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LARP1 on TOP of Ribosome Production

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Abstract

The ribosome is an essential unit of all living organisms that commands protein synthesis, ultimately fuelling cell growth (accumulation of cell mass) and cell proliferation (increase in cell number). The eukaryotic ribosome consists of four ribosomal RNAs and up to 80 ribosomal proteins (RPs). Despite its fundamental role in every living organism, our present understanding of how higher eukaryotes produce the various ribosome components is incomplete. Uncovering the mechanisms utilized by human cells to generate functional ribosomes will likely have far-reaching implications in human disease. Recent biochemical and structural studies revealed La-related protein 1 (LARP1) as a key new player in ribosomal protein production. LARP1 is an RNA-binding protein that belongs to the LARP superfamily; it controls the translation and stability of the mRNAs that encode ribosomal proteins and translation factors, which are characterized by a 5' terminal oligopyrimidine (5'TOP) motif and are thus known as TOP mRNAs. The activity of LARP1 is regulated by the mammalian target of rapamycin complex 1 (mTORC1): an eukaryotic protein kinase complex that integrates nutrient sensing with mRNA translation, particularly that of TOP mRNAs. In this review, we provide an overview of the role of LARP1 in the control of ribosome production in multicellular eukaryotes.

Graphical Abstract

Ribosome production is a considerable energy investment for the cell—rRNAs and ribosomal proteins are amongst the most abundant classes of RNAs and proteins—and their production must, therefore, be synchronized temporally and stoichiometrically to ensure efficient ribosome assembly. Multicellular eukaryotes control ribosomal protein production at the level of mRNA

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translation. La-related protein 1 (LARP1) is a novel target of mammalian target of rapamycin complex 1 (mTORC1) and key repressor of ribosomal protein mRNA translation. LARP1 binds the 7-methyl guanosine triphosphate (m^7Gppp) cap and adjacent 5' terminal oligopyrimidine (5'TOP) motif of mRNAs encoding ribosomal proteins and translation factors. In doing so, LARP1 prevents binding of the eukaryotic initiation factor 4E (eIF4E) to the m^7Gppp cap and blocks the assembly of the eIF4F complex and the subsequent recruitment of the 40S ribosomal subunit to ribosomal proteins mRNAs. mTORC1 controls TOP mRNA translation by regulating the phosphorylation and association of LARP1 with the m^7Gppp cap and the characteristic adjacent 5'TOP motif of TOP mRNAs.

1 Introduction

The ribosome is a fundamental unit of life. The eukaryotic ribosome (80S) is formed by a large subunit (60S) and a small subunit (40S). In mammals, the large subunit comprises three ribosomal RNAs (28S, 5S and 5.8S) and 46 ribosomal proteins (RPs), while the small subunit contains a single ribosomal RNA (18S) and the remaining 33 RPs. Ribosomes are highly abundant in eukaryotic cells, *e.g.* a single yeast cell is estimated to have ~200,000 ribosomes¹¹⁶. Mass-production of ribosomes is an energetically costly cellular process and, thus, eukaryotic cells exert precise control over ribosomal protein expression to ensure that the stoichiometry of each component is appropriate for the assembly of translationally-competent ribosomes¹¹⁶. Unicellular and multicellular eukaryotes employ distinct strategies to attain proper ribosome production while minimizing unwarranted energy expenditure. *Saccharomyces cerevisiae* controls ribosome protein abundance through, predominantly, transcriptional control. In yeast, ribosomal protein mRNAs have a short half-life¹¹⁶ and therefore require continuous transcription to sustain the high ribosome demand for yeast growth. Large-scale ribosome production in yeast is therefore achieved through mass-transcription of ribosomal protein mRNAs, while ribosomal protein turnover compensates for subtle ribosomal protein expression imbalances¹¹². Artificial overexpression of ribosomal genes is counteracted by increased ribosomal protein turnover¹¹². Importantly, in yeast, overexpressed ribosomal protein mRNAs are translated at rates similar to those of endogenous ribosomal protein mRNAs indicating that translation control does not play a crucial role in the synthesis of the yeast ribosome¹¹². This is in stark contrast to higher eukaryotes (*e.g.* vertebrates), in which ribosomal protein production is controlled, primarily, at the level of mRNA translation^{28, 71, 72, 87}. In higher eukaryotes, as noted for yeast, ribosomal protein mRNAs are also mass-transcribed as evidenced by their

sheer steady-state abundance: *e.g.* RPL32 and RPS6 mRNAs are 50–100× more abundant than β -actin or GAPDH mRNAs in human fibroblasts (our unpublished data) but, unlike their yeast counterparts, human ribosomal protein mRNAs are remarkably stable⁸⁷. The corollary of this is that, in higher eukaryotes, the translation of ribosomal protein mRNAs must therefore be tightly regulated and mostly repressed to minimize unwarranted energy expenditure. Ample evidence suggests that this is indeed the case: higher eukaryotes engage the production ribosomes only under conditions where nutrients (namely amino acids, growth factors, oxygen) and energy sources (ATP) are abundant. The translation of ribosomal protein mRNAs is otherwise switched off; even under nutrient-rich conditions, a significant percentage of ribosomal protein mRNAs is found in subpolysomal fractions indicating that a fraction of these transcripts is repressed under favourable growth conditions^{71, 72}. This is in stark contrast with the translation of other housekeeping mRNA (*e.g.* GAPDH and β -actin), nutrient-free conditions still accommodate the translation of the latter mRNAs. This phenomenon was first observed by Meyuhas *et al.* in 1987⁷³ and, extensively reproduced by others^{5, 13, 24, 73, 106¹}.

