THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 290, NO. 26, pp. 15996–16020, June 26, 2015

La-related Protein 1 (LARP1) Represses Terminal Oligopyrimidine (TOP) mRNA Translation Downstream of mTOR Complex 1 (mTORC1)*[S]

Received for publication, November 4, 2014, and in revised form, April 27, 2015 Published, JBC Papers in Press, May 4, 2015, DOI 10.1074/jbc.M114.621730

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Background: mTORC1 plays an important role in the regulation of TOP mRNA translation.
Results: LARP1 is a target of mTORC1 that associates with TOP mRNAs via their 5′TOP motif to repress their translation.
Conclusion: LARP1 represses TOP mRNA translation downstream of mTORC1.
Significance: We elucidate an important novel signaling pathway downstream of mTORC1 that controls the production of ribosomes and translation factors in eukaryotic cells.

The mammalian target of rapamycin complex 1 (mTORC1) is a critical regulator of protein synthesis. The best studied targets of mTORC1 in translation are the eukaryotic initiation factor-binding protein 1 (4E-BP1) and ribosomal protein S6 kinase 1 (S6K1). In this study, we identify the La-related protein 1 (LARP1) as a key novel target of mTORC1 with a fundamental role in terminal oligopyrimidine (TOP) mRNA translation. Recent genome-wide studies indicate that TOP and TOP-like mRNAs compose a large portion of the mTORC1 translatome, but the mechanism by which mTORC1 controls TOP mRNA translation is incompletely understood. Here, we report that LARP1 functions as a key repressor of TOP mRNA translation downstream of mTORC1. Our data show the following: (i) LARP1 associates with mTORC1 via RAPTOR; (ii) LARP1 interacts with TOP mRNAs in an mTORC1-dependent manner; (iii) LARP1 binds the 5′TOP motif to repress TOP mRNA translation; and (iv) LARP1 competes with the eukaryotic initiation factor (eIF) 4G for TOP mRNA binding. Importantly, from a drug resistance standpoint, our data also show that reducing LARP1 protein levels by RNA interference attenuates the inhibitory effect of rapamycin, Torin1, and amino acid deprivation on TOP mRNA translation. Collectively, our findings demonstrate that LARP1 functions as an important repressor of TOP mRNA translation downstream of mTORC1.

The mammalian target of rapamycin (mTOR)[5] protein kinase plays a fundamental role in cellular homeostasis. mTOR is found in two multiprotein complexes termed mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (1). Each complex includes a distinct subset of protein components. mTORC1 includes the mTOR kinase, the scaffolding protein regulatory-associated protein of mTOR (RAPTOR) (2–5), the mammalian lethal with Sec13 protein 8 (mLST8) (4, 6), the proline-rich AKT substrate of 40 kDa (PRAS40) (7–12), and the DEP domain-containing mTOR-interacting protein (DEPTOR) (13). mTORC2 shares common components with mTORC1 (mTOR, mLST8, and DEPTOR) but lacks RAPTOR and PRAS40. The latter are specific to mTORC1. Conversely, mTORC2 includes unique components, *e.g.* rapamycin-insensitive companion of mTOR (RICTOR) (4, 14), mammalian stress-activated protein kinase-interacting protein (mSIN1) (15, 16), and protein observed with RICTOR-1 and -2 (PROTOR-1 and PROTOR-2) (11, 17, 18). mTORC1 and mTORC2 also differ in their sensitivity to the allosteric inhibitor, rapamycin. Acute rapamycin treatment selectively inhibits mTORC1, whereas prolonged incubation with the drug impairs

* The authors declare that they have no conflicts of interest with the contents of this article.
[S] This article contains supplemental Statistical Workbook.
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[5] The abbreviations used are: mTOR, mammalian target of rapamycin; mTORC1, mammalian target of rapamycin complex 1; TOP, terminal oligopyrimidine; PABP, poly(A)-binding protein; sqPCR, semiquantitative PCR; qPCR, quantitative PCR; RNC, RAPTOR N-terminal conserved; 4E-BP, eukaryotic initiation factor binding protein; S6K, S6 kinase; E.V., empty vector; TOS, TOR signaling.

both mTORC1 and mTORC2 activity (19). Despite its ability to acutely inhibit mTORC1, rapamycin does so only partially as certain mTORC1 outputs are insensitive to rapamycin (20). mTORC1 and mTORC2 are, however, fully blocked by specific ATP mimics (*e.g.* Torin1) that target the active site on mTOR (21).

mTORC1 controls mRNA translation through the phosphorylation of multiple substrates (22). The translational repressor family of proteins, eukaryotic translation initiation factor 4E-binding proteins (4E-BPs) and ribosomal S6 kinases (S6Ks), are arguably the best characterized targets of mTORC1. The eIF4F complex (formed by the mRNA m^7Gppp cap-binding protein eIF4E, the scaffold protein eIF4G, and the RNA helicase eIF4A) recruits the 43S pre-initiation complex to the 5′end of the mRNA (23). In their hypophosphorylated form, 4E-BPs bind the eukaryotic initiation factor 4E (eIF4E), precluding the binding of the latter to eIF4G and the assembly of the eIF4F complex. Sequential phosphorylation of multiple residues on 4E-BP1 by mTORC1 releases the former from eIF4E, thus allowing for eIF4F to assemble and translation initiation to ensue (24). S6Ks also play an important role in translation, but unlike 4E-BPs, they employ various mechanisms to regulate multiple steps in protein synthesis, *e.g.* S6K1 phosphorylates the eukaryotic initiation factor 4B (eIF4B) at Ser-422, which enhances the association of eIF4B with eIF3 at the initiation step of protein synthesis (25–29). S6K1 also phosphorylates eukaryotic elongation factor 2 kinase (eEF2K) at serine 366 and impairs its activities (30), which in turn negates phosphorylation of the eukaryotic elongation factor 2 (eEF2), a key translation factor that controls ribosomal transit during the elongation step of the protein synthesis (31).

4E-BPs and S6Ks play important roles in the control of protein synthesis, but recent studies suggest they do not act alone in the coordinated regulation of mRNA translation downstream of mTORC1. Adequate control of mRNA translation likely engages additional proteins and signaling pathways downstream of mTORC1. A number of recent studies emphasize this idea (32–37); these studies point to the existence of numerous additional mTORC1 substrates, some of which likely execute important functions in translation control of cellular mRNAs. Foremost among these is the synthesis of ribosomal proteins and associated components of the translation apparatus. Ribosomal proteins (and a number of translation factors) are encoded by a subgroup of mRNAs containing a 5′terminal oligopyrimidine tract (5′TOP motif) at the 5′end of the mRNA immediately downstream of the m^7Gppp mRNA cap. The presence of the 5′TOP motif within these mRNAs has previously been shown to confer translation repression in conditions of nutrient or oxygen deprivation (38). mTORC1 plays a seminal role in the regulation of TOP mRNA translation (22, 39–42). Analysis of the mTORC1 translatome using allosteric and active site mTOR inhibitors indicates that the mTORC1 pathway preferentially regulates the translation of TOP and TOP-like mRNAs via 4E-BPs/eIF4E/eIF4G (43, 44). However, these proteins may not be the sole mTORC1 targets controlling TOP mRNA translation (45). Several *trans*-acting factors have been implicated as regulators of TOP mRNA translation (46–51), but the mechanism and relation to mTORC1 activity remain unclear (42, 45, 51). More recently, the La-related protein 1 (LARP1) has been suggested to regulate TOP mRNA gene expression (52–54).

In this study, we establish that LARP1 is a target of mTORC1 and repressor of TOP mRNA translation. We demonstrate that LARP1 associates with mTORC1 via RAPTOR and that the interaction between LARP1 and RAPTOR is regulated by mTOR kinase activity. Our data also show that LARP1 binds RAPTOR preferentially in the absence of mRNA. In addition to binding RAPTOR, our data show that LARP1 also interacts with the poly(A)-binding protein (PABP). However, in contrast to the association with RAPTOR, the binding of LARP1 to PABP is largely unaffected by the presence of mRNA and is not regulated by mTORC1 activity.

LARP1 has previously been shown to associate with various mRNAs (52, 55). In this study, we show that LARP1 binds TOP mRNAs in an mTORC1-regulated manner. Specifically, LARP1 binding to TOP mRNAs is enhanced upon mTORC1 inhibition. LARP1 has previously been shown to bind the 3′-hydroxyl (OH) group of the terminal adenosine within the poly(A)-tail (52). In this study, we show that LARP1 also directly associates with TOP mRNAs via their 5′TOP motif at the 5′end of the 5′UTR, likely forming a translation-inactive mRNA loop.

Mechanistically, our data demonstrate that LARP1 functions as a repressor of TOP mRNA translation and that association of LARP1 with the 5′TOP motif is a pre-requisite to block TOP mRNA translation. Our data further show that there is an inverse relationship between LARP1 and eIF4G binding to TOP mRNAs and that LARP1 competes with eIF4G for TOP mRNA association, thus precluding the assembly of a functional eIF4F complex. Incubation with mTORC1 inhibitors results in increased association of LARP1 with TOP mRNAs, decreased eIF4G binding, and reduced TOP mRNA translation.

Interestingly, from a drug efficacy standpoint, we observe that depletion of LARP1 by RNA interference attenuates the inhibitory effects of pharmacological inhibitors of mTOR (*e.g.* rapamycin or Torin1) on TOP mRNA translation, suggesting that LARP1 may potentially be used as a biomarker for drug efficacy in the clinic. In summary, the findings reported here establish that LARP1 functions as a critical repressor of TOP mRNA translation downstream of mTORC1 with potential future implications in anti-cancer drug development.

Experimental Procedures

Chemicals and cDNAs—Rapamycin (catalogue no. 553211) was purchased from Calbiochem and Torin1 (catalogue no. 4247) from Tocris Bioscience. The pRK5 empty vector and pRK5-Myc human RAPTOR (transcript variant 1) were kindly provided by Dr. David Sabatini. pCMV6-entry-Myc/FLAG-tagged human LARP1 (transcript variant 1) (catalogue no. RC200935) and pCMV6-AC-GFP-tagged human LARP1 wild type (transcript variant 1) (RG200935) were purchased from Origene. pLKO1 vectors listed in Fig. 7*A* were purchased from Sigma. pcDNA5-FLAG vector was previously described (51). shRNA silencing was carried out using lentiviral vectors (mission shRNA, Sigma). A scrambled sequence (SHC002) (Sigma) was used as a nontargeting shRNA negative control. The accession numbers for each shRNA used against human LARP1

(Sigma) are listed in Fig. 7*A*. The shRNA against human RAPTOR (plasmid 1857) was obtained from Addgene.

Generation of Lentiviruses and Transduction of HEK293T Cells—Each shRNA vector was co-transfected with lentivirus packaging plasmids pLP1, pLP2, and pLP/vesicular stomatitis virus-G (pLP/VSV-G) into HEK293T cells using Lipofectamine 2000 (Invitrogen, Life Technologies, Inc.). Supernatants containing viral particles were collected at 48 and 72 h post-transfection and filtered through a 0.45-μm nitrocellulose membrane. Filtered supernatants were then applied to HEK293T target cells. Cells were incubated with virus for 24 h and then re-infected with fresh virus for a further 24 h prior to selection with 5 μg/ml puromycin (catalogue no. PUR333.25, Bioshop Canada Inc.). Puromycin selection medium was prepared in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS), 100 μg/ml streptomycin sulfate, and 100 units/m penicillin G. Cells were selected in puromycin for 2 days and then transferred to growth medium without puromycin for the experiments.

Mammalian Cell Culture, Cell Lysis, and Western Blot—HEK293T were used in every figure except Figs. 5*C* and 12, *A*–*E* (where HeLa cells were used instead). HEK293T and HeLa cells were propagated in Dulbecco's modified Eagle's medium (DMEM) (catalogue no. 319-005-CL) supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin G, and 100 μg/ml streptomycin sulfate at 37 °C and 5% (v/v) CO_2 humidified incubator. Amino acid deprivation experiments were carried out by switching cells to DMEM lacking all amino acids (catalogue no. 319-004-CL, Wisent Inc.) supplemented with dialyzed fetal bovine serum (catalogue no. 26400-036, Gibco Life Technologies, Inc.) for the times indicated (see legends to Figs. 4*C* and 9). Cell lysates were prepared by washing cells once with ice-cold PBS followed by incubation with extraction buffer (40 mM HEPES (pH 7.5) at room temperature, 0.3% (w/v) CHAPS, 120 mM NaCl, 1 mM EDTA, 10 mM sodium pyrophosphate, 10 mM β-glycerophosphate, 50 mM sodium fluoride, 1.5 mM sodium orthovanadate, 1 μg/ml RNase A (catalogue no. 10109169001, Roche Applied Science), 0.5 mM DTT, and complete EDTA-free protease inhibitors mixture tablets (catalogue no. 04693 132 001, Roche Applied Science)) for 30 min on ice followed by scraping, as originally described (1). RNase A was omitted from extraction buffer for RNA immunoprecipitation experiments. A range of RNase A concentrations (1, 10, and 100 μg/ml) were used for the experiment in Fig. 4*D*. Where indicated in Fig. 1*A*, 0.3% (v/v) Triton X-100 was used in place of CHAPS. Varying concentrations of NaCl were also used in Fig. 1*A*; low salt denotes 120 mM NaCl and high salt denotes 500 mM NaCl. Lysates were pre-cleared by centrifugation at 21,000 $\times$ *g* for 10 min at 4 °C. Phosphorylation and total protein levels were analyzed by resolving samples of lysates on a 10% (w/v) acrylamide gel containing 0.1% (w/v) methylene bisacrylamide, transferred onto a 0.2-μm nitrocellulose membrane (catalogue no. NBA083C001EA, PerkinElmer Life Sciences) at 100 V for 90 min followed by Western blot with the following antibodies: anti-phospho-Thr-389 S6K1 (catalogue no. 9234, Cell Signaling Technologies); anti-phospho-Ser-473 AKT (catalogue no. 2965, Cell Signaling Technologies); anti-S6K1 C18 (catalogue no. SC-230, Santa Cruz Biotechnology); anti-AKT (catalogue no. 4691, Cell Signaling Technologies); anti-RAPTOR (catalogue no. 09-217, Millipore); anti-RICTOR (catalogue no. 104838, Abcam); anti-mTOR (catalogue no. 2983, Cell Signaling Technologies); anti-PRAS40 (catalogue no. 2691, Cell Signaling Technologies); anti-mLST8 (catalogue no. 3274, Cell Signaling Technologies); anti-LARP1 (catalogue no. ab86359, Abcam); anti-PABP (catalogue no. ab21060, Abcam); anti-RPS6 C-8 (catalogue no. SC-74459, Santa Cruz Biotechnology); anti-β-actin (catalogue no. A5441, Sigma); anti-RPL7a H-26 (catalogue no. sc-98618, Santa Cruz Biotechnology); anti-DDX6 N-20 (catalogue no. SC-51416, Santa Cruz Biotechnology); anti-phospho-Thr-37/46 4E-BP1 (catalogue no. 2855, Cell Signaling Technologies); anti-phospho-Ser-65 4E-BP1 (catalogue no. 9451, Cell Signaling Technologies); anti-phospho-Thr-70 4E-BP1 (catalogue no. 9455, Cell Signaling Technologies); anti-4E-BP1 (catalogue no. 9644, Cell Signaling Technologies); anti-phospho-Thr-56 eEF2 (catalogue no. 2331, Cell Signaling Technologies); anti-eEF2 (catalogue no. 2332, Cell Signaling Technologies); anti-eIF4G C45A4 (catalogue no.2469, Cell Signaling Technologies); anti-eIF4E (catalogue no. 610269, BD Transduction Laboratories); anti-FLAG® M2 (catalogue no. F1804, Sigma); and anti-Myc (catalogue no. TAG003, Bioshop Canada Inc.).

Site-directed Mutagenesis and Cloning—Site-directed mutagenesis on pCMV6-human LARP1-Myc/FLAG was performed as per the manufacturer's instructions (Stratagene). The following oligonucleotides were designed with PrimerGenesis automated primer design software (and used for site-directed mutagenesis of human LARP1: LARP1 ΔPAM2_forward (5′-GATTATTCACAGACTGATTTCCGTCAGCACTACCAAAAGGAG-3′) and LARP1 ΔPAM2_reverse (5′-CTCCTTTTGGTAGTGCTGACGGAAATCAGTCTGTGAATAATC-3′); LARP1 ΔTOS_forward (5′-AGTTATGGCCTGGAAAAGAAGTTCAAGG-3′) and LARP1 ΔTOS_reverse (5′-TTCCTCCTGAAAATCCTTGAACTTCTTTTC-3′); LARP1_F889A_forward (5′-GTTATGGCCTGGAAAAGAAGGCCCGGCT-3′) and LARP1_F889A_reverse (5′-CTTGAATATGTCCAGCCGGGCCTTCTTTT-3′); LARP1_F505A_forward (5′-CTCAACTGCCCTGAAGCTGTTCCCCGTCAGCA-3′) and LARP1_F505A_reverse (5′-TGCTGACGGGGAACAGCTTCAGGGCAGTTGAG-3′). The following primers were used for sequencing human LARP1: LARP1_seq1_forward (5′-ATGCTTTGGAGGGTGCTTTTG-3′); LARP1_seq2_forward (5′-GTTCCTAAACAGCGCAAAGGC-3′); LARP1_seq3_forward (5′-TGCCAGCGAGGCGGGCAGAAG-3′); LARP1_seq4_forward (5′-GACCAGGATGAGACATCGAGTG-3′); LARP1_seq5_forward (5′-GTGGATCAGGAACTGCTCAAAG-3′); LARP1_seq6_forward (5′-GAGGAACCAGAAAAGTGGCCTC-3′); LARP1_seq7_forward (5′-ATTGAAGTGAAGAAGAGGCCTC-3′); LARP1_seq8_forward (5′-AGGGATGTCAACAAGATCCTC-3′); LARP1_seq9_forward (5′-GAGCAGTTTGACACACTGACC-3′); LARP1_seq10_forward (5′-TCACGGTTTTACCCAGTGGTG-3′); LARP1_seq11_forward (5′-GAACTGCTCAAGGAAAATGGC-3′); LARP1_seq12_forward (5′-TACAGTTATGGCCTGGAAAAG-3′); LARP1_seq13_forward (5′-CGACACTCAGTGGTAGCAGGAG-3′); and LARP1_seq14_reverse (5′-AGGGAATGGCAATGGCTTCTC-3′). The following

primers were used to clone RAPTOR fragments (1–904, 526–904, and 904–1335) by restriction digestion (5′-SalI and 3′NotI) and ligation using pRK5-Myc human RAPTOR isoform 1 as a template (numbering refers to amino acid sequence) as follows: RAPTOR_1_forward (5′-ACGCGTCGACGATGGAGTCCGAAATGCTGC-3′) and RAPTOR_904_reverse (5′-TTTTCCTTTTGCGGCCGCGCCAGAAGGCAAGTCTCGGC-3′); RAPTOR_526_forward (5′-ACGCGTCGACGTACATGCCAGCTGAACACCG-3′); and RAPTOR_904_forward (5′-ACGCGTCGACGCCGGCCGGGCACCACAGGC-3′) and RAPTOR_1335_reverse (5′-TTTTCCTTTTGCGGCCGCCTATCTGACACGCTTCTCCACC-3′). The following primers were used to sequence human RAPTOR (numbering refers to nucleotide sequence): SP6_forward (5′-ATTTAGGTGACACTATAG-3′); RAPTOR_430_forward (5′-GTCGCAACGCCAAGGAGG-3′); RAPTOR_923_forward (5′-GAACGACAGGAGGACGCC-3′); RAPTOR_1438_forward (5′-CCTACGTGCTGAAGCTGC-3′); RAPTOR_1914_forward (5′-GGACGGACCACTCCACC-3′); RAPTOR_2526_forward (5′-CATCGCCTACAAGGCCACC-3′); RAPTOR_2938_forward (5′-CCCAGAAGAGCACGACC-3′); RAPTOR_3378_forward (5′-CGACGCGAGGAGCTGGG-3′); and RAPTOR_3772_forward (5′-CGTGAAGGGGCTGACGG-3′).

