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**Discovering links between
elongation factors and general amino
acid control in
*Saccharomyces cerevisiae***

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This thesis is presented in partial fulfilment of the requirements for the
degree of **Doctor of Philosophy (PhD)** in **Biochemistry** at Massey
University, Auckland, New Zealand

2011

Abstract

Continuous protein synthesis is essential for life; hence, a steady supply of amino acids must be maintained. In order to respond appropriately to amino acid shortages, cells need to constantly monitor their availability. Cells have a signal transduction pathway, called the general amino acid control (GAAC), for sensing and ameliorating amino acid shortages. Since the sensing occurs on translating ribosomes, the objective of this study was to investigate links between translation elongation and the general amino acid control in *S. cerevisiae*. In all eukaryotes, Gcn2 and its effector Gcn1 are responsible for monitoring amino acid availability. Active protein synthesis requires ekaryotic translation elongation factors (eEFs) to associate with translating ribosomes. This study focussed on two eEFs, eEF3 and eEF1A, and their potential role in GAAC.

Gcn1 has homology to eEF3, which suggests that both proteins utilise overlapping binding sites on the ribosome. Supporting this idea, it was found that over-expression of eEF3 caused sensitivity to amino acid analogues (AAA^s), suppressed the growth defect associated with constitutively active Gcn2, and impaired Gcn2 function. The C-terminal domain in eEF3 was found to be responsible for affecting Gcn2 function. Over-expression of this domain was sufficient for ribosome binding and for causing AAA^s. These findings suggest that eEF3 influences Gcn1 negatively.

For signal perception, Gcn1 and Gcn2 need to access the ribosomal A-site where eEF1A is functional. This suggests a link exists between eEF1A and GAAC. This link was confirmed by the discovery that eEF1A interacts with Gcn2 *in vivo*. The Gcn2 C-terminal domain was sufficient to precipitate eEF1A, independent of ribosomes, other yeast proteins and RNA. The interaction was lost under amino acid starvation conditions, suggesting that eEF1A is a negative regulator of Gcn2 activation under replete conditions.

This study reveals a link between translation elongation and GAAC. As eEF3 and eEF1A are known to interact with each other it is proposed here that they act in concert to inhibit Gcn1 and Gcn2 under replete conditions, hence suggesting a novel mechanism of Gcn2 regulation.

~ श्रद्धा सवुरी ~

(Faith & Perseverance)

- Sai Baba

Acknowledgements

No woman is an island – adapted from John Donne

Firstly I would like to thank my supervisors Dr. Evelyn Sattlegger and Prof. Barry Scott for taking me on. Thanks Evelyn for providing resources, advice, encouragement and guidance throughout my research. The projects have intellectually challenged me and allowed me to push my own boundaries. I'd like to thank Prof Barry Scott for guidance with administrative matters and advice.

I would like to acknowledge that this project was funded by the Marsden Fund council administered by Royal Society of New Zealand and Massey University Research Fund. I would like to also acknowledge Massey University for a doctoral scholarship and bursary fund. I would like to thank Dr. Evelyn Sattlegger for a year of financial support. I acknowledge travel grants from Institute of Molecular BioSciences and Education New Zealand to attend conferences where I presented my work.

I would like to thank Dr. Alan Hinnebusch for strains, antibodies and advice with my work. Thanks to Prof. Terri Kinzy for strains and antibodies. I also thank Dr. Beatriz Castilho for the Gcn2 antibodies, without which most of my work would not be possible.

I would like to thank Bei Cao my summer student for help with the eIF2-P time course experiments. I would like to thank Martina Dautel and Su Jung Lee for support in the lab with experiments and for doing my lab duties when I couldn't. I would also like to acknowledge Martina Dautel and Dr. Andrew Cridge for helpful scientific discussions. I thank the past and present members of the Sattlegger group for their support in the lab.

A special thanks to Jarod Young from whom I "borrowed" numerous chemicals, glass ware and equipment.

I am grateful to Martina Dautel, Dr. Andrew Cridge, Dr. Lutz Gehlen, Gabrielle Beans and Ralph Grand who tirelessly read through my thesis offering much needed and helpful suggestions. I would also like to thank Saumya Agrawal for help with formatting the thesis.

I want to thank the past and present members of building 11 who have made working in the lab a more pleasant experience. Thanks to my friends Martina, Gabby, Eli, Katie, Monique, Jarod and Lutz for keeping me sane through the thesis. A special thanks to Martina for tolerating me through my thesis writing. I'd like to thank Kanoj for believing in me and for never letting me give up ("If it was easy everyone would be doing one").

I am highly indebted to my parents for letting me pursue my dreams. Without their support, encouragement and love I would not have been able to get through. I thank my sister for always being there and for encouraging me to go on.

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Abbreviations

In addition to the chemical symbols from the periodic table of elements and the *système international d'unités* (SI), the following abbreviations are used:

3AT	3 amino triazole
aa	amino acid
aa-tRNA	amino acylated tRNA
ABC	ATP-binding cassette
APS	ammonium persulphate
A-site	acceptor-site
ATP	adenosine triphosphate
bp	base pair
BSA	Bovine Serum Albumin
CIA	chloroform: iso-amyl alcohol
CIP	calf intestinal phosphatase
CTD	C-terminal domain
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DTT	dithiothreitol
EDTA	ethylenediamine tetra acetic acid
eEF1A	eukaryotic elongation factor 1A
eEF3	eukaryotic elongation factor 3
EF2	elongation factor 2
eIF2	eukaryotic initiation factor 2
E-site	exit-site
GAAC	general amino acid control
Gcn	general control non derepressible
GDP	guanosine diphosphate
GTP	guanosine triphosphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
His-RS	histidyl-tRNA synthetase
IPTG	isopropyl- β -D-thiogalactopyranoside
LB	luria-bertani
Ni-NTA	nickel-nitrilo triacetic acid
OD	optical density
ORF	open reading frame
p	plasmid
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEG	polyethylene glycol
PMSF	phenylmethanesulphonyl fluoride
PRS	post ribosomal supernatant
P-site	peptidyl donor site
PVDF	polyvinylidene difluoride
rpm	revolutions per minute
RNase	ribonuclease
RWD	RING finger proteins, WD-repeat-containing proteins, yeast DEAD-like helicases
SD	synthetic dropout
SDS	sodium dodecyl sulfate

SM	sulfometuron methyl
tRNA _i ^{Met}	Methionyl-tRNA
Tris	tris(hydroxymethyl)aminomethane
TAE	tris-acetate EDTA
TBS	tris-buffered saline
TBS-T	TBS-Tween
TE	Tris EDTA
TEMED	N,N,N',N'-Tetramethylethylenediamine
TOR	target of rapamycin
v/v	volume/ volume
WCE	whole cell extract
w/v	weight/ volume

Chapter 1 Introduction

Organisms (and cells alike) must adapt to their environment in order to grow and flourish. Hence they constantly monitor their environment and adapt their metabolism appropriately to suit their environment. This stress sensing and appropriate cellular response is known as signal transduction. There has been considerable interest among the scientific community in dissecting the signal transduction cascades in order to understand an array of cellular processes operating in diverse physiological conditions such as cellular response to stress.

