The Function of the Fission Yeast LARP1 Ortholog Slr1p in mRNA Translation and Decay

Joudi Kanaan

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Abstract:

Gene expression is the process by which information encoded in DNA is used to produce functional products. Protein-coding genes are transcribed into messenger RNA (mRNA), which is translated into proteins. Translation is highly regulated, with initiation, elongation, and termination influenced by cellular conditions and regulatory proteins. In *Schizosaccharomyces pombe*, the LARP1 ortholog Slr1p binds mRNAs and regulates translation, although its molecular functions remain incompletely understood. This study investigates the role of Slr1p in translation regulation and translation-associated mRNA decay under stressed and unstressed conditions. Using dual-luciferase assays, and co-immunoprecipitation, we examined the effects of Slr1p on translation initiation, elongation, programmed ribosomal frameshifting, and its interaction with the 5′–3′exonuclease Exo2p. We hypothesized that Slr1p promotes translation while coordinating mRNA decay through Exo2p, thereby linking protein synthesis with mRNA turnover. Understanding Slr1p function may reveal conserved regulatory mechanisms and provide insight into the roles of LARP1 proteins in disease, including cancer.

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List of Abbreviations

3-AT: 3-amino-1,2,4-triazole

5′DUR: 5′ Distal U-Rich

5′PAC: 5′ Proximal AC-rich

5′TOP: 5′ terminal oligopyrimidine

CDS: coding sequence

CTD: C-terminal domain

eIF: eukaryotic initiation factor

EMM: Edinburgh Minimal Media

EMSA: electrophoretic mobility shift assay

hLARP1: human LARP1

KO: knockout

LaM: La motif

LARP: La-related protein

LARP1-L: long-form LARP1

LARP1-S: short-form LARP1

m^7G: 7-methylguanosine

mRNA: messenger RNA

mtDNA: mitochondrial DNA

mTOR: mammalian target of rapamycin

NTD: N-terminal domain

OD: optical density

ORF: open reading frame

PABP: poly(A)-binding protein

Slr1p: S. pombe La-related protein 1

tRNA: transfer RNA

UTR: untranslated region

WT: wild type

YES: Yeast Extract plus Supplement

Chapter 1: Introduction

1.1 Gene expression is highly regulated to control protein production

Gene expression is the process by which the information stored in DNA is used to produce functional products that carry out the activities and essential functions within the cell. This process begins with transcription, during which the information stored in DNA is copied to RNA. For protein-coding genes, the resulting messenger RNA (mRNA) can serve as a template to produce a protein through the process of translation. Those steps are highly regulated to control when and how much protein of a particular gene is produced[1,2].

In eukaryotic cells, coding genes are transcribed by RNA polymerase II in the nucleus. The produced pre-mRNA then undergoes several processing steps to become a mature mRNA. These include the addition of a 5′ 7-methylguanosine cap, removal of introns through splicing, and cleavage and polyadenylation of the 3′ end. Together, these steps contribute to the stability of the mRNA, its export from the nucleus, and its subsequent translation in the cytoplasm[3,4]. Once the mature mRNA is in the cytoplasm, it can be recognized by the translation machinery and used for protein synthesis.

The amount of mature mRNA present in the cells does not necessarily reflect how much protein will ultimately be made. In the cytoplasm, an mRNA can have different fates. It may actively get translated, temporarily get stored in a translationally inactive state, get localized to a particular region or organelle of the cell, or be targeted for degradation. This explains why mRNA and protein levels do not always show a direct relationship within the cell[1,5].

This control level is essential because cells are constantly adjusting how much protein they are producing as their needs change. For instance, some proteins are required continuously, whereas others are only needed under particular conditions. Also, cells must respond to surrounding

changes such as nutrient availability, oxidative stress, and other environmental challenges. Instead of changing the expression of every gene at the level of transcription, cells can also regulate the use of mRNAs that have already been produced.

Therefore, translation is a major point at which gene expression can be controlled. Regulating translation is particularly important during cellular stress, when overall protein synthesis can change while the translation of specific mRNAs is maintained or increased to support adaptation and survival[6]. Given the importance of translational control in determining protein production, understanding the mechanisms that regulate translation plays an important role in understanding gene expression as a whole.

1.2 Protein synthesis occurs through the translation of mRNA

Translation is the process through which the information carried by an mRNA is decoded to produce a protein. This process is carried out by ribosomes, which are large complexes made up of ribosomal RNAs (rRNAs) and ribosomal proteins[7]. As the ribosome moves along the mRNA in the 5′ to 3′ direction, it reads the sequence three nucleotides at a time. Each group of three nucleotides is referred to as a codon, and each codon is recognized by a tRNA carrying a corresponding amino acid. In this way, amino acids are added in the correct order to the growing polypeptide chain[8].

In eukaryotic cells, the ribosome is made up of a small 40S subunit and a large 60S subunit that come together to form the 80S ribosome. Each subunit has a different role during translation. The 40S subunit interacts with the mRNA and is responsible for decoding its sequence, while the 60S subunit contains the peptidyl transferase centre. This is where peptide bond formation occurs between amino acids[9]. The ribosome also contains three sites through which tRNAs move during protein synthesis: the aminoacyl (A), peptidyl (P), and exit (E) sites. During translation, tRNAs

move through these sites while the polypeptide chain is extended one amino acid at a time. These coordinated steps allow the sequence of an mRNA to be accurately translated into its corresponding protein[8].

1.3 Protein synthesis is highly regulated at the stage of translation initiation

Translation begins with initiation, during which the mRNA, initiator tRNA, and ribosomal subunits are brought together at the start codon[10,11]. One of the early steps in this process is the formation of the ternary complex. During this step, the initiation factor eIF2 binds GTP and associates with the initiator methionyl-tRNA (Met-tRNAi). This complex is then recruited to the 40S ribosomal subunit along with other initiation factors, forming what is known as the 43S pre-initiation complex (PIC)[10,12]. The 43S PIC is then recruited to the 5′ end of the mRNA through interactions involving eIF3 and the eIF4F complex, which includes the cap-binding protein eIF4E, the scaffold protein eIF4G, and the RNA helicase eIF4A. Once bound, the ribosome starts searching for a suitable start codon (AUG) to recruit the 60S and start translation[11,12].

The 40S subunit scans along the 5′ untranslated region (5′ UTR) in the 5′ to 3′ direction until it encounters a suitable start codon. Recognition of the start codon also depends on the surrounding nucleotide sequence, known as the Kozak sequence, which influences the efficiency of start codon recognition and allows the initiator tRNA to base-pair with it[10]. Once the start codon has been recognized, several initiation factors are released. The 60S ribosomal subunit then joins the 40S subunit. This results in the formation of an 80S ribosome positioned at the start codon and ready to enter the elongation phase[10,11].

Since initiation determines whether a ribosome is successfully recruited to an mRNA, it is a major stage at which protein synthesis can be regulated. This regulation allows cells to adjust

protein production in response to stress, nutrient availability, and other changes in cellular conditions[6,12].

1.4 Controlling translation during stress

1.4a Cis-regulatory mechanisms of selective translation: uORFs

During stress, overall protein synthesis is often reduced. However, some mRNAs continue to be translated or are translated more efficiently. One mechanism that allows this selective regulation involves upstream open reading frames (uORFs). uORFs are short open reading frames (starting with an AUG and ending with a stop codon) located upstream of the main protein-coding sequence and can be recognized and translated by the ribosome. Because ribosomes encounter these regions before reaching the main coding sequence, uORFs can influence how efficiently the main protein is produced. In general, translation of a uORF reduces translation of the downstream main coding region. However, under certain conditions (such as during cellular stress), the change in translation initiation can reduce uORF translation and increase translation of the main coding region[13].

A well-studied example of this type of regulation is the activating transcription factor 4 (ATF4), which is an important regulator of the cellular response to stress in humans. The translation of this transcription factor is controlled by two uORFs found in its 5′ leader. Under normal conditions, translation of these uORFs and associated dissociation of ribosomes limits the amount of ATF4 protein that is produced. However, during amino acid starvation, uncharged tRNAs accumulate and activate the eIF2α kinase GCN2. GCN2 then phosphorylates eIF2α, and thus reduces the availability of the ternary complex required for translation initiation. Although this generally leads to a reduction in protein synthesis, it has the opposite relative effect on expression of ATF4. The reduced availability of the ternary complex changes where ribosomes

reinitiate, after translating the first uORF. This allows more ribosomes to bypass the inhibitory second uORF and initiate at the ATF4 coding region instead (Figure 1). As a result, ATF4 translation increases during amino acid starvation, and cells are allowed to produce proteins needed to adapt to nutrient stress[14,15].

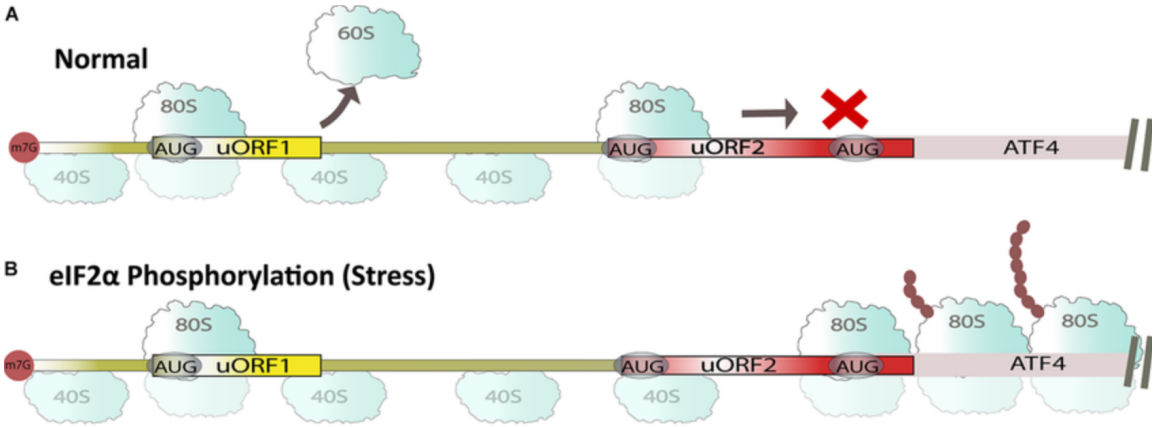


Figure 1: uORF-mediated regulation of ATF4 translation during normal and stress conditions. Under normal conditions, ribosomes translate uORF1 and subsequently reinitiate at the inhibitory uORF2, limiting translation of the downstream ATF4 coding sequence. During cellular stress, phosphorylation of eIF2α reduces ternary complex availability, delaying translation reinitiation and allowing ribosomes to bypass uORF2 and initiate at the ATF4 coding sequence, resulting in increased ATF4 expression. Adapted from Chukka et al. (2021).

This provides an example of how uORFs can regulate the translation of specific mRNAs. This regulation allows cells to reduce overall protein synthesis while maintaining or increasing the production of proteins needed to respond to changing cellular conditions. The ability of uORFs to selectively regulate translation in response to cellular conditions has made them an active area of study, particularly for understanding how cells rapidly adapt gene expression to stress and changes in nutrient availability.

1.4b Trans-acting regulation of mRNA translation: RNA-binding proteins (RBP)

In addition to cis-regulatory features found within the mRNA itself, such as uORFs, the fate of an mRNA can also be influenced by trans-acting factors, including proteins that bind to

the RNA. These proteins are known as RNA-binding proteins (RBPs), and they can interact with all different types of RNA, including mRNAs, rRNAs, tRNAs, and non-coding RNAs. RBPs are involved in many steps of RNA regulation, including RNA processing, localization, stability, translation, and degradation[16,17].

RBPs can interact with RNA using different RNA-binding regions. Some examples are RNA recognition motifs (RRMs), K-homology (KH) domains, and zinc-finger domains. Each of these regions can recognize certain RNA sequences or structures. Thus, different RBPs can interact with different RNA targets and influence what happens to them[15]. For example, some RBPs can increase or decrease its translation by mainly affecting its interaction with the translation machinery. Others can influence how long the mRNA remains in the cell by protecting it from degradation or promoting its decay. They can also help move or localise mRNAs to specific parts of the cell, where they can be stored or translated when needed[17,18].

These interactions between RBPs and mRNAs can also change depending on the conditions of the cell. This is especially important during stress, when cells need to change protein production quickly. In other words, RBPs can help control which mRNAs remain available for translation and which are removed from the translating pool[17].

One group of RBPs that has been linked to both RNA stability and translation is the La-related protein (LARP) family. LARPs share some conserved regions involved in RNA binding, but different members of the family can bind different RNA targets and have different functions within the cell[19,20].

1.5 La-related proteins are a conserved family of RNA-binding proteins

The La-related proteins (LARPs) are a conserved family of RNA-binding proteins found in all eukaryotes. They are named after the La protein, which was first identified as an

autoantigen in patients with autoimmune diseases such as systemic lupus erythematosus, Sjogren's syndrome and neonatal lupus[19]. Later, these proteins were found to have important roles in RNA metabolism. La binds newly synthesized RNA polymerase III transcripts, including precursor tRNAs, through their common UUU-3′OH containing ends. This interaction protects these newly produced RNAs from exonucleolytic decay and helps them fold and mature correctly[19,21].

A characteristic feature of genuine La proteins is the presence of two neighbouring RNA-binding domains: the N-terminally located La motif (LaM), which is followed by the RNA recognition motif (RRM). Together, these domains form what is known as the La module. The La motif is highly conserved and is also found in a larger group of proteins that became known as La-related proteins. Although LARPs share this connection to La, they have evolved to recognize different RNA targets and carry out different functions within the cell[19,22].

The LARP family is generally divided into five major groups: LARP1, LARP3 (the genuine La proteins), LARP4, LARP6, and LARP7. These groups can be distinguished by differences in their protein sequences and domain organization. During evolution, the different LARP families acquired additional domains and changes within their RNA-binding regions that altered the types of RNA they recognize. Thus, members of the LARP family are involved in a wide range of RNA-related processes[21,23].

For instance, LARP7 proteins are more closely related to genuine La. They mainly interact with specific non-coding RNAs, including 7SK RNA in humans[21]. In contrast, LARP1, LARP4, and LARP6 are mainly associated with mRNAs in the cytoplasm and have been linked to the control of mRNA stability and translation[21]. For example, LARP6 is best known for regulating the translation of mRNAs encoding the most abundant protein in the human body, type I collagen.

LARP4 binds poly(A) RNA and interacts with poly(A)-binding protein (PABP), contributing to mRNA stability and translation[24,25]. These findings show that although LARPs share a common evolutionary origin, individual family members have developed specialized roles in RNA regulation.

Some of these gained special functions come from regions outside the La module. In other words, different LARP families contain additional domains that contribute to their specific RNA-binding properties and interactions with other proteins. For instance, most LARP1 family members contain a conserved C-terminal region known as the DM15 domain. The DM15 domain in LARP1 family members is an additional RNA-binding surface that contributes to their ability to recognize specific mRNAs[21,22]. LARP1 is of particular interest because of its important role in the regulation of protein synthesis. It has been especially linked to the regulation of mRNAs encoding components of the translation machinery such as ribosomal proteins. By binding these mRNAs and influencing their translation, LARP1 provides a direct link between mRNA regulation and protein synthesis**.** Understanding how LARP1 recognizes and regulates these transcripts has therefore become an important area of research in translational control.

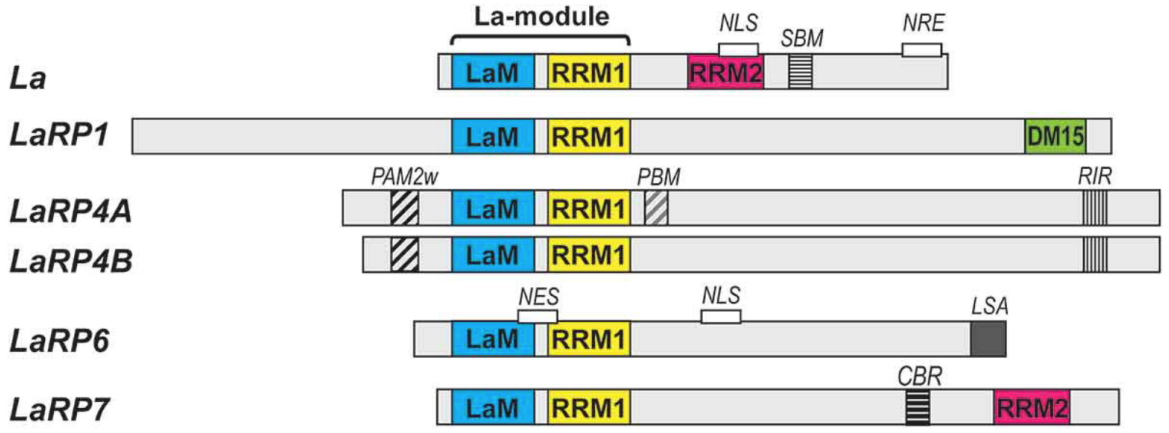


Figure 2: Domain organization of La and La-related proteins (LARPs). The conserved La motif (LaM) and RRM1, along with family-specific domains such as the DM15 region in LARP1, are shown. Adapted from Maraia et al. (2017).

1.6 LARP1 is an important regulator of mRNA translation.

LARP1 has been strongly linked to the regulation of mRNAs that encode components of the protein synthesis machinery. Many of these transcripts belong to a group known as 5′ terminal oligopyrimidine (5′TOP) mRNAs. These mRNAs contain a characteristic sequence at their 5′ end that begins with an invariant cytidine followed by a stretch of 5-15 pyrimidines. This group includes many mRNAs encoding ribosomal proteins, translation factors, and other proteins needed for ribosome production and function. Since producing ribosomes and maintaining the translation machinery require a large amount of cellular energy, translating these mRNAs is highly regulated according to the growth and nutrient conditions of the cell[10,26,27].

LARP1 plays an important role in this regulation through its ability to bind directly to 5′TOP mRNAs. This binding is mediated by its C-terminal DM15 region, which recognizes the 5′ end of these transcripts and bind to the 7-methylguanosine cap and the nearby TOP sequence[28]. This interaction is important because the 5′ cap is also recognized by eIF4E. When LARP1 binds this region, it can compete with eIF4E for access to the cap. This in turn limits the formation of the eIF4F complex and therefore reduces translation initiation on these mRNAs (Figure 3b) [28,29].

Moreover, the ability of LARP1 to regulate these transcripts is connected to the nutrient state of the cell. One of the main pathways involved in this regulation is the mechanistic target of rapamycin complex 1 (mTORC1). This pathway is highly responsive to signals such as nutrient and amino acid availability, and it promotes cell growth and protein synthesis when conditions are favourable.

LARP1 is also involved in regulating the stability of the mRNAs that it binds. Studies in mammalian cells have shown that reducing LARP1 levels can decrease the abundance or stability of some TOP mRNAs. This suggests that LARP1 can help protect these transcripts from

degradation or decay [30,31]. Thus, reducing the translation of a transcript does not necessarily lead to its degradation. Instead, LARP1 can help maintain these mRNAs in the cell while their translation is reduced. Consequently, they remain available to be used again when cellular conditions become more favourable.

Although LARP1 family proteins are conserved across all eukaryotes, some of the features involved in RNA recognition are not present in all LARP1 orthologs. One important difference is the presence of the DM15 region. DM15 has been identified in most LARP1 orthologs studied so far but is absent from yeast orthologs and from two of the three orthologs in *Arabidopsis*. LARP1 proteins that lack the DM15 region are referred to as short-form LARP1 proteins (LARP1-S). This is particularly interesting given the established role of the DM15 region in recognizing 5′TOP mRNAs. In addition, some organisms, including *Schizosaccharomyces pombe*, do not have the classical 5′TOP motif found in mammals. Therefore, the conservation of LARP1 proteins in organisms that lack both the DM15 region and classical 5′TOP mRNAs raises an important question: how do these LARP1 orthologs recognize and interact with their target mRNAs?[32]

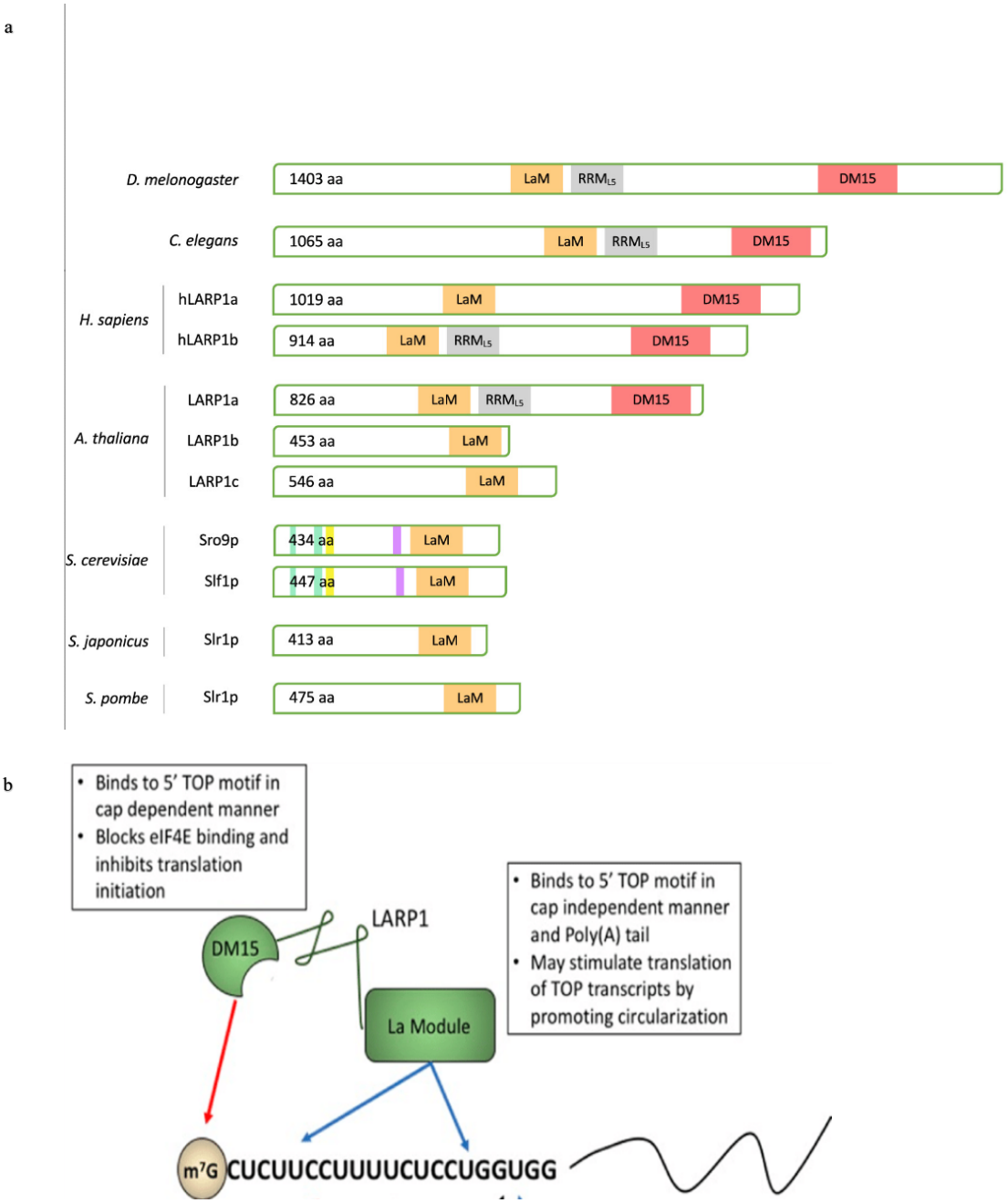


Figure 3: LARP1 domain conservation and the role of the DM15 domain in 5'TOP regulation
(A) Domain organization of LARP1 homologs across different species. Adapted from Mansouri-Noori et al. (2023).
(B) Proposed interactions of the LARP1 DM15 domain and La module with 5′TOP mRNAs. Adapted from Al-Ashtal et al. (2020).

1.7 The *S.pombe* LARP1 ortholog Slr1p is important for translation regulation of proto-5′TOP mRNAs

The fission yeast *Schizosaccharomyces pombe* provides an interesting system for understanding how LARP1 proteins function in the absence of the DM15–5′TOP interaction. *S. pombe* contains a single LARP1 ortholog, Slr1p, which shares the conserved La motif found in other LARPs, but lacks the DM15 region found in mammalian LARP1. Additionally, *S. pombe* ribosomal protein mRNAs do not contain the 5′TOP motif found in mammals. Despite these differences, Slr1p has been linked to the regulation of mRNAs similar to those regulated by LARP1 in higher eukaryotes. Specifically, many Slr1p target mRNAs encode ribosomal proteins and other components involved in protein synthesis[26].

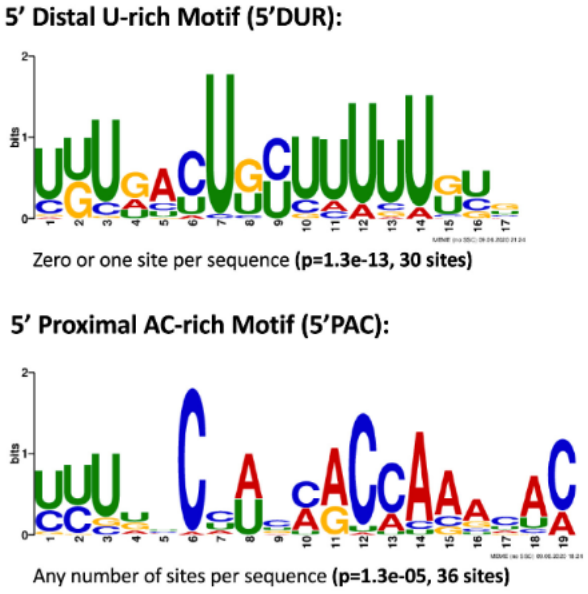
Previous analysis of the 5′ untranslated regions of *S. pombe* ribosomal protein mRNAs revealed that, although they lack a classical 5′TOP sequence, many of these transcripts still contain pyrimidine-rich sequences near their 5′ ends. These sequences were described as proto-5′TOP motifs because they resemble some features of the mammalian TOP sequence. This finding suggested that the connection between LARP1 proteins and pyrimidine-rich mRNAs may have appeared earlier in evolution, before the development of the classical 5′TOP system found in higher eukaryotes[26].

