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Ilona Patursky-Polischuk  
Hebrew University-Hadassah Medical School

Judith Kasir  
The Hebrew University-Hadassah Medical School

Rachel Miloslavski  
The Hebrew University-Hadassah Medical School

See next page for additional authors

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Comments
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Reassessment of the Role of TSC, mTORC1 and MicroRNAs in Amino Acids-Meditated Translational Control of TOP mRNAs

Ilona Patursky-Polischuk1, Judith Kasir1, Rachel Miloslavski1, Zvi Hayouka1, Mirit Hausner-Hanochi1,2, Mirit Stolovich-Rain1,2, Pinchas Tsukerman2, Moshe Biton2, Rajini Mudhasani3, Stephen N. Jones3, Oded Meyuhas1

1 Department of Biochemistry and Molecular Biology, The Institute for Medical Research – Israel-Canada, The Hebrew University-Hadassah Medical School, Jerusalem, Israel, 2 Lautenberg Center for General and Tumor Immunology, The Institute for Medical Research – Israel-Canada, The Hebrew University-Hadassah Medical School, Jerusalem, Israel, 3 Department of Cell Biology, University of Massachusetts Medical School, North Worcester, Massachusetts, United States of America

Abstract

TOP mRNAs encode components of the translational apparatus, and repression of their translation comprises one mechanism, by which cells encountering amino acid deprivation downregulate the biosynthesis of the protein synthesis machinery. This mode of regulation involves TSC as knockout of TSC1 or TSC2 rescued TOP mRNAs translation in amino acid-starved cells. The involvement of mTOR in translational control of TOP mRNAs is demonstrated by the ability of constitutively active mTOR to relieve the translational repression of TOP mRNA upon amino acid deprivation. Consistently, knockdown of this kinase as well as its inhibition by pharmacological means blocked amino acid-induced translational activation of these mRNAs. The signaling of amino acids to TOP mRNAs involves RagB, as overexpression of active RagB derepressed the translation of these mRNAs in amino acid-starved cells. Nonetheless, knockdown of raptor or rictor failed to suppress translational activation of TOP mRNAs by amino acids, suggesting that mTORC1 or mTORC2 plays a minor, if any, role in this mode of regulation. Finally, miR10a has previously been suggested to positively regulate the translation of TOP mRNAs. However, we show here that titration of this microRNA failed to downregulate the basal translation efficiency of TOP mRNAs. Moreover, Drosha knockdown or Dicer knockout, which carries out the first and second processing steps in microRNAs biosynthesis, respectively, failed to block the translational activation of TOP mRNAs by amino acid or serum stimulation. Evidently, these results are questioning the positive role of microRNAs in this mode of regulation.

Introduction

TOP mRNAs encode more than ninety proteins of the translational apparatus and are characterized by the presence of an oligopyrimidine tract at their 5’ terminus (5’TOP motif), which comprises their core translation cis-regulatory element [1]. The translation of these mRNAs is selectively mitigated by mitogenic and oxygen signals that converge at the tuberous sclerosis complex (TSC) dimer, TSC2-TSC2 [2,3]. The TSC1-TSC2 acts as a GTPase-activating protein (GAP) for Ras-homolog enriched in brain (Rheb), and thereby blocks Rheb activity toward its own target, mammalian target of rapamycin (mTOR) [4,5]. mTOR operates within two functionally and structurally distinct complexes, mTOR complex 1 (mTORC1) and mTORC2 (reviewed in [6,7]). The essential core components of mTORC1 are raptor (regulatory-associated protein of mTOR) and mLST8 (mammalian lethal with SEC thirteen 8), whereas, those of mTORC2 are rictor (rapamycin-insensitive companion of mTOR), SIN1 (SAPK-interacting 1) and mLST8.

Rheb-GTP operates as an activator of mTORC1 (reviewed in [8]). Accordingly, deficiency in either TSC1 or TSC2 renders mTORC1 activity completely refractory to mitotic arrest or anoxic signal [3,9]. Most of the effects of mTORC1 are abolished by rapamycin, an allosteric inhibitor, which exerts its inhibitory effect when complexed with its intracellular receptor, the immunophilin FKBP12 (FK506-binding protein) [10].

Once mTORC1 is activated it regulates protein synthesis by direct phosphorylation of: (a) eukaryotic initiation factor (eIF) 4E-
binding proteins (4E-BPs) at multiple sites, which consequently dissociates from and derepresses eIF4E [11]; and (b) ribosomal protein S6 kinase (S6K) at T389 [12], which becomes fully active and affects protein synthesis machinery [1]. A third protein, eukaryotic elongation factor 2 kinase (eEF2K), seems to be indirectly phosphorylated and inactivated by mTORC1, thus leading to dephosphorylation and activation of its substrate eEF2 (reviewed in [13]).