As discussed above, the translation of ribosomal protein mRNAs is sensitive to nutrient availability. Unsurprisingly, the synthesis of ribosomes is coupled to signalling pathways that sense nutrient availability. Chief among these pathways is the mammalian target of rapamycin complex 1 (mTORC1) pathway, which plays a fundamental role in the control of translation of the transcripts that encode ribosomal proteins^{23, 24, 40, 110}. The mechanism by which mTORC1 controls the translation of ribosomal protein transcripts remained elusive up until the recent discovery of a novel signalling axis of mTORC1: the mTORC1-LARP1 signalling axis^{24, 107}. LARP1 (the acronym for La-related protein 1) is a novel target of mTORC1^{24, 41, 119}. LARP1 specifically associates with the mTORC1-specific component RAPTOR (regulatory-associated protein of mTOR), but not RICTOR (rapamycin-insensitive companion of mTOR; an mTORC2-specific component)^{24, 107}. LARP1 was first connected to ribosomal protein transcript regulation by Natsume and colleagues in 2013³; Aoki *et al.*, reported that LARP1 protein selectively associates with ribosomal protein transcripts (among a few other mRNAs)³. Natsume's group early observations prompted us²⁴ and others¹⁰⁷ to investigate a potential connection between mTORC1, LARP1 and ribosomal protein mRNA translation. Compelling evidence now demonstrates that LARP1 functions as a repressor of ribosomal protein mRNA translation downstream of mTORC1²⁴. Recent structural data provide important insights into the mechanism by which LARP1 represses ribosomal proteins mRNA translation: specifically, these data show that LARP1 recognizes the N7-methyl guanosine triphosphate (m⁷Gppp) cap moiety and the adjacent 5' terminal oligopyrimidine (5'TOP) sequence found in ribosomal protein mRNAs⁵⁷. Binding of

¹It is important to point out that oxygen availability or the nutritional status of a cell are not the sole defining factors in ribosomal protein mRNA translation, *i.e.* nutrients, ATP and oxygen are necessary but not sufficient to engage ribosomal proteins mRNA translation. The ability of cells to divide, regardless of nutritional supply and oxygen availability, defines the ribosomal protein mRNA translation response. Cells undergoing mitotic arrest, due to terminal differentiation, contact inhibition, or pharmacological inhibition in the progression of the cell cycle at any stage do not engage ribosomal protein mRNA translation – even under a surplus supply of nutrients and oxygen. 1. Aloni R, Peleg D, Meyuhas O. Selective translational control and nonspecific posttranscriptional regulation of ribosomal protein gene expression during development and regeneration of rat liver. *Mol Cell Biol* 1992, 12:2203–2212, 95. Shama S, Avni D, Frederickson RM, Sonenberg N, Meyuhas O. Overexpression of initiation factor eIF-4E does not relieve the translational repression of ribosomal protein mRNAs in quiescent cells. *Gene Expr* 1995, 4:241–252, 103. Stolovich M, Lerer T, Bolker Y, Cohen H, Meyuhas O. Lithium can relieve translational repression of TOP mRNAs elicited by various blocks along the cell cycle in a glycogen synthase kinase-3- and S6-kinase-independent manner. *J Biol Chem* 2005, 280:5336–5342.

LARP1 to the m⁷Gppp cap moiety of ribosomal protein mRNAs precludes the binding of eukaryotic initiation factor 4E (eIF4E) to this class of transcripts⁵⁷, thus blocking the assembly of the eIF4F complex on ribosomal protein mRNAs^{24, 57}. These structural data provide a direct mechanism that corroborates the model for LARP1-mediated ribosomal protein mRNA translation repression first postulated in 2015²⁴. Recent data by Philippe et al.⁹⁰ confirm that LARP1 functions as a repressor of TOP mRNA translation²⁴ and that it binds the m⁷Gppp cap of TOP mRNAs⁵⁷. Moreover, Thoreen and colleagues demonstrated that LARP1 represses TOP mRNA translation *in vitro* through the combined recognition of both the m⁷Gppp cap moiety and the adjacent 5' TOP motif in ribosomal proteins mRNAs⁹⁰. Preliminary findings indicate that mTORC1 controls the association of ribosomal protein transcripts with LARP1²⁴ through phosphorylation of the latter³⁸. Additional work will be required to elucidate the precise nature of the signalling mechanism connecting mTORC1 to LARP1. At present, the biological function of LARP1 is not known; however, a recent study by Thomas and colleagues linked LARP1 to pathophysiologies associated with impaired ribosome biogenesis²⁷, consistent with a central role for LARP1 in ribosomal protein production. LARP1 has also been implicated in human cancer progression^{39, 77} (reviewed in¹⁰²); and its protein levels correlate with cancer prognosis and chemotherapy response³⁹. This article reviews the latest advances in the rapidly developing field of mTORC1, LARP1, and ribosomal protein production.

2 mTORC1: a sensor for nutrient availability and master regulator of ribosome production

The mammalian target of rapamycin complex 1 (mTORC1) is a hub for sensing nutrient and mitogen availability^{12, 31, 94, 97, 117} by integrating signals from the Sestrin/Gator/Ragulator/Rag GTPase and the PI3K/Akt/TSC/Rheb pathways, respectively^{94, 117} and relaying this information to the translation machinery^{21, 23, 109}. mTORC1 plays a role in both global and transcript-specific translation. mTORC1 controls the translation of a myriad of mRNAs, most notably terminal oligopyrimidine (TOP) transcripts^{23, 24, 40, 110}. TOP mRNAs constitute a class of transcripts that encode, among other proteins, ribosomal proteins and are so-named because they contain a unique *cis*-regulatory mRNA element defined by a cytosine at the +1 position followed by an uninterrupted stretch of 4–15 pyrimidines⁸. This element is invariably located at the very 5' end of the message immediately downstream of the m⁷Gppp mRNA cap; it is, therefore, coined the 5' terminal oligopyrimidine (5' TOP) motif. The 5' TOP motif is present in every one of the 80 mRNAs that encode human ribosomal proteins and it coordinates their translation^{23, 72}. The same motif is also found in mRNAs encoding components of the translation machinery, such as some translation initiation and all elongation factors, and a number of RNA-binding proteins^{23, 43, 72}. The presence of this common *cis*-regulatory element in every ribosomal protein mRNA ensures that all components are synthesized in a co-regulated fashion resulting in proper stoichiometry. Because every ribosomal protein mRNA contains a 5' TOP motif, hereafter we will refer to ribosomal protein mRNAs simply as TOP mRNAs^{60, 2}.

