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The amino acid transporter PAT1 regulates mTORC1 in a nutrient-sensitive manner that requires its transport activity

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ABSTRACT

The proton-coupled amino acid transporter PAT1 has been postulated to regulate the amino acid-stimulated mTORC1 through two different mechanisms, either it activates mTORC1 by sensing and transducing the lysosomal amino acid signal to mTORC1, or it inhibits mTORC1 by decreasing the signal level, as increased PAT1 has been shown to either activate or inactivate mTORC1 in the human embryonic kidney HEK293 cells. The current study aims to clarify the cause of these controversial observations, which is promoted by the recent discovery that the lysosomal PAT1 can be induced by starvation. Here, we show that under the normal culture condition, overexpression of PAT1 did not apparently change the mTORC1 activity in the fast proliferating cells. However when these cells were synchronized by starvation, followed by nutrient replenishment for a short period of time, the mTORC1 activity was decreased by PAT1 overexpression; if the nutrient stimulation lasted for longer time, the mTORC1 activities could be recovered in the PAT1-overexpressing cells. In addition, we showed the starvation-induced lysosomal PAT1 was gradually decreased during the nutrient replenishment. These results reveal that the influence of PAT1 on mTORC1 seems to be affected by the nutrient condition and the level of lysosomal PAT1. We further demonstrate that suppressing the transport activity of PAT1 abolished its inhibitory effect on mTORC1. Our data support a mechanism that PAT1 can negatively regulate mTORC1 by controlling the cellular nutrient signal level.

1. Introduction

The membrane-bound transporters play important roles in the homeostasis of the contents in both the cytosol and the membrane-enclosed compartments. The proton-coupled amino acid transporter PAT1 belongs to the solute carrier family SLC36, which can co-transport protons and other substrates, including amino acids and the amino acids-related compounds, across the membranes down the proton gradient [15]. PAT1 has been detected in a broad range of tissues with different subcellular localizations. For example, in the gastrointestinal epithelium, PAT1 is localized on the cell surface and participates in the absorption of nutrients and other substances from the gut lumen [1,3]; while in many other cell types, PAT1 is enriched on the lysosome and controls the nutrient recycling. These results indicate that PAT1 can exert multiple physiological functions, depending on to its specific subcellular localizations.

The lysosome is the primary catabolic compartment in eukaryotic cells [13]. Many macromolecules, such as proteins, lipids, and nucleic acids, are sent to the lysosomes for degradation, and the degradation products will be reused during the anabolic reactions. This process is especially important under the starvation condition when cells cannot obtain sufficient nutrients from the environment. Accordingly, the lysosome has been considered as a center of the cellular nutrient homeostasis [12]. This view can be evidenced by the discovery that the lysosome plays critical roles in the activation of the amino acid-stimulated mTORC1 signaling pathway [2].

The mechanistic target of rapamycin complex 1, or mTORC1 in abbreviation, is a multicomponent protein complex controlling many cellular processes in response to the fluctuations of environmental stimuli, including amino acids, energy, oxygen, and growth factors. There is growing evidence that lysosome is a key site for mTORC1 activation. First, the amino acids within the lysosome are an important signal to stimulate mTORC1; second, the lysosomal surface is anchored with a chain of proteins that can sense the luminal amino acids, recruit mTORC1 to the lysosomal surface, and directly activate mTORC1 [16]. According to this model, the level of lysosomal amino acids, to a large extent, determines the mTORC1 activity. In the recent years, researchers have gained much knowledge about the signal transduction

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process. In contrast, the mechanisms controlling the signal level within the lysosome remain poorly understood.

In addition to its well-characterized role in nutrient absorption, PAT1 has been identified as a regulator of mTORC1. For example, knockdown of PAT1 by RNA interference (RNAi) decreased the mTORC1 activity in both the breast cancer cells (MCF-7) and the human embryonic kidney HEK293 cells [6]. Conversely, overexpression of PAT1 could slightly increase the mTORC1 activity in HEK293 cells following the starvation and nutrient re-stimulation treatment [6]. These results suggest PAT1 positively regulates mTORC1. However in a different study, PAT1 was found to inhibit mTORC1 because increasing the PAT1 expression level was found to decrease the mTORC1 activity in HEK293 cells following the similar starvation and nutrient replenishment treatment [18]. Based on these controversial observations, two different mechanisms have been proposed. In one mechanism, PAT1 was considered as a so called transceptor, meaning it can directly bind the signal molecules and transfer the signal to mTORC1. In this process, the transport activity of PAT1 seems to be dispensable [6]. In the other mechanism, PAT1 absolutely requires its transport activity to decrease the signal level and therefore down-regulates mTORC1 [18].

In a previous study, we discovered that the localization of PAT1 on the lysosome was suppressed by nutrient. This provides a hint that PAT1 may regulate mTORC1 in a context-dependent manner [17]. Here, we attempted to test this hypothesis by addressing two important questions. First, how does overexpression of PAT1 affect mTOCR1? Second, does PAT1 require its transport activity to regulate mTORC1? In the results, we found that under the normal culture condition, increasing the PAT1 level did not apparently change the mTORC1 activity in the fast proliferating cells. When the cells were treated with starvation, followed by nutrient replenishment for a short period of time (10 min), the mTORC1 was reactivated in the wild-type cells but not in the PAT1-overexpressing cells. Interestingly, extending the nutrient replenishment (> 20 min) could fully reactivate mTORC1 even in the PAT1-overexpressing levels. Consistent with these findings, we found the lysosomal PAT1 was increased upon starvation, but was decreased by the following nutrient replenishment. These results suggest that PAT1 can regulate mTORC1 in a nutrient-sensitive manner, which is likely linked with its level on the lysosomal surface. Moreover, we demonstrated that blocking PAT1's transport activity, by expressing either the amino acid- or proton-binding mutant form, or pretreatment of cells with the competitive inhibitors, abolished its inhibitory effect on mTORC1. Our data support a mechanism that PAT1 can inhibit mTORC1 on the lysosome by controlling the luminal nutrient signal level.

