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Effect of the intersubunit bridges on the eukaryotic ribosome functionality

Η επίδραση των γεφυρών των υπομονάδων στη λειτουργικότητα των ευκαρυωτικών ριβοσωμάτων

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Abstract

Ribosome is responsible for the translation of the mRNA nucleotide sequence to the amino acid sequence of the protein. Structurally ribosome is the largest cellular molecular complex composed of the rRNA molecules and the ribosomal proteins. Eukaryotic 80S ribosome consists of two unequal ribosomal subunits, the small ribosomal subunit (40S) and the large ribosomal subunit (60S). These subunits are being held together by 17 intersubunit bridges. These are rRNA-rRNA, rRNA-protein and protein-protein type interactions between ribosomal subunits. Twelve bridges are conserved among all life domains. The rest of the five bridges are eukaryote-specific interactions made by eukaryote-specific rRNA expansion segments and eukaryote-specific protein domains. These bridges are mostly localized in the periphery of the ribosome and are thought to stabilize the structure of the ribosome and transmit signals between the small and large subunit during translation. The structure of the intersubunit bridges and their dynamics are therefore of particular importance for an understanding of the mechanism of protein synthesis. In the current thesis the function and cooperation of the eukaryote specific bridges eB13, eB14 and of the conserved bridge B6 was investigated under environmental stresses (temperature stress and amino acid deprivation). A *GCN4-lacZ* reporter analysis was also conducted. The results indicate that none of the intersubunit bridges that were studied are essential for coping with stress conditions. However, protein eL24, which is the main component of bridges eB13 and B6, was found to be necessary for the survival of the cells under stress.

Περίληψη

Το ριβόσωμα είναι υπεύθυνο για τη μετάφραση της νουκλεοτιδικής αλληλουχίας του mRNA στην αμινοξική αλληλουχία των πρωτεϊνών. Δομικά το ριβόσωμα είναι το μεγαλύτερο κυτταρικό, μοριακό σύμπλεγμα που αποτελείται από μόρια rRNA και ριβοσωμικές πρωτεΐνες. Το ευκαρυωτικό 80S ριβόσωμα αποτελείται από δύο άνισες ριβοσωμικές υπομονάδες, τη μικρή ριβοσωμική υπομονάδα (40S) και την μεγάλη ριβοσωμική υπομονάδα (60S). Αυτές οι υπομονάδες συγκρατούνται από 17 γέφυρες μεταξύ των υπομονάδων. Αυτές οι γέφυρες αποτελούνται από αλληλεπιδράσεις rRNA-rRNA, rRNA-πρωτεΐνη και πρωτεΐνη-πρωτεΐνη μεταξύ των υπομονάδων. Δώδεκα από αυτές είναι συντηρημένες σε όλους τους ζωντανούς οργανισμούς. Οι υπόλοιπες πέντε γέφυρες είναι αποκλειστικά ευκαρυωτικές αλληλεπιδράσεις που αποτελούνται από ευκαρυωτικά τμήματα επέκτασης του rRNA και ευκαρυωτικά τμήματα πρωτεϊνών. Οι γέφυρες εντοπίζονται κυρίως στην περιφέρεια του ριβοσώματος και θεωρούνται πως σταθεροποιούν τη δομή του ριβοσώματος καθώς και ότι μεταδίδουν σήματα μεταξύ της μεγάλης και της μικρής υπομονάδας κατά τη διάρκεια της μετάφρασης. Συνεπώς, η δομή των γεφυρών των υπομονάδων και η δυναμική τους έχουν ιδιαίτερη σημασία για την κατανόηση του μηχανισμού της πρωτεϊνικής σύνθεσης. Στην παρούσα διπλωματική εργασία εξετάστηκε η λειτουργία και η συνεργασία των ευκαρυωτικών γεφυρών eB13, eB14, καθώς και της συντηρημένης B6 αφού υποβλήθηκαν σε περιβαλλοντικά στρες (θερμικό στρες και στέρηση αμινοξέων). Επίσης πραγματοποιήθηκε ανάλυση του δείκτη *GCN4-lacZ.* Τα αποτελέσματα ενδεικνύουν ότι καμία από τις γέφυρες των υπομονάδων δεν είναι απαραίτητη για την αντιμετώπιση των συνθηκών στρες. Παρόλα αυτά η πρωτεΐνη eL24, βασικό συστατικό των γεφυρών eB13 και B6, αποδείχθηκε απαραίτητη για την επιβίωση των κυττάρων κατά το στρες.

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1. Introduction

1.1. Structure of the eukaryotic 80S ribosome

1.1.1. The conserved core

The ribosome is the largest ribonucleoprotein assembly in the cell and is responsible for the translation of the genetic code into proteins (Ramakrishnan, 2002). This basic cellular function is essential for life, which explains why ribosomes can be found across all kingdoms of life.

Even though the prokaryotic and eukaryotic ribosomes share a universally conserved core, eukaryotic ribosomes are at least 40% larger than their bacterial counterparts as a result of additional ribosomal RNA (rRNA) elements called expansion segments (ESs) and extra protein moieties (Spahn *et al.*, 2001). The core is comprised from 34 conserved proteins (15 in the small subunit and 19 in the large subunit) and ~4,400 RNA bases. It also harbors the major functional centers of the ribosomes: the decoding site, the peptidyl transferase center and tRNA-binding sites (Spahn *et al.*, 2001; Smith, T.F *et al.,* 2008).

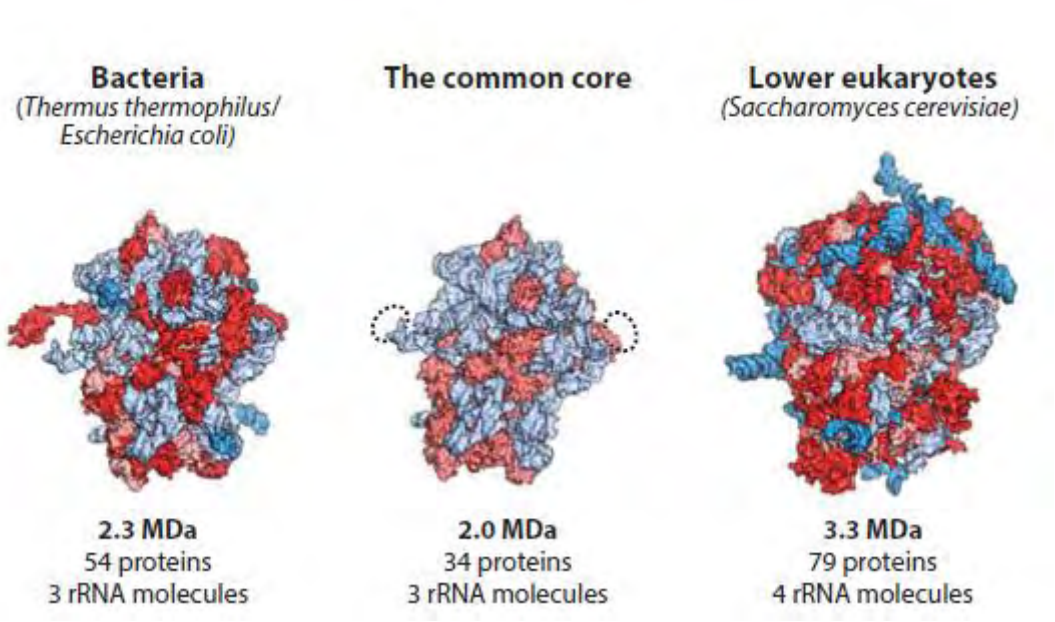

Figure 1. *Composition of bacterial and eukaryotic ribosomes and the common core. Bacterial and eukaryotic ribosomes share a conserved core built of RNA (light blue) and proteins (light red). In addition to the common core ribosomes from each domain of life contain their own set of proteins: extensions in conserved proteins (both in red) and extensions in ribosomal RNA (blue) (adapted from Yusupova & Yusupov, 2014).*

The eukaryotic 80S ribosome contains 46 eukaryote – specific proteins (18 in the small subunit, 28 in the large subunit), extensions and insertions in most of the proteins of the core. The rRNA also contains several extensions in the conserved rRNA chains, with a total length of 900 or more bases. Most of these rRNA and protein moieties envelop the core from the solvent side and therefore are accessible for potential interactions with molecular partners, such as translation factors and chaperone proteins (Melnikov *et al.*, 2012).

1.1.2. The ribosomal subunits

All ribosomes consist of two subunits. The ribosomes found in bacteria and archaea consist of a large 50S subunit, which itself is composed of 5S and 23S rRNAs and 33 ribosomal proteins (r-proteins), and a small 30S subunit, with one 16S ribosomal RNA (rRNA) and 21 r-proteins. Together they constitute the 70S ribosome. In eukaryotes, the 80S ribosome is composed of a large 60S subunit, consisting of three rRNAs (25S, 5.8S, 5S) and 46 different ribosomal proteins (r proteins), and a small 40S subunit, consisting of 18S rRNA and 33 r proteins (Wilson and Doudna Cate, 2012).

The 40S ribosomal small subunit includes structural landmarks known as the 'head', 'body', 'platform', 'beak' and 'shoulder' (Figure 2). The messenger RNA (mRNA) and the three tRNA – binding sites (A, P and E) are all located on the subunit interface. The mRNA enters through a tunnel located between the head and the shoulder and wraps around the neck of the 40S subunit. The mRNA exit site (5' end of the mRNA) is located between the head and the platform. The decoding center of the small subunit, where the codon and anticodon are paired and convey fidelity to mRNA decoding is located on the interface surface and is comprised of three domains from the head, the shoulder and the penultimate stem.

The 60S ribosomal large subunit has an overall crown-like shape, which includes the 'central protuberance', 'L1-stalk' and the 'P-stalk' (Figure 2). On the subunit, 27 eukaryote-specific proteins, multiple insertions and extensions of conserved proteins and the numerous rRNA expansion segments are concentrated on the periphery of the subunit, forming a nearly continuous ring-shaped assembly which envelopes the core. Located on the interface side of the large ribosomal subunit are the three (A, P and E) tRNA-binding sites and the peptidyl transferase center where the peptide bond formation is catalyzed. This peptidyl transferase center is adjacent to the entrance of a tunnel along which nascent proteins progress before they emerge from the ribosome on the solvent side. On the central regions of both the solvent and interface sides of the subunit, the complete absence of bacteria- and eukaryote-specific moieties is consistent with the universally conserved functions of these areas. This conservation is seen at the peptidyl transferase center on the intersubunit surface as well as

around the peptide tunnel on the solvent side, which is used for ribosome association with membranes during protein synthesis (Melnikov *et al.*, 2012; Yusupova & Yusupov, 2017).

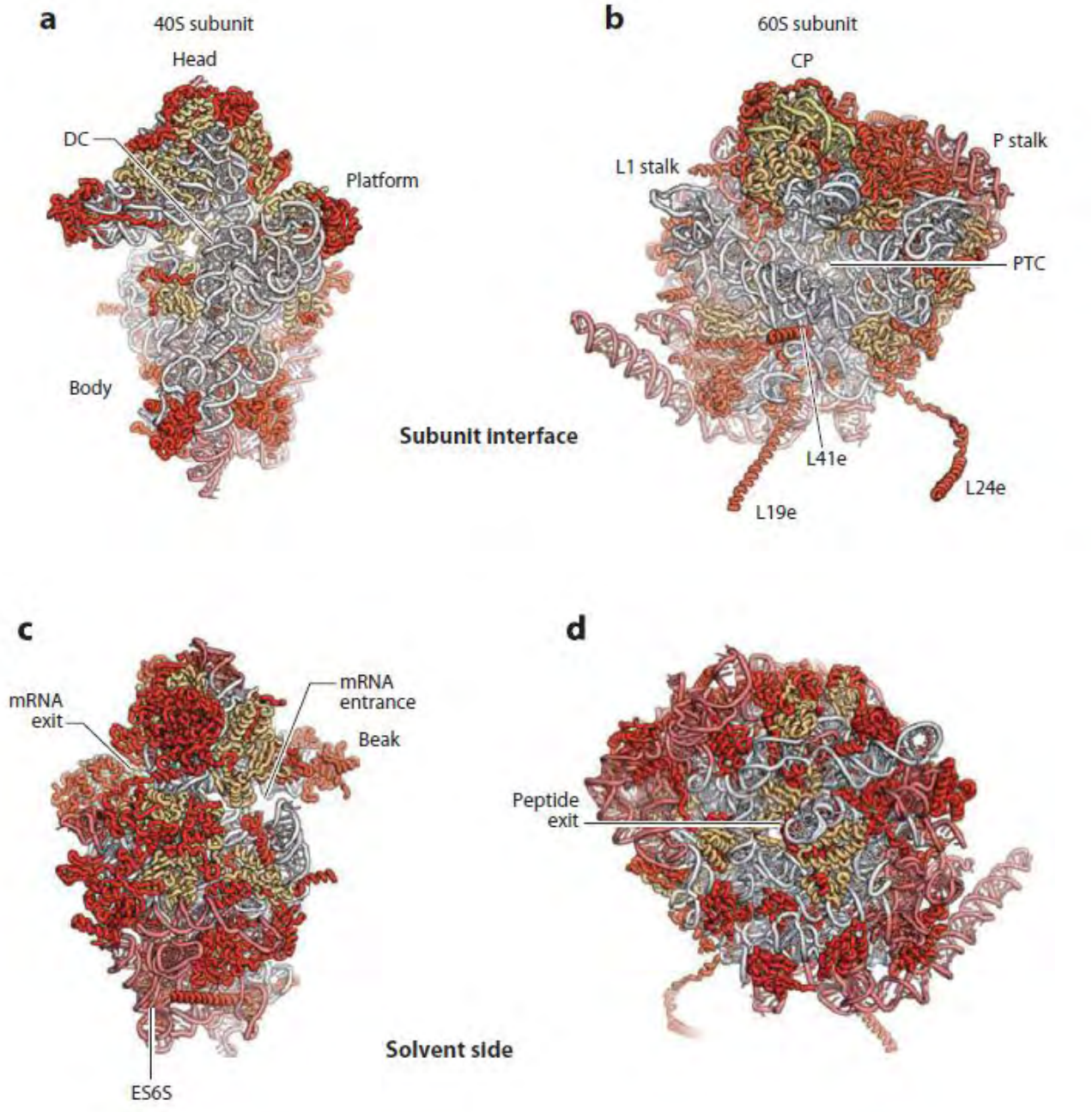

Figure 2. *View from (a,b) the interface and (c,d) the solvent side of ribosomal subunits of the yeast ribosome, showing the decoding center (DC), head, body, platform and beak in the small subunit and the central protuberance (CP), peptidyl transferase center (PTC), L1 stalk, and P stalk in the large subunit. The common core consists of ribosomal RNA (white) and proteins (light orange); eukaryote-specific moieties are shown in red. Abbreviation: mRNA, messenger RNA (Yusupova & Yusupov, 2014).*

1.1.3. The intersubunit bridges

Interaction between the two ribosomal subunits is maintained by several contact points of the interface, named bridges. Intersubunit bridges play an important structural role, in addition to maintaining communication pathways between the subunits during protein synthesis and helping to coordinate their activities. During translation, the ribosome undergoes global conformational rearrangements that are required for mRNA and tRNA translocation, termination, and other processes. These changes involve intersubunit rotation and swiveling of the small subunit's head domain. The interactions between the ribosomal subunits change with each rearrangement and are dynamic in composition (Ben Shem *et al.*, 2011; Yusupova & Yusupov, 2017).

