이학석사학위논문

KIF11에 의한 mTORC1 신호전달체계 조절에 관한 연구

Molecular functions of KIF11 in regulating mTORC1 signaling pathway

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최 유 진

Abstract

Molecular functions of motor protein KIF11 in regulating mTORC1 signaling pathway

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The mechanistic target of rapamycin complex 1 (mTORC1) is a central regulator that coordinates eukaryotic cell growth and metabolism by responding to diverse environmental inputs, including nutrients and growth factors. Amino acid stimulation activates mTORC1 signaling by allowing Rag GTPases to recruit mTORC1 to the site of activation, the lysosome. Here I report an essential role of KIF11 and microtubules in mTORC1 signaling through regulation of mTORC1 localization regarding amino acid availability. Motor protein KIF11 depletion and microtubule depolymerization result in

aberrant mTORC1 activity upon amino acid deprivation. Moreover, cells lacking KIF11 and microtubule exhibit mislocalization of mTORC1 to the lysosome in the absence of amino acid. Also, KIF11, as a transporter, is associated with mLST8, which is a component of mTORC1, through its motor domain. These data demonstrate that KIF11 is required to move mTORC1 out of the lysosome along microtubules upon amino acid scarcity. I propose not only a novel mechanism of the regulation regarding amino acid availability for mTORC1 but also a new function of KIF11 and microtubules in mTORC1 signaling.

Key words: KIF11, lysosome, microtubule, mTORC1

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INTRODUCTION

The essential ability of cells is to regulate cell growth and metabolism in response to various environmental signals such as nutrients and growth factors. The mechanistic target of rapamycin (mTOR) is a core kinase of the signaling pathway that senses numerous environmental inputs and coordinates both anabolism and catabolism. mTOR belongs to two kinds of protein complexes, mTORC1 and mTORC2 (Kim et al., 2013; Saxton and Sabatini, 2017). mTORC1 mediates fundamental cell processes with multiple critical substrates as downstream targets. For example, mTORC1 promotes protein synthesis by phosphorylating ribosomal protein S6 kinase (S6K1) and eukaryotic initiation factor 4E-binding protein (4E-BP) and inhibits autophagy by phosphorylating Unc-51-Like Autophagy Activating Kinase 1 (ULK1) (Burnett et al., 1998; Chung et al., 1992; Kim et al., 2011). Malfunction of mTORC1 signaling pathways leads to the development of metabolic diseases such as cancer, diabetes and aging processes (Kim et al., 2013; Saxton and Sabatini, 2017).

mTORC1 signaling pathway is regulated by growth factors, such as insulin and insulin-like growth factor (IGF-1), which stimulate a pathway

involving class I phosphatidyl-3-kinase (PI3K) and its downstream effector Akt. Tuberous sclerosis complex (TSC) complex, a negative regulator of mTORC1 signaling pathway, is inhibited by Akt-dependent phosphorylation upon insulin/IGF-1 stimulation (Inoki et al., 2002). The TSC complex has GTPase-activating proteins (GAP) activity of small G protein Ras homolog-enriched in brain (Rheb), which directly binds to and activates mTORC1 at the lysosome (Inoki et al., 2003; Long et al., 2005). After the TSC complex inhibited by Akt is dispersed from the lysosome, Rheb can activate mTORC1 recruited to the lysosome by Rag GTPases (Menon et al., 2014).

As illustrated in Figure 1, in addition to regulation through growth factors, regulation via amino acids is necessary to fully activate mTORC1 signaling fully (Hara et al., 1998; Xuemin et al., 1998). This modulation in response to amino acids occurs through Rag GTPases, central regulators of mTORC1. RagA or B form a heterodimer with RagC or D. In the presence of amino acids, GTP-bound RagA/B and GDP-bound RagC/D forms interact more strongly with mTORC1 compared to the opposite forms in the absence amino acids. These interactions are required to activate mTORC1 signaling (Kim et al., 2008; Sancak et al., 2008). Ragulator complex, a scaffold protein complex interacting with Rags, induces Rags to recruit mTORC1 to the lysosome (Sancak et al., 2010). The lysosomal v-ATPase bound to Ragulator

regulates the interaction of Ragulator and Rag by amino acid, promoting the guanine nucleotide exchange factor (GEF) activity of Ragulator toward RagA/B (Bar-Peled et al., 2012; Zoncu et al., 2011). While GTPase-activating proteins toward Rags complex (GATOR1) is reported to act as a GAP of RagA/B, GATOR2 interacting with GATOR1 is a positive regulator whose function is unknown (Bar-Peled et al., 2013). Sestrin2, a negative regulator of mTORC1 signaling, is a leucine sensor that is dissociated from GATOR2 by leucine to regulate mTORC1 signaling (Wolfson et al., 2016). Most studies of mTORC1 signaling have focused on the amino acid sensors and the components that regulate the nucleotide state of Rags (Wolfson and Sabatini, 2017). The precise mechanism of how mTORC1 moves in and out of the lysosome, however, has not fully been elucidated.

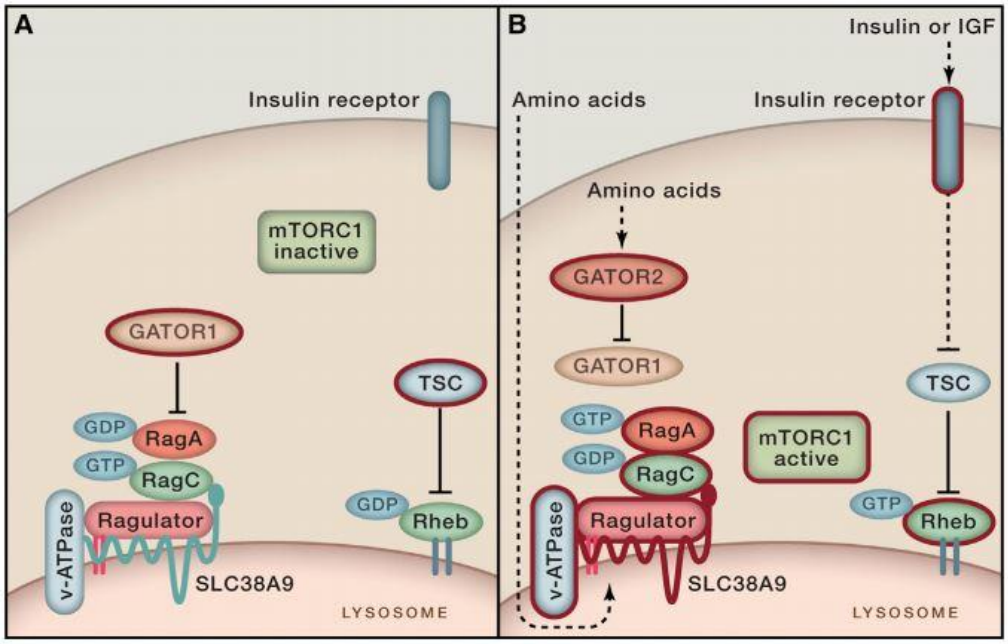


Figure 1. An overview of mTOR signaling pathway.

Adopted from Chantranupong, L., Wolfson, R.L., and Sabatini, D.M. (Chantranupong et al., 2015).

Microtubules are hollow tubes composed of heterodimers of α-tubulin and ß-tubulin, which undergo dynamic polymerization and depolymerization at the end. Microtubules are reported to require many cytoskeletal activities, including mitosis, cell division, intracellular transport, cell motility, and the regulation of cell organization (Akhmanova and Steinmetz, 2008). For these cellular processes, they serve as polarized tracks for molecular motor proteins that transport large protein complexes or intracellular components such as subcellular organelles. In the cell, microtubules have minus-end on the nucleus side and plus-end on the cell periphery side, and two classes of motor proteins move along the microtubule toward each end (Gross, 2004). Kinesin motor proteins mainly move along toward plus-end, and dynein motor proteins move along toward minus-end (Paschal and Vallee, 1987; Porter et al., 1987; Vale et al., 1985).