Additionally, sequences within Slr1p target mRNAs were further investigated. The UTRs of mRNAs stabilized by Slr1p were found to have a generally higher GC content. Also, two distinct motifs were enriched within these transcripts. One was a U-rich motif and was more commonly found toward the 5′ end of the UTR. The other was an AC-rich motif and was more likely to be found closer to the start codon. Based on their sequence and location, these were named the 5′ distal U-rich (5′DUR) and 5′ proximal AC-rich (5′PAC) motifs, respectively[32] (Figure 4a). Further experiments showed that Slr1p could directly interact with mRNAs containing the 5′PAC motif in *vitro.* Previous electrophoretic mobility shift assays (EMSAs) revealed that this

interaction was sequence specific, in that binding was disrupted upon mutating the most highly conserved nucleotides. For example, Slr1p- 5′PAC binding was lost when the conserved cytosines (C6, C12 and C19) within the motif were changed to guanines (Figure 4b). This indicates that these nucleotides are essential for Slr1p binding. Therefore, the 5′PAC motif may provide an alternative way for Slr1p to recognize its target mRNAs in the absence of the DM15-dependent 5′TOP recognition used by mammalian LARP1[26].

Together, these findings provide a possible explanation for how Slr1p recognizes specific mRNAs despite lacking the DM15 region found in mammalian LARP1. However, identifying the RNAs that Slr1p binds is only part of understanding its function. An important additional question is how this interaction affects the translation and stability of these mRNAs.

a



b

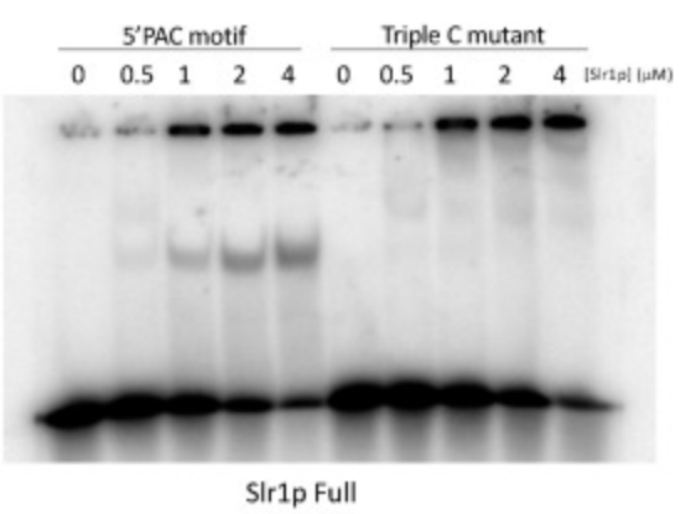


Figure 4: Identification of Slr1p-bound 5′ UTR motifs. (A) 5′DUR and 5′PAC motifs identified in Slr1p-associated mRNAs. **(B)** EMSA showing Slr1p binding to the 5′PAC motif, which is disrupted by mutation of three conserved cytosines. Adapted from Mansouri-Noori et al. (2023).

1.8 Slr1p is involved in regulating translation initiation

Previous studies have linked Slr1p to translation initiation and suggested that it helps support efficient protein synthesis. One important piece of evidence comes from its interaction with translation initiation factors. Slr1p was shown to interact with Tif471p, an eIF4G protein in *S. pombe*. This interaction was maintained following RNase treatment. This suggests that the two proteins interact independently of their association with RNA. Slr1p was also found to interact with Sum1p, the *S. pombe* homolog of eIF3i. Since eIF4G and eIF3 are both involved in recruiting the ribosome to mRNA during translation initiation, these interactions support a role for Slr1p at this stage of translation[26].

Additionally, polysome profiling was performed in wild-type and *slr1*- cells in the presence of the translation initiation inhibitor harringtonine. In *slr1*- cells, a diminishing pattern of heavy polysomes relative to light polysomes was observed, along with an 80S peak that was higher than the 60S and 40S subunit peaks. In contrast, wild-type cells exhibited more sustained heavy

polysomes and lower subpolysomes. This was indicated by an 80S peak that was lower than the 60S peak (Figure 5)[26]. Together, these findings support the involvement of Slr1p in translation initiation. However, the exact mechanism through which Slr1p contributes to this process and whether its effects are specific to particular groups of mRNAs remain unclear.

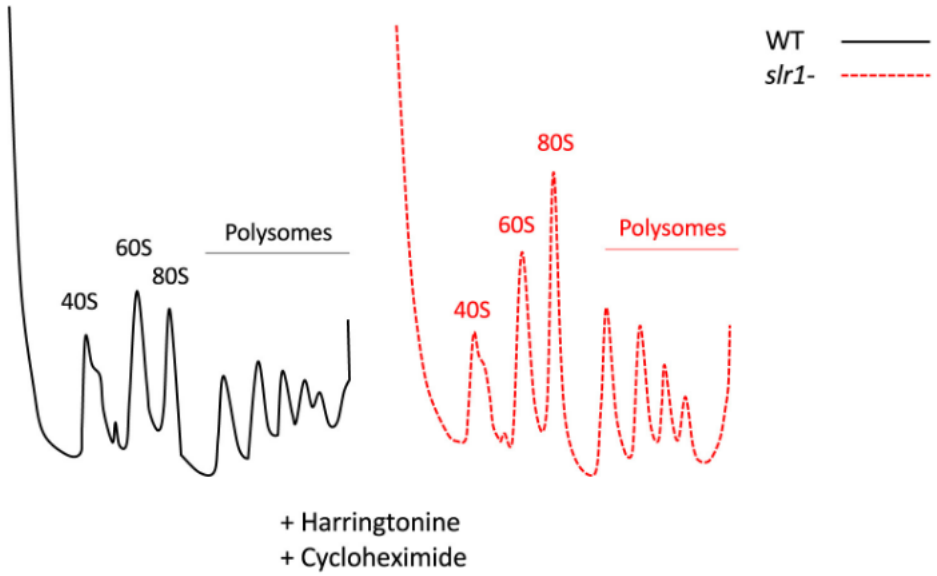


Figure 5: Polysome profiling for WT and slr1Δ following harringtonine treatment. Polysome profiling for wild-type (WT) and *slr1Δ* cells following harringtonine and cycloheximide treatment. The *slr1Δ* profile shows increased 80S peaks and reduced polysomes compared with WT. Adapted from Mansouri-Noori et al. (2023).

1.9 Slr1p may also be involved in translation elongation and maintaining translational fidelity

Although Slr1p has been linked to translation initiation, additional evidence has recently suggested that its role may extend to translation elongation. One observation supporting this possibility is the sensitivity of cells lacking Slr1p to 3-aminotriazole (3-AT). 3-AT is considered a translation elongation inhibitor. It inhibits histidine biosynthesis, which can limit the availability of charged histidyl-tRNAs and cause ribosomes to stall at histidine codons. In yeast spotting assays, *slr1-* cells showed reduced growth when 3-AT treatment was combined with allowing the cells to reach stationary phase compared to wild-type cells (Figure 6). This

increased sensitivity suggested that Slr1p may become particularly important under conditions where translation elongation is disrupted[33].

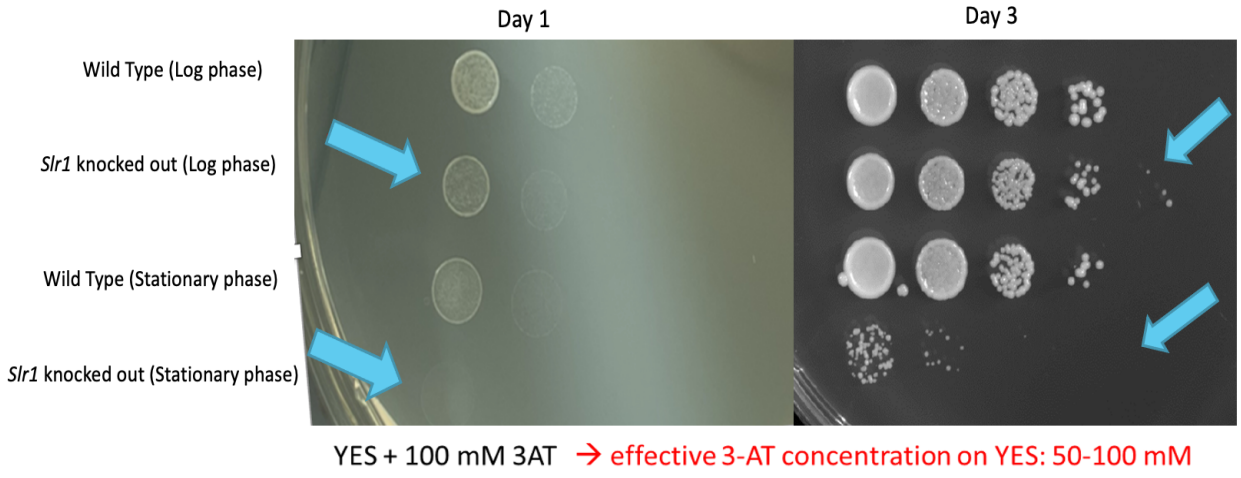


Figure 6: Loss of Slr1p increases sensitivity to 3-AT. Yeast spotting assay comparing the growth of wild-type and slr1Δ S. pombe cells on YES medium containing 100 mM 3-AT. slr1Δ cells showed reduced growth compared with wild-type cells. (Mansouri-Noori, unpublished)

Evidence from other yeast LARP1 orthologs also supports the possibility that this family of proteins can function during elongation. *Saccharomyces cerevisiae* contains two related proteins, Slf1p and Sro9p, which share several features with Slr1p. Like Slr1p, they belong to the shorter forms of LARP1 proteins. In other words, they also lack the DM15 region found in most LARP1 orthologs. Both proteins are mainly found in the cytoplasm and associate with translating ribosomes and polysomes, and have been connected to the translation of mRNAs needed during cellular stress[41]. Slf1p, in particular, has been shown to support the expression of mRNAs involved in the response to oxidative stress. Interestingly, these mRNAs were found to remain associated with translating ribosomes in the absence of Slf1p (Figure 7). However, the amount of protein produced from them was reduced. This suggests that ribosomes are still recruited to these mRNAs, but translation is less efficient after initiation has already occurred. These observations

provided early evidence that Slf1p may act during translation elongation rather than only during ribosome recruitment or initiation[35].

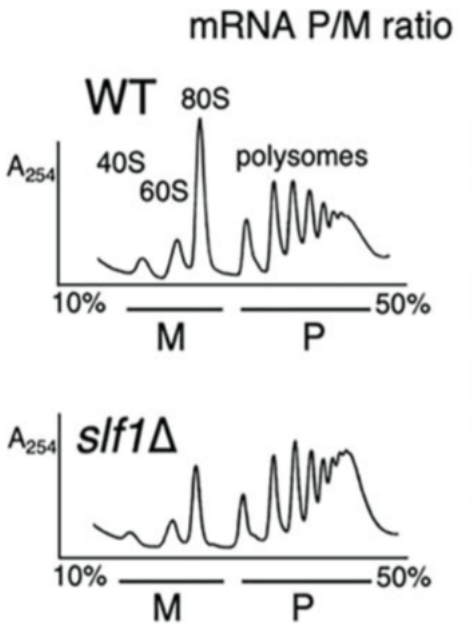


Figure 7: Ribosome association with mRNAs in WT and slf1Δ cells. Polysome profiles of WT and *slf1Δ S. cerevisiae* cells showing that mRNAs remain bound to ribosomes in the presence and absence of slf1. Adapted from Jennings et al. (2023).

More recent work has provided a clearer picture of where Slf1p and Sro9p are located during translation. PAR-CLIP analysis showed that both proteins bind mainly within the coding regions of their target mRNAs, localised to regions that are actively being translated by ribosomes. This suggests that those proteins can influence translation after initiation has already occurred[35].

The position they occupy became even more interesting during oxidative stress. Following H_2O_2 treatment, Slf1p and Sro9p binding sites were frequently found in the regions between neighbouring ribosome footprints. This means that those proteins were localised between ribosomes at a time when oxidative stress can interfere with normal ribosome movement (Figure

8). Therefore, their position between two ribosomes suggested that these proteins may help reduce ribosomal collisions and contribute to the maintenance of translational fidelity[35].

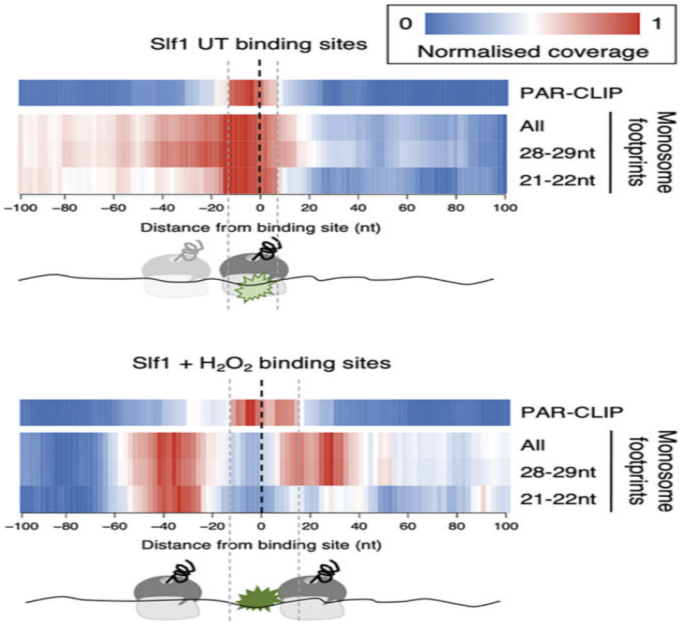


Figure 8. Slf1p binding sites relative to translating ribosomes during oxidative stress. Distribution of Slf1p PAR-CLIP binding sites relative to ribosome footprints under untreated and H_2O_2-treated conditions. Adapted from Jennings et al. (2023).

This connection was further supported by examining collided ribosomes. When one ribosome stalls during translation, a following ribosome can catch up with it and collide. This produces a pair of ribosomes known as a disome. Disome profiling showed that mRNAs bound by Slf1p and Sro9p contained significantly more disomes than mRNAs that were not targeted by these proteins. Importantly, the positions of these disomes also overlapped with the PAR-CLIP binding sites of Slf1p and Sro9p (Figure 9b). This places the proteins directly at regions where ribosomes tend to stall or collide. Slf1p was further detected in isolated disome and trisome fractions and was more abundant in these fractions relative to monosomes (Figure 9a). Together, these

findings provide stronger evidence that Slf1p associates with collided ribosomes and suggest that Slf1p and Sro9p may function at sites where elongating ribosomes encounter difficulties[35].

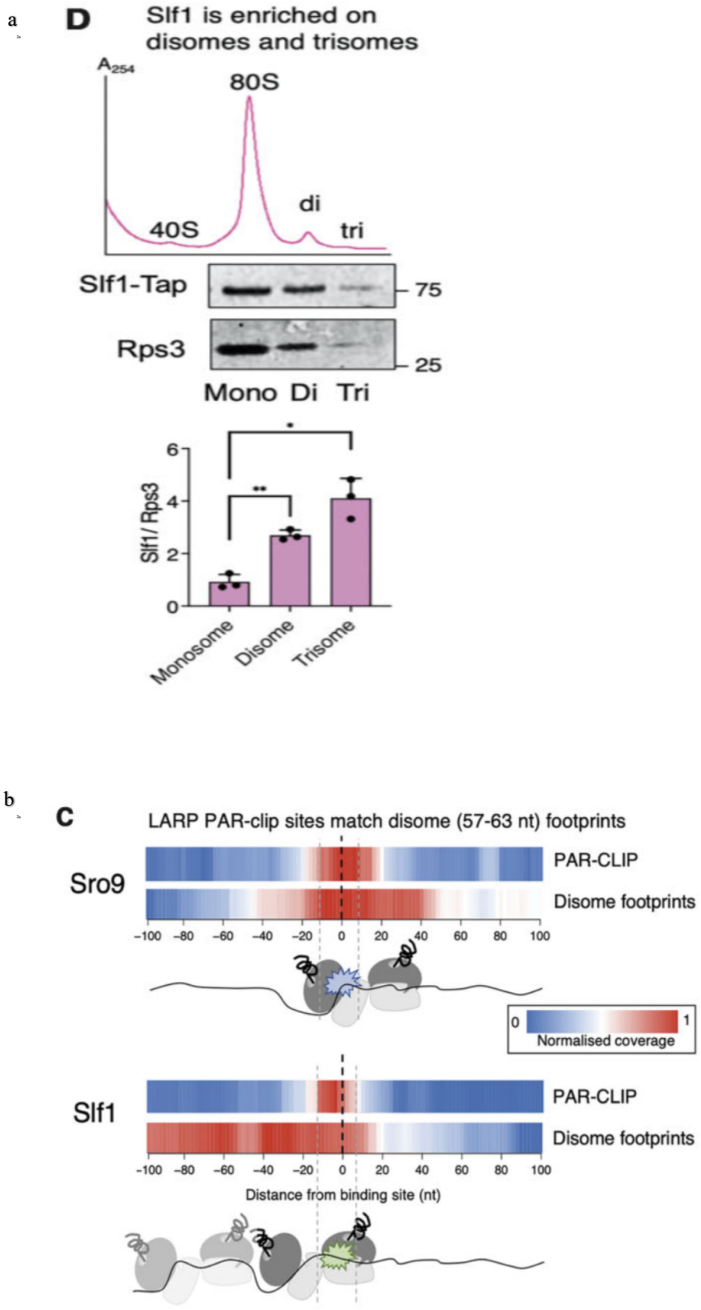


Figure 9: Association of Slf1 with collided ribosomes.
(A) Slf1 enrichment in monosome, disome, and trisome fractions
(B) Disome footprints mapped around Sro9 and Slf1 PAR-CLIP binding sites. Adapted from Jennings et al. (2023).

Ribosome stalling and collisions can also create problems for translational fidelity. During normal elongation, the ribosome must maintain the correct reading frame as it moves along the mRNA. However, under certain conditions, the ribosome can shift by one nucleotide in either the −1 or +1 direction, which changes how the downstream sequence is decoded. This process is known as ribosomal frameshifting. While programmed frameshifting is used by some viruses as a mechanism of gene regulation, maintaining the correct reading frame is generally important for accurate protein synthesis.

The involvement of Slf1p in this process was examined using reporters containing sequences that promote programmed frameshifting. These included an L-A double-stranded RNA virus sequence, which normally promotes −1 ribosomal frameshifting. The second contained a sequence from the Ty1 retrotransposon, which promotes +1 ribosomal frameshifting. In the absence of Slf1p, −1 frameshifting was increased under non-stressed conditions, suggesting that Slf1p normally helps prevent ribosomes from shifting into the −1 reading frame (Figure 10). Slf1p was also found to influence and inhibit +1 frameshifting under oxidative stress[35]. Together with its association with stalled and collided ribosomes, these findings support a role for Slf1p in maintaining translational fidelity during elongation.

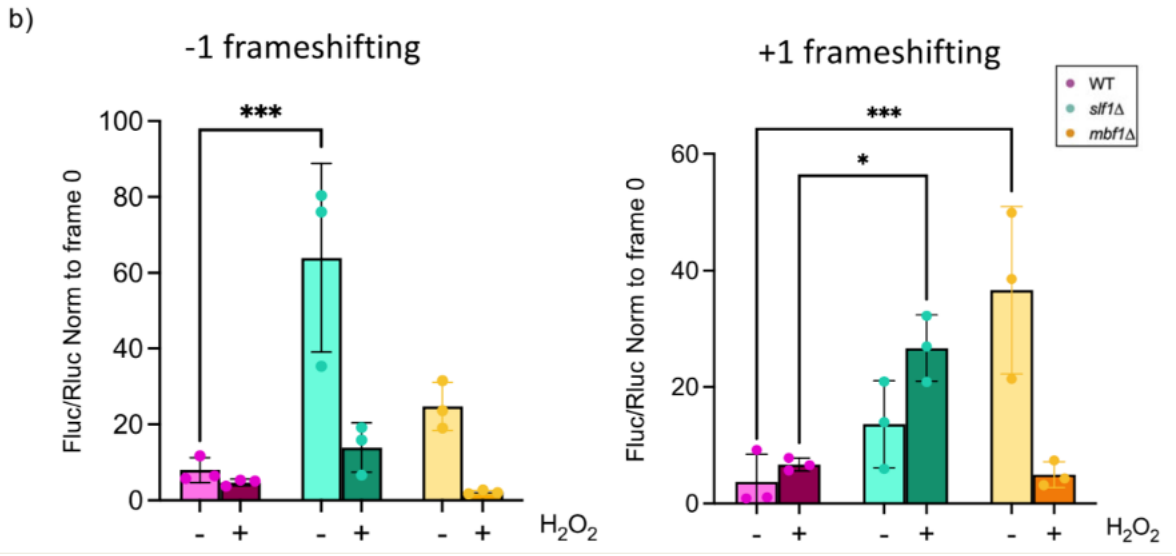


Figure 10: Slf1 contributes to translational reading-frame maintenance. −1 and +1 frameshifting in WT and *slf1Δ* cells under untreated (−) and H_2O_2-treated (+) conditions. Frameshifting was measured using dual-luciferase reporters and normalized to the frame 0 control. Adapted from Jennings et al. (2023).

Overall, these studies suggest that the functions of La-related proteins during translation are not necessarily limited to initiation. Slf1p and Sro9p remain associated with mRNAs during active translation and are positioned near stalled and collided ribosomes. Furthermore, Slf1p has been also linked to maintaining the correct reading frame. Therefore, the increased sensitivity of *slr1-* cells to conditions that promote elongation stalling raises the possibility that Slr1p may have a related function in *S. pombe*. However, whether Slr1p contributes to maintaining translational fidelity and limiting ribosomal frameshifting remains unclear.

1.10 Slr1p may have an essential function in mRNA decay

In addition to its role in translation, Slr1p has also been linked to mRNA stability and decay. This is particularly interesting because translation and mRNA degradation are often closely connected. The association of an mRNA with ribosomes and RNA-binding proteins can influence whether it continues to be translated or becomes available to the mRNA decay machinery, and several mRNA decay processes, like nonsense-mediated decay (NMD), depend on translation[36].

Previous co-immunoprecipitation (co-IP) and yeast two-hybrid analyses independently showed that Slr1p was also found to interact with Exo2p, the *S. pombe* homolog of the conserved and primary mRNA 5′–3′ exoribonuclease XRN1 (Figure 11). XRN1-family proteins have a major role in cytoplasmic mRNA decay, where they degrade mRNAs from the 5′ end following removal of the 5′ cap. Co-IP between Slr1p and Exo2p persisted following RNase treatment, indicating that the association was not simply caused by both proteins binding to the same RNA molecule[30].

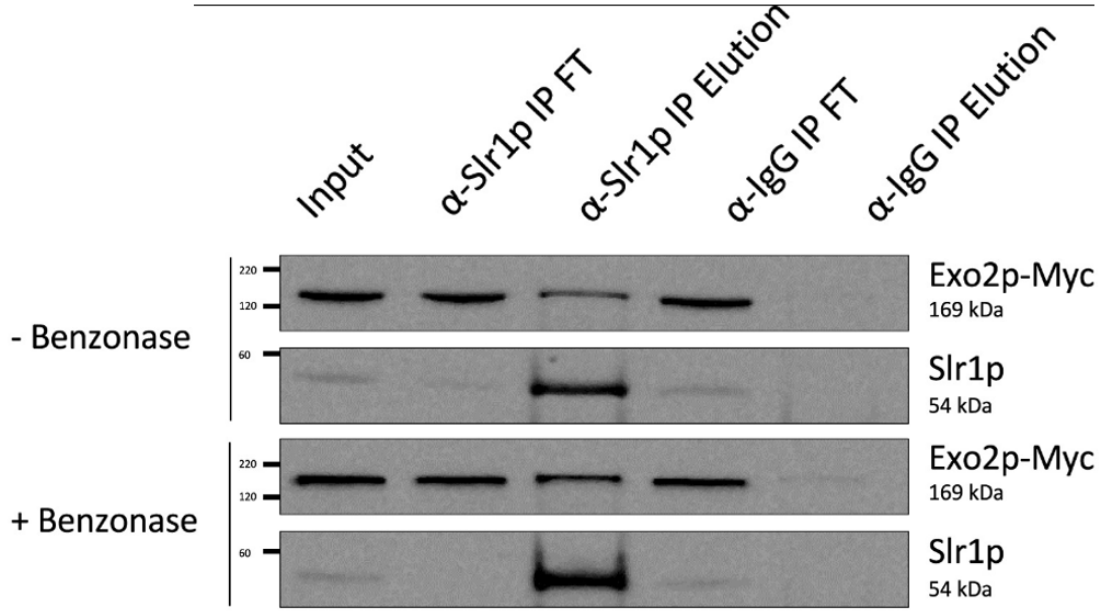


Figure 11: Slr1p interacts with Exo2p independently of RNA. Co-immunoprecipitation of Slr1p and Exo2p-Myc in the absence (−) or presence (+) of Benzonase. The interaction was maintained following Benzonase treatment, suggesting that the Slr1p–Exo2p interaction is RNA-independent. Adapted from Mansouri-Noori et al. (2023).