All the bridges that have been described in the crystal structure of the bacterial 70S ribosome have a corresponding bridge in the yeast 80S ribosome. This significant evolutionary conservation demonstrates the importance of these bridges (Yusupov *et al.*, 2001). Nevertheless, the interaction surface between the two subunits is nearly doubled in eukaryotes due to the formation of additional bridges (Figure 3). There are seventeen intersubunit bridges in total. Twelve of them are conserved in all three domains of life and five of them are eukaryote – specific. In practically all the added bridges, almost all the participating components on both subunits are eukaryote-specific. Intersubunit bridges form three kinds of contacts: rRNA – rRNA, rRNA – protein and protein - protein. Unlike bacteria, whose bridges are composed by rRNA – rRNA contacts, proteins have a dominant role in the formation of eukaryote-specific bridges. (Ben Shem *et al.*, 2011)

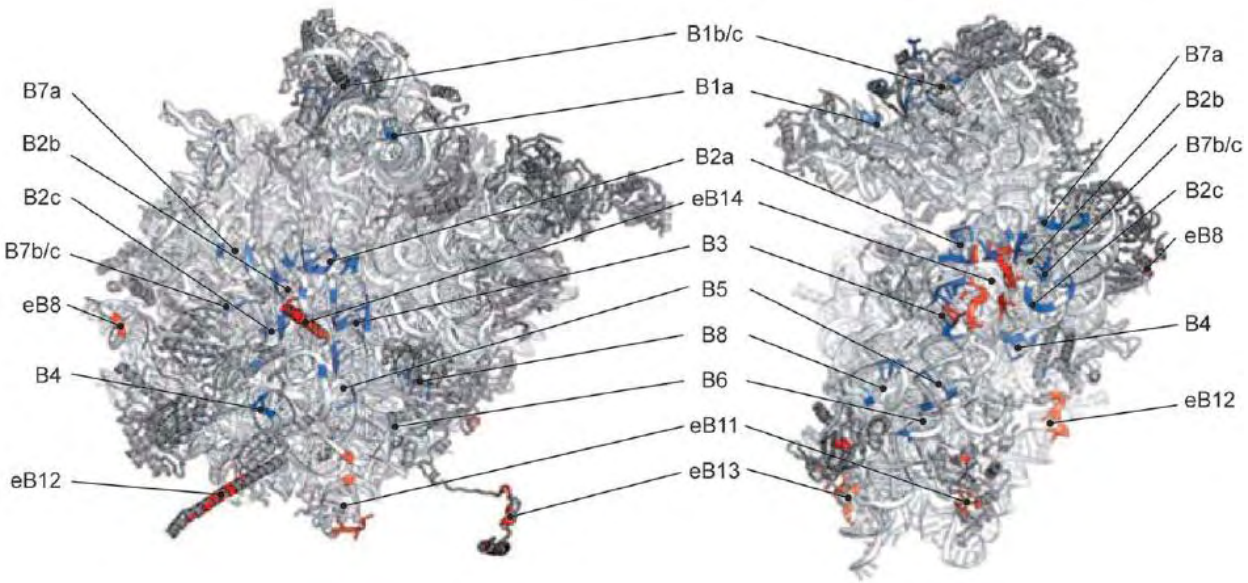

Figure 3. *Interface view showing residues forming eukaryote-specific bridges (red) and conserved ones (blue) (adapted from Ben Shem et al., 2011).*

The eukaryote-specific bridges are positioned at the periphery of the subunit interface and on the solvent sides of both subunits. The only exception is bridge eB14, which is located in the center. The existence of these additional bridges in the outer edge of the ribosome increases considerably the interaction surface between subunits and is probably the reason for the preferential rotated state of eukaryotic ribosomes. Therefore, adaptation of the bridges to the different states of the ribosome requires considerable structural plasticity of their components. (Ben Shem *et al.*, 2011; Yusupova & Yusupov, 2017).
The eB8 intersubunit bridge is constructed by the eukaryote specific protein eS1, which defines the far end of the 40S platform, and 25S rRNA ES31L (Figure 4). Bridge eB8 is located proximal to the mRNA exit tunnel, part of which is formed by interactions between protein eS1 with h26, S11, and eS26. (Ben Shem *et al.*, 2011)

The eukaryote-specific bridge eB11 is created by interactions between the small subunit protein eS8, the 18S rRNA ES3S and the 25S rRNA ES41L (Figure 4). The protein eS8 is sandwiched by ES3S and ES41L. The location of the bridge eB11 is within a large continuum of eukaryote-specific elements, ~100 Å in length, which starts on the platform of the small subunit, right under the mRNA exit tunnel. (Ben Shem *et al.*, 2011)

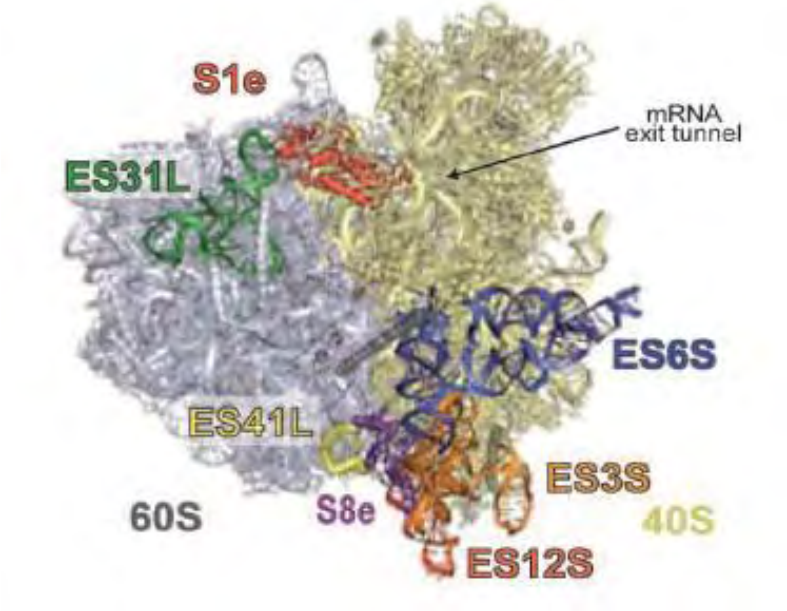

Figure 4. *The location of bridge eB8 in proximity to the mRNA exit tunnel (arrow) and the location of bridge eB11 within a continuum of eukaryote specific elements at the bottom of 80S. (adapted from Ben Shem et al., 2011).*

Bridge eB12 is mainly formed by multiple interactions between its core component, the eukaryote – specific protein eL19, the 18S rRNA ES6S, eS7 and uS17 (Figure 5). The bridge is accessible from the solvent side and is located underneath the mRNA exit tunnel. Even though the bridge is expendable for the cell it is involved in the last stages of translation, specifically, providing structural support for ribosomal subunit joining and shedding/releasing factors. Moreover, the N-terminal domain of eL19 is involved in ribosome biogenesis. (Kisly *et al.*, 2016; Ben Shem *et al.*, 2011)

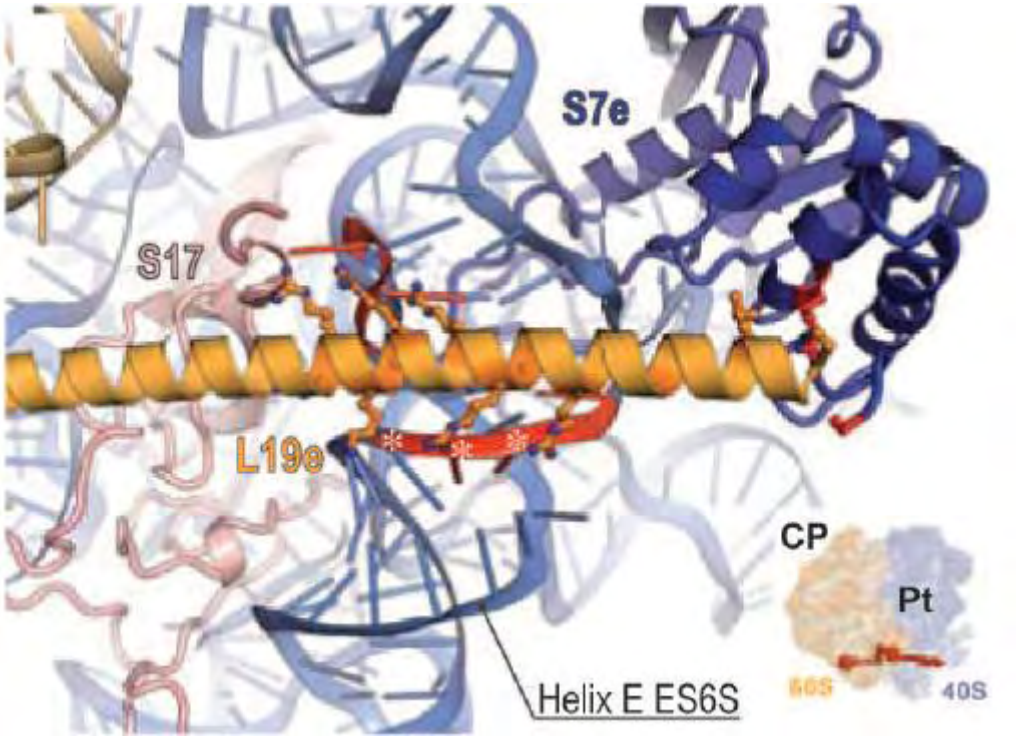

Figure 5. *The structure of the eB12 bridge. Residues of protein eL19 (orange) that contact the helix ES6S (blue) are depicted as orange sticks. The contacts with proteins S17 and eS7 are also shown. Asterisks (along the red ribbon) highlight ES6S residues that are close to the binding site for eIF4G, a protein that plays a central role in assembling the pre-initiation complex. In the right corner, the location of the bridge on the 80s ribosome is shown. (adapted from Ben Shem et al., 2011)*

The eukaryote – specific bridges eB13 and eB14 and the conserved bridge B6 will be discussed in more detail, because their role in ribosome functionality was studied in this work.

1.1.4. Bridge eB13

The core component of bridge eB13 is protein eL24, which consists of an N-terminal domain that is located in the 60S subunit, followed by a long, flexible linker that projects deep into the side of the 40S subunit body and a C-terminal domain that reaches the back of the 40S subunit (Figure 6a). Bridge eB13 is formed by the contacts between the C-terminal α-helix of protein eL24 with eS6, assisted by uL3 in both conformational states. During the post-translocational state an additional interaction between eL24 and 18S rRNA helix 10 takes place (Figure 6b). Similar to eB12, bridge eB13 is accessible from the solvent, where its long α-helix extends from the A-site side of the 60S subunit (Kisly *et al.*, 2016; Ben Shem *et al.*, 2011)

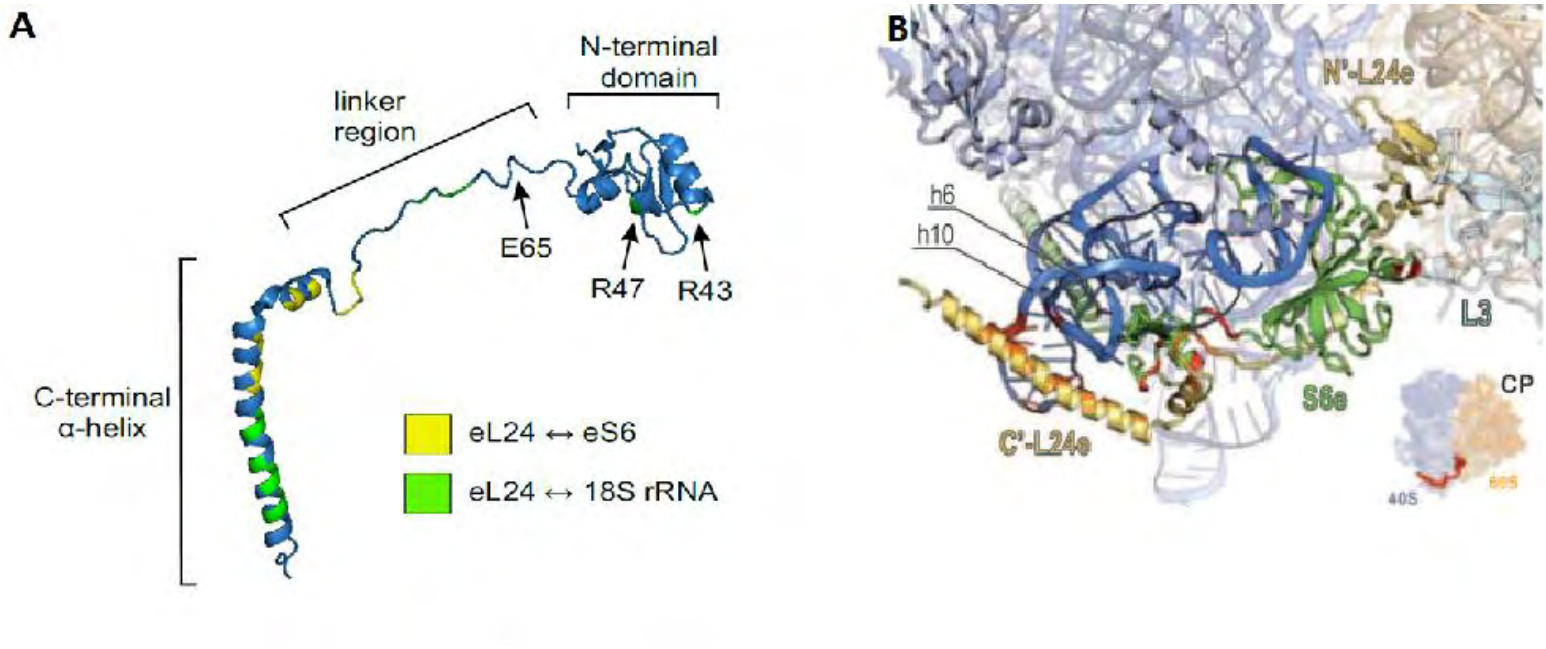

Figure 6. *(A) Protein eL24 is shown. The contact points with eS6 are shown in yellow and with 18S rRNA in green. The arrows show the 65th amino acid (E65) and the location of arginines 47 and 43 (R47, R43). (B) Intersubunit bridge eB13. Protein eL24 extends from the 60S subunit body and interacts with eS6 on the 40S subunit. The 18S rRNA and protein residues that participate in the bridge formation are depicted in red. The rRNA and protein residues of the large subunit that are involved in the bridge formation are in orange. In the lower right corner the location of the bridge within the 80S ribosome is shown. (adapted from Tiina Tamm presentation (A), Ben Shem et al., 2011 (B))*

Protein eL24 can be found only in archaeal and eukaryotic ribosomes (Lecompte *et al.,* 2002). The eukaryote-specific protein eL24 is encoded by the two paralogous genes *RPL24A* and *RPL24B*, which differ in only 35 of 467 nucleotides, thus producing two almost identical proteins

with only 5 out of 155 amino acid residues replaced. When both paralogous genes are deleted, the growth rate of the cells is only slightly decreased. Therefore, protein eL24 is dispensable to the cell and the eB13 bridge not essential for cell viability (Baronas-Lowell and Warner, 1990; Steffen *et al.,* 2012).

