

Biochemical and Structural Characterization of the *S. pombe* La-Related Protein 1 (Slr1p)

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Abstract

La and La-related proteins (LARPs) are conserved RNA-binding proteins that share a characteristic La motif (LaM) and have important functions in RNA metabolism. Several LARP families exist where members possess specific domains that allow for binding to unique substrates. The LARP1 family, which includes a DM15 domain, binds a cohort of mRNAs encoding factors involved in the process of mRNA translation. These mRNAs contain a sequence of 5-15 pyrimidines in their 5'UTRs, immediately following the m⁷G cap, and are characterized as 5' terminal oligopyrimidine (5'TOP) mRNAs. The DM15 domain of human LARP1 has been suggested to specifically recognize this motif, thereby affecting the translation and stability of 5'TOP mRNAs. However, the specific function of LARP1 in this context remains unclear. Intriguingly, the 5'TOP motif does not exist in yeast, and LARP1 orthologs in this system lack the characteristic DM15 domain. In this work, we characterize the *S. pombe* La-related protein 1, Slr1p, as an RNA-binding protein with conserved functions relative to other LARP1 orthologs. Through proteomic and transcriptomic studies, we show that Slr1p regulates the translation and stability of mRNAs that encode ribosomal proteins and other components of the translational apparatus in *S. pombe*. We further identify a 5' Proximal AC-rich (5'PAC) motif, residing close to the start AUG, in these mRNAs and propose that they represent a cohort of proto-5'TOP mRNAs. Through structural analyses and mutagenesis studies, we show that the LaM of Slr1p is responsible for binding to the 5'PAC RNA motif, where mutagenesis of a single amino acid in our characterized binding site disrupts this interaction. We further demonstrate that Slr1p plays a role in adaptation to translation stress and that its phosphorylation status may allow for several

functional states. Our findings are consistent with the conservation of LARP1 function across eukaryotes, and suggest that the involvement of this protein family in ribosomal protein mRNA translation and stability is influenced by a more fundamental LaM-dependent form of LARP1 regulation that is utilized in *S. pombe*.

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List of Abbreviations

3-AT: 3-amino-1,2,4-triazole	ORF: open reading frame
4EBP: eIF4E-binding protein	PABP: poly(A)-binding protein
4EBP1/2: eIF4E-binding proteins 1 and 2	PAC10: 10-mer Proximal AC-rich motif
5'DUR: 5' Distal U-Rich	PAC15: 15-mer Proximal AC-rich motif
5'PAC: 5' Proximal AC-rich	PAC19: 19-mer Proximal AC-rich motif
5'TOP: 5' terminal oligopyrimidine	PAM2: PABP-interacting motif
CDS: coding sequence	PIC: pre-initiation complex
CTD: C-terminal domain	PrA: protein-A
eIF: eukaryotic initiation factor	PRTE: pyrimidine-rich translational element
EMM: Edinburgh Minimal Media	RATE-Seq: RNA approach to equilibrium sequencing
EMSA: electrophoretic mobility shift assay	RNA-Seq: RNA sequencing
GO: Gene Ontology	RIP-Seq: RNA-immunoprecipitation and sequencing
hLARP1: human LARP1	RNP: ribonucleoprotein
KO: knockout	RP: ribosomal protein
LaM: La motif	RRM: RNA Recognition Motif
LARP: La-related protein	rRNA: ribosomal RNA
LARP1-L: long-form LARP1	Slr1p: <i>S. pombe</i> La-related protein 1
LARP1-S: short-form LARP1	TCP-Seq: translation-complex profiling and sequencing
m⁷G: 7-methylguanosine	tRNA: transfer RNA
mRNA: messenger RNA	UTR: untranslated region
mtDNA: mitochondrial DNA	WT: wildtype
mTOR: mammalian target of rapamycin	YES: Yeast Extract plus Supplements
NMR: Nuclear Magnetic Resonance	
NTD: N-terminal domain	
OD: optical density	

Chapter 1: Introduction

LARP1 function across eukaryotes: A review

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Portions of this chapter are in preparation for publication as a review.

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1.1. Preamble

Gene expression needs to be maintained with high fidelity in all living organisms. Different factors, including RNA molecules, are involved in this process. Messenger RNAs (mRNAs) code for a series of amino acids, and the synthesis of a functional protein requires an mRNA template. mRNAs are transcribed as products of genes and must be decoded through a process called translation. This is made possible with the help of many translation factors and other RNAs, such as transfer RNAs (tRNAs), which supply the growing chain of amino acids, and ribosomal RNAs (rRNAs), which function as components of ribosomes. The need for the synthesis of different types of proteins is dynamic and depends on several signalling events. Therefore, the process of translation is tightly regulated to ultimately regulate gene expression.

Cells need to respond to stress and other physiological cues, and this is often controlled by maintaining levels of mRNAs, which is a combination of their transcription, stability and the degree to which they are translated. mRNA translation is controlled at multiple levels and through several cis- and trans-acting factors. Aside from the coding sequence, mRNAs contain non-coding regions upstream and downstream of the open reading frame (ORF): these regions are referred to as the 5' and 3' untranslated regions (UTRs), respectively. In general, the UTRs play important roles in the localization, translation and stability of mRNAs. They can also mediate protein-protein interactions, influence the formation of mRNA-protein complexes and behave as individual units of information independent of the mRNA's coding region¹. These functions are achieved through the recruitment of several effector proteins to regulatory elements contained within the UTRs¹. These include RNA-binding

proteins which can influence mRNA translation as well as stability to effectively modulate gene expression.

Studying how the cell coordinates these processes in response to stress and understanding how RNA-binding proteins enable different forms of regulation are central to studying gene expression and its implications for human health. While the complexity of these interconnected processes makes this more challenging to study in human cells, model organisms have been a powerful tool for studying conserved mechanisms of gene expression, as well as how RNA-binding proteins function in these processes, in simpler systems. Traditionally, yeast models have been effective in studying the mechanisms of eukaryotic translation and RNA decay. This is due to their short life cycles, ease of genetic manipulation, well-annotated genomes and the strong conservation of biological and biochemical pathways in these systems².

1.2. Principles of cap-dependent mRNA translation

The initiation step of mRNA translation is highly regulated. While this step commonly occurs through cap-dependent mechanisms, it can also happen through cap-independent processes. mRNA translation requires many translation factors, including ribosomes which consist of approximately 80 ribosomal proteins and four rRNAs that are assembled into two ribosomal subunits. The eukaryotic small (40S) ribosomal subunit includes the 18S rRNA, and the large (60S) ribosomal subunit contains the 5S, 5.8S and 28S rRNAs. In cap-dependent mRNA translation, the 7-methylguanosine (m⁷G) containing mRNA 5' cap is

initially complexed with components of the eukaryotic initiation factor 4F (eIF4F). These include a cap-binding protein, eIF4E, a large scaffold protein, eIF4G, and a helicase, eIF4A (Figure 1). An interaction between eIF4E and eIF4G is essential for cap-dependent mRNA translation initiation, which can be occluded by unphosphorylated eIF4E-binding proteins (4EBPs). These proteins are substrates of the mammalian target of rapamycin complex 1 (mTORC1) and their phosphorylation by mTORC1 leads to their dissociation from eIF4E. When eIF4E directly contacts eIF4G, eIF3 and the 40S small ribosomal subunit are recruited to form the 43S pre-initiation complex (PIC). Following this, the 43S PIC will begin scanning the 5'UTR until it reaches a start codon. At this point, the 60S large ribosomal subunit will be recruited to form the 80S ribosome and translation will proceed^{3,4}.

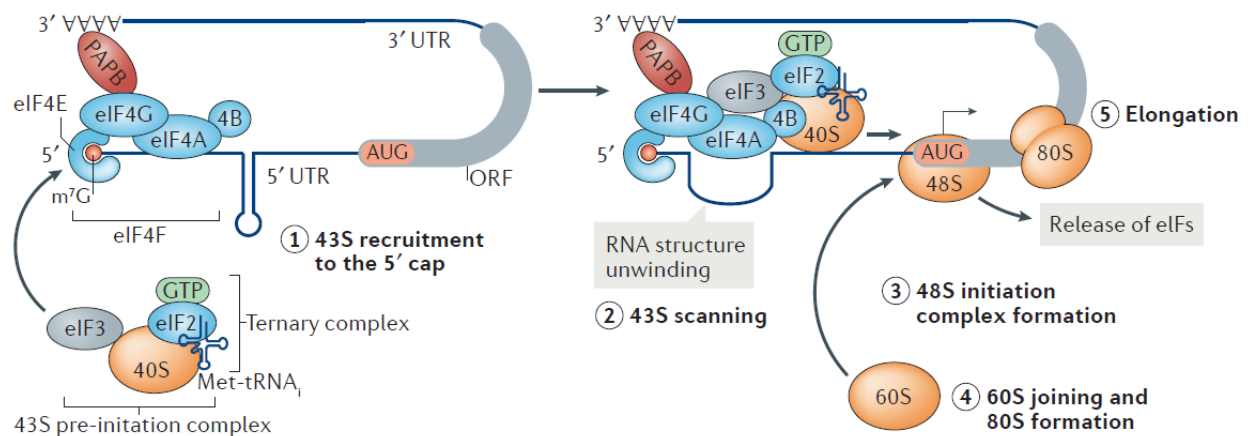


Figure 1: Canonical cap-dependent mRNA translation initiation. Several translation initiation factors and ribosomal subunits are recruited to an mRNA during this process. (Adapted from Leppek *et al.*, 2018)⁴

1.3. Principles of mRNA stability

mRNAs undergo different processing events; amongst which the addition of a 5' m⁷G cap and a 3' poly(A) tail are crucial in yielding a mature mRNA that is stable⁵. These structures serve to protect the mRNA from exonuclease activity and need to be removed prior to mRNA decay. The length of the poly(A) tail is dynamic: an mRNA goes through progressive deadenylation first by the Pan2/Pan3 deadenylases, which trim the poly(A) tail, and then by the CCR4-NOT deadenylase complex (or poly(A)-specific ribonuclease (PARN) found only in vertebrates), which remove the poly(A) tail (Figure 2)⁶⁻⁸. When the poly(A) tail is critically short such that its association with poly(A)-binding proteins (PABP) is disrupted, 3'-5' mRNA decay is triggered. This process is carried out by the exosome⁹. The 5' cap can then be hydrolyzed by scavenger decapping enzymes such as DcpS¹⁰.

Following deadenylation, the mRNA can also be decapped to undergo 5'-3' degradation. Decapping in yeast involves the DCP1/DCP2 complex, and a third factor, Hedls or Ge-1, which is also present in higher eukaryotes¹¹⁻¹³. Following decapping, the XRN1 exonuclease, which remains the best characterized factor in degradation from the unprotected 5' end, will digest the mRNA in a 5'-3' manner^{5,14}. Sm-like (Lsm) proteins can further enhance the efficiency of decapping and degradation in this case^{15,16}. An understanding of multiple levels of quality control has brought forward studies in mRNA surveillance where the mechanisms for degradation of defective mRNAs, leading to problems with translation, have also been characterized. These include nonsense-mediated decay (NMD), where mRNAs with premature translation termination codons are targeted for decay, and no-go decay (NGD),

which is triggered by stalling ribosomes and leads to endonucleolytic cleavage of the mRNA^{17,18}.

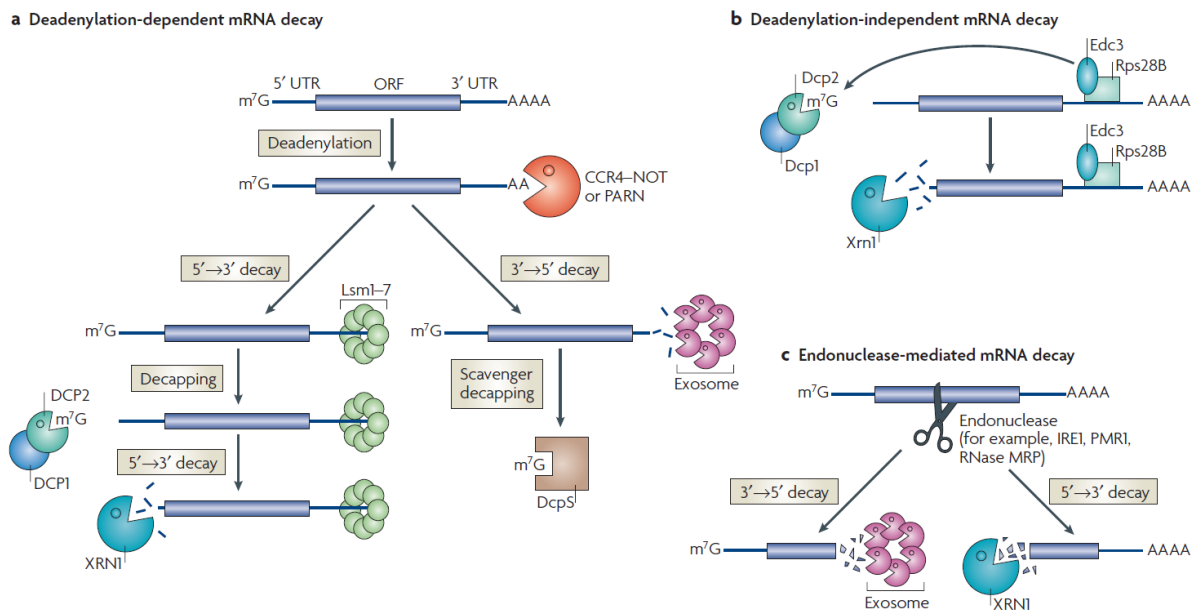


Figure 2: Mechanisms of cytoplasmic mRNA turnover. mRNA decay can happen through deadenylation-dependent or -independent processes. (Adapted from Garneau *et al.*, 2007)¹⁴

1.4. 5' Terminal oligopyrimidine sequences and their regulation by mTOR

While the first transcribed nucleotide of most mRNAs is usually a purine^{19,20}, a special subset of cellular mRNAs harbour a stretch of 5-15 pyrimidines in the 5' UTR, immediately after the m⁷G cap, and are consequently named 5' terminal oligopyrimidine (5'TOP) mRNAs²¹⁻²³. The first nucleotide in the 5'TOP motif is an invariant cytidine^{21,24}, and this unique +1C has been hypothesized to drive the regulation of 5'TOP mRNA expression through trans-acting factors. 5'TOP mRNAs code for proteins that are important in the process of mRNA translation,

including ribosomal proteins and translation initiation and elongation factors. These mRNAs are efficiently transcribed and translated in an evolutionary conserved manner²⁵. The unique motif is found in humans, other vertebrates and *Drosophila*, while a partial variant of it has been suggested to exist in *Arabidopsis*²⁶. On the other hand, the 5'TOP sequence is not reported to exist in *C. elegans*^{23,27} or yeast²³. Given that *Arabidopsis*, yeasts and humans all share a common ancestor, it is intriguing to consider that selective pressures may have driven the evolution of a refined 5'TOP sequence in fruit flies, which has persisted through to humans, while it appears to have been lost in *C. elegans* and yeasts (Figure 3). Furthermore, 5'TOP sequences have been suggested to link mRNA translation and stability²⁵. Given that 5'TOP mRNAs mainly code for ribosomal proteins, downstream events such as protein production, cell proliferation and growth are, therefore, modulated by how often these mRNAs are translated as well as how different trans-acting factors regulate this process.

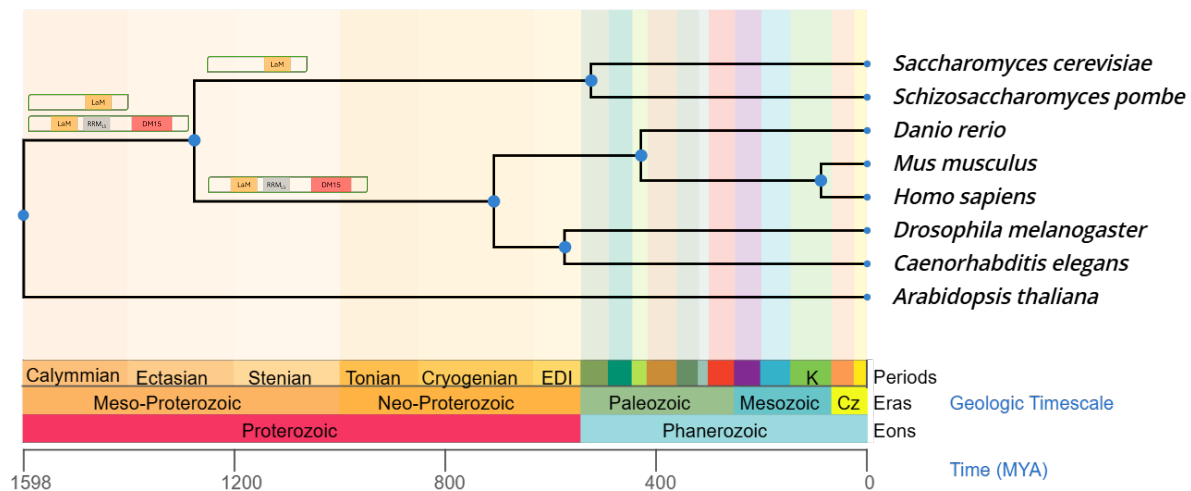


Figure 3: An evolutionary tree depicting common ancestry. *Arabidopsis*, which contains both short and long forms of LARP1, shares a common ancestor with yeasts and humans. The evolutionary tree was drawn using TimeTree of Life (TToL5)²⁸.

Translation of 5'TOP mRNAs is intimately linked to mTOR signalling. Treatment with mTOR inhibitors such as rapamycin selectively inhibits the translation of mRNAs coding for ribosomal proteins and other factors involved with translation^{29,30}. Thus, 5'TOP and 5'TOP-like mRNAs account for the majority of the mTOR signalling pathway substrates^{31,32}. mTOR is the kinase subunit of the multi-subunit mTORC1 and mTORC2^{33,34}. The two complexes have different responses to allosteric inhibition by rapamycin: an acute exposure to rapamycin only blocks mTORC1, whereas both complex 1 and complex 2 are fully inhibited by compounds that are structurally similar to adenosine triphosphate (ATP), including Torin1 and Torin2³⁵. Functionally, the activity of mTORC1 is central to phosphorylation of S6 kinases (S6K) and 4EBPs. Therefore, mTOR signalling and how it regulates 5'TOP mRNA translation is important for cell survival and organismal growth. Also, many RNA-binding proteins are phosphorylated by mTOR complexes and have regulatory functions that are involved with related processes. The presence of a trans-acting factor that significantly and selectively inhibits 5'TOP mRNA translation has been reported as early as three decades ago³⁶.

1.5. The La and La-related protein superfamily

La and La-related proteins (LARPs) are a conserved family of RNA-binding proteins with important and diverse functions in RNA metabolism. These proteins are classified into five subfamilies including genuine La (LARP3), LARP1a/b (previously known as LARP1/2), LARP4a/b (previously known as LARP4/5), LARP6 and LARP7³⁷. With the exception of genuine La proteins from ciliates^{38,39}, La contains two main RNA binding motifs: an N-terminally

located La motif (LaM) and an adjacent RNA recognition motif (RRM1). These two regions give rise to the La module which folds into an RNA binding pocket. Unlike PABP and *Drosophila*'s Sex-lethal (Sxl) protein, which use adjacent RRM1s to bind continuous stretches of RNA, genuine La uses its RRM1 to contact spatially separate regions on an RNA substrate^{40,41}. The La module in genuine La forms a tight V-shaped binding cleft which is best characterized to accommodate terminal uridylates (UUU-3'OH) found in nascent polymerase III transcripts⁴²⁻⁴⁴.

Several distinct LARPs have been characterized since the original classification of genuine La. The LaM is the unifying structural element found in all LARPs, but these proteins also harbour other motifs that are specific to each sub-family and allow for interaction with unique substrates (Figure 4). Amongst the four LARP families (LARP1, LARP4, LARP6 and LARP7), LARP1, 4 and 6 interact with mRNAs, whereas LARP7 interacts with non-coding RNAs. Structural studies characterizing the LaM of human LARP1 (hLARP1)^{45,46}, hLARP4⁴⁷, hLARP7⁴⁸⁻⁵¹ and hLARP6⁵² have confirmed an overall similarity between these and the LaM of hLa⁵³.

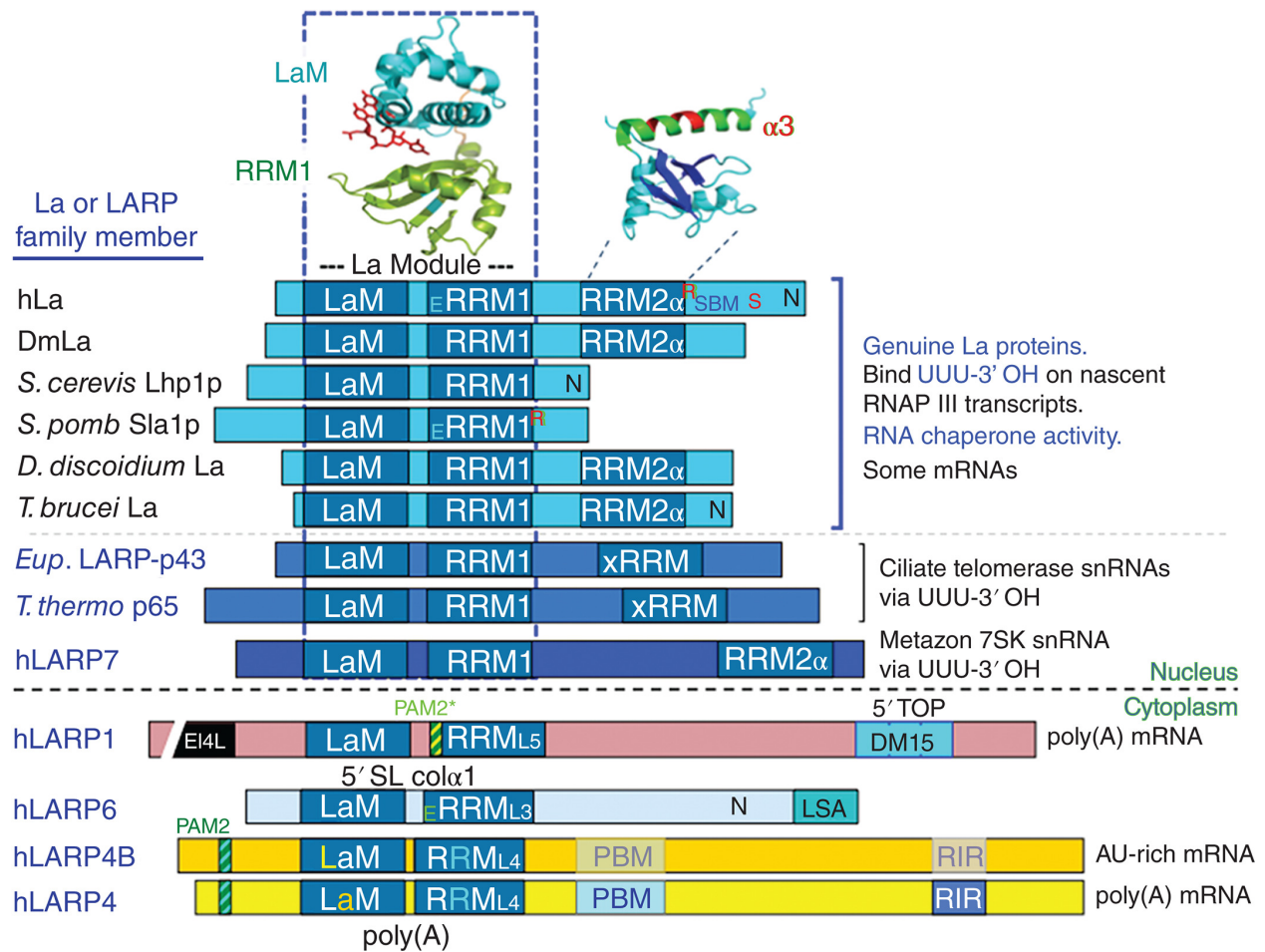


Figure 4: Schematic representations of La and La-related proteins (LARPs). Genuine La proteins from several species and several human LARPs are depicted. (Adapted from Maraia *et al.*, 2017)⁴¹

hLARP1 is predominantly cytoplasmic but it can also be found in the nucleus⁵⁴. hLARP1 is further hypothesized as the primary factor controlling 5'TOP mRNA translation and stability, specifically binding to the characteristic m⁷G/+1C sequence found in these mRNAs^{55–57}. hLARP4 is cytoplasmic and has been suggested to interact with mRNAs to promote their translation. Also, hLARP4 binds RACK1, a kinase that interacts with the 40S ribosomal subunit, and PABP, while it can further localize to stress granules^{58–60}. hLARP6, which is also

cytoplasmic, interacts with conserved stem loops found in the 5'UTRs of mRNAs that encode α -collagens I and III. hLARP6 was initially suggested to modulate the translation of these mRNAs^{52,61}, but more recent work has also described functions related to 5'TOP mRNA metabolism and epithelial to mesenchymal transition in cancer cells⁶². The nuclear hLARP7, which is structurally the closest family member to genuine La, interacts with the 7SK small nuclear RNA and contributes to its proper fold and stability^{48,50,51,63}.

1.6. LARP1 family

Our understanding of LARP1 function is recent compared to what has been known for genuine La and other LARPs⁶⁴. Also, proposed functions for LARP1 family members have been controversial⁶⁵. With respect to hLARP1 which harbours a functionally important and C-terminally located DM15 region^{37,64,66,67}, most studies have linked this family to the inhibition of 5'TOP mRNA translation initiation as it binds the extreme 5' end of these mRNAs, sequestering the m⁷G cap and the 5'TOP sequence which immediately follows. Even as this suggests the interaction between hLARP1 and 5'TOP mRNAs, which code for ribosomal proteins and translational factors, is inhibitory, other works have shown hLARP1 can rather promote mRNA translation.

hLARP1 contains multiple phosphorylation sites^{68,69} and interacts with mTORC1^{55,56,68} as one of its direct substrates. Therefore, phosphorylation of hLARP1, downstream of mTORC1, may play a role in differential regulation of 5'TOP mRNA translation. Additionally, altered modes of association of the DM15 region with the m⁷G cap/5'TOP sequence may allow for hLARP1

to toggle between various roles. Interestingly, some LARP1 orthologs in *Arabidopsis*, *S. cerevisiae* and *S. pombe* do not possess a DM15 region. Reports in humans suggest that the N-terminal region of the protein (containing the LaM) is dispensable for the inhibition of 5'TOP mRNA translation⁷⁰, raising an intriguing possibility where the LARP1 LaM may have ancestral roles that are utilized in eukaryotes missing the DM15 region. Furthermore, 5'TOP mRNAs are not characterized in all eukaryotes. Given the strong evolutionary conservation of LARP1 proteins, despite key structural and substrate differences, their distinct yet connected proposed roles in mRNA translation and stability are an interesting area of study.

1.6.1. Summary of the LARP1 family: members across eukaryotes

Related to the arrangement of RNA binding domains, LARP1 family members from different species are classified into two types including the LARP1-L (long-form) and LARP1-S (short-form) sub-categories (Figure 5). The long-form contains a La module (LaM + RRM_{L5}) and a C-terminal DM15 motif and the short-form only contains a C-terminal LaM⁶⁴. Humans contain two LARP1-L copies: hLARP1a and hLARP1b which were previously characterized as LARP1 and LARP2, respectively. There are two isoforms of hLARP1a encoding 1019 and 1096 amino acid proteins, and three isoforms of hLARP1b encoding proteins of 914, 512 and 358 amino acids long⁷¹. hLARP1a is the predominant form in humans which is described to be 40 times more abundant in HeLa cells⁷². While several LARP1 orthologs in higher eukaryotes were originally suggested to possess the La module, consisting of the LaM and an adjacent RRM, recent structural work in humans has revealed that hLARP1a is missing the RRM region⁴⁵.

Drosophila and *C. elegans* each possess a single LARP1-L ortholog, containing the La module and a DM15 region. *Arabidopsis* has three LARP1 orthologs: one of which has the La module and the DM15 region, while the other two belong to the LARP1-S sub-category. Similarly, the two paralogs of LARP1 in *S. cerevisiae* (Slf1p and Sro9p) and the one ortholog in *S. pombe* (Slr1p) only possess a LaM (Figure 5). While LARP1 orthologs in *Drosophila*, *C. elegans*, *Arabidopsis* and *S. cerevisiae* have been studied, the *S. pombe* Slr1p has not been characterized yet.

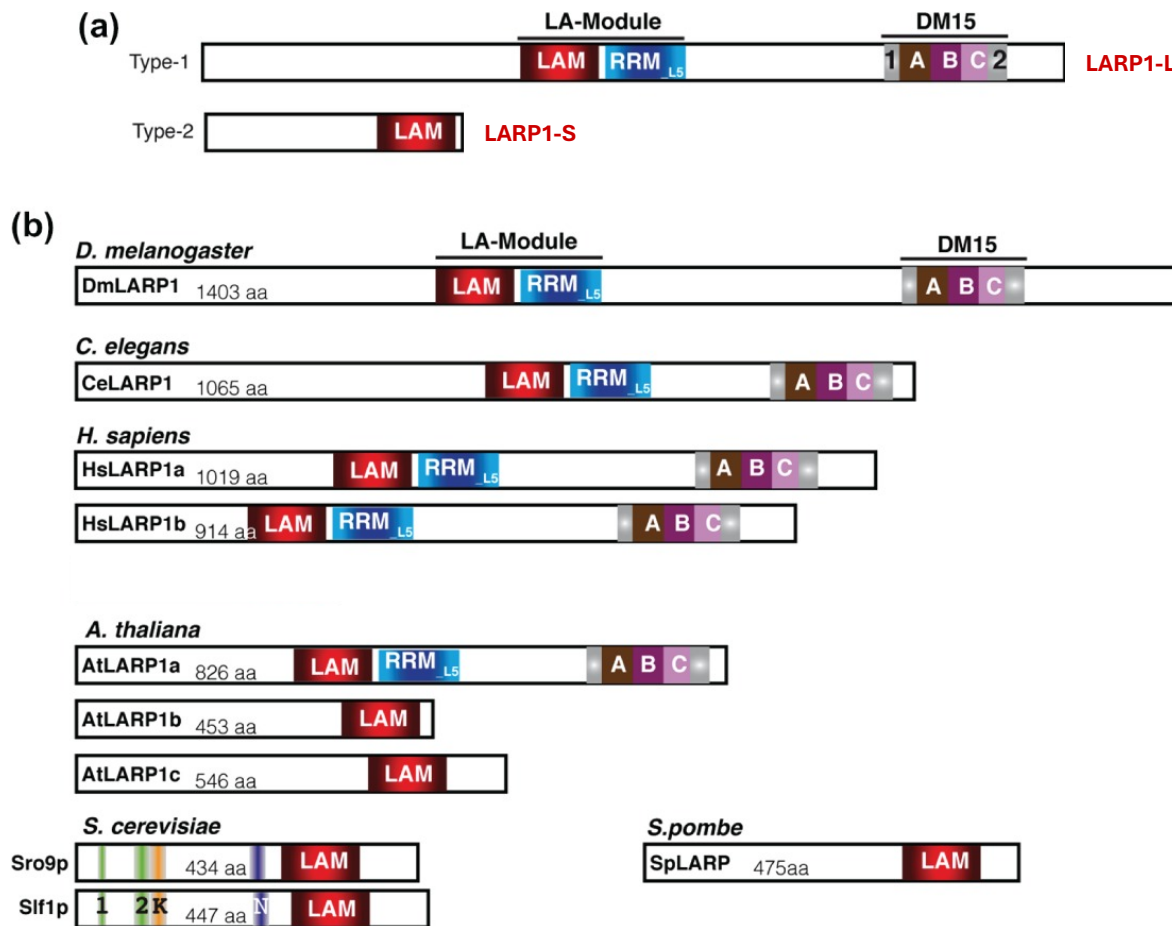


Figure 5: Structural features of different LARP1 orthologs. LARP1 proteins from several different species are depicted. These members either belong to a LARP1-L (long-form) or LARP1-S (short-form) sub-category. (Adapted from Deragon and Bousquet-Antonelli, 2015)⁶⁴

1.6.2. LARP1 characterization in different models

Following the characterization of genuine La, the existence of other LaM-containing proteins was first reported in *S. cerevisiae*⁷³. These findings initiated further studies on “La-related proteins” or LARPs. The two LaM-containing proteins in *S. cerevisiae* were named Slf1p and Sro9p, which were grouped into the LARP1 family³⁷. In contrast to genuine La, which is mainly nuclear, Slf1p and Sro9p were shown to largely function in the cytoplasm and associate with translating ribosomes⁷³. Both proteins bind highly expressed mRNAs, and Slf1p is proposed to enhance the stability of target mRNAs in a LaM-dependent manner⁷⁴. Yeast strains lacking either Slf1p or Sro9p are less sensitive to the effects of paromomycin and cycloheximide which affect the elongation phase of translation^{73,75}. Before its characterization as a LARP1 family member, Slf1p was also linked to copper detoxification since, in a dose-dependent manner, it increases the ability of yeast cells to process copper sulfide and allow for its depletion^{74,76}. Sro9p was also reported to function as a chaperone that represses the yeast heme activator protein Hap1⁷⁷. Furthermore, functions in transcription⁷⁸ and cytoskeleton organization^{79,80} have been reported for Sro9p. Additional functions for the *S. cerevisiae* LARP1 orthologs have been described more recently and they will be discussed later.

Two LARP species have been identified in *Drosophila*: dLarp (CG42551) belonging to the LARP1 family and CG11505 belonging to the LARP5 (LARP4b) family. Early studies in *Drosophila* characterized the LARP1 ortholog in this system, dLarp, as a cytoplasmic Hox target protein with functions related to cytoskeleton structure and integrity⁸¹. dLarp, which contains a La module and two tandem repeats of a DM15 motif at its C-terminal end, was also reported to function in male meiosis and mitochondrial inheritance⁸². Detached spindle

microtubules which were seen in dLarp mutant flies resembled observed phenotypes in mutants of PABP⁸². Interestingly, later studies confirmed that dLarp exists in a complex with PABP⁸³. dLarp functions in mRNA translation during cell cycle progression and it interacts with PABP in an RNA-independent manner. PABP mutant and dLarp mutant flies show similar meiotic defects and double mutants show an enhanced phenotype. While downregulation of dLarp does not affect DNA content, downregulation of PABP leads to a substantial increase in cell death. Therefore, dLarp was proposed to interact with a subset of PABPs in the cell, and potentially a specific cohort of mRNAs⁸³.

Following the initial characterization of dLarp⁸¹⁻⁸³, further studies have proposed additional roles for this long-form LARP1 ortholog in mRNA translation^{84,85}. dLarp is suggested to be recruited as a translational stimulator by a *Drosophila* A-Kinase Anchoring Protein (AKAP) named MDI, for “Mitochondrial DNA Insufficient”, to enhance the localized translation of a subset of nuclear-encoded mitochondrial proteins by cytosolic ribosomes⁸⁴. MDI’s top three interactors include eIF4G, dLarp and PABP. It is suggested that the MDI-dLarp complex promotes the translation of mitochondrial DNA (mtDNA) replication factors, mitochondrial ribosomal proteins and electron-transport chain subunits⁸⁴. In MDI knockout cells, several nuclear-encoded mitochondrial factors showed significantly reduced mRNA levels in polysomes and decreased de novo protein synthesis. However, mRNA and protein levels were restored to normal levels in MDI mutant cells expressing a Tom20-dLarp fusion protein constitutively targeted to the mitochondrial outer membrane. dLarp is further phosphorylated by the *Drosophila* PINK1 kinase, which accumulates with defective mitochondria. While phosphorylation of dLarp does not affect its stability or localization, it

was proposed to inhibit its function as a translational activator⁸⁵. Therefore, these findings suggest that in *Drosophila* ovaries, dLarp and other translational factors can be recruited by MDI, which acts as a scaffold protein, to stimulate local protein synthesis required to drive mtDNA replication and biogenesis.

With respect to *C. elegans*, two distinct LARPs exist in this system: one that clusters with the LARP1 family and another which is related to a metazoan-specific branch including LARP4 and LARP5 (LARP4a/LARP4b). The *C. elegans* LARP-1 was described to include a “LARP1 domain”⁸⁶ which is now known as the DM15 motif. LARP-1 was shown to bind RNA *in vitro* both through the LaM and the DM15 region in isolation. Biological roles described for the protein involve functions related to gene expression in the germline and RNA stability. *larp-1* null mutant worms have a defect in oogenesis reminiscent of elevated RAS-MAPK signalling which is up- or down-regulated with *let-60* (encodes the single *C. elegans* RAS protein⁸⁷) gain of function or reduction of function mutations, respectively⁸⁶. Also, LARP-1 is cytoplasmic and colocalizes with P bodies or sites of mRNA turnover⁸⁸ together with the *C. elegans* decapping enzyme homolog DCAP-1^{89,90}. LARP-1 and CAR-1, an RNA-binding protein and Scd6 homolog^{91,92} thought to act as a translational repressor, were further shown to colocalize in P bodies. The abundance of specific and some conserved mRNAs (i.e., *lip-1* (MAPK phosphatase), *let-60* (RAS), *mpk-1* (MAPK kinase) and *fem-3* (sex determination protein)) was additionally shown to increase in *larp-1* null worms, further arguing for functions related to RNA stability for *C. elegans* LARP-1^{86,93}.

Initial studies in *Arabidopsis* showed that overexpression of LARP1c, one of the three orthologs of LARP1 in this system missing a DM15 region, leads to precocious leaf senescence and upregulation of several senescence-associated and defense-related genes⁹⁴. LARP1c-GFP foci were sensitive to cycloheximide treatment suggesting that LARP1c, potentially aggregated due to overexpression, could be involved with processes related to translation. Additionally, long-form LARP1a in *Arabidopsis* (referred to as LARP1 hereafter) functions in coupling mRNA translation and decay during heat stress⁹⁵. In response to the stress, *Arabidopsis* reprograms gene expression by downregulating translation⁹⁶, which in turn triggers the decay of 25% of its transcriptome. LARP1 forms a complex with XRN4, the *Arabidopsis* ortholog of the 5'-3' exonuclease XRN1, to target XRN4 to translating polysomes. Also, LARP1 and XRN4 co-immunoprecipitate in an RNase-independent manner. Interestingly, treatment with RNase I, which cleaves single-stranded RNAs, suggested that their interaction in polysomes is not mediated by the presence of RNA or poly(A) tails. Furthermore, XRN4 cannot be recruited to polysomes in *larp1* mutant plants under heat stress, but this is rescued in *larp1* mutant cells expressing transgenic LARP1.

More recent studies, performed under normal physiological conditions, suggest that *Arabidopsis* LARP1 is involved with mRNA translation in a TOR-dependent manner^{26,97}. The classic 5'TOP motif is not characterized in *Arabidopsis*. However, several mRNAs, involved in diverse steps of ribosome biogenesis (i.e., rDNA transcription, rRNA processing and ribosome complex assembly) along with a few cytosolic ribosomal protein mRNAs and plant-specific mRNAs, were suggested to contain TOP-like motifs that are regulated by TOR signalling in a LARP1-dependent manner^{26,97,98}. Here, TOR inhibition resulted in a significant

reduction in 91% of the cytosolic ribosomal protein mRNAs and 37% of the mitochondrial mRNAs, broad translational repression of ribosomal protein mRNAs, decreased phosphorylation of LARP1 and hyperphosphorylation of eIF4G, altogether highlighting repression of mRNA translation. Importantly, the translational efficiency of mRNAs involved in translation, particularly those encoding cytosolic ribosomal proteins, was significantly reduced in *larp1* mutant seedlings. Therefore, the TOR-LARP1-5'TOP axis is proposed to be conserved in *Arabidopsis*, linking LARP1 activity to TOR signalling and regulation of TOP-like mRNAs in this system.

Other evolutionary studies have identified members of the La, LARP1 and LARP6 families across various plant species, while LARP4 and LARP7 family members were absent^{99,100}, consistent with earlier findings³⁸. In an analysis of 16 plant species, 188 La and LARP genes were identified⁹⁹, consisting of 32 genuine La proteins, 80 LARP1 proteins and 76 LARP6 proteins. As discussed earlier, this study further classified LARP1 members into two sub-categories: LARP1-L (long-form) and LARP1-S (short-form). While the long-form contains both a LaM and a C-terminal DM15 domain, the short-form only contains a C-terminal LaM. Notably, amongst the identified LARP1 orthologs in plants, the short-form protein is more abundant, suggesting that it may represent an ancestral type. Therefore, it is interesting to note that plants, which share a common ancestor with both yeasts and humans (Figure 3), possess both the long and short forms of LARP1. Given that LARP1-S is the only form to exist in yeasts⁶⁴ and LARP1-L in humans, *Arabidopsis* may be an interesting model organism for studying the evolutionary divergence of LARP1 members with a DM15 region, which could have initially evolved in plants.

The first human LARP1 study reported that hLARP1 interacts with PABP in an RNase-independent manner and that it associates with ribosomal subunits⁷². Depletion of hLARP1 leads to a considerable increase in the ratio of subpolysomes (40S, 60S and 80S) to polysomes; therefore, it was suggested that hLARP1 is involved with mRNA translation initiation. Since overexpression of hLARP1 in HeLa cells contributes to mesenchymal-like or migratory phenotypes, it was further proposed that hLARP1 facilitates the synthesis of proteins required for cancer-related phenotypes. hLARP1 was also shown to associate with mRNA poly(A) tails, specifically recognizing the 3' termini, to stabilize mRNAs¹⁰¹. Additionally, the depletion of hLARP1 in HEK293 cells led to a considerable decrease in expression levels of several 5'TOP mRNAs, further suggesting that hLARP1 selectively stabilizes this subset of mRNAs.

In summary, studies on LARP1s from various systems converge on themes of mRNA translation and stability. Since their initial characterizations, several LARP1 orthologs have been suggested to be involved with these processes; however, the precise mechanisms by which these conserved proteins coordinate involvement with such processes remain elusive. This is especially puzzling in the more complex human system, which will be discussed further, since despite a growing body of research shaping our current understanding of hLARP1, proposed functions for the protein are still controversial⁶⁵. Therefore, deeper insights into the specific roles of LARP1 in different contexts are of special interest.

1.6.3. Connections to disease states

Several studies have linked hLARP1 to tumor survival and cancer progression. Reports by Hopkins *et al.*¹⁰² and Mura *et al.*¹⁰³ suggest that hLARP1 binds BCL2, BIK and mTOR mRNAs and differentially regulates their stability through contacts to pyrimidine-rich sequences in the 3'UTR. Furthermore, in SKOV3 ovarian cancer cells treated with sodium arsenite, hLARP1 colocalizes with DCP1A, a P-body marker, and PABP, a stress granule marker, suggesting that hLARP1 is involved with both mRNA stability and translation in cancer cells¹⁰². The RNA interactome of hLARP1 includes 3000 transcripts enriched for 5'TOP mRNAs as well as others involved in cancer pathways¹⁰³. Several groups have shown that hLARP1 is overexpressed in cancer cells with a net effect that leads to a pro-growth enhanced protein expression and a poor prognosis in epithelial cancers¹⁰³, colorectal cancer^{104,105}, hepatocellular carcinoma¹⁰⁶, multiple myeloma¹⁰⁷ and gastric carcinoma¹⁰⁸.

In colorectal cancer cells, hLARP1 is proposed to bind to approximately 400 transcripts that contain binding sites in their 3'UTRs; importantly, these transcripts are not enriched for 5'TOP-containing ribosomal protein mRNAs¹⁰⁵. Thus, dysregulated hLARP1 may be targeted to different mRNA 3'UTRs in cancer cells, while its phosphorylation state may also differ from its normal state^{68,105}. Other reports have identified hLARP1 as a target of the oncogenic transcription factor MYC^{105,107}, and a GCN2-dependent target of ATF4 during a sustained response to nutrient starvation. hLARP1 is also suggested to colocalize with GCN1^{109,110} which is the upstream activator of GCN2¹¹¹. Therefore, abnormal hLARP1 function downstream of some transcription factors may allow for sustained phenotypes under disease states.

hLARP1 has additionally been implicated in mitochondrial fitness and regulation of oxidative phosphorylation as it's linked to cancer cell survival¹¹². Knockdown of hLARP1 in A2780 ovarian cancer cells disrupts the translation of the mitochondrial transcription initiation factor TFB2M and respiratory chain complex II subunit SDHB, leading to mitochondrial dysfunction following PKI-402 treatment, which inhibits PI3K and mTORC1/2. This was affected by hLARP1's role in stabilizing TFB2M mRNA through its LaM. Interestingly, overexpression of a hLARP1 Q333A LaM point mutant failed to restore TFB2M mRNA levels, while full-length hLARP1 and a mutant lacking the DM15 domain partially restored mRNA levels. Therefore, hLARP1, which was suggested to be recruited to the mitochondrial outer membrane, could be involved in maintaining mitochondrial function during PI3K/mTOR inhibition or mitigating the effects of their pharmaceutical inhibition¹¹². Furthermore, hLARP1 may regulate the expression of mtDNA encoded proteins¹¹³ similar to reports in *S. cerevisiae*¹¹⁴ and *Drosophila*⁸⁴. hLARP1 is also required for full oxidative phosphorylation capacity as its knockout in erythroleukemic K562 cells leads to a partial reduction in oxygen consumption and increased cell death¹¹³.

Additionally, several studies have suggested that hLARP1 is involved with viral replication. RyDEN, an interferon-stimulated gene that inhibits replication of the dengue virus, was found in a complex with hLARP1 and PABP¹¹⁵. RyDEN interferes with the translation of the dengue virus by interacting with the 3'UTR of the viral RNA, potentially blocking cellular RNA-binding proteins such as hLARP1 and PABP, which may stabilize the viral RNA and promote its expression. Other reports characterizing the SARS-CoV-2 RNA-protein interactome and upregulated cellular RNAs during viral infection suggest that hLARP1 has antiviral functions

in this context^{116,117}, while it may also interact with the SARS-CoV-2 nucleocapsid protein¹¹⁸. eCLIP analysis revealed that hLARP1 binds the SARS-CoV-2 RNA at multiple locations enriched in oligopyrimidine sequences. Binding sites mainly occur within the first 70 nucleotides of the RNA or the viral 5' leader, which contains TOP-like motifs¹¹⁶. Knockout of hLARP1 led to higher levels of viral RNA and a corresponding increase in the production of infectious virus, while transient hLARP1 overexpression rescued the phenotype to near wildtype levels. Additionally, inhibiting mTORC1/2 results in a reduction in viral replication, and phospho-proteome studies have shown that hLARP1 undergoes active phosphorylation as a result of SARS-CoV-2 infection^{119,120}.

Other studies have indicated that genes with 5'TOP tracts escape global suppression of translation by the SARS-CoV-2 Nsp1 protein, an essential protein for the translation of the SARS-CoV-2 RNA genome, which shuts down cellular translation¹²¹. However, cellular mRNAs, including those coding for the translational machinery, mitochondrial components and factors important for viral replication, were preferentially translated in cells expressing Nsp1 in a hLARP1-dependent manner. In response to the ectopic expression of Nsp1, which increased 5'TOP mRNA expression, reporters with a TOP motif in their 5'UTR were less expressed when hLARP1 was knocked out. Therefore, several findings suggest that SARS-CoV-2 infection interferes with ribosome biogenesis and its regulation^{117,121,122}, which hLARP1 is also involved with. hLARP1 has further been linked to viral particle production^{123,124} and viral entry¹²⁵ during HCV infection. A proteomic survey, identifying novel host factors involved with Zika virus infection, also reported that hLARP1 is dephosphorylated due to the downregulation of Akt/mTOR signalling in this context¹²⁶. Therefore, hLARP1's role in viral

replication appears to be context-dependent and potentially influenced by the presence of TOP-like motifs within viral genomes. Given that hLARP1 is either dysregulated in cancer or that it is involved with the cellular response to viral infection, a mechanistic understanding of its function is valuable where hLARP1 can be considered as a therapeutic target.

1.6.4. hLARP1 functions in mRNA translation and stability

The specific function of hLARP1 has been the subject of some debate, starting with work reporting that it either enhances⁵⁵ or represses⁵⁶ the translation of 5'TOP mRNAs. These studies identified hLARP1 in a proteomic screen for factors that associate with the mRNA 5' cap in an mTOR-dependent manner⁵⁵ using m⁷GTP affinity chromatography coupled with quantitative mass spectrometry¹²⁷. Furthermore, hLARP1 co-immunoprecipitates with eIF4A, but not eIF4E or eIF4G. eIF4A is also reported to enhance the interaction of 5'TOP mRNAs with hLARP1 through helicase-independent functions¹²⁸. hLARP1 knockdown followed by polysome profiling results in an increase in the height of the 80S peak and a decrease in polysomes⁵⁵, similar to other reports by Burrows *et al.*⁷², indicative of defects in translation initiation caused by hLARP1 knockdown. Therefore, it was concluded that hLARP1 depletion significantly decreases the abundance of 5'TOP mRNAs, while treatment with mTOR inhibitors did not lead to an equally robust effect on 5'TOP mRNAs⁵⁵. These observations have been further suggested to recapitulate basal levels of 5'TOP mRNA translation, which instead of being translationally silenced during starvation, could undergo low baseline translation with reduced initiation rates¹²⁹.

In contrast to findings by Tcherkezian *et al.*⁵⁵, Fonseca *et al.*⁵⁶ proposed a model where hLARP1 acts as a cap-binding protein that sequesters the mRNA cap under mTOR inhibition, preventing eIF4F assembly and therefore mRNA translation initiation. Their findings suggested that hLARP1 competes with eIF4G for binding to 5'TOP mRNAs, directly binding to the cap and the adjacent 5'TOP motif. Knockdown of hLARP1 also decreased the inhibitory effects of rapamycin/Torin1 and amino acid starvation on 5'TOP mRNA translation. Therefore, hLARP1 was proposed to play a crucial role in inhibiting the translation of these mRNAs downstream of mTORC1, while also having a positive role in maintaining 5'TOP mRNA stability. Notably, overexpression of hLARP1 significantly increased the half-lives of mRNAs coding for RPL29, RP36 and PABP. It was concluded that hLARP1 acts as a bimodal regulator of 5'TOP mRNAs, repressing their translation during mTOR inhibition while also stabilizing them for active translation under favourable conditions.

While the 5'TOP sequence is sufficient for blocking mRNA translation during mTOR inhibition^{130,131}, hLARP1 knockout in HeLa cells may either fully⁶⁹ or only partially¹³¹ rescue the translation of 5'TOP mRNAs. The inhibitory effects of hLARP1 on 5'TOP mRNA translation therefore may be secondary to the effects of eIF4E-binding proteins 1 and 2 (4EBP1/2) during mTOR inhibition, while loss of hLARP1 results in selective destabilization of 5'TOP mRNAs independent of 4EBP1/2¹³¹. Related to its functions in mRNA stability, hLARP1 is also crucially important for anchoring 5'TOP mRNAs to stress granules and to some extent P-bodies¹³². Furthermore, pyrimidine-rich sequences in the 5'UTR, regardless of whether they are at the extreme 5' end or not, are sufficient to drive 5'TOP mRNA localization across the apicobasal axis of epithelial cells in a hLARP1-dependent manner¹³³. Therefore, several

studies have linked hLARP1 to both promotion and inhibition of 5'TOP mRNA translation and decay.

In addition to interacting with 5'UTRs of specific and diverse mRNAs, hLARP1 has been proposed to interact with the mRNA poly(A) tail. While the DM15 region is thought to be mainly responsible for binding to the cap and 5'TOP motif, residues within the LaM, known for recognizing terminal uridylates in genuine La^{43,44}, enable hLARP1 to bind poly(A) RNA^{45,46}. Interestingly, LaM amino acids (310-540) have been implicated in interacting with both the 5'TOP motif and mRNA poly(A) tail¹³⁴. Given that the amino acid boundary defined for the LaM in binding to poly(A) RNA spans residues 323-410⁴⁵, it's possible that additional amino acids beyond this region, particularly 410-540, contribute to hLARP1's capacity to bind to the 5'TOP motif. Notably, a co-crystal structure of the LaM bound to oligo(U) RNA revealed that the same amino acids responsible for binding to U6 are also involved in binding to A6⁴⁶. The isolated LaM, along with a PABP-interacting motif (PAM2) and a few additional C-terminal residues which follow it, also mediate 3' poly(A) tail length protection and mRNA stabilization in HEK293 cells, dependent on an interaction between the PAM2 motif and PABP¹³⁵. hLARP1 further participates in global poly(A) tail lengthening which most substantially stabilizes 5'TOP mRNAs¹³⁶⁻¹³⁹.

Moreover, the reports that suggest the LaM of hLARP1 recognizes the 5'TOP sequence, independently of the mRNA cap, while it also binds poly(A) sequences imply that hLARP1 can simultaneously engage both ends of 5'TOP mRNAs¹³⁴. Regarding the hLARP1 LaM (amino acids 310-540), a 20-mer 5'TOP containing RNA competes off poly(A) RNA in *in vitro* binding

assays, whereas the poly(A) RNA does not compete off the 5'TOP RNA, suggesting a higher affinity of the LaM for the 5'TOP sequence. This supports a model where binding of hLARP1 to the 5'TOP motif and the poly(A) tail of the same mRNA could enhance translation, while the DM15 region specifically binding the Cap/+1C leads to repression of translation. Notably, LARP1 orthologs missing the DM15 region are still involved with mRNA translation^{73,74,114,140}. Structural studies^{45,141} have confirmed that the LaM of hLARP1 (amino acids 323-410) binds a poly(A) RNA⁴⁵, where a penultimate guanine at the 3' end of the poly(A) sequence led to a three-fold increase in binding⁴⁶ hence providing evidence for guanylation of 5'TOP mRNA poly(A) tails which may protect them from rapid deadenylation^{142,143}.

Other studies suggest that hLARP1's interaction with its target mRNAs, here including 5'TOP mRNAs, non-5'TOP mRNAs and mitochondrial function-related transcripts, increases under mTOR inhibition when these mRNAs are translationally repressed, and that this interaction is specifically dependent on PABP¹⁴⁴. Mutations to the hLARP1's PAM2 motif led to an overall decrease in RNA binding while mutations to the DM15 region did not, supporting a model where the LaM/PAM2 and DM15 domains interact with distinct regions of a target mRNA. These findings suggest that a dominant mode of RNA binding through the LaM/PAM2 may be in effect, while the DM15 domain may serve as an additional regulatory region that is activated when binding to certain mRNAs such as the 5'TOPs. Notably, inhibiting PABP's interaction with hLARP1 decreased RNA binding by hLARP1, indicating that its association with PABP may allow for broader mRNA binding beyond transcripts containing 5'TOP motifs.

Evidently, the distinct functions of the LaM and the DM15 domain of hLARP1 are central to these conclusions. The DM15 binding mode is specific, allowing for binding to 5'TOP mRNAs through their characteristic Cap/+1C motif^{57,145}. The structure of the DM15 region consists of several evolutionarily conserved¹⁴⁶ helix-turn-helix modules within three monomers resembling HEAT-like repeats¹⁴⁵. It's been suggested that the DM15 fold is highly dynamic and exists in a collapsed or open state corresponding to either a disordered loop or a helical ligand-ready state^{147,148}, respectively, which could explain positive or negative modes of regulation through this region. The co-crystal structure of the DM15 with a capped cytidine (m⁷GpppC) has further revealed that hLARP1 is a special cap-binding protein that requires the m⁷G to bind to the adjacent 5'TOP motif⁵⁷. This presented evidence for hLARP1 effectively blocking the cap structure, preventing eIF4E from accessing it and therefore inhibiting the assembly of the eIF4F complex. The LaM and regions immediately C-terminal to it, including an adjacent PAM2 domain, on the other hand are dispensable for repressing 5'TOP mRNA translation⁷⁰ even though these regions have been proposed to still bind the 5'TOP motif¹³⁴.

1.6.5. Phosphorylation of hLARP1

The phosphorylation status of hLARP1 has been proposed to allow for the protein to toggle between different regulatory functions. hLARP1 is a direct substrate of mTORC1 and Akt/S6K1. Upon phosphorylation, hLARP1 is suggested to dissociate from the 5'UTR, relieving its inhibitory effects on mRNA translation, and to associate with the 3' UTR⁶⁸. PAR-CLIP data indicates hLARP1 binds at the 3' end of the 5'UTR, where a pyrimidine-rich

translational element (PRTE) resides closer to the AUG of 5'TOP mRNAs³², as well as within the 3'UTR of translation-related and ribosomal protein mRNAs⁶⁸. Furthermore, hLARP1 contains 26 serine/threonine phosphorylation sites distributed across seven rapamycin-sensitive clusters⁶⁹. Cluster 6 residues, located upstream of the DM15 domain, are particularly sensitive to rapamycin which impairs RNA binding and hLARP1's inhibitory effects on mRNA translation. In contrast, residues in clusters 4 and 5, located in the mid-region of hLARP1, are suggested to enhance hLARP1's association with mTORC1. Therefore, phosphorylation of different identified clusters may differentially regulate the LaM and DM15 domain of hLARP1, leading to distinct functional outcomes. Thus, while the inhibitory effects of hLARP1 on mRNA translation are primarily attributed to its C-terminal region, including the DM15 domain, hLARP1 may remain bound to 5'TOP mRNAs under growth-stimulating conditions to act as a nucleation site for recruitment of translational factors^{55,68,130,134,147,149}.

1.6.6. Diversity of hLARP1 RNA targets

hLARP1 is further proposed to selectively mediate 5'TOP mRNA stability through interaction with free 40S ribosomal subunits¹³⁰. Under mTOR inhibition, the 40S-hLARP1 complex stabilizes classic 5'TOP mRNAs, such as ribosomal protein mRNAs, along with those involved in ribosome biogenesis, metabolic processes and mitochondrial functions¹⁴⁹. Notably, transcription start site sequencing has shown that hLARP1's function extends to transcripts with TOP-like motifs that are not classified as 5'TOP mRNAs. Similar findings have led to the description of a metric called TOPscore, which quantifies pyrimidine-richness at

transcription start sites⁹⁸. TOPscore was defined as a mean length of consecutive C/U residues up to 6 nucleotides at each analyzed position, since TOP sequences longer than 6 nucleotides failed to increase the degree of translation repression by hLARP1. Intriguingly, it was proposed that the 5'TOP sequence is an extreme example of a broader set of 5' regulatory sequences. Thus, hLARP1 recognizes a wide range of 5' sequences, with a requirement for a +1 C or U and as few as two additional pyrimidines. Interestingly, an increase in the length of the polypyrimidine sequence (up to 6 nucleotides) correlates with an increased level of repression by hLARP1, a finding that challenges the common notion that 5'TOP motifs have a binary function. Therefore, mRNAs with 5'TOP or TOP-like sequences can be regulated to varying degrees, potentially by different regions of hLARP1, and this may have led to some contradictory conclusions regarding the functions of hLARP1.

1.6.7. Recent studies in yeast

Yeasts possess the simplest forms of LARP1. The *S. cerevisiae* LARP1 orthologs, Slf1p and Sro9p, were the first La-related proteins to be identified⁷³. These proteins belong to the short-form LARP1 sub-category, which also includes *Arabidopsis* LARP1b and LARP1c, as well as the *S. pombe* Slr1p. While studies in *S. cerevisiae* have provided the field with valuable insights, the other short-form LARP1 orthologs have not been extensively studied. Several findings from human models suggest that the LARP1 LaM may play an independent role in RNA binding; however, it is challenging to study LaM-specific functions of hLARP1 due to the presence of a DM15 domain in this system. Yeast models, therefore, offer a unique system

where the function of the LaM can be studied in isolation, and the DM15-independent RNA binding modes of LARP1 can be characterized.

The two *S. cerevisiae* LARP1 paralogs, which have evolved as a result of gene duplication¹⁵⁰, share a 37% overall, and 57% LaM, similarity in amino acid sequence¹¹⁴. Slf1p is predominantly cytoplasmic, while Sro9p has been suggested to shuttle between the nucleus and the cytoplasm, potentially bound to its target mRNAs^{74,151}. Key point mutations in the LaM, or its deletion, lower Slf1p's association with polysomes¹¹⁴. Other functions such as RNA binding, target mRNA stabilization and copper detoxification are also LaM-dependent for Slf1p⁷⁴. Polysome profiling shows that while Slf1p primarily sediments with monosomes and polysomes, Sro9p tends to associate with subpolysomes and polysomes^{73,114}. This may indicate that Slf1p preferentially interacts with elongating ribosomes, whereas Sro9p could affect initiation as well as elongation events¹¹⁴. Additionally, Sro9p binds more RNAs, including all that Slf1p binds to; however, Sro9p is also approximately 13.5 times more abundant than Slf1p¹⁴⁰. Interestingly, in nearly every study, both Slf1p and Sro9p have been reported to bind highly expressed mRNAs, including those coding for ribosomal protein mRNAs which are functionally similar to 5'TOP mRNAs^{73,74,114,140}. Despite these findings, their interaction with ribosomal protein mRNAs has not yet been specifically studied in *S. cerevisiae*. Thus, this remains an intriguing area for further investigation.

Slf1p and Sro9p have also been reported to associate with RNAs that are upregulated in response to copper toxicity^{74,76,140} and oxidative stress^{114,140}, events which involve reprogramming of the cellular protein synthesis. Both proteins immunoprecipitate

components of the mRNA translation machinery, such as eIF4E, eIF4G and PABP, as well as ribosomal proteins Rps3 and Rpl35. Notably, Slf1p and Sro9p remain associated with these factors during oxidative stress¹¹⁴. Slf1p plays a crucial role in this context, involving the translation and stability of the upregulated stress response mRNAs^{74,114,140}. Acute stress leads to an increased expression of specialized mRNAs and recruitment of many ribosomes to each translating mRNA, which are then likely to collide during the elongation phase of mRNA translation. Slf1p is proposed to play a role in resolving ribosome collisions and preventing frameshifting, such that stress response mRNAs could be rapidly translated in this context^{114,140}. Slf1p and Sro9p bind within coding regions of target mRNAs, closer to the stop codon. Target transcripts, encoding stress-regulated antioxidant enzymes and other highly translated mRNAs, were suggested to contain a YGSU consensus sequence that was reported in approximately 50% of the binding sites within the main ORF¹⁴⁰.

Analysis of the transcripts bound by both Slf1p and Sro9p also suggests that their targets are enriched for mRNAs involved with mitochondrial functions, in addition to those related to protein synthesis¹¹⁴. Slf1p and Sro9p are further involved in promoting survival fitness in *S. cerevisiae*, which is chronically infected with a double-stranded RNA virus called L-A^{152,153}. Despite being presumed to be harmless, the infection leads to proteotoxic stress and conditional lethality when viral attenuation pathways are suppressed. In cooperation with other proteins, Slf1p alleviates the consequences of proteotoxic stress to increase cell survival, while Sro9p suppresses viral protein accumulation in response to high levels of viral translation¹⁵⁴. The promotion of survival fitness and antiviral functions proposed for Slf1p and Sro9p suggest that, in addition to their ancient roles in regulating mRNA translation and

stability, LARP1 family members have conserved functions related to cell survival and innate immunity.

1.6.8. Proposed work in *S. pombe*

Pioneering research in *S. pombe* has shaped our understanding of genuine La proteins and their conserved functions. Despite its usefulness as a model organism for studying La and La-related proteins, the *S. pombe* LARP1 ortholog, Slr1p, remains uncharacterized. Exploring LARP1 orthologs in different eukaryotes can provide us with critical insights into the function of hLARP1, which is implicated in human disease states. Given the scarcity of information on short-form LARP1 orthologs, Slr1p is of particular interest because it's the only ortholog found in *S. pombe*, where the DM15-independent functions of the LaM with respect to RNA binding can be studied. In contrast, the only other short-form LARP1 orthologs found in *Arabidopsis* and *S. cerevisiae*, present challenges for studying LARP1-S. Neither model is optimal because *Arabidopsis* contains two short-forms and a long-form LARP1, making it difficult to study one form in isolation, and *S. cerevisiae* contains two LARP1 paralogs with overlapping functions. Therefore, characterizing Slr1p would contribute to our better understanding of LARP1 function, and insights into its mechanisms of action could inform future research in human models, where multiple and context-dependent proposed functions for hLARP1 remain perplexing.