1.1 Translational Control

Cells need to adapt and overcome various stress conditions to survive. They need to respond very quickly to sudden changes in the environment especially during cellular stress. Gene expression can be regulated at different stages like at the transcriptional or translational levels. Regulation of gene expression at the translational level allows instant and rapid response to stress compared to the transcriptional level. Considering the time required for synthesis and processing of *de novo* mRNA, using pre-existing mRNA by a controlled translational mechanism is better for dealing instantly with cellular stress (Holcik and Sonenberg, 2005). Among the known translational control mechanisms, one mechanism is mediated via the reversible phosphorylation of the eukaryotic translation initiation factor eIF2 α at a conserved Ser residue (Ser 51 in yeast) (Chen, 2000; Dever et al., 1992; Harding et al., 1999). Phosphorylation of eIF2 α is a crucial mechanism for adjusting the rate of protein synthesis in response to stress. This phosphorylation is catalysed by a family of eIF2 α kinases each activated by different cellular stress. Each member of the eIF2 α kinase family harbours a conserved protein kinase domain which is controlled by its own unique regulatory domain. This suggests that response to different stimuli is channelled through the same downstream events in this signalling cascade (Figure 1.1). This feature allows each kinase to respond to a specific kind of stress and hence the cell is sensitive to a wide variety of stresses (Figure 1.1). The eIF2 α kinases best studied so far in eukaryotes are:

- ❖ **Heme Regulated Inhibitor (HRI)**, activated under conditions of heme deficiency, oxidative stress, heat/osmotic shock (Chen, 2000).
- ❖ **Double Stranded (ds) RNA dependent Protein Kinase (PKR)**, activated by interferon and dsRNA during viral infection (Clemens, 1996).
- ❖ **General Control Non-repressible 2 (Gcn2)**, activated upon amino acid starvation/ serum deprivation (Berlanga et al., 1999; Ramirez et al., 1991; Sood et al., 2000), UV irradiation (Deng et al., 2002; Jiang and Wek, 2005) and RNA viruses (Berlanga et al., 2006).
- ❖ **PKR like Endoplasmic Reticulum Kinase (PERK/ PEK)**, is activated by unfolded proteins in the endoplasmic reticulum (Harding et al., 1999; Shi et al., 1998).

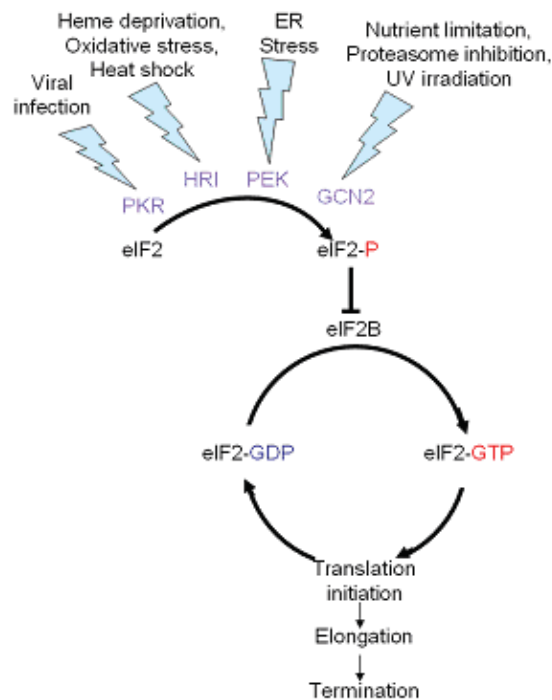


Figure 1.1: Representation of protein kinases being activated by different stress conditions to regulate the rate of protein synthesis via eIF2 α phosphorylation. (Adapted from (Wek et al., 2006))

1.2 Role of eIF2 α in translational initiation

eIF2 α , a translation initiation factor, along with initiator methionyl-tRNA (tRNA^{Met}) and GTP form a ternary complex (TC). This ternary complex binds to the 40S ribosomal subunit, resulting in the formation of a 43S pre-initiation complex (Figure 1.2) which binds near the 5' end of the mRNA (Hershey, 2000). The 43S pre-initiation complex then scans the mRNA in a 5'→3' direction searching for the AUG start codon (Kozak, 1989). Upon recognition, the GTP associated with the TC is hydrolyzed and is released as an inactive eIF2 α -GDP complex (Kimball, 1999). In order to participate in another round of initiation, the GDP bound to eIF2 α must be

exchanged with GTP. This is done by the guanidine nucleotide exchange factor eIF2B (Hershey, 2000) (Figure 1.2).