Nevertheless, eL24 is not unremarkable. Mutants lacking eL24 form stalled translation initiation complexes, which combined with the steady ratio of 60S to 40S subunits indicates the protein's involvement in the initiation of protein synthesis. The mutants are defective in ribosome assembly but without having an apparent effect on translational accuracy. Moreover, cells carrying a different mutation, leaving the N-terminal 70 amino acids intact, have a severely reduced growth rate. The amino acid sequence of the N-terminal region of ribosomal protein eL24 is highly conserved in archaea and eukaryotes. This short version of the protein is far more disturbing to the cell than the complete absence of it. Considering that protein eL24 has no major impact on other functions, it might improve on an existing ribosomal function instead of adding a new one (Baronas-Lowell and Warner, 1990; Dresios *et al.,* 2000).

1.1.5. Bridge eB14

There is only one eukaryote-specific bridge that is located at the center of the ribosome – bridge eB14. The bridge is formed by the smallest protein in yeast cells (25 amino acids), protein eL41. Protein eL41 is highly positively charged and consists of a single α helix that is enveloped by rRNA of the conserved core (Figure 7). The protein extends beyond the 60S subunit into the 40S subunit close to the decoding center and is almost buried in a binding pocket that consists of helices h27, h45, and h44 (Ben Shem *et al.,* 2011).

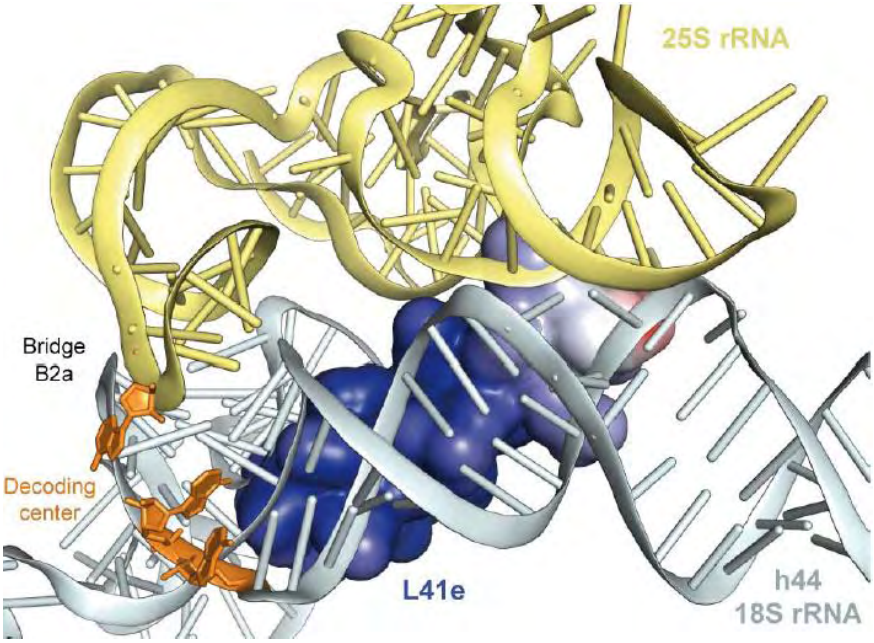

Figure 7. *Intersubunit bridge eB14. Representation of the highly positively charged eL41 (blue) that protrudes from 60S (yellow) into 40S (light blue) in proximity of the decoding center (orange)(adapted from Ben Shem et al. supplementary, 2011)*

There are two characteristics of this bridge that stand out. The first one is that the binding pocket of protein eL41 in the 40S subunit is highly conserved in eukaryotes and bacteria. The second is that even though protein eL41 forms only minor contacts with the 60S subunit, it remains part of the large subunit upon dissociation. It is therefore much more strongly associated with the 40S subunit than with the 60S, in the context of the full ribosome (Yusupova & Yusupov, 2014).

Protein eL41 is the smallest and most basic eukaryotic protein with 17 of its 25 amino acids being arginines or lysines. The protein is highly conserved in eukaryotes and can also be found in certain archaea (Dresios *et al.,* 2002). In *Saccharomyces cerevisiae*, eL41 is encoded by two linked genes, *RPL41A* and *RPL41B* (Suzuki *et al.,* 1990). Protein eL41 is also dispensable for viability, even though it is conserved from the archaea through the mammalia. Nevertheless there were some effects in the mutants where the protein was deleted. After deletion of both genes *S. cerevisiae* doubling times were a little longer and there was a slight hyperaccuracy. The most prominent effects were on the peptidyl transferase activity, which was decreased 3-fold, and the translocation process of protein synthesis, where its absence increased spontaneous elongation. Even though removal of eL41 did not affect the 60:40 S ratio, it reduced the amount of 80S, suggesting a role in ribosomal subunit association (Dresios *et al.,* 2002).

1.1.6. Bridge B6

As previously stated, each intersubunit bridge that has been described in the crystal structure of the bacterial ribosome has a corresponding bridge in the eukaryotic ribosome. One of these conserved bridges is bridge B6 (Figure 3). This bridge is formed through electrostatic interactions between the phosphate backbone of helix 44 of 18S rRNA and the arginine residues 43 and 47 (Figure 6a) from the large subunit protein eL24 (Ben Shem *et al.* supplementary, 2011). B6 has been previously observed in the *T. thermophilus* 70S ribosome, and since eL24 has no homologous protein in the bacteria, the bridge is formed by an analogous protein, L19 (Spahn *et al.,* 2001; Yusupov *et al.,* 2001).

The formation of the B6 intersubunit bridge is sterically obstructed by eIF6. The 40S subunit and eIF6 share a common binding region on the 60S subunit and cannot be present simultaneously. This region on the 60S subunit coincides exactly with the dynamic intersubunit bridge B6. Thus, in the presence of eIF6, the bridge B6 can no longer be formed (Gartman *et al.,* 2010).

1.2. Eukaryotic translation initiation

Gene expression is regulated at multiple levels, including the translation of mRNAs into proteins. The process of translation can be divided into initiation, elongation, termination, and ribosome recycling. Protein synthesis is mainly regulated at the initiation stage (Sonenberg & Hinnebusch, 2009). Translation initiation is the process of assembly of elongation-competent 80S ribosomes, in which AUG start codon is base-paired with the methionyl tRNA specialized for initiation (Met-tRNA$_i$) in the ribosomal P-site (Figure 8). At least eleven eukaryotic initiation factors (eIFs) are required to initiate translation on an mRNA. Conceptually, this process can be divided in the following steps: formation of the ternary complex (TC), formation of the 43S pre-initiation complex (PIC), activation of mRNA, formation of 48S PIC and joining of the 60S subunit (Dever *et al.,* 2016).

In addition to the 40S and 60S ribosomal subunits, Met-tRNA$_i^{Met}$, 11 translation factors and mRNA features also contribute to the formation of a translating 80S ribosome. A ternary complex is formed when Met-tRNA$_i^{Met}$ interacts with the GTP-bound factor eIF2. This ternary complex associates with the 40S ribosome assisted by the factors eIF1, eIF1A, eIF3 and possibly eIF5 and forms the 43S preinitiation complex (PIC). The eIF4 family of factors, including factors eIF4B, eIF4A, eIF4E and eIF4F is considered to prepare the mRNA (activated mRNA) for binding the 43S PIC and thus forming the 48S PIC. Activated mRNAs bear eIF4E at the 5’ cap, poly(A) binding protein Pab1 bound to the poly(A) tail, bridged by eIF4G to form a loop along with eIF4A and eIF4B. Following binding near the 5’ end of the mRNA, the ribosomal complex scans the mRNA 5' untranslated region to localize the initiator AUG. During scanning, the 43S PIC is in an open conformation, where the Met-tRNA$_i^{Met}$ is not fully base paired within the P site (P_{out}). Selection of the translation start site is accompanied by completion of GTP hydrolysis by eIF2, release of many of the bound factors and reorganization of the 43S PIC to a closed state with Met-tRNA$_i^{Met}$ in the P_{in} conformation and tightly bound to the complex. The factor eIF5B, a second GTPase, promotes joining of the 60S subunit to the AUG-bound PIC to form an 80S ribosome. Subsequent GTP hydrolysis by eIF5B leads to its release from the 80S monosome, which is poised to begin translation elongation (Dever *et al.,* 2016).

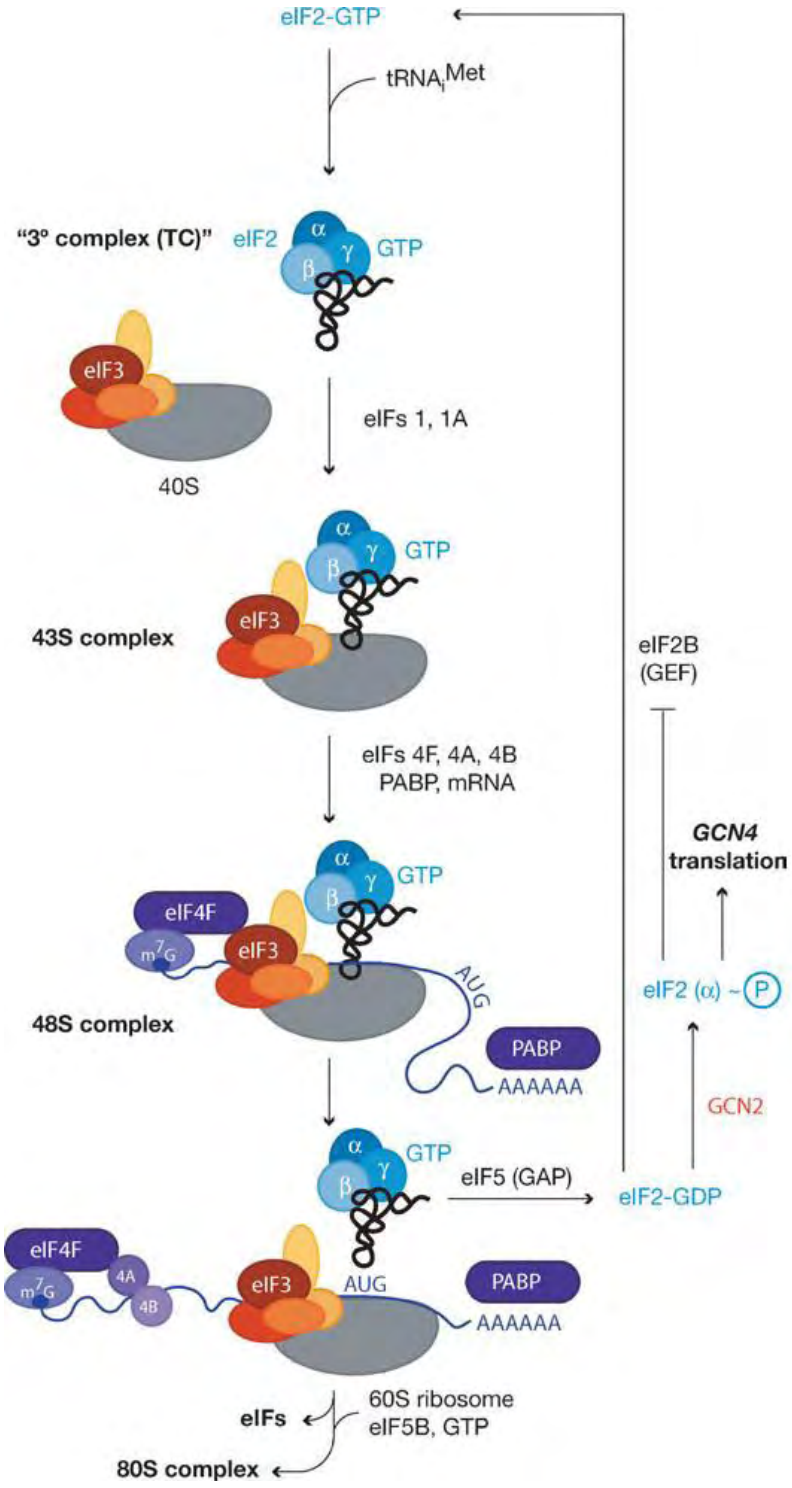

Figure 8. *Schematic of the translation initiation pathway in eukaryotes. The initiation of translation begins with the formation of the ternary complex (TC) containing GTP-bound eIF2 and the initiator tRNA (tRNAiMet). The ternary complex is recruited to the 40S subunit with the help of eIFs 1, 1A, and 3 to form the 43S preinitiation complex. The mRNA bound by the eIF4 factors and poly(A) binding protein (PABP) joins and forms the 48S complex, which scans the mRNA to locate the start (AUG) codon. PABP can interact with eIF4F such that both ends of the mRNA are bound to eIF4F. On start codon recognition, eIF5*

binds and triggers GTP hydrolysis by eIF2, followed by the release of the rest of the factors and the joining of the 60S subunit mediated by eIF5B. Subunit joining is followed by GTP hydrolysis by eIF5B and factor dissociation to form the 80S initiation complex (IC). Recycling of eIF2-GDP to eIF2-GTP is catalyzed by the GEF eIF2B, a reaction inhibited by phosphorylation of eIF2-GDP on the α-subunit by Gcn2p (adapted from Asano et al., 2001).

1.3. Translational regulation in yeast by *GCN4*

The control of protein synthesis has been studied extensively in yeast. Perhaps the best-described example of protein synthesis regulation in yeast is *GCN4* (General Control Nondepressible), which is widely viewed as primary evidence to support the scanning mechanism of translation initiation (Dever *et al.*, 2016). Gcn4p is a basic leucine zipper (bZIP) transcriptional activator of more than 30 genes, which take part in 12 different pathways, and are required for amino acid biosynthesis in response to starvation for amino acids. This regulatory response is known as general amino acid control (GAAC). In addition to the derepression of genes involved in the amino acid biosynthetic pathways, Gcn4p may directly or indirectly control the expression of genes involved in purine biosynthesis, organelle biosynthesis, autophagy, glycogen homeostasis, and multiple stress responses (Natarajan *et al.,* 2001).