One of the well-known mitotic motor proteins is KIF11, a member of kinesin 5 family (Lawrence et al., 2004). KIF11 mainly regulates the assembly of the mitotic spindle (Cole et al., 1994; Enos and Morris, 1990; Slangy et al., 1995). KIF11 forms a bipolar homo-tetramer, which allows itself to help the two anti-parallel microtubules be cross-linked, aligned, and able to slide with each other (Kapitein et al., 2005; Kashlna et al., 1996; Sharp et al., 1999). Another study, however, suggested a different role of KIF11 in intracellular

trafficking. In the study, KIF11 was proved to be involved in delivering CARTS [carriers of the trans-Golgi network (TGN) to the cell surface] from the TGN to the cell surface in non-mitotic cells (Wakana et al., 2013). According to the protein-protein interaction database, Biological General Repository for Interaction Datasets (BioGRID), KIF11 physically interacts with mLST8, the component of mTORC1, however, the mechanism of how KIF11 regulates mTORC1 signaling remains poorly understood.

In a genetic screening for searching an unknown regulator of mTORC1, a previous study by my colleague has found that microtubule depolymerization increases mTORC1 activity (Jang, 2018). Here, this study reveals that mTORC1 activity increases and has resistance to amino acid scarcity when microtubule and motor protein KIF11 are inhibited in both genetically and pharmacologic ways. Additionally, I discover that microtubule destabilization or KIF11 depletion results in the mislocalization of mTORC1 to the lysosome in amino acid-starved conditions. Taken these findings into account, I propose a new mechanism that KIF11, moving to the plus-end direction through microtubule, translocates mTORC1 from the lysosome to the cytoplasm in the absence of amino acids.

SPECIFIC AIM

To understand the mechanism that regulates the subcellular localization of mTORC1

- To identify KIF11 and microtubules as new regulators of mTORC1 signaling
- To decipher the role of KIF11 in mTORC1 signaling
- To understand how KIF11 transports mTORC1 from the lysosome to the cytoplasm along the microtubule

RESULTS

Depolymerization of microtubule dynamics results in increased mTORC1 activity

The previous study selected 26 genes from the genes reported to interact with Akt or the members of the TSC complex through Biophysical Interactions of ORFeome-based complexes (BioPlex), a protein-protein interaction network database (Jang, 2018). In the study, *dorsal eye*-GAL4 was used to induce expression of RNAi targeting each candidate gene specifically in the dorsal region of the eye disc to find out genes that enhance or suppress TORC1 activity when knocked down. As a result, an increased phosphorylation of ribosomal protein S6 (RpS6) was observed in eye discs with *Tubulin-binding cofactor C (TBCC)* knockdown (Fig 2A). Consistently, TORC1 activity was higher in cells containing clones lacking TBCC (Fig 2B). This study proposed a possibility that TBCC (hTBCC in mammalian cells), a protein required for microtubule polymerization (Nithianantham et al., 2015), is one of the regulators of mTORC1 signaling (Jang, 2018). To investigate whether hTBCC plays a role in the regulation of mTORC1 signaling, I measured the mTORC1 activity upon small interfering RNA (siRNA)-

mediated knockdown of *hTBCC* in HEK293E cells. The phosphorylation of RpS6 and S6K increased when hTBCC was decreased (Fig 3A). Thus, I concluded that the lowered expression of *hTBCC* caused an increase in mTORC1 activity. Taking the role of TBCC into account, I administered colchicine, a drug that induces depolymerization of microtubules in order to identify whether the destruction of microtubule dynamics was the reason of the phenotype I observed (Fig 3C and D). After treating colchicine, the phosphorylation of RpS6 and S6K increased in HEK293E cells (Fig 3B), demonstrating that the elevated activity is strongly associated with disruption of microtubule polymerization via *hTBCC* knockdown or treating colchicine.

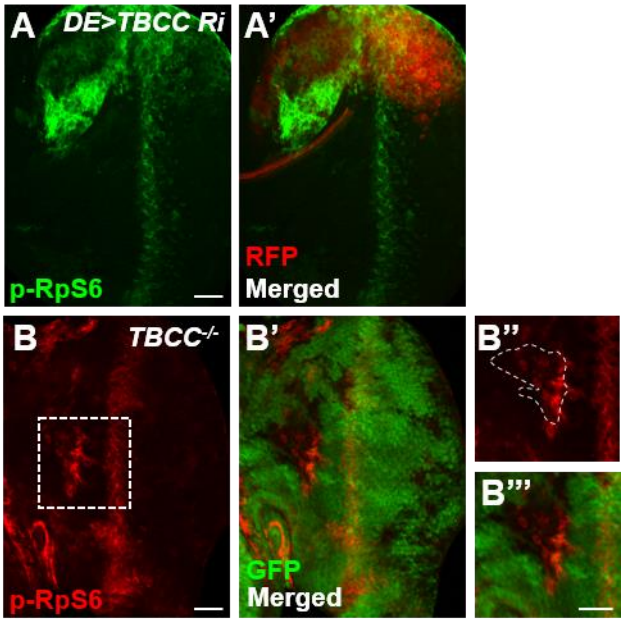


Figure 2. Disrupting microtubule dynamics increases mTORC1 activity in *Drosophila*.

(A) *TBCC* was knocked down (*TBCC Ri*) in dorsal region of the eye disc by dorsal-eye (*DE*) GAL4, labeled by RFP (red), and the tissue was immunostained with anti-p-*Drosophila* RpS6 antibody (green). The scale bar represents 30 μm.

(B) Clones of cells with homozygous *TBCC* mutation indicated by the absence of GFP (green) were generated and the eye discs were immunostained with anti-p-*Drosophila* RpS6 antibody (red). Inset is magnified in the rightmost panels. The scale bar represents 30 μm.

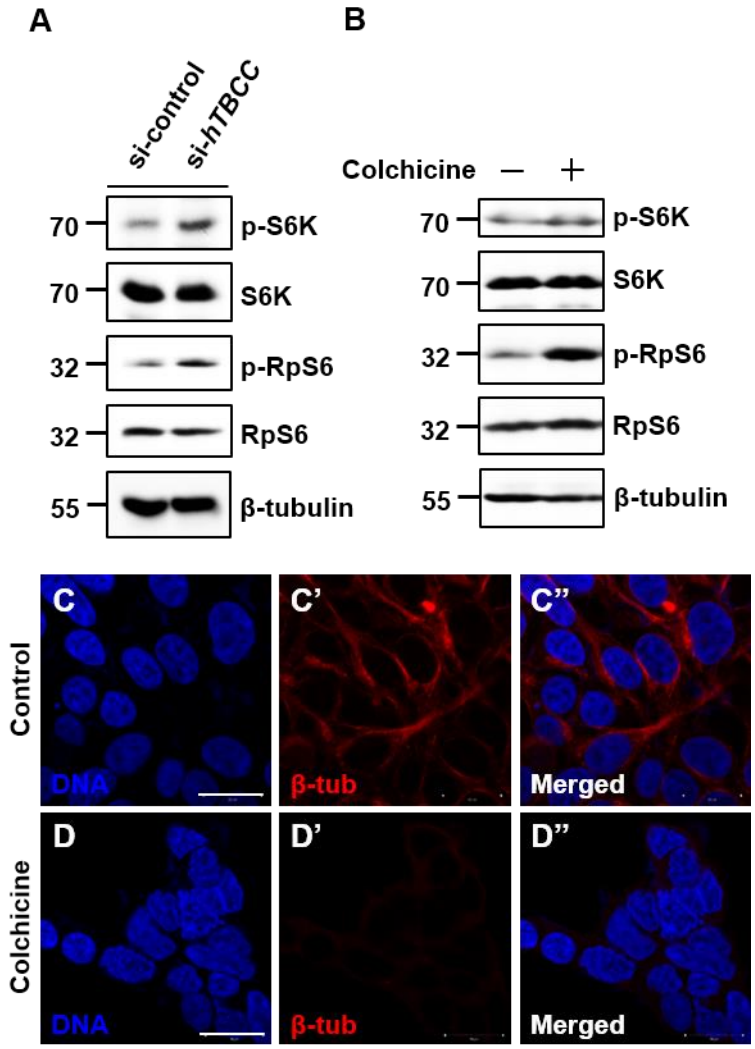


Figure 3. Disrupting microtubule dynamics increases mTORC1 activity in mammalian cells.