2. Results

2.1. Overexpression of PAT1 does not clearly change the mTORC1 activity under the normal culture condition

In order to explore why mTORC1 was either activated or inactivated upon PAT1 overexpression, we carefully examined the mTORC1 activities with different culture conditions. To this end, we generated stable HEK293 cell lines constitutively expressing myc-PAT1, which has been shown to be localized on the lysosome in both HEK293 [6,18] and the mouse embryonic fibroblast cells (Matsui and Fukuda, 2010), and probably endows the normal PAT1 functions (Dorn et al., 2009; [6,18]). To test its subcellular localization, we performed the double immunostaining experiment using antibodies against the myc tag and LAMP1, which is an integral protein of the lysosomal membranes. The overlaps between myc-PAT1 and LAMP1 were calculated as the Pearson's correlation coefficient (r). Consistent with the previous reports, we found the myc-PAT1 was mainly localized on the LAMP1-marekd endosomes in HEK293 cells (Fig. 1A).

We firstly investigated the influence of PAT1 overexpression on the mTORC1 activity under the normal culture condition. About 3×10^5 cells of each cell type were seeded into a single well of the 6-well plate and cultured with the full medium (DMEM + 8% FBS). After 36 h, we noticed that both the wild-type HEK293 and the myc-PAT1 stable cells have reached about 80–90% confluence. These cells were then collected and the whole cell lysates were analyzed by the western blotting analyses. We checked two specific readouts of the mTORC1 activity, including the phosphor-ribosomal protein S6 kinase 1 (at T389, pS6K1) and the phosphor-ribosomal protein 4EBP1 (at S65, p4EBP1). As a result, we found the signals of both pS6 K1 and p4EBP1 remained at similar levels in both the wild-type HEK293 and three different myc-PAT1 stable cell lines (Fig. 1B). This result indicates overexpression of PAT1 does not necessarily change the mTORC1 activity at least in the fast proliferating cells under the normal culture condition.

2.2. The influence of overexpressed PAT1 on mTORC1 in affected by the nutrient condition

In the previous studies, researchers often synchronized the cells with starvation, followed by nutrient replenishment for a short period of time, before the mTORC1 activity was examined. We noted that we and Sabatini's group re-stimulated the starved cells with nutrient medium for 10 min [17,18], while Goberdhan's group performed the re-stimulation for a longer time (30 min, [6]). Because starvation could induce the accumulation of PAT1 on the lysosome, we speculated that the duration of the nutrient replenishment perhaps determined the level of lysosomal PAT1 and the mTORC1 readout [17].

To test this hypothesis, we starved the fast proliferating cells (~90% confluence) with the AA-free RPMI1640 medium (−AA, −serum) for 50 min. After that, the cells were cultured with nutrient medium (+AA, +serum) for different periods of time. Consistent with the previous observations [17,18], with 10 min of nutrient replenishment, the mTORC1 activities were decreased in three different myc-PAT1 stable cell lines (Fig. 2A, compare lane 1 with 2–4). Interestingly, when the nutrient replenishment was lasted above 20 min, the mTORC1 activities were recovered to the similar levels between the control and the PAT1-overexpressing cells (Fig. 2B, lane 5–8).

These results reveal that the influence of overexpressed PAT1 on the mTORC1 activity is sensitive to the nutrient condition. To explain the previous controversial observations, we propose that with 10 min of nutrient replenishment, the lysosomal PAT1 was reduced below certain level in the wild-type cells, allowing the full reactivation of mTORC1 (Fig. 2B, compare lane 1 with 3); while for the PAT1-overexpressing cells, this duration might be insufficient to relocate the lysosomal PAT1, as a consequence, the mTORC1 activity was decreased (Fig. 2B, lane 3 and 4; [17,18]). In support of this hypothesis, among the three different myc-PAT1 stable cell lines, the one with relatively low myc-PAT1 level (#5) showed a little bit higher mTORC1 activity than the other two lines (Fig. 2A, compare lane 2–4). However, extending the nutrient replenishment will further decrease the lysosomal PAT1. Eventfully, the mTORC1 could be recovered even in the cells with high PAT1 expression levels (Fig. 2B, lane 5–8; [6]).

To gain more evidence of this proposal, we purified the lysosomes by the cell fractionation method (see the Materials and methods section) and examined the lysosome-bound PAT1 by western blotting assays. Consistent with the pervious discovery [17], we found the lysosomal myc-PAT1 was increased by starvation (Fig. 2C, compare lane 1 and 2). Importantly, we found the lysosomal myc-PAT1 was gradually decreased as the nutrient replenishment continued (Fig. 2C and D).

2.3. Overexpression of the amino acid-binding mutant of PAT1 could not inhibit mTORC1

The next question is how PAT1 inhibits mTORC1 on the lysosome. We speculate that either PAT1 inhibits the transduction of the amino acid signal to mTORC1, or PAT1 promotes the decrease of the lysosomal amino acid signal level. To differentiate these two possibilities, it is