The single short-form *S. pombe* LARP1 contains a C-terminally located LaM, and we have named it the *S. pombe* La-Related protein 1 or Slr1p (PomBase: *slr1*, Gene ID:

SPAC1527.03)¹⁵⁵. This protein is non-essential, making knock out experiments possible in fission yeast. Conversely, overexpression of Slr1p results in a senescent phenotype, similar to the overexpression of LARP1c in *Arabidopsis*. Studies on LARP1 orthologs in different model organisms suggest that these proteins are primarily involved with mRNA translation and stability, arguing for the conservation of function across eukaryotes. While the main RNA substrates of hLARP1 are a subset of mRNAs classified as 5'TOP mRNAs, yeasts are not known to have mRNAs that are characterized as such. However, yeasts still possess functionally equivalent mRNAs which code for ribosomal proteins and other factors important in the process of mRNA translation. Considering previous studies on LARP1 family members and the conservation of the superfamily across different eukaryotes, **we hypothesize that Slr1p functions as an RNA-binding protein that regulates the translation and stability of mRNAs encoding ribosomal proteins and other components of the translational apparatus in *S. pombe***. In order to characterize Slr1p, we propose to:

1) investigate processes that it may be involved with by identifying its protein partners and RNA targets and querying these RNAs for common features or motifs (**Chapter 2 – Published**),

2) perform a structural analysis of Slr1p LaM and examine its interaction with target RNAs (**Chapter 3 – In review**)

and

3) explore Slr1p's role in adaptation to stress and how its phosphorylation state may influence its functions (**Chapter 4 – In preparation for publication**).

This work aims to uncouple the various confounding RNA binding modes and functions that are suggested for various LARP1 orthologs. By characterizing Slr1p in *S. pombe* we plan to contextualize earlier findings from other systems. This approach is particularly significant given that hLARP1 has been implicated in diverse cellular roles and disease states, including cancer, where its overexpression predicts a poor prognosis. Therefore, by studying a simpler LARP1 ortholog, we seek to gain insights into the function of hLARP1 which could serve as a promising therapeutic target.

1.7. References

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Chapter 2: The LARP1 homolog Slr1p controls the stability and expression of proto-5'TOP mRNAs in fission yeast

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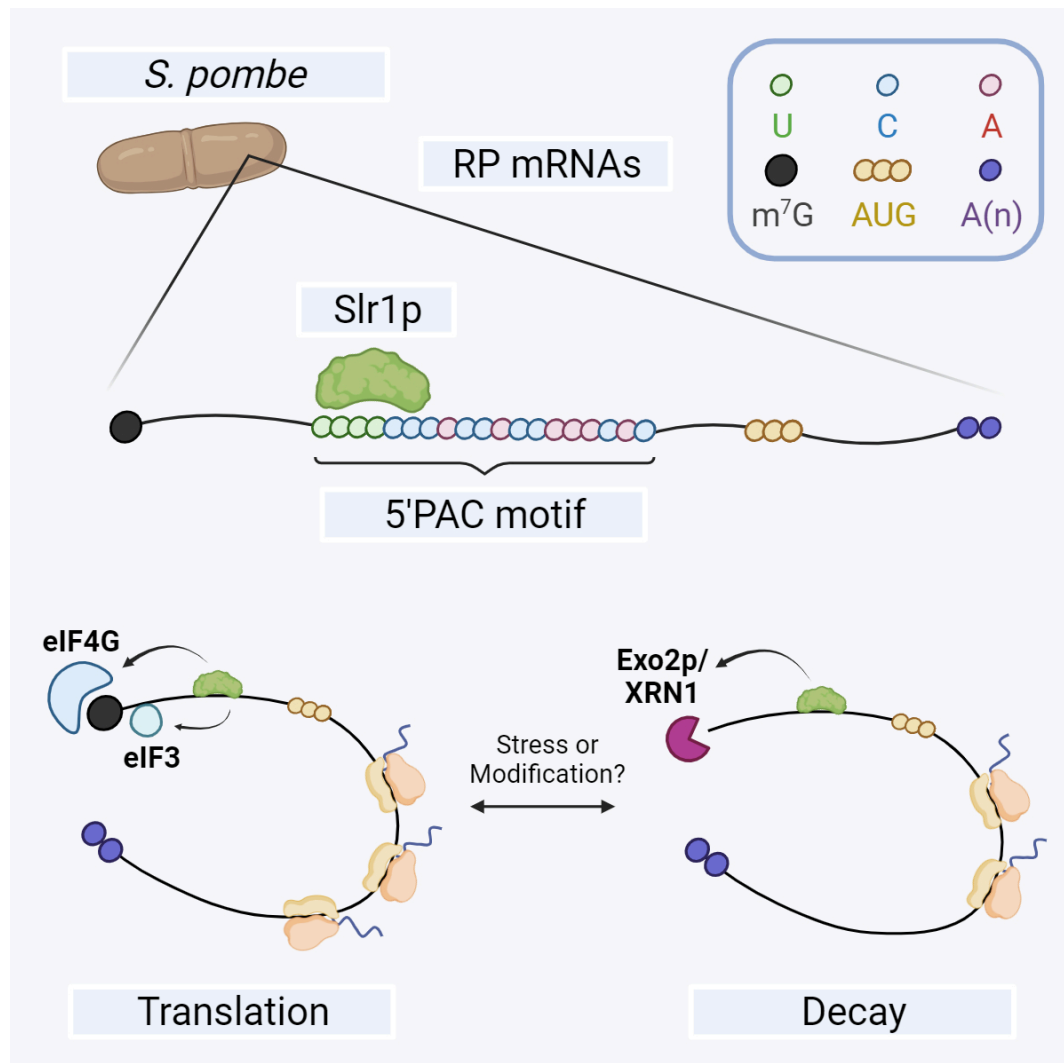
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Contributions: A.P. (previous lab member) conducted the RNA-Seq, J.G. (collaborator) performed the bioinformatics, F.M. analyzed the data and made figures. F.M. prepared RNA samples for the RATE-Seq, D.B. and O.S.R. (collaborators) conducted the RATE-Seq and performed the bioinformatics, D.B. made Figure S2, F.M. analyzed the RATE-Seq data, performed motif discovery search, designed and conducted relevant experiments, validated results and made the corresponding figures. L.S. (previous lab member) performed the mass spectrometry, F.M. analyzed and validated the results and made figures. With respect to all other presented work, F.M. conceptualized the studies, designed and conducted experiments, collected data, conducted formal analysis, made figures and wrote the first draft of the manuscript. M.A.B. supervised the project, helped with designing experiments and formal analysis, reviewed and revised the original draft of the manuscript. F.M. and M.A.B. edited the manuscript.

The sections and headings in this chapter are structured to align with the formatting guidelines of *Cell Reports* journal.

2.1. Graphical Abstract



In brief:

Expression of human ribosomal protein mRNAs is tightly linked to cellular growth and controlled by hLARP1 binding a 5'TOP sequence. In Mansouri-Noori *et al.*, the authors demonstrate that the *S. pombe* LARP1 homolog Slr1p controls expression of proto-5'TOP motif-containing ribosomal protein mRNAs, supporting a conserved function of LARP1 proteins.

Highlights:

S. pombe ribosomal protein (RP) mRNAs are enriched for a proto-5'TOP motif
 RP mRNAs have a 5' Proximal AC-rich (5'PAC) motif which resides close to the initiator AUG
 Slr1p interacts with translation (eIF4G, eIF3) and decay (Exo2p/XRN1) factors
 Slr1p binds the 5'PAC motif and controls RP mRNA translation and stability

2.2. Summary

Messenger RNAs (mRNAs) in higher eukaryotes that encode proteins important for the assembly of the translational apparatus (e.g. ribosomal proteins) often harbour a pyrimidine-rich motif at the extreme 5' end known as a 5' terminal oligopyrimidine (5'TOP) sequence. Members of the La-related protein 1 (LARP1) family control 5'TOP expression through a conserved DM15 motif, but the mechanism is not well understood. 5'TOP motifs have not been described in many lower organisms, and fission yeast harbours a LARP1 homolog that also lacks a DM15 motif. In this work, we show that the fission yeast LARP1 homolog, Slr1p, controls the translation and stability of mRNAs encoding proteins analogous to 5'TOP mRNAs in higher eukaryotes, which we thus refer to as proto-5'TOPs. Our data suggest that the LARP1 DM15 motif and the mRNA 5'TOP motif may be features that were scaffolded over a more fundamental mechanism of LARP1-associated control of gene expression.

2.3. Introduction

The La and La-related proteins (LARPs) are a family of conserved RNA-binding proteins, ubiquitous in nearly all eukaryotes, with important functions in RNA metabolism¹⁻³. Genuine La proteins are best characterized in promoting the maturation of precursor tRNAs (pre-tRNAs) through the binding and protection of a terminal poly-uridylate (UUU-3'OH) sequence⁴. La is an essential protein in all metazoans examined but it is dispensable in *Saccharomyces cerevisiae* (*S. cerevisiae*) and *Schizosaccharomyces pombe* (*S. pombe*)⁵⁻¹⁰.

Much of the work done to characterize La and its functions, therefore, has been established in yeast models. Genuine La (except in alveolates^{11,12}) contains two main RNA binding motifs: an N-terminally located La motif (LaM) and an RNA recognition motif (RRM1) which follows the LaM. These two regions constitute what is known as the La module which cooperate for binding to the UUU-3'OH motif in a special way^{13,14}. There are also several distinct La-related proteins (LARPs) that have been described since the original classification of genuine La. The La module is also commonly found within the LARPs, but for several of these it is no longer linked to UUU-3'OH binding. Furthermore, many LARPs also harbour other motifs which are important and specific to each family's function.

La-related protein 1 (LARP1) family members have been identified in a number of model organisms, with recurring functional themes relating to mRNA translation and mRNA stability. A DM15 region is characteristic of all studied LARP1 family members except the yeast orthologs and two of the three *Arabidopsis* orthologs¹⁵ (Figure 1A). The *S. cerevisiae* Sro9p and Slf1p are known to be mainly cytoplasmic, they associate with translating ribosomes, and are suggested to be important for oxidative stress response and metal ion metabolism¹⁶⁻²¹. The *C. elegans* LARP1 is important for proper oogenesis and localizes to P-bodies or sites of mRNA decay, suggesting that it has a role in mRNA degradation^{22,23}. *Arabidopsis* LARP1 recruits XRN4 to polysomes, facilitating the 5'-3' degradation of housekeeping and growth-coding mRNAs under heat stress²⁴. There are two other LARP1 isoforms in *Arabidopsis*, which similar to the *S. pombe* LARP1 only contain a LaM and no RRM1 or DM15 region, and they have been shown to induce precocious leaf senescence if overexpressed²⁵. The *Drosophila* dlarp was characterized as an essential Hox target protein

that binds the poly(A)-binding protein and is important for spermatogenesis^{26–28}. The more extensively characterized human LARP1 (hLARP1) protein binds the 7-methylguanosine (m⁷G) cap and the invariant +1 cytidine of 5' terminal oligopyrimidine (5'TOP) mRNAs through the DM15 region^{29–31}. 5'TOP mRNAs encode proteins that are important in the process of mRNA translation and are identified by a sequence of 5-15 pyrimidines in their 5'UTRs, immediately following the m⁷G cap^{32–34}. Accumulating evidence shows that hLARP1 function involves the stability and translation of 5'TOP mRNAs, but this has been controversial and is still poorly understood^{35–43}. In addition, the specific interaction of hLARP1 with 5'TOP mRNAs is suggested to indirectly modulate events such as overall protein production and cell proliferation, as well as disease states such as cancer^{44–48}.

Studies on LARP1 in different model organisms hint at functions mainly involving mRNA translation and decay, arguing for the conservation of these functions for this family throughout evolution. *S. pombe* has been a useful model for studying La function, and even though LARP1 orthologs in many model organisms have either been studied or characterized, the *S. pombe* LARP1 ortholog remains uncharacterized. Based on previous studies on LARP1 family members and the conservation of the superfamily across different eukaryotes, as well as the usefulness of the *S. pombe* system in characterizing genuine La, we aimed to gain a holistic understanding of the function of the *S. pombe* LARP1 (**Slr1p**, PomBase: *slr1*, Gene ID: SPAC1527.03⁴⁹).

Notable to *S. pombe* is that its single LARP1 ortholog possesses neither a DM15 region, nor have 5'TOP mRNAs been described in this system as they are characterized in higher eukaryotes. *S. pombe* still bears the mRNAs that are functionally similar to 5'TOP mRNAs,

in that they code for ribosomal proteins and other factors important in the process of mRNA translation, but these do not have a 5' terminal oligopyrimidine sequence (i.e., 5-15 pyrimidines with an invariant +1 C following the m⁷G cap). Given the link established between the DM15 and 5'TOP motifs described in higher organisms, the conservation of LARP1 family members in species that lack these, such as *S. pombe*, raises the intriguing possibility that there may be additional mechanisms by which LARP1 family members control mRNA translation and decay.

In this work, we show that Slr1p indeed regulates the translation and stability of mRNAs associated with translation, such as those encoding ribosomal proteins (RP mRNAs). We demonstrate that Slr1p physically associates with factors linked to mRNA translation and stability, and that it specifically binds an AC-rich motif enriched in the 5'UTRs, proximal to the start codon, of RP mRNAs. Interestingly, *slr1*- cells show both decreased translation and increased stability of RP mRNAs, which we validate using AC-rich motif-containing reporters. Our data suggest that the involvement of LARP1 family members in RP mRNA metabolism might predate the emergence of the 5'TOP sequence and the DM15 region, and that such mRNAs may thus functionally group as proto-5'TOP mRNAs. These data highlight the potential importance of DM15-independent RNA binding modes in the possible deconvolution of confounding results previously observed for hLARP1.

2.4. Results

2.4.1. Slr1p interacts with factors linked to mRNA translation and decay

LARP1 family members in higher eukaryotes have been hypothesized to contain a La module comprised of a winged-helix fold La motif (LaM) and an adjacent RNA recognition motif fold (RRM, although hLARP1a was shown to lack the RRM recently⁵⁰), as well as a more C-terminal DM15 motif (Figure 1A). A co-crystal of the hLARP1 DM15 and a 5'TOP sequence has demonstrated that the DM15 binds the invariant “+1C” residue immediately downstream of the m⁷G cap in 5'TOP mRNAs^{15,30}. Despite lacking an RRM immediately C-terminal to the LaM as well as a DM15 motif, an *S. pombe* LaM-containing protein (SPAC1527.03, which we name here *S. pombe* La-related protein 1, or Slr1p) has been phylogenetically grouped into the LARP1 family^{1,15}. In an attempt to determine the function of Slr1p, we set out to identify factors that associate with Slr1p by integrating a protein-A (PrA) tag⁵¹ into the endogenous Slr1p locus followed by immunoprecipitation and characterization of Slr1p complexes by LC/MS-MS. In addition, we also performed a yeast two-hybrid analysis of a) full length Slr1p, b) the Slr1p NTD (amino acids 1-323) and c) the Slr1p CTD (amino acids 312-475, including the LaM).

Our LC/MS-MS analysis revealed that Slr1p-PrA co-immunoprecipitated several components of the mRNA translation machinery relative to an untagged strain, including the translation initiation factor eIF4G (Tif471p), several components of eIF3 including eIF3i (Sum1p), as well as cytoplasmic poly(A)-binding protein (PABP) (Figure 1B; Tables S1A and S1B). While our yeast two-hybrid screen with the full-length protein was unsuccessful, our

yeast two-hybrid using the Slr1p CTD resulted in hits for a number of proteins that included eIF3i (Sum1p) and the 5' to 3' mRNA exonuclease and XRN1 homolog Exo2p (Table S2). Notably, the *Arabidopsis* XRN1 homolog XRN4 has previously been demonstrated to form a complex with a LARP1 homolog in this system²⁴. To confirm a number of these putative interactions, we performed co-immunoprecipitation experiments in the presence or absence of nuclease (benzonase) in order to determine whether prospective interactions relied on the presence of nucleic acids (Figure 1C). Western blotting confirmed that Slr1p immunoprecipitated PABP but in a manner that was sensitive to benzonase treatment. In contrast eIF4G (Tif471p), eIF3i (Sum1p) and Exo2p co-precipitated with Slr1p in a nuclease-insensitive manner, although enrichment of Sum1p was less apparent above background, especially without nuclease treatment, and the persistence of these factors in the flowthrough are indicative that Slr1p only binds a subset of the cellular cohort of these proteins. Together, our proteomic data link Slr1p to functions in mRNA translation and decay, as has been proposed for LARP1 homologs in higher eukaryotes.

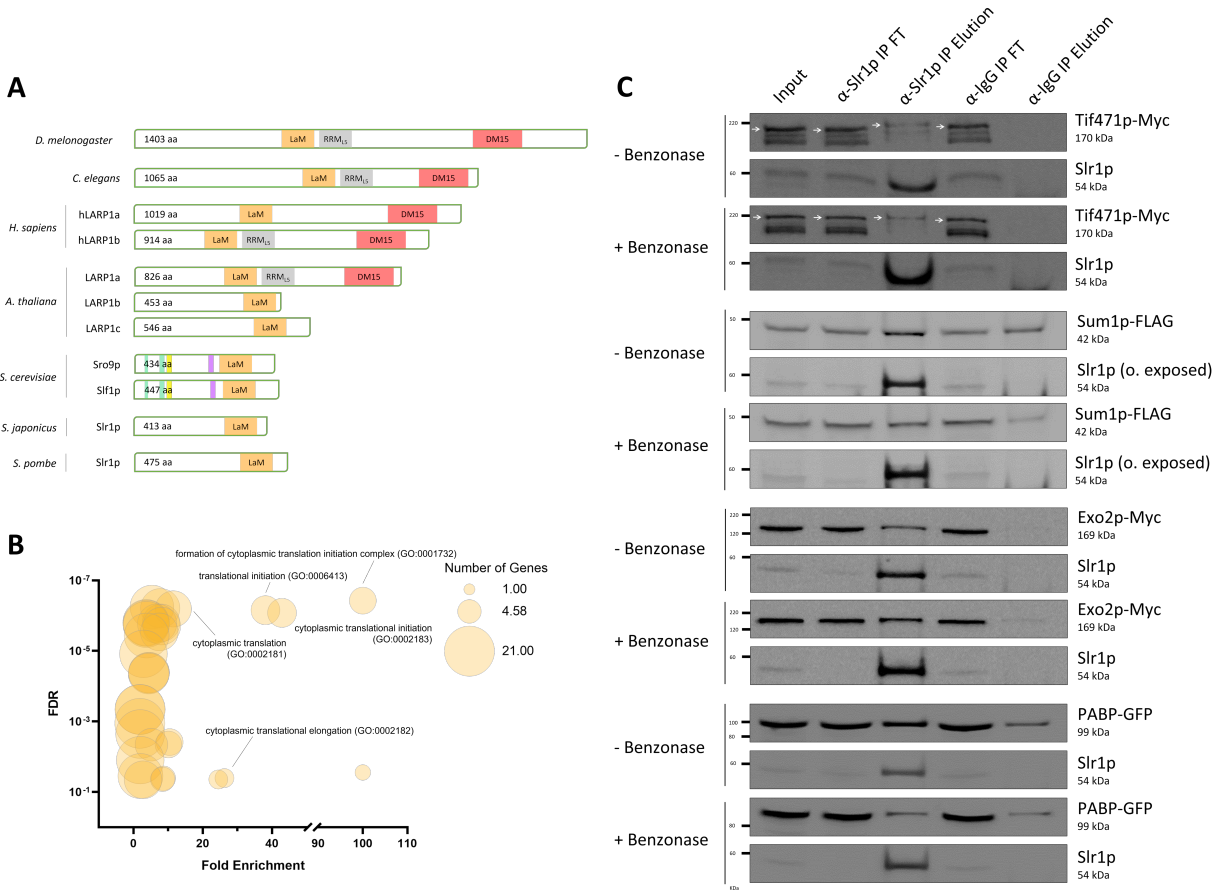


Figure 1: Slr1p interacts with mRNA translation and decay factors. A) Schematic representations of LARP1 proteins across several eukaryotes, all of which have a La motif (LaM) with some containing an RNA Recognition Motif (RRM_{L5}) and/or a DM15 region. The *S. cerevisiae* proteins were proposed to have two conserved patches (green) plus a lysine-rich (yellow) and an asparagine-rich (purple) region located upstream of their LaM (adopted from Deragon and Bousquet-Antonelli, 2015)¹⁵. **B)** Proteins that interact with Slr1p were identified by LC/MS-MS (n=2 biological replicates). These are enriched for Gene Ontology (GO) biological processes related to mRNA translation and translation initiation. GO categories with a fold enrichment of >100 were plotted at 100. FDR=False Discovery Rate as calculated by GO enrichment analysis. See also Tables S1A and S1B. **C)** Slr1p interacts with eIF4G (Tif471p, arrows point to the full-length protein), eIF3i (Sum1p) and the *S. pombe* XRN1 ortholog, Exo2p, in an RNA-independent manner while its interaction with poly(A)-binding protein (PABP) is sensitive to benzonase treatment. See also Tables S1 and S2. Tif471p and Exo2p were endogenously tagged with Myc, Sum1p was tagged with FLAG and PABP was tagged with GFP. Slr1p was visualized using a custom antibody. Each set of co-immunoprecipitations were performed for a minimum of two times and representative blots are shown (n=2 biological replicates). 15 µl of each of input and FT was loaded per lane. For elutions: 25 µl of beads were used for each IP, all of which was resuspended in 45 µl 5X Laemmli buffer+10% β-mercaptoethanol, of which 15 µl was loaded per lane. α=Anti-,

IP=Immunoprecipitation, FT=Flowthrough, o. exposed=Overexposed to visualize the band corresponding to Slr1p in input.

2.4.2. Slr1p controls the stability of mRNAs that encode factors important for the translational apparatus

Based on previous studies on La-related protein 1 (LARP1) family members and the conservation of the superfamily across different eukaryotes, we hypothesized that the *S. pombe* LARP1 ortholog is linked to mRNA translation^{1,15,35,36}. To assess how Slr1p might function in mRNA translation, we compared transcriptome-wide mRNA abundance in *slr1+* versus *slr1-* cells by RNA-Seq of polysome-associated mRNAs and total mRNAs. We observed that a subset of mRNAs enriched for the Gene Ontology (GO) biological process “cytoplasmic translation” showed increased abundance in *slr1-* cells (Figure 2A; Table S3). Intriguingly, we noted that such transcripts had similar polysome occupancy in *slr1-* versus *slr1+* cells, as opposed to a commensurate increase in polysome occupancy relating to the increase in their total mRNA abundance in *slr1-* cells (Figure 2B and S1). This is consistent with a possible positive effect of Slr1p on ribosomal protein mRNA translation in wildtype cells. Notably, these transcripts appear to be functionally related to 5'TOP motif-containing (and hLARP1-linked) mRNAs as described in higher eukaryotes, in that they are highly expressed (Figure S6) and code for ribosomal proteins and other factors important in the process of mRNA translation, despite a lack of evidence for the 5'TOP motif in *S. pombe* or other single-celled eukaryotes. For simplicity, we hereto refer to this enriched cohort of mRNAs as ribosomal protein (RP) mRNAs.

We hypothesized that the decreased steady state abundance of RP mRNAs might be due to decreased transcript stability in the presence of Slr1p. To test this, we performed a RATE-Seq or 4-thiouracil (4tU) *in vivo* metabolic labelling to measure transcriptome-wide mRNA half-lives and decay rates in wildtype versus *slr1*- cells^{52,53}. We identified 208 RNA transcripts that were stabilized and 44 RNAs that were destabilized at least 2-fold in *slr1*- cells (Figure S2A; Table S4). Similar to our RNA-Seq dataset, we noted that the cohort of stabilized mRNAs were significantly enriched for the GO biological process “cytoplasmic translation” (Figure 2C and S2B; Table S4), while the destabilized cohort were not significantly enriched for a GO process. These data are thus consistent with the increased abundance of RP mRNAs in *slr1*- cells being linked to post-transcriptional mRNA decay.

In order to investigate if these effects on RP mRNA stability might be due to a direct interaction, we generated custom polyclonal antibodies and immunoprecipitated Slr1p after formaldehyde crosslink and sequenced associated RNAs (RIP-Seq) relative to an IgG isotype control. Similar to our RNA-Seq and RATE-Seq datasets, our RIP-Seq results indicated that Slr1p is associated with mRNAs that are enriched for GO processes related to translation (Figure 2D; Table S5). Comparison of the three independently derived RNA-Seq, RATE-Seq and RIP-Seq datasets revealed a substantial degree of overlap between the enriched transcripts, which were then also enriched for the GO process “cytoplasmic translation” (Figure 2E; Table S6). Together, these data are consistent with Slr1p being associated with translation complexes and having a role in the stability of RP mRNAs in *S. pombe*, which are functionally related to 5'TOP mRNAs that are controlled by LARP1

proteins in higher eukaryotes, despite the lack of this motif described in yeast mRNAs or the DM15 motif in Slr1p.

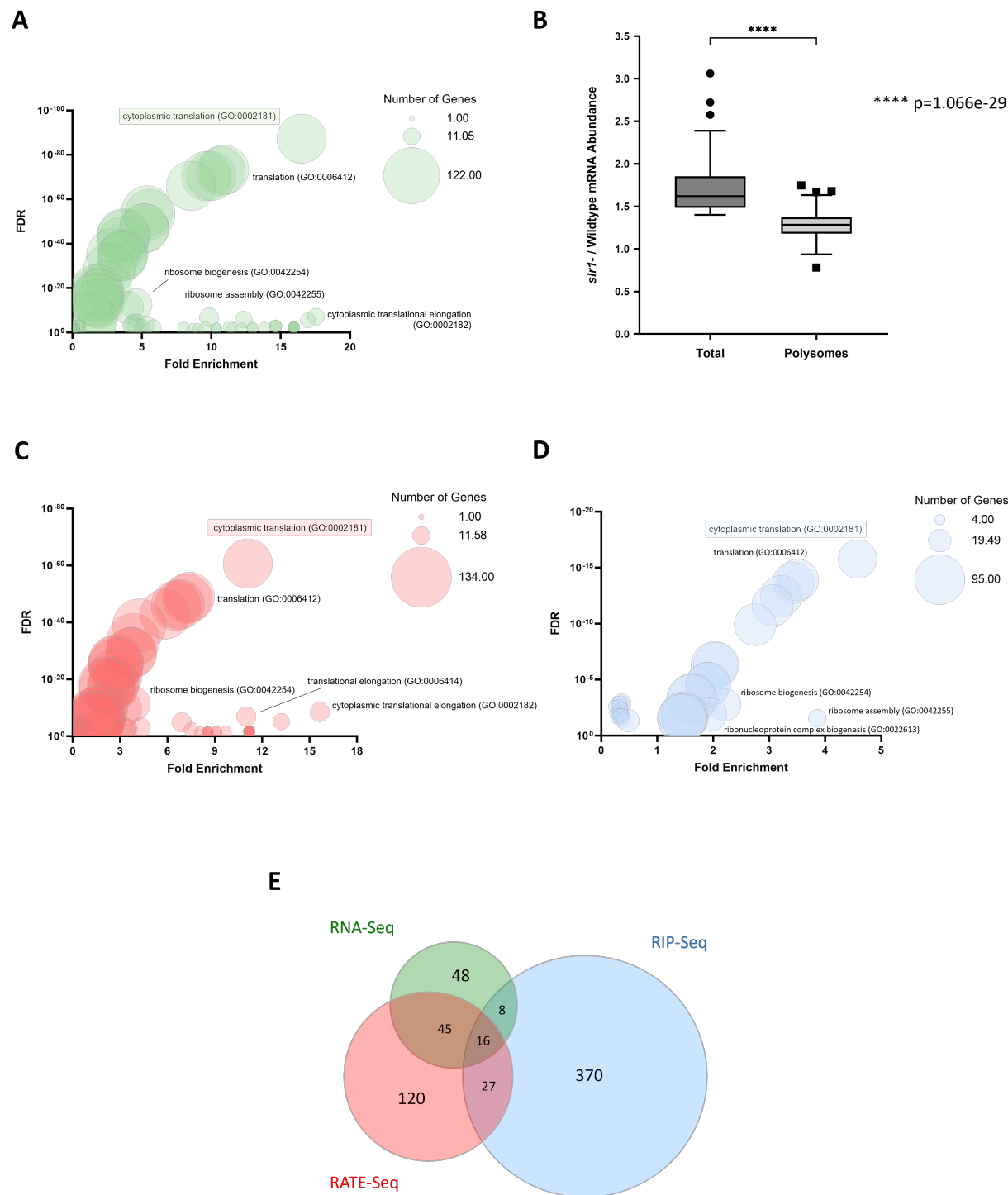


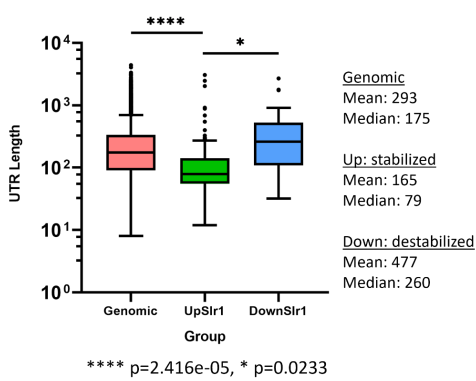
Figure 2: Slr1p interacts with and affects the mRNA stability of a cohort of mRNAs enriched for GO processes related to translation. **A)** Translational profiling followed by RNA-Seq analysis shows that mRNAs enriched for translation-related GO processes have an increased total RNA abundance in *slr1*- cells (n=2 biological replicates). FDR=False Discovery Rate as calculated by GO enrichment analysis. See also Table S3. **B)** mRNA transcripts with increased total RNA abundance in *slr1*- cells are significantly less enriched in polysomes (normalized to wildtype RNA levels). Tukey boxplots were generated using Prism; statistical analysis performed: unpaired two-tailed t-tests with Welch's correction (unequal SDs) with Alpha=0.05. See also Table S3, and Figure S1. **C)** RATE-Seq: stabilized RNAs in *slr1*- cells are enriched for translation-related GO processes (n=3 biological replicates). FDR=False Discovery Rate as calculated by GO enrichment analysis. See also Table S4, and Figure S2. **D)** RIP-Seq: Slr1p-immunoprecipitated mRNAs are enriched for GO processes related to cytoplasmic translation (n=2 biological replicates). FDR=False Discovery Rate as calculated by GO enrichment analysis. See also Table S5. **E)** Overlap between transcripts enriched in each of the three sequencing experiments. The 16 mRNAs common to all three datasets are most highly enriched for the GO process "cytoplasmic translation". See also Table S6.

2.4.3. Slr1p-linked mRNAs are enriched for U-rich and AC-rich motifs in their 5'UTRs

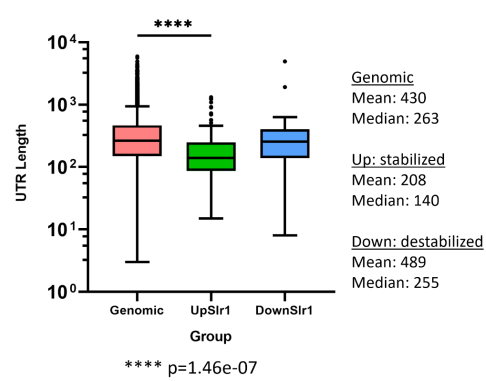
We hypothesized that the Slr1p-affected RP mRNA cohort might be enriched for particular features or motifs in their 5' or 3' UTRs. To test this, we analyzed their UTRs for length and GC content, and performed a MEME motif discovery search⁵⁴. We observed that mRNAs that are stabilized in absence of Slr1p have significantly shorter 5' and 3' UTRs relative to general transcripts in the *S. pombe* transcriptome (Figure 3A). We also noted that the stabilized transcripts have a higher GC content in their 5'UTRs in comparison to mRNAs more generally (Figure 3B). Our MEME motif discovery searches revealed two enriched motifs in the 5'UTRs of the stabilized transcripts, which we will refer to as the U-rich and the AC-rich motifs (Figure 3C). We also queried where these motifs were localized in the endogenous identified RP mRNAs and found that the AC-rich motif was more likely to be found proximal to the start AUG, while the U-rich was more likely to be found closer to the transcriptional start site

(Figure 3D; Tables S7 and S7B). Therefore, we have decided to name these motifs the 5' Distal U-Rich (5'DUR) and the 5' Proximal AC-rich (5'PAC) motifs. In order to investigate the distribution of the Slr1p-linked 5'UTR motifs another way, we selected mRNAs whose function is linked to the GO term “cytoplasmic translation” (GO:0002181) and reran the MEME motif discovery search on their 5'UTRs. Interestingly, the MEME search retrieved a 15-nucleotide motif as well as a 19-nucleotide motif that were nearly identical to the 5' end or the entirety of the originally obtained 5'PAC motif (Figure 3E), consistent with this motif being a general feature of RP mRNAs in this system (full list found in Tables S7 and S7B). We also tried to look for 5'UTR motifs in the mRNAs whose function is again linked to the GO term “cytoplasmic translation” (GO:0002181) in a related fission yeast, *Schizosaccharomyces japonicus*⁵⁵, and found AC-rich motifs that closely resemble the 5'PAC motif described here in *S. pombe* (Figure S3A) arguing for the broader existence of this motif in *Schizosaccharomyces* species.

A 5'UTR Length

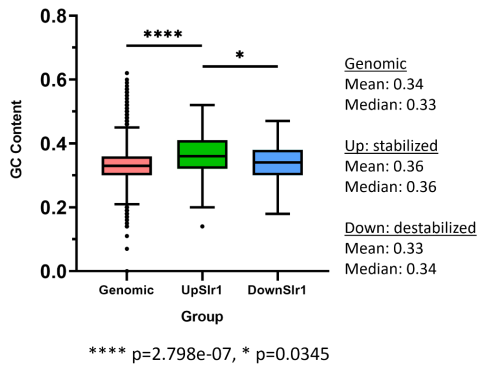


3'UTR Length

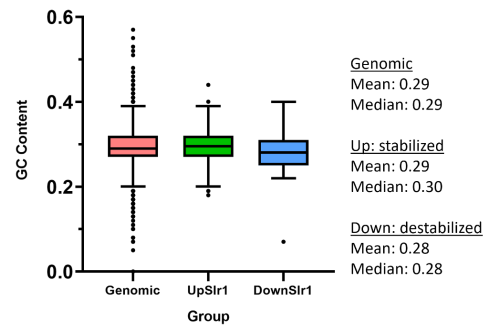


B

5'UTR GC Content

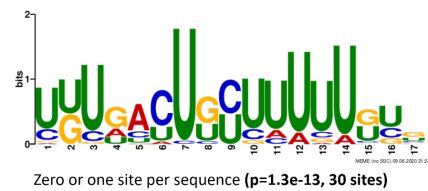


3'UTR GC Content

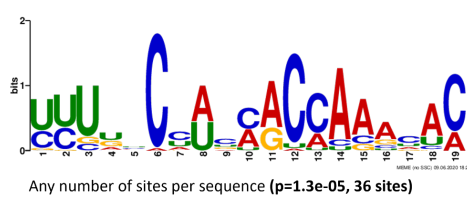


C

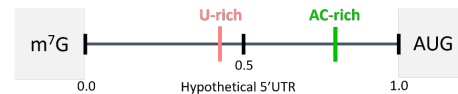
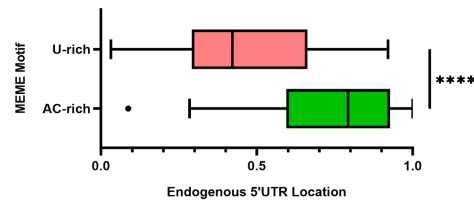
5' Distal U-rich Motif (5'DUR):



5' Proximal AC-rich Motif (5'PAC):



D



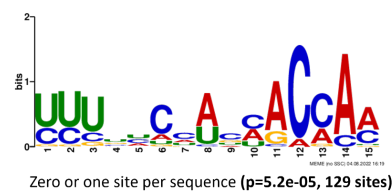
U-rich
Mean: 0.471
Median: 0.422

AC-rich
Mean: 0.737
Median: 0.792

**** p=5.679e-06

E

RP 15-mer 5' Proximal AC-rich Motif (PAC15):



RP 19-mer 5' Proximal AC-rich Motif:

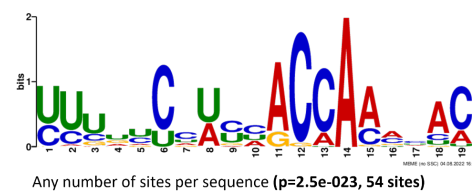


Figure 3: 5' & 3' UTR analysis of transcripts that change in stability in *slr1*- cells. **A)** Transcripts identified with increased stability in absence of Slr1p (UpSlr1) have shorter 5' and 3' untranslated regions (UTRs) and a **B)** higher GC content in their 5'UTRs. UTR length and GC content were statistically analyzed using unpaired two-tailed t-tests without assuming equal SDs (Welch's correction) for each of the following groups: UpSlr1 versus Genomic, DownSlr1 versus Genomic and UpSlr1 versus DownSlr1 with Alpha=0.05. Statistical tests and Tukey boxplots were generated using Prism. **C)** Enrichment of a U-rich (5'DUR) and an AC-rich (5'PAC) motif in the 5'UTRs of stabilized messages in *slr1*- cells. See also Table S7 and S7B. p values were generated by MEME Suite 5.4.1. **D)** The U-rich motif resides closer to the m⁷G cap and the AC-rich motif closer to the initiator AUG. An unpaired two-tailed t-test was performed, Alpha=0.05, and Tukey boxplots were generated using Prism. **E)** 265 mRNAs annotated for the GO process "cytoplasmic translation" in *S. pombe* (GO:0002181) included in a motif discovery search are enriched for an RP 15-mer 5' Proximal AC-rich motif (PAC15) and an RP 19-mer 5' Proximal AC-rich motif. p values were generated by MEME Suite 5.5.3.

To test whether these motifs might represent direct binding sites for Slr1p, we performed electrophoretic mobility shift assays (EMSAs) using RNAs corresponding to the 5'DUR or 5'PAC motif, or variants of these containing alterations at the most highly overrepresented nucleotides (Figure 4). Even though a substantial portion of Slr1p aggregated and bound RNA non-specifically in the well, we observed that Slr1p indeed formed a discrete ribonucleoprotein with the 5'PAC motif that was lost upon alteration of the most highly enriched cytosines to guanines, consistent with the possibility that Slr1p may engage RP mRNAs through the 5'PAC motif directly *in vivo* (Figure 4A). Conversely, no interaction was observed with the 5'DUR motif (data not shown), suggesting that this motif may be incomplete in our tested RNA substrate, or that the link between this motif and Slr1p function may be indirect. We additionally tested whether mutating the 5' uracil residues (U1-4) in the 5'PAC motif to adenine residues affects binding by Slr1p. We observed that Slr1p no longer engaged the mutated RNA substrate, consistent with a role in binding pyrimidine-rich

regions analogous to the 5'TOP sequence in higher eukaryotes (Figure 4A). Notably, we made single C to G mutations to the 5'PAC motif as well and observed that a C6G mutation completely disrupted binding, where a C19G or a C12G mutation had less pronounced effects towards loss of binding (Figure S3B).

In order to investigate the interaction between Slr1p and the 5'PAC motif more comprehensively, we also compared binding of the wildtype protein as well as some Slr1p mutants to both the original 19-mer 5'PAC motif and the similar, shorter 15-mer (PAC15) identified from the 5'UTRs of mRNAs linked to cytoplasmic translation. We tested binding of these to full-length Slr1p mutants carrying either a point mutation to a tyrosine (Y335A) known to be essential for RNA binding via the classic La-motif dependent mode¹³ or to a lysine predicted to project from the same nucleic acid binding surface (K344A). We found that the wildtype protein bound the 19-mer with slightly higher affinity than the 15-mer (Figure 4B), and that Slr1p binding was also lost upon the introduction of the C6G mutation in the context of the 15-mer RNA substrate (Figure 4B). Interestingly, the point mutations Y335A and K344A had minor effects on binding to the 19-mer but showed more substantial defects in binding to the 15-mer, suggesting that the importance of these residues in RNA recognition becomes more apparent as fewer Slr1p-RNA contacts are permitted through the shortening of the substrate (Figure 4C). We also tested a shortened Slr1p variant comprising the LaM and regions C-terminal to it and found that this also bound the 19-mer 5'PAC motif specifically, in that the triple C mutation eliminated binding (Figure S3C). Together, these data indicate that Slr1p specifically recognizes the 5'PAC sequence, that the length of the

RNA is important for binding, and that canonical RNA binding surface of the La motif plays a role in 5'PAC recognition.

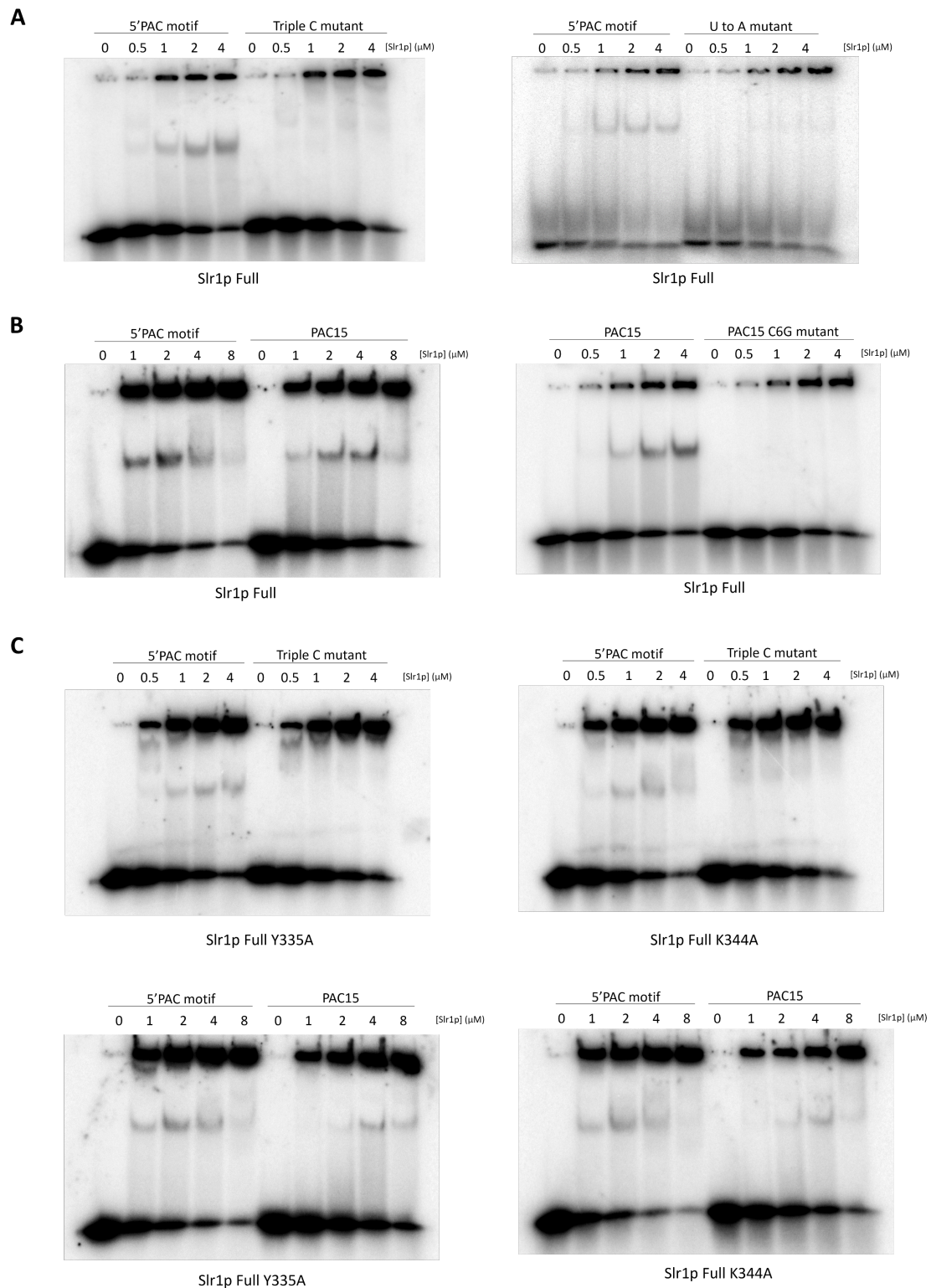


Figure 4: Slr1p binds the 5'PAC motif specifically *in vitro*. Electrophoretic mobility shift assays (EMSAs) for Slr1p binding to various RNA substrates. **A)** Slr1p makes a distinct ribonucleoprotein with the 5' Proximal AC-rich motif (5'PAC motif: UUUUCCCACCACCAAACAC) *in vitro*. This interaction is lost upon mutating the 5'PAC motif (Triple C mutant: UUUUC**G**CACCAGCAAACAG; C6G, C12G, C19G) or by mutating the 5' U residues to A's (U to A mutant: **AAA**ACCCACCACCAAACAC). **B)** Slr1p binds the shorter RP 15-mer 5' Proximal AC-rich motif (PAC15: UUUUUCCACCACCAA) and binding is lost upon a single mutation (PAC15 C6G mutant: UUUUU**G**CACCACCAA). **C)** Point mutants of Slr1p show normal binding to the 19-nucleotide 5'PAC motif but impaired binding to the 15-nucleotide 5'PAC motif (PAC15). EMSAs were performed at least twice in each case with representative experiments shown.

2.4.4. Slr1p promotes the expression of 5'PAC and 5'DUR motif-containing mRNAs even as these are destabilized

We next hypothesized that the 5'PAC and 5'DUR motifs might have regulatory roles with respect to Slr1p's function in mRNA translation and decay. To explore such roles, we generated a series of *Renilla* reporters (Figure 5A) where we placed the motifs either immediately upstream to the *Renilla* start codon (20N/(PAC or DUR motif)/AUG; 20N = twenty randomly selected nucleotides) or further upstream ((PAC or DUR motif)/20N/AUG). We also compared effects of differential expression by including the reporters on high expression (pRep3x-based) or low expression (pRep81x-based) plasmids. These reporters were introduced into wildtype and *slr1*- cells and compared to a 40N reporter (the previous randomly selected 20N sequence, repeated). We observed that the DUR-20N (but not the 20N-DUR) and the 20N-PAC (but not the PAC-20N) reporters had significantly higher reporter gene expressions in wildtype cells when the reporters were highly expressed (pRep3x-based; Figure 5B). Notably, these arrangements mimic the endogenous locations of the U-rich and AC-rich motifs in the Slr1p-destabilized RP mRNA cohort, with the 5'PAC

motif enriched closer to the AUG than the 5'DUR motif (Figure 3D). These differences in reporter gene expression between wildtype and *slr1*- cells were not observed in the context of the more lowly expressed pRep81x (Figure S4A and see Discussion). Based on our understanding of known LaM-substrate interactions^{4,56-58}, we also tested poly(A)- and poly(U)-containing versions of the same reporters in the context of more highly expressed pRep3x (Figure S4B). We noticed that the differences in reporter gene expression between wildtype and *slr1*- cells that we observed with the 5'PAC and 5'DUR motifs were lost in this case (Figure S4C). These data suggest that the function of the 5'PAC and 5'DUR motifs in Slr1p-dependent expression is specific to those motifs and cannot be recapitulated from more general homopolymers.

Our transcriptomic data showed that Slr1p may have a role in promoting the translation of RP mRNAs in wildtype cells even as they were destabilized. In order to study how RNA abundance corresponds with our reporter gene expression, we extracted total RNA from wildtype and *slr1*- cells and performed Northern blots for the reporter transcripts. We confirmed that the mRNAs for these reporters were indeed less abundant in wildtype cells relative to *slr1*- cells compared to the 40N control reporter (Figure 5C). Given both the cytoplasmic localization of Slr1p⁵⁹ and our observed Slr1p-associated effect on RNA stability of 5'PAC- and 5'DUR-enriched endogenous mRNAs (Figure 2C), it is likely that these reporter based data are consistent with our transcriptome-wide analyses suggesting that Slr1p promotes the expression of 5'PAC- and 5'DUR-containing mRNAs (Figure 2B) even as it also promotes their decay (Figure 2A and 2C), although an indirect effect on RNA transcription rates cannot be ruled out.

In order to investigate the effect that Slr1p may have on reporter gene expression more directly, we tethered Slr1p to either the 5' or 3' end of a *Renilla* reporter gene using the 5BoxB and LambdaN tethering system⁶⁰. *Renilla* reporter expression was thus measured in *slr1*-cells in which either Slr1p or β -galactosidase was tethered to the positions indicated (Figure 5D). Consistent with our 20N/PAC and DUR/20N reporter data, we observed higher reporter gene expression when Slr1p was tethered to the 5'UTR relative to the β -galactosidase control, while expression was lower when Slr1p was tethered to the 3'UTR (Figure 5E). However, the relative abundance of the mRNAs for the reporter genes decreased when Slr1p was tethered to either the 5'UTR or 3'UTR (Figure 5F). Together, these data suggest that Slr1p destabilizes bound mRNAs but has the added effect of promoting the expression of mRNAs when it is bound to the 5'UTR.

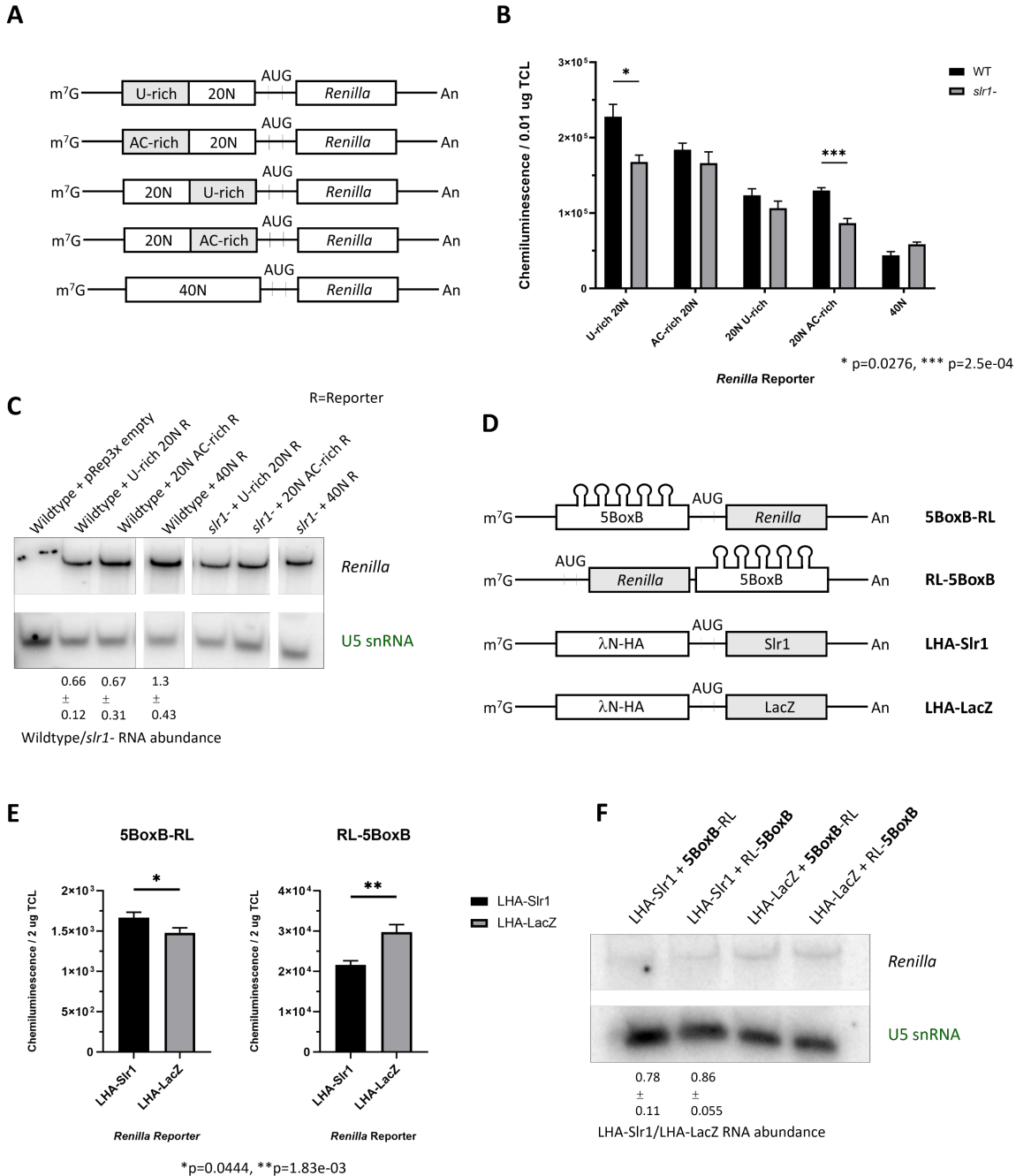


Figure 5: *Renilla* reporter analysis of *Slr1*p-associated 5'UTR motifs. **A)** Schematic representation of reporters containing specific motifs identified by RATE-Seq analysis including the 5'DUR (U-rich) and the 5'PAC (AC-rich) motif. 20N or 40N refers to 20 or 40 randomly selected nucleotides. See also Table S7. **B)** U-rich 20N and 20N AC-rich reporters are significantly more highly expressed in wildtype cells in comparison to *slr1*⁻ cells and the 40N control reporter. Data are represented as mean + SEM error bars. **C)** Abundance of transformed mRNA reporters as measured by Northern blot in wildtype versus *slr1*⁻ cells. Quantified reporter band intensities were normalized to intensity of U5 snRNA. Values for

abundance are represented as mean $\pm$ SEM. pRep3x empty: wildtype cells lacking reporter, transformed with vector control. **D)** Schematic representations of reporters cloned using the 5BoxB and LambdaN tethering system. 5BoxB elements were placed either in the 5'UTR or 3'UTR of *Renilla* reporters (5BoxB-RL and RL-5BoxB, respectively). The LHA-LacZ set up was used as a control. **E)** Reporters are significantly more highly expressed when Slr1p is tethered to the 5'UTR and less expressed when it is tethered to the 3'UTR, relative to the LHA-LacZ negative control. Data are represented as mean + SEM error bars. **F)** Abundance of transformed tethered mRNA reporters as measured by Northern blot in wildtype versus *slr1*-cells. Quantified reporter band intensities were normalized to intensity of U5 snRNA. Values for abundance are represented as mean $\pm$ SEM.

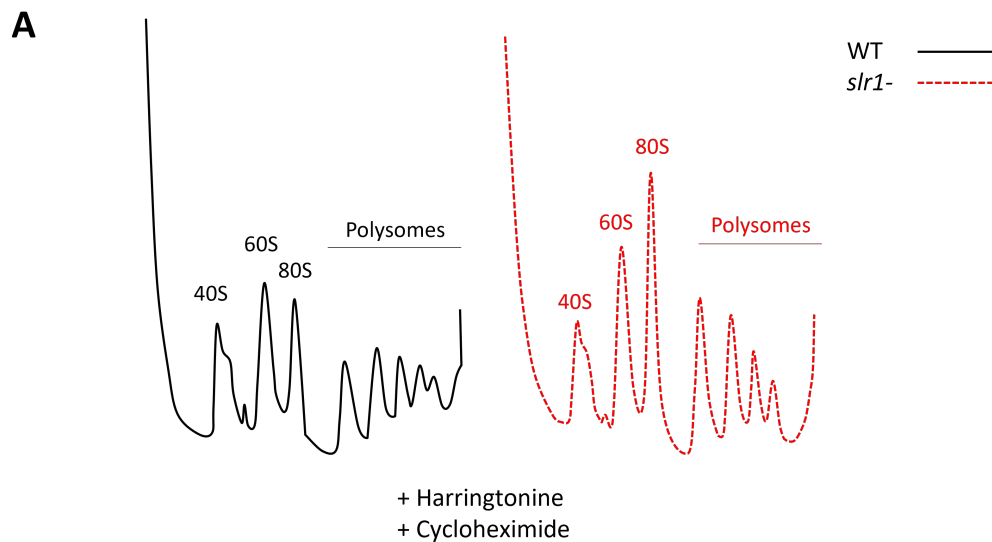
Reporter assays: n=3-4 biological replicates per reporter, 3 technical replicate readings per biological replicate, providing a total of 9 or 12 readings for each reporter. Multiple unpaired t-tests were performed by Prism without assuming consistent SDs and using the Holm-Šídák method with Alpha=0.05. Northern blots: n=4 biological replicates in each case. WT=Wildtype, R=Reporter, TCL=Total Cell Lysate, RL=*Renilla* Luciferase, LHA=LambdaN followed by HA tag, SEM=Standard Error of the Mean.

2.4.5. Slr1p promotes translation initiation and recruits Exo2p to translation complexes

We hypothesized that the function of Slr1p could be related to the regulation of mRNA translation initiation. To test for a possible defect related to the function of Slr1p when translation initiation is inhibited, we performed polysome profiling on wildtype and *slr1*-cells in presence of the translation initiation inhibitor harringtonine, which impedes initiating ribosomes⁶¹, in both rich and minimal media. In *slr1*- cells grown in minimal media, we reproducibly observed a diminishing trend of heavy polysomes relative to light polysomes, as well as an 80S peak higher than the 60S and 40S peaks. This was in contrast to wildtype cells, which showed more sustained heavy polysomes and lower subpolysomes, as evidenced by a lower 80S peak than 60S peak, consistent with a function for Slr1p in

promoting robust translation (Figure 6A; replicates in Figure S5A). We observed similar trends but with lesser effects in rich media (Figure S5B).

Our yeast two-hybrid and co-immunoprecipitation data indicated that Slr1p interacts with Exo2p in an RNA-independent manner. To explore the possibility that Slr1p promotes the co-translational decay of mRNAs, we probed western plots corresponding to *slr1*⁺ and *slr1*⁻ cells for Exo2p sedimentation after polysome profiling with cycloheximide treatment. Consistent with Slr1p function in mRNA decay, Exo2p was more highly recruited from 40S/60S subunits into monosomes in *slr1*⁺ cells relative to *slr1*⁻ cells, while PABP was not affected (Figure 6B; quantitation of Exo2p in fractionation in Figure 6C). Together, these data support a role for Slr1p in promoting translation initiation while also recruiting Exo2p to translation complexes, consistent with a role for Slr1p in promoting both mRNA translation and decay (Figure 7).



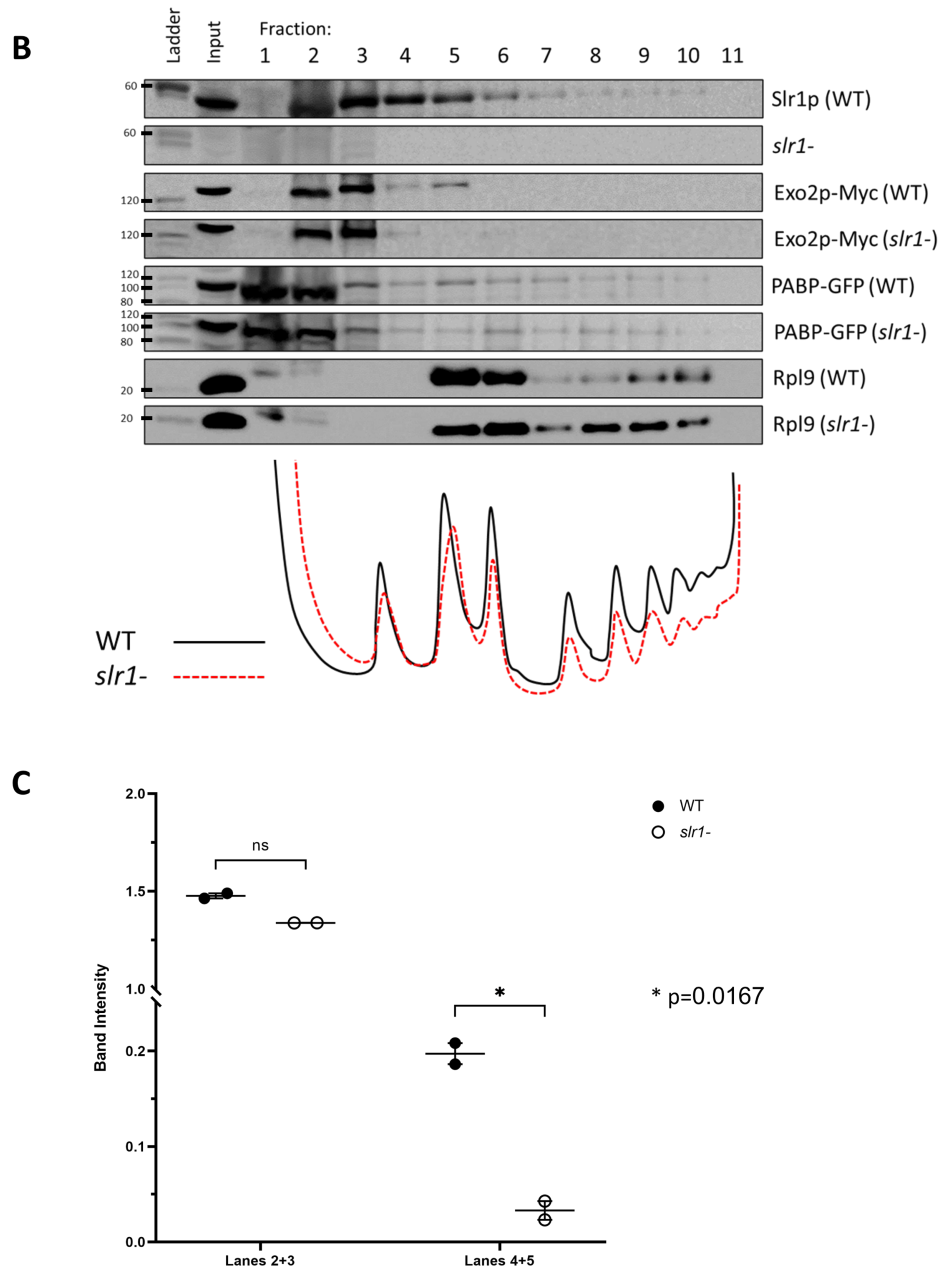
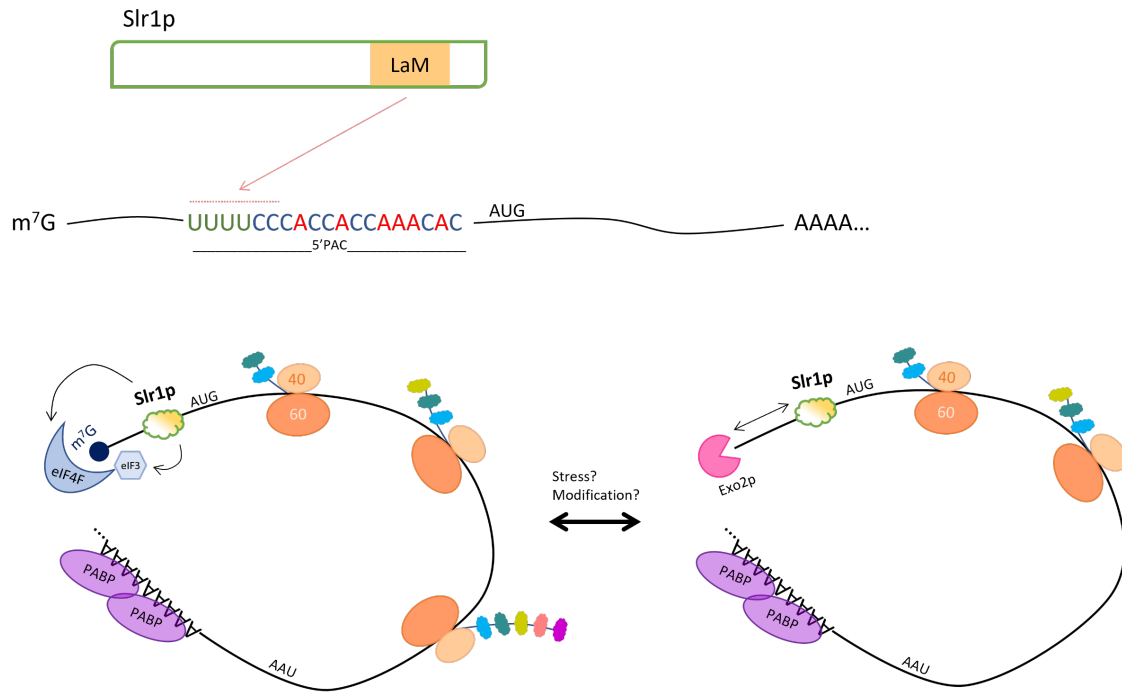


Figure 6: Polysome sedimentation patterns of Slr1p and associated factors. A) Polysomes of wildtype and *slr1*- cells grown in minimal media with harringtonine treatment (2 μ g/mL, 2 minutes) followed by cycloheximide (100 μ g/mL, 10 minutes). See also Figure S5. **B)** Western blots of polysome fractions from cycloheximide-treated wildtype and *slr1*- cells. **C)** Quantitation of Exo2p sedimentation comparing abundance in 40S/43S (lanes 2+3) to monosomes (4+5). Multiple unpaired t-tests, with Welch's correction assuming individual variance for each group, using the Holm-Šídák method were performed by Prism with Alpha=0.05. Individual values are shown in a scatter plot plus mean $\pm$ SEM error bars. Polysome profiles: n=4 biological replicates, western blots: n=2 biological replicates. WT=Wildtype.