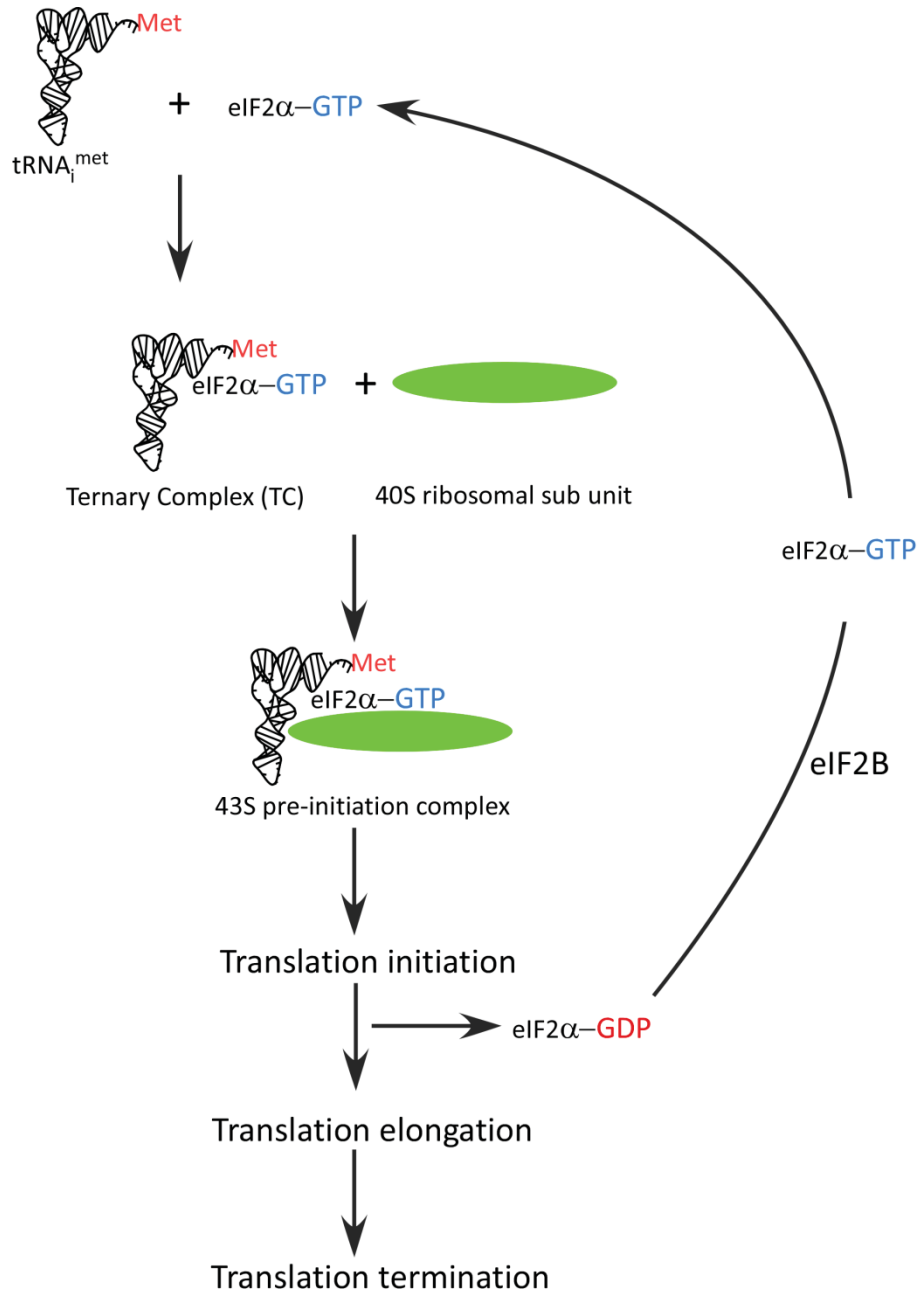


Figure 1.2: **Schematic representation of translation initiation.**

When phosphorylated, $eIF2\alpha$ is transformed to an inhibitor of $eIF2B$ (Pavitt et al., 1998), hence reducing the rate of GDP to GTP exchange on $eIF2\alpha$. As a result, there is a decrease in ternary complex levels leading to reduced global protein synthesis. Since the cellular levels of $eIF2\alpha$ molecules are higher than $eIF2B$, even a small

fraction of phosphorylated eIF2 α will have a remarkable effect on the ternary complex levels and hence on translation initiation (Holcik and Sonenberg, 2005).

1.3 Selective translation mediated by eIF2 α phosphorylation

Among the kinases described in section 1.1, HRI and PKR completely inhibit protein synthesis while PERK and Gcn2 reduce global protein synthesis and increase the expression of specific stress related genes. A well studied example of selectively increased translation is that of yeast transcriptional activator Gcn4 (Figure 1.3). A genome wide analysis (Jia et al., 2000; Natarajan et al., 2001) revealed that Gcn4 induces expression of about 10% of the genes in the yeast genome in amino acid starved cells, including genes coding for amino acid and vitamin biosynthesis, peroxisomal components and amino acid transporters. Gcn4 is a bZIP transcriptional activator of amino acid biosynthetic genes consequently leading to increased cellular levels of amino acids. In yeast this regulatory response has been well studied and is known as the general amino acid control (GAAC) (Hinnebusch, 2005) (Figure 1.3).

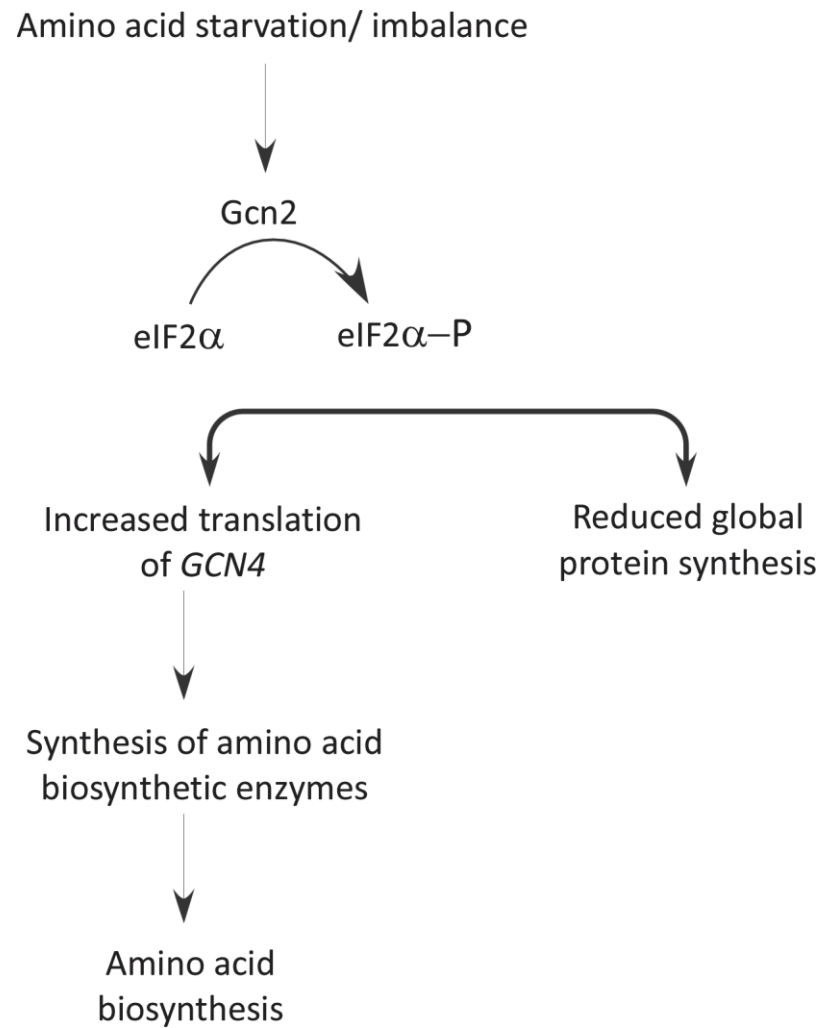


Figure 1.3: **Order of molecular events in the general amino acid control (GAAC).**