²The term TOP mRNA was originally coined by Levy et al. (60. Levy S, Avni D, Hariharan N, Perry RP, Meyuhas O. Oligopyrimidine tract at the 5' end of mammalian ribosomal protein mRNAs is required for their translational control. *Proc Natl Acad*

It has been appreciated for decades that mTORC1 controls the translation of TOP mRNAs. mTORC1 was first linked to the control of TOP mRNA translation in 1994^{49, 108}, following the observation that rapamycin, an allosteric inhibitor of mTORC1, decreases the translation of ribosomal proteins and elongation factors^{49, 108}. Meticulous studies by Meyuhas' group have advanced our understanding of the contribution of each component of the PI3K/Akt/TSC/Rheb/mTOR pathway to the control of TOP mRNA translation^{74, 83, 84, 104, 106}. More recently, genome-wide ribosome profile studies^{40, 110} confirm that mTORC1 does, indeed, play a central role in TOP mRNA translation. Ribosome profiling analyses revealed that TOP mRNAs account, in fact, for the largest class of mRNAs that are translationally-regulated by mTOR^{40, 110}. It makes physiological sense for a cell to connect a nutrient-signalling pathway, such as the amino acid-sensing mTORC1 signalling pathway, to the translation of ribosomal protein mRNAs. This is of particular importance because the production of ribosomes is an energy-costly cellular process and ought to be engaged only in conditions of amino acid abundance. But how does mTORC1 relay the amino acid availability signal to the translation machinery to promote ribosome biosynthesis?

Typically, mTORC1 elicits its effects on the translation machinery through the phosphorylation of multiple proteins directly or indirectly involved in translation (see²¹ for a comprehensive review on the various mechanisms by which mTORC1 directs mRNA translation across eukaryotes). The best-studied targets of mTORC1 are the eukaryotic initiation factor 4E-binding proteins (4E-BPs) and the ribosomal S6 kinases (S6Ks), which have prominent roles in the control of mRNA translation²³, although neither of these families of proteins plays a direct role in the translation regulation of TOP mRNAs^{74, 104}. The production of ribosomes is a fundamental cellular process and, as such, there is considerable interest in deciphering the mechanisms by which higher eukaryotes control the translation of ribosomal protein mRNAs, as evidenced by the vast literature on the subject. Research groups have rallied to address this vexing question and numerous candidate proteins have been proposed to regulate TOP mRNA translation (and, by extension, the translation of ribosomal protein mRNAs) downstream of mTOR (see⁷² for a recent review on the subject), but a key *trans*-acting factor that fulfilled the pre-requisites for (i) direct association with 5' TOP motif, (ii) repression of TOP mRNA translation and (iii) regulation by mTORC1 remained elusive until recently.

3 The discovery of La-related protein 1 (LARP1) as a target of mTORC1 and as a key repressor of TOP mRNA translation

In the last ~25 years since the discovery of the 5' TOP motif⁶⁰, several candidate *trans*-acting factors have been proposed to control TOP mRNA translation (and, by extension, ribosome production). Many of them did not fulfil essential criteria for TOP mRNA translation regulation and were either disregarded over time or eventually refuted (see review⁷² for a detailed analysis of each candidate). Recently, we²⁴ proposed that La-related protein 1 (LARP1) may function as the elusive *bona fide trans*-acting repressor of TOP mRNA

Sci U S A 1991, 88:3319–3323.) following the observation that ribosomal proteins mRNA contained a 5' terminal oligopyrimidine (5' TOP) motif.

translation. This section details the sequence of events that led to the discovery of LARP1 as a *trans*-acting repressor of TOP mRNA translation.

The LARP superfamily of proteins

LARP1 belongs to the LARP superfamily that comprises 7 members: LARP1/1a, LARP2/1b, La, LARP4/4a, LARP5/4b, LARP6 and LARP7. LARP proteins share common domain architecture but also unique features (see Fig. 1 for details and reference ⁶⁷ for an in-depth review on the LARP superfamily). AllLARPs contain an evolutionarily conserved La module ^{6, 9} that comprises the La motif (LaM) and one or more RNA-recognition motifs (RRMs). In addition to the La module, LARP1 and LARP2 (also referred to as LARP1a and LARP1b, respectively) contain a unique region known as the DM15. The DM15 motif was discovered in a computational search for repeat sequences in *Drosophila melanogaster*⁹¹. The DM15 is evolutionarily conserved in animals (vertebrate and invertebrate), plants and algae, protista, and some fungi ⁵⁸. *Saccharomyces cerevisiae* encodes 2 proteins (Sro9 and Slf1) ^{52, 98} with a conserved La-module; however, the DM15 is conspicuously absent in these proteins arguing against these proteins acting as functional homologs of LARP1 in yeast.

In early 2013, Natsume and colleagues ³ reported that LARP1 protein preferentially binds to TOP mRNAs and that LARP1 knockdown leads to decreased steady-state levels, suggesting a LARP1-mediated regulation of the abundance/stability of this class of transcripts. Around this time, we were pursuing the characterization of novel downstream targets of mTORC1 and identified LARP1 in mTORC1 immunoprecipitates by mass spectrometry ²⁴. The observation that LARP1 interacted with mTORC1 together with the Natsume's original report that LARP1 interacts with TOP mRNAs led us to hypothesize that LARP1 might function as the long-sought TOP mRNA *trans*-acting translation repressor ⁸ regulated by mTORC1. We set out to test this hypothesis and confirmed that LARP1 does indeed interact with TOP mRNAs *via* their 5' TOP motif and that LARP1 is required for TOP mRNA stabilization. Then, we tested whether LARP1 controls TOP mRNA translation utilizing rapamycin and Torin1 that preferentially repress the translation of TOP mRNAs. Importantly, stable depletion of LARP1 (by shRNA-mediated knockdown) relieves the repression of TOP mRNA translation by rapamycin and Torin1. Conversely, transient overexpression of LARP1 represses the translation of TOP mRNAs even in conditions of nutrient abundance. Together, these data led us to hypothesize that LARP1 functions as *trans*-acting repressor of TOP mRNA translation as it fulfilled the 3 pre-requisites: (i) association with the 5' TOP motif, (ii) repression of TOP mRNA translation, and (iii) regulation by mTORC1 ²⁴.