A



B

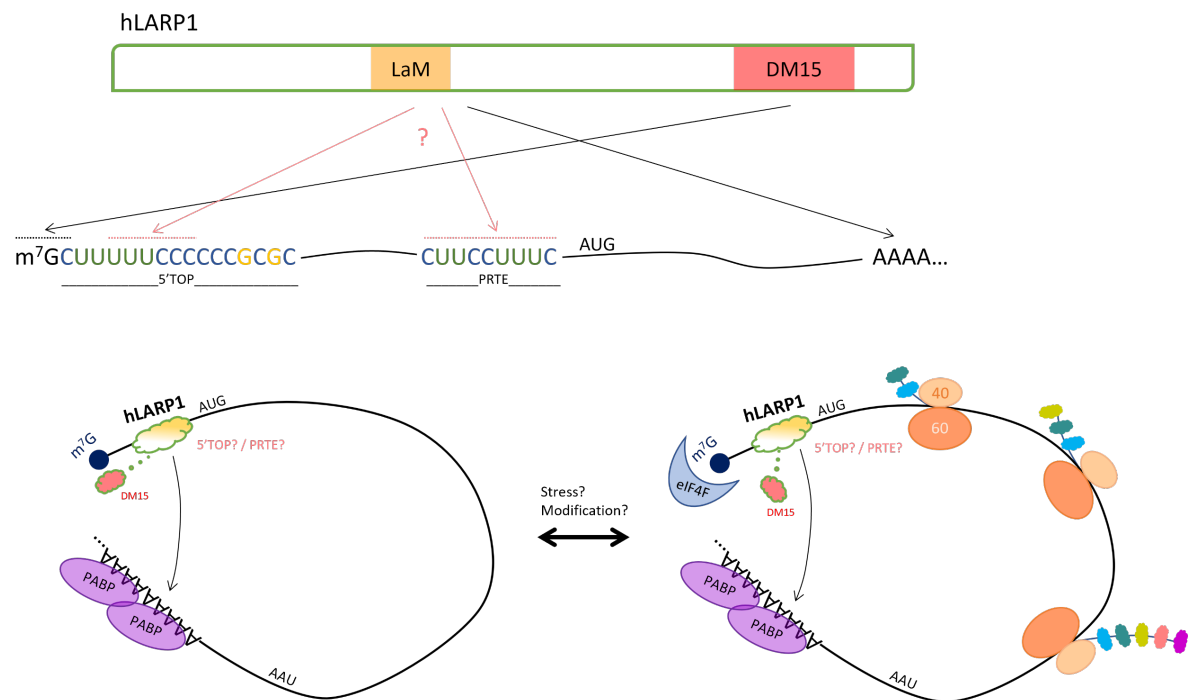


Figure 7: A model for various binding modes of LARP1 orthologs in *S. pombe* and humans. A) Slr1p interacts with the 5'PAC motif identified in close proximity to the start AUG in *S. pombe* ribosomal protein (RP) mRNAs. Slr1p also makes direct protein-protein contacts to components of eIF4F (eIF4G/Tif471p), eIF3 (eIF3i/Sum1p) and the 5'-3' exoribonuclease Exo2p (XRN1). Nutritional cues or environmental stressors may result in post-translational modifications of Slr1p and consequently affect its role in translational control and/or mRNA decay, as has been hypothesized for other LARP1 family members. **B)** Human LARP1 interacts with the m⁷G/+1C (Lahr *et al.*, 2017; Cassidy *et al.*, 2019)^{30,31}, the 5'TOP sequence (Al-Ashtal *et al.*, 2021)⁵⁸ and/or potentially the PRTE sequence (Hong *et al.*, 2017)⁶² as well as the poly(A) tail (Aoki *et al.*, 2013; Al-Ashtal *et al.*, 2021; Mattijssen *et al.*, 2021)^{56,58,64} of 5'TOP mRNAs in humans. LaM=La motif, 5'PAC=5' Proximal AC-rich, 5'TOP=5' Terminal Oligopyrimidine, PRTE=Pyrimidine Rich Translational Element.

2.5. Discussion

In this work we investigate the *S. pombe* La-related protein 1/Slr1p using a combination of proteomic and transcriptome-wide approaches. Overall, our data suggest that Slr1p plays a role in regulating both mRNA translation and decay. Our LC/MS-MS and yeast two-hybrid analyses identified direct interactions with factors important in these processes, including eIF4G, eIF3i and the 5'-3' exoribonuclease and XRN1 homolog Exo2p. Our three transcriptome-wide sequencing analyses showed that Slr1p affects ribosomal protein mRNAs (RP mRNAs) that are functionally similar to 5'TOP mRNAs in higher eukaryotes and we thus have referred to them as proto-5'TOP mRNAs. This is intriguing, as neither have RP mRNAs in *S. pombe* been characterized as 5'TOP mRNAs, lacking the characteristic 5-15 pyrimidines adjacent to the m⁷G cap, nor does Slr1p harbour a DM15 region, which in hLARP1 is paramount in how this factor exerts its regulatory function on 5'TOP mRNA metabolism. We show that in absence of Slr1p, RP mRNAs have a similar ribosomal occupancy yet an increased abundance compared to wildtype cells. Our RATE-Seq

confirms that this is due to an increased stability for such mRNAs in *slr1*- cells. Furthermore, our RIP-Seq indicates that Slr1p is enriched on such mRNAs, consistent with an Slr1p function affecting their translation and stability.

We further present biochemical data that confirm our transcriptome-wide analyses. Slr1p-affected mRNAs are enriched for two 5'UTR motifs that influence reporter gene expression *in vivo* and in an Slr1p-dependent fashion. Inclusion of the 5'PAC and 5'DUR motifs increases reporter gene expression, and we further show that this effect is dependent on the location of the motifs within the 5'UTR, which importantly resembles their locations within endogenous RP mRNA 5'UTRs. This elevated expression happens as there is also decreased reporter mRNA abundance, consistent with our RNA-Seq and RATE-seq datasets. It is notable that mRNAs which have an increased stability through loss of Slr1p are significantly more abundant than those that are not (Figure S6), which we hypothesize is linked to the importance of Slr1p-dependent reporter effects that rely on high reporter gene expression (Figure 5B and S4A). The apparent rescue of Slr1p-associated reporter effects when it is tethered to low expression reporters (Figure 5D and 5E) is consistent with a model in which the affinity of this factor for RNA has evolved so as to bind highly expressed transcripts, of which RP mRNAs are well established to represent a major cohort. We also show that Slr1p directly and specifically engages our identified 5'PAC motif in *in vitro* binding assays, further arguing for a specific role that Slr1p plays with respect to the metabolism of RP mRNAs in *S. pombe*. By tethering Slr1p to the 5' and 3' UTRs of reporter genes, we additionally show that the presence of Slr1p in the 5'UTR of a message increases its expression while its association with either the 5' or 3' UTR destabilizes the transcript.

Our data also indicate that in *slr1+* cells, Exo2p is recruited more prominently to subpolysomes and monosomes, reminiscent of work in *Arabidopsis* linking LARP1 to recruitment of the Exo2p homolog XRN4²⁴. Thus, Slr1p may promote Exo2p access to RP mRNAs, which is also consistent with our RATE-Seq determined stability and reporter mRNA abundance data. Overall, our data argue for a role for Slr1p in RP mRNA expression and turnover, consistent with Slr1p functioning as a LARP1 homolog even in the absence of the DM15 motif or 5'TOP sequence in *S. pombe* RP mRNAs.

Preceding work on human LARP1 has been controversial³⁷, with multiple functions proposed but with a preponderance of studies linking it to the inhibition of translation and the stabilization of 5'TOP mRNAs^{36,40,41,56,62,63}. Other works suggest that hLARP1 can rather promote 5'TOP mRNA translation^{35,44}, and altered association of the DM15 motif with the m⁷G/+1C 5'TOP, potentially through different phosphorylation states, has been proposed as a mechanism by which hLARP1 might toggle between these roles^{31,62,63}. Additionally, hLARP1 has been linked to variations in poly(A) tail length in a manner linked to PABP function^{38,64}, and while hLARP1 depletion results in decreased stability of 5'TOP mRNAs, hLARP1 has also been linked to both steady-state enrichment and depletion of non-5'TOP mRNAs⁴⁶. Furthermore LARP1 family members have hypothesized roles in mRNA degradation, i.e., in *Arabidopsis* and *C. elegans*²²⁻²⁴. This diversity of function is likely linked to the wide breadth of RNA binding modes hypothesized in LARP1 family members, most of which are still not well understood, as well as LARP1 specialization and protein-protein interactions in various systems. Besides hLARP1 binding the cap through the DM15 motif, the La motif and adjacent residues have been hypothesized to bind pyrimidine-rich sequences in a cap-

independent manner, as well as to the poly(A) tail⁵⁸. Notably, PAR-CLIP of hLARP1 has also revealed direct interactions to pyrimidine enriched (i.e., TOP-like) sequences at the 3' end of the 5'UTR, closer to the AUG, of RP mRNAs⁶², in a region reminiscent of the Pyrimidine-Rich Translational Element (PRTE) present in mTOR-responsive 5'TOP mRNAs⁶⁵ and the 5'PAC motif described in this work. Additionally, recent work identifying hLARP1 in the SARS-CoV-2 RNA-protein interactome, indicates hLARP1 binds regions in the first 70 nucleotides, corresponding to the viral 5' leader sequence of the SARS-CoV-2 RNA which contains several pyrimidine rich, TOP-like motifs⁶⁶. Therefore, hLARP1 may interact with pyrimidine-rich sequences closer to the AUG, as well as the poly(A) tail, and in addition to the characterized DM15 binding mode. Thus, it is possible that these interactions can each allow for varied levels of regulation. Our model is thus consistent with a framework in which LARP1 family members have multiple functions of which certain roles are favoured under specific circumstances and according to different metabolic requirements of the cell (Figure 7).

In comparing wildtype versus *slr1*- cells, the apparent function of Slr1p in promoting both mRNA decay and translation may initially seem contradictory. However, context dependent function in apparently opposing processes has been described for several mRNA binding factors, including PABP that is generally linked to mRNA stability despite also recruiting deadenylases to mRNA poly(A) tails (reviewed in⁶⁷). Given the regulatory roles proposed for LARP1 family members in higher systems, it thus seems likely that Slr1p may function in mRNA translation and/or decay to varying degrees in response to cellular stress, growth stage, or environmental cues for which future work will be required. Co-translational mRNA

decay may be a unifying theme in Slr1p function for these varied roles, as *Arabidopsis* LARP1 has been proposed to promote co-translational mRNA decay in response to heat stress through an interaction with XRN4²⁴, and we likewise detect direct protein-protein interactions with both translation initiation factors and Exo2p in *S. pombe*. Alternatively, it is also possible that translation is first inhibited by cellular mechanisms in place for preventing mRNA translation initiation, and Exo2p is recruited next. Considering mRNA stability, it is important to note that studies measuring the distribution of mRNA half-lives across the transcriptome indicate that mRNAs enriched for the GO process “Ribosome Biogenesis” are the least stable group, with “Cytoplasmic Translation” also being overrepresented among mRNAs with the shortest half-lives⁶⁸. This reaffirms the intricate nature of RP mRNA metabolism where their high expression and low stability must be tightly regulated potentially through a factor such as Slr1p, which through interactions with different translation- or decay-specific proteins has the ability to modulate the fate of a target mRNA in both processes and as a result of specific cellular cues.

Future work is needed to address how protein modifications, different types of stress or environmental cues may allow for Slr1p to differentially regulate RP mRNA metabolism. It should be noted that hLARP1, harbouring a DM15 motif, is able to block the m⁷G cap to inhibit eIF4F assembly, but the *S. pombe* ortholog lacks a similar motif, and thus presumably the ability to block initiation machinery at the cap. This work thus emphasizes that the role associated with LARP1 family members in RP mRNA regulation may predate the DM15 motif and the emergence of the 5'TOP sequence, which may have been features that were evolutionary selected for as a consequence of increasing complexity in higher

eukaryotes. RP mRNA expression is tightly regulated, and it is thus a major determining factor in cell growth. The work presented here considers and confirms the possibility that LARP1 family members are factors which act as molecular switches, modulating RP mRNA metabolism and therefore ribosome biogenesis, hence indirectly affecting all aspects of cell growth, proliferation and survival. This is consistent with proposed models in which the abnormal functioning of a molecular switch such as hLARP1 is linked to disease states^{44–48,62}, and where hLARP1 could serve as a promising therapeutic target.

Limitations of the Study

While our data argue strongly for a new motif that links RP mRNA translation and stability to a LARP1 homolog in fission yeast, future work will be required to understand the mechanism by which this occurs and any cues that cause an associated response. While our *in vitro* RNA binding data argue for a sequence-specific interaction between Slr1p and a proto-5'TOP motif, the insolubility of Slr1p on native gels make determination of a dissociation constant impossible.

2.6. Acknowledgements

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Author Contributions

F.M.: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Validation, Visualization, Writing – original draft, Writing – review & editing. **A.P.:** Conceptualization, Investigation, Methodology. **D.B.:** Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Validation, Visualization, Writing – review & editing. **L.S.:** Conceptualization, Investigation, Methodology. **J.G.:** Conceptualization, Data curation, Formal Analysis. **O.S.R.:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing. **M.A.B.:** Conceptualization, Formal Analysis, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing.

Declaration of Interests

The authors declare no competing interests.

Inclusion and Diversity

We support inclusive, diverse, and equitable conduct of research. One or more of the authors of this paper self-identifies as an underrepresented ethnic minority in their field of research. One or more of the authors of this paper self-identifies as a gender minority in their field of research.

2.7. Supplemental Information

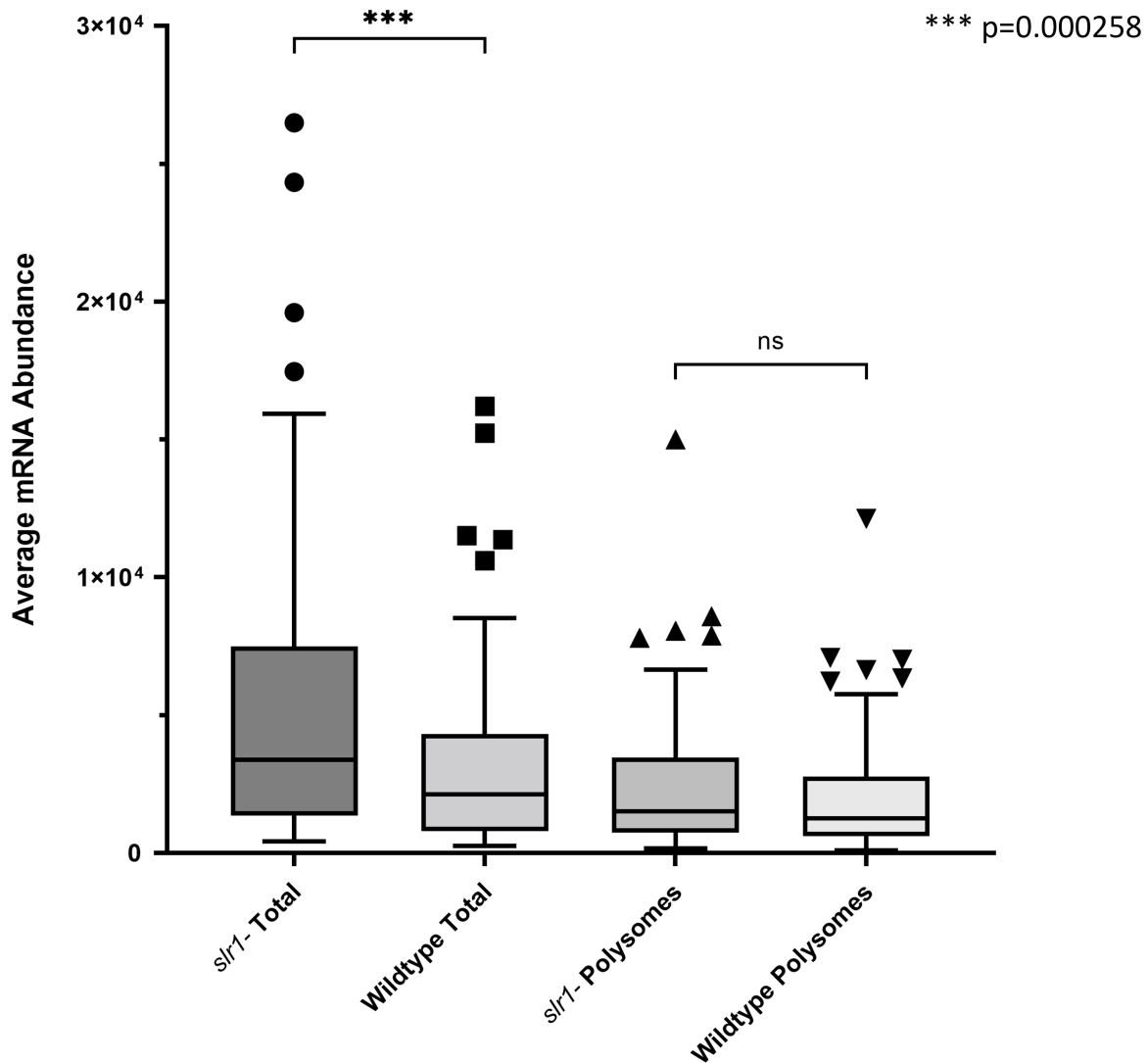


Figure S1: mRNAs enriched for “cytoplasmic translation” have increased total mRNA abundance in *slr1*- cells, Related to Figure 2. Transcripts enriched for the Gene Ontology (GO) process “cytoplasmic translation”, identified by polysome profiling followed by RNA-Seq, are significantly more abundant in *slr1*- cells when compared to wildtype cells even if their polysome occupancy does not differ significantly (n=2 biological replicates). Tukey boxplots were generated using Prism, statistical analysis performed: unpaired two-tailed t-tests with Welch’s correction (unequal SDs) with Alpha=0.05. ns=non-significant.

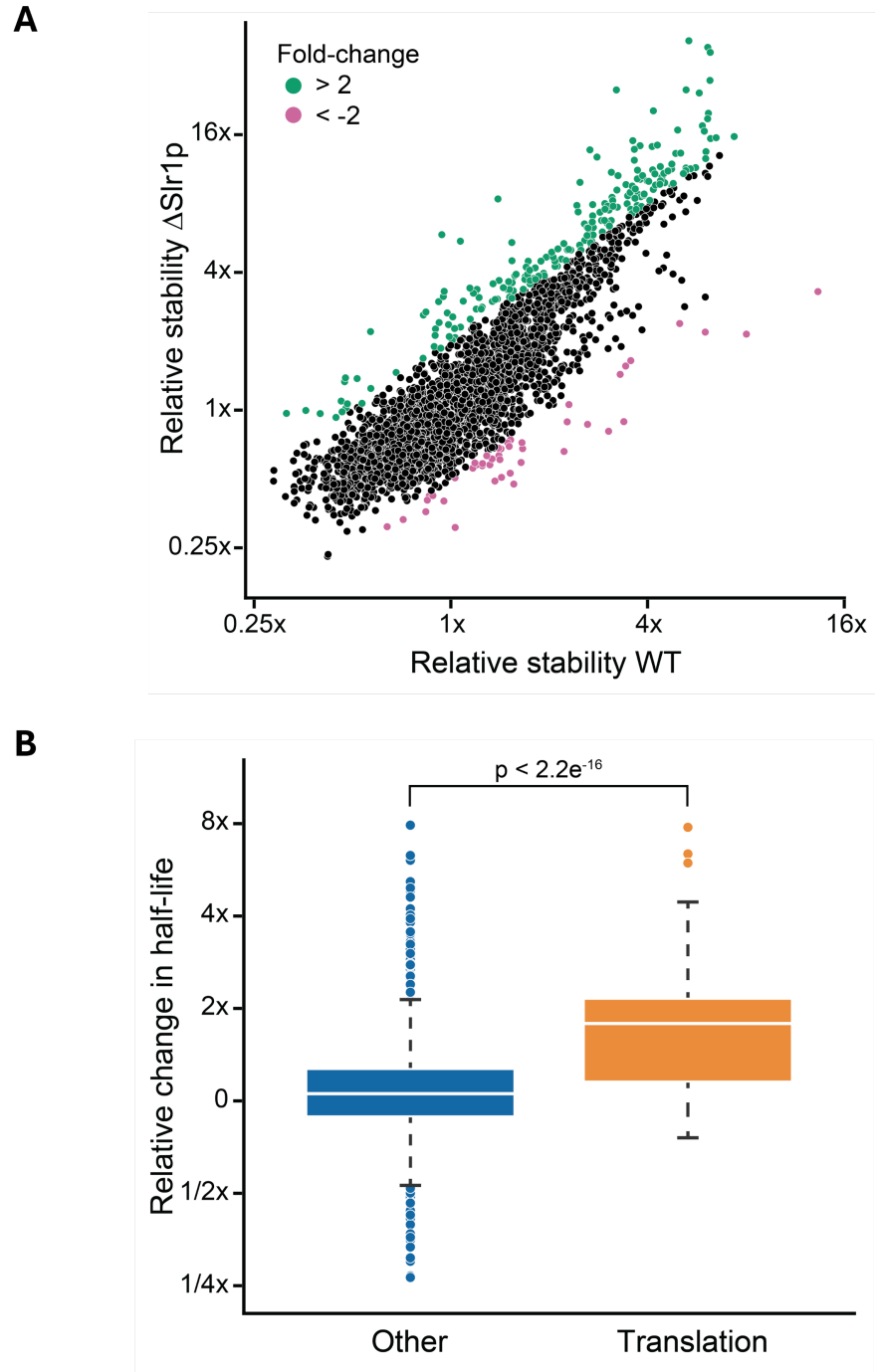


Figure S2: Stabilized mRNAs in *slr1*- cells are enriched for the GO process “translation”, Related to Figure 2. **A) Scatterplot of all transcripts that were compared in wildtype versus *slr1*- cells using a RATE-Seq analysis shows 208 RNAs are stabilized (green) while 44 are destabilized (pink) in absence of Slr1p (n=3 biological replicates). **B)** The stabilized cohort of RNAs are enriched for the GO process “translation” plotted relative to all other genes in *S. pombe* and their respective GO terms. Statistical tests and figures were generated using R (v.3.5.2).**

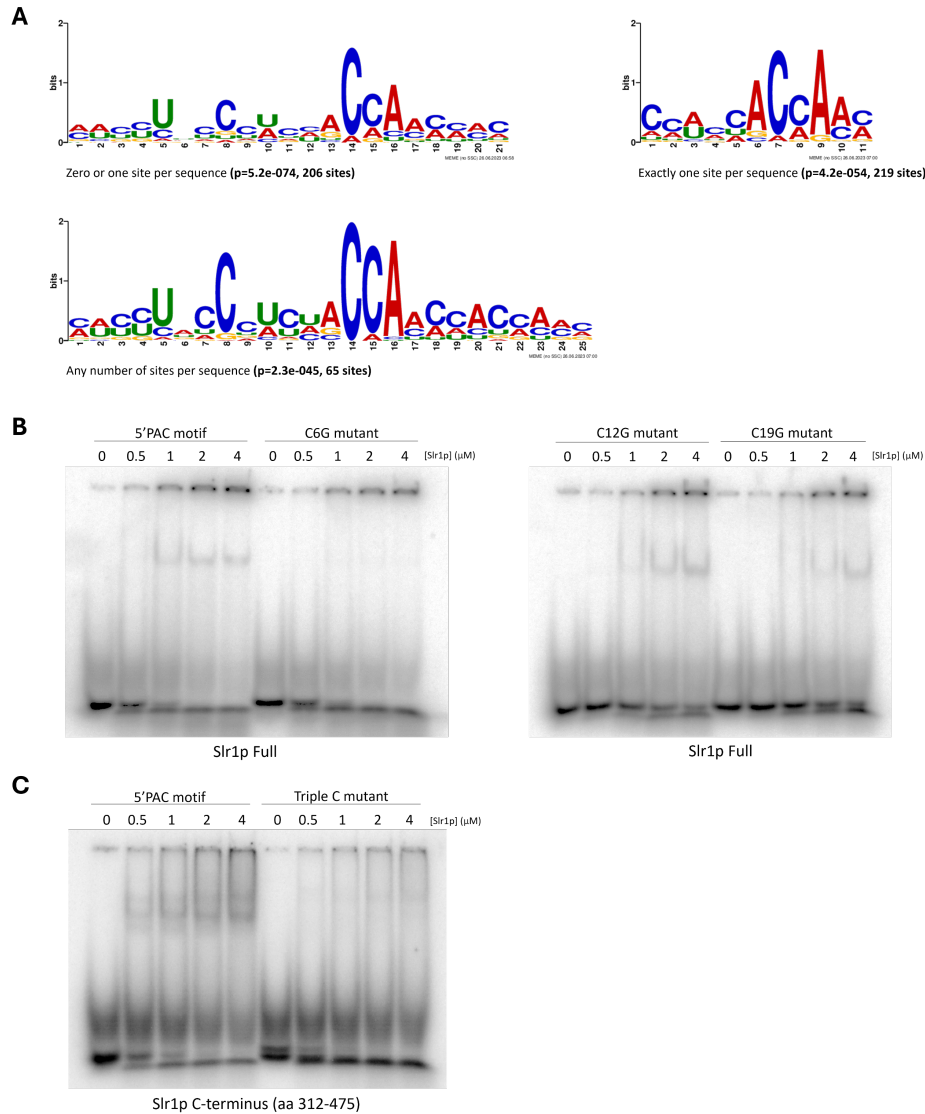
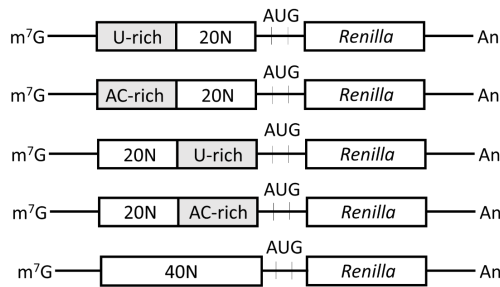
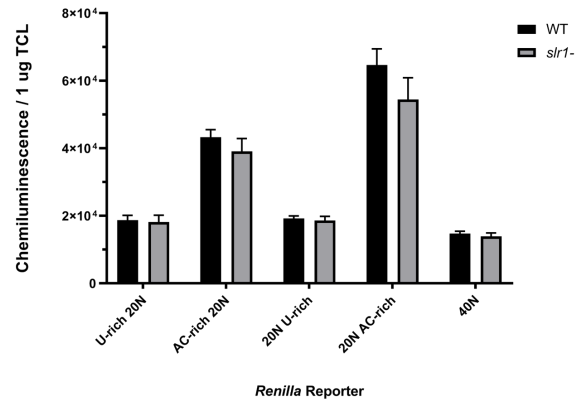
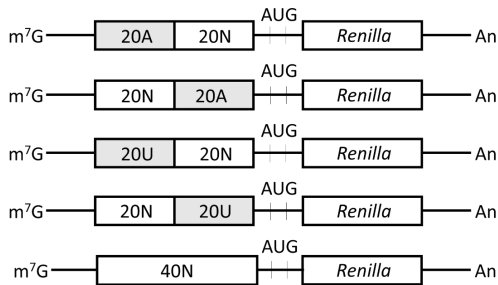
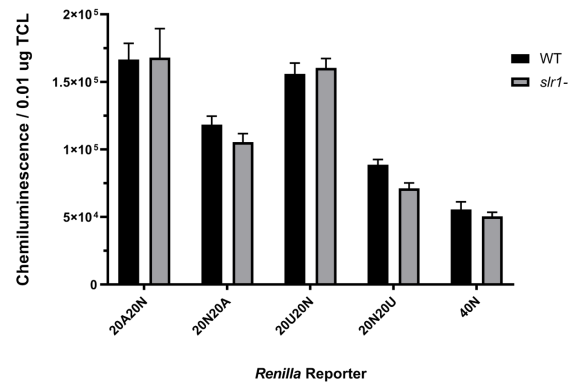


Figure S3: Slr1p's interaction with the 5'PAC motif and its mutants, Related to Figures 3 and 4. A) Genes enriched for the GO process “cytoplasmic translation” in *S. japonicas* (GO:0002181) were analyzed for the presence of any 5'UTR motifs. 261 protein coding genes were included in the search and three variations of an AC-rich motif (closely resembling the 5'PAC motif in *S. pombe*) were retrieved. p values were generated by MEME Suite 5.5.3. **B)** Slr1p makes distinct ribonucleoproteins with the 5' Proximal AC-rich motif (5'PAC motif: UUUUCCCACCACCAAACAC) *in vitro* and this interaction is lost upon mutating C6 to G (C6G mutant: UUUUUCGACCACCAAACAC). In contrast, the C12G and C19G single mutants do not lead to a similar complete loss of interaction (C12G mutant: UUUUCCCACCAGCAAACAC; C19G mutant: UUUUCCCACCACCAAACAG). **C)** The C-terminus of Slr1p corresponding to amino acids 312-475, including the LaM, binds the 5'PAC motif but not the Triple C mutant (UUUUUCGACCAGCAAACAG; C6G, C12G, C19G). EMSAs were performed for a minimum of two times in each case and representative experiments are shown. aa=amino acid.

A**pRep81x Reporters****B****C****pRep3x Reporters****Figure S4: Lower expression and homopolymer motif reporters, Related to Figure 5. A)**

Schematic representation of reporters containing specific motifs identified by RATE-Seq analysis including the 5'DUR (U-rich) and the 5'PAC (AC-rich) motif, see also Table S7, and *Renilla* reporter assays using pRep81x (low expression reporters). Data are represented as mean + SEM error bars. 20N or 40N refers to 20 or 40 randomly selected nucleotides. **B)** Schematic representation of reporters containing generalized motifs according to La and La-related proteins' known substrates. **C)** *Renilla* reporter assays using reporters containing generalized motifs (pRep3x based, high expression) show that such reporters are not differentially expressed in wildtype versus *slr1*- conditions. Data are represented as mean + SEM error bars.

Reporter assays: n=3-4 biological replicates per reporter transformed into either wildtype or *slr1*- cells, 3 technical replicate readings per biological replicate, providing a total of 9 or 12 readings for each reporter. Multiple unpaired t-tests were performed by Prism without assuming consistent SDs and using the Holm-Šidák method, Alpha=0.05. No statistical significance was reported. WT=Wildtype, TCL=Total Cell Lysate, SEM=Standard Error of the Mean.

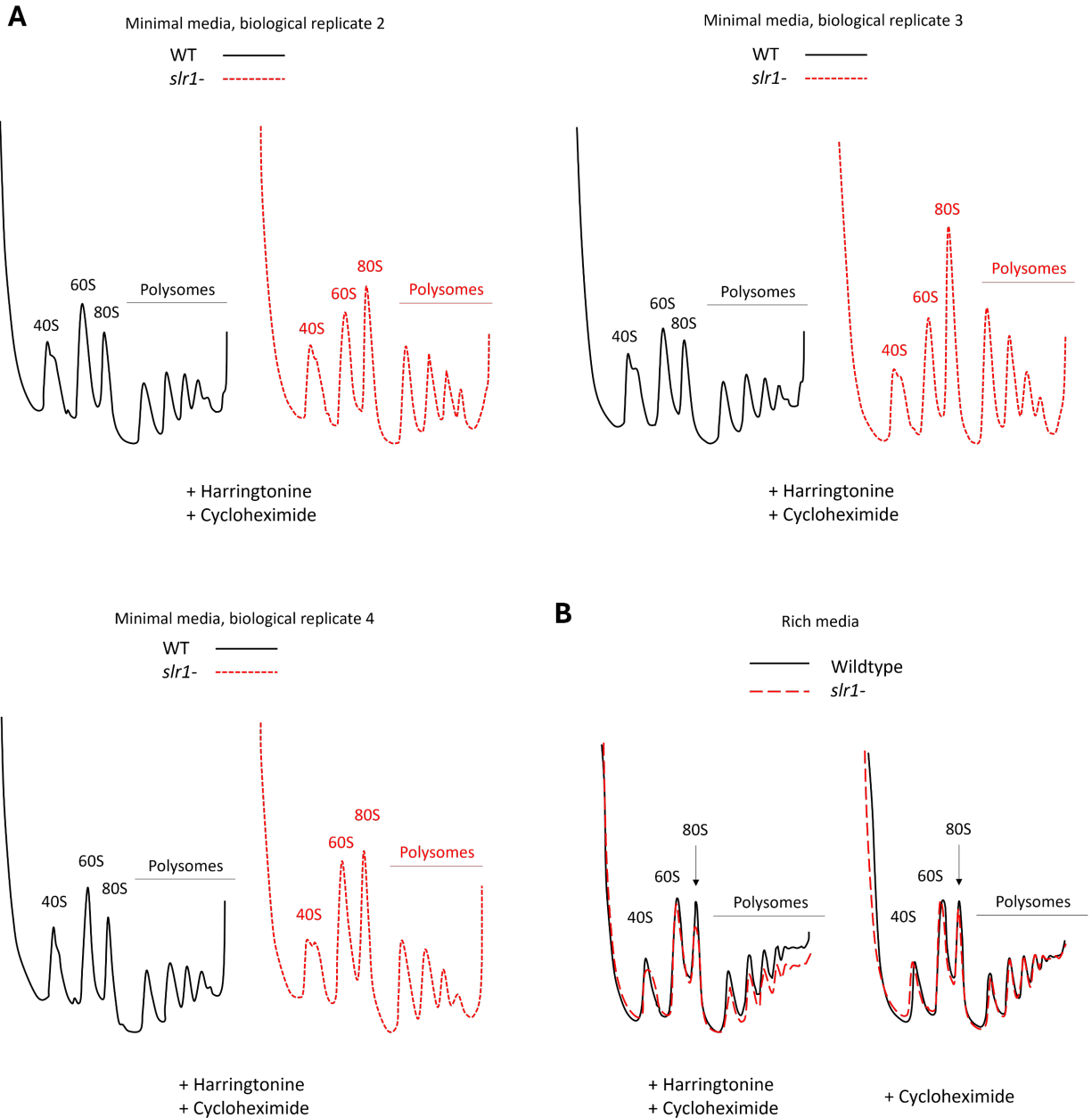


Figure S5: Biological replicates of polysome profiles in minimal media and profiles in rich media, Related to Figure 6. A) Polysome profiles after treating wildtype and *slr1*- cells with harringtonine (2 μ g/mL, 2 minutes) followed by cycloheximide (100 μ g/mL, 10 minutes) show there is an increase in the height of the 80S peak and a movement towards light polysomes in *slr1*- cells in comparison to wildtype cells. Biological replicate 1 is shown in Figure 6, biological replicates 2-4 are shown here. **B)** Polysome profiles in rich media for harringtonine- and cycloheximide-treated cells or cycloheximide-treated cells. Rich media profiles shown are representative of at least 2 biological replicates. WT=Wildtype.

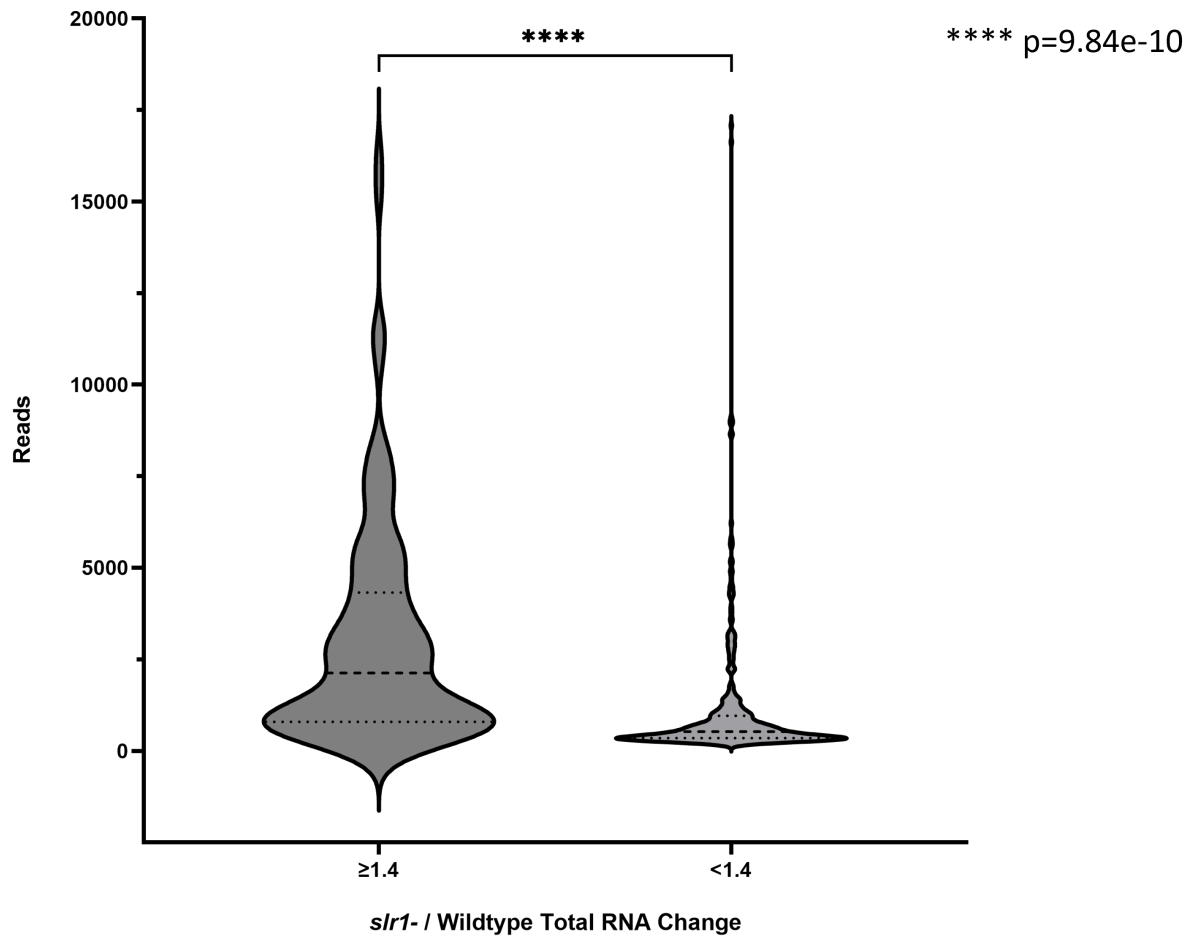


Figure S6: mRNAs whose stability is linked to Slr1p are abundant, Related to Figure 2. Comparison of average number of reads per mRNA (expression) for transcripts that have a ≥ 1.4 or < 1.4 fold increase in abundance in *slr1*- cells. Violin plots with median, 25th and 75th percentiles are shown. A two-tailed unpaired t-test with Welch's correction was performed using Prism and Alpha=0.05 (n=2 biological replicates for the RNA-Seq).

Table S1: Slr1p interacts with various components of the translational apparatus, Related to Figure 1. A) Slr1p-PrA was immunoprecipitated from *S. pombe* cells and the following protein interactions were determined by LC/MS-MS. Gene names are italicized. Proteins with significant enrichment in both replicates are listed. **B)** Slr1p-interacting proteins are enriched for translation-related GO processes. Proteins interacting with Slr1p, as determined by LC/MS-MS, are enriched for various GO processes ranked according to False Discovery Rate (FDR). (See “Note” on page 89)

Systematic ID	Gene name	Molecular weight	Human orthologs	Fisher's Exact Test, replicate 1	Fisher's Exact Test, replicate 2
SPAC1527.03	<i>slr1</i>	53.23	LARP1, LARP1B	1.80479E-57	1.87752E-22
SPAC926.09c	<i>fas1</i>	230.56		1.00138E-27	7.8814E-21
SPAC4A8.11c	<i>fas2</i>	202.17		3.84588E-26	4.93697E-19
SPBC17D11.05	<i>tif301</i>	107.07	EIF3A	7.79556E-08	7.36523E-14
SPAC25G10.08	<i>tif302</i>	84.03	EIF3B	1.10764E-08	2.87877E-10
SPAC17C9.03	<i>tif471</i>	154.03	EIF4G2	2.60509E-29	6.60473E-08
SPAC4A8.16c	<i>tif303</i>	104.37	EIF3C, EIF3CL	6.21526E-09	6.66729E-08
SPAC4D7.05	<i>sum1</i>	36.81	EIF3I	0.002359803	8.79923E-06
SPBC1778.01c	<i>zuo1</i>	50.21	DNAJC2	0.001454621	3.3257E-05
SPAC1751.03	<i>tif313</i>	45.08	EIF3M	4.41182E-05	0.000160139
SPAC29A4.04c	<i>cbf5</i>	53.11	DKC1	0.014615917	0.000160139
SPBC11G11.05	<i>rpa34</i>	28.01	POLR1G	0.000176515	0.000770442
SPBP4H10.15	<i>aco2</i>	99.9		1.39827E-08	0.001972004
SPAC22G7.06c	<i>ura1</i>	248.31	CAD	0.02762298	0.008353698
SPAC140.02	<i>gar2</i>	52.99	NCL	5.60759E-07	0.010369854
SPCC285.17	<i>spp27</i>	26.65		0.038324878	0.016647543
SPBC4C3.07	<i>tif306</i>	33.25	EIF3F	0.033619068	0.022483831
SPAC17A5.13	<i>fol2</i>	26.49	GCH1	0.043239383	0.025135659
SPAC1071.08	<i>rpp203</i>	11.11	RPLP2	0.002541914	0.028919518
SPAC57A7.04c	<i>pabp</i>	71.51	PABPC1, PABPC3, PABPC4	4.94161E-07	0.043314708
SPBP23A10.07	<i>rpa2</i>	131.69	POLR1B	0.019711831	0.046355532

Table S2: Yeast two-hybrid analysis of Slr1p NTD and CTD reveals various protein-protein interactions, Related to Figure 1. PBS categories include A: very high confidence in the interaction, B: high confidence in the interaction, and C: good confidence in the interaction. Gene names are italicized.

Systematic ID	Gene name	Global PBS	Molecular weight	Human orthologs
Slr1p NTD (amino acids 1-323):				
SPAC1687.05	<i>pli1</i>	A	80.72	PIAS1, PIAS2, PIAS3, PIAS4
Slr1p CTD (amino acids 312-475, including the LaM):				
SPBC25B2.06c	<i>btb2</i>	B	31.78	GMCL1, GMCL2
SPBC30D10.17c	<i>smi1</i>	C	55.56	
SPAC17A5.14	<i>exo2</i>	B	152.59	XRN1
SPBC211.08c	<i>mtr3</i>	A	28.44	EXOSC6
SPBP19A11.06	<i>lid2</i>	C	172.26	KDM5A, KDM5B, KDM5C, KDM5D
SPCP1E11.11	<i>puf6</i>	A	72.63	PUM3
SPBC119.15	<i>npa3</i>	B	41.64	GPN1
SPAC29B12.01	<i>ino80</i>	B	183.05	INO80
SPAC4D7.05	<i>sum1</i>	C	36.81	EIF3I

Table S3: RNA-Seq reads corresponding to total and polysome-associating RNAs in wildtype and *slr1*- cells, Related to Figure 2. Reads are sorted according to the change in total RNA abundance. GO processes for RNAs with a change in total RNA abundance equal to or greater than 1.4 are included. (See “Note” below)

Table S4: Half-life differences for RNAs in wildtype versus *slr1*- cells as determined by a RATE-Seq, Related to Figure 2. Upregulated gene IDs correspond to genes stabilized in *slr1*- cells whereas downregulated gene IDs correspond to genes destabilized in *slr1*- cells. GO processes in which the upregulated genes are enriched in are included. (See “Note” below)

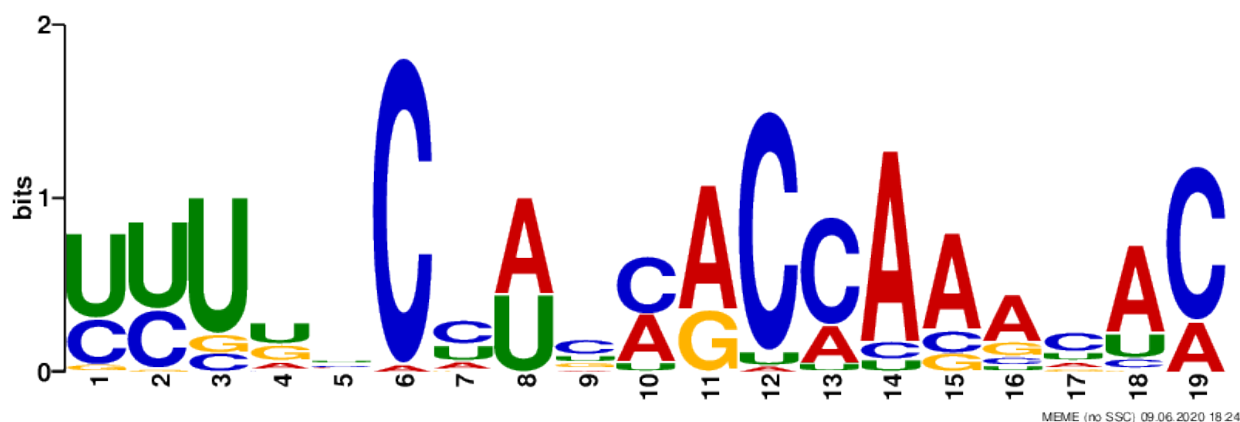
Table S5: RIP-Seq analysis of RNAs that co-immunoprecipitate with Slr1p in comparison to an IgG control, Related to Figure 2. Reads were sorted according to log2FoldChage (≥ 1.5) first and padj next (> 0.05). GO processes in which the significant hits are enriched in are included. (See “Note” below)

Table S6: Overlap between the genes enriched in transcriptomic datasets, Related to Figure 2. Gene lists of Slr1p-affected transcripts determined by each of the sequencing experiments and their overlap as well as GO processes in which the common genes are enriched in are provided. (See “Note” below)

Note: Tables **S1B**, **S3-S6** and **S7B** (Excel files) can be found online at [Cell Reports Journal](#).

Table S7: 5'UTR motifs determined by MEME motif discovery search, Related to Figures 3 and 5. A) Gene names, start sites, p-values determined by the MEME Suite (5.4.1 or 5.5.3), and the literal motif found within each of the 5'UTR sequences that contain the 5'PAC and/or the 5'DUR motif are shown. **B)** Gene lists for the transcripts containing the 5'PAC, 5'DUR, RP 15-mer 5' Proximal AC-rich motif (PAC15) or RP 19-mer 5' Proximal AC-rich motif as well as overlaps are included. (See “Note” on page 89)

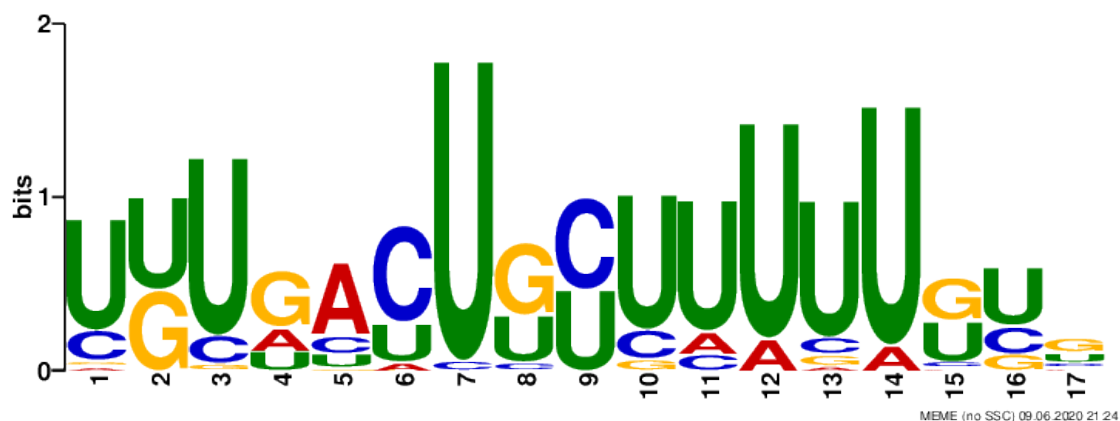
5' Proximal AC-rich motif (5'PAC):



Name	Start	p-value	Sites
151. SPCC1322.11 SPCC1322.11.1	36	9.21e-8	GGUUCGACGU UCCUUCUACAACCAAAACAC CAGCAGAAAA
54. SPAC3A12.10 SPAC3A12.10.1	57	1.25e-7	AACAAGUCAA CCUGUCCAGAGCCAGAGAC AUCAAA
22. SPAC11E3.15 SPAC11E3.15.1	44	1.95e-7	AUUCUCUUUG UUUAUCCUCCACCACAUAUC UCGUAGAGAG
3. SPAC4F10.14c SPAC4F10.14c.1	49	2.59e-7	CAAGUUCAAG UCCUUCACAAACCAAAAAAC GUUUGGUCAA
149. SPCC1393.03 SPCC1393.03.1	59	3.42e-7	AAAAGAUACG UUGUACCAUCACCAACUAC GGCAUAAAAU
30. SPAC8C9.04 SPAC8C9.04.1	41	3.42e-7	AUUUACAACU UCUACCAACCACCAAAACAA GAACGCAGAG
150. SPCC1450.04 SPCC1450.04.1	19	3.91e-7	UCUAAAAUUU UCUGCCAAACAACCAACUAC UUUAAAA
71. SPAC27E2.03c SPAC27E2.03c.1	60	6.57e-7	UAUUUAAGUU CGUGACCAGAGCCAAAGCAC UUGUUGCGUA
80. SPAC1805.12c SPAC1805.12c.1	21	8.43e-7	UAAAUGACAU UUGUUCGAGUGCCAAACAC CACAUUCCCA
44. SPAC26A3.07c SPAC26A3.07c.1	50	8.43e-7	UGCUAAAUAU UUGGCAUCCACCACAAAGAA AAGGCACAAG

154. SPBC56F2.02 SPBC56F2.02.1	56	9.53e-7	UUAAGAAAGA UUGUCCGUCCACCAAAAAAC AGCAAGAAAA
124. SPBC577.02 SPBC577.02.1	7	1.36e-6	GAAUUC UUGGUUUUUACCAAGCUC AACAAA
133. SPCC1795.06 SPCC1795.06.1	848	1.92e-6	CAUUGUGUAC CCUUUCCAGCACCAGGACC GCCUAAAAACA
78. SPAC959.08 SPAC959.08.1	43	2.14e-6	CCGUGCGUAC UUGGCCUUUAACCAGGAAC AGCA
39. SPAC4F10.20 SPAC4F10.20.1	48	2.66e-6	AUAAUACAUA UUGGCCUACCACAUACCAC CAAAUUUAAA
7. SPAC31G5.03 SPAC31G5.03.1	16	3.65e-6	ACUGAGCUGU CUUUUCUACAGCAACACAA G
34. SPBC19G7.19 SPBC19G7.19.1	58	4.48e-6	UCCAACUUUAU UCCAGCUUUGCCAAACAC ACAAGCUCUU
129. SPCC794.09c SPCC794.09c.1	5	4.95e-6	CGCC UUGUCAUUCACCACACGC CAAAAAUUU
62. SPAC1805.02c SPAC1805.02c.1	38	6.03e-6	AAUUCAGUC UUGACCACCGCAUAGCAA UAUCGCAAAA
72. SPBC17G9.07 SPBC17G9.07.1	28	8.03e-6	GAGAGCCUUU CCUUAACCACCAUCAGUCAC CAAAAUUUCU
113. SPCC970.05 SPCC970.05.1	24	8.81e-6	CGCAGUAAAGU UUUCCCCUACACCAAUUC AC
91. SPBC19G7.02 SPBC19G7.02.1	233	8.81e-6	AGCAAUCGAU UUUUUGCUACCACUAACUAC AUCAGUUGAU
14. SPBC1773.07c SPBC1773.07c.1	24	9.66e-6	GUACACUCUU CCUUCCAUCAGCACAUAAC CACA
100. SPBC2F12.04 SPBC2F12.04.1	49	1.06e-5	AGCACUAGCC UUUUACCACAACAAAAGUA GCA
50. SPBC16A3.16 SPBC16A3.16.1	57	1.06e-5	UAAGGUUCAC CUUACCUUACGCCAAAUC UAUUUUUCUAC
76. SPAC2F7.17 SPAC2F7.17.1	41	1.16e-5	GGAUAUCGAU GCUUCCUACAACCAAAUA UUUUUUCUUG
142. SPBC800.04c SPBC800.04c.1	32	1.64e-5	UUACCUUCAA GUUACCAACGCAAAACAC CCUUAUAGUC
10. SPAC343.12 SPAC343.12.1	345	1.78e-5	GUUGAUUCUA CCUUAACUUAAUAAAACAC UAUCGCCUCU
97. SPAC23C11.05 SPAC23C11.05.1	121	1.94e-5	CACAUUGCAA CCUGUACUUAAACAAAAAC ACUUUUUCAGU
38. SPAC26F1.06 SPAC26F1.06.1	6	1.94e-5	AGUCA CUUUACUUACACCACACUA UACAACGUUA
106. SPCC777.03c SPCC777.03c.1	1574	2.11e-5	UUCAGCAAUG CCGAGCCUGAGCCAAUAAC CAAGCCAGUG
119. SPCC1223.05c SPCC1223.05c.1	66	2.29e-5	AUGACAAUA UUUUGCCAGCAACAAACUA GCAAAU
73. SPBC16C6.11 SPBC16C6.11.1	62	2.29e-5	UACUUGGUUC CUUUUCCACUGCAACGGUC GAAAAC
37. SPAC23D3.17 SPAC23D3.17.1	161	3.14e-5	UCUCACUGUA UUUACCCAUAAGCACCACUAC CAAAACAGUG
152. SPCC576.09 SPCC576.09.1	11	3.95e-5	CUUCAACAAC UUCUUUUUACUAAACCC CAUCGUGGUC
46. SPAC5H10.03 SPAC5H10.03.1	956	4.58e-5	UCCACUCGGC UUGUCCAUGAACCCGGCAA AAUUUACAUI

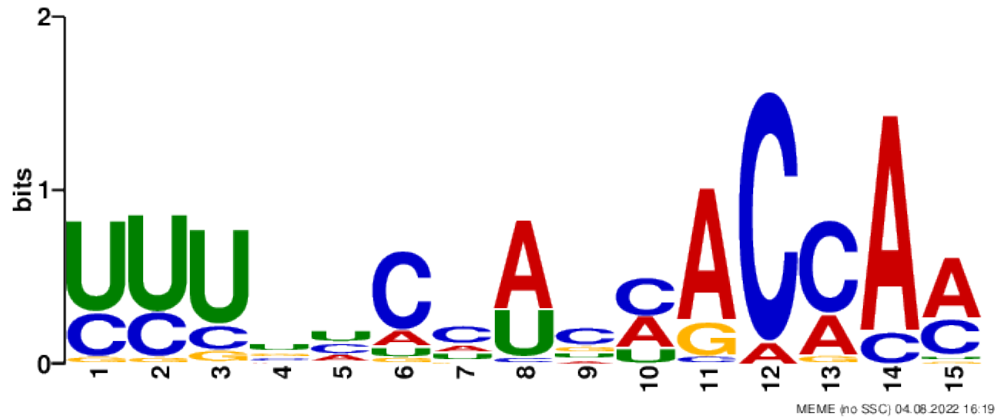
5' Distal U-rich motif (5'DUR):



Name	Start	p-value	Sites		
14. SPAC22A12.04c SPAC22A12.04c.1	188	5.88e-10	UUCGUAGACA	UGUGACUGC UUUUUUUUG	AGAACAAACA
41. SPCC962.04 SPCC962.04.1	22	1.25e-9	CCUAUUUAUU	UGUGACUGUUUUUUUGG	UAAGAAGAGC
29. SPCC1223.05c SPCC1223.05c.1	19	8.57e-9	CCCUGAGAAA	UGUGACUGUUUUUUUGG	UCGAAAAGAA
11. SPAC17G6.06 SPAC17G6.06.1	3	6.80e-8	CU	UGUGACUGCCUUCUUUG	GGGUAAAAUU
6. SPAC1071.07c SPAC1071.07c.1	26	2.23e-7	UUUGCGUACA	CGUGACUGUUUUUUUGU	UUACGAAAAA
26. SPCC285.15c SPCC285.15c.1	67	9.33e-7	UAAAAUAUAU	CGUGACUGUCAUUUUUG	AACACAACAA
2. SPAC343.12 SPAC343.12.1	84	9.33e-7	UCGCAAAUUG	UGUGACUUUGUUUUUGCC	CUUGUUUAAG
15. SPAC5H10.03 SPAC5H10.03.1	662	1.34e-6	UGAUGAACGU	UUUAACUGCGUUUUUGCU	GAAGCUCAUU
21. SPAC19B12.04 SPAC19B12.04.1	82	1.68e-6	GAAUCCAAAA	UGUGACUGCUUAGUUUC	UUGUUUUUUG
40. SPCC1393.03 SPCC1393.03.1	5	1.88e-6	UUUA	CGUGACUGUUUAUAGUG	ACCAAAACUU
22. SPAP7G5.05 SPAP7G5.05.1	44	2.36e-6	UGUUUAUAGU	GGUGACUGCUUUUUUCUU	CCAAAGUUUG
36. SPBC428.11 SPBC428.11.1	21	3.61e-6	UCUUUUUUUU	UUUUCCUUUUUUUUUG	UCAUUUUCUC
5. SPAC1F12.02c SPAC1F12.02c.1	2	4.92e-6	C	UGUGACUGCUUUUAUCUC	AUUAAACCUG
16. SPAC19B12.09 SPAC19B12.09.1	161	5.44e-6	UUCCUAAAUU	CGUUUUUGCUUUUAGCG	AAUUUCAAAA
23. SPAC27E2.03c SPAC27E2.03c.1	138	6.01e-6	AAAGAGUAAC	UUCAACUUUUUUUUUGUU	UGUCUUGAUU
33. SPBC3E7.07c SPBC3E7.07c.1	80	1.28e-5	UAUUUACAAU	UUUUCUUUCUUUUUGUU	ACCUUACCUA
12. SPBC106.18 SPBC106.18.1	8	1.53e-5	GGUAGGG	CUGGACUGCUUUUUUGA	AUUCAGGAC

30. SPBC29B5.03c SPBC29B5.03c.1	12	1.82e-5	GCAAAAUCAG	UGUGACUGUCAACUGUC	ACACAACAAA
32. SPCC191.05c SPCC191.05c.1	3	1.98e-5	UU	UUUGUUUUUUUUUUUUUU	UUUUUCGUAU
31. SPCC1795.06 SPCC1795.06.1	2239	1.98e-5	UAUUUGUUUA	UUCUCCUCUUUUUUUGCG	UGCUGCAUCU
10. SPBC17G9.10 SPBC17G9.10.1	5	3.24e-5	AACA	AGUGACUGUUAAUUUCG	AAUGCAAACA
34. SPCC1183.03c SPCC1183.03c.1	191	5.13e-5	AAAAUAUCA	UUUAUUUUUCUUUCUUUG	UACCUAGCAA
3. SPAC1805.03c SPAC1805.03c.1	214	5.13e-5	UGAUUCCGUA	UUUGAAUUUUUUUUUAUG	AGCACGCCAA
1. SPAC13G6.02c SPAC13G6.02c.1	11	5.13e-5	ACGGGUUCAA	UUUGGCUCUUUUUAGCA	UUUGUACUAU
37. SPBC660.06 SPBC660.06.1	153	5.94e-5	GCCUAUAACA	UUUUUCUUUUUUUUUGGU	UAGAAUCUUU
42. SPCC830.11c SPCC830.11c.1	284	6.38e-5	AAACACAUAC	UUUAUUUCCUUUUUUUCU	UGUUUUUUUU
39. SPBC21C3.13 SPBC21C3.13.1	70	9.67e-5	UACCUUACGC	UUCACUUGCUUUUGUGUA	AAAGAAAAUU
25. SPBC1685.17 SPBC1685.17.1	26	9.67e-5	UUUUUUUCUU	UUUAUUUUCCAUUUUCC	UUCAUUUUUU
38. SPCC576.17c SPCC576.17c.1	181	1.71e-4	GUCCUUUCGU	CUCACUCUCUUUUUUGUG	CGUCAGUGGG
7. SPAC11E3.15 SPAC11E3.15.1	29	1.93e-4	AAUUUCGAAU	UUUUUAUUCUCUUUGUU	UAUCCUCCAC

RP 15-mer 5' Proximal AC-rich motif (PAC15):



Name	Start	p-value	Sites		
48. SPAC17C9.03 SPAC17C9.03.1	57	7.75e-8	UAUUGCUCUCU	CUUGACCACCACCAC	UAACCCAACC
102. SPAC4A8.16c SPAC4A8.16c.1	55	2.21e-7	CGAAAAAAAAA	CUUGCCAACCACCAC	C
164. SPAPJ698.02c SPAPJ698.02c.1	7	3.69e-7	GGAAUU	UCUCCAACCACCAC	GAGGUGCACG
167. SPAPB1E7.12 SPAPB1E7.12.1	56	7.42e-7	AGAAUAGGAA	UUUCCAACCACCAC	G
245. SPCC1450.04 SPCC1450.04.1	19	1.15e-6	UCUAAAAUUU	UCUGCCAACAACCAC	CUACUUUAAA
236. SPCC1393.03 SPCC1393.03.1	59	1.15e-6	AAAAGAUACG	UUUGACCAUCACCAC	CUACGGCAUA
94. SPAC11E3.15 SPAC11E3.15.1	44	1.74e-6	AUUCUCUUUG	UUUAUCCUCCACCAC	AUACUCGUAG
213. SPCC970.05 SPCC970.05.1	24	3.06e-6	CGCAGUAAGU	UUUCCCCUACACCAC	UCUCACA
206. SPBP8B7.03c SPBP8B7.03c.1	48	3.06e-6	UAAACUGAUU	UUUUCCAACCACAAA	AGCAUACUAA
1. SPAC1006.07 SPAC1006.07.1	36	3.06e-6	CCUUCAACUU	UUUUUCCAACAACCCA	CUCACCUAGC
84. SPAC3F10.18c SPAC3F10.18c.1	13	3.72e-6	UACAAUACAU	UUUUACCAACUGCCA	G
29. SPAC23C4.06c SPAC23C4.06c.1	424	3.72e-6	UCACCAAGAU	CCUUACCAUCACCAC	GAAUUACGAU
38. SPAC18G6.05c SPAC18G6.05c.1	1	4.43e-6		CCUGUCCAUAACCAC	CAUUUCUUUC
37. SPAC1F7.13c SPAC1F7.13c.1	26	4.43e-6	CAAUACCGUA	UUCUCCAACAACCAC	AACACCAGAA
177. SPBC3B9.13c SPBC3B9.13c.1	44	5.33e-6	AAUAAGAUAU	CUUUACCAACACCAC	A
145. SPBC2F12.04 SPBC2F12.04.1	49	5.33e-6	AGCACUAGCC	UUUUACCAACAACAAA	AGUAGCA
93. SPAC29A4.15 SPAC29A4.15.1	97	5.33e-6	AUGUCUUAUC	CUUUACCAACACCAC	CUCGUUGAAA

