AWARD NUMBER: W81XWH-15-1-0222

TITLE: Mechanism of ribosomes biogenesis in TSC

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Ann Arbor, MI 48109-2216

REPORT DATE: September 2019

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
September 2019	Final	07/01/2015–06/30/2019

4. TITLE AND SUBTITLE

Mechanism of ribosome biogenesis in TSC

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH–15–1–0222

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The Regents of the University of Michigan Ann Arbor, MI 48109-2216

8. PERFORMING ORGANIZATION REPORT

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Tuberous Sclerosis Complex (TSC) is a genetic disorder caused by mutations in either TSC1 or TSC2 gene. TSC is characterized by widespread benign tumor formation in a variety of organs. Mutations in either TSC1 or TSC2 tumor suppressor gene are responsible for TSC. The gene products of TSC1 and TSC2, also known as hamartin and tuberin, respectively, form a physical and functional complex. We have found that TSC complex functions as a tumor suppressor to inhibit mammalian target of rapamycin complex1 (mTORC1) signaling. mTORC1 is an important protein kinase complex that stimulates cell growth and proliferation. Hyper-activation of mTORC1 contributes to the development of TSC diseases as well as many cancers. mTORC1 dominantly stimulates translation of an essential class of mRNAs such as ribosome protein subunits thereby increasing cellular ribosomes and enhancing the capacity of protein synthesis in cells. The number of ribosomes in the cell determines how fast they can make proteins. Increased ribosome biogenesis is thus important in rapidly dividing cells such as cancer cells or for cell growth in post-mitotic cells. However, the mechanisms by which mTORC1 stimulates these mRNA translations remain elusive. Our main goal is to understand how mTORC1 preferentially stimulates ribosome biogenesis through a novel mTORC1 substrate LARP1 and its functional importance in cell growth and proliferation in TSC diseases. In this proposal, we will investigate the molecular mechanisms by which mTORC1 regulates LARP1 through its phosphorylation, and how LARP1 contributes to aberrant proliferation and growth in cells lacking the functional TSC complex in both in vitro and in vivo. We believe that completion of this project will provide important and novel molecular insights into how LARP1 executes critical functions in ribosome biogenesis and tumorigenesis in response to aberrant mTORC1 activation. In addition, these studies will establish a new direction for future mechanistic studies in the regulation of mTORC1-mediated protein synthesis and provide a basis for new therapeutic approaches targeting specific translation downstream of mTORC1 for the TSC-related diseases and cancer.

15. SUBJECT TERMS: NONE LISTED

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	93	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)

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1. INTRODUCTION

The TSC-mTORC1 signaling pathway plays essential roles in the regulation of cell growth and proliferation. Loss of functional Tuberous Sclerosis Complex (TSC) gene products causes hyperactivation of mTORC1 and causes the development of TSC-associated diseases. Hyper-activation of mTORC1 leads to aberrant ribosome biogenesis by stimulating the translation of an essential class of mRNAs such as ribosome protein mRNAs (RP mRNAs) encoding ribosome proteins, thereby enhancing global protein synthesis and cell growth.

We have identified that La-related protein 1 (LARP1) is a direct substrate of mTORC1 and plays essential roles in stimulating ribosome biogenesis and cell proliferation in response to mTORC1 activation. In this proposal, we have explored the molecular mechanisms underlying mTORC1-LARP1-dependent RP mRNA translation and ribosome biogenesis, and functional importance of LARP1 for aberrant ribosome biogenesis, cell proliferation/growth control in TSC both in vitro and in vivo.

2. KEYWORDS

La-related protein 1, Akt, S6K, mTOR, mTORC1, TSC complex, TSC1, TSC2, ribosomal protein, ribosome, mRNA, translation, podocyte, glomerular function, rapamycin

3. ACCOMPLISHMENTS

A) What were the major goals of the project?

The major goal of this project was to investigate the role of LARP1, a substrate of mTORC1, in the regulation of ribosome biogenesis and protein synthesis in wild-type and TSC cells both in vitro and in vivo. The effect of LARP1 ablation on cellular mTORC1 activity and podocyte development and renal function in wild-type and the podocyte-specific

B) What was accomplished under these goals?

1) Major activities

a) Measurement of the rate of ribosome biogenesis in TSC cells by using the established ribosome protein (RP) mRNA reporters.

Based on the observation in HEK293T cells and other cancer cell lines, loss of LARP1 significantly reduced cellular mTORC1 activity and ribosome biogenesis. We hypothesized that the ablation of LARP1 also reduced mTORC1 activity as well ribosome biogenesis in TSC cells and block their growth and proliferation.

b) Determination of LARP1 interacting sites on the RP mRNAs by PAR-CLIP analyses.

We have determined the direct LARP1 interacting sequences by performing PAR-CLIP analyses and confirmed their interactions by RIP (RNA-interacting protein IP) assay using the synthetic RP mRNA bearing the mutation in the binding regions.

c) Measurement of cell size of podocytes in podocyte-specific LARP1 KO, TSC1 KO, LARP1/TSC1 double KO mice. We have

d) Monitoring glomerular histological phenotypes and glomerular function in podocyte-specific LARP1, TSC1, and LARP1/TSC1 KO mice.

2) Specific objective:

Aim 1: Determine the roles of LARP1 in the regulation of mTORC1-mediated ribosome biogenesis and cell growth/proliferation in TSC cells.

Aim 2: Determine the in vivo roles for LARP1 in ribosome biogenesis and cell growth control in a tissue-specific TSC animal model.

3) Significant results:

We have elucidated the molecular mechanisms by which mTORC1 and Akt positively regulate LARP1 function in the regulation of RP mRNA translation and cell growth, and these results were published in eLife journal in 2017 (Hong et al. eLife 2017 appendix). The studies raised the possibility that hyperactivation of mTORC1 may lead to aberrant activation of LARP1 thereby stimulating ribosome biogenesis and increasing the capacity of cellular protein synthesis, which contribute to abnormal cell growth in cells lacking the functional TSC complex. In support of this hypothesis, we have reported that 1) RP mRNA (RpL32) translation was significantly enhanced in TSC1 deficient HEK293T cells (Figure 1), 2) The expression of the LARP1 mutant, which is not phosphorylated by mTORC1 and Akt (LARP1 4A), decreased RpL32 mRNA translation in TSC1 deficient HEK293T cells (Figure 2), and 3) The ablation of LARP1 significantly blocked cell proliferation in TSC1 deficient MEF cells (Figure 3) as the results in Aim1. These observations indicated that LARP1 plays key roles in the stimulation of ribosome biogenesis and cell proliferation in cultured cells lacking the functional TSC complex. To test if LARP1 also functions as a key growth-promoting factor in vivo (Aim 2), we have developed LARP1 flox/flox mice and tested if the ablation of LARP1 in glomerular podocytes attenuates cell growth and glomerular dysfunction in wild-type and podocyte-specific TSC1 KO mice (podo-TSC1 KO mice). We previously demonstrated that the podocyte lacking functional TSC1 led to aberrant cell growth, the detachment of the cells from the glomerular basement membrane, and massive proteinuria in podo-TSC1 KO mice (Inoki et al JCI 2011, 121, 2181). However, in contrast to our predictions, we have observed that the ablation of LARP1 had little effect on aberrant cell growth (Figure 4), proteinuria (Figure 5), and glomerular sclerosis (Figure 6) seen in podo-TSC1 KO mice (Figure 1A, 1B, and 1C). These in vivo observations suggested that LARP1 has little role in the growth of glomerular podocytes and glomerular dysfunction in the podocyte lacking the functional TSC complex. Although these results were unexpected, it is likely that podocytes may express other redundant LARP-related proteins such as LARP2 and LARP7, which compensate loss of LARP1 functions in

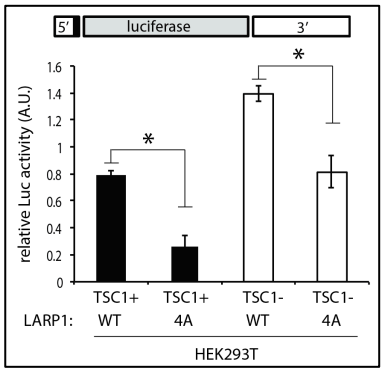

Figure 2. The LARP1 4A mutant reduced the level of RpL32 mRNA translation in both wild-type and TSC1-deficient HEK293T cells. The RpL32 reporter mRNA was expressed in the indicated cells with either wild-type LARP1 or LARP1 4A mutant.

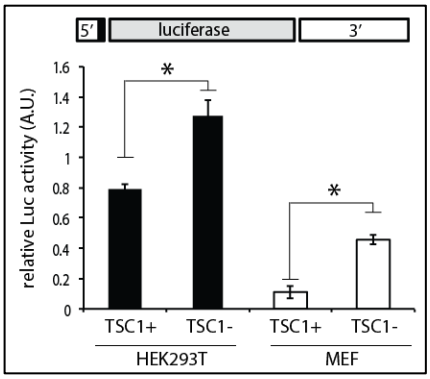

Figure 1. The levels of RpL32 mRNA translation in the wild-type and TSC1-deficient HEK293T or MEF cells. The RpL32 reporter mRNA was expressed in the indicated cells, and levels of its translation were monitored by measuring luciferase activity.

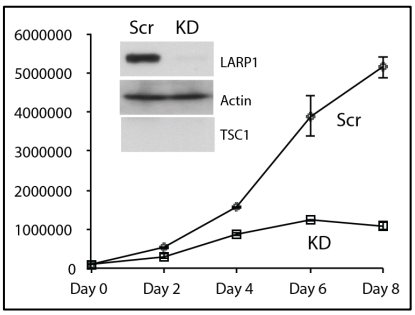

Figure 3. LARP1 plays an important role in cell proliferation in TSC1-deficient MEF cells. Endogenous LARP1 was knocked down, and levels of cell proliferation were monitored in TSC1-deficient MEF cells. Scr: control shRNA.

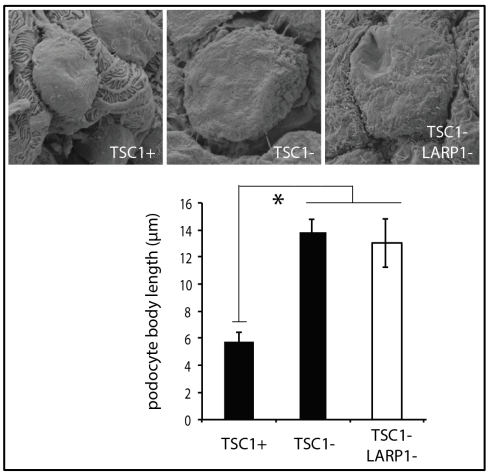

Figure 4. The effect of LARP1 ablation on podocyte growth in podo-TSC1 KO mice. Glomerular podocytes were visualized by SEM, and the diameter of podocyte cell body was monitored in the indicated mice.

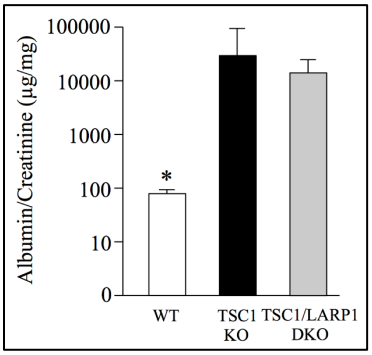

Figure 5. The effect of LARP1 ablation on glomerular function (albuminuria) in podo-TSC1 KO mice. 24 hr-urine were collected from the indicated mice, and levels of albumin in their urine were monitored and normalized by urine creatinine.

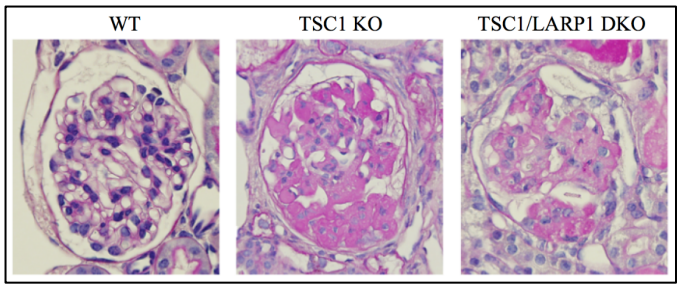

Figure 6. The effect of LARP1 ablation on glomerular sclerosis in podo-TSC1 KO mice. Glomeruli from the indicated mice were stained by PAS and Hematoxylin

podocytes. It is worth noting that LARP1 has also been proposed as a suppressor of 5'TOP mRNA translation, which include RP mRNA translation. However, we did not observe any growth phenotypes in podocytes lacking endogenous LARP1 expression in the podo-specific LARP1 KO mice (data no shown). Further experiments, especially LARP1 knockout in other tissues and organs, will be necessary to address whether LARP1 indeed plays a key role in stimulating or inhibiting RP mRNA translation and cell growth.

4) Other achievements:
To understand further regulations and roles of LARP1, we have performed mass spectrometry analyses to identify LARP1 interacting proteins and found a number of LARP1-interacting proteins (unpublished observations).

C) What opportunities for training and professional development has the project provided?
Nothing to report.

D) How were the results disseminated to communities of interest?
At the LARP society meeting in 2016, we presented a part of the data generated under this proposal. In the publication, we demonstrated details of the regulation of LARP1 by mTORC1 activity, and the roles of LARP1 in the regulation of mTORC1-dependent ribosome biogenesis.

E) What do you plan to do during the next reporting period to accomplish the goals? Nothing to report.

4. IMPACT
A) What was the impact on the development of the principal discipline(s) of the project?
We identified that LARP1 as a substrate of mTORC1 kinase, Akt, and S6K1. We also mapped specific phosphorylation sites of LARP1 by these kinases. We also determined that specific LARP1 interacting mRNAs, which mainly encode proteins contributing to ribosome biogenesis and protein synthesis. Our data indicated that LARP1 functions as a molecular switch for mTORC1- and Akt-dependent translation of LARP1-interacting mRNAs as while the non-phosphorylated LARP1 blocked the translation, the phosphorylated LARP1 stimulated the translation of LARP1-interacting mRNAs. As expected, LARP1 activity was enhanced in cells lacking functional TSC complex, and LARP1 positively contributed to cell proliferation of TSC1-deficient cells. However, the roles of LARP1 in the regulation of ribosome biogenesis and cell growth control in vivo remained elusive. By completing this proposal, it was clear that at least in glomerular podocytes, LARP1 had little effect on aberrant cell growth induced by hyper-mTORC1 activity in TSC1-deficient podocytes. Further studies will be required to address if LARP1 indeed contribute to mTORC1-dependent ribosome biogenesis and cell growth in other tissues such as brain, lung, and renal tubular cells where the TSC-related tumor often occur. In addition, it will also be necessary to study a possible compensation mechanism by other LARP family member including LARP2 in LARP1-deficient podocytes.

B) What was the impact on other disciplines?
Nothing to report.

C) What was the impact on technology transfer?
Nothing to report.

D) What was the impact on society beyond science and technology?
Nothing to report.

5. CHANGES/PROBLEMS
Not applicable.

6. PRODUCTS
Publications supported by this grant are listed.

1) LARP1 functions as a molecular switch for mTORC1-mediated translation of an essential class of mRNAs.
Hong S, Freeberg MA, Han T, Kamath A, Yao Y, Fukuda T, Suzuki T, Kim JK, Inoki K. (2017), *Elife*. pii: e25237 doi: 10.7554/eLife.25237.
2) Evaluating the mTOR Pathway in Physiological and Pharmacological Settings.
Hong S, Inoki K. (2017), *Methods Enzymol*. 587:405-428. doi: 10.1016/bs.mie.2016.09.068.

3) Lysosomal Regulation of mTORC1 by Amino Acids in Mammalian Cells.
Yao Y, Jones E, Inoki K. (2017), *Biomolecules*. pii: e51. doi: 10.3390/biom7030051.
4) Macropinocytosis, mTORC1 and cellular growth control.
Yoshida S, Pacitto R, Inoki K, Swanson J. (2018), *Cell Mol Life Sci*. 75: 1227-1239. doi: 10.1007/s00018-017-2710-y.
5) Microphthalmia-associated transcription factors activate mTORC1 through RagD GTPase gene expression. Jones E, Inoki K. (2017), *Transl Cancer Res*. Supple 7: S1234-S1238. doi: 10.21037/tcr.2017.09.31.

Other Products:
We have developed LARP1 flox and TSC1/LARP1 double flox animals.
We have generated reporter mRNA systems related to RpL32 mRNAs.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATION

A) What individuals have worked on the project during the period 7/1/18-6/30/19

Name:	*Ken Inoki*
Project Role:	*PD/PI*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*2.4 CM*
Contribution to Project:	*Dr. Inoki is responsible for in vivo experiments and all the design and analyses.*
Funding Support:	*n/a*

Name:	*Sung Ki Hong*
Project Role:	*Research Assistant Scientist*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*5.2 CM*
Contribution to Project:	*Dr. Hong is responsible for in vitro experiments using cultured cells.*
Funding Support:	*n/a*

Name:	*Yao Yao*
Project Role:	*Research Assistant Scientist*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*5.0 CM*
Contribution to Project:	*Dr. Yao is responsible for in vitro experiments using cultured cells.*
Funding Support:	*n/a*

B) Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period? Dr. Inoki's R01s ended 12/31/18 and 7/31/19.

C) What other organizations were involved as partners?
Dr. Kim was not involved in the project no-cost extension.

8. SPECIAL REPORTING REQUIRMENTS

Nothing to report.

9. APPENDICES

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RESEARCH ARTICLE

LARP1 functions as a molecular switch for mTORC1-mediated translation of an essential class of mRNAs

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Competing interests: The authors declare that no competing interests exist.

Funding: See page 22

Received: 19 January 2017
Accepted: 15 June 2017
Published: 26 June 2017

Reviewing editor: Elisa Izaurralde, Max Planck Institute for Developmental Biology, Germany

Abstract The RNA binding protein, LARP1, has been proposed to function downstream of mTORC1 to regulate the translation of 5'TOP mRNAs such as those encoding ribosome proteins (RP). However, the roles of LARP1 in the translation of 5'TOP mRNAs are controversial and its regulatory roles in mTORC1-mediated translation remain unclear. Here we show that LARP1 is a direct substrate of mTORC1 and Akt/S6K1. Deep sequencing of LARP1-bound mRNAs reveal that non-phosphorylated LARP1 interacts with both 5' and 3'UTRs of RP mRNAs and inhibits their translation. Importantly, phosphorylation of LARP1 by mTORC1 and Akt/S6K1 dissociates it from 5'UTRs and relieves its inhibitory activity on RP mRNA translation. Concomitantly, phosphorylated LARP1 scaffolds mTORC1 on the 3'UTRs of translationally-competent RP mRNAs to facilitate mTORC1-dependent induction of translation initiation. Thus, in response to cellular mTOR activity, LARP1 serves as a phosphorylation-sensitive molecular switch for turning off or on RP mRNA translation and subsequent ribosome biogenesis.
DOI: 10.7554/eLife.25237.001

Introduction

Mechanistic target of rapamycin complex 1 (mTORC1) functions as a positive regulator of translation initiation and protein synthesis to promote cell growth and proliferation (***Bhat et al., 2015***; ***Dibble and Manning, 2013***). Short-term treatment with rapamycin, an allosteric mTORC1 inhibitor, only partially inhibits global protein synthesis but effectively blocks the translation of certain 5' terminal oligopyrimidine tract (5'TOP) mRNAs (***Hinnebusch et al., 2016***; ***Jefferies et al., 1997***; ***Meyuhas and Kahan, 2015***). In contrast, recent studies using newly developed specific mTOR kinase inhibitors such as Torin1 demonstrate that complete inhibition of cellular mTOR kinase activity results in strong suppression of nearly all mRNA translation (***Hsieh et al., 2012***; ***Thoreen et al., 2012***). However, the sensitivity of translation inhibition by mTOR kinase inhibitors still varies significantly among different mRNAs, and the translation of mRNAs containing pyrimidine-enriched sequence (PES) in their 5'UTRs (i.e., 5'TOP, TOP-like, and pyrimidine rich translation element (PRTE) sequences) is much more effectively inhibited. Moreover, the sensitivity of translation inhibition by mTOR inhibitors also varies within PES-containing mRNAs.

The 4EBP family of proteins have been proposed to play a key role in suppressing the translation of PES-containing mRNAs (***Thoreen et al., 2012***). However, the molecular mechanisms by which

inhibition of active eIF4F complex formation by 4EBPs further potentiates translation inhibition of PES-containing mRNAs remain elusive (**Miloslavski et al., 2014**). Recent studies demonstrate that La-related proteins 1 (LARP1), an evolutionarily conserved RNA binding protein, interacts with components of the active eIF4F complex and mTORC1 and regulates the translation of TOP mRNAs (**Tcherkezian et al., 2014**). LARP1 directly interacts with the TOP sequences of 5'TOP mRNAs such as those that encode ribosome proteins (RP) in vitro and stabilizes RP mRNAs in vivo (**Aoki et al., 2013**; **Fonseca et al., 2015**; **Lahr et al., 2015**). However, the roles of LARP1 in mTORC1-mediated RP mRNA translation remain controversial because previous studies propose conflicting models wherein LARP1 functions as either a positive or negative regulator of RP mRNA translation (**Fonseca et al., 2015**; **Tcherkezian et al., 2014**). Furthermore, how LARP1 involves in mTORC1-mediated RP mRNA translation also remains unclear.

In this report, we investigated the molecular mechanisms of LARP1 function in the mTORC1-mediated translation of RP mRNAs. We first identified mRNAs and sequences directly bound by endogenous LARP1 in vivo under normal growing and mTORC1-inhibited conditions using photoactivatable ribonucleoside–enhanced crosslinking and immunoprecipitation (PAR-CLIP) (**Hafner et al., 2010**). As predicted, LARP1 directly interacts with pyrimidine-enriched sequences (PES) of mRNAs such as RP mRNAs that significantly overlap with those regulated by mTOR activity. However, LARP1 interacts with the 3'UTR of RP mRNAs under growth conditions while it also binds to specific PES at the 3'end of their 5'UTRs when mTOR activity is inhibited. Thus, LARP1 may not be a bona fide 5'TOP binding protein in vivo. We identified that these dynamic LARP1 interactions with RP mRNAs are regulated through direct phosphorylations of LARP1 by mTORC1 and Akt/S6K1. Phosphorylation of LARP1 induces its dissociation from the PES in 5'UTRs but enhances its binding to 3'UTRs of RP mRNAs. Importantly, phosphorylated LARP1 also functions as a scaffolding protein for mTORC1 on translationally-competent LARP1-interacting mRNAs to facilitate mTORC1-dependent phosphorylation of its substrate proteins, 4EBP1 and S6K1, processes that are essential for translation initiation and elongation. Thus, the spatial recruitment of mTORC1 by LARP1 to specific translational machinery may provide significant advantages for the translation of LARP1-associated RP mRNAs. As a unique substrate of mTORC1 and Akt/S6K1, we propose that LARP1 functions as a phosphorylation-sensitive molecular switch in the translation of an essential class of mRNAs as well as an important regulator of mTORC1 itself.

Results

Dynamic LARP1 interaction with RP mRNAs in an mTOR activity-dependent manner

While several recent studies have indicated that LARP1 associates with 5'TOP mRNAs through their TOP sequences or polyA tails (**Aoki et al., 2013**; **Lahr et al., 2015**), the comprehensive identity and sequence characteristics of mRNAs that preferentially interact with LARP1 have not been defined. To address this gap, we performed PAR-CLIP of endogenous LARP1 in HEK293T cells in the presence or absence of an mTOR inhibitor (PP242), followed by deep sequencing of the LARP1-bound RNA substrates (The data set was deposited: GEO: GSE59599). One advantage of PAR-CLIP over conventional UV crosslinking methodologies is the signature of specific T-to-C conversions in the resulting sequencing reads that mark where the incorporated 4-thiouracil of RNAs form covalent linkages with the interacting protein (**Hafner et al., 2010**). Sequenced reads were mapped using Bowtie (**Langmead et al., 2009**) to the human transcriptome, clustered to derive LARP1 binding sites, and filtered to retain clusters containing 0–2 T-to-C conversion events and passing an empirically-derived reads per million mapped reads (RPM) threshold (details in Materials and methods; ***Figure 1—figure supplement 1A***; ***Supplementary file 1***). In parallel, replicate mRNA-seq experiments were performed in the presence or absence of PP242 to quantify gene expression and normalize LARP1 binding sites to mRNA abundance levels. We identified 1200 and 1,900 LARP1 binding sites on 1000 and 1500 mRNAs in the presence or absence of PP242, respectively (***Figure 1—figure supplement 1A and B***).

Gene ontology (GO) term enrichment analysis of genes bound by LARP1 in growing conditions revealed enrichment for terms related to translation. LARP1 was bound to 137 translation-related genes, including 42 genes encoding ribosomal proteins (RP), as well as genes involved in cellular

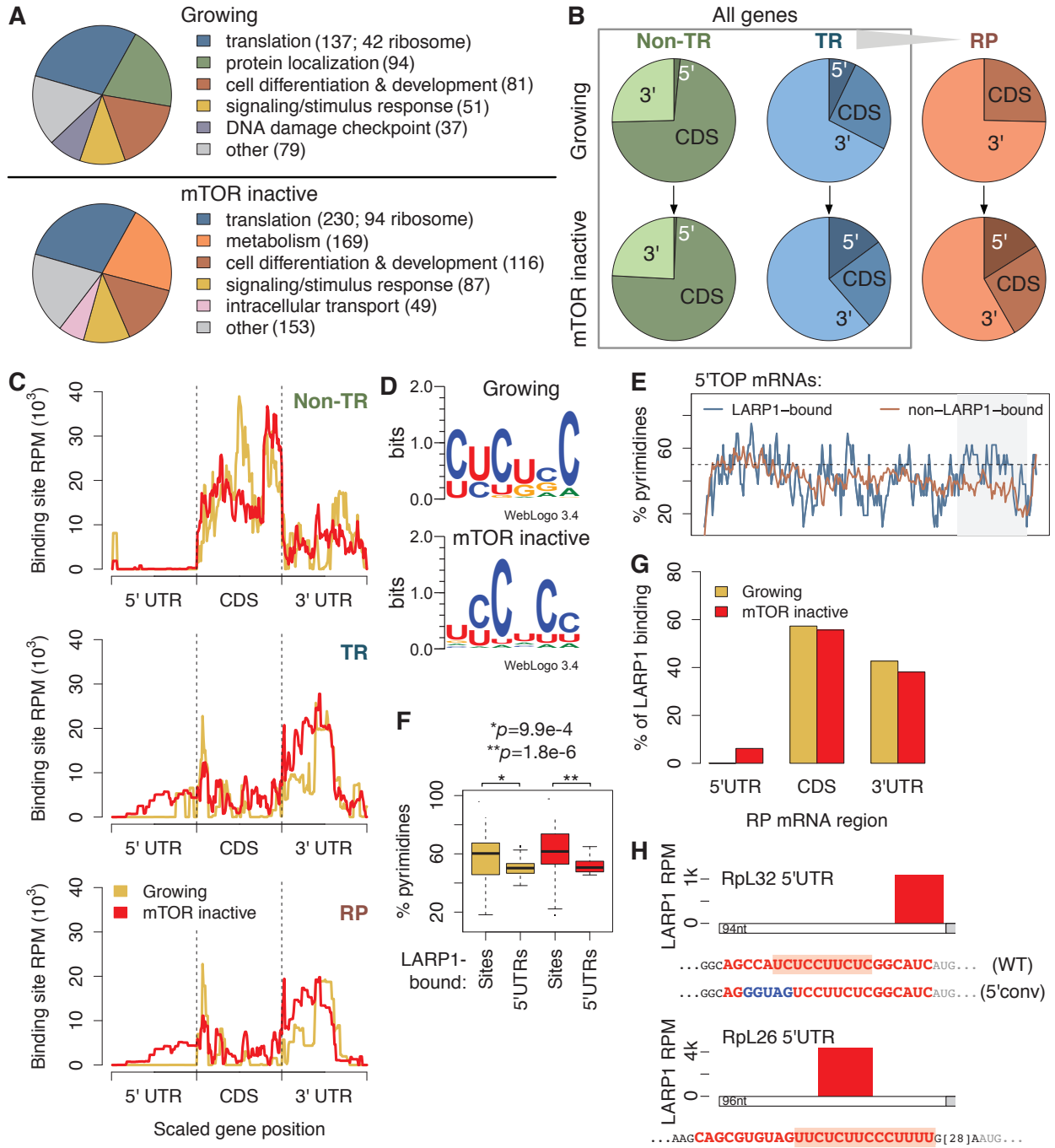

Figure 1. LARP1 binds pyrimidine-rich 5'UTR regions of translation-related transcripts. (**A**) LARP1-bound genes are most enriched for GO terms related to translation including RP genes. (**B**) Upon mTOR inactivation, LARP1 binding at 5'UTRs increases on TR and RP genes. (**C**) LARP1 binding at TR and RP 5'UTRs under mTOR-inactive conditions tends to occur at the 3' end. (**D**) LARP1 binds directly to pyrimidine-enriched sequences in 5'UTRs. (**E**) The LARP1 binding sites at the 3' end of 5'TOP-containing 5'UTRs are enriched for pyrimidines. (**F**) LARP1-bound sites on 5'UTRs are enriched for pyrimidines compared to the rest of the 5'UTR sequence. Welch's two-tailed *t*-test: *p=1.4e-15 and **p=1.2e-18. (**G**) LARP1 binding on RP-encoding mRNAs is gained at 5'UTRs upon mTOR inactivation and slightly decreased at CDSs and 3'UTRs. (**H**) The locations (red box), sequences (red color), and

Figure 1 continued on next page

Figure 1 continued

motifs (orange background) of the 5′ UTR LARP1 binding site in RpL32 and RpL26 mRNAs under mTOR-inactive conditions. Substituted nucleotides are highlighted by blue color.

DOI: 10.7554/eLife.25237.002

The following figure supplement is available for figure 1:

Figure supplement 1. LARP1 binds 5′UTR pyrimidine-rich regions of translation-related transcripts.

DOI: 10.7554/eLife.25237.003

differentiation and development (***Figure 1A***; ***Supplementary file 2***). Under mTOR-inactive conditions, more (230) translation-related genes were bound by LARP1, including 94 RP-encoding genes (***Figure 1A***; ***Supplementary file 2***). These results indicate that LARP1 substrates are enriched for mRNAs encoding factors involved in translation, and that this interaction is enhanced under conditions of mTOR inactivation.

To identify where LARP1 binds across a transcript, we summed LARP1 binding coverage across the 5′UTR, CDS, and 3′UTR regions of its targets, which were separated into non-translation-related (non-TR) genes, translation-related (TR) genes, and the subset of TR genes encoding RPs (***Figure 1B***; ***Supplementary file 3***). Strikingly, LARP1 binding at 5′UTRs of TR genes more than doubled upon mTOR-inactivation, and binding on RP genes increased from 0% to 17% (***Figure 1B***). To further explore this observation, we plotted the accumulation of LARP1 binding under growing and mTOR-inactive conditions along normalized gene lengths (***Figure 1C***). Across non-TR mRNAs, LARP1 preferentially associated with CDSs and 3′UTRs, but was almost completely absent from 5′UTRs. In contrast, LARP1 bound most strongly to 3′UTRs of TR and RP mRNAs under growing conditions. Importantly, under conditions of mTOR inactivation, LARP1 accumulated at 5′UTRs, with the majority of 5′UTR binding occurring on RP transcripts (***Figure 1C***).

Since LARP1 regulates PES-containing mRNAs, including 5′TOP sequences, we searched the 58 and 92 5′UTR LARP1 binding sites under growing and mTOR-inactive conditions, respectively, for a consensus motif using MEME. We identified six consecutive pyrimidines in all 5′UTR LARP1 binding sites, suggesting that LARP1 binds directly to PESs (***Figure 1D***). Surprisingly, 5′UTR LARP1 binding sites rarely overlapped with 5′TOP sequences, which are located at the 5′-most end of 5′UTRs; instead, LARP1 binds predominantly at the 3′-most end of 5′UTRs (***Figure 1C and H***; ***Supplementary file 4***). In fact, 5′TOP-containing 5′UTRs bound by LARP1 are more pyrimidine-rich at their 3′ ends than those not bound by LARP1 (***Figure 1E***). To confirm that LARP1 binds PESs within target 5′UTRs, we compared pyrimidine-richness of LARP1-bound regions to non-LARP1-bound regions of these 5′UTRs and observed a significantly higher proportion of pyrimidines in LARP1-bound regions under both growing (Welch's two-tailed *t*-test: p=1.4e-15) and mTOR-inactive (p=1.2e-18) conditions (***Figure 1F***). Taken together, our data suggest that LARP1 specifically recognizes and binds PESs at the 3′-end of 5′UTRs for a subset of TR and RP transcripts and that LARP1 is not a genuine 5′TOP RNA binding protein (RBP) in vivo.

LARP1 also binds CDSs of non-TR genes and 3′UTRs of TR and RP mRNAs. We identified GA-rich motifs in 9–15% of these sites under both conditions (***Figure 1—figure supplement 1C***). LARP1-bound 3′UTR regions are slightly, but significantly, enriched for higher G-content than non-LARP1-bound regions on the same 3′UTRs (***Figure 1—figure supplement 1D***). These motifs are similar to ones identified for RRM domain-containing RBPs in a recent systematic in vitro study characterizing the sequence-specific recognition sites for RBPs across 24 eukaryotes (***Ray et al., 2013***), suggesting a possible role of LARP1's RRM domain (***Bayfield et al., 2010***) to interact with CDSs and 3′UTRs.

The relationship between LARP1 binding and decreases in translational efficiency (TE) upon mTOR inactivation are paralleled in mouse embryonic fibroblasts. We obtained measurements of changes in mouse transcript TE upon treatment of cells with Torin1 (***Thoreen et al., 2012***). Thirty-three percent of human homologs of mouse genes exhibiting decreased TE were bound by LARP1 in mTOR-inactive conditions compared to only 12% and 14% of genes showing no change in or increased TE, respectively (***Figure 1—figure supplement 1E***). We next wondered if increased pyrimidine richness observed at 3′ ends of LARP1-bound 5′UTRs is functionally linked to mTOR-dependent changes in TE rates. We compared 5′UTR pyrimidine content of mouse RP-encoding mRNAs exhibiting the greatest changes in TE to those exhibiting the least and saw no difference at the 5′-most region of the 5′UTRs (***Figure 1—figure supplement 1F***). Strikingly, however, the 3′-most

5'UTR region of the most affected genes contained a significantly higher proportion of pyrimidines compared to the least affected genes (Welch's two-tailed *t*-test: p=0.036), indicating that pyrimidine richness at LARP1-interacting regions of 5'UTRs is correlated with strong decreases in TE upon mTOR inactivation. Together, these results suggest that the relationship between LARP1 binding and decreased TE is conserved from mouse to human.

Of the 88 annotated human ribosomal proteins, mRNAs encoding 84 were expressed in our mRNA-seq libraries under both conditions. Summing LARP1 binding site coverage of these genes confirms increased LARP1 binding at 5'UTRs and slightly decreased LARP1 binding at CDS and 3'UTR upon mTOR inactivation (***Figure 1G***). We verified the specific interaction between endogenous LARP1 and mRNAs encoding RpS6, S3A, S18, L26, and L32 by RNA immunoprecipitation (RIP) assays followed by quantitative PCR (qPCR) (***Figure 1—figure supplement 1G***). Taken together, these observations raise the intriguing possibility that the function of LARP1 in regulating RP mRNA translation may be context-dependent: the interaction of LARP1 with PESs in the 5'UTRs of RP mRNAs may have an inhibitory role, whereas its interaction with 3'UTRs may exert a positive role in RP mRNA translation.