The *GCN4* gene is itself regulated by amino acid availability and the regulation occurs at the translational level. The derepression of *GCN4* mRNA translation in nutrient-starved cells is mediated by Gcn2p, a protein kinase that phosphorylates the α subunit of translation initiation factor 2 (eIF2α). eIF2 is responsible for transferring the charged methionyl initiator tRNA (Met-$tRNA_i^{Met}$) to the 40S subunit in the first step of translation initiation, as indicated above. Then, it binds to the ribosome forming a ternary complex (TC), which consists of Met-$tRNA_i^{Met}$ and the GTP-bound form of eIF2. Subsequently, it is released as an inactive eIF2-GDP binary complex and must be recycled to active eIF2-GTP by the guanine nucleotide exchange factor eIF2B (GEFeIF2B) to reform the TC. This exchange reaction is inhibited by Gcn2p, which phosphorylates Ser- 51 in the *α*-subunit of eIF2 and converts eIF2-GDP from a substrate to an inhibitor of eIF2B, thus impeding the formation of the TC. The reduction in TC formation evoked by Gcn2p activation in amino acid starved cells activates *GCN4* translation while decreasing the rate of general translation initiation (Hinnebusch & Natarajan, 2002).

Four short upstream open reading frames (uORFs), of only two to three codons in length, in the 5’ region of the *GCN4* mRNA leader mediate the translational induction of *GCN4* (Hinnebusch, 1984). With only the first (uORF1) and fourth (uORF4) uORFs present the translational control is almost like wild type (Mueller & Hinnebusch, 1986). Typically, after the translation of an uORF, reinitiation of translation downstream is inefficient, therefore uORFs function as translational barriers (Kozak, 1983). Accordingly when all four uORFs were removed, the translation of *GCN4*

was increased dramatically. However, some uORFs allow ribosomes to resume scanning after they have been translated. This depends on a roughly 15-nucleotide long sequence immediately downstream of the uORF stop codon. The first (uORF1) and second (uORF2) have this element, and are weak translational barriers that allow ribosomes to remain on the *GCN4* mRNA after their translation, while the third (uORF3) and fourth uORFs (uORF4) act like translational barriers, forcing almost all of the ribosomes to disassociate from the *GCN4* mRNA after their translation (Hinnebusch, 2005).

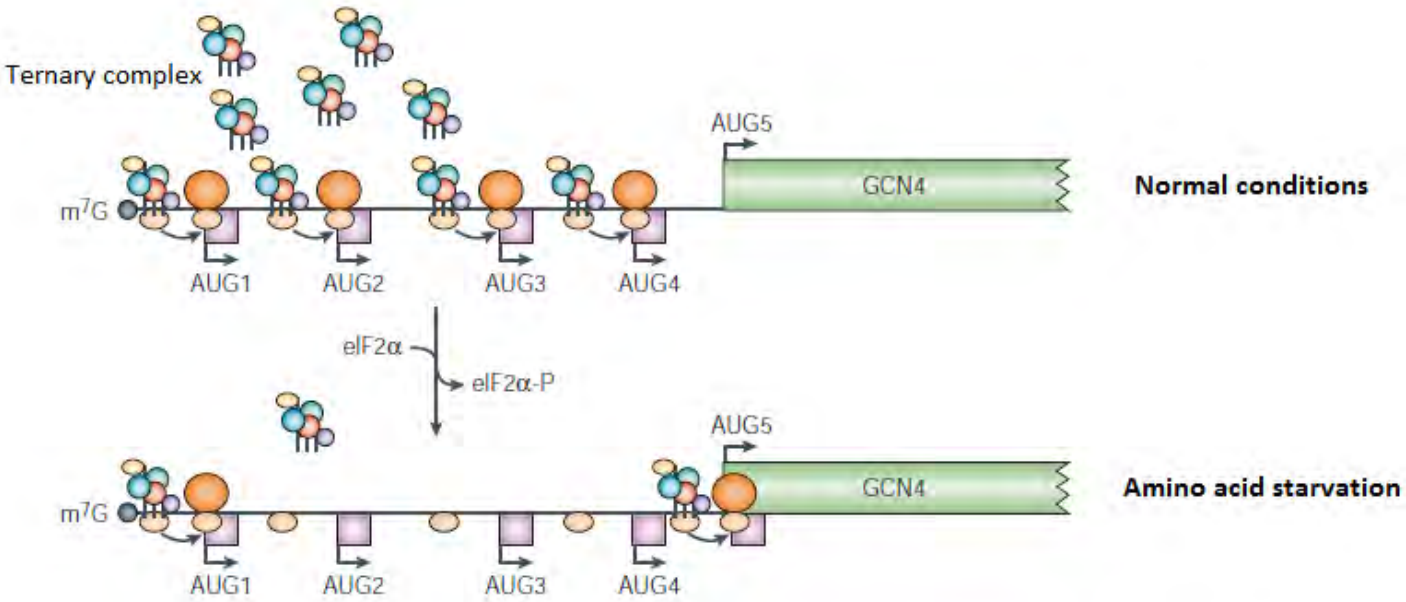

Figure 9. *Model for GCN4 translational control. The translation of GCN4 is regulated by four uORFs. Under non inducing conditions, when eIF2α is not phosphorylated and there are sufficient levels of TC, ribosomes initiate at uORF1 and quickly rebind the TC to reinitiate at uORF2, uORF3 or uORF4. The ribosomes that translate uORFs 2 to 4 cannot continue scanning and they dissociate, thereby lowering the probability to reinitiate at the GCN4 start codon. Under amino acid starvation conditions, the increased levels of phosphorylated eIF2α lower the concentration of TC, and the rescanning 40S ribosomes fail to rebind the TC until scanning past uORF4 and they reinitiate at GCN4 instead (adapted from Holcik and Sonenberg, 2005).*

According to the current model of the scanning mechanism of translation initiation, in non starved cells, where TC concentration is high, nearly all 40S subunits scanning from the 5' end of the mRNA translate uORF1 and continue scanning downstream. Then they rebind the TC before reaching the uORFs 2, 3 or 4, reinitiate translation at these uORFs and dissociate from the mRNA, leaving *GCN4* untranslated. In starved cells, when phosphorylation of eIF2α reduces

the concentration of TC, only ~50% of the 40S subunits scanning downstream from uORF1 will rebind the TC before reaching uORFs 2, 3, or 4 and reinitiate translation at one of these uORFs (Figure 9)(Hinnebusch, 2005). The rest of these 40S subunits, which lack the TC when they reach uORFs 2 to 4, bypass the start codons at these uORFs and continue scanning because the recognition of the AUG initiation codon requires base pairing with Met-tRNA$_i^{Met}$ (Cigan *et al.,* 1988). Subsequently, most of these ribosomes rebind the TC before reaching *GCN4* main ORF AUG codon and reinitiate translation there instead (Abastado *et al.,* 1991; Dever *et al,.* 1992). Thus, a reduction in TC formation produced by Gcn2p activation enables some of the reinitiating ribosomes to bypass the inhibitory uORF4 and reinitiate at *GCN4* main ORF instead (Hinnebusch & Natarajan, 2002).

As mentioned earlier, when cells are starving for an amino acid the Gcn2p kinase is activated and phosphorylates eIF-2a. It was observed that the carboxy-terminal portion of Gcn2p bears significant similarity to the entire sequence of histidyl-tRNA synthetases (HisRSs). Aminoacyl-tRNA synthetases bind uncharged tRNA as a substrate and uncharged tRNA is a direct signal for the general control, therefore the HisRS-related domain in Gcn2p could monitor the concentration of uncharged tRNA that accumulates during amino acid starvation. Thus, binding of any uncharged tRNA to the synthetase-like domain would produce a conformational change in Gcn2p that stimulates kinase function and increases Gcn2p phosphorylation of the substrate eIF-2a (Wek *et al.,* 1989).

2. Aim of the study

The aim of this study is to reveal the functional importance of the intersubunit bridges at the biological level. The study is based on the hypothesis that the loss of even one of these bridges leads to decreased ribosome functionality. This results in the activation of the general stress response and induces cross protection against environmental stresses. Yeast mutant strains with one or more bridges deleted are analyzed. The study consists of three parts:

1. Temperature sensitivity analysis by serial dilutions spot test to study the growth characteristics of the deletion mutants.
2. Analysis of the sensitivity to amino-acid starvation by serial dilutions spot test.
3. Testing the translational regulation and fidelity by analyzing the *GCN4*-reporter.

3. Materials and Methods

3.1. Yeast strains and plasmids used

The *Saccharomyces cerevisiae* strains and plasmids that were used in the study are presented in Table 1 and Table 2 respectively. The yeast strains with a functional *HIS3*$^{+}$ locus and their counterparts are demonstrated in Table 3.

Table 1. Yeast strains that were used

Strain	Genotype	Description	Source
TYSC309	*MAT*a *ura3-52 his3Δ200 trp1Δ36 leu2Δ1 Δarg4 Δlys1*	wild type	Lab collection
TYSC488	*MAT*a *ura3-52 his3Δ200 trp1Δ36 leu2Δ1 Δarg4 Δlys1 Δrpl24A::hphMX6 Δrpl24B::hphMX6*	deletion of protein eL24 encoding genes (ΔeL24)	Lab collection
TYSC532	*MAT*a *ura3-52 his3Δ200 trp1Δ36 leu2Δ1 Δarg4 Δlys1 Δrpl41A::natMX6 Δrpl41B::natMX6*	deletion of protein eL41 encoding genes (ΔeB14)	Lab collection

TYSC561	*MAT*a *ura3-52 his3Δ200 trp1Δ36 leu2Δ1 Δarg4 Δlys1 Δrpl41A::natMX6 Δrpl41B::natMX6 Δrpl24A::hphMX6 Δrpl24B::hphMX6*	deletion of proteins eL41 and eL24 encoding genes (ΔeB14 ΔeL24)	Lab collection

Table 2. Plasmids that were used

Plasmid	Description
pRS314	plasmid with the *TRP1* auxotrophic marker, no insert
pRS314 - eL24 (wt)	plasmid expressing the wild type eL24
pRS314 - eL24 (Arg43Ala; Arg47Ala)	plasmid expressing the mutant eL24 (arginines 43 and 47 are substituted with alanines)
pRS314 - eL24$_{1-65}$	plasmid expressing the truncated version of eL24 (only the amino acids 1-65 maintained)
pRS314 - eL24$_{1-65}$ (Arg43Ala; Arg47Ala)	plasmid expressing the truncated version of eL24 with arginines 43 and 47 mutations
p180	plasmid bearing a *GCN4 – lacZ* fusion containing all four uORFs of *GCN4*
p227	plasmid bearing a *GCN4 – lacZ* fusion lacking all four uORFs of *GCN4*
pM226	plasmid bearing a *GCN4 – lacZ* fusion with uORF1 elongated such that it overlaps the *GCN4* ORF in an alternate reading frame

Table 3. *S. cerevisiae* strains with a functional *HIS3*$^{+}$ locus

***his3Δ-200* Strain**	***HIS3*$^{+}$ strain**	**Source**
TYSC309	TYSC606	Lab collection
TYSC488	TYSC612	Lab collection
TYSC532	TYSC608	Lab collection
TYSC561	TYSC610	Lab collection

A strain with a *gcn* deletion (TYSC616) was also used as a control strain for the Gcn phenotype.

3.2. Media used

The composition of media that was used in the study is presented in table 4. All plates were made with an addition of 2% agar.

Media	**Media composition**
LB	1.6% Tryptone, 1% Yeast extract, 0.5% NaCl
YPD	1% Yeast extract, 2% Bacto-peptone, 2% Glucose
SC-HIS	0.2% mixture of amino acids without histidine, 0.67% Yeast nitrogen base, 2% Glucose
SC-TRP	0.2% mixture of amino acids without tryptophan, 0.67% Yeast nitrogen base, 2% Glucose
SC-HIS-TRP	0.2% mixture of amino acids without histidine and tryptophan, 0.67% Yeast nitrogen base, 2% Glucose
SC-URA-HIS	0.2% mixture of amino acids without uracil and histidine, 0.67% Yeast nitrogen base, 2% Glucose or 2% Galactose
SC-URA-TRP-HIS	0.2% mixture of amino acids without uracil, tryptophan and histidine, 0.67% Yeast nitrogen base, 2% Glucose or 2% Galactose
Synthetic Minimal (Sm)	0.2% mixture of minimal amino acids (0.1% lysine, 0.1% tryptophan, 0.1% arginine, 0.5% leucine), 0.67% Yeast nitrogen base, 2% Galactose

3.3. Transformation of E. coli cells

For the transformation of bacterial cells 1 µl (approximately 200 ng) of DNA was mixed with 40 µl of competent E. coli cells (DH5α), stirred gently and incubated on ice for 30 minutes. The cells were heat shocked by incubating them for 90 seconds at 42^{o}C and then immediately immersing them in ice for 3 minutes. Afterwards, 400 µl of LB medium is added and the cells are incubated at 37^{o}C for 1 hour. Then 150 µl of the mix is plated on LB agar containing 100 µg/ml ampicillin and grown overnight at 37^{o}C.

3.4. Plasmid DNA purification from E. coli cells

For the plasmid purification the GeneJET Plasmid Maxiprep Kit (Thermo Scientific) was used. 250 ml of overnight E. coli culture was grown in LB medium containing 100 µg/ml ampicillin. Cells were centrifuged at 4500 rpm at +4^{o}C for 10 minutes and the supernatant was discarded. The pelleted cells were resuspended by vortexing in Resuspension Solution containing RNAase A. The Lysis Solution was added, mixed gently and incubated for 3 minutes at room temperature. The Neutralization Solution and the Endotoxin Binding Reagent was then added, stirred gently and incubated for another 5 minutes at room temperature. Then 96% Ethanol was added, the cells were centrifuged at 4500 rpm for 40 minutes at room temperature. The supernatant was transferred to a clean tube, 96% Ethanol was added again and mixed gently. Next, part of the sample was moved to the column, centrifuged it for 3 minutes at 2000 x g and discarded the flow – through. This step was repeated for the remaining lysate. The plasmid DNA that was bound to the column was washed with Wash Solution 1 (containing isopropanol), centrifuged at 3000 x g for 2 minutes and discarded the flow through. Following these steps, the column was then washed two times using Wash Solution 2 (containing ethanol). The column was transferred to a fresh tube and 700 µl of Elution Buffer was added. Then it was incubated for 10 minutes at 37^{o}C and centrifuged for 30 minutes at 3000 x g to elute plasmid DNA. The DNA concentration was measured using Nanodrop 2000.