Cloning of human LARP1 (isoform 1019 amino acids) into a pcDNA5-FLAG vector (51) for generation of stable HeLa Flp-in cell lines was done using the following primers: LARP1_forward (5′-ATTAAAGCTTATGCTTTGGAGGGTGCTTTTG-3′) and LARP1_reverse (5′-AATAGCGGCCGCTCACTTTCCCAAAGTCTGTG-3′).

RNA-Electrophoretic Mobility Shift Assay (RNA-EMSA)—PAGE-purified synthetic RNA oligonucleotides corresponding to the 53-nucleotide-long 5′UTR of RPL32 mRNA and 42-nucleotide-long 5′UTR of RPS6 (listed in Fig. 11*A*) were purchased from Integrated DNA Technologies. The oligonucleotides were radiolabeled at their 5′ends using [γ-^{32}P]ATP (40 μCi) and polynucleotide kinase (8 units; MBI, Fermentas) in a total reaction volume of 20 μl as recommended by the manufacturer. Prior to use, the probes were purified by centrifugation through Chroma Spin-10 Columns (BD Biosciences). Standard binding reaction mixtures (20 μl) contained 8 fmol (~40,000 cpm) of 5′-labeled oligonucleotides, 10 μl of 2× incubation buffer (40 mM HEPES-KOH (pH 7.3), 200 mM KCl, 4 mM $MgCl_2$, 2 mM DTT, 0.1% (v/v) Nonidet P-40, 10% (v/v) glycerol, and 0.2 mg/ml acetylated bovine serum albumin), 0.2 μg of tRNA (*Escherichia coli*), and 30 or 60 ng of LARP1 (Abnova). Following incubation at 30 °C for 30 min, the samples were supplemented with 2 μl of 50% (v/v) glycerol and analyzed by electrophoresis in 7% nondenaturing polyacrylamide gel (prepared with 0.5× TBE buffer and 5% (v/v) glycerol) at 4 °C. Bands were visualized by autoradiography of the dried gels.

RPL32-β-Globin Polysomal Reporter Assay—HeLa Tet-Off cells (Clontech) were seeded in 10-cm plates 24 h prior to transfection with a mixture of 300 ng of pTET2-β-globin/pTET2-RPL32-β-globin (51), 300 ng of ptTA (Clontech), and 1.4 μg of empty vector (E.V.) or pcDNA5-FLAG-LARP1 in the presence of 40 ng/ml tetracycline using Lipofectamine 2000 (Life Technologies, Inc.) according to the manufacturer's instructions. The inclusion of tetracycline inhibits transcription of the β-globin reporter, while allowing expression of FLAG-tagged LARP1. Twenty four hours post-transfection tetracycline was removed, and cells were washed in PBS, and medium was replaced with DMEM containing 10% (v/v) certified Tet-free serum (Life Technologies, Inc.) for 8 h to allow reporter expression. Cells were harvested and subjected to polysome profiling as described below.

RAPTOR Immunoprecipitation and C_{18} Liquid Chromatography/Tandem Mass Spectrometry—HEK293T cells were transfected with 5 μg of Myc-tagged human RAPTOR and lysed in low salt (120 mM NaCl) CHAPS-based buffer containing 1 μg/ml RNase A. Two milligrams of lysate were used for immunoprecipitation with anti-Myc antibody. Immunoprecipitated samples were loaded onto a 4–12% gradient pre-cast SDS-polyacrylamide gel, resolved by electrophoresis, and visualized by SYPRO® Ruby staining. Each lane was cut into 10 slices. Each slice was then reduced with dithiothreitol (DTT) in ammonium bicarbonate and alkylated in iodoacetamide. The samples were subsequently trypsinized overnight at 37 °C. Peptides were extracted and dried by speed vacuum. Dried samples were resuspended and injected onto an Agilent Q-TOF 6550. Data were extracted with Peak Distiller (Matrix Science) and a search against a human database using the Mascot search engine ("transproteomic pipeline"). Results were visualized using the Scaffold 3 viewer software. Hits were selected from Scaffold list with basis on the total number of spectra in Myc RAPTOR immunoprecipitation *versus* Myc empty vector control. A minimum threshold of two unique peptides (spectra) was used for validation of hits. A minimum of a 2-fold ratio of Myc-RAPTOR to E.V. spectra was used for validation of hits.

LARP1 and eIF4G RNA Immunoprecipitation—HEK293T cells were lysed in CHAPS extraction buffer described above. Typically, 1–2 mg of total protein lysate were used for immunoprecipitation by incubating lysate with 4 μl of LARP1, FLAG, or 10 μl of eIF4G antibody for 1 h at 4 °C mixing end-over-end. Thirty microliters of magnetic Dynabeads® protein G (catalogue no. 10003D, Life Technologies, Inc.) were then added to the antibody/lysate mixture, and samples were incubated for 45 min mixing end-over-end. Beads were pelleted and washed twice with 1 ml of lysis buffer and then resuspended in 500 μl of TRIzol® reagent (catalogue no.15596018, Life Technologies, Inc.) followed by brief vortexing. RNA was extracted as per the manufacturer's protocol. Input RNA was resuspended in 100 μl, and immunoprecipitated material was resuspended in 10 μl of RNase-free diethyl pyrocarbonate-treated water. Ten microliters were used from each sample for cDNA synthesis. cDNA synthesis was performed using the iScript™ select cDNA synthesis kit as follows: 4 μl of 5× iScript select reaction mix, oligo-$(dT)_{20}$ primer, 10 μl of RNA template, 1 μl of iScript reverse transcriptase, and 3 μl of RNase-free diethyl pyrocarbonate-treated water. cDNA synthesis product was diluted 1:10, and 8 μl were used for quantitative PCR (qPCR). qPCR was performed as per the manufacturer's instruction using the iQ™ SYBR® Green Supermix (catalogue no. 170-882, Bio-Rad). Briefly, 10 μl of iQ™ SYBR® Green Supermix, 10 μM primer forward and reverse, 8 μl of cDNA template, and RNase-free diethyl pyrocarbonate-treated water up to a 20-μl final volume mix was prepared. The following conditions were used in a

two-step qPCR: denaturation at 95 °C for 15 s and extension at 61 °C for 30 s (RPS6 and RPL32) and 64.5 °C for 30 s (RPS20 and PABP).

m^7GTP Cap Binding Assay—HEK293T cells were lysed in CHAPS extraction buffer described above. Typically, 500–1000 μg of total protein lysate were used for m^7GTP pulldown by incubating lysate with 20–30 μl of immobilized γ-aminophenyl-m^7GTP (C10 spacer) beads (catalogue no. AC-155S, Jenna Biosciences) for 1 h at 4 °C mixing end-over-end. Unbound material was washed three times with lysis buffer prior to elution with 1 mM free m^7GTP or by addition of 50 μl of 4× sample buffer. Samples were resolved by SDS-PAGE/Western blot and probed with the indicated antibodies.

Polysome Profile Assay—Preparation of gradients was as follows: 10–50% sucrose gradients were prepared in 20 mM HEPES/KOH (pH 7.6) at room temperature; 100 mM KCl, 5 mM $MgCl_2$, 100 μg/ml cycloheximide, EDTA-free protease inhibitors mixture tablets (catalogue no. 04693 132 001, Roche Applied Science), and 200 units/ml RNasin ribonuclease inhibitor (catalogue no. N2515, Promega). Isolation of polysomes was as follows: cells were cultured to ~70% confluence and then stimulated with serum for 3–6 h by replacing culturing media with fresh media (DMEM supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin G, and 100 μg/ml streptomycin sulfate at 37 °C and 5% (v/v) CO_2 humidified incubator). Where indicated, cells were simultaneously incubated with 0.1% (v/v) DMSO (vehicle), 100 nM rapamycin, or 300 nM Torin1. Cells were then incubated with 100 μg/ml cycloheximide for 5 min prior to lysis. Cells were washed once with ice-cold phosphate-buffered saline (PBS) in the presence of 100 μg/ml cycloheximide and then scraped with a cell scraper in 10 ml of ice-cold PBS containing 100 μg/ml cycloheximide. Cells were centrifuged at 1200 rpm for 5 min at 4 °C and resuspended in 425 μl of hypotonic buffer (5 mM Tris (pH 7.5) at room temperature), 2.5 mM $MgCl_2$, 1.5 mM KCl, EDTA-free protease inhibitors mixture tablets (catalogue no. 04693 132 001, Roche Applied Science), 100 μg/ml cycloheximide, 2 mM DTT, 200 units/ml RNasin, 0.5% (v/v) Triton X-100, and 0.5% (w/v) sodium deoxycholate) and vortexed for 5 s. Lysates were precleared by centrifugation at 21,000 × *g* for 5 min at 4 °C. Supernatant was collected and $A_{260\,nm}$ determined for each sample. Five hundred micrograms of total RNA were typically loaded onto each gradient. Gradients were subjected to ultracentrifugation at 36,000rpm for 2 h at 4 °C on an SW41Ti rotor in a Beckman Coulter (Optima L80 XP) ultracentrifuge. Brake was set at 5. Centrifuged samples were subjected to fractionation (Teledyne ISCO), and fractions were collected at 35-s intervals. $A_{254\,nm}$ was monitored with an UV-visible detector (Brandel). Data were analyzed with TracerDAQ software. Seven hundred and fifty microliters were used for RNA extraction and 250 μl for protein analysis from each fraction.

Real Time Quantitative PCR and Semi-quantitative PCR and Northern Blot for Polysome Profile Samples—Equal volumes of each fraction were used for RNA extraction. RNA from each fraction was resuspended in 20 μl and, where indicated, pooled into subpolysomal and polysomal fractions. Three microliters from each pool (corresponding to ~500 ng to 1 μg of RNA) were used for cDNA synthesis. Reverse transcription reaction was carried out by mixing 1 μl of oligo(dT) single-stranded 18-mer oligonucleotide (catalogue no. SO142, Thermo Scientific™ Fermentas) and 1 μl of dNTPs completed with 13 μl of RNase-free water. Reactions were incubated for 5 min at 65 °C followed by a few minutes on ice, and then we added 4 μl of 5× buffer, 1 μl of dithiothreitol, 1 μl of RiboLock RNase inhibitor (catalogue no. EO0381, Thermo Scientific™ Fermentas), and 1 μl of Superscript III® reverse transcriptase (catalogue no. 18080044, Life Technologies, Inc.). Samples were mixed and incubated at 50 °C for 60 min then at 70 °C for 15 min. Quantitative PCR was performed as per the manufacturer's instruction using the iQ™ SYBR® Green Supermix (catalogue no. 170-882, Bio-Rad). Two-step qPCR was used as follows: denaturation at 95 °C for 15 s and extension at 61 °C for 30 s (RPS6 and RPL32) and 64.5 °C for 30 s (RPS20 and PABP). The following cycle numbers were used for sqPCR: RPS6 (22 cycles), RPS20 (22 cycles), RPL32 (22 cycles), PABP (25 cycles). The following oligonucleotides were used for qPCR and sqPCR analysis: human RPL32_forward (5′-AGCCATCTCCTTCTCGGCAT-3′) and human RPL32_reverse (5′-TCAATGCCTCTGGGTTTCCG-3′); human RPS20_forward (5′-CCAGTTCGAATGCCTACC-AAGACTT-3′) and human RPS20_reverse (5′-ACTTCCACC-TCAACTCCTGGCTCA-3′); human PABP_forward (5′-TAC-GAGCTGTTCCCAACCCT-3′) and human PABP_reverse (5′-TTTGGAATGGATGAGGTCTGGC-3′); and human RPS6_forward (5′-CTGGGTGAAGAATGGAAGGGTT-3′) and human RPS6_reverse (5′-TGCATCCACAATGCAAC-CAC-3′). Northern blotting analyses and specific RNA-probe generation was performed as described previously (51).

mRNA Decay and Northern Blot—mRNA decay assays were performed using either HeLa Flp-in or HeLa Flp-in Myc-LARP1 cell lines. Briefly, cells were plated at ~60% confluency in 6-well plates and cultured for 24 h under standard conditions as described above. Cells were subsequently treated with actinomycin D at a concentration of 5 μg/ml prior to harvest after 0, 3, 6, and 9 h. Cells were lysed directly in the cell culture plate using 1 ml of TRIzol (Life Technologies, Inc.), and RNA was purified according to the manufacturer's protocol. Northern blotting analyses and specific RNA-probe generation were described previously (51).

Statistical Analysis—Appropriate statistical tests were performed within Prism (GraphPad). For comparison between two independent samples, a one-tailed unpaired *t* test was used. For comparisons between more than two independent samples, a one-way analysis of variance was performed with Dunnett's or Bonferroni's post hoc tests, and adjusted *p* values were reported. *p* values are reported as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; *n.s.* denotes nonsignificant *p* values. At least two replicates were performed for each experiment.

Results

LARP1 Interacts with mTORC1 via RAPTOR—Although 4E-BPs and S6Ks play important functions in mRNA translation, other uncharacterized mTORC1 targets in translation likely exist. Recent proteomic studies support this notion (32–37). Numerous proteins are phosphorylated in an mTORC1-sensitive manner (32–37), some of which likely execute important roles in mRNA translation. A persistent challenge of cell