LARP1 is a direct substrate of Akt/S6K1 and mTORC1

To investigate the mechanisms by which site-specific LARP1 interaction with RP mRNAs is regulated by the activity of mTOR, we examined the roles of post-translational modifications of LARP1 regulated by mTOR activity. Previous genome-wide phospho-mass spectrometry analyses showed that LARP1 is a highly phosphorylated protein and has Torin1-sensitive phosphorylation sites (***Hsu et al., 2011***; ***Kang et al., 2013***; ***Yu et al., 2011***), suggesting that phosphorylation of LARP1 may regulate the configurations of LARP1-RP mRNA interaction. Phospho-mass spectrometry analysis targeting endogenous LARP1 revealed that more than 10 LARP1 phosphorylation sites, eight (highlighted by red color) of which were significantly sensitive to short-term Torin1 treatment (***Figure 2A*** and ***Figure 2—figure supplement 1A***), were identified. Among the Torin1-sensitive phosphorylation sites, two serine residues (Ser770 and Ser979), which follow the typical consensus motif (RxRxx[S/T]) for AGC kinases (***Figure 2B***), were directly phosphorylated by S6K1 or Akt in vitro (***Figure 2C and D***, and ***Figure 2—figure supplement 1B***). Further, in vitro kinase assays using multiple polypeptides containing these Torin1-sensitive phosphorylation sites identified that Ser689 and Thr692, which do not match the AGC kinase phosphorylation motif (***Figure 2B***), were directly phosphorylated by mTOR (***Figure 2E***). These observations indicate that LARP1 is a direct substrate of mTOR, S6K, and Akt. Overexpression of active S6K1 or Akt induced phosphorylation of the RxRxx[S] motifs of wild type LARP1 but not the LARP1 2A mutant, where both Ser770 and Ser979 were substituted with alanine, in vivo (***Figure 2F and G***). Active Akt-induced LARP1 phosphorylation was not inhibited by rapamycin or S6K1 inhibitor (PF-4708671: PF), confirming that Akt is also able to directly phosphorylate LARP1 in vivo (***Figure 2—figure supplement 1C***). The phosphorylation of AGC kinase sites of LARP1 is sensitive to amino acids or amino acids/growth factor stimulation (***Figure 2H***). To investigate the functional roles of endogenous S6K1 and Akt in the phosphorylation of LARP1, we examined the phosphorylation status of LARP1 under acutely stimulated (growth factor/amino acid starvation for 60 min then stimulation with growth factor/amino acid for 10 min) or steady state growth conditions with or without the addition of rapamycin, Torin1, or a specific Akt inhibitor, MK-2206 (***Figure 2I***). Upon growth factor/amino acid stimulation, the phosphorylation of both Akt and S6K1 was enhanced compared to those under starvation conditions. Simultaneously, the phosphorylation of LARP1, as detected by both the Akt substrate antibody and the pS979 LARP1 antibody, was enhanced. Importantly, under this acutely stimulated condition, rapamycin, which completely inhibits S6K1, but not Akt, had little effect on S770/S979 phosphorylation of LARP1. In contrast, the Akt inhibitor MK-2206, as well as Torin1, which inhibit both Akt and S6K1, largely inhibited S770/S979 phosphorylation of LARP1.

Under the steady state growth condition, inhibition of S6K1 with rapamycin or PF 4708671, a S6K inhibitor, equally and significantly decreased LARP1 phosphorylation, and Torin1 and MK-2206 further blocked these phosphorylations. Taken together, these observations indicate that Akt is a physiologically relevant primary kinase for S770/S979 phosphorylation of LARP1 especially under acute stimulatory conditions. Under steady state conditions, S6K plays a major role while Akt also partially contributes to the phosphorylation of LARP1 (***Figure 2J***).

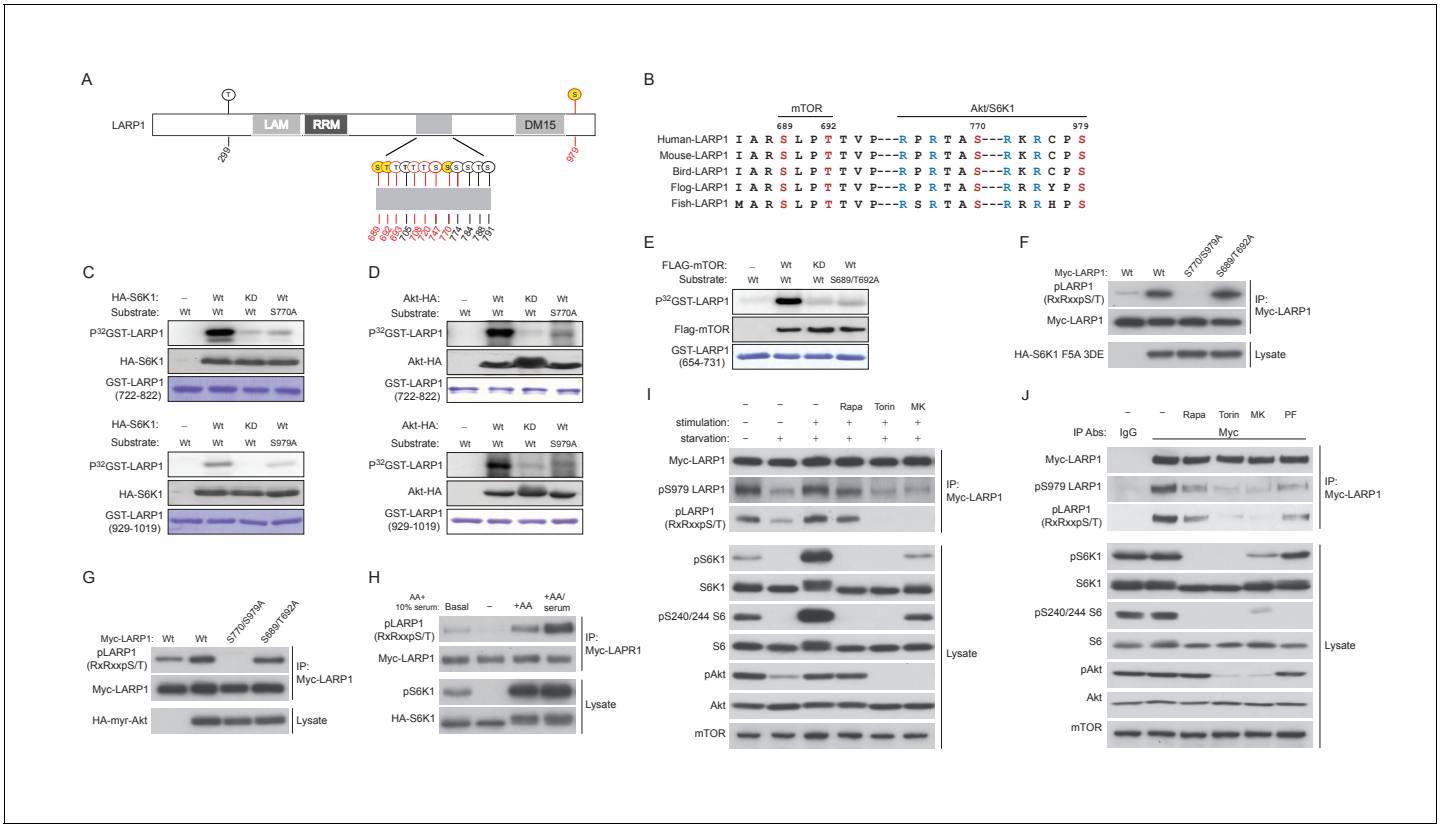

Figure 2. LARP1 is a direct substrate of mTOR, Akt, and S6K1. (**A**) Schematic position of LARP1 phosphorylation sites identified by liquid chromatography coupled to electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS). (**B**) Location and sequence conservation of LARP1 phosphorylation sites. (**C–E**) S6K1 (**C**), Akt (**D**), and mTOR (**E**) directly phosphorylate LARP1 in vitro. In vitro kinase assay (IVK) were performed with the indicated wild type kinase (WT) and inactive kinase (KD) purified from HEK293T cells using the indicated GST-LARP1 fragments. (**F–G**) Active S6K1 (**F**) or Akt (**G**) enhances phosphorylation of wild-type LARP1 but not the S770A/S979A LARP1 mutant in HEK293T cells. Phosphorylation of LARP1 was detected by phospho-specific-Akt substrate antibody. (**H**) Levels of LARP1 phosphorylation sites of AGC kinases are enhanced by amino acids or amino acids/growth factors. (**I**) Amino acids/growth factors-inducible S770/S979 phosphorylation of LARP1 is partially inhibited by rapamycin but largely inhibited by Torin1 or MK-2206. HEK293T cells were serum starved over night and incubated with HBSS with or without the indicated inhibitors for 1 hr before stimulation with DMEM containing 10% FBS for 10 min. (**J**) Levels of S770/S979 phosphorylation of LARP1 are decreased by rapamycin or S6K1 inhibitor (PF 470861) and further decreased by Torin1 or MK-2206 under steady state growth conditions.

DOI: 10.7554/eLife.25237.004

The following figure supplement is available for figure 2:

Figure supplement 1. LARP1 is a direct substrate of mTOR, Akt, and S6K1.

DOI: 10.7554/eLife.25237.005

LARP1 functions as a phosphorylation-sensitive molecular switch for RP mRNA translation

Our PAR-CLIP data indicated that while LARP1 primarily interacts with 3'UTRs of RP mRNAs under growth conditions, it also binds to 5'UTRs of RP mRNAs upon mTOR inactivation (***Figure 1C***). To investigate the roles of LARP1 phosphorylation in binding RP mRNAs, we examined specific interactions between LARP1 and the 5' or 3'UTR of RpL32 mRNA as a representative example of RP mRNAs bound by LARP1 identified in our PAR-CLIP analyses (***Figure 1H***). In agreement with our PAR-CLIP data, RIP assays demonstrated that endogenous LARP1 interacted with both the 5'UTR (closed bar) and 3'UTR (open bar) of RpL32 under amino acid starvation conditions (***Figure 3A***). In addition, in response to amino acids, which enhances mTORC1 activity, the interaction between LARP1 and the RpL32 5'UTR was decreased in a manner dependent on cellular mTOR kinase activity. In contrast, the binding of LARP1 to the RpL32 3'UTR was increased in response to mTOR activation.

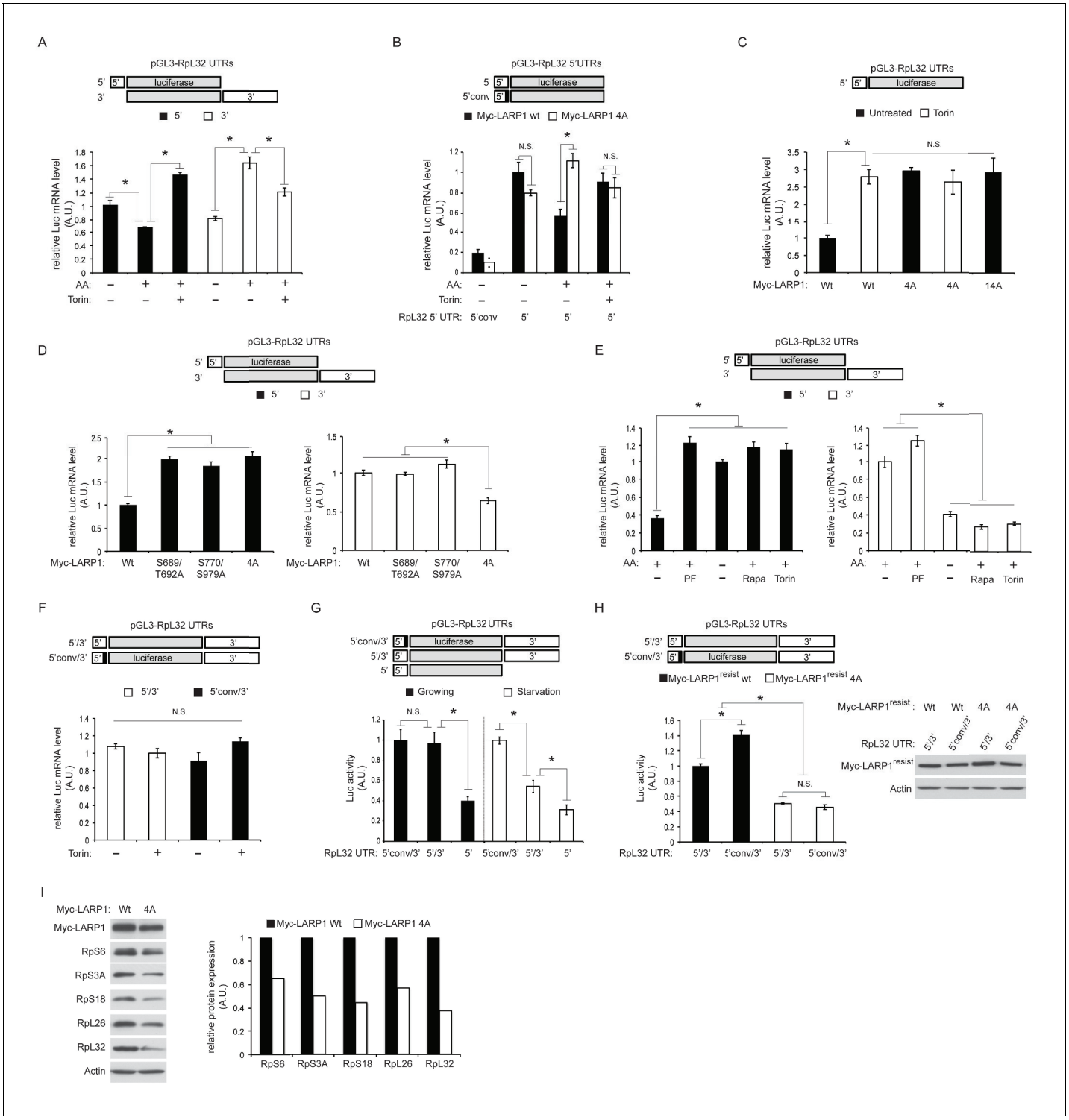

Figure 3. Dynamic rearrangement of LARP1 biding to the UTRs of RpL32 mRNA is regulated by the phosphorylation of LARP1. (**A**) The effect of amino acid and mTOR inhibitor on the levels of endogenous LARP1 binding to the 5′ and 3′UTR of RpL32 mRNA. HEK293T cells were transfected with the indicated reporter mRNAs. Endogenous LARP1 was IPed, and the levels of co-IPed luciferase mRNA were determined by qPCR. Data were normalized by input luciferase mRNAs and the amount of IPed LARP1. *p<0.05, mean±SEM (n = 3). (**B**) LARP1 phosphorylation by mTOR and S6K1/Akt induces its dissociation from the PES in the 5′UTR of RpL32 mRNA. The wild type and the LARP1 4A mutant were transfected with the indicated reporter mRNAs. Data were expressed as ***Figure 3A***. N.S. denotes 'not significant'. *p<0.05, mean±SEM (n = 3). (**C**) The effect of alanine substitutions of all the phosphorylation sites of LARP1 identified in this study on the binding to the 5′UTR of RpL32 mRNA under growth conditions. N.S. denotes 'not significant'. *p<0.05, mean±SEM (n = 3). (**D–E**) Both mTOR- and S6K1/Akt-dependent LARP1 phosphorylation are necessary for its dissociation from the

Figure 3 continued on next page

Figure 3 continued

5'UTR of RpL32 mRNA, while either mTOR or S6K1/Akt phosphorylation of LARP1 is sufficient to maintain its binding to the 3'UTR. The wild type and the indicated LARP1 mutants were IPed, and the levels of co-IPed 5' or 3' reporter mRNA were determined by qPCR (**D**). HEK293T cells were starved with amino acids or treated with the indicated inhibitors for 1 hr, and levels of co-IPed 5' or 3' reporter mRNA with endogenous LARP1 were determined (**E**). Data were normalized by input luciferase mRNAs and the amount of IPed LARP1. *p<0.05, mean±SEM (n = 3). (**F**) LARP1 constitutively interacts with the RpL32 reporter RNA containing both the 5' and 3' UTRs in a manner independent of the PES in the 5'UTR and mTOR activity. Endogenous LARP1 PAR-CLIP was performed in the presence or absence of Torin1 treatment. Levels of LARP1-bound reporter mRNA were determined by qPCR. Data were normalized by input luciferase mRNAs and the amount of IPed LARP1. N.S. denotes 'not significant', mean±SEM (n = 3). (**G**) The PES motif on 5'UTR of RpL32 is the cis-acting element necessary for translation inhibition in response to mTORC1 inhibition. HEK293T cells were transfected with the indicated reporter mRNAs and grown in normal growth media (10% serum) or mild starvation media (1% serum). After 24 hr, luciferase activity was measured and normalized by luciferase mRNA levels. *p<0.05, mean±SEM (n = 3). (**H**) Phosphorylation of LARP1 and its dissociation from the 5'UTR are critical for the translation of RpL32. HEK293T cells lacking endogenous LARP1 were transfected with the indicated shRNA-resistant LARPs and reporter mRNAs. 48 hr post-transfection, luciferase activity was measured and normalized by luciferase mRNA levels (left panel). *p<0.05, mean±SEM (n = 3). Levels of transfected Myc-LARP1s were shown (right panel). (**I**) Phosphorylated LARP1 plays a positive role in ribosomal protein translation. Myc-tagged wild type LARP1 and the 4A mutant LARP1 were stably expressed in HEK293T cells by retrovirus-mediated infection to achieve lower levels of LARP1 expression, and endogenous LARP1 was knockdown by LARP1 shRNA targeting its 5'UTR. Levels of RP proteins were determined by western blotting and the intensity of the bands was quantified.

DOI: 10.7554/eLife.25237.006

Importantly, phosphorylation of LARP1 (S689/T692 and S770/S979) identified in ***Figure 2*** plays key roles in LARP1 binding to both the 5' and 3' UTRs of RpL32 mRNA. Similar to endogenous LARP1, the interaction of wild-type myc-LARP1 (closed bar) with the 5'UTR of RpL32 is decreased by amino acid stimulation in a manner dependent on mTOR activity (***Figure 3B***). However, alanine substitutions of mTOR (S689/T692) and S6K1/Akt (S770/S979) phosphorylation sites confer the LARP1 4A mutant (open bar) resistant to its dissociation from the 5'UTR in response to amino acid stimulation. Furthermore, the PES located at the 3' end of the RpL32 5'UTR was essential for its interaction with LARP1 as substitutions of pyrimidines with guanines (5'conv) in the PES region within the 5'UTR (***Figure 1H***) dramatically reduced its interaction with both wild type LARP1 and LARP1 4A mutant even under amino acid starvation conditions (***Figure 3B***). The LARP1 14A mutant, which bears an additional 10 alanine substitutions in the phosphorylation sites identified in ***Figure 2A***, also displayed enhanced binding to the 5'UTR of RpL32 mRNA, similar to the LARP1 4A mutant compared to wild type LARP1 under growth culture conditions (***Figure 3C***). These results indicate that phosphorylation of these four serine/threonine residues plays a key role in reducing the interaction of LARP1 with the 5'UTR of RpL32 mRNA through the PES. Further analyses revealed that both mTOR- and S6K1-dependent LARP1 phosphorylation were required to reduce its binding to the 5'UTR of RpL32 mRNA, while either mTOR- or S6K1-dependent phosphorylation of LARP1 was sufficient to maintain its association with the 3'UTR under steady state growth condition (***Figure 3D***). Consistently, the inhibition of S6K1 activity with PF-4709671 sufficiently blocked the reduction of LARP1's binding to the 5' UTR of RpL32 mRNA as did amino acid starvation, rapamycin or Torin1 treatment, which inhibits both S6K1 and mTORC1 activity. However, PF-470961 treatment alone failed to induce the dissociation of LARP1 from the 3'UTR of RpL32 mRNA (***Figure 3E***). Together with our PAR-CLIP data, these observations indicate that the affinity of LARP1 binding to the 5' and 3' UTR of RpL32 mRNA dynamically changes in a manner dependent on mTOR and S6K1/Akt-dependent phosphorylation.

To examine the configuration of LARP1 binding to the 5'UTR and 3'UTR of RpL32 mRNA, we performed PAR-CLIP assays using endogenous LARP1 with the reporter mRNAs containing both the 5' and 3'UTR of RpL32 mRNA. Interestingly, under cross-linking conditions, LARP1 can bind the wild type (5'/3') or the 5'UTR-mutated (5'conv/3') reporter mRNA equally well in the presence or absence of Torin1 treatment, respectively (***Figure 3F***). These observations suggest that the same amount of LARP1 constantly interacts with the UTRs of RpL32 mRNA under both high and low mTOR activity conditions, although the affinity of LARP1 binding to the 5' and 3' UTR of RpL32 mRNA significantly changes in response to cellular mTOR/Akt activity. This raises the possibility that non-phospohrylated LARP1 may simultaneously interact with both the 5' and 3' UTR of the same RpL32 mRNA under low mTOR activity conditions while phosphorylated LARP1 dissociates from the 5'UTR and mainly interacts with the 3'UTR of RpL32 mRNA.

To examine the significance of LARP1 interaction with the 5′ or 3′UTR in the translation of RpL32 mRNA, we measured luciferase production from the indicated reporters (***Figure 3G***). Luciferase protein expression from the reporter containing both 5′ and 3′UTRs of RpL32 was consistently higher compared to the reporter with just 5′UTR, suggesting that the 3′ UTR is important for stimulating RpL32 translation. Both the 5′ UTR-mutated reporter (5′conv/3′) and the wild type reporter (5′/3′) generate similar levels of luciferase protein under growth conditions (***Figure 3G***, filled bars) that induce LARP1 dissociation from the 5′UTR. Importantly, under mild serum starvation conditions, the 5′UTR-mutated reporter (5′conv/3′) produces more luciferase protein compared to the wild type reporter (5′/3′) (***Figure 3G***, open bars). Furthermore, phosphorylatable wild type Myc-LARP1 showed some advantages in stimulating translation of the 5′conv/3′ reporter compared to the wild type 5′/3′ reporter in cells lacking endogenous LARP1 (***Figure 3H***). In contrast, the phopho-defective Myc-LARP1 4A mutant significantly and equally inhibited the translation of both reporters, regardless of the existence (5′/3′) or absence (5′conv/3′) of the LARP1 binding site in the 5′UTR in these cells. These observations indicate that the dissociation of LARP1 from the PES motif in the 5′UTR and the phosphorylation of LARP1, which enhances LARP1 binding to the 3′UTR, are important processes in stimulating RpL32 mRNA translation. Moreover, the data also suggest that the phosphorylation of LARP1 has an additional key role in stimulating the translation of RpL32 mRNA at the 3′UTR (see Figures 5 and 6). Note that the translation of the 5′conv/3′ mutant reporter was slightly, but significantly, enhanced compared to the 5′/3′ wild type reporter in cells expressing ectopic Myc-LARP1, which was not seen in the cells expressing just endogenous LARP1 (***Figure 3G***). We speculate that overexpressed Myc-LARP1 may not be fully phosphorylated in our experimental conditions. As a consequence, non-phosphorylated wild type LARP1 may still bind to the PES in the 5′UTR and reduce the translation of the wild type 5′/3′ reporter. Accordingly, cells expressing the LARP1 4A mutant, which strongly binds to the 5′UTR of RpL32 mRNA irrespective of cellular mTOR activity, have reduced expression of RP proteins including RpL32 (***Figure 3I***). Together with our PAR-CLIP data (***Figure 1***), these observations suggest that LARP1 stimulates RP mRNA translation through its interaction with the 3′UTR and that its association with the 5′UTR has a negative role in RP mRNA translation.

LARP1 recruits mTORC1 to LARP1-interacting mRNPs in a manner dependent of mTORC1 activity

To stimulate the translation of 5′TOP mRNAs, previous studies have proposed that LARP1 directly interacts with initiation factors and polyA-binding protein (PABP) in response to mTORC1 activity (***Aoki et al., 2013***; ***Tcherkezian et al., 2014***). In contrast, a more recent study proposed that active mTORC1 interacts with LARP1 and inhibits LARP1's function to suppress translation initiation of LARP1-interacting 5′TOP mRNAs (***Fonseca et al., 2015***). Thus, the functional importance of LARP1-mTORC1 interaction and the role of LARP1 in the regulation of translation of LARP1-interacting mRNAs remain unclear. We also observed that endogenous as well as exogenous LARP1 specifically and stably interacts with mTORC1 through Raptor under growth conditions (***Figure 4A*** and ***Figure 4—figure supplement 1A–C***). LARP1 exclusively interacts with mTORC1 but not with mTORC2 (***Figure 4A*** and ***Figure 4—figure supplement 1A***). mTOR sufficiently co-IPs LARP1 only in the presence of endogenous Raptor (***Figure 4—figure supplement 1B***). In addition, Raptor is able to co-IP LARP1 in the presence of non-ionic detergent (NP-40), which is known to disrupt the interaction between Raptor and mTOR (***Figure 4—figure supplement 1C***). These results indicate that LARP1 association with mTORC1 requires Raptor.

LARP1 also co-IPs PABP1, a polyA tail and eIF4G1 binding protein, as previously reported (***Blagden et al., 2009***; ***Burrows et al., 2010***; ***Tcherkezian et al., 2014***). However, whether LARP1 directly interacts with PABP1 or indirectly, perhaps through a common mRNA substrate, remains unresolved. To address the nature of LARP1 interaction with PABP1 and mTORC1, we treated cell lysates with RNaseA prior to or after LARP1 immunoprecipitation (***Figure 4B*** and ***Figure 4—figure supplement 1D***). As expected, LARP1 co-IPed not only mTORC1 and PABP1, but also eIF4E, a 5′CAP mRNA binding initiation factor. While LARP1 interaction with mTORC1 was resistant to RNaseA treatment, its interaction with PABP1 and eIF4E was markedly sensitive to RNaseA treatment. (***Figure 4B*** and ***Figure 4—figure supplement 1D***). In addition, eIF4G1, a scaffolding protein for forming the eIF4F complex, co-IPed other eIF4F components and LARP1 (***Figure 4B***). Although the interaction between eIF4G1 and other eIF4F components such as eIF4E, eIF3B, and PABP1 was

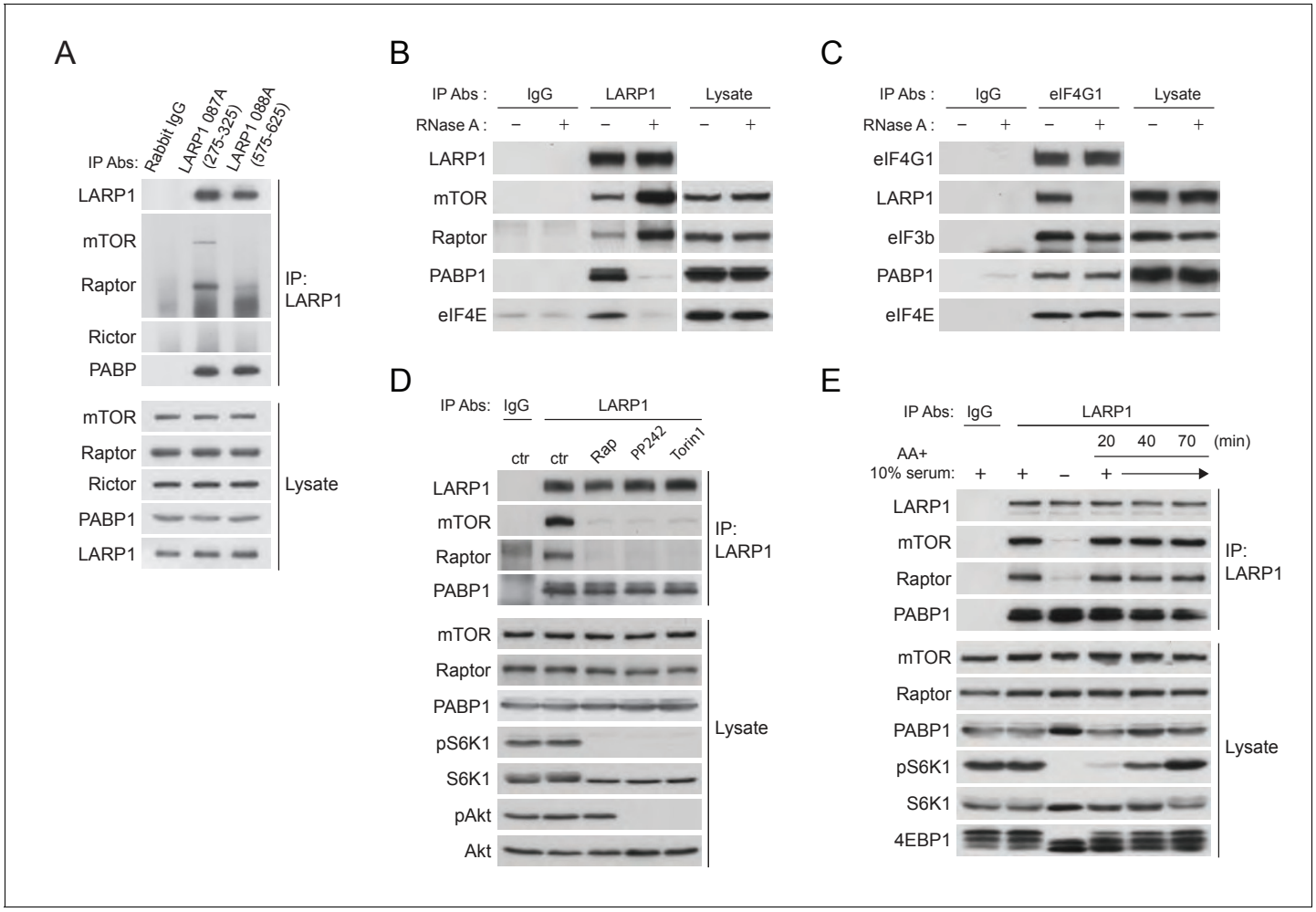

Figure 4. LARP1 recruits mTORC1 to LARP1-interacting mRNPs in a manner dependent of mTORC1 activity. (**A**) Endogenous LARP1 co-IPs endogenous mTORC1 in HEK293T cells. LARP1 antibody 087A, but not 088A, co-IPs mTORC1. The LARP1 antibody, 087A or 088A recognizes amino acids 275–325 or 575–625 of LARP1, respectively. (**B**) The effect of RNAse A on the interaction of LARP1 with mTORC1 and other mRNA binding proteins (PABP1 and eIF4E). Endogenous LARP1 was IPed from the lysates treated with RNAse A. (**C**) The effect of RNase A on the interaction of LARP1 with the components of the initiation complex. Endogenous eIF4G1 was IPed from the lysates treated with RNAse A. (**D**) LARP1 interacts with mTORC1 in a manner dependent on mTORC1 activity. (**E**) LARP1 co-IPs mTORC1 in a growth factor/amino acid stimulation-dependent manner.

DOI: 10.7554/eLife.25237.007

The following figure supplement is available for figure 4:

Figure supplement 1. LARP1 interacts with mTORC1 in an RNAse A insensitive manner.

DOI: 10.7554/eLife.25237.008

RNaseA resistant, the binding of eIF4G1 to LARP1 was abolished by the treatment with RNaseA. Interestingly, LARP1 coIPed more mTORC1 from lysates treated with RNaseA than in untreated lysates (***Figure 4B*** and ***Figure 4—figure supplement 1D***), suggesting that a substantial pool of the mTORC1-LARP1 complex is refractory to IP with the LARP1 antibody under standard lysis conditions and may exist in an RNaseA-sensitive pool. Taken together, these data indicate that LARP1 indirectly associates with PABP1 and initiation factors through binding of common mRNAs, while LARP1-mTORC1 interaction occurs through direct protein-protein contacts.

Importantly, the activity of mTORC1 plays a key role in LARP1's binding to mTORC1 but not to PABP1. LARP1 failed to interact with mTORC1 but maintained its interaction with PABP1 subjected to mTOR inhibitors, suggesting that LARP1 associates with mRNAs regardless of cellular mTOR activity (***Figure 4D***), consistent with our PAR-CLIP data (***Figure 1C***). Furthermore, the interaction between endogenous LARP1 and mTORC1 was quickly and fully restored by replenishment of amino

acids and growth factors after starvation of these activating cues, whereas the interaction between LARP1 and PABP1 was not affected by these cues (***Figure 4E***). Furthermore, recovery of the interaction between LARP1 and mTORC1 by growth factor/nutrient stimulation occurred concomitantly with 4EBP1 phosphorylation but precedes full phosphorylation of S6K1.