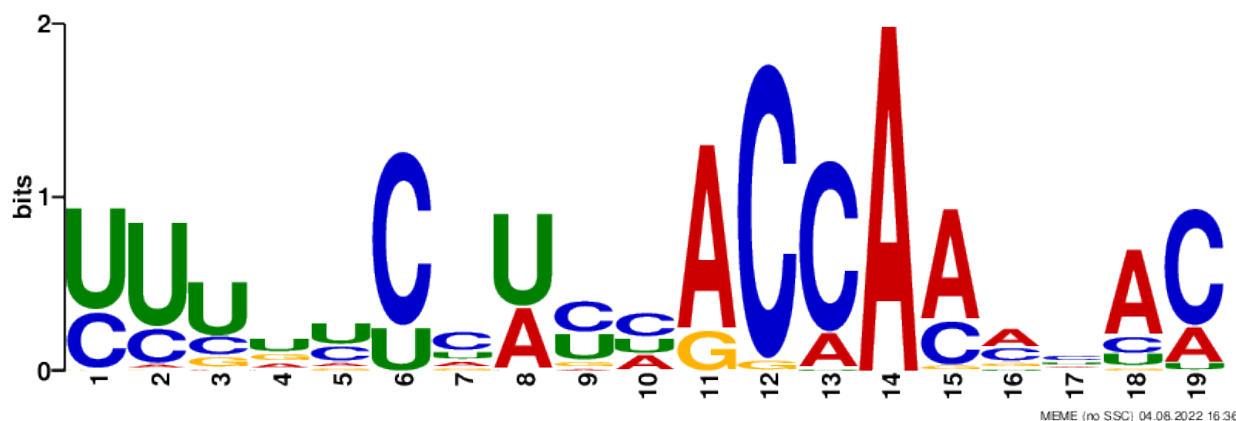
Name	Start	p-value	Sites		
229. SPCC74.03c SPCC74.03c.1	28	7.45e-6	AUACUAUGGC	UUUGCCUUCUACCAA	AUUAUAUAUU
181. SPBC23G7.05 SPBC23G7.05.1	18	7.45e-6	UUUACUACAU	CCUCCUAGAACCAC	CACCGUUUAA
151. SPBC56F2.02 SPBC56F2.02.1	56	7.45e-6	UUAAGAAAGA	UUGUCCGUCCACCAA	AAACAGCAAG
19. SPAC26A3.07c SPAC26A3.07c.1	50	7.45e-6	UGCUAAAUAC	UUUGGCAUCCACCAA	AGAAAAGGCA
178. SPBC17D1.02 SPBC17D1.02.1	68	1.03e-5	CAAACUUUUU	UUUGCGCAGAACCAA	GUGGAGGCAA
247. SPCC1322.11 SPCC1322.11.1	36	1.21e-5	GGUUCGACGU	UCCUUCUACAACCAC	ACACCAGCAG
218. SPCC794.09c SPCC794.09c.1	5	1.42e-5	CGCC	UUUGUCAUUCACCAC	ACGCCAAAAA
231. SPCC663.04 SPCC663.04.1	204	1.65e-5	GAUGCAACAC	UCUGUCUUCACAAA	
116. SPBC216.07c SPBC216.07c.1	93	1.65e-5	CAGGUGCUUG	UUUGGUCACCACCAA	CCACAUUUAA
98. SPAC1751.03 SPAC1751.03.1	12	1.65e-5	CCAAGUCCUA	CUCUCCAACUACCAC	UAACAGCGAA
248. SPCC31H12.04c SPCC31H12.04c.1	178	1.91e-5	ACUUUGCGUA	UUUUUCUUGAACCAC	GCAAGAGUAA
171. SPBC16C6.11 SPBC16C6.11.1	62	1.91e-5	UACUUGGUUC	CUUUUCCACUGCAAC	GGUCGAAAAC
246. SPCC18B5.06 SPCC18B5.06.1	58	2.22e-5	UUCACUUAUA	UUCUUCGUAACCAC	AUUCAAU
228. SPCC1795.11 SPCC1795.11.1	211	2.22e-5	GCCAACUUA	CCUAACCUCACCAA	ACACUUCAAC
45. SPAC1805.11c SPAC1805.11c.1	53	2.22e-5	GGGUGAAACU	UUUCCUCUGCACCAC	C
203. SPAC9G1.03c SPAC9G1.03c.1	3	2.55e-5	AA	GUUUUCUAGCACCAC	CUUUCUCCAA
28. SPAC11G7.04 SPAC11G7.04.1	62	2.55e-5	GAAGGAAUCC	UUUGUCCACCACAAU	C
18. SPAC17A5.03 SPAC17A5.03.1	63	2.55e-5	CAGCAAAAUG	UCGUUCCAUAACCAC	CUAAGUGGCC
8. SPAC1805.12c SPAC1805.12c.1	21	2.55e-5	UAAAUGACAU	UUUGUCGAGUGCCAA	ACACCACAUU
136. SPBC17G9.03c SPBC17G9.03c.1	37	2.95e-5	GGUAAAUAUU	CUCUUCUCCACGAA	AAUUUCUCUG
189. SPBC15C4.05 SPBC15C4.05.1	41	3.38e-5	AUAUACACUU	UCUCUCUACAACCCA	CAAGAGAAUA
214. SPCC622.18 SPCC622.18.1	1	3.90e-5		UCUCUACACCACCCA	AAAACUCCAA
87. SPAC3G9.06 SPAC3G9.06.1	45	3.90e-5	CGCCAGUUAC	UUUACCUACUGCCAA	CACACCAACA
75. SPAC3A12.10 SPAC3A12.10.1	57	3.90e-5	AACAAGUCAA	CCUGUCCAGAGCCAG	AGACAUCAAA
42. SPAC31G5.03 SPAC31G5.03.1	16	4.43e-5	ACUGAGCUGU	CUUUUCUACAGCAAC	ACAAG
210. SPBC29B5.03c SPBC29B5.03c.1	63	5.07e-5	GAACUACCAU	UUUCACCAGACCCAC	GACACAAA
2. SPAC23A1.11 SPAC23A1.11.1	52	5.07e-5	UGGUCAACAU	UUCUUCACGACACA	
66. SPAC513.01c SPAC513.01c.1	8	5.75e-5	UUGAAAU	UGUUUCGUCCGCCAC	UGACAUUUAA
148. SPBC18E5.04 SPBC18E5.04.1	220	6.55e-5	AUACAAAUAG	UCCUCCGUUGCCAA	CCACUUCUUU
124. SPBC577.02 SPBC577.02.1	7	6.55e-5	GAAUUC	UUGGUCUUUCACCAA	GCUCAACAAA

Name	Start	p-value	Sites		
62. SPAC637.07 SPAC637.07.1	97	6.55e-5	UCGAAGAAAA	CUUCACCACUCCCAA	
223. SPCC1259.01c SPCC1259.01c.1	48	7.40e-5	CAGCUAAAAC	UUUGUCCACAGCGCA	UUAGCCAGUA
219. SPCC1223.05c SPCC1223.05c.1	66	7.40e-5	AUGACAAUA	UUUUGCCAGCAACAA	ACUAGCAAUA
150. SPBC646.09c SPBC646.09c.1	60	7.40e-5	AGUUGUGCUA	UCCUCCCCACACCAC	GCACUUUUUU
67. SPAC821.05 SPAC821.05.1	285	7.40e-5	GUACAGCAAU	UUGCACUUAACCAA	CUACUUGGCA
221. SPCC576.11 SPCC576.11.1	39	8.40e-5	UUUAGGAUCA	GUUGCACAGAACCAC	CACCACCGCU
108. SPAC26F1.13c SPAC26F1.13c.1	50	8.40e-5	UGGAUUCUCU	CCUUGCCAACACAAA	CUACUAAUAU
95. SPAC6F6.07c SPAC6F6.07c.1	64	8.40e-5	UCACUCAAAAG	UUGCACCUUAACCAA	ACAAAUUAAA
216. SPCC584.04 SPCC584.04.1	22	9.45e-5	UUCAACAUCA	CCGUCCCCUUACCAC	AUAUUUUAAU
35. SPAC23A1.08c SPAC23A1.08c.1	32	9.45e-5	GAAAAGGAAA	CCUGCGCAGAACAAA	CGAAAAAAAU
21. SPAC3G9.09c SPAC3G9.09c.1	28	9.45e-5	CACACAAAUU	UUUACUCAGAACCAA	ACACUCAACA
152. SPBC18H10.04c SPBC18H10.04c.1	45	1.06e-4	AAAUAUUAAA	UCUGUCGAGAACGAC	GAAGAAAACG
51. SPAC589.10c SPAC589.10c.1	36	1.06e-4	GAAAAACAUU	UCUAAGCACAAACCAA	A
40. SPAC2C4.16c SPAC2C4.16c.1	24	1.06e-4	UUUCUAGCCA	UCUCCAAAUCACCAA	C
224. SPCC364.03 SPCC364.03.1	24	1.34e-4	CCAUACAAAU	UUUGUACUGCGCAA	CGCCUUGACA
220. SPCC18.14c SPCC18.14c.1	1089	1.34e-4	AUUUAUUUUC	UUCACAAACCACCAA	ACCAGCAAAA
190. SPBC1709.02c SPBC1709.02c.1	9	1.34e-4	CGAACAAU	UCUUUCAACAACACA	CUUUUCCA
252. SPCC417.08 SPCC417.08.1	37	1.50e-4	ACCAGGACCU	CCGCUCAUCUACCAA	CAACCAUUUC
111. SPBC12C2.06 SPBC12C2.06.1	62	1.50e-4	UAAGGAGUAA	CUUAGCAAUCACCAA	CUACAUUGCC
20. SPAC21E11.06 SPAC21E11.06.1	7	1.50e-4	CUACGC	UUCUACCAAAACAAA	CCAAUUUAAU
175. SPBC14F5.06 SPBC14F5.06.1	69	1.68e-4	UAAGUUGUCC	UUUGCCAACUGCACC	CUACUCAACA
160. SPBC8D2.06 SPBC8D2.06.1	56	1.68e-4	GUACAUAUUC	CUCUCCUUCACACC	UAAACUUCUU
41. SPAC3H5.10 SPAC3H5.10.1	37	1.68e-4	CAAGUGCUAA	CUUUUCCACCCACAC	AACAGAA
185. SPBC4B4.04 SPBC4B4.04.1	12	1.88e-4	CAAAUAAGAC	UUCUACAACCAACAA	UCGCUUUCGU
183. SPBC17D11.05 SPBC17D11.05.1	31	1.88e-4	AGGGGGAAGU	UUUCAACUGCACAAC	
123. SPBC1921.01c SPBC1921.01c.1	80	1.88e-4	ACACUAAGAG	UUCCGCCUUUACCAA	GUCAAA
36. SPAC13G6.02c SPAC13G6.02c.1	864	1.88e-4	ACAACGAAGG	CUUCUCCUACACCAU	ACCGCA
52. SPAC823.15 SPAC823.15.1	61	2.08e-4	CUUUAUGUAU	CCUGUCUACUACCAU	ACCUUUCAAC
212. SPCC4G3.08 SPCC4G3.08.1	32	2.32e-4	AACUUGUGAU	CCUUCACAGUACAAA	UUGCUCAAAG
194. SPAC959.08 SPAC959.08.1	8	2.32e-4	CACAAAG	UUUCACCACAAACCA	UAUGGAAUUU

Name	Start	p-value	Sites		
168. SPBC685.07c SPBC685.07c.1	108	2.32e-4	CCACCUAUGA	UU <u>G</u> UCCU <u>A</u> CAACACA	ACA
182. SPBC36B7.09 SPBC36B7.09.1	483	2.57e-4	GCGAGAAACU	UCU <u>U</u> CCUCCAGCAA	UUCGCGGCAA
106. SPAC328.10c SPAC328.10c.1	31	2.57e-4	CACAAGCUAC	<u>C</u> <u>G</u> UCUGAACCACCAC	AGAA
99. SPAC15A10.04c SPAC15A10.04c.1	232	2.57e-4	UUAUUCUGUA	<u>C</u> UU <u>G</u> UCCU <u>U</u> AAACAC	AUCUAAUCAA
60. SPAC664.05 SPAC664.05.1	47	2.57e-4	CGAGCACCAA	<u>C</u> UU <u>A</u> CCU <u>U</u> CCACACC	AACUCACCUG
147. SPBC21C3.13 SPBC21C3.13.1	157	2.85e-4	AUUGGAGAAG	<u>C</u> UU <u>U</u> <u>G</u> UAA <u>A</u> CAACCAC	ACUGCACA
109. SPBC800.04c SPBC800.04c.1	32	2.85e-4	UUACCUUCAA	<u>G</u> UU <u>C</u> ACCAAC <u>G</u> CAA	ACACCCUUUAU
59. SPAC23A1.12c SPAC23A1.12c.1	116	2.85e-4	UUCAGGCAAG	CCCA <u>U</u> C <u>G</u> UU <u>C</u> ACCAC	AGAUCUUUUG
58. SPAC3G6.13c SPAC3G6.13c.1	28	2.85e-4	UAGUCUGCUA	<u>G</u> UCAUCCAUAACCAC	A
166. SPAP27G11.03 SPAP27G11.03.1	292	3.15e-4	UGGCGCUUGG	UU <u>G</u> UUCAUUCAAA	AUAUAAGCAU
78. SPAC3A11.03 SPAC3A11.03.1	54	3.15e-4	AAACAGUUA	<u>C</u> UAUUCCACUGCCAA	UUACAGACUA
144. SPBC17G9.07 SPBC17G9.07.1	25	3.49e-4	UGUGAGAGCC	UUUCCUUACCACCAU	CAGUCACCAA
126. SPBC29A3.12 SPBC29A3.12.1	30	3.84e-4	AAAGGUAGGU	UGCGCAAAGCACCAA	ACAAACA
230. SPCC1183.08c SPCC1183.08c.1	57	4.23e-4	UCCUCACGCA	UCCGCACAACACCAG	CAUUGCACC
142. SPCC1183.02 SPCC1183.02.1	51	4.23e-4	AGGCCAGCGA	<u>C</u> UCACUCUCCACAAA	CCAACCUCAA
107. SPAC3H5.12c SPAC3H5.12c.1	27	4.23e-4	AAAUAGCAAU	UCGACACACUGCCAA	A
16. SPAC521.05 SPAC521.05.1	62	4.23e-4	ACUAGUUCAU	<u>C</u> <u>G</u> UAACCACAGCAA	UCUCUUUCUG
92. SPAC16E8.15 SPAC16E8.15.1	51	4.64e-4	GUAAAUUUUC	UUUUACAUAACAGAC	CAGUUCCAG
239. SPCC16C4.13c SPCC16C4.13c.1	36	5.10e-4	CACUCUUGCU	UGAUCCCACAGCCAC	AACCCAAG
44. SPAC26A3.04 SPAC26A3.04.1	44	5.10e-4	AAAGUGAAAC	UUUUACGAACAACAC	CAAUUGGGGA
17. SPAC25G10.08 SPAC25G10.08.1	14	5.10e-4	GGUGAAGAAA	<u>C</u> UUGACUAGAGACAA	CGGCACUACA
193. SPBC106.18 SPBC106.18.1	54	5.59e-4	UUCCGAAUGA	UCUGUCACGAGCAAC	AACCAACUCU
43. SPAC18G6.14c SPAC18G6.14c.1	13	5.59e-4	CGAAACAAUU	UCUUUUUCUACACAAC	AGCA
33. SPAC3A12.13c SPAC3A12.13c.1	75	5.59e-4	UACAAUAAAU	UCCUUAAU <u>G</u> AACCAC	AUACCUUUUU
27. SPAC222.07c SPAC222.07c.1	218	5.59e-4	CCUUUUUCAC	UUCCAUCAUACAAA	U
22. SPAC15E1.03 SPAC15E1.03.1	49	5.59e-4	AUGCUGUCGA	<u>G</u> UUCCUAAU <u>C</u> ACCAC	CAUCUAACCA
217. SPCC576.09 SPCC576.09.1	16	6.12e-4	ACAACUUCUU	<u>C</u> UUUCACUAAACCCC	AUCGUGGUCA
4. SPAC3G9.03 SPAC3G9.03.1	19	6.12e-4	GUUUGAGAGC	UUUCACAUCUACACA	AUAUCACG
117. SPBC18H10.14 SPBC18H10.14.1	50	6.68e-4	CUUUUUUGGC	UUUCAGGACUACAAC	CCAGUAAAAC
105. SPAC30D11.12 SPAC30D11.12.1	104	6.68e-4	GGAAAUCCUA	CCUGUGCACAGCAAG	UUACGAGAGU

Name	Start	p-value	Sites		
89. SPAC23C11.02c SPAC23C11.02c.1	39	6.68e-4	UUCGCCGCAA	CCUCUAUACUACAAA	
65. SPAC1071.07c SPAC1071.07c.1	66	6.68e-4	AGUCACAACG	UUUCAAGAGAACAAC	GAAAUUGGUU
250. SPCC1393.09c SPCC1393.09c.1	123	7.29e-4	CCCAUUUGCA	UUCACCUUUCACGCA	A
53. SPAC6G10.11c SPAC6G10.11c.1	23	7.29e-4	AUCAUAUUUU	UCCUCCAUCGCGAG	GAAGAGGCAA
198. SPAP7G5.05 SPAP7G5.05.1	97	7.94e-4	AAUUACUUUC	CUGAAUCAGAACCAC	AUUUUUCACA
76. SPAC25H1.02 SPAC25H1.02.1	58	7.94e-4	CCGCUGGUCA	UUUUACUAGCGAAAA	AACAACAUUU
242. SPCC1682.14 SPCC1682.14.1	21	8.64e-4	AAAGCACAAU	CGUCCCCAACCCAA	CCAUAUUAAC
79. SPAC2F7.05c SPAC2F7.05c.1	30	8.64e-4	GGCUGCAACU	UUUGUUUUUUACCAA	ACACCUUACC
170. SPBC21B10.10 SPBC21B10.10.1	125	9.39e-4	CGUAAUCCA	CCUUAUCCAACCAA	AGCUAUUGAA
64. SPAC1805.13 SPAC1805.13.1	1	9.39e-4		UCUUCCCCUAACAAG	UCACCACCAG
63. SPAC4D7.09 SPAC4D7.09.1	60	9.39e-4	UCAUCAAAUA	UUGGCGUACUGCAAA	AAAACCCAAU
132. SPBC839.04 SPBC839.04.1	49	1.02e-3	CCGUUAGGAA	GUUUUCUAUCGCCAU	CUAACAACC
83. SPAC6B12.15 SPAC6B12.15.1	65	1.02e-3	GCUACCUACA	UCUCUCCUUAAGAC	AUCCCGACCA
201. SPBC1709.18 SPBC1709.18.1	1	1.10e-3		UUUCCAAUCAAACAA	AUAAUUUACU
47. SPAC4D7.05 SPAC4D7.05.1	35	1.10e-3	AACUAAAGAC	UUUUCGCUCAAACCC	UCUACCAGCA
34. SPAC29A4.02c SPAC29A4.02c.1	46	1.19e-3	CCCUUUACAA	UUCUUUCUCUCCCAA	CAUCUUAGUA
155. SPBC776.11 SPBC776.11.1	50	1.39e-3	ACAUCUUUUU	GCUUCAGACUACCCA	CUCUAUUAAG
61. SPAC19B12.04 SPAC19B12.04.1	132	1.39e-3	UCCACUUAUA	UUUAUCAAGAACAUA	AAA
30. SPAC17A5.07c SPAC17A5.07c.1	14	1.39e-3	GUAGUAGGCU	UCUCUAUCUCGCCAA	AACAGGUAUG
82. SPAC22H12.04c SPAC22H12.04c.1	21	1.50e-3	AACAACUAAU	UUUUACGAAACCAA	CUUCUCCACA
187. SPBC1685.10 SPBC1685.10.1	39	1.62e-3	GCACACACAU	CUCAGCUUCUACCCC	GAGCAAUUCA
14. SPAC1071.08 SPAC1071.08.1	5	1.87e-3	GCAG	UUUGCAAUAAACGAA	CGCACGCCGU

RP 19-mer 5' Proximal AC-rich motif:



Name	Start	p-value	Sites		
94. SPAC11E3.15 SPAC11E3.15.1	44	2.13e-8	AUUCUCUUUG	UUUAUCCUCCACCACAUAAC	UCGUAGAGAG
116. SPBC216.07c SPBC216.07c.1	93	4.59e-8	CAGGUGCUUG	UUUGGUCACCACCAACCAC	AUUUAAUAAU
236. SPCC1393.03 SPCC1393.03.1	59	1.10e-7	AAAAGAUACG	UUUGACCAUCACCAACUAC	GGCAUAAAAU
151. SPBC56F2.02 SPBC56F2.02.1	56	1.30e-7	UUAAGAAAGA	UUGUCCGUCACCAAAAAAC	AGCAAGAAAA
148. SPBC18E5.04 SPBC18E5.04.1	220	2.10e-7	AUACAAAUAG	UCCUCCGUUCGCCAACCAC	UUCUUUAACU
247. SPCC1322.11 SPCC1322.11.1	36	4.41e-7	GGUUCGACGU	UCCUUCUACAACCAAAACAC	CAGCAGAAAA
245. SPCC1450.04 SPCC1450.04.1	19	4.41e-7	UCUAAAAUUU	UCUGCCAACAACCAACUAC	UUUAAAA
228. SPCC1795.11 SPCC1795.11.1	211	5.85e-7	GCCAACUUAA	CCUAACCUCAAACCAAAACAC	UUCAACUCAG
93. SPAC29A4.15 SPAC29A4.15.1	91	8.77e-7	AUGGGCAUGU	CUUAUCCUUUACCAACACC	ACCUCGUUGA
8. SPAC1805.12c SPAC1805.12c.1	21	8.77e-7	UAAAUGACAU	UUUGUCGAGUCCAAACAC	CACAUUCCCA
79. SPAC2F7.05c SPAC2F7.05c.1	30	1.00e-6	GGCUGCAACU	UUUGUUUUUUACCAAAACAC	CUUACCUUGA
218. SPCC794.09c SPCC794.09c.1	5	1.14e-6	CGCC	UUUGUCAUUCACCACACGC	CAAAAAUUUU
48. SPAC17C9.03 SPAC17C9.03.1	57	1.29e-6	UAUUGCUCU	CUUGACCACCACCACUAAC	CCAACCACUU
189. SPBC15C4.05 SPBC15C4.05.1	38	1.46e-6	UAUAUAUACA	CUUUCUCUCUACAACCCAC	AAGAGAAUUA
124. SPBC577.02 SPBC577.02.1	7	1.46e-6	GAAUUC	UUGGUCUUUACCAAGCUC	AACAAA
19. SPAC26A3.07c SPAC26A3.07c.1	50	1.46e-6	UGCUAAAUAC	UUUGGCAUCCACCAAGAA	AAGGCACAAG
162. SPAC8C9.05 SPAC8C9.05.1	18	2.37e-6	AACAGUAUAA	UAUACCCUUUACCAAAACAC	ACUACCUGCU

Name	Start	p-value	Sites		
111. SPBC12C2.06 SPBC12C2.06.1	62	2.37e-6	UAAGGAGUAA	CUUAGCAAUCACCAACUAC	AUUGCCCACC
21. SPAC3G9.09c SPAC3G9.09c.1	28	2.37e-6	CACACAAAUU	UUUACUCAGAACCAAAACAC	UCAACACUUU
1. SPAC1006.07 SPAC1006.07.1	33	2.99e-6	AACCCUUCAA	CUUUUUUUCCACAACCCAC	UCACCUAGCU
248. SPCC31H12.04c SPCC31H12.04c.1	178	4.66e-6	ACUUUGCGUA	UUUUUCUUGAACCAAGCAA	GAGUAAG
246. SPCC18B5.06 SPCC18B5.06.1	58	5.19e-6	UUCACUAAAA	UUCUUUCGUAACCACAUUC	AAU
18. SPAC17A5.03 SPAC17A5.03.1	63	5.19e-6	CAGCAAAAUG	UCGUUCCAUAACCAACUAA	GUGGCCGUUG
252. SPCC417.08 SPCC417.08.1	37	5.77e-6	ACCAGGACCU	CCGCUCAUCUACCAACAAC	CAUUUCCCCG
194. SPAC959.08 SPAC959.08.1	43	6.40e-6	CCGUGCGUAC	UUUGCCUUUAACCAGGAAC	AGCA
229. SPCC74.03c SPCC74.03c.1	28	7.10e-6	AUACUAUGGC	UUUGCCUUCUACCAAAUUA	UAUAUUUAUC
182. SPBC36B7.09 SPBC36B7.09.1	482	7.10e-6	AGCGAGAAAC	UUCUUCCUCCAAGCAAUUC	GCGGCAAACA
67. SPAC821.05 SPAC821.05.1	285	7.86e-6	GUACAGCAAU	UUGCACUUCACCAACUAC	UUGGCAGACU
98. SPAC1751.03 SPAC1751.03.1	12	8.69e-6	CCAAGUCCUA	CUCUCCAACUACCACUAA	AGCGAAUAUU
249. SPCC613.05c SPCC613.05c.1	50	9.60e-6	UGUCGCUGAG	UUUACUCUUGACCAACGAC	GACGACGAG
142. SPCC1183.02 SPCC1183.02.1	51	1.06e-5	AGGCCAGCGA	CUCACUCUCCACAAACCAA	CCUCAAUUCA
181. SPBC23G7.05 SPBC23G7.05.1	18	1.17e-5	UUUACUACAU	CCUUCUAGAACCACCACC	GUUUAAAGGUC
75. SPAC3A12.10 SPAC3A12.10.1	57	1.17e-5	AACAAGUCAA	CCUGUCCAGAGCCAGAGAC	AUCAA
213. SPCC970.05 SPCC970.05.1	24	1.28e-5	CGCAGUAAAGU	UUUCCCCUACACCAAUUC	ACA
95. SPAC6F6.07c SPAC6F6.07c.1	64	1.28e-5	UCACUCAAAAG	UUGCACCUUAACCAAAACAA	AUUAUUAUCAA
216. SPCC584.04 SPCC584.04.1	22	1.86e-5	UUCAACAUCA	CCGUCCCUUUACCACAUAU	UUUAAUAAAG
150. SPBC646.09c SPBC646.09c.1	60	1.86e-5	AGUUGUGCUA	UCCUCCCCACACCACGCAC	UUUUUUUUUG
220. SPCC18.14c SPCC18.14c.1	1082	2.03e-5	GGUAAGAAUU	UAUUUUUUUACAAACCAC	CAAACCAGCA
108. SPAC26F1.13c SPAC26F1.13c.1	50	2.03e-5	UGGAUUCUCU	CCUUGCCAACACAAACUAC	UAAUAUUAAG
37. SPAC1F7.13c SPAC1F7.13c.1	26	2.03e-5	CAAUACCGUA	UUCUCCAACAACCAAAACA	CCAGAA
78. SPAC3A11.03 SPAC3A11.03.1	54	2.22e-5	AAACAGUUAA	CUAUUCCACUGCCAUAUAC	AGACUAACCC
175. SPBC14F5.06 SPBC14F5.06.1	62	2.42e-5	CGCACCUUAA	GUUGUCCUUUGCCAACUGC	ACCCUACUCA
87. SPAC3G9.06 SPAC3G9.06.1	45	2.42e-5	CGCCAGUUAC	UUUACCUACUGCCAACACA	CCAACAAAGC
66. SPAC513.01c SPAC513.01c.1	8	2.42e-5	UUGAAAU	UGUUUCGUCCGCCACUGAC	AUUUAAAAAU
206. SPBP8B7.03c SPBP8B7.03c.1	48	2.64e-5	UAAACUGAUU	UUUUCCAACCACAAAAGCA	UACUAACAGC
171. SPBC16C6.11 SPBC16C6.11.1	62	2.64e-5	UACUUGGUUC	CUUUUCCACUGCAACGGUC	GAAAC
42. SPAC31G5.03 SPAC31G5.03.1	16	2.87e-5	ACUGAGCUGU	CUUUUCUACAGCAACACAA	G

Name	Start	p-value	Sites		
217. SPCC576.09 SPCC576.09.1	11	3.12e-5	CUUCAACAAC	UUCUUCUUUCACUAAACCC	CAUCGUGGUC
29. SPAC23C4.06c SPAC23C4.06c.1	424	3.39e-5	UCACCAAGAU	CCUUAACCAUACCAAGAAU	UACGAUUUUC
123. SPBC1921.01c SPBC1921.01c.1	80	3.69e-5	ACACUAAGAG	UUCCGCCUUUACCAAGUCA	AA
59. SPAC23A1.12c SPAC23A1.12c.1	116	4.33e-5	UUCAGGCAAG	CCCAUUCGUUACCAACAGAU	CCUUUGAAGC
95. SPAC6F6.07c SPAC6F6.07c.1	32	5.91e-5	ACCUAGAAUU	UUAUUUUUCUACGACACAC	CUCUCACUCA
9. SPAC806.03c SPAC806.03c.1	68	5.91e-5	AUUGUACAUG	CUUAUUAUUACAAAAAAC	AGU
171. SPBC16C6.11 SPBC16C6.11.1	31	6.37e-5	AGUCACAUAG	UUCGUUCAUAGCCAAAACA	UUUACUUGGU

Additional Tables

RNA substrates for EMSAs (Related to STAR Methods):

5' Proximal AC-rich (5'PAC)	UUUUUCCCACCACCAAACAC
5' Proximal AC-rich (5'PAC) Triple C Mutant	UUUUUCGCACCAGCAAACAG C6G, C12G, C19G
5' Distal U-Rich (5'DUR) (data not shown)	UUUGACUGCUUUUUUGUG
5' Distal U-Rich (5'DUR) Triple U Mutant (data not shown)	UUUGACGGCUUGUGGUG U7G, U12G, U14G
5' Proximal AC-rich (5'PAC) U to A Mutant	AAAACCCACCACCAAACAC
5' Proximal AC-rich (5'PAC) C6G Mutant	UUUUUCGCACCACCAAACAC
5' Proximal AC-rich (5'PAC) C12G Mutant	UUUUUCCCACCAGCAAACAC
5' Proximal AC-rich (5'PAC) C19G Mutant	UUUUUCCCACCACCAAACAG
RP 15-mer 5' Proximal AC-rich motif (PAC15)	UUUUUCCACCACCAA
PAC15 C6G mutant UUUUUGCACCACCAA	UUUUUUGCACCACCAA

Cloning primers for *Renilla* reporters (Related to STAR Methods):

20N AC-rich XhoI For	GCGCCTCGAGCGGCCGGATCAAGAGCTACCTTTCCACCACCA AACACATGACTTCGAAAGTTTATGATCCAGAACAAAGG
AC-rich 20N XhoI For	GCGCCTCGAGTTTTCCACCACCAAACACCGGCCGGATCAAGAG CTACCATGACTTCGAAAGTTTATGATCCAGAACAAAGG
20N U-rich XhoI For	GCGCCTCGAGCGGCCGGATCAAGAGCTACCTTTGACTGCTTTTTGT GATGACTTCGAAAGTTTATGATCCAGAACAAAGG
U-rich 20N XhoI For	GCGCCTCGAGTTTGACTGCTTTTTGTGCGGCCGGATCAAGAGCTAC CATGACTTCGAAAGTTTATGATCCAGAACAAAGG
20A20N XhoI For	GCGCCTCGAGAAAAAAAAAAAAAAAAAAAAACGGCCGGATCAAGAG CTACCATGACTTCGAAAGTTTATGATCCAGAACAAAGG
20N20A XhoI For	GCGCCTCGAGCGGCCGGATCAAGAGCTACCAAAAAAAAAAAAAA AAAAAATGACTTCGAAAGTTTATGATCCAGAACAAAGG
20T20N XhoI For	GCGCCTCGAGTTTTTTTTTTTTTTTTTTTCGGCCGGATCAAGAGCTAC CATGACTTCGAAAGTTTATGATCCAGAACAAAGG
20N20T XhoI For	GCGCCTCGAGCGGCCGGATCAAGAGCTACCTTTTTTTTTTTTTTTT TTATGACTTCGAAAGTTTATGATCCAGAACAAAGG

40N XhoI For	GCGCCTCGAGCGGCCGGATCAAGAGCTACCCGGCCGGATCAAG AGCTACCATGACTTCGAAAGTTTATGATCCAGAACAAAGG
<i>Renilla</i> XmaI Rev	GCGCCCCGGGCTATTGTTTATTTTGAGAACTCGCTCAACGAACGA TTTG

Cloning primers for the tethering reporters (Related to STAR Methods):

For cloning LambdaN-Ago2 into pRep81x	
LambdaN-Ago2/LacZ For (XhoI)	GCGCCTCGAGATGGACGCACAAACACGACGACGTGAGC GTCGCGCTGAGAAACAAGCTCAATGGAAAGCTGCAAACC CACCGCTGGAGGCTGCCCAAGCTTACACCATGGCCTACC CATA
LambdaN-Ago2 Rev (XmaI)	GCGCCCCGGGGGACAGGGGTGCAATAGCTTTATTCCTG CCCCCGT
For subcloning Slr1 into the above, for pRep81x-LHA-Slr1	
LambdaN-Slr1 For (BglII)	GCGCAGATCTCATGTCTCATAAAGAACCAAGTGCTGCTGA AAC
LambdaN-Slr1 Rev (XmaI)	GCGCCCCGGGCTAAACAGTAAGTTTATCCTCAACCTCAGT TT
For cloning LambdaN-LacZ into pRep81x, for pRep81x-LHA-LacZ	
LambdaN-Ago2/LacZ For (XhoI)	GCGCCTCGAGATGGACGCACAAACACGACGACGTGAGC GTCGCGCTGAGAAACAAGCTCAATGGAAAGCTGCAAACC CACCGCTGGAGGCTGCCCAAGCTTACACCATGGCCTACC CATA
LambdaN-LacZ Rev (XmaI)	GCGCCCCGGGTTATTTTGGACACCAGACCAACTGGTAATG
For cloning <i>Renilla</i> -5BoxB into pRep82x (3' end stem-loops, for pRep82x-RL-5BoxB)	
<i>Renilla</i> -5BoxB 3' For (XhoI)	GCGCCTCGAGATGGCTTCCAAGGTGTACGACCCCGAG
<i>Renilla</i> -5BoxB 3' Rev (XmaI)	GCGCCCCGGGTCGAGATAATATCCTCGATAGGGCCCTTC T
For cloning 5BoxB into pRep82x	
5BoxB 5' For (XhoI)	GCGCCTCGAGTCTAGATAAGTCCAATACTAACTGGGGA TTCC
5BoxB 5' Rev (XmaI, BglII)	GCGCCCCGGGGCGCAGATCTTCGAGATAATATCCTCGAT AGGGCCCTTCT
For subcloning <i>Renilla</i> into the above (5' end stem-loops, for pRep82x-5BoxB-RL)	
<i>Renilla</i> -5BoxB 5' For (BglII)	GCGCAGATCTATGGCTTCCAAGGTGTACGACCCCGAGCA ACGC

<i>Renilla</i> -5BoxB 5' Rev (XmaI)	GCGCCCCGGGTTACTGCTCGTTCTTCAGCACGCGCTCCA CGAAGCT
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Northern blot probes (Related to STAR Methods):

<i>Renilla</i>	CGCCATAAATAAGAAGAGGCCGC
U5 snRNA	TAGCACACCTTACAAACGGCTGT

Strains and genotypes (Related to STAR Methods):

Strain	Genotype	Source
yAS99	Wildtype, <i>ade6-704</i> , <i>ura4-D18</i> , <i>leu1-32</i> , <i>h-</i>	Intine <i>et al.</i> , 2000 ¹
y12088	Wildtype, <i>ura4-D18</i> , <i>leu1-32</i> , <i>h+</i>	Boone lab, Toronto, Canada
yMAB001	Made in yAS99; <i>slr1::G418R</i>	This paper
yLS001	Made in yAS99; <i>slr1-PrA::G418R</i>	This paper
yFM001	Made in y12088; <i>pabp-GFP::PhleomycinR</i>	This paper
yFMN20	yFM001xyMAB001; <i>pabp-GFP::PhleomycinR</i> , <i>slr1::G418R</i> , <i>h+</i>	This paper
JU915	<i>exo2-13xMyc::NatR</i> , <i>h-</i>	Duncan and Mata, 2014 ²
yFM009	yFM001xJU915; <i>pabp-GFP::PhleomycinR</i> , <i>exo2-13xMyc::NatR</i> , <i>h+</i>	This paper
yFMN90	yFMN20xJU915; <i>pabp-GFP::PhleomycinR</i> , <i>slr1::G418R</i> , <i>exo2-13xMyc::NatR</i> , <i>leu1-32</i> , <i>h+</i>	This paper
yFM006	Made in y12088; <i>tif471-13xMyc::G418R</i>	This paper
yFM012	Made in yAS99; <i>sum1-5xFLAG::G418R</i>	This paper
yFM013	yFM009xyAS99; <i>pabp-GFP::PhleomycinR</i> , <i>exo2-13xMyc::NatR</i> , <i>ura4-D18</i> , <i>leu1-32</i> , <i>h-</i>	This paper
yFMN100	yFMN90xyAS99; <i>pabp-GFP::PhleomycinR</i> , <i>slr1::G418R</i> , <i>exo2-13xMyc::NatR</i> , <i>ura4-D18</i> , <i>leu1-32</i> , <i>h+</i>	This paper

Key Resources Table (Related to STAR Methods):

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-Slr1p antibody	This paper	AB2648
Anti-Slr1p antibody	This paper	AB2756
Anti-Myc antibody	Cell Signalling Technology	Cat#2276S
Anti-GFP antibody	Takara Bio Clontech	Cat#632381
Anti-Rpl9 antibody	Abcam	Cat#ab182556

Anti-FLAG antibody	Abcam	Cat#ab49763
Anti-mouse IgG HRP-linked antibody	Cell Signalling Technology	Cat#7076S
Anti-rabbit IgG HRP-linked antibody	Cell Signalling Technology	Cat#7074S
Non-immune rabbit IgG	Invitrogen	Cat#02-6102
Mouse Anti-rabbit IgG (Conformation Specific) antibody	Cell Signalling Technology	Cat#3678S
Chemicals, Peptides, and Recombinant Proteins		
Yeast Extract	Bioshop	Cat#YEX401.205
Adenine	Bioshop	Cat#ADA001.100
L-histidine	Bioshop	Cat#HIS200.100
L-leucine	Bioshop	Cat#LEU222.100
L-uracil	Bioshop	Cat#URA241.100
L-lysine monohydrochloride	Bioshop	Cat#LYS101.500
Edinburgh Minimal Media (EMM) - Glucose	Sunrise Science Products	Cat#2020-500
Cycloheximide	Sigma	Cat#C7698-5G
Nuclease-free water	Invitrogen	Cat#10977-015
Polyvinylpyrrolidone	Sigma	Cat#PVP40-50G
HEPES	Fisher	Cat#BP310-500
Halt™ Protease Inhibitor Cocktail	Thermo Scientific	Cat#78425
PMSF	Bioshop	Cat#PMS123.5
DTT	Bioshop	Cat#DTT002.25
SUPERase•In™ RNase Inhibitor	Invitrogen	Cat#AM2694
Heparin	Sigma	Cat#H4784-1G
Polypropylene centrifuge tubes	Beckman Coulter	Cat#331372
Trichloroacetic acid (TCA)	Fisher	Cat#A322-500
Acetone	Fisher	Cat#A18-200
β-mercaptoethanol	Sigma	Cat#M6250-100ML
ECL	Thermo Scientific™	Cat#32106
TRIzol	Invitrogen	Cat#15596018
4-thiouracil (4tU)	Sigma	Cat#440736-1G
Formaldehyde	Fisher	Cat#BP531-500
Glycine	Fisher	Cat#BP381-5
TurboDNase	Invitrogen	Cat#2238

Dynabeads™ Protein A magnetic beads	Invitrogen	Cat#10001D
Proteinase K	Invitrogen	Cat#25530049
Acidic phenol	Fisher	Cat#BP1751I-400
Phenol/Chloroform/Isoamyl Alcohol (25:24:1 mixture)	Fisher	Cat#BP1752I-400
Chloroform/Isoamyl Alcohol (24:1 mixture)	Sigma	Cat#25666-100ML
IPTG	Fisher	Cat#BP1755-100
Imidazole	Bioshop	Cat#IMD508.250
Amicon® Ultra-4 Centrifugal Filter Unit	Millipore Sigma	Cat#UFC803024
γ - ³² P ATP	Perkin Elmer	Cat#BLU502A250UC
T4 Polynucleotide Kinase (T4 PNK)	NEB	Cat#M0201S
Dynabeads™ M-270 Epoxy	Invitrogen	Cat#14301
Critical Commercial Assays		
Ribo-Zero Gold rRNA Removal Kit	Illumina	Yeast
TruSeq Stranded Total RNA Kit	Illumina	
TruSeq Stranded mRNA Sample Preparation Kit	Illumina	
Ultra II Directional mRNA library prep Kit	NEB	
<i>Renilla</i> Luciferase Assay System	Promega	Cat#E2820
Decade™ Markers System	Invitrogen	Cat#AM7778
Deposited Data		
RNA-Seq	This paper	GEO: GSE237860
RATE-Seq	This paper	GEO: GSE200633
RIP-Seq	This paper	GEO: GSE237526
LC/MS-MS	This paper	PRIDE: PXD043979
Experimental Models: Organisms/Strains		
<i>S. pombe</i> : yAS99: <i>ade6-704, ura4-D18, leu1-32, h-</i>	Intine <i>et al.</i> (2000) ⁷⁹	Wildtype
<i>S. pombe</i> : y12088: <i>ura4-D18, leu1-32, h+</i>	Boone lab, Toronto, Canada	Wildtype
<i>S. pombe</i> : yMAB001: <i>slr1::G418R</i>	This paper	Made in yAS99
<i>S. pombe</i> : yLS001: <i>slr1-PrA::G418R</i>	This paper	Made in yAS99
<i>S. pombe</i> : yFM001: <i>pabp-GFP::PhleomycinR</i>	This paper	Made in y12088

<i>S. pombe</i> : yFMN20: <i>pabp-GFP::PhleomycinR, slr1::G418R, h+</i>	This paper	yFM001 x yMAB001
<i>S. pombe</i> : JU915: <i>exo2-13xMyc::NatR, h-</i>	Duncan and Mata (2014) ⁷⁸	
<i>S. pombe</i> : yFM009: <i>pabp-GFP::PhleomycinR, exo2-13xMyc::NatR, h+</i>	This paper	yFM001 x JU915
<i>S. pombe</i> : yFMN90: <i>pabp-GFP::PhleomycinR, slr1::G418R, exo2-13xMyc::NatR, leu1-32, h+</i>	This paper	yFMN20 x JU915
<i>S. pombe</i> : yFM006: <i>tif471-13xMyc::G418R</i>	This paper	Made in y12088
<i>S. pombe</i> : yFM012: <i>sum1-5xFLAG::G418R</i>	This paper	Made in yAS99
<i>S. pombe</i> : yFM013: <i>pabp-GFP::PhleomycinR, exo2-13xMyc::NatR, ura4-D18, leu1-32, h-</i>	This paper	yFM009 x yAS99
<i>S. pombe</i> : yFMN100: <i>pabp-GFP::PhleomycinR, slr1::G418R, exo2-13xMyc::NatR, ura4-D18, leu1-32, h+</i>	This paper	yFMN90 x yAS99
Oligonucleotides		
RNA substrates, primers, probes	See Supplemental Information	
Recombinant DNA		
pFA6a-GFP-bleMX6	Gadaleta <i>et al.</i> (2013) ⁷⁷	Addgene Plasmid #33141
pFA6a-13Myc-kanMX6	Bahler <i>et al.</i> (1998) ⁷⁶	Addgene Plasmid #39294
pFA6a-G11-5FLAG-kanMX6	Gadaleta <i>et al.</i> (2013) ⁷⁷	Addgene Plasmid #49182
Software and Algorithms		
Trimmomatic (version 0.36)	Bolger <i>et al.</i> (2014) ⁷²	http://www.usadellab.org/cms/index.php?page=trimmomatic
STAR (version 2.5.2a)	Dobin <i>et al.</i> (2013) ⁷³	https://code.google.com/archive/p/rna-star/
HTSeq (version 0.6.1p2)	Anders <i>et al.</i> (2015) ⁷⁴	https://pypi.org/project/HTSeq/
DRUID pipeline	Lugowski <i>et al.</i> (2018) ⁵³	https://github.com/risslandlab/DRUID

GraphPad Prism (version 9.3.1 and 9.5.0)	GraphPad Software	www.graphpad.com
DESeq2 (version 1.26.0s)	Love <i>et al.</i> (2014) ⁸²	https://bioconductor.org/packages/release/bioc/html/DESeq2.html and http://master.bioconductor.org/packages/release/workflows/vignettes/rnaseqGene/inst/doc/rnaseqGene.html
Scaffold (version 5.1.1)	Proteome Software, Inc.	https://www.proteomesoftware.com/products/scaffold-5
ImageJ	Schneider <i>et al.</i> (2012) ⁸³	https://imagej.net/ij/index.html

2.8. STAR Methods

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Mark A. Bayfield (bayfield@yorku.ca).

Materials Availability

All plasmids/strains generated in this study will be available on request from the lead contact, but we may require a completed Materials Transfer Agreement.

Data and Code Availability

- RNA-Seq, RATE-Seq and RIP-Seq data have been deposited at GEO⁶⁹ and are publicly available as of the date of publication. Accession numbers are listed in the key resources table. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE⁷⁰ partner repository and are publicly available as of the date of publication. The dataset identifier is listed in the key resources table.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

General yeast growth conditions

S. pombe strains were streaked on YES (5 g/L Yeast Extract (Bioshop YEX401.205), 225 mg/L adenine, L-histidine, L-leucine, L-uracil and L-lysine monohydrochloride (Bioshop ADA001.100, HIS200.100, LEU222.100, URA241.100, LYS101.500), 30 g/L Dextrose (D-Glucose) Anhydrous (Fisher D16-10)) or EMM-ura or EMM-leu (11.77 g/L Edinburgh Minimal Media - Glucose (Sunrise Science Products 2020-500) plus other substrates and minus uracil or leucine, 20 g/L Dextrose (D-Glucose) Anhydrous (Fisher D16-10)) plates, and incubated at 32°C until colonies formed. For each biological replicate, a single colony was used to inoculate 10 mL of the respective liquid media. Cultures were incubated at 32°C overnight with shaking (200 rpm). Each overnight culture was subsequently used as a seed culture to inoculate fresh media. Fresh cultures were next grown at 32°C, 200 rpm, to $OD_{600} \sim 0.6$ at the time of harvest.

METHOD DETAILS

Polysome profiling

S. pombe yFM009 (*slr1+*) and yFMN90 (*slr1-*) liquid cultures were grown in YES, 32°C, 200 rpm, to $OD_{600} \sim 0.6$. 100 µg/mL cycloheximide (Sigma C7698-5G) was added to each culture which was incubated again for 10 minutes, 32°C, 200 rpm. *S. pombe* yFM013 (*slr1+*) and yFMN100 (*slr1-*) strains were used for polysome profiles in minimal media (EMM) and were grown at 32°C, 200 rpm.

Harringtonine treatment was performed as described (Ingolia *et al.*, Nat Protoc., 2012)⁶¹. Briefly, cultures were grown in YES or EMM, 32°C, 200 rpm, to $OD_{600} \sim 0.6$ and harringtonine (Abcam ab141941) was added to each at a final concentration of 2 µg/mL. Cultures were mixed quickly and returned to the incubator for 2 minutes. This was quickly followed by cycloheximide treatment as described above.

Cells were then harvested, washed with sterile nuclease-free water (Invitrogen 10977-015), washed with Resuspension buffer (12 mg/mL Polyvinylpyrrolidone (Sigma PVP40-50G), 20 mM HEPES pH 7.4 (Fisher BP310-500), 1% v/v Halt™ Protease Inhibitor Cocktail (Thermo Scientific 78425), 1 mM PMSF (Bioshop PMS123.5), 1 mM DTT (Bioshop DTT002.25); ~7.5 mL/L of cells), and pelleted to be flash-frozen in liquid nitrogen as described (Oeffinger *et al.*, Nature Methods, 2007)⁵¹ with some modifications. Briefly, each yeast pellet was transferred to a 20 mL syringe using a cooled down spatula, and using a syringe further transferred into a conical filled with liquid nitrogen. Frozen yeast noodles were then ground into a fine powder in presence of liquid nitrogen and using a cooled down marble pestle and mortar. Grindates were then stored at -80°C until the day of polysome profiling. Prior to polysome profiling, grindates were resuspended in Polysomal Lysis buffer (20 mM Tris-HCl pH 7.5 (Fisher BP152-5), 10 mM MgCl₂ (Bioshop MAG520.500), 50 mM KCl (Fisher BP366-1), 100 µg/mL cycloheximide (Sigma C7698-5G), 1 mM DTT (Bioshop DTT002.25), 0.0067% Halt™ Protease Inhibitor Cocktail (Thermo Scientific 78425), 6.7 U SUPERase•In™ RNase Inhibitor (Invitrogen AM2694); 5 mL/g of grindate) by vortexing three times, 30 seconds on

and off ice each time. The suspension was spun down next to obtain a clear lysate. 26 OD₂₆₀ units from the cleared lysate were then diluted in 500 µl of Polysomal Lysis buffer + 0.2 mg/mL heparin (Sigma H4784-1G) and layered onto a linear sucrose (Fisher BP220-212) gradient (20-50% in polypropylene centrifuge tubes (Beckman Coulter 331372)). Gradients were then spun at 39,000 rpm for 4 hours in a Beckman ultracentrifuge (Optima™ XL-100) using a SW4Ti rotor (acceleration: max, deceleration: no break) and fractionated (Brandel, sensitivity: 0.5, speed: 60 cm/hr). 11 fractions were collected at 1 mL each.

TCA precipitation and western blotting

Trichloroacetic acid (TCA, 100% (w/v), Fisher A322-500) was added to 1 mL of protein sample to a final concentration of 20%. Next, samples were incubated at -20°C for 75 minutes. Samples were then spun down at max speed for 15 minutes. Supernatants were decanted, and pellets were washed with 500 µl of ice-cold acetone (Fisher A18-200). Two more acetone washes were performed per sample. Then, pellets were dried in a 95°C heat block for 5 minutes followed by an overnight incubation at room temperature. The next day, pellets were heated in a 95°C heat block for 5 minutes again followed by a 30-minute incubation at room temperature. Dried pellets were finally resuspended in 20 µl of ddH₂O plus 20 µl of 5X Laemmli buffer (312.5 mM Tris-HCl pH 6.8 (Fisher BP152-5), 50% Glycerol (Bioshop GLY001.500), 5% SDS (Bioshop SDS003.1), 0.05% Bromophenol Blue (Sigma B8026); and 10% β-mercaptoethanol (Sigma M6250-100ML) added just before use). 5 µl of 1 M Tris-HCl pH 8.0 was then added to each sample to adjust the pH. Samples were heated at 95°C again for 10 minutes, before 15 µl of each being loaded onto a 10% SDS-Polyacrylamide (Bioshop SDS003.1, Fisher BP1406-1) gel followed by electrophoresis and western blotting.

For westerns, blots were blocked with 5% Skim Milk (Bioshop SKI400.1) for 1 hour at room temperature or overnight at 4°C. Blots were then incubated with primary antibodies (anti-Slr1p (custom AB2648), anti-Myc (Cell Signalling Technology 2276S), anti-GFP (Takara Bio Clontech 632381), or anti-Rpl9 (Abcam ab182556)) followed by either secondary anti-mouse IgG HRP-linked antibody (Cell Signalling Technology 7076S) or anti-rabbit IgG HRP-linked antibody (Cell Signalling Technology 7074S), for 1 hour at room temperature with washes in between. Blots were probed for one primary antibody followed by the respective secondary antibody at a time, before being treated with ECL (Thermo Scientific™ 32106) for 2 minutes. A MicroChemi (DNR Bio-Imaging Systems) was used to visualize bands on each blot. The block solution and antibody dilutions were made in 1X TBST buffer (20 mM Tris Base (Fisher BP152-5), 150 mM NaCl (Fisher S271-10), 0.1% Tween-20 (Sigma P9416-100ML)). The 1X TBST buffer was also used for washes.

Sequencing Experiments

RNA-Seq:

S. pombe yAS99 (wildtype) and yMAB001 (*slr1*-) liquid cultures were grown in YES at 32°C, 200 rpm, to OD₆₀₀ ~0.6. 100 µg/mL cycloheximide (Sigma C7698-5G) was added to each culture which was incubated again for 10 minutes, 32°C, 200 rpm. Cells were harvested and lysed using a mortar and pestle under liquid nitrogen. Lysates were resuspended in 20 mM

HEPES-KOH pH 7.4 (Fisher BP310-500), 100 mM KOAc (Bioshop POA303.1), 2 mM MgOAc₂ (Bioshop MAG512.250), 0.5 mM DTT (Bioshop DTT002.25), 1 mM PMSF (Bioshop PMS123.5) and 100 µg/mL cycloheximide (Sigma C7698-5G). 13 OD₂₆₀ units were layered onto a 20-50% sucrose gradient and centrifuged and extracted as described earlier. Fractions corresponding to the trisome peak onwards were pooled for RNA extraction. TRIzol (Invitrogen 15596018) RNA extraction was performed on total RNA and polysome RNA samples for 2 biological replicates prior to library preparation and sequencing at The Centre for Applied Genomics (TCAG, Toronto, Canada). Ribo-Zero Gold rRNA Removal Kit (Yeast, Illumina) was used followed by library preparation using TruSeq Stranded Total RNA Kit (Illumina) according to manufacturer's protocol. Sequencing was performed on an Illumina HiSeq 2500 for SE 50bp reads. Analysis was performed using the assembly *Schizosaccharomyces pombe* (972h-). Alignment and quantitation of FASTQ files was performed using RSEM/Bowtie and differential translation analysis was performed by utilizing the R Babel framework as described (Olshen *et al.*, Bioinformatics, 2013)⁷¹.

RATE-Seq:

yAS99 (wildtype) or yMAB001 (*slr1*-) strains were streaked on fresh YES plates. For each of the 3 biological replicates per strain, a single colony was used to inoculate 10 mL of YES liquid media. Cultures were incubated at 32°C overnight with shaking (200 rpm). The overnight culture was subsequently used as a seed culture to inoculate 700 mL of fresh media at a final OD₆₀₀=0.2. Fresh cultures were next grown at 32°C, 200 rpm, to OD₆₀₀~0.4, at which point (time=0) 100 mL of the culture was removed. Cells were washed with sterile nuclease-free water (Invitrogen 10977-015) and pelleted before being flash frozen in a dry ice/ethanol bath. To the rest of the 600 mL culture, 5 mM final concentration of 4-thiouracil (4tU) (Sigma 440736-1G) was added. Cultures were then incubated for 5 minutes at 32°C, 200 rpm, at which point (time=5 minutes) another 100 mL of cells were removed, washed, pelleted and flash frozen as described. Subsequently, 100 mL of cells were removed at each of the following time points: 20, 50, 105 and 125 minutes. Flash frozen cells were stored at -80°C until hot phenol RNA extraction.

For RNA extraction, previously flash frozen cells were briefly thawed on ice before adding 250 µl of Complete Buffer A (50 mM NaOAc pH 5.2 (Sigma S2889-1KG), 10 mM EDTA pH 8.0 (Sigma E5134-100G), 1% SDS (Bioshop SDS003.1), kept at room temperature) to each cell pellet. Cells were vortexed next to mix. To each resuspension then, 750 µl of warm Buffer A Phenol was added. To prepare the Buffer A Phenol, an equal volume of saturated acidic phenol pH 4.3 (Fisher BP1751I-400) was mixed with Buffer A (50 mM NaOAc pH 5.2 (Sigma S2889-1KG), 10 mM EDTA pH 8.0 (Sigma E5134-100G)) for 20 minutes at room temperature, twice. Phases were allowed to separate after each 20-minute incubation and before removing and discarding the top layer. The phenol layer is referred to as Buffer A Phenol which was then kept warm at 37°C and added to cells mixed with Complete Buffer A as described above. Cells were vortexed to mix again and subsequently incubated at 65°C for 4 minutes, with vortexing every minute. Samples were then spun down for 3 minutes at 21000xg, room temperature. The aqueous top layer was removed to a new microfuge tube and kept aside. To ensure complete RNA extraction, another 250 µl of Complete Buffer A was added to the cell pellet tube and mixed using vortexing again. The incubation at 65°C for

4 minutes was repeated and samples were again spun down to remove the top layer which was added to the new microfuge tube from the previous step. To the collected RNA sample then, an equal volume (~500 μ l) of Phenol/Chloroform/Isoamyl Alcohol (25:24:1 mixture, Fisher BP1752I-400) was added. Samples were vortexed and spun down for 3 minutes at 21000xg. The top layer containing extracted RNA was removed to a fresh tube, to which 1/10th of the volume 3M NaOAc pH 5.2 (Sigma S2889-1KG) was added along with 1 mL of ice-cold 100% absolute ethanol (Greenfield Global P006EAAN). Extracted RNAs were then precipitated at -80°C until sequencing.

Sequencing and analysis of 4tU containing RNA was completed according to methods described in (Lugowski *et al.*, Methods, 2018)⁵³. First, 4tU containing RNA was purified by *in vitro* biotinylation and biotin-streptavidin pull down, and purified RNA was used to prepare libraries using the TruSeq Stranded mRNA Sample Preparation Kit (Illumina) according to manufacturer's instructions. Libraries were sequenced at the Genomics and Microarray Shared Resource at the University of Colorado Denver Cancer Center. Reads were first trimmed and clipped for Illumina adaptors using Trimmomatic v0.36 (Bolger *et al.*, Bioinformatics, 2014)⁷² and then aligned to the *S. pombe* genome using STAR version 2.5.2a (Dobin *et al.*, Bioinformatics, 2013)⁷³. HTSeq 0.6.1p2 (Anders *et al.*, Bioinformatics, 2015)⁷⁴ was used to calculate coverage per gene and the DRUID pipeline (Lugowski *et al.*, Methods, 2018)⁵³ was then used to calculate half-lives.

RIP-Seq:

Anti-Slr1p custom antibody generation

Anti-Slr1p antibody was raised in rabbit using a commercially produced peptide region of the protein (Pierce). The peptide contained 18 amino acids corresponding to the C-terminus of Slr1p: amino acids 458-475 with the sequence DSIKRDVQTEVEDKLTV. This region was chosen based on the highest score achieved on the Kyte-Doolittle hydrophobicity and Hopp-Woods hydrophilicity scale (Pierce). This antibody may have been referred to as **AB2756** throughout the manuscript. Another antibody was also raised in rabbit against Slr1p (Pierce) using recombinantly expressed and purified full-length Slr1p. This antibody may have been referred to as **AB2648** throughout the manuscript and was only used for western blotting.

Crosslinking of ribonucleoproteins, RNA immunoprecipitation and extraction

yAS99 wildtype cells were grown at normal conditions (YES medium, 32°C, 200 rpm) to OD₆₀₀~0.6. Prior to harvesting, cells were cross-linked using 0.05% final concentration of formaldehyde (Fisher BP531-500). Cultures were incubated at room temperature with occasional shaking for 20 minutes. The cross-linking reaction was then quenched using 0.2 M final concentration of glycine (Fisher BP381-5). Cultures were incubated at room temperature for 5 minutes with shaking after the addition of glycine. Cells were harvested next by centrifugation at 4000xg, 4°C, for 15 minutes. Cell pellets were washed twice using 40 mL of cold sterile nuclease-free water (Invitrogen 10977-015) next and resuspended in Resuspension buffer (12 mg/mL Polyvinylpyrrolidone (Sigma PVP40-50G), 20 mM HEPES pH7.4 (Fisher BP310-500), 1% v/v Halt™ Protease Inhibitor Cocktail (Thermo Scientific 78425), 1 mM PMSF (Bioshop PMS123.5), 1 mM DTT (Bioshop DTT002.25); ~7.5 mL/L of cells)

prior to being flash frozen in liquid nitrogen and cryogenic lysis. Cell lysates were prepared in 1X RNP buffer (20 mM HEPES pH 7.4 (Fisher BP310-500), 110 mM KOAc (Bioshop POA303.1), 100 mM NaCl (Fisher S271-10), 0.5% Triton X-100 (Sigma T8787-100ML), 0.1% Tween-20 (Sigma P9416-100ML), 1mM PMSF (Bioshop PMS123.5), 1% v/v Halt™ Protease Inhibitor Cocktail (Thermo Scientific 78425), 20 U/mL SUPERase•In™ RNase Inhibitor (Invitrogen AM2694), 2 U/mL TurboDNase (Invitrogen 2238); 9 mL/g of cells) next and prior to RNA immunoprecipitation. Lysates were precleared for 1 hour at 4°C with nutation, using Dynabeads™ Protein A magnetic beads (Invitrogen 10001D) coupled to non-immune rabbit IgG (Invitrogen 02-6102). Generally, 25 µg of antibody was used for each 100 µl of beads. Meanwhile, Dynabeads™ Protein A magnetic beads (Invitrogen 10001D) were coupled to anti-Slr1p antibody or non-immune rabbit IgG for immunoprecipitations. Precleared lysates were then divided equally between beads coupled with anti-Slr1p antibody or non-immune rabbit IgG for RNA immunoprecipitations. Samples were then incubated for 1 hour at 4°C, with nutation. Following this, flowthroughs were removed, and beads bound to ribonucleoproteins were washed three times using ice-cold 1X RNP buffer. Following the last wash, beads were resuspended in 400 µl 1X RNP buffer to which 1 µg/µl final concentration of proteinase K (Invitrogen 25530049) solution and 0.125% final concentration of SDS solution (Bioshop SDS003.1) was added. Samples were then incubated at 37°C for 1 hour to digest immunoprecipitated proteins first, and 65°C for another hour to reverse crosslinking. This was followed by phenol-chloroform extraction of RNA and ethanol precipitation. Briefly, equal volumes of acidic phenol pH 4.3 (Fisher BP1751I-400) was added to each sample which was vortexed to mix and centrifuged at 21000xg for 10 minutes at 4°C. The top aqueous layer was removed to a new tube to which equal volumes of Phenol/Chloroform/Isoamyl Alcohol (25:24:1 mixture, Fisher BP1752I-400) was added following by vortexing and centrifugation at 21000xg for 10 minutes at 4°C. The top aqueous layer was removed to a new tube again and to this, equal volumes of Chloroform/Isoamyl Alcohol (24:1 mixture, Sigma 25666-100ML) was added. The centrifugation step above was repeated and the top layer was removed to a new tube again. Finally, 20 µg glycogen (Sigma G8751), 1/10th of the volume 3M NaOAc pH 5.2 (Sigma S2889-1KG) and 1 mL ice-cold 100% absolute ethanol (Greenfield Global P006EAAN) was added to each RNA sample which was precipitated next at -80°C until sequencing.