4 LARP1 binds to the 5' TOP motif through the DM15 region

Following the identification of LARP1 as a *bona-fide* repressor of TOP mRNA translation and the observation that LARP1 associates with the 5' TOP motif ²⁴, we sought to identify the domain of LARP1 that mediates 5' TOP motif binding. Two publications ^{3, 79} had suggested that the C-terminal region of LARP1 (that comprises the DM15) might be involved in RNA binding. We performed electrophoretic mobility shift assays (EMSAs)

using a recombinant fragment of human LARP1 spanning residues 796–946 of the DM15 motif in combination with the 5'UTR of RPS6 mRNA to investigate this assertion⁵⁸. These data revealed that the DM15 directly binds the 5'TOP motif with moderate affinity (mid-nanomolar K_d value)⁵⁸. To further understand the role of DM15 in 5'TOP motif binding, we solved the X-ray crystal structure of the DM15 region from human LARP1 to 1.86 Å resolution. The *apo* crystal structure of DM15 from human LARP1 revealed that this domain folds in to three HEAT-repeat-like structures akin to those found in other proteins including Huntingtin, Elongation Factor 3, the A subunit of protein phosphatase 2A and mTOR, from which the HEAT module derived its name. These studies revealed that these tandem HEAT-like repeats may extend N-terminally to include an additional helical repeat. Found in 3–36 tandem arrays, these structural repeats form a concave surface that most commonly facilitates protein-protein interactions in Huntingtin, EF3, PP2A and mTOR. In the case of LARP1, however, the DM15 has been repurposed as an RNA binding domain that directly interacts with the 5'TOP motif⁵⁸. Interestingly, the ribosomal protein mRNAs in budding yeast lack a 5'TOP motif; this is consistent with the observations that the DM15 region is not present in Sro9 and Slf1 (the closest-related LARP1 homologs) and that the synthesis of ribosomes in yeast is not subject to translation control¹¹². Together, these data suggest that LARP1 may exert its inhibitory effect of TOP mRNA translation in higher eukaryotes *via* the association of the DM15 with the 5'TOP motif, but what is the molecular mechanism underlying DM15-mediated TOP mRNA translation repression?

5 LARP1 binds the m⁷Gppp cap moiety and adjacent 5'TOP motif

The 5' m⁷Gppp mRNA cap is a hallmark of all nuclear-encoded mRNAs that is co-transcriptionally synthesized by three enzymes recruited to the 25–30-nt nascent transcript by specific phosphorylation of the RNA polymerase II C-terminal domain (reviewed in⁹⁶). The m⁷Gppp mRNA cap functions as a key regulatory element in each step throughout the life of a transcript from RNA processing, export, storage, degradation and translation²⁵. Cap-binding proteins bind to the mRNA cap structure and mediate these processes¹¹¹. Several eukaryotic cap-binding proteins have been identified to date. These include, the eukaryotic initiation factor 4E family that comprises 3 members: eIF4E1 (or simply as eIF4E)^{20, 99, 100}, eIF4E-homologous protein (4EHP, also known as eIF4E2)⁴, and eIF4E3⁸⁰; the cap-binding protein complex (CBP) that consists of CBP20 (the cap-binding protein) and CBP80 (an accessory protein)^{46, 47}; the family of decapping proteins DCP1^{7, 56, 65}, DCP2^{18, 65, 115}, DCPS⁶³ and DXO,⁵⁰ and the poly(A)-specific ribonuclease (PARN)^{14, 54}.

Cap-dependent translation initiates in the cell cytoplasm when eIF4E recognizes the m⁷Gppp mRNA cap structure¹¹¹. eIF4E assumes a cupped hand-shaped architecture whereby the concave (or ventral) face binds the m⁷Gppp moiety^{68–70} and the convex (or dorsal) face mediates interactions with a number of proteins including the eukaryotic initiation factor 4G (eIF4G), a high-molecular weight scaffolding protein, and eIF4E-binding proteins (4E-BPs), a family of low molecular weight phosphoproteins. Importantly, the binding of eIF4G to eIF4E stabilizes the association of eIF4E with the mRNA cap^{35, 114}.

LARP1 interacts with the 5'TOP motif of RPS6 and RPL32 mRNAs^{24, 58} and this interaction is mediated by the LARP1-specific DM15 region⁵⁸. Recent structural and

biochemical and studies from our groups⁵⁷ revealed, however, that the interaction of the DM15 region of LARP1 with TOP mRNAs not only includes the 5' TOP motif, but also incorporates the adjacent 5' m⁷Gppp cap moiety. These findings demonstrate that LARP1 is a novel mRNA cap-binding protein and provide unprecedented important insights into the mechanism by which LARP1 represses TOP mRNA translation⁵⁷. LARP1 can, thus, be added to the list of cap-binding proteins described above. The events that led to identification of LARP1 as a cap-binding protein are described below.

In an effort to elucidate the molecular mechanism by which LARP1 represses TOP mRNA translation, we utilized X-ray crystallography to determine the RNA-bound structure of human LARP1 DM15 region (amino acids 796–946) resolved to 2.6 Å. The RNA oligonucleotide utilized mimics a segment of the 5' TOP motif of human RPS6 mRNA (5'-CUUUUCCG-3') (Fig. 2). The sequence and length choice were guided by the *apo* DM15 structure and the results of nuclease protection assays with the RPS6 TOP RNA⁵⁸. Selection of this particular sequence was particularly serendipitous, as it allowed us to make some expected and unexpected observations.