LARP1 scaffolds mTORC1 to LARP1-interacting mRNAs in a manner dependent on LARP1 phosphorylation

To investigate the mechanisms underlying the formation of the LARP1-mTORC1 complex, we determined regions of LARP1 necessary to associate with mTORC1. Interestingly, serial truncations of the LARP1 carboxyl terminus revealed that the DM15 motif and an N-terminal region adjacent to the DM15 motif (***Figure 5A***), where the majority of Torin1-sensitive LARP1 phosphorylations occur (***Figure 2A***), were critical for its association with mTORC1. The LARP family of proteins consists of

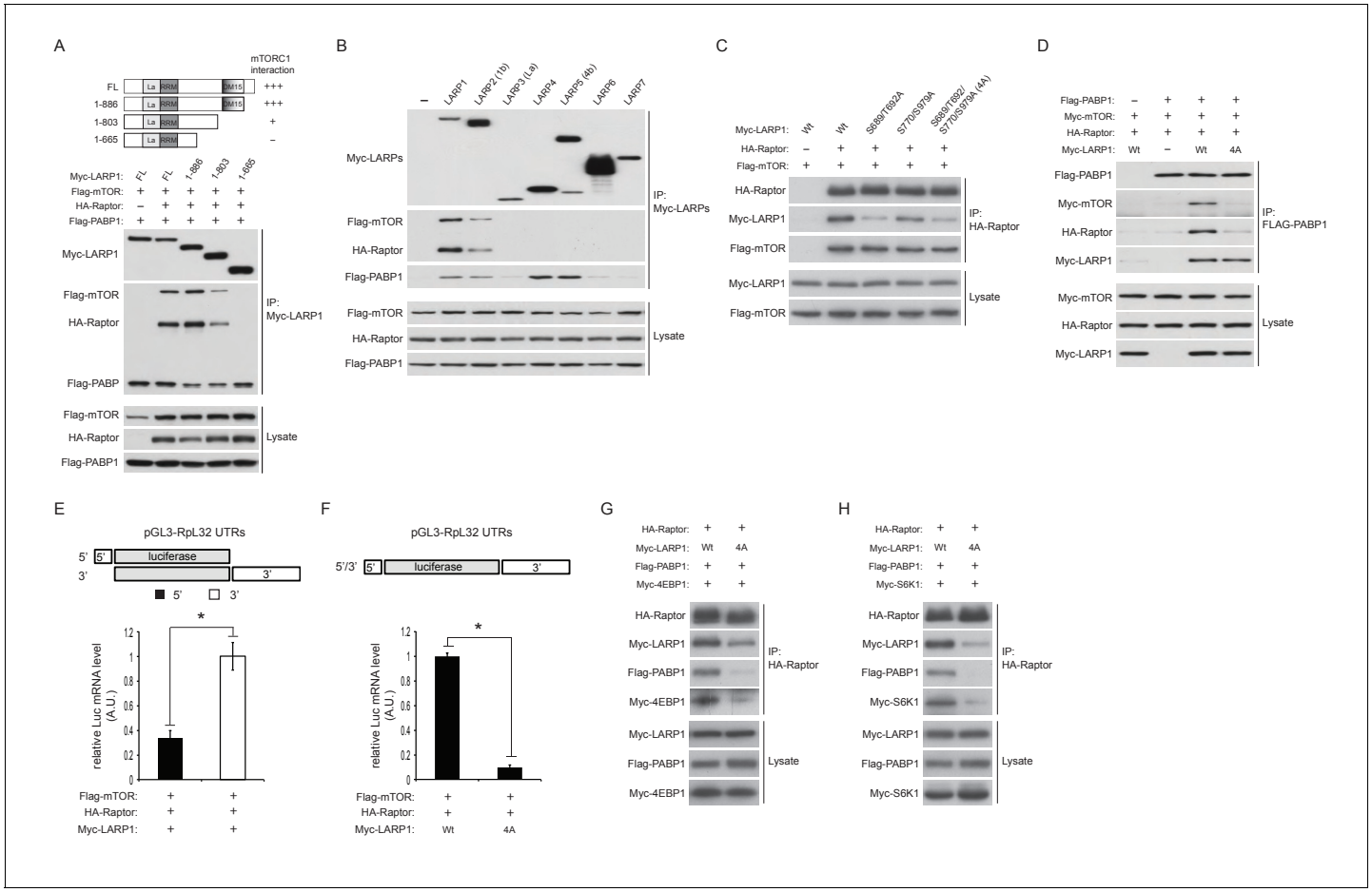

Figure 5. LARP1 scaffolds mTORC1 to LARP1-interacting mRNAs in a manner dependent on LARP1 phosphorylation. (**A**) The DM15 domain and its adjacent N-terminal region of LARP1 are required for the interaction with mTORC1. (**B**) LARP1 and LARP2 but not other LARP family members interact with mTORC1. (**C**) mTORC1-dependent LARP1 phosphorylation (S689/T692) plays a major role in the interaction between phosphorylated LARP1 and mTORC1. (**D**) mRNPs containing wild type LARP1 (Wt), but not the phospho-defective LARP1 (4A), associate with mTORC1. (**E**) mTORC1 preferentially interacts with the 3'UTR than the 5'UTR of RpL32 mRNA. HA-Raptor RIP assays were performed in the presence of wild type LARP1 with the indicated reporter mRNAs. Data were expressed as ***Figure 3A***. (**F**) mTORC1 interacts with the RpL32 reporter mRNA in the presence of wild type but not LARP1 4A mutant. HA-Raptor RIP assays were performed. (**G–H**) mTORC1 more interacts with its substrates, 4EBP1 (**G**) and S6K1 (**H**) in the presence of wild type LARP1 compared to the LARP1 4A mutant.

DOI: 10.7554/eLife.25237.009

The following figure supplement is available for figure 5:

Figure supplement 1. Schematic structure of human LARP family.

DOI: 10.7554/eLife.25237.010

six members comprised of LARP1, 2 (1B), 4, 5 (4B), 6, and 7 (***Figure 5—figure supplement 1***) (***Bousquet-Antonelli and Deragon, 2009***). Consistently, only LARP1 and LARP2, both of which possess the DM15 motif and an adjacent N-terminal region, interacted with mTORC1 (***Figure 5B***). Although LARP2, a close LARP1 paralog (59% amino acid sequence identity), interacted with mTORC1, its affinity for mTORC1 was significantly weaker than LARP1. These data suggest that the phosphorylations of LARP1 may also play an important role in maintaining LARP1-mTORC1 interactions, in addition to their roles in the binding of LARP1 with RP mRNAs.

To examine the role of LARP1 phosphorylation in the regulation of interaction between LARP1 and mTORC1, we first assessed mTORC1 binding with a series of phospho-defective LARP1 mutants (S689/T692A: mTOR sites, S770/S979A: Akt/S6K1 sites, and 4A: mTOR and Akt/S6K1 sites). While the replacement of LARP1 Akt/S6K1 phosphorylation sites with alanines only slightly reduced LARP1 interaction with mTORC1, mutations in the LARP1 mTOR phosphorylation sites largely abrogated its interaction with mTORC1 (***Figure 5C***). Importantly, both the wild type and LARP1 4A mutant were able to associate with PABP1 equally well through their binding to common mRNAs; however, PABP1 co-IPed mTORC1 only in the presence of wild-type LARP1 but not the LARP1 4A mutant (***Figure 5D***). These observations suggest that LARP1 phosphorylation by mTORC1 is critical for tethering mTORC1 to the LARP1-mRNP complex. In support of this hypothesis, under growth conditions where phosphorylated LARP1 mainly binds to the 3'UTRs of RP mRNAs (***Figures 1B, C*** and ***3A***), mTORC1 associated more with the 3'UTR than the 5'UTR of RpL32 mRNA in Raptor CLIP assays (***Figure 5E***). mTORC1 association with RpL32 mRNA was dependent on wild type LARP1 but not the LARP1 4A mutant (***Figure 5F***). Moreover, wild type LARP1 supports greater association of mTORC1 with its substrates S6K1 and 4EBP1 than the LARP1 4A mutant (***Figure 5G and H***). Taken together, these data suggest that while non-phosphorylated LARP1 blocks RP mRNA translation through its interaction with the 5'UTR of RP mRNAs, phosphorylated LARP1 is converted into a scaffolding protein for mTORC1 on the 3'UTRs of RP mRNAs to facilitate the accessibility of mTORC1 with 4EBP1 and S6K1.

To further investigate the roles of LARP1 in mTORC1-dependet phosphorylation of its substrates, endogenous LARP1 was knocked down in mammalian cells with varying degrees of insulin sensitivity. In HEK293T cells (insulin insensitive), ablation of LARP1 slightly decreased phosphorylation of S6K1, S6, and 4EBP1 compared to those in control cells under normal growth culture conditions (***Figure 6—figure supplement 1A***). However, in response to growth factor/amino acids stimulation, LARP1 knockdown significantly delayed and attenuated phosphorylation of these proteins in HEK293T cells (***Figure 6A*** and ***Figure 6—figure supplement 1A***), LnCap (prostate cancer cell line) (***Figure 6—figure supplement 1B***), and HEK293E cells (insulin sensitive) (***Figure 6—figure supplement 1C***). In contrast, Akt phosphorylation (Ser473) was not affected by the ablation of LARP1, indicating that the activity of PI3K and mTORC2 was intact in the LARP1 knockdown cells. In addition, LARP1 knockdown had little effect on intrinsic mTOR kinase activity monitored by mTOR auto-phosphorylation (Ser2481) (***Figure 6A***, ***Figure 6—figure supplement 1A–C***) (***Copp et al., 2009***; ***Peterson et al., 2000***), or on the integrity of mTORC1 and its kinase activity as measured by in vitro kinase assays (***Figure 6—figure supplement 1D***). The inhibitory effects of LARP1 knockdown on inducible S6K1 and 4EBP1 phosphorylation were not due to a disruption of eIF4F-mediated translation because knockdown of eIF4G1, which plays a key role in mTORC1-dependent translation (***Thoreen et al., 2012***), did not affect S6K1, S6, or 4EBP1 phosphorylation (***Figure 6B***).

Immunostaining of LARP1 and phosphorylated forms of 4EBP1 and S6 revealed that endogenous LARP1 was predominantly expressed in the cytoplasm where 4EBP1 and S6 phosphorylation occurs in response to growth factor and nutrient stimulation (***Figure 6C***). In addition, LARP1 did not colocalize with LAMP2 on the lysosomal membrane, where mTORC1 is activated. Consistent with the biochemical observations (***Figure 6A*** and ***Figure 6—figure supplement 1A–C***), LARP1 knockdown significantly reduced growth factor/nutrient-induced phosphorylation of 4EBP1 and S6 (***Figure 6C***). Notably, LARP1 knockdown (***Figure 6—figure supplement 1E***) or ectopic expression (***Figure 6D***) did not alter amino acid-induced mTORC1 localization on the lysosomal membrane, suggesting that, in addition to growth factor-induced PI3K/Akt activity, the amino acid-sensing mechanism on lysosomal membranes also remains intact in LARP1 knockdown and overexpressing cells. In response to amino acids, co-localization of Flag-LARP1 with mTOR became more obvious in cytosolic regions but not on the lysosomal membrane (***Figure 6D***, lower panels). Taken together, these data support

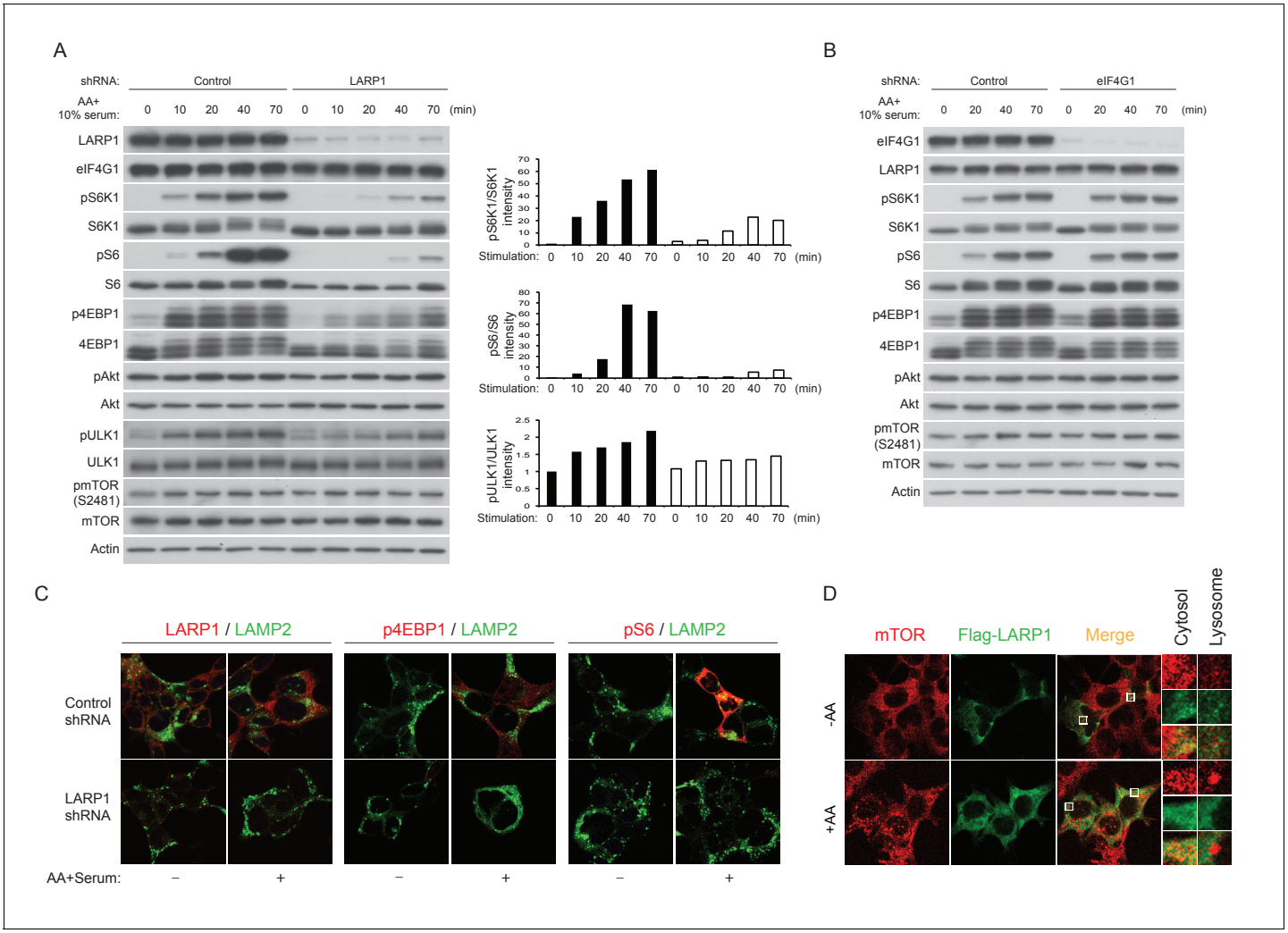

Figure 6. LARP1 enhances mTORC1-dependent phosphorylation of its substrates. (**A–B**) LARP1, but not eIF4G1, is required for growth factor/amino acid-induced phosphorylation of mTORC1 substrates in HEK293T cells. (**C**) LARP1 expresses in cytosolic region and supports growth factor/amino acid-induced S6 and 4EBP1 phosphorylation by mTORC1 in the cytosol. Note that neither LARP1 nor phosphorylated 4EBP1 co-localize with LAMP2, a lysosomal marker. (**D**) LARP1 co-localizes with mTOR at the cytosolic region in response to amino acid. Note that amino acid-inducible mTOR-positive punctate structures were shown as lysosomes.

DOI: 10.7554/eLife.25237.011

The following figure supplement is available for figure 6:

Figure supplement 1. LARP1 enhances mTORC1-dependent phosphorylation of its substrates.

DOI: 10.7554/eLife.25237.012

the idea that phosphorylated LARP1 facilitates mTORC1-dependent phosphorylation of S6K1 and 4EBP1 on the LARP1-containing mRNPs by scaffolding mTORC1.

Loss of LARP1 function causes inefficient RP translation elongation

In agreement with the results of ***Figure 6A***, m^7GTP pull down assays showed that the interaction of 4EBP1 with eIF4E was increased in LARP1 knockdown cells (***Figure 7A***), suggestive of increased levels of non-phosphorylated 4EBP1 in LARP1 knockdown cells. Consistently, LARP1 knockdown partially decreased eIF4E in fractions containing pre-initiation complex (43S and 48S: fraction 5 and 6) while Torin 1, an mTOR kinase inhibitor, largely eliminated eIF4E from these fractions (***Figure 7B***). These data indicate that a fraction of cellular translation initiation is decreased in LARP1 knockdown cells. However, intriguingly, RP mRNAs that interact with LARP1 accumulated more in lighter polysome fractions in LARP1 knockdown cells compared to control cells under normal growth conditions

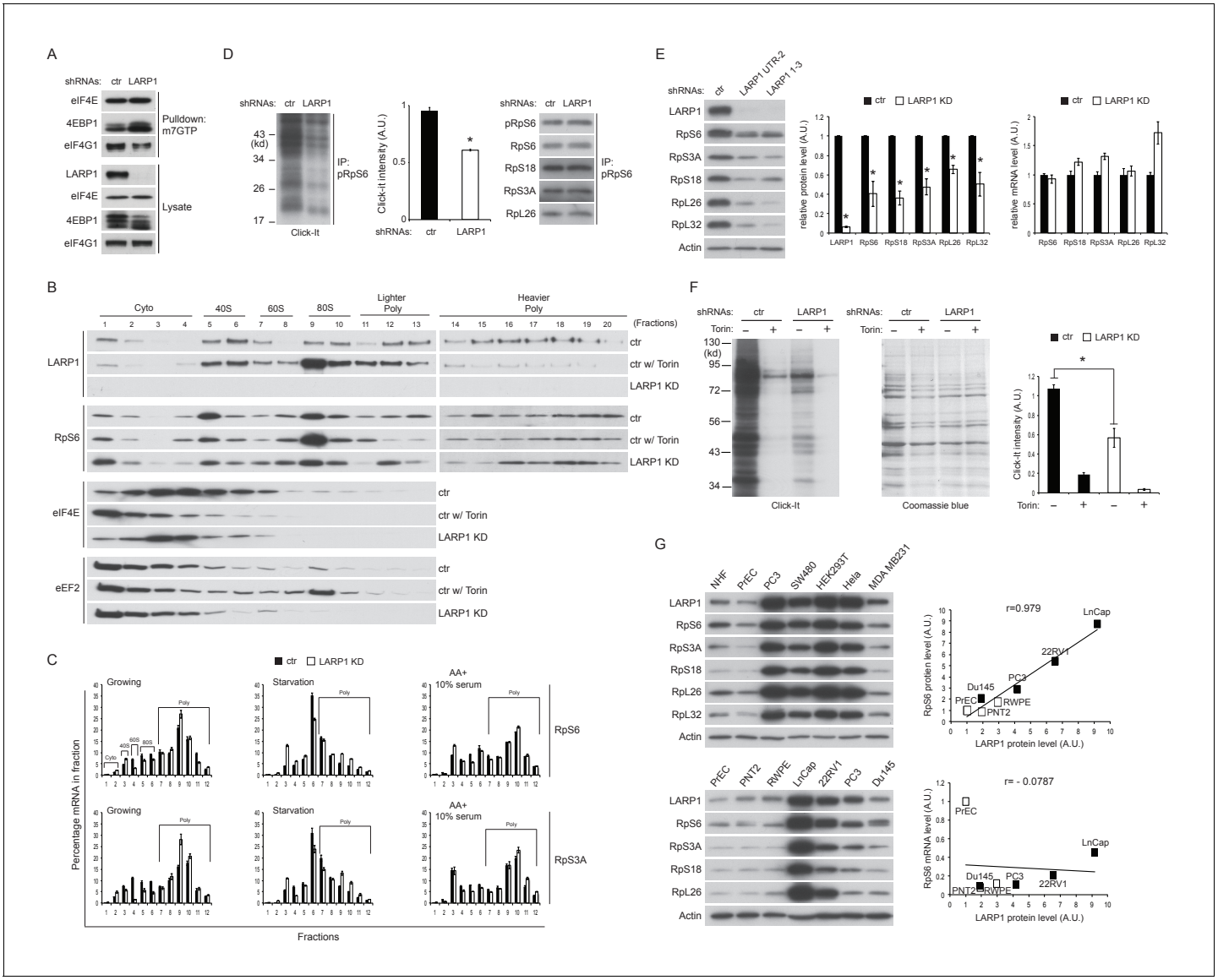

Figure 7. Loss of LARP1 causes defects in the multiple steps of RP mRNA translation. (**A**) LARP1 knockdown enhances 4EBP1 binding to the eIF4E precipitated with m^7GTP sepharose beads. (**B**) Loss of LARP1 decreases the expression of eEF2 in the fractions containing active monosomes (80S) and polysomes. (**C**) RP mRNAs are accumulated in the lighter polysome fractions in LARP1 knockdown cells. (**D**) Loss of LARP1 decreases the translation of RP mRNAs. Equal amount of ribosomes were immunoprecipitated by phopho-S6 antibody from normal growing HEK293T cells in the presence or absence of LARP1 expression (right panel). Newly synthesized ribosome subunits were visualized (left panel) and quantified (middle panel). *p<0.05, mean±SEM (n = 3). (**E**) Prolonged LARP1 knockdown (96 hr) decreases the expression of RP proteins (left panels). Levels of RP proteins (middle panel) were quantified and mRNA levels of RP proteins were monitored by qPCR (right panel). Newly synthesized RP proteins were monitored by the Click-It assy *p<0.05 vs control shRNA treatment, mean±SD (n = 3). (**F**) Prolonged LARP1 knockdown (96 hr) decreases global protein synthesis. p<0.05, mean±SD (n = 3). (left panel). Equal amount of protein loading was visualized by Coomassie blue staining (middle panel). Click-It reaction was quantitated (right panel). *p<0.05 vs control shRNA treatment, mean±SD (n = 3). (**G**) The expression of LARP1 and RP proteins is enhanced in multiple cancer and transformed cell lines (left panels). Correlation between LARP1 protein vs. RpS6 protein (right upper) or RpS6 mRNA (right lower) in prostate epithelial cells. Open or close square indicates normal or benign prostate epithelial cells or metastatic prostate cancer cells, respectively.

DOI: 10.7554/eLife.25237.013

The following figure supplements are available for figure 7:

Figure supplement 1. Loss of LARP1 causes defects in the multiple steps of RP mRNA translation.

DOI: 10.7554/eLife.25237.014

Figure supplement 2. Hypothetical model for mTORC1-dependent LARP1-interacting RP mRNA translation.

DOI: 10.7554/eLife.25237.015

(*Figure 7C*). In control cells, acute nutrient/growth factor starvation selectively redistributes RP mRNAs from the polysome fractions to the monosome (80S) fraction (*Figure 7C*, middle panels). In contrast, under the same starvation conditions, LARP1 knockdown results in retention of a substantial portion of RP mRNAs in the polysomes fractions while attenuating the accumulation of RP mRNAs in the monosome fraction (*Figure 7C*, middle panels). These observations suggest that RP mRNA translation is less sensitive to the inhibition caused by growth factor/nutrient depletion in LARP1 knockdown cells. Furthermore, replenishing growth factors and nutrients following identical starvation conditions showed similar RP mRNA distribution in polysome fractions in both control and LARP1 knockdown cells (*Figure 7C*, right panels). These results suggest that both ribosome dissociation from RP mRNAs and ribosome loading on RP mRNAs are inefficient, and that ribosome stalling may occur during RP mRNA elongation in LARP1 knockdown cells. However, non-LARP1 interacting mRNAs such as FOXO1 and YBX1 did not accumulate in polysome fractions in LARP1 knockdown cells, indicative of the specific roles of LARP1 in preventing ribosome stalling during the translation of LARP1 interacting mRNAs (*Figure 7—figure supplement 1A*). Indeed, in cells subjected to short-term LARP1 knockdown, the expression of eEF2 in the 80S monosome and polysome fractions was reduced (*Figure 7B*). Taken together, these data suggest that the efficiency of translation elongation of LARP1-interacting mRNAs is likely to be compromised in LARP1 knockdown cells.

To assess directly the flux of RP mRNA translation, we measured the rate of de novo RP protein synthesis. In vivo labeling experiments revealed that knockdown of LARP1 significantly reduced the levels of newly synthesized RP proteins (*Figure 7D*). Furthermore, prolonged LARP1 knockdown decreased RP protein expression (*Figure 7E*) and reduced global protein synthesis (*Figure 7F*) without significantly affecting levels of RP mRNAs. In multiple cancer cell lines (PC3 (prostate), SW40 (colon), HeLa (cervix), and MDA MB231 (breast)), expression levels of LARP1 and RP proteins were well correlated and often enhanced compared to those in non-transformed normal cells (human normal fibroblasts: HNF, and human normal prostate epithelial cells: PrEC) (*Figure 7G*, left upper panels). Furthermore, there was an especially clear trend of higher LARP1 and RP protein expression in multiple prostate cancer cell lines (LnCap, 22RV1, PC3, and Du145) compared with those in non-transformed (PrEC) or transformed (PTN2 and RWPE) normal prostate epithelial cells (left lower panels). Again, there was clear positive correlation between the expression of LARP1 and the RP proteins (e.g. RpS6, right upper panel) but not between LARP1 protein and RpS6 mRNA (right lower panel) in prostate cancer and normal cells. Finally, as previously demonstrated in other cancer cell lines (*Burrows et al., 2010*; *Hopkins et al., 2016*; *Tcherkezian et al., 2014*), the ablation of LARP1 largely blocked cell proliferation in multiple cancer cell lines including prostate cancer cells (*Figure 7—figure supplement 1B*). Together, these observations indicate that LARP1 functions as a phosphorylation-sensitive switch for inhibiting or stimulating the translation of RP mRNAs in response to the mTOR activity (*Figure 7—figure supplement 2*), thereby titrating cellular ribosomes and the capacity of cellular protein synthesis.

Discussion

In this study, we demonstrate the critical role of LARP1 in the mTORC1-dependent translation of PES-containing mRNAs, especially those that encode ribosome proteins. Key mechanistic insights from this study reveal that (1) LARP1 is a direct substrate of mTOR and S6K1/Akt; (2) the phosphorylation of LARP1 by the mTORC1 pathway triggers the dissociation of LARP1 from the 5'UTR of the RP mRNAs; (3) concomitantly, phosphorylation of LARP1 induces its stable interaction with mTORC1 and recruits mTORC1 to the 3'UTR of RP mRNAs; (4) LARP1 facilitates mTORC1-dependent phosphorylation of 4EBP1 and S6K1, a process essential for inducing translation initiation of RP mRNAs; and (5) while LARP1 is necessary for the blockade of RP mRNA translation in response to starvation, it is required for efficient translation elongation of RP mRNAs. Thus, LARP1 is a unique mTORC1 substrate and regulator, and functions as a molecular switch for turning off or on the translation of RP mRNAs (*Figure 7—figure supplement 2*).

Our data reveal that LARP1 mainly associates with the 3'UTR of mRNAs including RP mRNAs under high mTOR activity conditions, while it also directly binds to PES regions of RP 5'UTRs when mTOR activity is inhibited. Intriguingly, recent reports demonstrate that recombinant LARP1 binds to the TOP sequence of RpL32 and RpS6 in vitro (*Fonseca et al., 2015*; *Lahr et al., 2015*). However, our PAR-CLIP analyses using endogenous LARP1 showed that LARP1 binding within 5'UTRs of

5'TOP mRNAs, such as RpL32 mRNA, occurs predominantly at PES regions in the 3' end of the 5'UTRs and not at the 5'TOP sequences themselves in vivo, when mTORC1 activity is inhibited (***Figure 1C***). We posit that while LARP1 has a strong affinity for pyrimidine cluster sequences, the 5'TOP sequences of RP mRNAs are likely to be occupied by other 5'TOP-binding proteins in vivo. Alternatively, it is possible that mutations or deletions of 5'TOP sequences might structurally affect the function of the PES motifs of RP mRNAs in vitro. Our data demonstrate that the mutation of the PES in the 3' end of the 5'UTR largely reduce LARP1's binding to the 5'UTR of RpL32 mRNA and renders RpL32 mRNA translation resistant to starvation, suggesting that LARP1 binding to the PES regions in the 5'UTRs of RP mRNAs inhibits their translation. Furthermore, the dissociation of LARP1 from the 5'UTR of RpL32 mRNA requires both mTORC1- and S6K1/Akt-dependent LARP1 phosphorylation (***Figure 3D***), indicating that LARP1 phosphorylation by mTORC1 and S6K1/Akt relieves its inhibitory role in translation at the 5'UTRs of RP mRNAs.

Importantly, our data also demonstrate that phosphorylated LARP1 serves as a nucleation site onto which mTORC1 can associate with translationally-competent mRNAs. mTORC1-dependent LARP1 phosphorylation plays a key role in scaffolding mTORC1 on LARP1-interacting RpL32 mRNA (***Figure 5C***). Furthermore, phosphorylated LARP1 and mTORC1 mainly associates with the 3'UTR of RP mRNAs under high mTOR activity conditions. In contrast, phospho-defective mutant LARP1 interacts with the 5'UTR of RP mRNAs regardless of the status of mTOR activity and fails to scaffold mTORC1. Therefore, mTORC1 association with the 3'UTRs of RP mRNAs via LARP1 interaction may allow for a local enhancement of mTORC1 activity to activate the eIF4F complex and thus secure intact translation of LARP1-interacting RP mRNAs. This model provides the mechanistic underpinning for how translation of PES containing mRNAs, such as RP mRNAs, is regulated by LARP1 and may explain why the translation of these mRNAs is sensitive to mTOR activity (***Figure 7—figure supplement 2***).

Given that mTORC1-dependent LARP1 phosphorylation to scaffold mTORC1 is important for mTORC1 phosphorylation of S6K1, mTORC1 may phosphorylate LARP1 prior to S6K1-dependent LARP1 phosphorylation. In support of this model, formation of the LARP1-mTORC1 complex precedes full S6K1 activity/phosphorylation by growth factor/nutrient stimulation (***Figure 4E***). Alternatively, Akt may first phosphorylate LARP1 to prepare mTORC1 phosphorylation of LARP1 for its dissociation from the 5'UTR of RP mRNAs. This functional redundancy of S6K1 and Akt in the phosphorylation of LARP1 may explain previous studies where translation of certain 5'TOP mRNAs was shown to be intact in S6K1/2 null cells but still rapamycin sensitive (***Pende et al., 2004***).

Tcherkezian et al. recently proposed that upon mTOR activation, LARP1 associates with the initiation complex as well as polysomes through PABP (***Tcherkezian et al., 2014***). In addition, they proposed that the DM15 repeats of LARP1 play important roles for its interaction with PABP, eIF4E, and polysomes. Although Tcherkezian et al. and our study both demonstrate important positive roles of LARP1 in the translation of certain 5' TOP mRNAs, our data indicate that the direct binding of LARP1 to a subset of mRNAs does not depend on direct binding to PABP. Instead, we demonstrate that endogenous LARP1 interactions with PABP1 and initiation factors are sensitive to RNase A treatment and thus occur indirectly through binding of common shared mRNA substrates. Intriguingly, our biochemical data suggest that the region containing the DM15 motif is necessary for LARP1 to interact with mTORC1. We speculate that reduced translation in cells expressing mutant LARP1 lacking the complete DM15 motif demonstrated by Tcherkezian et al. may be in part due to a loss of LARP1's scaffolding role for mTORC1, thereby mitigating translation initiation of mRNAs that bind to the mutant LARP1. Moreover, Tcherkezian et al. demonstrated that LARP1 knockdown significantly enhanced the level of 80S monosomes, indicative of a strong inhibition of initiation similar to short-term mTOR inhibitor treatment. They also observed that levels of RP mRNAs associated with polysomes were significantly reduced in LARP1 knockdown cells compared to those in control cells. In contrast, we observed that levels of RP mRNAs in fractions containing polysomes are rather increased in LARP1 knockdown cells, which is consistent with the data more recently published by Fonseca et al., although our data disagreed with the conclusion that LARP1 is a simple suppressor of translation as proposed by ***Fonseca et al. (2015)***. While the reasons for these discrepancies remain unclear, our multiple lines of biochemical evidence indicate that loss of LARP1 causes defects in translation at multiple steps, including abnormal translation initiation and inefficient translation elongation likely due to ribosome stalling on LARP1-interacting mRNAs. Therefore, we propose that LARP1 function is context dependent: non-phosphorylated LARP1 acts as a suppressor of translation

initiation of RP mRNAs when mTOR activity is inhibited, whereas upon mTOR activation, phosphorylated LARP1 promotes translation of RP mRNAs at both translation initiation and elongation steps.

Circularization of linear mRNAs through the interaction between the 5'Cap-binding translation initiation factors and PABPs has been recognized as the active conformation for their translation (***Wells et al., 1998***). mTORC1-dependent phosphorylation of 4EBPs plays a critical role in the induction of this topological change. Our characterization of LARP1 interaction with RpL32 mRNA indicates that LARP1 interacts with both the 5' and 3'UTR of RpL32 mRNA under low mTOR activity conditions. This suggests that certain RP mRNAs that interact with LARP1 under low mTOR activity conditions may form an inactive circular conformation (***Figure 7—figure supplement 2***). Although visualization or/and stoichiometric analysis of the LARP1-RP mRNA complex will be necessary to elucidate this proposed inactive circular conformation, it is reasonable to speculate that retaining such a circular conformation with LARP1 may provide an efficient way to rapidly toggle between translationally on and off states.

Matherials and methods

Antibodies

Antibodies were purchased from the following sources: Antibodies to mTOR (cat. # 2983, RRID:AB_2105622 for western blotting and immunostaining), Raptor (cat. # 2280, RRID:AB_561245), pT389 S6K1 (cat. # 9206, RRID:AB_2285392), S6K (cat. # 9202, RRID:AB_823592), pS473 Akt (cat. # 9270, RRID:AB_329824), Akt (cat # 9272, RRID:AB_329827), phospho-Akt Substrate (RXXS/T) (cat. # 9614, RRID:AB_2225188), pT37/46 4EBP1 (cat. # 2855, RRID:AB_560835), 4EBP1 (cat. # 9644, RRID:AB_10691384), S6 (cat. # 2217, RRID:AB_331355), pS235/236 S6 (cat. # 4856, RRID:AB_2181037 for western blotting), pS240/244 S6 (cat. # 5364, RRID:AB_10694233 for co-IP), RpL13a (cat. # 2765, RRID:AB_916223), PABP1 (cat # 4992, RRID:AB_2156887), mLST8 (cat. # 3274, RRID:AB_823685), PRAS40 (cat. # 2610, RRID:AB_916206), pULK1 (cat. # 6888, RRID:AB_10829226), and ULK1 (cat. # 6439, RRID:AB_11178933) from Cell Signaling Technology; antibodies to LARP1 (cat. # A302-087A, RRID:AB_1604274 for co-IP and western blotting, cat. # A310-088A, RRID:AB_2632225 for IP and western blotting and cat. # IHC-00559, RRID:AB_10631280 for immunostaining), Rictor (cat. # A300-458A, RRID:AB_420924), eIF4G (cat. # A301-776A, RRID:AB_1211011), eIF3D (cat. # A301-758A, RRID:AB_1210970), and eIF3B (cat. # A301-761A, RRID:AB_1210995) from Bethyl Laboratories; antibodies to eIF4E (cat. # sc-9976, RRID:AB_627502) and mTOR/FRAP (cat. # sc-1549, RRID:AB_631981 for co-IP) from Santa Cruz Biotechnology; antibodies to β-Actin (cat. # A1978, RRID:AB_476692), and Flag M2 antibodies (cat. # F1804, RRID:AB_262044) from Sigma-Aldrich; antibodies to HA tag (cat. # MMS-101P, RRID:AB_2314672), and Myc tag (cat. # MMS-150P, RRID:AB_291322) from Covance; antibody to LAMP2 (cat. # H4B4, RRID:AB_528129) from the Developmental Studies Hybridoma Bank at the University of Iowa; HRP-conjugated mouse secondary antibody (cat. # NA931-1ML, RRID:AB_772210) and HRP-conjugate rabbit secondary antibody (cat. # NA934-1ML, RRID:AB_772206) from GE Healthcare; Alexa fluor 594 goat anti-rabbit IgG (cat. # A11012, RRID:AB_141359) and Alexa fluor 488 goat anti-mouse IgG (cat. # A11001, RRID:AB_2534069) from Invitrogen.

Cell culture and treatment

HEK293T and HEK293E (a generous gift from Dr. Diane Fingar, University of Michigan) cells (***Tee et al., 2002***) were cultured in DMEM with 10% fetal bovine serum (FBS) and 100 units/ml penicillin/streptomycin (Invitrogen). Human breast cancer cell line: MDA-MB-231 cells (kindly provided by Dr. Shaomeng Wang, University of Michigan) (***Lu et al., 2008***), human colon cancer cell line: SW480 cells (kindly provided by Dr. Eric Fearon, University of Michigan) (***Mazzoni et al., 2015***), and human prostate cancer cell lines: LNCaP, 22RV1 and PC3 and benign prostate cell lines: PNT2 and RWPE (generous gifts from Dr. Arul Chinnaiyan, University of Michigan) (***Ateeq et al., 2011***; ***Helgeson et al., 2008***; ***Tomlins et al., 2007***) were cultured in RPMI 1640 with 10% FBS and 100 units/ml penicillin/streptomycin and RWPE cells were cultured in keratinocyte serum-free medium (Invitrogen) with Supplements for Keratinocyte-SFM (Invitrogen). Primary prostate cell line: PrEC was purchased from LONZA and cultured in the PrEGM media (LONZA). Each cell line was tested for mycoplasma contamination and confirmed the absence of mycoplasma using fluorescence- and

PCR-based methods (Invitrogen) before subjecting to the experiments. In order to inhibit kinases involved in the PI3K pathway we treated cells with 250 nM Torin-1, 20 μM PF 470861 (Tocris bioscience), 2.5 μM PP242 (Sigma-Aldrich), 100 μM rapamycin (LC laboratories), 1 μM Akt1/2 inhibitor (Sigma-Aldrich), or 2 μM MK-2206 (Active BioChem) for indicated times. For growth factors/nutrients starvation, cells were incubated with either HBSS (Hank's Balanced Salt Solution, Invitrogen) or DPBS (Dulbecco's Phosphate-Buffered Saline, Invitrogen) for the indicated times. For stimulation of these, growth media were added for the indicated times.