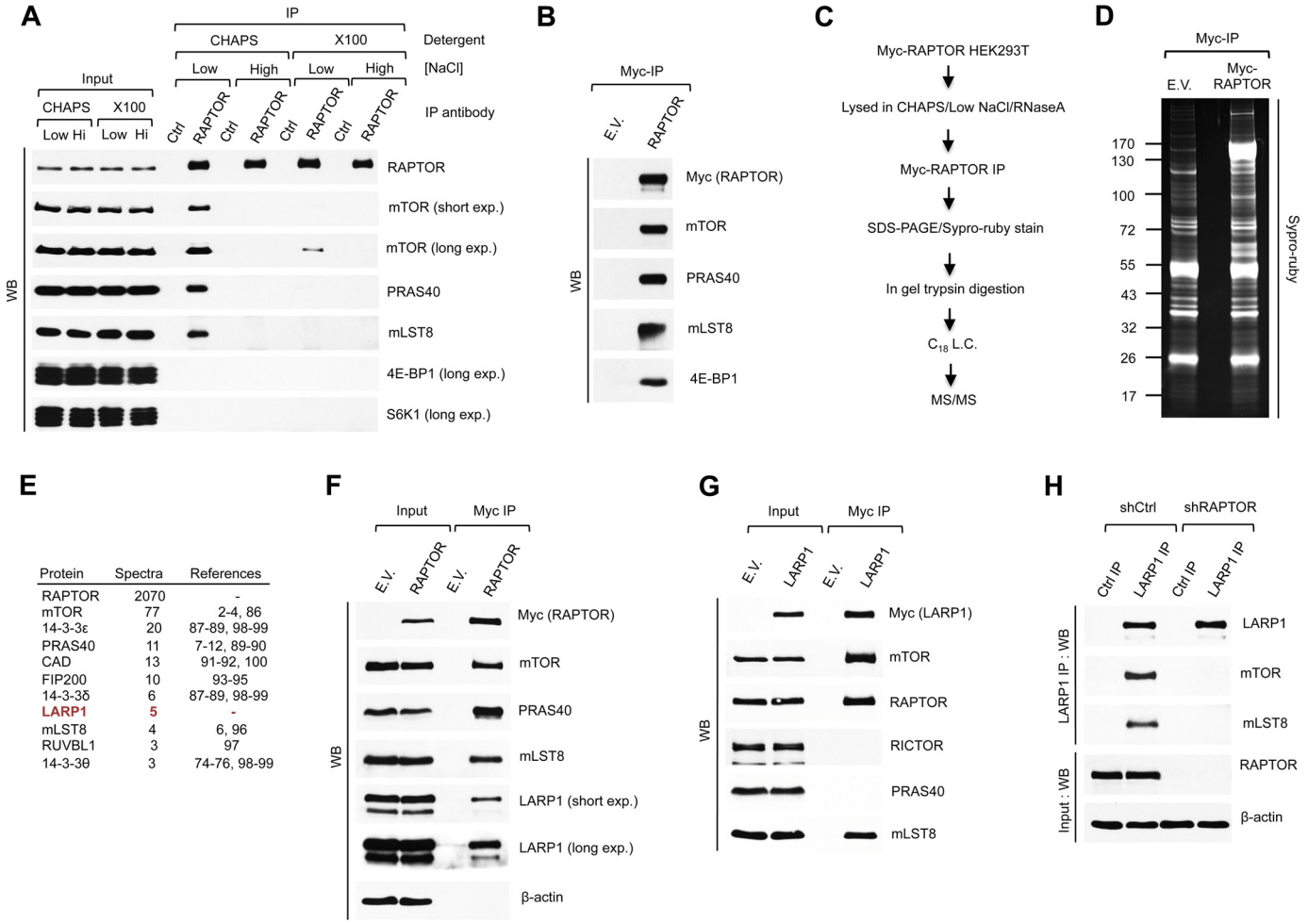


FIGURE 1. **Identification of LARP1 as a novel RAPTOR-binding partner.** *A,* optimization of lysis conditions for preservation of an intact endogenous mTORC1. HEK293T cells were lysed in extraction buffer containing either 0.3% (w/v) CHAPS or 0.3% (v/v) Triton X-100 and low salt (120 mM NaCl) or high salt (500 mM NaCl). See under "Experimental Procedures" for additional buffer components and extraction conditions. Samples of lysates and immunoprecipitates (*IP*) were probed with antibodies against known mTORC1 components and substrates by SDS-PAGE/Western blot (*WB*). *B,* validation of lysis conditions for preservation of an intact exogenous mTORC1 complex. HEK293T cells were transfected with Myc-RAPTOR and lysed in extraction buffer containing 0.3% (w/v) CHAPS and low (120 mM) salt (NaCl). The Myc-RAPTOR immunoprecipitates were probed with the indicated antibodies. *C,* flow chart for affinity purification/mass spectrometry identification of LARP1 as a RAPTOR-binding partner. *D,* SYPRO Ruby-stained SDS-PAGE for RAPTOR-associated proteins and respective spectral count. HEK293T cells were transfected with 5 μg of Myc-tagged human RAPTOR and lysed in 0.3% (w/v) CHAPS, 120 mM NaCl buffer containing 1 μg/ml RNase A (see "Experimental Procedures" for complete buffer composition). 2 mg of lysate were used for immunoprecipitation with anti-Myc antibody. Immunoprecipitates were loaded onto a 4–12% gradient pre-cast SDS-polyacrylamide gel, resolved by electrophoresis, and visualized by SYPRO Ruby staining. *E,* list of previously validated RAPTOR-binding proteins identified in our Myc-RAPTOR immunoprecipitation LC-MS/MS screen and respective references (2–4, 6–13, 86–100). (Refer to Table 1 for complete list of mTORC1 targets identified by mass spectrometry.) *F,* exogenous RAPTOR interacts with endogenous LARP1. HEK293T cells were transfected with Myc-RAPTOR, and samples of lysates were subjected to immunoprecipitation with anti-Myc antibody. Lysates and immunoprecipitates were probed by SDS-PAGE/Western blot with the indicated antibodies. *G,* exogenous LARP1 interacts with endogenous RAPTOR. HEK293T cells were transfected with Myc/FLAG LARP1, and samples of lysates were subjected to immunoprecipitation with anti-Myc and analyzed as described in *F*. *H,* endogenous LARP1 interacts with endogenous mTORC1 via RAPTOR. HEK293T cells were stably depleted of RAPTOR using lentiviral shRNA. Samples of lysates were used for immunoprecipitation of endogenous LARP1. Lysates and immunoprecipitates were analyzed by SDS-PAGE/Western blot with the indicated antibodies.

signaling phospho-proteomic screens is to differentiate between proximal (direct) and distal (indirect) phospho-substrates. A number of the mTORC1-sensitive phospho-substrates reported thus far may be indirect targets of mTORC1.

In an effort to identify proximal novel targets of mTORC1, we have opted for investigating the mTORC1 interactome, using the mTORC1-specific component RAPTOR as bait. First, we tested the optimal conditions for immunoprecipitation of intact mTORC1. Consistent with earlier reports (2), cell lysis with a low salt CHAPS-based extraction buffer successfully preserved the association of integral endogenous mTORC1 components (mTOR, RAPTOR, mLST8, and PRAS40) (Fig. 1*A*). We used these optimized conditions for immunoprecipitation of transiently expressed Myc-tagged RAPTOR and subjected the immunoprecipitated samples to C_{18} liquid chromatography-tandem mass spectrometry (LC-MS/MS), as shown in Fig. 1, *B–D*. One hundred and fifteen unique proteins were identified (Table 1), several of which have already been characterized as *bona fide* mTORC1 targets (Fig. 1*E*); other identified proteins have previously been listed as candidate mTORC1 targets but not verified biochemically. One such protein, LARP1 (previously shown to be phosphorylated in an mTORC1-dependent manner (34, 35, 56)), was selected for further analysis.

TABLE 1
List of putative RAPTOR-binding partners identified by mass spectrometry

Protein	Molecular mass	E.V.	Spectral count, Myc-RAPTOR
	kDa		
MUC19	598	2	4
mTOR	289	0	77
CAD	243	5	13
SNRNP200	245	0	2
CNOT1	241	0	8
EVPL	232	0	2
MYH9	227	0	12
HELZ	219	0	2
RB1CC1	183	0	10
EIF4G1	175	0	2
GPRASP1	157	0	2
Myc-RAPTOR	149	0	2070
PUM1	126	0	8
ESYT1	123	0	2
LARP1	124	0	5
PUM2	114	0	6
C2CD5	110	0	10
TNOP2	102	1	3
PSMD2	100	0	3
KPNB1	97	1	4
ZNF512B	97	0	4
UNK	88	0	3
MAGED1	86	0	8
RFWD3	85	0	2
HSP90AA1	85	37	110
HSP90AB1	83	18	49
HPS6	83	0	2
CCD170	82	0	3
TCF25	77	0	2
PABPC1	71	5	27
PABPC4	71	0	3
HSPA2	70	1	5
XRCC6	70	0	4
CNX	68	1	5
TKT	68	1	3
RARS	67	0	13
SNX9	67	6	20
NOP56	66	2	4
HAS1	65	0	3
GPI	63	0	6
DCP1A	63	0	3
CCT3	61	2	81
TCP1	60	2	84
CCT5	60	0	76
CCT8	60	3	116
CCT7	59	3	62
CCT4	58	0	100
CCT6A	58	2	66
KPNA2	58	1	4
PKM	58	5	10
CCT2	57	1	101
PTBP1	57	2	5
PHGDH	57	3	9
VIM	54	3	6
ZIM3	54	0	2
CAP1	52	0	3
PP2A	52	0	3
DNAJA3	52	0	2
HSPA13	52	0	2
RUVBL1	50	0	3
TUBA1A	50	1	10
TUBA1B	50	61	220
TUBB	50	77	210
TUBB4B	50	1	14
TUBB8	50	0	2
TUFM	50	0	3
DDX39B	49	1	2
RGMA	49	0	4
RPL4	48	3	13
MRPS27	48	0	3
RBBP4	48	0	2
ENO1	47	6	15
PSMC4	47	0	3
PAICS	47	1	3
eIF4A1	46	0	5
DNAJB1	46	0	2
NAP1L1	45	1	3
PA2G4	44	0	3
FIBP	42	0	5
HSPA2	41	0	2
LDHA	40	6	13
PSMD8	40	0	2
PCBP2	39	0	3
PPP1CB	37	0	2
LDHB	37	8	17
ANXA5	36	0	7
MLST8	36	0	4
LRRC59	35	0	2
RACK1	35	2	11
CDK5	33	0	8
PPA1	33	0	4
RPL7a	30	8	16
RPS4X	30	6	12
PSMA1	30	0	3
TPM3	29	0	3
14-3-3ϵ	29	7	20
PGM1	29	1	6
CA2	29	2	5
SRSF1	28	1	4
14-3-3ζ	29	0	6
14-3-3θ	28	0	3
AKT1S1	27	0	11
TPI1	27	1	7
CACYBP	26	0	2
UBE4B	26	3	24
EEF1B2	25	1	8
RAN	24	3	7
HMGB1P1	24	0	2
PSMB3	23	0	2
IGLL5	23	1	4
NACA	23	1	3
GSTP1	23	0	3
PTGES3	19	1	3
RPL31	14	1	5
RPS17	9	0	2

To confirm whether LARP1 associates with mTORC1, lysates from HEK293T cells overexpressing Myc-tagged human RAPTOR were subjected to immunoprecipitation with anti-Myc antibody (Fig. 1*F*) and subsequently probed for LARP1 and other known mTORC1 components (mTOR, PRAS40, and mLST8). LARP1,[6] but not β-actin, efficiently co-immunoprecipitated with mTORC1 (Fig. 1*F*). To further validate the interaction between LARP1 and mTORC1, we performed the converse experiment using Myc-LARP as bait. We found that Myc-LARP1[7] successfully pulled down mTOR, RAPTOR, and mLST8 (Fig. 1*G*), confirming earlier findings in Fig. 1*F*. Interestingly, RICTOR did not interact with Myc-LARP1 indicating that LARP1 specifically associates with mTORC1 but not mTORC2 (Fig. 1*G*). PRAS40 also did not to associate with LARP1 (Fig. 1*G*), consistent with a model in which PRAS40 functions as an mTORC1 substrate competitor (8, 9, 57). We considered it to be important to test whether endogenous LARP1 interacted with endogenous components of the mTORC1 complex. To test this, endogenous LARP1 was immunoprecipitated using an anti-LARP1 antibody. Endogenous LARP1 efficiently co-purified endogenous RAPTOR along with endogenous mTOR and mLST8 (Fig. 1*H*), indicating

[6] Two bands can be detected by the LARP1 antibody (ab86359) raised against residues 275–325, likely corresponding to full-length LARP1 (1096 amino acids) and a shorter LARP1 isoform (1019 amino acids). Both isoforms associate with mTORC1.

[7] The Myc-LARP1 construct employed here encodes for the short (1019 amino acids) LARP1 isoform.

that this interaction is also conserved between the endogenous proteins. Importantly, shRNA-mediated knockdown of RAPTOR abolished the interaction of LARP1 with both mTOR and mLST8 (Fig. 1*H*), indicating that RAPTOR bridges the interaction between LARP1 and mTOR/mLST8. To further validate the interaction between LARP1 and mTORC1, we used two commercially available anti-RAPTOR antibodies to immunoprecipitate endogenous RAPTOR and then probed for endogenous LARP1. The RAPTOR antibody (09-217) successfully immunoprecipitated endogenous RAPTOR and the mTORC1 components (mTOR, mLST8, and PRAS40) but failed to co-immunoprecipitate LARP1 (Fig. 2, *A* and *B, lane 3*). This was also the case for the commercial RAPTOR (05-140) antibody (Fig. 2*B*, *lane 4*). One possibility is that the binding site for the RAPTOR commercial antibodies overlapped with the LARP1 interaction site on RAPTOR. To circumvent this possibility, we generated anti-RAPTOR rabbit sera and tested each bleed for its ability to co-immunoprecipitate RAPTOR with LARP1. Bleeds 91.4, 91.5, and 91.7 efficiently co-immunoprecipitated RAPTOR with LARP1 (Fig. 2*B*, *lanes 16–18*), further supporting the notion that endogenous RAPTOR specifically interacts with endogenous LARP1. In addition to binding RAPTOR, LARP1 has previously been reported to associate directly with PABP (58, 59). Immunoprecipitation of endogenous LARP1 co-immunoprecipitated PABP in the presence of RNase A (Fig. 2*A*), confirming earlier findings that LARP1 interacts directly with PABP (58, 59). We also observed that GFP-LARP1 co-localizes with endogenous RAPTOR and PABP to subcellular punctate structures resembling stress granules (Fig. 2*C*) (60–62), further supporting the interaction of LARP1 with these proteins.

LARP1 Binds RAPTOR via the RAPTOR N-terminal Conserved (RNC) Domains and the WD40 Repeats—How does LARP1 associate with RAPTOR and PABP? To investigate the domains in RAPTOR to which LARP1 binds, we expressed fragments of RAPTOR encompassing the following: the RNC caspase-like domain, spanning amino acids 1–526 (63); the mid-domain comprising three HEAT (Huntingtin, EF3, PR65/A, mTOR) repeats, spanning residues 526–697; and seven C-terminal WD40 repeats (Trp/Asp repeats 40 amino acids long), spanning amino acids 904–1335 (Fig. 3*A*). Myc/FLAG-LARP1 was transiently co-expressed with full-length Myc-tagged RAPTOR or fragments 1–904, 904–1335, or 526–904 in HEK293T cells and lysates subjected to immunoprecipitation with FLAG-specific antibody (Fig. 3*B*). All RAPTOR fragments expressed to similar levels, but LARP1 preferentially associated with the WD40 C-terminal domain (904–1335 fragment) in FLAG-LARP1 immunoprecipitates (Fig. 3*B*). Of note, fragment 1–904, but not fragment 526–904, also contributed to LARP1 binding indicating that the RNC domains but not the HEAT repeats also play a role in the association with LARP1 (Fig. 3*B*).

Which domain(s) in LARP1 contribute to RAPTOR binding? Direct mTORC1 substrates typically possess a TOR signaling (TOS) motif, with Gbr10 being a noteworthy exception (34, 35), through which RAPTOR binds each mTORC1 substrate (64–66). The TOS motif is a 5-amino acid stretch invariably defined by a phenylalanine at position 1 and a combination of hydrophobic, charged, and/or polar uncharged amino acids between positions 2 and 5. The consensus for the TOS motif has previously been established as F(DEVMQPRA)(IMLE)(DVEL)(ELIQDA) (67). Careful examination of the LARP1 amino acid sequence revealed a potential TOS motif (Fig. 3*C*). To investigate whether LARP1 associates with RAPTOR via its predicted TOS motif, we generated LARP1 mutants lacking amino acids 889–893 (Fig. 3*C*). Deletion of the putative TOS motif impaired the binding of LARP1 to RAPTOR (Fig. 3*D*). The phenylalanine residue within the putative TOS motif has previously been shown to be critically important for the binding of 4E-BPs, S6Ks, and PRAS40 to RAPTOR. Single amino acid substitution of this residue in 4E-BPs and S6Ks abolishes RAPTOR binding (65, 66). Similarly, mutation of Phe-129 to alanine within PRAS40's TOS motif markedly reduces RAPTOR binding (8, 9). However, mutation of the corresponding Phe-889 to an alanine in LARP1 did not affect its association with RAPTOR (Fig. 3*E*), arguing against the FRLDI functioning as a *bona fide* TOS motif.

LARP1 Interacts with PABP via a PAM2-like Motif—In addition to binding RAPTOR, we have shown earlier that LARP1 also directly binds PABP (Fig. 2). PABP interacting proteins (*e.g.* PABP-interacting protein 1 and 2 (PAIP1 and PAIP2) and eukaryotic release factor 3 (eRF3)) associate with PABP via classical PAM2 motifs (68). LARP4, another member of the LARP family of proteins, has been previously shown to associate with PABP via a noncanonical PAM2 motif that comprises a tryptophan in place of the critical phenylalanine typically found in classical PAM2 motifs (69, 70). Thus, we investigated whether LARP1 also possessed a PAM2 motif. Close inspection of the LARP1 amino acid sequence revealed a PAM2-like motif within the La motif (Fig. 3*C*). Deletion of the 11 amino acids comprising the putative PAM2 motif in LARP1 reduced PABP binding (this was done in the presence of RNase A to minimize RNA-dependent protein-protein interactions) (Fig. 3*D*). Similarly, substitution of the critical phenylalanine for an alanine achieved a comparable reduction in PABP binding (Fig. 3*E*). Taken together, these data suggest that the PAM2-like motif in LARP1 contributes to PABP binding.

mTORC1 Activity Regulates the Association of LARP1 with RAPTOR but Not with PABP—Having established that LARP1 binds RAPTOR and PABP and defined the various domains involved in these interactions, we investigated whether the interaction between LARP1 and RAPTOR or PABP is subject to the control of mTORC1. To address this question, we treated HEK293T cells with rapamycin or Torin1. Acute (30 min) rapamycin or Torin1 treatment markedly decreased LARP1 association with both RAPTOR and mTOR (Fig. 4*A*). It did not, however, affect the interaction between LARP1 and PABP. We then tested whether longer rapamycin and Torin1 exposure affected the LARP1-PABP association. HEK293T cells were stimulated with serum in the presence of DMSO (vehicle), rapamycin, or Torin1 for the indicated times (Fig. 4*B*). Rapamycin and Torin1 efficiently reduced mTORC1 and/or mTORC2 activities, as noted by the decrease in phosphorylation of 4E-BP1, S6K1, and AKT and an increase in phosphorylation of eEF2. Rapamycin and Torin1 also impaired the interaction of LARP1 with RAPTOR and mTOR as described in Fig. 4*A*. These drugs did not, however, affect LARP1 interaction with