Moreover, Slr1p was also found to influence where Exo2p associates with the translation machinery. Following polysome profiling, more Exo2p was recruited from the 40S and 60S fractions into monosome fractions in wild-type cells compared with cells lacking Slr1p. In contrast, the distribution of PABP was not similarly affected (Figure 12). These findings

suggested that Slr1p may help recruit Exo2p to translating ribosomes and raised the possibility that Slr1p connects its role in promoting translation with translation-associated mRNA decay[32].

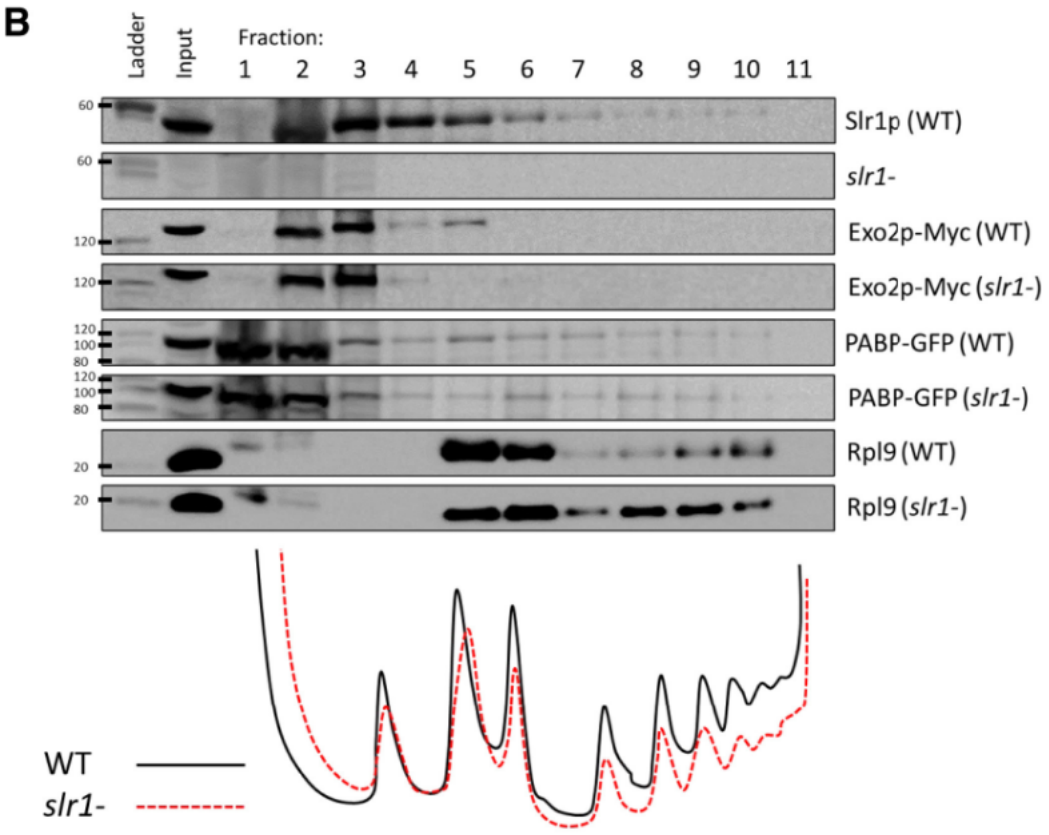


Figure 12: Slr1p affects Exo2p association with ribosomes. Distribution of Slr1p and Exo2p-Myc across polysome fractions in WT and *slr1−* cells. Adapted from Mansouri-Noori et al. (2023).

1.11 Phosphorylation as a potential switch between Slr1p functions in translation and mRNA decay

How Slr1p is regulated between its different functions is not yet fully understood. One mechanism that has been proposed is phosphorylation. Phosphorylation is a reversible post-translational modification that can alter protein activity, structure, localization, or interactions with other proteins. It can therefore allow the function of a protein to change in response to different cellular conditions without requiring a change in the amount of protein produced.

Phosphorylation is already well established as an important regulator of mammalian LARP1. Human LARP1 contains several phosphorylation sites and is regulated downstream of signalling pathways including mTORC1. Changes in its phosphorylation state have been linked to differences in its interaction with 5′TOP mRNAs and components of the translation machinery. Phosphorylation has therefore been proposed to allow LARP1 to shift between different functional states depending on the growth and nutrient conditions of the cell[37,38,39].

A similar mode of regulation may also occur in Slr1p, as several phosphorylation sites have been identified within the protein, raising the possibility that phosphorylation may contribute to the regulation of Slr1p function. This is particularly interesting given that Slr1p has been linked to several processes, including translation regulation and mRNA stability. Phosphorylation may therefore provide a mechanism for modulating Slr1p activity in response to different cellular conditions[33].

Among the phosphorylation sites identified in Slr1p are serine 410 (S410) and serine 437 (S437). These residues were previously reported to be phosphorylated in a TOR-dependent manner[40]. To explore the potential roles of phosphorylation at these sites, previously generated Slr1p phospho-mutants were used in this study. In the phospho-ablative mutants, serine was replaced with alanine to prevent phosphorylation, generating S410A and S437A. In contrast, serine was replaced with the negatively charged amino acid aspartic acid in the phosphomimetic mutants, generating S410D and S437D to mimic some properties of the phosphorylated state. The functional significance of phosphorylation at these sites remains unclear, particularly in terms of how it may influence Slr1p function.

Since phosphorylation can alter protein–protein interactions, changes in the phosphorylation state of Slr1p could potentially influence its association with proteins involved in translation or

mRNA decay, such as Exo2p. Phosphorylation could therefore provide a possible switch between these different functions of Slr1p. Whether phosphorylation at specific sites affects the interaction between Slr1p and Exo2p remains unknown. Examining this relationship may help explain how the roles of Slr1p in translation and mRNA decay are regulated within the cell.

1.12 Research objectives and hypotheses

Previous work has linked Slr1p to both the initiation and elongation phases of mRNA translation, but several aspects of its function remain unclear. Control of gene expression through the use of uORFs is notable in that they are linked to both translation initiation and elongation, but it is not known whether Slr1p contributes to the translation of stress-responsive mRNAs containing regulatory uORFs. It is also not known whether its role extends beyond translation initiation to maintaining translational fidelity during elongation, or whether Slr1p may provide a connection between translation and mRNA decay. Thus, the overall aim of this study was to further define the role of Slr1p in mRNA translation and to investigate its potential involvement in translation-associated mRNA decay.

The first objective was to determine whether Slr1p contributes to the translation of stress responsive genes mRNAs containing uORFs and whether this role changes under different cellular conditions. Dual-luciferase reporters containing the 5′ leaders of the well-characterized uORF containing *S. cerevisiae GCN4* and the candidate uORF containing *Hri2* from *S. pombe* were used to compare reporter expression between WT and *slr1Δ* cells under non-stressed conditions and following heat shock, oxidative stress, or amino acid starvation. **It was hypothesized that loss of Slr1p would alter expression from mRNAs containing uORF-regulated 5′ leaders and that this effect may differ between stressed and non-stressed conditions.**

The second objective was to investigate whether Slr1p contributes to translational fidelity during elongation. Previous evidence linking LARP1-family proteins to translating ribosomes and their positioning between neighbouring ribosomes suggested that these proteins may contribute to proper ribosome behaviour during translation. To test whether Slr1p influences maintenance of the reading frame, dual-luciferase frameshifting reporters were used to measure −1 and +1 programmed ribosomal frameshifting in WT and *slr1Δ* cells. **It was hypothesized that loss of Slr1p may increase frameshifting, consistent with a role for Slr1p in maintaining translational fidelity.**

Finally, the third objective was to investigate whether Slr1p may connect mRNA translation with mRNA decay. This was addressed by examining the interaction between Slr1p and the 5′–3′ exonuclease Exo2p. Because phosphorylation may provide a mechanism through which Slr1p changes its interactions or functions, phosphorylation sites S410 and S437 were also investigated using phosphoablative and phosphomimetic mutants. **It was hypothesized that Slr1p interacts with Exo2p and that this interaction may be influenced by the phosphorylation state of Slr1p.** Together, these experiments were designed to determine whether Slr1p functions across multiple stages of mRNA regulation, from controlling translation to maintaining translational fidelity and potentially linking translation with mRNA decay.

Chapter 2: Materials and Methods

2.1 Molecular cloning

2.1a Cloning the uORF reporter constructs

The *Renilla luciferase* (*Rluc*) gene was first cloned into the *Schizosaccharomyces pombe* expression vectors pRep4x and pRep82x following the protocol described by the National Library of Medicine[41]. The *Renilla luciferase (Rluc)* expression cassette containing the constitutive *adh1* promoter derived from the *Schizosaccharomyces pombe alcohol dehydrogenase 1 (adh1)* gene (which has previously been used to drive gene expression in *S. pombe*[42]*),* and the *adh2* terminator was synthesized as a gene block (Integrated DNA Technologies, IDT) and amplified by polymerase chain reaction (PCR) using forward and reverse primers containing *SacI* restriction sites (Appendix, Table A1, 2443 & 2444). The pRep4x and pRep82x yeast expression vectors were also digested with *SacI* (NEB, R3156S) to generate compatible cloning sites. Both vectors contain the *ura4+* selectable marker, allowing growth of transformed yeast cells on uracil-deficient medium, while pRep4x provides stronger promoter-driven expression than pRep82x[43]. The digested vectors were separated by agarose gel electrophoresis, and the linearized vector backbones were purified using a gel extraction kit (Thermo Scientific, K0691) following the manufacturer's protocol[44]. Ligation reactions were prepared by combining 2 µL of gel-purified vector DNA (200–400 ng/µL), 6 µL of PCR-amplified Rluc insert DNA (1,000 ng/µL), 1 µL of 10× T4 DNA ligase buffer, and 1 µL of T4 DNA ligase (NEB, M0202S). Reactions were incubated at room temperature for 15 min and subsequently held at 4 °C overnight until transformation. This generated pRep4x-Rluc and pRep82x-Rluc constructs that served as internal normalization controls for subsequent dual-luciferase assays.

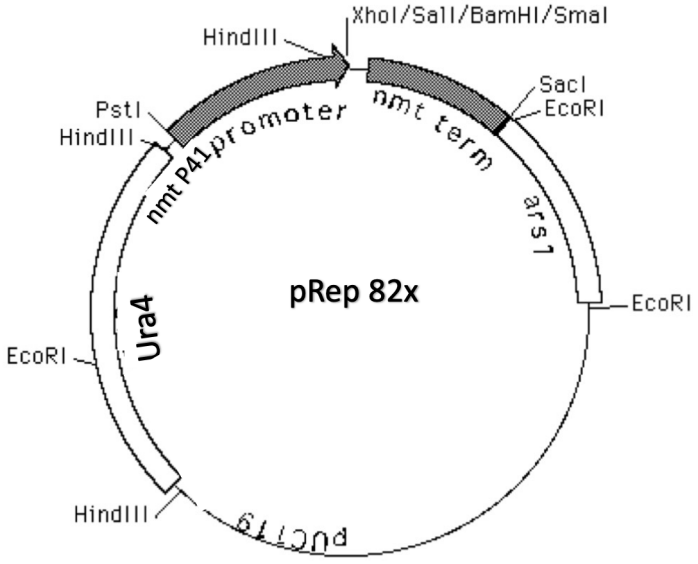
Following ligation, the recombinant plasmids were transformed into chemically competent *Escherichia coli* XL1 cells according to a previously described chemical transformation protocol[45]. Transformants were selected by growing them on Luria–Bertani (LB) agar plates containing 0.1 mg/ml ampicillin at 37 °C. Numerous colonies were observed on plates containing the pRep4x-*Rluc* and pRep82x-*Rluc* constructs, whereas few or no colonies were detected on the negative control plate which contained the linearized vector without the *Rluc* insert to assess vector religation, suggesting successful cloning. Six colonies from each construct were selected for plasmid purification using the Thermo Scientific GeneJET Plasmid Miniprep Kit[46]. Purified plasmids were digested with *SacI* and analyzed by agarose gel electrophoresis. The presence of DNA fragments corresponding to the expected *Rluc* insert size confirmed successful cloning. Sequencing was performed at The Center for Applied Genomics (TCAG, Toronto, Canada). Each sequencing sample contained 1 μL of miniprep DNA, 0.7 μL of a 1:10 dilution of the sequencing primer (prepared from a 200 μM stock) (Appendix, Table A1, 2443& 2444), and 6 μL of nuclease-free water, in accordance with the facility's submission requirements.

Before cloning the uORF–Fluc reporter cassettes, the multiple cloning site (MCS) of pRep82x was modified to facilitate insertion of the reporter sequences. The original arrangement of restriction sites within the pRep82x MCS is shown in Figure 13a. A synthetic oligonucleotide containing an expanded MCS was designed and ordered from Integrated DNA Technologies (IDT). The sequence, 5′-GCGCCTCGAGGTCGACAGATCTGCGGCCGCGGATCCACTAGTCCCGGGGCGC-3′, introduced restriction sites for XhoI, SalI, BglII, NotI, BamHI, SpeI, and XmaI/SmaI. This sequence was cloned into the original pRep82x MCS using XhoI and XmaI restriction enzymes

to generate a modified vector, referred to as pREP-J. This modification increased the spacing between the XhoI and BamHI restriction sites and provided additional restriction sites for subsequent cloning.

Similarly, the sequences containing the upstream open reading frame (uORF) reporters for *gcn4* and *hri2*, together with the Firefly luciferase (*Fluc*) reporter gene, were synthesized as gene blocks and cloned into the MCS of the verified pRep4x-Rluc and modified pREP-J plasmids using *XhoI* (NEB, R0146S) and *BamHI* (NEB, R3136S) restriction enzymes. Following ligation, the recombinant plasmids were transformed into *E. coli* XL1 competent cells, and transformants were selected on ampicillin-containing LB agar plates. For each construct, six colonies were selected and cultured for plasmid purification. Correct insertion of each reporter construct was verified by restriction digestion with *XhoI* and *BamHI* followed by agarose gel electrophoresis. The integrity of all cloned constructs was subsequently confirmed by whole-plasmid sequencing (*Plasmidsaurus)*[48]. The overall cloning strategy used to generate the dual-luciferase reporter constructs is illustrated in Figure 13b.

a



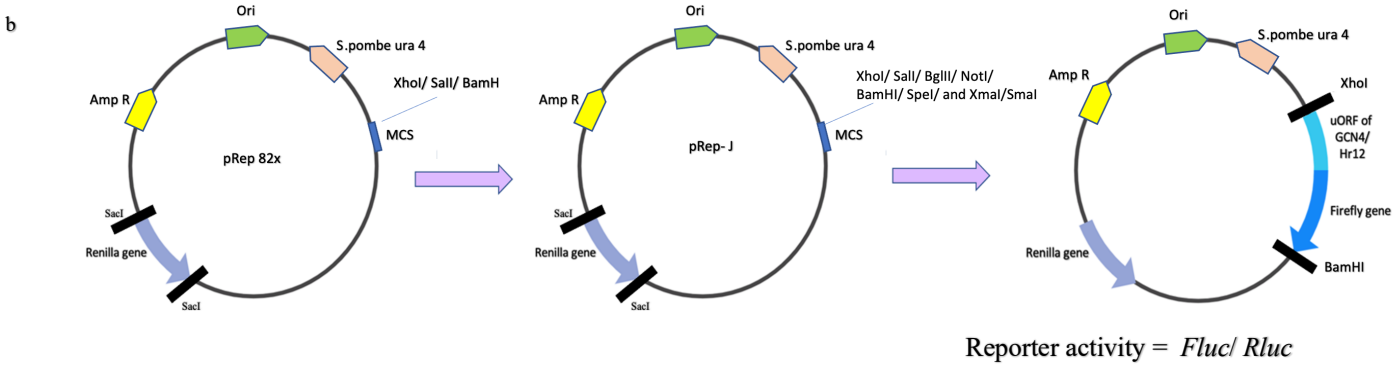


Figure 13: **Construction of the modified pRep82x dual-luciferase reporter plasmid. (A)** Map of the original pRep82x vector showing the multiple cloning sites containing XhoI, SalI, BamHI, and SmaI downstream of the nmt1 p41 promoter. The original arrangement of these restriction sites was modified to generate pREP-J for cloning of the uORF–Fluc reporter cassette**. (B)** Schematic representation of the sequential cloning steps used to generate the final dual-luciferase reporter construct. The *Renilla luciferase* (*Rluc*) reporter cassette was first cloned into pRep82x using SacI. The original pRep82x multiple cloning site (MCS) was then redesigned to generate pRep-J with an expanded MCS containing additional restriction sites. The *GCN4* or *Hri2* 5′ leader containing regulatory uORFs, followed by *Firefly luciferase* (*Fluc*), was subsequently cloned using XhoI and BamHI. *Rluc* served as an internal control, and reporter activity was calculated as *Fluc/Rluc.*

2.1b Cloning the frameshift constructs

The three frameshifting reporter plasmids used in the study conducted in *Saccharomyces cerevisiae* by Jennings *et al.* were obtained, including PAV2789 (0-frame/control reporter), PAV2790 (−1 frameshift reporter), and PAV2791 (+1 frameshift reporter). PAV2789 contained *Rluc* and *Fluc* in the same reading frame and therefore represented 100% in-frame translation. PAV2790 contained the L-A viral frameshifting sequence, derived from the *Saccharomyces cerevisiae* L-A double-stranded RNA virus, inserted between the *Rluc* and *Fluc* genes to induce −1 programmed ribosomal frameshifting, whereas PAV2791 contained the Ty1 frameshifting sequence, derived from the *S. cerevisiae* Ty1 retrotransposon, which induces +1 programmed ribosomal frameshifting[35]. These plasmids used ADH1 and CYC1 as their promoters and terminators, respectively.

Each plasmid was amplified by polymerase chain reaction (PCR) using two different forward primers: 40N XhoI Forward, containing 40 random nucleotides within the 5′ untranslated region (UTR) and an *XhoI* restriction site (**5'-CTCGAG-3'**), and PAC XhoI Forward, containing the proximal AC-rich (PAC) motif within the 5′ UTR, a sequence preferentially bound by Slr1p together with an *XhoI* restriction site (Appendix, Table A1, 1239 & 1403). A single reverse primer (AgeI Reverse) (NEB, R3552S) was used for all reactions (Appendix, Table A1, 2442), resulting in six unique reporter constructs (Figure 14).

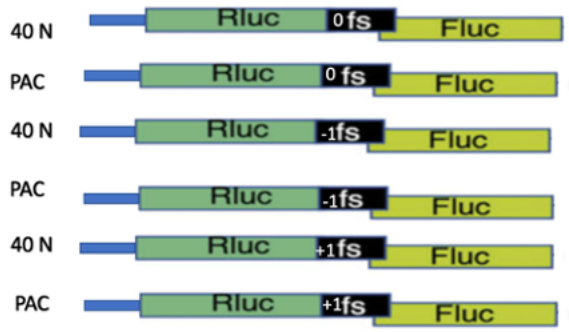


Figure 14: Schematic of the dual-luciferase frameshifting reporter constructs. Reporters contained either a 40N or PAC 5′ UTR and a control (0-frame), −1 frameshift, or +1 frameshift sequence positioned between the upstream Rluc and downstream Fluc coding sequences, generating six reporter constructs used to measure translational frameshifting.

The PCR-amplified reporter constructs were cloned into the *Schizosaccharomyces pombe* expression vectors pRep4x and pRep82x. Both the inserts and vectors were digested with XhoI to generate the 5′ cloning site. For the 3′ cloning site, the PCR-amplified inserts were digested with AgeI, while the vectors were digested with XmaI (NEB, R0180S). Because AgeI and XmaI generate compatible cohesive ends, the inserts were successfully ligated into the vectors (Figure 15).

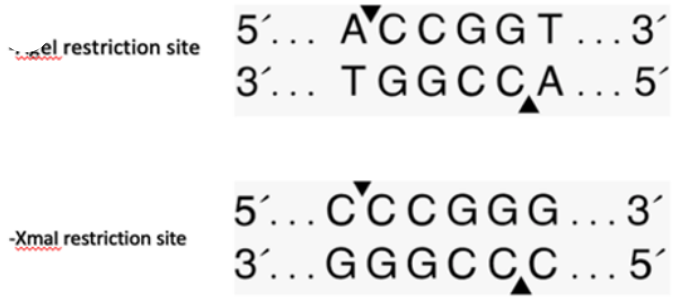


Figure 15: Schematic illustrating the compatible cohesive ends generated by AgeI and XmaI restriction enzymes. Digestion of the PCR-amplified reporter inserts with AgeI and the pRep vectors with XmaI produced compatible overhangs, allowing directional ligation of the inserts into the vectors despite the use of different restriction enzymes.

All six reporter constructs were successfully cloned into both pRep4x and pRep82x using standard molecular cloning procedures described earlier[41]. The integrity of the recombinant plasmids was verified by whole-plasmid sequencing (*Plasmidsaurus*)[48].

2.2 Yeast Transformation

The pRep4x and pRep82x empty vectors and vectors expressing the cloned constructs were introduced into wild-type and *slr1Δ Schizosaccharomyces pombe* strains using a lithium acetate/polyethylene glycol (LiAc/PEG) transformation protocol[49]. Briefly, yeast cultures were grown in 10 ml YES medium per transformant (5 g/L yeast extract supplemented with 225 mg/L each of adenine, histidine, leucine, lysine, and uracil, and 20% dextrose) at 32°C. On the day of experiment cells were seeded down to an OD of 0.2 and incubated at 32°C/ 180 RPM until mid-log phase. Cells were harvested by centrifugation at 1,900 × *g* for 10 min at 4°C, washed with sterile distilled water, and pelleted again under the same conditions.

The cell pellets were resuspended in 200 µL chilled LiAc-TE buffer (1× LiAc, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA), and 100 µg salmon sperm DNA was added as carrier DNA. For each transformation, 100 µL of the cell suspension was mixed with 2 µL of purified plasmid DNA

and 700 µL LiAc-TE-PEG solution (1× LiAc, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 40% PEG). Samples were vortexed thoroughly and heat shocked at 42°C for 15 min. Cells were then collected by centrifugation at 5,500 × *g* for 1 min, resuspended in 200 µL sterile distilled water, and spread onto EMM-URA agar plates supplemented with 11.8 g/L Edinburgh Minimal Medium (EMM) glucose base supplemented with 225 mg/L each of adenine, histidine, leucine, and lysine, while uracil was omitted to maintain selection for plasmids carrying the ura4+ selectable marker using sterile glass beads. Plates were incubated at 32°C until transformant colonies appeared.

2.3 Yeast growth conditions

2.3a Normal Growth Conditions (Unstressed)

A single colony from each transformation plate was inoculated into 10 mL of Edinburgh Minimal Medium lacking uracil (EMM–URA) and incubated at 32 °C with shaking at 200 rpm for 48 h. On the day of the experiment, the overnight cultures were diluted to an optical density at 600 nm (OD_{600}) of 0.2 in 20 mL of medium and grown at 32 °C with shaking until reaching an OD_{600} of 0.6–0.8 (mid-log phase). Cells were then harvested by centrifugation at 4,000 rpm for 10 min at 4 °C and washed with 40 mL of sterile distilled water. Following a second centrifugation under the same conditions, the supernatant was discarded, and the cell pellets were washed once more using the residual water. The pellets were subsequently transferred to 2 mL microcentrifuge tubes with flat screw-caps (VWR 16466-060) and pelleted again at 8,000 rpm for 1 min. Excess supernatant was discarded and cells were stored at −80 °C until protein extraction[32].

2.3b Heat shock stress

A single colony from each transformation plate was inoculated into 10 mL of Edinburgh Minimal Medium lacking uracil (EMM–URA) and incubated at 32 °C with shaking at 200 rpm for 48 h. On the day of the experiment, the overnight cultures were diluted to an optical density at 600 nm (OD_{600}) of 0.2 and grown at 32 °C with shaking until reaching an OD_{600} of 0.6–0.8 (mid-log phase). A 20 mL aliquot was collected immediately before heat shock and served as the unstressed control (0 min). The remaining culture was then subjected to heat shock at 42 °C, and additional 20 mL aliquots were collected after 10, 20, and 30 min of incubation to monitor stress-dependent changes in reporter expression[22].

2.3c Oxidative Stress (H_2O_2)

A single colony from each transformation plate was inoculated into 10 mL of Edinburgh Minimal Medium lacking uracil (EMM–URA) and incubated at 32 °C with shaking at 200 rpm for 48 h. On the day of the experiment, the overnight cultures were diluted to an optical density at 600 nm (OD_{600}) of 0.2 and grown at 32 °C with shaking. Oxidative stress treatment was performed using 0.8 mM H_2O_2 for 2 h when cultures reached an optical density at 600 nm (OD_{600}) of 0.4–0.5, based on stress conditions previously described by Jennings et al. Prior to H_2O_2 treatment, a 20 mL aliquot was collected to serve as the untreated control (0 h). The remaining culture was then treated with H_2O_2, and samples were collected after 2 h for analysis[35].

2.3d Amino acid Starvation Stress (3-AT)

A single colony from each transformation plate was inoculated into 10 mL of Edinburgh Minimal Medium lacking uracil (EMM–URA) and incubated at 32 °C with shaking at 200 rpm for 48 h. On the day of the experiment, the overnight cultures were diluted to an optical density at 600 nm (OD_{600}) of 0.2 and grown at 32 °C with shaking. For amino acid starvation

experiments, cultures were grown to an optical density at 600 nm (OD_{600}) of 0.4–0.5 and harvested by centrifugation at 3,200 rpm for 10 min at room temperature. Cell pellets were washed once with sterile water, centrifuged again under the same conditions, and resuspended in histidine-deficient EMM medium. 3-Amino-1,2,4-triazole (3-AT) (Sigma-Aldrich, A81803) was then added to a final concentration of 10 mM to induce amino acid starvation. Samples were collected after 1 and 2 h of treatment for reporter analysis[23].