As anticipated by previous mutagenesis studies, the three tandem helix-turn-helix structural repeats of the DM15 engage the negatively charged phosphate backbone of the RNA through a positively charged concave surface that is created by a swath of highly conserved residues. Surprisingly, the crystal lattice revealed that one DM15 molecule simultaneously bound two RNA molecules: nucleotides 1–5 of one RNA and the 3' guanosine reaching from the neighboring DM15 molecule in the crystal lattice. This crystal packing positioned the terminal guanosine of one RNA next to the first cytosine of the TOP-like sequence of another RNA in the RNA-binding site of DM15; this observation led us to hypothesize that the guanosine bound 5' to the first cytosine of the TOP-like motif could represent the 5'-7-methylguanosine cap of a TOP transcript.

The DM15 region recognizes this guanosine in a guanosine recognition pocket whose architecture is similar to that of other cap-binding proteins (Fig. 3). Two conserved tyrosine residues stabilize this nucleotide with cation- π interactions. Additionally, the Watson-Crick face of the guanosine is recognized by a conserved acid amino acid residue (E886). To confirm that these residues form a 7-methylguanosine-recognition pocket, we determined the co-crystal structure of DM15 bound to the cap analog 7-methylguanosine triphosphate (m⁷GTP) to 1.8 Å resolution; indeed, m⁷GTP is stabilized in this pocket, confirming that the DM15 region of LARP1 indeed binds the m⁷Gppp cap structure.

To further establish the specificity of DM15 binding to methylated/capped RPS6 mRNA, we performed electrophoretic mobility shift assays (EMSAs) using a recombinant construct of LARP1 that contains the DM15 region and a radiolabeled 7-methylguanosine triphosphate-capped RNA oligo spanning the entire 5' UTR (42-mer) of RPS6 mRNA. The data revealed that the DM15 region of human LARP1 binds to the m⁷Gppp-RPS6 42-mer with high affinity (K_d in the low nanomolar range). Moreover, these data indicated that, unlike most cap-binding proteins that prefer a purine at the +1 position (non-TOP transcripts), the DM15 region of LARP1 has ~90-fold greater affinity for a capped RNA substrate with a cytosine immediately following the cap. This could explain why previous RNA pulldowns with

capped oligonucleotides having a purine in the +1 position were not enriched for LARP1. Together, these data suggest that LARP1 preferentially interacts with capped mRNAs that contain a cytosine at the +1 position. To confirm this notion, we determined the crystal structure of the dinucleotide m⁷GpppC bound to the DM15 of human LARP1 at 1.7 Å resolution⁵⁷. The resulting structure supports the model of specific recognition of the invariant +1 cytosine that characterizes the 5'TOP motif of every TOP mRNA. This result is of particular importance because the +1 cytosine is essential for efficient repression of TOP mRNA translation⁸. The crystal structures provide a strong molecular basis for the TOP-specific cap recognition of TOP mRNAs by LARP1, but how does LARP1 recognition of the mRNA cap and 5'TOP motif confer translation repression of TOP mRNAs?

6 LARP1 blocks the binding of eIF4E to the m⁷Gppp cap of TOP mRNAs

A key rate-limiting step to mRNA translation initiation is the binding of eIF4E to the mRNA m⁷Gppp cap structure and its vicinity⁵⁵. Despite it recognizing the cap of all nuclear-encoded mRNAs, eIF4E binds to capped-mRNAs with varying affinities in accordance with the cap-proximal nucleotides^{53, 105}. In contrast to LARP1, eIF4E exhibits lower affinity for a 5' cytosine than for a 5' guanosine¹⁰⁵. To better understand the mechanism by which LARP1 inhibits TOP mRNA translation, we compared the affinity of recombinant human eIF4E with that of the DM15 fragment of human LARP1 for capped m⁷Gppp-RPS6 5'UTR by EMSA and noted that LARP1 binds with higher affinity than eIF4E to this TOP mRNA⁵⁷, consistent with LARP1 showing a preference for transcripts beginning with a cytosine and eIF4E a preference for transcripts beginning with a guanosine. Moreover, we observed that the DM15 efficiently displaces eIF4E from m⁷Gppp-RPS6 5'UTR, even when added substoichiometrically, while eIF4E cannot dissociate the DM15-m⁷Gppp-RPS6 complex, even when added in molar excess⁵⁷. Notably, replacement of the first cytosine for a guanosine within the 5'TOP motif of RPS6 or omission of the m⁷Gppp-cap structure considerably reduces the affinity of the DM15 region for the RPS6 5'UTR, confirming that the DM15 interacts with both the 5'TOP sequence as well as the m⁷Gppp-mRNA cap structure⁵⁷.

7 LARP1 blocks the assembly of eIF4F on TOP mRNAs

While the ventral face of eIF4E mediates mRNA binding to the m⁷Gppp cap (section 6), its dorsal face mediates interactions with a number of proteins. For instance, eIF4E associates with eIF4G^{59, 66, 93, 118} through the lateral surface of eIF4G³³. In turn, eIF4G interacts with eIF4A (an ATP-dependent RNA helicase)^{17, 19, 45, 78, 88} that unwinds secondary structures within the 5'UTR of mRNAs with long and complex 5'UTRs⁸². Together, eIF4E, eIF4G, and eIF4A form the heterotrimeric eIF4F complex that recruits the 43S pre-initiation complex (PIC) to the mRNA for translation⁴⁸. Mammals express three eIF4G proteins: eIF4G1, eIF4G2 and eIF4G3 (also known as p97 or DAP5). eIF4G1 and eIF4G2 share 46% identity at the amino acid level and show functional homology; both isoforms interact with eIF4E and eIF4A to mediate cap-dependent translation³². On the other hand, eIF4G3 does not bind eIF4E and mediates cap-independent translation^{36, 61}. Data by Sabatini and colleagues¹¹⁰ indicate that eIF4G1 (but not eIF4G2) stimulates the translation initiation of TOP transcripts; stable knockdown of eIF4G1, but not eIF4G2,