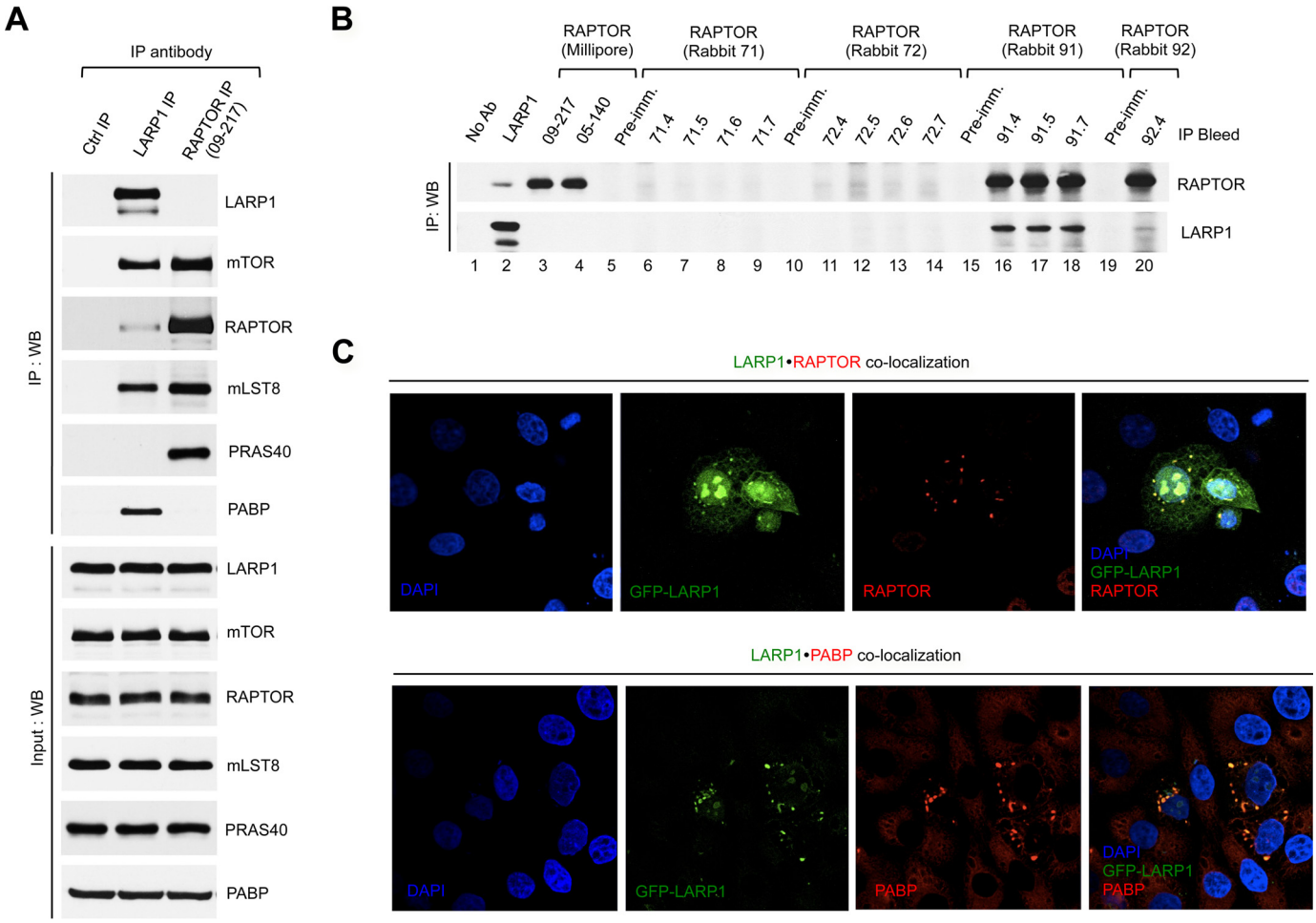


FIGURE 2. **LARP1 interacts and co-localizes with RAPTOR and PABP.** *A,* lysate from HEK293T cells was used for immunoprecipitation (*IP*) of endogenous LARP1 and endogenous RAPTOR with commercial antibodies. LARP1 and RAPTOR immunoprecipitates were probed for LARP1, PABP, RAPTOR, and various mTORC1 components by SDS-PAGE/Western blot (*WB*). *B,* endogenous LARP1 co-immunoprecipitates with endogenous RAPTOR, using homemade RAPTOR antisera. HEK293T cell lysate was used for immunoprecipitation of endogenous RAPTOR using in-house anti-RAPTOR bleeds. Immunoprecipitates were resolved by SDS-PAGE/Western blot and probed with anti-LARP1 and anti-RAPTOR antibodies. *C,* GFP-LARP1 co-localizes with endogenous RAPTOR and PABP to stress granule-like structures.

PABP at any of the time points tested (Fig. 4*B*), confirming that, in contrast to the LARP1/RAPTOR interaction, the association between LARP1 and PABP is not regulated by mTORC1.

mTORC1 signaling is also subject to control by nutrients such as amino acids and growth factors (71, 72). Thus, we next tested whether amino acids and/or dialyzed serum also regulated the association of LARP1 with mTORC1. Selective deprivation of amino acids and/or dialyzed serum in HEK293T cells reduced the association of LARP1 with mTORC1 components RAPTOR and mTOR (Fig. 4*C*), consistent with the earlier findings for rapamycin and Torin1 (Fig. 4, *A* and *B*). Moreover, nutrient deprivation enhanced the binding of LARP1 to PABP. This is in contrast to the lack of an effect of rapamycin and Torin1 on the LARP1-PABP interaction and likely reflects pleiotropic effects of nutrient deprivation on LARP1 via mTORC1-independent pathways.

RAPTOR Competes with RNA for LARP1 Binding—LARP1 has recently been reported to associate with mRNA via the terminal 3′OH group of their poly(A)-tail (52), which prompted us to investigate whether mRNA modulates RAPTOR binding to LARP1. All the experiments described thus far were performed in the presence of RNase A to exclude mRNA-mediated protein-protein interactions. Interestingly, omission of RNase A reduced LARP1/RAPTOR association (Fig. 4*D*), suggesting that the presence of mRNA hinders the binding of LARP1 to RAPTOR and, consequently, that mRNA and RAPTOR compete for LARP1 binding.

LARP1 Interacts with TOP mRNAs in an mTORC1-regulated Manner—A study by Aoki *et al.* (52) first proposed that LARP1 binds TOP mRNAs. Given the prominent role of mTORC1 in the control of TOP mRNA translation, we explored further the possibility that LARP1 associated with TOP mRNAs. To test this, we performed immunoprecipitation of endogenous LARP1 protein and monitored the association of TOP mRNAs with LARP1 by RT-qPCR. Our data (Fig. 5*A*) demonstrate that LARP1 associates with TOP mRNAs encoding ribosomal proteins (RPS6, RPS20, and RPL32) and translation factors (PABP). Moreover, the association of LARP1 with TOP mRNAs was markedly enhanced upon mTORC1 inhibition with both rapamycin and Torin1 (Fig. 5*A*). In contrast, mTORC1 inhibition resulted in reduced association of eIF4G with all TOP mRNAs tested, consistent with the ability of mTORC1 inhibitors to dis-

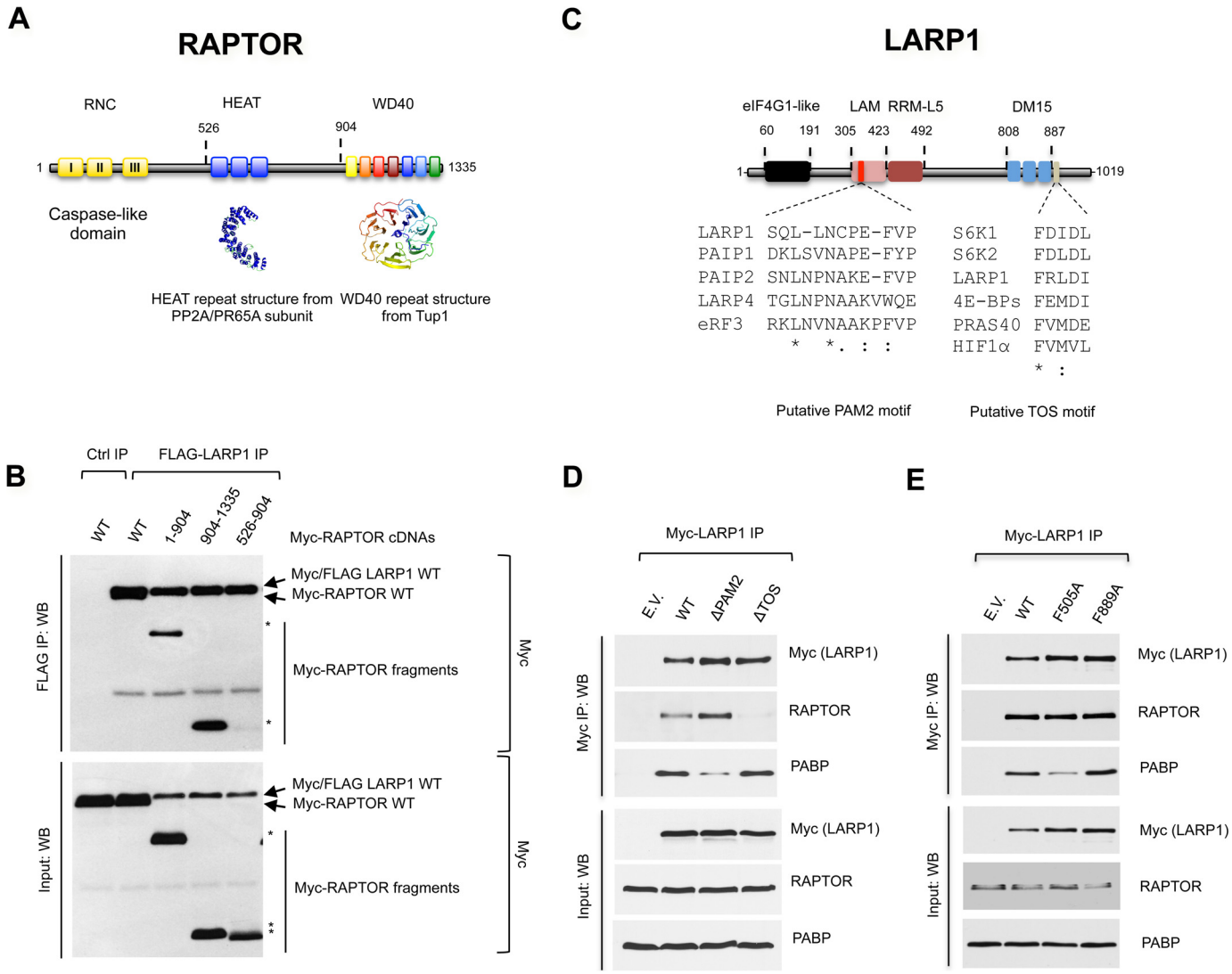


FIGURE 3. **LARP1 binds to RAPTOR via WD40 repeats and the RNC domains and to PABP via a PAM2 motif.** *A,* schematic illustration of various RAPTOR domains. *B,* HEK293T cells were transfected with 2 μg of Myc-RAPTOR WT, 1.5 μg of Myc-RAPTOR(1–904), 4 μg of Myc-RAPTOR(526–904), 4 μg of Myc-RAPTOR(526–1335), and 6 μg of Myc/FLAG LARP1 WT. Twenty four hours following transfection, cells were stimulated with 10% (v/v) fetal bovine serum for 3 h and then lysed in CHAPS buffer in the presence of 1 μg/ml RNase A, and lysates were subjected to immunoprecipitation (*IP*) with FLAG antibody. Immunoprecipitates and inputs were analyzed by SDS-PAGE/Western blot (*WB*) with anti-Myc antibody. *Asterisks* denote Myc-RAPTOR fragments. *C,* schematic illustration of various LARP1 domains and the predicted PAM2-like and TOS-like motifs. LARP1 contains an N-terminal eIF4G-like motif spanning residues 60–191 (numbering refers to human sequence) as predicted by BLASTp search. It possesses an RG repeat region of unknown function and an La motif (*LAM*) spanning residues 305–429 (human sequence numbering). The La motif encompasses a PAM2-like motif that possesses the residues required for PABP binding. At the C-terminal region, LARP1 has a domain of unknown function (DM15) spanning residues 808–887. *D* and *E,* PAM2 motif in LARP1 is required for its interaction with PABP. HEK293T cells overexpressing wild type or the indicated mutants of LARP1 were stimulated with 10% (v/v) fetal bovine serum and subsequently lysed in low salt CHAPS buffer in the presence of 1 μg/ml RNase A, as described in *B*. LARP1 immunoprecipitates and lysates were analyzed by SDS-PAGE/Western blot using the antibodies indicated.

sociate the eIF4F complex (Fig. 5*A*). Control Western blots were performed to monitor mTORC1 inactivation in response to drug treatment in these samples (Fig. 5*B*). We considered it important to validate our RT-qPCR findings by Northern blot and found that FLAG-LARP1 associated with endogenous RPL29 and PABP mRNA but not 28S or 18S rRNA (Fig. 5*C*). LARP1 associated with RPL29 and PABP mRNAs under conditions of serum deprivation or rapamycin treatment and was released upon serum stimulation (Fig. 5*C*). Taken together, these results demonstrate that LARP1 associates with TOP mRNAs, and this interaction is enhanced upon pharmacological inhibition of mTORC1, whereas serum stimulation triggers the release of LARP1.

LARP1 Co-sediments with Polysomes and Suppresses the Translation of TOP mRNAs—The observation that LARP1 associates with the translation factor PABP and TOP mRNAs (Figs. 2–5) (58, 59) prompted us to investigate a possible role for LARP1 in the control of TOP mRNA translation. Western blot analysis of LARP1 protein distribution in polysome profile fractions revealed that LARP1 co-sediments with PABP throughout the polysome gradient (Fig. 6, *A* and *B*), consistent with the earlier findings that LARP1 binds PABP (Figs. 2–4). Treatment of cells with Torin1 or EDTA (both of which cause polysome dissociation to different extents (43, 73)) shifted LARP1 and PABP from heavy to light polysomal fractions (Fig. 6, *A* and *B*). This indicates that LARP1 and PABP co-sediment with poly-

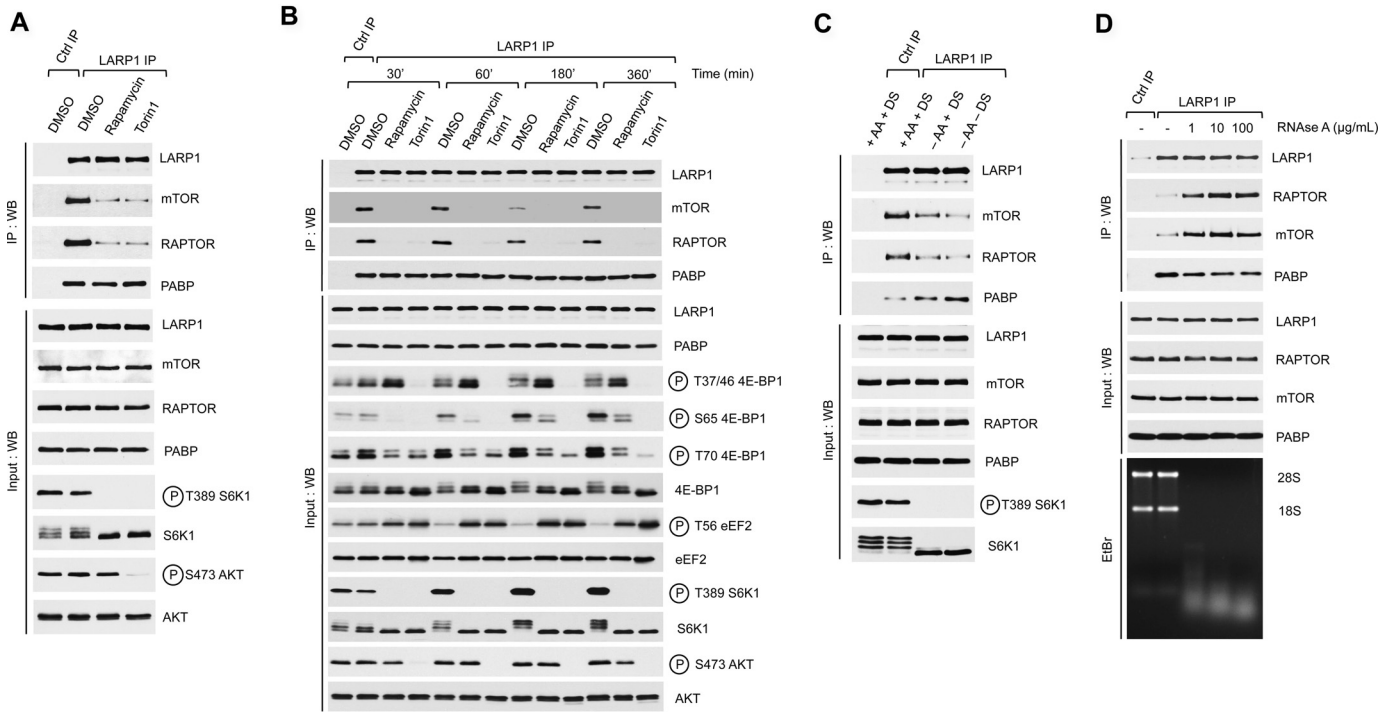


FIGURE 4. **LARP1 association with mTORC1 is regulated by mTOR inhibitors and mRNA.** *A,* rapamycin and Torin1 reduce binding of endogenous LARP1 to endogenous RAPTOR and endogenous mTOR. HEK293T cells were incubated with 10% (v/v) fetal bovine serum for 30 min in the presence of 0.1% (v/v) DMSO, 100 nM rapamycin, or 300 nM Torin1 and subsequently lysed as described under "Experimental Procedures." Samples of lysates were used for immunoprecipitation (*IP*) with anti-LARP1 antibody and then probed with the indicated antibodies by SDS-PAGE/Western blot (*WB*). *B,* interaction between endogenous LARP1 and endogenous PABP is insensitive to mTORC1 inhibition (even after prolonged incubation with rapamycin or Torin1). HEK293T cells were stimulated with 10% (v/v) fetal bovine serum in the presence of 0.1% (v/v) DMSO (vehicle), 100 nM rapamycin, or 300 nM Torin1 for the times indicated and subsequently lysed in extraction buffer containing 0.3% (w/v) CHAPS, 120 mM NaCl, and 1 μg/ml RNase A. Lysates were subjected to immunoprecipitation with LARP1 antibody. Inputs and immunoprecipitates were analyzed by SDS-PAGE/Western blot with the indicated antibodies. *C,* amino acid deprivation reduces binding of endogenous LARP1 to endogenous RAPTOR and endogenous mTOR. HEK293T cells were starved of amino acids and/or dialyzed serum for 1 h in DMEM without amino acids in the presence or absence of 10% (v/v) dialyzed fetal bovine serum. *D,* mRNA impairs the interaction between endogenous LARP1 and mTORC1 but not between endogenous LARP1 and PABP. HEK293T cells were stimulated with 10% (v/v) fetal bovine serum and subsequently lysed in the absence or presence of various concentrations of RNase A. Lysates were subjected to immunoprecipitation with anti-LARP1 antibody. Lysates and immunoprecipitates were analyzed by SDS-PAGE/Western blot with the indicated antibodies. RNA degradation was monitored by agarose gel electrophoresis.

somal mRNAs that are actively engaged in translation. In contrast, mTOR and RAPTOR are only found in mRNPs and 40S fractions, suggesting that these proteins play a more distal role in translation regulation (Fig. 6, *A* and *B*).