Cell lysis, immunoprecipitation and immunostaining

Cells were harvested with CHAPS lysis buffer (40 mM HEPES [pH 7.5], 120 mM NaCl, 1 mM EDTA, 0.3% CHAPS, and 20 mM glycerophosphate, 10 mM NaF, 10 mM sodium pyrophosphate [Sigma-Aldrich] and EDTA-free protease inhibitor [Roche]) by incubating on ice for 15 min. The soluble fractions were isolated by centrifugation at 14,000 rpm for 15 min at 4°C. For immunoprecipitation, 1 μg of antibodies was added and incubated with gentle rocking for 3–6 hr at 4°C. 20 μl of 50% slurry of protein G sepharose (GE Healthcare) was added and incubated for additional 1 hr. Immunoprecipitates were washed with CHAPS lysis buffer for five times and then denatured at 100°C for 5 min in 1X SDS sample buffer. For RNase A treatment, lysates were incubated with 20 μg of RNase A (Affymetrix) for 1 hr at room temperature with gentle rocking. After spinning at 14,000 rpm at 4°C for 15 min to remove protein aggregates, remaining supernatant was applied for further analysis. For immunostaining, cells on round cover slips (Fisher Scientific) were fixed with 4% paraformaldehyde for 20 min and washed with PBS three times. Fixed cells were permeabilized with 0.2% TX-100 for 10 min at room temperature and blocked in 2% BSA in PBS for 1 hr. After washing with PBS three times, cells were incubated with indicated primary antibodies for three hours at room temperature and washed with PBS three times. Cells were incubated with Alexa fluor 488/594 goat anti-mouse/rabbit IgG for 1 hr at room temperature. Cells were washed with PBS three times and once with water. The cover slips were mounted on the glass slides using ProLong Gold antifade reagent with DAPI (Life technologies) and imaged with 63X oil-immersion objective by using a Leica TCS SP5 confocal microscope.

Capture and sequencing of LARP1-bound RNA fragments using PAR-CLIP-seq

To label cells with 4-thiouridine (4-SU), cells were seeded in two 15 cm plates to grow overnight to reach 70% confluency. On the next day, cells were incubated with 100 μM 4-SU for 14 hr. After washing with cold PBS, cells were irradiated in the CL-1000 ultraviolet Crosslinker (UVP) on ice with 150 mJ/cm^2. Cross-linked cell pellets were collected by scraping and lysed with 600 μl PAR-CLIP lysis buffer (0.1% SDS, 0.5% deoxycholate, 0.5% NP-40 in PBS without Mg^{2+}, Ca^{2+}) on ice for 10 min. To remove DNA, 10 μl of RQ1 DNAse was added into each tube and tubes were incubated at 37°C for 10 min with gentle rocking. Afterward, 1 U/μl of RNase T1 (Fermentas) was added and the lysates were incubated at room temperature for 15 min with gentle rocking. Lysates were spun at 4°C for 10 min at 14,000 rpm. The soluble fractions were incubated with LARP1 antibody-Dynabeads Protein A for 1 hr at 4°C. Immunoprecipitates were collected on a magnetic stand and were then washed three times with IP-wash buffer (50 mM HEPES-KOH [pH 7.5], 300 mM KCl, 0.05% NP-40, 0.5 mM DTT, and EDTA-free protease inhibitor). Immunoprecipitates were resuspended in 40 μl of IP wash buffer containing 50 U/μl RNase T1 (Fermentas) and incubated at room temperature with gentle rocking for 15 min followed by incubation on ice for 5 min. Immunoprecipitates were washed with high-salt wash buffer (50 mM HEPES-KOH [pH 7.5], 500 mM KCl, 0.05% NP-40, 0.5 mM DTT, and EDTA-free protease inhibitor), three times with the PAR-CLIP lysis buffer, and twice with high-salt wash buffer (0.1% SDS, 0.5% deoxycholate, 0.5% NP-40 in 5X PBS without Mg^{2+}, Ca^{2+}) followed by washing twice with PNK (polynucleotide kinase) buffer (50 mM Tris-Cl pH 7.4, 10 mM $MgCl_2$, 0.5% NP-40). For visualization of crosslinked RNAs, immunoprecipitates were incubated in 40 μl of the PNK mixture (1 μl of $P^{32}\gamma$ATP, 4 μl of 10X PNK buffer [NEB], 2 μl of T4 PNK enzyme [NEB], 33 μl of water) for 30 min at 37°C. Labeled immunoprecipitates were washed three times with PNK buffer and resuspended with 30 μl of 2X NuPAGE LDS sample buffer (15 μl of 1 X PNK with 15 μl of Novex LDS sample buffer). Denatured samples were resolved in 4–12% NuPAGE Bis-Tris gel and transferred to nitrocellulose membrane at 30 V for 1 hr using NuPAGE transfer buffer. The membrane was exposed

to X-ray film at −80˚C for 4 hr to visualize crosslinked RNAs. Molecular biology procedures for cloning LARP1-bound RNA fragments was described previously (**Freeberg et al., 2013**).

Sequence read processing

PAR-CLIP-seq and mRNA-seq reads were processed to remove linkers. All reads were mapped to the human transcriptome version GRCh37 using Bowtie (**Langmead et al., 2009**) allowing for up to three mismatches with the following parameters: -v 3 k 100 –best –strata –phred33-quals. mRNA-seq reads mapping perfectly to the transcriptome were kept; reads mapping perfectly to multiple loci were distributed evenly among the mapped positions. Transcript RPKM values were calculated as the number of reads per million mapped reads aligning to a transcript normalized to transcript length in kilobases. Replicate mRNA-seq libraries had a high Pearson correlation coefficient (R^2 = 0.99975), so transcript RPKM values were averaged from the two libraries. PAR-CLIP-seq reads with 0–2 T-to-C mismatches were clustered into peaks with at least one overlapping nucleotide. Clusters were smoothed with a Gaussian smoothing technique as described in (**Freeberg et al., 2013**) Clusters with at least one read containing a T-to-C conversion event were kept as LARP1 binding sites, and all reads containing 0–2 T-to-C conversion events were summed per binding site and normalized to the number of million mapped reads per library (RPM). The RPM of binding sites located in genic regions were additionally normalized to the gene RPKM and multiplied by 1000 to account for the kilobase normalization of gene RPKM values.

Gene ontology term enrichment analysis

GO term enrichment analysis was performed using the topGO Bioconductor package for R. The background gene list was restricted to genes with reads in at least one of our replicate mRNA-seq libraries. The Fisher's exact test was used to measure the significance of enriched GO terms, and *p*-values were corrected for multiple testing using the Bonferroni correction method. GO terms with adjusted p-values<0.001 were submitted to REVIGO (**Supek et al., 2011**) which summarizes long lists of GO terms (accompanied by enrichment p-values) by removing redundant terms and grouping terms into larger categories. REVIGO parameters were: allowed similarity = 0.9, database with GO term sizes: Homo sapiens, semantic similarity: SimRel. From these REVIGO-derived categories, GO terms were manually grouped into the following categories: translation, cell differentiation and development, protein localization to the ER, regulation of signaling response, response to stimulus, antibody production, metabolism, and other (**Supplementary file 2**). For example, the categories 'translational elongation', 'translational initiation', and 'translation' were all grouped into the 'translation' super-category. Genes are considered 'translation-related' if they are annotated to at least one of the GO terms in our translation super-categry.

Identification of sequence motifs

Sequence motifs were searched for within the 198 and 186 5'UTR LARP1 binding sites found under growing and mTOR inactive conditions, respectively, using MEME with default parameters (**Bailey et al., 2009**) Binding site sequences were extended by 15nt up- and down-stream for this search. Additionally, sequence motifs were searched for within CDS and 3'UTR LARP1 binding sites under growing and mTOR inactive conditions using the same parameters.

Measurement of newly synthesized protein (Click-IT labeling)

Cells were seeded in 6-well plates a day before labeling to reach 70% confluency on the day of labeling. Cells were washed twice with warm methionine/cysteine free DMEM with 1 x L-glutamine (Invitrogen) and incubated with methionine/cysteine free DMEM with 1 X L-glutamine and 10% dialyzed FBS (Invitrogen) for 1 hr. Cells were labeled with 25 µM Click-IT AHA (L-Azidohomoalanine) for 4 hr in a CO_2 incubator. Labeled cells were washed once with PBS and lysed with NP-40 lysis buffer (10 mM Tris-Cl [pH 7.5], 2 mM EDTA, 100 mM NaCl, 1% NP-40, 50 mM NaF, 10 mM sodium pyrophosphate and EDTA-free protease inhibitor) for 10 min on ice. The soluble fractions isolated by centrifugation were transferred into new tubes and the Click-IT reaction was performed in 1 X Click-IT reaction buffer according to the manufacturer's guide. The Click-IT reaction was stopped by addition of SDS sample buffer and denatured by boiling. Samples were resolved by SDS-PAGE and transferred to PVDF membrane for immunoblot analysis. Labeled proteins were probed with Avidin-HRP

(BioRad) and membranes were visualized by ECL on x-ray film. Intensity of signal was quantified by Image J.

Polysome analysis

Prior to lysis, cells were treated with 100 µg/mL cycloheximide (CHX) for 5 min and washed with cold PBS containing 100 µg/mL CHX. Cells were harvested in 1 ml of cold PBS containing 100 µg/mL CHX. Cell pellets were resuspended in hypotonic buffer (5 mM Tris [pH 7.5], 2.5 mM $MgCl_2$, 1.5 mM KCl, 0.3% CHAPS, 20 mM glycerophosphate, 10 mM NaF, 10 mM sodium pyrophosphate and EDTA-free protease inhibitor) containing 100 µg/mL CHX, 2 mM DTT, and 200 U/ml RNasin (Promega) by vortexing for 5 s. Supernatants were collected by centrifugation for 10 min at 14,000 rpm, 4˚C, and the A_{260} of lysates was measured to normalize RNA levels. Lysates of 30 $O.D._{260}$ were loaded on 10–50% sucrose density gradients (100 mM HEPES [pH 7.6], 1M KCl, 50 mM $MgCl_2$, 100 µg/mL CHX, 200 U/ml RNasin, and EDTA-free protease inhibitor) and centrifuged at 35,000 rpm for 3 hr at 4˚C. Gradients were fractionated and the optical density at 254 nm was recorded using an ISCO fractionator (Teledyne ISCO). For immunoblotting, the fractions were concentrated by Vivaspin concentrator (Sartorius). For quantitative PCR normalization, 5 ng of luciferase control mRNA (Promega) was added to each fraction. RNA was extracted using TRIzol and cDNA was synthesized by SuperScript III First-Strand Synthesis System (Invitrogen) following the manufacturer's instructions.

In vitro kinase assay

For mTOR in vitro kinase assays, HEK293T cells were lysed with CHAPS lysis buffer and endogenous mTOR, Flag-mTOR Wt (wild type), or Flag-mTOR KD (kinase defective) was immunoprecipitated and subjected to in vitro kinase assays. For LARP1 phosphorylation, the kinase reaction was performed in kinase reaction buffer (25 mM HEPES [pH 7.4], 50 mM KCl, 10 mM MgCl2, 200 mM cold ATP and 5 µCi of ^{32}PγATP) using 2 µg of GST-LARP1 fragment (654–731 aa) purified from bacteria. For 4EBP1 phosphorylation, 200 ng of GST-4EBP1 (full length) was used as a substrate for the kinase reaction without using ^{32}PγATP, and the phosphorylation of 4EBP1 was detected by the indicated phospho-specific 4EBP1 antibody. For AKT and S6K1 kinase assays, AKT-HA, AKT-HA KD, HA-S6K1 F5A 3DE (constitutive active), or HA-S6K1 KD was immunoprecipitated from HEK293T cells and kinase reactions were performed in the AKT/S6K1 kinase reaction buffer (75 mM Tris-Cl [pH 7.5], 15 mM $MgCl_2$, 1.5 mM DTT, 1.5 µg/ml BSA, 200 mM cold ATP and 5 µCi of P^{32}γATP) using 2 µg of GST-LARP1 fragments (722–822 aa or 929–1019 aa).

shRNAs, lentivirus production, and stable knock-down

Short-hairpin RNAs (shRNAs) against LARP1 or eIF4G1 were cloned into the lentivirus plasmid pLKO.1-puro. HEK293T cells were transfected with pLKO.1-puro cloned with shRNA, psPAX2 (packaging plasmid) and pMD2 (envelope plasmid) using the calcium phosphate method. Lentivirus-containing supernatants were collected and spun at 23,000 rpm for 90 min. Virus pellets were resuspended with Opti-MEM (Invitrogen). Cells were infected for 24 hr and selected with 2 µg/ml puromycin for additional 24 hr. 72 hr post-infection, stably knocked-down cells were harvested and processed for further analysis.

shRNA scramble (control):
Sense:
5'CCGGCCTAAGGTTAAGTCGCCCTCGCTCGAGCGAGGGCGACTTAACCTTAGGTTTTTC3'
Antisense:
5'AATTGAAAAACCTAAGGTTAAGTCGCCCTCGCTCGAGCGAGGGCGACTTA
ACCTTAGG3' shRNA LARP1 UTR-2:
Sense:
5'CCGGGGTGAGGACTTCATCTCAACACTCGAGTGTTGAGATGAAGTCCTCACCTTTTTC3'
Antisense:
5'AATTGAAAAAGGTGAGGACTTCATCTCAACACTCGAGTGTTGAGATGAAGTCCTCACC3'
shRNA LARP1-3
Sense:
5'CCGGGCCAGTCTCAGGAGATGAACACTCGAGTGTTCATCTCCTGAGACTGGCTTTTTC3'
Antisense:

5'AATGAAAAAGCCAGTCTCAGGAGATGAACACTCGAGTGTTCATCTCCTGAGACTGGC3'

shRNA eIF4G1-1

Sense:

5'CCGGGGATCCCACTAGACTACAAGGCTCGAGCCTTGTAGTCTAGTGGGATCCTTTTTC3'

Antisense:

5'AATTGAAAAAGGATCCCACTAGACTACAAGGCTCGAGCCTTGTAGTCTAGTGGGATCC3'

Oligonucleotides used for qPCR

RpS6:

Forward: 5'TGTCCGCCTGCTACTGAGTAA3'

Reverse: 5'GCAACCACGAACTGATTTTCTC3'

RpS3A:

Forward: 5'AGGGTCGTGTGTTTGAAGTGA3'

Reverse: 5'CATGGAAGTTAGTCAGGCAGTTT3'

RpS18:

Forward: 5'GCGGGAGAACTCACTGAGG3'

Reverse: 5'CGTGGATTCTGCATAATGGTGAT3'

RpL26:

Forward: 5'GACTTCCGACCGAAGCAAGAA3'

Reverse: 5'TGCACCCGTTCAATGTAGATAAC3'

RpL32:

Forward: 5'GCCCAAGATCGTCAAAAAGAGA3'

Reverse: 5'TCCGCCAGTTACGCTTAATTT3'

Actin:

Forward: 5'CATGTACGTTGCTATCCAGGC3'

Reverse: 5'CTCCTTAATGTCACGCACGAT3'

GAPDH:

Forward: 5'TTGCCATCAACGACCCCTTC3'

Reverse: 5'TTGTCATGGATGACCTTGGC3'

Firefly luciferase:

Forward: 5'CTCACTGAGACTACATCAGC3'

Reverse: 5'TCCAGATCCACAACCTTCGC3'

Oligonucleotides used for generating pGL3-RpL32 5'conv

Forward: 5'gcctacggaggtggcag**GGTAG**tccttctcggcatc3'

Reverse: 5'gatgccgagaagga**CTACC**ctgccacctccgtaggc3'

Statistical analysis

Data are representative of at least two independent experiments. All values were given as means±SEM from three independent biological replicates. Comparisons were performed using Student's t-test or one-way factorial ANOVA followed by Bonferroni's post-hoc analysis.

Acknowledgement

We thank Arul Chinnaiyan and Shaomeng Wang for providing breast and prostate cancer cell lines, respectively, Eric Fearon for helpful advice, Ivan Topisirovic for instructions on polysome fractionation, Aristotle Mannan for technical assistance, and Philip Gafken (Fred Hutchinson Cancer Research Center) for proteomic analyses. This work was supported by grants from the NIH (DK083491/GM110019 to KI, GM088565 to JKK), DOD (TS140055 to KI), and ACS Research Scholar Grant (to JKK) and the Ruddon Research Fund in Cancer Biology (to KI).

Additional information

Funding

Funder	Grant reference number	Author
National Institute of General Medical Sciences	GM088565	John K Kim
National Institute of General Medical Sciences	GM110019	Ken Inoki
U.S. Department of Defense	TS140055	Ken Inoki
National Institute of Diabetes and Digestive and Kidney Diseases	DK083491	Ken Inoki

The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

Author contributions

SH, Resources, Formal analysis, Validation, Investigation, Writing—original draft; MAF, Software, Formal analysis, Methodology, Writing—original draft, Writing—review and editing; TH, Formal analysis, Investigation, Methodology; AK, Investigation, This author contributed to clone all the cDNAs of LARP family proteins and LARP1 mutants, which was essential to generate specific figures (e.g. Fig. 5B) and other figures using LARP1 mutants; YY, Formal analysis, Investigation, Visualization; TF, Investigation, This author also contributed to clone cDNAs for LARP1 mutants, which were essential to generate specific figures (e.g. Fig. 5A, 5G, and 5H); TS, Investigation, This author originally identified LARP1 as a mTORC1 interacting protein using proteomics approaches (data not shown), which was essential part of work for initiating this project; JKK, Supervision, Funding acquisition, Validation, Writing—review and editing; KI, Supervision, Funding acquisition, Validation, Writing—original draft, Writing—review and editing

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Additional files

Supplementary files

- Supplementary file 1. LARP1 binding sites.
DOI: 10.7554/eLife.25237.016
- Supplementary file 2. Gene ontology terms enriched in LARP1-bound genes under growing and mTOR inactive conditions.
DOI: 10.7554/eLife.25237.017
- Supplementary file 3. LARP1-bound genes classified as non-TR, TR, and RP-encoding genes.
DOI: 10.7554/eLife.25237.018
- Supplementary file 4. Location of 5'TOP/5'TOP-like and LARP1 binding site in select 5'UTRs.
DOI: 10.7554/eLife.25237.019

Major datasets

The following dataset was generated:

Author(s)	Year	Dataset title	Dataset URL	Database, license, and accessibility information
Hong S, Freeberg MA, Avani K, Ting H, Yao Y, Tomoko F, Andy K, Tsukasa S, Kim JK, Inoki K	2014	LARP1 mRNP scaffolds mTORC1 to stimulate translation initiation of an essential class of mRNAs	https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE59599	Publicly available at the NCBI Gene Expression Omnibus (accession no: GSE59599)

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CHAPTER TWENTY-FOUR

Evaluating the mTOR Pathway in Physiological and Pharmacological Settings

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Contents

Abstract

Mammalian/mechanistic target of rapamycin (mTOR) is an evolutionarily conserved genuine protein kinase, which phosphorylates serine/threonine in response to growth factors and nutrients. It functions as a catalytic core in two distinct multiprotein complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 promotes cell growth and proliferation by positively regulating translation, transcription, and lipid biosynthesis in response to growth factors and amino acids, whereas it inhibits autophagy, an essential degradation and recycling pathway. mTORC2 regulates cell survival and cytoskeleton organization. Mechanistic insights into the function and regulation of mTOR complexes have been provided in various experimental settings and monitoring mTOR activity has been a most valuable way to judge whether levels of

Methods in Enzymology, Volume 587
ISSN 0076-6879
http://dx.doi.org/10.1016/bs.mie.2016.09.068

environmental cues such nutrients and growth factors can satisfy cellular needs for cell growth, proliferation, and autophagic response. Here, we describe useful methods to access mTOR activity in different experimental settings.

1. INTRODUCTION

Mammalian/mechanistic target of rapamycin (mTOR) is a master kinase that regulates autophagy, cell growth, proliferation, and survival in response to growth factors and nutrients such as amino acids. It forms two structurally and functionally distinct multiprotein kinase complexes named mTORC complex 1 (mTORC1) and mTORC complex 2 (mTORC2) (Dibble & Cantley, 2015; Guertin & Sabatini, 2007; Wullschleger, Loewith, & Hall, 2006). While mTORC1 consists of mTOR, Raptor (regulatory-associated protein of mTOR), mLST8 (mammalian lethal with SEC Thirteen 8), PRAS40 (proline-rich Akt substrate of 40 kDa), and Deptor (DEP domain-containing mTOR-interacting protein), mTORC2 comprises of mTOR, Rictor (rapamycin-insensitive companion of mTOR), SIN1 (SAPK-interacting 1), mLST8, Protor (protein observed with rictor), and Deptor (Laplante & Sabatini, 2012). In mTORC1, Raptor functions as a scaffold for several specific mTORC1 substrates, including S6 kinase (S6K), eIF4E binding protein (4EBP), and ULK1 (Unc-51-like kinase 1), as well as for tethering mTORC1 to the endosomal membrane for its activation (Hara et al., 2002; Kim et al., 2002; Sancak et al., 2008). In contrast, PRAS40 and Deptor negatively regulate the activity of mTORC1 (Peterson et al., 2009; Sancak et al., 2007; Vander Haar, Lee, Bandhakavi, Griffin, & Kim, 2007). In mTORC2, Rictor, SIN1, and mLST8 play an essential role in the activity of mTORC2 to phosphorylate its substrates, including Akt, PKCα, and SGK1 (Jacinto et al., 2006; Su & Jacinto, 2011; Yang, Inoki, Ikenoue, & Guan, 2006).

Bacteria-produced rapamycin is a macrolide and its pharmaceutical derivatives are drugs approved by the FDA for organ transplantation, coronary artery stenosis, and several types of cancer (Cargnello, Tcherkezian, & Roux, 2015; Geissler, 2015). mTORC1 is defined as the rapamycin-sensitive complex, whereas mTORC2 is insensitive. Rapamycin forms a complex with FKBP12 to interact with the FKBP12–rapamycin-binding (FRB) domain of mTOR kinase in mTORC1 and allosterically suppresses the mTOR kinase activity by blocking the accessibility of substrates to the

active site of mTOR kinase and ultimately disrupts the formation of mTORC1 (Brown et al., 1994; Hara et al., 2002; Kim et al., 2002). It has been demonstrated that the FRB domain of mTOR in mTORC2 is hindered by Rictor once mTORC2 is established (Gaubitz et al., 2015). However, prolonged rapamycin treatment often decreases the expression of mTORC2 and inhibits its functions (Sarbassov et al., 2006). Thus, it is likely that the rapamycin–FKBP12 complex may gain access to newly synthesized mTOR and prevent mTOR from forming mTORC2. Recently, several specific mTOR kinase inhibitors have been synthesized and now are commercially available. These second-generation mTOR inhibitors function as an ATP-competitive inhibitor for mTOR kinase that potently inhibits the kinase activity of both mTORC1 and mTORC2 (Feldman et al., 2009; Hsieh et al., 2012; Thoreen et al., 2009).

Two important environmental cues have long been studied in the regulation of mTORC1 activation: growth factors such as insulin and nutrients such as amino acids. The activity of mTORC1 is stimulated by growth factors and nutrients through two distinct Ras-related small guanosine triphosphatases (GTPases): monomeric ras homolog enriched in the brain (Rheb) and the heterodimeric Rag complex, respectively, on the lysosomal membrane (Dibble & Manning, 2013; Jewell, Russell, & Guan, 2013; Sancak et al., 2010).

Growth factors activate Rheb by inhibiting the trimeric TSC1/TSC2/TBC1D7 complex (hereafter called the *TSC complex*), a well-known tumor suppressor and the specific GTPase-activating protein (GAP) for Rheb, through inhibitory phosphorylation of TSC2 by Akt (Dibble et al., 2012; Garami et al., 2003; Inoki, Li, Xu, & Guan, 2003; Inoki, Li, Zhu, Wu, & Guan, 2002; Manning, Tee, Logsdon, Blenis, & Cantley, 2002; Potter, Pedraza, & Xu, 2002; Zhang et al., 2003). Akt-dependent TSC2 phosphorylation induces the dissociation of the TSC complex from the lysosomal membrane, thereby maintaining lysosomal active Rheb, which directly activates mTORC1 (Demetriades, Doumpas, & Teleman, 2014; Menon et al., 2014).

Amino acids, especially leucine and arginine, activate mTORC1 through the activation of Rag GTPases (Kim, Goraksha-Hicks, Li, Neufeld, & Guan, 2008; Sancak et al., 2008). There are four mammalian Rag proteins, which form obligate heterodimers. RagA and RagB are functionally redundant and form heterodimeric complexes with either RagC or RagD (Nakashima, Noguchi, & Nishimoto, 1999; Sekiguchi, Hirose, Nakashima, Ii, & Nishimoto, 2001). Intriguingly, when Rag complexes

are active, RagA and RagB are GTP-bound forms, whereas RagC and RagD are GDP-bound forms (Kim et al., 2008; Sancak et al., 2008). Another unique feature of Rags is their lack of a lipid moiety, even though they reside on lysosomes. The lysosomal expression of Rags is dependent on the lysosome-anchored Ragulator (Bar-Peled, Schweitzer, Zoncu, & Sabatini, 2012; Sancak et al., 2010). The Ragulator is a pentameric protein complex consisting of five subunits, p18 (LAMTOR1), p14 (LAMPTOR2), MP1 (LAMTOR3), C7orf59 (LAMTOR4), and HBXIP (LAMTOR5). Importantly, Ragulator functions as not only a scaffold but also a guanine nucleotide exchange factor (GEF) for RagA and RagB (Bar-Peled et al., 2012). In response to amino acids, Ragulator is activated through v-ATPase on the lysosomal membrane, thereby stimulating the activity of Rags. In contrast, GATOR1, a trimetric protein complex consisting of DEPDC5, NPRL2, and NPRL3, functions as a GAP for both RagA and RagB. GATOR1 is inhibited by another pentameric protein complex, GATOR2, which interacts with Sestrins and CASTORs (Bar-Peled et al., 2013; Chantranupong et al., 2014, 2016; Kim et al., 2015; Parmigiani et al., 2014). Importantly, recent studies have revealed that Sestrin2 and CASTOR1 directly interact with leucine and arginine, respectively (Chantranupong et al., 2016; Wolfson et al., 2016). Both leucine-binding to Sestrin2 and arginine-binding to CASTOR1 are required for leucine and arginine to activate mTORC1 through GATOR2 activation. Although it has been proposed that lysosomal v-ATPase transmits lumenal amino acid signal to the Rag complexes through Ragulator (Wolfson et al., 2016), newly identified cytosolic amino acid sensors such as Sestrins and CASTORs are not expressed on the lysosomal membrane (Chantranupong et al., 2016; Wolfson et al., 2016). Therefore, these observations suggest that essential amino acids are sensed at the lysosome and cytosol for mTORC1 activation.

In addition to the role of amino acids in recruiting mTORC1 to lysosomal membranes, recent studies have revealed other roles of amino acids in the regulation of the TSC complex. Under amino acid starvation conditions, TSC2 interacts with the inactive form of RagA on lysosomes, and this interaction is required for complete inactivation of mTORC1 upon amino acid starvation (Demetriades et al., 2014). Furthermore, amino acids, especially arginine, disrupt the interaction between TSC2 and Rheb, which enhances the accessibility of mTORC1 to active Rheb on the lyososomal membrane (Carroll et al., 2016). Presumably, this is why the activity of mTORC1 is relatively insensitive to amino acid depletion in cells lacking a functional TSC complex.

Compared to mTORC1, molecular mechanisms of mTORC2 activation have not been clearly shown, although a recent study proposed that ribosomes are required for mTORC2 activation (Zinzalla, Stracka, Oppliger, & Hall, 2011). mTORC2 interacts with ribosomal proteins (including RpL26) in a manner dependent on the activity of PI3K, and reduction of ribosomal proteins mitigates cellular mTORC2 activity.

Given that mTOR, especially mTORC1, plays a critical role in suppressing the induction of autophagy, monitoring cellular mTOR activity is a valuable tool to determine the status of cellular autophagic activity. Here, we summarize established methods for monitoring the activity of mTOR, its subcellular localization, and the activity of Rheb to determine cellular mTOR activity.

2. FUNCTIONAL READOUTS AND INHIBITORS FOR THE mTOR PATHWAY

There are a plethora of substrates that have been shown to be phosphorylated by the mTOR complexes. These substrates include S6K1, 4EBP1, PRAS40, and ULK1 (UNC-51-like kinase 1) as mTORC1 substrates, and serum- and glucocorticoid-induced kinase 1 (SGK1) and Akt (Laplante & Sabatini, 2012). For monitoring the activity of mTORC1 in vivo and in vitro, levels of S6K1 phosphorylation on Thr389 (hydrophobic motif) and 4EBP1 phosphorylation on Thr37/Thr46 and Ser65 have been widely used. These substrates are known to play essential roles in mTORC1-dependent mRNA translation (Fig. 1) (Moschetta, Reale, Marasco, Vacca, & Carratu, 2014). In addition, ULK1 phosphorylation on Ser757 can be monitored to determine cellular mTORC1 activity in the regulation of autophagy (Kim, Kundu, Viollet, & Guan, 2011). Along with the abovementioned biochemical approaches, monitoring lysosomal localization of mTOR has begun to be accepted as a new biological method for assessing mTORC1 activation.

Akt phosphorylation on Ser473 (hydrophobic motif) has been widely used for monitoring mTORC2 activity both in vivo and in vitro (Fig. 1) (Sarbassov, Guertin, Ali, & Sabatini, 2005).

To assess functions of mTOR in a variety of experimental settings, two types of mTOR inhibitors have been well used; allosteric inhibitors such as rapalogs, and ATP-competitive inhibitors, including Torin1, PP242, and INK128. Since ATP-competitive inhibitors directly inhibit the kinase activity of mTOR, a catalytic core in both mTORC1 and mTORC2, these inhibitors completely block the activity of both mTORC1 and mTORC2

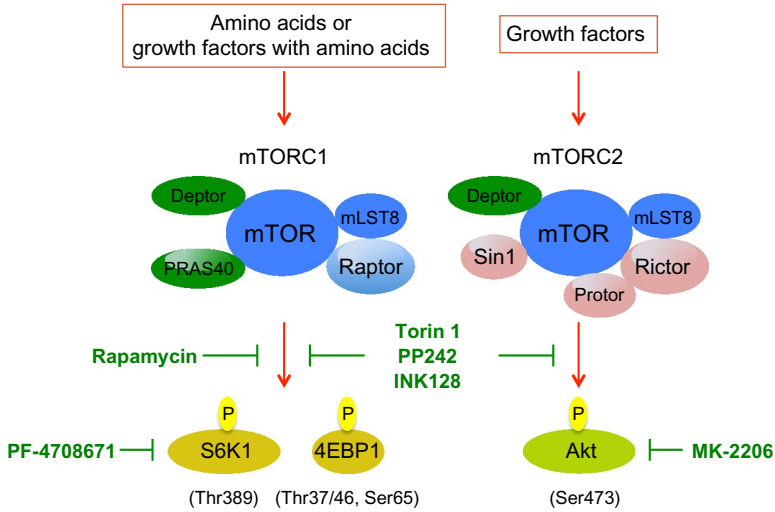

Fig. 1 mTORC1 and mTORC2 and mTOR pathway inhibitors. Schematic illustration shows the key components of mTORC1 and mTORC2, essential cellular cues activating these complexes, inhibitors for the mTOR pathway; rapamycin for mTORC1, Torin1, PP242, and INK128 for mTORC1 and mTORC2, PF-470861 for S6K1, and MK-2206 for Akt. Major phosphorylation sites of mTORC1 and mTORC2 substrates are indicated.

(Figs. 1 and 3). While rapamycin and its derivatives, rapalogs are well-known mTORC1 inhibitors, and the effect of these allosteric inhibitors on mTORC1 inhibition is widely acknowledged, they do not completely suppress the phosphorylation of some mTORC1 substrates, such as 4EBP1 and ULK1. In addition, S6K1 and Akt, which are downstream kinases and substrates of mTORC1 and mTORC2, respectively, can be inhibited by PF-4708671 (S6K1 inhibitor) and MK-2206 (Akt inhibitor) (Figs. 1 and 3).

mTORC1 receives at least two essential signals from growth factors and amino acids for its activation. These two signals impinge on the lysosomal membrane for mTORC1 activation through Rheb and Rag small GTPases. Thus, monitoring the active status of these two small GTPases provides important information for the molecular mechanisms by which mTORC1 regulators stimulate mTORC1 activity (Fig. 2).

3. METHODS

3.1 Cell Culture and Treatments

The signal transductions from growth factors such as insulin and amino acids to mTOR are well conserved in mammalian cells. Representative

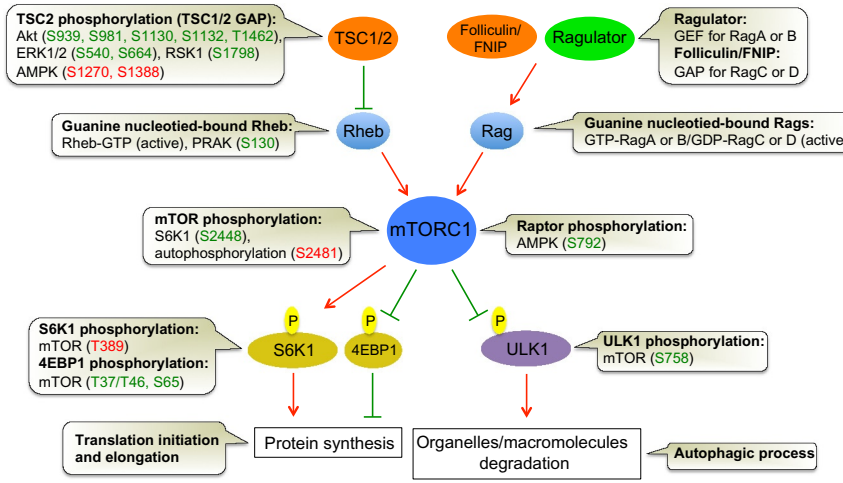

Fig. 2 Diagnostic posttranslational modifications in the mTORC1 pathway. Diagnostic phosphorylation events and critical regulators such as small GTPases and their GAPs and GEF are shown. The phosphorylation sites depicted in red have a positive role, whereas those in *green* play a negative role for the phosphorylated proteins.

mammalian cells widely used in the research of mTOR signaling include HEK293T (human embryonic kidney 293T), MEFs (mouse embryonic fibroblasts), and some cancer cell lines such as HeLa cells. These cells can be cultured in standard culture media such as DMEM (Dulbecco's modified eagle medium) with fetal bovine serum (FBS). However, it is noteworthy that the mTOR pathway in HEK293T and some cancer cells is less sensitive to growth factor stimulation or depletion, in part due to a lack of the activity of phosphatase and tensin homolog (PTEN), a key lipid phosphatase that removes the phosphate in the D3 position of inositol rings from a variety of phophstidylinositols. In addition, HeLa cells that lack the expression of serine/threonine-protein kinase STK11 (LKB1), a master kinase for the T-loop of AMPK family of proteins, show less sensitive to glucose stimulation or depletion in the regulation of the mTOR pathway (Lizcano et al., 2004; Shaw et al., 2004).