3.5. Sodium Acetate precipitation of plasmid DNA

A solution of 3M sodium acetate (NaOAc, pH 5), was added to the sample to bring to a final concentration of 10%. Thereafter 2.5 volumes of 96% ethanol were added and the DNA was precipitated by centrifugation at 13000 rpm for 15 minutes. The supernatant was discarded and the pellet was washed with 70% ice cold ethanol and centrifuged again at 12000 rpm for 5 minutes. The supernatant was discarded and the pellet was suspended in 100 µl of previously used Elution Buffer.

3.6. Restriction digest of plasmid DNA

Single and double digests were done in a final volume of 20 µl, using 2.5 µl of plasmid DNA, 0.5 µl of enzyme and 2 µl of the recommended Restriction Buffer (Thermo Scientific). For the single digest of plasmid *pRS314* the restriction enzyme *Xba*I was used. The rest of the plasmids were all double digested using the enzymes *Xho*I and *Pst*I as well as *Xho*I and *Eco*RI. The digests were mixed by vortex and incubated for almost 2 hours at 37^{o}C. The digests were analyzed in 0.8% agarose-TBE gel.

3.7. Agarose gel electrophoresis

All plasmid DNA samples were mixed with 4µl of loading dye viewed on a 0.8% agarose gel (TBE buffer 1X, 10 mg/ml EtBr) which was electrophorised at 120V for adequate time. For determination of the size of the molecules, samples were compared to the 1 kb DNA ladder marker (Solis BioDyne). All gels were viewed using the Uvitec system. The plasmids were also verified by sequencing.

3.8. Transformation of yeast cells (adapted from Knop *et al.,* 1999)

For the transformation of yeast cells the lithium acetate method was used. Single *S. cerevisiae* colonies were inoculated in 25 ml of YPD liquid medium each and were grown overnight at 30^{o}C. The cell cultures were diluted with fresh media to an OD_{600}=0.3 and grown to an OD_{600} = 0.6-0.8. The cells were collected by centrifugation at 3200 rpm for 2 minutes at room temperature. After centrifugation, the supernatant was discarded and the cells were washed with sterile H_2O, recentrifuged at the same conditions and the supernatant was removed. The cells were washed with LiOAc-TE 1X (0.1 M LiOAc, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA), centrifuged in the same conditions and discarded the supernatant. The cells were resuspended in 150 µl of LiOAc-TE 1X. Denatured carrier DNA (10 mg/ml) was added to the cells and mixed by vortex. 50 µl of competent yeast cells were mixed with 2µl of plasmid DNA and incubated for 15 minutes at room temperature. Then, added 300 µl of PEG-LiOAc-TE (40% PEG, LiOAc-TE 1X), mixed by pipeting up and down and incubated for 15 minutes at room temperature. 30 µl of DMSO was added, mixed using vortex and incubated for 10 minutes at 42^{o}C. The transformation mixture was centrifuged at 3000 rpm for 3 minutes at room temperature and the supernatant was removed. The cells were then resuspended in 150 µl of sterile H_2O and plated on synthetic media plates lacking the appropriate amino acids for selection.

3.9. *S. cerevisiae* spot test dilutions

Single S*. cerevisiae* colonies were inoculated in the appropriate liquid media and were grown overnight at 30^{o}C. The cell cultures were diluted to reach an OD_{600} = 0.4-0.7 and then diluted in H_2O so that 5 µl of culture would contain 5000 cells. Subsequent twofold, fivefold, tenfold and fivefold dilutions were made so that 5 µl would contain 2500, 500, 50 and 10 cells. 5 µl was plated from the lowest concentration to the highest on the designated plates. The cells were incubated at 16 ^{o}C, 20 ^{o}C, 25 ^{o}C, 30 ^{o}C and 36^{o}C for 2-7 days (temperature sensitivity analysis) or at 30^{o}C for 2-4 days (histidine starvation response). The pictures were taken using the Uvitec system and were edited with Adobe Photoshop CS6.

3.10. Growth conditions for histidine starvation

Yeast strains with a *HIS3*$^{+}$ locus and the p180 plasmid were grown at 30^{o}C in liquid synthetic media lacking a specific amino acid to an OD_{600}=0.7 when the inhibitor of His3p enzyme 3AT (10mM) was added. The cells were starved for 6 hours and then they were collected. The *HIS3*$^{+}$ strains with the p227 or pM226 plasmids were not starved and were collected when they reached an OD_{600}=0.9-1.0. To collect the cells, 5 ml of culture was spinned down at 3200 rpm for 3 minutes at +4^{o}C and the supernatant was discarded. The pelleted cells were resuspended in cold H_2O and centrifuged at 13000 rpm for 1 minute at +4^{o}C and the supernatant was removed. Then they were resuspended again in Breaking Buffer (100 mM Tris-HCl [pH 8], 1 mM DTT, 20% Glycerol), immersed in liquid and nitrogen and stored at -80^{o}C. The reporter plasmids were analyzed using the Bradford method and the β-Galactosidase assay to calculate Miller Units.

3.11. Cell lysate preparation

To prepare the cell lysates the cells were thawed and put on ice. Breaking Buffer (100 mM Tris-HCl [pH 8], 1 mM DTT, 20% Glycerol), PMSF (100 mM) and glass beads were added to the cells, which then were homogenized using the Precellys 24 homogenizer at 6000 rpm, 3x60 seconds, 60 seconds pause. Another 100 µl of Breaking Buffer was added to the cells, mixed and the samples were centrifuged at 13000 rpm for 15 minutes at +4^{o}C. The lysates (supernatant) were transferred to new Eppendorf tubes.

3.12. Bradford protein assay

To perform the Bradford protein assay the lysates were diluted threefold in Breaking Buffer without glycerol. 5 µl of the diluted samples were mixed with 250 µl of Coomassie Reagent (Thermo Scientific) and incubated for 10 minutes at room temperature. Absorbance was measured at 595 nm using the Omega microplate reader and was normalized to a control containing a tenfold dilution of the Breaking Buffer without glycerol and Coomassie Reagent.

3.13. β – Galactosidase assay (adapted from Rose and Botstein, 1983)

For β – Galactosidase assay the lysates were mixed with Z-buffer (60 mM Na_2HPO_4-$7H_2O$, 40 mM NaH_2PO_4-H_2O, 10 mM KCl, 1 mM $MgSO_4$-$7H_2O$, 14 M β-Mercaptoethanol, pH to 7, Miller, 1972) and were incubated for 5 minutes at 30°C. The reaction was initiated by adding 100 µl of ONPG (4 mg/ml in Z-buffer) and noted precisely the time the addition was made. Incubated at 30°C until the mixture acquired a pale yellow color and the reaction was terminated by adding 250 µl of sodium carbonate 1M (Na_2CO_3). The exact time that the reaction was terminated was also noted. The optical density was measured at 420 nm using the Omega microplate reader. The measurement was normalized to a control containing Breaking Buffer, PMSF, Z-buffer, ONPG and Na_2CO_3. The Miller Units were calculated using the following equation:

$$Miller\ Units = \frac{OD420 * 0.85}{0.0045 * protein\ concentration * extract\ volume * time}$$

4. Results

4.1. Transformation of yeast strains with eL24 and eL41 proteins deleted

The eukaryote specific proteins eL24 and eL41 are not essential to cell viability, therefore the genes encoding these proteins, *RPL24A, RPL24B* and *RPL41A, RPL41B* respectively, could be deleted directly from the genome. The strain TYSC561 which is lacking both proteins has four genes deleted from its genome. The mutants were transformed with the plasmids, from which the protein was expressed after the transformation. The transformations that were performed and the acquired transformants with their names are presented in Table 5. The TYSC309 strain with the *pRS314* plasmid was used as a control. *TRP1* was used as a selection marker for the strains containing the *pRS314* plasmid, so SC-TRP plates were used to plate the transformants.

Table 5. Transformations that were used for the temperature sensitivity analysis

***S. cerevisiae* strain**	**Plasmid introduced**	**Name**
TYSC309	pRS314	control; wt
TYSC532	pRS314	ΔeB14
TYSC488	pRS314	ΔeL24
TYSC488	pRS314 – eL24	Δel24 + eL24
TYSC488	pRS314 – eL24 (R43A;R47A)	ΔB6
TYSC488	pRS314 – $eL24_{1-65}$	ΔeB13
TYSC488	pRS314 – $eL24_{1-65}$ (R43A;R47A)	ΔeB13ΔB6
TYSC561	pRS314	ΔeL24 ΔeB14
TYSC561	pRS314 – eL24	ΔeL24 ΔeB14 + eL24 (wt)
TYSC561	pRS314 – eL24 (R43A;R47A)	ΔB6ΔeB14
TYSC561	pRS314 – $eL24_{1-65}$	ΔeB13 ΔeB14
TYSC561	pRS314 – $eL24_{1-65}$ (R43A;R47A)	ΔeB13 ΔB6 ΔeB14

4.2. Temperature sensitivity analysis

Temperature sensitive (Ts) mutations have proven to be an essential tool for gene identification and for defining essential gene function. Temperature sensitive mutations are the ones in which there is a noticeable decrease in the level or activity of the gene product above a certain temperature (non permissive temperature). At a standard (permissive) low temperature, the activity of the mutant is very similar to that of the wild type (Ben-Aroya *et al.,* 2010). In

contrast, cold sensitive (Cs) mutants behave like loss-of-function mutants at temperatures lower than the permissive one, but have the wild type phenotype at higher temperatures (Baliga *et al.,* 2016).

Protein eL24 and eL41 are the main components of bridges eB13 and eB14, respectively (Ben Shem *et al.,* 2011). Protein eL24 has an additional role in the formation of the conserved intersubunit bridge B6 (Spahn *et al.,* 2001). Mutations in the arginines 43 and 47 of protein eL24 result in the loss of B6. Moreover, the mutant ΔeB13, where only the first 65 amino acids of eL24 are expressed, imitates the archaeal version of the protein (Ben Shem *et al.* supplementary, 2011).

One of the objectives of this work is to study how the deletion of the intersubunit bridges eB13, eB14 and B6 affect the growth phenotype. For this, temperature sensitivity analysis by serial dilutions spot test on rich medium at different temperatures was performed. Three different sets of spot tests were made on YPD plates and they were repeated (including the transformations). The results are presented in figures 10, 11 and 12.

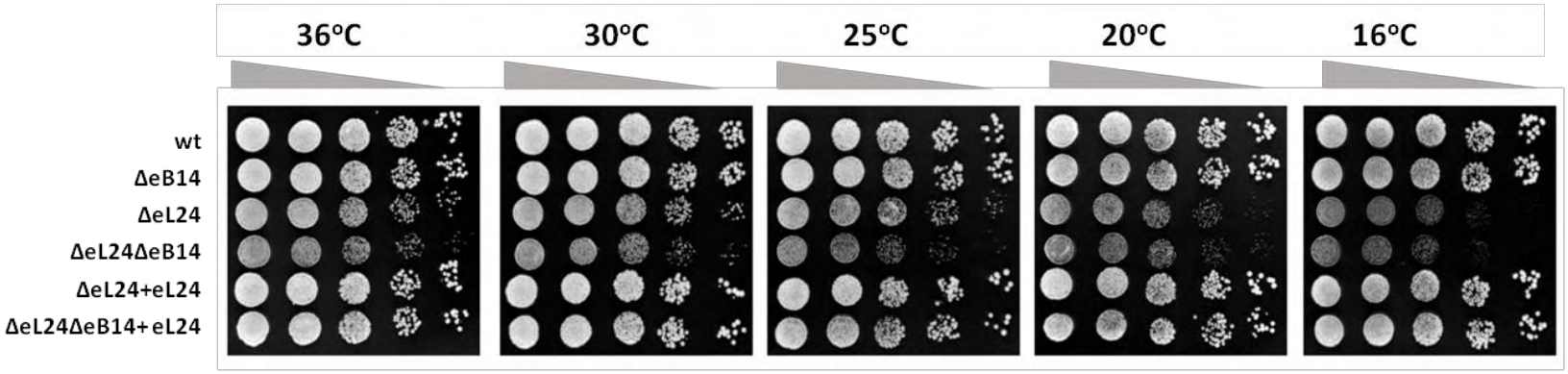

Figure 10. *Temperature sensitivity analysis by spot test (1st set). The mutants were grown for 2-7 days on YPD plates at different temperatures. The spots are from highest to lowest concentration (gray bar).*

The first three strains that were analyzed in the 1st set are those where the entire protein is missing (eL41, eL24 or both) and is not complemented from the plasmid. The ΔeB14 mutant is not affected by the deletion of the protein and is behaving like wild type, while the deletion of eL24 causes a reduction of growth and at 16^{o}C the cells have a cold sensitive phenotype. The phenotype of ΔeL24ΔeB14 is the same as in ΔeL24 and is probably caused by the deletion of eL24. The last two strains have eL24 expressed from the plasmid and therefore have a phenotype similar to wild type (Figure 10).

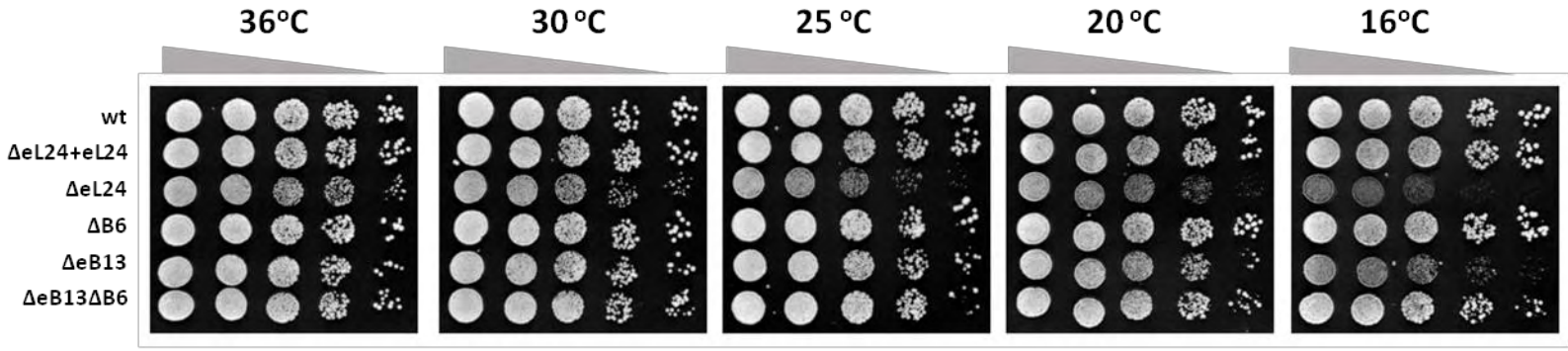

Figure 11. *Temperature sensitivity analysis by spot test (2nd set). The mutants were grown for 2-7 days on YPD plates at different temperatures. The spots are from highest to lowest concentration (gray bar).*

In the 2nd set only the strains with eL24 removed (TYSC488) were tested. The mutant where the conserved bridge B6 is missing is growing like wild type. ΔeB13 with the truncated eL24 protein being expressed from the plasmid is growing slower than wild type and has a cold sensitive phenotype at 16^{o}C. Interestingly, the mutant where both bridges are missing is growing slower than wild type but better than ΔeB13. The deletion of B6 seems to be "rescuing" the phenotype of ΔeB13 (Figure 11).