2.4 Protein Extraction

Frozen yeast pellets were thawed on ice, and 100–150 µL of NET-2 buffer (50 mM Tris-HCl, pH 7.5 (Fisher BP152-5), 50 mM NaCl (Fisher S271-10), 0.05% Igepal (Bioshop NON999.500), and 0.1 mM PMSF (Bioshop PMS123.5)) was added to each sample. The cell pellets were resuspended, and 100–150 µL of 0.5 mm acid-washed glass beads (Sigma G8772-100G) were added to each tube. Cells were lysed using a bead beater (BioSpec Products) in a cold room by performing three 20 s cycles of bead beating, with a 2 min incubation on ice between each cycle. Following lysis, an additional 100 µL of NET-2 buffer was added to each sample, and the lysates were centrifuged at 4,000 rpm for 15 min at 4 °C. The resulting supernatants were transferred to fresh microcentrifuge tubes. An additional 100 µL of NET-2 buffer was then added to the remaining pellet and glass beads to recover residual protein. Following mixing, the samples were centrifuged again for 30 min at 4 °C. The resulting supernatants were mixed with a 10% final concentration of glycerol (Bioshop GLY001.500) and collected as whole-cell protein lysates and stored at −80 °C until further analysis[32].

2.5 Bradford Assay

Protein concentrations were determined using the Bradford protein assay. One milliliter of Bradford reagent (Thermo Scientific 23238), equilibrated to room temperature, was added to

each cuvette, followed by the addition of 10 µL of protein lysate. After incubation for 10 min at room temperature, concentrations were measured in µg/µL using a spectrophotometer.

2.6 Dual-Luciferase Assay

Luciferase activity was measured using the Renilla Luciferase Assay System (Promega, E2820) according to the manufacturer's instructions[50]. Luminescence measurements were recorded in triplicate using a Synergy H4 Hybrid Multi-Mode Microplate Reader, with each sample measured for 2 s in luminescence mode. Three independent biological replicates were analyzed for each reporter construct transformed into either wild-type (WT) or *slr1Δ Schizosaccharomyces pombe* strains.

2.7 Growth, RNA extraction, and qPCR

Total RNA was extracted from *Schizosaccharomyces pombe* cultures using an acid phenol extraction protocol. Briefly, single transformant colonies were inoculated into 10–15 mL of Edinburgh Minimal Medium lacking uracil (EMM–URA) and incubated overnight at 32 °C with shaking at 200 rpm. Overnight cultures were diluted to an optical density at 600 nm (OD_{600}) of 0.2–0.3 in 50 mL of fresh EMM–URA medium and grown to mid-logarithmic phase (OD_{600} = 0.6–0.8).

Cells were harvested by centrifugation at 1,800 × *g* for 10 min and washed once with sterile distilled water. Following a second centrifugation, the remaining cell suspension was transferred to a microcentrifuge tube, pelleted, and the supernatant was completely removed. Cell pellets were resuspended in 250 µL of complete RNA extraction buffer A (50 mM sodium acetate, pH 5.2, 10 mM EDTA, pH 8.0, and 1% SDS), followed by the addition of 750 µL of pre-warmed buffer A-saturated acid phenol (acid-saturated phenol equilibrated with RNA extraction buffer

A). Samples were mixed by vortexing and incubated at 65 °C for 4 min with intermittent vortexing.

Following centrifugation at maximum speed for 3 min, the aqueous phase was transferred to a fresh tube. A second extraction was performed by adding 250 µL of complete RNA extraction buffer A to the phenol phase, followed by incubation at 65 °C for 4 min and centrifugation at 20,000 × *g* for 3 min. The aqueous phases were combined and extracted once with an equal volume of phenol:chloroform alcohol. Following centrifugation at 20,000 × *g* for 3 min, the aqueous phase was transferred to a new tube.

RNA was precipitated by the addition of 2 µL of 20 mg/mL glycogen, 40 µL of 3 M sodium acetate (pH 5.2), and 1 mL of 100% ethanol, followed by incubation at −80 °C for at least 1 h. Subsequent washing, drying, and resuspension of the RNA pellet were performed according to the laboratory's standard RNA extraction protocol.

To eliminate residual genomic DNA, RNA samples were treated with TURBO DNase (Thermo Fisher Scientific**,** AM2238). Briefly, 10 µg of total RNA was combined with 10 µL of 10× TURBO DNase buffer, 1 µL of TURBO DNase enzyme, and nuclease-free water to a final volume of 50 µL. The reaction mixture was incubated at 37 °C for 30 min. Following DNase treatment, the reaction volume was adjusted to 200 µL with nuclease-free water, after which an equal volume of phenol:chloroform alcohol (PCI) was added. Samples were vortexed for 15 s and centrifuged at 20,000 × *g* for 10 min. RNA was subsequently precipitated by the addition of 20 µL of 3 M sodium acetate (pH 5.2), 1 mL of 100% ethanol, and 2 µL of GlycoBlue, and stored at −80 °C until further processing.

Complementary DNA (cDNA) was synthesized from DNase-treated RNA using the iScript™ cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's instructions using 1,000 ng of

total RNA in a final reaction volume of 30 µL. Reverse transcription was performed using the following thermocycling conditions:

5 min	25°C
20 min	46°C
1 min	95°C
Hold	4°C

cDNA was stored at -20°C until use.

Gene expression was quantified by real-time quantitative PCR (RT-qPCR) using SYBR Green chemistry. Expression of the firefly luciferase (Fluc) reporter gene was measured, and act1 was used as the reference (housekeeping) gene for normalization. Each 12.5 µL reaction contained 6.25 µL of SYBR Green Master Mix, 1.25 µL of 10× primer pair, and 5 µL of cDNA template. All reactions were prepared in technical triplicate. cDNA prepared from wild-type cells carrying the empty vector was included as a negative control for amplification of the reporter gene. Amplification was performed using the following thermocycling conditions:

Initial activation step

5 min	95°C

Two-step cycling (35-40 cycles)

5s	95°C
15s	60°C

+ melt 60-99C

2.8 Growth of Large-Scale Yeast Cultures and Cryogenic Cell Grinding

Single colonies were inoculated into 10 mL of the appropriate growth medium and incubated overnight at 32 °C with shaking at 200 rpm. The overnight cultures were diluted to an

optical density at 600 nm (OD_{600}) of 0.2 in 100 mL of fresh medium and grown to mid-logarithmic phase. The cultures were subsequently transferred into 1 L of fresh medium and incubated overnight under the same conditions to obtain sufficient biomass for co-immunoprecipitation experiments until they reached an OD of ~0.6-0.8.

Cells were harvested by centrifugation at 4,500 × *g* for 5–10 min and washed once with sterile distilled water. The cell pellets were resuspended in 2–5 mL of pelleting buffer (20 mM HEPES, pH 7.4, 1 mM PMSF, 1 mM DTT, and 1.2% polyvinylpyrrolidone [PVP]) and collected by centrifugation at 4,500 × *g*. The resulting pellets were maintained on ice until further processing.

To prepare cryogenically frozen cell material, a 20 mL syringe was pre-chilled in liquid nitrogen. Small holes were created in the cap of a 50 mL conical tube using a needle to allow excess liquid nitrogen to escape during processing. The syringe containing the cell pellet was positioned over the tube filled with liquid nitrogen, and the cell suspension was extruded directly into the liquid nitrogen to generate frozen cell strands ("noodles"). Excess liquid nitrogen was decanted through the holes in the tube cap, and the frozen cell strands were either stored at −80 °C or processed immediately.

The frozen cell strands were transferred to a pre-chilled mortar containing liquid nitrogen and ground with a pre-cooled pestle until a fine frozen powder was obtained. Liquid nitrogen was replenished throughout the grinding process to ensure that the sample remained frozen at all times. The resulting cryogenically ground yeast powder was transferred into pre-chilled 50 mL conical tubes maintained in liquid nitrogen and stored at −80 °C until use for co-immunoprecipitation experiments.

2.9 Co-immunoprecipitation

2.9a Slr1p immunoprecipitation

Co-immunoprecipitation (co-IP) assays were performed to evaluate the interaction between Exo2p and Slr1p in wild-type, *slr1Δ,* and phospho-mutant Slr1p (S410A, S410D, S437A, and S437D). Cryogenically ground yeast material was maintained at −80 °C until use. For each immunoprecipitation, approximately 0.3 g of frozen yeast powder was transferred to a pre-chilled 15 mL conical tube and allowed to thaw on ice for approximately 15 min.

A 1× ribonucleoprotein (RNP) lysis buffer was prepared containing 20 mM HEPES (pH 7.4) (Fisher BP310-500), 110 mM potassium acetate (Bioshop POA303.1), 100 mM NaCl (Fisher S271-10), 0.5% Triton X-100 (Sigma T8787-100ML), and 0.1% Tween-20 (Sigma P9416-100ML). The buffer was supplemented immediately before use with 5.55 µL/mL yeast protease inhibitor cocktail (Sigma P8215-5ML), 1 mM phenylmethylsulfonyl fluoride (PMSF) (Bioshop PMS123.5), and 4U/mL RNase inhibitor (Invitrogen, AM2694). The prepared buffer was maintained on ice throughout the procedure.

Approximately 1.8 mL of ice-cold RNP buffer was added to each 0.3 g sample of frozen yeast powder. Samples were mixed by vortexing for three 30 s intervals, with incubation on ice between each interval. Insoluble cellular material was removed by centrifugation at approximately 1,200 × *g* for 10 min at 4 °C. The supernatant was transferred to a fresh pre-chilled tube and centrifuged again under the same conditions for 5–10 min to obtain a clarified whole-cell lysate.

Protein A magnetic beads (Invitrogen 10001D) were used for both lysate pre-clearing and immunoprecipitation. Before use, the beads were washed three times with phosphate-buffered saline containing 0.1% Tween-20 (PBS-T). For pre-clearing, Protein A beads were coupled to non-immune rabbit IgG (Invitrogen 02–6102) by incubation with rotation at room temperature for 40–

60 min. The antibody-coupled beads were subsequently washed once with PBS-T and one to two times with RNP buffer.

Clarified yeast lysates were added to the non-immune rabbit IgG-coupled beads and incubated with rotation at 4 °C for 1–2 h. This pre-clearing step was performed to reduce non-specific binding of proteins to the beads and rabbit immunoglobulins. Following incubation, the beads were collected using a magnetic rack, and the pre-cleared lysates were transferred to fresh tubes. A portion of each lysate was retained as the input sample for subsequent Western blot analysis.

For immunoprecipitation, 25 µL of Protein A magnetic beads was used for each reaction. The beads were coupled either to 6.25 µg of anti-Slr1 antibody (AB2756) or to 6.25 µg of non-immune rabbit IgG. The non-immune rabbit IgG immunoprecipitation was included as a negative control to identify proteins that bound non-specifically to the beads or antibody. Antibodies were coupled to the beads by incubation with rotation at room temperature for 40–60 min. The beads were then collected magnetically and washed once with PBS-T, followed by two washes with RNP buffer.

Equal volumes of pre-cleared lysate, approximately 800 µL per reaction, were added to the anti-Slr1- and rabbit IgG-coupled beads. Immunoprecipitation reactions were incubated with rotation at 4 °C overnight to allow Slr1p and associated proteins to bind to the antibody-coupled beads. Following incubation, the beads were collected using a magnetic rack, and the unbound supernatant was retained as the flow-through fraction.

The beads were washed three times with 1 mL of RNP buffer to remove proteins that were not specifically associated with the immunoprecipitated complexes. The beads were collected magnetically after each wash, and the wash buffer was carefully removed. After the final wash, all residual buffer was removed from the beads.

Immunoprecipitated proteins were eluted by resuspension in 20 µL of ddH_2O 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 then heated at 95 °C for 10 min and collected using a magnetic rack. The resulting eluates, together with the corresponding input and flow-through fractions, were stored at −20 °C or analyzed immediately by loading 15 µL of each sample onto 10% SDS-polyacrylamide gel (Bioshop SDS003.1, Fisher BP1406-1) followed by western blotting.

2.9b Exo2p-Myc immunoprecipitation

Co-immunoprecipitation of Exo2p-Myc was performed as described above with minor modifications. Clarified yeast lysates were prepared using the same RNP lysis buffer and pre-cleared as described for Slr1 immunoprecipitation. For each reaction, 30 µL of Protein G magnetic beads (Invitrogen 10004D) was coupled to 2.5 µL of mouse anti-Myc antibody **(**Cell Signaling Technology 2276S**)** by incubation with rotation at room temperature for 40–60 min. Equal volumes of pre-cleared lysate (approximately 800 µL) were added to the antibody-coupled beads and incubated overnight at 4 °C with rotation. The beads were washed three times with RNP buffer, and bound proteins were eluted in 20 µL ddH_2O and 20 µL 5× Laemmli buffer supplemented with β-mercaptoethanol. Following the addition of 5 µL of 1 M Tris-HCl (pH 8.0), samples were heated at 95 °C for 10 min. Eluates, together with the corresponding input and flow-through fractions, were analyzed by SDS-PAGE and Western blotting.

2.10 SDS-PAGE and Western Blotting

Protein samples obtained from the input, flow-through, and immunoprecipitation eluate fractions were analyzed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% SDS-PAGE) followed by Western blotting to evaluate protein expression and protein-protein interactions.

Protein samples were mixed with 5× Laemmli sample buffer containing 312.5 mM Tris-HCl (pH 6.8), 50% glycerol, 5% SDS, and 0.05% bromophenol blue. β-Mercaptoethanol was added immediately before use to a final concentration of 10%, and samples were denatured by heating at 95 °C for 10 min prior to SDS-PAGE. Equal volumes of each sample were separated by SDS-PAGE using precast polyacrylamide gels at 100 V for approximately 60–90 min.

Following electrophoresis, proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane using a wet transfer system in transfer buffer containing 25 mM Tris, 192 mM glycine, and 15% (v/v) methanol. Protein transfer was carried out at 50 V for 90 min.

Successful protein transfer was verified by staining the membrane with Ponceau S (ThermoFisher Scientific, A40000279), followed by destaining with distilled water or TBS-T (20mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% Tween-20). Membranes were blocked for 1 h at room temperature in 0.5% w/v skim milk powder (Bioshop SK1400) prepared in TBS-T, then washed 3 times for 10 min in TBS-T.

Following blocking, membranes were incubated overnight at 4 °C with anti-Slr1 antibody (custom AB2648) diluted 1:5,000 in TBS-T. Membranes were then washed three times for 10 min each with TBS-T before incubation with a horseradish peroxidase (HRP) conjugated mouse anti-rabbit IgG (light chain specific) (Cell Signaling Technology 93702) diluted 1:10,000 for 1 h at room temperature. Following secondary antibody incubation, membranes were washed three additional times with TBS-T.

Protein bands were detected using an enhanced chemiluminescence (ECL) detection system by mixing the two substrate components at a 1:1 ratio according to the manufacturer's instructions. Membranes were incubated with the ECL substrate (ThermoFisher Scientific, 32106) for approximately 2 min before chemiluminescent signals were visualized using an Azure Biosystems imaging system (DNR Bio-Imaging Systems).

Following detection of the initial target, the membrane was stripped using a mild stripping buffer containing 15 mg/mL glycine, 0.1% SDS, and 1% Tween-20, adjusted to pH 2.2. A sufficient volume of stripping buffer was added to completely cover the membrane, and the membrane was incubated at room temperature for 10 min with gentle agitation. The stripping buffer was discarded, and the membrane was incubated for an additional 10 min with freshly prepared stripping buffer.

The membrane was subsequently washed twice with phosphate-buffered saline (PBS) for 10 min per wash, followed by two washes with Tris-buffered saline containing Tween-20 (TBST) for 5 min per wash. The membrane was then blocked in 0.5% w/v skim milk powder prepared in TBS-T.

To detect Myc-tagged Exo2, the membrane was incubated for 1 h with a mouse anti-Myc primary antibody (Cell Signaling Technology 2276S) diluted 1:1,000 in 0.5% skim milk prepared in TBST. Following primary antibody incubation, the membrane was washed with TBS-T and incubated with anti-mouse IgG HRP-linked antibody (Cell Signaling Technology 7076S) diluted 1:1,000 in TBST for 1 h at room temperature. The membrane was washed again with TBST, and the signal was detected using an enhanced chemiluminescence (ECL) substrate.

Chapter 3: Results

3.1: Slr1p function in uORF mRNA translation

To investigate whether Slr1p contributes to the expression of mRNAs containing upstream open reading frames (uORFs), dual-luciferase reporter constructs were generated in the pRep4x and pRep82x expression vectors, which express genes of interest under the control of strong or weak promoters, respectively[43].

The reporter system was generated using two synthesized gene blocks (Integrated DNA Technologies, IDT). The first contained *Renilla luciferase (Rluc)* under the control of the ADH1 promoter and ADH2 terminator and was cloned into both vectors using the SacI restriction enzyme. The ADH1 promoter and ADH2 terminator are regulatory sequences derived from *Schizosaccharomyces pombe* alcohol dehydrogenase genes, allowing *Rluc* to be expressed independently of the uORF reporter. Therefore, *Rluc* served as an internal control for normal cap-dependent translation.

Following cloning of the ADH1–*Rluc*–ADH2 cassette, successful insertion was confirmed by restriction digestion with SacI and gel electrophoresis (Figure 16). Digestion released a fragment of approximately 1.1–1.2 kb, consistent with the expected size of the *Rluc*-containing cassette. Successful cloning was further confirmed by sequencing performed at The Centre for Applied Genomics (TCAG, Toronto, Canada).

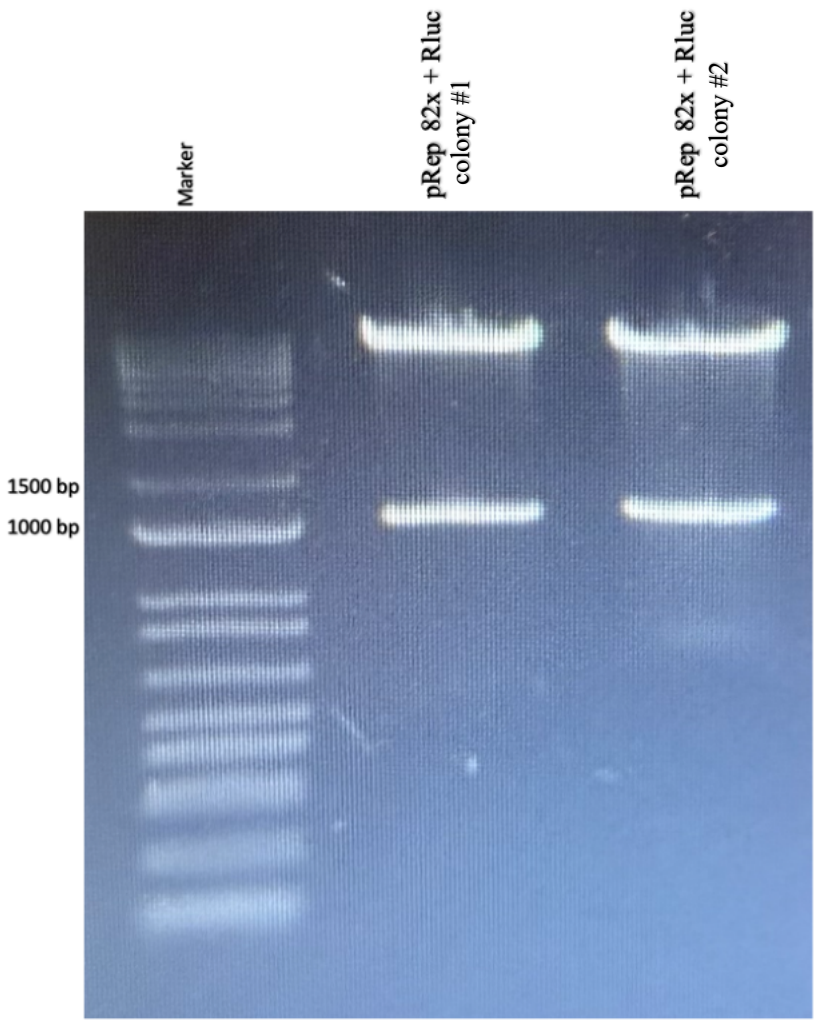


Figure 16: **Restriction digest confirmation of the ADH1–Rluc–ADH2 cassette.** Following cloning of the ADH1–*Rluc*–ADH2 cassette, plasmids were digested with SacI to confirm successful insertion. The released fragment at approximately 1.1–1.2 kb corresponds to the expected size of the *Rluc*-containing cassette. A 1 kb DNA ladder is shown in the leftmost lane as a molecular size marker.

The second gene block contained the 5′ leader of either *GCN4* or *Hri2*, including its regulatory uORFs, followed by *Firefly luciferase (Fluc)*. These uORFs were selected because of their established roles in regulating translation in response to cellular stress and nutrient availability[52]. This cassette was introduced into the multiple cloning sites of pRep4x or pRep82x using XhoI and BamHI, thereby placing its expression under the control of the nmt1 p41 and nmt1 promoters in pRep82x and pRep4x, respectively.

Following cloning of the uORF–*Fluc* reporter cassette, successful insertion was confirmed by restriction digestion with XhoI and BamHI (Figure 17). Digestion produced a larger fragment corresponding to the vector backbone and a smaller fragment of approximately 1,700 bp corresponding to the uORF-containing 5′ leader together with the *Fluc* reporter sequence (circled in red). This was consistent with successful insertion of the reporter cassette.

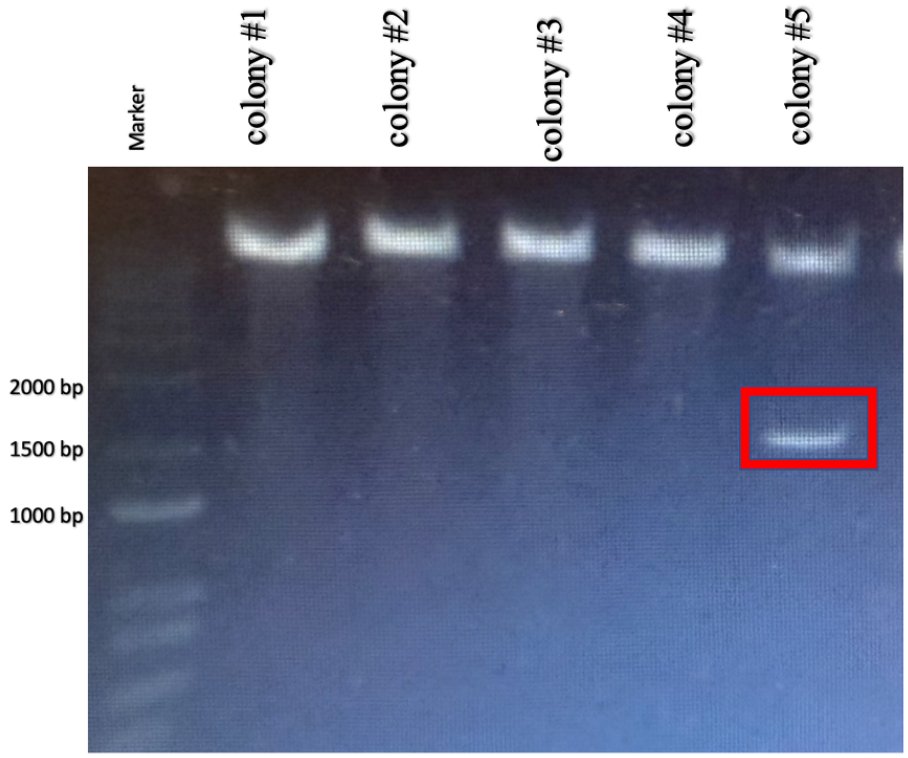


Figure 17: **Restriction digest confirmation of the uORF–Fluc reporter cassette**. Following cloning of the uORF–Fluc cassette, plasmids were digested with XhoI and BamHI to confirm successful insertion. Digestion produced a larger fragment corresponding to the vector backbone and a smaller fragment of approximately 1,700 bp corresponding to the uORF-containing 5′ leader and Fluc reporter cassette. A 1 kb DNA ladder is shown in the leftmost lane as a molecular size marker.

Following confirmation by restriction digestion, the reporter plasmids were submitted to *Plasmidsaurus* for whole-plasmid sequencing to confirm the final plasmid sequence and

organization. The resulting plasmid maps of the pRep82x- and pRep4x-derived dual-luciferase reporter constructs are shown in Figure 18.

Overall, this design allowed translation through the uORF-containing 5′ leader to be monitored through *Fluc* activity, while the independently expressed *Rluc* served as an internal control for normalization of *Fluc* reporter activity. Reporter activity was therefore expressed as the *Fluc/Rluc* ratio, allowing changes in uORF-mediated translation to be compared between WT and *slr1Δ* cells.