In order to inhibit mTOR activity by suppressing upstream inputs, cells need to be starved with growth factor, amino acids, or both by culturing growth factor-free DMEM, PBS containing dialyzed FBS, and PBS containing calcium, magnesium, and glucose (DPBS, ThermoFisher, Waltham, MA, USA, cat# 14040216), respectively. By depleting growth factors in medium, the activity of both mTORC1 and mTORC2 is inhibited. By depleting amino acids in medium, the activity of mTORC1

but not mTORC2 is greatly inhibited. Media lacking a specific amino acid such as leucine or glutamine are also commercially available. To ensure complete removal of growth factors or amino acids, cells should be washed at least once with growth factor- or amino acid-free media before culturing with the starvation media. To inhibit mTORC1 activity by suppressing Akt activity, specific and potent pan-Akt inhibitors such as MK-2206 (IC_{50} = 8/12/65 n*M* for Akt1/2/3, respectively), AZD5363 (IC_{50} = 3/8/8 n*M*), and GSK690693 (IC_{50} = 2/13/9) are commercially available.

In order to inhibit mTORC1 activity directly, cells are treated with rapamycin (sirolimus) or RAD001 (everolimus). To inhibit both mTORC1 and mTORC2, ATP-competitive inhibitors such as Torin 1 (IC_{50} = 2–10 n*M*, 1000-fold selectivity for mTOR than PI3K), KU-006379 (IC_{50} = ~10 n*M*) AZD8055 (IC_{50} = ~1 n*M*, 1000-fold selectivity), INK128 (IC_{50} = 1 n*M*, 200-fold selectivity), and Torkinib (PP242) (IC_{50} = 8 n*M*, 10- to 100-fold selectivity) can be used (Figs. 1 and 3).

3.2 Transfection

Treatments with physiological cues including mitogens, growth factors, and nutrients, or pharmacological compounds such as inhibitors generally produce their effects in all of cultured cells. Therefore, it is able to examine the mTOR signaling by analyzing endogenous proteins. However, in order to determine the role of an exogenous protein in the regulation of mTORC1 or mTORC2, coexpression of the exogenous protein with mTORC1 substrate (e.g., S6K1) or mTORC2 substrate (i.e., Akt) as a reporter helps to analyze its effects on the mTOR pathway in cells that have low transfection efficiency.

Liposome-mediated transfection is a common and efficient method for introducing negatively charged nucleic acid molecules, including cDNA and RNA. Lipofectamine (Invitrogen, Carlsbad, CA, USA) and fugene (Promega, Madison, WI, USA) are two representative commercially available transfection reagents with less cytotoxicity and high efficiency of cDNA transfection. However, due to the cost of these transfection reagents, calcium phosphate transfection is a more attractive method, especially for large-scale transfections such as shRNA-expressing virus production. In addition, PEI (polyethylenamine) can be used as a transfection reagent.

3.2.1 Calcium Phosphate Transfection

All the solutions should be warmed at room temperature before transfection.

1. Grow cells to 70% confluent in 10-cm plates: less confluent cells can die due to cytotoxicity of transfection mixtures.

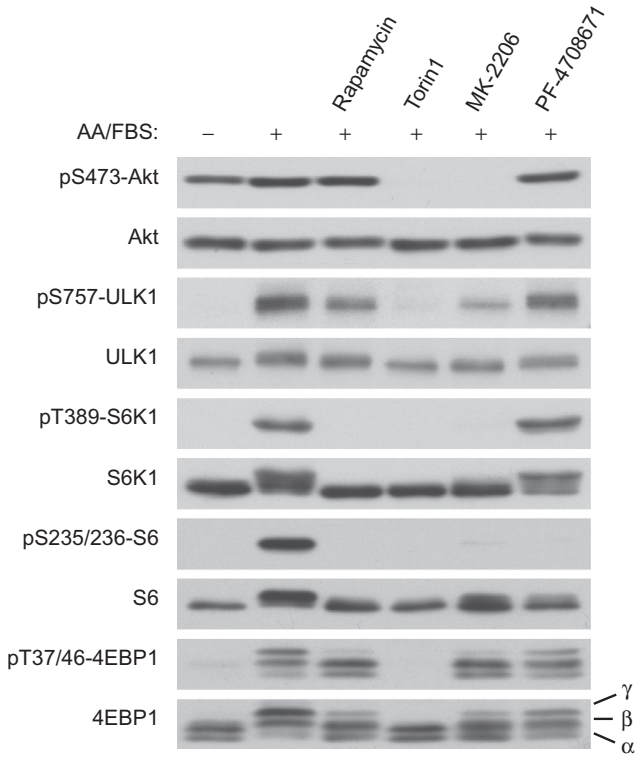

Fig. 3 Pharmacological and physiological inhibition of mTOR and downstream effector kinases. Regulation of the mTOR pathway by amino acids and growth factors. HEK293T cells were starved in HBSS (with calcium and magnesium) for 60 min, followed by amino acids and serum stimulation for another 30 min in the absence or presence of various inhibitors [rapamycin (mTORC1 inhibitor) 100 n*M*, Torin 1 (mTORC1 and mTORC2 inhibitor) 250 n*M*, MK2206 (Akt inhibitor) 2 μ*M*, or PF-4708671 (S6K1 inhibitor) 20 μ*M*]. The indicated proteins were analyzed in Western blotting using the indicated antibodies. α, β, and γ denote non/hypo-, less-, and hyperphosphorylated forms of 4E-BP1, respectively.

2. Change with fresh growth media 3 h before transfection.
3. Add 10–50 μg of DNA into a 50-mL disposable tube and add autoclaved water to 1095 μL.
4. Add 155 μL of 0.22-μm-filtered 2 *M* calcium chloride (stored at 4°C) and mix by gentle swirling.
5. Add 1250 μL of 2 × HBS dropwise within 1 min to evenly form calcium phosphate particles: 2 × HBS: For 500 mL, 8 g NaCl, 0.2 g Na_2HPO_4, 6.5 g HEPES, pH 7.0 stored at –80°C.

6. After 12 h, remove the media, wash once with media to remove calcium phosphates and add fresh growing media.
7. Cells can be harvested and expression can be assessed 36–48 h after transfection.

3.2.2 Transfection Using Liposome (Lipofectamine)

1. Grow cells to 60% confluent in one well of a six-well plate.
2. Before transfection, wash and change media with 0.85 mL of Opti-MEM (ThermoFisher, Waltham, MA, USA, cat# 31985062).
3. In a microcentrifuge tube, add 150 μL of Opti-MEM and DNA constructs.
4. Mix by gentle vortex.
5. Add 5 μL of lipofectamine into the same tube. (This step is different from the manufacturer's instructions.)
6. Mix by gentle vortex and incubate at room temperature for 30 min.
7. Add the transfection mixture from step 6 dropwise and do not disturb attached cells.
8. Incubate cells in the CO_2-humidified incubator for 4–12 h.
9. Change with growing media.
10. Cells can be harvested and levels of an exogenous protein can be assessed 36–48 h after transfection.

3.2.3 Transfection Using PEI

1. Grow cells to reach 90% confluent in a 10-cm plate.
2. Change media with 10 mL of serum-free DMEM (without antibiotics and FBS).
3. In a microcentrifuge tube, add 800 μL of serum-free DMEM, and DNA constructs (less than 50 μg) and mix by vortex.
4. In another microcentrifuge tube, add 800 μL of serum-free DMEM and 60 μL of PEI (DNA: PEI ratio is 1:3) and vortex; 2 mg/mL PEI pH 7.0 stored at −80°C.
5. Mix the abovementioned two tubes and incubate at room temperature for 20 min.
6. Add the PEI transfection mixture dropwise and incubate for 6–8 h.
7. Change media with growing media.
8. Cells can be harvested and levels of an exogenous protein expression can be assessed 36–48 h after transfection.

3.3 Protein Extraction for Western Blotting

Since mTORC1 senses intracellular nutrient levels, we recommend that cells are lysed promptly with lysis buffer without washing with nutrient-free solution such as PBS buffer.

1. For six-well plates, remove media completely by aspiration and immediately add 300 μL of cold NP-40 lysis buffer (10 m*M* Tris–Cl pH 7.5, 2 m*M* EDTA, 150 m*M* NaCl, 1% NP-40, 10 m*M* pyrophosphate, 10 m*M* glycerophosphate, 50 m*M* NaF, and EDTA-free protease inhibitors [Roche]). For a 10-cm plate, add 1 mL of lysis buffer.
2. Incubate on ice for 15 min with occasional tapping.
3. Transfer suspension into 1.5-mL microcentrifuge tubes.
4. Spin at maximum rpm for 15 min at 4°C.
5. Transfer 150 μL of supernatant into a new tube.
6. Add 50 μL of 4 × SDS sample buffer (200 m*M* Tris–Cl pH 6.8, 8% SDS, 40% glycerol, 20% β-mercaptoethanol, and 0.4% bromophenol blue) and vortex briefly.
7. Denature proteins in a 95°C heating block for 5 min.
8. Keep the samples at room temperature for 10 min.
9. Use 10 μL of each sample to apply to a well of SDS-PAGE, followed by Western blot analysis. Samples can be stored at –20°C for several years.

3.4 Assessing Cellular mTOR Activity Using Phosphospecific Antibodies

Since mTORC1 is sensitive to nutrients and growth factors (Figs. 1 and 3), washing cells with buffers without them is not desirable, which may lower the activity of mTORC1 during washing or harvesting. If it is necessary to remove unwanted components in media from cell surfaces, cells should be rinsed quickly. As described earlier, the starvation of nutrients such as amino acids inhibits the activity of mTORC1 within 30 min in most cells. However, the inhibition of mTORC1 and mTORC2 caused by growth factor starvation varies among cells. For instance, the activity of mTORC2 can be inhibited by serum depletion in MEFs within 60 min, whereas it takes much longer in HEK293T or certain cancer cells with diminished PTEN or TSC2 activity. Both the mTORC1 and mTORC2 activity suppressed by nutrient- and growth factor-starvation can be regained within 15 min by the replenishment of nutrients and growth factors (Fig. 3).

To determine the activity of mTORC1 in vivo, Western blot analysis with phosphospecific antibodies against mTORC1 substrates is the most accurate and straightforward method. In addition, monitoring mobility shift caused by protein phosphorylation in SDS-PAGE is an alternative way when the phosphospecific antibody is not available. However, the appropriate percentage of separating gel in SDS-PAGE needs to be empirically determined to obtain a clear mobility shift of target proteins by phosphorylation. For example, to detect the mobility shift of S6K1 or 4E-BP1, generally 8% or 13% SDS-PAGE, respectively, is considered as the ideal setting for obtaining clear mobility shift of these proteins. 4EBP1 is a representative protein that can be phosphorylated as multiple residues and detected as three major bands (α, β, and γ form) in Western blotting. The α, β, and γ 4EBP1 correspond to non/hypo-, less-, and hyperphosphorylated form, respectively (Fig. 3).

To monitor cellular mTORC2 activity, levels of Akt phosphorylation on serine 473 (hydrophobic site) can be determined by Western blotting with phospho-Ser473 Akt antibody. In this section, we describe methods to monitor the activity of mTORC1.

1. Cells grow 70% confluent in a 10-cm plate.
2. Wash with HBSS with calcium and magnesium twice.
3. Add 10 mL of HBSS with calcium and magnesium and incubate in the humidified CO_2 incubator for 1 h to inhibit mTORC1 activity completely.
4. Remove HBSS by aspiration and add 10 mL of DMEM media containing amino acids and 10% FBS for maximum activation of mTORC1. For only amino acid stimulation, add DMEM without FBS.
5. Incubate for additional 15–30 min.
6. Extract proteins as mentioned earlier: For the Western blotting, NP-40 lysis buffer is preferred.
7. In Western blotting, levels of phosphorylation of S6K1 and its total protein can be determined using phosphospecific S6K1 (phospho-T389) and S6K1 antibody, respectively. Levels of S6 phosphorylation and its total protein can also be monitored for determining cellular S6K activity using phospho-S6 (phospho-S240/244) and S6 antibodies. Note that Ser235/236 phosphorylation of S6 can be induced by not only mTORC1-S6K pathway, but also other kinases, including RSK. The activity of mTORC1 or S6K1 can be determined by the ratio of pS6K1/S6K or pS6/S6, respectively.

3.5 Accessing Levels of mTOR Complexes and Their Activities by Western Blotting

Although Western blotting is one of the most widely used techniques, the task of obtaining a clear band of high-molecular-weight proteins such as mTOR (288 kDa) and the components of mTOR complexes by Western blotting needs some extra effort. To detect mTOR, Raptor, and Rictor, approximately 8% SDS-PAGE can be used.

1. During SDS-PAGE, briefly rinse a PVDF membrane with water.
2. Activate a membrane with MeOH until it becomes transparent: this takes a couple of seconds.
3. Remove MeOH and incubate with transfer buffer. The transfer buffer can be prepared a day before the experiment; keep it at 4°C to enhance transfer efficiency (for 4 L, Tris 12.1 g, glycine 57.6 g, MeOH 800 mL).
4. Incubate the membrane in transfer buffer for at least 5 min at room temperature by shaking.
5. Transfer proteins to the membrane at 4°C for 180 min at fixed 350 mA for mTOR, Raptor, S6K1, Rictor, and Akt in a 8% SDS-PAGE gel, and for 120 min at the same mA for S6 and 4EBP1 in a 13% SDS-PAGE gel.
6. After transfer, briefly wash the membrane three times with TBST.
7. Block the membrane with blocking buffer (5% nonfat dry milk in TBST) for 20–60 min.
8. Wash the membrane as in step 6 to remove excess milk from the membrane.
9. Incubate the membrane in antibody solution overnight at 4°C with gentle rocking: primary antibodies are diluted in 10 mL of TBST containing 5% BSA and 0.02% sodium azide.
10. The next day, collect primary antibodies in polystyrene tubes to recycle these antibodies and keep them at –20°C.
11. Wash the membrane three times with TBST for 30 min.
12. Incubate the membrane with secondary antibodies conjugated with HRP (1:5000) in blocking solution and incubate for 2 h at room temperature with rocking.
13. Wash the membrane with TBST four times for 40 min (longer than 40 min washing is also acceptable).
14. Visualize target protein bands using ECL mixture onto X-ray film.

3.6 Membrane Stripping and Reprobing Membrane

1. After visualizing proteins by ECL reagents, recover the membrane and wash with water several times to remove TBST and ECL: if membrane is dried, activate with MeOH and wash with water.
2. Wash the membrane with stripping buffer (25 m*M* glycine, 1% SDS, pH 2) for 40 min (each 10 min × four times).
3. Briefly wash the membrane with water several times to remove any trace of SDS in the stripping buffer and wash three times with TBST for 30 min.
4. Repeat Western blotting from the blocking stage to probe other proteins.

3.7 Coimmunoprecipitation of mTORC1 and mTORC2

mTOR immunoprecipitation using mTOR antibodies can pull-down all the key components of mTORC1 and mTORC2, since mTOR is a common component in both mTORC1 and mTORC2. If specific isolation of mTORC1 or mTORC2 is desired, immunoprecipitation of Raptor for mTORC1 or Rictor for mTORC2 is necessary. For the coimmunoprecipitation (co-IP) of mTOR complexes, CHAPS lysis buffer must be used because other nonionic detergents such as NP-40 and TX-100 disrupt the integrity of these complexes.

1. Grow cells 70% confluent in 10-cm plates.
2. Remove media completely by aspiration and add 1 mL of CHAPS lysis buffer [40 m*M* HEPES pH 7.5, 120 m*M* NaCl, 1 m*M* EDTA, 0.6% CHAPS 10 m*M* pyrophosphate, 10 m*M* glycerophosphate, 50 m*M* NaF, and EDTA-free protease inhibitors (Roche)] immediately.
3. Incubate on ice for 15 min with occasional tapping.
4. Collect suspension and spin at maximum rpm for 15 min at 4°C.
5. Transfer 800 μL for immunoprecipitation and 150 μL for input (lysate).
6. Add 1–2 μg of mTOR antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA, cat# SC1549) into 800 μL of extract and incubate for 2–3 h at 4°C with gentle rocking.
7. Add 20 μL of protein G sepharose beads (50% slurry in CHAPS lysis buffer, GE Healthcare, Little Chalfont, UK, cat# 17-068-01) and incubate for another hour.
8. Wash five times with CHAPS lysis buffer.
9. Remove the lysis buffer completely and denature immunoprecipitated proteins with 50 μL of 1 × SDS sample buffer for 5 min at 95°C.

10. After 10 min of incubation at room temperature, spin samples for 10 s and analyze those in 8% or lower SDS-PAGE, followed by Western blotting.
11. In Western blotting, co-IPed mTORC1 and mTORC2 components can be detected by using specific antibodies (Cell Signaling Technology, Danvers, MA, USA), such as mTOR (cat# 2983), Raptor (cat# 2280), mLST8 (cat# 3274), and Rictor (cat# 9476). All these antibodies are diluted 1/1000 in 5% BSA TBST and stored at –20°C. Frozen diluted antibodies are thawed at room temperature before use and incubated at 4°C for overnight.

3.8 In Vitro Kinase Assay

In order to measure the kinase activity of mTORC1 or mTORC2 directly, an in vitro kinase (IVK) assay can be performed using S6K1 or 4EBP1 as a substrate for mTORC1, and Akt for mTORC2 kinase assay.

3.8.1 Preparation of GST-S6K1 from Mammalian Cells

1. Grow HEK293T cells 60% confluent in 5 × 15-cm plates.
2. Transfect with 20 μg of mammalian expression GST-S6K1 using the calcium phosphate method.
3. Next, 48 h after transfection, starve cells with HBSS for 2 h or treat cells with 250 n*M* of torin1 or 100 n*M* of rapamycin for 1 h to completely dephosphorylate GST-S6K1 within the cells.
4. Rinse cells with ice-cold PBS one time.
5. Lyse cells with PBST buffer (PBS with 0.3% Tween-20, 1 mL per plate) with protease inhibitors.
6. Incubate on ice for 15 min.
7. Collect suspension into microcentrifuge tubes.
8. Spin at 4°C for 15 min at maximum rpm.
9. Transfer the supernatant into a new 15-mL tube.
10. Add 50 μL of PBST-washed 50% slurry of glutathione sepharose beads and rock it at 4°C for 4 h.
11. Wash three times with HNTG buffer (20 m*M* HEPES pH 7.5, 150 m*M* NaCl, 10% glycerol, 0.1% Triton-X100).
12. Wash twice with cold PBS.
13. Elute with 10 mg/mL of GSH solution (reduced glutathione in 100 m*M* Tris pH 8) for 4 h.
14. Dialyze the eluent with cold dialysis buffer (10 m*M* Tris pH 7.5, 50 m*M* NaCl, 0.5 m*M* EDTA, and 0.05% beta-mercaptoethanol).

15. Determine the concentration by SDS-PAGE and snap-freeze in liquid nitrogen and store at –80°C until use.

Note that GST-4EBP1 or GST-Akt can be prepared using the same method. For GST-Akt purification, cells transfected with GST-Akt should be treated with mTOR kinase inhibitors such as Torin1 before harvesting. Low-molecular-weight substrates such as GST-4EBP1 can also be prepared from bacteria using a bacterial GST expression vector.

3.8.2 mTOR in vitro Kinase Assay Using Phosphospecific Antibody

For kinase assays, mTORC1 can be immunoprecipitated by Raptor antibodies, while mTORC2 is immunoprecipitated by Rictor antibodies. For in vitro kinase assays using exogenous mTORC1 and mTORC2, each component of the complexes needs to be transfected.

1. Grow cells (e.g., HEK293T, MEF) at 80% confluency in 10-cm plates.
2. Remove media and briefly wash cells with cold PBS.
3. Lyse cells in CHAPS lysis buffer containing protease inhibitors and phosphatase inhibitors (1 mL per 10-cm plate) on ice for 15 min.
4. Collect lysates and spin at 4°C for 15 min at maximum rpm.
5. Transfer supernatant into a new tube.
6. Add 1 μg of Raptor or Rictor antibodies and rock it for 3 h at 4°C.
7. Add 20 μL of 50% slurry of protein G sepharose beads in CHAPS buffer.
8. Rock it for 1 h at 4°C.
9. Wash three times with CHAPS lysis buffer and wash once with HEPES washing buffer (25 m*M* HEPES–KOH, 20 m*M* KCl, pH 7.4).
10. Wash once with 1 × kinase reaction buffer without ATP (20 m*M* Tris–HCl pH 7.5, 10 m*M* $MgCl_2$).
11. Add 25 μL of kinase reaction mixture: 5 μL of 5 × kinase reaction buffer, 120 ng of GST-S6K1 for mTORC1 and GST-AKT for mTORC2, 200 μ*M* ATP.
12. Incubate at 37°C for 20 min with gentle rocking.
13. Terminate the reaction by adding 10 μL of 4 × SDS sample loading buffer and denature the samples at 95°C for 5 min.
14. Analyze the samples by Western blotting.

For mTORC1 kinase assays, levels of GST-S6K1 phosphorylation can be detected by using phospho-Thr389 S6K1 antibodies. Similarly, phospho-Ser473 Akt antibodies can be used for mTORC2-dependent GST-Akt phosphorylation.

3.9 mTOR Immunofluorescence Staining

Lysosomal localization of mTORC1 is necessary for its activation and stimulated in a manner dependent on Rag GTPases activity. Thus, monitoring cellular mTORC1 localization can be used for an indirect measurement of Rag and mTORC1 activity. To determine mTOR localization on lysosomes, cells need to be starved with amino acid-free media such as HBSS or DPBS (with calcium and magnesium) for 50 min to dissipate mTORC1 from lysosomal membranes. Replenishment of amino acids (DMEM without FBS) sufficiently induces lysosomal mTOR localization within 5 min in MEF cells.

1. Grow cells on a round-cover slide in a 12-well plate.
2. Wash with PBS once to remove media.
3. Fix cells with 1 mL of warmed PBS containing 4% paraformaldehyde at 37°C for 5 min.
4. Wash with PBS twice and permeabilize with 1 mL of permeabilizing buffer (PBS containing 0.05% Triton X-100) at room temperature for 5 min.
5. Incubate cells with PBS containing 0.25% BSA at room temperature for 1 h for blocking.
6. Add mTOR antibody (Cell Signaling Technology, Danvers, MA, USA, cat# 2983, 1:100 dilution) into blocking solution and incubate at room temperature for 1 h or at 4°C for 16 h.
7. Wash four times with PBS.
8. Incubate with fluorescence-conjugated secondary antibodies (1:1000 dilution) at room temperature for 30 min in the dark.
9. Wash four times with PBS and once with water.
10. Mount slides and keep in a slide box at room temperature until analysis using a microscope.

3.10 GTP Loading Assay of Small GTPase

To investigate events upstream of mTORC1, it is important to measure the activity of Rheb or Rag to dissect the molecular mechanism underlying mTORC1 activation (Fig. 4). The activity of these small GTPases can be assessed by determining amounts GTP and GDP that bind to Rheb or Rag in vivo. Generally, an increased ratio of GTP/GDP indicates the activation of small GTPases. This assay can be done for both endogenous and exogenous small GTPases. Next, we introduce the assay for measuring the

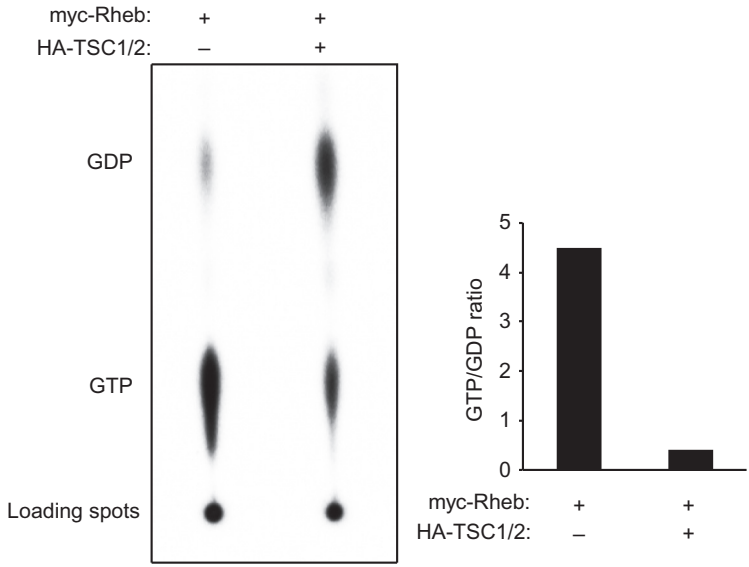

Fig. 4 GTP loading assay for Rheb GTPase. HEK293T cells were transfected with the indicated cDNA constructs. 48 h after transfection, cells were labeled with radioactive ^{32}P phosphate, and immunoprecipitated myc-Rheb was analyzed in the GTP loading assay. Rheb-bound radioactive GTP and GDP were visualized by a PhosphoImager and the volumes of Rheb-bound GTP and GDP were quantified by an ImageQuant. The data were expressed as a ratio (GTP moles/GDP moles, GTP moles = GTP volume/3, GDP moles = GDP volume/2) (*right panel*). Coexpression of HA-TSC2 with myc-Rheb largely stimulated GTP hydrolysis of Rheb.

activity of exogenous Rheb (myc-Rheb) and discuss appropriate approaches to measure the activity of Rag small GTPases.

3.10.1 Accessing Guanine Nucleotide Loading Status on Rheb In Vivo

1. On a six-well plate, transfect 50 ng of myc-Rheb construct using lipofectamine, as previously described.
2. Wash cells once with phosphate-free DMEM (ThermoFisher, Waltham, MA, USA, cat# 11971-025).
3. Incubate cells with 0.8 mL/well of phosphate-free DMEM at the humidified CO_2 incubator for 60 min.
4. During the incubation, prepare a labeling master mix: 245 μL of phosphate-free DMEM, 105 μL of ^{32}P orthophosphate (5 mCi/mL) per well of a six-well plate.
5. Add 50 μL of the mixture per well.
6. Incubate for 4 h.

7. Prepare antibody-protein G sepharose bead conjugates: Add 1–2 μg of myc antibodies into 10 μL of the beads (50% slurry in the lysis buffer) per well and rock it at 4°C for 2 h.
8. Remove labeling media and lyse the cells with 250 μL of lysis buffer (50 m*M* Tris pH 7.5, 0.5% NP-40, 100 m*M* NaCl, 10 m*M* $MgCl_2$, 1 m*M* DTT, and protease inhibitors) per well.
9. Gently rock the plate on ice for 30 s and transfer the lysate into a tube.
10. Centrifuge for 15 min with maximal rpm at 4°C.
11. Take 200 μL of supernatant and add 10 μL of the antibody-protein G bead conjugates and NaCl to a final concentration of 0.5 *M* to block any GAP activity in the immunoprecipitants.
12. Rock the tube for 2 h at 4°C.
13. Wash three times with washing buffer I (50 m*M* Tris–Cl pH 8.0, 500 m*M* $MgCl_2$, 500 m*M* NaCl, 1 m*M* DTT, 0.5% TX-100).
14. Wash three times with washing buffer II (50 m*M* Tris–Cl pH 8.0, 100 m*M* NaCl, 5 m*M* $MgCl_2$, 1 m*M* DTT, 0.1% TX-100).
15. Add 20 μL of elution buffer (2 m*M* EDTA, 0.2% SDS, 1 m*M* GDP, 1 m*M* GTP) and rock it at 68°C for 10 min.
16. Spin shortly and recover supernatants for analysis.
17. Apply 10 μL of each sample onto a PEI cellulose plate (approximately 3 cm above the bottom of the plate) and dry it completely with a regular hairdryer.
18. Soak the plate in MeOH and dry it.
19. Immerse the bottom portion of the plate (below where the samples are loaded) with MeOH.
20. Stand the plate in the TLC chamber that is filled to a depth of 1 cm with TLC running buffer (1 *M* LiCl, 1 *M* formic acid).
21. Close the chamber lid to keep humidified in the chamber and remove the plate from the chamber when the solvent ascends to the top of the plate.
22. Dry the plate with a hairdryer.
23. Expose the TLC plate on a PhosphorImager screen for 6 h and read the radioactive GTP and GDP in a PhosphorImager.
24. Determine the amount of radioactive GTP and GDP by the using Imagequant software and calculate GTP/GDP ratio using the formula. GTP moles (=GTP signal/3), GDP moles (=GDP signal/2). Note that overexpression of Rheb sufficiently activates mTORC1 in HEK293T cells, indicating that excess expression of Rheb overcomes the activity of endogenous TSC2, a specific GAP for Rheb. Therefore,

endogenous or lower levels of exogenous Rheb that do not affect basal mTORC1 activity need to be analyzed.

3.10.2 Accessing in vivo Guanine Nucleotide Loading Status on Rag

RagA or RagB forms an obligate heterodimer with RagC or RagD. In addition, in the most active state, RagA/B is GTP-charged, whereas RagC/D is the GDP-bound form. A Rag heterodimer is very stable; therefore, it is difficult to determine the amount of GTP and GDP that bind to one of the Rags within a heterodimer in vivo. Therefore, to analyze endogenous Rag activity in vivo, it may need to establish special cell lines. For instance, the RagC S75L mutant (Oshiro, Rapley, & Avruch, 2014), which is unable to bind guanyl nucleotides, can be stably expressing in cells lacking both RagC and RagD expression, and then endogenous RagA can be IPed and monitored levels of bound GTP and GDP.

4. CONCLUDING REMARKS

Over the past several decades, tremendous efforts to reveal molecular mechanisms underlying the regulation of mTOR signaling have been made, and hence numerous new members in the mTOR pathway have been discovered. Identification of new members in the mTORC1 signaling has shed light on the molecular mechanism by which mTORC1 is activated by amino acids. During the last decade, more than 20 new proteins have been identified in the regulation of amino acids-induced mTORC1 activation. Identifications of the molecular mechanisms by which mTORC1 receives signals from amino acids such as leucine and arginine are the most significant discoveries in the last few years. However, there are still unanswered questions. For example, the molecular mechanism by which glutamine induces mTORC1 activation remains elusive. It has been reported that glutamine is transported into cells through the SLC1A5 amino acid transporter; hence cellular glutamine in turn is used to import leucine via the antiporter SLC7A5-SLC3A2, thereby stimulating mTORC1 through Rag activation (Nicklin et al., 2009). In addition, α-ketoglutarate, a glutamine metabolite, can stimulate GTP loading of RagB. However, a recent study has shown that glutamine induces lysosomal mTORC1 localization and its activation in a manner independent of Rag small GTPases (Jewell et al., 2015). Secondarily, GEFs for Rheb and RagC/D have not been identified. It is possible that GEFs for Rheb or RagC/D may not be necessary for the regulation of these small GTPases. For instance, Rheb

has little its own GTPase activity of its own, and high concentrations of cellular GTP may be spontaneously loaded to Rheb. Therefore, the regulation of the TSC complex may fulfill the sole mechanism of Rheb-induced mTORC1 activation. Finally, another interesting topic that has not been fully elucidated is where mTORC1 can physically interact with its distinct substrates, including S6K, 4EBPs, and ULK1. For instance, upon mTORC1 activation, the majority of these substrates can be sufficiently and fully phosphorylated. However, these substrates are not exclusively expressed at the surface or surrounding of the lysosomes where mTORC1 is activated. It remains unclear whether mTORC1 leaves lysosomes to find its substrates, and if it does, how the trafficking of mTORC1 from lysosomes is regulated.

In terms of the mechanism of mTORC2 activation, it has been shown that ribosomes play an important role in the PI3K-dependent mTORC2 activation (Zinzalla et al., 2011). However, it has not been fully understood how the association of mTORC2 with ribosomes stimulates mTORC2. In this chapter, we described the basic but critical techniques and methods to examine cellular mTOR activity, and hopefully, these methods can help to elucidate these questions that are still in mystery.

ACKNOWLEDGMENTS

We thank members of the Inoki lab for helpful discussions and this work was supported by National Institutes of Health (NIH) Grants (DK083491 and GM110019) and the Department of Defense CDMRP grant (DOD TS140055).

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Review

Lysosomal Regulation of mTORC1 by Amino Acids in Mammalian Cells

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Academic Editors: Kazuhiro Shiozaki and Ted Powers
Received: 1 June 2017; Accepted: 4 July 2017; Published: 7 July 2017

Abstract: The mechanistic target of rapamycin complex 1 (mTORC1) is a master regulator of cell growth in eukaryotic cells. The active mTORC1 promotes cellular anabolic processes including protein, pyrimidine, and lipid biosynthesis, and inhibits catabolic processes such as autophagy. Consistent with its growth-promoting functions, hyper-activation of mTORC1 signaling is one of the important pathomechanisms underlying major human health problems including diabetes, neurodegenerative disorders, and cancer. The mTORC1 receives multiple upstream signals such as an abundance of amino acids and growth factors, thus it regulates a wide range of downstream events relevant to cell growth and proliferation control. The regulation of mTORC1 by amino acids is a fast-evolving field with its detailed mechanisms currently being revealed as the precise picture emerges. In this review, we summarize recent progress with respect to biochemical and biological findings in the regulation of mTORC1 signaling on the lysosomal membrane by amino acids.

Keywords: mTOR; mTORC1; rapamycin; Rheb; Rag; TSC; lysosome; amino acid; growth factor

1. Overview of Mechanistic Target of Rapamycin Complex 1

During evolution, cells in different species developed diverse strategies to sense extracellular cues and adapt to environmental changes. Among these extracellular cues, nutrient availability is the most fundamental element in determining cell survival and growth. In multicellular eukaryotic organisms, growth factor signaling has impinged on the nutrient signal to establish scrupulous regulation of cellular nutrient usage in a spatiotemporal manner. Thus, cells in different tissues systemically sense nutrients and use these signals to control their growth, proliferation, quiescence, or survival. Recent studies demonstrate that the cellular multiunit protein complex, mechanistic target of rapamycin (mTOR) complex 1, functions as a central regulator of cell growth in response to nutrients and growth factors.

mTOR is an evolutionarily conserved serine/threonine protein kinase, which belongs to the phosphoinositide-3-kinase (PI3K)-related family of protein kinases [1]. mTOR forms large protein complexes with other proteins, and the configurations of these mTOR complexes (mTORCs) are also evolutionarily conserved from yeast to mammals [2,3]. Two mTOR-containing multi-protein complexes have been identified, named mTOR complex 1 (mTORC1) and mTORC2 [4–8]. mTORC1 and mTORC2 have their specific accessory components: regulatory-associated protein of mTOR (Raptor) and proline-rich Akt substrate 40 (PRAS40) are specific to mTORC1 while the rapamycin-insensitive companion of mammalian target of rapamycin (Rictor), stress-activated protein kinase-interacting

protein 1 (Sin 1), and the protein observed with Rictor-1 (Protor) are specific for mTORC2. Two other proteins, mammalian lethal with Sec13 protein 8 (mLST8) and Dishevelled, Egl-10, and Pleckstrin (DEP) domain containing mTOR interacting protein (Deptor), are common mTOR interacting proteins found in both mTORC1 and mTORC2 [9–14]. In addition, both mTORC1 and mTORC2 form an obligate dimer [15–17]. The major cellular role of mTORC1 involves its cell growth control, while mTORC2 regulates cytoskeleton organization and cell survival.

mTORC1 activity is sensitive to rapamycin, a macrolide originally developed as an antifungal agent [18]. Rapamycin strongly interacts with FK506-binding protein 12 (FKBP12), and this drug–protein complex binds to the FKBP12–rapamycin-binding (FRB) domain of mTOR kinase [19]. The FRB domain acts as a gatekeeper since its rapamycin binding site interacts with substrates to grant them access to the restricted active site of mTOR kinase. The rapamycin–FKBP12 complex therefore allosterically inhibits mTOR kinase by blocking substrate recruitment and further restricts the accessibility of substrates to the active site of mTOR kinase [20]. However, the sensitivity of rapamycin varies significantly among different mTORC1 substrates. Interestingly, the FKBP12–rapamycin complex not only restricts the accessibility of substrates to the active site of mTOR kinase but also weakens the mTOR–Raptor interaction and destabilizes the dimeric structure of mTORC1 [6,15,21]. It has been postulated that the dimeric conformation of mTORC1 is required for its phosphorylation of eukaryotic translation initiation factor 4E binding protein 1 (4EBP1) but not ribosomal S6 kinase 1 (S6K1) [15]. Thus, in addition to the size of substrates, the integrity of the mTORC1 dimer determines the sensitivity of rapamycin to inhibit mTORC1-dependent phosphorylation of its substrates.