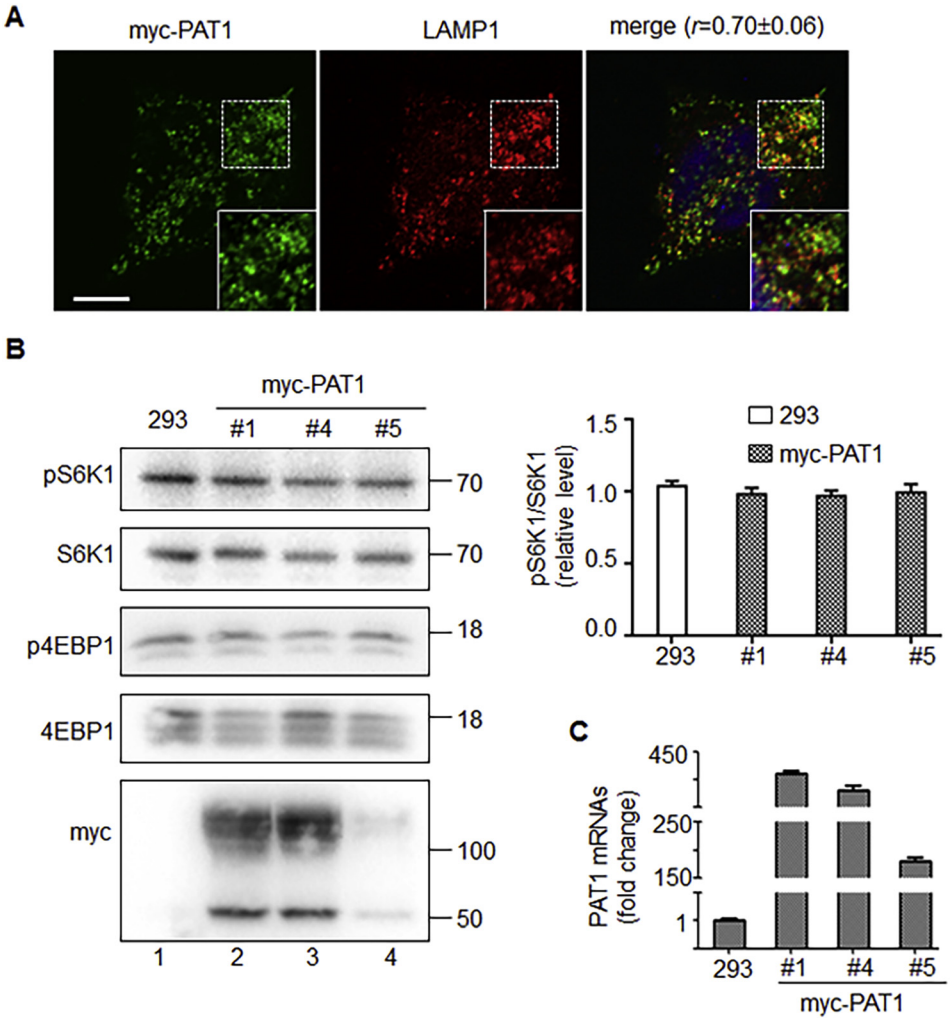

Fig. 1. Increasing the PAT1 level does not clearly change the mTORC1 activity under the normal culture condition. A. a confocal microscopy image shows myc-PAT1 is mainly localized on the lysosome in HEK293 cells. The overlaps of myc-PAT1 and LAMP1 were calculated as the Pearson's correlation coefficient (r), $n = 15$ cells. The DNA was stained with DAPI (blue). Bar: 10 μM. B, the whole cell lysates of the fast proliferating cells (80–90% confluence) were analyzed by western blotting using the indicated antibodies. Note the signals of pS6 K1 and p4EBP1 remain at similar levels between the wild-type HEK293 and three different myc-PAT1 stable cell lines. The quantification of the relative pS6 K1 levels was based on three different experiments. The myc-PAT1 was revealed by two major bands, the lower band (~55 kDa) is the monomers and the upper band (> 100 kDa) represents the aggregates due to strong hydrophobic interactions [5] and the putative glycosylated forms [18]. C, qRT-PCR assay of the relative PAT1 mRNA levels, $n = 3$ experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

important to uncover the significance of PAT1's transport activity during this process.

We assume that if the transport activity of PAT1 is dispensable for mTORC1 regulation, overexpression of the transport activity-deficient mutant of PAT1 should still be able to inhibit mTORC1. It has been shown that the loading of amino acid on PAT1 requires an internal disulfide bond formed between two cysteine residues (C180 and C329; [4]) in its extracellular region (Fig. 3A). Disruption of this disulfide bond by introducing the cysteine to alanine (C-A) mutations decreased the affinities between PAT1 and its amino acids substrates; leading to the missing of the transport activity. However the mutated PAT1 could still be transported to the lysosome in the human retinal pigment epithelial (HRPE) cells [4]. We constructed a plasmid encoding an amino acid-binding mutant form, myc-PAT1^{C180A}, and transiently expressed it in HEK293 cells. In the double immunostaining experiments, we found myc-PAT1^{C180A} was co-localized with LAMP1 (Fig. 3B), suggesting the C180A mutation did not interfere with the intracellular transport of PAT1, which is consistent with the observation in HRPE cells.

Next, we generated the stable cells constitutively expressing myc-PAT1^{C180A}. The cells (~90% confluence) were starved with RPMI1640 medium for 50 min, followed by re-stimulation with full medium for 10 min. With such treatment, the mTORC1 activity was decreased in the cells overexpressing the wild-type myc-PAT1. Interestingly, we found the mTORC1 activities remained at the similar levels between the wild-type and two different myc-PAT1^{C180A} cell lines (Fig. 2C). This means blocking the amino acid-binding ability of PAT1 abolished its inhibitory effect on mTORC1.

2.4. The proton-binding mutant of PAT1 could not inhibit mTORC1

However, disruption of the C180–329 disulfide bond may bring conformational changes and hence impair the general PAT1 functions. To address this concern, we introduced a different point mutation on histidine 55, which is close to the end of the first cytosolic region (Fig. 2A). Mutation of it into an alanine (H55A) abolished the proton binding process and inhibited the co-transport of protons and amino acids across membranes, but did not affect its lysosomal localization in HRPE cells [11].

We generated the stable myc-PAT1H55A cell lines. The immunostaining experiment revealed that myc-PAT1H55A was successively localized on the lysosome in HEK293 cells (Fig. 4A). Next, we investigated if overexpression of myc-PAT1H55A could affect the mTORC1 activity. Following the starvation and nutrient re-stimulation treatment (10 min), we found the mTORC1 activity was not decreased in two different myc-PAT1H55A stable cell lines. Together with the results of myc-PAT1C180A, we propose that PAT1 requires its transport activity to regulate mTORC1.

2.5. The competitive inhibitors of PAT1's transport activity could antagonize its inhibitory effect on mTORC1

We attempted to use a different strategy to block PAT1's transport activity. PAT1 preferentially transports the small non-essential amino acids, such as glycine, proline, and alanine. Although PAT1 also binds tryptophan and the derivatives, such as serotonin, it could not