(A) HEK293E cells were transfected with *hTBCC*-targeting siRNA (si-*hTBCC*) or a non-targeting control siRNA (si-control). The cell lysates were

immunoblotted with anti-p-S6K, -S6K, -p-RpS6, -RpS6, and -β-tubulin antibodies.

(B) HEK293E cells were treated with 10 μM colchicine (+) or distilled water (-) for 2 hours and the cell lysates were immunoblotted with the same antibodies as in (A).

(C-D) HEK293E cells were treated with distilled water (C) or 10 μM colchicine (D) for 2 hours and immunostained with anti-β-tubulin antibody (red). The DNA was labelled by Hoechst 33342 (blue). Scale bars represent 20 μm.

mTORC1 activity is resistant to amino acid starvation when microtubules are disrupted

The activity of mTORC1 requires amino acids (Hara et al., 1998; Saxton and Sabatini, 2017). Since the removal of amino acid suppresses mTORC1 activity, I examined whether microtubule disassembly has an impact on the mTORC1 activity regulated by amino acid availability. An amino acid-free medium was used to starve HEK293E cells of amino acids for a series of time. I detected the phosphorylation of RpS6 and S6K for mTORC1 activity and compared those in control and colchicine-treated cells. In control cells, the phosphorylation of RpS6 reduced with increasing time of amino acid starvation. In the colchicine-treated cells, however, mTORC1 activity reduced significantly less (Fig 4). Apart from this, when I re-added normal medium containing amino acids to carry out amino acid stimulation, mTORC1 activity increased regardless of colchicine treatment (Fig 4). These data collectively suggested that mTORC1 activity is sustained upon amino acid deficiency when microtubules are disrupted.

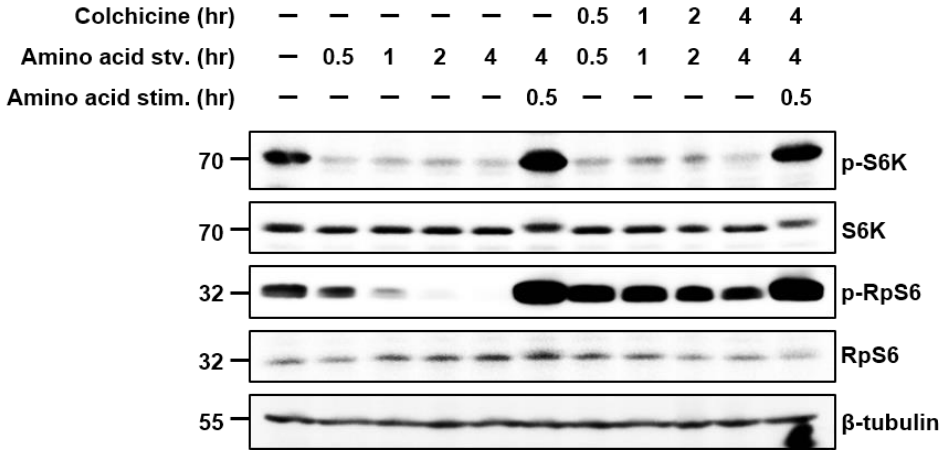


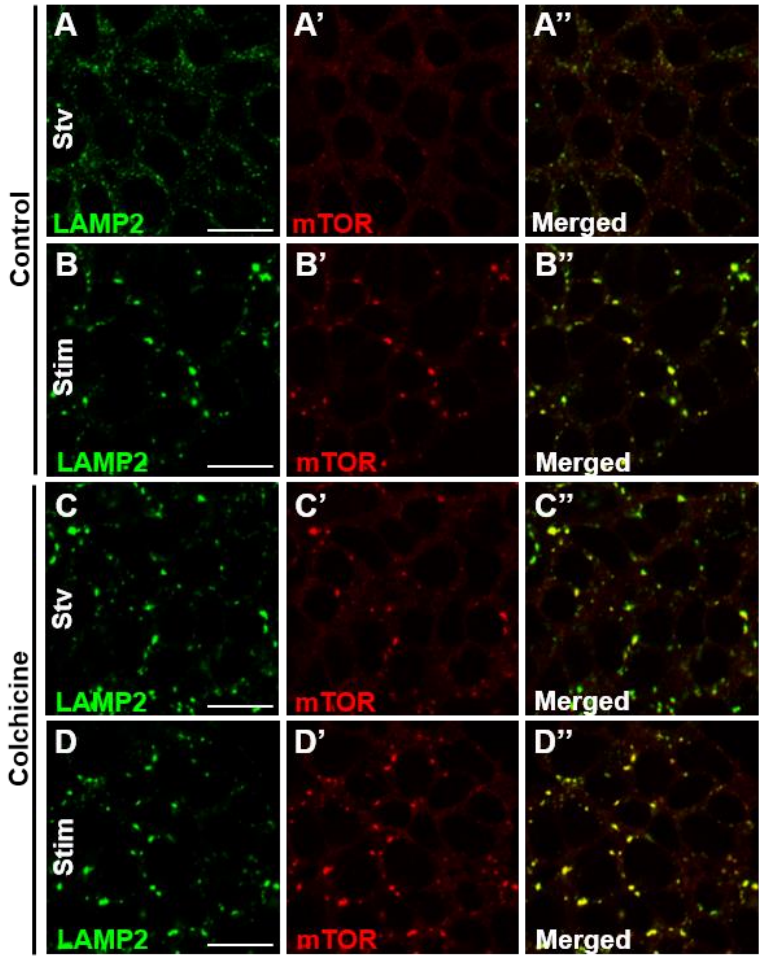
Figure 4. Microtubule disruption leads mTORC1 to be resistant to amino acid-starvation.

HEK293E cells were given amino acid-starvation (Amino acid stv.) with or without 10 μM colchicine as indicated and lysed. Amino acid stim. indicates that the cells were provided with amino acids for 0.5 hour after 4 hours of amino acid-starvation. The cell lysate samples were immunoblotted with anti-p-S6K, -S6K, -p-RpS6, -RpS6, and -β-tubulin antibodies.

mTORC1 localizes to the lysosome under amino acid starvation when microtubules are disrupted

The mTORC1 activity that is altered by amino acid stimulation is known to depend on the positioning of the complex to lysosomes. Rag GTPases accept amino acid stimuli and recruit mTORC1 to lysosomes so that the complex gets in proximity with Rheb (Kim et al., 2008; Sancak et al., 2008). On the other hand, amino acid deprivation causes mTORC1 to move from lysosomal membranes to the cytoplasm. As I assumed that the microtubule destabilization would affect the lysosomal localization of mTORC1 regulated by the presence of amino acids, HEK293E cells were immunostained with antibodies against mTOR and Lysosomal-associated membrane protein 2 (LAMP2), a lysosome marker. In control conditions, the lack of amino acids induced mTOR to be dispersed from LAMP2 and the addition of amino acids made mTOR co-localize strongly with LAMP2 (Fig 5A and B). Strikingly, in colchicine-treated condition, amino acid starvation did not inhibit co-localization of mTOR with LAMP2 (Fig 5C). Consistently, the quantification data showed that the rate of co-localization coefficient was higher in the colchicine-treated compared to the non-treated condition when amino acids were unavailable (Fig 5E). In addition, I transfected siRNA