The mTORC2 complex has been implicated in the activation of Akt and protein kinase C [14,15]. Although only mTORC1 is acutely sensitive to the allosteric inhibitor, rapamycin, newly developed competitive inhibitors that target the catalytic site of mTOR have been shown to potently and directly inhibit both complexes (reviewed in [16]).

Translational control of TOP mRNAs by mitogens or oxygen relies on TSC1, TSC2 and Rheb, as deletion of either of the two TSC proteins or overexpression of Rheb, renders TOP mRNAs refractory to serum deprivation [3,17,18]. Accordingly, mTOR is essential for transduction of mitogenic or oxygen signals to translation efficiency of TOP mRNAs, as its knockdown represses the insulin-induced translational activation of TOP mRNAs. However, this mode of regulation appears to rely neither on mTORC1 nor on mTORC2, as deficiency of raptor or rictor exerts marginal or no inhibitory effect, respectively, on the translation efficiency of TOP mRNAs [3,18].

TOP mRNAs are translationally regulated also by amino acid sufficiency [19,20]. Amino acid starvation leads to rapid dephosphorylation of S6K1, which can be restored upon readdition of amino acids in an mTORC1-dependent fashion [6] and references therein). Members of the Rag subfamily of Ras small GTPases (RagA, B, C and D) and the trimeric complex, Ragulator, are essential transducers of amino acids signals to mTORC1 activity [8]. Amino acid stimulation elicits movement of mTORC1 to the lysosomal surface, where Rheb and Ragulator reside. The latter recruits Rag GTPases to the lysosomes in a p62- and vacuolar H+-ATPase-dependent manner [21,22], and thereby participates in mTORC1 activation [9,23]. In contrast, the pentameric complex, GATOR1, inhibits the mTORC1 pathway by functioning as a GAP for RagA, whereas the trimeric complex, GATOR2 negatively regulates GATOR1 [24].

MicroRNAs (miRs) are short oligonucleotides that function as major regulators of gene expression and function at the posttranscriptional level [25]. It has previously been reported that miR-10a binds the CG-rich sequence immediately downstream of the 5’TOP motif in TOP mRNAs. Furthermore, overexpression of miR-10a selectively enhanced the synthesis of ribosomal proteins in untreated cells and increased the polysomal association of the respective mRNAs in amino-acid-starved cells [26]. These results, therefore, imply that overexpressed miR-10a exerts its positive role in the translational control of TOP mRNAs in a 5’TOP motif-dependent manner.

Here, we set out to establish the pathway that transduce amino acid signal to translation efficiency of TOP mRNAs. Our results show that this pathway relies on TSC and mTOR, exhibits distinct requirement for the small GTPase - RagB. Moreover, amino acid-induced translational activation of these mRNAs, does not depend on either of the two canonical complexes, mTORC1 and mTORC2, or on microRNAs.

**Materials and Methods**

Materials used for treating cells, FK506 (20 μM) and puromycin (3 μg/mL) were obtained from Sigma-Aldrich. Rapamycin (20 nM) was from Sigma-Aldrich or Calbiochem. Torin-1 (50 nM) was kindly provided by N. Gray and D. Sahatini, Whitehead Institute for Biomedical Research, Cambridge, MA.

**Cell culture**

Human embryonic kidney (HEK) 293 [27], HEK293T cells, as well as MEFs from TSC2+/−/p53−/−, TSC2−/−/p53−/− [28], TSC1−/− and TSC1−/−/p53−/− [29], MDA-MB-231 (human breast adenocarcinoma) [30] and KKO (human colon carcinoma) [31], Dicer−/− and Dicer−/−/hemangiosarcoma cells [3] were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum, 2 mM glutamine, 100 u/ml penicillin, and 0.1 mg/ml streptomycin. Mitotic arrest was achieved by incubation in serum-free medium for 48 h. Amino acid starvation was carried out as previously described [20]. Serum and amino acid starvation was attained by keeping the cells in DMEM (without serum) for 32 h (TSC1 and TSC2) or 18 to 21 h (HEK293) and then for additional 16 h (TSC1 and TSC2) or 3 to 6 h (HEK 293) in Earle’s salt solution, MEM/Eagle vitamin solution, 0.37% NaHCO3, 100 u/ml penicillin, and 0.1 mg/ml streptomycin. HEK293 and HEK293T cells were transfected using polyethyleneimine (PEI) procedure. Briefly, 25 μl of 2 mg/ml PEI (Sigma-Aldrich, average molecular weight 25,000) were added to 750 μl of serum-free medium containing 12.5 μg of DNA. The solution was mixed and kept for 5 min at room temperature prior to its addition to 60 to 70% confluent cell culture in a 100 mm plate containing 10 ml complete medium. The medium was changed the next morning and cells were harvested about 48 h post-transfection.