selectively decreases the translation of RPS20 and eEF2 TOP mRNAs as monitored by polysome profiling¹¹⁰. Motivated by this observation, we tested whether eIF4G1 physically associates with TOP mRNA²⁴. To this end, we performed RNA-IP experiments using an antibody against endogenous eIF4G1 protein from human fibroblasts stimulated with serum²⁴. Quantitative analysis of the TOP mRNAs co-precipitated with eIF4G1 revealed that this class of transcripts associates with eIF4G *in vivo*²⁴. This finding lends support for a role for the eIF4F complex in the control of TOP mRNA translation¹¹⁰.

Since LARP1 and eIF4E/eIF4G associate with TOP mRNAs, we asked whether these two complexes compete for TOP mRNA binding. To address this question, we carried out RNA IP experiments targeting endogenous eIF4G1 in extracts from human fibroblasts that had been stably depleted of LARP1 by RNAi; we observed that knocking down LARP1 enhances the co-precipitation of TOP mRNAs with eIF4G1²⁴, suggesting that LARP1 and eIF4G compete for association with TOP mRNAs. To further validate this possibility, we performed the same experiment using extracts from human fibroblasts transiently overexpressing LARP1; consistent with the knockdown experiments, transient overexpression of LARP1 markedly reduces the co-precipitation of TOP mRNAs with eIF4G1²⁴. Notably, overexpression of a LARP1 mutant (R840E/Y883A), defective for m⁷Gppp cap- and 5' TOP motif-binding, does not impair the interaction between eIF4G1 and TOP mRNAs⁵⁷. Collectively, these findings lead us to propose a model (illustrated in the Graphical Abstract) in which LARP1 represses the translation of TOP mRNAs by blocking the assembly of the eIF4F complex on the 5' end of TOP mRNAs^{24, 57}. But how is LARP1 release/eIF4F assembly on TOP mRNAs achieved on a molecular level?

8 mTORC1 coordinates LARP1 release and eIF4F assembly on TOP mRNAs

The assembly of the eIF4F complex, specifically the interaction between eIF4E and eIF4G1, is regulated by reversible phosphorylation events of eIF4E-binding proteins (4E-BPs), a family of low-molecular weight translation inhibitor proteins. In vertebrates, the 4E-BP family consists of three closely-related members: 4E-BP1, 4E-BP2 and 4E-BP3^{15, 16, 42, 62, 85, 92} that exert their inhibitory effects on translation initiation through a common mechanism: 4E-BPs block the assembly of the eIF4F complex^{2, 34} by competing with eIF4G for binding to overlapping regions on the dorsal surface of eIF4E^{33, 44, 89}. The binding of 4E-BP1 and 4E-BP2 to eIF4E is regulated by sequential phosphorylation of Thr46, followed by Thr37, Thr70, and lastly Ser65^{29, 30, 37, 64}. mTORC1 catalyzes the phosphorylation of all four phosphorylation sites *in vitro*^{10, 11} and *in vivo*^{75, 76}, while 4E-BP3 is transcriptionally induced upon mTORC1 inhibition¹¹³. mTORC1-mediated phosphorylation of 4E-BPs releases them from eIF4E^{75, 76}, thus allowing eIF4G1 to bind to eIF4E.

mTORC1 signaling plays a prominent role in the control of TOP mRNA translation^{23, 40, 110}. Rapamycin, an allosteric mTORC1 inhibitor, was first reported to inhibit TOP mRNA translation in 1994¹⁰⁸. Polysome profiling studies^{26, 40, 110} confirmed this notion and revealed that TOP mRNA transcripts comprise the class of transcripts that is most sensitive to translation repression by mTOR inhibitors studies. Given that eIF4G1 is required for TOP mRNA translation¹¹⁰ and that 4E-BPs regulate eIF4F assembly, it was originally

postulated that 4E-BPs may function as the primary point of regulation of TOP mRNA translation downstream of mTORC1¹¹⁰. Subsequent work by Meyuhas and colleagues showed that genetic deletion of 4E-BPs alone is not enough to stimulate TOP mRNA translation in conditions of mTORC1 inhibition⁷⁴. More likely, release of 4E-BPs from eIF4E is necessary but insufficient for eIF4F assembly on TOP mRNAs. It follows that mTORC1 must control TOP mRNA translation through an additional target, other than 4E-BPs. A plausible explanation for why genetic loss of 4E-BPs is insufficient for engaging TOP mRNA translation in conditions of mTORC1 inhibition is that LARP1 remains tightly associated with the m⁷Gppp cap and the 5'TOP motif under these conditions²⁴. RNA immunoprecipitation experiments demonstrated that eIF4G1, as is the case for LARP1, interacts with TOP mRNAs in an mTORC1-dependent manner²⁴. Specifically, treatment of human embryo kidney fibroblasts with rapamycin or Torin1 releases TOP mRNAs from eIF4G1²⁴. Contrary to the binding of eIF4G1 to TOP transcripts, the binding of LARP1 to TOP mRNAs is *enhanced* (rather than diminished) by rapamycin and Torin1²⁴. This observation suggested to us that the translation of TOP mRNAs alternates between two states: (1) a translationally-competent state, in which they are bound to eIF4E and eIF4G1 and (2) a translationally inactive state, in which they are sequestered by LARP1. While this review was in preparation, a study by Philippe et al.⁹⁰ confirmed our model²⁴ that LARP1 functions as a repressor of TOP mRNA translation; specifically, the authors show that the DM15 region of LARP1 represses TOP mRNA translation *in vitro* and it does so *via* the combined recognition of both the m⁷Gppp cap moiety and the adjacent 5'TOP motif of TOP mRNAs.