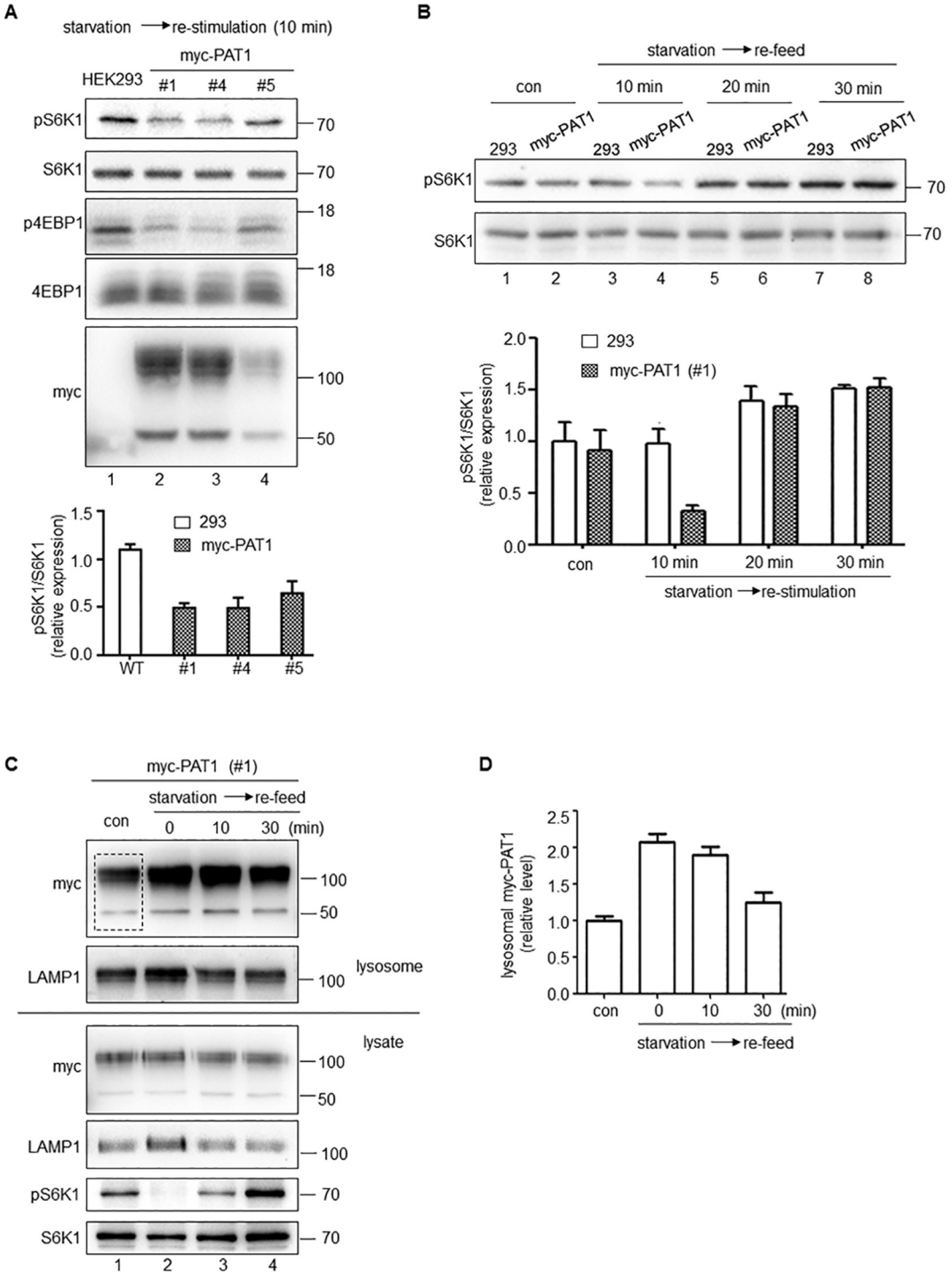

Fig. 2. PAT1 regulates mTORC1 in a nutrient-sensitive manner. A, cells were starved (−AA, −serum) for 50 min, followed by re-stimulation with nutrient medium (+AA, +serum) for 10 min. B, longer term of nutrient re-stimulation could recover the mTORC1 activity in the PAT1-overexpresisng cells (lane 5–8). Note for the wild-type cells, 10 min of nutrient re-stimulation reactivated mTORC1 to the level similar to that of untreated cells (compare lane 1 with 3); while long term of re-stimulation (above 20 min) somehow increased the mTORC1 activity (compare lane 3 with 5 and 7). C, the stable myc-PAT1 cells (#1) were either cultured with nutrient medium (con) or starved and replenished for different periods of time. The lysosome fractions and whole cell lysates were analyzed by WB. D, quantification of the relative amounts of lysosomal myc-PAT1 shown in C. The whole PAT1 signals (within the dashed box as shown in C) were measured. The quantifications were explained in the Materials and Methods section and were based on 3 experiments. Note the lysosomal myc-PAT1 was increased by starvation, but was decreased during the nutrient replenishment.

efficiently transport them across the membranes. Indeed, tryptophan and serotonin have been found to be the competitive inhibitors of PAT1's transport activity in both Caco-2 cells and *Xenopus laevis* oocytes [10].

We wonder if tryptophan or serotonin could antagonize PAT1's inhibitory effect on mTORC1. To test this, we pretreated cells with the full medium supplemented with high levels of tryptophan or serotonin for 2 h. After that, the cells were starved with RPMI1640 medium for 50 min, followed by incubation with nutrient medium for 10 min. Interestingly, we found the mTORC1 activity was not decreased in the