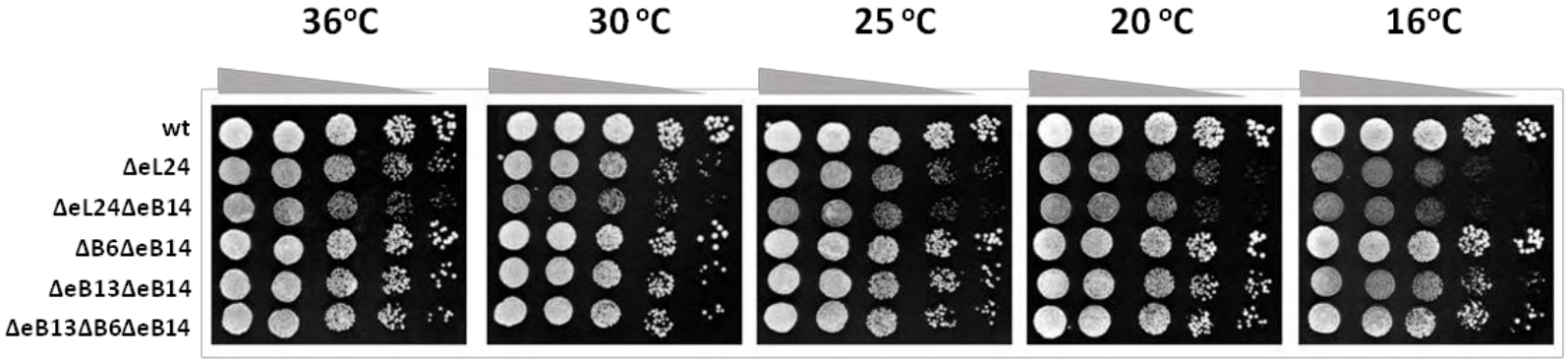

Figure 12. *Temperature sensitivity analysis by spot test (3rd set). The mutants were grown for 2-7 days on YPD plates at different temperatures. The spots are from highest to lowest concentration (gray bar).*

In the 3rd set mutants with both eL24 and eL41 deleted (TYSC561) were tested. The growth rate of mutant ΔB6ΔeB14 is the same with wild type and the combination of their deletion has no visible effect on the phenotype. Mutant ΔeB13ΔeB14 is growing slower than wild type, while the mutant ΔeB13ΔB6ΔeB14 grows better than ΔeB13ΔeB14, but still slower than the wild type. Once more it appears that the deletion of B6 is improving the growth rate of the mutant (Figure 12). In Addition, the deletion of protein eL41 does not enhance any of the previously observed phenotypes (Figure 11).

4.3. Analysis of the sensitivity to histidine starvation

Yeast overcomes the growth inhibition caused by amino acid starvation by increasing the expression of the *GCN4* gene. Gcn4p is the transcriptional activator of at least 40 genes encoding enzymes involved in amino acid biosynthesis. The translation of *GCN4* is regulated by 4 uORFs in the 5' leader region. Under non inducing conditions, ribosomes scanning the *GCN4* mRNA will translate the first uORF encountered and then they will reinitiate translation at the subsequent uORFs and be unable to reinitiate again at *GCN4* main ORF. Under starvation conditions the increased synthesis of the Gcn4 protein is dependent upon the Gcn2 kinase. Gcn2p phosphorylates the eIF-2α and inhibits its GTP binding, thereby decreasing the ternary complex levels. Consequently, ribosomes that have translated uORF1 will ignore the start codons at uORF2 to uORF4 and will reinitiate at the *GCN4* start codon instead (Ramirez *et al.,* 1992; Hinnebusch, 2005).

To induce amino acid starvation we used 3-aminotriazole (3AT), a competitive inhibitor of the histidine biosynthetic enzyme His3p, on *HIS3*$^{+}$ strains. Yeast strains, which were prototroph for histidine, were needed to test for 3AT sensitivity, for this reason *HIS3*$^{+}$ strains were produced from their *his3Δ-200* parents (Table 3) and they were transformed with the same plasmids as their counterparts in Table 5. The same names were also used for the transformants. Δ*gcn2* strains are sensitive to amino acid starvation since eIF-2α is not phosphorylated and the ternary complex formation is not low enough to induce *GCN4* translation. Therefore, Δ*gcn2* strains were used as an additional control.

To test how the loss of bridges eB13, eB14 and B6 affect the cells during amino acid starvation, serial dilution spot tests were carried out. Again, three different sets were spotted on SC-HIS and SC-HIS-TRP plates with different concentrations of 3AT (10 mM, 30 mM, 40 mM). The cells were incubated at 30^{o}C for 2-4 days. A second biological replicate was created. The results are presented in figures 13, 14 and 15.

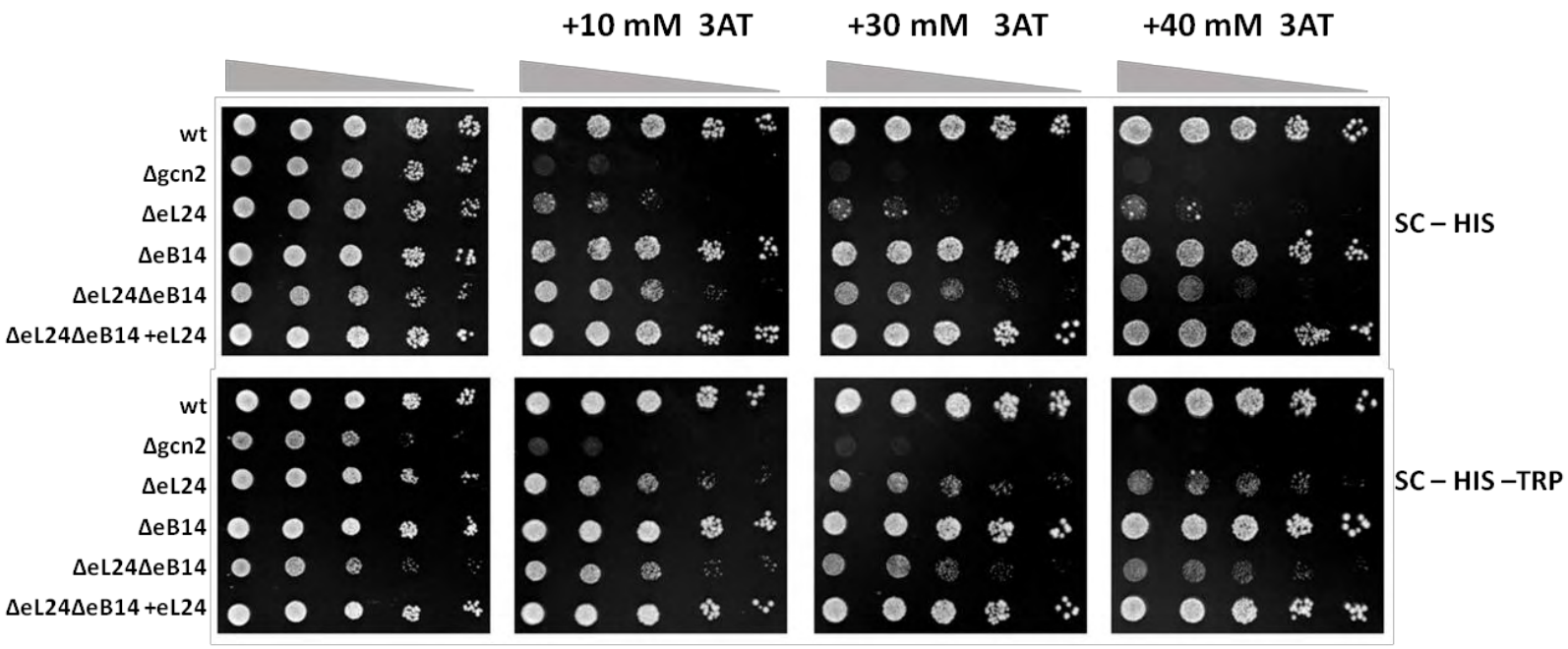

Figure 13*. Sensitivity analysis to histidine starvation by serial dilution spot test (1st set). The mutants were plated on selective medium plates (SC-HIS and SC-HIS-TRP) supplemented with different concentrations of 3AT and incubated at 30°C for 2-4 days. Serial dilutions were spotted from highest concentration to lowest (gray bar on top).*

The first 2 strains (wt and Δ*gcn2*) are both used as controls in this assay. As expected, the Δgcn2 was unable to form any colonies in the presence of 3AT, while wild type cells were not hindered by the drug. Similarly to the temperature sensitivity analysis, in the first set the strains with the entire protein removed (eL24, eL41 or both) were analyzed along with one strain where both proteins are missing and eL24 is expressed from the plasmid. ΔeB14 mutant is growing like wild type. The strain with protein eL24 removed displays 3AT sensitivity as well as the mutant with both eL24 and eL41 missing, although not as strongly. It appears that the deletion of eB14 is slightly "rescuing" the phenotype of ΔeL24ΔeB14. Accordingly, when protein eL24 is expressed in ΔeL24ΔeB14 mutant (ΔeL24ΔeB14+eL24), it is growing like wild type and is not sensitive to histidine starvation. Moreover, the strains seem to grow better when spotted on SC-HIS-TRP plates (Figure 13).

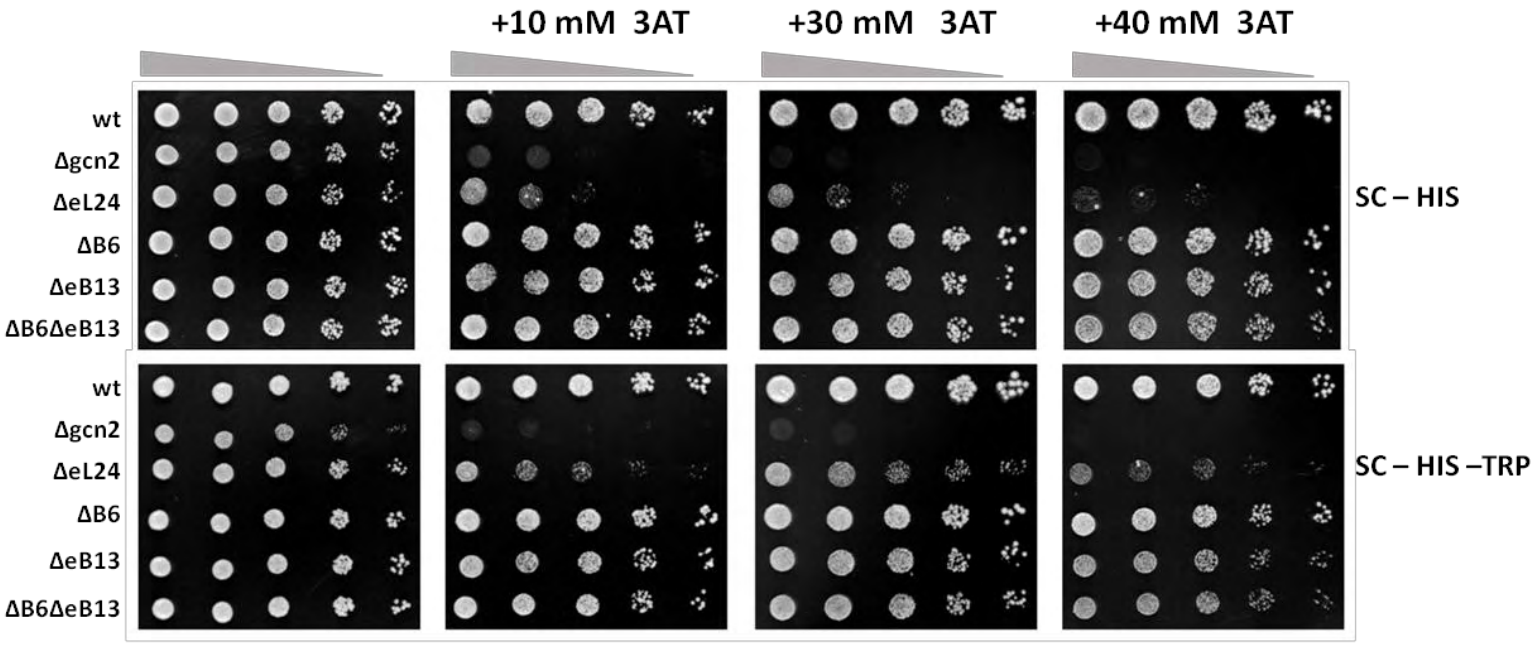

***Figure 14**. Sensitivity analysis to histidine starvation by serial dilution spot test (2nd set). The mutants were plated on selective medium plates (SC-HIS and SC-HIS-TRP) supplemented with different concentrations of 3AT and incubated at 30°C for 2-4 days. Serial dilutions were spotted from highest concentration to lowest (gray bar on top).*

In the 2nd set were analyzed the mutants, in which only protein eL24 was deleted from the genome (TYSC612) and the 65 amino acid long version of it or/and the version carrying the alanine mutations were expressed from the plasmid. The strain carrying the alanine mutations (ΔB6) is growing like wild type. ΔeB13 mutant exhibits a slight sensitivity to 3AT when SC-HIS-TRP with 40 mM 3AT was used, and so does the ΔB6ΔeB13 mutant, probably caused by the deletion of eB13. ΔeL24 strain seems to be growing better on the SC-HIS-TRP plates, whereas the rest of the strains have no visible difference (Figure 14).