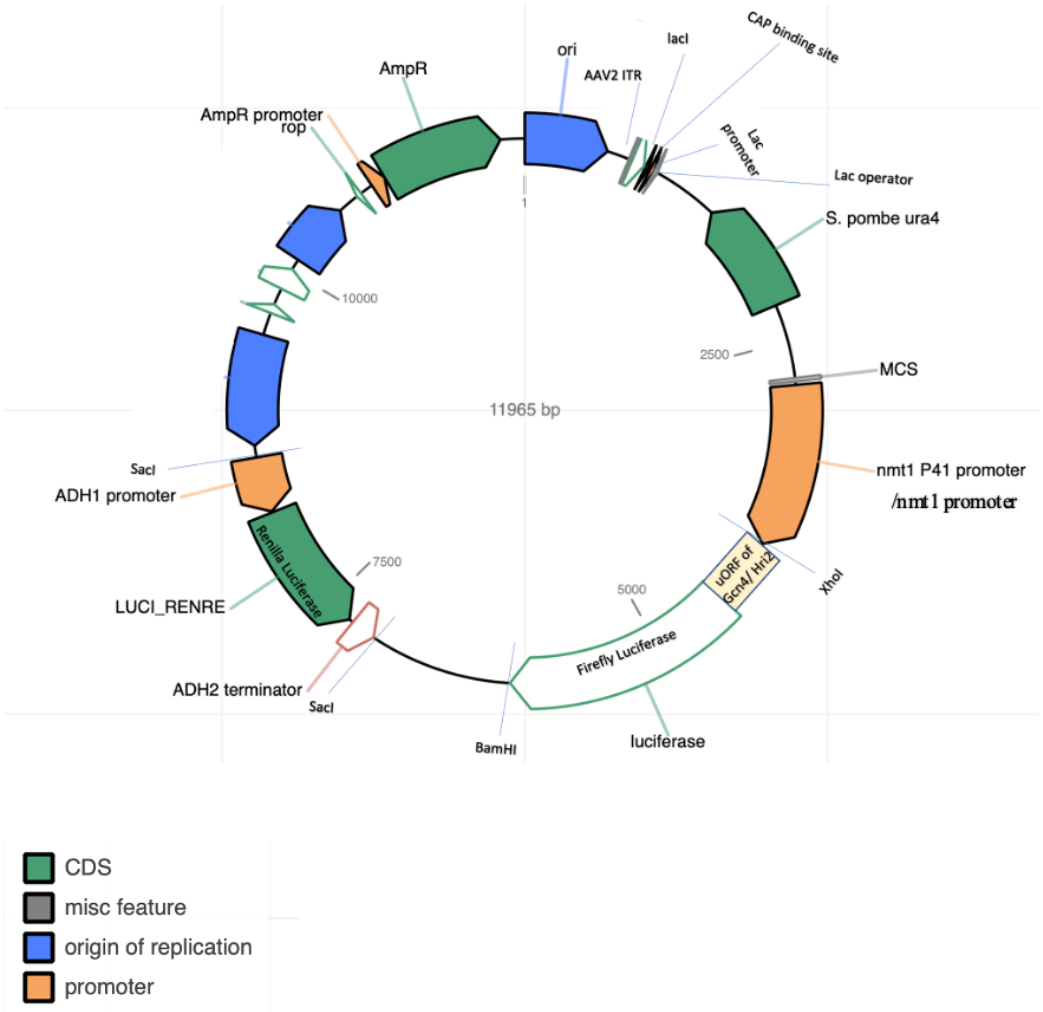


Figure 18: Map of the pRep82x and pRep4x dual-luciferase reporter constructs used to investigate Slr1p function in uORF mRNA translation. Firefly luciferase (Fluc) served as the experimental reporter and was positioned downstream of the 5′ UTR containing the uORFs of either GCN4 or HRI2, depending on the reporter construct. Fluc expression was driven by the attenuated nmt1 P41 promoter in pRep82x or the stronger nmt1 promoter in pRep4x. Renilla luciferase (Rluc), expressed from the ADH1 promoter, served as an internal control for

normalization. The *S. pombe ura4* gene was used as a selectable marker in *S. pombe*, while AmpR provided ampicillin resistance for selection in *E. coli*. The plasmid backbone and principal annotated features are shown. The plasmid map was adapted from whole-plasmid sequencing performed by Plasmidsaurus.

3.1.1 Slr1p promotes expression from the uORF-containing GCN4 5'UTR:

The *GCN4* 5′ UTR was selected as a model to investigate whether Slr1p contributes to the translation of mRNAs containing upstream open reading frames (uORFs). In *S. cerevisiae*, Gcn4p is a transcription factor that plays a central role in the response to amino acid starvation by promoting the expression of genes involved in amino acid biosynthesis. Its translation is tightly controlled by four uORFs located within the *GCN4* 5′ leader[52]. Under amino acid-rich conditions, these uORFs limit translation of the main *GCN4* coding region. During amino acid starvation, Gcn2-dependent phosphorylation of eIF2α reduces ternary complex availability and changes the pattern of ribosome reinitiation. This in turn allows more ribosomes to bypass the inhibitory uORFs and translate the downstream *GCN4* ORF[51]. While the *GCN4* uORF studied here is endogenous to *S. cerevisiae*, it has been previously used as a uORF positive control for study in *S. pombe*, alongside the candidate *S. pombe* uORF *HRI2*[51].

Given the role of Slr1p in translational regulation, we asked whether Slr1p is also required for efficient expression of mRNAs containing regulatory uORFs. **We hypothesized that loss of Slr1p might alter expression from the *GCN4* uORF-containing 5′ UTR and that this effect might become more pronounced under conditions that place different stresses on the translational machinery**. Reporter activity was therefore compared between WT and *slr1Δ* cells under non-stressed conditions and following amino acid starvation, oxidative stress, and heat shock.

To induce amino acid starvation, cells were treated with 10mM 3-aminotriazole (3-AT) in a media that lacked histidine. 3-AT inhibits histidine biosynthesis, creating histidine starvation and activating the Gcn2/eIF2α pathway. This made 3-AT particularly relevant to the expression of

the *GCN4* reporter, since amino acid starvation is a well-established signal for uORF-mediated regulation of *GCN4* translation[53].

Moreover, to induce oxidative stress, cells were treated with 0.8mM H_2O_2. for 2 h. La-related proteins have previously been linked to the regulation of translation under oxidative stress conditions in yeast. In *S. cerevisiae*, deletion of the LARP1 ortholog *slf1* causes increased sensitivity to H_2O_2. Also, Slf1p was found to remain associated with translating ribosomes and promotes the expression of a group of stress-responsive proteins, including antioxidant proteins[35,54]. These findings provided a strong reason to ask whether Slr1p also contributes to translational regulation of such genes during oxidative stress in fission yeast.

Furthermore, previous literature showed that increasing temperature rapidly changes translational regulation in *S. pombe***.** Heat shock activates stress-responsive eIF2α kinases and increases eIF2α phosphorylation. This in turn provides a mechanism for reducing overall translation initiation during heat stress[53]. Thus, heat shock at 42 °C was included to examine whether heat stress influences the effect of Slr1p on regulating the expression of genes with multiple uORFs.

Under non-stressed conditions, analysis of *Fluc/Rluc* ratios revealed a substantial difference between strains, with wild-type (WT) cells exhibiting approximately a 6.2-fold higher expression of the uORF-associated *Fluc* relative to *slr1Δ* cells (Figure 19a). This pronounced decrease in expression in the absence of Slr1p suggests that Slr1p may play an important role in promoting *GCN4* translation under basal conditions, likely through mechanisms involving uORF-mediated regulation.

However, the stress treatments shown in Figures 19b–d did not appear to strongly alter the relationship between WT and *slr1Δ* cells, suggesting that Slr1p may contribute to basal *GCN4* translational regulation rather than acting specifically during heat shock, oxidative stress, or 3-

AT-induced amino acid starvation. Therefore, these results suggest that Slr1p is generally required for efficient *GCN4* reporter expression, but further biological replicates and optimization of stress conditions are needed to determine whether Slr1p has a stress-specific regulatory role.

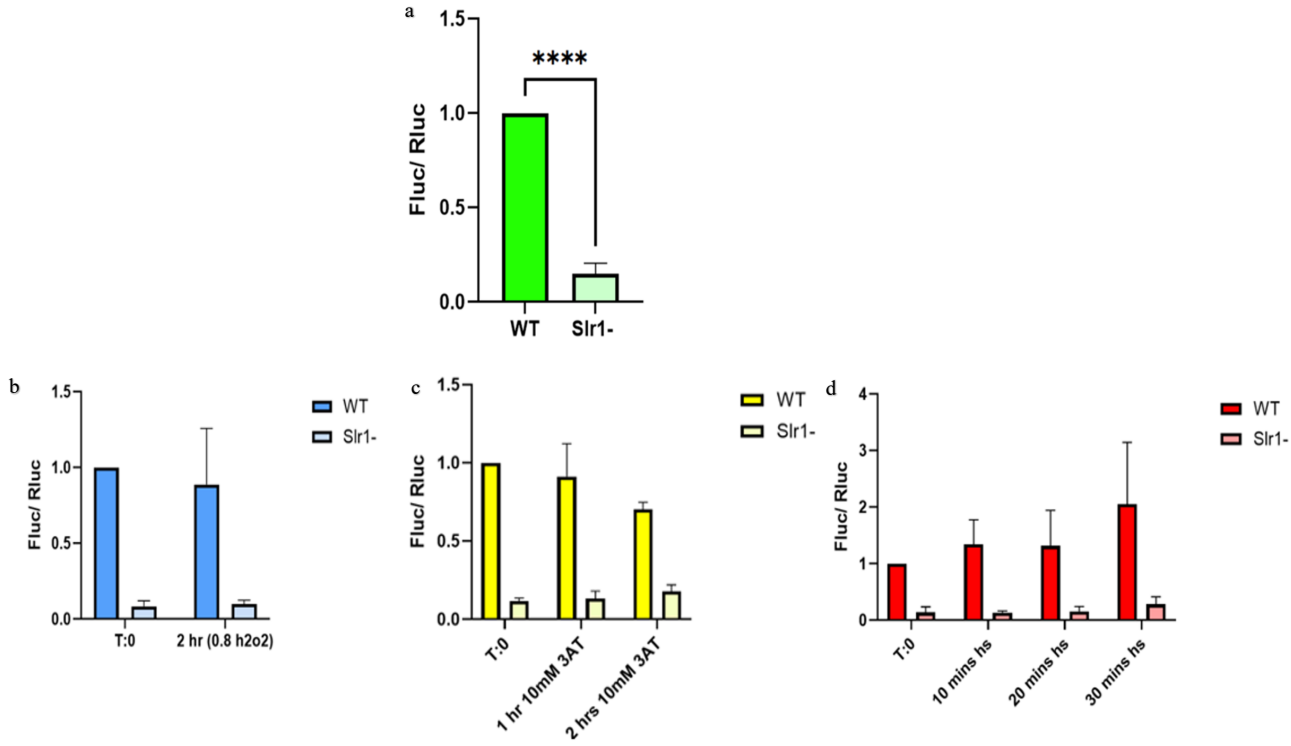


Figure 19: Analysis of Slr1p-dependent regulation of GCN4 uORF-mediated translation under different stress conditions using dual luciferase reporter assays. (A) Fluc/Rluc ratios of the GCN4 reporter in wild-type (WT) and *slr1Δ* cells under non-stressed conditions. Values were normalised to WT, which was set to 1. Data represent the mean ± SD of four biological replicates. Statistical significance was determined using a two-tailed unpaired Welch's t-test (p <0.0001). (B) Fluc/Rluc ratios of the GCN4 reporter in WT and slr1Δ cells following treatment with 10 mM 3-AT for 1 h and 2 h. (C) Fluc/Rluc ratios of the GCN4 reporter in WT and slr1Δ cells following treatment with 0.8 mM H_2O_2 for 2 h. (D) Fluc/Rluc ratios of the GCN4 reporter in WT and slr1Δ cells during heat shock treatment at 42 °C at the indicated time points.

3.1.2 Restoration of GCN4 Reporter Expression Following Reintroduction of Slr1p

To further investigate whether Slr1p directly contributes to *GCN4* reporter expression, *slr1Δ* cells were transformed with either an empty pRep81x vector control or pRep81x vector expressing Slr1p. Reintroduction of Slr1p into *slr1Δ* cells resulted in a partial restoration of

GCN4 reporter activity, with *Fluc/Rluc* levels increasing by approximately 2.6-fold compared with the empty vector control (Figure 20). This restoration supports the idea that the reduced *GCN4* reporter expression observed in *slr1Δ* cells is at least partially linked to the absence of Slr1p. However, additional biological replicates would be required to further confirm the reproducibility and magnitude of this effect.

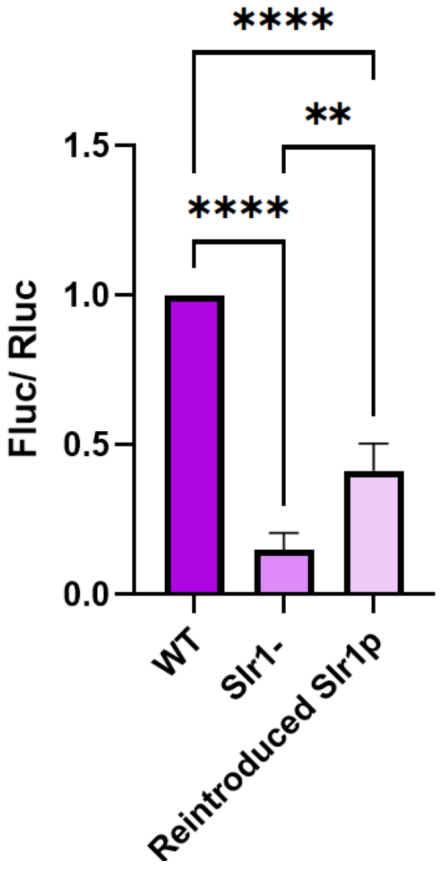


Figure 20: Reintroduction of Slr1p partially restores GCN4 reporter activity in slr1Δ cells. Fluc/Rluc ratios were normalized to WT, which was set to 1. Data are presented as mean ± SD from four independent biological replicates for WT and *slr1Δ* and two independent biological replicates for Slr1p-reintroduced cells. Statistical significance was assessed using one-way ANOVA with Šídák's multiple-comparisons test. ****P < 0.0001; **P ≤ 0.001

3.1.3 Slr1p Expression Following Reintroduction in *slr1Δ* Cells

To determine whether the partial restoration of *GCN4* reporter activity could be related to the level of Slr1p expression following reintroduction, Slr1p protein levels were examined by Western blotting. Slr1p was detected in wild-type cells and in *slr1Δ* cells expressing plasmid-

borne Slr1p, but was absent from *slr1Δ* cells and *slr1Δ* cells containing the empty pRep81x vector, as expected (Figure 21). Ponceau staining showed relatively even protein loading across the samples. Notably, the level of plasmid-expressed Slr1p appeared slightly lower than the endogenous Slr1p level in wild-type cells. This slightly lower expression could contribute to the partial, rather than complete, restoration of *GCN4* reporter activity observed following Slr1p reintroduction.

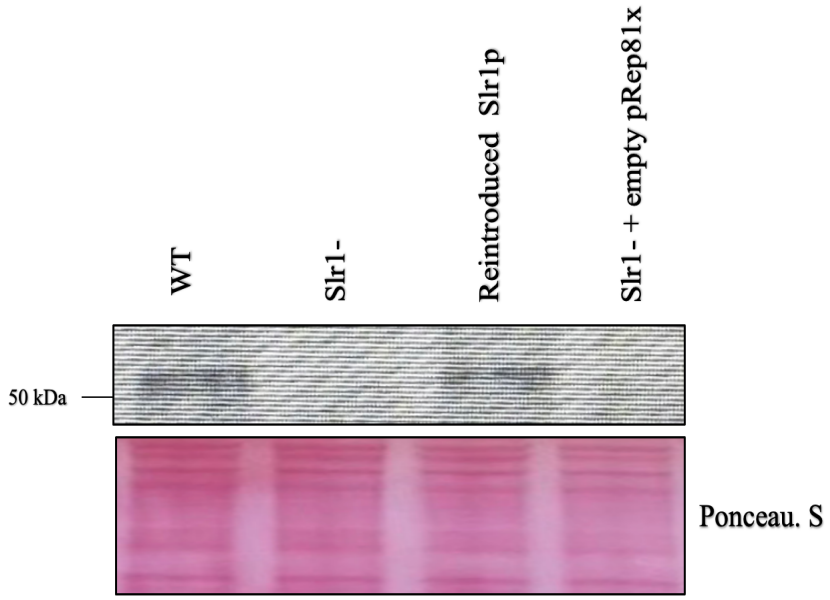


Figure 21: Western blot analysis of Slr1p expression following reintroduction in *slr1Δ* cells. Slr1p was detected in wild-type (WT) and Slr1p-reintroduced cells, but not in *slr1Δ* or *slr1Δ* cells carrying the empty pRep81x vector. Plasmid-expressed Slr1p appeared slightly lower than endogenous Slr1p in WT cells. Ponceau staining is shown as a loading control.

3.1.4 Reduced GCN4 reporter activity in *slr1Δ* cells is not linked to decreased mRNA abundance

Slr1p has previously been linked to promoting both mRNA decay and mRNA translation[32]. To determine whether the reduced *GCN4* reporter activity observed in *slr1Δ* cells was a consequence of decreased reporter mRNA abundance rather than impaired translation, quantitative PCR (qPCR) was performed on the *Fluc* reporter transcript. *Fluc* expression was

normalized to the housekeeping gene *Act1* and analyzed using the comparative ΔΔCt method, where:

$$\Delta Ct = Ct_{Fluc} - Ct_{Act1}$$

$$\Delta\Delta Ct = \Delta Ct_{slr1\Delta} - \Delta Ct_{WT}$$

$$\text{Relative expression} = 2^{-\Delta\Delta Ct}$$

Using this approach, the relative *Fluc* mRNA expression did not differ significantly between WT and *slr1Δ* cells (Figure 22) indicating that reporter transcript levels were essentially unchanged between the two strains.

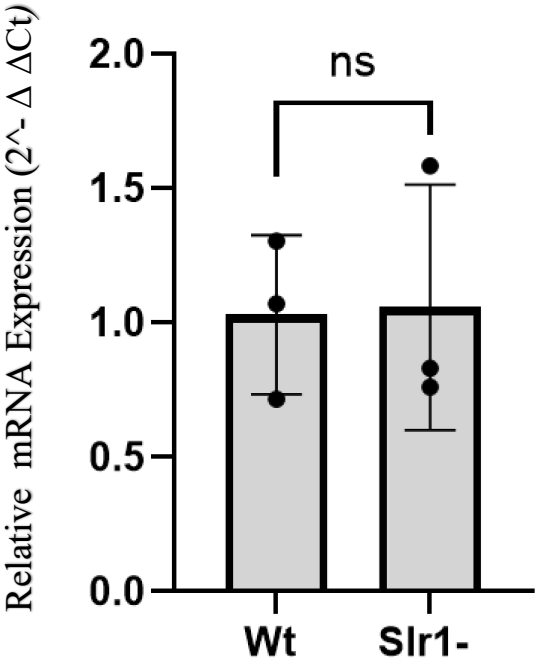


Figure 22: Relative Fluc mRNA expression in wild-type (WT) and slr1Δ cells. Relative *Fluc* mRNA levels were quantified by RT-qPCR and normalized to the reference gene *act1*. Expression levels were calculated using the 2^-ΔΔCt method. Data represent the mean ± SD of three biological replicates, with individual data points shown. No significant difference in *Fluc* mRNA expression was detected between WT and *slr1Δ* cells (Welch's unpaired two-tailed t-test, $p = 0.9340$; ns).

In contrast, the dual-luciferase assays demonstrated an approximately 6.2-fold reduction in *GCN4* reporter activity in *slr1Δ* cells under non-stressed conditions. The absence of a corresponding decrease in *Fluc* mRNA abundance indicates that the reduced reporter activity cannot be explained by changes in steady-state transcript levels. Instead, these findings suggest that Slr1p promotes efficient translation of the *GCN4* reporter**,** with loss of Slr1p resulting in a defect at the level of protein synthesis rather than reporter transcript abundance. Although qPCR does not directly measure mRNA stability, the comparable *Fluc* mRNA levels between WT and *slr1Δ* cells support the conclusion that the decrease in reporter activity primarily reflects impaired translation rather than reduced steady-state mRNA levels.

3.1.5: Slr1p has a minor role in controlling expression from the Hri2 uORFs

Previous studies in *S. pombe* have shown that Hri2p is an eIF2α kinase involved in the cellular response to stress, with an important role during heat shock[53]. The *Hri2* transcript also contains regulatory uORFs within its 5′ leader, providing another example of a transcript whose expression can be controlled at the level of translation. Importantly, *Hri2* translation has been shown to increase under stress conditions through uORF-dependent regulation[52]. We therefore selected the *Hri2* 5′ leader as a second reporter to determine whether the effect of Slr1p observed with *GCN4* extends to another uORF-containing transcript. **We hypothesized that loss of Slr1p may alter *Hri2* reporter expression, particularly under stress conditions in which *Hri2* regulation becomes important.**

Under basal conditions, the *Hri2* reporter showed only a modest difference between WT and *slr1Δ* cells in Fluc/Rluc activity (Figure 23a). In contrast to the much stronger difference observed with the *GCN4* reporter, this suggests that expression from the *Hri2* uORF-containing 5′ leader is less dependent on Slr1p under basal conditions. Similarly, H_2O_2 and 3-AT treatment

did not produce a clear change in the relationship between WT and *slr1Δ* cells (Figures 23b and 23c).

The clearest response in reporter expression was observed following heat shock. Hri2p has previously been identified as an important eIF2α kinase during the heat-stress response in *S. pombe*, making heat shock particularly relevant to this reporter[53]. Consistent with this known role, *Hri2* reporter activity increased strongly over the course of heat shock, reaching approximately a 5–6-fold increase after 30 min (Figure 23d). Importantly, this induction occurred in both WT and *slr1Δ* cells, and the two strains followed a similar pattern throughout the heat-shock treatment**.** This shows that the *Hri2* reporter remained strongly responsive to heat stress in the absence of Slr1p and suggests that Slr1p is not required for this response. Together, these results suggest that the dependence on Slr1p differs between uORF-containing transcripts. While Slr1p was found to be a very important regulator of *GCN4* expression, its effect on *Hri2* expression is comparatively small.

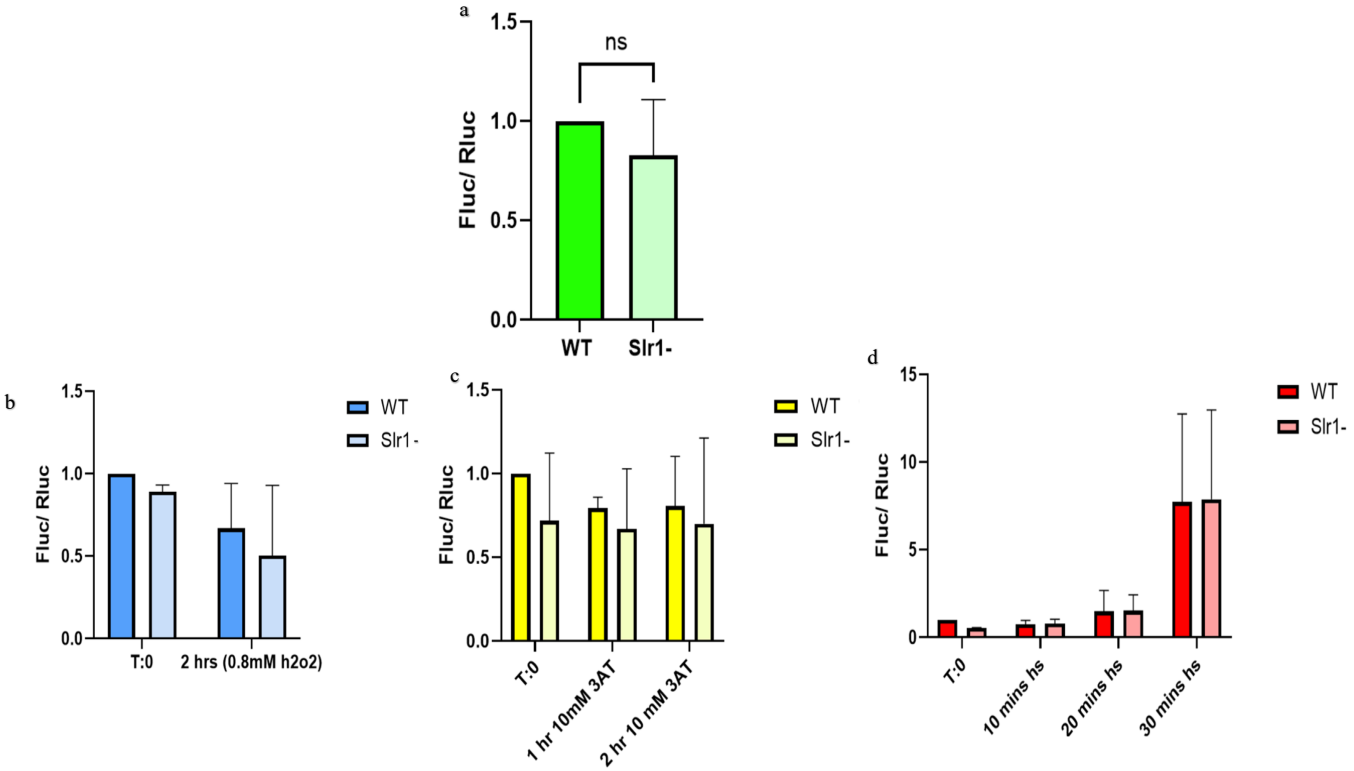


Figure 23: Analysis of Slr1-dependent regulation of Hri2 uORF-mediated translation under different stress conditions using dual luciferase reporter assays. (A) *Fluc/Rluc* ratios of the *Hri2* reporter in wild-type (WT) and *slr1Δ* cells under non-stressed conditions. Values were normalised to WT, which was set to 1. Data represent the mean ± SD of four biological replicates. Statistical significance was determined using a two-tailed unpaired Welch's t-test, ns ($p = 0.9258$). **(B)** *Fluc/Rluc* ratios of the *Hri2* reporter in WT and s*lr1Δ* cells following treatment with 10 mM 3-AT for 1 h and 2 h. **(C)** *Fluc/Rluc* ratios of the *Hri2* reporter in WT and *slr1Δ* cells under basal conditions and following treatment with 0.8 mM H_2O_2 for 2 h. **(D)** *Fluc/Rluc* ratios of the *Hri2* reporter in WT and *slr1Δ* cells during heat shock treatment at the indicated time points. Error bars represent standard deviation from biological replicates.