Polysomal fractionation and RNA analysis were performed as previously described [3]. Typical polysomal profiles of cells with or without amino acids and detailed distribution of rpL32 and actin mRNA along these profiles have been previously shown [20].

**Molecular probes**

The isolated fragment probes used in the Northern blot analysis were: a 0.97-kb fragment bearing the rpL32 processed gene, 4A [32]; a 0.29-kb EcoRI-HindIII fragment containing mouse rps16 cDNA; a 0.85-kb PCR generated fragment containing mouse rps6 coding and flanking sequences [33]; a 1.15 kb PstI fragment containing mouse α-actin cDNA [34]; 1.05 PstI fragment containing rat β-tubulin cDNA [35] and a 0.8-kb HindIII fragment containing a hGH cDNA.

**Western blot analysis**

Immunoblotting was performed as described [36], using antibodies against rps6 (#2217), phospho rps6 (Ser235/236 [2211] or Ser240/244 [2215]), phospho S6K1(Thr389) [9206], phospho Akt (ser473) (#4058), P-4E-BP (Thr37/46 [2853], β-actin (#4967), α-actin (#2144), mTOR (#2972), rictor (#2114) and raptor (#2280) (Cell Signaling Technology, Beverly, MA, USA), as well as FLAG (F3165, Sigma-Sigma Aldrich) and myc (SC-40, Santa Cruz). All antibodies were diluted 1:1000. Exposures were chosen so that the chemiluminescent signals were within the linear range of the film and were quantified by ImageMaster VDS (Amersham Pharmacia Biotech).

**Knocking down and overexpression using lentiviral vectors**

Cloning of an shRNA for the non-relevant HcRed mRNA and knockdown of mTOR and raptor were performed as previously described [18]. The sequences of the HcRed sense and antisense oligonucleotides are:
were used. MDA-MB-231 cells expressing anti-miR-10b sponge or binding site (designated miR-10), or a negative control with a place. Solution of 400 µg of miR-10 or miR-10mut DNA (in a ratio of 3:1) were excised and cloned into the lentiviral vector SIN18-pRLL-hEFIap EGFP-WRPE instead of the GFP as previously described [37].

Sponges anti-miR-10b and anti-miR-BART 1–5p (control) were excised and cloned into the lentiviral vector SIN18-pRLL-hEFIap EGFP-WRPE, downstream to the GFP cassette. Each sponge consists of 6 adjacent binding sites for the relevant viral miRNA, separated by a 4-nucleotide (AGAG) spacer [38].

Transfection and Luciferase assay
Dual luciferase PsiCheck2 reporter vectors containing either a fragment from the 3’-UTR of NCOA2 that bears miR-10a/10b binding site (designated miR-10), or a negative control with a mutated miR-10a/10b seed region (designated miR-10mut) [39] were used. MDA-MB-231 cells expressing anti-miR-10b sponge or a control sponge were grown in RPMI medium and plated in 6-well format, at 6 × 10⁵ cells/well, just before the transfection took place. Solution of 400 µl serum-free medium, linear PEI and either 6 µg of miR-10 or miR-10mut DNA (in a ratio of 3:1) were prepared and kept for 15 min. at RT before added to the cells. Medium was changed in the next morning and 4 hours later cells were washed twice with PBS. 150 µl of 1× Passive Lysis Buffer (Promega) were added to each well and cells were scrapped vigorously with rubber policeman. The lysates were kept on ice for 10 minutes and then frozen at −70°C. Thawed lysates were centrifuged at 9,500 g (4°C) for 10 minutes and aliquots containing 20 µg of protein were used for the assay. Luciferase assay was performed using the Dual-Luciferase Reporter (DLR) Assay System (Promega, USA) and Mithras LB 940 microplate (Berthold Technologies).

Quantification of MICB and GFP expression
The expression of MICB on the cell surface was quantified by flow cytometry using anti-MICB antibody (ABclonal, USA), whereas that of GFP by its own fluorescent, using a FACScan (Becton Dickinson Immunocytometry Systems, San Jose, California, USA)

Quantification of miRs
Total RNA from Dicer+/+ and Dicer−/− was prepared using Ultraspec RNA (Biotecx Laboratories, Houston, Texas). miScript Reverse Transcription kit and miScript SYBR Green PCR kit (Qiagen, Duesseldorf, Germany) were used for relative quantification of mature miRNA expression levels. Reverse transcription for individual miRNAs or U6 snoRNA were performed according to manufacturer’s instructions. Quantitative real-time PCR (qPCR) of the cDNA products were performed using LightCycler 480 Real-Time PCR System (Roche). Analysis was performed using the LightCycler 480 Software. A dilution series using a known cDNA sample was used to generate a standard curve for each assay. The threshold cycle (CT) was determined by automatic assignment of the threshold at the exponential phase of the amplification curves. The following primers were used for qPCR of miR-10a TACCCCTGTAGATCCGAATTTGT, miR-140 CAGTAGGGTTTACCCATATGATAG, and U6 GATGACAAGCAAATTCGAGGA. Quantitative PCR analysis of mature miRNA in RKO cells as described [40].