Sequencing

Sequencing was performed for 2 biological replicates at The Centre for Applied Genomics (TCAG, Toronto, Canada). The NEB Ultra II Directional mRNA library prep Kit was used for library preparations. 1 µg of RNA was used for each of the Slr1p RNA immunoprecipitation samples with 9 cycles of PCR. For the IgG control samples, approximately 0.2 µg of RNA was immunoprecipitated all of which was used for library preparation using 16 cycles of PCR. Sequencing was performed on an Illumina NovaSeq SP flow cell for PE 2x50bp reads. Analysis was also performed by TCAG using the assembly *Schizosaccharomyces pombe* (972h-).

In vitro binding assays

Recombinant protein expression and purification:

Full-length Slr1p-6xHis was recombinantly expressed in *E. coli* Rosetta cells and purified using IMAC chromatography. Cells were induced at $OD_{600}=0.6-0.8$ using 1 mM final concentration of IPTG (Fisher BP1755-100). They were subsequently incubated at 32°C for 2.5-3 hours with shaking (200 rpm). Cells were harvested next and pellets were resuspended in Pellet Eluting buffer (50 mM Tris-HCl pH 7.4 (Fisher BP152-5), 100 mM NaCl (Fisher S271-10); 40 mL/L of cells) followed by centrifugation. Dry pellets were stored at -80°C until purification. On the day of purification, pellets were thawed on ice for approximately 15-30 minutes and then resuspended in 10 mL of Lysis buffer (50 mM Tris-HCl pH 7.6 (Fisher BP152-5), 500 mM NaCl (Fisher S271-10), 0.05% Igepal (Bioshop NON999.500), 20 mM Imidazole (Bioshop IMD508.250), 5 mM β -mercaptoethanol (Sigma M6250-100ML)) plus 100 μ L of Halt™ Protease Inhibitor Cocktail (Thermo Scientific 78425) and 100 μ L of 100 mM PMSF (Bioshop PMS123.5). Lysis was performed by vortexing the resuspension three times, 30 seconds each time, interspersed with 30 seconds of rest on ice. Following lysis, the resuspension was sonicated while being kept on ice and using an Ultrasonic Liquid Processor (Misonix XL-2000 series) for 6 minutes consisting of 5-second pulses at 0.15 Watts followed by 10-second rest periods. Lysates were then spun down at 20000xg using a Beckman centrifuge (Avanti J-E Centrifuge) for 40 minutes at 4°C. Cleared lysates were then loaded onto a previously equilibrated (using Lysis buffer) 1 mL Ni^{2+} HisTrap™ Fast Flow column (Millipore Sigma GE17-5319-01). Next, the column was washed three times using 5-7 mL of Wash buffer (50 mM Tris-HCl pH 7.6 (Fisher BP152-5), 500 mM NaCl (Fisher S271-10), 20 mM Imidazole (Bioshop IMD508.250), 1 mM β -mercaptoethanol (Sigma M6250-100ML)) each time. Slr1p-6xHis was then eluted using 5 mL of Elution buffer (50 mM Tris-HCl pH 7.6 (Fisher BP152-5), 500 mM NaCl (Fisher S271-10), 300 mM Imidazole (Bioshop IMD508.250), 5 mM β -mercaptoethanol (Sigma M6250-100ML)). The eluted protein was then concentrated using a pre-soaked 30 KDa Amicon® Ultra-4 Centrifugal Filter Unit (Millipore Sigma UFC803024) and centrifugation at 3000xg, 4°C, into a final volume of 500 μ L. At this point the sample was buffer-exchanged into 1X EMSA buffer (20 mM Tris-HCl pH 7.6 (Fisher BP152-5), 800 mM KCl (Fisher BP366-1), 0.2 mM EDTA pH 8.0 (Sigma E5134-100G), 1 mM DTT (Bioshop DTT002.25)) by bringing up the volume to 5 mL and centrifugation again. This step was performed twice. Subsequently, the sample was concentrated into a final volume of 50-200 μ L. 10% final concentration of glycerol (Bioshop GLY001.500) was then added to the concentrated protein before storage at -80°C. The protein sample was quantified against Albumin Standards (Thermo Scientific™ 23209) later and prior to binding assays.

Radioactive labelling of RNA substrates:

RNA substrates (Supplemental Information) were purchased from Integrated DNA Technologies (IDT) and radioactively labelled using γ -³²P ATP (Perkin Elmer BLU502A250UC) and T4 Polynucleotide Kinase (T4 PNK, NEB M0201S). Lyophilized RNAs were first reconstituted to 1 mM stocks using nuclease-free water (Invitrogen 10977-015), from which 10 pmol was used in a final reaction volume of 20 μ L also containing 2 μ L T4 PNK Reaction Buffer (10X), 1.5 μ L γ -³²P ATP and 1 μ L T4 PNK enzyme. Labelling reactions were carried out at

37°C for 2 hours. Following this, 5 µl of Formamide loading dye (80% Deionized Formamide (Bioshop FOR001.500), 10 mM EDTA pH 8.0 (Sigma E5134-100G), 0.06% Bromophenol Blue (Sigma B8026), 0.06% Xylene Cyanol (Abcam ab146296)) was added to the 20 µl reaction which was heated to 95°C for 10 minutes next. For gel extraction, samples were then loaded onto a 12% TBE-Urea gel (7 M Urea (Bioshop URE001.5), 12% Polyacrylamide (Fisher BP1406-1), 1X TBE buffer, 0.08% APS (Sigma A3678-25G), 0.037% TEMED (Fisher BP150-20)) that had pre-ran for 20 minutes at 4°C, using 1X TBE buffer (130 mM Tris Base (Fisher BP152-5), 45 mM Boric Acid (Fisher BP168-1), 2.5 mM EDTA (Sigma E5134-100G)). After a desired separation was achieved, based on the size of the RNA substrates loaded and according to the migration of the Bromophenol Blue and Xylene Cyanol dyes, the radioactive RNAs were visualized using autoradiography. Using the exposed autoradiography film (Denville Scientific E3012) as reference, pieces of the gel containing the full-length radioactively labelled RNAs were excised and moved into a fresh 0.7 mL microfuge tube cut at the bottom and inserted into a 1.5 mL microfuge tube. The set up was then spun down at 9000xg for 2 minutes to crush and collect the gel pieces into the 1.5 mL tube. A volume of 0.5 M NaCl (Fisher S271-10) was then added to the gel pieces such that they were covered. The RNA was then eluted overnight at room temperature and later transferred to -20°C for future use. RNA samples were counted prior to binding assays, by dilution in scintillation fluid (Ecoscint™ A LS-273) and using a scintillation counter (Hidex 300 SL). 3000 cpm of radioactive RNA was used per lane for every different concentration of protein indicated for EMSA reactions.

Electrophoretic Mobility Shift Assays (EMSAs):

For EMSAs, RNA master mixes were prepared using 10X EMSA buffer (200 mM Tris-HCl pH 7.6 (Fisher BP152-5), 1 M KCl (Fisher BP366-1), 10 mM EDTA pH 8.0 (Sigma E5134-100G), 50 mM β-mercaptoethanol (Sigma M6250-100ML)) or 5X High Salt EMSA buffer (100 mM Tris-HCl pH 7.6 (Fisher BP152-5), 2 M KCl (Fisher BP366-1), 5 mM EDTA pH 8.0 (Sigma E5134-100G), 25 mM β-mercaptoethanol (Sigma M6250-100ML)). Master mixes were prepared per lane as follows: 1X EMSA buffer, 10% glycerol (Bioshop GLY001.500), 3000 cpm of labeled RNA, 8 µl of Bromophenol Blue dye (Sigma B8026, dissolved in nuclease-free water), plus nuclease-free water (Invitrogen 10977-015) to a final volume of 20 µl. Each master mix containing a specific type of radioactive RNA was then heated to 95°C for 5 minutes and slow cooled at room temperature until it reached 25°C. Meanwhile, protein dilutions were prepared using La Dilution Buffer (LDB: 1X EMSA buffer, 10% glycerol (Bioshop GLY001.500)). 10X protein dilutions were made for the following concentrations: 0.5, 1, 2, 4 and 8 µM. For each EMSA reaction, 18 µl of RNA master mix was combined with 2 µl of a 10X protein dilution in a final reaction volume of 20 µl. A reaction with no protein was also prepared using 18 µl of RNA master mix plus 2 µl of LDB in order to visualize the free RNA band as a control. Binding reactions were then incubated at 32°C for 35 minutes, then snap-chilled on ice for 15 minutes. Meanwhile a 6% TBE-Native gel (6% Polyacrylamide (Fisher BP1406-1), 1X TBE buffer, 0.08% APS (Sigma A3678-25G), 0.037% TEMED (Fisher BP150-20)) was pre-run for at least 20 minutes at 4°C with 1X TBE buffer (130 mM Tris Base (Fisher BP152-5), 45 mM Boric Acid (Fisher BP168-1), 2.5 mM EDTA (Sigma E5134-100G)). Binding reactions corresponding to 0 to 4 or 8 µM of protein were then loaded onto the TBE-Native

gel in an increasing order. The gel was then allowed to run at 100 Volts for about 60 minutes or until the Bromophenol Blue dye had moved $\frac{3}{4}$ of the way to the bottom of the gel. Gels were transferred onto a piece of Whatman Chromatography Paper (Cytiva 3030-861) next to be dried using a Gel Drier (Bio-Rad, Model 583), for 45 minutes at 80°C. Dried gels were then exposed to a Storage Phosphor Screen (GE Healthcare) in an autoradiography cassette, overnight. The Storage Phosphor Screen was scanned the next day using a Typhoon FLA 9000 Imager (GE Healthcare) and the phosphor-imaging setting.

***Renilla* reporter assays**

Cloning:

5'PAC and 5'DUR as well as 20A and 20U *Renilla* reporters (Supplemental Information) were cloned into either pRep3x (*leu+*, high expression) or pRep81x (*leu+*, low expression) vectors for expression in *S. pombe* and transformed into wildtype (yAS99) or *slr1-* (yMAB001) cells. Transformants were selected for by plating on EMM-leu plates. With respect to the tethering reporters (constructs used as templates were kindly provided by the Fabian lab), pRep82x (*ura+*) and pRep81x (*leu+*) backbones were used. Either pRep81x-LHA-LacZ or pRep81x-LHA-Slr1 were transformed together with either pRep82x-5BoxB-RL or pRep82x-RL-5BoxB into *slr1-* cells. Transformants were selected for by plating on EMM-ura-leu plates. Yeast transformations were performed using standard lithium acetate procedures (Okazaki, NAR, 1990)⁷⁵.

Growth:

For each biological replicate, a single colony was diluted in 10 mL of the respective minimal media using sterile techniques. The 10 mL culture was incubated for two nights at 32°C with shaking (200 rpm). Seed cultures were then used to inoculate 10 mL of fresh media at OD₆₀₀=0.2. These cultures were incubated again at 32°C, 200 rpm, and harvested at OD₆₀₀=0.5-0.8 on the same day. Cells were pelleted (1000xg, 5 minutes, 4°C), washed with 1 mL of sterile ddH₂O, transferred to 2 mL microcentrifuge tubes with flat screw-caps (VWR 16466-060) and pelleted again. Excess supernatant was discarded and cells were stored at -80°C until protein extraction.

Cell lysis and protein extraction:

For protein extraction, cells were briefly thawed on ice and resuspended in 100 µl of ice-cold NET-2 buffer (50 mM Tris-HCl pH 7.5 (Fisher BP152-5), 150 mM NaCl (Fisher S271-10), 0.05% Igepal (Bioshop NON999.500), 0.1 mM PMSF (Bioshop PMS123.5)). 100-150 µl of 0.5 mm acid-washed glass beads (Sigma G8772-100G) were then added to the resuspended cells. The remaining steps were performed in a cold room. A beadbeater (BioSpec Products) was used to lyse the cells by vortexing on “homogenize” setting for three 20-second pulses (total of 1 minute) interspersed by 2 minutes of resting on ice. This was followed by adding another 100 µl of ice-cold NET-2 buffer to the lysed cells and mixing. The cell lysate was then collected by centrifugation at 21000xg for 5 minutes at 4°C. 100 µl of ice-cold NET-2 buffer was added to the pelleted cell debris again and mixed by shaking to ensure sufficient protein lysate recovery. This was spun down again and the supernatant was pooled with the lysate

collected in the previous step. This was followed by centrifugation at 21000xg for 30 minutes at 4°C. The cleared lysate was transferred to a new tube after, mixed with a 10% final concentration of glycerol (Bioshop GLY001.500) and stored at -80°C.

Normalization to total proteins:

Cell lysates were thawed on ice to measure total protein concentration by Bradford assay. For each measurement, 7.5 µl of lysate was diluted in 750 µl of Bradford Reagent (Thermo Scientific™ 23238). For blank, 7.5 µl of NET-2 buffer was used instead. Concentrations were obtained in µg/µl and consistent dilutions were made for each set of reporters. 1 µg of total protein lysate/technical replicate (total of 3) was used for each of the pRep81x reporters. For the pRep3x reporters, 0.01 µg of total protein lysate was used per technical replicate. For the tethering reporters, 2 µg of total protein lysate was used per technical replicate. Therefore, luciferase reads obtained were normalized to the same amount of total protein for wildtype and *slr1*- conditions, per replicate, for any of the reporter series.

Reporter assays:

Luciferase assays were performed using the *Renilla* Luciferase Assay System (Promega E2820) and according to the manufacturer's guidelines. Briefly, *Renilla* Luciferase Assay Reagent (Substrate + Buffer) was prepared to perform the desired number of *Renilla* luciferase assays (100 µl of reagent per assay). 1 volume of 100X *Renilla* Luciferase Substrate was added to each 100 volumes of *Renilla* Luciferase Assay Buffer. The prepared reagent was kept on ice and wrapped in aluminum foil until aliquoting into a 96-well plate (Thermo Scientific™ 7571). Using a multi-channel pipette, 100 µl of the *Renilla* Luciferase Assay Reagent was added to the desired number of wells on the 96-well plate. To measure *Renilla* luciferase activity for each reporter being studied, 20 µl of the corresponding cell lysate dilution made previously was added to three wells containing 100 µl of the *Renilla* Luciferase Assay Reagent at a time. Readings for 3 technical replicates were then initiated using a Synergy H4 Hybrid Reader (Agilent) under the luminescence setting and measured over 2 seconds. Analysis was performed for 3-4 biological replicates per reporter transformed into either wildtype or *slr1*- cells. 3 technical replicate readings were acquired for each biological replicate, providing a total of 9 or 12 readings for each reporter. Readings were recorded and later analyzed using Prism (GraphPad, Version 9.3.1), refer to "QUANTIFICATION AND STATISTICAL ANALYSIS".

Reporters' RNA abundance

Growth, RNA extraction and Northern blot analysis:

To measure RNA abundance for the 5'PAC and 5'DUR motif-containing *Renilla* reporters in wildtype versus *slr1*- backgrounds, yeast cells were transformed with either the U-rich/20N, 20N/AC-rich or 40N (control) reporter (pRep3x-based). Transformants were selected for by plating on EMM-leu plates. A single colony was diluted in 10 mL liquid minimal media and allowed to grow overnight at 32°C with shaking (200 rpm). The next day, the overnight cultures were used to inoculate 10 mL of fresh media. New cultures were grown again at 32°C, 200 rpm, until harvest at OD₆₀₀=0.5-0.8. This was followed by total RNA extraction

using the hot phenol RNA extraction method explained earlier, for a total of 4 biological replicates. To measure RNA abundance for the tethering reporters, *slr1*- cells were transformed with the combination of reporters tested (LHA-Slr1 + **5BoxB**-RL, LHA-Slr1 + RL-**5BoxB**, LHA-LacZ + **5BoxB**-RL or LHA-LacZ + RL-**5BoxB**). Transformants were selected for by plating on EMM-ura-leu plates. Later, RNA extraction was performed for 4 biological replicates per condition as described here before. Prior to Northern blotting, RNA samples precipitating at -80°C were spun down for 10 minutes at 21000xg, 4°C, to pellet the RNA. Supernatants were discarded and the RNA pellet was washed with 500 µl of ice-cold 70% ethanol (diluted 100% absolute ethanol (Greenfield Global P006EAAN) with nuclease-free water (Invitrogen 10977-015)). Samples were again spun down for 5 minutes at 21000xg, 4°C, and supernatants were removed. RNA pellets were then air-dried for approximately 5 minutes on ice in a fume hood. Each RNA pellet was resuspended in nuclease-free water (Invitrogen 10977-015) after. RNA samples' concentrations and A260/A280 measurements were obtained using a Nanodrop 2000 Spectrophotometer (Thermo Scientific™).

For Northern blots, 5% TBE-Urea gels (7 M Urea (Bioshop URE001.5), 5% Polyacrylamide (Fisher BP1406-1), 1X TBE buffer, 0.08% APS (Sigma A3678-25G), 0.037% TEMED (Fisher BP150-20)) were used to adequately resolve RNA bands corresponding to our *Renilla* reporters and endogenous U5 snRNA used as a loading control. Gels were pre-ran at 4°C, using 1X TBE buffer (130 mM Tris Base (Fisher BP152-5), 45 mM Boric Acid (Fisher BP168-1), 2.5 mM EDTA (Sigma E5134-100G)), for at least 20 minutes prior to electrophoresis. Depending on recovery yield for each of the 4 biological replicates, between 1-10 µg of total RNA were loaded per lane for the 5'PAC and 5'DUR motif-containing *Renilla* reporters. With respect to the 4 biological replicates examined for the tethering reporters, 12.5 µg of total RNA were loaded per lane. 10 µl dilutions were made for each RNA sample such that it could be mixed with Formamide loading dye (80% Deionized Formamide (Bioshop FOR001.500), 10 mM EDTA pH 8.0 (Sigma E5134-100G), 0.06% Bromophenol Blue (Sigma B8026), 0.06% Xylene Cyanol (Abcam ab146296)) in a 1:1 ratio. 20 µl RNA samples were then heated to 95°C for 5 minutes to resolve any secondary structures. Samples were then snap-chilled on ice and loaded on TBE-Urea gels. Electrophoresis was performed at 75 Volts, 4°C, using 1X TBE buffer until a desired separation was achieved, estimating based on the migration of the Bromophenol Blue and Xylene Cyanol dyes. Gels were then transferred onto nitrocellulose membranes using iBlot 2 Dry Blotting System (Invitrogen IB21001) and iBlot 2 Transfer Stacks (Invitrogen IB23001), according to the manufacturer's instruction and using the setting "P0". RNAs were then UV crosslinked to the membrane using a Fotodyne DNA Transfer Lamp (Model 2-1500), for 90 seconds. The membrane was dried at 80°C for 10 minutes next using a Bio-Rad Gel Dryer (Model 583). Following this, the membrane was incubated in Pre-Hybridization buffer (6X Saline-Sodium Citrate (SSC), 2X Denhardt's Solution (Invitrogen 750018), 1% SDS (Bioshop SDS003.1), 0.1 mg/mL Yeast RNA (Invitrogen AM7118)) for 2-5 hours. Radioactively labelled DNA probes, to detect *Renilla* and U5 snRNA, were then added to the buffer followed by an overnight incubation period at 48°C (10 degrees below the melting temperature of the probes), in a hybridization oven. 20 mL Pre-Hybridization buffer was used per blot plus 25 µM of each of the radioactively labelled probes. The next day, Pre-Hybridization buffer was discarded and blots were washed three times using approximately 30 mL of Wash buffer (2X SSC, 0.1% SDS (Bioshop SDS003.1)),

20 minutes each time. First wash was performed at probing temperature, second wash at probing temperature decreasing to room temperature and the third wash was performed at room temperature. Membranes were then exposed to a Storage Phosphor Screen (GE Healthcare) in an autoradiography cassette. The screen was later scanned using a Typhoon TRIO+ Variable Mode Imager (GE Healthcare) and the phosphor-imaging setting.

Labelling probes:

Complementary DNA probes (Supplemental Information) were radioactively labelled to detect the RNAs of interest on Northern blots. This was done using T4 Polynucleotide Kinase (T4 PNK, NEB M0201S). Briefly, 50 μ M of a DNA probe was mixed with 2 μ l T4 PNK Reaction Buffer (10X), 2 μ l γ -³²P ATP (Perkin Elmer BLU502A250UC) and 1 μ l T4 PNK enzyme in a final reaction volume of 20 μ l. Labelling reactions were carried out at 37°C for 2 hours. 10 μ l of the reaction mixture was then heated to 95°C for 5 minutes and added to the Pre-Hybridization buffer as explained previously. 25 μ M of each probe was used per membrane during the overnight incubation, i.e., for two membranes incubating together, 50 μ M of probe was used. Where applicable, radioactively labelled probes were stored at -20°C and heated to 95°C for 5 minutes prior to use.

Decade marker:

To prepare the RNA ladder used for Northern blots, the Decade™ Markers System (Invitrogen AM7778) was used according to the manufacturer's instructions. The following were mixed in a microfuge tube: 1 μ l Decade™ Marker RNA (100 ng/ μ L), 1 μ l 10X Kinase Reaction Buffer, 1 μ l γ -³²P ATP (Perkin Elmer BLU502A250UC), 1 μ l T4 Polynucleotide Kinase (10 U/ μ L), 6 μ l nuclease-free water (Invitrogen 10977-015). The mixture was incubated at 37°C for 1 hour. 8 μ l nuclease-free water (Invitrogen 10977-015) and 2 μ l 10X Cleavage Reagent were then added to the mixture. This was followed by another incubation at room temperature for 5 minutes. Following this, 20 μ l of the Gel Loading Buffer II provided in the kit was added to the mixture. 5 μ l of the mixture was heated to 95°C for 5 minutes and loaded on a gel. The remainder of the labelled ladder was stored at -20°C.

Proteomics and co-immunoprecipitation analyses

Strain maintenance and tag integration:

Yeast glycerol stocks (Supplemental Information) were kept at -80°C for long-term storage. Fresh plates were streaked on either rich (YES) or minimal media (EMM) prior to experiments. Different colonies from the same plate were used for biological replicates. Endogenous tagging of the proteins of interest was performed according to Bahler *et al.* (Yeast, 1998)⁷⁶ using the pFA6a plasmids as templates described by Bahler *et al.* (Yeast, 1998)⁷⁶ and Gadaleta *et al.* (BioTechniques, 2013)⁷⁷. Specifically, pFA6a-GFP-bleMX6 (Addgene Plasmid #33141) was used to tag PABP (major poly(A)-binding protein, SPAC57A7.04c) with GFP (referred to as yFM001 here), pFA6a-13Myc-kanMX6 (Addgene Plasmid #39294) was used to tag Tif471p (translation initiation factor eIF4G, SPAC17C9.03) with Myc (referred to as yFM006 here) and pFA6a-G11-5FLAG-kanMX6 (Addgene Plasmid #49182) was used to tag Sum1p (translation initiation factor eIF3i, SPAC4D7.05) with FLAG

(referred to as yFM012 here). The strain JU915, i.e., Exo2p-Myc (exonuclease II, SPAC17A5.14) was kindly provided by the Mata lab (Duncan and Mata, BMC Genomics, 2014)⁷⁸. The yLS001 (Slr1p-PrA) strain used for our LC/MS-MS analysis was made as described by Oeffinger *et al.* (Nature Methods, 2007)⁵¹.

The wildtype strains that were used are yAS99 (*h*-, *ura*-, *leu*-)⁷⁹ and y12088 (*h*+, *ura*-, *leu*-). The *slr1*- strain yMAB001 was generated using yAS99. yFM001 was made in y12088 and crossed with yMAB001 to generate the strain yFMN20. The wildtype and *slr1*- strains that were used for polysome profiling (yFM009 and yFMN90 described earlier) were made by crossing either yFM001 or yFMN20 by JU915. yFM013 and yFMN100 were made by crossing either yFM009 or yFMN90 by yAS99.

Mass spectrometry:

Mass Spectrometry was performed according to Oeffinger *et al.* (Nature Methods, 2007)⁵¹. *S. pombe* yAS99 and yLS001 liquid cultures were grown in YES at 32°C, 200 rpm, to OD₆₀₀~0.6. Cells were harvested and lysed using a mortar and pestle under liquid nitrogen as explained earlier. 0.5 g of yAS99 or 1.5 g of yLS001 yeast grindate was used per immunoprecipitation per biological replicate. Dynabeads™ M-270 Epoxy (Invitrogen 14301) conjugated to rabbit IgG (Sigma I5006-100MG) were pre-washed 3 times with 1X RNP buffer (20 mM HEPES pH 7.4 (Fisher BP310-500), 110 mM KOAc (Bioshop POA303.1), 100 mM NaCl (Fisher S271-10), 0.5% Triton X-100 (Sigma T8787-100ML), 0.1% Tween-20 (Sigma P9416-100ML), 1mM PMSF (Bioshop PMS123.5), 1% v/v Halt™ Protease Inhibitor Cocktail (Thermo Scientific 78425), 4 U/mL SUPERase•In™ RNase Inhibitor (Invitrogen AM2694), 0.02% Antifoam B (Sigma A5757-250ML)) by pipetting up and down. 25 µl of beads were used per 0.5 g of grindate. 4.5 mL of room temperature 1X RNP buffer was used to resuspend 0.5 g of grindate by vortexing for 30 seconds followed by centrifugation at 1500xg for 10 minutes, 4°C. Clear lysates were added onto beads previously washed with 1x RNP buffer. This was followed by nutation at 4°C for 30 minutes. Flowthroughs were removed next, and beads were washed in 1 mL of 1X RNP buffer, 4 times. Beads were then washed with 1 mL of Wash buffer (100 mM NH₄OAc (Sigma A1542-250G), 0.1 mM MgCl₂ (Bioshop MAG520.500), 0.02% Tween-20 (Sigma P9416-100ML)) with 5 minutes of nutation. Sample preparation for mass spectrometry: 4 more washes (last one with 5 minutes of nutation) were performed in Wash buffer without Tween-20. Lastly, immunoprecipitated Slr1p-PrA were eluted twice with 500 µl of Elution buffer (500 mM NH₄OH (Sigma 221228-100ML-A), 0.5 mM EDTA (Sigma E5134-100G)) each time, by nutation at room temperature for 20 minutes. Pooled elutions were lyophilized using a SpeedVac Concentrator (Thermo Scientific, Savant DNA 120) overnight without heat or radiant cover. Lyophilized samples were then subjected to mass spectrometry (in-solution trypsin digestion, LC/MS-MS) at Montreal Clinical Research Institute (IRCM, Montréal, Canada). Exclusive Spectrum Count indicated by Scaffold (Version: 5.1.1) was used to compare interactions mapped in each yAS99 and yLS001 sample, refer to “QUANTIFICATION AND STATISTICAL ANALYSIS”.

Yeast two-hybrid assay:

The ULTimate Y2H SCREEN was performed by Hybrigenics (Paris, France) using three constructs: a) Gal4-Slr1 1-475 as bait which failed, and b) Slr1 1-323-LexA and c) LexA-Slr1

312-475 as baits which both worked (Table S2). In this paper, we have followed up on hits from the 312-475 construct. A minimum Global PBS (for interactions represented in the screen) cut-off score of “C” (good confidence in the interaction) was used to follow up on potential interactions.

Co-immunoprecipitations:

Wildtype (*slr1+*) strains of *S. pombe* with the hypothesized protein partners of Slr1p tagged endogenously, i.e., PABP-GFP (SPAC57A7.04c), Exo2p-Myc (SPAC17A5.14), Tif471p-Myc (SPAC17C9.03) and Sum1p-FLAG (SPAC4D7.05) were grown in normal conditions (YES, 32°C, 200 rpm), harvested at OD₆₀₀=0.6-0.7, resuspended in Resuspension buffer (12 mg/mL Polyvinylpyrrolidone (Sigma PVP40-50G), 20 mM HEPES pH 7.4 (Fisher BP310-500), 5.55 uL/mL Yeast PIC (Sigma P8215-5ML), 1 mM PMSF (Bioshop PMS123.5), 1 mM DTT (Bioshop DTT002.25); ~7.5 mL/L of cells), noodled and ground as explained before. 2 x 0.2 g of grindate (for +/- benzonase co-immunoprecipitations) were weighed out and transferred to separate 15 mL conicals while working on liquid nitrogen. Each 0.2 g of grindate was either resuspended in 1X RNP buffer + RNase inhibitor **or** 1X RNP buffer + benzonase nuclease (20 mM HEPES pH 7.4 (Fisher BP310-500), 110 mM KOAc (Bioshop POA303.1), 100 mM NaCl (Fisher S271-10), 0.5% Triton X-100 (Sigma T8787-100ML), 0.1% Tween-20 (Sigma P9416-100ML), 1mM PMSF (Bioshop PMS123.5), 5.55 uL/mL Yeast PIC (Sigma P8215-5ML), 4 U/mL SUPERase•In™ RNase Inhibitor (Invitrogen AM2694) **or** 100 U/mL Benzonase Nuclease (Sigma E8263-5KU); 9 mL/g of cells). Specifically, 1.8 mL + or - benzonase 1X RNP buffer was added to each 0.2 g of grindate after allowing it to thaw on ice for 15 minutes. The resuspension was then vortexed three times, 30 seconds on and off ice each time. Lysates were then spun down to remove any cellular debris and pre-cleared using 50 µL of Dynabeads™ Protein A magnetic beads (Invitrogen 10001D) coupled to non-immune rabbit IgG (Invitrogen 02-6102). Pre-clearing was performed by nutation at 4°C for 1 hour. An equal volume of each pre-cleared lysate (+/- benzonase) was then added to 25 µL of Dynabeads™ Protein A magnetic beads (Invitrogen 10001D) coupled to either anti-Slr1p antibody (custom AB2756) or non-immune rabbit IgG (Invitrogen 02-6102). Generally, 25 µg of antibody was used for each 100 µL of beads. This was followed by nutation at 4°C for 1 hour. Next, flowthroughs were removed and the beads were washed with the respective 1X RNP buffer + RNase inhibitor or benzonase nuclease, three times, using 1 mL of buffer each time. Following the last wash, beads were resuspended in 45 µL Laemmli buffer (312.5 mM Tris-HCl pH 6.8 (Fisher BP152-5), 50% Glycerol (Bioshop GLY001.500), 5% SDS (Bioshop SDS003.1), 0.05% Bromophenol Blue (Sigma B8026); and 10% β-mercaptoethanol (Sigma M6250-100ML) added just before use). The resuspension was then heated to 95°C for 10 minutes. 15 µL of this was then loaded on SDS-Polyacrylamide (Bioshop SDS003.1, Fisher BP1406-1) gels as “Elution” for western blot analysis. To blot for Slr1p, anti-Slr1p antibody (custom AB2648) was used first followed by Mouse Anti-rabbit IgG (Conformation Specific) antibody (Cell Signalling Technology 3678S), and lastly by anti-mouse IgG HRP-linked antibody (Cell Signalling Technology 7076S). Exo2p, Tif471p, PABP and Sum1p were visualized using anti-Myc (Cell Signalling Technology 2276S), anti-GFP (Takara Bio Clontech 632381) or anti-FLAG (Abcam ab49763) antibodies, respectively, followed by anti-mouse IgG HRP-linked antibody (Cell Signalling Technology 7076S). All antibody dilutions were

made in 1X TBST buffer (20 mM Tris Base (Fisher BP152-5), 150 mM NaCl (Fisher S271-10), 0.1% Tween-20 (Sigma P9416-100ML)). Blots were visualized using ECL (Thermo Scientific™ 32106) and a MicroChemi (DNR Bio-Imaging Systems). Co-immunoprecipitations were performed at least twice in each case and each biological replicate was prepared using a single colony of yeast.

QUANTIFICATION AND STATISTICAL ANALYSIS

RNA-seq

Differences in amounts of total RNA in *slr1*- cells compared to wildtype cells were calculated using the ratio of averaged reads for *slr1*- total RNA to averaged reads for wildtype total RNA. Genes with a total change ≥ 1.4 were considered for Gene Ontology analysis using the GO Consortium (<http://geneontology.org/>)^{80,81}, Biological Process, *Schizosaccharomyces pombe*. GO terms that were computed were sorted according to False Discovery Rate (FDR). A Bubble Plot was generated using Prism (GraphPad, Version 9.3.1) and the following setting: Graph Type: Multiple Variables, X-axis variable: Fold Enrichment according to GO analysis, Y-axis variable: FDR according to GO analysis, Bubble size (area): number of genes in our analysis that fell under the computed GO term. Tukey boxplots in Figure 2B and Figure S1 were generated using Prism (GraphPad, Version 9.3.1). For statistical analyses, unpaired two-tailed t-tests with Welch's correction (without assuming equal standard deviations (SDs)) were performed with Alpha=0.05. To generate Figure S6, the average number of reads per mRNA (expression) for transcripts with a ≥ 1.4 or < 1.4 fold increase in abundance in *slr1*- cells (compared to wildtype cells) were considered. Violin plots with median, 25th and 75th percentiles were generated and a two-tailed unpaired t-test with Welch's correction was performed using Prism (GraphPad, Version 9.5.0) and Alpha=0.05.

RATE-Seq

Half-lives for *pombe* transcripts in our dataset were calculated followed by mean differences in half-lives per transcript which were then used to determine stability in *slr1*- cells compared to wildtype cells. 208 RNAs were stabilized for at least 2-folds in *slr1*- cells while 44 RNAs were destabilized for at least 2-folds, results being consistent across 3 biological replicates. A GO analysis was performed as explained earlier, for both the stabilized and destabilized cohort of transcripts, with the stabilized set yielding a number of GO terms sorted according to False Discovery Rate (FDR). A Bubble Plot was generated using Prism (GraphPad, Version 9.3.1) as explained earlier. Figures S2A and S2B were generated using R (v.3.5.2).

RIP-Seq

Following pre-processing, alignment and obtaining gene counts, two-condition differential gene expression analysis was performed with DESeq2 (<https://bioconductor.org/packages/release/bioc/html/DESeq2.html> and <http://master.bioconductor.org/packages/release/workflows/vignettes/rnaseqGene/inst/>

doc/rnaseqGene.html)⁸² v. 1.26.0s, using R (v.3.6.1). Initial minimal filtering of 10 read counts per gene for at least 3 samples was applied to the dataset. Log2FoldChange values ≥ 1.5 for biological duplicates of Slr1p-immunoprecipitated RNAs compared to an IgG control were considered and sorted based on p values. Significantly enriched genes ($p > 0.05$) were subjected to a GO analysis as explained before. Using the computed GO terms, a Bubble Plot was graphed by Prism (GraphPad, Version 9.3.1) as described.

RNAs immunoprecipitated using anti-Slr1p antibody (custom AB2756) and non-immune rabbit IgG (Invitrogen 02-6102) were also independently analyzed over input RNAs. We confirmed that genes enriched in anti-Slr1p IP were unique from genes enriched in anti-IgG IP (except for a single non-RP gene: SPNCRNA.51).

Mass Spectrometry

Significance was determined using a Fisher's Exact Test per biological replicate (total of 2). 21 interactions were deemed significantly enriched over the untagged control in both biological replicates and are reported in Table S1. A Gene Ontology analysis was also performed using the GO Consortium as explained earlier. GO terms that were computed were sorted according to False Discovery Rate (FDR). A Bubble Plot was generated using Prism (GraphPad, Version 9.3.1) and the following setting: Graph Type: Multiple Variables, X-axis variable: Fold Enrichment according to GO analysis, Y-axis variable: FDR according to GO analysis, Bubble size (area): number of genes in our analysis that fell under the computed GO term.

Venn diagram

A Venn diagram was generated using datasets for enriched genes from each of the three sequencing experiments using BioVenn (<https://www.biovenn.nl/>). A GO analysis was also performed for the common genes.

MEME motif discovery search

FASTA sequences of UTRs from transcripts identified as having increased stability in *slr1*- cells were retrieved from Ensemble Fungi (<https://fungi.ensembl.org/index.html>)'s Biomart (Ensembl Fungi Genes 51/*Schizosaccharomyces pombe* genes ASM294v2). DNA sequences retrieved were then converted to RNA (<http://biomodel.uah.es/en/lab/cybertory/analysis/massager.htm>) prior to MEME motif discovery search using the MEME Suite 5.4.1 or 5.5.3⁵⁴ (<https://meme-suite.org/meme/tools/meme>). The following criteria were considered: Classic mode, RNA alphabet, "Zero or One Occurrence Per Sequence" or "One Occurrence Per Sequence" or "Any Number of Repetitions", default Advanced options with "Search given strand only." Selected. P values were generated by MEME Suite 5.4.1 (5'DUR and 5'PAC motifs, Figure 3) or 5.5.3 (RP 15-mer 5' Proximal AC-rich motif (PAC15) and RP 19-mer 5' Proximal AC-rich motif (Figure 3), and the three *S. Japonicas* motifs in Figure S3).

265 mRNAs annotated under the GO process "cytoplasmic translation" in *S. pombe* (GO:0002181) were identified using PomBase (<https://www.pombase.org/>)'s advanced search (Ontology queries: GO). FASTA sequences of their 5'UTRs were retrieved from Ensemble Fungi (<https://fungi.ensembl.org/index.html>)'s Biomart (Ensembl Fungi

Genes 57/*Schizosaccharomyces pombe* genes ASM294v2) followed by steps described above for MEME motif discovery search.

261 mRNAs annotated under the GO process “cytoplasmic translation” in *S. japonicus* (GO:0002181) were identified using JaponicusDB (<https://www.japonicusdb.org/><https://www.pombase.org/>)’s advanced search (Ontology queries: GO). FASTA sequences of their 5'UTRs were retrieved from Ensembl Fungi (<https://fungi.ensembl.org/index.html>)’s Biomart (Ensembl Fungi Genes 57/*Schizosaccharomyces japonicus* genes (SJ5)) followed by steps described above for MEME motif discovery search.

Motif locations

Motif start sites were obtained through the MEME motif discovery search explained earlier (MEME Suite 5.4.1)⁵⁴. To obtain motif location with respect to the start AUG for each transcript, we added the length of the motif, i.e., either 19 (for the 5'PAC motif) or 17 (for the 5'DUR motif) to the start site indicated by the MEME Suite (5.4.1). The ratio of the adjusted start site to the length of the entire 5'UTR (obtained from Ensembl Fungi as explained) was calculated for each transcript per motif set. An unpaired two-tailed t-test was performed using Prism (GraphPad, Version 9.3.1) and Alpha=0.05. A Tukey boxplot was graphed using Prism (GraphPad, Version 9.3.1).

UTR length and GC-richness

FASTA sequences of UTRs from transcripts identified as having increased (UpSlr1) or decreased (DownSlr1) stability in *slr1*- cells were retrieved from Ensembl Fungi (<https://fungi.ensembl.org/index.html>)’s Biomart (Ensembl Fungi Genes 51/*Schizosaccharomyces pombe* genes ASM294v2) and lengths were compared to genome-wide UTR lengths using a custom script in R. GC-richness for the UTRs of the described transcripts as well as all genomic transcripts were calculated by geecee (https://www.bioinformatics.nl/cgi-bin/emboss/geecee?_pref_hide_optional=0).

UTR length and GC content were statistically analyzed using an unpaired two-tailed t-test without assuming equal SDs (Welch’s correction) for each of the following groups: UpSlr1 versus Genomic, DownSlr1 versus Genomic and UpSlr1 versus DownSlr1, with Alpha=0.05. Tests were performed and Tukey boxplots were generated using Prism (GraphPad, Version 9.3.1).

Reporter assays

For motif reporters, analysis was performed for 3-4 biological replicates per reporter transformed into either wildtype or *slr1*- cells. 3 technical replicate readings were acquired for each biological replicate, providing a total of 9 or 12 readings for each reporter. Readings were recorded and later analyzed using Prism (GraphPad, Version 9.3.1). Multiple unpaired t-tests were performed between values obtained per reporter transformed into wildtype versus *slr1*- cells, without assuming consistent SDs and using the Holm-Šidák method with Alpha=0.05. For tethering reporters, t-tests were performed between values obtained for LHA-Slr1 versus LHA-LacZ conditions. Figures were plotted using Prism (GraphPad, Version

9.3.1) as well. Mean values are plotted with error bars corresponding to Standard Error of the Mean (SEM) values.

Reporters' RNA abundance

Following visualization, the intensity of the bands corresponding to *Renilla* or U5 RNAs were measured using the ImageQuant TL software (v2005). An empty region of the blot close to either the *Renilla* or U5 RNA bands was also measured as background. The intensity measure for each RNA band was determined following background subtraction. Next, each *Renilla* band intensity was normalized to the same lane U5 band intensity by using the ratio *Renilla*/U5. *Renilla*/U5 intensities for the wildtype cells were then divided by the respective *slr1*⁻ intensities. Wildtype/*slr1*⁻ ratios corresponding to 4 biological replicates were averaged next and are indicated in Figure 5C and F. The corresponding SEM values were also calculated and are indicated alongside the average.

Exo2p sedimentation

Following polysome profiling, fractionation, TCA precipitation and western blotting (2 biological replicates), sedimentation of Exo2p with 40S/43S (Figure 6B, lanes 2+3) or monosomes (4+5) was quantified using ImageJ⁸³ to compare abundance in wildtype versus *slr1*⁻ cells. Multiple unpaired t-tests with Welch's correction assuming individual variance for each group using the Holm-Šídák method were performed by Prism (GraphPad, Version 9.5.0) with Alpha=0.05.

2.9. References

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Chapter 3: Structural analysis of the *S. pombe* LARP1 homolog Slr1p reveals conserved amino acids in the La motif function in binding to proto-5'TOP mRNAs

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Contributions: F.M. optimized expression and purification protocols, purified proteins, conducted *in vitro* binding assays and the mutagenesis studies. L.W.D. (collaborator) did the NMR study and solved the structure. F.M., L.W.D. and M.A.B. conceptualized the studies and designed the experiments. F.M. and L.W.D. conducted experiments, collected data, conducted formal analysis and made figures. F.M. wrote the first draft of the manuscript. L.W.D. and M.A.B. supervised the project, reviewed and revised the original draft of the manuscript. F.M., L.W.D and M.A.B. edited the manuscript.

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3.1. Abstract

La-related protein 1 (LARP1) family controls the expression of 5' terminal oligopyrimidine (5'TOP) ribosomal protein (RP) mRNAs. Fission yeast RP mRNAs lack a 5'TOP motif but are enriched for a 5' proximal AC-rich (5'PAC) motif found closer to the AUG and thus are referred to as proto-5'TOP mRNAs. The *S. pombe* LARP1 ortholog, Slr1p, binds the 5'PAC motif and affects the translation and stability of these mRNAs. While the DM15 region of human LARP1 specifically recognizes the 5'TOP motif, it is unclear how Slr1p, lacking a DM15 region, binds the 5'PAC motif. Here we present the NMR structure of the La motif (LaM) of Slr1p and report that its RNA binding interface conforms to an expected La motif/winged-helix fold. Inclusion of a few C-terminal amino acids increased LaM's solubility with no observed critical contacts with RNA. We show that conserved amino acids within the protein's RNA binding site participate in interactions with the fission yeast 5'PAC motif. Mutagenesis studies further confirm single amino acid mutations in this region disrupt 5'PAC RNA binding. Our data are consistent with the LaM in LARP1 family members playing a crucial role in recognition and binding of RP mRNAs, affecting their expression and stability.

3.2. Introduction

La and La-related proteins (LARPs) are a family of conserved RNA-binding proteins with multiple described functions and characterized binding modes^{1,2}. While genuine La is best characterized to bind terminal uridylates found in nascent polymerase III transcripts³⁻⁵, it has also been shown to bind mRNAs and viral RNAs^{6,7,16-20,8-15}. Early structural analyses of

genuine La proteins have informed our understanding of how La and LARPs contact their substrates while also characterizing the amino acid residues responsible for these interactions^{4,5,21,22}. The highly conserved²³ La motif (LaM) is a winged-helix domain^{4,21} and the defining feature of proteins in this superfamily. The N-terminally located LaM is often followed by an RNA recognition motif (RRM). These two regions constitute the La module and form a V-shaped binding cleft to accommodate a UUU-3'OH sequence in genuine La and LARP7 family members^{4,5,24}.

Phylogenetic and structural analysis of LARPs have led to their classification into five subfamilies, which in addition to genuine La (LARP3) include LARP1, LARP4, LARP6 and LARP7²³. With respect to the LARPs, structural studies characterizing the LaMs of human LARP7 (hLARP7)^{24–27}, hLARP6²⁸ and hLARP4A²⁹ have confirmed an overall similarity with LaM of hLa, with some variations³⁰. Structural work related to human LARP1 (hLARP1) first characterized the C-terminally located DM15 region of this protein, which is a family-specific motif found in humans and higher eukaryotes but is missing from the yeast LARP1 orthologs. The DM15 region of hLARP1 is positively charged, adopts a tandem helix-turn-helix HEAT-like repeat and allows for the specific recognition of the m⁷G cap and invariant +1 cytidine of the 5' terminal oligopyrimidine (5'TOP) sequence found in 5'TOP mRNAs³¹. These specialized mRNAs code for ribosomal proteins and factors important in the process of mRNA translation and are regulated downstream of the mammalian target of rapamycin complex 1 (mTORC1)³². hLARP1 is phosphorylated downstream of mTORC1^{33–37} and was hypothesized as the bona fide trans-acting factor that regulates the expression as well as stability of 5'TOP mRNAs^{31,38,47,39–46}. By binding to the mRNA cap upon mTORC1 inhibition, hLARP1 selectively

prevents the assembly of the eIF4F complex on 5'TOP mRNAs, and in doing so directly affects ribosome biogenesis.

The LaM of hLARP1 was previously shown to simultaneously bind to the TOP motif and poly(A) RNA⁴², suggesting the possibility of varied binding modes. Other studies have linked hLARP1 to the protection of poly(A) tail and association with poly(A) binding protein (PABP)^{45,48–53}. An initial series of high-resolution structures have shown that the hLARP1 LaM is versatile not only in its sequence recognition, but also in the mode of RNA binding⁵⁴ despite lacking an RRM domain after the LaM⁵⁴. A follow up structural study later showed that substitution of a single penultimate guanine in a poly(A) sequence leads to a three-fold improvement in binding⁵⁵. Therefore, in contrast to the other LARPs, hLARP1 is suggested to bind oligo(A) RNA, which can be guanylated, only through contacts in the LaM.

Despite lacking a DM15 region, the *S. pombe* La-related protein 1 (Slr1p; PomBase: *slr1*, Gene ID: SPAC1527.03)⁵⁶, which contains a LaM as its only predicted RNA binding domain, is able to bind a variant proto-5'TOP motif enriched in ribosomal protein mRNAs in fission yeast, even as the classic 5'TOP sequence described in higher eukaryotes does not exist in yeasts⁵⁷. In a functionally analogous manner in which the hLARP DM15 region recognizes the +1C of the human 5'TOP sequence, Slr1p was shown to recognize a cytidine at position 6 found in a 5' Proximal AC-rich (5'PAC) sequence which resides closer to the start AUG of ribosomal protein mRNAs in *S. pombe*. This observation presents an alternative binding mode for LARP1 proteins and an interesting possibility that the regulation they exert on

ribosomal protein mRNA translation and stability, and therefore ribosome biogenesis, may predate the emergence of the DM15 region and the 5'TOP sequence.

Here we present the NMR structure of the LaM of Slr1p, plus mutational studies coupled with electrophoretic mobility shift assays (EMSAs), characterizing the amino acids involved in RNA binding. While amino acids C-terminal to the predicted LaM boundaries greatly enhance expression and stability, tested mutants in this region have minimal to moderate effects on RNA binding. Consistent with the sequence and structural conservation among La and LARPs, we demonstrate that amino acids previously linked to RNA binding in LaM-containing proteins remain critical for RNA binding in Slr1p. Notably, we observed a minor conformation for Q332 that is similar to the bound state of the analogous glutamine in other reported LaM structures.

3.3. Results

3.3.1. LARP1 proteins are preconfigured to bind RNA

Human LARP1 (hLARP1) is a member of the La-related protein superfamily, and unlike genuine La appears to mainly bind to and regulate the expression of mRNAs. Previous structural analysis showed that the DM15 region of hLARP1 interacts with and specifically recognizes a group of specialized mRNAs called 5' terminal oligopyrimidine (5'TOP) mRNAs that code for ribosomal proteins³¹. Other structural studies have characterized the La motif (LaM) of hLARP1 to bind poly(A) RNA^{54,55}. Recently, the *S. pombe* La-related protein 1 (Slr1p) was shown to also bind a variant proto-5'TOP motif enriched in ribosomal protein mRNAs in

fission yeast, despite lacking a DM15 region or the presence of the classic 5'TOP sequence in this system which is found in human mRNAs⁵⁷. Given the interesting possibility that the LaM of Slr1p is responsible for this binding mode and the scarcity of structural information on the LaM of LARP1 proteins found in other eukaryotes, including how they could contact different types of RNA, we solved the NMR structure of the LaM of Slr1p (Figure 1B, Table 1, Supplemental Figure 1).

In order to choose the amino acid boundaries that correspond to the LaM of Slr1p, we used Clustal-W alignment of the yeast (Sro9p and Slf1p in *S. cerevisiae*, Slr1p in *S. pombe*) and hLARP1 proteins (Figure 1A) in addition to the AlphaFold predicted model of Slr1p and made three initial constructs that we have referred to as Slr1a, Slr1b and Slr1c (Supplemental Figure 2A). Recombinant expression followed by nickel affinity chromatography suggested Slr1b, version 1 (Slr1b V1 or V1, amino acids 324-431) was our most suitable candidate, where the addition of a few amino acids C-terminal to the predicted LaM enhanced its stability and expression relative to the LaM in isolation (Slr1a; Supplemental Figure 2A). Subsequently, we made solubility enhancing mutations targeting non-conserved hydrophobic amino acids and cysteines and arrived at a third version (Slr1b V3, hence referred to as V3, Supplemental Figure 2A) which was better expressed and purified at ten-fold higher concentrations, enabling a high-resolution NMR study. Both V1 and V3 were used for NMR analysis and treated as wildtype.

The Slr1p LaM adopts an $\alpha 1\text{-}\alpha 2\text{-}\alpha 3\text{-}\alpha 4\text{-}\alpha 5\text{-}\alpha 6\text{-}\beta 1\text{-}\alpha 7\text{-}\beta 2$ fold (Figure 1B) which resembles hLARP1's LaM ($\alpha 1\text{-}\alpha 2\text{-}\alpha 3\text{-}\alpha 4\text{-}\alpha 5\text{-}\beta 1\text{-}\beta 2$)⁵⁴ but differs from genuine human La (hLa, $\alpha 1\text{-}\alpha 1'\text{-}\alpha 2\text{-}$

$\beta 1$ - $\alpha 3$ - $\alpha 4$ - $\alpha 5$ - $\beta 2$ - $\beta 3$)²¹. Thus, Slr1p is missing a beta strand between $\alpha 2$ and $\alpha 3$ (Supplemental Figure 3), originally characterized in the LaM of hLa^{21,22}, however this is similar to what has been reported for hLARP1's LaM⁵⁴. Since Clustal-W alignment showed a region of highly conserved amino acids (332-336 in Slr1p) in the LaM of yeast and hLARP1 proteins (Figure 1A), we compared side chain positions in this region between our NMR structure, an AlphaFold predicted structure of Slr1p and the previously solved structure of hLARP1 LaM (PDB: 7SOO). Upon closer inspection of the AlphaFold structure of Slr1p and our NMR structure, we observed that the amide side chain of Q332 was solvent exposed (Figure 1C), as well as differences in the positioning of β -strands and the loop that connects the two strands which contains a short α -helix ($\alpha 7$) in our NMR structure (red arrows in Figure 1C). We further used TM-align⁶³ to assess similarity between the two structures (TM-score: 0.84457, where 1 indicates a perfect match between two structures). Comparing our NMR structure of Slr1p and that of hLARP1, the R group of Q332 in Slr1p again points in a different direction, which is especially notable because the equivalent residue has been linked to nucleotide binding specificity for both hLARP1 (Q333)^{54,55} and hLa (Q20)^{4,5}. The difference in positioning of Q332 in Slr1p and Q333 in hLARP1 was observed in multiple PDB entries for hLARP1 in complex with poly(A) RNA (PDB: 7SOP, 7SOQ, 7SOS; Supplemental Figure 2B). In contrast to the AlphaFold prediction, the β -strands are superimposable between Slr1p and hLARP1 yet the loop between $\beta 1$ and $\beta 2$ is notably longer in Slr1p and includes an extra helical element ($\alpha 7$, red arrows in Figure 1D). Comparing Slr1p's and hLARP1's LaM rendered a TM-score of 0.77387, which suggests there are notable differences between the two

structures, related to those highlighted here such as positioning of Q332 (Slr1p)/Q333 (hLARP1) and the loop connecting $\beta 1$ and $\beta 2$.

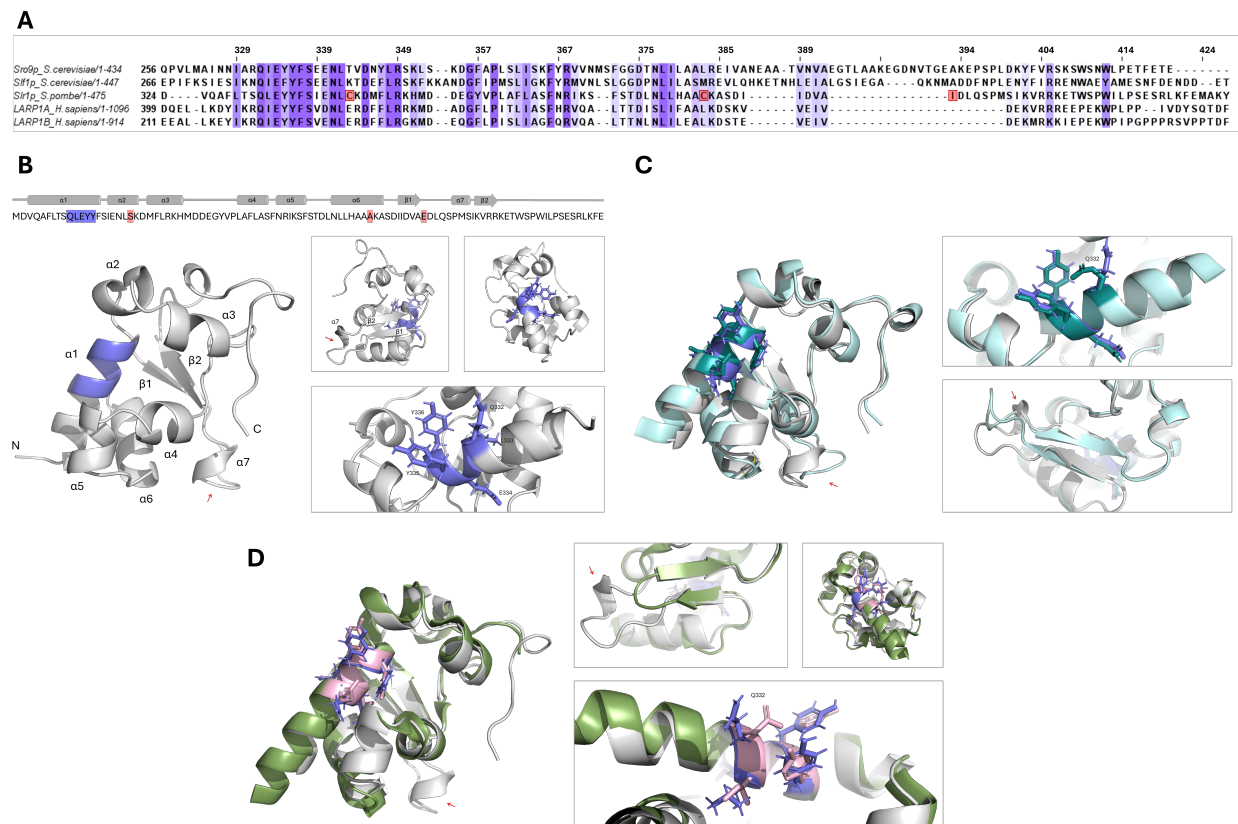


Figure 1: LARP1 proteins are preconfigured to bind RNA. A) Clustal-W alignment of the yeast and human LARP1 (hLARP1) proteins. A region containing highly conserved amino acids, which corresponds to the La motif (LaM), for each protein is shown (parameters used: 50% consensus, 30% conservation). Full amino acid length for each protein is displayed as part of the identifier on the left. Numbers preceding the first shown amino acid mark the amino acid number in the native protein sequence. Numbers above the alignment correspond to the amino acid in the native Slr1p sequence. Purple shadings indicate the level of conservation with dark purple being identical. Amino acids numbered 332-336 in the alignment correspond to a highly conserved binding site in La and La-related proteins. **B)** NMR structure of Slr1p with the conserved amino acids in the binding site (QLEYY) coloured in purple. Slr1p's LaM adopts an $\alpha 1$ - $\alpha 2$ - $\alpha 3$ - $\alpha 4$ - $\alpha 5$ - $\alpha 6$ - $\beta 1$ - $\alpha 7$ - $\beta 2$ fold. Numbering is according to the full-length protein. Amino acids boxed in red in A) were mutated to the following solubility-enhancing residues: C to S, C to A, I to E and are highlighted red in the amino acid sequence shown here (also see Supplemental Figure 2). **C)** Superimposition of Slr1p's LaM structure (grey) with its predicted AlphaFold structure (light blue, binding site in teal). Inset (top): R groups of Q332 point in different directions. Inset (bottom): The NMR structure is

different in positions of the β -strands and an extra α -helix between β 1 and β 2 (α 7, red arrow) that appears to be a loop in the predicted AlphaFold structure. TM-score: 0.84457 (where TM-Score = 1 indicates a perfect match between two structures). **D)** Overlay of Slr1p's LaM (NMR) with that of hLARP1's LaM (green, binding site in pink; PDB: 7SOO). Alignment of the amino acids in the binding site is shown where Q332 (Q333 in hLARP1) points in a different direction. β -strands appear to superimpose (inset top left) although α 7 (red arrow) in Slr1p appears to be a short loop in hLARP1's structure (TM-score: 0.77387).

Table 1: Statistics for the unbound Slr1p LaM ensemble.

Table 1: Statistics for the unbound Slr1p LaM ensemble.

NOE distance restraints in the ensemble	2031 / 1484
intraresidue (observed/unique)	967 / 665
sequential (observed/unique)	435 / 312
medium (observed/unique)	288 / 241
long (observed/unique)	341 / 266
NOE distance restraint violations ^a	
0.1-0.2 Å	1.65
0.2-0.5 Å	1.45
> 0.5 Å	0.75
Hydrogen bond distance restraints	
HN-O / N-O pairs	29
Torsion angle restraints	
backbone (Φ/Ψ)	80
Torsion angle restraint violations ^a	
1-10°	0
> 10°	0
Structural quality analysis ^b	
close contacts	4
RMS deviation of bond angles (°)	0.3
RMS deviation of bond lengths (Å)	0.009
RMS deviation of ordered residues to the mean ^c	
backbone (Å)	0.49 ± 0.14
heavy atoms (Å)	0.70 ± 0.12
Ramachandran plot (%)	
residues in the most favored regions	96.2
residues in the additional allowed regions	3.8
residues in the generously allowed regions	0.0
residues in the disallowed regions	0.0

^aDetermined using PSVS.

^bDetermined using PROCHECK.

^cOrdered residues (2-83, 91-96) are defined by a dihedral angle order parameter with $S(\phi)+S(\psi)>1.8$ as determined by PSVS.

3.3.2. Amino acids in the binding site of Slr1p are essential for RNA binding

In order to test the importance of each amino acid in Slr1p's binding site, we made single mutants in this region and performed electrophoretic mobility shift assays (EMSAs)⁵⁸. To compare binding affinities of the mutants to wildtype proteins, we first tested binding of Slr1p's LaM to an RNA corresponding to a 15-nucleotide long 5' Proximal AC-rich (5'PAC) motif found in the *S. pombe* ribosomal protein mRNAs for which we have previously shown specific interactions (PAC15: UUUUCCACCACCAA)⁵⁷. We additionally tested binding to the 19-mer 5'PAC RNA and a 10-mer oligonucleotide corresponding to the first ten nucleotides of the 19-mer sequence. Similar to our previous work, we saw binding with the 19-mer but not the shorter 10-mer RNA (Supplemental Figure 4A), suggesting that the Slr1p LaM RNA binding mode has a substantial length dependence, similar to full-length Slr1p and in contrast to the LaM of hLARP1 and hLa. Both Slr1b V1 and V3 formed ribonucleoproteins (RNPs) at similar concentrations that were also higher than those observed for the full-length protein⁵⁷, starting at 7 μ M concentration of protein with the 15-mer RNA (Figure 2A). These data suggest that regions outside of the LaM still contribute to RNA binding affinity, as has been described for other LARPs, even if the LaM represents the only predicted RNA binding domain in Slr1p. V3 was then mainly used for making our mutants and subsequent binding assays as it was far better expressed and more stable compared to V1, and produced better RNPs as visualized by EMSAs. Mutating Q332 to an alanine led to a complete loss of binding up to 28 μ M of protein (Figure 2B), in agreement with previous work noting that the equivalent residues in hLARP1 and hLa have an important role in ligand binding. E334A and Y336A mutants also showed a substantial loss of binding. Importantly, these three residues (Q332,

E334A and Y336A) are invariant amino acids in LARP1 proteins compared in Figure 1A as well as genuine La proteins (Figure 2D in Dong *et al.* (2004)²², also see Supplemental Figure 5). Additionally, single mutants L333A, Y335A (conserved amino acids in the binding site, Figure 1A) and the double mutant F347L348>AA (Figure 2C) showed an intermediate decrease in binding affinity. We made more mutants spanning several alpha helices in the structure (K344A, D345A, D345R, K350A and R368A) and some in the C-terminal amino acids following the LaM (R419A, K421A, F422A, K426A and Y427A) and further tested their affinity for the 15-mer 5'PAC RNA using EMSAs (Supplemental Figure 4B). The extent to which they influenced RNA binding is depicted in Figure 2D, with the amino acids leading to most loss of binding coloured red, those with intermediate effects coloured orange and those with minimal effects coloured green. We then surveyed amino acids in the proximity of Q332 since mutating this amino acid showed the most severe loss of binding. Consistent with a primary RNA binding site in this region and mutations to residues in this site which showed various binding defects, we noticed that Y335, Y336 and R368 may cooperate with Q332 to form an RNA binding groove (Figure 2E, also see Supplemental Figure 7). Interestingly, Q332 is in closest contact with L329 (2.5 Å) that instead is a conserved isoleucine in hLARP1 and the two budding yeast LARP1 orthologs, which may be relevant to Q332's altered side-chain arrangement in Slr1p (Figure 1B-D).