targeting *hTBCC* and treated amino acids the same way in order to examine that the knockdown of *hTBCC* also affects the localization of mTOR under amino acid starvation. In consistent with the previous results, endogenous mTOR significantly localized more on lysosome in cells with *hTBCC* knockdown (Fig 6A and C). Meanwhile, I found that the microtubule depolymerization had no detectable effect on the co-localization of mTOR with lysosomes when the starved cells were stimulated by amino acids (Fig 5B, 5D, 6B, and 6D). In the experimental groups, the mTORC1 activity, which was determined via the localization of mTOR and the phosphorylation of RpS6, was observed to be increased compared to control groups when cells were starved with amino acids, but not when the retrieved amino acids activated mTORC1. Thus, these data suggested that the microtubule disruption may play an important role in regulating mTORC1 distribution from lysosomes to cytoplasm in one direction.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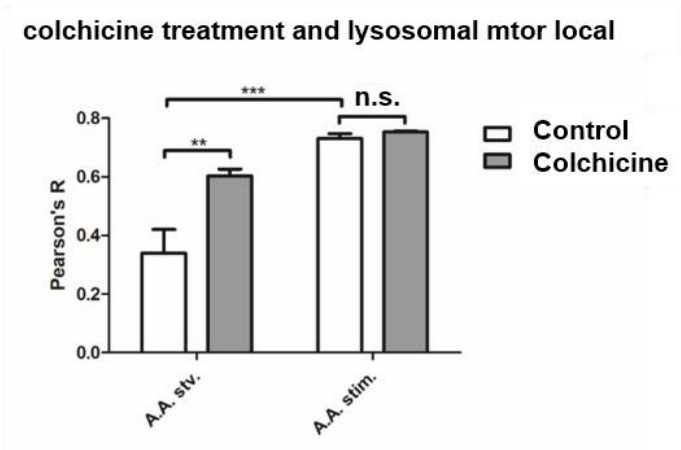
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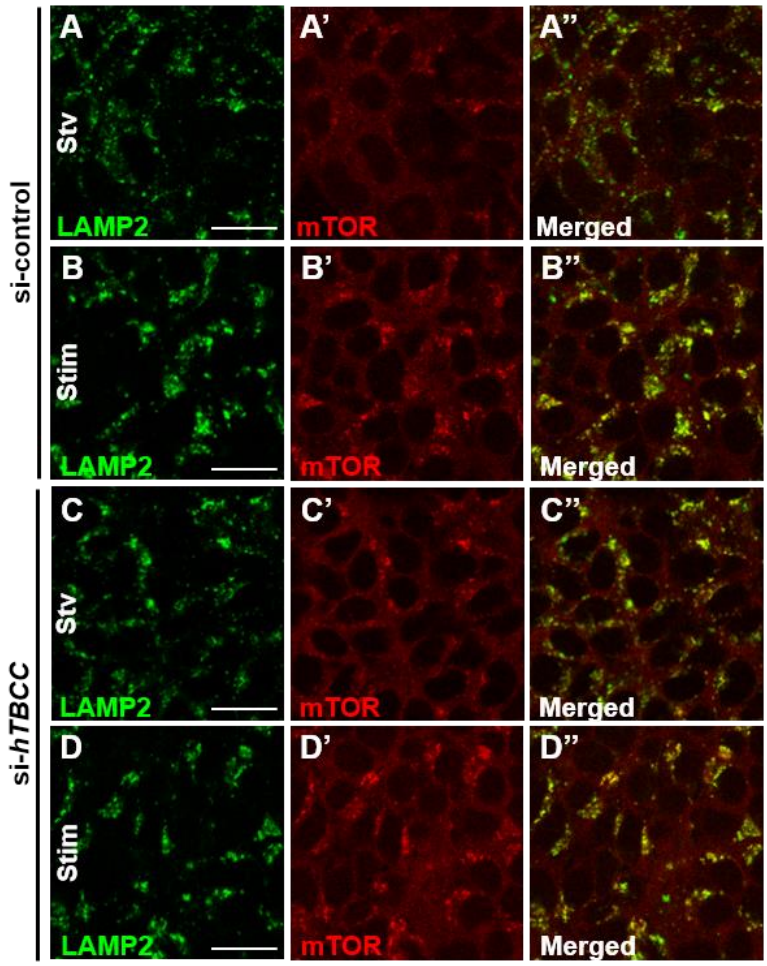
Figure 5. mTORC1 is forced to the lysosome upon amino acid-starvation when microtubules are destabilized by colchicine.

(A-D) HEK293E cells were under amino acid-starvation for 1 hour (Stv.; A and C) or provided with amino acids for 30 minutes after being starved for 1 hour (Stim.; B and D). Distilled water (Control; A and B) or 10 μM colchicine (Colchicine; C and D) was treated for 1 (Stv.) or 1.5 (Stim.) hour(s) and the cells were immunostained with anti-LAMP2 (green) and -mTOR (red) antibodies. Scale bars represent 20 μm.

(E) The degree of co-localization between mTOR and LAMP2 was quantified in 300 cells from three independent experiments in each group. All results were expressed as mean ± standard deviation (SD). Probabilities: ***$p<0.001$, **$p<0.01$, and *$p<0.05$, calculated by Student's two-tailed *t*-test.



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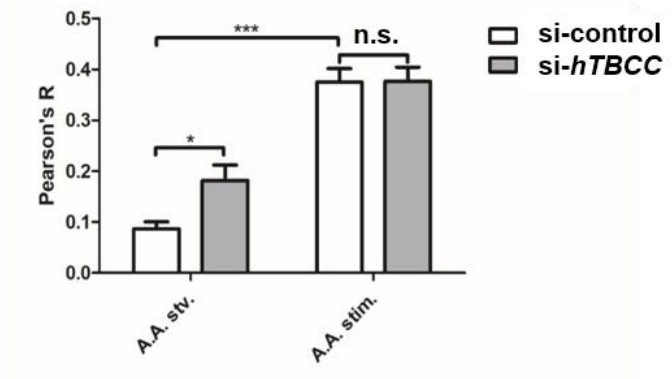


Figure 6. mTORC1 is forced to the lysosome upon amino acid-starvation when microtubules are destabilized by *TBCC* knockdown.

(A-D) HEK293E cells were transfected with a non-targeting control siRNA (si-control; A and B) and *hTBCC*-targeting siRNA (si-*hTBCC*; C and D), and given amino acid-starvation for 1 hour (Stv.; A and C) or provided with amino acids for 30 minutes after being starved for 1 hour (Stim.; B and D). The cells were immunostained with anti-LAMP2 (green) and -mTOR (red) antibodies. Scale bars represent 20 μm.

(E) The degree of co-localization between mTOR and LAMP2 was quantified in 500 cells from three independent experiments in each group. All results were expressed as mean ± SD. Probabilities: ***$p<0.001$, **$p<0.01$, and *$p<0.05$, calculated by Student's two-tailed *t*-test.

mTORC1 becomes resistant to amino acid starvation when *KIF11* is knocked down

The previous results let me conclude the translocation of mTORC1 to cytoplasm requires intact microtubules. It is then plausible that a motor protein associated with the cargo trafficking would be required for this microtubule-dependent mTORC1 regulation. Among many genes and proteins reported to interact with the components of mTORC1 based on an online database BioGRID, I chose KIF11, a plus-end-directed microtubule-based motor protein (Mann and Wadsworth, 2019). As KIF11 interacts with mLST8 according to BioGRID, I performed a co-immunoprecipitation assay to show that indeed KIF11 binds to mLST8 (Fig 7A). To define whether KIF11 does affect mTORC1 activity, I knocked down *KIF11* in HEK293E cells and measured the phosphorylation of RpS6 and S6K. As a result, *KIF11* knockdown elevated phosphorylation of RpS6 and S6K (Fig 7B). Then, I examined whether depleting *KIF11* enhances mTORC1 activity under amino acid starvation. Consistent results were obtained as mTORC1 activity was reduced much more slowly in cells with *KIF11* knockdown than in control upon serially increasing time of amino acid starvation (Fig 8 lane 1-5 and 7-11). When stimulated by amino acid, however, mTORC1 activity was elevated

to the same extent in both groups (Fig 8 lane 6 and 12). These results demonstrated that KIF11 plays an important role on regulating mTORC1 activity both in normal cells and amino acid-starved cells.

To understand whether KIF11 regulates the translocation of mTOR under amino acid deprivation, cells were transfected with *KIF11* siRNA and starved with amino acids. I observed that the deprivation of amino acid led mTOR disperse from lysosomes in control cells (Fig 9A). When KIF11 was declined, the ratio of cells containing lysosomal mTOR after amino acid starvation was increased (Fig 9C). As showed in the quantification plot, *KIF11* knockdown resulted in a higher degree of co-localization in amino acid-starved cells (Fig 9E). However, the effect of KIF11 depletion on the co-localization of mTOR and lysosomes after stimulating the cells with amino acids was negligible (Fig 9B and D). Therefore, I concluded that KIF11 is crucial for the translocation of mTORC1 from lysosomes to cytoplasm.