Results
The translation of TOP mRNAs is resistant to amino acid deprivation in cells deficient of TSC1 or TSC2
The TSC1-TSC2 complex appears to mediate the translational repression of TOP mRNAs upon starvation for serum or oxygen [3,18]. However, conflicting results regarding the role of this complex in transduction of amino acid signal to mTORC1 activity [41–44], rendered questionable its involvement also in transducing the signal of amino acid starvation to translational repression of TOP mRNAs. To directly address this issue, we monitored the translation efficiency of TOP mRNAs, as can be inferred from their relative polysomal association, when TSC2−/− or TSC1−/− MEFs were deprived of amino acids. Fig. 1A demonstrates that the translation of rpS6 mRNA was refractory to amino acid starvation in TSC2−/− MEFs and rpL32 mRNAs in either of these cell lines, in contrast to the apparent sensitivity of these mRNAs in the wild-type counterparts. It is worth noting that the pronounce decrease in the translation efficiency of actin or tubulin mRNA upon amino acid starvation of TSC2−/− and TSC1−/− MEFs (Fig. 1A), relative to that observed in other cell types examined in this study, might simply reflect a cell type-specific response.

Interestingly, the phosphorylation of 4E-BP as well as that of rpS6, was rescued in amino acid-starved TSC2−/− MEFs (Fig. 1B). Previous experiments with the same cell lines have shown an inconsistent sensitivity of the phosphorylation of S6K1 and rpS6 when TSC2−/− MEFs were deprived of both serum and amino acids [42–44]. Hence, we set out to examine the translational behavior of rpL32 mRNA in TSC2−/− MEFs that underwent combined starvation. Figs. 1 clearly shows that the deficiency of either TSC1 or TSC2 can rescue the translation efficiency of this mRNA in the absence of both serum and amino acids. These results imply that translational repression of TOP mRNAs by starvation for amino acids, or for amino acids and serum, relies on both TSC1 and TSC2. It is likely therefore, that silencing of the TSC1-TSC2 complex constitutes a critical step toward translational activation of TOP mRNAs by amino acids.

mTOR is involved in translation control of TOP mRNAs
Rapamycin is a widely used tool for establishing the role of mTOR in many biological processes. However, while this drug inhibited mTORC1 activity with a half-time of about 2 min (Fig. 2A and [45]), it repressed the translation of rpL32 mRNA much more slowly, reaching its maximal effect after 2 h (Fig. 2B). This and previous conflicting reports on the translational repression of TOP mRNAs by rapamycin [2,18,20], prompted us to verify the role of mTOR in signaling toward these mRNAs. mTOR knockdown, using lentivirus expressing mTOR shRNA, resulted in downregulation of both mTORC1 activity, as can be judged by the phosphorylation status of S6K and translational activation of rpL32 mRNA upon amino acid stimulation (Figs. 2C and 2D).

The critical role played by mTOR during mouse development has been demonstrated by the death of mTOR-deficient mice shortly after implantation, due to impaired cell proliferation in both embryonic and extraembryonic compartments [46]. It can be argued, therefore, that the repressed translation of TOP mRNAs might reflect a secondary response, due to a mitotic arrest in...
mTOR knocked down cells. To directly address this issue, we utilized a hyperactive mutant of mTOR (FLAG-mTORSL1\textsuperscript{1+1T}) that contains four amino acid substitutions within the kinase domain (I2017T, V2198A, L2216H and L2260P), previously shown to rescue mTORC1 activity in amino acid-starved HeLa cells [47]. Indeed, expression of this mutant, but not of wild-type mTOR, alleviated the inhibition of mTORC1 in amino acid-starved HEK 293 cells (Fig. 2E), as well as relieved the translational repression of rpL32 mRNA (Fig. 2F). Taken together, these results imply that mTOR is involved in amino acid signaling to translational efficiency of TOP mRNAs.