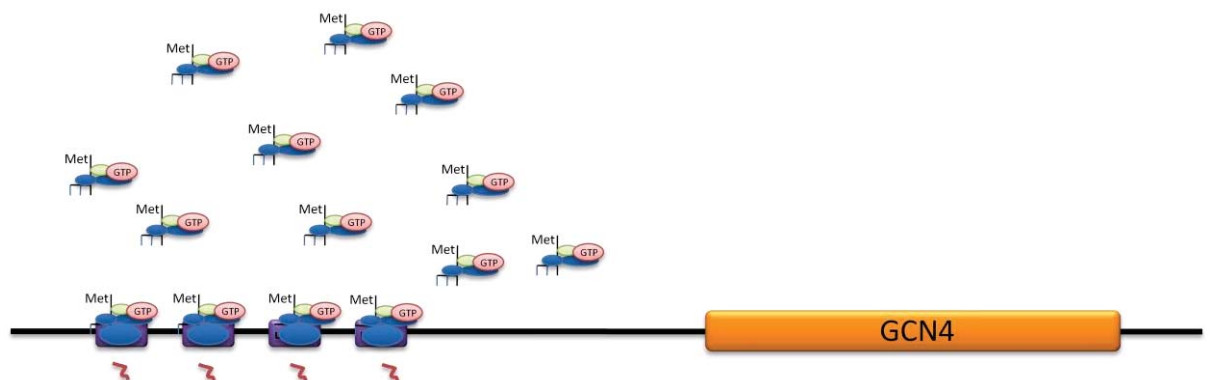
As mentioned earlier Gcn2 is found in all eukaryotes and is activated by amino acid starvation or imbalance. Upon activation Gcn2 phosphorylates eIF2 α , a consequence of which is the reduction in global translation while translation of *GCN4* mRNA is increased (Hinnebusch, 1988; Lanker et al., 1992; Rolfes and Hinnebusch, 1993). This increase in translation of *GCN4* mRNA is attributed to 4 short upstream open reading frames (uORFs) present in the leader sequence of *GCN4* mRNA (Mueller and Hinnebusch, 1986).

1.4 Mechanism of augmented *GCN4* mRNA translation

Although global translation is reduced, there is a selective translation of the *GCN4* mRNA when eIF2 α is phosphorylated. The mechanism of this augmented *GCN4* translation is outlined below.

Pre-initiation complexes bind near the 5' cap of *GCN4* mRNA and scan downstream. Upon finding the first AUG codon, that of pre-initiation open reading frame (uORF) 1, the pre-initiation complex forms an 80S initiation complex to translate uORF1. Most ribosomes that translate uORF1 do not dissociate from the mRNA immediately, but instead continue scanning the mRNA as 40S subunits without a ternary complex. Under replete conditions, the GTP bound eIF2 α (and hence ternary complex) is abundant. The ternary complex can now rapidly bind the 40S subunit, thereby allowing re-initiation of translation at the next AUG codon. Most of these pre-initiation complexes will reinitiate translation at uORFs 2, 3 and 4. However, unlike at uORF1, most 80S initiation complexes that translate uORF2, 3 or 4 dissociate from the mRNA and fail to reach the *GCN4* start codon. Hence *GCN4* translation is very minimal. This state of *GCN4* expression is called “repressed” (Figure 1.4 A).

A.



1.5 eIF2 α kinase: Gcn2

In yeast, under non-starvation conditions Gcn2 is an inactive kinase. Most eIF2 kinases dimerise upon activation, unlike Gcn2 which exists as an inactive dimer (Qiu et al., 1998). It is activated by deacylated tRNA, which is the signal for amino acid starvation. All forms of deacylated tRNAs investigated so far can bind the HisRS (Histidyl tRNA Synthetase like) domain of Gcn2 (Dong et al., 2000) (Figure 1.5), a regulatory domain that resembles histidyl tRNA synthetase (Wek et al., 1995). The interaction of the deacylated tRNA with the HisRS domain is known to induce a conformational change in Gcn2 which is necessary for its activation (Dong et al., 2000). In yeast, it has been shown that the extreme C-terminal segment binds the ribosomes (Ramirez et al., 1991; Zhu and Wek, 1998) and also contains the main dimerisation determinants (Qiu et al., 1998). N terminal to the kinase domain (Figure 1.5) is the Pseudo Kinase (Ψ PK) domain of unknown function that resembles a truncated kinase domain. N terminal to this is a highly charged region (+/-) of unknown function. The N-terminal domain is highly conserved and binds the Gcn1/20 complex (Garcia-Barrio et al., 2000).

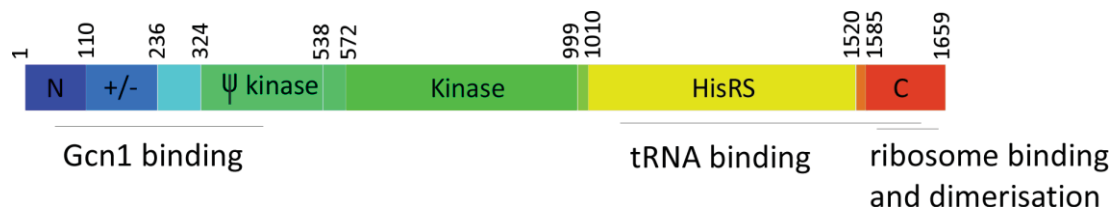


Figure 1.5: **Schematic representation of domains in Gcn2**

The different domains of Gcn2 are represented with various colours starting at the N terminal domain (dark blue) and a highly charged region (+/-, blue) bind Gcn1. The pseudo kinase domain (Ψ kinase, green) has homology to a truncated kinase domain and is non-functional as a kinase. The HisRS domain (yellow) is involved in binding uncharged tRNAs, the signal for amino acid starvation. The C terminal domain (orange) of Gcn2 is known to bind the ribosome, participates in tRNA binding and is responsible for dimerisation of Gcn2 (Hinnebusch, 2005).

1.6 Effector proteins Gcn1 and Gcn20

For the phosphorylation of eIF2 α , Gcn2 requires two additional proteins called Gcn1 and Gcn20. Gcn1 is a very large protein (296kDa) with its central region having a strong sequence similarity to the N terminal segment of fungal ELongation FFactor 3 (eEF3) (Marton et al., 1993). eEF3 assists the release of deacylated tRNAs from the ribosomal exit (E) site. This stimulates the delivery of amino acylated tRNAs to the acceptor (A) site by eEF1A/GTP (Chakraborty, 1999).