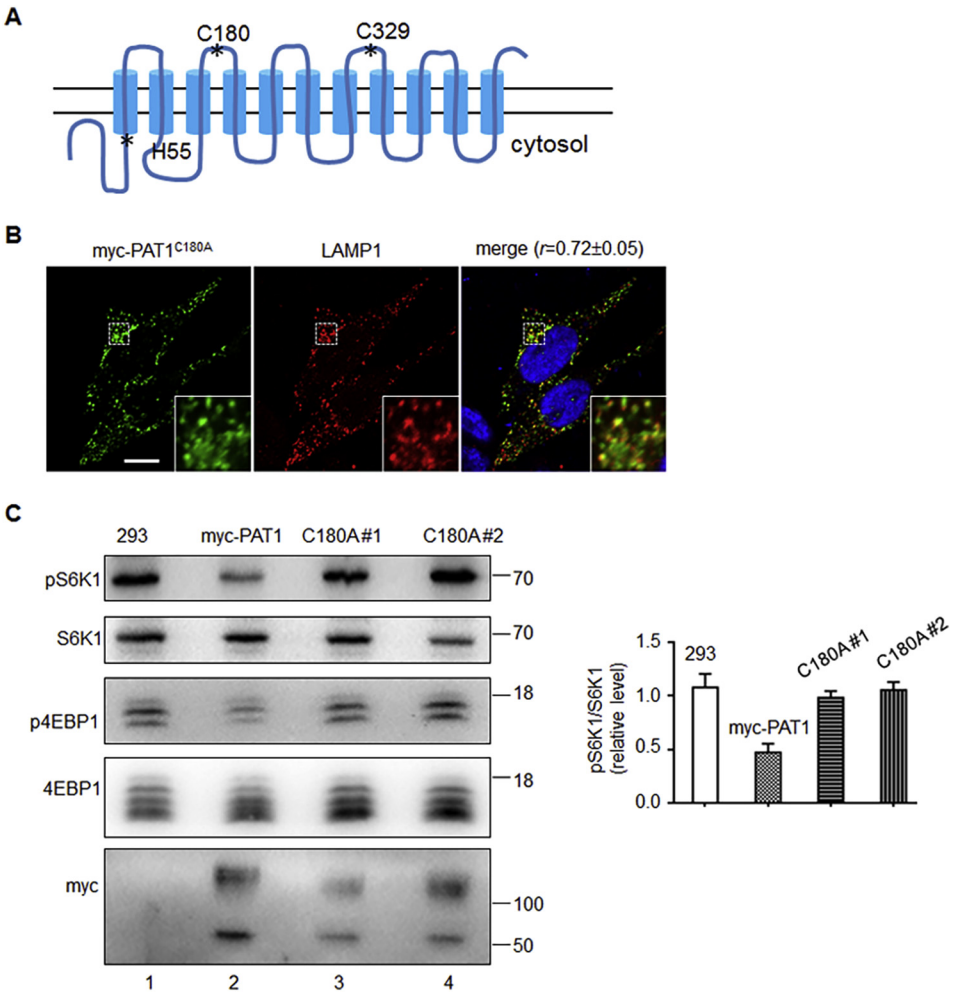

Fig. 3. The amino acid-binding mutant of PAT1 (C180A) could not inhibit mTORC1. A, the structure of PAT1 protein. For the Pearson's correlation coefficient (r), $n = 15$ cells. The blue columns indicate the transmembrane domains. B, a confocal image shows the lysosomal localization of myc-PAT1^{C180A} in HEK293 cells. Bar: 10 μM. C. the cells were starved for 50 min and replenished with full medium for 10 min. Following such treatment, the mTORC1 activity was decreased in the cells overexpressing myc-PAT1 (WT, #1), but not in the cells overexpressing myc-PAT1^{C180A} (amino acid-binding mutant). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

PAT1-overexpresing cells that had been pre-incubated with tryptophan or serotonin (Fig. 5A, C). However, we could not completely exclude the possibility that tryptophan or serotonin can activate mTORC1 through mechanisms independent of PAT1, although tryptophan or serotonin could not stimulate mTORC1 under the normal culture condition (Fig. 5B, D).

2.6. PAT1 promotes the starvation-induced decrease of mTORC1 activity

Based on the above findings, two predications could be made. First, the starved cells with high level of PAT1 need long term of nutrient replenishment to remove PAT1 from the lysosome. This will result in a slow recovery of the mTORC1 activity. Indeed, this has already been confirmed by the results shown in Fig. 2. Second, in response to starvation, the mTORC1 activity would decline fast in the cells with high PAT1 levels.

To test the second prediction, we analyzed the mTORC1 activities at different time points following starvation. Initially, we used the strong starvation condition with the RPMI1640 medium (−AA, −serum). With this treatment, the mTORC1 activities were decreased quickly in both the wild-type and the PAT1-overepressing cells (Fig. 6A). As a result, it was difficult to find clear differences on the mTORC1 activities between the two cell types. Serum (particularly the growth factors) is another important stimulator of mTORC1. We tried the amino acid-free medium supplemented with full serum (−AA + 8% FBS). With this condition, the mTORC1 activity was decreased slowly in both the wild-type and PAT1-overexpressing cells, and no clear differences could be detected within the examined time window (Fig. 6B). Interestingly, when the serum supply was reduced by 50% (−AA + 4% FBS), we found the mTORC1 activity was decreased clearly faster in the PAT1-overexpressing cells than the wild-type cells (Fig. 6C). This result reveals that overexpression of PAT1 promotes the starvation-induced decrease of the mTORC1 activity.

On the other hand, one could further assume that reducing the PAT1 level may inhibit the mTORC1 down-regulation induced by starvation. To test this, we performed the following experiment. It has been shown that with the normal culture condition, knockdown of PAT1 by RNAi inhibited mTORC1, a result that is probably caused by the low cellular amino acid pool due to the extended decrease of amino acid uptake [6,17]. To circumvent this problem, we carried out a mild PAT1 suppression experiment by transfecting cells with PAT1 siRNA (si160 in [6,17]) for a short period of time (24 h). With such treatment, the cells had reduced PAT1 transcripts but still retained considerable mTORC1 activities (Fig. 6D). Upon starvation, mTORC1 was down-regulated in both control and these PAT1-konckdown cells. However, we noted that mTORC1 was down-regulated slowly in the siPAT1-treated cells, especially at the early stage of starvation (at 10 min, Fig. 6D). This result demonstrates that under certain condition, reducing the PAT1 level can somehow resist the starvation-induced mTORC1 suppression.

3. Discussion

In summary, our data support a model that PAT1 regulates mTORC1 by controlling the homeostasis of the lysosomal nutrients, and highlight the importance of its subcellular localization on the mTORC1 activity. There is evidence that the lysosomal amino acids are a critical signal to