Translational activation of TOP mRNAs by amino acids is resistant to raptor or rictor deficiency

Establishing the role of mTOR in amino acid-mediated translational activation of TOP mRNAs has further underscored the discrepancy between the promptness and sensitivity of mTORC1 response to rapamycin and the relative rapamycin resistance exhibited by TOP mRNAs (Fig. 2 and [20]). To examine whether mTORC1 is involved at all in amino acid-induced translational activation of these mRNAs, we knocked down its core constituent, raptor, in HEK293 cells. This silencing, indeed, led to a pronounced decrease in raptor level and mTORC1 activity, as exemplified by the hypophosphorylation of S6K1 and rpS6 (Fig. 3A). However, despite this efficient elimination of raptor, the translational activation of rpL32 mRNA by amino acids was unaffected (Fig. 3B).

The lack of an effect of raptor knockdown prompted us to examine the role of mTORC2 in this mode of regulation. The results presented in Figure 3A and 3B clearly show that amino acid-induced translational activation of rpL32 mRNA was completely refractory to the loss of rictor, and consequently to that of mTORC2 activity (as can be judged by the hypophosphorylation of Ser473 in Akt in rictor knocked down cells). Notably, we have previously utilized inducible raptor and rictor cell lines (iRapKO and iRicKO, respectively) to demonstrate the dispensability of mTORC1 or mTORC2 in insulin or oxygen induced mTOR translational activation of TOP mRNAs [3,18]. However, these cell lines exhibit an inherent resistance to prolonged amino acid starvation (data not shown), and therefore could not be used here. Nevertheless, collectively our results imply that amino acid-stimulated translational activation of TOP mRNAs does not rely on raptor or rictor.
mTOR regulates TOP mRNA translation through its kinase activity

One plausible explanation for the apparent ability of mTOR to regulate TOP mRNA translation in an either mTORC1- or mTORC2-independent fashion might be its role as a scaffold protein, rather than an active kinase. Indeed, such a role has previously been proposed for regulation of dystrophin gene expression by mTOR [48]. Two complementary experimental approaches were used to directly address this possibility: a) HEK293 cells were transfected with wild type mTOR [49], rapamycin-resistant mTOR mutant (mTOR-rr) that contains S2035I substitution, or catalytically inactive version of mTOR-rr that contains an additional D2338A substitution (mTOR-rr-kd) [50]. Cells were amino acid-starved for 3 h followed by 3 h amino acid stimulation on day 4 post-infection. The abundance of mTOR and its activity were monitored by Western blot analysis of cytoplasmic proteins with the indicated antibodies. (D) Cytoplasmic extracts from cells described in (C) were subjected to polysomal analysis. The percentage of mRNA in polysomes at each time point is presented as an average of at least 2 measurements. (E) HEK293 were transiently transfected with plasmid-based vectors expressing either wild-type (WT) mTOR or enhanced (En) mTOR. 48 h later cells were amino acid-starved for 3 h and harvested. Cytoplasmic proteins were subject to Western blot analysis (E) and polysomal analysis (F). The percentage of mRNA in polysomes is presented as an average ± SEM of three experiments.

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Collectively, our results indicate that rapamycin suppresses the amino acid-induced translational activation of TOP mRNAs by the availability of FKBP12, as it does for mTORC1 and mTORC2.
mTORC2 activity [52], even though both raptor and rictor seems dispensable for the translational activation.

Over expression of constitutively active RagB can derepress the translation of TOP mRNAs in amino acid-starved, but not in oxygen-deprived cells

Rag GTPases bind raptor and thus mediate amino acid signaling to mTORC1 [9]. Accordingly, expression of constitutively active mutant forms of RagA or B (GTP-bound) can protect mTORC1 activity in amino acid-deprived cells [9,53]. However, the establishment of raptor as dispensable for amino acid- or oxygen-mediated translational activation of TOP mRNAs (Fig. 3B and [3]), raised a question regarding the role of Rag in this mode of regulation. To examine this issue, we measured the effect of FLAG-tagged wild type RagB or a mutant [RagB (Q99L)] that constitutively binds GTP (RagBGTP), on the translation efficiency of TOP mRNAs under stress conditions. Our results show that RagBGTP, but not wild type RagB, exerts complete, partial or no protective effect on rpS6 phosphorylation in cells that were amino acid-starved, starved for both serum and amino acids, or oxygen-deprived cells, respectively (Figs. 6A). Notably, RagBGTP exerts a similar stress-specific relief of the translational repression of rpL32 and rpS6 mRNAs (Fig. 6B), suggesting that RagB primarily mediates signals emanating from amino acids.