The N-terminal domain of Gcn20 (first 117 amino acids) binds the eEF3 like region in Gcn1 (Marton et al., 1993). The rest of Gcn20 has sequence similarity to the C-terminal portion of eEF3 (Dealdana et al., 1995). Thus the Gcn1/Gcn20 complex that is formed has domains juxtaposed such that it resembles an entire eEF3 molecule (Figure 1.6).

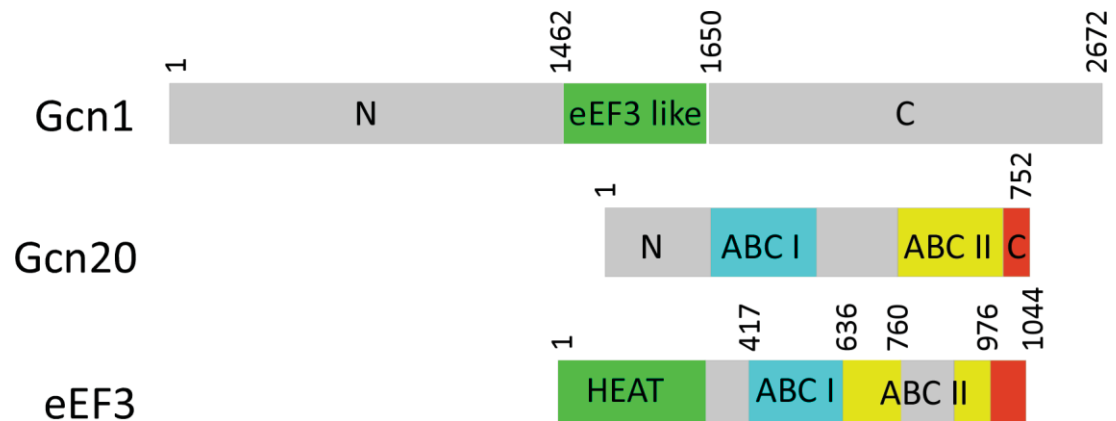


Figure 1.6: Representation of the domains in Gcn1 and Gcn20. The domains are juxtaposed when in a Gcn1/20 complex, resembling the fungal Elongation Factor 3 (eEF3)

Different domains of Gcn1 and Gcn20 that show homologies to eEF3 are depicted in colour. Non homologous regions are depicted in grey. HEAT repeats are shown in green and are found in the central region of Gcn1 (eEF3 like) and N-terminal region of eEF3 (HEAT). The turquoise colour represents the ATP Binding Cassette I (ABC I) and yellow represents ATP Binding Cassette II (ABC II) found in the C-terminal regions of both Gcn20 and eEF3. However in the case of eEF3 there is a Chromodomain (grey insert flanked by yellow) inserted within the ABC II.

Yeast *GCN1*, like *GCN2*, was found to be non-essential under non-starvation conditions but is required for wild-type growth when cells are starved for amino acids (Marton et al., 1993). Gcn1 was shown to be a translational activator of *GCN4* (Marton et al., 1993), since loss of Gcn1 function abolished *GCN4* derepression, suggesting that Gcn1 may have a positive role in the GAAC. It was also shown that Gcn1 is required *in vivo* for eIF2 α phosphorylation catalyzed by Gcn2, further confirming the positive role of Gcn1 in assisting Gcn2 function.

Gcn1 and Gcn20 have been shown to be necessary for full Gcn2 activation by genetic studies in *S. cerevisiae*. Deletion of *GCN1* abolished eIF2 α phosphorylation by Gcn2 (Marton et al., 1993), while deletion of *GCN20* reduced the phosphorylation of eIF2 α by Gcn2 (Dealdana et al., 1995), hence preventing *GCN4* translation and its derepression. Lack of Gcn4-mediated gene expression can be scored by the inability of cells to grow on medium causing amino acid starvation.

This phenotype is called Gcn⁻ phenotype. Gcn1 and Gcn20 are not required for the expression (Dealdana et al., 1995; Marton et al., 1993) of *GCN2* or for its *in vitro* kinase activity (Dealdana et al., 1995; Marton et al., 1993) since the kinase was active from a *GCN1* deletion strain. This suggests that Gcn1 and Gcn20 must be involved in transmitting the starvation signal to Gcn2 *in vivo*. Further support for this hypothesis, is provided by the observation that Gcn1 binds Gcn20 to form a protein complex (Dealdana et al., 1995) and this complex binds Gcn2 at the N-terminal domain (Garcia-Barrio et al., 2000). The interaction of Gcn1 and Gcn20 complex (Gcn1/20) with Gcn2 is required for Gcn2 to function *in vivo* (Garcia-Barrio et al., 2000). Moreover, the N-terminal fragment of *Drosophila* Gcn2 was shown to interact with *S. cerevisiae* Gcn1 (Garcia-Barrio et al., 2000) indicating that the interaction is conserved in evolution. Hence the mechanism of responding to nutrient limitation seems to be conserved among eukaryotes.

1.7 Role of Gcn1 in activation of Gcn2

Some regions in Gcn1 have known functions. It was shown that Gcn2 binds Gcn1 via its N-terminal domain both *in vitro* and *in vivo* (Garcia-Barrio et al., 2000). This N-terminal domain was called the GI domain (Kubota et al., 2000) (Gcn2 and IMPACT, two proteins found to share such a domain), now known as the RWD domain. The amino acids 2052-2428 in Gcn1 are sufficient in binding the N-terminus of Gcn2 *in vitro* (Sattlegger and Hinnebusch, 2000). For Gcn2 to be activated, the binding of Gcn1 to Gcn2 is essential. This suggests that Gcn1 has a positive role in activating Gcn2 possibly by “holding” it in a correct conformation, or to recruit Gcn2 at the ribosome, or to channel deacylated tRNAs over to Gcn2. Mutating a single amino acid (Arg 2259) in Gcn1 nearly abolished Gcn2 binding both *in vivo* and *in vitro*, demonstrating that Arg 2259 in Gcn1 is a major contact point between Gcn1 and Gcn2 (Sattlegger and Hinnebusch, 2000). This Gcn1-Gcn2 interaction is necessary for Gcn2 function *in vivo* (Sattlegger and Hinnebusch, 2000).