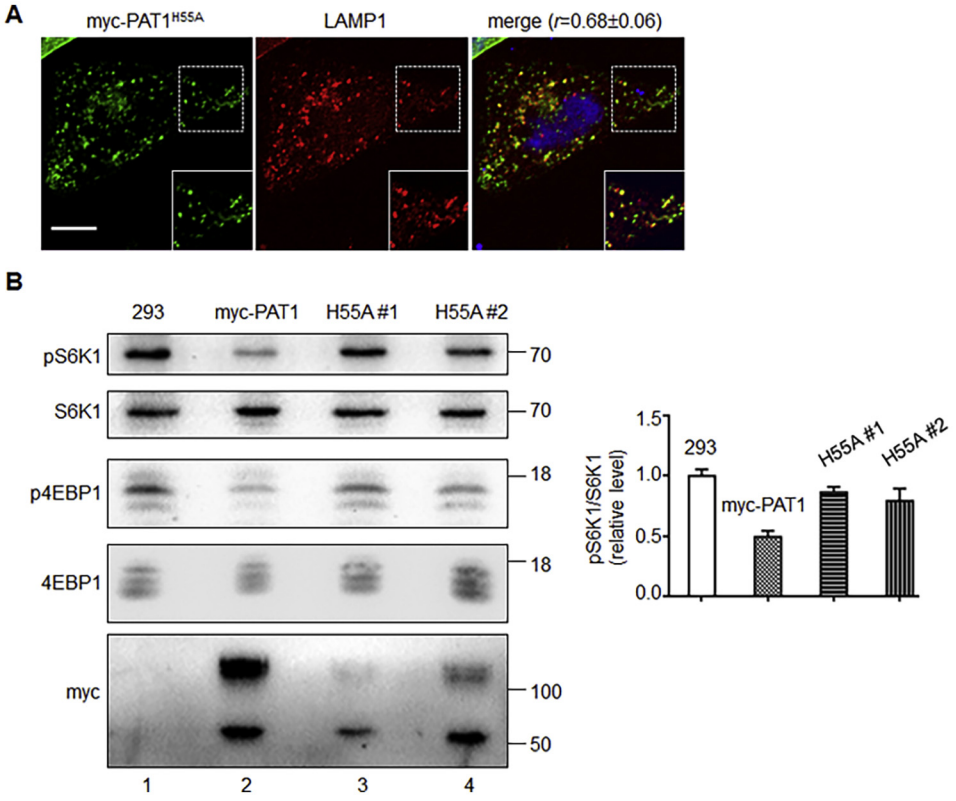

Fig. 4. The proton-binding mutant of PAT1 (H55A) could not inhibit mTORC1. A, a confocal image shows myc-PAT1^{C180A} was localized on the lysosomes. For the Pearson's correlation coefficient (r), n = 15 cells. B, overexpression of myc-PAT1^{H55A} (proton-binding mutant) could not inhibit mTORC1. Cells were starved with RPMI1640 medium for 50 min, followed by incubation with nutrient medium for 10 min.

stimulate mTORC1. Decreasing the lysosomal amino acids, by either puncturing the lysosomal membranes or overexpressing PAT1, inactivated mTORC1 in HEK293 cells [18]. In support of this view, we demonstrated in the current study that PAT1 requires its transport activity to inhibit mTORC1. However, it was also reported that PAT1 can activate mTORC1, as overexpression of PAT1 increase the mTORC1 activity in HEK293 cells [6]. Due to these controversial observations, the mechanism of how PAT1 regulates mTORC1 remains elusive. Here, we provide evidence that the influence of overexpressed PAT1 on mTORC1 is sensitive to the nutrient condition.

Using the stable PAT1-overexpressing cell lines, we demonstrate that increasing the PAT1 level does not necessarily change the mTORC1 activity at least under the nutrient environment. We suspect the overexpressed PAT1 might have reached a balance of its distribution on the lysosome and other locations, such as the cell surface. However, this balance can be disrupted by starvation, which induces the accumulation of PAT1 on the lysosome. On one hand, the increased lysosomal PAT1 promotes the nutrient recycling; on the other hand, it downregulates mTORC1 by decreasing the nutrient signal level. Both of these two reactions are important for cells to survive the stress condition. Once the nutrient is replenished, the lysosomal PAT1 will be reduced until its subcellular distribution is rebuilt. We found that for the wild-type cells, it takes a relatively short term of the nutrient stimulation (10 min) to relocate the lysosomal PAT1, but for the PAT1-overexpressing cells, long term of nutrient stimulation (> 20 min) is needed. Our data demonstrate that the effect of PAT1 overexpression on the mTORC1 activity varies in response to the nutrient fluctuations.

Then, why does knockdown of PAT1 by RNAi inhibit mTORC1 [6,17]? This is probably because PAT1 can also stimulate mTORC1 by promoting the nutrient absorption into the cell. Using the cell fractionation method, we have shown that PAT1 was localized on both the cell surface and the lysosome in HEK293 cells [8,9]. Suppressing the glycosylation modifications [9] or destroying a lysosomal targeting signal [8] decreased the level of PAT1 on the lysosome and increased its level on the cell surface. This kind of PAT1 mislocalization tends to activate mTORC1. Based on the available evidence, it seems that PAT1 can either activate or inactivate mTORC1 depending on its intracellular localizations. On the plasma membrane, it may stimulate mTORC1 by moving the environmental amino acids into the cell, while on the lysosome it tends to inhibit mTORC1 by releasing the luminal amino acids. When the expression level of PAT1 was suppressed by RNAi, the general amino acids absorption from the environment might be decreased. As a result, it may lead to the low cellular amino acid pool that tends to inactivate mTORC1. We suspect that the relative abundance of PAT1 on different locations, instead of its absolute level on the lysosome, affects the mTORC1 activity.

Notably, PAT1 was found to interact with RagC/D. This raises the hypothesis that PAT1 may be a component of the signal transduction cascade and regulate mTORC1 through certain transport activity-independent mechanisms [14]. To confirm this, it might be necessary to investigate the significance of this interaction, for example, if PAT1 affects the assembly of the signal transduction machinery. PAT1 was also found to be enriched on the plasma membrane of the gut epithelial cells and in the nuclei of rat smooth muscle cells [7]. This indicates the influence of PAT1 on the mTORC1 activity might be cell type specific. As previously suggested [6], new tools, such as the PAT1 knockout cells or animals, and high quality PAT1 antibodies, are needed to study the roles and localizations of the endogenous PAT1 in different cell contexts.

4. Materials and methods

4.1. Cell cultures

Cells were normally cultured with the full medium: Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 8% fetal bovine serum, 4 mM L-glutamine, 4500 mg/L glucose, and sodium pyruvate, at 37 °C and 5% CO2.

For the starvation treatment, about 3×10^5 cells were seeded in the 6-well plate. When the cells grew up to about 80% confluence, the full