TOP mRNAs are not positively regulated by microRNAs

The demonstration that overexpression of miR-10a increases the polysomal association of TOP mRNAs in amino acid-starved cells has implied that this miR positively regulates the translation of TOP mRNAs [26]. However, based on multiple examples of erroneous conclusions regarding the function of an overexpressed protein [19], we set out to examine the role of miRs in this mode of regulation by a loss-of-function approach. Notably, miR-10a and miR-10b differ by just one nucleotide at a non-seed sequence, yet display a similar enhancing effect on the translation of a reporter TOP mRNA [26]. Likewise, miR-10a and miR-10b are potent inducers of neuroblastoma cell differentiation through targeting of nuclear receptor corepressor 2 (NCOR2) [39]. Hence, we initially used the miRNA sponge technique that can block the activity of miR-10b and conceivably that of miR-10a as well [54].

The location of the sponge sequence downstream of the GFP open reading frame enabled us to assess the sponge activity. Thus, the sequestration of the relevant miRNA in MDA-MB-231 cells indeed led to reduced GFP fluorescence intensity (Fig. 7A). Moreover, miR-10b has been implicated in downregulation of the stress-induced cell surface molecule, MICB (MHC class I chain related gene B) [38], and therefore, titrating out this miR resulted in increased expression of MICB (Fig. 7A). Next, we set to examine the ability of miR-10b sponge to derepress the expression of a luciferase encoded by an mRNA that contained in its 3′ untranslated region (3′ UTR) the wild-484 bp-long 3′ UTR from NCOR2 mRNA (designated as miR-10a) [39]. To this end, MDA-
MB-231 cells were transiently transfected with miR-10a luciferase reporter. This reporter construct showed a small (about 19%), yet statistically significant, increase in luciferase activity expressing mir-10b sponge, but not a control sponge. Moreover, a reporter containing NCOR2 3′ UTR with mutated miR-10a binding motif (designated miR-10a mut), failed to respond to the expression of the miR-10b sponge (Fig. 7B).

Collectively, these results indicate that miR-10b sponge can titrate out both miR-10a and miR-10b, and thereby set the stage for verifying the role of these miRs in controlling the translation efficiency of TOP mRNAs. Fig. 7C, clearly demonstrates that expression of a mir-10b sponge exerted no effect on the translation efficiency of rpL32 mRNA, when compared to untreated cells or cells transfected with a control sponge. It appears therefore, that miR-10a or miR-10b are not critical for efficient basal translation of TOP mRNAs. It can be argued that the positive effect of these miRs can only be detected under stress conditions. Hence, we set out to examine the effect of global deficiency of miRs on translation efficiency of TOP mRNAs under such conditions.

First, we knocked down Drosha, the nuclear RNase III enzyme that initiates the processing of miRs. The efficient silencing of Drosha (Fig. 7D) indeed, nearly nullified the abundance of representative miRs (Fig. 7E). This global deficiency in miRs had no appreciable effect on the proliferation rate of the respective cells (data not shown), yet it slightly decreased the basal translational efficiency of rpL32 mRNA (see Control in Fig. 7F). Nonetheless, it failed to block the translational activation of this mRNA in response to refeeding of starved cell with either amino acids or serum (Fig. 7F). These results imply that miRs are not required for the recovery of TOP mRNA translation from the nutritional stress. Unexpectedly, however, cells infected with the empty virus were the ones that exhibited minor or incomplete recovery of the translation efficiency of rpL32 mRNA following amino acid or serum refeeding, respectively. The reason for this effect of the empty virus is currently unclear.
Translational Control of TOP mRNAs by Amino Acids

Figure 5. Rapamycin represses the translation of TOP mRNAs in an FKBP-12-dependent fashion. (A) HEK293 cells were amino acid-starved for 3 h and then refed for 3 h in the absence or presence of rapamycin (20 nM), FK506 (20 mM), or both. Cytoplasmic proteins were subjected to Western blot analysis. (B) HEK293 cells were amino acid-starved for 3 h (→ AA), refed for 3 h (→ AA→+AA) in the absence or presence of rapamycin (20 nM), FK506 (20 mM) or both. Cytoplasmic extracts were subjected to polysomal analysis. doi:10.1371/journal.pone.0109410.g005

Discussion

Biogenesis of the protein synthesis machinery, and particularly of ribosomes, is a highly resource-consuming process [57]. Thus, cells that encounter unfavorable conditions attenuate the production of components of the translational machinery and cease to grow [58]. Indeed, the present report demonstrates that mTOR-sensitive translational repression of TOP mRNAs is one mechanism that is exploited by cells to selectively downregulate wasteful biogenesis of the protein synthesis machinery under nutritional stresses.

mTOR operates as a translation activator of TOP mRNA translation outside of its canonical complexes