3.2 Slr1p inhibits frameshifting and helps maintaining translation fidelity

Maintaining the correct reading frame during translation is essential for producing the intended protein. However, ribosomes can sometimes shift by one nucleotide while moving along an mRNA, resulting in translation in an alternative reading frame. This process is known as ribosomal frameshifting and can occur in either the −1 or +1 direction. Although programmed ribosomal frameshifting is used naturally by some viruses and cellular elements to produce alternative proteins, changes in frameshifting efficiency can also provide a useful measure of how well the ribosome maintains the correct reading frame during translation[24]. Notably, the

frequency of ribosome frameshifting has also been hypothesized to serve as a proxy for measuring the frequency of ribosome collisions/disome formation, which have been linked to translation stress and the function of the *S. cerevisiae* LARP1 homologs Slf1p and Sro9p[35].

To determine whether Slr1p contributes to this aspect of translational fidelity, dual-luciferase frameshifting reporters originally developed for *S. cerevisiae* were used[55]. These reporters contain *Renilla luciferase (Rluc)* followed by a defined frameshifting sequence and *Firefly luciferase (Fluc)*. The −1 reporter contains a frameshifting signal derived from the L-A double-stranded RNA virus of *S. cerevisiae*, which promotes movement of the ribosome by one nucleotide in the −1 direction. The +1 reporter instead contains a sequence derived from the *Ty1* retrotransposon, which promotes movement of the ribosome by one nucleotide in the +1 direction. These L-A and Ty1 sequences are well-established reporters for measuring −1 and +1 programmed ribosomal frameshifting in yeast, respectively[55].

A separate 0-frame control was included. This places *Rluc* and *Fluc* in the same reading frame, meaning that no frameshift is required for *Fluc* production (even if this still requires stop codon readthrough). Frameshifting efficiency was calculated by comparing the *Fluc/Rluc* ratio of the −1 or +1 reporter with the *Fluc/Rluc* ratio of the corresponding 0-frame control. Thus, the ratio obtained represents the proportion of translation events that result in successful readthrough of the reporter through a −1 or +1 frameshift relative to the in-frame control[55].

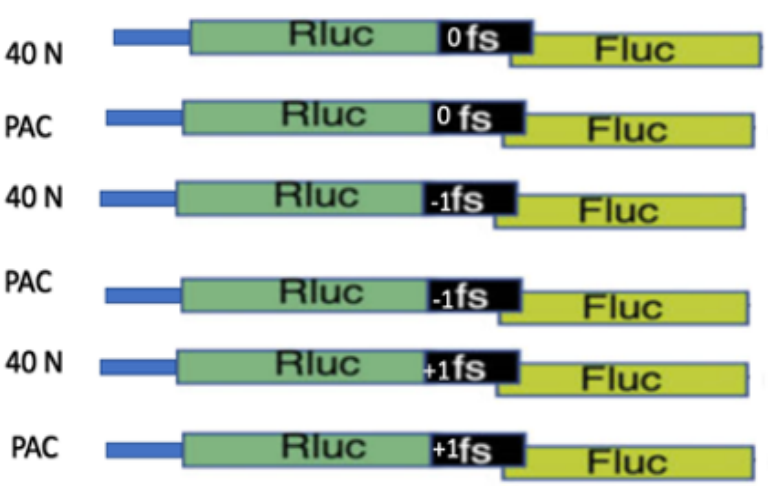
This dual luciferase system was previously studied in *S. cerevisiae*. The results showed that loss of the Slr1p-related protein Slf1p increased −1 frameshifting under non-stressed conditions, and +1 frameshifting under oxidative stress conditions. These findings suggested that this family of RNA-binding proteins may contribute to maintaining the correct reading frame. **We therefore hypothesized that Slr1p may have a similar function in *S. pombe*. To test this, we analysed**

whether Slr1p inhibits ribosomal -1 and +1 frameshifting under stressed versus non-stressed conditions.

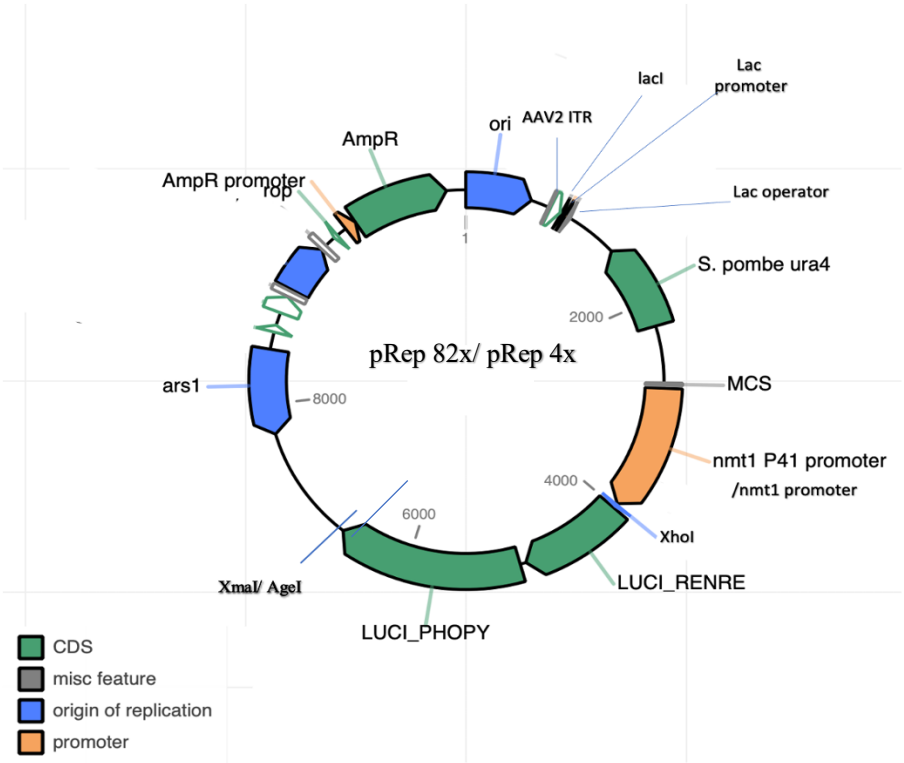
We also wanted to determine whether this effect was influenced by an RNA sequence previously identified to be overrepresented in Slr1p-associated mRNAs, known as the 5′ proximal AC-rich (5′PAC) motif in Slr1p-regulated mRNAs. The PAC motif is located close to the translation start site of many Slr1p targets, and Slr1p has been shown to bind PAC-containing RNA *in vitro*[32]. To test whether the presence of this Slr1p-associated sequence influences frameshifting, each reporter was generated in two versions: one containing the PAC motif and another containing a 40-nucleotide randomized sequence (40N) as a comparison. This allowed us to ask two related questions: whether Slr1p affects frameshifting and whether any Slr1p-dependent effect changes when the reporter contains a sequence associated with Slr1p binding.

The frameshifting reporter cassettes were cloned into the multiple cloning sites of both pRep4x and pRep82x. The reporter inserts were amplified using primers containing XhoI and AgeI restriction sites, while the pRep vectors were digested with XhoI and XmaI. AgeI and XmaI generate compatible cohesive ends, allowing the reporter cassette to be introduced into the vector. This produced 0-frame, −1 L-A, and +1 Ty1 reporters containing either PAC or 40N. The resulting six reporter constructs were cloned into both pRep4x, the higher-expression system, and pRep82x, the lower-expression system, allowing the effect of Slr1p on frameshifting to be examined at different reporter expression levels. The plasmid maps of the 0-frame, -1, and +1 dual luciferase frameshifting reporters used in this study are shown below (Figure 24).

a


40 N
Rluc
0fs
Fluc
PAC
Rluc
0 fs
Fluc
40 N
Rluc
-1fs
Fluc
PAC
Rluc
-1fs
Fluc
40 N
Rluc
+1fs
Fluc
PAC
Rluc
+1fs
Fluc

b


lacI
Lac promoter
ori
AAV2 ITR
AmpR
AmpR promoter
rop
Lac operator
1
S. pombe ura4
2000
pRep 82x/ pRep 4x
ars1
8000
MCS
nmt1 P41 promoter
/nmt1 promoter
4000
6000
XhoI
XmaI/ AgeI
LUCI_RENRE
LUCI_PHOPY
CDS
misc feature
origin of replication
promoter

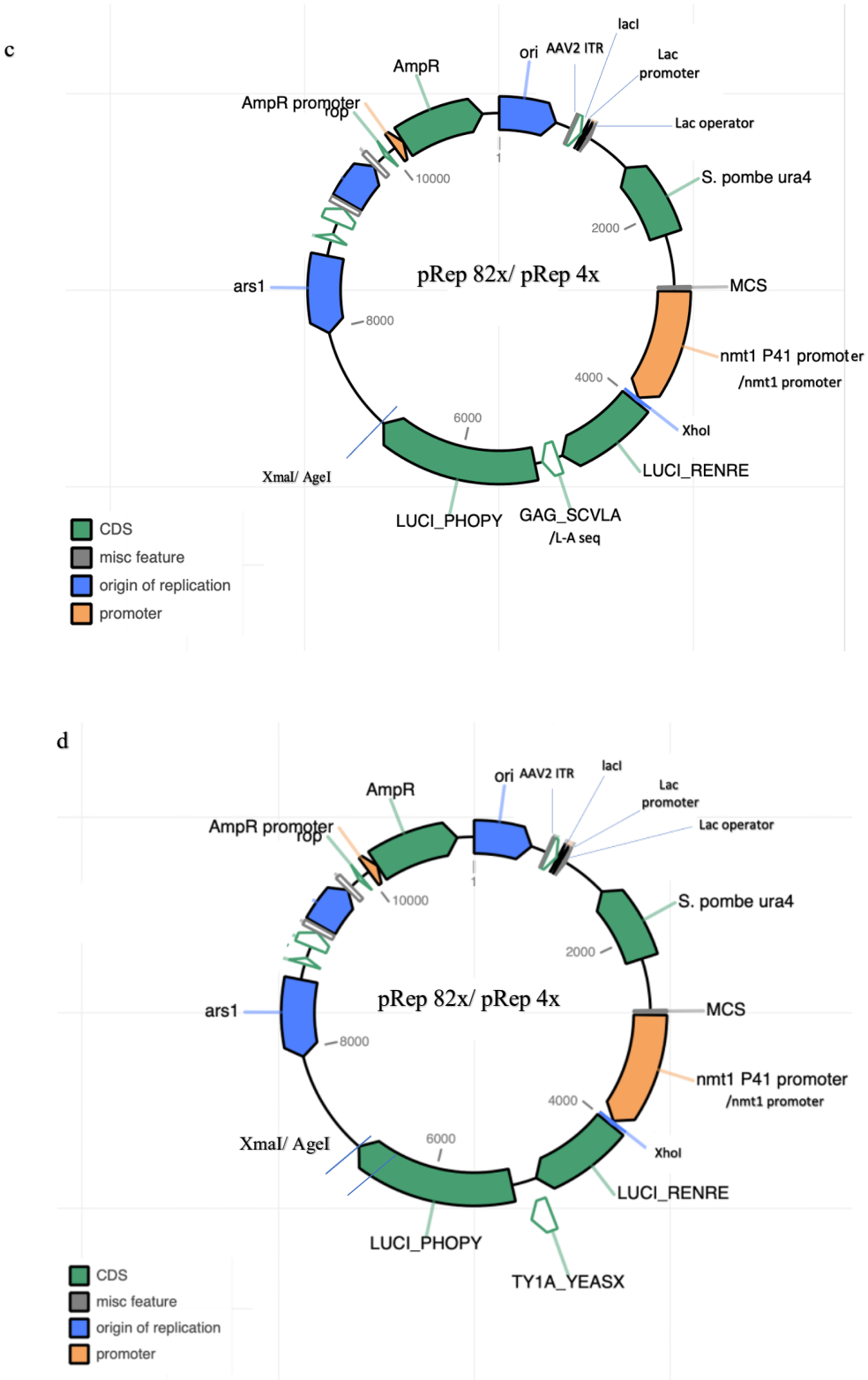


Figure 24: Dual-luciferase reporter constructs used to measure programmed ribosomal frameshifting. (A) Schematic representation of the dual-luciferase frameshifting reporters containing either a 40-nucleotide randomized

sequence (40N) or the PAC motif. Renilla luciferase (*Rluc*) is positioned upstream of Firefly luciferase (*Fluc*), with the corresponding frameshifting sequence located between the two reporters. **(B)** Plasmid map of the 0-frame control, in which *Rluc* and *Fluc* are maintained in the same reading frame, allowing Fluc production without a frameshifting event and representing 100% readthrough. **(C)** Plasmid map of the −1 frameshifting reporter containing the L-A frameshifting sequence derived from the *S. cerevisiae* L-A double-stranded RNA virus, which promotes −1 programmed ribosomal frameshifting. **(D)** Plasmid map of the +1-frameshifting reporter containing the Ty1 retrotransposon frameshifting sequence, which promotes +1 programmed ribosomal frameshifting. Plasmid maps in (B–D) were generated from whole-plasmid sequencing data obtained from Plasmidsaurus.

The frameshifting experiment results suggest that Slr1p may play a role in maintaining translational fidelity, particularly by regulating −1 frameshifting events. Under non-stressed conditions in the *pRep4x* reporter system (Figure 25b), *slr1Δ* cells generally displayed higher frameshifting efficiencies compared to WT cells in the −1 frameshift reporters. However, only modest differences were observed in the +1 frameshift constructs. This suggests that Slr1p may have a stronger influence on −1 frameshifting than on +1 frameshifting.

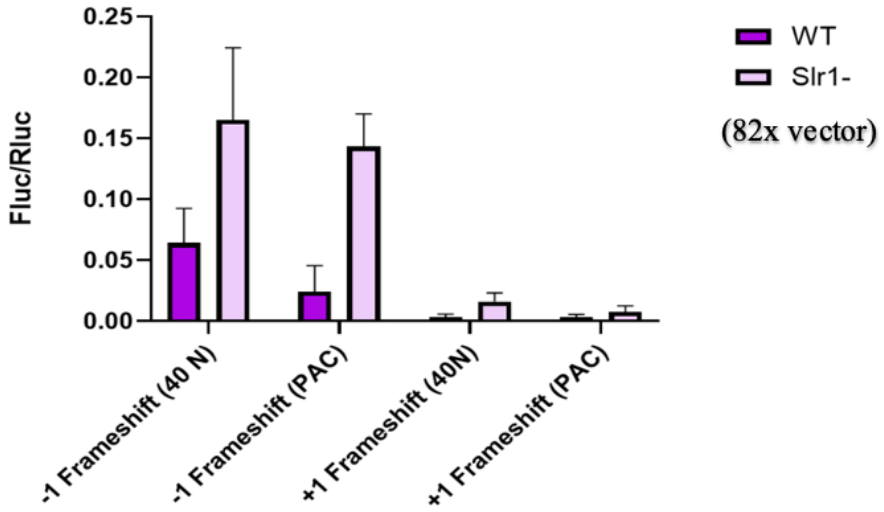
A similar but more noticeable trend was observed in the *pRep82x* reporter system (Figure 25a), where the absence of Slr1p resulted in increased −1 frameshifting relative to WT cells. The most pronounced effect was observed in the −1 PAC construct, where *slr1Δ* cells showed approximately a 6-fold increase in frameshifting efficiency compared to WT cells. In contrast, the +1 frameshift reporters showed only small changes between WT and *slr1Δ* cells. Together, these findings suggest that Slr1p may normally function to suppress −1 frameshifting during translation.

Under oxidative stress conditions induced by 2 hrs of 0.8 mM H_2O_2 treatment in the *pRep4x* reporter system, the differences between WT and *slr1Δ* cells became weaker (Figure 25c). Although *slr1Δ* cells still tended to show slightly higher frameshifting efficiencies in several constructs, the magnitude of the changes was smaller compared to non-stressed conditions. This may suggest that oxidative stress broadly alters translational regulation and may mask more subtle Slr1p-dependent effects.

Overall, these findings support a potential role for Slr1p in translational regulation and suggest that loss of Slr1p can increase frameshifting efficiency, particularly during −1 frameshifting events in lower expression vectors (*pRep 82x*). This trend is consistent with the findings reported in *Saccharomyces cerevisiae* that the Slr1p homolog of Slf1p, suppresses −1 frameshifting under non-stressed conditions. The similarity between these observations suggests that the role of this protein family in limiting −1 frameshifting may be conserved across yeast species.

Since PAC-dependent regulation was not consistently observed across all conditions tested, these findings may suggest that the PAC motif is more strongly associated with translation initiation or early ribosome recruitment rather than directly regulating elongation-dependent frameshifting. It should be noted that these observations are based on only two biological replicates. Therefore, while the results suggest that Slr1p may suppress −1 frameshifting, additional biological replicates will be required to enable robust statistical analysis and to confirm the reproducibility of these findings.

a



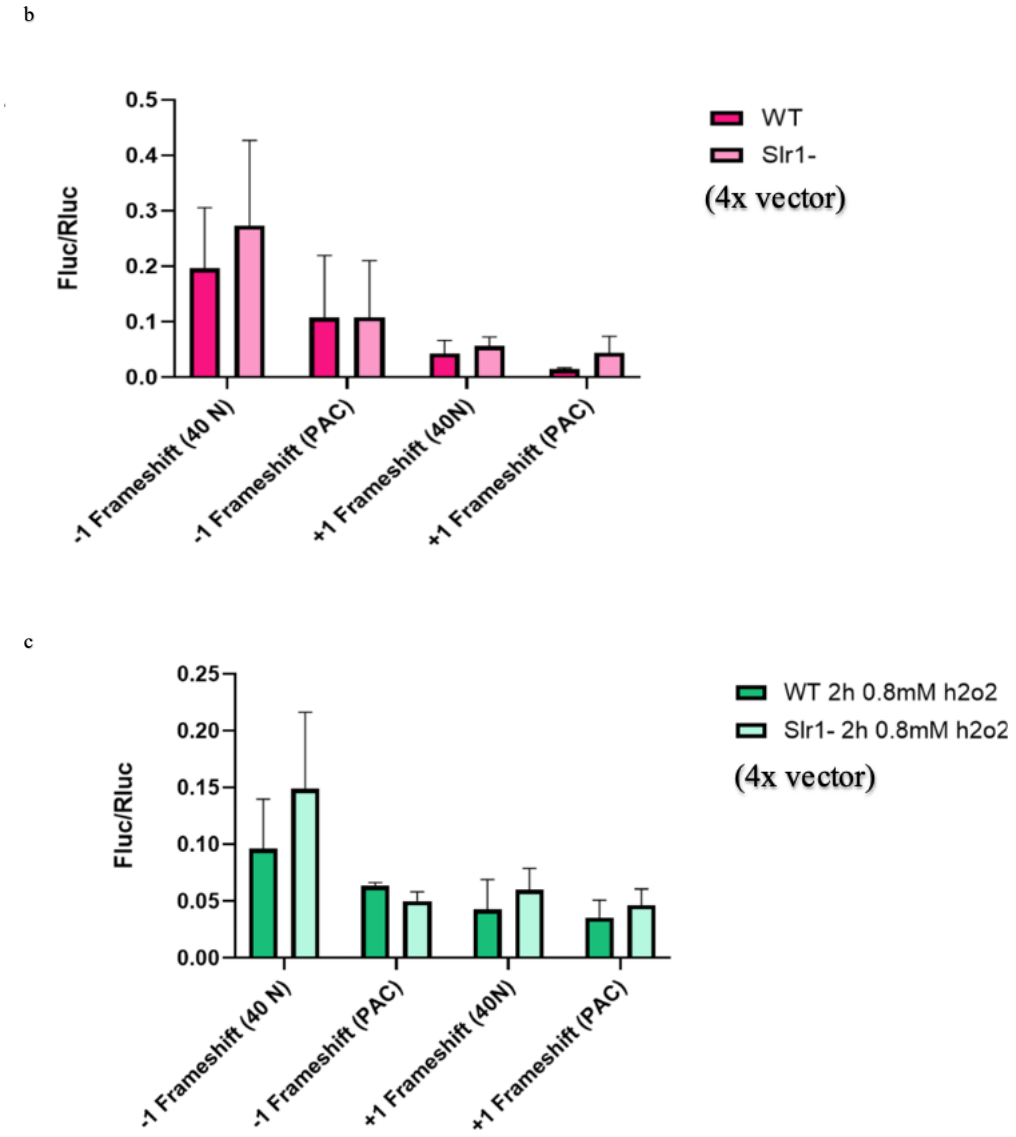


Figure 25*:* **Frameshifting reporter constructs and dual-luciferase analysis of frameshifting activity in WT and slr1Δ cells. (A)** Relative luciferase activity (*Fluc/Rluc)* of the pRep82x vector frameshifting constructs in WT and *slr1Δ* cells under non-stressed conditions. **(B)** Relative luciferase activity (*Fluc/Rluc)* of the pRep4x vector frameshifting constructs in WT and slr1Δ cells under non-stressed conditions**. (C)** Relative luciferase activity *(Fluc/Rluc)* of the pRep4x vector frameshifting constructs in WT and *slr1Δ* cells following treatment with 0.8 mM H_2O_2 for 2 h. Error bars represent standard deviation from biological replicates.

3.3 Slr1p interacts with Exo2p and may be involved in mRNA decay

3.3.1: Co-immunoprecipitation of Exo2p-Myc with wild-type and phospho-mutant Slr1p

To investigate whether Slr1p phosphorylation influences its interaction with the mRNA decay factor Exo2p, co-immunoprecipitation assays were performed using wild-type Slr1p,

phosphoablative (S410A and S437A), and phosphomimetic (S410D and S437D) mutants. These residues were selected because phosphorylation at S410 and S437 has been linked to TOR signalling, a pathway that coordinates cell growth and translation in response to nutrient availability. S410 has also been reported as a highly phosphorylated site in Slr1p. In addition, both residues are located within or near the RNA-binding La motif (LaM; amino acids 323–416), making them particularly relevant candidates for investigating whether phosphorylation influences Slr1p function and its protein interactions[40]. As phosphorylation is known to regulate the function of RNA-binding proteins, **we hypothesized that changing the phosphorylation state of S410 or S437 may alter the interaction between Slr1p and Exo2p, potentially providing a mechanism for regulating the different functions of Slr1p in translation and mRNA decay.**

Immunoprecipitation of Slr1p was performed using a custom anti-Slr1p antibody (AB2756) raised against the C-terminal region of Slr1p. Successful immunoprecipitation of Slr1p was subsequently confirmed by Western blotting using a second anti-Slr1p antibody (AB2648), raised against the full-length Slr1p protein. As shown in Figure 26, Exo2p-Myc co-immunoprecipitated with wild-type Slr1p but was not detected in the empty vector control, indicating that the interaction was dependent on the presence of Slr1p. Among the phospho-mutant proteins, the phosphomimetic S410D mutant exhibited a weaker Exo2p signal than S410A which interacted with Exo2p at a level comparable to or higher than that of wild-type Slr1p. Neither the S437A nor S437D mutations produced an obvious change in Exo2p co-immunoprecipitation.

Together, these results suggest that phosphorylation at serine 410 may modulate the interaction between Slr1p and Exo2p, whereas phosphorylation at serine 437 does not appear to influence

this interaction under the conditions tested. Given the established roles of LARP1 family proteins in translation regulation and RNA stability/decay, the observed interaction between Slr1p and Exo2p is consistent with a model in which Slr1p may contribute to coordinating translation with translation-associated mRNA decay. However, additional biological replicates and further validation experiments will be required to confirm the reproducibility and functional significance of these observations.