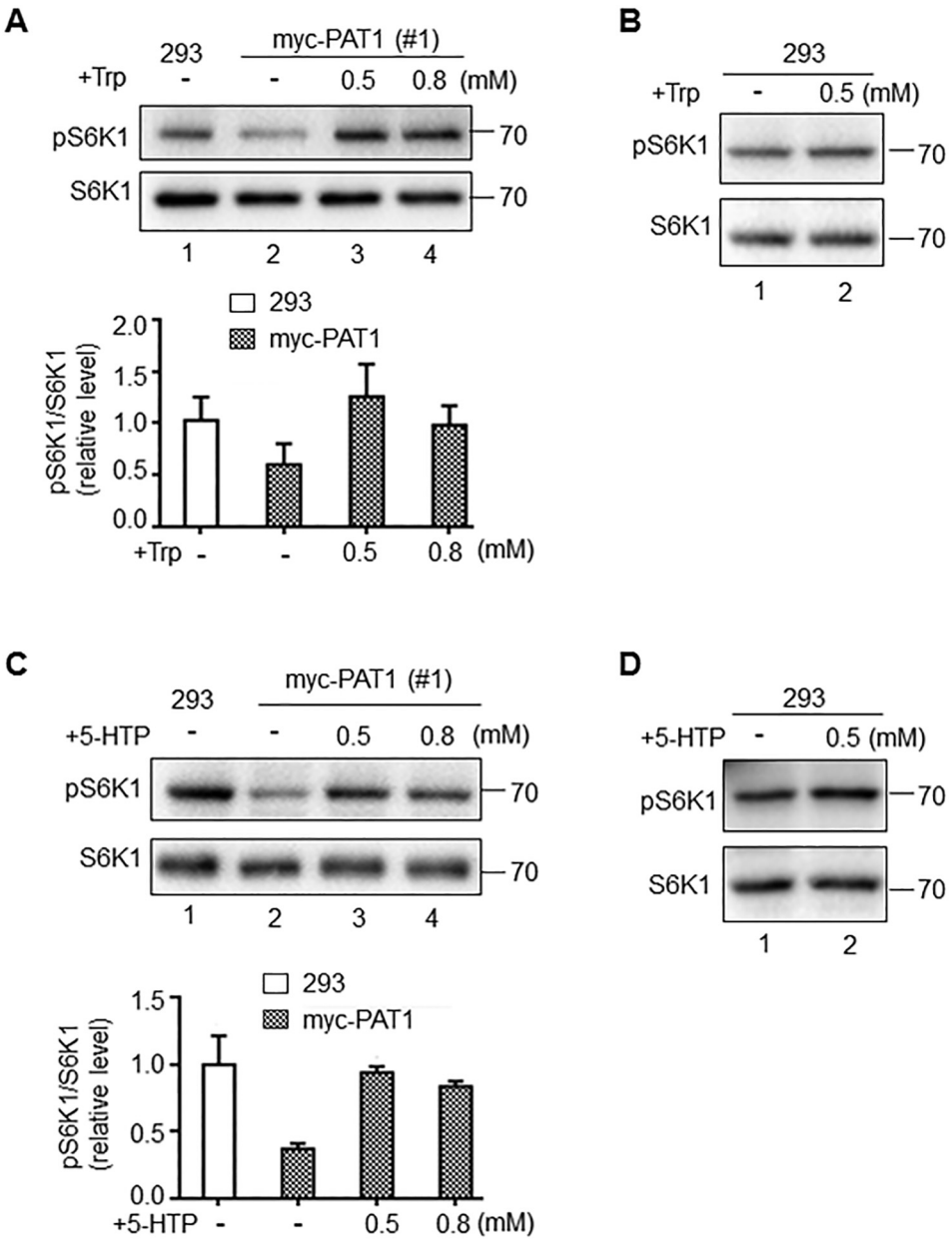

Fig. 5. The inhibitory effect of PAT1 overexpression on mTORC1 could be antagonized by the competitive inhibitors. A and C, overexpression of PAT1 could not inhibit mTORC1 in the cells that were pre-treated with Trp or 5-HTP. The HEK293 or myc-PAT1 stable cells were cultured with the full medium (−) or stimulated with the full medium supplemented with high levels of Trp (A) or 5-HTP (C) for 2 h, followed by starvation (50 min) and re-stimulation with the full medium (10 min) treatment. B and D, the wild-type HEK293 cells were cultured with either full medium or stimulated with 0.5 mM of Trp or 5-HTP for 2 h. Note mTORC1 was not stimulated by Trp or 5-HTP.

medium was removed and the cells were washed with PBS twice, followed by incubation with the AA-free RPMI1640 medium (−AA, −serum), or AA+ RPMI1640 medium supplemented with full (8%) or half (4%) amount of FBS for the indicated periods of time. For the starvation and nutrient re-stimulation treatment, the fast proliferating cells were starved with AA-free RPMI1640 medium for 50 min, washed with PBS twice, and then incubated with AA+ RPMI1640 medium supplemented with 8% FBS. To knockdown PAT1, RNAiMax diluted in OptiMEM (Life Techonology, Carlsbad, CA, USA) was used to deliver the PAT1 siRNA (si160) that has been described in previous studies [6,17].

4.2. Plasmid construction and stable cell line selection

The coding regions of myc-PAT1 (WT), myc-PAT1^{C180A} (amino acid-binding mutant) and myc-PAT1^{H55A} (proton-binding mutant) were amplified by PCR and inserted into the pcDNA3.1 vector, which can express the inserted gene under the control of the constitutive CMV promoter. All the insertions were confirmed by sequencing. The primers are available upon request to the authors. Stable cell lines expressing the related transgenes were obtained by selection with G418 (Invitrogen).

4.3. Antibodies

The following antibodies were used: mouse monoclonal to LAMP1 (H4A3) was from Developmental Studies Hybridoma Bank (Iowa City, Iowa); mouse monoclonal to beta-actin (KM9001T) was from Sungene Biotech (Tianjin, China); pS6 K1 (T389, #9205), S6 K1 (#9202), p4EBP1 (D9G1Q) and 4EBP1(53H11) were from Cell Signaling Technology (Danvers, MA, USA).

4.4. Immunofluorescent staining

Cells were fixed with 4% formaldehyde (in PBS) for 20 min, rinsed with PBS. Incubation with the primary or second antibody was performed at room temperature for 2 h or at 4 °C overnight. Nuclei were counterstained with DAPI. Images were captured using the confocal