Raptor is an essential component of mTORC1, forming an obligate dimer with an overall rhomboid shape and a central cavity. The dimeric interfaces are formed by interlocking interactions between mTOR and Raptor [15]. Raptor functions as a scaffold protein to recruit mTORC1 substrates such as S6K1 and 4EBP1. These substrates are recognized by mTORC1 through their TOR signaling (TOS) motif, which is a conserved five amino acid sequence and is crucial for their interaction with Raptor [22–24]. In addition to its role in substrate recognition, recent studies reveal the role of Raptor in determining mTORC1 subcellular localization. In response to amino acids, Raptor interacts with the lysosomal Ras-related guanosine 5′-triphosphate (GTP)-binding protein (Rag small GTPase protein complex tethering mTORC1 to the lysosomal membrane, where it encounters another small GTPase, Ras homolog enriched in brain (Rheb) that directly interacts with the mTOR kinase and stimulates the activity of mTORC1 [25,26].

Rheb resides at the cellular endomembrane system [26–28]. Previous studies suggested that Rheb localizes on different cellular compartments, including endoplasmic reticulum (ER), Golgi, mitochondria, peroxisome, and importantly, lysosome [29–32]. Rheb localizes to their membranes through its farnesylation on the "CAAX" motif [33]. The mutation of cysteine in the CAAX motif disrupts the membrane localization of Rheb and disables the ability of Rheb for mTORC1 activation, suggesting that appropriate membrane localization of Rheb and mTORC1 are required for mTORC1 activation. As a small GTPase protein, the GTP/guanosine 5'-diphosphate (GDP) loading status of Rheb is important for its activity. When GTP is loaded, Rheb functions as a potent stimulator for mTORC1 kinase activity [11,34]. However, the precise molecular mechanisms by which Rheb specifically stimulates mTORC1 have not been well understood. It has been demonstrated that the tuberous sclerosis complex (TSC) consisting of TSC1, TSC2, and the Tre2-Bub2-CDC16 (TBC) 1 domain family member 7 (TBC1D7) also localizes on the membrane of lysosomes and peroxisomes and inhibits Rheb activity by functioning as a GTPase activating protein (GAP) complex [31,35–40]. Among these subunits, TSC2 bears a GAP domain, which specifically converts Rheb from the GTP-bound active form to GDP-bound inactive form [35,41,42]. The activity of TSC2 is regulated by the growth factor-dependent PI3K–Akt pathway. Akt directly phosphorylates at least four serine and threonine residues of TSC2 and induces the dissociation of the TSC complex from lysosomal membranes [27,43]. Although the molecular mechanisms by which TSC2 phosphorylation by Akt dislocates the entire TSC complex away from the lysosome remain unclear, the absence of its GAP activity on the lysosomal

membrane provides the permissive conditions for Rheb GTP-loading and its activation [27] (refer to the subsequent section for details). Interestingly, recent studies demonstrated that lysosomal localization of TSC is also diminished by amino acids [43,44] (see the details in the following section). Thus, the coordinated spatial regulations of both mTORC1 and the TSC complex establish the machinery for sensing multiple environmental cues to regulate cell growth control.

2. The Lysosome is the Major Cellular Compartment for mTORC1 Activation

The lysosome is a major catabolic organelle and is responsible for the degradation of all kinds of biomolecules [45]. Over 60 digestive enzymes are found in the lysosomal lumen and are used for macromolecule hydrolysis (i.e., proteins, lipids, and polysaccharides) breaking them down into their constitutive monomers (i.e., amino acids). These digested monomers are exported to the cytosol from lysosomes via diffusion and/or through specific transporters as fuels for various metabolic and biosynthetic pathways in response to cellular demands.

Although mTORC1 can be activated at the Golgi apparatus and the peroxisome [30,31,46], recent studies demonstrated that the lysosomal membrane is the major site for mTORC1 activation [25,26,47,48]. In response to amino acid availability, mTORC1 is recruited to the lysosomal membrane from unidentified cytosolic foci. The disruption of lysosomal mTORC1 localization largely diminishes its activation by amino acids. In contrast, artificially tethering mTORC1 to lysosomes renders constitutive mTORC1 activation, regardless of amino acid availability. This constitutive mTORC1 activation depends on Rheb, as its deletion abolishes mTORC1 activation even though mTORC1 is localized on the lysosomal membrane. Thus, the major role of amino acid input for mTORC1 activation is recruitment of mTORC1 to the place where Rheb, a direct activator of mTORC1, is localized. In support of this idea, tethering both mTORC1 and Rheb to other membrane compartments such as the plasma membrane sufficiently induces mTORC1 activation [25]. These observations clearly indicate that the lysosomal membrane functions as a key physiological platform to merge mTORC1 and Rheb for mTORC1 activation in response to amino acid availability.

3. Amino Acid-Sensing Signaling to the Lysosomal Membrane

3.1. The Rag GTPase and the Ragulator Complex Form a Super Complex with Vacuolar-ATPase, which Recruits mTORC1 to the Lysosomal Membrane in Response to Amino Acid Availability

Diverse upstream signals including growth factors, hypoxic stress, energy, and amino acids impinge on the TSC complex to regulate Rheb–mTORC1 activity. Among these signal inputs, amino acids also exert a crucial role in supporting mTORC1 activation independent of the regulation of the TSC complex. mTORC1 activity is still inhibited upon amino acid withdrawal while it is resistant to growth factor starvation in cells lacking functional TSC complex [49,50].

By using genetic and biochemical approaches, studies from different labs have identified that an evolutionarily conserved Ras-related small GTPase (Rag), plays a key role in enhancing mTORC1 activity in response to amino acids [26,51]. Mammalian cells contain four members of Rag proteins (RagA, B, C, and D), which are expressed on the lysosomal membrane [52]. RagA and B, like RagC and D, are highly similar to each other and functionally redundant. Rags form obligate heterodimers of either RagA or RagB with either RagC or RagD. Interestingly, in the active Rag heterodimer, RagA or RagB binds to GTP while RagC or RagDs binds to GDP. In addition, these nucleotide-loading states are tightly regulated by lysosomal luminal and cytoplasmic amino acids although the precise measurement of the in vivo nucleotide-loading status of each Rag small GTPase in the Rag heterodimer is challenging. Indeed, Oshiro et al. failed to detect any significant changes of GTP-charging toward RagA and RagC in response to amino acid stimulation [53]. Thus, further efforts to develop relevant methods (e.g., active/inactive-Rag antibodies) are necessary for monitoring in vivo Rag activity. Upon amino acid availability, the active Rag heterodimer interacts with mTORC1 through Raptor, an essential component of mTORC1, thereby recruiting mTORC1 to the lysosomal

membrane [26]. Loss of functional Rag heterodimer largely disrupts lysosomal mTORC1 localization and significantly reduces acute induction of mTORC1 activity in response to amino acid stimulation. In contrast, the expression of a constitutive active Rag heterodimer (e.g., RagB–GTP/RagC–GDP) confers constitutive lysosomal mTORC1 localization with its activity being resistant to amino acid starvation (Figure 1).

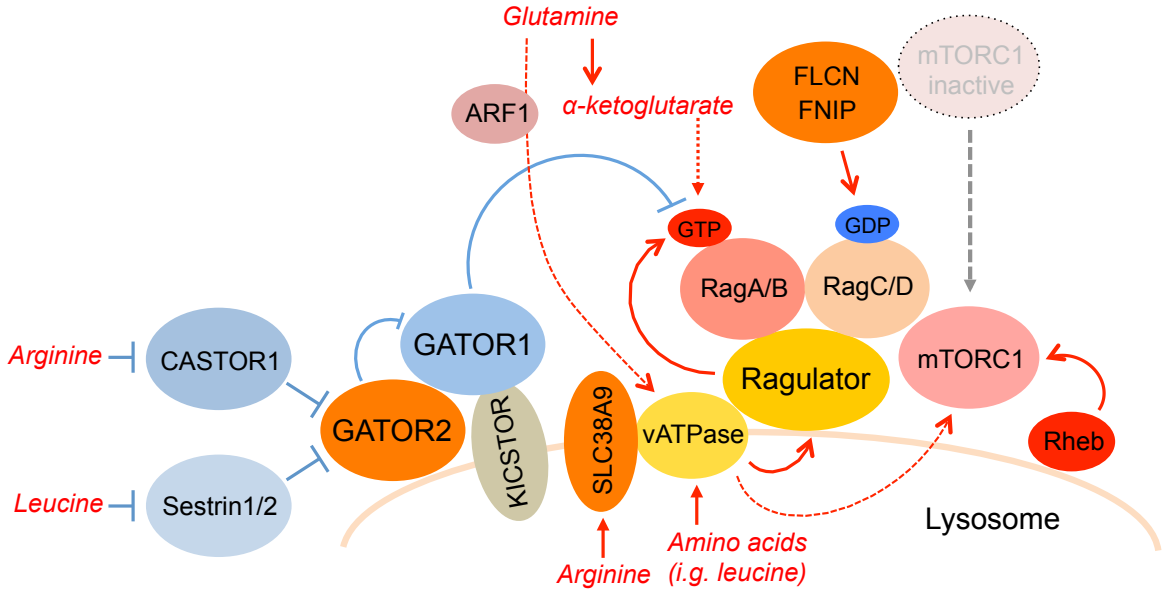

Figure 1. Amino acid-sensing mechanisms that recruit mechanistic target of mTORC1 to the lysosomal membrane. Cytosolic arginine and leucine activate GATOR2 by CASTOR1 and Sestrin1/2, respectively, leading to the inhibition of GATOR1, the GAP for the RagA/B small GTPases. Lysosomal luminal arginine activates vATPase through SLC38A9, leading to the activation of Ragulator, the guanine exchange factor (GEF) for RagA/B. Upon the activation of the Rag heterodimer, mTORC1 is recruited to the lysosomal membrane and is then activated by the small Rheb GTPase. CASTOR: cellular arginine sensor for mTORC1; Rheb: Ras homolog enriched in brain; GATOR: GTPase activating protein (GAP) activity toward Rags; KICKSTOR: Kaptin (KPTN), Integrin alpha phenylalanyl-glycyl-glycyl-alanyl-prolyl (FG-GAP) repeat containing 2 (ITFG2), chromosome 12 open reading frame 66 (C12orf66) and seizure threshold 2 homolog (SZT2)-containing regulator of mTORC1; Rag: Ras-related GTP binding; mTORC1: mechanistic target of rapamycin complex 1; GDP: guanosine 5′-diphosphate; GTP: guanosine 5′-triphosphate; ARF1: adenosine diphosphate-ribosylation factor 1; FLCN: folliculin; FNIP: folliculin interacting protein; vATPase: vacuolar H^+-ATPase; SLC38A9: solute carrier family 38 member 9.

Although the Rag heterodimer localizes on the lysosomal membrane, Rags do not possess membrane localization signals, unlike other small GTPases. Importantly, lysosomal Rag expression depends on Ragulator, a pentameric protein complex anchored on the lysosome [25,26,47]. Ragulator consists of p18 (late endosomal/lysoosmal adaptor and mitogen-activated protein kinase and mTOR activator 1 (LAMTOR1)), p14 (LAMTOR2), mitogen-activated protein kinase (MAPK) /extracellular signal-regulated kinase (ERK) kinase (MEK) binding partner 1 (MP1/LAMTOR3), C7ORF59 (LAMTOR4), and hepatitis B virus X-interacting protein (HBXIP/LAMTOR5) and interacts with the Rag heterodimer. One of the Ragulator subunits, LAMTOR1, is myristoylated and palmitoylated at its N-terminus and anchors the Ragulator complex and Rag heterodimer to the lysosomal membrane [25,47,54]. In support of this model, the Rag heterodimer is unable to localize on the lysosomal membrane in cells lacking LAMTOR1. As expected, amino acid-induced lysosomal mTORC1 localization and its activation are largely diminished in LAMTOR1 deficient cells as seen

in RagA/B knockout cells. These observations indicated that the Ragulator complex is an essential component in Rag-dependent mTORC1 activation in response to amino acids (Figure 1).

Importantly, in addition to its scaffolding role for the Rag heterodimer on the lysosomal membrane, the Ragulator complex also functions as a GEF for RagA and RagB [47]. Upon amino acid stimulation, the GEF activity of Ragulator promotes GTP loading to RagA and RagB in a manner dependent on lysosomal vATPase activity. Moreover, four out of five components of Ragulator (LAMTOR2–5) contain a putative roadblock domain, which is often observed in GTPase regulatory proteins [55,56]. However, the precise molecular mechanism as to which each subunit plays a critical role for the Ragulator complex GEF activity remains elusive. As all of the components of Ragulator are indispensable for its function as a Rag A/B GEF, it is possible that a tertiary structure composed by these subunits is required for the GEF activity towards RagA/B. This specific GEF activity toward Rag A/B but not Rag C/D likely stems from the difference between the RagA/B and RagC switch I/II regions, which are known to be a critical recognition motif on a GTPase for its cognate GEF [57]. As a GEF preferentially interacts with GDP-bound or nucleotide-free small GTPases, Ragulator also binds to the nucleotide-free RagA/B mutant with higher affinity compared to the wild type RagA/B. Accordingly, the interaction between RagA/B and Ragulator is weakened by amino acid stimulation whereas it is strengthened by amino acid starvation. These observations raise the possibility that GTP-loading to RagA/B incurs a conformational change of the Rag–Ragulator complex, which has a higher affinity for mTORC1. Other possibilities include that the GTP–Rag heterodimer interacting with mTORC1 may dissociate from the Ragulator complex upon amino acid stimulation to ferry mTORC1 from the lysosomal membrane to the cytosol [58]. Further studies will be required to determine the molecular mechanisms underlying the dynamics of Ragulator–Rag–mTORC1 interactions in response to amino acid stimulation or starvation.

By using an in vitro system, Zoncu et al. proposed that the lysosome contains all the machinery required for mTORC1 recruitment in response to amino acids as amino acid stimulation in vitro induces the association of Raptor with immunopurified lysosomes [48]. Intriguingly, the study also demonstrated that the amino acids inside of the lysosomal lumen play a key role in initiating a signal for mTORC1 recruitment to the lysosomal membrane and subsequent mTORC1 activation. Zoncu et al. also demonstrated that vATPase directly interacts with Ragulator, and that the structural rearrangement of vATPase but not the lysosomal proton gradient is important for lysosomal mTORC1 localization and activation. Treatment of isolated lysosomes with Streptolysin O or Triton X-100, which induce lysosomal luminal leakage, largely inhibits amino acid-induced Raptor interaction with the Ragulator–Rag complex in vitro. Furthermore, cells overexpressing H+/amino acid transporter 1 (PAT1/SLC36A1), a lysosome-specific proton-coupled amino acid transporter that exports amino acids out of the lysosomal lumen, inhibit amino acid-induced mTORC1 activation, though physiological levels of PAT1 are required for intact lysosomal function and mTORC1 activation [59]. Based on these observations, a lysosome-centric inside-out model of amino acid-sensing by mTORC1 has been proposed. This model states that amino acids within the lysosomal lumen initiate the signal for mTORC1 recruitment to the lysosomal membrane through the vATPase–Ragulator–Rag complex [48] (Figure 1).

Although the above lysosome-centric inside-out model clearly demonstrated how RagA and RagB are GTP-loaded through the Ragulator complex by luminal amino acids in the lysosome, recent studies have also identified other non-lysosomal amino acid sensors such as Sestrins (leucine sensor) and CASTOR proteins (arginine sensor), which regulate RagA/B GTP hydrolysis through GATOR1 [60,61] (refer to Sestrin and CASTOR sections). It is important to note that while the lysosomal Rag heterodimer plays a pivotal role on amino acid-induced lysosomal mTORC1 localization and its acute activation, cells seem to possess alternative mechanisms to sense amino acid availability in a manner independent of the Ragulator–Rag system. For instance, glutamine is able to stimulate lysosomal mTORC1 localization and its activity in RagA/RagB double knockout cells in an ADP-ribosylation factor 1 (ARF1)-dependent manner [62]. Furthermore, amino acids also stimulate GTP-charging to Rab1A, which stimulates the interaction between Rheb and mTORC1 at the Golgi and mTORC1 activation [30].

The molecular mechanisms by which Arf1 and Rab1 sense amino acids remain elusive and need further investigations.

3.2. GATOR1 is a GTPase Activating Protein for Rag A/B, while GATOR2 is a Negative Regulator of GATOR1

The Ragulator complex stimulates RagA/B through its GEF activity in response to lysosomal luminal amino acids. By contrast, recent studies identified GATOR, an octomeric protein complex, as a key regulator of RagA/B [63] (Figure 1).

GATOR (GTPase-activating protein activity toward Rags) is composed of two sub-complexes, GATOR1 and GATOR2, and localizes on the lysosome. Three proteins, DEP domain containing 5 (DEPDC5), nitrogen permease regulator 2-like protein (Nprl2), and Nprl3 comprise GATOR1, which inhibits the activity of Rag A/B, while the pentameric GATOR2 complex consisting of protein subunits, meiosis regulator for oocyte development (Mios), tryptophan-aspartic acid (WD) repeat-containing protein 24 (WDR24), WDR59, SEH1-like nucleoporin (Seh1L), and Sec13, functions as a suppressor of GATOR1 through unknown molecular mechanisms [63]. Loss of functional GATOR1 confers mTORC1 resistance to amino acid starvation and allows for its constitutive lysosomal localization. In contrast, loss of functional GATOR2 renders mTORC1 insensitive to amino acid stimulation and unable to localize to the lysosomal membrane even under amino acid availability conditions. Importantly, GATOR1 complex possesses specific GAP activity toward Rag A/B.

Interestingly, mutations in genes encoding GATOR1 components such as DEPDC5 and Nprl2 have been found in several cancer cells such as astrocyte tumors with chromosome 22 rearrangements, lung cancers with homozygous deletion on chromosome region 3p21.3, and NPRL2/G1 homozygous deletion in renal, lung and cervical cell lines [64–66]. In these cancer cell lines, as expected, mTORC1 constitutively localizes to the lysosomal membrane and therefore maintains its activity even under amino acid starvation conditions. Re-introduction of intact GATOR1 into these cells renders mTORC1 sensitive to amino acid starvation. Notably, the proliferation of these cells with loss of function GATOR1 mutations is highly sensitive to the mTORC1 inhibitor rapamycin compared to other cancer cell lines including HeLa and PC3, which bear phosphatase and tensin homolog (PTEN) loss of function mutations.

3.3. SLC38A9 is a Lysosomal Arginine Sensor for mTORC1 Activation

Among the twenty classical L-amino acids, arginine and leucine are two essential amino acids that potently stimulate the activity of mTORC1 in mammalian cells. However, the molecular mechanisms by which these specific amino acids stimulate mTORC1 activity have not been clearly understood. By searching amino acid transporters that localize on the lysosomal membrane or regulatory proteins that interact with the Ragulator–Rag complex, SLC38A9 was identified as a lysosomal amino acid transporter that interacts with the Ragulator–Rag complex [67,68]. SLC38A9 is a previously uncharacterized trans-membrane protein with sequence similarity to the SLC38 class of sodium coupled amino acid transporters [69]. SLC38A9 is predicted to have 11 trans-membrane domains with a cytosolic N-terminal region of 119 amino acids and a lysosomal luminal trans-membrane region [67,69]. SLC38A9 interacts with both the Ragulator complex and vATPase through its distinct regions on the lysosomal membrane [54,67]. Interestingly, the interaction between SLC38A9 and Ragulator is regulated by amino acid availability, as amino acid stimulation or starvation weakens or strengthens their interaction, respectively. Ablation of SLC38A9 suppresses the activation of mTORC1 by amino acids, whereas overexpression of wild-type SLC38A9 or the N-terminal 119 amino acids (Ragulator-binding domain) confers the mTORC1 activation resistant to amino acid starvation [67,68]. Epistatic analyses suggest that SLC38A9 functions upstream of the small Rag GTPases, as ectopic SLC38A9-induced mTORC1 activation is largely blocked by the expression of the dominant negative Rags [68]. These observations suggest that conformational rearrangements induced by amino acids between SLC38A9 and the Ragulator complex are necessary to stimulate the Ragulator–Rag system.

Importantly, ablation of SLC38A9 specifically attenuated arginine-induced but not leucine-induced mTORC1 activation [67]. Taken together, the studies suggest that SLC38A9 functions as a lysosomal membrane-resident arginine sensor for mTORC1 activation. However, it remains unclear how SLC38A9 specifically conveys a signal from arginine to the vATPase–Ragulator–Rag complex. More recently, Castellano et al. demonstrated that SLC38A9 also interacts with cholesterol through its cholesterol recognition motifs within the trans-membrane domain 8 and Niemann-Pick C1 (NPC1), which regulates cholesterol export from the lysosome [70]. SLC38A9 is required for mTORC1 activation by cholesterol in a manner independent of its arginine-sensing function. In contrast, NPC1 binds to SLC38A9 and inhibits mTORC1 activity through its sterol transport function. Thus, SLC38A9 functions as a key sensor for both arginine and cholesterol availability to instigate mTORC1 activation through the vATPase–Ragulator–Rag system on the lysosomal membrane.

3.4. CASTOR Proteins are Cytosolic Arginine Sensors for mTORC1 Activation

Although SLC38A9 plays a key role in sensing arginine availability to stimulate mTORC1 through the Ragulator complex, a GEF for RagA/B on the lysosomal membrane, a recent study also identified the cytosolic arginine sensor, cellular arginine sensor for mTORC1 (CASTOR), which activates GATOR1, a GAP for RagA/B, by inhibiting GATOR2, the upstream suppressor of GATOR1 [60] (Figure 1). CASTOR was originally identified as a GATOR2 interacting protein [60]. In vertebrates, two CASTOR proteins (CASTOR1 and CASTOR2 also known as stromal antigen (STAG) 3 opposite strand protein like 3 (GATSL3) and GATSL2, respectively) are found as cytosolic proteins. Intriguingly, both CASTOR1 and CASTOR2 bear four tandem aspartate kinase, chorismate mutase and TyrA (ACT) domains [60,71], which are known to interact with diverse small molecules such as amino acids and nucleotides [72–75]. CASTOR1 and CASTOR2 form a homo- or heterodimer [60]. Interestingly, amino acid depletion significantly enhances the interaction between GATOR2 and the CASTOR complex containing CASTOR1, whereas amino acids induce the dissociation of these complexes. Critically, CASTOR1 but not CASTOR2 specifically binds to arginine with a dissociation constant (Kd) of approximately 35 μM, which is similar to the half maximal concentration of arginine that induces the dissociation of GATOR2 from CASTOR1 in vitro and activates mTORC1 in vivo. Overexpression of CASTOR1 largely inhibits amino acid-induced mTORC1 activation, whereas ablation of CASTOR1 in cells confers mTORC1 activity substantially insensitive to deprivation of arginine. Furthermore, the CASTOR1 mutant that is unable to interact with arginine constitutively binds to GATOR2, rendering mTORC1 insensitive to arginine stimulation [71]. These results suggest that arginine binding to CASTOR1 triggers its dissociation from GATOR2 and relieves CASTOR1's inhibitory effect on GATOR2 (Figure 1). Notably, while dimerization of CASTOR1 is dispensable for arginine binding, CASTOR1 mutants are unable to form a dimer and only weakly interact with GATOR2 and lose their inhibitory effect on mTORC1 activity, indicating that dimerization of CASTOR1 is critical for its inhibitory effect on GATOR2.

The structure of CASTOR1 revealed that CASTOR1 forms a dimer, consistent with observations in previous biochemical analyses [60]. Among the four ACT domains in each CASTOR1 monomer, ACT2 and ACT4 generate an arginine-binding pocket at the interface of these domains [71,76,77]. The bound arginine forms extensive hydrophilic and hydrophobic interactions with the surrounding residues composing the binding pocket. Importantly, the critical residues of CASTOR1 for its interaction with GATOR2 cluster along the surface of the ACT2–ACT4 interface, adjacent to the arginine-binding pocket. One of these important residues is buried deep in the ACT2–ACT4 interface in the arginine-bound conformation of CASTOR1, potentially explaining why GATOR2 is unable to interact with the arginine-bound form of CASTOR1. Taken together, SLC38A9 and CASTOR1 have unique subcellular localizations and receive arginine signals from different cellular compartments thus regulating mTORC1 through distinct molecular mechanisms. SLC38A9 localizes on the lysosome and likely senses lysosomal luminal arginine to activate the Ragulator–Rag pathway in a vATPase dependent manner. On the other hand, CASTOR1 senses cytosolic arginine to regulate the GATORs–Rag pathway.

3.5. Sestrin2 is a Leucine Sensor for the mTORC1 Pathway, and Regulates the Activity of Rags through GATORs

Importantly, three recent independent studies have identified that Sestrins also interact with GATOR2 and inhibit mTORC1 activity [78–80] (Figure 1). The mammalian Sestrins comprise three related proteins, Sestrin1, 2 and 3. The expression of Sestrins is induced by several stress-responsive transcription factors such as p53, CCAAT/enhancer binding protein (C/EBP) bata, activating transcription factor 4 (ATF4), and forkhead box O proteins (FoxOs) [81–83]. Consistent with the roles of these transcription factors, Sestrins maintain cellular homeostasis in response to DNA damage, amino acid insufficiency, energy starvation, and oxidative stress [81–83]. Importantly, Sestrin1 and Sestrin2 strongly interact with GATOR2 under amino acid-deficient conditions while Sestrin3 constitutively interacts with GATOR2, irrespective of amino acid availability [61]. Furthermore, leucine is the only amino acid able to disrupt the interaction between Sestrin2 and GATOR2 within its physiological concentration both in vivo and in vitro. 20~40 μM leucine shows half-maximal effects on both the Sestrin2–GATOR2 interaction and mTORC1 activation in cultured cells. Moreover, Sestrin2 directly interacts with leucine but not arginine with a dissociation constant of ~20 μM.

The structure of Sestrin2 revealed that Sestrin2 bears an evolutionarily unique leucine-binding pocket, which specifies a leucine with several hydrophobic residues and holds it with adjacent charged residues [84]. In addition, the bound-leucine is concealed by the hydrophilic threonine residues adjacent to the leucine-binding pocket. Importantly, the study also identified the binding site for GATOR2 in close proximity to the leucine-binding pocket of Sestrin2 [84]. It is conceivable that similar to the nature of arginine bound-CASTOR1, leucine binding to Sestrin2 induces conformational changes of the structure adjacent to the leucine-binding pocket, which may cause the transformation of the moiety of the GATOR2 binding site, thereby disrupting the interaction between GATOR2 and Sestrin2. Together, these studies indicate that Sestrin1 and Sestrin2 are physiological cytosolic leucine sensors that inhibit mTORC1 through GATOR2.

3.6. SZT2-Containing KICSTOR Recruits GATOR1 to the Lysosome and Inhibits Amino Acid-Induced mTORC1 Activation

Previous studies have indicated that GATOR1 inhibits RagA/B through its GAP activity while GATOR2 functions as a suppressor of GATOR1 through unknown mechanisms [63] (Figure 1). It has not been clearly understood how RagA/B, which lacks membrane-anchoring motifs are regulated by GATOR1. Two recent independent studies have identified that seizure threshold 2 homolog (SZT2) or the KICSTOR complex consisting of kaptin (KPTN), integrin alpha phenylalanyl-glycyl-glycyl-alanyl-prolyl (FG-GAP) repeat containing 2 (ITFG2), chromosome 12 open reading frame 66 (C12 or f66) and SZT2, plays an important role in tethering GATOR1 to the lysosome, thereby inhibiting the activity of RagA/B and mTORC1 [85,86]. Wolfson et al. identified that the SZT-containing KICSTOR complex localizes on the lysosome and interacts with GATOR1 independently of amino acid availability [85] (Figure 1). Deletion of any of the KICSTOR components blocks lysosomal localization of GATOR1 and disperses it throughout the cytoplasm without affecting levels of GATOR2 lysosomal localization. GATOR1 fails to interact with its substrates the Rag GTPases as well as its regulator, GATOR2 in cells lacking an intact KICSTOR complex. Importantly, as expected, in KICSTOR-deficient cells, amino acid deprivation fails to block lysosomal mTORC1 localization and activity. Thus, the study indicated that KICSTOR is an important scaffolding protein complex that tethers GATOR1 to the lysosomal membrane thereby not only inhibiting RagA/B activity but also maintaining intact amino acid-sensing mechanisms through CASTOR– and Sestrin–GATOR2 pathways (Figure 1).

In a parallel study from Li's group, STZ2 was also identified as a key interacting protein with both GATOR1 and GATOR2 [86]. Consistent with the observations reported in the study by Wolfson et al., amino acid deprivation fails to diminish lysosomal mTORC1 localization and activity in cells lacking STZ2. However, Peng et al. demonstrated that ablation of STZ2 diminishes both GATOR1 and GATOR2 localization on the lysosome [86]. Furthermore, ablation of either GATOR1 or GATOR2

also reduces lysosomal STZ2 localization, indicating that the integrity of the STZ2-orchestrated GATOR1–GATOR2 (SOG) complex is necessary for lysosomal localization of both GATOR complexes as well as STZ2 and for intact amino acid sensing to mTORC1 signaling. Intriguingly, while ablation of WDR59, a component of GATOR2, strongly inhibits amino acid-induced mTORC1 activation, artificially tethering WDR59 (lyso-WDR59) to the lysosomal membrane inhibits amino acid-insensitive mTORC1 activation in GATOR1/STZ2 or GATOR2/STZ2 deficient cells. These observations suggest that lysosomal WDR59 exerts an unexpected inhibitory function in the regulation of mTORC1 activity. Given that Sestrin2 interacts with GATOR2 under amino acid starvation conditions, and the lysosome-targeted Sestrin2 (lyso-Sestrin2) sufficiently inhibits mTORC1 activity in cells lacking the SOG complex, the study proposed that WDR59-contaning GATOR2 complex may have a key scaffolding role in recruiting Sestrin2 to inhibit RagA/B–mTORC1 activity independently of GATOR1. Indeed, these results support the idea that Sestrin2 functions as the guanine nucleotide dissociation inhibitor (GDI) for RagA/B through a putative GDI motif of Sestrin2 as previously proposed by the same group [87]. However, two recent independent Sestrin2 structure studies demonstrated that two of three key charged residues important for Sestrin2's GDI activity are buried inside their structure, and Sestrin2 shows no structural similarity to known GDI proteins [84,88]. Thus, it remains unclear as to whether lyso-Sestrin2 inhibits mTORC1 activity in SGO-deficient cells through its GDI activity toward RagA/B. Although these two studies proposed slightly different models in terms of the role of SZT2/KICSTOR in the regulation of lysosomal GATOR2 localization, both studies demonstrated that the SZT2 or SZT2-containing protein complex, KICSTOR, is an essential component, which works cooperatively with GATORs and functions upstream of Rag A/B in the amino acid sensing pathway for the regulation of mTORC1. In addition, these studies highlighted aberrant activation of mTORC1 as a potential pathomechanism underlying the onset and development of epilepsy as well as macrocephaly since mutations in SZT2 and other components of KICSTOR (KPTN) have been identified in patients with these disorders [89–93]. In support of this idea, epileptic seizure or macrocephaly are major symptoms seen in patients with tuberous sclerosis complex (TSC) mutations in either TSC1 or TSC2 [94], or Cowden syndrome with PTEN mutations [95], respectively.

3.7. The FLCN–FNIP Complex Functions as a GAP for Rag C/D

The activation of mTORC1 on the lysosomal membrane is regulated through not only RagA/B but also RagC/D. The active Rag complex is established by the hetero-dimerization of GTP-bound RagA/B and GDP-bound RagC/D. Recent studies identified that folliculin (FLCN) and its binding partner, FLCN interacting protein 1 (FNIP1) and 2 function together as a specific GAP for RagC/D [96,97] (Figure 1). The FLCN–FNIP protein complex is evolutionarily conserved from yeast to mammal. Importantly, loss of function mutations in the *FLCN* gene cause Birt–Hogg–Dube (BHD) syndrome, which is characterized by the formation of benign or malignant tumors in hair follicles (fibrofolliculomas), kidney, and lung, suggesting that FLCN is a tumor suppressor [98,99]. Tsun et al. demonstrated that the FLCN–FNIP complex localizes on the lysosome in an amino acid sensitive manner: amino acid starvation stimulates its lysosomal localization whereas amino acid stimulation dissociates the FLCN–FNIP complex from the lysosome [96]. Accordingly, the FLCN–FNIP complex preferentially interacts with the Rag heterodimer under amino acid starvation conditions [100]. It remains elusive why the FLCN–FNIP complex, which activates the Rag heterodimer, resides on the lysosomal surface under amino acid starvation conditions. However, the fact that FLCN functions as a GAP for RagC/D indicates that the FLCN–FNIP complex is a key activator of the Rag heterodimer and mTORC1. Thus, it also remains unclear how the FLCN–FNIP complex functions as a tumor suppressor. Intriguingly, while in most cell-based systems, acute loss of FLCN inhibits mTORC1 activation [101–103], ablation of FLCN in tissues causes the enhancement of mTORC1 activity in vivo [104–107]. These seemingly inconsistent observations suggest that other compensatory mechanisms for RagC/D activation may exist [108] and/or FLCN may have other biological functions that suppress tumorigenesis. How the FLCN–FNIP senses amino acids or the existence of upstream

regulators of the FLCN–FNIP complex in amino acid signaling remains unknown. Han et al. previously reported that leucyl-tRNA synthetase (LRS) functions as a specific GAP for RagD by sensing cellular leucine [108]. However, the possibility of LRS as a GAP for RagD has been questioned by the study reported by Tsun et al. [96]. Instead, a more recent study demonstrated that LRS stimulates vacuolar protein sorting 34 (VPS34), an evolutionarily conserved class III-PI3K, which is known to activate mTORC1, in response to leucine availability [109].