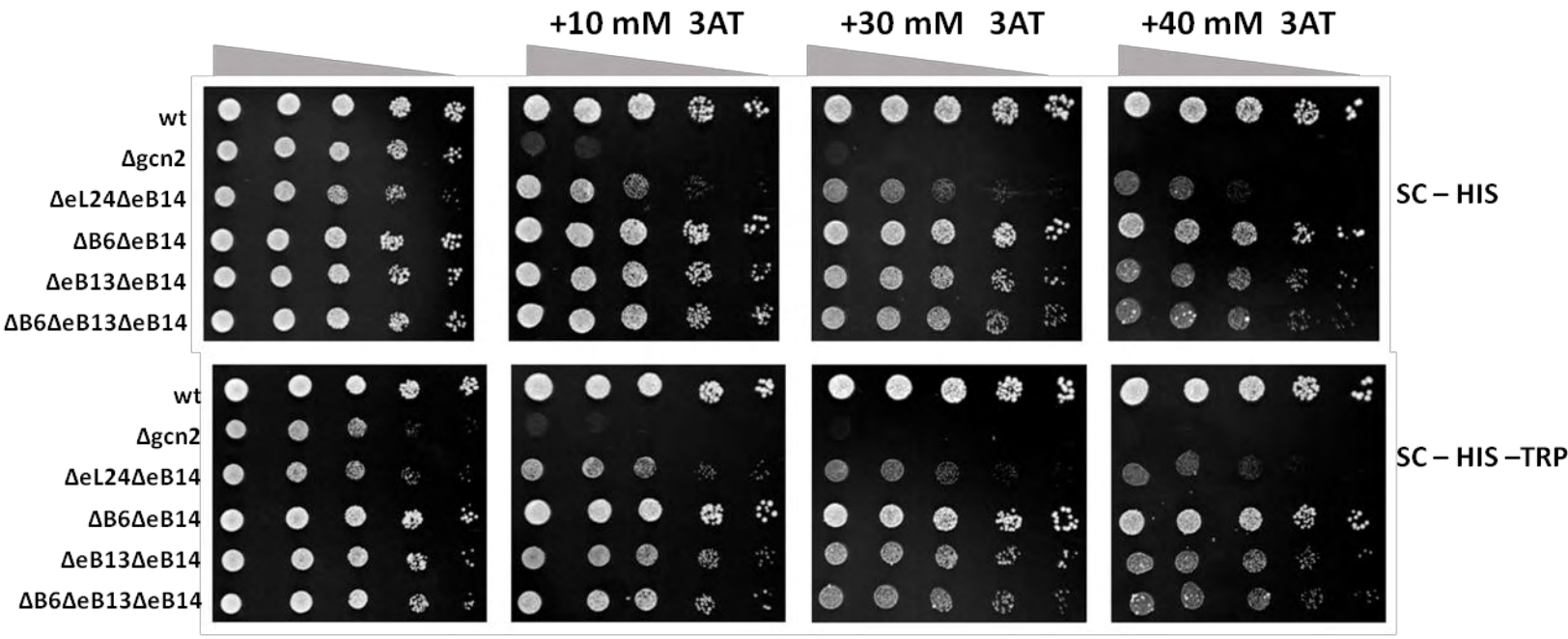

***Figure 15**. Sensitivity analysis to histidine starvation by serial dilution spot test (3rd set). The mutants were plated on selective medium plates (SC-HIS and SC-HIS-TRP) supplemented with different concentrations of 3AT and incubated at 30°C for 2-4 days. Serial dilutions were spotted from highest concentration to lowest (gray bar on top).*

In the 3rd set the strains with both eL24 and eL41 proteins deleted from their genome (TYSC610) were analyzed. The mutant ΔeL24ΔeB14 displays sensitivity to 3AT, while ΔB6ΔeB14 is growing like wild type. The last two strains are both growing slower than the wild type but better than ΔeL24ΔeB14 (Figure 15). This phenotype could be attributed to the deletion of bridge eB13, since ΔB6ΔeB14 mutants have a phenotype similar to wild type. This is also consistent with the results of figure 14, where eB13 mutants are growing slower than wild type and better than eL24 mutants. The deletion of protein eL41, as in the temperature sensitivity analysis, does not confer to an enhanced phenotype of the strains in figure 14. The mutants are growing similarly on both media.

4.4. Analysis of translation regulation using *GCN4-lacZ* reporter constructs

The translational regulation of *GCN4* expression is mediated by uORFs 1 to 4 and is linked to the levels of ternary complex (TC) in the cell. When uORFs 3 and 4 are translated the ribosomes do not reinitiate in the *GCN4* main ORF start codon, while translation of uORF1 enables ribosomes to overcome the translational barrier at uORFs 3 and 4 and express *GCN4* in starved cells where

ternary complexes are limited (Hinnebusch, 2005). Thus, *GCN4* can serve as a specific reporter for translation initiation activities. The best method to assess *GCN4* expression and verify translational regulation is through the use of *GCN4–lacZ* reporter constructs.

The *GCN4-lacZ* fusion plasmids with a modified leader region that were used for this assay are p180, p227 and pM226 (Figure 16). Plasmid p180 contains the wild type *GCN4* mRNA leader with all 4 uORFs. Plasmid p227 is a derivative of p180 containing substitution mutations in the initiation codon of each of the four uORFs of *GCN4* (Mueller *et al.,* 1987). In the absence of all uORFs, high-level translation of *GCN4* occurs independently of eIF2 phosphorylation. The expression of this reporter indicates overall expression efficiency. In plasmid pM226 only uORF1 remains and is extended to a site 130 nucleotides downstream of the *GCN4* start codon, making the ribosome incapable to reinitiate translation at *GCN4* after translating the modified uORF1. Therefore, *GCN4* can be translated only by the ribosomes that skipped translation at uORF1 and increased expression of this reporter would indicate increased frequency of leaky scanning of uORF1 (Lee *et al.,* 2007). These plasmids use the *URA3* gene as a selectable marker in yeast, therefore all media used for this assay does not contain uracil and the transformations with the reporter plasmids were plated on SC-URA or SC-URA-TRP (for the double transformations with the pRS plasmids).

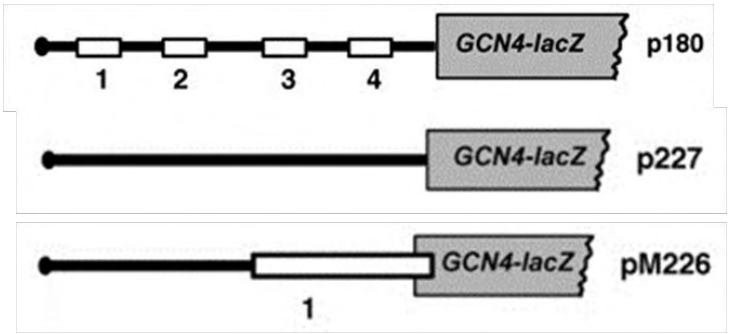

Figure 16. *The structure of the GCN4-lacZ constructs and their leader regions. The uORFs are represented as white boxes (adapted from Ghosh et al, 2014).*

The cells were grown at 30°C in the absence of 3AT or presence of 10 mM 3AT for 6 hours and then assayed for β-galactosidase activity, as described in Materials and Methods. A concentration of 30 mM of 3AT was also tested but the induction of the Gcn4-LacZ was not significantly increased. Different media were tested on the strains before choosing which ones would be used. In particular, SC-URA-HIS and SC-URA-TRP-HIS, containing either glucose or galactose, and Synthetic Minimal (SM) with galactose were tested on wild type (TYSC606) cells

with the p180 plasmid and on wild type (TYSC606) cells with the p180 and pRS314 plasmids. The results are presented in figure 17.

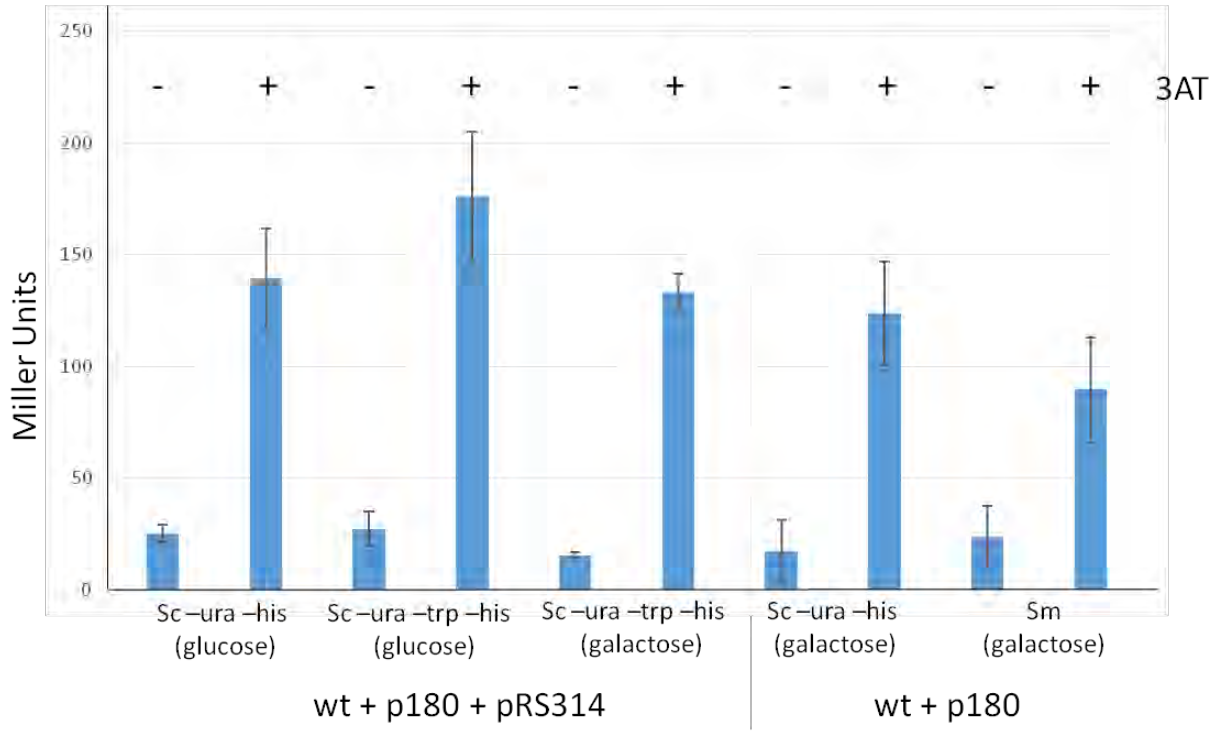

Figure 17. *Different media tested on wild type cells transformed with the p180 plasmid and the pRS314. The absence or presence of 10 mM 3AT is shown as (-) and (+) respectively. The activity of β-galactosidase is expressed in Miller Units. The number of independent cell cultures that were analyzed for each media is N=3-9. The bars are indicating standard errors.*

The cells were growing faster on glucose media and the induction of Gcn4-LacZ reporter was similar to the galactose media (Figure 17). Thus, it was decided to proceed using the media containing glucose, SC-URA-HIS and SC-URA-TRP-HIS.

Since the strains with protein eL24 missing were the ones that suffered in the histidine starvation assay, it was decided they would be assayed for β-galactosidase activity. To determine if expression of *GCN4* is impaired in ΔeL24 mutants, and therefore translational regulation is affected, the cells were transformed with the p180 plasmid. Specifically, wild type (TYSC606) and ΔeL24 (TYSC612) yeast strains were both transformed with the p180 and pRS314 plasmids. The results of the assay are shown in figure 18.

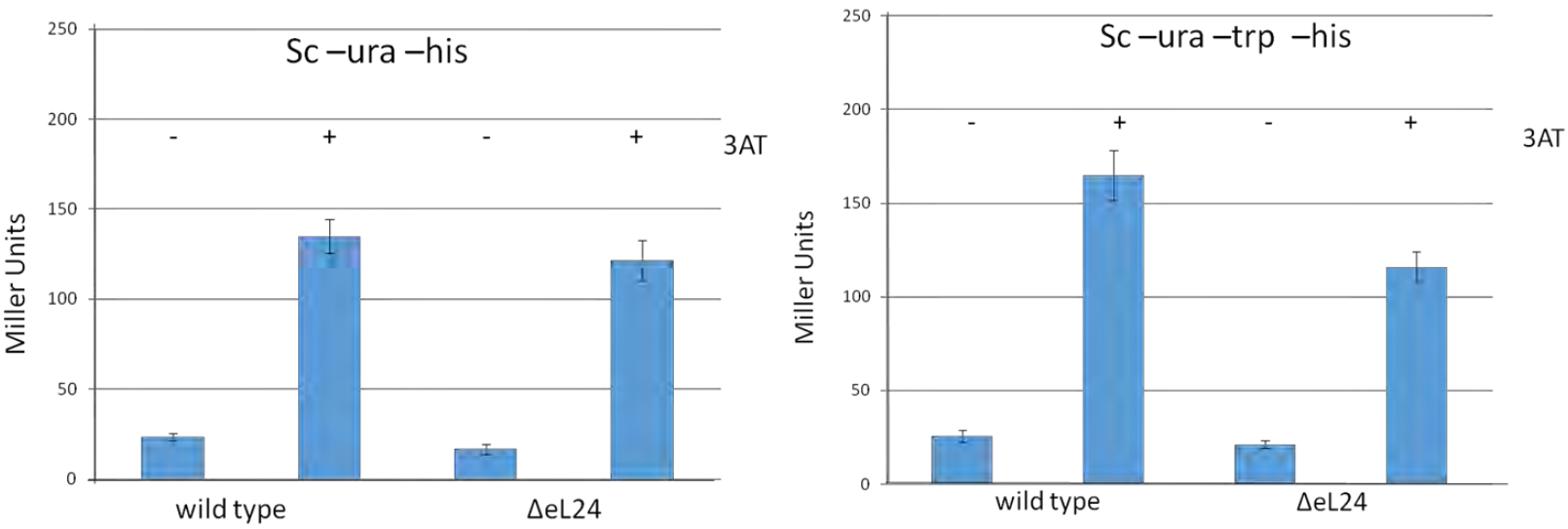

Figure 18*. Analysis of GCN4-reporter in ΔeL24 strains. Transformants carrying p180 (GCN4-lacZ) were grown in the absence (-) or presence (+) of 10 mM 3AT. Two different glucose media were used to grow the cells (SC-URA-HIS and SC-URA-TRP-HIS). The activity of the reporter is expressed in Miller Units. The number of independent cell cultures that were analyzed is n=12. The error bars indicate 95% confidence intervals of at least three independent experiments.*

When the cells are grown in SC-URA-HIS medium the reporter activity of the wild type was increased 5.7 times after histidine starvation, while in SC-URA-TRP-HIS medium the induction was increased 6.3 times. ΔeL24 mutants had a 7-fold rise in the expression of the reporter when grown in SC-URA-HIS medium. Interestingly, when the ΔeL24 cells were grown in SC-URA-TRP-HIS medium the induction of the reporter was only 5.4 times higher after starvation. When grown in SC-URA-HIS medium the induction of the reporter in ΔeL24 cells did not differ significantly compared to the induction in wild type cells. However, when grown in SC-URA-TRP-HIS medium the induction of the reporter in ΔeL24 cells did have a statistical difference compared to the induction in wild type cells. The statistical significance was determined using t-test.

Plasmid p227, the derivative of p180 lacking all four uORFs, was also assayed to eliminate any possible effects on the transcription of *GCN4-lacZ*. Wild type (TYSC606) strains were transformed with the p227 and pRS314 plasmids. ΔeL24 (TYSC612) yeast strains were transformed with the p227 and pRS314, pRS314 – eL24 and pRS314 – $eL24_{1\text{-}65}$ plasmids. The results are presented in figure 19.