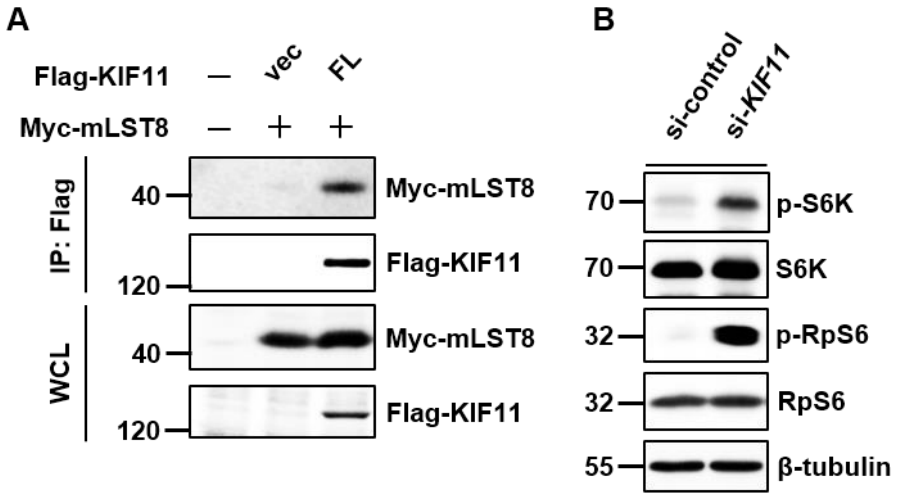


Figure 7. KIF11 binds to mLST8 and knockdown of *KIF11* increases mTORC1 activity.

(A) Flag-tagged KIF11 and Myc-tagged mLST8 were co-expressed in HEK293E cells. The lysate samples were immunoprecipitated with anti-Flag antibody and immunoblotted with anti-Myc and -Flag antibodies. Whole cell lysate (WCL) samples were loaded to confirm protein expression levels.

(B) HEK293E cells were transfected with *KIF11*-targeting siRNA (si-*KIF11*) or a non-targeting control siRNA (si-control) and the cell lysate samples were immunoblotted with anti-p-S6K, -S6K, -p-RpS6, -RpS6, and -β-tubulin antibodies.

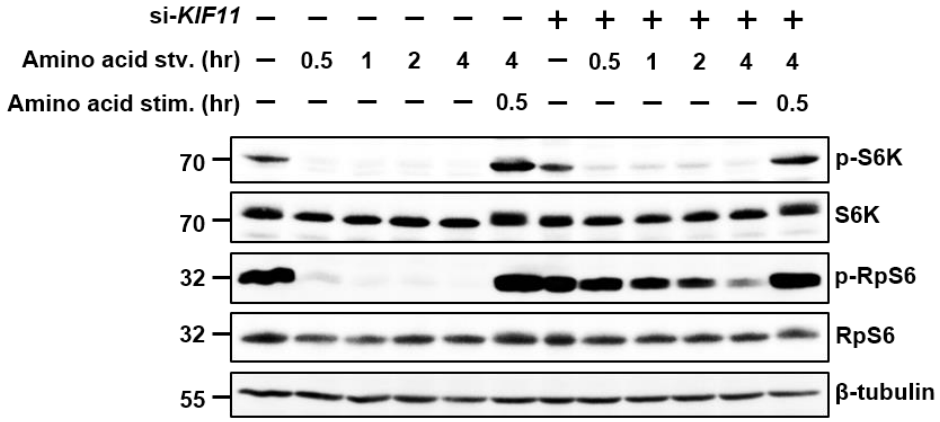
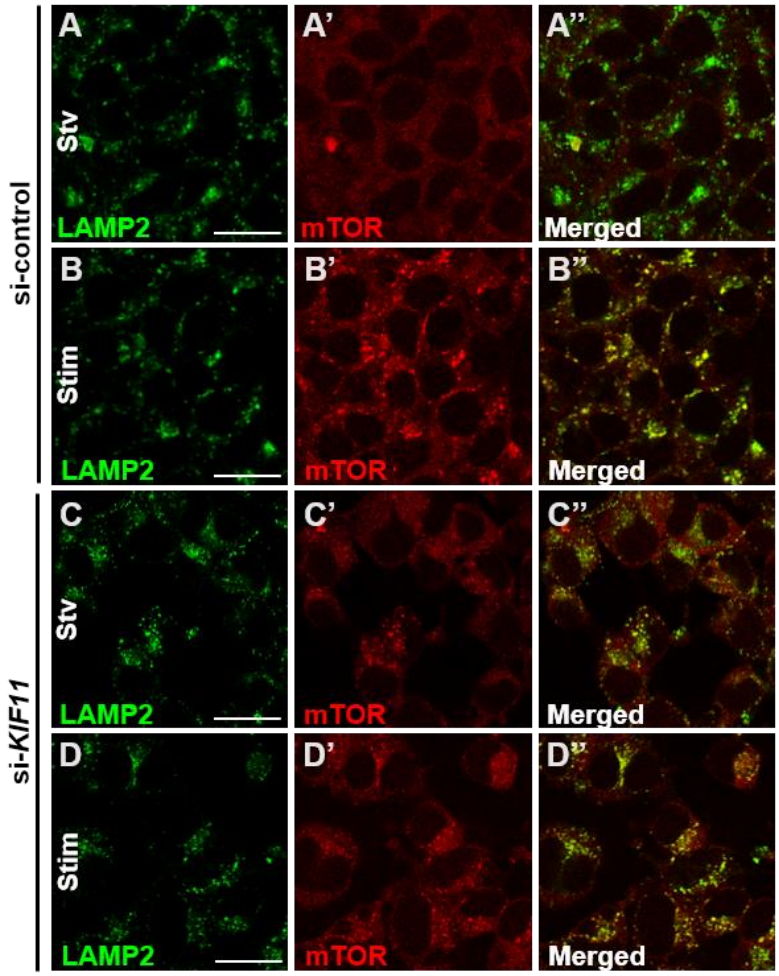


Figure 8. Knockdown of *KIF11* results in sustained mTORC1 activity after amino acid-starvation in a similar fashion to microtubule destabilization.

HEK293E cells were transfected with a non-targeting control (lane 1-6) and *KIF11*-targeting siRNA (lane 7-12) and given amino acid-starvation (Amino acid stv.) for indicated time. Amino acid stim. indicates that the cells were provided with amino acids for 0.5 hour after 4 hours of amino acid-starvation. The cell lysate samples were immunoblotted with anti-p-S6K, -S6K, -p-RpS6, -RpS6, and -β-tubulin antibodies.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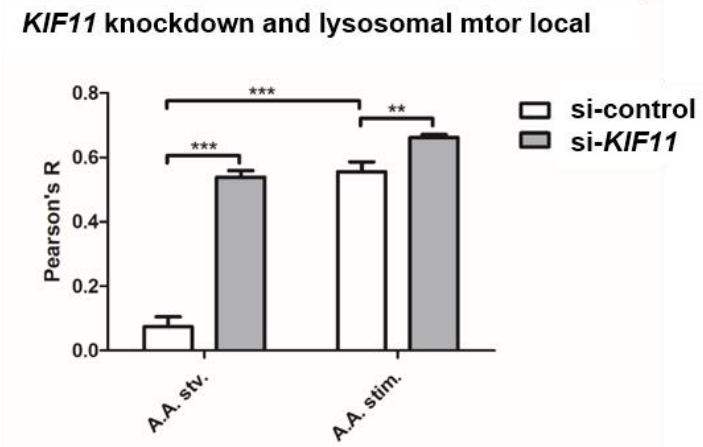
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A.A. stim.

Figure 9. mTORC1 is forced to localize to lysosomes under amino acid starvation when *KIF11* is depleted.

(A-D) HEK293E cells were transfected with a non-targeting control (si-control; A and B) or *KIF11*-targeting siRNA (si-*KIF11*; C and D), and given amino acid-starvation for 1 hour (Stv.; A and C) or provided with amino acids for 30 minutes after being starved for 1 hour (Stim.; B and D). The cells were immunostained with anti-LAMP2 (green) and -mTOR (red) antibodies.

(E) The degree of co-localization between mTOR and LAMP2 was quantified in 300 cells from three independent experiments in each group. All results were expressed as mean ± SD. Probabilities: ***$p<0.001$, **$p<0.01$, and *$p<0.05$, calculated by Student's two-tailed *t*-test.