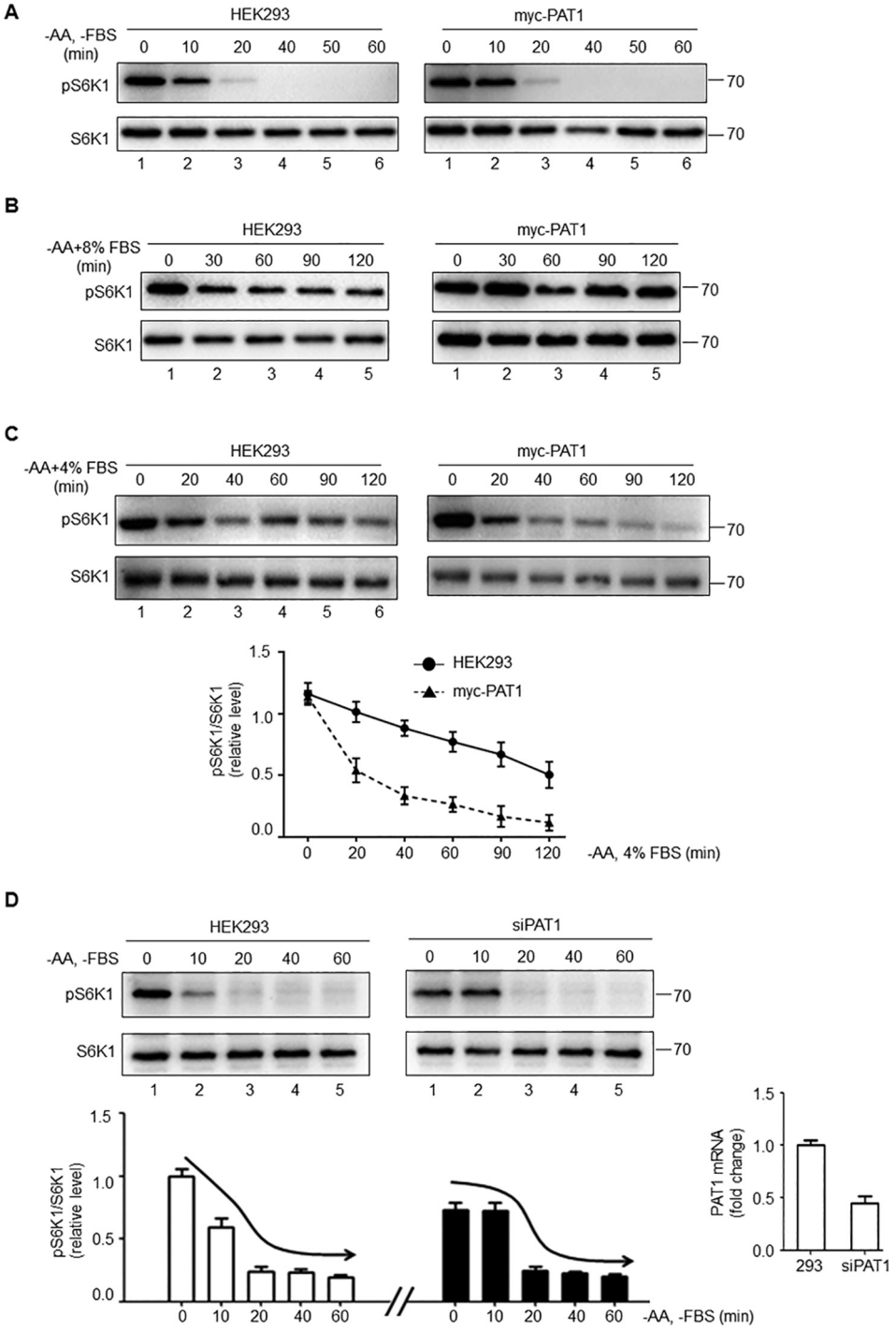

Fig. 6. PAT1 promotes the starvation-induced decrease of the mTORC1 activity. A-C, the fast proliferating cells (about 80–90% confluence) were starved with AA-free RPMI1640 medium supplemented with 0 (A), full (B, 8%) or half (C, 4%) amount of FBS. In D, the control cells or the cells treated by siPAT1 for 24 h were starved with the AA-free RPMI1640 medium (−AA, −serum). The PAT1 mRNA level was measured by qRT-PCR (right). Note under certain starvation conditions, the down-regulation of mTORC1 was promoted by PAT1 overexpression (C) but was inhibited by PAT1 knockdown (D).

microscopy system (Nikon A1R-si). For the co-localization assays, two-channel stacks of each image were analyzed using the Nikon NIS-Element confocal microscope program and the Pearson's correlation coefficient was calculated. At least 15 cells from three repeated experiments were analyzed in each assay, with one way ANOVA followed by Fisher's least significant difference test (Fisher's LSD) using the SPSS software (20.0, SPSS, Inc., Chicago, IL, USA).

4.5. Western blotting

Cells were washed once in ice-cold 1 × PBS, harvested and lysed in RIPA lysis buffer (Nanjing KeyGen Biotech, Nanjing, China) containing 25 mM Tris-HCl pH 7.6, 150 mM NaCl, 0.5 mM PMSF, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, protease and phosphatase inhibitors. The lysates were cleared by centrifugation at 12,000 rpm at 4 °C for 15 min. Protein concentration of the supernatant was measured by BCA assay. The supernatant was fixed with SDS loading buffer and boiled for

5 mins. For western blotting, the samples were separated by SDS-PAGE and transferred to PVDF. The PVDF membranes were blocked in 5% non-fat milk, incubated with primary antibodies and the then HRP-conjugated secondary antibodies; immunoreactivity was detected using ECL and chemiluminescence reagents. The quantification of WB results were conducted using the Bio-Rad Quantity One software, based on at least three repeated experiments. Data are presented as mean ± S.E.M., Student's *t*-test.

4.6. qRT-PCR

Total RNA was extracted using TRIzol as previously described [9]. The qRT-PCR reactions were performed using the QuantStudio™ 12 K Flex Real-Time PCR System (Life Technologies). The actin mRNA was used as a normalization control. The PCR primers are available upon request. $n = 3$ experiments.

4.7. Lysosome purification and calculation of the lysosomal PAT1

Lysosomes were isolated using LYSISO1 (Sigma-Aldrich, USA) following to the manufacture's instruction. Briefly, ~1.5×10^8 cells were collected by centrifugation at 600 ×*g* for 5 min. The following steps were carried out on ice. The cells were suspended in 200 μl of extraction buffer and homogenized with 5 gentle strokes in a 2 ml dounce glass tissue grinder. After centrifugation at 1000 ×*g* for 10 min, the supernatant was collected, and the pellet was homogenized for four more rounds. The supernatants from all homogenizations were then collected (~1 ml) and centrifuged at 20,000 ×*g* for 20 min. The total pellets were re-suspended in 50 μl of RIPA lysis buffer to yield the lysosome fraction.

To calculate the relative abundance of lysosomal PAT1, we used the following method: {lysosomal (PAT1)/lysosomal (LAMP1)}/{lysate (PAT1)/lysate (LAMP1)}.

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Author contributions

WL conceived and supervised the study; XZ, LZ, XJ performed the experiments; YJ provided reagents and supervised the study; LZ, XZ analyzed the data; WL wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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