Having observed that LARP1 co-sediments with polysomal mRNAs, we asked whether LARP1 plays an active role in mRNA translation. LARP1 has been recently linked to TOP mRNA gene expression (52). Given the prominent role of mTOR in the control of TOP mRNA translation, we investigated whether LARP1 is implicated in the control of this cellular process. To address this question, we stably depleted HEK293T cells of LARP1 using four distinct shRNAs against the coding sequence of LARP1 mRNA (designated shLARP1_1 to 1_4), thus controlling for potential off-target effects of an individual shRNA (Fig. 7*A*). Western blot analysis indicated that shLARP1_1 had little or no effect on LARP1 protein levels. shLARP1_2 and shLARP1_4 displayed a moderate knockdown effect, whereas shLARP1_3 strongly inhibited LARP1 protein expression (Fig. 7*A*). Silencing LARP1 with these shRNAs had only minor effects on polysome distribution suggesting that LARP1 likely regulates the translation of specific mRNAs in the cell, rather than global protein synthesis (Fig. 7*B*). More importantly, silencing LARP1 led to a significant accumulation of TOP mRNAs (RPS6, RPS20, RPL32, and PABP) in heavier polysomal fractions as monitored by RT-qPCR (Fig. 7*D*), indicating that LARP1 functions to repress TOP mRNA translation. The *left panels* in Fig. 7*D* show the percent of TOP mRNA level distributions between subpolysomal and polysomal fractions, and the *right panels* display the translation efficiency (*i.e.* the ratio of polysomal to subpolysomal mRNA abundance) of shRNA LARP1 knockdown cells relative to shRNA control cells (asterisks denote degree of significance (see supplemental Workbook for details)). Interestingly, we have noted that depletion of LARP1 with either shLARP1_2 or shLARP1_3 resulted in reduced steady-state TOP mRNA levels (Fig. 7*C*), consistent with a potential role for LARP1 in stabilizing TOP mRNAs as previously suggested by Aoki *et al.* (52).

mTORC1 Controls TOP mRNA Translation via LARP1—The data presented thus far indicate that LARP1 is a target of mTORC1 and that it plays a role in the repression of TOP mRNA translation. This raised the following intriguing question. Does mTORC1 regulate TOP mRNA translation via LARP1? To test this, HEK293T cells stably knocked down for LARP1 were incubated with rapamycin or Torin1, and TOP mRNA translation was analyzed by polysome profiling/RT-sqPCR of individual fractions (Fig. 8, *A* and *B*). As observed earlier (Fig. 7), knocking down LARP1 caused the re-distribution of TOP mRNAs from light subpolysomal to heavy poly-

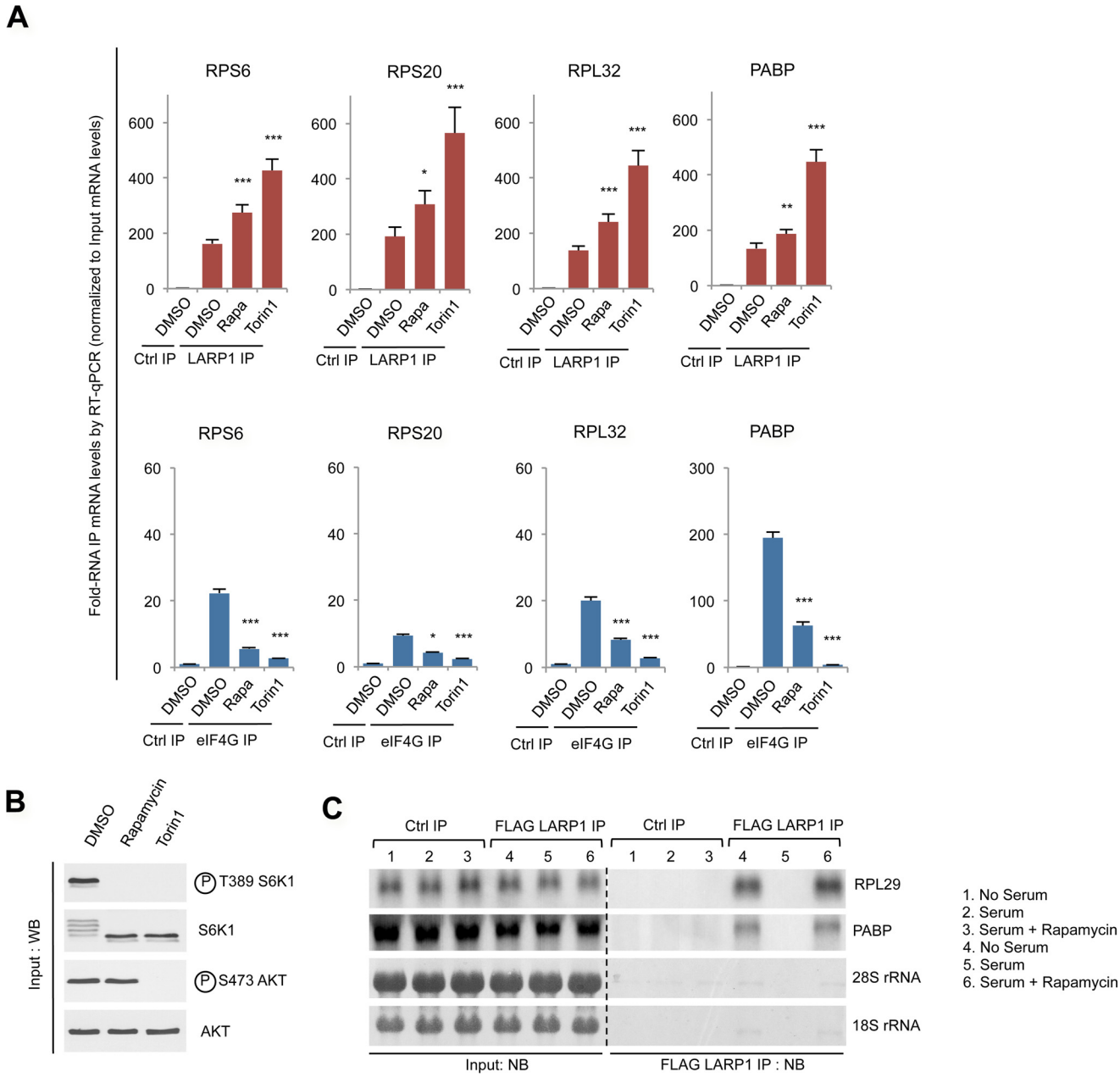


FIGURE 5. **LARP1 associates with TOP mRNAs, and this interaction is enhanced upon mTORC1 inactivation.** *A*, HEK293T cells were incubated with 100 nM rapamycin, 300 nM Torin1 or vehicle (DMSO) for 3 h in the presence of 10% (v/v) fetal bovine serum and lysed, and samples of lysates were subjected to RNA-immunoprecipitation (*RIP*) with anti-LARP1 or anti-eIF4G1 antibody conjugated to protein G-coated magnetic beads. RNA was extracted from immunoprecipitates, and cDNA was generated using oligo(dT) priming. TOP mRNA abundance for each immunoprecipitate was determined by RT-qPCR. Statistical significance was determined as detailed under the "Experimental Procedures" and the supplemental Workbook. *B*, samples of lysates were analyzed for mTORC1 and mTORC2 activation by SDS-PAGE/Western blot with the indicated antibodies. *C*, exogenous LARP1 binds RPL29 and PABP mRNAs in an mTORC1-dependent manner. HeLa cells stably overexpressing FLAG-LARP1 were starved for serum overnight, incubated with 100 nM rapamycin (where indicated), and subsequently stimulated with 10% (v/v) fetal bovine serum. Lysates were subjected to RNA immunoprecipitation and probed by Northern blot as detailed under "Experimental Procedures."

somal fractions in growing (DMSO) conditions (Fig. 8*B*). Rapamycin and (to a larger extent) Torin1 reduced translation of RPS6, RPS20, and RPL32 mRNAs (Fig. 8*B*, *shCtrl panels*). Notably, the ability of these drugs to reduce TOP mRNA translation was compromised upon knockdown of LARP1; the shift of ribosomal protein mRNAs to subpolysomal fractions was less pronounced in LARP1 knockdown cells (shLARP1_2) than in the control knockdown counterpart (shCtrl) (Fig. 8*B*). Together these data indicate that mTORC1 inhibitors repress TOP mRNA translation specifically via LARP1.

To confirm the effect of LARP1 on TOP mRNA translation in a quantitative manner, we performed RT-qPCR analysis on pooled subpolysomal and polysomal fractions. LARP1 knockdown enhanced translation of RPS6, RPS20, and RPL32 under basal conditions (Fig. 8*C*, *DMSO*). Notably, LARP1 knockdown rendered TOP mRNA translation resistant to the inhibitory effects of rapamycin and Torin1 (Fig. 8*C*), corroborating our sqPCR data (Fig. 8*B*). The *left panels* in Fig. 8*C* display the percent levels of TOP mRNA distribution between subpolysomal and polysomal fractions, and the *right panels* show translation

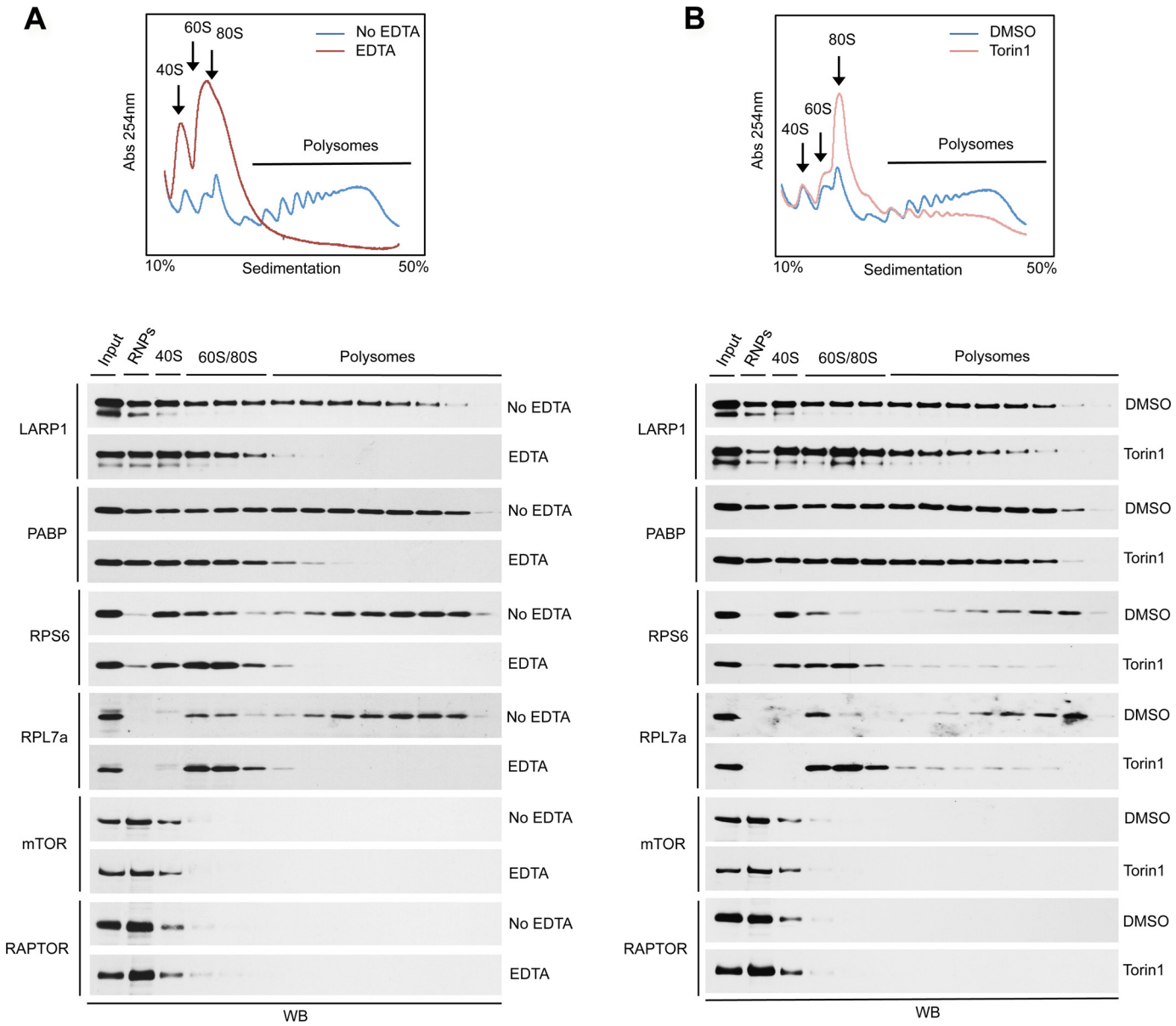


FIGURE 6. **LARP1 and PABP, but not mTORC1, co-sediment with polyribosomes.** *A,* polysome profile traces of HEK293T cells lysed in the presence or absence of EDTA. HEK293T cells were stimulated with 10% (v/v) serum for 6 h and subsequently lysed in hypotonic buffer containing or lacking EDTA. Lysates were fractionated by sucrose density gradient ultracentrifugation as detailed under the "Experimental Procedures." Polysome profile fractions were subjected to SDS-PAGE/Western blot (*WB*) analysis with antibodies against LARP1, PABP, mTORC1 components, and ribosomal protein markers. *B,* polysome profile traces of HEK293T cells in the presence or absence of DMSO/Torin1. HEK293T cells were stimulated with 10% (v/v) serum for 6 h in the presence or absence of 300 nM Torin1. Lysates were prepared and analyzed as described in *A*.

efficiency (*i.e.* the ratio of polysomal to subpolysomal TOP mRNA levels normalized to DMSO-treated cells). Asterisks denote a degree of significance (refer to supplemental Workbook for detailed statistical analyses).

In addition to monitoring the translation of ribosomal protein TOP mRNAs, we tested the effect of LARP1 depletion on the ability of rapamycin and Torin1 to repress PABP TOP mRNA translation. Interestingly, although LARP1 depletion enhanced the translation of PABP mRNA, it remained fully sensitive to rapamycin and Torin1, suggesting that mTORC1 can also control PABP translation via the LARP1-independent mechanism. Collectively, these data show that LARP1 functions as a repressor of ribosomal protein mRNA translation downstream of mTORC1.

Next, we tested whether LARP1 controls TOP mRNA translation in response to nutrient (amino acid) availability. HEK293T cells stably transduced with lentiviruses encoding shRNA against LARP1 (shLARP1_2) or shRNA control were subjected to amino acid starvation and subjected to polysome profiling followed by RT-qPCR analysis. Amino acid depletion reduced the amount of total mRNA associated with the polysome fractions in shCtrl and shLARP1_2 cells (albeit to a lesser extent in the latter case) (Fig. 9*A* and knockdown validated in *B*). Notably, the polysomal association of mRNAs encoding ribosomal proteins (RPS6, RPS20, and RPL32) and PABP was significantly higher in shLARP1_2 knockdown cells compared with shRNA control cells (Fig. 9*C*, translation efficiency of shRNA LARP1 knockdown cells is shown as a function of trans-