Gcn1 seems to harbour several ribosome contact points that are spread over its first 2052 amino acids (Sattlegger and Hinnebusch, 2000). The binding of Gcn1 to the ribosome appears to be necessary for full Gcn2 activation. This was observed in a

mutated Gcn1 that was defective in ribosome binding but was still able to bind Gcn2 and Gcn20; however, it was unable to fully activate Gcn2 upon starvation for amino acids. This reduced activation was due to the inability of Gcn1 to bind ribosomes and not because of reduced protein expression of either Gcn1 or Gcn20 (Sattlegger and Hinnebusch, 2005). The mutations in *GCN1* that most severely affected Gcn2 function seem to be found in the region with similarity to the N-terminal domain of eEF3. This is rather interesting, because eEF3 is known to stimulate the release of deacylated tRNAs from the E-site and hence the delivery of aminoacylated tRNAs to the A-site (Trianaalonso et al., 1995). Similarly, Gcn1 may stimulate transfer of the deacylated tRNAs from the A-site to the HisRS domain of Gcn2. The observation that mutations in *GCN1*'s "eEF3 like" region affects Gcn2 activation supports the idea that Gcn1 may stimulate transfer of the deacylated tRNA from the A-site to the HisRS domain of Gcn2 (scenario 2 in Figure 1.7).

The sequence similarity of the Gcn1/20 complex to eEF3 suggested that the Gcn1/20 complex functions on the ribosome to mediate Gcn2 activation by channelling deacylated tRNA (Marton et al., 1997). In conjunction with this hypothesis Marton *et. al* (1997) showed that Gcn1 and Gcn20 associate with polysomes (elongating ribosomes). They saw that this association was enhanced when studies were performed in the presence of excess ATP. They attributed this ATP dependent association of the Gcn1/20 complex to the polysomes, to the ATP binding cassettes (ABCs) in Gcn20.

With regions on Gcn1 mapped and based on other observations, Sattlegger and Hinnebusch (2000) proposed a model for Gcn2 activation (Figure 1.7) wherein Gcn1 and Gcn2 are bound to the ribosome in a complex along with Gcn20. The role of Gcn1 in this complex could be to

- (a) aid in transfer of deacylated tRNA to the ribosomal A-site (1 in Figure 1.7).
- (b) aid transfer of deacylated tRNA from the A-site to the HisRS domain of Gcn2 for its activation (2 in Figure 1.7).
- (c) act as a scaffolding protein to hold Gcn2 in a conformation such that other accessory proteins can bind Gcn2 directly to transfer the deacylated tRNA.
- (d) hold Gcn2 in a correct position for binding deacylated tRNAs.

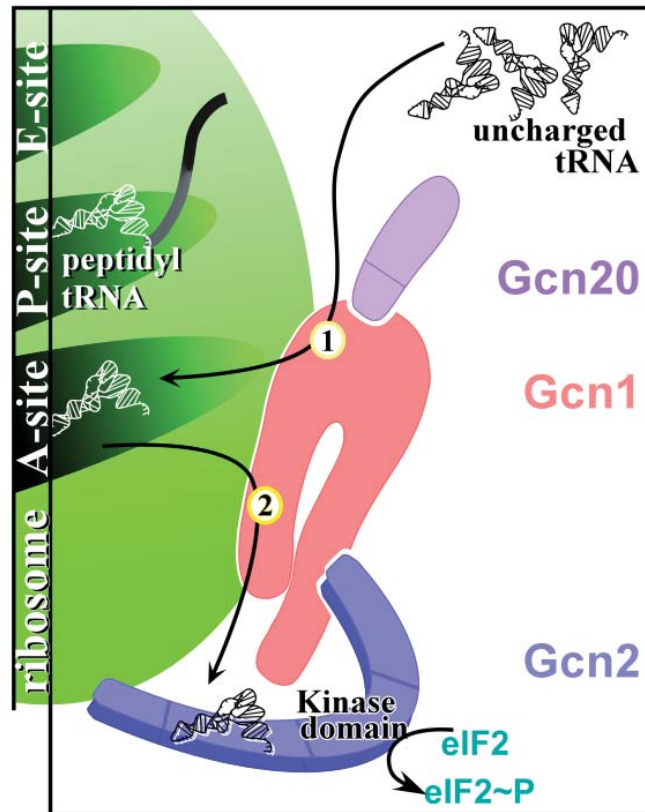


Figure 1.7: Model for Gcn1 mediated amino acid depletion sensing and activation of Gcn2 by deacylated tRNA- (Sattlegger and Hinnebusch, 2000)

1.8 Translational elongation

Protein synthesis occurs on the ribosome in three phases - initiation, elongation, and termination of translation. Protein production occurs during translation elongation. Each phase is driven by conserved and essential soluble factors that must cycle on and off the ribosome in an orderly fashion. Translation elongation is a cyclic process where during each cycle one amino acid is added to the growing peptide chain. The growing peptide chain is covalently attached to a tRNA bound in the ribosomal peptidyl-tRNA binding site (P-site). Each translation elongation cycle starts with the eukaryotic translation Elongation Factor 1A (eEF1A) delivering one aminoacylated tRNA to the ribosomal acceptor site (A-site) in a codon specific manner (Carvalho Da da et al., 1984). During peptide bond formation the peptide chain is transferred from the P-site bound tRNA to the amino acid attached to the A-site bound aminoacyl tRNA, resulting in a peptidyl-tRNA in the A site, and a

deacylated tRNA in the P-site. eukaryotic Elongation Factor 2 (eEF2) is essential for translocating the ribosome along the mRNA by one triplet codon, thereby placing the peptidyl-tRNA again in the P-site, and the deacylated tRNA in the ribosomal exit site (E-site) (Spahn et al., 2004). This results in a vacant A-site allowing the next elongation cycle to commence, i.e. codon-specific delivery of aminoacyl tRNA by eEF1A. The delivery of aminoacyl tRNA to the A-site is coupled with the release of deacylated tRNA from the E-site (Trianaalonso et al., 1995). In yeast, it has been shown that this coupled mechanism is triggered by a soluble ATPase called eukaryotic Elongation Factor 3 (eEF3) that binds close to the E-site (Dasmahapatra and Chakraburttty, 1981; Skogerson and Wakatama, 1976; Trianaalonso et al., 1995).