KIF11 binds to mLST8 through its motor domain

Similar to other kinesin structures, KIF11 also consists of a motor domain, a C-terminal domain that binds to the microtubule, and a stalk domain between them (Fig 10A). To discover which region of KIF11 is responsible for interaction with mLST8, I prepared truncated forms of KIF11 for co-immunoprecipitation assays (Fig 9A): (1) ΔMotor (ΔM) which lacks motor domain, (2) ΔC-term (ΔC) which lacks C-terminal domain, (3) ΔMotor&C-term (ΔM&C), which lacks both motor domain and C-terminal domain, and (4) Motor (M), which specifically contains motor domain (Fig 10A). Co-immunoprecipitation experiments using these truncated forms and mLST8 showed that the interaction between mLST8 and KIF11 decreases when KIF11 lacked its motor domain (Fig 10B). Thus, this result demonstrated that the motor domain is required for KIF11-mLST8 binding.

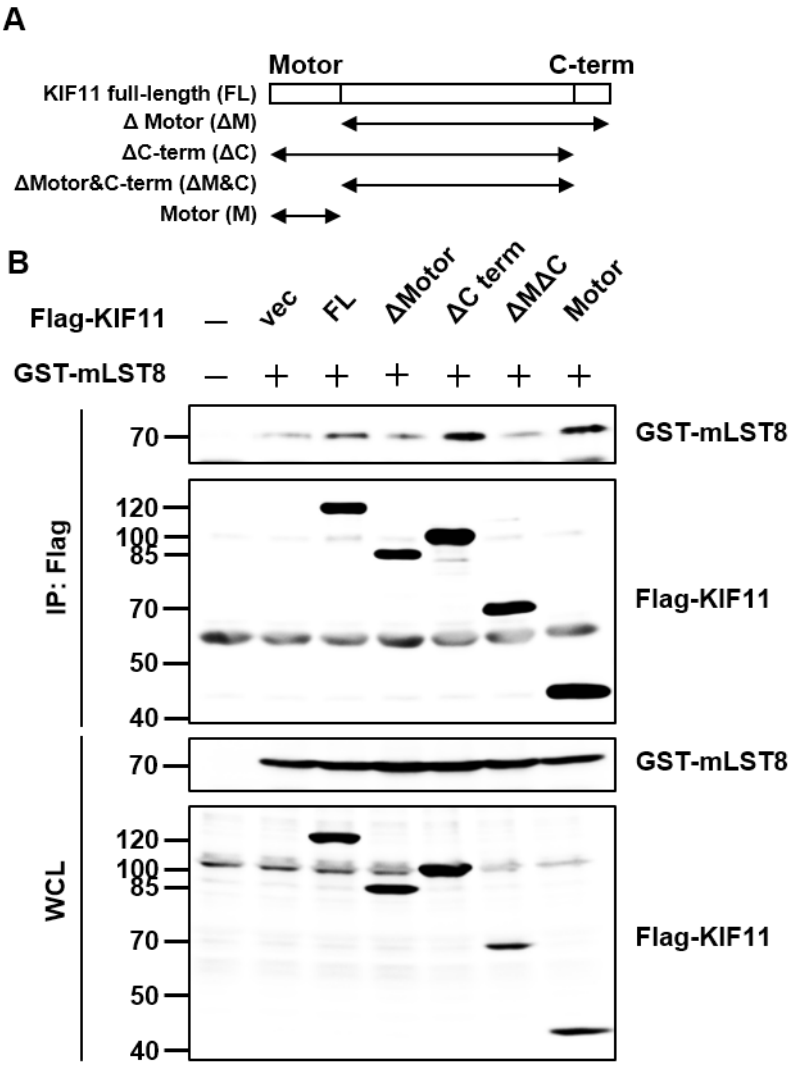

Figure 10. KIF11 binds to mLST8 through its motor domain.

(A) The model depicts how each truncated form of KIF11 was generated.

(B) Full-length or truncated form of Flag-tagged KIF11 was co-expressed with

GST-tagged mLST8 in HEK293E cells. The lysate samples were immunoprecipitated with anti-Flag antibody and immunoblotted with anti-GST and –Flag antibodies. Whole cell lysate (WCL) samples were loaded to confirm protein expression levels.

Increased phosphorylation of RpS6 and S6K by microtubule destabilization and *KIF11* knockdown is dependent on mTORC1

Since mTORC1 signaling requires PI3K-TSC complex-Rheb axis to be activated (Menon et al., 2014), I questioned whether microtubule polymerization and KIF11 affect insulin growth factor-mediated activation of mTORC1. In order to verify this possibility, I observed mTORC1 activity upon serum starvation or insulin treatment. Although I knocked down *hTBCC* and *KIF11*, mTORC1 was inactivated by the removal of serum and reactivated upon the addition of insulin (Fig 11A and B). These results supported that microtubule disruption and *KIF11* knockdown have no effect on mTORC1 activation regulated by the growth factor. Instead, it is plausible to conclude that PI3K-TSC complex-Rheb axis activation is still required for mTORC1 activity when *hTBCC* or *KIF11* is knocked down.

To investigate which one of the two mTOR-containing complexes is responsible for the increased phosphorylation of RpS6 and S6K we observed under *KIF11* or *hTBCC* knockdown, we first treated rapamycin, an acute mTORC1-specific inhibitor (Heitman et al., 1991). Rapamycin dramatically reduced the phosphorylation of RpS6 and S6K increased by *KIF11* and *hTBCC* knockdown (Fig 12A). Interestingly, in condition in which *KIF11* or

hTBCC was knocked down, rapamycin treatment could not reduce RpS6 phosphorylation as much as the control condition did. The increased phosphorylation of RpS6 and S6K due to *KIF11* or *hTBCC* knockdown was also successfully decreased by treating torin1, a drug which inhibits both mTORC1 and mTORC2 (Fig 12B). To identify whether the activity of mTORC2 was changed upon microtubule destabilization or *KIF11* knockdown, we examined the phosphorylation on Ser473 of Akt, which is known to be catalyzed by mTORC2 (Sarbassov et al., 2005). While microtubule disruption and KIF11 decline alleviated RpS6 phosphorylation, the phosphorylation on Ser473 of Akt was unaffected by both conditions (Fig 13). Therefore, this result provided evidence that microtubule and KIF11 regulate the phosphorylation of RpS6 and S6K depending on mainly mTORC1, but not mTORC2.

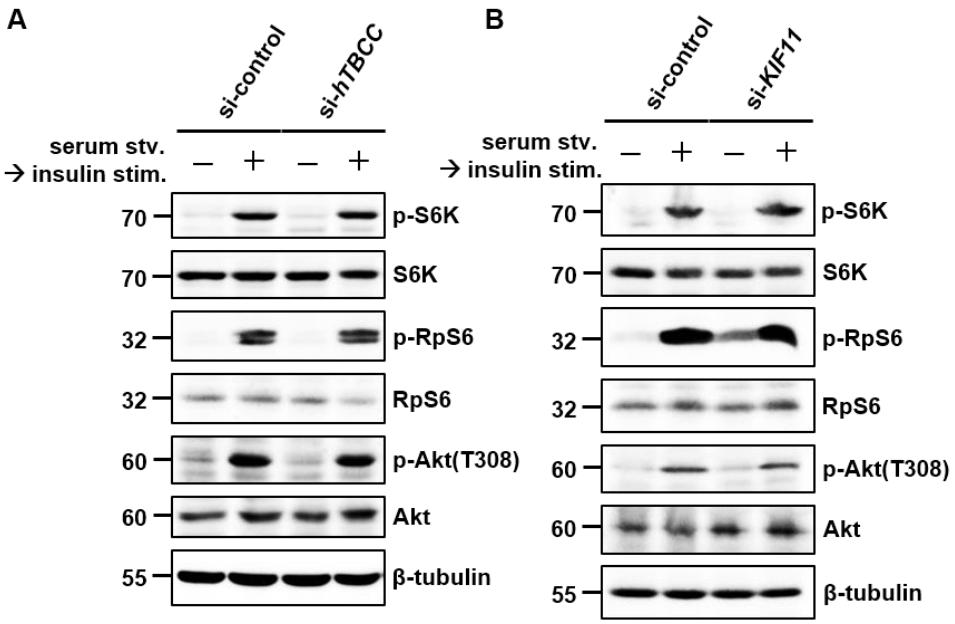

Figure 11. Knockdown of *hTBCC* or *KIF11* does not affect serum-dependent regulation of mTORC1.