The positive regulatory role of mTOR in translational activation of TOP mRNAs is supported by results obtained by two complementary experimental approaches: a) the inhibition of amino acid-induced recruitment of TOP mRNAs into polysomes in mTOR knockdown cells or by Torin 1 treatment (Figs. 2D, 4D); and b) the ability of a hyperactive mutant of mTOR to protect the translation of these mRNAs from amino acid deficiency (Fig. 4B). Moreover, the fact that mTOR must be enzymatically active to exert this role indicates that it does not function merely as a scaffold protein (Fig. 4B). However, data presented here demonstrate that the involvement of mTOR in amino acid-activation of TOP mRNA translation depends on neither of the two canonical complexes, mTORC1 and mTORC2 (Figs. 3B). These results coincide with our previous reports on the minor, if at all, reliance of insulin- and oxygen-induced translational activation of TOP on either of these complexes [3,18]. Not surprisingly, therefore, the role assigned to either S6K or 4E-BP, the two best-characterized mTOR effectors, in translational control of TOP mRNAs has been refuted [59,60]. A question can be raised as to how amino acid-induced translational activation of TOP mRNA is repressed by rapamycin (Fig. 2) if it does not involve raptor. However, we have already demonstrated that TOP mRNA translation is rendered rapamycin-hypersensitive in raptor-deficient cells [18]. Furthermore, mTOR has been shown to doubly phosphorylate IMP2, another mTOR target, in a raptor-independent fashion. Thus, knockdown of mTOR strongly inhibited IMP2 phosphorylation in cells, whereas depletion of raptor had no effect on this modification in vitro or in vivo [61]. In light of these supportive data, we propose that mTOR regulates TOP mRNAs translation, and possibly a subset of other targets, through as yet unidentified third complex, or in a complex-independent fashion, and therefore neither raptor nor rictor is critical for its activity toward these targets.

TSC mediates translational repression of TOP mRNAs under any stress, whereas the RagB transduces only a subset of stimuli to translation efficiency of TOP mRNAs

TOP mRNAs translation, like mTORC1 activity, is protected from oxygen or serum starvation in cells lacking either TSC1 or TSC2 [3,18]. Contrarily, it has been widely argued that the TSC1-TSC2 complex plays no role in transducing the negative signal resulting from amino acid starvation to mTORC1 activity (reviewed in [62,63]). However, a recent study has shown that inhibition of mTORC1 by amino acid deprivation is indeed mediated by the TSC1-TSC2 complex. The latter is required for the release of mTORC1 from its site of action, the lysosomal membrane, and therefore, cells lacking TSC fail to efficiently turn off mTORC1 and consequently their response to amino acid starvation is compromised [64]. Results presented here clearly demonstrate that both TSC1 and TSC2 are critical, not only for mTORC1 inhibition, but also for translational repression of TOP mRNAs upon amino acid starvation (Fig. 1). It should be pointed out, however, that since amino acid-induced translational activa-
tion of TOP mRNAs does not rely on mTORC1 (Fig. 3), it is conceivable that also TSC1-TSC2 complex exerts its repressive effect on TOP mRNA translation in an mTORC1-independent fashion.

Unlike TSC1-TSC2 complex, that convey inhibitory signals to TOP mRNA translation or to mTORC1 activity emanating from all examined stress conditions, RagBGTP can partially derepress TOP mRNAs in cells starved for amino acids or amino acids and serum, but not cells subjected to anoxic conditions (Fig. 6). Evidently, RagBGTP is able to confer on mTORC1 complete resistance to amino acid starvation, and it does so in a raptor-dependent fashion [9]. However, the hierarchical relationships between RagB and mTOR in transduction of amino acids to TOP mRNA translation, a pathway that does not rely on raptor, is yet to be established.

The nature of the translational trans-acting factor of TOP mRNAs is still elusive

The discrete translational behavior of TOP mRNAs implies that the 5' TOP motif is recognized by a specific translational trans-acting factor that transduces physiological cues into a selective translational control of these mRNAs. However, more than two decades of a search for such a regulator(s) have mostly yielded candidates that have failed to withstand the test of time.

The microRNA, miR-10a, was claimed to play both a selective and a positive role in translational control of TOP mRNAs in response to fluctuations in amino acid sufficiency [26]. Moreover, global microRNA deficiency in Drosha knockdown cells, failed to suppress translational activation of TOP mRNAs upon readdition of either amino acids or serum to starved cells (Fig. 7F). Likewise, experiments conducted with Dicer knockout cells revealed that translational activation of TOP mRNAs upon serum refeeding or resuming oxygen supply was not affected by microRNA deficiency (Fig. 8 and [3]). Collectively, these observations appear to disprove a positive role of micro-RNAs in translational control of TOP mRNAs. Nevertheless, based on the apparent higher translation efficiency of TOP mRNAs in Dicer−/− cells, we cannot exclude the possibility that the basal translation efficiency of these mRNAs is downregulated by one or more microRNAs. Indeed, we have previously shown that Dicer deficiency leads to constitutive activation of mTOR [3], and therefore the higher translation efficiency reported here (Fig. 8B) for Dicer−/− cells might reflect this phenotypic change.