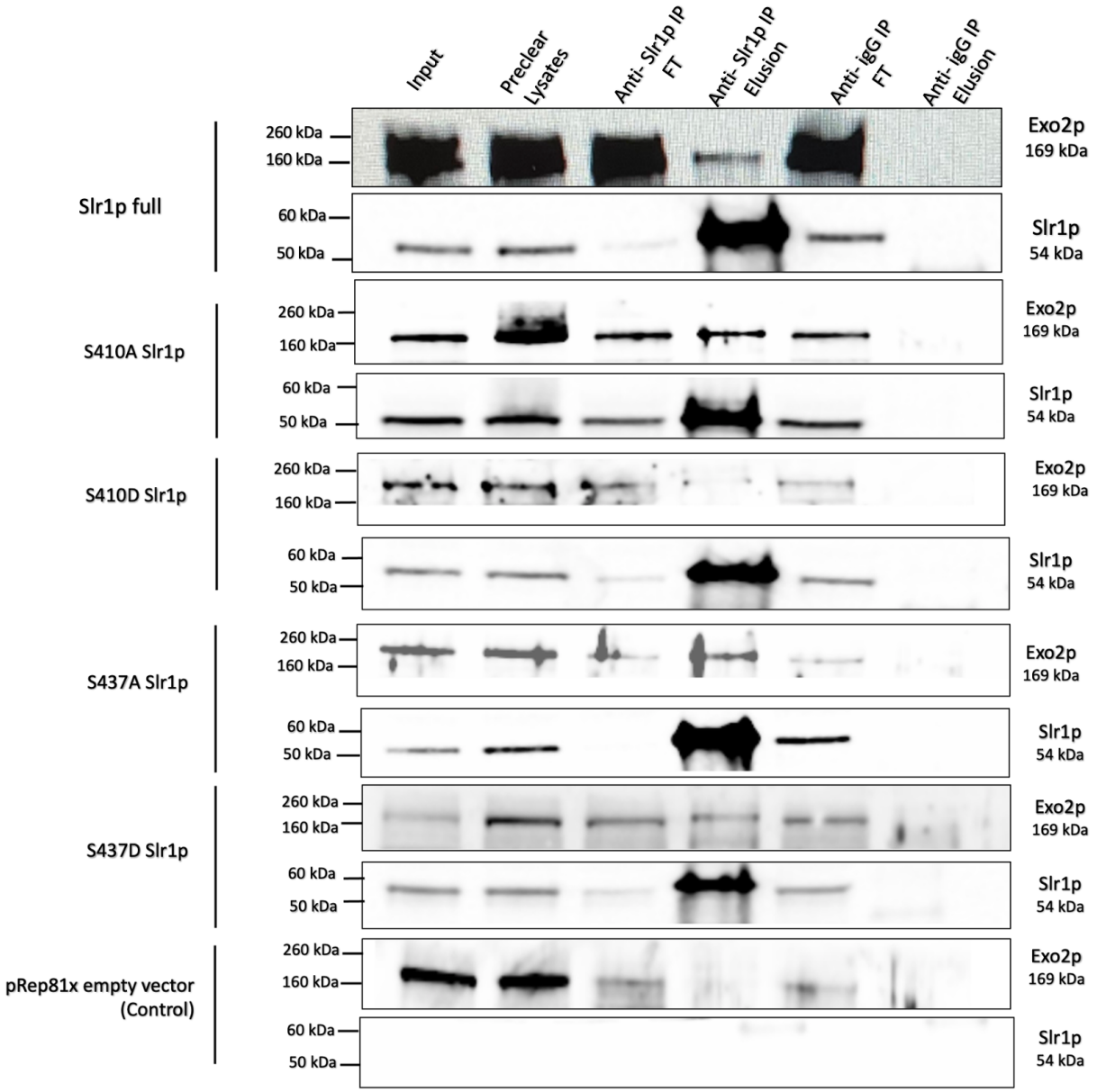


Figure 26: Co-immunoprecipitation of Exo2-Myc with wild-type and phospho-mutant Slr1p proteins. Approximately 0.3 g of cryogenically ground protein extracts expressing Exo2-Myc together with the

phosphoablative Slr1p mutants (S410A and S437A), phosphomimetic mutants (S410D and S437D), or the pRep81x empty vector control were subjected to immunoprecipitation using an anti-Slr1p antibody (AB2756). Exo2-Myc was detected by immunoblotting with an anti-Myc antibody. The corresponding Slr1p blots were probed using an anti-Slr1p antibody (AB2648) to confirm immunoprecipitation of Slr1p. Input, precleared lysate, anti-Slr1p immunoprecipitation flow-through (FT), anti-Slr1p immunoprecipitation eluate, anti-IgG immunoprecipitation flow-through (FT), and anti-IgG immunoprecipitation eluate fractions are shown. Exo2p and Slr1p were detected at approximately 169 kDa and 54 kDa, respectively. (n= 2 or 3)

3.3.2 Reciprocal co-immunoprecipitation of Exo2p-Myc

To further validate the interaction between Slr1p and Exo2p, a reciprocal co-immunoprecipitation was performed by immunoprecipitating Exo2p-Myc using an anti-Myc antibody and probing the eluates for Slr1p. As shown in Figure 27, Exo2p-Myc was successfully immunoprecipitated, as demonstrated by its enrichment in the anti-Myc elution fraction and its absence from the IgG control elution, confirming that the immunoprecipitation was successful and specific. However, Slr1p was not detected in the anti-Myc eluates of either the wild-type or phospho-mutant strains, despite being readily detected in the corresponding input and lysate samples. Consequently, the reciprocal co-immunoprecipitation did not independently confirm the interaction observed in the Slr1p immunoprecipitation experiments. One possible explanation is that Slr1p associates with Exo2p in close proximity to the C-terminal Myc epitope, thereby reducing antibody accessibility through steric hindrance and thus preventing efficient immunoprecipitation of the Slr1p-bound Exo2p complex. Under this model, the anti-Myc antibody would preferentially capture Exo2p molecules with an exposed Myc tag, whereas Exo2p molecules associated with Slr1p would remain in the flow-through. Although this hypothesis is consistent with the observed results, additional experiments using alternative epitope-tag positions or antibodies recognizing endogenous Exo2p will be required to determine whether steric hindrance accounts for the lack of detectable Slr1p in the reciprocal immunoprecipitation.

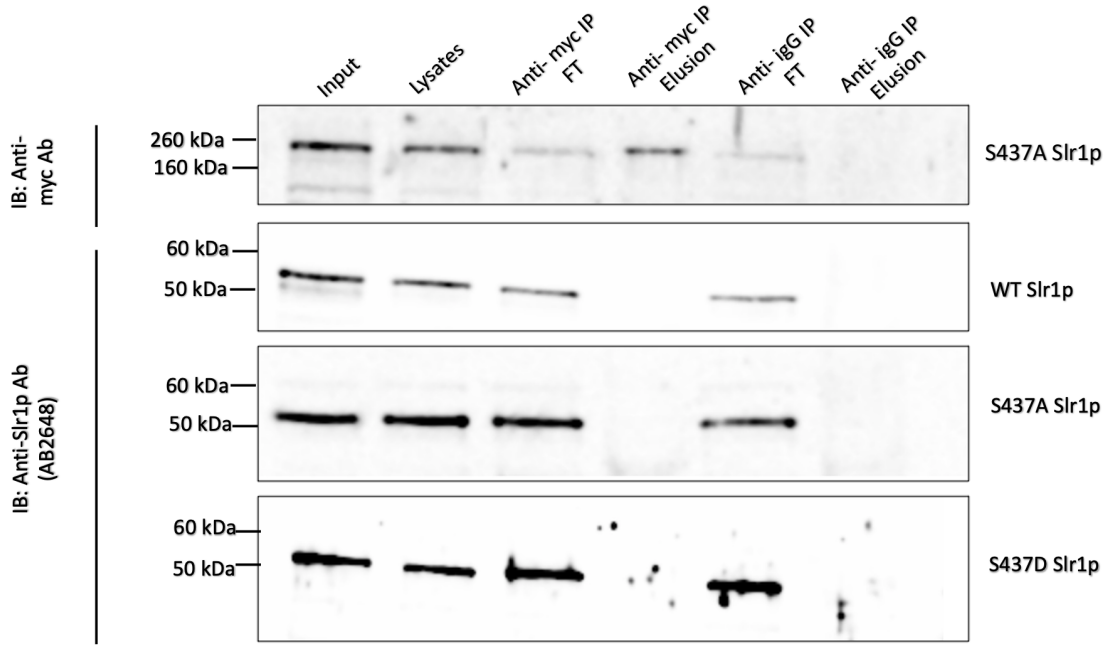


Figure 27: Reciprocal immunoprecipitation of Exo2p-Myc and Slr1p. Exo2p-Myc was immunoprecipitated using an anti-Myc antibody and the indicated fractions were analyzed by Western blotting with anti-Myc or anti-Slr1p antibodies. WT Slr1p and the indicated phospho-mutants are shown. IgG IP was included as a negative control.

Chapter 4: Discussion

Slr1p has previously been linked to the regulation of translation in *S. pombe*, particularly at the level of translation initiation. However, its role in the translation of specific mRNAs and whether it contributes to other stages of translation are still not fully understood. The results of this study add to this picture by suggesting that Slr1p may influence more than one aspect of mRNA translation. Interestingly, Slr1p was important for efficient expression of a *GCN4* uORF reporter, while having much less effect on an *Hri2* reporter. Loss of Slr1p was also associated with increased −1 frameshifting, suggesting a possible role in maintaining translation fidelity. Finally, the interaction observed between Slr1p and Exo2p raises the possibility that the function of Slr1p in translation may also be connected to mRNA decay. Together, these findings suggest that Slr1p has a broader role in mRNA regulation, influencing not only how specific mRNAs are translated, but also the accuracy of their translation and potentially their transition toward mRNA decay.

4.1 Slr1p contributes to efficient translation of the *GCN4* reporter

One of the most interesting observations from this study was the strong decrease in *GCN4* reporter activity when Slr1p was absent. Under non-stressed conditions, reporter expression was approximately 6.2-fold higher in WT cells than in *slr1Δ* cells. This suggests that Slr1p is important for efficient expression of this reporter even when the cells are not exposed to stress.

The qPCR results help explain where this difference may be coming from. Despite the large difference in luciferase activity, the amount of *Fluc* reporter mRNA was very similar between WT and *slr1Δ* cells. Therefore, the lower reporter activity in *slr1Δ* cells does not appear to simply result from having less reporter transcript available. Instead, the difference is more

consistent with Slr1p affecting how efficiently the mRNA is translated. This is also supported by the rescue experiment, in which reintroducing Slr1p into *slr1Δ* cells increased reporter activity compared with the empty vector control. Although the rescue was only partial and more biological replicates would strengthen this result, it supports the idea that the phenotype is linked to the absence of Slr1p.

An interesting part of this result is that translation of the reporter is controlled by the *GCN4* 5′ leader containing uORFs. uORFs can regulate whether scanning ribosomes reach the downstream coding sequence and are particularly important for controlling the translation of stress-responsive genes. The strong decrease in reporter expression in *slr1Δ* cells therefore raises the possibility that Slr1p helps ribosomes translate mRNAs containing these regulatory features. At this point, however, it is difficult to say how exactly Slr1p is involved. It could affect ribosome recruitment to the mRNA, scanning through the 5′ leader, or events that occur after a uORF has been translated, such as reinitiation at the downstream ORF. The current reporter measures the final outcome of these events rather than separating the individual steps.

Interestingly, the difference between WT and *slr1Δ* cells was already clear under basal conditions and did not become dramatically stronger following heat shock, H_2O_2 treatment, or 3-AT treatment. This was somewhat unexpected given the well-established role of *GCN4*-type regulation. In particular, we expected a stronger response to amino acid starvation, since ATF4, the mammalian homolog of *GCN4,* is also regulated through uORFs and its translation is strongly induced during amino acid starvation[14,15]. Based on this, we expected 3-AT treatment to produce a more pronounced change in the *GCN4* reporter. However, this was not clearly observed under the conditions tested. One interpretation is that Slr1p is not simply a stress-specific regulator of this reporter. Instead, it may contribute more generally to its efficient uORF-

dependent translation, with the effect remaining present when the cells are exposed to different stresses. It is also possible that the stress conditions used here did not produce the specific translational changes needed to reveal an additional Slr1p-dependent response. More biological replicates and further optimization of these conditions would help distinguish between these possibilities.

4.2 Slr1p does not affect all uORF-containing reporters in the same way

The results obtained with *Hri2* provide an important comparison to the strong phenotype observed with *GCN4*. Under basal conditions, the difference in *Hri2* reporter activity between WT and *slr1Δ* cells was small. A similar pattern was seen during H_2O_2 and 3-AT treatment. This suggests that the presence of uORFs alone is probably not enough to make an mRNA strongly dependent on Slr1p.

The heat shock experiment was particularly informative in this regard. *Hri2* reporter activity increased strongly over the course of heat shock in both WT and *slr1Δ* cells. Hri2p is an eIF2α kinase involved in the cellular response to heat stress[53]. Thus, the strong induction of the *Hri2* reporter during heat shock was consistent with its known role in this pathway. Importantly, WT and *slr1Δ* cells responded similarly, with no clear defect in the heat shock response when Slr1p was absent. This suggests that *Hri2* can respond normally to heat stress in *slr1Δ* cells and that Slr1p is not essential for this response.

The different behaviour of the *GCN4* and *Hri2* reporters suggests that Slr1p is unlikely to regulate all uORF-containing mRNAs through one general mechanism. Instead, other features of the transcript may determine whether its translation depends on Slr1p. These could include the number and position of the uORFs, their surrounding sequences, the distance between the uORFs and the main coding region, or other features within the 5′ leader. This fits more broadly with the

idea of Slr1p acting on a particular group of mRNAs rather than simply increasing translation globally. An alternative but not mutually exclusive possibility relates to the degree of characterization of these uORFs: the *GCN4* uORF from *S. cerevisiae* is one of the most highly studied uORFs in the scientific literature, while the *HRI2* uORF from *S. pombe* is not nearly as well characterized. Thus, our results showing Slr1p associated effects predominantly on the *GCN4* uORF may alternatively be due to its expression simply being more uORF-dependent than *HRi2.*

These considerations also raise an important question for future work: what makes one uORF-containing transcript Slr1p-dependent while another is largely unaffected? Comparing additional reporters and systematically changing features of the *GCN4* 5′ leader could help answer this. For example, removing individual uORFs would make it possible to determine whether the strong Slr1p-dependent phenotype requires a particular uORF or whether other sequences in the leader are responsible for the observed difference in *GCN4* expression between WT and *slr1Δ* cells.

4.3 Slr1p may also function during translation elongation and maintaining translation fidelity

Although the *GCN4* experiments support a role for Slr1p in translational regulation during 40S scanning/ initiation, the frameshifting experiments suggest that its function may not be limited to events occurring at the beginning of translation. The loss of Slr1p generally resulted in higher −1 frameshifting, while the differences in +1 frameshifting observed were much smaller. Also, the effect was particularly noticeable with the pRep82x reporters, where the −1 PAC (the sequence that Slr1p likes binding to) construct showed the largest difference between WT and *slr1Δ* cells.

Frameshifting occurs when the ribosome moves away from the correct reading frame while translating an mRNA. Therefore, the increased −1 frameshifting observed in *slr1Δ* cells suggests that Slr1p may help ribosomes maintain the correct reading frame during elongation. Rather than simply helping translation begin efficiently, Slr1p may remain important once the ribosome has entered the elongation phase.

This interpretation fits with previous observations that connect Slr1p and related proteins to actively translating ribosomes and elongation. It is also consistent with previous findings that showed that the LARP1 homolog in *S. cerevisiae* Slf1p, suppresses −1 frameshifting under non-stressed conditions. Seeing a similar phenotype following loss of Slr1p in *S. pombe* is interesting because it suggests that a role in maintaining translation fidelity may be shared between these related proteins.

At the same time, the frameshifting phenotype was not equally strong in every experimental condition. The difference between WT and *slr1Δ* cells was more pronounced with the lower-expression pRep82x vector than with the higher-expression pRep4x vector. One possible explanation is that when the reporter is expressed at a lower level, the amount of Slr1p present in the cell may be sufficient to regulate a larger proportion of the translating reporter mRNAs. This in turn makes the effect of Slr1p easier to detect. In contrast, the higher expression driven by pRep4x may result in a much larger number of reporter transcripts being translated, potentially exceeding the stoichiometric amount of Slr1p available to regulate them. This could reduce the difference between WT and *slr1Δ* cells, even though a Slr1p-dependent effect might still be present**.** This may explain why the frameshifting phenotype was still observed with pRep4x, but was less pronounced than with pRep82x.

The Slr1p-dependent difference was further reduced when cells carrying the pRep4x reporters were exposed to oxidative stress using 0.8 mM H_2O_2 for 2 h. Although *slr1Δ* cells still tended to show higher frameshifting in several constructs, the difference from WT was smaller than under non-stressed conditions. One possibility is that oxidative stress causes broader changes in translation that make the contribution of Slr1p less apparent. Under these conditions, stress-induced changes in ribosome activity or translational regulation may have a stronger overall effect, which could partially mask the difference caused by the loss of Slr1p.

The strong effect seen with the −1 PAC reporter is also interesting given the previous association between Slr1p and PAC-containing transcripts. However, a PAC-specific effect was not consistently seen across the different reporter systems. Therefore, it is still too early to conclude that the PAC motif itself is responsible for the frameshifting phenotype. PAC sequences may be more important for Slr1p-dependent regulation at translation initiation, while the frameshifting phenotype could reflect a separate function of Slr1p during elongation. Future work will be required to separate these possibilities.

Overall, the frameshifting results broaden the possible role of Slr1p in translation. Together with previous evidence linking Slr1p to translation initiation, they suggest that Slr1p may influence translation at more than one stage. This would make sense for an RNA-binding protein that was found to remain associated with particular mRNAs as they are being translated, rather than acting only during the initial recruitment of the ribosome[35]. However, since the frameshifting experiments were performed with a limited number of biological replicates, additional replicates will be needed to determine whether these patterns are reproducible and to strengthen the conclusions regarding the role of Slr1p in maintaining translational fidelity.

4.4 Slr1p interaction with Exo2p may provide a link between translation and mRNA decay

In addition to its role in translation, the interaction between Slr1p and Exo2p raises the possibility that Slr1p may also be connected to mRNA decay. Exo2p is a 5′–3′ exoribonuclease involved in RNA degradation, making its association with Slr1p particularly interesting given the role of Slr1p in translation. Rather than acting only while an mRNA is being translated, Slr1p may also be involved in connecting translation with what happens to the transcript afterward[30]. Translation and mRNA decay are closely interconnected. The fate of an mRNA can depend on which regulatory proteins associate with it. Some interactions allow an mRNA to be translated, while others can promote its degradation. As an RNA-binding protein associated with translation, Slr1p could potentially provide a connection between these processes by interacting with different proteins at different stages in the lifetime of an mRNA. The interaction observed between Slr1p and Exo2p is consistent with this possibility and suggests that the role of Slr1p may extend beyond translation itself.

Importantly, previous experiments showed that the interaction between Slr1p and Exo2p was maintained in the presence of Benzonase[32]. This suggests that their association is RNA-independent and makes it less likely that the two proteins are simply recovered together because they are bound to the same RNA molecule. Instead, Slr1p and Exo2p may interact directly or may be brought together through other proteins in the same complex. Although this strengthens the evidence for an association between the two proteins, it still does not tell us what this interaction is doing in the cell. In particular, the current results do not show that Slr1p recruits Exo2p to its target mRNAs or that their interaction directly promotes mRNA degradation.

The phospho-mutant experiments provide an interesting clue as to how this interaction might be regulated. The difference in Exo2p- Slr1p interaction was observed at serine 410, a site that is known to be highly phosphorylated in Slr1p. The phosphomimetic S410D mutant showed a

weaker interaction with Exo2p compared with S410A and WT Slr1p. However, S437 mutants did not show a clear change. This suggests that the phosphorylation state of S410 may influence how strongly Slr1p associates with Exo2p.

The difference between S410A and S410D also raises an interesting possibility for how Slr1p may move between its different roles. S410A, which mimics the unphosphorylated state, showed a stronger association with Exo2p, while the phosphomimetic S410D mutant showed a weaker interaction. One possible interpretation is that when Slr1p is unphosphorylated at S410, its association with Exo2p is favoured, potentially linking this form of Slr1p more closely with mRNA decay. In contrast, phosphorylation at S410 may weaken the interaction with Exo2p and favour a more translation-associated state. In this way, phosphorylation at S410 could provide a mechanism for shifting Slr1p between translation- and decay-associated functions. Based on these findings, we propose a model in which phosphorylation at S410 may act as a molecular switch that regulates Slr1p between translation- and decay-associated functions (Figure 28).

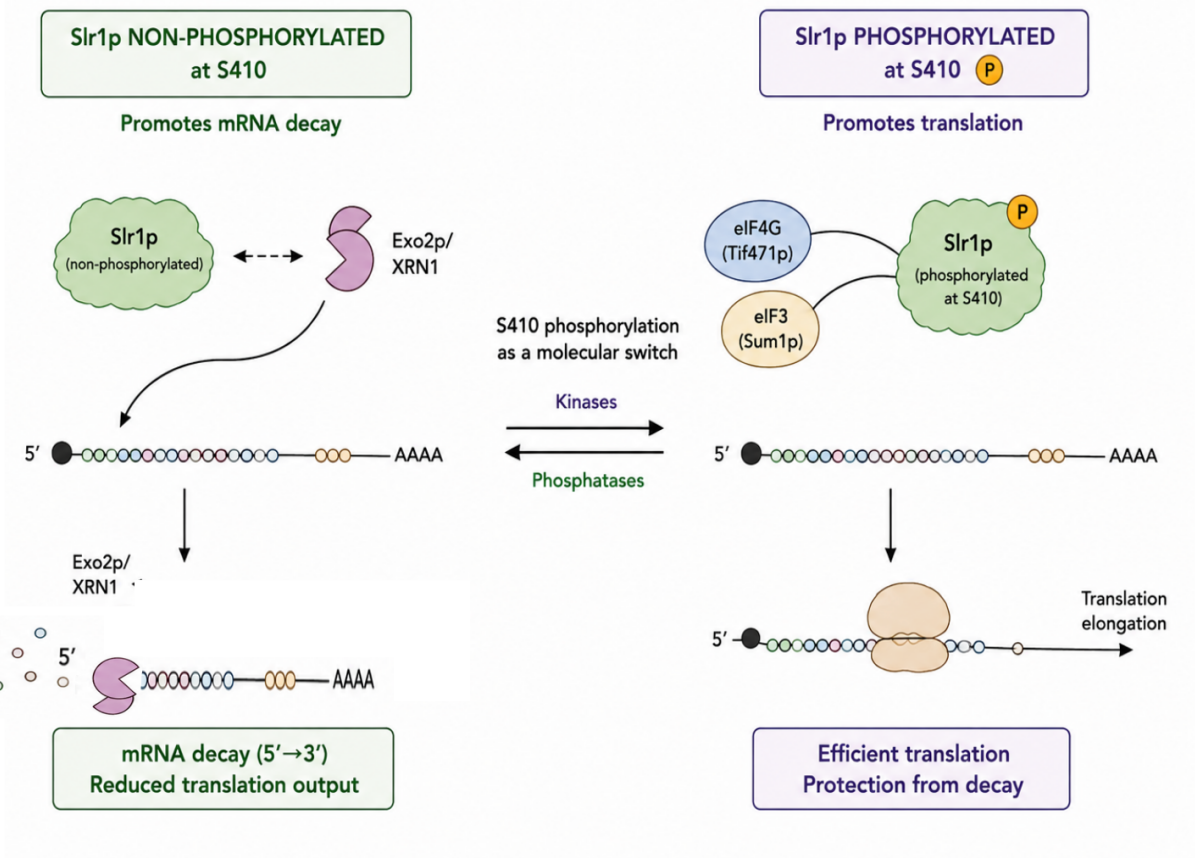


Figure 28: **Proposed model for phosphorylation-dependent regulation of Slr1p between translation and mRNA decay.** Phosphorylation of Slr1p at S410 is proposed to act as a molecular switch between translation- and decay-associated functions. In the non-phosphorylated state, Slr1p may favour association with Exo2p and promote mRNA decay, whereas phosphorylation at S410 may reduce its association with Exo2p and favour a translation-associated state. This model is based on the reduced Exo2p interaction observed with the S410D phosphomimetic mutant together with the translation-associated functions of Slr1p identified in this study.

Although this model requires further experimental testing, it provides a possible explanation for how a single RNA-binding protein could participate in different stages of mRNA regulation. It also can provide an interesting connection between the different findings in this study. Slr1p appears to contribute to the efficient and accurate translation of particular mRNAs, while its interaction with Exo2p suggests a potential role for Slr1p in mRNA decay. A change in phosphorylation could potentially alter the proteins that Slr1p interacts with, which in turn can

change its function. However, the current experiments only show that the S410 mutants may differ in their association with Exo2p. Future work is required to demonstrate that phosphorylated Slr1p is preferentially involved in translation or that the unphosphorylated form directly promotes mRNA decay. Future work will thus involve performing similar Slr1p co-IPs using the Slr1p phosphomimetic and phosphoablative mutants but probing for the (endogenously FLAG-tagged) Sum1p (eIF3i) protein. Sum1p is a component of the eIF3 translation initiation factor complex and has also been previously demonstrated to form an interaction with Slr1p by both co-IP and yeast two-hybrid[32].

The reciprocal co-immunoprecipitation also needs to be considered when interpreting the Slr1p–Exo2p interaction. Although Exo2p-Myc was successfully immunoprecipitated using the anti-Myc antibody, Slr1p was not detected in the Exo2p-Myc eluate. One possible explanation is the accessibility of the Myc tag. If Slr1p associates with Exo2p close to its C-terminal Myc tag, binding of Slr1p could make the epitope less accessible to the anti-Myc antibody. Therefore, the antibody may preferentially immunoprecipitate Exo2p molecules that are not associated with Slr1p, while the Slr1p-bound population remains in the flow-through. This could explain why Exo2p itself was successfully immunoprecipitated but Slr1p was not recovered with it. A proposed model illustrating how Slr1p binding could interfere with accessibility of the C-terminal Myc epitope during reciprocal immunoprecipitation is shown in Figure 29. However, this remains one possible explanation, and the reciprocal experiment alone cannot show that steric hindrance is responsible for what we have observed.