9 LARP1 is a direct target of mTORC1

mTORC1 elicits its effects on mRNA translation through the phosphorylation of multiple targets. LARP1 interacts with mTORC1²⁴ and phosphoproteomic studies suggest that it undergoes phosphorylation on multiple serine, threonine and tyrosine residues^{41, 119}. Over 26 rapamycin-sensitive phosphorylation sites have been reported for LARP1¹¹⁹, some of which are directly phosphorylated by mTORC1 *in vitro*^{38, 51}. Two residues in particular, Ser774 and Ser766 (amino acid numbering according to the 1096-amino acid isoform of human LARP1),³ were phosphorylated *in vitro* by mTORC1⁵¹. Though both sites were verified as direct mTORC1 phosphorylation sites, they are phosphorylated by mTORC1 to different extents⁵¹. Ser689, which lies within the peptide sequence Thr-Ile-Ala-Arg-Ser-Leu-Pro-Thr-Thr, is more efficiently phosphorylated by mTORC1 *in vitro* than is Ser697, located within the peptide sequence Thr-Val-Glu-Ser-Pro-Asn-Tyr-Arg. Ser689 lies within an Xaa-Ala-Lys/Arg-Ser-Leu-Pro amino acid sequence (where Xaa denotes any amino acid), which is also the context of another confirmed mTORC1 phosphorylation site, Ser183 in PRAS40^{22, 81}; Ser697, on the other hand, is immediately followed by a Pro residue, as is the case for many other confirmed mTORC1 phosphorylation sites (*e.g.* Thr37, Thr46 and Ser65 in 4E-BPs, Ser758 in ULK1, Ser150 and Ser476 in Grb10⁵¹). Notably, Ser689 and Ser697 display differential sensitivity to rapamycin and Torin1: phosphorylation of Ser697 is inhibited by both rapamycin and Torin1, while phosphorylation of Ser689 is sensitive to Torin1 but insensitive to rapamycin⁵¹. A recent study by Hong et al.³⁸ confirmed that Ser689 is a direct target of mTORC1. This suggests a preferred

or context-dependent mTORC1 activity and some kinase redundancy with these two sites. Other kinases (namely AKT and S6K1) have also been implicated in the phosphorylation of LARP1: Inoki and colleagues³⁸ mapped Ser770 within the Arg-Pro-Arg-Thr-Ala-Ser peptide and Ser979 within the Arg-Lys-Arg-Cys-Pro-Ser peptide as substrates of AKT and S6K1 *in vitro* and *in vivo*. The significance of these phosphorylation sites remains unclear. Nonetheless, particularly interesting from the perspective of TOP mRNA recognition, these phosphorylation sites reside just N-terminal to the LARP1-specific DM15 domain that mediates binding to the m⁷Gppp cap and the 5' TOP motif^{57, 58}, suggesting that mTORC1 regulates LARP1 association with the 5' end of TOP mRNAs through multisite phosphorylation. Further work is required to elucidate the role of mTORC1-mediated phosphorylation of LARP1 on the binding of LARP1 to TOP mRNAs and its significance in the context of TOP mRNA translation repression.

10 LARP1 controls TOP mRNA stability

Though this review focuses primarily on the role of LARP1 in the control of TOP mRNA translation, LARP1 has been shown to play additional roles on TOP gene expression, namely in the control of TOP mRNA stability^{3, 24, 27}. LARP1 was, in fact, first implicated in the stabilization of TOP mRNAs³ and only subsequently linked to the repression of TOP mRNA translation²⁴. Aoki et al.³ used RNA immunoprecipitation to show that LARP1 preferentially interacts with highly abundant TOP mRNAs. They also noted that transient depletion of LARP1 by RNAi results in a decreased expression of mature TOP mRNAs as compared to their precursors. This latter observation led Natsume and colleagues to conclude that LARP1 positively regulates TOP gene expression (likely *via* preferential stabilization of TOP mRNA transcripts)³. Spurred on by Natsume's findings, we tested the effect of transient overexpression of LARP1 on the stability of TOP mRNAs in actinomycin D-treated human fibroblasts; consistent with Natsume's original proposal, we observed that LARP1 indeed stabilizes TOP mRNAs in human cells²⁴. Together these results demonstrate that LARP1 plays a dual role in TOP gene expression: on the one hand, LARP1 negatively regulates (represses) TOP mRNA translation by preventing the assembly of the eIF4F complex on TOP mRNAs. On the other hand, upon binding to TOP mRNAs LARP1 stabilizes this class of transcripts²⁴.

11 LARP1 and Ribosomopathies

What is the physiological significance of LARP1 binding to TOP mRNAs for regulation of TOP gene expression? A recent paper by Thomas and colleagues²⁷ delved into a possible physiological meaning for LARP1-mediated TOP mRNA stabilization. Thomas and colleagues confirmed earlier findings^{3, 24} that transient LARP1 depletion by RNAi with small interfering RNAs destabilizes TOP mRNAs and remarked that increased TOP mRNA decay in these cells is accompanied by a small, but reproducible accumulation of p53 protein and induction of cell-cycle arrest at G1 phase²⁷. This led Thomas and colleagues to speculate that LARP1 RNAi-mediated destabilization of TOP mRNAs induces an impaired

³Numbering according to the 1096 amino acid-long human LARP1 splice variant. The corresponding numbering for residues Ser774 and Ser766 in the 1019 amino acid-long human LARP1 splice variant are Ser689 and Ser697, respectively.

ribosome biogenesis checkpoint (IRBC) response in which p53 becomes stabilized and cells undergo cell-cycle arrest. An earlier study^{86, 101} demonstrated that myelodysplastic syndrome (MDS) patients with deletions in the 5q chromosome arm exhibit reduced TOP mRNA levels. The relative proximity of the *LARP1* gene locus at 5q33.2 to the commonly deleted region in 5q⁻ myelodysplastic patients suggested that loss of LARP1 may be associated with 5q⁻ myelodysplastic syndrome, a type of blood cancer characterized by the inability of blood cells to undergo full maturation, resulting in low red blood cell counts and anaemia. Interestingly, in support of this notion, depletion of LARP1 by RNAi leads to a ~20–30% reduction in the induction of globin mRNAs²⁷, suggesting that LARP1 deletion in 5q⁻ myelodysplastic syndrome (MDS) may be intimately related to the pathological development of this disease.

