysate preparation. Protein extracts were separated in 7.5%, 12% or 15% SDS-polyacrylamide gels and transferred to PDVF membranes. Blocking was at room

temperature for 1 h in 5% fat-free milk, and membranes were incubated overnight at 4°C with primary antibody, and then for 1 h at room temperature with a secondary antibody, and developed with enhanced chemiluminescence method (ECL) and visualized by Kodak Image Station 2000MM. Images were employed for densitometric analysis.

Preparation of S-100 Fraction from human HeLa cells. We set up HeLa cells at 5×10^5 cells per 100 mm dish in DMEM medium supplemented with 10% FBS and 1% penicillin-streptomycin. After incubation for 48 h at 37°C in a 5% CO₂ incubator, the cells were harvested, collected by centrifugation (1000 *g* for 10 min at 4°C). The cell pellets of HeLa cells were washed once with ice-cold PBS and resuspended in 5 volume of ice-cold buffer Q [20 mM Hepes-KOH (pH 7.5), 10 mM KCl, 1.5 mM MgCl₂, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM dithiothreitol (DTT), 10% glycerol, and 0.1 mM PMSF] supplemented with protease inhibitors (5 µg/ml pepstatin A, 10 µg/ml leupeptin, and 2 µg/ml aprotinin). After sitting on ice for 15 min, the cells were broken by passing 15 times through a G22 needle. After centrifugation in a microcentrifuge for 5 min at 4°C, the supernatants were further centrifuged at $10^5 \times g$ for 30 min in a benchtop ultracentrifuge (Beckman). The resulting supernatant (S-100) was stored at -80°C and used for the in vitro cell-free assay.

Purification of autophagosome-enriched membrane from human HeLa cells. Human HeLa cells were cultured with DMEM (10% FBS, and 1% penicillin-streptomycin). The media were changed into EBSS for starvation when cell confluence was up to 80-90%. After incubation for 3 h at 37°C in a 5% CO₂ incubator, the cells were harvested, and collected by centrifugation (1000 *g* for 10 min at 4°C). The cell pellets of HeLa cells were washed three times with ice-cold PBS and resuspended in 5 volume of ice-cold buffer Q supplemented with protease inhibitors. After sitting on ice for 30 min, the cells were broken by passing 15 times through a G25½ needle. After centrifugation (500 *g*) in a microcentrifuge for 5 min at 4°C, the supernatants were collected and centrifuged (21,000 rpm) for 30 min at 4°C. After removing the supernatant, the pellets were washed three times using ice-cold PBS and stored at -80°C for the in vitro cell-free assay.

Recombinant Barkor protein expression and purification. The recombinant ZZ-Barkor-His₆-Flag protein was overexpressed using suspension cultures of Sf9. One liter Sf9 cells were infected with recombinant baculovirus at a multiplicity of infection (MOI) of 5 and allowed to incubate at 27°C for ~48 h. Cells were collected by centrifugation, washed three times with PBS and resuspended in 20 ml of hypotonic buffer [20 mM TRIS-HCl (pH 7.6), 5 mM KCl; 2 mM MgCl₂, 0.5% NP-40] supplemented with protease inhibitors cocktail (Roche, 04693124001). The resuspended cells were lysed by treating (20 times) with Dounce cell homogenizer and centrifuged at 12000 *g* for 15 min. Both supernatant and the pellet were collected. The pelleted fraction was treated with 16 ml of high salt buffer [20 mM TRIS-HCl (pH 7.6), 450 mM NaCl, 0.1% NP-40, 25% glycerol, 100 U DNase I/ml, protease inhibitor cocktail] and vortexed vigorously and then centrifuged at 12000 *g* for 20 min. The

supernatant was combined with the first supernatant (collected earlier) to generate the final lysate. The recombinant His₆-Flag-tagged Barkor protein was purified from the soluble supernatant fraction by absorption to IgG-Sepharose resin (GE Healthcare, 17096901) according to the manufacturer's instructions. Briefly, 1 ml of IgG-Sepharose resin pre-equilibrated with 20 ml TEV protease cleavage buffer [10 mM TRIS-HCl (pH 8.0), 150 mM NaCl, 0.1% NP-40, 1 mM DTT], was added to the cell lysate and rotate at 4°C for 1 to 2 h at 30 rpm. Beads were then pelleted by centrifugation for 10 min at 1000 *g*, washed three times with TEV protease cleavage buffer to remove unbound proteins and then the recombinant protein was eluted from the resin by incubating at 4°C overnight with homemade TEV protease (20 U/ml) to cleave the ZZ domain from the recombinant Barkor. IgG-Sepharose eluent was collected after centrifugation at 500 *g* for 10 min at 4°C, and then incubated with 0.5 ml Ni-NTA Sepharose (Qiagen, 30210), and incubated at 4°C for 2 h rotating at 30 rpm. The beads were pelleted by centrifugation at 500 *g* for 5 min at 4°C, and then the beads were washed with 20 ml buffer containing 20 mM imidazole. Barkor-His₆-Flag was eluted with 3 ml buffer containing 250 mM imidazole. The final eluent was dialyzed against 500 ml storage buffer [25 mM TRIS-HCl (pH 7.5), 125 mM NaCl, 1 mM DTT, 10% glycerol].

Cell-free assay for Barkor membrane association in vitro. The autophagosome-enriched membranes were prepared from human HeLa cells. The membrane fractions were incubated with Barkor protein (1 µM) and S-100 in a final volume of 100 µl of buffer Q for 30 min at 37°C with gentle shaking (300 rpm). The reaction was stopped by transferring the reaction tubes on ice. The reaction mixture was subsequently subjected to centrifugation (21,000 rpm) in order to separate the autophagosomal pellet from the soluble proteins and washed twice with 100 µl buffer Q. The binding of Barkor to autophagosome-enriched membrane was assessed by western blotting using rabbit anti-Flag antibody.

Statistical analysis. Experiments were performed at least three times, with each condition run in duplicate. Statistical significance was determined by paired Student's t-test and/or with analysis of variance (ANOVA) test. Differences were considered significant with a p-value < 0.05.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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