A previous report has demonstrated that two stress granule (SG)-associated RNA-binding proteins, T-cell intracellular antigen-1 (TIA-1) and TIA-1-related (TIAR), assembled onto the first 39 nucleotides of TOP mRNAs in amino acid-starved cells. This interaction resulted in a selective accumulation of TOP mRNAs and their unloading from polysomes, as well as decreased synthesis of TOP mRNA-encoded proteins [65]. Furthermore, simultaneous knockdown of both proteins derepressed TOP mRNA translation in amino acid-, but not oxygen-, deprived cells, implying their critical, yet selective, role in amino acid starved cells [3,65].

The translational repressor, 4E-BP, is yet another candidate proposed to carry out the translational repression of TOP mRNAs [66]. However, a recently published report, has shown that the
Figure 7. Knockdown of miRs fails to suppress translational activation of TOP mRNAs.

(A) MDA-MB-231 cells were infected with lentivirus expressing either anti-miR 10b sponge or anti-miR-BART 1–5p sponge (control). The fluorescent signals of MICB and GFP were analyzed by FACS. The mean intensity of MICB or GFP in the control miR–transduced cells was arbitrarily set up to be 1, and the relative increase in the MICB expression or the decrease in the GFP fluorescence in sponge-10b–transduced cells was calculated accordingly (individual numbers are presented within the bars).

(B) MDA-MB-231 cells infected with lentiviruses described in (A) were transiently transfected with Dual luciferase PsiCheck2 reporter vectors. These vectors contained within the 3′-UTR of the Renilla luciferase either a fragment from the 3′-UTR of NCOR2 that bears miR-10a/10b binding site (designated miR-10), or a negative control with a mutated miR-10a/10b seed region (designated miR-10mut). The Renilla to Firefly activity ratio (R/FF) was calculated for each sample and the average obtained for the miR-10b sponge-infected cells was normalized to that obtained for the control sponge-infected cells, which arbitrarily was set at 1.0. (*) p < 0.001 versus miR-10a transfected cells (n = 8).

(C) MDA-MB-231 cells that were either kept uninfected (None), expressed anti-miR 10b sponge or control sponge (Control) were kept untreated and cytoplasmic extracts from these cells were subjected to polysomal analysis. (D) and (F) RKO cells infected with Sin 18, an empty lentiviral vector (EV), or by lentivirus expressing shDrosha RNA. Cells were either untreated (Control in (F)), starved for serum for 19 h and during the last 3 h also for amino acids and then either kept without serum and amino acids [− AA in (D); − Ser in (F)] or refed for just amino acid for additional 2 h [+ AA in (D); − Ser → + Ser in (F)]. Similarly infected cells were serum starved for 48 h [−Ser in (D); −Ser in (F)] or serum refed for 3 h [+Ser in (D); − Ser → + Ser in (F)]. Cells were harvested and subjected to Western blot analysis with the indicated antibodies (D) or subjected to polysomal analysis (F).

(E) Total RNA was prepared from RKO cells infected with either empty lentiviral vector (EV) or lentivirus expressing shDrosha RNA. The abundance of each of the indicated miRs in Drosha knockdown cells was normalized to that in cells infected with empty vector, which was arbitrarily set at one.

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TOP mRNAs are translationally regulated by physiologically relevant stresses, like anoxia or serum starvation, in a 4E-BP-independent manner [3]. Finally, LARP1, an RNA-binding protein, has been implicated as a positive regulator of TOP mRNA translation [67]. Thus, LARP1 deficiency led to a selective reduced recruitment of TOP mRNAs to polysomes, relative to non-TOP mRNAs, which was accompanied by a decrease in the abundance of TOP mRNA-encoded proteins. However, the selectivity of LARP1 is still questionable, since its silencing reduced the rate of global protein synthesis by 2 to 2.5-fold [67]. Moreover, its activity has not been examined under any physiological stress.

In summary, in light of the conflicting results and the missing information, the nature of the proximal trans-acting factor and its mode of action in translational control of TOP mRNAs still remains elusive.

References


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Author Contributions

Conceived and designed the experiments: IPP OM. Performed the experiments: IPP JK R. Miloslavski ZH MHH MSR PT MB. Analyzed the data: IPP JK R. Miloslavski ZH MHH MSR PT MB. Contributed reagents/materials/analysis tools: R. Mudhasani SJ. Wrote the paper: IPP OM.