1.9 Fungal elongation factor 3 (eEF3)

eEF3 belongs to the family of ATP-binding cassette (ABC) proteins which consists of proteins involved in transport across membranes, DNA repair and translation. The membrane proteins of this class contain ATP/ADP-binding ABC domains, which transduce chemical energy derived from binding ATP or its hydrolysis into a 'burst' of mechanical energy (Jones and George, 2002). ABC proteins function as either homodimers or as twin-cassette proteins with two ABC domains within the same polypeptide. eEF3 belongs to the latter group and assists with the release of deacylated tRNAs from the ribosomal exit (E) site. This stimulates eEF1A-dependent binding of aminoacyl tRNA to the ribosomal A-site (Trianaalonso et al., 1995). The E-site release is coupled to A-site delivery such that there are always 2 tRNAs on the ribosome at any given time to maintain the reading frame. ATP hydrolysis by eEF3 is required in every elongation cycle to allow release of deacylated tRNA from the E-site. This ATPase activity of eEF3 is stimulated by ribosomes (Trianaalonso et al., 1995). eEF3 is known to interact with both ribosomal subunits (Andersen et al., 2006; Gontarek et al., 1998; Kambampati et al., 2000; Kovalchuke et al., 1998) and it competes with eEF2 for ribosomal binding (Kovalchuke et al., 1998). According to the three-site model of the ribosomal elongation cycle, E-site release is required for efficient A-site binding, hence it has been suggested that eEF3 functions at the ribosomal E-site (Kamath and Chakraburttty, 1989; Trianaalonso et al., 1995).

In non-fungal eukaryotes the factor equivalent to eEF3 has not been found, hence making eEF3 a unique factor in fungi. Conservation of the translational process implies that the coupling of E-site tRNA release with the A-site aminoacyl tRNA delivery is also highly conserved. This suggests that the function of the factor triggering this process is also highly conserved (e.g. ATPase activity) while other non-essential properties may not be. Supporting this idea it was found that metazoan ribosomes have an intrinsic ATPase activity, but not yeast ribosomes (Elskaya et al., 1997), suggesting that in metazoans a ribosome bound factor executes the eEF3-like function rather than a soluble factor. Interestingly, in *Escherichia coli* the protein RbbA (ribosome-bound ATPase) was identified which exhibits ATPase activity and is tightly associated with ribosomes (Kiel et al., 1999; Kiel and Ganoza, 2001), and thus may have an eEF3-like function.

eEF3 consists of several domains (Andersen et al., 2006) (Figure 1.8 A). The N-terminal domain contains repeats that were first found in the Huntingtin protein, eEF3, protein phosphatase 2A, and TOR, therefore called HEAT repeats (Andrade and Bork, 1995). HEAT repeats are predicted to be interaction surfaces for other proteins and/or nucleic acids (Andrade and Bork, 1995). In fact an eEF3 fragment encompassing this domain was reported to bind 18S rRNA (Gontarek et al., 1998), and structural analyses of ribosome bound eEF3 revealed that the eEF3 HEAT repeats interact with rRNA and ribosomal proteins of the small ribosomal subunit (Andersen et al., 2006). C terminal to the HEAT repeat domain is a 4 helix bundle (4HB) of unknown function. The eEF3 C-terminus contains two ATP binding cassettes (ABC) each with a Walker A and B motif. Unique to the ABC protein eEF3 is the insertion of a chromodomain within the C-terminal ABC (ABC2) cassette (Andersen et al., 2006). The chromodomain as well as the ABC2 portion N terminal to this domain bind to the small and large ribosomal subunit (Andersen et al., 2006).

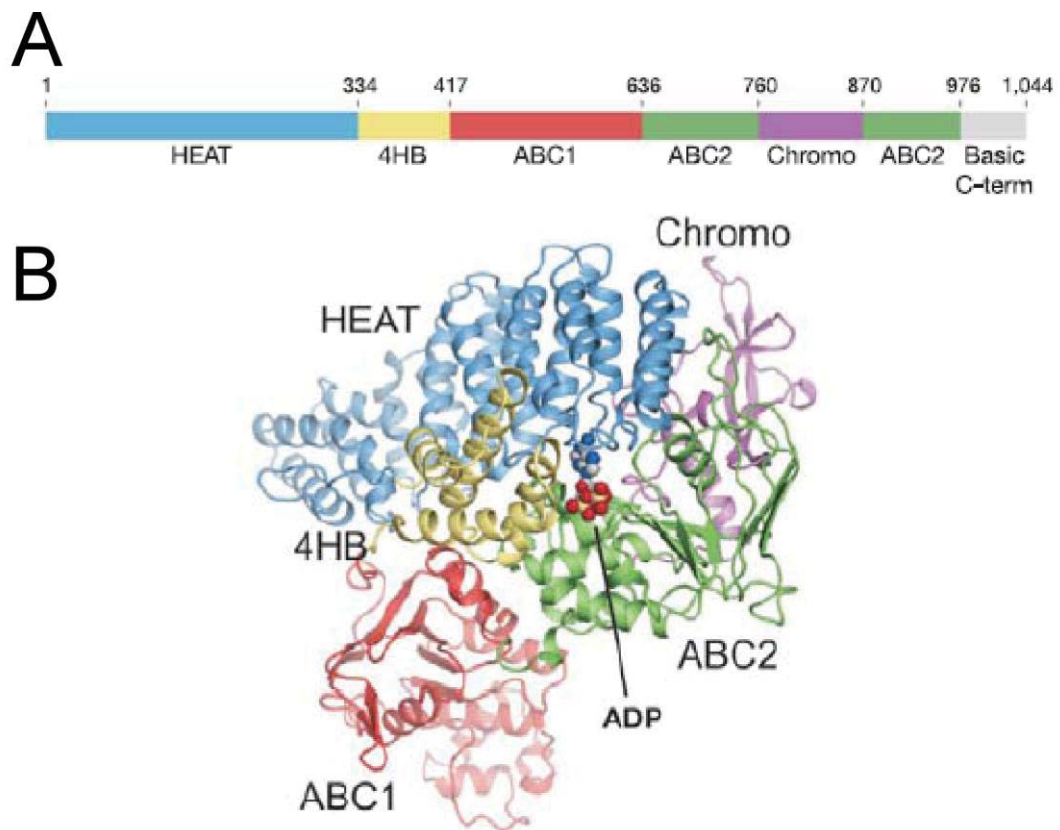


Figure 1.8: **(A) Representation of domains in eEF3, (B) Crystal structure of eEF3-ADP taken from (Andersen et al., 2006)**

1.10 Eukaryotic elongation factor 1 A (eEF1A)

A translation elongation cycle starts with the delivery of aminoacylated tRNA to the ribosomal A-site by the eukaryotic elongation factor 1 A (Carvalho Da da et al., 1984). eEF1A is homologous to its prokaryotic counterpart EF-Tu and belongs to the GTPase super family. Once the anticodon-codon match is made, the ribosome stimulates GTP hydrolysis resulting in the release of inactive GDP-bound eEF1A from the ribosome.