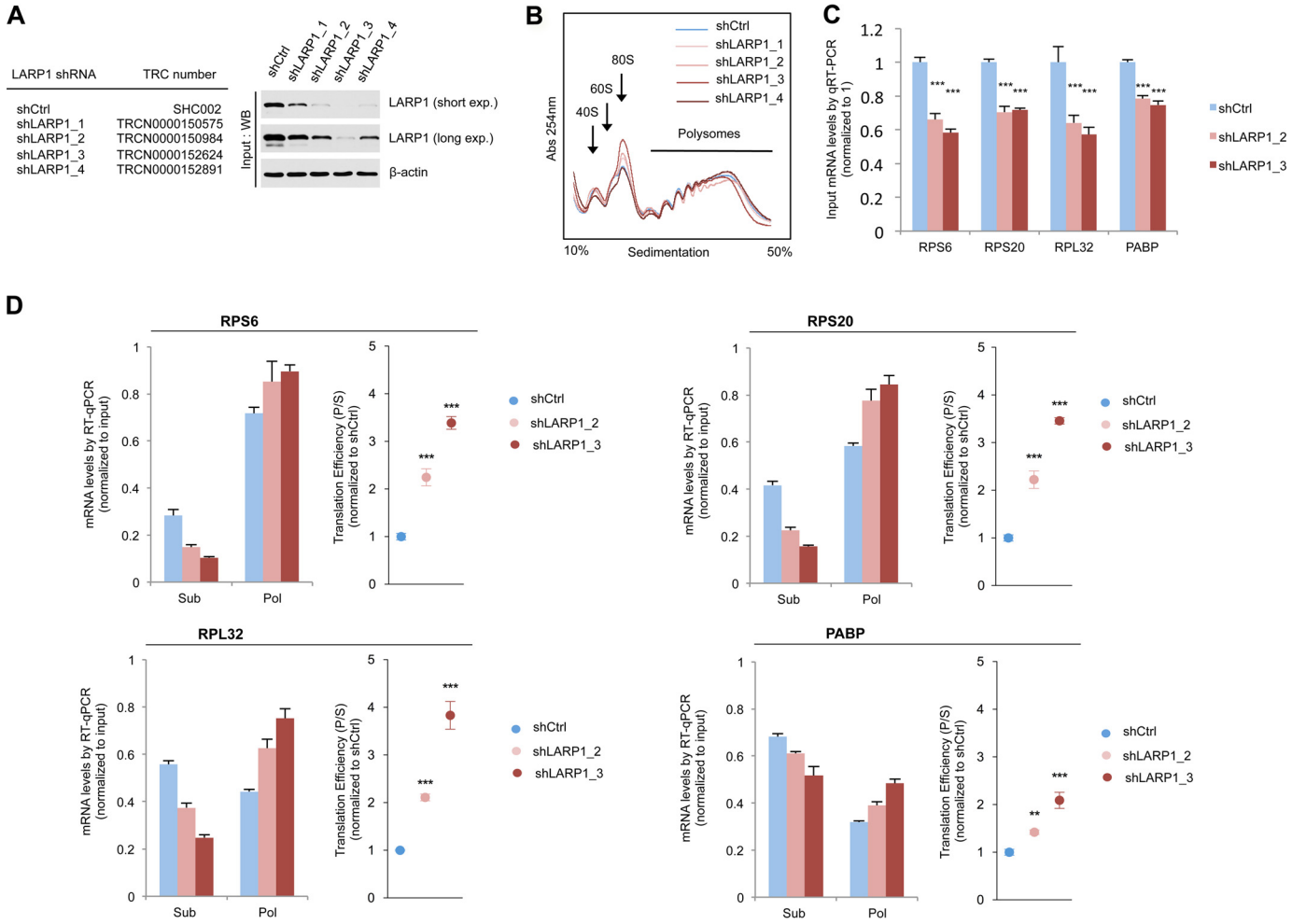
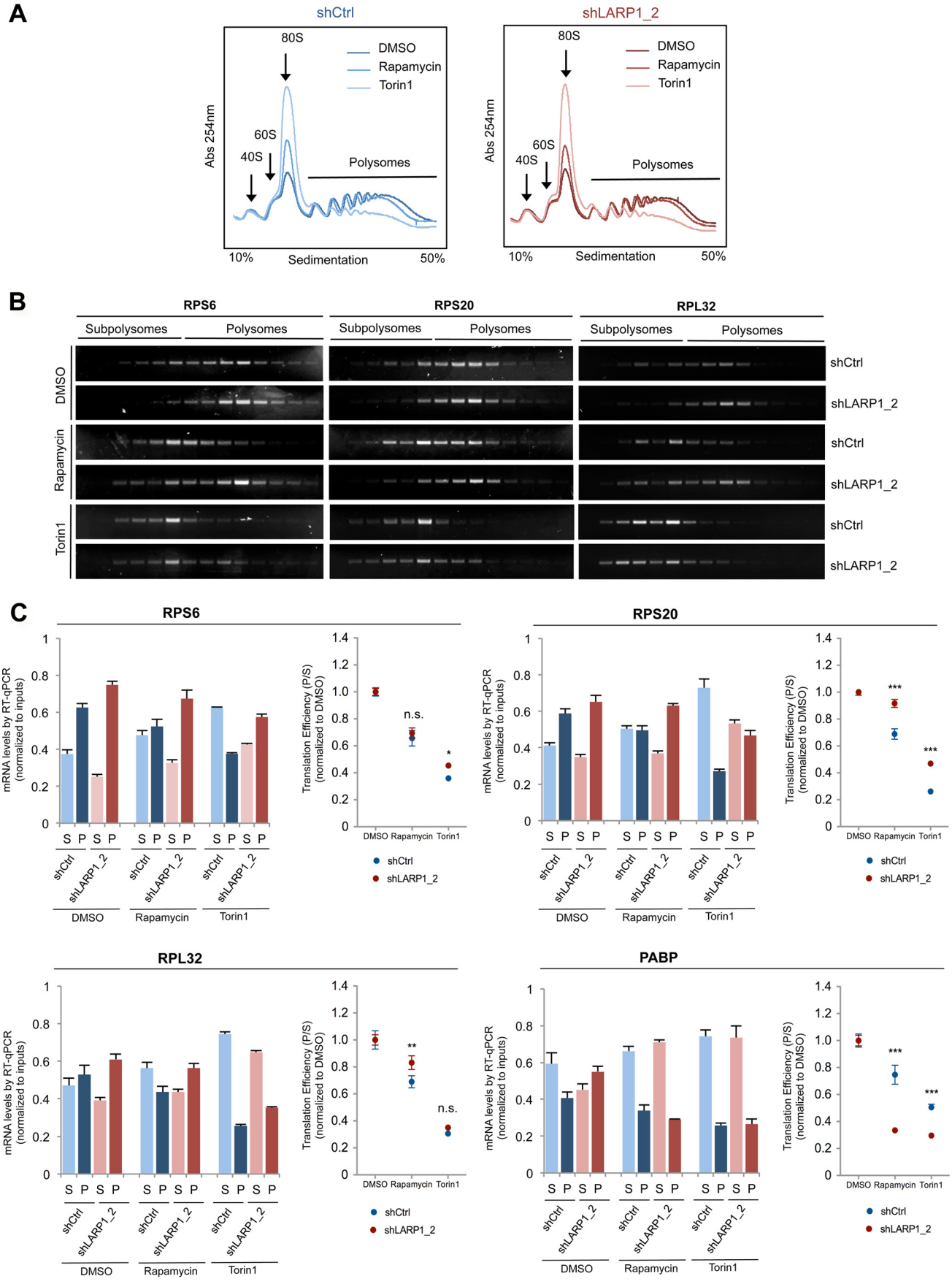


FIGURE 7. **Endogenous LARP1 represses TOP mRNA translation under basal conditions.** *A,* HEK293T cells were stably depleted of LARP1 by transduction of lentiviral particles encoding various shRNAs against the listed human LARP1. Lysates were analyzed by SDS-PAGE/Western blot (*WB*) for knockdown efficiency. *B,* stably knocked down LARP1 cells were propagated to 70% confluence and subsequently stimulated with 10% (v/v) fetal bovine serum for 6 h, following which they were lysed in hypotonic buffer and lysates fractionated by sucrose density gradient ultracentrifugation. Polysome profile traces for shCtrl, shLARP1_1, shLARP1_2, shLARP1_3, and shLARP1_4 are shown. *C,* RNA was extracted from lysates, and RPS6, RPS20, RPL32, and PABP mRNA levels were quantitated by RT-qPCR. Statistical analyses were performed as described under "Experimental Procedures" (see also supplemental Workbook for details). *D,* subpolysomal (*Sub*) and polysomal (*Pol*) fractions were pooled and subsequently analyzed for RPS6, RPS20, RPL32, and PABP mRNA abundance by RT-qPCR. Statistical analyses were performed as described under "Experimental Procedures" (see also supplemental Workbook for details).

lation in shRNA control cells). These findings are consistent with our earlier conclusion that LARP1 functions as a repressor of TOP mRNA translation downstream of mTORC1.

Transient Overexpression of LARP1 Represses TOP mRNA Translation—Because depletion of LARP1 enhances translation of TOP mRNAs, we asked the following question. Does overexpression of LARP1 conversely suppress TOP mRNA translation? HEK293T cells were transiently transfected with a plasmid encoding FLAG-tagged LARP1. Although mild overexpression of LARP1 did not alter general polysome distribution (Fig. 10, *A* and *B*), it did reduce the association of TOP mRNAs with heavier polysomes (Fig. 10*D*). Translation efficiency in LARP1-overexpressing cells is shown as a function of translation efficiency in empty vector cells, consistent with our earlier findings that LARP1 knockdown enhances TOP mRNA association with heavier polysomes (Figs. 8 and 9). Interestingly, overexpression of LARP1 appeared to stabilize steady-state total mRNA levels (Fig. 10*C*), in line with earlier findings (Fig. 7*C*).

LARP1 Represses TOP mRNA Translation via Binding of the 5′TOP Motif—What confers the specific binding of LARP1 to TOP mRNAs? One possibility is that LARP1 directly binds the 5′TOP motif on TOP mRNAs. To test this hypothesis, we examined the ability of recombinant purified LARP1 protein to bind synthetic RNA oligonucleotides spanning the complete 5′UTRs of RPL32 and RPS6 by EMSA, as depicted in Fig. 11*A*. RNAs lacking the 5′TOP motif were used as negative controls (Fig. 11*A*). As predicted, LARP1 physically associated with both the 5′UTRs of RPL32 and RPS6 (Fig. 11*B*). Moreover, deletion of the 5′TOP motif reduced the binding of LARP1 to RPL32 and RPS6 mRNA leaders, indicating that LARP1 specifically interacts with the 5′TOP motif at the very 5′end of the mRNA. The first pyrimidine (invariably a C) within the 5′TOP motif is known to play a key role in the translational repression of TOP mRNAs (74). Interestingly, single nucleotide substitution of the critical 5′-C for a G within the 5′TOP motif of RPL32 sufficed to significantly reduce LARP1-RNA association (Fig. 11*C*).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shCtrl
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40S
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Sedimentation
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B
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RPS20
RPL32
Subpolysomes
Polysomes
DMSO
Rapamycin
Torin1
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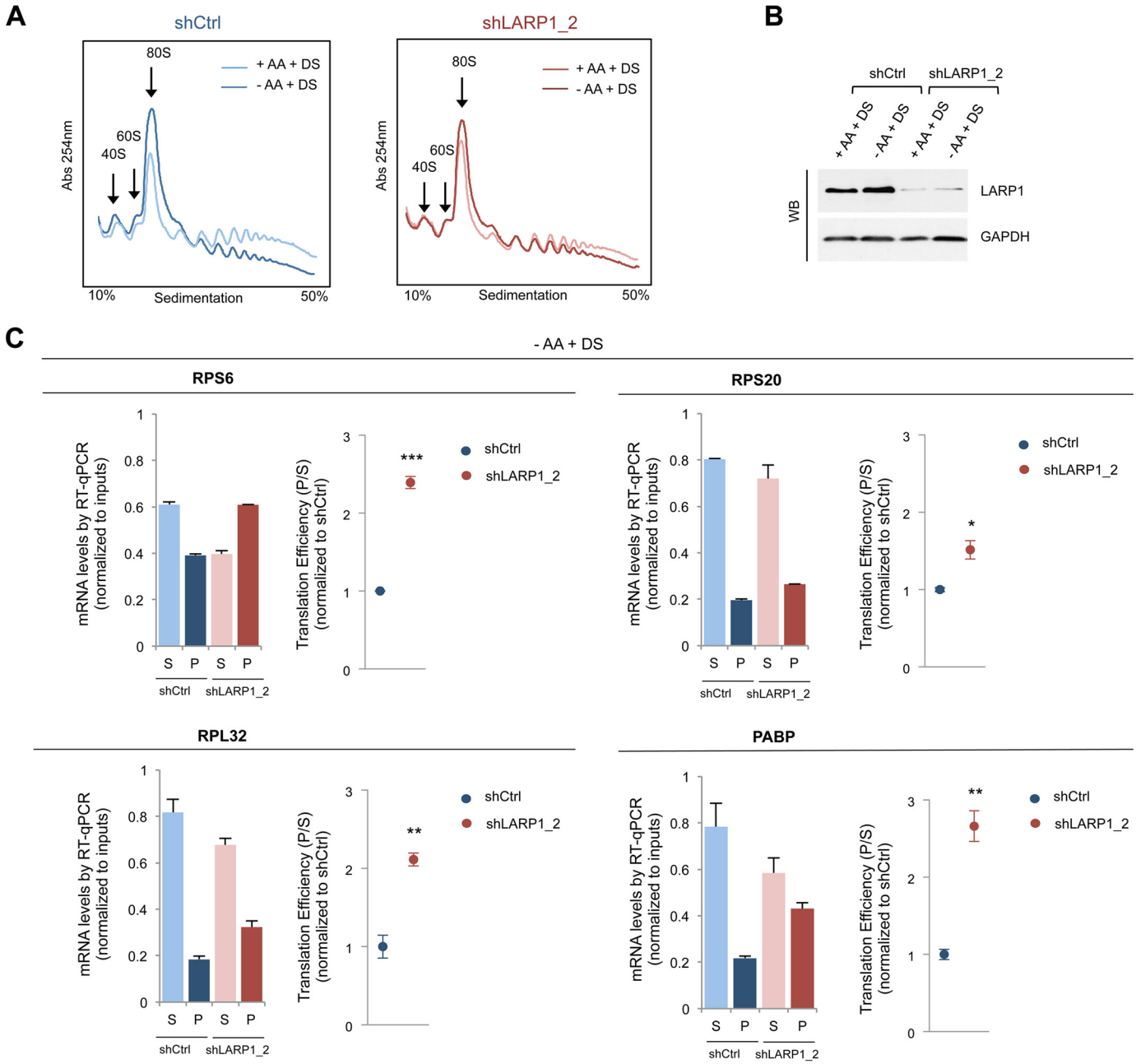

FIGURE 9. **Endogenous LARP1 is required for repression of TOP mRNA translation upon amino acid starvation.** *A,* shLARP1_2 HEK293T cells described in Fig. 7 were further used to investigate the effect of LARP1 knockdown on the ability of amino acid deprivation to inhibit TOP mRNA translation by polysome profile analysis. Cells were incubated for 3 h in DMEM containing or lacking amino acids in the presence of 10% (v/v) dialyzed serum. Polysome profiling was performed by an identical number of $A_{260\ nm}$ units (a crude estimate of total RNA amounts) onto each gradient. Polysome profile traces are shown. *B,* Western blot analysis of LARP1 and GAPDH protein levels. *C,* RT-qPCR analysis of subpolysomal (*S*) and polysomal (*P*) mRNA abundance for RPS6, RPS20, RPL32, and PABP, and statistical analyses were performed as described under "Experimental Procedures" and the supplemental Workbook.

Our data thus far show that LARP1 represses TOP mRNA translation and directly binds the 5′TOP motif, which suggests that LARP1 binding to the 5′TOP motif is potentially required for TOP mRNA translation repression. To further investigate this, we used a tetracycline-inducible reporter construct of the 5′-proximal region of the untranslated region (UTR) of RPL32

FIGURE 8. **Endogenous LARP1 is required for repression of TOP mRNA translation by mTORC1 inhibitors.** *A,* shLARP1_2 HEK293T cells described in Fig. 7 were further used to investigate effect of LARP1 knockdown on the ability of rapamycin and Torin1 to inhibit TOP mRNA translation by polysome profile analysis. Cells were stimulated with 10% (v/v) fetal bovine serum for 6 h in the presence of 0.1% (v/v) DMSO (vehicle), 100 nM rapamycin, or 300 nM Torin1 prior to lysis in hypotonic buffer. Polysome profiling was performed by loading an identical number of $A_{260\ nm}$ units (a crude estimate of total RNA amounts) onto each gradient. Polysome profile traces are shown. RT-sqPCR analysis for RPS6, RPS20, RPL32, and PABP mRNA was performed for each fraction. *B,* RT-qPCR analysis of subpolysomal (*S*) and polysomal (*P*) mRNA abundance for RPS6, RPS20, RPL32, and PABP was performed as follows: RNA from fractions 1 to 6 and 7 to 14 (shown in *A*) were pooled and designated *S* and *P*, respectively. cDNA was synthesized from each pool, and qPCR was performed as detailed under "Experimental Procedures." *C,* RT-qPCR analysis of subpolysomal (*S*) and polysomal (*P*) mRNA abundance for RPS6, RPS20, RPL32, and PABP and statistical analyses was performed as described under "Experimental Procedures" and the supplemental Workbook.

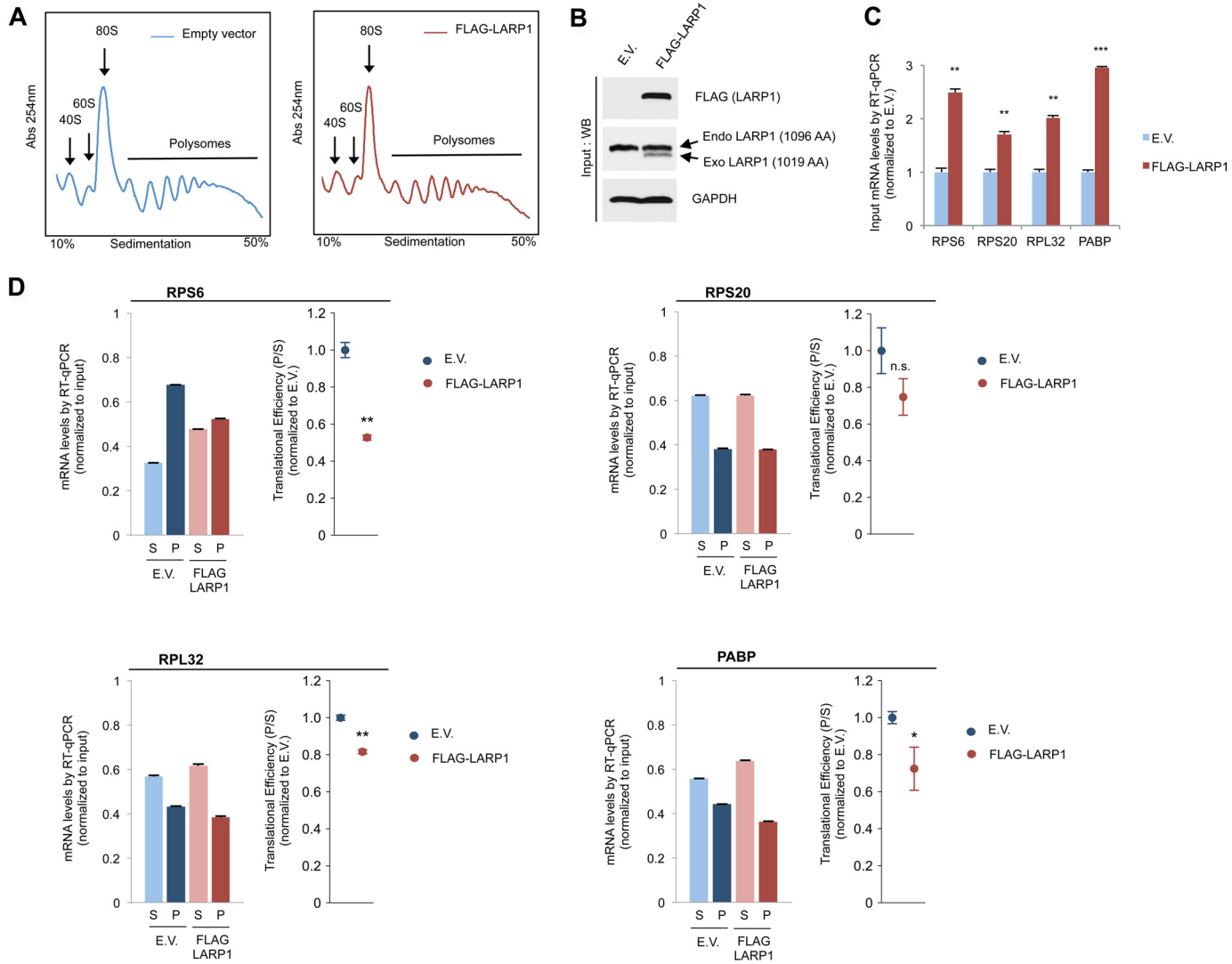

FIGURE 10. **Ectopic LARP1 represses TOP mRNA translation.** *A,* HEK293T cells were transiently transfected with 6 μg of Myc/FLAG LARP1 wild type into 2 × 10-cm plates (6 μg each). Thirty six hours after transfections, cells were stimulated with 10% (v/v) fetal bovine serum for 3 h and then lysed in hypotonic buffer as described under "Experimental Procedures." Polysome profiling was performed by loading identical amounts of total RNA onto each gradient. Polysome profile traces are shown. *B,* inputs were analyzed for ectopic LARP1 expression by SDS-PAGE/Western blot. *C,* inputs were analyzed for specific mRNA levels by RT-qPCR, following normalization by total RNA concentration. *D,* RT-qPCR analysis of subpolysomal (*S*) and polysomal (*P*) mRNA abundance for RPS6, RPS20, RPL32 and PABP, and statistical analyses were performed as detailed under "Experimental Procedures" and the supplemental Workbook.