(A-B) HEK293E cells with *hTBCC* (A) or *KIF11* (B) knockdown were starved with serum for 16 hours and stimulated by 100 nM insulin for 15 minutes. The cell lysate samples were immunoblotted with anti-p-S6K, -S6K, -p-RpS6, -RpS6, -p-Akt (Thr308), -Akt and -β-tubulin antibodies.

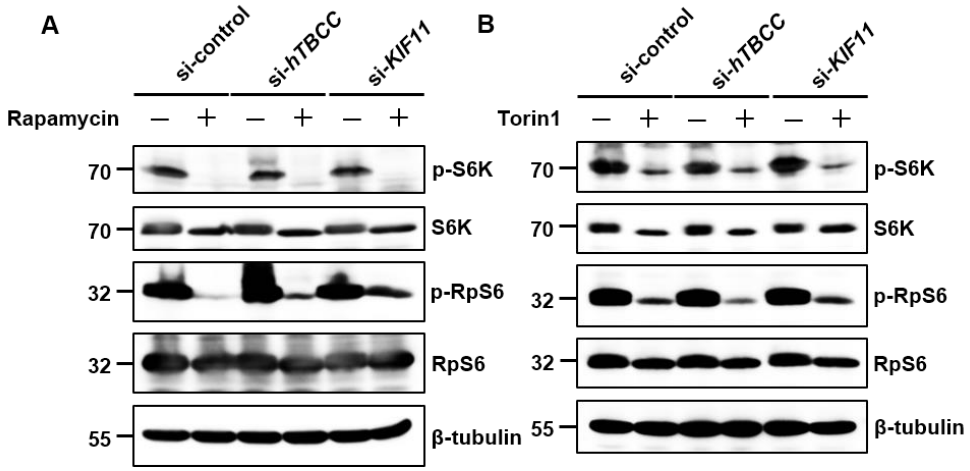

Figure 12. mTORC1 and mTORC2 inhibitors reduce elevated mTORC1 activity by knockdown of *hTBCC* or *KIF11*.

(A) HEK293E cells with *hTBCC* or *KIF11* knockdown (si-*hTBCC* and si-*KIF11*, respectively) were treated with DMSO (-) or 200 nM rapamycin (+) for 1 hour. The cell lysate samples were immunoblotted with anti-p-S6K, -S6K, -p-RpS6, -RpS6, and -β-tubulin antibodies.

(B) HEK293E cells with *hTBCC* or *KIF11* knockdown (si-*hTBCC* and si-*KIF11*, respectively) were treated with DMSO (-) or 250 nM torin1 (+) for 1 hour. The cell lysate samples were immunoblotted with the same antibodies as in (A).

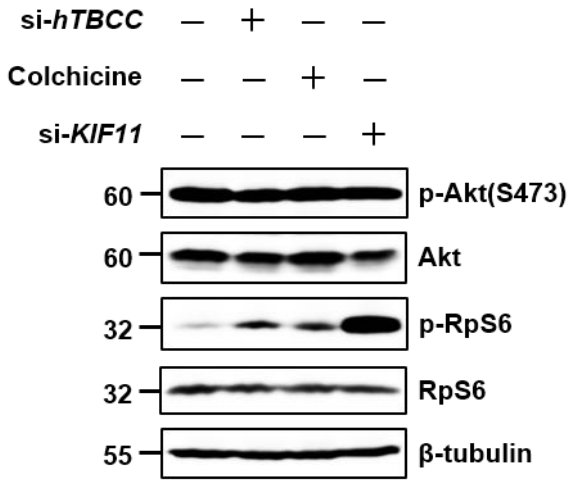

Figure 13. Microtubule disruption and *KIF11* knockdown do not affect mTORC2 activity.

HEK293E cells were transfected with *hTBCC* or *KIF11* knockdown (si-*hTBCC* and si-*KIF11*, respectively) or treated with 10 μM colchicine for 1 hour. The cell lysate samples were immunoblotted with anti-p-S6K, -S6K, -p-RpS6, -RpS6, -p-Akt (Ser473), -Akt, and -β-tubulin antibodies.

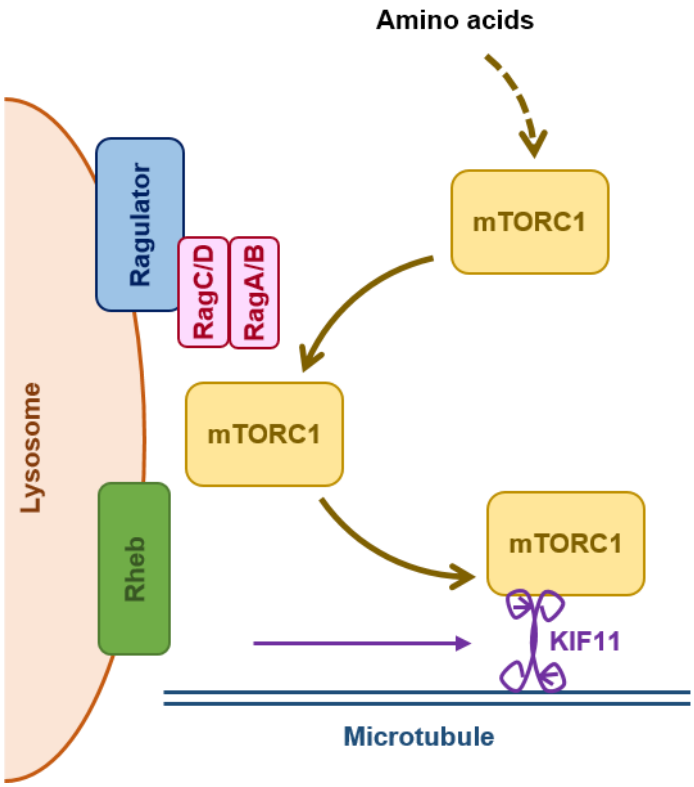

Figure 14. A proposed model of microtubules and KIF11 regulating mTORC1 activity.

A model illustrating my working mechanism. mTORC1 is translocated to the lysosome by the Rag complex for activation and when it must move out of the lysosome, the motor protein KIF11 binds to mTORC1 via mLST8 to carry it along microtubules.

DISCUSSION

This present study focused on the discovery of a new role for KIF11 as a regulator of the mTORC1 signaling pathway. After confirming that impairment of both microtubule and KIF11 increases the phosphorylation of RpS6 and S6K, I found that the mislocalization of mTORC1 induces aberrant mTORC1 activity. Although the amino acid starvation distributes mTORC1 from the lysosome to the cytoplasm and reduces mTORC1 activity, microtubule destabilization and KIF11 depletion provide resistance to this condition to sustain mTORC1 activity. Considering all of the results, this study reports that microtubules and the motor protein KIF11 are required for the proper dissociation of mTORC1 from the lysosome upon amino acid deprivation.

As KIF11 is well known for its ability to move and align the mitotic spindle, previous researches have actively studied various upstream regulators that activate and regulate KIF11 to function in mitosis (Mann and Wadsworth, 2019). Little is known, however, about the ability of KIF11 to transport cellular cargo in non-mitosis situations, and it is not clear what factors induce KIF11 to act as the transporter. This study proposes a novel function of KIF11 that translocate mTORC1 in response to amino acid scarcity in order to

mediate mTORC1 signaling. An important question for further studies is to explore the upstream regulator that detect environmental cues and regulates KIF11.

In contrast to my expectation that mTORC1 would bind to the C-terminus of KIF11 as observed in typical cargo trafficking by a kinesin motor dimer, mLST8 was found to bind to the motor domain of KIF11 (Fig 9B). As KIF11 is known to work in a homo-tetrameric manner when functioning in mitosis (Mann and Wadsworth, 2019), it can be suspected that when trafficking mTORC1, one end of the tetramer would bind to the microtubule and the other to mLST8, although precise regulation and mechanism further remains to be revealed. Especially, an electron microscopy explaining the binding of the two would provide valuable information. Indeed, another research reported that the bipolar assembly (BASS) domain of KLP61F is critical in the formation of homo-tetramer (Scholey et al., 2014). This research also noticed that multiple KLP61F mutants in the BASS domain form monomer or dimer, but not a tetramer. Therefore, it would be worthy to verify that mTORC1 translocation is affected by KIF11/KLP61F mutants which are unable to form tetramers.