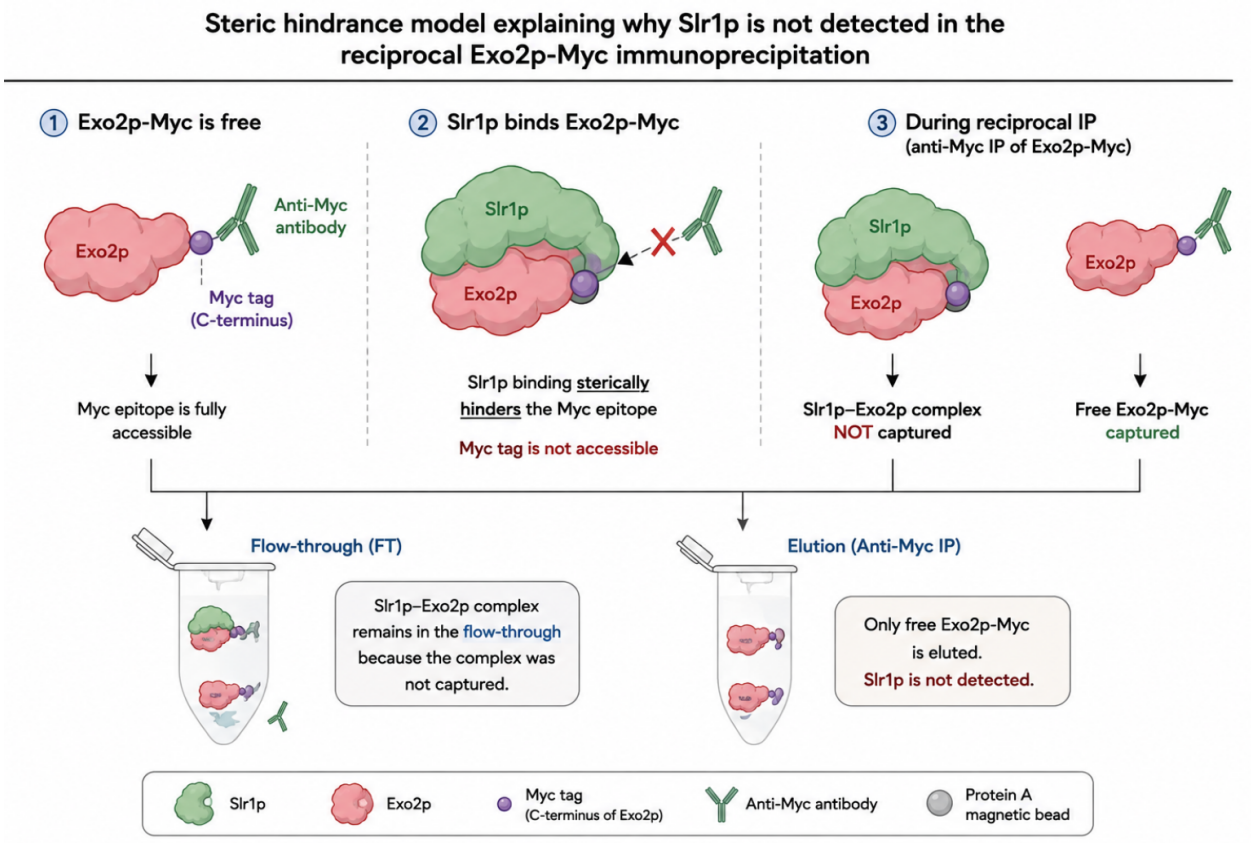


Figure 29*:* **Proposed steric hindrance model for the reciprocal Exo2p-Myc immunoprecipitation.** Slr1p binding to Exo2p may reduce accessibility of the Myc epitope, preventing efficient capture of the Slr1p–Exo2p complex by the anti-Myc antibody and resulting in preferential immunoprecipitation of unbound Exo2p-Myc.

Further experiments could help determine whether the failure to detect Slr1p in the reciprocal co-immunoprecipitation is related to accessibility of the Myc epitope. For example, using a different Exo2p tag position or an antibody against endogenous Exo2p could help test whether the C-terminal Myc tag interferes with Slr1p binding or prevents efficient antibody recognition.

4.5 Limitations and future directions

While this study provides new insight into the role of Slr1p in translation and its potential connection to mRNA decay, some of the observations presented here would benefit from further

validation. For some experiments, the number of biological replicates was limited, which reduced the statistical power to evaluate the observed trends. Additional biological replicates will therefore be important to confirm the reproducibility of these observations and further strengthen the conclusions drawn from them.

Although the findings from this study provide new insight into the role of Slr1p in translation, they also raise several questions about how Slr1p carries out these functions. The strong decrease in *GCN4* reporter activity in *slr1Δ* cells, together with the lack of a corresponding change in reporter mRNA levels, points toward an effect at the level of translation. However, the exact step affected by Slr1p remains unclear. Since the *GCN4* reporter contains multiple regulatory uORFs, modifying or removing individual uORFs would help determine whether the Slr1p-dependent phenotype is directly linked to uORF-mediated regulation. Testing additional Slr1p target transcripts would also be important for understanding why some transcripts, such as *GCN4*, show a strong dependence on Slr1p while others, such as *Hri2*, are much less affected. This could help identify the RNA features that make a transcript particularly dependent on Slr1p for efficient translation.

The frameshifting results open another direction for understanding the role of Slr1p during translation. The increase in −1 frameshifting in *slr1Δ* cells suggests that Slr1p may contribute to maintaining the correct reading frame as ribosomes move along an mRNA. However, additional biological replicates are needed to confirm the reproducibility of this phenotype, particularly the stronger effect observed with the lower-expression pRep82x reporters. Once this is established, it would be interesting to examine more directly how Slr1p influences elongating ribosomes. This

could help determine whether the frameshifting phenotype reflects a broader role for Slr1p in translation elongation and fidelity.

The interaction with Exo2p also provides an important direction for future work. Since this interaction was maintained in the presence of Benzonase, it is unlikely to simply result from Slr1p and Exo2p being associated with the same RNA molecule. Therefore, an importatnt next question is what this interaction means functionally. In particular, directly comparing WT, S410A, and S410D under the same conditions would help establish whether the difference in Exo2p association at S410 is reproducible. Moreover, further validation of the reciprocal interaction, using an alternative Exo2p tag position or an antibody against endogenous Exo2p, would also help strengthen the evidence for this association. It would also be informative to perform co-immunoprecipitation experiments with WT and the different Slr1p phosphomutants to compare their interaction with Sum1p, the *S. pombe* eIF3i translation initiation factor. Sum1 was previously shown to interact with Slr1p[32]. If S410 phosphorylation status contributes to shifting Slr1p between translation- and decay-associated functions, changes in Exo2p association may be accompanied by corresponding changes in its association with Sum1p. Thus, comparing these interactions across the same phosphorylation mutants could help determine whether S410 contributes to regulating the balance between Slr1p association with translation and mRNA decay factors.

Overall, the findings of this study expand our understanding of the role of Slr1p beyond a protein involved only in translation initiation. Slr1p appears to influence several stages in the life of an mRNA: it supports efficient translation of specific transcripts such as *GCN4*, may help ribosomes maintain the correct reading frame during elongation, and associates with Exo2p, providing a possible connection to mRNA decay. Rather than representing separate functions,

these findings may reflect a broader role for Slr1p in coordinating how particular mRNAs are translated and what happens to them afterward. The possible regulation of the Slr1p–Exo2p interaction by S410 phosphorylation adds another layer to this model and suggests that Slr1p may move between different functional states depending on its phosphorylation status. Together, these findings provide a model in which Slr1p helps connect the efficiency and accuracy of translation with the later fate of an mRNA. This extends our understanding of how this RNA-binding protein contributes to post-transcriptional regulation in *S. pombe*

References

[1] Buccitelli, C., & Selbach, M. (2020). mRNAs, proteins and the emerging principles of gene expression control. Nature Reviews Genetics, 21, 630–644. https://doi.org/10.1038/s41576-020-0258-4

[2] Crick, F. (1970). Central dogma of molecular biology. Nature, 227, 561–563. https://doi.org/10.1038/227561a0

[3] Hocine, S., Singer, R. H., & Grünwald, D. (2010). RNA processing and export. Cold Spring Harbor Perspectives in Biology, 2(12), a000752. https://doi.org/10.1101/cshperspect.a000752

[4] Moore, M. J., & Proudfoot, N. J. (2009). Pre-mRNA processing reaches back to transcription and ahead to translation. Cell, 136(4), 688–700. https://doi.org/10.1016/j.cell.2009.02.001

[5] Vogel, C., & Marcotte, E. M. (2012). Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. Nature Reviews Genetics, 13, 227–232. https://doi.org/10.1038/nrg3185

[6] Sonenberg, N., & Hinnebusch, A. G. (2009). Regulation of translation initiation in eukaryotes: Mechanisms and biological targets. Cell, 136(4), 731–745. https://doi.org/10.1016/j.cell.2009.01.042

[7] Schuller, A. P., & Green, R. (2018). Roadblocks and resolutions in eukaryotic translation. Nature Reviews Molecular Cell Biology, 19, 526–541. https://doi.org/10.1038/s41580-018-0011-4

[8] Dever, T. E., Dinman, J. D., & Green, R. (2018). Translation elongation and recoding in eukaryotes. Cold Spring Harbor Perspectives in Biology, 10(8), a032649. https://doi.org/10.1101/cshperspect.a032649

[9] Melnikov, S., Ben-Shem, A., Garreau de Loubresse, N., Jenner, L., Yusupova, G., & Yusupov, M. (2012). One core, two shells: Bacterial and eukaryotic ribosomes. Nature Structural & Molecular Biology, 19(6), 560–567. https://doi.org/10.1038/nsmb.2313

[10] Hinnebusch, A. G. (2014). The scanning mechanism of eukaryotic translation initiation. Annual Review of Biochemistry, 83, 779–812. https://doi.org/10.1146/annurev-biochem-060713-035802

[11] Jackson, R. J., Hellen, C. U. T., & Pestova, T. V. (2010). The mechanism of eukaryotic translation initiation and principles of its regulation. Nature Reviews Molecular Cell Biology, 11, 113–127. https://doi.org/10.1038/nrm2838

[12] Merrick, W. C., & Pavitt, G. D. (2018). Protein synthesis initiation in eukaryotic cells. Cold Spring Harbor Perspectives in Biology, 10(12), a033092. https://doi.org/10.1101/cshperspect.a033092

[13] Wethmar, K. (2014). The regulatory potential of upstream open reading frames in eukaryotic gene expression. Wiley Interdisciplinary Reviews: RNA, 5(6), 765–778. https://doi.org/10.1002/wrna.1245

[14] Harding, H. P., Novoa, I., Zhang, Y., Zeng, H., Wek, R., Schapira, M., & Ron, D. (2000). Regulated translation initiation controls stress-induced gene expression in mammalian cells. Molecular Cell, 6(5), 1099–1108. https://doi.org/10.1016/S1097-2765(00)00108-8

[15] Vattem, K. M., & Wek, R. C. (2004). Reinitiation involving upstream ORFs regulates ATF4 mRNA translation in mammalian cells. Proceedings of the National Academy of Sciences of the United States of America, 101(31), 11269–11274. https://doi.org/10.1073/pnas.0400541101

[16] Gerstberger, S., Hafner, M., & Tuschl, T. (2014). A census of human RNA-binding proteins. Nature Reviews Genetics, 15(12), 829–845. https://doi.org/10.1038/nrg3813

[17] Hentze, M. W., Castello, A., Schwarzl, T., & Preiss, T. (2018). A brave new world of RNA-binding proteins. Nature Reviews Molecular Cell Biology, 19(5), 327–341. https://doi.org/10.1038/nrm.2017.130

[18] Keene, J. D. (2007). RNA regulons: Coordination of post-transcriptional events. Nature Reviews Genetics, 8(7), 533–543. https://doi.org/10.1038/nrg2111

[19] Bayfield, M. A., Yang, R., & Maraia, R. J. (2010). Conserved and divergent features of the structure and function of La and La-related proteins (LARPs). Biochimica et Biophysica Acta (BBA) – Gene Regulatory Mechanisms, 1799(5–6), 365–378. https://doi.org/10.1016/j.bbagrm.2010.01.011

[20] Bousquet-Antonelli, C., & Deragon, J.-M. (2009). A comprehensive analysis of the La-motif protein superfamily. RNA, 15(5), 750–764. https://doi.org/10.1261/rna.1478709

[21] Maraia, R. J., Mattijssen, S., Cruz-Gallardo, I., & Conte, M. R. (2017). The La and related RNA-binding proteins (LARPs): Structures, functions, and evolving perspectives. Wiley Interdisciplinary Reviews: RNA, 8(6), e1430. https://doi.org/10.1002/wrna.1430

[22] Bohnsack, M. T., Regener, K., Schwappach, B., Saffrich, R., Paraskeva, E., Hartmann, E., & Görlich, D. (2002). Sum1, a component of the fission yeast eIF3 translation initiation complex, is rapidly relocalized during environmental stress and interacts with components of the 26S proteasome. Molecular Biology of the Cell, 13(5), 1626–1640. https://doi.org/10.1091/mbc.01-06-0301

[23] Benard, L. (2004). Inhibition of 5′ to 3′ mRNA degradation under stress conditions in Saccharomyces cerevisiae: From GCN4 to MET16. RNA, 10(3), 458–468. https://doi.org/10.1261/rna.5183804

[24] Cai, L., Fritz, D., Stefanovic, L., & Stefanovic, B. (2010). Binding of LARP6 to the conserved 5′ stem-loop regulates translation of mRNAs encoding type I collagen. Journal of Molecular Biology, 395(2), 309–326. https://doi.org/10.1016/j.jmb.2009.11.020

[25] Yang, R., Gaidamakov, S. A., Xie, J., Lee, J., Martino, L., Kozlov, G., Crawford, A. K., Russo, A. N., Conte, M. R., Gehring, K., & Maraia, R. J. (2011). La-related protein 4 binds poly(A), interacts with the poly(A)-binding protein MLLE domain via a variant PAM2w motif, and can promote mRNA stability. Molecular and Cellular Biology, 31(3), 542–556. https://doi.org/10.1128/MCB.01162-10

[26] Mansouri-Noori, F., & Bayfield, M. A. (2025). Conserved functions of LARP1 proteins in eukaryotes. WIREs RNA, 16(6), e70033. https://doi.org/10.1002/wrna.70033

[27] Meyuhas, O., & Kahan, T. (2015). The race to decipher the top secrets of TOP mRNAs. Biochimica et Biophysica Acta (BBA) – Gene Regulatory Mechanisms, 1849(7), 801–811. https://doi.org/10.1016/j.bbagrm.2014.08.015

[28] Lahr, R. M., Fonseca, B. D., Ciotti, G. E., Al-Ashtal, H. A., Jia, J.-J., Niklaus, M. R., Blagden, S. P., Alain, T., & Berman, A. J. (2017). La-related protein 1 (LARP1) binds the mRNA cap, blocking eIF4F assembly on TOP mRNAs. eLife, 6, e24146. https://doi.org/10.7554/eLife.24146

[29] Philippe, L., Vasseur, J.-J., Debart, F., & Thoreen, C. C. (2018). La-related protein 1 (LARP1) repression of TOP mRNA translation is mediated through its cap-binding domain and controlled by an adjacent regulatory region. Nucleic Acids Research, 46(3), 1457–1469. https://doi.org/10.1093/nar/gkx1237

[30] Aoki, K., Adachi, S., Homoto, M., Kusano, H., Koike, K., & Natsume, T. (2013). LARP1 specifically recognizes the 3′ terminus of poly(A) mRNA. FEBS Letters, 587(14), 2173–2178. https://doi.org/10.1016/j.febslet.2013.05.035

[31] Gentilella, A., Morón-Duran, F. D., Fuentes, P., Zweig-Rocha, G., Riaño-Canalias, F., Pelletier, J., et al. (2017). Autogenous control of 5′TOP mRNA stability by 40S ribosomes. Molecular Cell, 67(1), 55–70.e4. https://doi.org/10.1016/j.molcel.2017.06.005

[32] Mansouri-Noori, F., Pircher, A., Bilodeau, D., Siniavskaia, L., Grigull, J., Rissland, O. S., & Bayfield, M. A. (2023). The LARP1 homolog Slr1p controls the stability and expression of proto-5′TOP mRNAs in fission yeast. Cell Reports, 42(10), 113226. https://doi.org/10.1016/j.celrep.2023.113226

[33] Mansouri-Noori, F. (2025). Biochemical and structural characterization of the S. pombe La-related protein 1 (Slr1p) [Doctoral dissertation, York University].

[34] Sobel, S. G., & Wolin, S. L. (1999). Two yeast La motif-containing proteins are RNA-binding proteins that associate with polyribosomes. Molecular Biology of the Cell, 10(11), 3849–3862. https://doi.org/10.1091/mbc.10.11.3849

[35] Jennings, M. D., Srivastava, P., Kershaw, C. J., et al. (2023). Interaction of the La-related protein Slf1 with colliding ribosomes maintains translation of oxidative-stress responsive mRNAs. Nucleic Acids Research, 51(11), 5755–5773. https://doi.org/10.1093/nar/gkad272

[36] Kurosaki, T., & Maquat, L. E. (2016). Nonsense-mediated mRNA decay in humans at a glance. Journal of Cell Science, 129(3), 461–467. https://doi.org/10.1242/jcs.181008

[37] Fonseca, B. D., Zakaria, C., Jia, J.-J., Graber, T. E., Svitkin, Y., Tahmasebi, S., Healy, D., Hoang, H.-D., Jensen, J. M., Diao, I. T., Lussier, A., Dajadian, C., Padmanabhan, N., Wang, W., Matta-Camacho, E., Hearnden, J., Smith, E. M., Tsukumo, Y., Yanagiya, A., Morita, M., Petroulakis, E., González, J. L., Hernández, G., Alain, T., & Damgaard, C. K. (2015). La-related protein 1 (LARP1) represses terminal oligopyrimidine (TOP) mRNA translation downstream of mTOR complex 1 (mTORC1). Journal of Biological Chemistry, 290(26), 15996–16020. https://doi.org/10.1074/jbc.M114.621730

[38] Hong, S., Freeberg, M. A., Han, T., Kamath, A., Yao, Y., Fukuda, T., Suzuki, T., Kim, J. K., & Inoki, K. (2017). LARP1 functions as a molecular switch for mTORC1-mediated translation of an essential class of mRNAs. eLife, 6, e25237. https://doi.org/10.7554/eLife.25237

[39] Jia, J.-J., Lahr, R. M., Solgaard, M. T., et al. (2021). mTORC1 promotes TOP mRNA translation through site-specific phosphorylation of LARP1. Nucleic Acids Research, 49(6), 3461–3489. https://doi.org/10.1093/nar/gkaa1239

[40] Harris, M.A., Rutherford, K.M., Hayles, J., Lock, A., Bähler, J., Oliver, S.G., Mata, J., and Wood, V. (2022). Fission stories: using PomBase to understand Schizosaccharomyces pombe biology. Genetics 220. 10.1093/GENETICS/IYAB222.

[41] Joska, T. M., Mashruwala, A., Boyd, J. M., & Belden, W. J. (2015). A universal cloning method based on yeast homologous recombination that is simple, efficient, and versatile. Journal of Microbiological Methods, 117, 86–90. https://doi.org/10.1016/j.mimet.2015.07.015

[42] Bröker, M., & Bäuml, O. (1989). New expression vectors for the fission yeast Schizosaccharomyces pombe. FEBS Letters, 248(1–2), 105–110. https://doi.org/10.1016/0014-5793(89)80441-7

[43] Forsburg, S. L. (1993). Comparison of Schizosaccharomyces pombe expression systems. Nucleic Acids Research, 21(12), 2955–2956. https://doi.org/10.1093/nar/21.12.2955

[44] Thermo Fisher Scientific. (2020). GeneJET gel extraction kit user guide (Publication No. MAN0012661).

[45] Green, R., & Rogers, E. J. (2013). Chemical transformation of E. coli. Methods in Enzymology, 529, 329–336. https://doi.org/10.1016/B978-0-12-418687-3.00028-8

[46] Thermo Fisher Scientific. (2021). GeneJET Plasmid Miniprep Kit user manual (Publication No. K0502).

[47] Dominguez, D., Freese, P., Alexis, M. S., Su, A., Hochman, M., Palden, T., Bazile, C., Lambert, N. J., Van Nostrand, E. L., Pratt, G. A., Yeo, G. W., Graveley, B. R., & Burge, C. B. (2018). Sequence, structure, and context preferences of human RNA binding proteins. Molecular Cell, 70(5), 854–867.e9. https://doi.org/10.1016/j.molcel.2018.05.001

[48] Plasmidsaurus. (n.d.). Whole plasmid sequencing service. https://plasmidsaurus.com/whole-plasmid-sequencing

[49] Gietz, R. D., & Schiestl, R. H. (2007). High-efficiency yeast transformation using the LiAc/SS carrier DNA/PEG method. Nature Protocols, 2(1), 31–34. https://doi.org/10.1038/nprot.2007.13

[50] Promega Corporation. (2021). Renilla Luciferase Assay System (E2820) technical manual (Publication No. TM052). Promega Corporation.

[51] Chikashige, Y., Kato, H., Thornton, M., Pepper, W., Hilgers, M., Cecil, A., Asano, I., Yamada, H., Mori, C., Brunkow, C., Moravek, C., Urano, T., Singh, C. R., & Asano, K. (2020). Gcn2 eIF2α kinase mediates combinatorial translational regulation through nucleotide motifs and uORFs in target mRNAs. Nucleic Acids Research, 48(16), 8977–8992

[52] Hinnebusch, A. G. (2005). Translational regulation of GCN4 and the general amino acid control of yeast. Annual Review of Microbiology, 59, 407–450. https://doi.org/10.1146/annurev.micro.59.031805.133833

[53] Zhan, K., Narasimhan, J., & Wek, R. C. (2004). Differential activation of eIF2 kinases in response to cellular stresses in Schizosaccharomyces pombe. Genetics, 168(4), 1867–1875. https://doi.org/10.1534/genetics.104.031443

[54] Kershaw, C. J., Costello, J. L., Castelli, L. M., Talavera, D., Rowe, W., Sims, P. F. G., Ashe, M. P., Hubbard, S. J., Pavitt, G. D., & Grant, C. M. (2015). The yeast La related protein Slf1p is a key activator of translation during the oxidative stress response. PLoS Genetics, 11(1), e1004903

[55] Harger, J. W., & Dinman, J. D. (2003). An in vivo dual-luciferase assay system for studying translational recoding in the yeast Saccharomyces cerevisiae. RNA, 9(8), 1019–1024. https://doi.org/10.1261/rna.5930803

[56] Burrows, C., Abd Latip, N., Lam, S.-J., Carpenter, L., Sawicka, K., Tzolovsky, G., Gabra, H., Bushell, M., Glover, D. M., Willis, A. E., & Blagden, S. P. (2010). The RNA binding protein Larp1 regulates cell division, apoptosis and cell migration. Nucleic Acids Research, 38(16), 5542–5553. https://doi.org/10.1093/nar/gkq294

[57] Okazaki, K., Okazaki, N., Kume, K., Jinno, S., Tanaka, K., & Okayama, H. (1990). High-frequency transformation method and library transducing vectors for cloning mammalian cDNAs by trans-complementation of Schizosaccharomyces pombe. Nucleic Acids Research, 18(22), 6485–6489. https://doi.org/10.1093/nar/18.22.6485

[58] Tcherkezian, J., Cargnello, M., Romeo, Y., Huttlin, E. L., Lavoie, G., Gygi, S. P., & Roux, P. P. (2014). Proteomic analysis of cap-dependent translation identifies LARP1 as a key regulator of 5′TOP mRNA translation. Genes & Development, 28(4), 357–371. https://doi.org/10.1101/gad.231407.113

[59] Yang, L., Li, Y., Zhang, Q., & Zhang, L. (2022). Preparation of competent Escherichia coli and yeast cells for transformation. Bio-protocol, 12(22), e4526. https://doi.org/10.21769/BioProtoc.4526

[60] Chukka, P. A. R., Wetmore, S. D., & Thakor, N. (2021). Established and emerging regulatory roles of eukaryotic translation initiation factor 5B (eIF5B). Frontiers in Genetics, 12, 737433. https://doi.org/10.3389/fgene.2021.737433

Appendix

Table A1: Oligonucleotide Primers Used for Cloning, Sequencing, and qPCR

#	Primers	Sequence 5' to 3'
2443	ADH1 Renilla SacI For	GCG CGA GCT CCG GAT TCT TTT CTT TTT TTT TCT TTT CTC TCT CCC CCG TTG
2444	ADH1 Renilla SacI Rev	GCG CGA GCT CGG GTA CAA CTC ACA GGC GAG G
2440	ADH Promoter Seq For	GTT TCC TCG TCA TTG TTC TCG TTC CC
2441	CYC1 Term Seq Rev	GCG TGA ATG TAA GCG TGA C
2469	Renilla into Firefly For	GGT GCC AAG AAG TTT CCT AAT ACT G
2470	Firefly before Start Rev	GCCG GGA GGT AGA ATG AAA TGT G
2471	Firefly after Stop For	GGA GGA GTT GTG TTT GTG GAC G
2442	Firefly Rep AgeI Rev	GCG CAC CGG TTT ACA ATT TGG ACT TTC CGC CCT TCT TGG CC
1239	pRep 40N XhoI For	GCG CCT CGA GCG GCC GGA TCA AGA GCT ACC CGG CCG GAT CAA GAG CTA CCA TGA CTT CGA AAG TTT ATG ATC CAG AAC AAA GG
1403	20N AC- rich Meme2 Ren Rep For	GCG CCT CGA GCG GCC GGA TCA AGA GCT ACC TTT TCC CAC CAC CAA ACA CAT GAC TTC GAA AGT TTA TGA TCC AGA ACA AAG G
365	pREP For	TGA ATA AGT TGA ATT AAT TAT TTC
366	pREP Rev	AGC TTG AAT GGG CTT CCA TAG TTT G
2179	Fluc qPCR For 3	
2180	Fluc qPRCR Rev 2	CAG TAT CCG GAA TGA TTT GAT TGC
2183	Act1 qPCR For 2	CGT TGG TGA TGA AGC TCA AAG C
2184	Act1qPCR Act1 Rev 2	CTC GTT GTA GAA AGT GTG ATG C
2500	Gcn4 Geneblock XhoI For	GCG CCT CGA GAC AGC TCA CTC ATC TAC TTC GCA ATC
2501	Gcn4 Geneblock BamHI Rev	GCG CGG ATC CTC ACA ATT TGG ACT TTC CGC CC
2504	Hri2 Geneblock XhoI For	GCG CCT CGA GCG ACC ATA GTC AGT AGA AAC CAT G
2505	Hri2 Geneblock BamHI Rev	GCG CGG ATC CTC ACA ATT TGG ACT TTC CGC CC
2537	pREP-J XhoISalI Bgl2Not1 BamSpeX	GCG CCT CGA GGT CGA CAG ATC TGC GGC CGC GGA TCC ACT AGT CCC GGG GCG C