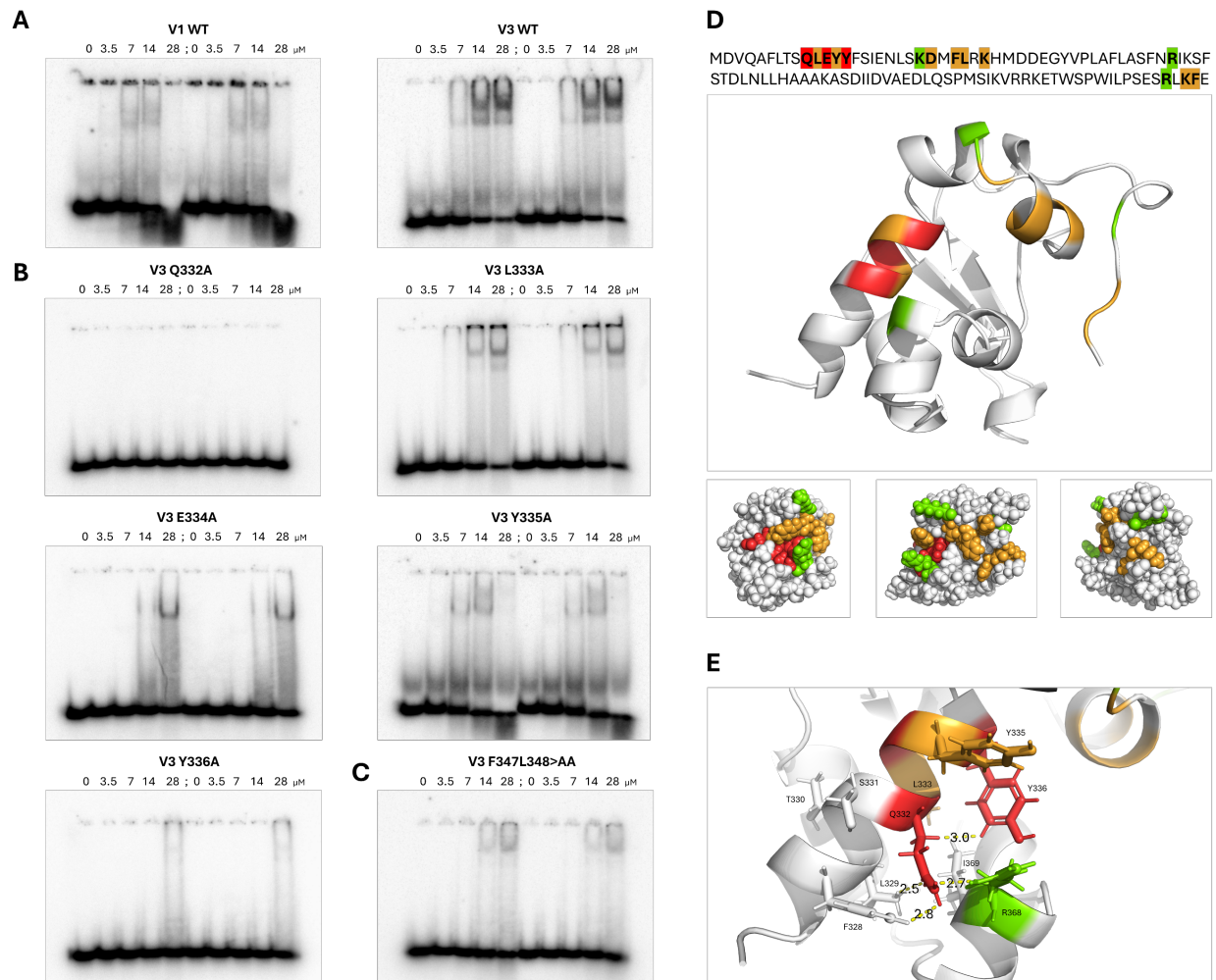


Figure 2: Single amino acid mutations in Slr1p's binding site impair RNA binding. **A)** *In vitro* binding assays or electrophoretic mobility shift assays (EMSAs) were performed to test interactions between recombinantly expressed and purified Slr1b V1 and V3 (both considered as “wildtype”) and an RNA corresponding to a 15-nucleotide long 5' Proximal AC-rich (5'PAC) motif found in the *S. pombe* ribosomal protein mRNAs (PAC15: UUUUCCACCACCA)⁵⁷. RNPs start to form at 7 μ M concentration of protein in both cases. Two replicates are shown on the same gel per protein. WT=wildtype. **B)** V3 constructs containing single mutations in the binding site were expressed in *E. coli* and purified. EMSAs were performed with the PAC15 RNA and indicated concentrations of protein. Binding affinity is lowered to varying extents in L333A, E334A, Y335A and Y336A mutants with complete loss of binding in the Q332A mutant. Two replicates are shown on the same gel per protein. **C)** F347 and L348 are also involved in RNA binding. Both amino acids were mutated to alanines and EMSAs were performed with the PAC15 RNA where binding affinity was observed to be decreased. Two replicates are shown on the same gel. **D)** A representation of assessed mutants and their relative position in the structure of Slr1p's LaM is shown. Mutated amino acids having the most severe effect in terms of loss of binding are coloured

in red (Q332A, E334A and Y336A). Amino acids with intermediate effects on binding are coloured orange (L333A and Y335A, also see Supplemental Figure 4B: D345A, D345R, K350A, K421A and F422A) and those with minimal effects on binding are coloured green (Supplemental Figure 4B: K344A, R368A and R419A). Two more mutants were also made in the extreme CTD, with minimal effects on binding, that are not coloured in the shown sequence here: K426A and Y427A (Supplemental Figure 4B). Bottom three panels show a space-filling model of the mutated amino acids and their original R groups which span the binding site and the middle portion of the protein. **E)** All amino acids within 3 Å of Q332 are labelled with their R groups shown. The distance measured between four of which (L329 at 2.5 Å, R368 at 2.7 Å, F328 at 2.8 Å and y336 at 3 Å) and Q332 is indicated.

The amide group protons of Q332 (HE21/HE22) have distinct upfield resonances likely due to their proximity to F328 and Y335. As expected, NOEs are observed between the amide protons of Q332 and the aromatic ring protons of F328, in particular (Figure 3A). If the spectra are contoured closer to the noise, an additional set of resonance are apparent. While weak in intensity, one peak can be unambiguously assigned to the methyl group of I369. The I369 peak, plus others assigned to R368 and Y336, complete the set of weak NOEs from the Q332 side chain. If the weak and strong NOEs are used together in a structure calculation, unresolvable violations occur since no single conformation of the Q332 side chain can satisfy all of the NOE assignments. When the strong and weak sets of NOEs are used independently in a structure calculation, each set is satisfied by the resulting solution. In one solution shown in the Figure 3B, the side chain of Q332 is solvent facing and in another solution shown in Figure 3C, the side chain of Q332 is positioned away from the solvent in an orientation that is analogous to what was observed for the similar glutamine in several high-resolution structures of the RNA bound form of hLARP1. A χ_1 rotamer flip of Q332 at

slow exchange relative to the chemical shift timescale provides a plausible explanation for the NOEs observed and the two resulting structure solutions.

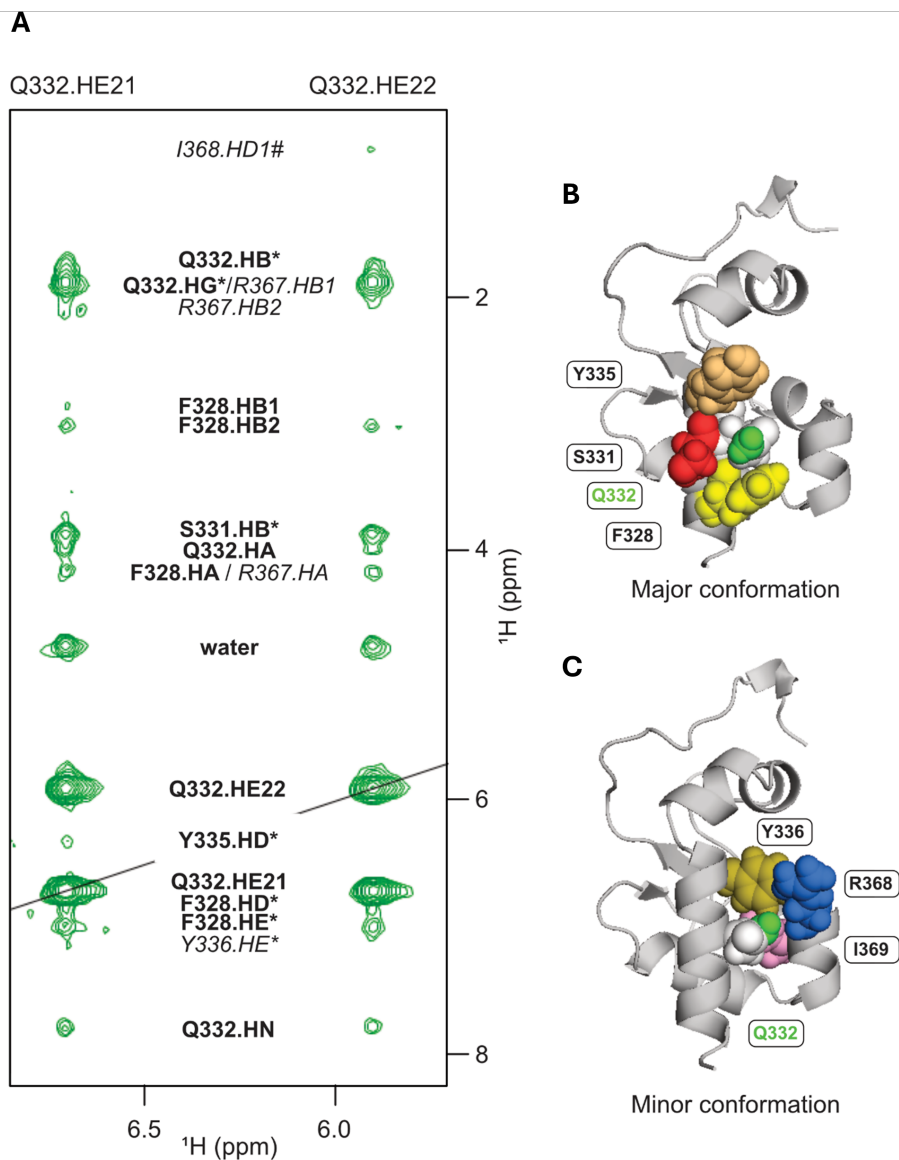


Figure 3: Two possible conformations for the side chain of Q332. A) One plane from a 3D ^{15}N NOESY spectrum ($^{15}\text{N}=114.6$ ppm) highlighting the side chain NH_2 protons of Q332. Assigned resonances in boldface are consistent with a major conformation where the Q332 side chain projects towards the solvent. Assigned resonances in italics are consistent with a minor conformation where the Q332 side chain projects towards the RNA binding pocket. **B, C)** Structure solutions of the major and minor conformations.

3.3.3. Molecular docking predicts a high confidence binding mode between Slr1p and PAC15 RNA

In order to predict the positioning of the PAC15 RNA when in complex with Slr1p, we used a docking server (HDOCK Server)⁶⁴ and derived a number of high confidence binding models. Our NMR structure of Slr1p was used as input and the RNA structure was modeled by the server. Given that PDB entries for protein-protein, protein-RNA and protein-DNA complexes normally have a docking score of around -200 or lower⁶⁴, we considered top 10 models predicted with a docking score of less than -200. Amino acids in the binding site of Slr1p LaM were predicted to be involved in RNA binding in every case. Previous work showed that C6 of the PAC15 RNA is important as mutating it to G fully disrupts binding⁵⁷. Consistent with this, a model predicted C6 to come into contact with Q332, Y335 and Y336. Stacking is apparent between Y335 and the pyrimidine ring of C6 while some stacking appears to occur between the RNA bases U4, U5 and to some extent C7 (Figure 4A). R368, F347, F366 and N367 appear to surround the stacking bases. Notably, previous work has suggested contacts to 5' uridines in the 5'PAC RNA are important for establishing binding to this motif⁵⁷. Moreover, A8 and C9 participate in some stacking near R368 on one side and N367 on the back side (Figure 4B). This model also predicts N367 and K370 to be in close proximity of RNA bases A11 and C12 in addition to K421, F422 and E423 being in the proximity of A14 in the RNA (not shown). Slr1p does not bind a short RNA (i.e., PAC10 in Supplemental Figure 4A) and therefore these contacts to a long substrate, although far from the binding site, might be necessary to fold the RNA in such a way that orients C6 towards the binding site.

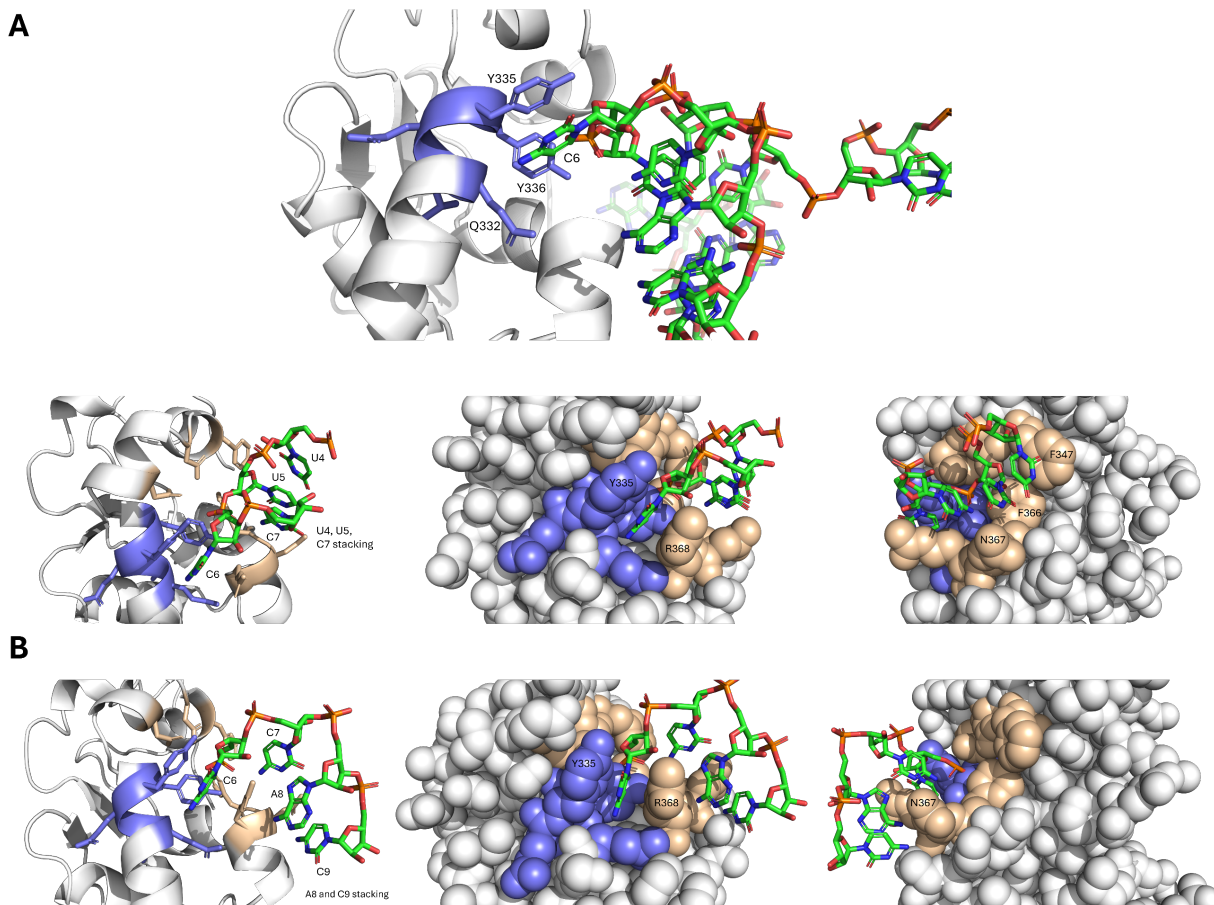


Figure 4: Molecular docking predicts high confidence binding modes plus amino acids involved in interactions between Slr1p and the PAC15 RNA. A) A molecular docking server (HDOCK Server)⁶⁴ was used to predict binding modes between our solved structure of Slr1p and the PAC15 RNA where the RNA structure was modeled by the server. The top panel shows helix 1 with amino acids in the binding site (QLEY) in purple. C6 of the PAC15 RNA, previously shown to be important where its mutation to G fully disrupts binding⁵⁷, is surrounded by Q332, Y335 and Y336. Stacking is apparent between Y335 and the pyrimidine ring of C6. Bottom panels show a portion of the 15-mer RNA: UUCC, where U4, U5 and to some extent C7 are stacking to allow for C6 to point into the binding site. Y335 and R368 appear to be flanking C6 on either side. On the back side F347, F366 and N367 appear to contribute to the stability of the stacking between U4 and U5. **B)** A different portion of the 15-mer RNA is shown: CCAC, where C6 is still in contact with the binding site but C7 appears to be disordered. A8 and C9 participate in some stacking near R368 on one side and N367 on the other side. Docking score: -229.43 (protein-protein/-RNA/-DNA complexes in the PDB normally have a docking score of around -200 or lower), confidence score: 0.8304 (>0.7 means the two molecules would be very likely to bind).

Calculating the predicted free energy difference as a consequence of an alanine mutation on the entire binding surface of Slr1p with respect to a 3-nucleotide or a 4-nucleotide oligo(A) substrate suggests that there is good overlap between predicted residues that contact RNA and our EMSA data. Therefore, it also appears that there is an overall similarity between binding modes of hLARP1 and Slr1p LaM, even if there is not complete overlap which is expected as we propose Slr1p is binding an internal proto-5'TOP sequence instead of a terminal AAA-3'OH (Supplemental Figure 6). Considering the solvent accessible surface of Slr1p, the positioning of Q332, Y335, Y336 and R368 side-chains appear to support the formation of a binding groove that surrounds base 6 of the 15-mer 5'PAC RNA (Supplemental Figure 7A). The corresponding amino acids in hLARP1 don't seem to form a similarly shaped binding groove that could accommodate a pyrimidine ring as well (Supplemental Figure 7B). Similarly, Slr1p doesn't seem to best accommodate a purine in its binding cleft (Supplemental Figure 7C). Further examination of how hLARP1 contacts an AAAA RNA and Slr1p contacts PAC15 RNA suggests stacking between Y336 of hLARP1 and A3 of AAAA is more favourable (Supplemental Figure 7D) while Y335 of Slr1p is better positioned to stack with a pyrimidine (Supplemental Figure 7E). Additionally, Q333 of hLARP1 could be in a steric clash relative to C6 of the PAC15 RNA, such that Q332 of Slr1p initially pointing away from the binding site may better accommodate the RNA base (Supplemental Figure 7E, right inset).

3.4. Discussion

In this work, we present the NMR structure of the La motif (LaM) of the *S. pombe* La-related protein 1, Slr1p. We report that the addition of a few amino acids C-terminal to the predicted LaM enhances its stability and expression relative to the LaM in isolation even though the added region doesn't appear to adopt a certain fold. We show notable differences in positioning of key amino acid residues in the RNA binding site (specifically Q332), and a loop (referred to as $\alpha 7$ here) connecting $\beta 1$ and $\beta 2$ which is missing from previously reported LaM structures including hLARP1's LaM and relative to the AlphaFold predicted model. Through mutagenesis studies, we show that invariant and highly conserved amino acids in helix 1 of the LaM are predominantly important for making contacts to a 5' Proximal AC-rich (5'PAC) motif found in the *S. pombe* ribosomal protein mRNAs representing a proto-5'TOP sequence in this system⁵⁷. Mutating each of three invariant amino acids in the binding site leads to severe losses of RNA binding, with Q332A having the most severe effect followed by the E334A and Y336A mutations. Interestingly, the invariant glutamine residue (Slr1p Q332) in the LaM is linked to nucleotide binding specificity for both hLARP1 (Q333)^{54,55} and hLa (Q20)^{4,5}. In addition to Q332, E334 and Y336, we characterize the influence of 13 other amino acids in the LaM and C-terminal regions to it in RNA binding, with most mutations leading to a relatively moderate but observable loss of binding (Figure 2, Supplemental Figure 4). Our molecular docking predictions suggest that the PAC15 RNA folds in a specific manner that orients an invariable C6 in the sequence towards a modeled binding groove. Interestingly, other amino acids outside of the binding site could be making additional contacts to the RNA to potentially further stabilize it, suggesting an explanation for the substantial RNA length

dependence of the isolated LaM, which we've also previously observed for the full-length Slr1p⁵⁷.

LaM containing proteins are highly versatile RNA binders with varied reported substrates. In genuine hLa, the LaM is linked to the binding of terminal uridylates^{4,5} and poly(A) RNA²⁰, while the protein is also linked to the binding of histone mRNAs⁸, 5'TOP mRNAs^{15,17} and cellular^{12,13} and viral^{6,7,9-11,16,18,19} mRNAs containing IRESs. The N-terminal RRM1 and C-terminal xRRM/RRM2 of hLa have not been linked to any sequence-specific binding modes and the protein doesn't possess any other characteristic motifs (such as the DM15 in hLARP1) that bind RNA in a sequence-specific manner. Therefore, characterized sequence-specific contacts for La proteins occur through the LaM, albeit with the additional help or facilitation from the RRMs. Similarly, hLARP1 has been linked to a breadth of substrates such as polypyrimidine tracks in the SARS-CoV-2 RNA genome containing TOP-like motifs⁷⁶, poly(A) RNA^{45,48-50,52-54} and guanylated poly(A) RNA (through amino acids 323-410 from the 1019-residue isoform)⁵⁵. Also, pyrimidine-rich sequences within 5'UTRs of ribosomal protein mRNAs have been shown to drive basal RNA localization in human epithelial cells and mouse neuronal cells where proper RNA localization is dependent on the presence of LARP1, but the regulatory effects of the examined pyrimidine-rich sequences are independent of their position within the 5'UTR⁷⁷. Other studies have shown that hLARP1's LaM plus amino acids which follow this region (amino acids 310-540 from the 1019-residue isoform previously thought to include an RRM_{L5}) can simultaneously engage TOP motifs and poly(A) RNAs⁴². Given that the established m⁷G/+1C specific binding mode is associated with the DM15 motif, and that hLARP1's interaction with TOP-like motifs^{42,76,77} occur outside

of the m⁷G/+1C context, it seems likely that it is the hLARP1's LaM that is linked to the diversity of hLARP1 RNA binding modes, including that which relates to pyrimidine-rich sequences beyond the 5'TOP motif found right after the m⁷G mRNA cap.

Our studies on the single *S. pombe* LARP1 ortholog, which binds and affects the expression/stability of proto-5'TOP containing ribosomal protein mRNAs, further expands the context dependent roles for LARP1 proteins across eukaryotes. While hLARP1 is best characterized as an effector of 5'TOP expression and stability, it is notable that 5'TOP mRNAs make up a small fraction of hLARP1's targets in cancer cells^{36,43,78,79}, and hLARP1 appears to interact with viral mRNAs^{76,80–82}. Additionally, hLARP1 and orthologs in other systems have been suggested to bind mRNAs coding for mitochondrial factors^{83–86} and mRNAs upregulated in response to heat stress⁸⁷, oxidative stress^{83,88} and copper detoxification^{88–90}. The LaM of hLARP1 has been linked to poly(A) binding^{48,54,55}, but this is insufficient to explain how hLARP1 is capable of recognizing the specialized subsets of mRNAs (other than 5'TOPs) which it specifically binds to in certain contexts or in response to stress, especially since previous studies have ruled out associations for some LARP1 members through the mRNA poly(A) tail⁸⁷. Thus, it seems likely that LARP1 orthologs are recruited to diverse groups of mRNAs through special features that are as of yet uncharacterized, and that the LaM may be linked to several binding modes which may be related to confounding functions reported for hLARP1⁴³.

In the current study, we report that conserved amino acids in the LaM of Slr1p are utilized to contact the 5'PAC motif, previously identified in *S. pombe* ribosomal protein mRNAs, which

is consistent with earlier work highlighting the evolutionary importance of these amino acids in how La and LARPs contact RNA. While the amino acids linked to 5'PAC motif binding show strong similarity to those linked to terminal uridylate binding in hLa LaM and terminal adenylate binding in hLARP1 LaM, we also show that there are notable differences in the structure of the Slr1p LaM relative to these proteins, which could be related to the binding of Slr1p to internal RNA sequences versus terminal RNA binding modes described for hLa and hLARP1. Given established links between hLARP1 and internal pyrimidine-rich sequences (discussed earlier), it would be interesting to determine in the future whether the Slr1p LaM structure observed here could be related to alternative binding modes linked to the hLARP1 LaM. Thus, studying LARP1 proteins in several eukaryotes and in complex with various RNAs may reveal distinct binding modes, providing important functional implications for LARP1 in RNA metabolism, stress conditions and disease states.

Data Availability

Coordinates of the ensemble were deposited in the Protein Data Bank with accession number 9CB2. Chemical shifts were deposited in the BioMagResBank with accession number 31178.

3.5. Acknowledgements and Funding

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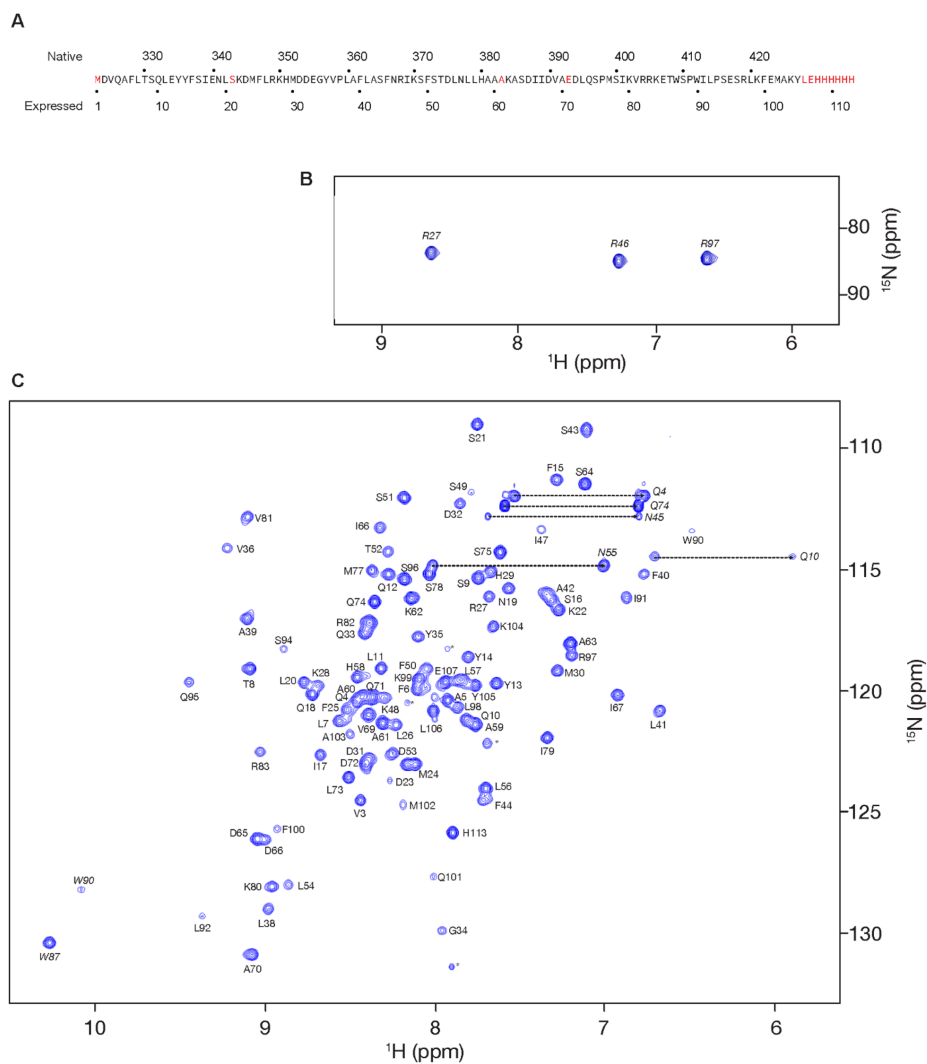
Declaration of Interests

The authors declare no competing interests.

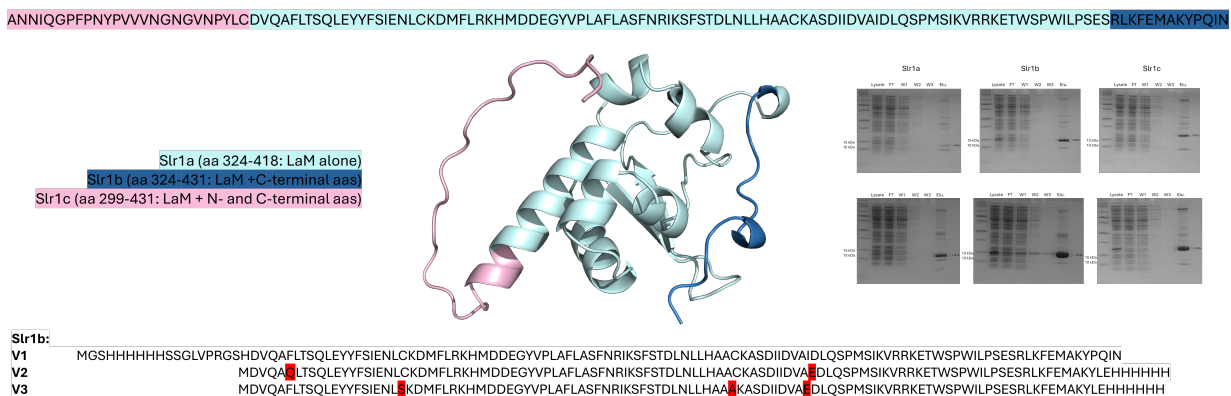
Author Contributions

Conceptualization: F.M., M.A.B., L.W.D.; Data Curation: F.M., L.W.D.; Formal analysis: F.M., M.A.B., L.W.D.; Funding acquisition: F.M., M.A.B., L.W.D.; Investigation: F.M., L.W.D.; Methodology: F.M., M.A.B., L.W.D.; Project administration: F.M., M.A.B., L.W.D.; Software: F.M., L.W.D.; Resources: M.A.B., L.W.D.; Supervision: M.A.B., L.W.D.; Validation: F.M., M.A.B., L.W.D.; Visualization: F.M.; Writing – original draft: F.M.; Writing – review & editing: F.M., M.A.B., L.W.D.

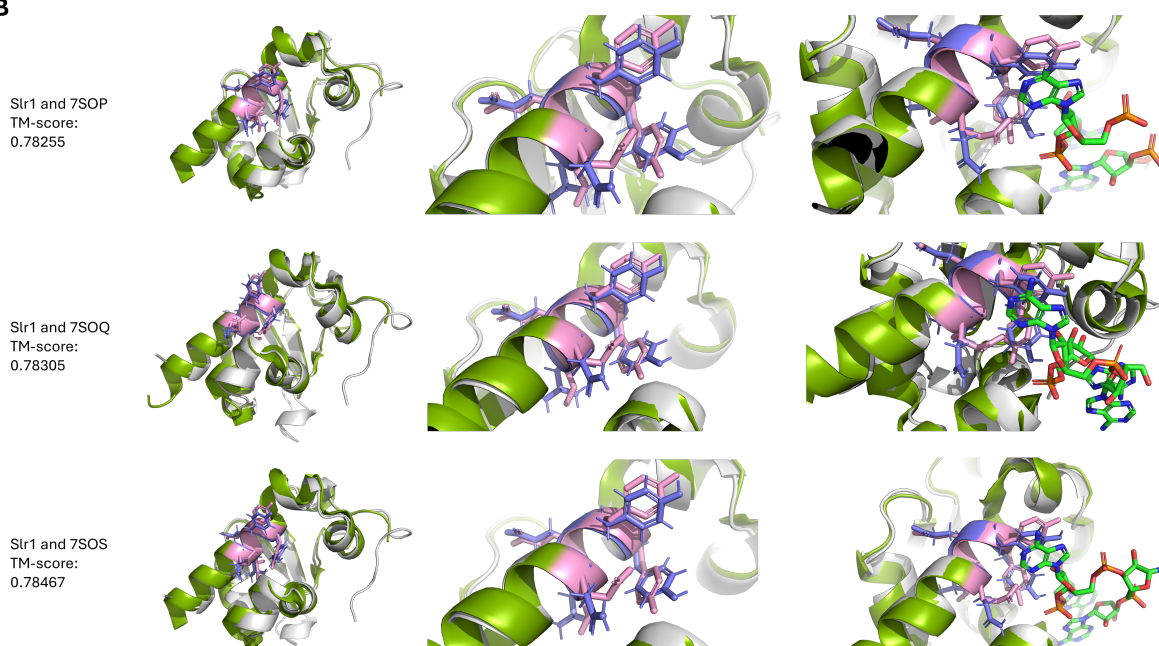
3.6. Supplemental Information



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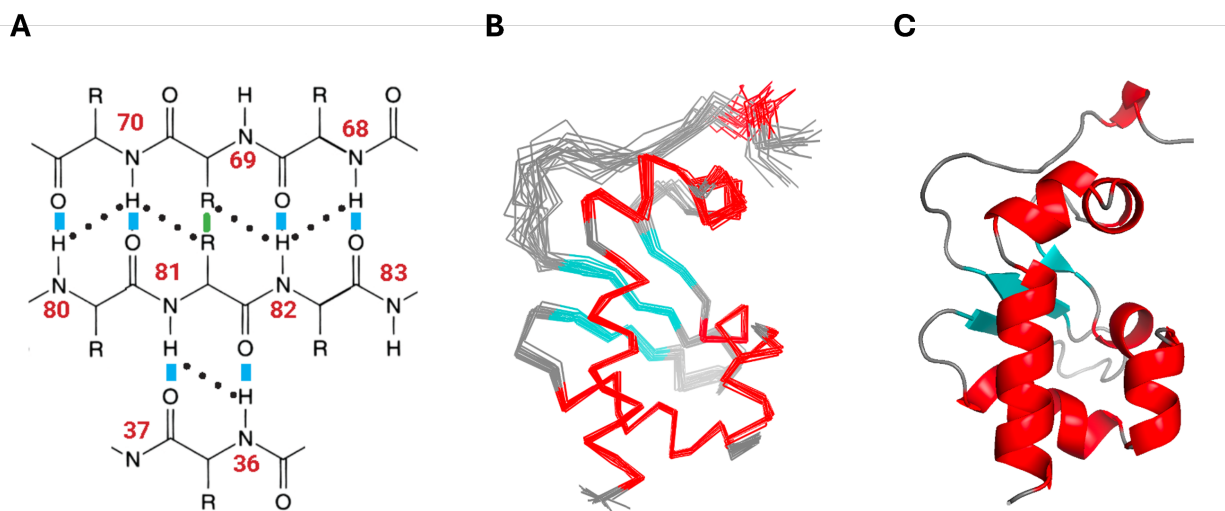


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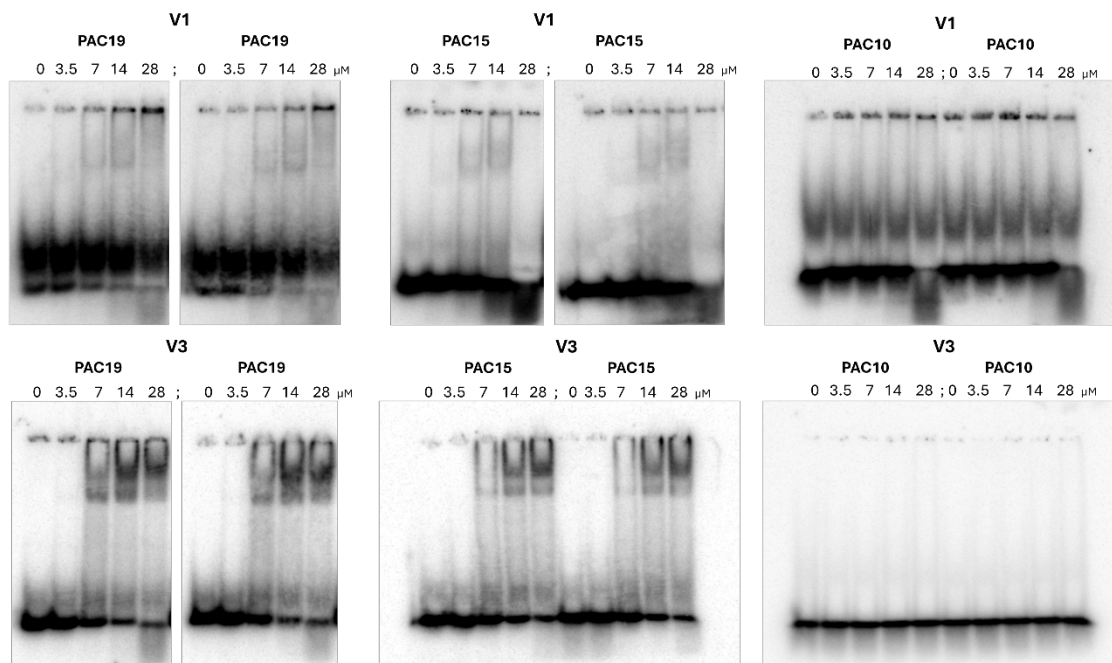
Supplemental Figure 2: Amino acids C-terminal to Slr1p LaM aid in protein expression and stability. **A)** Amino acid boundaries for three Slr1p constructs initially considered for structural analysis are shown. Slr1a (light blue) and c (all) were poorly expressed: purification gels show 3 h expression at 37°C (top panels) and overnight expression at 18°C (bottom panels), FT=flowthrough, W1-3=wash 1-3, Elu.= elution. Slr1b consisting of the LaM plus a few C-terminal amino acids was chosen as the best expressed candidate for further work. This is also referred to as Slr1b version 1 or V1. V2 and V3 were subsequently considered for increased solubility and expression. Amino acids highlighted in red were mutated to the ones currently shown in the sequence. **B)** Alignments between the NMR structure of Slr1p (white, binding site in purple) and PDB entries for the LaM of hLARP1 (green, binding site in pink⁵⁴) – 7SOP: LaM domain of hLARP1 in complex with AAUAA RNA, 7SOQ: LaM domain of hLARP1 in complex with AAA RNA, 7SOS: LaM domain of hLARP1 in complex with AAAA RNA. TM-scores are provided where 1 indicates a perfect match between

two structures. Far-right panels show the positioning of an adenine nucleotide between Y336 and Q333 of hLARP1 (pink) relative to side chains of the same amino acids in Slr1p (purple).

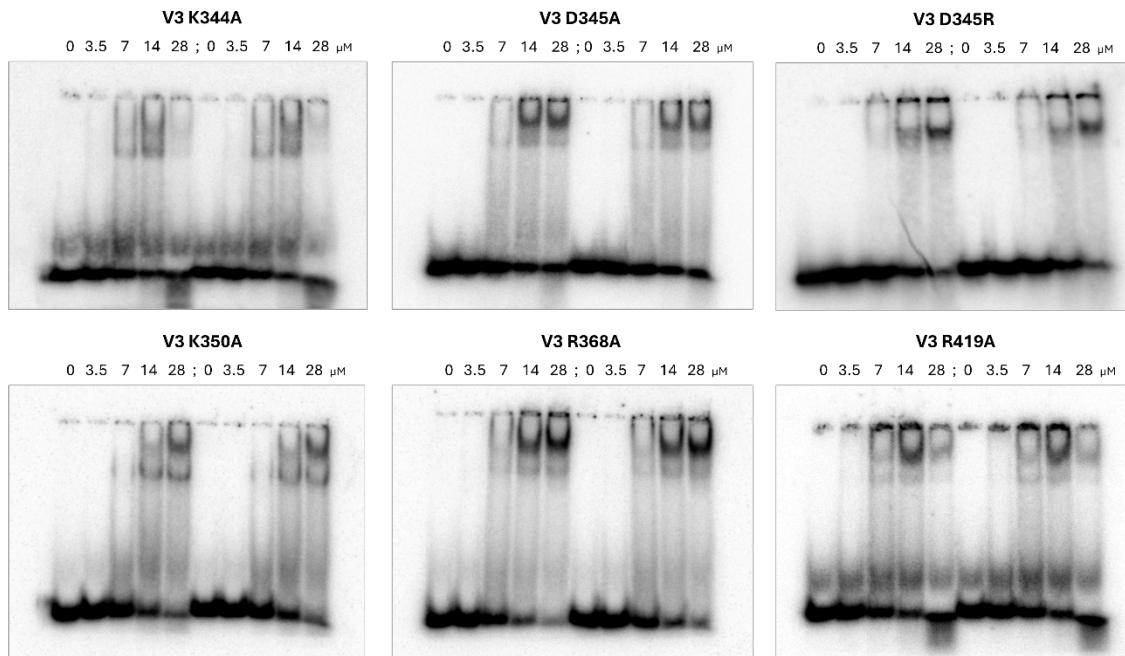


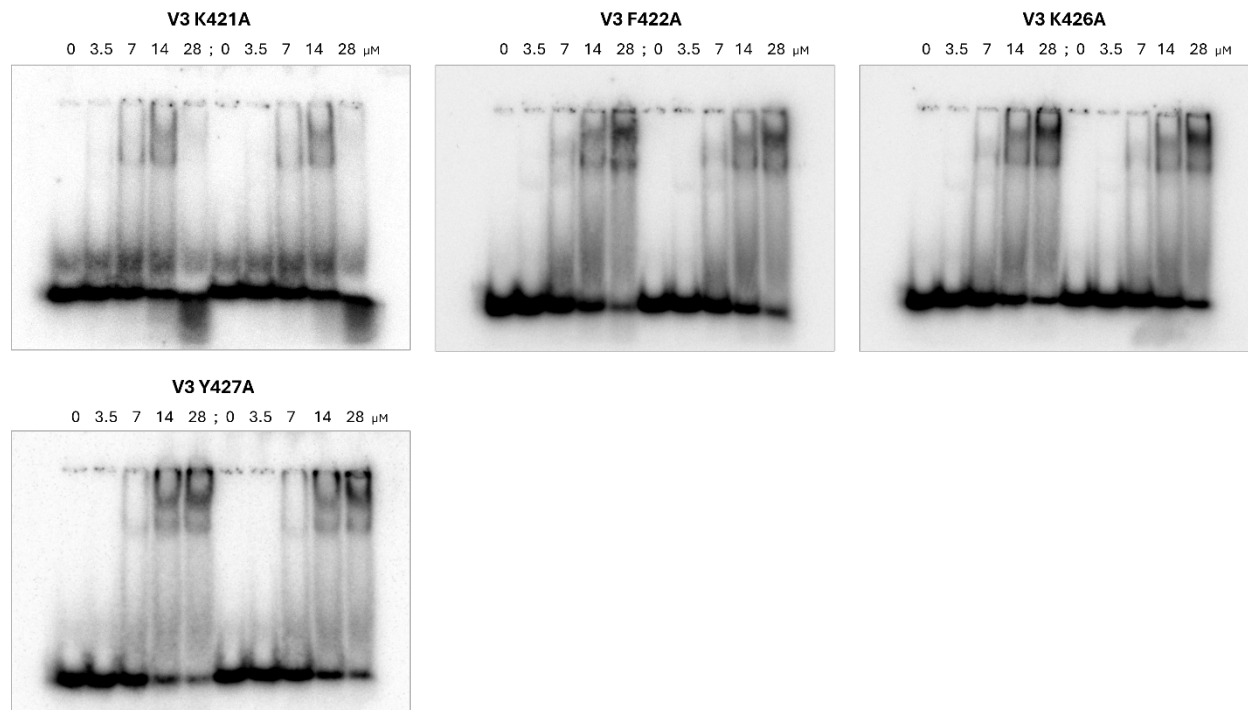
Supplemental Figure 3: Structural features of the Slr1p LaM domain. A) Inferred beta strands from NOE observations. Dashed lines represent HN-HN and HN-HA NOEs. Green lines represent HA-HA NOEs. Blue lines represent hydrogen bond restraints used in the structure calculation. See Supplemental Figure 1 for native numbering. **B)** Superposition of the top 20 lowest energy solutions. **C)** Cartoon representation of the lowest energy solution.

A

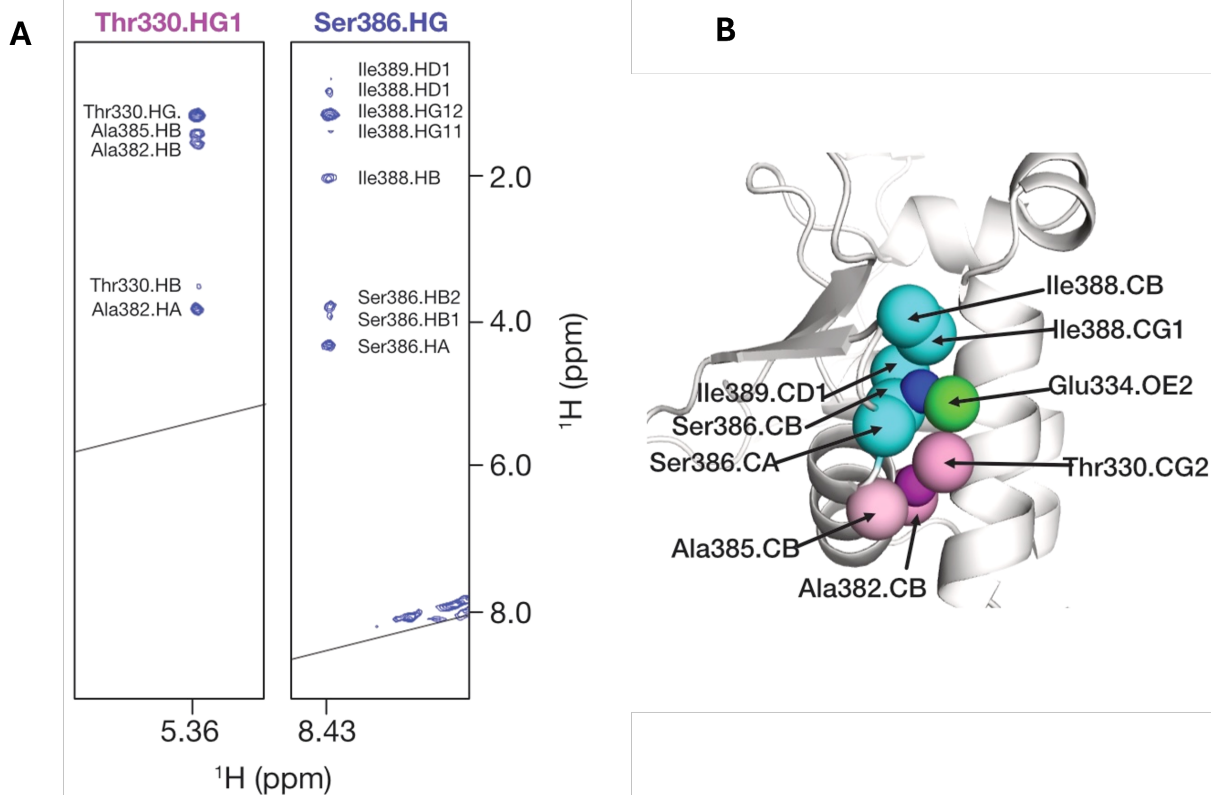


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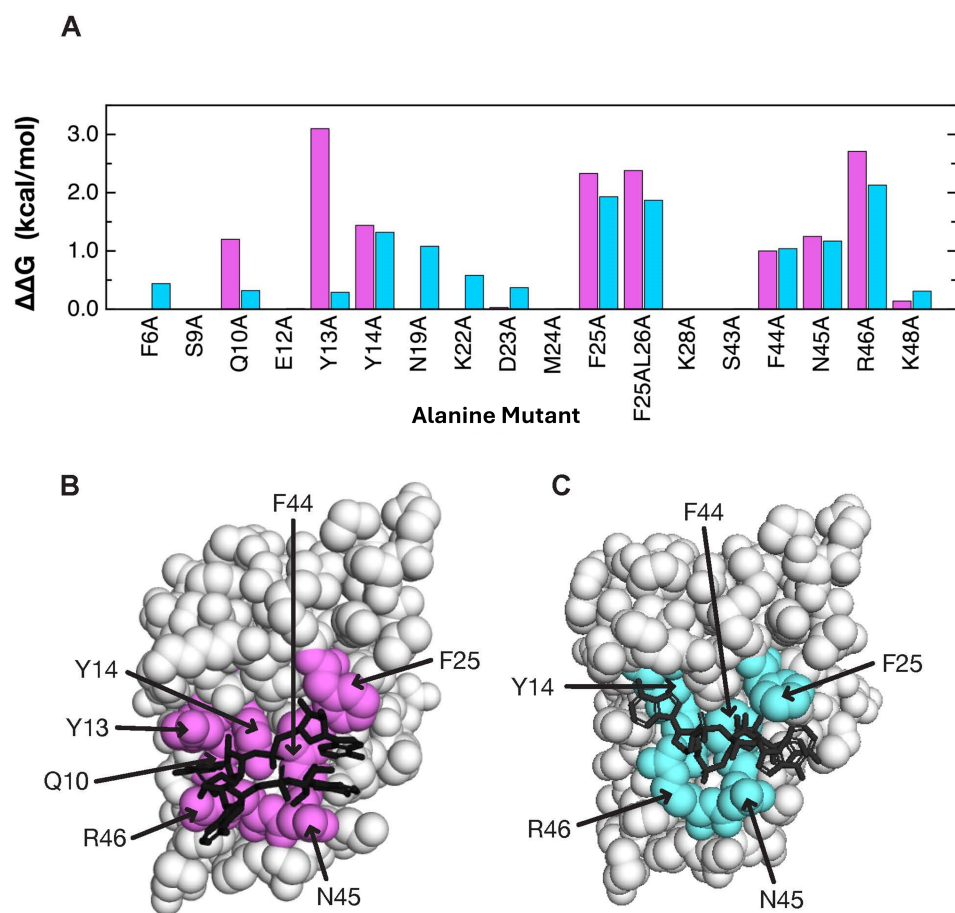




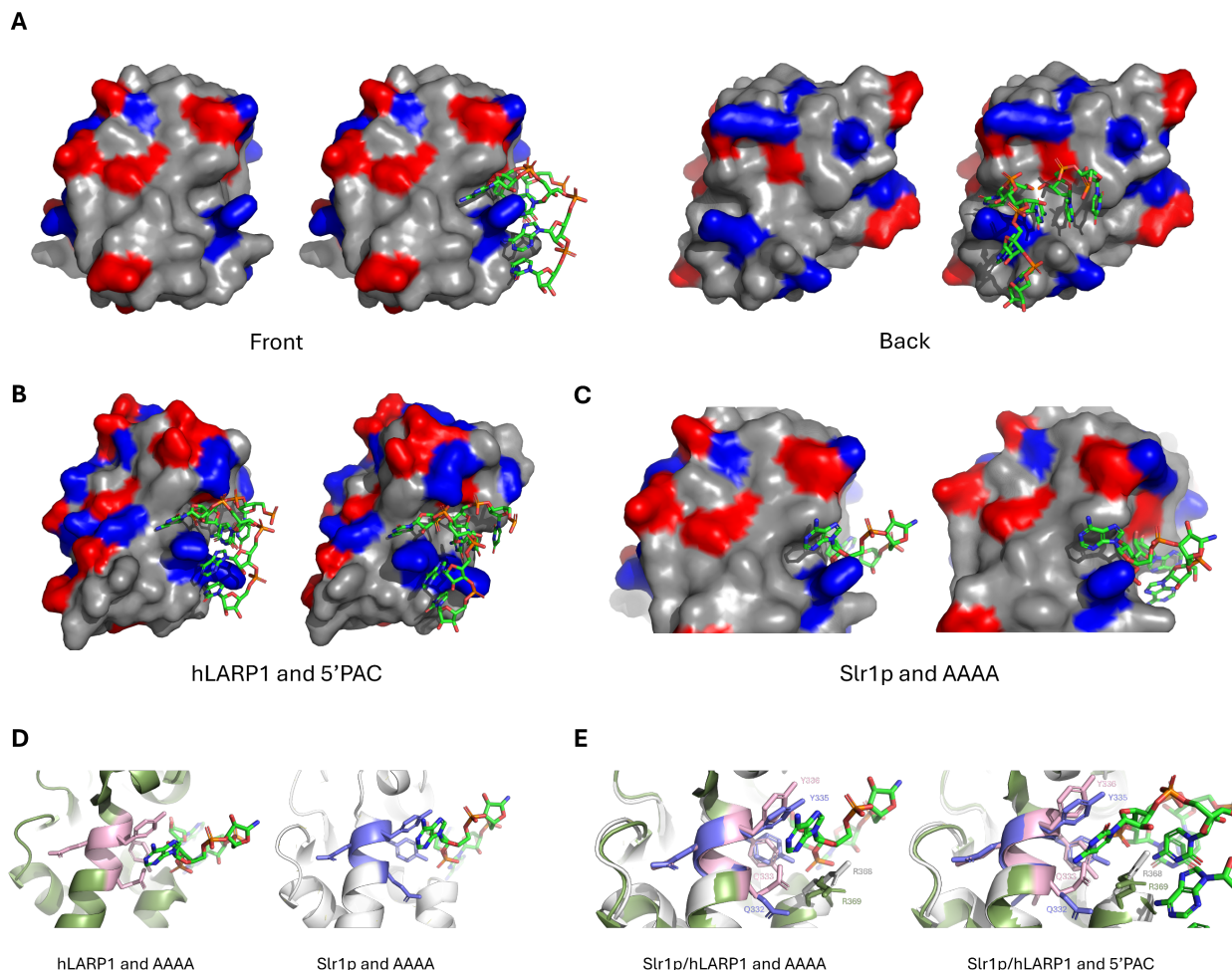
Supplemental Figure 4: Electrophoretic mobility shift assays for Slr1b V1, V3 and V3 mutants. A) EMSAs showing binding between recombinantly expressed and purified Slr1b V1 and V3 (both considered as “wildtype”) and RNAs corresponding to 19- and 15-nucleotide long 5' Proximal AC-rich motifs found in the *S. pombe* ribosomal protein mRNAs (PAC19: UUUUCCCCACCACCAAACAC, PAC15: UUUUUCCACCACCAA)⁵⁷. PAC10 refers to an RNA containing the first 10 nucleotides of the PAC19 RNA. Neither V1 nor V3 bind to the shorter PAC10 RNA. Two replicates are shown per RNA. **B)** EMSAs for a number of V3 single mutants (K344A, D345A, D345R, K350A, R368A, R419A, K421A, F422A, K426A and Y427A) are shown (see also Figure 2D). PAC15 RNA was used for all EMSAs here. Two replicates are shown on the same gel per mutant.



Supplemental Figure 5: Two hydroxyl protons are protected from solvent exchange in the Slr1p LaM. A) Sections from a ^{12}C , ^{13}C filtered-selected 2D NOE spectrum highlighting NOEs arising from T330_HG1 and S386_HG. **B)** Immediate vicinity of the T330_HG1 (dark blue ball) and NOEs observed (cyan balls) along with S386_HG (purple ball) and NOEs observed (pink balls). The side chain of E334 (green ball) is positioned to make a hydrogen bond with S386_HG.



Supplemental Figure 6: Decoding potential binding modes of the Slr1p LaM domain by free energy difference. A) The free energy difference between the base protein and an alanine substituted protein at a given position at the RNA binding surface for a 4-nucleotide ligand (purple) and a 3-nucleotide ligand (blue) assuming an analogous interaction as observed in the crystal structures of hLARP1. **B, C)** Sites on an Slr1p / 4-nucleotide and an Slr1p / 3-nucleotide model with a predicted free energy difference > 1 kcal/mol. See Supplemental Figure 1 for native numbering.



Supplemental Figure 7: Comparisons of binding pockets and ligands between Slr1p and hLARP1 LaMs. **A)** The solvent accessible surface of Slr1p (NMR) is shown where hydrophobic and polar residues are coloured grey, negative amino acids are red and positive amino acids are blue. The “Front” shows a binding groove formed by amino acids Q332, Y335, Y336 and R368 which surround C6 of the 15-mer 5'PAC RNA modeled by HDock Server to point into this region (Figure 4). In the “Back”, the positioning of the rest of the RNA (only UUC6CAC is shown) is presented. **B)** Having the structures of Slr1p and hLARP1 (PDB: 7SOS) aligned by PyMol, it appears that hLARP1 with its surface coloured in the same manner as in A), lacks a similarly shaped binding groove to accommodate the UUC6CAC of the 15-mer 5'PAC RNA. **C)** Slr1p and an AAAA RNA which hLARP1 binds to (in PDB: 7SOS) are shown, where the positioning of the purine base relative to Slr1p's binding groove is highlighted. **D)** Stacking between Y336 of hLARP1, Y335 of Slr1p and A3 of AAAA in PDB: 7SOS is shown. **E)** Superimposed structures of hLARP1 (green, binding site in pink) and Slr1p (grey, binding site in purple) and contacts to AAAA in PDB: 7SOS (left) as well as C6 of the 15-mer 5'PAC RNA (right) are shown. Relative positions of Slr1p/hLARP1 Q332/3, Y335/6 and R368/9 are labeled.

Supplemental Table 1: Mutagenesis primers. FL=full length (native numbering), Slr1b V3 (expressed). Related to Figure 2, Supplemental Figures 2 and 4.

FL Q332A_Slr1b V3 Q10A	For	TCTGACCAGCGCGCTGGAATATTATTTTAG
	Rev	AAGGCCTGCACATCC
FL L333A_Slr1b V3 L11A	For	GACCAGCCAGGCGGAATATTATTTTAGTATTG
	Rev	AGAAAGGCCTGCACATC
FL E334A_Slr1b V3 E12A	For	CAGCCAGCTGGCGTATTATTTTAGTATTG
	Rev	GTCAGAAAGGCCTGC
FL Y335A_Slr1b V3 Y13A	For	CCAGCTGGAAGCGTATTTTAGTATTGAAAACC
	Rev	CTGGTCAGAAAGGCC
FL Y336A_Slr1b V3 Y14A	For	GCTGGAATATGCGTTTAGTATTGAAAACCTG
	Rev	TGGCTGGTCAGAAAG
FL F347L348>AA_Slr1b V3 F25L26>AA	For	CAAGGATATGGCGGCGCGCAAACACATG
	Rev	CTCAGGTTTTCAATACTAAAATAATATTC
FL K344A_Slr1b V3 K22A	For	AAACCTGAGCGCGGATATGTTTCTG
	Rev	TCAATACTAAAATAATATTCCAGC
FL D345A_Slr1b V3 D23A	For	CCTGAGCAAGGCGATGTTTCTGC
	Rev	TTTTCAATACTAAAATAATATTCCAG
FL D345R_Slr1bV3 D23R	For	CCTGAGCAAGCGCATGTTTCTGC
	Rev	TTTTCAATACTAAAATAATATTCCAG
FL K350A_Slr1b V3 K28A	For	GTTTCTGCGCGCGCACATGGATG
	Rev	ATATCCTTGCTCAGGTTTTTC
FL R368A_Slr1b V3 R46A	For	CAGTTTTAATGCGATTAAGAGTTTTAGCACCGATCT GAATC
	Rev	GCCAGAAAGGCCAGC
FL R419A_Slr1b V3 R97A	For	GAGCGAAAGTGCGCTGAAATTTGAAATGGC
	Rev	GGCAGAATCCACGGG
FL K421A_Slr1b V3 K99A	For	AAGTCGTCTGGCGTTTGAAATGGCCAAATATC
	Rev	TCGCTCGGCAGAATC
FL F422A_Slr1b V3 F100A	For	TCGTCTGAAAGCGGAAATGGCCAAATATCTC
	Rev	CTTTCGCTCGGCAGAATC
FL K426A_Slr1b V3 K104A	For	TGAAATGGCCGCGTATCTCGAGCACC
	Rev	AATTTTCAGACGACTTTTCG
FL Y427A_Slr1b V3 Y105A	For	AATGGCCAAAGCGCTCGAGCACC
	Rev	TCAAATTTTCAGACGACTTTTC

3.7. Materials and Methods

Cloning, protein expression and purifications

Slr1a (324-418: LaM alone), Slr1b (324-431: LaM +C-terminal amino acids) and Slr1c (299-431: LaM + N-and C-terminal amino acids) were each cloned into pET-28a(+) (*NdeI* (CATATG), *XhoI* (CTCGAG)) with an N-terminal 6x His tag by Gene Universal Inc. (Item NO:724861-1/2/3; Newark, DE, USA). A solubility- and expression-enhanced variant of Slr1b termed Slr1b V3 (please see Supplemental Figure 2A) was cloned into pET-29b(+) (*NdeI* (CATATG), *XhoI* (CTCGAG)) with a C-terminal 6x His tag by Gene Universal Inc. (Item NO: 7A036820-1; Newark, DE, USA). All cloned sequences were codon optimized for expression in *E. coli*. Mutants were made in Slr1b V3 using the Q5 Site-Directed Mutagenesis Kit (NEB E0554S) and according to the manufacturer's instructions.

Proteins were recombinantly expressed in *E. coli* BL21:DE3 cells and purified using nickel affinity chromatography as described previously (Mansouri-Noori *et al.*, Cell Rep., 2023)⁵⁷ with some modifications: Slr1a, Slr1b (V1) and Slr1c were incubated further at 18°C overnight (shaking at 200 rpm) following induction with 1 mM IPTG (Fisher BP1755-100). Since Slr1b V3 (as well as mutants made in V3) were better expressed and more stable, they were incubated at 37°C for 3 h with shaking (200 rpm) post-induction with 1 mM IPTG. Following growth, cells were pelleted and stored at -80°C until purification. Purifications were performed as described previously (Mansouri-Noori *et al.*, Cell Rep., 2023)⁵⁷.

For EMSAs, eluted proteins were buffered exchanged into low salt 1X EMSA buffer (20 mM Tris-HCl pH 7.6 (Fisher BP152-5), 100 mM KCl (Fisher BP366-1), 0.2 mM EDTA pH 8.0 (Sigma E5134-100G), 1 mM DTT (Bioshop DTT002.25)) using a pre-soaked 10 kDa Amicon Ultra-4 Centrifugal Filter Units (Millipore Sigma UFC803024) and centrifugation at 3,000xg, 4°C, as described earlier (Mansouri-Noori *et al.*, Cell Rep., 2023)⁵⁷. Each sample was concentrated into a final volume of 50–200 µl. 10% final concentration of glycerol (Bioshop GLY001.500) was added to the concentrated protein before storage at -80°C. Concentrations were obtained by measuring absorbance at 280 nm as described in (Mansouri-Noori and Bayfield, STAR Protoc., 2024)⁵⁸.

For NMR, uniformly isotopically labeled proteins were prepared from a 1 L culture containing M9 minimal media salts supplemented with 3 g of ¹³C (99%) glucose (Sigma-Aldrich), 1 g of ¹⁵N (99%) ammonium chloride (Sigma-Aldrich) and 0.5 g of ¹³C, ¹⁵N (99%, 99%) Celtone algal extract (Cambridge Isotopes). At an A600_{nm} of 0.8, protein expression was induced with 1 mM IPTG for 3 h with shaking (200 rpm) at 30 °C. A bacterial crude extract was prepared by lysing cells with a French press followed by 30 s of sonication in T300 buffer (20 mM Tris-Cl pH 7.6, 300 mM NaCl, 1 mM PMSF). After centrifugation (20,000xg for 15 min), the soluble protein was applied to a 5 mL nickel affinity column (Ni-INDIGO resin; Cube Bio, Monheim Germany) and washed extensively with T1300 buffer (20 mM Tris-Cl pH 7.6, 1300 mM NaCl, 1 mM PMSF). The protein was eluted by competition with T300 buffer supplemented with 400 mM imidazole. The protein was desalted and further purified on a HiPrep 16/60 S-100

HR gel filtration column (Cytiva) in NMR buffer (10 mM sodium phosphate pH 7.4, 60 mM NaCl). Protein concentration was estimated by UV absorbance at 280 nm.

Electrophoretic mobility shift assays (EMSAs)

PAC19 RNA (UUUUCCCACCACCAAACAC), PAC15 RNA (UUUUUCCACCACCAA) and PAC10 RNA (UUUUCCCACC) were radioactively labelled using $\gamma^{32}\text{P}$ ATP (Revvity BLU502A250UC) and T4 Polynucleotide Kinase (T4 PNK, NEB M0201S) and EMSAs were performed for Slr1b V1 and V3 wildtypes and all mutant V3 proteins as described previously (Mansouri-Noori *et al.*, Cell Rep., 2023; Mansouri-Noori and Bayfield, STAR Protoc., 2024)^{57,58}. Each set of EMSAs were performed for at least four times. RNA substrates were purchased as a standard RNA synthesis (5'OH) from Integrated DNA Technologies (IDT).

Protein alignments, software and programs

Clustal-W⁵⁹ was used for multiple sequence alignments and Jalview (Version 2)⁶⁰ was used for visualization. PyMol (Version 3.0, Schrödinger, LLC) was used for all structural visualizations and alignments. AlphaFold 2⁶¹ and AlphaFold 3⁶² were used for structure predictions. TM-align was used for protein structure comparisons and generating TM-Scores⁶³. The HDock Server was used for protein/RNA docking predictions⁶⁴.