Strikingly, KIF11 knockdown-mediated RpS6 phosphorylation was not completely blocked by serum starvation and rapamycin treatment (Fig 10B

and 11A). This result can be interpreted as that when KIF11 is knocked down, sustained phosphorylation of RpS6 results from the regulation independent on mTORC1 and growth factor signaling. Several studies have reported other kinases phosphorylating RpS6; p90 RpS6 kinase (RSK), Protein kinase A (PKA), and Casein kinase 1 (CK1), yet not S6K (Hutchinson et al., 2011; Moore et al., 2009; Roux et al., 2007). From among these kinases, I speculated whether U0126, which is an inhibitor of a MAP and ERK kinases (MEK) (phosphorylating RSK) (Favata et al., 1998), could alter the KIF11-mediated phosphorylation of RpS6. The treatment of U1026 could not abolish the elevated phosphorylation of RpS6 resulting from KIF11 knockdown, supporting the idea that KIF11 induced the phosphorylation of RpS6 independent on RSK (data are not shown). In addition to the ability of KIF11 to translocate mTORC1, non-canonical pathways that regulate the phosphorylation of RpS6 by affecting these kinases or phosphatases through KIF11 may exist.

Not only the mechanism that detects nutrients in the environment thereby activating the mTORC1 signaling is important, but also the manipulation of the activity of mTORC1 signaling under the absence of amino acids is essential in the context of homeostasis. This study opens the door to further investigate these unanswered questions by suggesting a mechanism in

which mTORC1 escapes from the lysosome to the cytoplasm in amino acid deficiency via the motor protein KIF11.

MATERIALS and METHODS

Mammalian cell culture, transfection and plasmids

HEK293E cells were cultured in DMEM (Welgene) supplemented with 10% fetal bovine serum (Invitrogen) at 37°C in a humidified atmosphere of 5% CO_2. For amino acid-starvation or stimulation, cells were seeded in 9.6 cm^2 6-well plates and incubated in media without or with amino acids, respectively. Cells were treated with 10 nM colchicine, 100 nM insulin, 200 nM rapamycin, or 250 nM torin1 for indicated hour(s). For immunostaining cells, cells were seeded in 3.5 cm^2 12-well plates and deprived of serum for 16 hours before amino acid-starvation or stimulation. Cells were seeded in 9.6 cm^2 6-well plates and incubated in serum starved media for 16 hours to identify serum-dependency. siRNAs for control (Bioneer, 1003), *hTBCC* (Bioneer, 6903-1), or *KIF11* (Bioneer, 3832-1) were transfected to HEK293E cells using the RNAiMAX reagent (Invitrogen) according to the manufacture's protocol. For co-immunoprecipitation experiment, plasmids were transfected to HEK293E cells with polyethylenimine (Sigma-Aldrich, 408727) according to the manufacturer's protocol. The mammalian expression plasmids for human KIF11 were kind gifts from Dr. Wen H. Shen. KIF11 and mLST8 were cloned

in pcDNA3.1 Flag, pcDNA3 Myc, or pEBG GST vectors. Plasmids for truncated forms of KIF11 were generated by PCR and cloned in pcDNA3.1 Flag expression vectors. All the constructs generated were confirmed by DNA sequencing.

Immunoblotting and immunoprecipitation assay

Cells were lysed in a lysis buffer (150 mM NaCl, 20 mM Tris, pH 7.4, 1 mM EGTA, 1 mM EDTA, 1% NP-40, 2.5 mM sodium pyrophosphate, 1 mM Na_3VO_4, 10% glycerol, and protease inhibitors pepstatin A, PMSF, and leupeptin). Equivalent protein quantities were subjected to SDS-PAGE, and transferred to nitrocellulose membranes. Membranes were blocked with 5% bovine serum albumin-containing PBS for 1 hour at room temperature and then probed with the indicated primary antibodies, followed by the appropriate HRP-conjugated anti-mouse/rabbit secondary antibodies. Immuno-reactive bands were visualized with enhanced chemiluminescence (ECL) reagent. For immunoprecipitation assay, cells were collected and lysed in 0.5 ml lysis buffer plus protease inhibitors for 30 minutes at 4°C. After 12,000*g* centrifugation for 15 minutes, the lysates were immunoprecipitated with 2 μg of specific antibody overnight at 4°C, and 30 μl A/G agarose beads were

washed and then added for an additional 1 hour. Thereafter, the precipitants were washed five times with the lysis buffer, and the immune complexes were boiled with loading buffer for 5 minutes and analyzed by SDS–PAGE. The following antibodies were used for immunoblotting and immunoprecipitation: antibodies against Flag (MBL life science, M185-3L), Myc (MBL life science, M192-3), GST (CST, #2625), p-S6K T389 (CST, #9205), S6K (CST, #9202), p-RpS6 (CST, #2211), RpS6 (CST, #2217), p-Akt Thr308 (CST, #13038), p-Akt Ser473 (CST, #4060), Akt (CST, #9272), and β-tubulin (DSHB, E7).

Immunostaining

For immunostaining mammalian cells, cells were seeded on coverslips. Cells were fixed with 4% paraformaldehyde for 10 minutes at room temperature, and then permeabilized with 0.1% Triton X-100. After blocking in goat serum for 1 hour, slides were incubated with primary antibody for 1 hour at room temperature or at 4°C overnight, washed 3 times with phosphate-buffered saline (PBS), and then incubated with FITC- or TRITC-conjugated secondary antibodies (Jackson, 1:100) and Hoechst (Invitrogen, 33342, 1:10000) for 1 hour at room temperature. The primary antibodies against LAMP2 (1:100, Santa Cruz, sc-18822), mTOR (1:100, CST, #2983), and β-

tubulin (1:100, Abcam, ab6046) were used for immunofluorescence. The slides were then washed 3 times with PBS and mounted. Cell images were captured with a confocal microscope (Zeiss).

Quantification and statistical analysis

All experiments were repeated at least three times, and all results were expressed as mean ± SD. Student's two-tailed *t*-test was used to determine statistical significance and Pearson's R value was used to determine the degree of co-localization. Software Prism 7 (Graphpad) and Fiji were used for the statistical analyses.

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국문 초록

KIF11에 의한 mTORC1 신호전달체계 조절에 관한 연구

최유진

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Mechanistic target of rapamycin complex 1 (mTORC1) 은 영양소와 성장 인자를 포함하는 다양한 환경적 신호에 반응함으로서 진핵세포의 생장과 대사를 조절하는 중요 인자이다. 아미노산 자극은 Rag GTPases 가 mTORC1 을 활성화되는 장소인 라이소좀으로 소환하는 것을 가능케 하여 mTORC1 신호 전달 경로를 활성화한다. 본 연구는 KIF11 과 미세소관이 mTORC1 신호전달체계에서 아미노산의 가용에 따라 mTORC1 의

위치를 조절하는 필수적인 역할을 한다는 것을 시사한다. 키네신 운동 단백질인 KIF11 이 감소하거나 미세소관의 중합이 해체되면 아미노산이 부족하더라도 mTORC1 의 활성이 높은 이상 현상을 초래한다. 더욱이, KIF11 과 중합된 미세소관이 부족한 세포는 아미노산이 없는 경우 mTORC1 이 라이소좀에 잘못 위치해 있는 것을 확인할 수 있다. 또한 운반자로서 KIF11 은 mTORC1 의 구성 요소 중 하나인 mLST8 과 결합관계를 가지며 이러한 관계에 KIF11 의 모터 부위가 중요하다는 것을 알 수 있다. 이러한 결과들은 KIF11 이 아미노산이 부족한 경우에 미세소관을 따라 라이소좀으로부터 mTORC1 을 운반하는데 필요하다는 것을 설명한다. 본 연구는 아미노산 가용에 따른 mTORC1 의 새로운 조절 기작 뿐만 아니라 mTORC1 신호전달체계에 있어서 KIF11 과 미세소관의 새로운 기능을 제안한다.

주요어: KIF11, 라이소좀, 미세소관, mTORC1

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