12 Other key factors in TOP mRNA translation: TIA proteins play an important role in starvation-mediated TOP mRNA repression

So far, we have discussed the critical function(s) of LARP1 in TOP gene expression with an emphasis on its role in repressing TOP mRNA translation²⁴. The importance of understanding the molecular mechanisms underlying ribosome biosynthesis has raised considerable interest in the study of TOP mRNA translation and in the identification of the elusive *trans*-acting repressor of TOP mRNA translation⁷². The available data indicate that LARP1 likely plays a critical role in TOP mRNA repression^{24, 57}. These findings do not rule out the existence of additional *trans*-acting repressors. It is conceivable that repression of TOP mRNA translation (during various stresses that stall cell growth and proliferation) is not mediated by a single master-regulator, a concept that has been articulated for decades in the field. An alternative view that we favour and will expound here is that full repression of TOP mRNA translation is orchestrated by, not one, but several *trans*-acting factors, whose localization, abundance, and activity are heavily regulated in different cellular contexts. In support of this hypothesis, we have previously identified the stress granule-associated TIA-proteins, TIA-1 and TIAR (TIA/R) as important factors in TOP-mRNA repression during amino acid starvation¹³, which are conditions under which mTORC1 is deactivated and GCN2 signalling becomes activated. These findings were confirmed by Meyuhas and co-workers⁷⁴, who went on to demonstrate that the repressive function of the TIA-proteins is restricted to amino acid starvation, but does not occur under other stresses such as hypoxia or serum starvation. Hence, even though amino acid starvation and hypoxia (but not serum starvation) can induce stress granule formation, in which TOP mRNAs can accumulate¹³, these stresses rely differently on the integral stress granule factors TIA/R⁷⁴. We, and others have observed a minimal effect on the ability of the TIA proteins to repress TOP mRNA translation upon mTORC1 pathway inhibition¹¹⁰ (and our unpublished results), indicating that these repressors function indirectly and temporally downstream of mTORC1. So how do the TIA-proteins act if not directly regulated by mTORC1? One attractive possibility is that the TIA-proteins sustain an initial repression exerted by other primary 5' TOP *trans*-acting factors (*e.g.* LARP1) by physical sequestration from translationally-competent pools of ribosomes, which likely depends on GCN2 activation¹³. Further work is required to explore a possible interplay between LARP1 and TIA proteins.

13 Conclusion

The synthesis of large macromolecular complexes requires the concerted expression of several components in a temporal and stoichiometric manner. The synthesis of the eukaryotic ribosome is subject to precise translational control to ensure the production of translationally-competent ribosomes. Higher eukaryotes have devised a simple mechanism for the precise synthesis of each ribosomal protein component in proportion to its stoichiometry in the ribosome^{71, 72, 87} such that the resultant ribosomes can be correctly assembled and translationally-competent. This mechanism involves a *cis*-regulatory RNA element (the 5' TOP motif) and a cognate *trans*-acting factor (LARP1 protein) that binds to and represses the translation of these mRNAs^{24, 58}. Structural studies^{57, 58}, confirmed by recent biochemical experiments⁹⁰, provide important insights into the molecular mechanism underlying LARP1-mediated TOP mRNA translation repression that was initially proposed in 2015²⁴: in an mTORC1-dependent manner, LARP1 recognizes the m⁷GpppC characteristic of and specific to 5' TOP mRNAs, excluding access of eIF4E to the cap, and limiting the translation of bound transcripts. Thus, the majority of published data suggests that LARP1 plays the role of a translational repressor of TOP mRNA translation.

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Further Reading

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Figure 1. LARP superfamily *Homo sapiens* (Hs) and *Saccharomyces cerevisiae* (Sc) evolutionary tree

Abbreviations: *LaM*, La motif; *LARP1*, La-related protein 1; *LARP2/1b*, La-related protein 2/1b; *La*, lupus autoantigen; *LARP4/4a*, La-related protein 4/4a; *LARP5/4b*, La-related protein 5/4b; *LARP6*, La-related protein 6; *LARP7*, La-related protein 7; *RRM*, RNA-recognition motif; *RRM-L4*, RNA recognition motif-like 4; *RRM-L5*, RNA recognition motif-like 5; *PBM*, PABP binding motif; *PAM2*, PABP interaction motif 2; *LSA*, LaM and S1 associated motif; *5' TOP*, 5' terminal oligopyrimidine; *SBM*, short basic motif; *sn7SK RNA*, small nuclear 7SK RNA; *SL*, stem loop.

Figure 2. Crystal packing revealed a G-binding pocket 5' of the +1C binding pocket in the LARP1 DM15 region

Crystals are comprised of molecules packed into unit cells that relate to each other by symmetry operations; three unit cells are shown (PDBID 5V7C). The red DM15 molecule (cartoon representation) binds nucleotides C1–U5 of the yellow oligonucleotide (stick representation). Nucleotide G8 from the RNA bound to the neighboring molecule of DM15 (RNA, C1–C7 shown as a black line connected to G8 shown as sticks; DM15, grey cylinders) binds the red DM15 molecule upstream of the +1C of the yellow oligonucleotide.

Figure 3. Conservation of cap-binding architecture in LARP1

A. m⁷GpppC bound to the DM15 region of LARP1 (PDBID 5V87). **B.** m⁷GpppG bound to eIF4E (PDBID 3HXL).