NMR sample preparation and NMR spectroscopy

The protein concentration of samples for NMR spectroscopy ranged from 0.8-1.2 mM supplemented with 10% (v/v) D₂O. A series of 2D and 3D heteronuclear NMR spectra were acquired at 298 K on a 700 MHz Bruker AvanceIII spectrometer equipped with a nitrogen chilled probe. Datasets for backbone and side chain assignments included 2D-¹⁵N-HSQC, 2D-¹³C-HSQC, 3D-HNCACB, 3D-CBCACONH, 3D-HNCA, 3D-HNCOCA, 3D-HNCO, 3D-HNCACO, 3D-CCONH, 3D-HCCONH, 3D-HBHACONH, and 3D-CCH-TOCSY. The 3D experiments were acquired with a 10-15% sine-burst sampling schedule⁶⁵. NMR datasets were processed with NMRPipe⁶⁶ and HMSist⁶⁷. Distance restraints were derived from 3D-¹⁵N-NOESY and 3D-¹³C-NOESY experiments with a mixing time of 100 ms and 25% sparse sampling.

Structure determination

NMR spectra were analyzed with CCPN Analysis 2.52⁶⁸. Backbone dihedral angles were predicted from chemical shifts with TALOS-N⁶⁹. An initial ensemble of 100 structures sorted by the lowest number of NOE violations were calculated from a set of 5000 structures using CYANA 3⁷⁰. The ensemble was refined using Rosetta release 371 with distance, angle and hydrogen bond restraints converted to Rosetta .cst format⁷¹. The final ensemble of 20

structure solutions was selected based on lowest Rosetta score. Structural quality was assessed with PSVS⁷² and PROCHECK⁷³.

Free energy calculations

Initial models of Slr1p LaM domain with a 3-nucleotide (AAA) and 4-nucleotide (AAAA) RNA were built from the human LARP1 structures (PDB: 7SOR and 7SOV, respectively). Following a published Rosetta-based protocol^{74,75}, an ensemble of 100 refined models were calculated and scored with a function containing additional terms for RNA and RNA-protein interactions. The sample with the lowest energy score was selected for a $\Delta\Delta G$ calculation describing the free energy difference between the free and RNA-bound states.

Mutagenesis primers

Back-to-back primers, which were used with the Q5 Site-Directed Mutagenesis Kit (NEB E0554S), were designed using NEBaseChanger (NEBaseChanger.neb.com). See Supplemental Table 1.

3.8. References

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Chapter 4: Slr1p function in mRNA translation is related to a role in adaptation to stress and its phosphorylation states

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Contributions: F.M. and C.D.S.D. (collaborator) conducted the TCP-Seq, C.D.S.D. and J.M. (collaborator) performed the bioinformatics, F.M. analyzed the data and made figures. With respect to all other presented work, F.M. conceptualized the studies, designed and conducted experiments, collected data, conducted formal analysis, made figures and wrote the first draft of the manuscript. M.A.B. supervised the project, helped with designing experiments and formal analysis, reviewed and revised the original draft of the manuscript. F.M. and M.A.B. edited the writing.

The sections and headings in this chapter are structured to align with the formatting guidelines of *Nucleic Acids Research* journal.

4.1. Abstract

La and La-related proteins are a family of conserved RNA-binding proteins with various functions in RNA metabolism. LARP1 family members have been widely suggested to function in mRNA translation and stability. Their main RNA substrates include a special subset of mRNAs known as 5' terminal oligo pyrimidine (5'TOP) mRNAs which code for factors involved in the process of mRNA translation such as ribosomal proteins. The *S. pombe* LARP1 ortholog, Slr1p, lacks the characteristic DM15 region that in humans allows for specific binding to 5'TOP mRNAs. Nevertheless, Slr1p functions in translation and stability of the same cohort of mRNAs in *S. pombe* even as these mRNAs also lack the 5'TOP motif. How Slr1p may be involved with these processes under stress or different modification states remains unclear. We aimed to identify stress conditions where *slr1*- cells exhibit growth defects, and tested how Slr1p's phosphorylation states at two serine residues may affect this. Our data indicate that *slr1*- cells are sensitive to the effects of the translation initiation inhibitor harringtonine and the histidine biosynthesis inhibitor 3-AT. Interestingly, *slr1*- cells transformed with phosphoablative mutants of Slr1p at serine-410 and serine-437 appear to be partially resistant to the negative effects of 3-AT. Additionally, we've employed selective translation-complex profiling coupled with sequencing (TCP-Seq) to outline Slr1p's function in different stages of mRNA translation. Our findings suggest that Slr1p is involved with both mRNA translation initiation and elongation, interacting with different cohorts of mRNAs at each stage. Our data are consistent with an evolutionary conserved function for Slr1p in mRNA translation that is related to its role in adaptation to stress and different phosphorylation states.

4.2. Introduction

La-related protein 1 (LARP1) family members regulate the translation and stability of mRNAs that code for factors important in the process of mRNA translation in several experimental systems. These mRNAs are characterized in higher eukaryotes to contain a 5' terminal oligopyrimidine (5'TOP) sequence immediately after the m⁷G mRNA cap, but the motif is missing in yeast and *C. elegans* mRNAs coding for translational factors^{1,2}. Human LARP1 (hLARP1) contains a C-terminally located DM15 domain that specifically recognizes the 5'TOP sequence³. However, the short form of LARP1, found in yeasts and *Arabidopsis*, is missing the DM15 region and only contains a La motif (LaM)⁴. LARP1 proteins have also been reported to have functions related to stress adaptation⁵⁻⁸ and mitochondrial fitness⁹⁻¹⁴. In *S. cerevisiae*, the two LARP1 paralogs, Slf1p and Sro9p, have been implicated in resolving ribosome collisions in response to oxidative stress¹⁵. We have previously demonstrated that Slr1p has important functions in the process of mRNA translation specifically with respect to regulating the translation and stability of mRNAs that are functionally similar to 5'TOP mRNAs, coding for ribosomal proteins in *S. pombe*¹⁶. We've hypothesized that the dual function of Slr1p in mRNA translation and decay, plus its interaction with decay factors such as XRN1, may be modulated through modification states or stress conditions, even as *slr1*-cells do not exhibit any growth defects under normal conditions. In order to test how *slr1*-cells respond to stress, we examined growth under oxidative stress, glucose starvation, heat shock or amino acid starvation. We also treated *slr1*-cells with translation inhibitors such as harringtonine and Torin1. Our results suggest that *slr1*-cells survive an acute stress similarly to wildtype cells while prolonged stress leads to detectable growth defects. When *slr1*-cells

were grown in the presence of the translation initiation inhibitor harringtonine or the histidine biosynthesis inhibitor 3-amino-1,2,4-triazole (3-AT), especially when these cells were grown past stationary phase, they were more susceptible to the effects of these drugs in comparison to wildtype cells. Western blot analysis showed that Slr1p is expressed and phosphorylated at serine residues 410 and 437 during log phase growth. In contrast, during the stationary phase, Slr1p expression may be minimal and phosphorylation at these residues is undetectable.

We also performed selective translation profiling coupled with next-generation sequencing¹⁷ which revealed that Slr1p is involved with both the initiation and elongation stages of mRNA translation. Immunoprecipitation of Slr1p, when it sediments with 40S ribosomal fractions, enriches for transcripts that code for ribosomal proteins and other factors involved in the process of mRNA translation. Additionally, immunoprecipitation of Slr1p from the 80S ribosomal fractions leads to enrichment of transcripts coding for factors involved with mitochondrial functions. In this work, we present intriguing insights into the function of Slr1p in *S. pombe* with relevance to various roles described in other eukaryotes. Our studies elucidate the involvement of Slr1p in adaptation to stress which appears to be related to its functions in different stages of mRNA translation and therefore interaction with different subsets of mRNAs at each stage.

4.3. Results

4.3.1. *slr1*- cells are sensitive to the effects of translation inhibitors harringtonine and 3-AT

We've previously demonstrated that Slr1p has important functions in mRNA translation¹⁶ even as *slr1*- cells have no apparent growth defect. To test whether *slr1*- cells are sensitive to specific drugs or stresses we performed yeast spotting assays in various conditions. We first tested the recovery of wildtype yAS99 and *slr1*- cells on rich media (Yeast Extract + Supplements, YES) post oxidative stress (0.5 mM H₂O₂, 15 min at 32°C), glucose starvation (15 min growth in minus glucose media at 32°C), heat shock (1 hr at 37°C) or amino acid starvation (10 mM 3-AT, 1 hr at 32°C). Wildtype and *slr1*- cells growing at log phase (OD₆₀₀= 0.6) were treated as described in Figure 1A, harvested, and an OD₆₀₀= 1 equivalent (approximately 1.5 x 10⁷ cells) from each condition was diluted in 1 millilitre of sterile water. Following four successive 1 in 10 dilutions, equal volumes of cells from each dilution were spotted on YES plates. Comparison of growth between unstressed and stressed conditions suggested that *slr1*- cells recover to the same extent as wildtype cells after each indicated type of stress (Figure 1A). Additionally, wildtype and *slr1*- yeast cells appear to grow to the same extent at elevated temperatures (37°C) even after oxidative stress, glucose or amino acid starvation (Figure 1B). Furthermore, wildtype and *slr1*- cells harvested at log phase (OD₆₀₀= 0.6) or past stationary phase (3 days in YES at 32°C, shaking at 200 rpm) grew to the same extent on normal YES plates, containing 3% glucose, or low glucose plates with 0.75% glucose. These results suggest that in the absence of Slr1p, yeast cells that undergo the indicated types of stress can recover and grow normally if returned to optimal conditions.

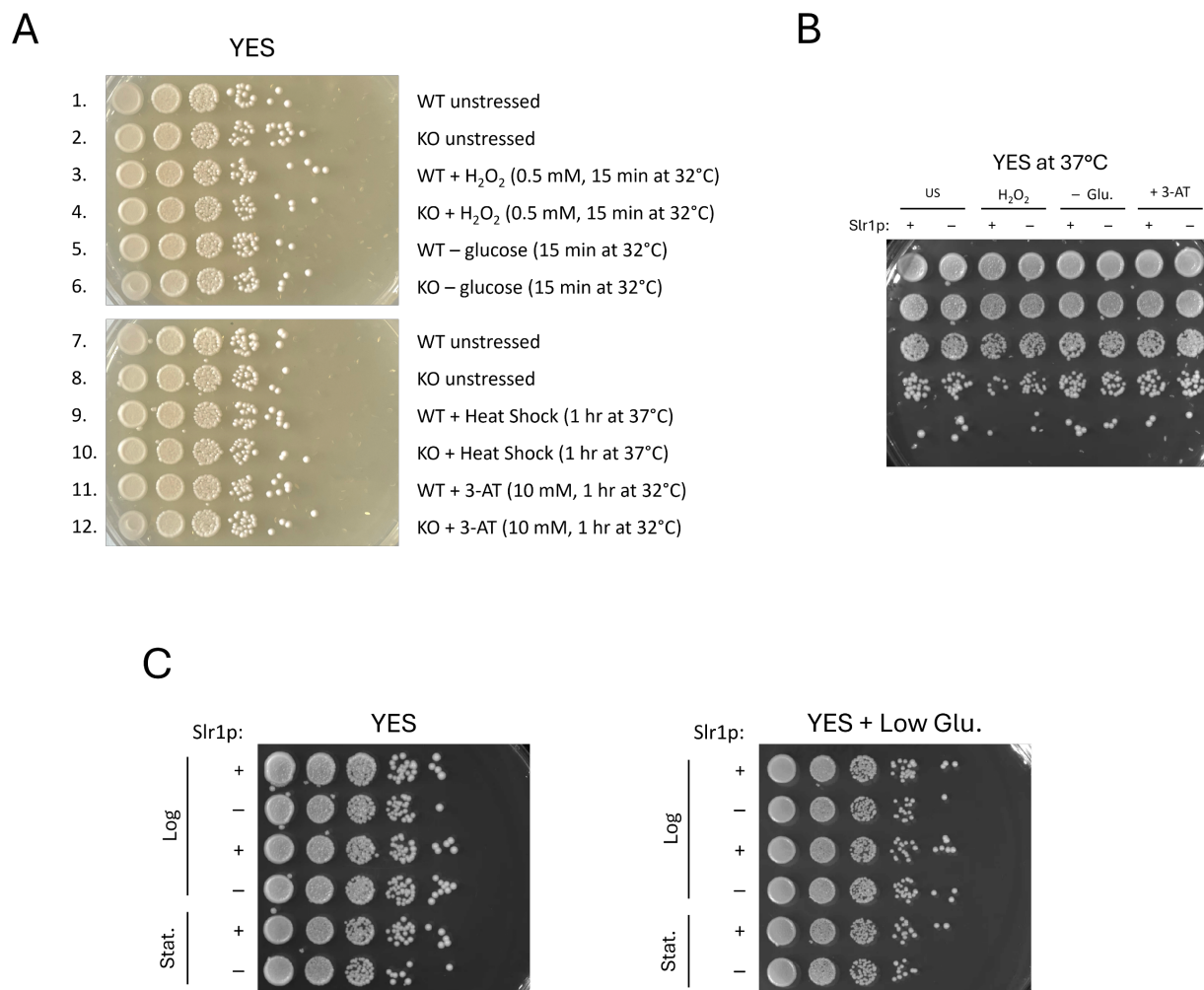
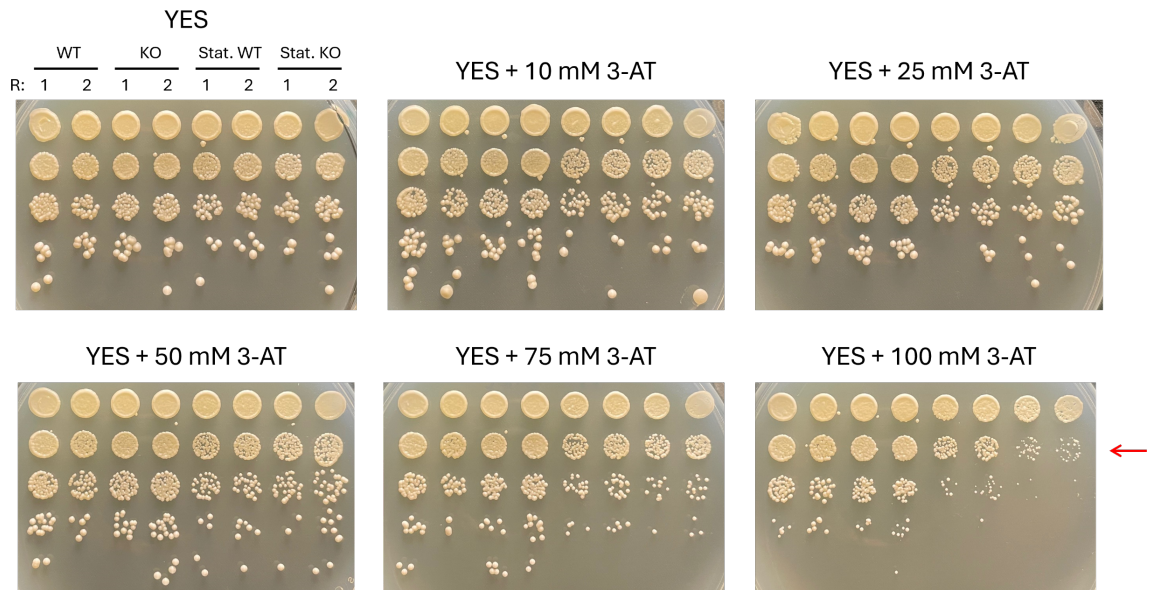


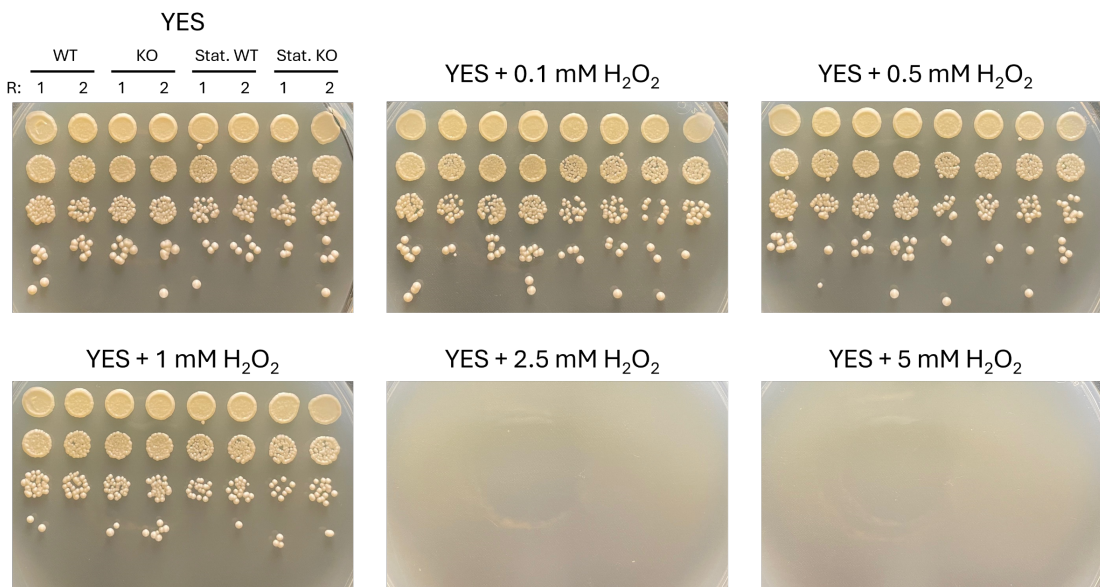
Figure 1: Spotting assays of wildtype and *slr1*- cells and recovery on rich media (YES) post stress. **A) Recovery after several stress conditions was tested where wildtype (WT) and *slr1*- (KO) cells were grown in YES with shaking (32°C, 200 rpm) and stressed as indicated. An OD₆₀₀ = 1 equivalent (approximately 1.5 x 10⁷ cells) from each condition was diluted in 1 millilitre of sterile water. Following four successive 1 in 10 dilutions, equal volumes of cells from each dilution were spotted from left to right. For the minus glucose condition (rows 5 and 6), cells were removed out of YES + glucose, washed with sterile water and grown in YES – glucose media for 15 minutes. Cells in rows 3, 4 and 9-12 were harvested after the incubation time indicated. **B)** Unstressed (US) or stressed (as indicated in A) WT and KO cells were spotted top to bottom on a YES plate that was incubated at 37°C. **C)** Two biological replicates of each of WT and KO cells harvested at log phase (OD₆₀₀ = 0.6) and one replicate of each of WT and KO cells grown past stationary phase (3 days in YES at 32°C, 200 rpm) were spotted on normal YES plates containing 3% glucose (left) or low glucose plates with 0.75% of glucose (right). Cells were spotted from left to right.**

We then wanted to test how *slr1*- cells would grow in comparison to wildtype cells during prolonged stress. For this, we performed spotting assays using YES plates containing increasing concentrations of various drugs. Two biological replicates of each of yAS99 and *slr1*- strains either growing at log phase ($OD_{600} = 0.6$) or past stationary phase (3 days in YES at 32°C, 200 rpm) were harvested and diluted as described above. Spotting assays were performed on YES + 3-AT (10-100 mM), YES + H_2O_2 (0.1-5 mM), YES + harringtonine (2-50 μ g/mL) and YES + Torin1 (2-4 μ M) plates (Figure 2). Treatment with 3-AT leads to histidine starvation and consequently translation elongation defects due to uncharged histidyl-tRNAs¹⁸ while harringtonine affects the initiation step of mRNA translation¹⁹. Torin1 inhibits both target of rapamycin complexes TORC1 and TORC2²⁰ in yeast, and therefore inhibits overall protein synthesis, cell growth and proliferation. Our spotting assay results showed that on rich media, *slr1*- cells that were grown past stationary phase are more sensitive to the effects of 3-AT (Figure 2A) and harringtonine (Figure 2C) compared to wildtype cells. We've previously shown that treatment with harringtonine leads to polysome profiles of *slr1*- cells which show an increase in the height of the 80S peak and a diminishing trend of heavy polysomes¹⁶. Therefore, our spotting assays further confirm that *slr1*- cells are sensitive to harringtonine, consistent with Slr1p functioning in mRNA translation initiation. Intriguingly, the phenotype we observed with 3-AT suggests that in addition to initiation, Slr1p may be involved with the elongation step of mRNA translation.

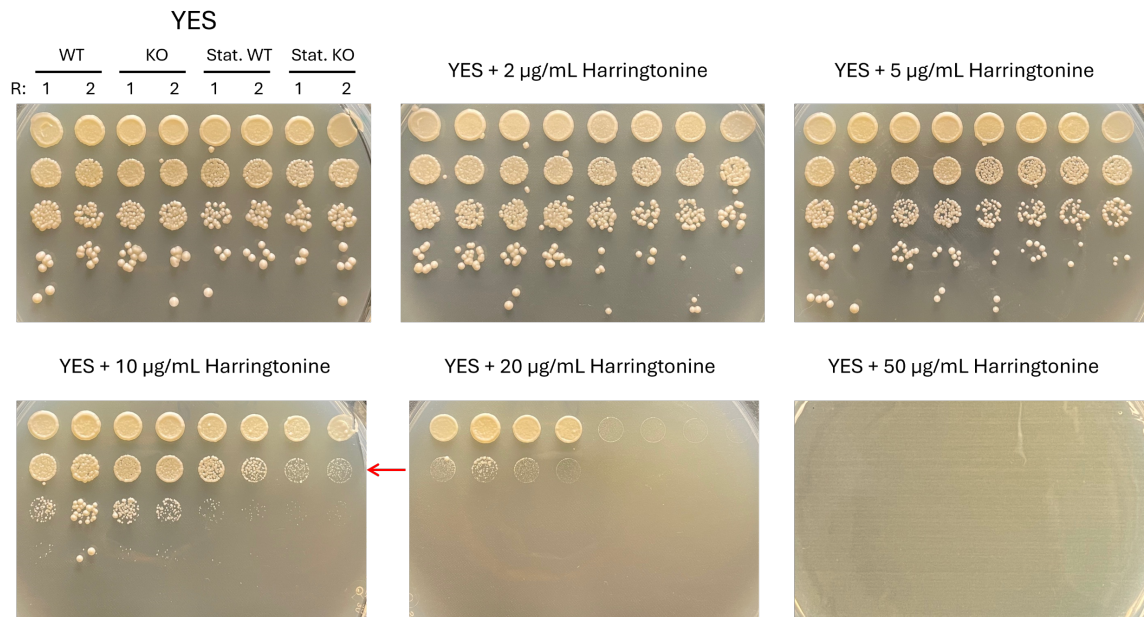
A



B



C



D

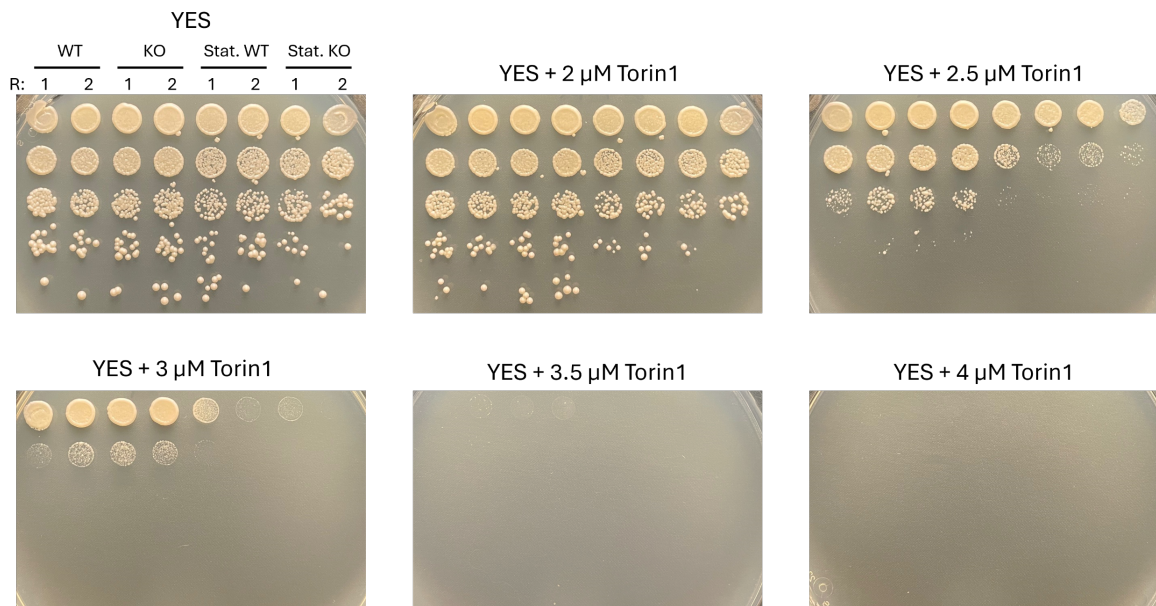


Figure 2: Slr1p knockout cells are sensitive to the effects of translation inhibitors harringtonine and 3-AT when grown in rich media. A) YES plates containing increasing concentrations of 3-AT were spotted with two biological replicates of each of wildtype (WT) and *slr1*- (KO) cells plus two replicates of WT and KO cells grown past stationary phase (3 days in YES at 32°C, 200 rpm). An OD₆₀₀ = 1 equivalent (approximately 1.5 x 10⁷ cells) from each condition was diluted in 1 millilitre of sterile water. Following four successive 1 in 10 dilutions, equal volumes of cells from each dilution were spotted from top to bottom. The

red arrow indicates a visible difference in *slr1*- growth. **B)** spotting assays with YES plates containing increasing concentrations of H₂O₂, **C)** harringtonine **D)** and Torin1. R= Replicate.

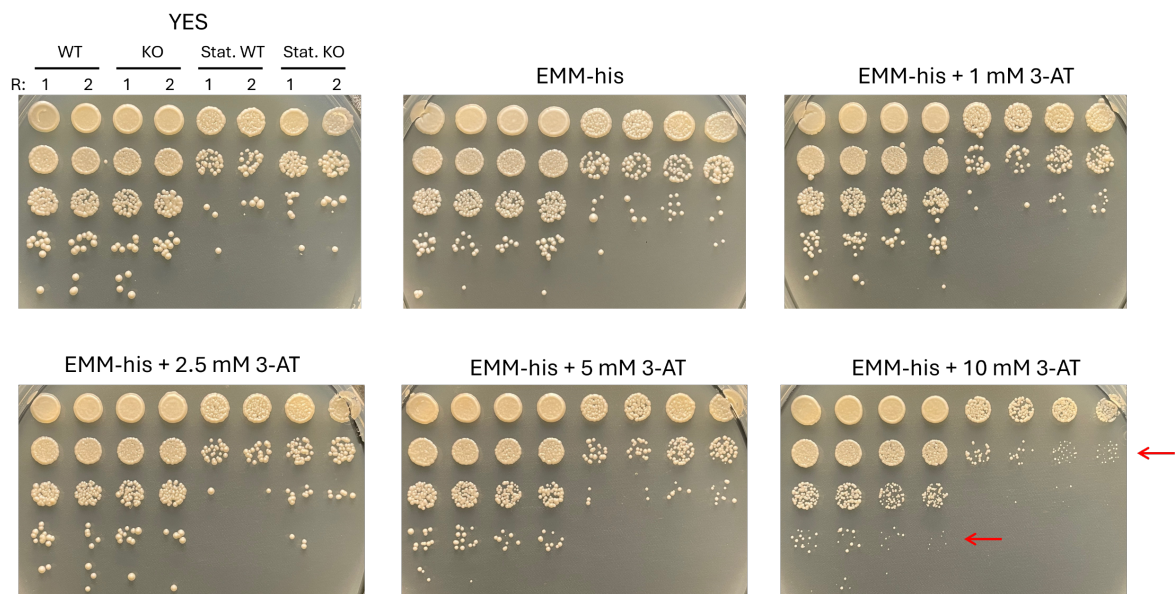
Since our YES media is supplemented with histidine, we wanted to test if the effects we observed with 3-AT are recapitulated in minimal media (Edinburgh Minimal Media, EMM) lacking histidine. We repeated our spotting assays with two biological replicates of each of yAS99 and *slr1*- strains either growing at log phase (OD₆₀₀= 0.6) or past stationary phase (7 days in EMM at 32°C, 200 rpm) which were harvested and diluted as explained before. Diluted samples were spotted on YES (for control) and EMM minus histidine (EMM-his) plates (Figure 3A). Interestingly, we noticed that a lower concentration of 3-AT led to the same growth defect we observed with stationary *slr1*- cells growing on YES + 3-AT plates and that at a concentration of 10 mM 3-AT, we also start to see a growth defect for log phase *slr1*- cells. These results confirm that *slr1*- cells are sensitive to the effects of 3-AT and that Slr1p may be involved with mRNA translation elongation as it has been suggested for the *S. cerevisiae* LARP1 orthologs, Slf1p and Sro9p.

4.3.2. Unphosphorylated Slr1p may aid in cell survival during stress

In order to test if the phosphorylation state of Slr1p affects the phenotype we observed with 3-AT, we transformed *slr1*- cell with pRep81x vector (leu+) or pRep81x vector containing wildtype full-length Slr1p, or full-length Slr1p mutated at serine-410 (“S410A”) or serine-437 (“S437A”). These residues were previously reported to be highly phosphorylated (S410) or phosphorylated specifically in a TOR-dependent manner (S437)²¹. We repeated our spotting

assays on EMM-leu-his plates as described earlier and using transformed *slr1-* cells. We tested growth on plates containing 5-20 mM 3-AT and observed that *slr1-* cells transformed with empty vector exhibit a growth defect at 10 mM as previously shown. Further increasing the concentration of 3-AT exacerbated this phenotype (Figure 3B). Interestingly, *slr1-* cells transformed with Slr1p S410A and S437A phosphoablative mutants appeared to survive better in the presence of increasing concentrations of 3-AT even in comparison to *slr1-* cell transformed with wildtype Slr1p. These observations suggest that in the absence of phosphorylation at serine-410 or serine-437, Slr1p may have a protective pro-survival function in response to 3-AT.

A



B

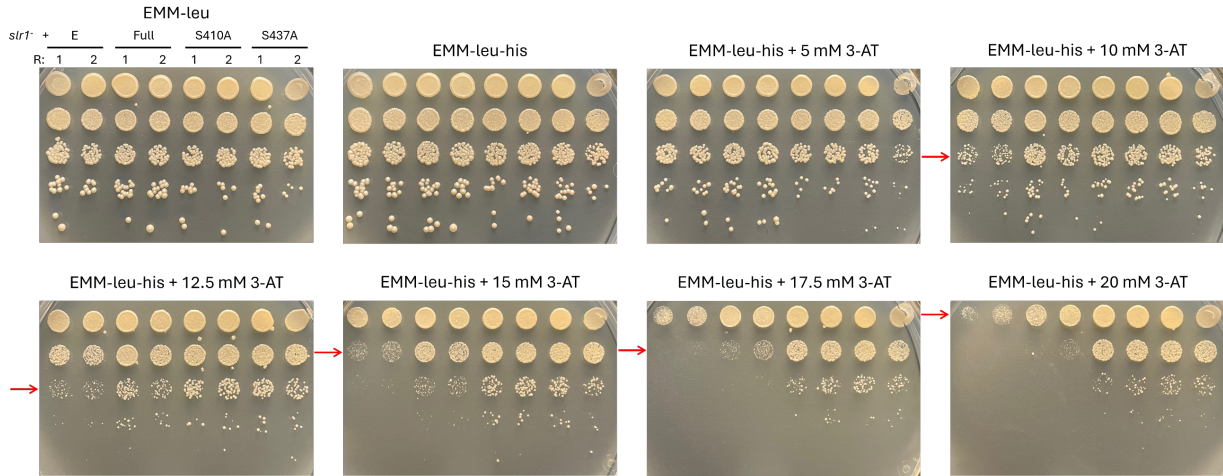


Figure 3: Phosphoablative mutants of Slr1p appear less sensitive to the effects of 3-AT.

A) Two biological replicates of each of wildtype (WT) and *slr1*- (KO) cells harvested at OD₆₀₀= 0.6 plus two replicates of WT and KO cells grown past stationary phase (7 days in EMM at 32°C, 200 rpm) were spotted on EMM-his plates and increasing concentrations of 3-AT. A defect in growth is visible at 10 mM 3-AT with both log phase and stationary *slr1*- cells (red arrows). As a control, spotting assays were also performed on a rich media YES plate. R= Replicate. **B)** *slr1*- cells were transformed with pRep81x empty vector (leu+; “E”), pRep81x-Slr1p Full WT (“Full”), pRep81x-Slr1p Full S410A (“S410A”) or pRep81x-Slr1p Full S437A (“S437A”) phosphoablative mutants. Two biological replicates of each were grown in normal conditions (EMM-leu, 32°C, 200 rpm), harvested at OD₆₀₀= 0.6 and spotted top to bottom on EMM-leu or EMM-leu-his plates containing increasing concentrations of 3-AT. An OD₆₀₀= 1 equivalent (approximately 1.5 x 10⁷ cells) from each condition was diluted in 1 millilitre of sterile water. Following four successive 1 in 10 dilutions, equal volumes of cells from each dilution were spotted from top to bottom. *slr1*- cells transformed with empty vector show diminishing growth at 10-20 mM 3-AT (red arrows) while cells transformed with either S401A or S437A mutant appear to survive better with increasing concentration of 3-AT.

We further wanted to query the expression levels of Slr1p during log and stationary phases, with and without 3-AT treatment. We first grew wildtype yAS99 cells in normal conditions (YES, 32°C, 200 rpm) to log phase (OD₆₀₀= 0.6) or past stationary phase (3 days at 32°C, 200 rpm) and treated them with 50 or 100 mM 3-AT (1 hr, 32°C, 200 rpm). Cells were harvested and snap-frozen prior to lysis and western blot analysis. We probed for Slr1p using two

antibodies that were previously raised against the protein¹⁶. Antibody 2648 was raised using recombinantly expressed and purified full-length Slr1p while antibody 2756 was raised using a synthetic peptide corresponding to the C-terminal end of the protein (amino acids 458–475 with the sequence DSIIKRDVQTEVEDKLTV). We also probed for phosphorylated Slr1p at either S410 or S437 using custom-made phospho-specific antibodies. Our western blot analysis suggested that Slr1p is expressed and phosphorylated at both S410 and S437 residues during log phase growth (Figure 4A). Treating the cells with an increased concentration of 3-AT (50 to 100 mM) did not seem to affect the expression or the phosphorylation status of Slr1p at the indicated residues. Conversely, it appeared that Slr1p is either not expressed in stationary phase or that it is present at low levels which cannot be detected using western blotting. We repeated these experiments using wildtype yAS99 cells grown in normal conditions (YES, 32°C, 200 rpm) to log phase (OD_{600} = 0.6) or past stationary phase (3 days at 32°C, 200 rpm) which were then either not treated or treated with 100 mM 3-AT (1 hr, 32°C, 200 rpm) (Figure 4B). Western blotting, following an overnight transfer, revealed a number of bands in lanes corresponding to cells harvested past stationary phase when we probed for Slr1p with antibody 2756 (Figure 4B). It is possible that these bands correspond to lowly expressed Slr1p in stationary phase. Furthermore, if Slr1p is expressed during the stationary phase, it still doesn't seem to be phosphorylated at S410 or S437. Overall, these data suggest that Slr1p is not expressed to the same extent as in the log phase during the stationary phase, and that the protein is phosphorylated at S410 and S437 during log phase growth.

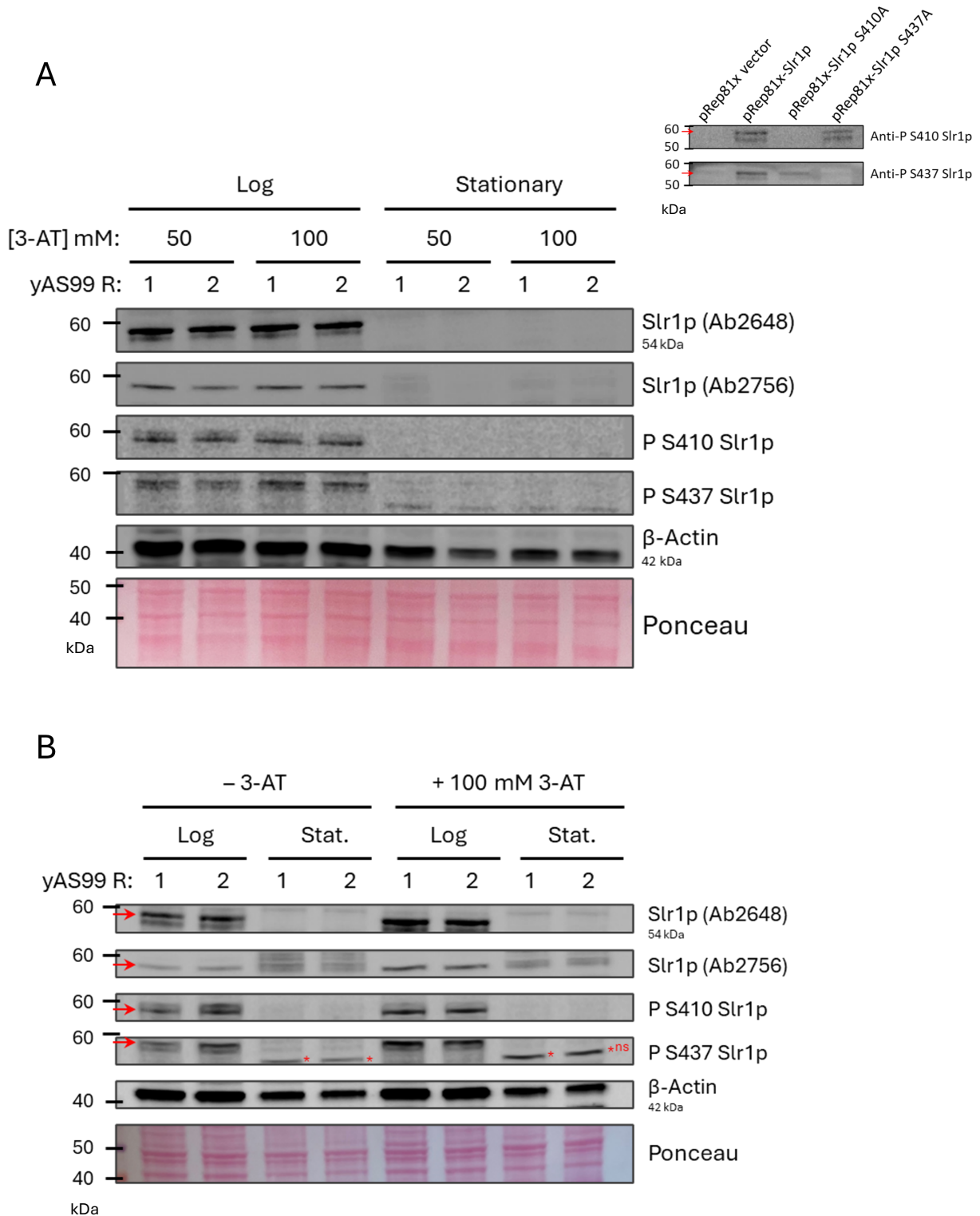


Figure 4: Western blot analysis of Slr1p expression in log and stationary phase plus 3-AT treatment. A) Wildtype yAS99 cells were grown in normal conditions (YES, 32°C, 200 rpm) to log phase (OD_{600} = 0.6) or past stationary phase (3 days at 32°C, 200 rpm) and treated with

the indicated concentration of 3-AT (1 hour, 32°C, 200 rpm). Cells were harvested and lysed, and an equal amount of total protein was loaded per lane for western blot analysis with a 90-minute transfer at room temperature. Two yAS99 biological replicates (R) are presented in each case. Blots were probed with two antibodies raised against Slr1p (Ab2648 and Ab2756) and two phospho-specific antibodies against phosphorylated serine-410 and serine-437. β -actin and the Ponceau stain are shown for loading controls. Top inset: validations for the phospho-specific antibodies. *slr1*⁻ cells were transformed with the indicated construct including the empty vector, full-length wildtype Slr1p and mutated Slr1p at S410 or S437. Blots were probed with either of the phospho-specific antibodies as indicated. Red arrows correspond to size of the full-length Slr1p. **B)** Wildtype yAS99 cells were grown in normal conditions (YES, 32°C, 200 rpm) to log phase (OD₆₀₀ = 0.6) or past stationary phase (3 days at 32°C, 200 rpm) and were either not treated or treated with 100 mM of 3-AT (1 hour, 32°C, 200 rpm). Harvested cells were lysed and an equal amount of total protein was loaded per lane for western blot analysis with an overnight transfer at 4°C. Red arrows point to the correct band representing full-length Slr1p. Asterisks mark non-specific bands (ns).

4.3.3. TCP-Seq shows Slr1p interacts with different subsets of mRNAs during various stages of mRNA translation

Since we've previously shown that Slr1p interacts with translation initiation factors and with 5'UTR motifs suggesting functions related to mRNA translation initiation, we wanted to explore its involvement with translation initiation or elongation more directly. For this reason, we employed translation-complex profiling coupled with sequencing (TCP-Seq)¹⁷. This is a ribosome profiling technique where factors of interest that sediment with either 40S or 80S fractions can be immunoprecipitated from the respective fraction to purify associating RNAs (Figure 5A). Extracted RNAs can be sequenced thereafter to inform whether the protein of interest is involved with the initiation or the elongation step of mRNA translation. Total yeast cell lysates were initially subjected to RNase I digestion, loaded onto sucrose gradients and fractionated post ultracentrifugation. Fractions corresponding to the 40S peak or the 80s peak were pooled prior to immunoprecipitation of Slr1p. We used antibody 2756 for

immunoprecipitation from each set of pooled fractions. Ribosome-protected RNA fragments co-immunoprecipitated with Slr1p were then extracted and sized selected, using gel purification, prior to library preparations and sequencing. As a control, *slr1*- cells were also subjected to the same pipeline where antibody 2756 was used for a mock immunoprecipitation. Two biological replicates were sampled in each case.

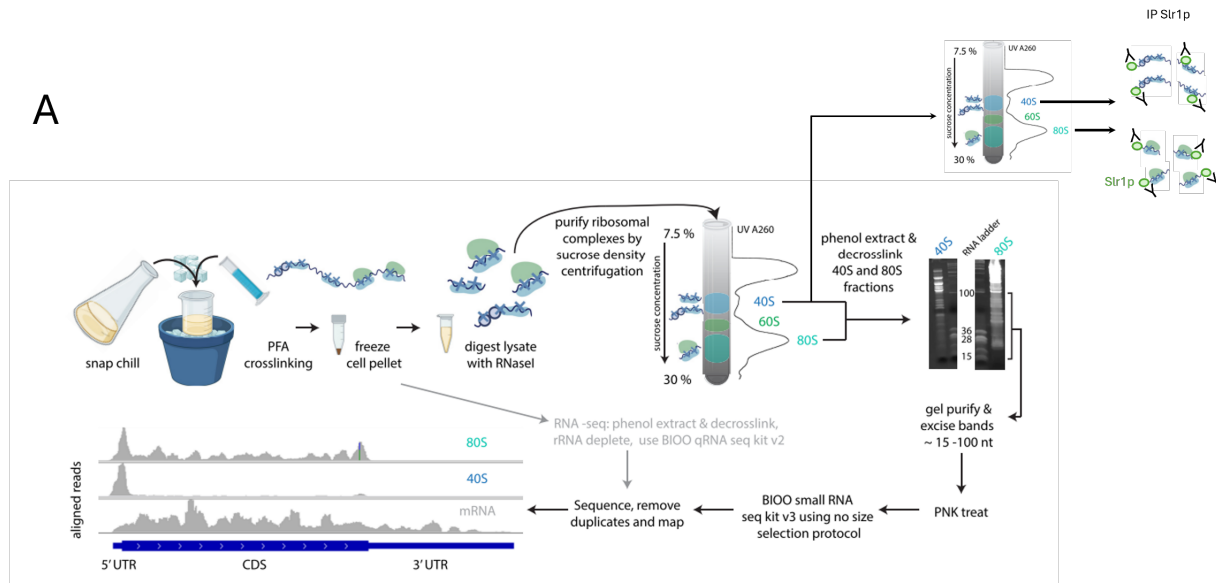
After subtraction of non-specific reads obtained with immunoprecipitation from *slr1*- cells, analysis of reads from wildtype cells showed that Slr1p- and 40S-associated ribosome footprints correspond to mRNA 5'UTRs and around the start AUG, as expected. Additionally, several reads were mapped to the coding sequence in this case. Previous studies report similar observations where this is thought to correspond to poor crosslinking of 60S subunits to recently initiated 40S subunits, that are consequently detached during purification processes^{17,22}. With respect to Slr1p immunoprecipitations from 80S fractions, the majority of the reads mapped to the coding sequence and closer to the stop codon (Figure 5B), as expected.

Gene ontology analysis suggested that transcripts immunoprecipitated with Slr1p, sedimenting with 40S fractions, with reads mapping to the 5'UTR are generally enriched for functions related to metabolism (Figure 5C), for which we've also previously seen related enrichments in our RNA-Seq, RATE-Seq and RIP-Seq analyses¹⁶. Intriguingly, transcripts with reads around the start AUG are enriched for translation-related GO terms. Most of these transcripts code for ribosomal proteins and we have previously shown that such mRNAs are enriched for a 5' Proximal AC-rich (5'PAC) motif, residing close to the AUG, that Slr1p

recognizes and directly binds to. Additionally, 40S-immunoprecipitated transcripts with reads in the coding sequence are enriched for GO terms related to translation, and as discussed, these correspond to initiated ribosomes that lost a recently recruited but poorly crosslinked 60S subunit. Therefore, transcripts with reads in the coding sequence in this case reflect the same GO terms observed for the AUG bin (Figure 5C).

A GO term analysis of transcripts that were enriched in Slr1p immunoprecipitations from 80S fractions, with reads in the coding sequence, returned terms for mitochondrial functions (Figure 5D). Interestingly, this is consistent with previous data reported for the *S. cerevisiae* LARP1 orthologs, Slf1p and Sro9p, which have been proposed to aid in resolving ribosome collisions during oxidative stress¹⁵. Therefore, our findings suggest that Slr1p may have an additional evolutionary conserved function, as reported in other systems, in mRNA translation elongation which is more specifically related to a subset of mRNAs such as those coding for mitochondrial factors. This may be also related to phenotypes we've observed for our *slr1*- strain which exhibits a growth defect with the drug 3-AT. Lastly, querying 12 enriched transcripts with reads around the stop codon returned translation-related GO terms (Figure 5D). Overall, our TCP-Seq analysis confirms that Slr1p functions in multiple steps of mRNA translation. Our data are informative in terms of identifying different subsets of mRNAs that Slr1p may interact with, depending on the region that it may be bound to on an mRNA. It is also possible that Slr1p interacts with ribosomes or other factors as they move along a transcript during translation.

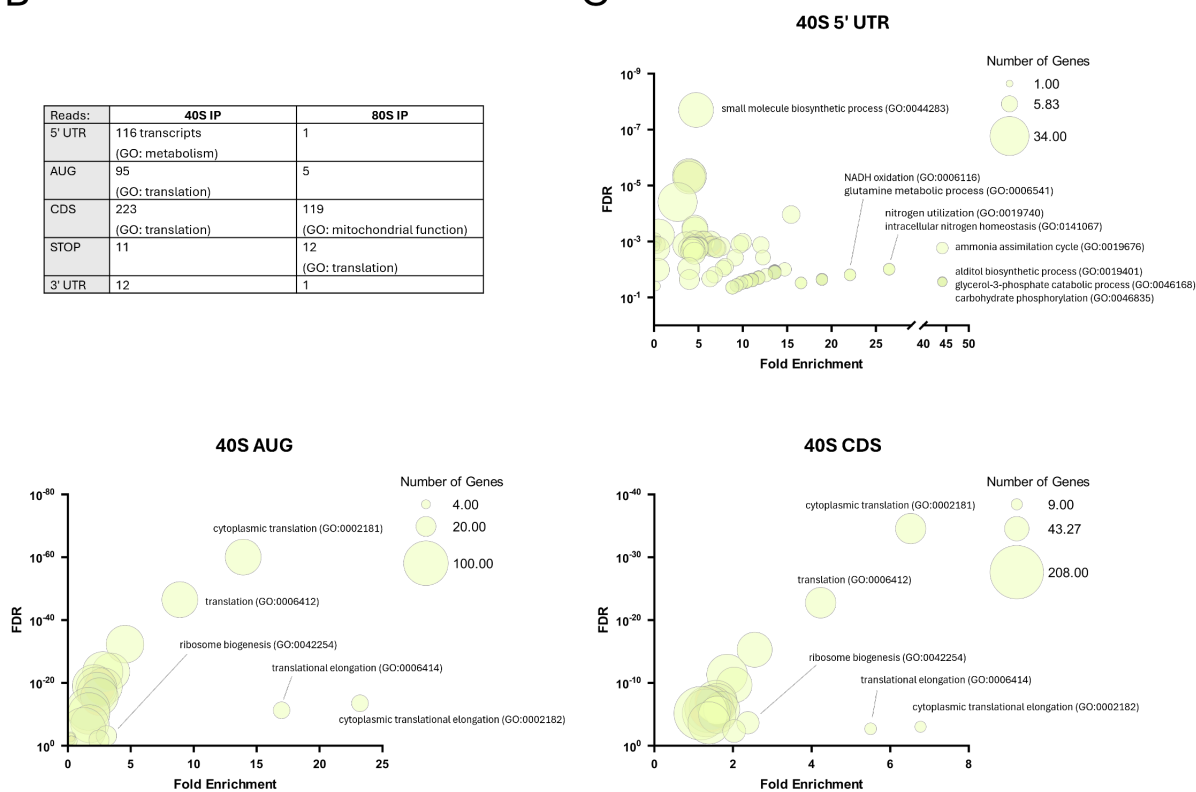
A



B

Reads:	40S IP	80S IP
5' UTR	116 transcripts (GO: metabolism)	1
AUG	95 (GO: translation)	5
CDS	223 (GO: translation)	119 (GO: mitochondrial function)
STOP	11 (GO: translation)	12 (GO: translation)
3' UTR	12	1

C



D

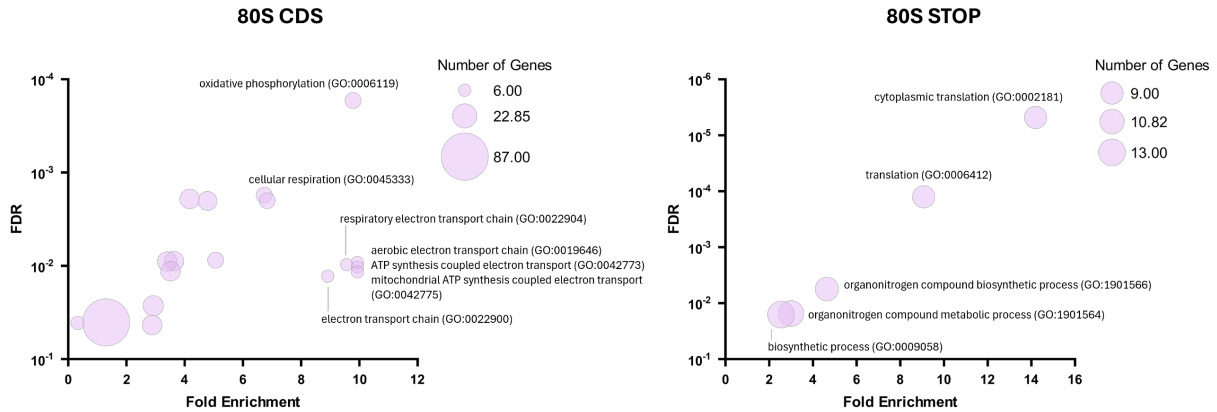


Figure 5: Translation-complex profiling coupled with sequencing (TCP-Seq) shows Slr1p interacts with different subsets of mRNAs depending on the region that it binds to on target RNAs. Prototrophic wildtype yAS99 cells were grown in normal conditions (EMM, 32°C, 200 rpm) and harvested at log phase ($OD_{600} = 0.6$). **A)** A ribosome profiling technique optimized for fission yeast (Adapted from Duncan and Mata, 2022)¹⁷ was employed to immunoprecipitate Slr1p from either 40S or 80S ribosomal fractions post polysome profiling. Whole-cell lysates were digested with RNase I prior to sucrose gradient ultracentrifugation. Slr1p-immunoprecipitated RNAs were extracted, gel purified and sequenced following library preparations. An equivalent ribosome profiling, immunoprecipitation and RNA extraction protocol was performed for *slr1*- cells as a control. Results shown are indicative of two biological replicates. **B)** Slr1p-immunoprecipitated RNAs (enriched in yAS99 cells over *slr1*- cells) from 40S fractions were aligned to sequences in 5' untranslated regions (5'UTR), closer to the start AUG and coding sequences (CDS) whereas RNAs immunoprecipitated from 80S fractions mainly aligned to the CDS and closer to the stop codon. Groups enriched for a main set of gene ontology (GO) terms are indicated. **C)** Transcripts with reads that were aligned to 5'UTRs in the 40S IPs were enriched for metabolism-related terms while those with reads closer to the start codon and CDS were enriched for translation-related GO terms. **D)** Transcripts with reads that were aligned to the CDS in the 80S IPs were enriched for terms related to mitochondrial function, and the 12 transcripts with reads closer to the stop codon were enriched for translation-related GO terms.

4.4. Discussion

Previous work on characterizing Slr1p suggests that it functions in controlling the translation and stability of a special subset of mRNAs coding for ribosomal proteins and translation

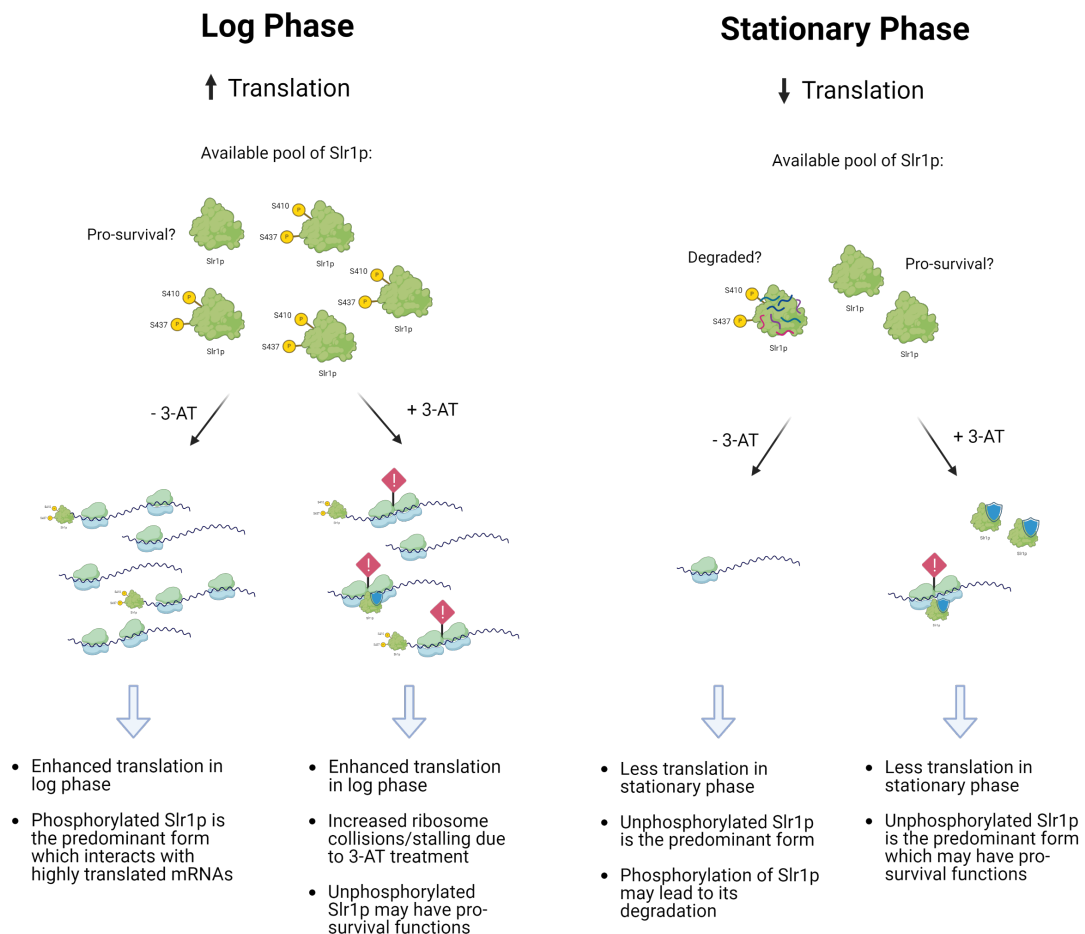
factors in *S. pombe*¹⁶. Data presented in this section further confirms these findings while also supporting a role for Slr1p in mRNA translation elongation in addition to initiation. To examine different phenotypes of *slr1*- cells, we assessed how this strain responds to several growth conditions, stresses and drugs. We tested how wildtype and *slr1*- cells grow following oxidative stress, glucose starvation, heat shock, amino acid starvation and in response to the translation initiation drug harringtonine or the TOR inhibitor Torin1. Our data suggest that *slr1*- cells recover to the same extent as wildtype cells following acute oxidative stress, glucose starvation, heat shock and amino acid starvation (Figure 1). However, *slr1*- cells, grown in rich media and past stationary phase, show a growth defect in increasing concentrations of harringtonine and 3-AT (Figure 2), arguing for Slr1p playing a role in mRNA translation initiation as well as elongation, respectively. We were also able to show that *slr1*- cells growing in minimal media, not supplemented with histidine, are more sensitive to lower concentrations of 3-AT (10-20 mM) even after being harvested at log phase (Figure 3A). Interestingly, *slr1*- cells transformed with phosphoablative mutants of Slr1p at S410 and S437 residues appear less sensitive to the effects of 3-AT, suggesting that the unphosphorylated form of Slr1p at these residues may have a pro-survival function (Figure 3B). Western blot analysis showed that Slr1p is expressed and phosphorylated at both serine residues during log phase growth (Figure 4), which is when mRNA translation happens at elevated levels. In contrast, Slr1p may be minimally expressed or mostly degraded during the stationary phase where phosphorylation at S410 and S437 residues was not detected by western blotting. This is consistent with our spotting assays where stationary phase wildtype cells, potentially expressing low levels of unphosphorylated Slr1p, exhibit a less substantial

growth defect in response to 3-AT compared to *slr1*- cells (Figure 2A). While the phosphorylated form of Slr1p may allow for enhanced translation of ribosomal protein mRNAs, a generally elevated rate of translation during the log phase could also lead to higher susceptibility of the cells to the effects of 3-AT (Figure 6). It is also possible that prior phosphorylation at the indicated serine residues leads to the degradation of the protein in stationary cells. Our data suggest that unphosphorylated Slr1p, at S410 and S437, leads to improved cell survival following prolonged stress caused by 3-AT treatment (Figure 3B; Figure 6). It would be interesting to test if ectopic expression of phosphomimetic mutants of Slr1p (S410D/E and S437D/E) in *slr1*- cells recapitulates the wildtype phenotype in Figure 3B.

We also performed a ribosome profiling analysis of Slr1p-associated 40S and 80S ribosomes. Immunoprecipitation of Slr1p from 40S fractions selects for ribosome-protected RNA fragments that map to transcript 5'UTRs, indicative of scanning small subunits, and around the start AUG. Additionally, some reads in the coding sequence were detected, which correspond to recently initiated mRNAs with a poorly crosslinked 60S subunit. In contrast, immunoprecipitation of Slr1p from 80S fractions leads to reads for transcripts with ribosome footprints mainly in the coding sequence and some closer to the stop codon (Figure 5). Gene ontology analysis of each set of transcripts suggests that when Slr1p is in the 5'UTR in a general manner, it interacts with metabolism-related mRNAs. When it is bound with 40S subunits found closer to the start AUG, through the 5'PAC motif presumably, it is more strongly associated with ribosomal protein mRNAs. Alternatively, when it is bound with 80S subunits further into the coding sequence, Slr1p is enriched with mRNAs encoding factors involved with mitochondrial functions, which has also been reported in humans^{13,14},

Drosophila^{9,10,12} and *S. cerevisiae*¹¹. These intriguing results present a delineated view of Slr1p function during different stages of mRNA translation which have relevant physiological implications in response to stress. These functions may be related to phosphorylation states of Slr1p and should be further explored in the future.

A



B

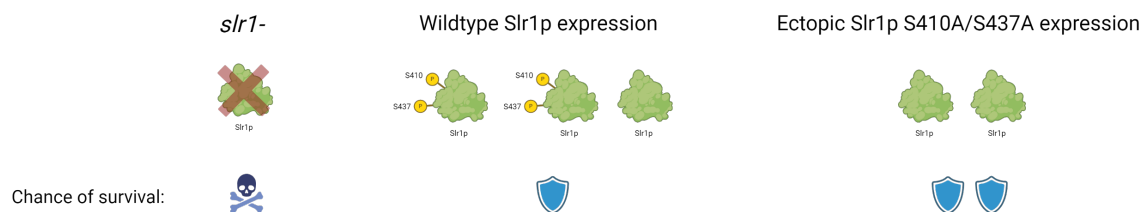


Figure 6: A model for the expression and function of Slr1p in wildtype *S. pombe* cells. A)

During log phase, translation is upregulated and Slr1p is well expressed. The majority of expressed Slr1p is phosphorylated at S410 and S437, which participate in mRNA translation, while the unphosphorylated form is also present. Under normal conditions (minus 3-AT), phosphorylated Slr1p promotes mRNA translation where it's recruited to highly translated mRNAs. Upon 3-AT treatment, instances of ribosome stalling/collision increase, and unphosphorylated Slr1p may be recruited to resolve them. During stationary phase, mRNA translation is downregulated and Slr1p is minimally expressed. The phosphorylated form of Slr1p at S410 and S437 could be subjected to degradation. The predominant unphosphorylated form of Slr1p may be recruited to sites of ribosome stalling/collision following 3-AT treatment or have a general pro-survival function that is triggered as part of the integrated stress response. **B)** The absence of Slr1p has detrimental effects on cell survival during translational stress caused by 3-AT treatment. Wildtype cells possessing both phosphorylated and unphosphorylated Slr1p have a higher chance of survival under prolonged 3-AT treatment compared to *slr1*⁻ cells. Ectopic expression of phosphoablative mutants of Slr1p (S410A and S437A) leads to improved survival, compared to wildtype expression, following 3-AT treatment. Please also see Figure 3B.

Data Availability

All sequencing files will be deposited online at GEO²³.

4.5. Acknowledgements and Funding

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Declaration of Interests

The authors declare no competing interests.

Author Contributions

Conceptualization: F.M., M.A.B.; Data Curation: F.M., C.D.S.D.; Formal analysis: F.M., M.A.B.; Funding acquisition: F.M., C.D.S.D., J.M., M.A.B.; Investigation: F.M., C.D.S.D.; Methodology: F.M., C.D.S.D., J.M., M.A.B.; Project administration: F.M., C.D.S.D., J.M., M.A.B.; Software: F.M., C.D.S.D., J.M.; Resources: J.M., M.A.B.; Supervision: J.M., M.A.B.; Validation: F.M., M.A.B.; Visualization: F.M.; Writing – original draft: F.M.; Writing – review & editing: F.M., M.A.B.