fused to the coding sequence of the β-globin gene or a control vector lacking the RPL32 5′UTR element (51). Cells were co-transfected with the reporter constructs and FLAG-LARP1 or E.V., and lysates were subjected to polysome profiling (Fig. 12*A*) followed by Northern blotting (Fig. 12*B*). As shown in Fig. 12*B*, ectopic expression of LARP1 caused a selective accumulation of RPL32-β-globin mRNA in lighter subpolysomal fractions, consistent with LARP1 being a translation suppressor. More importantly, the ability of LARP1 to suppress β-globin translation was largely lost for the mRNA lacking the RPL32 5′UTR (Fig. 12*B*). Having observed that LARP1 overexpression represses the translation of the RPL32-β-globin, next we determined the effect of LARP1 overexpression on the steady-state levels of RPL32-β-globin chimeric protein by Western blot. LARP1 had no discernible effect on the steady-state levels of endogenous PABP or DDX6; it did, however, slightly reduce the steady-state levels of RPL32-β-globin (monitored with an anti-β-globin antibody). Even though the reduction in RPL32-β-globin protein level is relatively small, LARP1 overexpression also increases the RPL32-β-globin steady-state mRNA levels (Fig. 12, *B* and *D*), likely masking the repressor effects of LARP1 on RPL32-β-globin mRNA translation and protein levels.

LARP1 Enhances TOP mRNA Stability—A study by Aoki *et al.* (52) has recently shown that LARP1 depletion by RNA interference reduces the steady-state levels of TOP mRNAs. Having observed similar effects of LARP1 on TOP mRNA abundance (Figs. 7*C*, 10*C*, and 12, *B* and *D*), we asked whether in addition to controlling TOP mRNA translation LARP1 played an additional role on TOP mRNA stability. To assess this, we monitored TOP mRNA decay in actinomycin D-treated cells stably expressing FLAG-LARP1 or a control E.V. (Fig. 12*E*). Notably, overexpression of LARP1 significantly increased the half-lives of RPL29, RP36, and PABP TOP mRNAs.

Together, these data demonstrate that LARP1 interacts with TOP mRNAs via their 5′TOP motif in an mTORC1-regulated manner, and the presence of the 5′TOP motif is required for

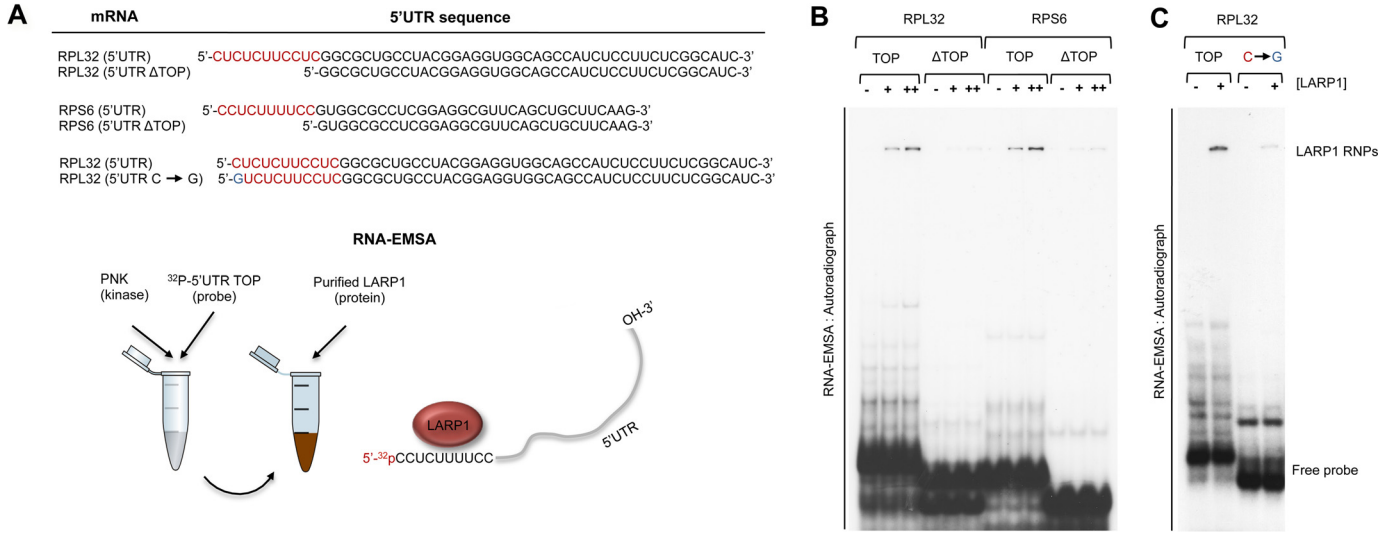

FIGURE 11. **LARP1 binds the 5′TOP motif.** *A*, schematic representation of RNA-EMSA. Synthetic RNA oligonucleotides were radiolabeled with [γ-^{32}P]ATP at the 5′end using polynucleotide kinase. Radiolabeled oligonucleotides were incubated with recombinant commercial (Abnova) human LARP1 protein purified from wheat germ extracts. Binding of LARP1 to RNA probes was monitored by a nondenaturing electrophoretic mobility shift assay. The *table* lists synthetic RNA oligonucleotides spanning the 5′UTRs of RPL32 and RPS6 used in the RNA-EMSA. *B* and *C*, recombinant purified LARP1 binds to 5′UTR of RPL32 and RPS6 via their 5′TOP motif.

efficient LARP1-mediated repression of TOP mRNA translation. In addition to repressing TOP mRNA translation, LARP1 plays a positive role in TOP mRNA stability.

LARP1 Does Not Bind eIF4E or eIF4G—Given the proximity of the TOP motif to the m^{7}Gppp mRNA cap, we tested whether LARP1 can bind the cap-binding protein eIF4E. To this end, we pulled down eIF4E using cap analog (m^{7}GTP)-Sepharose beads loaded with lysates from HEK293T cells stimulated with serum in the presence of rapamycin, Torin1, or DMSO (vehicle). eIF4E efficiently co-precipitated eIF4G in DMSO-treated cells. Rapamycin had only a minor effect on the association between eIF4E/eIFG, whereas Torin1 abolished eIF4E/eIF4G interaction (Fig. 13*A*). Conversely, the association of 4E-BP1 with eIF4E was enhanced upon mTORC1 inhibition with rapamycin, and more substantially with Torin1 (Fig. 13*A*). Importantly, LARP1 did not co-elute with eIF4E when free m^{7}GTP was used for elution (Fig. 13*A*, *left panel*). It did, however, elute nonspecifically and in an mTORC1-independent manner from the Sepharose resin when SDS-denaturing elution was used (Fig. 13*A*, *right panel*). Taken together, these data indicate that LARP1 associates nonspecifically with the m^{7}GTP resin and not with eIF4E.

Next, we tested whether LARP1 bound eIF4G as follows: HEK293T cells were stimulated with serum and lysed in CHAPS buffer with or without RNase A (see figure legend and under "Experimental Procedures" for details), and samples of lysates were subjected to immunoprecipitation with antibodies against LARP1 or eIF4G. As shown in Fig. 13*B*, LARP1 co-precipitated RAPTOR and PABP. As observed previously (Fig. 4*D*), the interaction between LARP1 and RAPTOR was augmented upon inclusion of RNase A in the lysis buffer, whereas the interaction of LARP1 with PABP was only slightly decreased in the presence of RNase A (Fig. 13*B*). Consistent with the data in Fig. 13*A*, LARP1 did not co-precipitate eIF4E nor did it pull down eIF4G (Fig. 13*B*). In a reverse eIF4G immunoprecipitation experiment, eIF4G pulled down eIF4E and PABP but not LARP1 or RAPTOR, reiterating the conclusion that LARP1 does not associate directly with eIF4G (Fig. 13*B*).

LARP1 Displaces eIF4G from TOP mRNAs Precluding TOP mRNA Translation—Having observed that mTORC1 inhibition has opposing effects on the association of LARP1 and eIF4G with TOP mRNAs, and given the proximity of these two noninteracting high molecular weight RNA-binding proteins at the extreme 5′end of the 5′UTR, we speculated that steric hindrance by LARP1 may occlude eIF4G if these factors compete for binding to TOP mRNAs. To test this hypothesis, we assessed whether the presence of LARP1 interfered with the binding of eIF4G to TOP mRNAs. To this end, we made use of HEK293T cells stably knocked down for LARP1, and we monitored the amount of TOP mRNA associated with eIF4G by RNA immunoprecipitation/RT-qPCR. Remarkably, depletion of LARP1 resulted in an up to 5-fold increased association of all tested TOP mRNAs with endogenous eIF4G in serum-stimulated HEK293T cells (Fig. 13*C*). Conversely, moderate overexpression of Myc/FLAG LARP1 decreased the association of the same TOP mRNAs with eIF4G (Fig. 13*D*). Together, these data demonstrate that LARP1 competes with eIF4G for TOP mRNA binding, effectively displacing or preventing the association of eIF4G with TOP mRNAs. This result is in line with an earlier report that eIF4G is required for TOP mRNA translation (43). Ultimately, these findings suggest that LARP1 functions as an eIF4G competitor and an important repressor of TOP mRNA translation downstream of mTORC1. Our proposed mechanism for LARP1-mediated TOP mRNA translation repression is diagrammatically illustrated in Fig. 14.

Discussion

In this study, we elucidate a mechanism by which LARP1 represses TOP mRNA translation downstream of mTORC1. Our data demonstrate that LARP1 physically associates with the 5′TOP element in close proximity with the eIF4F complex and disrupts the association of eIF4G with TOP mRNAs in

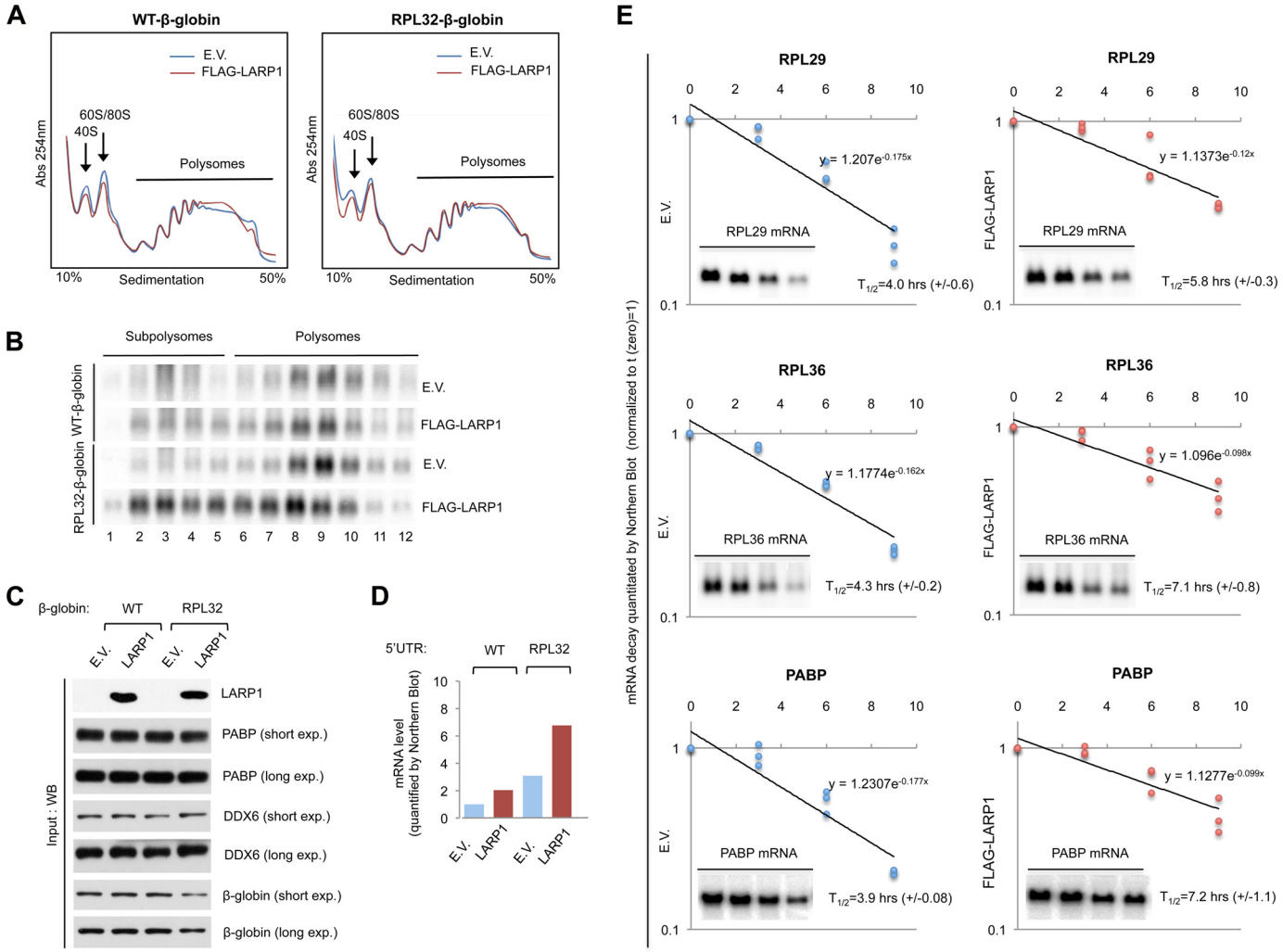

FIGURE 12. **Translational repression by LARP1 is dependent on the 5′TOP motif.** *A,* polysome profile traces of HeLa Tet-Off cells co-transfected with Tet-inducible β-globin reporters (expressing either their natural 5′UTR or the RPL32 5′UTR) and E.V. or Myc/FLAG-LARP1. *B,* distribution of β-globin reporter mRNAs in the sucrose gradients was monitored by Northern blot. *C,* Western blot (*WB*) analysis of steady-state protein levels of endogenous PABP, DDX6, and RPL32-β-globin fusion protein. *D,* Northern blot quantitation of steady-state protein levels of β-globin reporter mRNAs. *E,* effect of stable LARP1 overexpression on TOP mRNA stability.

conditions of mTORC1 inhibition, ultimately leading to inhibition of the initiation step of protein synthesis (43).

The mTOR pathway was first linked to the control of TOP mRNA translation 20 years ago, following the observation that rapamycin selectively inhibited the translation of TOP mRNAs encoding for elongation factors (eEF1α and eEF2) and ribosomal proteins (RPS3, RPS6, RPS14, and RPS24) (75, 76). Indeed, recent genome-wide ribosome profiling studies confirmed that TOP and TOP-like mRNAs account for a vast proportion of the mTOR translatome (43, 44), indicating that the mTOR pathway plays a prominent role in the control of the translation of this subset of mRNAs. Numerous environmental cellular cues (*e.g.* growth factors, amino acids, and oxygen levels) have previously been described to regulate the translation of TOP mRNAs via mTOR. Meticulous work from Meyuhas and co-workers (39–41, 45, 77) has, over the years, helped to carefully elucidate the precise contribution of various signaling molecules (that function upstream of mTOR) to the activation of TOP mRNA translation. We now know, for instance, that amino acids, serum, and oxygen signal via the tuberous sclerosis complex (TSC1 and TSC2) and its effector RHEB to activate TOP mRNA translation (41, 45, 77). mTOR itself plays a fundamental role in TOP mRNA translation as follows: knocking down mTOR diminishes the translation efficiency of TOP mRNAs in response to amino acids and insulin stimulation (41, 77). Moreover, the kinase activity of mTOR is also required for translational control of TOP mRNAs (77). Finally, the ability of rapamycin to repress TOP mRNA translation is also mediated by mTOR and its binding partner, FKBP12 (77).