4. The Spatial Regulation of TSC through Akt and Amino Acids

mTORC1 is recruited to the lysosomal membrane through Rag GTPases in response to amino acid availability. Subsequently, lysosomal mTORC1 is directly activated by Rheb, which is inhibited by TSC2, a specific GAP for Rheb. While it has been well demonstrated that active Akt phosphorylates and inhibits TSC2 GAP activity thereby stimulating the Rheb–mTORC1 pathway [110,111], the molecular mechanisms by which Akt-induced TSC2 phosphorylation inhibits its GAP activity are still not well understood. Strikingly, a recent paper from Manning's group revealed that the phosphorylation of TSC2 by Akt strongly induces the dissociation of the TSC complex from the lysosome [27]. In contrast, growth factor starvation or specific Akt inhibition strongly induces lysosomal localization of TSC2. Artificially tethering TSC2 to lysosomes (lyso-TSC2) confers mTORC1 activity insensitive to growth factor stimulation. Taken together, Akt stimulates Rheb–mTORC1 activity by repelling the TSC complex from lysosomal membranes through its phosphorylation of TSC2 (Figure 2). Interestingly, Rheb is required for lysosomal TSC localization as deletion of Rheb or disruption of lysosomal Rheb with a farnesyltransferase inhibitor disperses the TSC complex throughout the cytoplasm even under growth factor starvation conditions. Intriguingly, the TSC complex purified from serum-starved cells shows higher affinity to GDP-loaded Rheb than GTP-loaded Rheb, a property unusual among Ras family GAPs. It is possible that non-phosphorylated TSC2 or components in the TSC complex such as TSC1 and TBC1D7 may act as a GDI to block nucleotide exchange of GDP–Rheb on the lysosome under growth factor-deficient conditions.

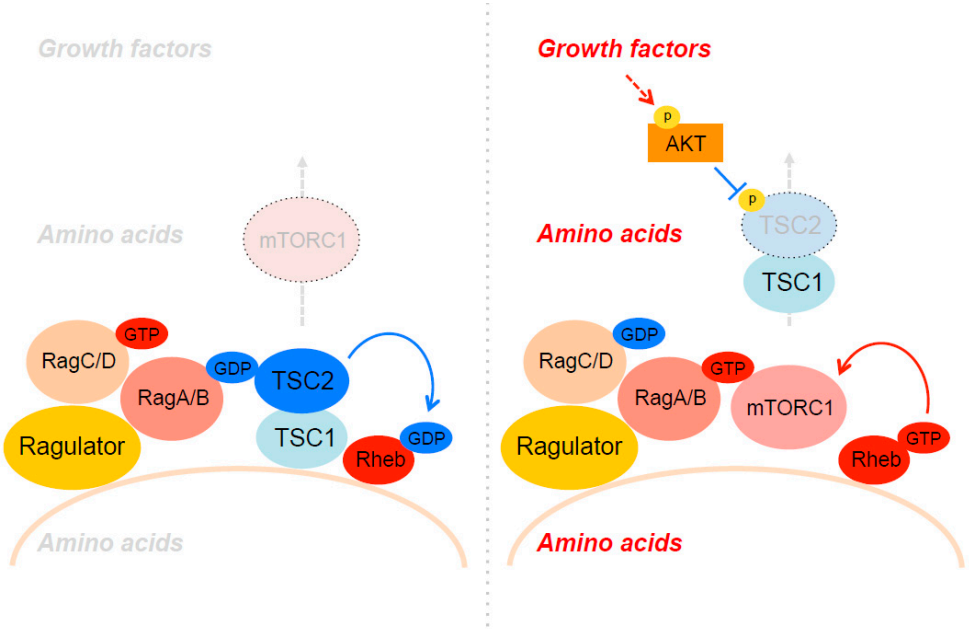

Figure 2. Spatial regulation of the tuberous sclerosis complex (TSC) complex on the lysosomes.The TSC complex preferentially interacts with both GDP-bound RagA and Rheb on the lysosomal membrane under growth factor and amino acid-deficient conditions. Upon growth factor and amino acid stimulation, TSC2 is phosphorylated by Akt and dissociates from the lysosomal membrane leading to the activation of Rheb.

Teleman's group also reported that the spatial regulation of the TSC complex is critical for the regulation of mTORC1 activity [43]. However, they demonstrated that lysosomal localization of the TSC complex is regulated by amino acids (Figure 2). Under amino acid-deprived conditions, the GDP-bound form of RagA strongly binds to TSC2 and recruits the TSC complex to lysosomes, thereby inhibiting Rheb–mTORC1 activity. Consistently, ablation of RagA/B or GATOR1, a RagA/B GAP, blocks lysosomal localization of the TSC complex even under amino acid-deprived conditions. Interestingly, in TSC2-deficient cells, mTORC1 remains localized on the lysosome in a manner dependent on active Rheb under amino acid-deprived conditions. Taken together, these observations suggest that mTORC1 localizes on the lysosomal membrane through both active GTP-bound Rag and Rheb under amino acid and growth factor enriched conditions, whereas the TSC complex takes over the place through inactive GDP-bound Rag and Rheb under amino acid and growth factor-deficient conditions (Figure 2). This swapping between mTORC1 and the TSC complex through "dual anchoring" mechanism explains how growth factor and amino acid stimulation impinge on lysosomal membranes and coordinately turn on or off the activity of mTORC1. In line with the above model, Carroll et al. reported that growth factors and arginine, a key amino acid that activates mTORC1, induce dissociation of the TSC complex from lysosomes [44]. Interestingly, arginine directly blocks the association between TSC2 and Rheb in vitro. These observations suggest that arginine contributes to mTORC1 activation through its direct action on the TSC complex–Rheb interaction in addition to the activation of Rag small GTPases via SLC38A9 and CASTORs. It is anticipated that more amino acid-sensing molecules and mechanisms likely exist and will be revealed by undergoing and future studies.

5. Concluding Remarks

The ability of cells to respond appropriately to nutrient availability is of fundamental importance for adaptation to the environment. In response to nutrient availability or metabolic stresses, cells modulate the rate of anabolism or catabolism, respectively. In these processes, mTORC1 is a central player that induces cell growth and proliferation by activating protein, pyrimidine, and lipid biosynthesis. In addition, mTORC1 also plays a key role in suppressing autophagy, a major cellular catabolic process. In this review, we summarized current knowledge and understanding of amino acid-sensing mechanisms that regulate mTORC1, especially on the lysosomal membrane of mammalian cells. Although emerging evidence indicates that leucine/arginine–Rag-dependent recruitment of mTORC1 to the lysosome and its subsequent binding to Rheb plays a pivotal role in the activation of mTORC1, it has not been clearly understood how other amino acids such as glutamine and lysine that have a potential to activate mTORC1 are sensed and lead to its activation. Glutamine has been reported to function as an efflux solute to increase influx of leucine through the SLC7A5–SLC3A2 heterodimeric antiporter expressed on the plasma and lysosomal membrane [112]. In addition, it has been reported that α-Ketoglutarate, a glutamine metabolite, stimulates the Rag–mTORC1 pathway [113]. However, glutamine is able to induce lysosomal mTORC1 localization and its activation in RagA/B knockout cells [62]. Thus, it appears that glutamine acts on multiple targets upstream of mTORC1 to enhance its activity.

Furthermore, it remains unclear where and how mTORC1 or the TSC complex localizes in the cytoplasm under amino acid-insufficient or -enriched conditions, respectively. Recent studies indicated that mTORC1 is tethered to or incorporated into unknown cytoplasmic punctate structures or stress granules under amino acid-deficient or severe metabolic stress conditions, respectively [26,114,115]. These observations suggest that there are undefined amino acids and/or growth factor-sensing mechanisms, which may relieve mTORC1 from stress-related compartments and support its trafficking to the lysosomes for reactivation.

In addition, it is also not well understood where mTORC1 phosphorylates its distinct substrates, which are expressed in different cellular compartments. Indeed, a recent study demonstrated that active mTORC1 phosphorylates its substrates in multiple cellular compartments. Using the subcellularly targeted specific mTORC1 reporter system, it was found that mTORC1 is able to phosphorylate its

substrates not only in cytosol and on the lysosomal membrane but also in the nucleus and plasma membrane. Interestingly, while growth factors widely enhance mTORC1 activity throughout these subcellular compartments, leucine-induced mTORC1 activity is more restricted to the lysosomal membrane and nucleus [116]. These observations raise the possibility that mTORC1 may be delivered to these compartments after its activation on the lysosome [58] or activated in the nucleus by amino acids and growth factors through undefined machineries. Answering these questions would provide further insights into the molecular mechanisms underlying mTORC1 regulation, and help to facilitate the identification of potential targets for treating mTORC1-associated health problems.

Acknowledgments: The research in the laboratory of K.I. is supported by grants from the National Institute of Health (NIH DK083491, GM110019) and the Department of Defense (DOD TS140055).

Author Contributions: Y.Y. wrote the first drat, E.J. and K.I. edited and completed the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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HHS Public Access

Author manuscript

Transl Cancer Res. Author manuscript; available in PMC 2019 June 10.

Published in final edited form as:

Transl Cancer Res. 2017 October ; 6(Suppl 7): S1234–S1238. doi:10.21037/tcr.2017.09.31.

Microphthalmia-associated transcription factors activate mTORC1 through RagD GTPase gene expression

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The mechanistic target of rapamycin complex 1 (mTORC1) is a major serine/threonine kinase that stimulates cellular anabolic processes including protein and lipid synthesis while suppressing catabolic processes such as autophagy in response to growth factors and amino acids (1). Upon mTORC1 activation, it phosphorylates multiple substrates including S6 kinase (S6K), eIF4E binding protein (4EBP), and unc-51-like kinase (ULK) (2). Both S6K and 4EBP are key regulators for mRNA translation and cell cycle progression (3). In addition to mTORC1's roles in stimulating these anabolic processes, mTORC1-dependent ULK phosphorylation inhibits its kinase activity, which is essential for autophagy induction (4). Thus, mTORC1 activation in response to growth factors and amino acids promotes key cellular anabolic processes while it suppresses major catabolic processes, to build biosynthetic molecules essential for cell growth and proliferation.

Both growth factor and amino acid signals impinge on the lysosomal membrane and coordinately stimulate the activity of mTORC1 by enhancing two distinct lysosomal small GTPases, Rheb and Rags, respectively. While the Rags recruit mTORC1 to the lysosomal membrane in response to amino acids such as leucine, arginine, and glutamine (5), Rheb, which directly interacts with mTORC1, stimulates the activity of mTORC1 on the lysosomal membrane in response to growth factors (6). Mammalian cells contain four members of Rag small GTPases (RagA, B, C, and D) and form obligate heterodimers of either RagA or RagB with either RagC or RagD (7). In the active Rag heterodimer, RagA or RagB binds to GTP while RagC or RagD binds to GDP. Upon amino acid availability, the Ragulator complex, a guanine nucleotide exchange factor (GEF) for RagA/B (8,9), stimulates RagA/B GTP loading in a manner dependent of lysosomal vATPase activity (10). Likewise the folliculin (FLCN)-FLIP complex, a GTPase activating protein (GAP) stimulates RagC/D GDP loading (11). Once the Rag heterodimer is in its active configuration, it localizes mTORC1 to the

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Conflicts of Interest: The authors have no conflicts of interest to declare.

Provenance: This is an invited Editorial commissioned by Section Editor Dr. Peng Zhang (Department of Urology, Zhongnan Hospital of Wuhan University, Wuhan, China).

Comment on: Di Malta C, Siciliano D, Calcagni A, *et al*. Transcriptional activation of RagD GTPase controls mTORC1 and promotes cancer growth. Science 2017;356:1188–92.

lysosomal membrane (5). On the other hand, growth factors instigate the activation of the PI3K (phosphoinositide 3-kinase)/Akt pathway leading to the activation of Rheb small GTPase, which directly stimulates mTORC1 on the lysosome (6).

It is not surprising that nutritional signals collide on the lysosomal membrane. The lysosome is responsible for breaking down macromolecular components through the process of autophagy. Autophagy allows cells to respond to stress conditions such as starvation by providing nutrients through the degradation of cellular components (12). Once nutrients become available, mTORC1 is activated, stimulating anabolism while ending the autophagy response. Thus the lysosome serves as a sensing platform where the extracellular and intracellular nutritional status is carefully monitored in order to maintain a balance between catabolic and anabolic processes.

mTORC1 is well known to inhibit the induction of autophagy by phosphorylation of ULK (4). In addition, mTORC1 has also been shown to negatively regulate two transcription factors, transcription factor EB (TFEB) and transcription factor E3 (TFE3) (13–15), which play a key role in inducing the expression of numerous genes encoding lysosomal hydrolases, membrane proteins, and essential proteins for autophagy. Both TFEB and TFE3 are members of the microphthalmia-associated transcription factor (MiTF) subfamily of transcription factors (16). Upon mTORC1 activation, it phosphorylates these transcription factors at key serine residues, which creates a binding site for the 14–3-3 cytosolic chaperone protein, leading to the blockade of nuclear translocation of these transcription factors (17). In contrast, under starvation conditions, mTORC1 is inactivated, thus the dephosphorylated form of TFEB and TFE3 dissociates from its interaction with the 14–3-3 protein and localizes to the nucleus. Here TFEB and TFE3 recognize the coordinated lysosomal expression and regulation (CLEAR) elements in the promoter region of genes responsible for lysosomal biogenesis and autophagy (16,18). Consequently, TFEB and TFE3 transcriptionally up-regulate the capacity of degradation machineries in cells to generate nutrients for their survival under starved conditions.

Previously Martina *et al*. reported that TFE3 could function as part of a feedback loop leading to mTORC1 activation by increasing expression of the FLCN and two FLCN interacting proteins FNIP and FNIP2, which form the FLCN-FNIP complex, a GAP that activates RagC/D small GTPases (19). Consistently, TFE3 overexpression stimulates Rag C/D GDP loading necessary for its activation, leading to lysosomal mTORC1 localization and its activation. The same observation was also made in a model of TFEB overexpression. Thus, continuous TFE3 and TFEB activation prepare the source and machinery for mTORC1 activation and might ensure the termination of autophagy-lysosomal-mediated catabolism once nutrients become available.

Understanding the molecular mechanisms by which TFEB/TFE3 regulate mTORC1 activity is particularly relevant as the mutations in TFEB/TFE3 genes have been found in renal cell carcinoma and amongst other cancers with high mTORC1 activity (16,17). Elucidation of the molecular mechanisms by which TFEB/TFE3 mutations lead to aberrant mTORC1 activation would provide insight into the development of possible therapeutic strategies.

In this context, the study recently published in *Science* by Di Malta *et al*. provided crucial roles of MiTF transcription factor family members, substrates of mTORC1, in the activation of mTORC1. Interestingly, the study indicates that even under amino acid sufficient conditions, the inhibition of TFEB or TFE3 leads to a decrease in cellular mTORC1 activity in a variety of mammalian cells. These results suggest that the transcription factors TFEB and TFE3 responsible for the initiation of lysosome biogenesis and autophagy under starvation conditions are indeed required for mTORC1 activity in response to amino acids.

Yu *et al*. have previously shown that upon amino acid starvation, the activity of mTORC1 is abolished as expected, however, prolonged amino acid starvation restores cellular mTORC1 activity (20). They proposed that mTORC1 is stimulated in response to the availability of newly synthesized nutrients restored by autophagy during prolonged starvation conditions, bringing to an end the autophagy response. In support of this model, genetic ablation of ATG5 or ATG7, key proteins for autophagy, inhibited the restoration of mTORC1 activity under prolonged starvation conditions (20).

The study by Di Malta *et al*., also observed that the restoration of mTORC1 activity in response to prolonged starvation was abolished when TFEB/TFE3 were genetically ablated, indicating that these transcription factors are crucial for mTORC1 re-activation under this condition. It could be argued that the loss of mTORC1 restimulation under prolonged starvation conditions is due to a decrease in the capacity of cellular autophagy-lysosome degradation system caused by the lack of TFEB/TFE3-dependent expression of lysosomal and autophagic proteins. However, the authors showed that TFEB overexpression lead to higher mTORC1 activity in cells lacking the essential autophagy genes, *ATG5* or *ATG7* compared to control cells. Based on these results the authors propose that the MIT-TFE transcription factors may stimulate mTORC1 activity in a manner independent of their role in the induction of autophagic machinery.

Leucine and arginine have been shown to be two amino acids particularly important for mTORC1 activation on the lysosomal membrane (21,22). The study demonstrated that the sensitivity of mTORC1 activation in response to leucine or arginine was increased in cells overexpressing TFEB, yet the complete starvation of leucine largely inhibited mTORC1 activity in these cells. These results suggested that TFEB/TFE3 overexpression might support the expression of positive regulators in amino acid sensing machinery responsible mTORC1 activation. Likely candidates include the FLCN complex and the subunits of v-ATPase, which is a positive regulator of the Ragulator complex that functions as a GEF for the RagA/B small GTPases.

As expected, enhanced gene expression in the TFEB overexpressing cells includes previously known TFEB targets important for amino acid-induced mTORC1 activation. Interestingly, the authors also identified *RAGD* as a putative TFEB target gene that encompasses the CLEAR element in its promoter. Strikingly, among 20 TFEB/TFE3 putative target genes that likely involve in the regulation of mTORC1 activity, *RAGD* was the most decreased transcript in TFEB or TFE3 silenced cells, whereas it was the most enhanced gene in cells overexpressing TFEB. Of note, consistent with the previous report by Martina *et al*. (19), *FLCN* expression was likewise affected by TFEB expression but to a

much lesser extent to that compared of *RAGD*. Through chromatin immunoprecipitation (CHIP) and luciferase assays, the authors confirmed that *RAGD* is a direct transcriptional target of TFEB, as the *RAGD* promoter has three CLEAR sites upstream of its transcriptional start site (Figure 1). Functional importance of the *RAGD* CLEAR element was confirmed by generating cells bearing a deletion of a key endogenous CLEAR site through CRISPR-CAS9-mediated genome editing (RagD^{promedit} cells). In RagD^{promedit} cells, amino acid-induced lysosomal mTORC1 localization and its activation were significantly decreased compared to the control cells that have the intact TFEB binding CLEAR element. These observations indicate that TFEB/TFE3-induced *RAGD* expression plays an important role in amino acid-induced lysosomal mTORC1 localization and its activation. Although the data indicate an important role of endogenous RagD in the activation of mTORC1, it remains unclear why transcriptional inhibition of the *RAGD* gene is so effective for mTORC1 inhibition in the presence of RagC of which expression is more ubiquitous and has a redundant function with RagD for mTORC1 regulation in response to amino acid availability. It is possible that the Rag heterodimer containing RagD might have higher activity and/or additional roles for lysosomal mTORC1 recruitment compared to the RagC containing heterodimer.

Based on these observations made in an *in vitro* system, the authors also addressed whether the over expression of TFEB or TFE3 had physiological relevance in tissues particularly important in adaptation to nutrient and starvation signals. In a liver specific TFEB over expression model, mTORC1 activity was indeed enhanced under nutrient rich conditions, but was inhibited under fasting conditions. In contrast, muscle specific TFEB knockout mice showed decreased mTORC1 activity in response to a post exercise leucine oral gavage, which was used to emulate the effect of a protein meal after exercise. These results pinpoint that TFEB is necessary to mediate leucine-mediated mTORC1 stimulation *in vivo*. Although their *in vitro* studies showed that TFEB is required for mTORC1 activation in response to leucine or full amino acid stimulation, it remains elusive if lack of TFEB also blocks full amino acid-induced mTORC1 activation in muscle tissues (23). In addition, the investigation of RagD expression and its role in mTORC1 activation in response to amino acid feeding in the exercised muscles will further clarify physiological relevance of the TFEB-RagD axis in the regulation of mTORC1 activity *in vivo*.

The TFEB/TFE3/MITF transcription factors belong to the MiT-TFE transcription factor family, and are well known oncogenes in various human tumors including renal cell carcinoma, melanoma, sarcoma and pancreatic ductal adenocarcinoma, in which aberrant mTORC1 activation is also observed. The study demonstrated positive correlations among the expression of TFE3/MITF, RagD expression, mTORC1 activity, cancer cell proliferation, and tumor development. Consistent with the other biochemical and biological observations demonstrated in this study, renal cancer cells carrying a chromosomal translocation of the *TFE3* gene, pancreatic ductal adenocarcinoma bearing high *MiT/TFE* genes, and melanoma cells with aberrant MITF expression all showed increased *RAGD* transcript accompanied with increased mTORC1 activity. Silencing either these transcription factors or RagD attenuated mTORC1 activity as well as cell proliferation in these cancer cells, implying that the MiT/TFE-RagD-mTORC1 axis plays an important role in cancer cell proliferation/survival *in vitro*. Importantly, xenotransplantation experiments performed using the

melanoma cell line showed significant reduction of xenografted tumor development upon *RAGD* silencing, highlighting a critical role of the MiT-TFE-RagD axis in promoting tumor development.

The MiT/TFE transcription factors are active in their dephosphorylated form under starvation conditions when mTORC1 is inactivated. However, the study reported by Di Malta *et al*. proposed a model where MiT/TFE-RagD-mTORC1-MiT/TFE feedback circuit is crucial for metabolic adaptation to nutrient availability. Dysregulation of this circuit such as constitutive activation of MiT/TFE leads to aberrant RagD-mediated mTORC1 activation and promotes cancer development (Figure 1). One likely physiological role of this feedback circuit is that under metabolic stress conditions, these transcription factors stimulate RagD expression and would prepare lysosomal mTORC1 localization and its activation once nutrients are replenished through extracellular influx or *de novo* production by autophagy. Alternatively, the MiT/TFE-RagD axis may play an emergent and specific role in keeping a low level of mTORC1 activity, maintaining cellular translational activity for the transcripts of lysosomal and autophagy components, as the restored mTORC1 activity after prolonged starvation is required for lysosomal biogenesis (20). In this regard, it is intriguing to examine the specific role of inducible RagD in the activation of mTORC1 under metabolic stress conditions.

In conclusion, this study provided a novel molecular mechanism by which oncogenic MiT/TFE transcription factors support cell growth/proliferation through their transcriptional regulation of the upstream of mTORC1 activator, RagD. The MiT/TFE-RagD-mTORC1-MiT/TFE feedback circuit precisely controls anabolic and catabolic processes with appropriate checkpoints and balances to maintain cellular homeostasis (Figure 1). Upon MiT/TFE overexpression as that observed in a variety cancers, a loss of anabolic/catabolic homeostasis occurs, leading to increased cell growth and proliferation even under metabolically stress conditions by enhancing RagD expression, and thus increased mTORC1 activity.

Acknowledgements

Funding: This work is supported by grants from the National Institute of Health (NIH DK083491, GM110019, T32GM008322) and the Department of Defense (DOD TS140055).

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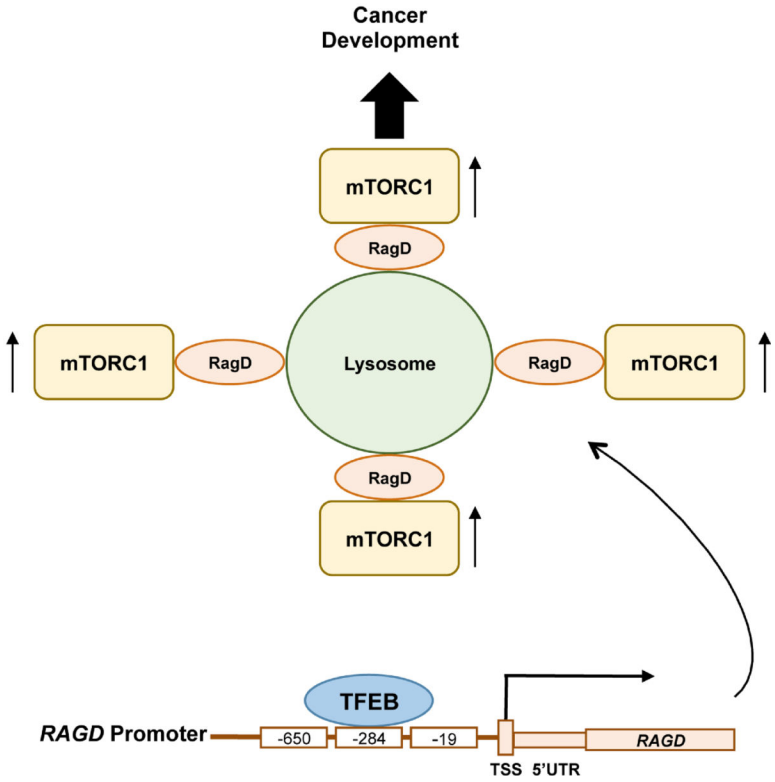

Figure 1.

Dysregulation of MiT/TFE-RagD-mTORC1-MiT/TFE feedback circuit leads to cancer development. Increased expression of MiT transcription factor family members such as TFEB, recognize the CLEAR elements in the *RAGD* promoter located −650, −284 and −19 base pairs upstream of its transcription start site (TSS) and enhance *RAGD* gene expression. Increased RagD protein in turn stimulates lysosomal mTORC1 localization and its activation leading to cell growth/proliferation and tumor development even under metabolically stress conditions.

Cell. Mol. Life Sci. (2018) 75:1227–1239
https://doi.org/10.1007/s00018-017-2710-y

Cellular and Molecular Life Sciences

REVIEW

Macropinocytosis, mTORC1 and cellular growth control

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Received: 10 August 2017 / Revised: 31 October 2017 / Accepted: 3 November 2017 / Published online: 8 November 2017

Abstract The growth and proliferation of metazoan cells are driven by cellular nutrient status and by extracellular growth factors. Growth factor receptors on cell surfaces initiate biochemical signals that increase anabolic metabolism and macropinocytosis, an actin-dependent endocytic process in which relatively large volumes of extracellular solutes and nutrients are internalized and delivered efficiently into lysosomes. Macropinocytosis is prominent in many kinds of cancer cells, and supports the growth of cells transformed by oncogenic K-Ras. Growth factor receptor signaling and the overall metabolic status of the cell are coordinated in the cytoplasm by the mechanistic target-of-rapamycin complex-1 (mTORC1), which positively regulates protein synthesis and negatively regulates molecular salvage pathways such as autophagy. mTORC1 is activated by two distinct Ras-related small GTPases, Rag and Rheb, which associate with lysosomal membranes inside the cell. Rag recruits mTORC1 to the lysosomal surface where Rheb directly binds to and activates mTORC1. Rag is activated by both lysosomal luminal and cytosolic amino acids; Rheb activation requires phosphoinositide 3-kinase, Akt, and the tuberous sclerosis complex-1/2. Signals for activation of Rag and Rheb converge at the lysosomal membrane, and several lines of evidence support the idea that growth factor-dependent endocytosis facilitates amino acid transfer into the lysosome leading to the activation of Rag. This review summarizes evidence that growth factor-stimulated macropinocytosis is essential for amino acid-dependent activation of mTORC1, and that increased solute accumulation by macropinocytosis in transformed cells supports unchecked cell growth.

Keywords Macropinocytosis · mTORC1 · Small GTPase · Phosphoinositide · Cancer

Introduction

Macropinocytosis is an endocytic process by which cells engulf relatively large volumes of extracellular fluid solutes, including nutrients, through movements of the plasma membrane [1, 2]. Subsequent organelle fusion reactions deliver internalized solutes into endolysosomal compartments, where macromolecules may be degraded by lysosomal hydrolases into constituent subunits for anabolic metabolism. Macropinocytosis was originally called pinocytosis [3, 4], but was later renamed to distinguish it from smaller endocytic vesicles such as clathrin-coated vesicles. Growth factors, cytokines, chemokines, pathogens, and the tumor promoter phorbol myristate acetate (PMA) can induce macropinocytosis. Macrophages and dendritic cells constitutively exhibit macropinocytosis, as do cells transformed by oncogenic mutations of K-Ras and v-Src [5, 6]. Aberrant activation of macropinocytosis has been implicated in cancer progression [7, 8], neurodegenerative diseases [9], atherosclerosis [10], and renal dysfunction [11].

Extracellular nutrients and growth factors can regulate cell growth, quiescence, and survival. In response to nutrient availability and growth factor stimulation, cells grow and proliferate by increasing anabolic metabolism. Mechanistic target of rapamycin (mTOR) is an evolutionarily conserved serine/threonine kinase that plays key roles in

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stimulating cellular anabolic processes and inhibiting catabolic processes such as autophagy in response to growth factors and nutrient availability. TOR was originally identified in yeast as a target protein of rapamycin, a macrolide compound that is now widely used in clinical settings as an immunosuppressant, anti-restenotic, and anti-cancer agent [12–15]. mTOR forms at least two distinct multiprotein complexes termed mTOR complex 1 (mTORC1) and mTORC2 [16–20]. Both complexes contain mTOR as a core kinase and the common subunits mLST8 (also known as GβL) [20] and DEPTOR [21]. mTORC1 [15] contains the specific subunits, raptor [18, 19] and PRAS40 [22–24], while mTORC2 contains rictor [17], mSIN1 [25, 26], and PROTOR [27]. While mTORC2 plays important roles in actin cytoskeleton reorganization, cell migration, survival, and glucose metabolism, mTORC1 has been shown to be essential in cell growth and a wide array of cellular metabolic processes [28–30]. In response to a variety of stimuli, including amino acids, glucose, growth factors, cytokines, and PMA [31–33], mTORC1 stimulates cell growth and proliferation by enhancing the rate of cellular protein synthesis, and lipid and pyrimidine/purine biogenesis [34]. Aberrant activation of mTORC1 plays key pathological roles in the development of diseases such as cancer, type 2 diabetes, atherosclerosis, and neurodegeneration [28, 29, 34–37]. Thus, the mechanism of mTORC1 activation and its roles in metabolic regulation have attracted intense interest in basic and clinical sciences.

Macropinocytosis and mTORC1 activation share many common mechanisms for their induction, and recent studies have demonstrated that macropinocytosis contributes to cell growth by stimulating mTORC1 activity [2, 7, 8, 38–42]. This review compares the molecular mechanisms underlying the induction of macropinocytosis and mTORC1 activity, and discusses crucial roles of macropinocytosis in the assimilation of nutrients for cell growth.

mTORC1 activity is regulated by Rag and Rheb

The small GTPases Rag and Rheb coordinately stimulate the activity of mTORC1 on the surface of the lysosome [43–45] (Fig. 1a). Mammalian cells contain four isoforms of Rag, Rag A, B, C, and D, which form heterodimers comprised of RagA or B with RagC or D in a functional conformation, and which are activated by amino acids such as leucine and arginine. The Rag heterodimer interacts with a pentameric protein complex called Ragulator, which consists of the proteins p18 (LAMTOR1), p14 (LAMTOR2), MP1 (LAMTOR3), C7ORF59 (LAMTOR4), and HBXIP (LAMTOR5), and associates with the lysosomal membrane [44]. Ragulator functions as a scaffold for the Rag heterodimer to localize on the lysosomal membrane and to stimulate GTP-binding by RagA or RagB through its guanine nucleotide exchange factor (GEF) activity. Amino acids in the lysosomal lumen play a key role in triggering a conformational change of the transmembrane vacuolar H^+-ATPase (v-ATPase), which activates the RagA/B GEF activity of Ragulator [46, 47]. In addition, SLC38A9, a lysosomal transmembrane protein, interacts with the v-ATPase and activates Ragulator by sensing luminal arginine [48–50]. Upon binding arginine, SLC38A9 transports leucine and other amino acids from the lysosomal lumen into cytoplasm [51]. Cytosolic arginine and leucine can activate the Rag heterodimer by inhibiting the inhibitory activity of a GTPase-activating protein (GAP) for RagA/B [52] (Fig. 1a). GATOR1, a trimeric protein complex consisting of DEPDC5, Nprl2, and Nprl3, is expressed on the lysosomal membrane and functions as a GAP for RagA/B. Furthermore, GATOR1 is inhibited by another pentametric protein complex, GATOR2 [53]. Thus, GATOR2 activates the Rag heterodimer by inactivating GATOR1. Sestrin1 and/or Sestrin 2 directly interact with and inhibit GATOR2, and suppress mTORC1 function [54, 55]. Sestrin bears a leucine-binding pocket in close proximity to its GATOR2 binding site, and the binding of leucine to Sestrin relieves its inhibitory effect on GATOR2. Thus, cytosolic leucine activates mTORC1 by inhibiting GATOR1 through its binding to Sestrin1/2. Similarly, cytosolic arginine activates mTORC1 by inhibiting GATOR1 through its binding to CASTOR1. CASTOR1 forms a homodimer or a heterodimer with CASTOR2 and inhibits GATOR2. Similar to the mode of Sestrins, arginine binding to CASTOR1 blocks its interaction with GATOR2 and relieves the CASTOR1 inhibitory effect on GATOR2, thereby activating RagA/B signaling [54–57]. Glutamine also stimulates mTORC1 [58]. However, it remains unclear whether glutamine itself functions as a signaling molecule for activating mTORC1. Rather, either glutamine stimulates the influx of leucine by acting as an efflux solute through a SLC7A5–SLC3A2 heterodimeric antiporter, or the glutamine metabolite α-ketoglutarate stimulates mTORC1 by activating the Rag heterodimer [59, 60]. It has also been reported that glutamine can activate mTORC1 in a manner dependent on Arf1 but not Rag small GTPase [58]. Thus, RagA/B-dependent activation of mTORC1 occurs by amino acids detected in the cytosol but reaching mTORC1 from within lysosomes or endolysosomes.