4.6. Materials and Methods

General yeast growth conditions

S. pombe yAS99 (wildtype) and yAS99 *slr1*- (referred to as *slr1*-) strains were initially streaked on YES (5 g/L Yeast Extract (Bioshop YEX401.205), 225 mg/L adenine, L-histidine, L-leucine, L-uracil and L-lysine monohydrochloride (Bioshop ADA001.100; HIS200.100; LEU222.100; URA241.100; LYS101.500), 30 g/L Dextrose (D-Glucose) Anhydrous (Fisher D16-10)) from glycerol stocks kept at -80°C for long term storage. Spotting assays or transformations were plated on YES or EMM plates minus an indicated metabolite (11.77 g/L Edinburgh Minimal Media minus Glucose (Sunrise Science Products 2020-500), 225 mg/L supplements, 20 g/L Dextrose (D-Glucose) Anhydrous (Fisher D16-10)). Plates were incubated at 32°C until colonies were visible. For each biological replicate, a single colony was used to inoculate 10 mL of liquid media. Cultures were incubated at 32°C overnight with shaking (200 rpm). Each overnight culture was subsequently used as a seed culture to inoculate fresh media using sterile techniques. Fresh cultures were grown at 32°C, 200 rpm, to OD₆₀₀ = ~0.6 at the time of harvest for log phase cells. For cells grown past stationary phase, cultures were seeded down to OD₆₀₀ = 0.2 and incubated for 3 days in YES at 32°C (200 rpm) or 7 days in EMM at 32°C (200 rpm).

Spotting assays

Cultures were spun down (1000xg, 5 min, 4°C), washed with 10 mL of sterile ddH₂O, pelleted, resuspended in 1 mL of sterile ddH₂O and transferred to 1.5 mL microcentrifuge tubes prior to UV spectrophotometry. An OD₆₀₀ reading was obtained for each set of cells before diluting them to an OD₆₀₀ = 1 (approximately 1.5 x 10⁷ cells in 1 mL) in sterile ddH₂O. A sterile flat-bottom 96-well plate (Sarstedt 82.1581.001) was used for subsequent 1 in 10 dilutions prior to spotting on plates +/- drugs. Plates containing 25 mL of agar-media (YES or EMM minus supplements) plus various concentrations of H₂O₂ (Sigma 216763-100ML), 3-AT (Sigma A8056-100G), harringtonine (Abcam ab141941) or Torin1 (Millipore 475991-10MG) were prepared prior to spotting assays.

Western blotting

For cell lysis, 10 mL cultures growing (32°C, 200 rpm) in liquid media were harvested, at log phase or after growth past stationary phase, and resuspended in 100 mL of ice-cold NET-2 buffer (50 mM Tris-HCl pH 7.5 (Fisher BP152-5), 150 mM NaCl (Fisher S271-10), 0.05% Igepal (Bioshop NON999.500), 0.1 mM PMSF (Bioshop PMS123.5)). Next, 100-150 mL of 0.5 mm acid-washed glass beads (Sigma G8772-100G) were added to the resuspended cells with NET-2. In a cold room, cells were lysed using a beadbeater (BioSpec Products) and the “homogenize” setting for three 20 s pulses (total of 1 min) interspersed by 2 min of rest on ice. Next, another 100 mL of the ice-cold NET-2 buffer was added to the lysed cells. After a

quick mix, samples were centrifuged at 21000xg for 5 min at 4°C and the supernatant was removed to a fresh tube. Another 100 mL of ice-cold NET-2 buffer was added to the pelleted cell debris and mixed by shaking. Samples were spun down again, and the supernatant was collected with the lysate from the previous step. Pooled lysates were centrifuged at 21000xg for 30 min at 4°C to obtain a cleared lysate which was mixed with a 10% final concentration of glycerol (Bioshop GLY001.500) for long-term storage at -80°C.

Total protein concentration per lysate was measured by Bradford assay. For each measurement, 7.5 µl of lysate was diluted in 750 µL of Bradford Reagent (Thermo Scientific 23238). For blank, 7.5 µl of NET-2 buffer was used instead. For preparing western blot samples, an equal concentration of total protein was diluted in NET-2 buffer to be mixed with 5X Laemmli buffer (312.5 mM Tris-HCl pH 6.8 (Fisher BP152-5), 50% Glycerol (Bioshop GLY001.500), 5% SDS (Bioshop SDS003.1), 0.05% Bromophenol Blue (Sigma B8026)) and 10% β-mercaptoethanol (Sigma M6250-100ML) added just before use. Samples were heated to 95°C for 10 min before an equal volume of each was loaded onto a 10% SDS-Polyacrylamide gel (Bioshop SDS003.1; Fisher BP1406-1) followed by electrophoresis and western blotting. Transfers were either performed for 90 minutes at room temperature or overnight at 4°C. Blots were Ponceau-stained (Sigma P7170-1L) and washed prior to being blocked with 5% Skim Milk (Bioshop SKI400.1) for 1 hr at room temperature. Blots were then incubated with custom primary antibodies: anti-Slr1p Ab2648 or Ab2756¹⁶, anti-phospho-specific S410 or S437 Slr1p (this work), or anti-β-actin (Abcam ab8224) for 1 hr at room temperature. Blots were probed for one primary antibody followed by the respective secondary antibody at a time, with washes in between, prior to treatment with ECL (Thermo Scientific 32106) for 2 min. A MicroChemi (DNR Bio-Imaging Systems) was used to visualize bands on each blot. The block solution and antibody dilutions were made in 1X TBST buffer (20 mM Tris Base (Fisher BP152-5), 150 mM NaCl (Fisher S271-10), 0.1% Tween 20 (Sigma P9416-100ML)). For washing blots, 1X TBST buffer was used.

TCP-Seq

Growth, profiling and sequencing were performed as discussed in Duncan and Mata (NAR, 2022)¹⁷. Prototrophic wildtypes yAS99 and *slr1*- cells were grown in normal conditions (EMM, 32°C, 200 rpm) to an OD₆₀₀ = 0.45-0.8. Cultures (800 mL) were snap-cooled by adding 250 g of crushed ice following which 100 mL of 30% paraformaldehyde (Sigma P6148) was added to the mixture with constant stirring for 10 min. The crosslinking reaction was quenched with the addition of 60 mL 2.5 M glycine (Fisher BP381-5). Cells were spun down for 2 min at 2000xg, 4°C, and washed twice before being flash frozen on dry ice. Cells were later resuspended as indicated by Duncan and Mata (NAR, 2022)¹⁷ and lysed using a beadbeater. Samples were digested with RNase I (Thermo Fisher Scientific AM2295) for 45 minutes at 23°C prior to loading up to 1200 A260 units on each sucrose gradient (7.5-30%). Ultracentrifugation was performed using a SW40Ti rotor at 37000xg for 4 hr at 4°C. Fractionation was carried out using a Brandel density gradient fractionator. Fractions were collected at 400 µl each and those corresponding to the 40S or 80S complexes were pooled.

For immunoprecipitations from 40S or 80S samples, Dynabeads Protein A magnetic beads (Invitrogen 10001D) were coupled to anti-Slr1p antibody (Ab2756) overnight at 4°C with nutation. Coupled beads were washed the next day and added to either pooled 40S or 80S fractions. Samples were then incubated with the beads overnight at 4°C with nutation. Beads were magnetized and washed 5x the following day prior to RNA extraction using 600 µl of hot Acid-Phenol:Chloroform pH 4.5 (with IAA, 125:24:1) (Thermo Fisher Scientific AM9722), 1% SDS (Bioshop SDS003.1) and 125 mM glycine (Fisher BP381-5) at 65°C for 45 min. Samples were then incubated on ice for 5 min and spun down at max speed (5 min, 4°C). The aqueous layer was removed to a fresh tube and mixed with room temperature Acid-Phenol:Chloroform pH 4.5 followed by another Chloroform:Isoamyl alcohol (24:1) (Sigma C0549) clean-up step (max speed, 2 min, 4°C). The aqueous layer, mixed with an equal volume of isopropanol and 1.5 µl of GlycoBlue (Thermo Fisher Scientific AM9516), was applied to a 100 kDa Amicon Ultra Centrifugal Filter Unit (Millipore Sigma UFC910024) to remove ribosomes or any remaining high molecular weight factors and precipitate isolated RNAs. Dried RNA pellets were resuspended in 15 µl of nuclease-free water, heated to 80°C for 2 min and loaded onto 10% TBE-Urea gels. Following gel electrophoresis and staining with SYBR Gold (Thermo Fisher Scientific S11494), RNA fragments between 15–100 nucleotides were gel extracted for 40S or 80S samples. Purified RNAs were treated with T4 Polynucleotide Kinase (Thermo Fisher Scientific EK0031) at pH 7.0 for 30 min followed by the addition of 10 mM ATP at pH 7.6 and incubation for another 30 min. Next-generation sequencing libraries were made with the NEXTFlex Small RNA-Seq Kit v3 following the no size selection protocol and using up to 100 ng of starting material. cDNA synthesis was performed with 10-16 PCR cycles and products were quantified using the Qubit dsDNA Quantification High Sensitivity Kit (Thermo Fisher Scientific Q32854) and the ProNex® NGS Library Quant Kit (Promega NG1201). Illumina NovaSeq 6000 sequencing was carried out by the CRUK-Cambridge Institute Genomics Core (Cambridge, UK). Sequencing results were analyzed as described by (Dundan and Mata, NAR, 2022)¹⁷. Transcripts with above 10 reads in both wildtype yAS99 biological replicates minus any that had reads in the *slr1*- biological replicates were subjected to a GO term analysis. Significant GO terms were plotted using a Bubble Plot generated by Prism (GraphPad Version 10.4.0). The following settings were used - Graph Type: Multiple Variables, X-axis variable: Fold Enrichment according to GO analysis, Y-axis variable: FDR according to GO analysis, Bubble size (area): number of genes enriched under each computed GO term.

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Chapter 5: Discussion and Future Directions

Contributions: F.M. wrote this section. M. A. B. reviewed this section and provided comments. F.M. revised this section. F.M. and M.A.B. edited the writing.

5.1. Key findings

RNA-binding proteins, among other factors, play important roles in regulating gene expression as part of a coordinated response to different environmental cues and stresses. mRNAs, as products of genes, need to be decoded in a process called translation. Specifically, the initiation step of mRNA translation is highly regulated and several initiation factors functioning downstream of conserved signalling pathways ensure its fidelity. Ribosomes, which are made up of around 80 ribosomal proteins and four rRNAs¹, are megadalton complexes that decode mRNAs in this process, and their production is energetically costly to cells. Therefore, a special subset of mRNAs called 5' terminal oligopyrimidine (5'TOP) mRNAs encode ribosomal proteins, and their translation and stability are proposed to be specifically regulated by a family of RNA binding proteins known as the La-related protein 1 (LARP1). LARP1 members are highly conserved across eukaryotes but their proposed functions have been controversial².

Given the conservation of the superfamily, we set out to characterize the single LARP1 ortholog in *S. pombe*, a simpler model organism, to uncouple the various confounding RNA binding modes and functions suggested for the human LARP1 (hLARP1). Studying the short-form *S. pombe* La-related protein 1, Slr1p, allowed us to investigate RNA binding modes via the La motif (LaM) of Slr1p in absence of a characteristic DM15 region found in other LARP1 orthologs. Our studies revealed that Slr1p functions in regulating both mRNA translation and stability of ribosomal protein (RP) and translation-related mRNAs. Importantly, these mRNAs lack the characteristic 5'TOP sequence in yeast. Therefore, we demonstrate that Slr1p binds mRNAs that are functionally similar to 5'TOP mRNAs in *S. pombe*, despite the

absence of this RNA motif in yeast and the DM15 region in Slr1p. Proteomic studies, including mass spectrometry and a yeast two-hybrid assay, showed that Slr1p interacts with several factors linked to mRNA translation such as translation initiation factors and PABP, as well as the ortholog of the 5'-3' exoribonuclease XRN1 in *S. pombe*, Exo2p. Co-immunoprecipitations in presence and absence of a nuclease (benzonase) confirmed that Slr1p associates with translation initiation factors eIF4G (Tif471p) and eIF3i (Sum1p) as well as Exo2p in an RNA-independent manner.

Translational profiling followed by RNA sequencing showed that mRNAs categorized under the Gene Ontology (GO) process “cytoplasmic translation” are less abundant in wildtype cells in comparison to *slr1-* cells. To test if the decreased steady-state abundance of RP mRNAs in presence of Slr1p is related to a role in mRNA stability, a RATE-Seq following 4-thiouracil *in vivo* metabolic labeling was performed. We measured transcriptome-wide mRNA half-lives and decay rates in wildtype versus *slr1-* cells and found that a cohort of mRNAs, significantly enriched for the GO process “cytoplasmic translation”, is stabilized in *slr1-* cells. A RIP-Seq analysis further confirmed that Slr1p binds these mRNAs. We identified two motifs in the 5'UTRs of mRNAs that are stabilized in absence of Slr1p: a 5' proximal AC-rich (5'PAC) motif found in close proximity to the start AUG and a 5' distal U-rich (5'DUR) motif found further upstream in relation to the AUG. A motif search using the 5'UTR sequences of all mRNAs categorized under the GO process “cytoplasmic translation” in *S. pombe* returned an almost identical sequence to our 5'PAC motif. *In vitro* binding assays such as electrophoretic mobility shift assays (EMSAs) confirmed that Slr1p binds the 5'PAC motif specifically since mutating a single cytidine or 5' uridines found in the motif fully disrupted

binding. These findings are consistent with a role for Slr1p in binding to pyrimidine-rich regions analogous to the 5'TOP sequence that is found in higher eukaryotes.

We also performed *Renilla* reporter assays which confirmed that the existence of a 5'PAC motif close to the start AUG of the *Renilla* gene and a 5'DUR motif further upstream from it, arrangements that mimic their endogenous locations, enhance the expression of our reporters in wildtype cells in comparison to *slr1*- cells. Furthermore, Northern blots measuring reporter mRNA abundance showed that these reporters were less abundant in wildtype cells relative to *slr1*- cells. Therefore, our data suggest that Slr1p enhances the translation of 5'PAC- and 5'DUR-containing mRNAs while it also promotes their decay. Other reporter assays, using the 5BoxB and LambdaN tethering system³, showed an increased reporter expression when Slr1p was tethered to the 5' end but a decreased reporter abundance when it was tethered to either the 5' or 3' end. These results further confirm that tethering Slr1p to a reporter leads to its destabilization while having the added effect of promoting expression when the protein is bound to the 5' end.

We further hypothesized that Slr1p is involved with the initiation step of mRNA translation. To test this, we performed polysome profiling in presence of a translation initiation inhibitor, harringtonine, and showed that *slr1*- cells display an increase in the height of the 80S peak and a diminishing trend of heavy polysomes relative to light polysomes, while wildtype cells displayed sustained heavy polysomes. Interestingly, western blots of protein fractions following polysome profiling (treated with cycloheximide) suggested that Exo2p sediments more with subpolysomes and monosomes in presence of Slr1p. This finding argues for Slr1p

potentially recruiting Exo2p to initiating ribosome, which may lead to co-translational decay, consistent with a role for Slr1p in promoting both mRNA translation and turnover.

Our structural work, analyzing the only characterized RNA binding domain found in Slr1p, suggested that the LaM is responsible for binding to the 5'PAC RNA, although with lower affinity compared to the full-length protein. This indicates that other regions of the protein could contribute to non-specific contacts to the RNA. We solved the NMR structure of the LaM and performed mutagenesis studies to characterize Slr1p's RNA binding site within this region. Our NMR structure suggests that Slr1p LaM adopts a fold similar to other characterized LaMs while also harbouring some distinct features, including an additional alpha helix which was not observed in the structure of the hLARP1 LaM. Our mutagenesis studies coupled with EMSAs showed that a highly conserved region in alpha helix 1 is responsible for making crucial contacts to the 5'PAC RNA, even though this region is characterized to bind terminal uridylates in genuine La proteins and terminal poly(A) sequences in hLARP1. Of note is that a single Q332A mutation in the LaM of Slr1p led to a complete loss of binding, up to 28 μ M of protein, to the 5'PAC RNA. Our findings therefore suggest that the LaM is a versatile RNA binding domain that in Slr1p allows for a unique, previously uncharacterized, RNA binding mode relative to genuine La proteins and hLARP1. These data further provide intriguing insights into multiple proposed functions for La and hLARP1 considering how they bind diverse sets of RNA targets.

Furthermore, a survey of stress conditions and drug treatments showed that *slr1-* cells are sensitive to the effects of translation inhibitors harringtonine and 3-AT. These results suggest

that Slr1p has critical functions in mRNA translation initiation as well as elongation. Interestingly, transforming *slr1*- cells with phosphoablative mutants of Slr1p at S410 and S437 residues led to an observed resistance to detrimental effects of 3-AT in these cells. These data further indicate that the unphosphorylated form of Slr1p, at the indicated residues, may have a pro-survival function. Using western blotting and phospho-specific antibodies, we have confirmed that Slr1p is phosphorylated at S410 and S437 during log phase growth which is when cells maximize mRNA translation. In contrast, our data suggest that Slr1p may be minimally expressed during stationary phase where phosphorylation at S410 and S437 was not detected.

Additionally, translation complex profiling followed by sequencing (TCP-Seq) suggested that Slr1p mainly interacts with different cohorts of mRNAs during the initiation and elongation phases of translation. Ribosome profiling followed by immunoprecipitation of Slr1p from 40S or 80S pooled fractions allowed us to query the identity of the co-purifying RNAs which Slr1p interacts with during different stages of mRNA translation. Consistent with our prior findings, we note that immunoprecipitation of Slr1p from 40S fractions enriches mRNAs coding for ribosomal proteins and other translational factors. Importantly, we've shown that these mRNAs contain a 5'PAC motif, found close to the start AUG, that Slr1p specifically binds to. On the other hand, immunoprecipitation of Slr1p from 80S fractions enriches mRNAs encoding factors involved with mitochondrial functions. These findings overlap with similar reports in other eukaryotes and argue for the conservation of LARP1 RNA targets across species. Furthermore, our data support a preference for Slr1p in enriching for specific

RNA targets at different steps of mRNA translation, which may have broader implications in adaptation to stress and cell survival.

5.1.1. Summary

Our studies in *S. pombe* signify the importance of model organism work where a simpler system such as yeast was used to elucidate various binding modes and confounding functions that have been suggested for hLARP1. **We characterized Slr1p as an RNA-binding protein which affects mRNA translation and stability by interacting with other factors known to be involved in these processes.** Our data suggest that Slr1p physically associates with such factors, and that it specifically binds an AC-rich motif enriched in the 5'UTRs proximal to the start codon of RP mRNAs. These mRNAs do not possess a 5'TOP sequence as in other eukaryotes but contain what we have characterized as a 5'PAC motif that allows for the regulation of their translation and stability in an Slr1p-dependent manner. Therefore, we suggest that these mRNAs represent proto-5'TOP mRNAs in *S. pombe*. **We also characterized the 5'PAC motif as a regulatory sequence that Slr1p binds to through contacts in the LaM.** Given that Slr1p lacks a DM15 region, we show that the LaM is a versatile RNA binding domain that recognizes a previously unknown motif in the *S. pombe* RP mRNAs. Therefore, our work highlights the importance of DM15-independent RNA binding modes for LARP1 proteins. **We further propose that Slr1p plays a role in adaptation to translation stress and that its phosphorylation status may allow for several functional states.** Since the LARP1 ortholog in *S. pombe* is involved with both mRNA

translation and decay, processes which other LARP1 family members have also been implicated in, our data argue for the conservation of LARP1 function across eukaryotes. This further indicates that the involvement of LARP1 family members in RP mRNA metabolism might predate the emergence of the 5'TOP sequence and the DM15 region, which may have been features that were scaffolded over a more fundamental form of LARP1-RP mRNA regulation that is still utilized in *S. pombe*. Thus, current and future studies in yeast can prove effective in further understanding hLARP1 function which is especially of interest in disease states.

5.2. Future directions

Future work in *S. pombe* should focus on:

5.2.1. Examining the unique way in which Slr1p binds a critically long RNA

The length requirement of the 5'PAC motif for Slr1p LaM is puzzling, as individual protein domains often bind much shorter RNAs. We've shown that Slr1p binds the 5'PAC RNA when it is a 19-mer or a 15-mer. However, both the full-length protein and the isolated LaM would not bind a 10-mer RNA corresponding to the 5' end of the 5'PAC motif. While chemical shift perturbations in our structural study confirmed that Slr1p LaM binds the longer RNA and not shorter versions of it, it is challenging to map which nucleotides in the longer RNA are being contacted by different amino acids using NMR. For this reason, it would be helpful to solve the crystal structure of the LaM of Slr1p in complex with our 15-mer RNA (UUUUUCCACCACCAA) corresponding to the 5'PAC motif. This study could aid in

understanding whether an RNA secondary structure is important for recognition by Slr1p and further characterizing amino acids, potentially outside of the main RNA binding groove in Slr1p LaM, that contact RNA.

5.2.2. Characterizing functions of Slr1p during mRNA translation elongation and in response to stress

Recent studies in *S. cerevisiae* suggest that LARP1 orthologs in this system are involved with resolving ribosome collisions and preventing programmed ribosome frameshifts⁴. Since we've also seen growth defects for *slr1*- cells in presence of 3-AT, a histidine mimic that leads to ribosome stalling, we have reason to believe that Slr1p is involved in mRNA translation elongation. Our TCP-Seq analysis has further indicated that Slr1p binds to a cohort of mRNAs coding for mitochondrial factors when it sediments with 80S subunits. In order to test if Slr1p is involved with preventing ribosome frameshifts, we've acquired a set of *Renilla*/firefly luciferase reporters used by Jennings et al. (2023)⁴ that employ two well-characterized systems, from the yeast *Ty1* and L-A elements⁵, to study +1 and -1 frameshifts. *Ty1* is a long-terminal repeat (LTR) retrotransposon element that leads to programmed +1 frameshifts and L-A is a viral element that leads to -1 frameshifts^{6,7}. Placing these elements between out-of-frame *Renilla* and firefly luciferase open reading frames, in comparison to a third control reporter without such elements, and measuring the activity of firefly relative to *Renilla* allows for quantitation of ribosome frameshifts. These reporters should be subcloned into *S. pombe* expression vectors and transformed into wildtype and *slr1*- cells. The activity of firefly luciferase in presence and absence of Slr1p can then be measured in unstressed and 3-AT-treated conditions. We hypothesize that in absence of Slr1p and

presence of 3-AT, firefly activity is increased in comparison to wildtype cells also treated with 3-AT. Our previously characterized 5'UTR motifs (5'PAC and 5'DUR) and a control sequence can also be incorporated into the 5'UTRs of these reporters to test if the 5'PAC and 5'DUR motifs better recruit Slr1p to these reporters.

It's also possible that Slr1p is involved with the general response to translation stress in a pro-survival manner. Another set of *S. pombe* reporters that measure upstream open reading frame (uORF) translation, as a response to translational stress induced by phosphorylation of eIF2 α ⁸, can be employed to measure activity in wildtype versus *slr1*-conditions. Under normal conditions, inhibitory uORFs, found in 5'UTRs of stress response genes, lead to lower downstream initiation of the main coding sequence. During stress, the inhibitory effects of such uORFs are alleviated when eIF2 α kinases, including the mammalian GCN2 and HRI1, are activated by stress stimuli⁹. This integrated stress response leads to elevated translation of stress response genes when eIF2 α phosphorylation is induced¹⁰. Dual luciferase reporters containing the 5'UTR of the *S. pombe gcn5* or *hri2* genes cloned upstream of firefly luciferase, relative to *Renilla* luciferase expressed from the same plasmid but with a normal 5'UTR, can be used to measure firefly expression in response to 3-AT and in presence and absence of Slr1p. We hypothesize that if the presence of Slr1p is important for the integrated stress response, we would have a higher expression of the firefly luciferase in wildtype cells when they are treated with 3-AT. This would suggest that, during stress, Slr1p promotes the expression of stress response genes through uORFs found within the 5'UTRs of transcripts such as *gcn5* and *hri2*.

5.2.3. Characterizing the phosphorylation status of Slr1p

We hypothesize that unphosphorylated Slr1p at S410 and S437 residues do not participate in mRNA translation. In order to test this, pRep81x vector, pRep81x-Slr1p, pRep81x-Slr1p S410A and pRep81x-Slr1p S437A can be transformed into *slr1*- cells to perform polysome profiling. Wildtype Slr1p is expected to sediment with subpolysomes and polysomes, whereas phosphoablative mutants of Slr1p at S410 and S437 (S410A and S437A) are not expected to sediment with polysomes or any fractions. This would suggest that unphosphorylated forms of Slr1p, at the indicated residues, may be involved with other processes, including cell survival (please refer to Chapter 4), as opposed to mRNA translation. Additionally, phosphomimetic mutants of Slr1p at these residues (S410D/E and S437D/E) are expected to restore wildtype phenotypes and should be tested. We've previously shown that Exo2p sediments better with subpolysomes in presence of Slr1p. The sedimentation patterns of Exo2p can also be reassessed with different phospho-mutants of Slr1p at S410 and S437. Given that characterization of Slr1p's protein partners was previously performed in wildtype conditions, it would be informative to test if phosphoablative mutants of Slr1p at S410 and S437 still co-immunoprecipitate with factors involved in mRNA translation and decay such as Tif471p, Sum1p, PABP and Exo2p. Lastly, 3-AT treatment could affect polysome sedimentation patterns of Slr1p and that of its phospho-mutants as well as interactions of Slr1p with its protein partners, which should also be examined following 3-AT treatment.

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Appendices

Appendix A: Electrophoretic mobility shift assays (EMSAs) for *in vitro* detection of protein-nucleic acid interactions

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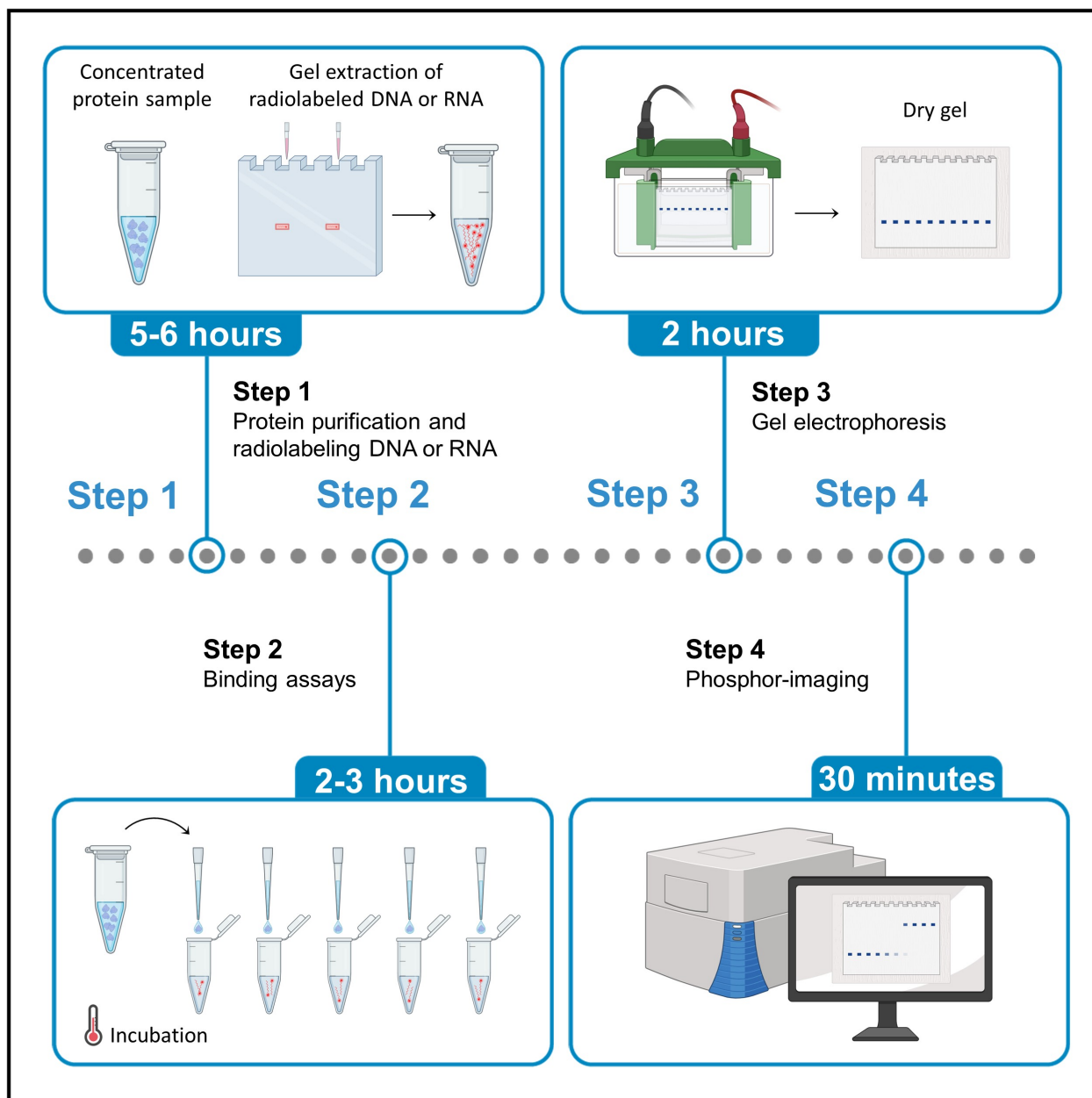
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The sections and headings in this chapter are structured to align with the formatting guidelines of *STAR Protocols* journal.

Graphical abstract



Highlights:

Protocol for radioactive labeling and gel extraction of desired nucleic acid substrates
In vitro binding assays at multiple protein concentrations for any number of substrates
Resolving reactions using nondenaturing gels to visualize free and protein-bound forms
Analysis of potential interactions between DNA-/RNA-binding proteins and their substrates

Summary

Protein-nucleic acid interactions drive some of the most important physiological events in cells. Here we present a protocol for detecting protein-DNA or protein-RNA interactions *in vitro*. We describe steps for labelling nucleic acid species and Electrophoretic Mobility Shift Assays (EMSAs). This protocol can be used to confirm suspected *in vivo* interactions using recombinantly expressed/purified proteins of interest and a nucleic acid substrate. It can further be used to investigate mutations that can disrupt interaction or compensatory mutations that restore it.

For complete details on the use and execution of this protocol, please refer to Mansouri-Noori *et al.*¹

Before you begin

The protocol below describes specific steps for radioactively labelling RNA substrates to be used for EMSAs. However, DNA substrates can also be labelled and used in the same manner.

1. Use standard protocols to recombinantly express, purify and concentrate your protein of interest.

- a. Obtain the concentration of your protein.

Note: This can be achieved through Bradford assays or gel electrophoresis of 1-5 microliter (μ l) of your protein sample against known concentrations of Bovine Serum Albumin (BSA).

Note: A NanoDrop spectrophotometer can also be used to measure the concentration of a pure protein sample (concentration = absorbance at 280 nm / extinction coefficient of the protein when path length is equal to 1 cm).^{2,3}

$c = A / \epsilon L$ (= A / ϵ when $L = 1$ cm)

Note: In this protocol concentrations are indicated in micromolar (μ M).

2. Obtain unlabeled RNA substrates.

Note: This can be done through in-house preparations such as *in vitro* transcription of RNA by T7 RNA polymerase or commercial means. If RNA substrates are to be prepared by *in vitro* transcription using T7 RNA polymerase, you can either a) dephosphorylate the generated 5'ppp using CIP prior to 5' PNK labelling, or b) use an α -³²P NTP during the transcription reaction, in which case, you don't need to perform 5' PNK labelling using γ -³²P ATP as outlined further.

Note: RNA substrates for this protocol were purchased as a standard RNA synthesis (5'OH) from Integrated DNA Technologies (IDT).

Note: A TBE-Urea gel will be required for gel extraction of labelled RNAs.

Optional: Fluorescently labelled RNA can alternatively be purchased, and this eliminates the need for radioactive labelling. However, in our experience, it is easier to

detect radioactively labelled RNA substrates. If estimations of dissociation constants (K_d s) are to be attempted, care must also be taken that the concentration of a fluorescently labelled RNA substrate is negligible (on the order of 100-fold less) relative to the concentrations of proteins being tested, as otherwise calculation of dissociation constants using this method may not be accurate (for more details on this, see section on Expected Outcomes, below). For this reason also, radiolabeled RNA substrates may be preferable.

3. Obtain radioactive γ - ^{32}P ATP for end-labelling RNA substrates.

Key Resources Table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Nuclease-free water	Invitrogen	Cat#10977-015
γ - ^{32}P ATP	Perkin Elmer	Cat#BLU502A250 UC
T4 Polynucleotide Kinase (T4 PNK)	NEB	Cat#M0201S
Urea	Bioshop	Cat#URE001.5
Polyacrylamide 19:1 Solution	Fisher	Cat#BP1406-1
Tris Base	Fisher	Cat#BP152-5
Boric Acid	Fisher	Cat#BP168-1
EDTA	Sigma	Cat#E5134-100G
APS	Sigma	Cat#A3678-25G
TEMED	Fisher	Cat#BP150-20
β -Mercaptoethanol	Sigma	Cat#M6250-100ML
Deionized Formamide	Bioshop	Cat#FOR001.500
Bromophenol Blue	Sigma	Cat#B8026
Xylene Cyanol	Abcam	Cat#ab146296
Autoradiography Markers, Glogos II	Agilent	Cat#420201
Autoradiography Film	Denville Scientific	Cat#E3012
NaCl	Fisher	Cat#S271-10
Scintillation Fluid	Ecoscint TM	Cat#A LS-273
KCl	Fisher	Cat#BP366-1
Glycerol	Bioshop	Cat#GLY001.500
Whatman Chromatography Paper	Cytiva	Cat#3030-861
SUPERase•In TM RNase Inhibitor	Invitrogen	Cat#AM2694
Other		
Scintillation Counter	Hidex 300 SL	
Gel Dryer	Bio-Rad, Model 583	
Storage Phosphor Screen	GE Healthcare	
Amersham Typhoon Biomolecular Imager	Cytiva	

Materials and equipment

This protocol uses a scintillation counter to count the activity of radioactively labelled RNA in Counts Per Minute (CPM), a gel dryer to dry polyacrylamide gels prior to exposure to a storage phosphor screen and a Typhoon Biomolecular Imager for phosphor-imaging. Autoradiography markers or Glogos II stickers can be replaced with any commercially available glow in the dark stickers which glow after being exposed to ambient light as these can also be visualized using autoradiography films.

5X TBE Buffer

Reagent	Final 1X concentration	Amount
Tris base	130 mM	108 g
Boric acid	45 mM	55 g
EDTA	2.5 mM	9.3 g
Total	n/a	2 L

Dissolve in 2 L ddH₂O. Store at 22-25°C for up to a month.

12% TBE-Urea Gel

Reagent	Final Concentration	Stock Concentration	Amount
Urea	7 M	n/a	8.4 g
Polyacrylamide solution (19:1)	12%	40%	6 mL
TBE Buffer	1X	5X	4 mL
APS	0.08%	10%	160 µl
TEMED	0.037%	100%	7.4 µl
Total	n/a	n/a	20 mL

Mix reagents in 20 mL nuclease-free water prior to adding APS and TEMED. Make sure urea is fully dissolved (the mixture can be heated in a 37°C water bath to speed the process). Add APS and TEMED and pour gels immediately after. Store solidified gels at 4°C for up to a week.

Formamide Loading Dye

Reagent	Final Concentration	Stock Concentration	Amount
Deionized formamide	80%	100%	8 mL
EDTA	10 mM	0.5 M (pH 8.0)	200 µl
Bromophenol blue	0.06%	n/a	6 mg
Xylene cyanol	0.06%	n/a	6 mg
Total	n/a	n/a	10 mL

Mix reagents in 10 mL nuclease-free water. Aliquot and store at -20°C for up to a year.

6% TBE Gel

Reagent	Final Concentration	Stock Concentration	Amount
Polyacrylamide solution (19:1)	6%	40%	3 mL
TBE Buffer	1X	5X	4 mL
APS	0.08%	10%	160 µl
TEMED	0.037%	100%	7.4 µl
Total	n/a	n/a	20 mL

Mix reagents in 20 mL nuclease-free water prior to adding APS and TEMED. Add APS and TEMED next and pour gels immediately after. Store prepared gels at 4°C for up to a week.

10X EMSA Buffer

Reagent	Final Concentration	Stock Concentration	Amount
Tris-HCl pH 7.2	200 mM	1 M	200 µl
KCl	1 M	3 M	333 µl
EDTA	10 mM	0.5 M (pH 8.0)	20 µl
β-mercaptoethanol	50 mM	14.3 M	3.5 µl
Nuclease-free water	n/a	n/a	443.5 µl
Total	n/a	n/a	1 mL

Prepare 10X EMSA Buffer on the day of Electrophoretic Mobility Shift Assays (EMSAs). Keep at 22-25°C for the day.

RNA Master Mix for 5+2 Lanes

Reagent	Final Concentration/20 µl Reaction	Stock Concentration	Amount
EMSA Buffer	1X	10X	14 µl
Glycerol	10%	80%	17.5 µl
Labelled RNA	3000 CPM*	Variable	21000 CPM
Protein**	1X	10X	14 µl**
Bromophenol blue dye***	n/a	n/a	8 µl
Nuclease-free water	n/a	n/a	Variable****
Total	n/a	n/a	140 µl

Prepare the RNA master mix on the day of EMSAs.

*We use the minimum amount of RNA possible that can be reliably quantitated and detected by phosphor-imaging. In our experience this works out to approximately 3000 CPM but this amount may vary depending on the phosphor screen and phosphor-imager instrument used.

**We account for the volume of protein that is to be added to the master mix – each 20 µl reaction would get 2 µl of a 10X protein stock, therefore, in a 140 µl master mix, we would

add 14 µl. We don't however add the protein at this stage since each master mix will be heated to 95°C first and slowly cooled next. After aliquoting 18 µl of RNA master mix/reaction tube, we then add 2 µl of each 10X protein stock to the RNA master mix aliquot.

***To make the bromophenol blue dye, dissolve 0.5% (w/v) of bromophenol blue powder in a volume of nuclease-free water (i.e., 5 or 10 mL).

****This depends on the volume of labelled RNA that is needed. Calculate the volume of nuclease-free water needed to fill up to 140 µl after subtracting the volume of labelled RNA added plus all other components in the recipe.

Protein Dilution Buffer (PDB)

Reagent	Final Concentration	Stock Concentration	Amount
EMSA Buffer	1X	10X	20 µl
Glycerol	10%	80%	25 µl
Nuclease-free water	n/a	n/a	155 µl
Total	n/a	n/a	200 µl

Prepare PDB on the day of EMSAs. Keep at 22-25°C for the day. Use the PDB to make 10X protein stocks and subsequent serial dilutions.

Step-by-step method details

Radioactive labelling of RNA substrates

In this step, RNA(s) of interest will be labelled prior to gel extraction for obtaining full-length species. All work using radioactive substrates should be performed behind a Plexiglass shield, and all waste should be disposed of in compliance with local radioactivity handling safety measures.

Timing: 2-3 h

1. Turn on a heat block at 37°C.
2. If frozen, thaw γ -³²P ATP.

CRITICAL: To protect yourself from radiation, take caution and make sure steps which involve handling radioactivity are performed behind a Plexiglass shield.

3. Reconstitute lyophilized RNA stock(s) to 1 mM using nuclease-free water.
 - a. Make a 10 µM dilution of each RNA substrate to be labelled using nuclease-free water.
 - b. Leave the dilutions on ice.

Note: RNA stocks can be kept at -80°C for long-term storage.

4. Set up the end-labelling reaction using the T4 Polynucleotide Kinase (T4 PNK) system.

CRITICAL: Work behind a Plexiglass shield after adding radioactivity.

- a. In a 1.5 mL microfuge tube, mix the following reagents in the order indicated:
 - i. 13.5 µl nuclease-free water
 - ii. 2 µl T4 PNK Reaction Buffer (10X)
 - iii. 1.5 µl γ -³²P ATP
 - iv. 2 µl 10 µM RNA dilution prepared in step 3
 - v. 1 µl T4 PNK enzyme
 - b. Mix the content by gentle vortexing followed by a quick spin to collect everything in the bottom of the tube.
5. Incubate reaction(s) at 37°C for 2 hours.

Pause point: following the incubation, labelled samples can be stored at -20°C for gel extraction at a later time.

6. Turn on a heat block at 95°C for the next step.

Note: a 12% TBE-Urea gel needs to be prepared prior to the following steps.

Gel extraction of ³²P-labelled RNA

Timing: 3 h

7. Pre-run a 12% TBE-Urea gel for at least 20 minutes using 1X TBE Buffer.
- a. Flush wells by pipetting up and down in each well a few times.
 - b. Pre-run gels at 100 V.
- Note:** To keep the set up cold, it is recommended that you do this in a cold room (4°C) or have the gel bucket placed in a dish filled with ice (Figure 1A).
8. Following the incubation in step 6, add 5 µl of Formamide Loading Dye to each 20 µl T4 PNK reaction.
- CRITICAL:** This and the following steps must be performed behind a Plexiglass shield.
- a. Heat each sample at 95°C for 10 minutes.
- Note:** This step should resolve any secondary structures prior to loading the RNA on a gel.
9. Load each 25 µl of labeled RNA + Formamide Loading Dye onto a well on the 12% TBE-Urea gel following pre-running in step 7 (Figure 1B).
- Note:** to make gel extraction easier, it is recommended to leave an empty lane between each sample.
10. Perform electrophoresis until the bromophenol blue and xylene cyanol dyes in the Formamide Loading Dye are sufficiently separated (Figure 1C).
- a. Run gels at 100 V.
- Note:** Sufficient separation depends on the size of the RNA loaded on the gel. Refer to Table 1.
11. Stop electrophoresis and crack gels open, leaving each gel on the glass plate that it falls onto.
- CRITICAL:** Keep track of the orientation of the lanes.

- a. Tape a piece of aluminum foil onto an autoradiography cassette.
 - b. Place the glass plate on top of the foil, having the gel side face up (Figure 1D).
 - c. Cover the gel with a piece of Saran wrap.
 - d. Add Glogos II/glow in the dark stickers to the sides of the gel and on top of the Saran wrap (Figure 1E).
Note: *Glow in the dark stickers will be used as reference for cutting areas of the gel containing full-sized labelled RNAs.*
 - e. In a dark room, expose the gel to autoradiography film.
 - f. Develop the film to visualize the bands corresponding to the migration of radioactive RNA (Figure 1F).
12. Cut off the bottom of a fresh 0.7 mL microfuge tube and insert it into a 1.5 mL microfuge tube (Figure 1G).
Note: *There should now be a tiny hole at the bottom of the 0.7 mL tube.*
- a. Label the 1.5 mL tube with your sample ID.
13. Using the developed autoradiography film as reference and a clean razor, cut pieces of the gel containing full-length radioactively labelled RNAs (Figure 1H).
14. Add excised pieces of the gel containing your radioactively labelled RNA into the 0.7 mL tube inserted inside the 1.5 mL tube (Figure 1I). Close the 0.7 mL tube's cap. Leave the 1.5 mL tube open.
15. Centrifuge the set-up at 9000 xg for 2 minutes (Figure 1J).
Note: *This will crush and collect the gel pieces into the 1.5 mL tube (Figure 1K).*
16. Remove the 0.7 mL tube and discard.
17. Add a volume of 0.5 M NaCl (prepared using nuclease-free water) to the gel pieces inside the 1.5 mL tube until the pieces are covered (Figure 1L).
Note: *This will require approximately 150-250 µl 0.5 M NaCl. Lower volumes are preferred for achieving a higher concentration.*
18. Leave the gel pieces in 0.5 M NaCl at 22-25°C for 16-20 hours.
Note: *This step allows for elution of radioactively labelled RNA into the solution.*
19. The next morning, store the tubes containing eluted RNA at -20°C for future use.
Note: *You don't need to remove the gel pieces as they tend to accumulate in the bottom of the tube.*
- Pause point:** *Labelled RNAs can be stored at -20°C until when EMSAs are to be performed.*

Electrophoretic Mobility Shift Assays (EMSAs): *in vitro* binding assays for detection of proteins-RNA interactions and visualization using gel electrophoresis

Timing: 4-5 h

In this step, protein(s) of interest will be allowed to incubate with potential RNA targets to form ribonucleoproteins (RNPs). Following binding assays, free RNA and RNPs will be resolved on a native TBE gel (no urea) using electrophoresis.

Note: a 6% TBE gel needs to be prepared prior to these steps.

Note: a phosphor screen and a Typhoon Biomolecular Imager or equivalent which allows phosphor-imaging will be required to detect RNPs.

CRITICAL: steps involving handling of radioactive RNA should be performed behind a Plexiglass shield.

20. Turn on a heat block at 95°C.

Note: You should use a heat block that allows you to remove a block with a thermometer inserted in it. This is to monitor changes in temperature.

21. Thaw radioactive RNAs (leaving them on top of some ice) if previously stored at -20°C. Flick to mix and spin down quickly using a tabletop centrifuge.

Note: This will help with collecting the gel pieces in the bottom of the tube. Keep tubes on ice.

22. Prepare RNA master mixes.

Note: A separate master mix must be prepared for each RNA substrate to be tested.

- a. Dilute 1-2 µl of each labelled RNA in 5 mL scintillation fluid previously aliquoted in a 20 mL scintillation vial.
- b. Use a scintillation counter to count the activity of your RNA. Obtain a value in Counts Per Minute (CPM). Calculate the CPM per microliter (CPM/µl) for each sample.
- c. Prepare 10X EMSA Buffer.
- d. Prepare the RNA master mixes by considering the number of protein concentrations to be tested.

Note: For example, if 5 different concentrations are to be tested, prepare enough master mix for 5 plus 2 extra reactions.

Note: For 5+2 or 7 reactions, prepare 140 µl of master mix. For 10 protein concentrations to be tested, i.e. 10 lanes on your gel, consider 10+2 reactions or simply multiply the provided recipe for 7 lanes by 2 for a total volume of 280 µl.

23. Heat each master mix at 95°C for 5 minutes.

24. Remove the block containing your master mixes from the heat block. Leave it on a countertop at 22-25°C.

Note: Keep the thermometer placed in the block.

- a. Allow the set up to slow cool to 30°C or below.

Note: This step should resolve any secondary structures and allow the RNA to fold naturally.

25. Set the heat block to 32°C or 37°C.

Note: We find incubation at 32°C works best with proteins with yeast origins.

Incubation at 37°C works best with proteins with mammalian origins.

26. Prepare Protein Dilution Buffer (PDB).

27. Prepare dilutions of your protein of interest at 10X concentrations in PDB. Keep on ice.

Note: These are the concentrations at which binding to your RNA substrate is to be tested. We recommend 0.1, 0.25, 0.5, 1 and 2 μM as a starting point. 10X protein dilutions should therefore be prepared for the following concentrations: 1, 2.5, 5, 10 and 20 μM .

Note: For 10 lanes, you can include more concentrations in this range, see Figure 2.

28. Prepare binding reactions for each protein concentration.

- a. Label the required number of 1.5 mL tubes with the protein ID+concentration and RNA substrate to be tested for interaction.
- b. Following step 24, aliquot 18 μL of each master mix per respective tube. Leave at 22-25°C until the next step.
- c. To each aliquoted master mix, add 2 μL of the respective 10X protein dilution in a final volume of 20 μL .
- d. Flick tubes to mix and spin to collect everything in the bottom of each tube. Leave each prepared binding reaction on ice after adding the protein.
- e. Prepare a reaction with no protein by adding 2 μL of the PDB to 18 μL of each RNA master mix. Leave this on ice as well.

Note: This will be used to visualize the migration of free RNA during gel electrophoresis.

29. Move the tubes corresponding to all binding reactions (kept on ice until this point) to the heat block at a temperature indicated in step 25 (32°C or 37°C).

30. Incubate binding reactions for 30-35 minutes.

31. Pre-run a native TBE gel for at least 20 minutes using 1X TBE Buffer.

- a. Flush wells.
- b. Pre-run at 100 V.

Note: To keep the set up cold, it is recommended that you do this in a cold room (4°C) or have the gel bucket placed in a dish filled with ice (Figure 1A).

32. Following step 30, remove tubes from the heat block and insert them in ice for 5-15 minutes.

- a. After incubation on ice, spin each tube to collect everything in the bottom of each tube.

33. Load each binding reaction onto a well on the TBE gel which had been pre-running from step 31. Start with the RNA only reaction and proceed with protein+RNA reactions in the manner of increasing protein concentration.

- a. Flush wells before loading.
- b. Set your pipette to 18 μL to avoid air bubbles in your tip.

Note: Pipette slowly into each well to avoid losing any of the sample.

34. After loading all reactions, perform electrophoresis until the bromophenol blue dye front has moved about $\frac{3}{4}$ of the way down.

- a. Run gels at 100 V.

Note: This will take approximately an hour but will depend on the percentage of the TBE gel being used.

Note: To keep the set up cold, it is recommended that you do this in a cold room (4°C) or have the gel bucket placed in a dish filled with ice (Figure 1A).

35. Stop electrophoresis and crack gels open one by one.

CRITICAL: *This and the following steps must be performed behind a Plexiglass shield as well.*

CRITICAL: *keep track of the orientation of the lanes.*

- a. Leave each gel on the glass plate that it falls onto.
- 36. Gently press the gel on the glass plate onto a pre-cut piece of Whatman chromatography paper.
 - a. Peel off the Whatman paper away from the glass plate, making sure the gel is stuck to the Whatman paper.

37. Dry the gels for 45-60 minutes at 80°C.

Note: *Drying time will depend on the thickness and percentage of each gel. The suggested duration is for a 1.0 mm 6% TBE gel.*

- a. Place the Whatman paper with the gel facing up on a gel dryer.
- b. Place a piece of Saran wrap on top of the gel.
- c. Start the gel dryer.

Note: *Make sure the suction mechanism is working properly. Poor suction will lead to gels cracking.*

38. Expose the dried gels to a storage phosphor screen for at least 16 hours.

- a. Stop the gel dryer.
- b. Remove the dried gels on Whatman papers.
- c. Tape down the dried gels onto an autoradiography cassette.

Optional: *You can tape down a piece of Saran wrap on top of the dried gels.*

- d. Place a storage phosphor screen face down on the dried gels.
- e. Close the cassette and leave to expose.

39. Scan the phosphor screen the next day using the phosphor-imaging setting on a Typhoon Biomolecular Imager or equivalent.

Expected outcomes

Scanning the phosphor screen should yield a replica image of the dried gel where free RNA and protein-RNA complexes were allowed to separate through electrophoresis. The free RNA band should be present in the RNA only lane and at the bottom of the next few lanes where the concentration of protein was not sufficient for formation of a protein-RNA complex. With increasing protein concentration, you should be able to see a ribonucleoprotein (RNP) band forming and the free RNA band disappearing. Super shifts may occur with increasing concentration of protein if multiple proteins interact with the RNA in your binding reaction (Figure 2).

Comparison of free RNA/RNP band shifting patterns between wild-type and mutant proteins can be used to identify candidate residues/regions linked to RNP formation. Alternatively, RNA substrates with substitutions at key nucleotides can also be tested to identify bases linked to RNP formation. Dissociation constants (K_{ds}) can be estimated as the protein concentration at which half of the RNA substrate is in the bound RNP, provided

the tested protein concentration is in large excess relative to the RNA substrate concentration (i.e., 100X more), observed RNPs are distinct species, and there is an absence of aggregation of protein/RNP complexes, often observed as RNA species trapped in the well. Dissociation constants estimated as the protein concentration at which half of the RNA substrate is in the bound RNP rely on the concentration of the free protein being negligibly different from the total protein concentration.⁴ In our experience, the amount of fluorescently labelled RNA required for a clear fluorescence detection signal can be in the same regime of protein concentrations required for a binding event, often making fluorescent ligands impractical for rigorous determination of dissociation constants by EMSAs. As a general rule, we only estimate a K_d when the RNA concentration is no more than 0.01X the concentration of protein required for binding. Specific fluorescent RNA/RBP pairs can be tested and optimized to see if they are amenable to K_d estimation; alternatively, use of fluorescently labelled RNAs may still be desirable when an estimate of K_d is not critical, such as simple characterization of protein mutants for loss of RNA binding.

If estimations of dissociation constants (K_d s) are to be attempted, we recommend doing each set of EMSAs in triplicates for statistical purposes.

Limitations

Possible limitations include protein sample's precipitation or aggregation in the wells, protein sample being contaminated with RNases, RNA sample's degradation and general RNase contamination during experimental set up. Improper drying of the gels may lead to cracked gels or diffused signal during phosphor-imaging.

Troubleshooting

Problem 1:

RNase contamination and RNA degradation (related to steps 3, 4, 17, 22 and 28)

Potential solution:

- Wear gloves: avoid touching your skin, hair or any surface that generally comes into contact with bare skin.
- Use RNase free water. Make sure all pipette tips and tubes are certified RNase-free. Take caution setting up reactions in an RNase-free manner. It is important that the pH of the solutions and buffers used is close to neutral pH. A basic pH combined with elevated temperatures lead to base hydrolysis of RNA.
- Make sure your protein sample is as pure as possible and not contaminated with RNases. An RNase inhibitor cocktail (refer to the KRT) can be added to your master mix after it has cooled down to 30°C or below (step 24) to counter the effects of RNase contamination in your protein sample.

Problem 2:

Protein aggregation or precipitation (related to Before You Begin step 1 and Method Details steps 28 and 34)

Potential solution:

- Make sure buffer conditions for your protein of interest is optimized such that it does not precipitate out of solution when concentrated.
- After mixing your RNA master mix with your protein, observe the reaction mixture and make sure you don't see any aggregates. Also monitor your gel during electrophoresis – some aggregation could accumulate in the well. Samples with some aggregation can still be loaded on the gel or allowed to go through electrophoresis as you may still be able to see some resolved RNPs.
- Running a low percentage TBE gel (i.e., 6%) can help with resolving aggregations accumulated in the wells during electrophoresis.
- Try using a different percentage acrylamide stock with a larger pore size (i.e., 37.5:1) when preparing your TBE gels.

Problem 3:

Diffused RNP bands (Related to step 39)

Potential solution:

- This could mean unstable binding between your protein of interest and RNA substrate but still indicates interaction.
- A higher percentage TBE gel (i.e., 8, 10 or 12%) could help with the formation of a more distinct RNP band.

Problem 4:

Cracked gels during the drying process (Related to Step 37)

Potential solution:

- Make sure the suction mechanism on your gel dryer is on and working properly prior to the drying process.
- Lower percentage gels tend to dry faster and are less likely to crack.

Problem 5:

Free RNA band not visible (Related to step 39)

Potential solution:

- You might have run the free RNA band off the gel. The amount of time required for optimal separation of your free RNA and RNPs depends on the size of the RNA and the percentage of your gels (Table 1). We suggest you stop electrophoresis when your dye front is about $\frac{3}{4}$ of the way down, but this step may require further optimization according to your experimental set up.

Problem 6:

No RNP visible (Related to step 39)

Potential solution:

- Repeat procedure but starting at a higher concentration of protein.

Resource availability

Lead contact: Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Mark A. Bayfield (bayfield@yorku.ca).

Technical contact: Technical questions on executing this protocol should be directed to and will be answered by the technical contact, Farnaz Mansouri-Noori (farnaz.mansouri@gmail.com).

Materials availability: This study did not generate new unique reagents.

Data and code availability: This study did not generate/analyze datasets/code.

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Author contributions

Conceptualization, F.M.; data curation, F.M.; formal analysis, F.M.; funding acquisition, M.A.B.; investigation, F.M.; methodology, F.M. and M.A.B.; project administration, F.M. and M.A.B.; validation, F.M.; visualization, F.M.; writing – original draft, F.M.; writing – review & editing, F.M. and M.A.B.; resources, M.A.B.; supervision, M.A.B.

Declaration of interests

The authors declare no competing interests.

Figures, tables and legends

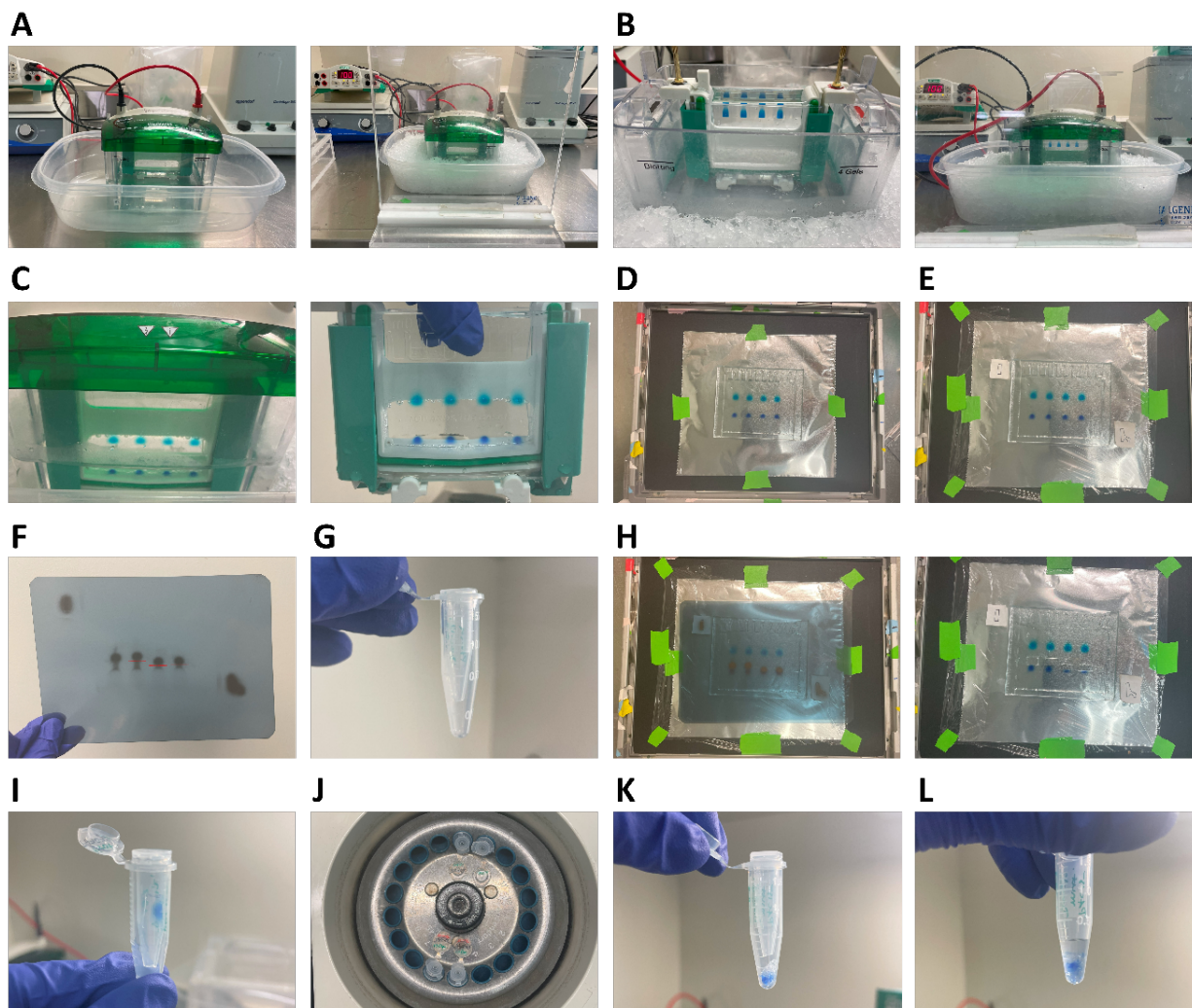


Figure 1: Set up for gel extraction of labelled RNA. **A)** The gel bucket can be placed in a dish filled with ice to keep the set up cold during gel electrophoresis. Alternatively, electrophoresis can be performed at 4°C or in a cold room. **B)** Load your RNA samples onto the gel, preferably leaving an empty lane between samples. **C)** Allow electrophoresis to continue until a sufficient separation of the bromophenol blue and xylene cyanole dyes is achieved. **D)** Keeping track of the lanes, crack the gel open, leaving it on the glass plate that it falls onto. Place the glass plate with the gel on a piece of aluminum foil taped onto an autoradiography cassette. **E)** Tape down a piece of Saran wrap on top of the gel, placing glow in the dark stickers on the sides. **F)** In a dark room, expose the gel to an autoradiography film and develop it to visualize the labelled RNA in each lane. Smears below the red dashed lines are degradation products which should not be excised in the following steps. **G)** Cut off the bottom of a fresh 0.7 mL microfuge tube and insert it into a 1.5 mL microfuge tube. **H)** Using the developed film as reference and a clean razor, cut pieces of the gel containing full-length

radioactively labelled RNAs (bands above the red dashed lines in F). **I)** Insert each excised gel piece into the respective 0.7 mL tube inserted into a 1.5 mL tube. **J)** Spin the set-up at 9000 xg for 2 minutes, with the 1.5 mL tube's cap open. **K)** This will crush and collect the gel pieces into the 1.5 mL tube. Remove the 0.7 mL tube and discard. **L)** Add a volume of 0.5 M NaCl to the gel pieces inside the 1.5 mL tube such that the pieces are covered. Elute RNA at 22-25°C for 16-20 hours. Place eluted RNA at -20°C the next day for future use.

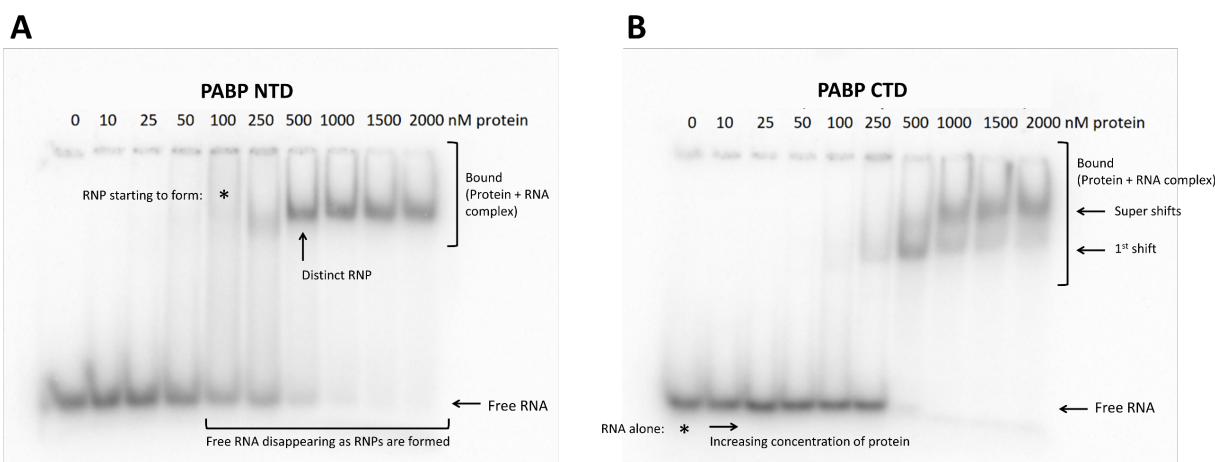


Figure 2: Examples of EMSAs. Ten different protein concentrations were tested for the N-terminal domain (NTD) and C-terminal domain (CTD) of the *S. pombe* poly(A)-binding protein (PABP, PomBase: SPAC57A7.04c⁵). A 20-nt poly(A) RNA (A20) was radioactively labelled and used for these EMSAs (3000 CPM/lane). **A)** The NTD of PABP binds A20. RNPs start to form at 100 nM concentration of protein and distinct RNPs are visible at 500 nM. Free RNA disappears as RNPs are forming. The asterisk marks the lane where the RNP is starting to form. **B)** The CTD of PABP also binds A20. Shifts start at 250 nM while super shifts start to appear at 1000 nM. The asterisk marks the lane with just RNA and no protein. A 6% native TBE gel was used for all presented EMSAs.

Percentage of Polyacrylamide	Xylene Cyanol	Bromophenol Blue
5%	130	35
6%	106	26
8%	75	19
10%	55	12
12%	28	8

Table 1: Migration of bromophenol blue and xylene cyanole dyes in denaturing polyacrylamide gels. Dyes will approximately run at the indicated nucleotide length at each respective percentage.⁶

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Appendix B: Research Contributions

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For Chapter 1, Figure 5



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Author: Duncan, Caia Deborah Suzanne; Mata, Juan

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For Chapter 4, Figure 5A