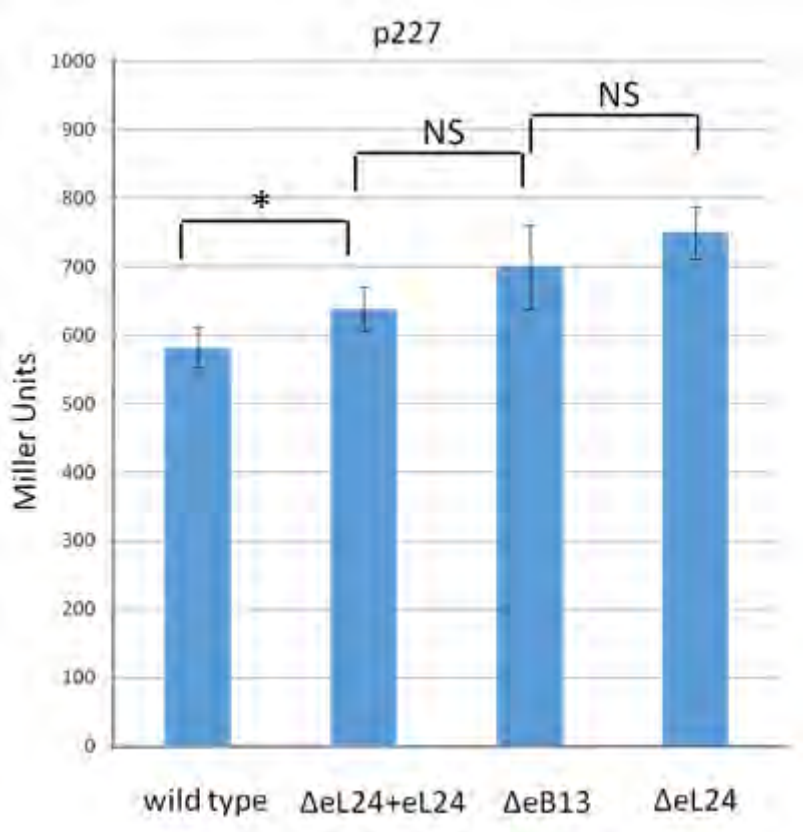

Figure 19. *Analysis of transcriptional induction using the reporter plasmid p227. Transformants carrying the p227 plasmid were grown in SC-URA-TRP glucose medium and were collected when they reached an* OD_{600}*=0.9. The activity of the reporter is expressed in Miller Units. The number of independent cell cultures that were analyzed is n=9-13. The error bars indicate 95% confidence intervals of at least four independent experiments. The non-significant differences are symbolized with NS. The significant difference is symbolized with an asterisk.*

As expected the induction with the p227 plasmid is very high (600-700 Miller Units) compared to the induction using the p180 plasmid. The induction from the mutant expressing protein eL24 is not significantly different from the eB13 mutant and the induction of eB13 mutant has no significant difference from that of Δel24 with the pRS314 plasmid. However, between the wild type and the ΔeL24 mutant with eL24 expressed from the plasmid there is a considerable difference in the induction. To better explain this phenomenon more experiments need to take place.

To investigate the possibility of a leaky scanning of uORF1, mutants containing plasmid pM226 with an elongated uORF1 were assayed. The same mutants as with plasmid p227 were created. The results are shown in figure 20.

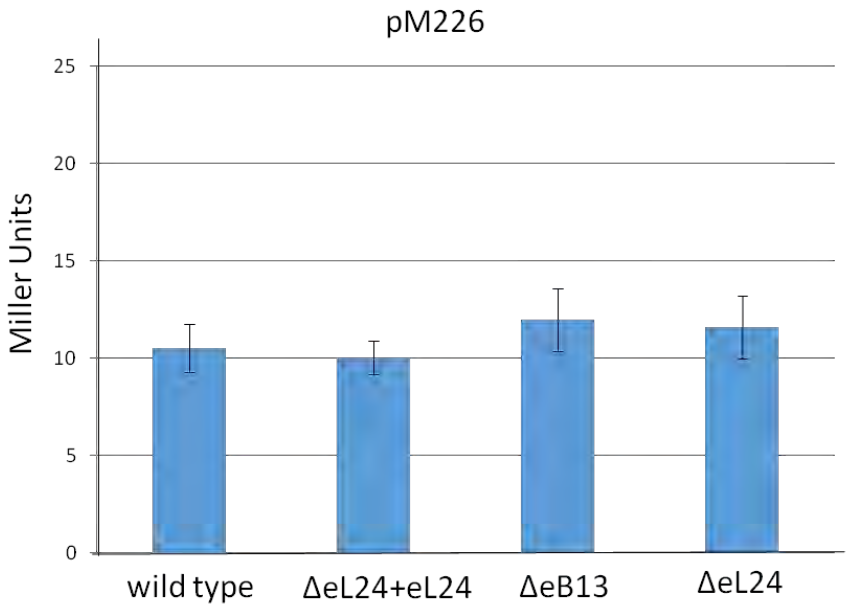

Figure 20. *Analysis for leaky scanning of uORF1 start codon. Transformants carrying the pM226 plasmid were grown in SC-URA-TRP glucose medium and were collected when they reached an OD600=0.9. The activity of the reporter is expressed in Miller Units. The number of independent cell cultures that were analyzed is n=6-9. The error bars indicate 95% confidence intervals of at least three independent experiments.*

Among the wild type and the mutants that were tested, there is no significant difference. Therefore, none of the mutants are accountable for leaky scanning. Nevertheless, the number of cultures that were tested is small to reach to a definite conclusion. Thus, more experiments should be carried out.

5. Discussion

In this work the importance of the eukaryote-specific intersubunit bridges eB13, eB14 and the conserved bridge B6 in ribosome functionality was examined. The core components of these bridges are the eukaryote-specific proteins eL24 and eL41. Specifically, protein eL24 makes up most of bridge eB13 as well as the conserved bridge B6 (Spahn *et al.,* 2001). Because eL24 is eukaryote-specific its bacterial counterpart that makes up B6 in prokaryotes is L19 (Yusupov *et al.,* 2001). Protein eL24 consists of an N-terminal globular domain, a flexible linker region in the middle and a long C-terminal α-helix (Figure 6a). The C-terminal α-helical domain forms the eB13 intersubunit bridge via its contacts with eS6. The conserved protein uL3 also helps to form the bridge (Kisly *et al.*, 2016; Ben Shem *et al.*, 2011). Additionally, eL24 contributes to the creation of the conserved bridge B6 in eukaryotes. Particularly, the arginine residues 43 and 47 in the N-terminal domain of protein eL24 contact the 18S rRNA helix 44, thus forming bridge B6 (Ben Shem *et al.* supplementary, 2011). Bridge eB14 is formed by protein eL41, the smallest yeast protein, and its interactions with the 18S rRNA. Protein eL41 consists of a single α helix that is enveloped by conserved core rRNA (Ben Shem *et al.*, 2011).

Mutants lacking one bridge or a combination of them were created and tested using different environmental stresses. Specifically, their responses were assessed after temperature stress and amino acid deprivation. The effects on translation regulation and translation initiation accuracy were also examined.

To assay for temperature sensitivity serial dilutions of the strains were spotted on YPD plates and incubated in 5 different temperatures (36^{o}C, 30 ^{o}C, 25 ^{o}C, 20 ^{o}C and 16 ^{o}C) for 2 to 7 days. From the obtained phenotypes it was apparent that mutants with the entire eL24 protein missing (ΔeL24) were affected the most and displayed cold sensitivity (Figure 10). However, when only the N-terminal domain of eL24 was expressed (ΔeB13) the strains were growing slower than wild type but better than ΔeL24 mutants and were cold sensitive only at 16^{o}C (Figure 11). Mutants with eB14 deletion (ΔeB14) had no evident phenotype and their growth rate was exactly like wild type (Figure 10). Moreover, in strains that more than one bridge was missing, with one of them being eB14, the phenotypes observed were similar to those where eB14 was present (Figure 12). Strains that were carrying the alanine mutations in arginines 43 and 47 of eL24 (ΔB6) were also growing like wild type (Figure 11). When combined with the deletion of the other bridges, ΔB6 appears to be rescuing the phenotype (Figures 11 and 12). This observation is suggestive of a phenomenon called cross-protection, in which adaptation to one form of stress often helps cells to survive other stress conditions (Berry & Gasch, 2008). Thus, in cells where ribosome functionality is compromised due to their inability to form bridge B6 a general stress response is activated. This activation may provide protection against various environmental stress conditions, such as temperature stress, in the B6 intersubunit bridge mutants.

To examine how amino acid starvation impacts the cells that lack one or more of the intersubunit bridges being studied, histidine deprivation was induced. Prototrophic for histidine cells, with an active *HIS3*$^{+}$ locus, were spotted on SC-HIS and SC-HIS-TRP plates containing different concentrations (10 mM, 30 mM, 40 mM) of 3 AT, a competitive inhibitor of imidazoleglycerol phosphate dehydratase (His3), and incubated for 2 to 4 days at 30^{o}C. Yeast can overcome amino acid starvation by translating the transcription factor Gcn4, which activates the transcription of genes required for amino acid biosynthesis. As a control for the General Amino Acid Control response a Δ*gcn2* strain was used, which is sensitive to amino acid starvation, since Gcn4 is not expressed in this strain (Hinnebusch and Natarajan, 2002). Accordingly, the Δ*gcn2* strain could not form any colonies in the presence of 3AT, while wild type cells were not hindered by the drug. The mutant that showcased the strongest sensitivity was ΔeL24 (Figure 13). When both eL24 and eL41 were deleted (ΔeL24ΔeB14), the mutant was sensitive to 3AT but the phenotype was not as strong as with ΔeL24 (Figure 13). However, the deletion of eL41 did not rescue any other phenotypes, when it was combined with the other mutations (Figure 15), and ΔeB14 was not sensitive and was growing like wild type (Figure 13). Strains with the linker region and the C-terminal domain of eL24 deleted (ΔeB13) were only slightly sensitive to 3AT (Figure 14). Mutants lacking the conserved bridge B6 (ΔB6) were not sensitive to histidine deprivation and had the same phenotype as wild type cells (Figure 14). Moreover, when deletion of bridge B6 was in conjunction with other mutations, there was no effect on the phenotypes of these mutations (Figures 14 and 15). A difference between the two media was also observed. On some plates the strains were growing better when spotted on SC-HIS-TRP medium than on SC-HIS.

Since the only strains that were affected by the previous environmental stresses were the ΔeL24 and the ΔeB13, the analysis of translational regulation using *GCN4–lacZ* reporter constructs was performed with these. The mutants were transformed with the p180 (all four uORFs), p227 (all four uORFs deleted) and pM226 (only elongated uORF1) plasmids containing the *GCN4-lacZ* fusion (Figure 16). The cells were grown in SC-URA-HIS and SC-URA-HIS-TRP media, as described in the materials and methods section, and the activity of the reporters was measured using a liquid β-galactosidase assay and expressed in Miller Units.

The ΔeL24 strain that was transformed with the p180 plasmid was assayed for *GCN4–lacZ* expression after growing under inducing conditions (histidine starvation). Only when the cells were grown in SC-URA-TRP-HIS medium, the induction of the reporter of ΔeL24 cells had a significant difference statistically compared to the induction in wild type cells (Figure 18). A possible explanation for the decrease of *GCN4–lacZ* expression in eL24 mutants could be due to ineffective ribosomal subunit joining. Protein eL24 is one of the later proteins to be incorporated into the large ribosomal subunit, where it then regulates the joining of the 60S subunit to the small 40S subunit. The absence of protein eL24 alters the protein-protein cross-linking patterns in the yeast 60S ribosomal subunit and this alteration is involved in 60S to 40S subunit interactions (Dresios *et al.*, 2000). Cryo-electron microscopy reconstruction of the *Saccharomyces cerevisiae* 60S indicates that eL24 resides on a surface of the 60S ribosomal subunit close to where the eukaryotic initiation factor 6 (eIF6) contacts the 60S (Gartman *et al.,*

2010). The anti-assembly factor, eIF6, binds to the pre-60S ribosomal subunit near eL24 and prevents premature association of the 40S and 60S ribosomal subunits. Following 60S maturation, eIF6 is released from the mature 60S, allowing for the joining of the 40S and 60S subunits to form the 80S ribosome and further assembly of polysomes (Ceci *et al.,* 2003; Brina *et al.*, 2011). Depletion of protein eL24 prevents eIF6 release and 80S formation (Wilson-Edell *et al.,* 2014). Therefore, the Gcn4 mRNA is not translated and the cells fail to induce the starvation response.

Mutants ΔeL24, ΔeB13 and ΔeL24+eL24 were transformed with plasmid p227 and were grown under non-inducing conditions. Plasmid p227 has nucleotide substitutions in the initiation codons of each of the four uORFs. These mutations render the upstream ORFs nonfunctional for translation control, and any increase of Gcn4p-LacZ enzyme activity from p227 would be attributable to transcriptional control (Hinnebusch, 1997). In this work, all mutants had a significantly increased expression of the reporter construct compared to the wild type (Figure 19). Therefore, this increased expression is attributable to transcriptional induction of *GCN4.* Interestingly, when protein eL24 is expressed from the plasmid (ΔeL24+eL24) the induction of the construct is higher compared to wild type, where the protein is expressed from the genome. In order to explain this phenomenon more experiments are required.

To examine the possibility of a failed initiation of translation at uORF1 (leaky scanning), mutants ΔeL24, ΔeB13 and ΔeL24+eL24 were transformed with plasmid pM226 and were grown under non-inducing conditions. Since the leader region of pM226 is modified, so that uORF1 is elongated and overlaps with the *GCN4* main ORF, *GCN4* can be translated only by the ribosomes that had failed to initiate translation at uORF1. Thus, increased expression from this reporter would indicate increased frequency of leaky scanning of uORF1 (Lee *et al.,* 2007). In this study, the mutants had no significant difference compared to wild type. Therefore, the loss of bridges does not affect the accuracy of translation initiation (Figure 20).

In summary, the effect of eukaryote specific intersubunit bridges eB13, eB14 and the conserved bridge B6 in ribosome functionality under two different stress conditions was studied. For the temperature sensitivity analysis serial dilution spot tests were used. For histidine starvation first serial dilution spot tests were performed and then the activity of the *GCN4-lacZ* reporter construct was analyzed. It was discovered that only eB13 intersubunit bridge is essential for coping with temperature stress or amino acid starvation conditions, since ΔeB13 cells were cold sensitive and slightly 3AT sensitive. Protein eL24 which is the main component of bridges eB13 and B6 is a nonessential protein (Steffen *et al.,* 2012). However, eL24 is required for surviving temperature stress and amino acid deprivation. The reporter activity of eL24 was assessed and was found not to be statistically different from wild type, when the cells were grown in SC-URA-HIS medium. However, when grown in SC-URA-TRP-HIS medium there was significant difference in the reporter activity of ΔeL24 cells compared to wild type. Mutants that contained only the N-terminal domain of the protein survived temperature stress and amino acid deprivation, although they had a reduced growth rate. Thus, the N-terminal part of the protein is essential for surviving the stresses that were tested.

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