Considerably less is known about how mTORC1 relays its effects on TOP mRNA translation (*i.e.* which downstream substrate(s) mediate this effect). The 4E-BPs·eIF4E complex has been proposed to play a role in the control of TOP mRNA translation (43, 44), but a recent study disputed this finding; eIF4E and 4E-BP1 were reported to be dispensable for TOP mRNA translational control, at least in response to amino acids and oxygen (45). Our data show that eIF4G association with TOP mRNAs is strongly enhanced once LARP1 is depleted and, conversely, diminished upon LARP1 overexpression. This suggests that both LARP1 and eIF4G play key mechanistic roles in

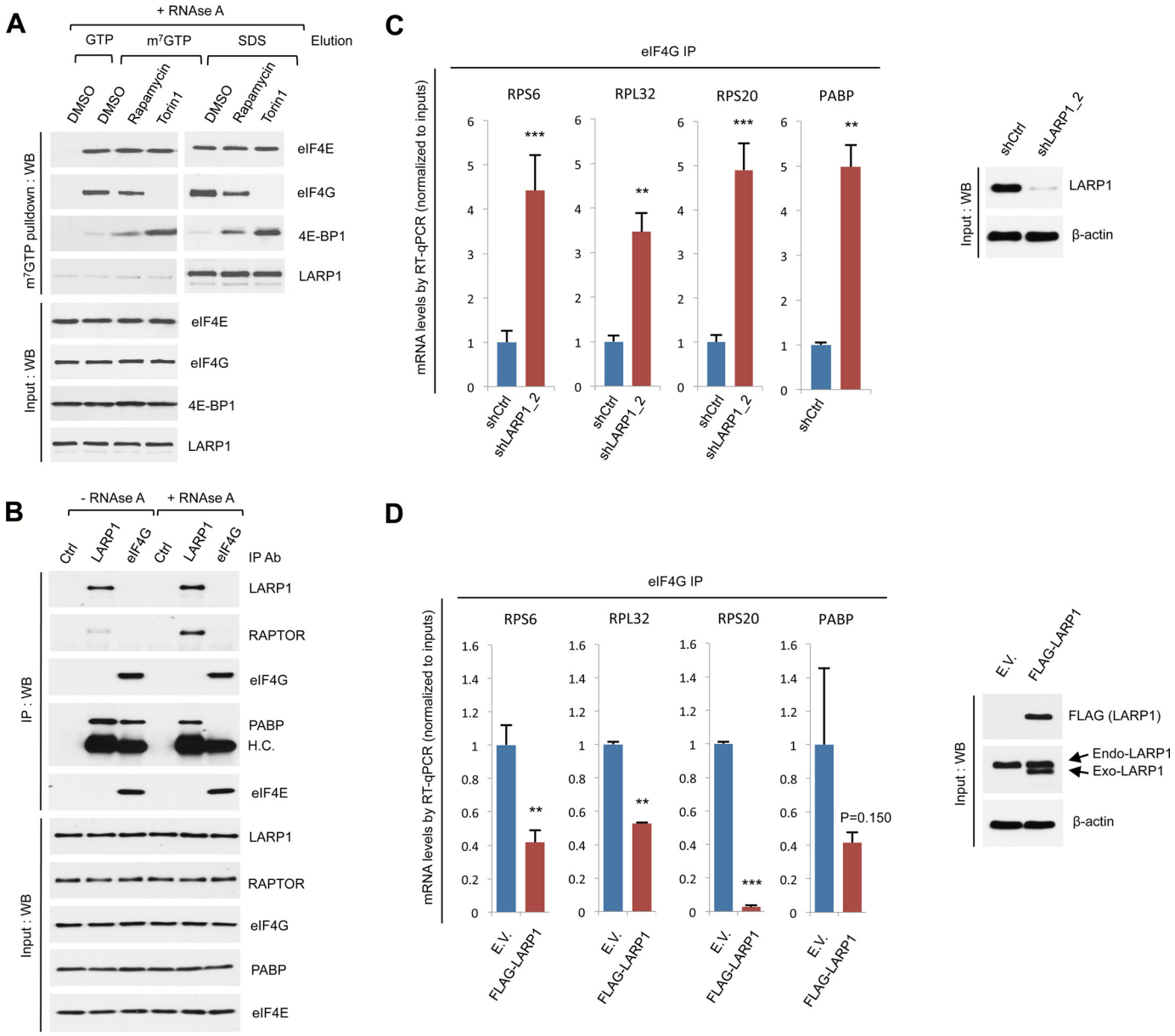

FIGURE 13. **LARP1 competes with eIF4G for TOP mRNA binding.** *A,* HEK293T cells were cultured to near-confluency (70–80%) and then stimulated with 10% (v/v) fetal bovine serum for 3 h in the presence of 0.1% (v/v) DMSO (vehicle), 100 nM rapamycin, or 300 nM Torin1, followed by lysis in CHAPS lysis buffer in the presence of 1 μg/ml RNase A. Lysates were subjected to m^{7}GTP chromatography and eIF4E-associated proteins probed by SDS-PAGE/Western blot (*WB*) with the antibodies indicated. *B,* HEK293T cells were propagated to near-confluency (70–80%) and then stimulated with 10% (v/v) fetal bovine serum for 3 h by media change. Cells were then lysed in CHAPS lysis buffer in the presence or absence of RNase A. Lysates were subjected to immunoprecipitation (*IP*) with LARP1 or eIF4G antibody, and immunoprecipitates were analyzed by SDS-PAGE/Western blot with the indicated antibodies. *C,* HEK293T cells were transduced with shCtrl or shLARP1_2 lentivirus and selected with puromycin was described under "Experimental Procedures." Following selection, pools of cells were seeded in 15-cm plates in complete growth media without puromycin. Cells were cultured for ~48 h until they reached 70–80% confluency at which point cells were stimulated with 10% (v/v) fetal bovine serum by media change for 3 h. Cells were then lysed in CHAPS lysis buffer, and lysates (2.5 mg of total protein) were subjected to immunoprecipitation with anti-eIF4G antibody (refer to "Experimental Procedures" for details). Immunoprecipitates were used for mRNA analysis by RT-qPCR and lysates for protein analysis by SDS-PAGE/Western blot. *D,* HEK293T cells were transiently transfected with 6 μg of wild type LARP1 in a 10-cm plate. Twenty one hours after transfection, cells were stimulated with 10% (v/v) fetal bovine serum for 3 h by media change. Cells were then lysed in CHAPS lysis buffer, and lysates were subjected to immunoprecipitation with anti-eIF4G antibody (1 mg of lysate was used per immunoprecipitation). Immunoprecipitates were used for mRNA analysis by RT-qPCR and lysates for protein analysis by SDS-PAGE/Western blot.

TOP mRNA translation. eIF4G has previously been proposed to be required for TOP mRNA translation (43). Several other proteins (*e.g.* La, ZNF9, and AUF1) have been proposed to control TOP mRNA translation (46–51, 78, 79), and the TIA proteins are potent repressors of TOP mRNA translation, specifically during amino acid starvation where the eIF2α-kinase GCN2 is active, although mTORC1 remains inactive (45, 51). However, whether any of these proteins function directly or indirectly downstream of mTORC1 is unclear. The missing link between mTORC1 and TOP mRNA regulation has remained elusive.

TOP mRNAs have long been known to be translationally repressed by a titratable *trans*-acting inhibitor. This is evinced by the observation that fusing a 5′TOP moiety to a translationally active non-TOP mRNA renders it translationally inactive (80, 81). Our observations that LARP1 is required for efficient

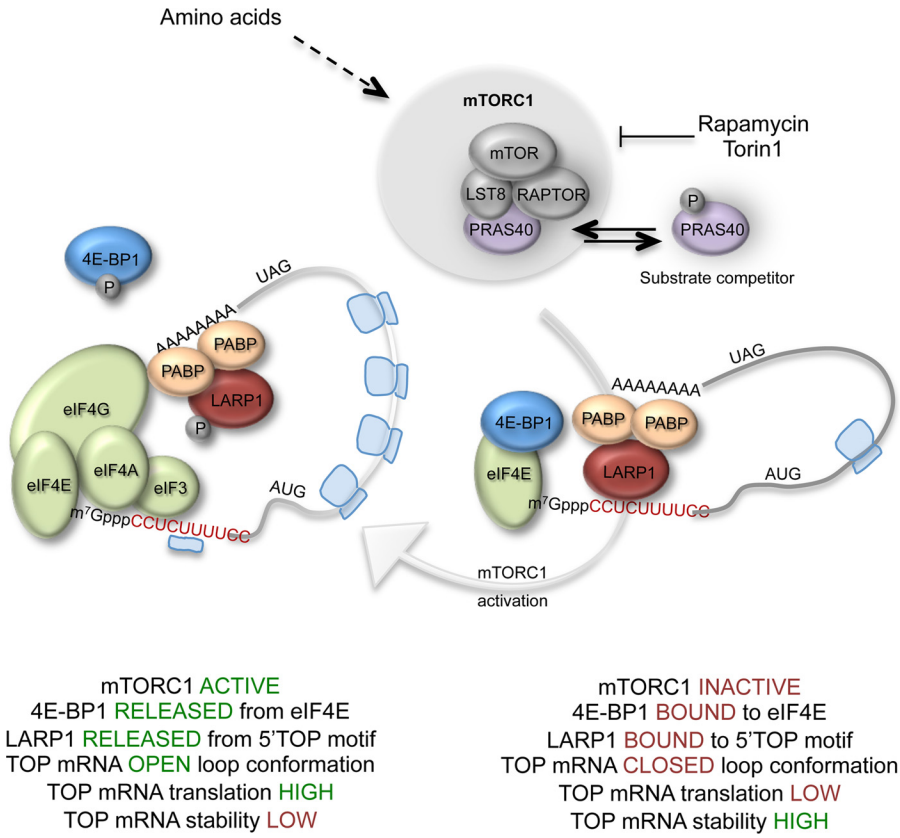

FIGURE 14. **Proposed model for LARP1-mediated repression of TOP mRNA translation downstream of mTORC1.** mTORC1 phosphorylates LARP1 at multiple residues (34), effectively releasing LARP1 from the TOP motif. mTORC1 also controls the phosphorylation of 4E-BP1, releasing it from eIF4E thus allowing for eIF4G to bind eIF4E and recruit the pre-initiation complex to the mRNA, and translation ensues.

repression of TOP mRNA translation as a result of mTOR inhibition and that it binds directly to the TOP motif *in vitro* places LARP1 as an important *trans*-acting TOP mRNA repressor.

While this manuscript was in preparation, a study by Tcherkezian *et al.* (53) linked LARP1 to TOP mRNA translation. Our findings confirm this report that LARP1 associates with PABP and TOP mRNAs but differs with respect to whether LARP1 functions as a positive or negative regulator of TOP mRNA translation. In contrast to the model put forth by Tcherkezian *et al.* (53), our data demonstrate that LARP1 functions as a repressor and *not* as an activator of TOP mRNA translation. One possible explanation for these apparently conflicting results may be that the high viral titers used for shRNA-mediated LARP1 knockdown in the study of Tcherkezian *et al.* (53) causes extensive cell death (we also observe that near-complete knockdown of LARP1 results in loss of cell viability).[8] Increased cell death in LARP1 knockdown cells is accompanied by transcriptome-wide mRNA decay and profound impairment of global protein synthesis (with massive disruption of polysome profiles) (53). In contrast, moderate silencing of LARP1 (as reported in our study) does not compromise cell viability as judged by global mRNA translation rates, because polysome profile traces are similar when equal rRNA concentrations are loaded onto the sucrose gradient. The use of healthy cells with moderate levels of LARP1 knockdown (as used in our study) revealed a *bona fide* inhibitory effect of LARP1 on TOP mRNA translation.

In support of a role as a repressor, our LARP1 RNA immunoprecipitation analyses demonstrate a preferential binding to TOP mRNAs and competition with eIF4G for association with this class of mRNAs. Importantly, the association of LARP1 with TOP mRNAs is markedly augmented upon mTORC1 inhibition (*i.e.* Torin1, rapamycin, or serum starvation) in agreement with the characteristics of a repressor. For example, 4E-BPs (well described targets of mTORC1 and inhibitors of translation initiation) also display increased association with eIF4E upon mTORC1 inhibition, in agreement with its role as a translation initiation repressor (82).

Importantly, we show here firsthand that LARP1 directly interacts with the 5′TOP motif of both RPL32 and RPS6 *in vitro* and that association of LARP1 with the 5′UTR is essential for LARP1-mediated TOP mRNA translation repression. Together, these data provide strong evidence in support of a role for LARP1 in the repression of TOP mRNA translation. We speculate that additional binding sites within the 5′UTR of these mRNAs contribute to LARP1 binding, as residual binding can be observed following complete removal of the 5′TOP motif. Notably, RPL32 and RPS6 contain additional TOP-like sequences within their 5′UTR that may also contribute to LARP1 binding. In addition to binding the 5′UTR, LARP1 has previously been shown to directly bind PABP as well as the 3′OH group of the terminal adenosine in the poly(A) tail. We

[8] B. D. Fonseca, C. K. Damgaard, and T. Alain, unpublished data.

confirmed these results, showing that LARP1 indeed binds PABP directly. Of note, we observe that the interaction of LARP1 with PABP is not regulated by mTORC1. This is in contrast to the association of LARP1 with TOP mRNAs. We therefore propose that mTORC1 specifically regulates the association of LARP1 with the 5′UTR but not the 3′UTR, as depicted by the model in Fig. 14. Additional work will be required to clarify the role of LARP1 association with PABP and the poly(A) tail. Further investigation will also be required to determine which specific domains within LARP1 mediate the interaction with the TOP motif and the poly(A) tail. Structural studies will likely shed more light on the exact mechanism by which this important new protein mediates its effect on TOP mRNA gene expression.

The 5′TOP motif immediately downstream of the m^7Gppp cap of TOP mRNAs allows for the concerted repression and de-repression of the translation of ribosomal proteins and translation factors. We show that the association of LARP1 (a *trans*-acting factor) with the 5′TOP motif allows for synchronized repression of the translation of both ribosomal proteins (RPL32, RPS6, and RPS20) and translation factors (PABP) when mTOR activity is challenged. Notably, our data suggest that not all TOP mRNA translation is equal; although silencing LARP1 enhances the translation of ribosomal proteins (RPL32, RPS6, and RPS20) and translation factors (PABP) alike, these mRNA subsets exhibit differential sensitivity to mTORC1 inhibition (Fig. 8). We observe that PABP translation remains fully sensitive to rapamycin or Torin1 in the absence of LARP1, whereas the translation of ribosomal proteins becomes partially resistant to these drugs. A number of reasons may explain the enhanced sensitivity of PABP mRNA to mTORC1 inhibitors in LARP1 knockdown cells as follows. (i) PABP mRNA displays an unusually long (505 nucleotides) 5′UTR (compared with ribosomal proteins and elongation factors) and an equally unusual 5′TOP motif bearing a high cytosine-to-uracil (C/U) content (83), which may explain differential translational control of this TOP mRNA.(ii) A *cis*-mRNA regulatory element has previously been shown to confer additional regulatory layers to PABP mRNA translation; PABP mRNA contains a small poly(A) tract within its 5′UTR that is subject to translational regulatory control by PABP protein itself in a negative auto-regulatory loop (84, 85). We speculate that the negative auto-regulatory loop of PABP protein on the PABP mRNA may also be subject to control by mTORC1 and may thus explain why PABP mRNA translation remains fully sensitive to mTORC1 inhibition by rapamycin and Torin1.

TOP mRNA translation is regulated by a myriad of stimuli (mitogens, growth factors, amino acids, and oxygen) and repressed by pharmacological inhibitors of PI3K (LY294002) and mTOR (rapamycin and Torin1). Translation of TOP mRNAs is strongly repressed even in growth conditions (*e.g.* upon serum stimulation). Pharmacological inhibition of mTORC1 further represses TOP mRNA translation. This study aimed to analyze the role of LARP1 in the control of TOP mRNA translation in both growth conditions as well as in response to pharmacological inhibition of mTORC1. We observed that LARP1 is required for both basal inhibition of TOP mRNA translation (*i.e.* in conditions of serum stimulation), for the enhanced translational suppression by mTORC1 inhibitors, and for repression upon nutrient (amino acid) insufficiency. Interestingly, we have noted differences between pharmacological inhibition of mTORC1 and nutrient-mediated inhibition of mTORC1 with regard to LARP1-PABP interaction; amino acid starvation, but not rapamycin and Torin1, enhances LARP1-PABP interaction. The mechanism for amino acid-mediated LARP1-dependent TOP mRNA repression may involve additional 3′UTR-dependent regulatory mechanisms. Future work will be required to understand how LARP1 may intersect with the action of the TIA proteins known to elicit TOP mRNA repression specifically during such conditions (37).

In the process of studying the role of LARP1 on TOP mRNA translation, we came across another important role for LARP1 on the control of TOP mRNA stability. Our data confirmed that LARP1 plays a positive role in TOP mRNA stability, originally proposed by Aoki *et al.* (52). The seemingly opposing effects of LARP1 on TOP mRNA translation (repressor), and TOP mRNA stability (activator) suggests that LARP1 functions as a bimodal TOP mRNA regulator; it represses TOP mRNA translation but simultaneously ensures that TOP mRNAs are preserved until they are ready to be translated once again. Such a mode-of-action would ensure maximal energy conservation in the production of ribosomal proteins and translation factors.

In conclusion, this study describes an important new link between mTORC1, LARP1, and TOP mRNA translation and stability. LARP1 likely regulates many other mRNAs as suggested by a recent study (55) and unpublished LARP1 data from our groups.[9] Detailed identification and characterization of the complete set of LARP1-associated mRNAs will likely contribute to a better understanding of how this important new mRNA translation repressor/mRNA stabilizing factor regulates gene expression and, ultimately, controls cell function(s). Finally, but perhaps most importantly, given the druggable nature of the mTORC1 signaling pathway and the prominent roles for ribosome biogenesis, protein synthesis, and mRNA decay in cancer, we anticipate that the LARP1 status of cancer cells may be a valuable marker for determining anti-cancer drug (*i.e.* rapamycin) resistance.

Acknowledgments—We thank Oded Meyuhas, Ken Inoki, Sungki Hong, George Thomas, Antonio Gentilella, Sarah Blagden, Manuela Mura, Tom Hopkins, Philippe Roux, Joseph Tcherkezian, Martin Bushell, Nahum Sonenberg, Ivan Topisirovic, Andrea Berman, and the members of the LARP society for helpful discussions. We also thank Kurt Dejgaard, Jing Liu, and Sophie Charbonneau for mass spectrometry analysis and Stephanie Swift for critical reading of the manuscript. Special thanks to Astrid Philipps for tireless support.

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[9] C. K. Damgaard, B. D. Fonseca, and T. Alain, unpublished data.

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