Activated Rag recruits mTORC1 to the lysosomal membrane through its interaction with Raptor [44, 61]. There, Rheb directly activates mTORC1 [15, 62, 63] (Fig. 1). Rheb itself is activated by signals from growth factor receptors [64] (Fig. 1b). Upon growth factor stimulation, active phosphoinositide 3-kinase (PI3K) synthesizes PIP_3, which recruits PDK1 and Akt to the plasma membrane where Akt is phosphorylated and activated by PDK1 and mTORC2. Subsequently, active Akt on the lysosomal membrane

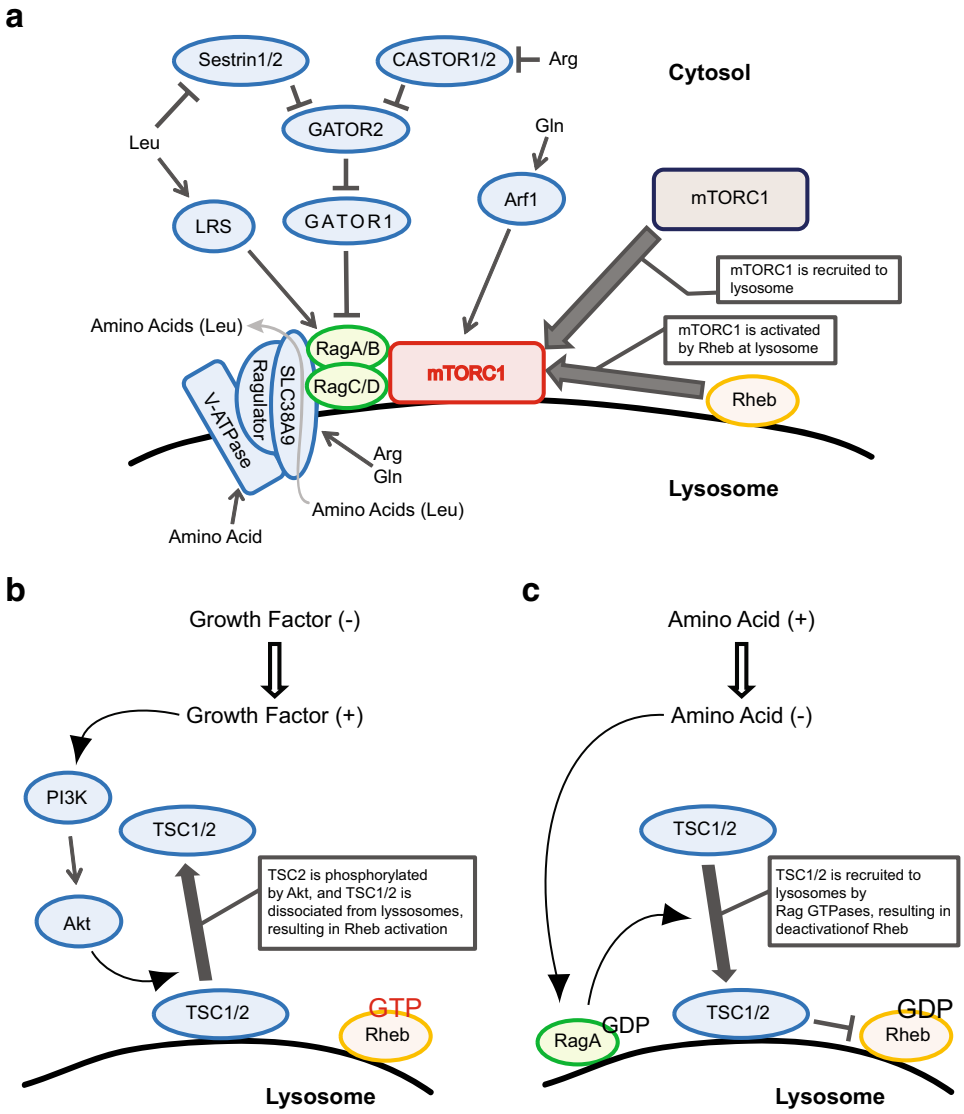

Fig. 1 Amino acid- and growth factor-induced mTORC1 activation. **a** The mechanism of amino acid-induced mTORC1 activation. mTORC1 is recruited to lysosomes by amino acid stimulation. Through V-ATPase and SLC38A9 on lysosomal membranes, amino acids such as arginine (Arg) and glutamine (Gln) modulate the function of protein complex Ragulator, leading to Rag activation. Arg and Gln are detected by SLC38A9. Once Rag is activated, mTORC1 is recruited to lysosomes via the interaction between Rag and raptor, followed by mTORC1 activation by Rheb. Upon binding arginine, SLC38A9 transports amino acids, such as leucine (Leu), from the lysosomal lumen into cytoplasm. GATOR1 and GATOR2 regulate Rag function. Rag is inhibited by GATOR1, which is inhibited by GATOR2. Sestrin1/2 and CASTOR1/2 inhibit GATOR2, and detect Leu and Arg, respectively, in cytosol. The interaction of these amino acids with their target proteins results in the reversal of inhibition by GATOR2. Leucyl-tRNA synthetase (LRS) can also activate Rag and detect Leu in the cytosol. Gln in the cytosol is detected by an Arf1-dependent mechanism, followed by Rag activation. **b** The mechanism of growth factor-induced Rheb activation. Growth factor stimulation induces the PI3K–Akt pathway. Akt phosphorylates TSC2, which is located at lysosomal membrane as a protein complex with TSC1. After phosphorylation, the TSC1/2 complex dissociates from the lysosome. TSC1/2 is a Rheb GAP, so loss of TSC1/2 complex from the lysosomal membrane allows Rheb to be activated (Rheb-GTP). **c** The mechanism of amino acid-modulated Rheb deactivation. Depletion of amino acids from culture medium induces deactivation of RagA (GDP form). Inactivated RagA triggers TSC1/2 recruitment to lysosomes, resulting in deactivation of Rheb (Rheb-GDP)

phosphorylates and inhibits tuberous sclerosis complex 2 (TSC2), a GAP for Rheb in a larger complex comprised of TSC1, TSC2 and TBC1D7 [Tre2–Bub2–Cdc16 (TBC)1 domain family number 7] [65–67]. Alternatively, the RAS–MEK–ERK–RSK pathway phosphorylates and inactivates the TSC complex in response to growth factors, cytokines, and PMA [31, 32, 68–72]. The phosphorylation of TSC2 by Akt induces the dissociation of the TSC complex from the lysosomal membrane, consequently permitting GTP-loading of Rheb and subsequent mTORC1 activation

[64, 72, 73]. The molecular mechanism by which Akt reaches the lysosome to phosphorylate TSC2, and how the phosphorylation of TSC2 leads to its dissociation from the lysosomal membrane are still unknown. Recent studies demonstrated that the dissociation of the TSC complex from lysosomes is also triggered by amino acid stimulation (Fig. 1c) [73, 74]. Under amino acid starvation conditions, the GDP-bound form of RagA (inactive) interacts with and recruits TSC2 to the lysosomal membrane. Conversely, GTP-bound RagA (active) is unable to retain the TSC complex on the lysosomal membrane. Thus, both growth factor-mediated TSC2 phosphorylation and amino acid-induced RagA activation induce the dissociation of the TSC complex and, consequently, stimulate Rheb-dependent mTORC1 activation. In addition to these mechanisms, a recent study demonstrated that arginine can directly inhibit the interaction between the TSC complex and Rheb, thereby supporting Rheb activation in response to amino acid availability [75].

Involvement of endocytosis and autophagy in mTORC1 activation

Given that the cytosolic face of the lysosomal membrane serves as a platform for numerous proteins and protein complexes that mediate amino acid- and growth factor signaling for mTORC1 activation, it can be hypothesized that processes important for endosomal and lysosomal trafficking play key roles in the regulation of mTORC1 activity [76–78]. In addition to Rag and Rheb, other small GTPases associated with endocytosis contribute to the activation of mTORC1. In *Drosophila* S2 cells [79], mTORC1 activation was decreased by knockdown of Rab5 or Arf, which are important for endocytic membrane trafficking. Similarly, knockdown of mammalian Rab5 or Arf1 decreased mTORC1 activity in HEK293 or murine embryonic fibroblast (MEF) cells. Ectopic expression of dominant-active Rab5(Q79L) in HEK293 cells specifically blocked activation of mTORC1 by amino acids but not glucose, implicating Rab5-related endocytic traffic in amino acid-dependent mTORC1 activation [79]. Ectopic expression of active Rab5 often generates unusual vesicles containing both the early endosome marker EEA1 and the late endosome/lysosome marker LAMP1, indicating that aberrant Rab5 activation causes a defect in early-to-late endosome conversion [80]. Consistent with this observation, ablation of hVps39, which plays a role in the early-to-late endosome conversion, produced hybrid endosomes and inhibited insulin-induced mTORC1 activation [80]. mTORC1 localized to these hybrid endosomes, suggesting that the maturation or integrity of the late endosome/lysosome was critical for proper activation of mTORC1. It remains unclear whether Rheb localizes to these hybrid endosomes, and whether the dissociation of the TSC complex from these organelles occurs in response to growth factor stimulation. Together, these reports suggest that the transition from early to late endosome, regulated by Rab5, is required for mTORC1 activation.

As noted above, the GTPase Ras functions as an upstream suppressor of TSC2 via the ERK pathway [31, 71]. Expression of dominant active Ras(Q61L) in HEK293T cells induced TSC2 phosphorylation [71], and stimulated mTORC1, as indicated by S6K1 phosphorylation. Thus, Ras functions upstream of Rheb to stimulate mTORC1 activity. mTORC1 activation by Ras(Q61L) was blocked by amino acid starvation in fibroblasts [65], suggesting that Ras does not act downstream of amino acid sensing machineries to activate mTORC1. However, these observations leave open the possibility that active Ras acts upstream of amino acid sensing machineries to induce mTORC1 activation. In addition, recent studies demonstrated that ablation of the GTPase Rac1 attenuated growth factor-induced mTORC1 and mTORC2 activation in MEFs and HeLa cells [40, 81]. Immunofluorescence staining showed that Rac1 co-localized with mTORC1 and mTORC2 at the plasma membrane in response to serum stimulation [81]. As both Ras and Rac regulate endocytic pathways, these reports also suggest the involvement of endosomal traffic in mTORC1 activation. Interestingly, active Ras acts upstream of Rac1 to stimulate actin cytoskeleton reorganization, membrane ruffling, and macropinocytosis [1, 82].

Another activity in which mTORC1 is responsive to lysosome function is macroautophagy, a process in which cytoplasm is sequestered into membranous autophagosomes that, like macropinosomes, fuse with lysosomes to allow macromolecule hydrolysis and nutrient recycling. Inhibition of cellular mTORC1 activity stimulates autophagy [30], and amino acids recovered by autophagy can activate mTORC1 [51, 83, 84]. Thus, both heterophagy—the assimilation of exogenous nutrients by endocytic activities—and autophagy—the degradation of cytoplasmic contents—can provide amino acids for activation or reactivation of mTORC1.

Mechanisms of macropinosome formation

Macropinocytosis was recognized long ago as a feature of growing cells [3, 85], but its essential role in growth was only established recently [7, 8, 40]. Many of the signaling molecules necessary for mTORC1 activation also contribute to macropinocytosis. The molecular mechanism of growth factor-induced macropinocytosis has been studied with a focus on the roles of small GTPases and phosphoinositides [1, 77, 86] (Fig. 2). Treatment of macrophages with their growth factor macrophage colony-stimulating factor (M-CSF) immediately induces irregular membrane

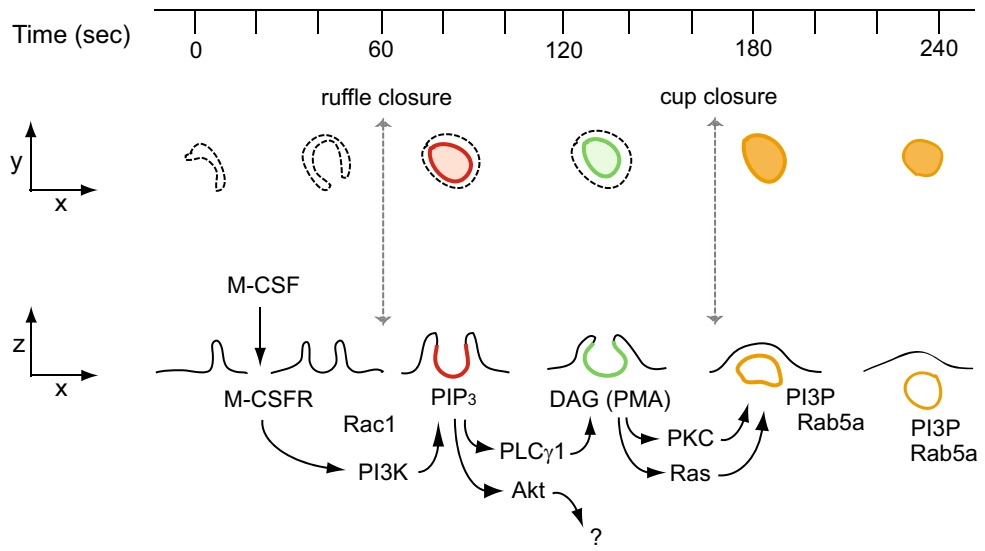

Fig. 2 M-CSF-induced macropinocytosis. Interaction between M-CSF and the M-CSF receptor in macrophages activates Rac1 followed by induction of membrane ruffling. Some ruffles change into cup-like structures, in which activated PI3K then transiently generates PIP_3 (red). PIP_3 generation in the cup triggers the activation of PLCγ and Akt. Akt is not involved in macropinosome formation. PLCγ generates DAG in the cup (green), leading to activation of PKC and Ras. Both pathways contribute to cup closure, in which the macropinosome pinches off into the cytoplasm from the plasma membrane. Following cup closure, PI3P and Rab5a are localized at the macropinosomes (orange). Macropinosomes with these signals (orange) then move toward the center of the cells

ruffles at the cell margins which transform into "C"-shaped ruffles and then "O" shaped, cup-like structures. The open area at the top of the cup later closes to form a complete macropinosome [87]. The first stage of the closing process (C- to O-shaped ruffle) is termed ruffle closure, and the second phase (cup to macropinosome) is termed cup closure [1]. Fully closed macropinosomes move toward the center of the cell via the microtubule network and fuse with the lysosome [88] or, rarely, recycle to the plasma membrane [89]. Imaging of cells expressing fluorescent protein chimeric protein probes revealed a cascade of signals corresponding to the various stages of macropinosome formation. These temporally arranged signals were all restricted to the bowl of the macropinocytic cup, likely by structural barriers to lateral diffusion in the inner leaflet of the cup membrane [90]. Förster resonance energy transfer (FRET) microscopy showed that Rac1 was active within the cup domain immediately following ruffle closure [87]. Ratiometric fluorescence microscopy showed that cyan fluorescent protein (CFP)-labeled Rab5a was recruited to the cup membrane during cup closure and persisted on the macropinosome during its movement toward the lysosome [87]. Similarly, yellow fluorescent protein (YFP)-tagged Ras-binding domain of Raf (YFP-RBD), a probe to detect activated Ras [91], was recruited to macropinocytic cups in macrophages, suggesting that Ras is active during cup closure [92]. Similar macropinocytosis signaling patterns were also reported in other cell types following stimulation with platelet-derived growth factor (PDGF) [93–97]. Thus, as for activation of mTORC1, GTPases associated with membrane traffic are required for macropinocytosis.

Phosphoinositides are also essential for macropinocytosis. PI3K is required for all macropinocytosis except that stimulated by PMA [98, 99]. Fluorescence microscopy of macrophages stimulated with M-CSF showed transient recruitment of YFP-Btk-PH, which localizes PIP_3, to the macropinocytic cup, indicating transient, localized PIP_3 generation (PIP_3 spike) [87, 92]. PI3K also regulates PDGF-induced macropinocytosis [100]. Live-cell imaging with fluorescent protein-tagged pleckstrin homology (PH)-domain chimeras demonstrated a signal transition from $PI(4,5)P_2$ to PIP_3 during epidermal growth factor (EGF)-induced macropinosome formation [86, 99]. Two well-known signal pathways are activated by PIP_3: Akt and phospholipase C-γ (PLCγ). PLCγ is involved in macropinosome formation; Akt is not [101]. Imaging YFP-C1δ as a probe for the PLCγ product diacylglycerol (DAG) revealed transient generation of DAG in the cup [87, 101]. Live-cell imaging also showed that YFP-tagged protein kinase C (PKC)-α, which is activated by DAG, was recruited to cups [92]. The DAG mimetic PMA stimulates macropinocytosis in macrophages [102]. PMA-induced macropinocytosis is blocked by inhibitors of PKC and Ras but not by inhibitors of PLCγ or PI3K [101]. Additionally, the PIP_3 spike was not observed in PMA-induced macropinocytic cups [40]. After cup closure, PI3P and Rab5a appeared on fully formed macropinosomes, which then moved toward the center of the cells [87]. The PKC inhibitor calphostin C blocked PDGF-induced

macropinocytosis in MEFs [40]. Diacylglycerol kinase-ζ (DGKζ), which phosphorylates DAG to yield phosphatidic acid, is also necessary for macropinocytosis [103]. Knockdown of DGKζ attenuated PDGF-induced macropinocytosis. Therefore, DAG is a key signaling molecule involved in macropinocytosis. Together, these observations suggest that growth factor (GF)-induced macropinosome formation results from a signal cascade comprised of many molecules essential to growth control (Fig. 2).

The role of Ras in macropinosome formation remains undefined. Ras-induced pinocytosis was first described as a cellular response to injection of H-Ras [85]. H-Ras(G12V) expression induced membrane ruffles and macropinocytosis in HeLa cells, which could be inhibited by the actin polymerization inhibitor cytochalasin D or by co-expression of dominant-negative Arf6(T27N) [104]. K-Ras-induced macropinocytosis in fibroblasts was blocked by cytochalasin E or by the PI3K inhibitors wortmannin and LY294002 [5]. H-Ras-induced macropinocytosis in BHK-21 cells was blocked by wortmannin or by expression of dominant negative Rab5(S34N), but not by dominant negative Rac1(S17N) [105]. The differential association of K-Ras with PI3K p110 isoforms suggests roles for Ras in ruffling and macropinosome closure. However, MEFs deficient in K-Ras, H-Ras and N-Ras are capable of generating macropinosomes in response to PDGF [106], which suggests that macropinocytosis induced by oncogenic Ras may be an aberrant cellular behavior.

Phosphoinositide signals on macropinosomes were also observed during H-Ras(G12V)-induced macropinocytosis. Live-cell imaging using YFP-AktPH and YFP-PLCδ1-PH to localize PIP_3 and $PI(4,5)P_2$, respectively, showed that H-Ras(G12V)-induced macropinosomes in COS7 cells recruited both probe proteins and indicated that, like macropinocytosis in macrophages, $PI(4,5)P_2$ was lost from macropinosomes before the PIP_3 spike appeared [104]. Live-cell imaging showed co-localization of GFP-Akt and monomeric red fluorescent protein (mRFP)-H-Ras(G12V) at macropinosomes in COS7 cells [104]. Immunofluorescence staining showed that cells co-expressing H-Ras(G12V) and Arf6(Q67L) formed macropinosomes containing phosphorylated Akt [104]. YFP-Akt-PH was recruited to M-CSF-induced macropinocytic cups in macrophages [101] and to EGF-induced macropinocytic cups in A431 cells [99]. Moreover, GFP-Akt localizes to macropinosomes in LPS-stimulated macrophages [107]. Thus, Akt is activated at the macropinocytic cup and/or macropinosomes.

Ras is also required for macropinocytosis and cell growth in axenic strains of the free-living ameba *Dictyostelium discoideum* which are capable of growth in nutrient broth. Those strains exhibit Ras activity localized to macropinocytic cups, which are larger than cups in wild-type amebas due to a mutation in the Ras GAP neurofibromin [108, 109]. Thus, active Ras contributes to the morphogenesis of large macropinosomes necessary for nutrient acquisition and cell growth.

Growth factor-induced macropinocytosis transfers amino acids into lysosomes to activate mTORC1

Macropinocytosis rapidly and efficiently delivers extracellular solutes into lysosomes [110]. Given that growth factors induce both mTORC1 activation and macropinocytosis, and that they share many common GTPases and signaling molecules for their induction, we proposed a model in which macropinocytosis-mediated delivery of extracellular amino acids or protein to lysosomes is essential for mTORC1 activation (Fig. 3) [40]. Biochemical studies in murine macrophages showed that M-CSF treatment induced the PI3K–Akt–TSC–Rheb–mTORC1 pathway. Live-cell imaging and quantitative fluorescence microscopy showed that M-CSF-induced macropinocytosis delivered small extracellular molecules rapidly into lysosomes, where mTORC1 was recruited and activated. Inhibition of macropinocytosis by ethyl isopropylamiloride (EIPA) [111] or with the cytoskeleton inhibitors jasplakinolide and blebbistatin (J/B) blocked M-CSF-induced mTORC1 activation without inhibiting the PI3K–Akt pathway. These results suggest that macropinocytosis provides rapid amino acid trafficking into lysosomes to activate mTORC1. Like M-CSF-induced macropinocytosis, PMA-induced macropinocytosis also increased amino acid-dependent mTORC1 activation, but without inducing Akt phosphorylation. A role for macropinocytosis in mTORC1 activation was also demonstrated in MEFs. PDGF-induced mTORC1 activation by leucine (in the absence of glucose) was blocked by EIPA, J/B, or by knock-down of Rac1, in a manner independent of the Akt–TSC pathway. PDGF treatment increased mTOR recruitment to lysosomes, as determined by the co-localization of mTOR with LAMP2, a lysosomal membrane protein.

Based on these observations, it was proposed that growth factor stimulation induces macropinocytosis, leading to efficient uptake of essential amino acids via macropinosomes and subsequent delivery to the lysosome for mTORC1 activation (Fig. 3). Accordingly, growth factor- dependent mTORC1 activation is established by two distinct pathways: a PI3K–Akt–TSC–Rheb (cytosolic) pathway and a PI3K–macropinocytosis–Rag (vesicular) pathway. The cytosolic pathway is the classical Akt-dependent mTORC1 activation pathway described above: activated Akt induces TSC phosphorylation (TSC deactivation) and consequent activation of Rheb. In the vesicular pathway, PIP_3 in macropinocytic cups localizes DAG synthesis and PKC activity, leading to macropinosome closure. Macropinosomes fuse with the tubular lysosomal network in macrophages or the

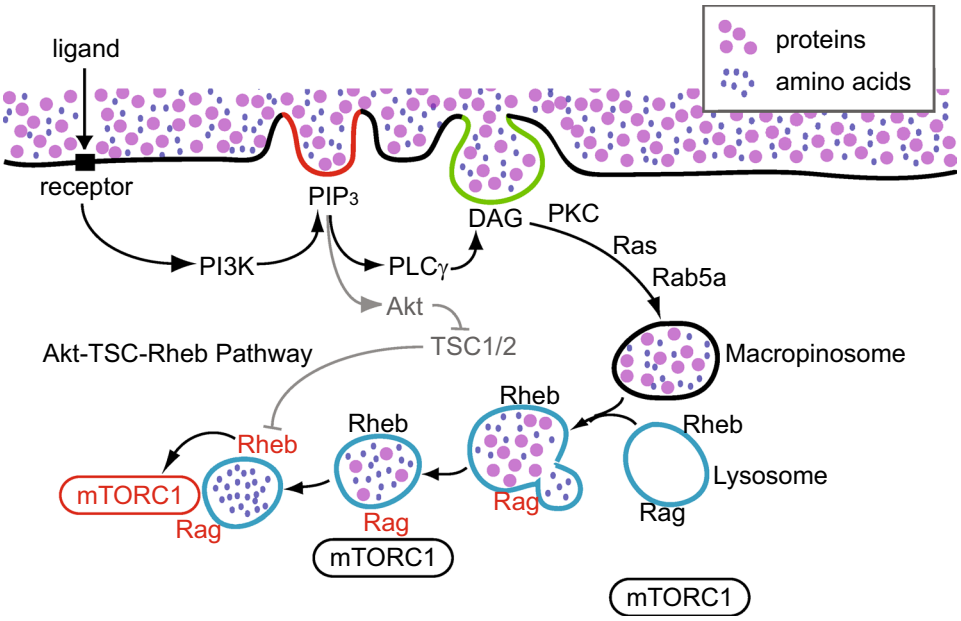

Fig. 3 Macropinocytosis triggers mTORC1 activation. PI3K-generated PIP_3 accumulates in macropinocytic cups (red line), activating Akt and PLCγ. PLCγ generates DAG in the cup (green line), leading to Ras- and PKC-dependent pathways that close the macropinosome. Extracellular nutrients internalized by the macropinosomes are delivered rapidly into lysosomes through fusion reactions. Nutrient transfer from macropinosomes to lysosomes induces Rag activation (black to red), followed by mTORC1 recruitment to lysosomes. Meanwhile, activated Akt inhibits TSC function in a cytosolic pathway independent of macropinocytosis, resulting in Rheb activation (black to red). Rheb directly activates mTORC1 on the lysosomal membranes (black to red)

lysosomes in MEFs, delivering ingested solutes such as proteins or amino acids. Amino acids transferred into the lysosome via macropinosome-lysosome fusion, or derived from hydrolysis of proteins in lysosomes, activate Ragulator and lead to subsequent activation of mTORC1 [40]. Therefore, growth factor receptor signaling organizes macropinosome formation, and the amino acids or proteins internalized by macropinocytosis signal to mTORC1 from inside lysosomes.

The macropinosome as a signal platform for mTORC1 signaling

Macropinocytic cups and macropinosomes may also serve as structural platforms of signaling for cell growth. In addition to small GTPases, phosphoinositides are common signaling molecules involved in mTORC1 activation and macropinocytosis [76, 112]. Phosphoinositide kinase FYVE-type zinc finger containing (PIKFYVE) catalyzes the synthesis of $PI(3,5)P_2$ from phosphatidylinositol 3-phosphate (PI3P) [113]. $PI(3,5)P_2$ interacts with raptor [114], indicating its involvement in mTORC1 activation [112]. In 3T3-L1 adipocytes, depletion of PIKFYVE blocked insulin-induced activation of mTORC1 (as measured by S6K phosphorylation) without affecting Akt phosphorylation [114]. Myotubularin-related phosphatase 3 (MTMR3) dephosphorylates PI3P to phosphatidylinositol [115]. Depletion of MTMR3 in HEK293T cells increased nutrient-induced mTORC1 activation, suggesting that MTMR3 suppresses mTORC1 activity by depleting PI3P [116]. Therefore, the synthesis of PI3P or $PI(3,5)P_2$ on macropinosomes could help recruit mTORC1 to the late endosome or lysosome.

The macropinocytic cup can also localize Akt phosphorylation. Like M-CSF, the chemokine CXCL12 induces both macropinocytosis and mTORC1 activation in macrophages [38]. Unlike the response to M-CSF, however, CXCL12-induced phosphorylation of Akt and S6K (a reporter of mTORC1 activity) was dependent on actin cytoskeleton rearrangement and the formation of macropinocytic cups. Live-cell imaging showed YFP-Akt-PH recruitment to the macropinocytic cup, and western blot analysis showed that the macropinocytosis inhibitors J/B and EIPA attenuated CXCL12-induced Akt phosphorylation. Thus, Akt phosphorylation in response to CXCL12 required the formation of a macropinocytic cup. Immunofluorescence microscopy showed that Akt was phosphorylated at membrane ruffles and macropinocytic cups. The PKCα/β-specific inhibitor Gö6976 blocked macropinocytosis and S6K phosphorylation without inhibiting membrane ruffling or cup formation, suggesting that PKCα and/or PKCβ are involved in cup closure. However, Gö6976 did not inhibit CXCL12-induced Akt phosphorylation. Together these studies indicated that CXCL12-induced macropinocytic cups are signal

platforms for the Akt phosphorylation required for mTORC1 activation.

To what extent does the cytosolic pathway (Akt–TSC1/2–Rheb) require macropinocytosis? The sensitivity of Akt activation by CXCL12 to cytoskeleton-inhibitors differed from Akt activation in response to M-CSF or PDGF, which was not affected by such inhibitors. The organization of the macropinocytic cup may allow localized amplification of signals from some receptors, perhaps those that require multiple inputs for signal amplification. Circular ruffles create isolated domains of plasma membrane where signal propagation can occur [92], indicating the presence of barriers to lateral diffusion in the inner leaflet of the plasma membrane of cups [90]. Maximal Akt phosphorylation observed in response to CXCL12 was less than the level of Akt phosphorylation measured in response to M-CSF. Acute stimulation of cells with M-CSF (or PDGF) may generate sufficiently high concentrations of PIP_3 that a spatially organized amplification is unnecessary. However, if receptors cannot generate high PIP_3 concentrations, then phosphorylation of Akt may require a mechanism based on spatial confinement of signal amplification to macropinocytic cups. Consistent with this model, a recent study identified a role for Rac-dependent macropinocytosis in the activation of the PI3K subunit p110β by G-protein coupled receptors [117].

As described above, the TSC complex inhibits Rheb function at the lysosome [64, 73, 74]. When Akt and Erk phosphorylate TSC2, the TSC complex subsequently loses its GAP activity for Rheb [31, 32, 72]. This suggests that, within a few minutes of stimulation, signal components that phosphorylate Akt and Erk reach lysosomal structures and phosphorylate TSC2. In cells co-expressing H-Ras(G12V) and Arf6(Q67L), Erk is recruited to and phosphorylated at macropinosomes [104]. Erk localizes to late endosomes and lysosomes via the protein complex p18/p14/MP1 [118]. Since macropinosomes show late endosome characteristics at this stage, growth factor/chemokine-induced macropinosomes should recruit Erk via the p18/p14/MP1 protein complex during the maturation process. Given that another important function of the p18/p14/MP1 complex is to recruit mTORC1 to the lysosome as a Ragulator, we speculate that late stage macropinosomes recruit mTORC1 directly. Together, these reports indicate that macropinosomes deliver signaling molecules to the lysosome.

How macropinocytosis could be essential to growth control

Macropinocytosis may be essential for the growth of metazoan cells [40]. Accordingly, when cells are growing in constant concentrations of growth factor, macropinosomes form stochastically as discrete units of growth factor signaling, and activation of mTORC1 follows after a bolus of extracellular protein or amino acids is delivered by macropinocytosis into the lysosomes. Moreover, Akt localization to cups and its continued association with fully formed macropinosomes could provide a route for Akt to reach its substrate tuberous sclerosis complex-1/2 (TSC1/2) on the lysosomal membrane. Thus, the magnitude of growth factor stimulation of mTORC1 may be determined in part by the volume of solute internalized by macropinocytosis, with feedback from a nutrient-sensing mechanism regulating the magnitude of Akt signaling on macropinosome membranes and the volume of nutrient delivered into the lysosome via macropinocytosis. This model predicts that macropinocytosis is necessary for cell growth and proliferation.

Pathogenic functions of macropinocytosis in K-Ras-induced cancer

Dysregulation of Ras and mTORC1 are involved in cancer development [15, 29]. Pathologic functions of macropinocytosis in oncogenic K-Ras-expressing cancer cells have been described. Human carcinoma cells expressing K-Ras(G12C) or H-Ras(G12V) showed increased macropinocytosis, similar to NIH 3T3 cells expressing K-Ras(G12V). Extracellular proteins ingested by macropinocytosis in cells expressing oncogenic K-Ras were degraded and their constituent amino acids were used for anabolic metabolism [7]. The macropinocytosis inhibitor EIPA blocked albumin-dependent cell proliferation [7], indicating that ingestion of albumin by K-Ras(G12D)-induced macropinocytosis and subsequent hydrolysis of proteins in lysosomes were sufficient to provide the essential amino acids (EAA) necessary for cell proliferation [39]. Moreover, the growth of cells in nutrient-poor regions of pancreatic tumors was supported by scavenging of extracellular proteins [119]. Other groups have reported that H-Ras(G12V)-induced macropinocytosis is necessary for albumin-dependent cell growth of MEFs and that inhibition of mTORC1 activation increases the rate of macropinocytosis in carcinoma cells (MIA PaCa-2 K Ras mutant) [41, 42]. Additionally, inhibition of DOCK1, a Rac-activating protein required for macropinocytosis, reduces survival of Ras-driven cell growth [120]. Thus, macropinocytosis-mediated ingestion of extracellular protein is now considered a hallmark of cancer metabolism [121].

However, unlike the responses observed in macrophages and MEFs, mTORC1 activation by EAA in K-Ras transformed cells was not inhibited by EIPA [8]. This indicates that macropinocytosis in Ras-transformed cells is not the primary route by which free amino acids reach the cytosolic SESTRIN1/2 and CASTOR detection systems.

In sum, these studies suggest that macropinosomes serve as organizational units of a signal transduction pathway that

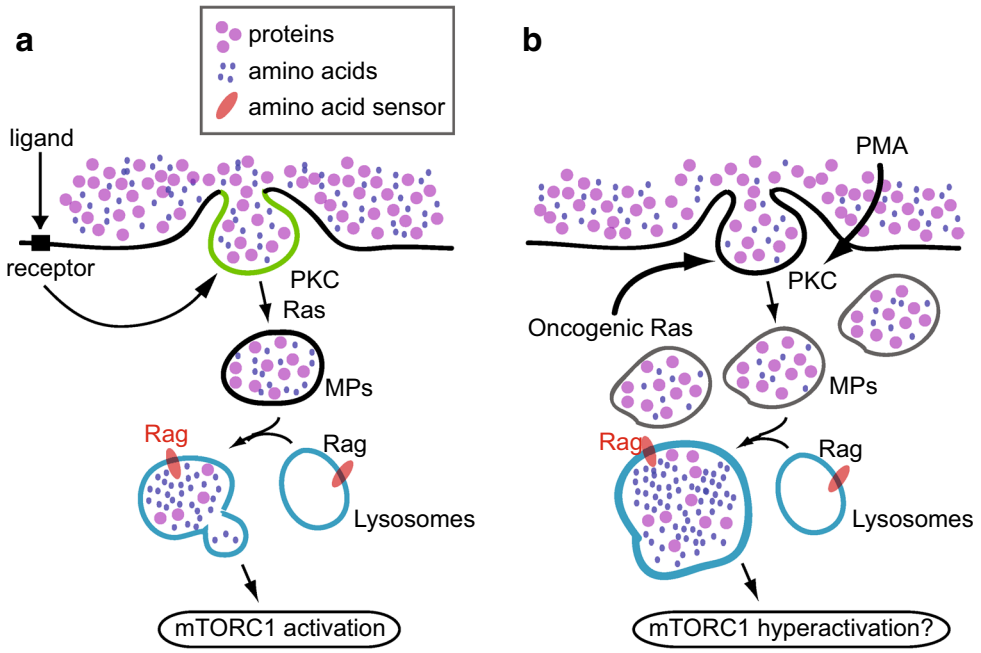

Fig. 4 Two models of macropinocytosis-regulated mTORC1 activation. **a** Role of macropinocytosis in ligand-induced mTORC1 activation. Signals derived from DAG (green) modulate macropinosome (MP) formation via the activation of PKC and Ras. Formed macropinosomes convey extracellular nutrients into lysosomes, where Rag is activated. **b** Proposed hypothesis of the function of oncogenic protein-induced macropinocytosis and mTORC1 activation. Overexpression of oncogenic Ras continuously induces macropinosomes, resulting in an overload of nutrients in the lysosomes. Because of this, following Rag activation, mTORC1 is hyperactivated. PMA treatment directly induces PKC activation, which would also lead to increased nutrient uptake via macropinocytosis

is induced by extracellular stimuli such as growth factors and chemokines (Fig. 4a). If this is the case, constitutive macropinocytosis induced by oncogenic K-Ras or cSrc may hyperactivate mTORC1, resulting in unrestrained growth (Fig. 4b). Similarly, the tumor promoting activity of PMA may be partly attributable to its activation of mTORC1 via macropinocytosis.

Future directions

Significant questions remain to be answered about the relationship between macropinocytosis and mTORC1. To what extent does macropinocytosis support growth of non-neoplastic cells? Why is mTORC1 activation by EAA in K-Ras-transformed cells independent of macropinocytosis? Does membrane traffic unrelated to macropinocytosis regulate mTORC1 activity? Does the activity of mTORC1 or the nutrient status of the cell regulate macropinosome formation or fusion with the lysosomes? The studies of Palm et al. [8, 106] indicated that active mTORC1 inhibits protein delivery into lysosomes via macropinocytosis, whereas Nofal et al. [122], showed that mTORC1 activation does not affect degradation of extracellular protein. These studies suggest that mTORC1 or the cytosolic concentrations of amino acids regulate the uptake and degradation of extracellular solutes by macropinocytosis (i.e., heterophagy) in a manner analogous to its role in protein recycling and degradation by autophagy.

Alternative macropinocytosis-specific inhibitors are needed, both for better understanding of macropinocytosis biology and for the potential therapeutic manipulation of the macropinocytosis signaling pathway. Although EIPA does not block other types of endocytosis, such as phagocytosis and clathrin-dependent endocytosis, it is reasonable to expect it to affect other signal pathways related to cell growth and differentiation. Drugs targeting macropinocytosis could attenuate growth of neoplastic cells or related mosaic disorders resulting from mutations in the signals leading to mTORC1 [123].

Acknowledgements The authors are grateful for the editorial suggestions of Dr. David Friedman. This work was supported by NIH Grants R01 GM110215 (J.S), GM110019 (K.I), DK083491 (K.I), and US Department of Defense Grant TS140055 (K.I).

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