There seems to be an allosteric connection between the ribosomal E-site and A-site which is thought to play a role in the accuracy of translation in *Escherichia coli* (Nierhaus, 1990). Supporting this idea in *S. cerevisiae*, it has been shown that the two factors that function at these sites (eEF3 and eEF1A) interact with each other (Anand et al., 2006; Anand et al., 2003).

The channelling hypothesis states that there is direct transfer of metabolites from one enzyme to another in a protein-protein complex, without dissociation of the metabolites to solution (Negrutskii and El'skaia, 2001). The channelling will positively influence translational efficiency since the number of random non-specific interactions is reduced. This hypothesis is strongly supported by the findings that (1) aminoacylated tRNA and deacylated tRNA are never free in the eukaryotic cytoplasm (Negrutskii and Deutscher, 1991; Negrutskii et al., 1994), (2) eEF1A binds deacylated tRNAs, although with a 1000 fold lower affinity than aminoacylated tRNA (Petrushenko et al., 2002), and (3) deacylated tRNA bound eEF1A was found in complex with aminoacyl-tRNA synthetase (Petrushenko et al., 2002).

Little is known about the fate of deacylated tRNAs exiting the ribosome, reaching the aminoacyl synthetases for recharging, after which it needs to be shuttled back to the ribosome for the next round of translation. Taking into account the above mentioned findings eEF1A seems to be an ideal candidate for channelling deacylated and aminoacylated tRNA in the cell.

Apart from its role in translation elongation eEF1A is emerging as a regulator involved in many cellular processes, for example actin bundling, nuclear export, apoptosis, protein quality control and degradation (Mateyak and Kinzy, 2010). The most extensively studied noncanonical function of eEF1A is the binding and bundling of actin, which was first observed in *Dictyostelium amoebae* (Yang et al., 1990). The eEF1A-actin interaction is conserved among species from yeast to mammals, suggesting the importance of eEF1A for cytoskeleton integrity. Studies have demonstrated that actin bundling by eEF1A is significantly reduced in the presence of aminoacylated tRNA while eEF1A bound to actin filaments is not in complex with aminoacylated tRNA (Liu et al., 1996). Hence, actin and aminoacylated tRNA binding to eEF1A must be mutually exclusive. Also, over-expression of eEF1A does not affect translation elongation and in actin-bundling deficient mutants translation is not affected (Gross and Kinzy, 2005, 2007; Liu et al., 1996), suggesting eEF1A-dependent cytoskeletal organisation is independent of its translation elongation function (Gross and Kinzy, 2005; Liu et al., 1996).

1.11 Aims and objectives

While the principal role of a ribosome is to synthesise polypeptides, it also acts as a platform for several non-ribosomal proteins involved in essential biological processes, including docking of the ribosome to cellular organelles and recruiting kinases engaged in various signalling pathways. One such signalling pathway is the general amino acid control pathway involving the kinase Gcn2. In order to respond to starvation instantaneously, amino acid availability within the cell needs to be constantly monitored. This sensing is thought to occur on the ribosomes, by Gcn2 together with Gcn1 and Gcn20 (Sattlegger and Hinnebusch, 2000). The Gcn complex is a huge complex, about 1/7th the size of the ribosome. The fact that translation of proteins is a constantly occurring process raises the question of whether there is a link between the two processes – translation and monitoring amino acid availability.

The aim of this study was to investigate if there is a link between translation elongation and the general amino acid control. In particular two translation elongation factors, eEF3 and eEF1A were studied. The objectives of this study were:

- to investigate if eEF3 affected the function of Gcn1 in Gcn2 activation. eEF3 has homology to Gcn1 (Marton et al., 1993) and hence eEF3 may affect Gcn1 function.
- to investigate if eEF1A associated with either Gcn1 or Gcn2. eEF1A has been shown to interact with eEF3 (Anand et al., 2003) and with aminoacyl tRNA synthetases (Petrushenko et al., 2002). Gcn1 has homology to eEF3 and Gcn2 has a histidyl tRNA synthetase like domain, suggesting that eEF1A could interact with either Gcn1 or Gcn2 or both.

1.12 Significance of the study

The control of protein synthesis plays a pivotal role in various physiological conditions including homeostatic mechanisms and long term memory formation. Poor regulation of protein synthesis is known to lead to diseases such as neurodegenerative disorders, epilepsy and diabetes (Abbott and Proud, 2004; Carnevalli et al., 2004; Kaufman, 2002; Kaufman et al., 2002; Klann and Dever, 2004; Kumar et al., 2001; Ron, 2002; Si et al., 2003).

In all eukaryotes, the general role of Gcn2 is in coping with nutritional limitation (Berlanga et al., 1999; Ramirez et al., 1991; Sood et al., 2000). However, in mammals it has also been shown to respond to UV irradiation (Deng et al., 2002; Jiang and Wek, 2005) and RNA viral infections (Berlanga et al., 2006). Gcn2 has been shown to be involved in long term memory formation (Costa-Mattioli et al., 2005) and feeding behaviour (Hao et al., 2005) in mice. Considering that Gcn2 plays a role in so many different functions, particularly in higher eukaryotes, it is astonishing that Gcn2 can perform the correct function, in the correct organ, at the right time. So far little is known about these mechanisms and how Gcn2 switches to the appropriate function whenever needed. To help resolve the ambiguity, this study will investigate if there is a link between translation and the general amino acid control pathway. To date *GCN1* and *GCN2* have been found in all eukaryotes, and their sequences are conserved, suggesting their functional importance in all eukaryotes. Hence studying their function at a molecular level in simple eukaryotes like yeast will help unravel their functions in higher eukaryotes. Furthermore it will help us understand how these basic proteins have evolved to execute more complicated processes like memory formation.

Chapter 2 Materials and Methods

2.1 Biological materials

The plasmids, bacterial strains and yeast strains used in this study are listed in Table 2.1 and Table 2.2.

2.2 Plasmid constructions

2.2.1 pJV01

The Gcn1-myc fragment under an endogenous promoter was excised using restriction endonucleases *Ngo*MI and *Sal*I from p2367. The digestion was checked for completion by agarose gel electrophoresis. The fragment was extracted and purified from the agarose gel. It was then inserted into a pRS314 which was also digested with *Ngo*MI and *Sal*I. The plasmid was verified by restriction digestion (Appendix A).

2.2.2 pJV02

A 393 base pair fragment of Gcn2 (nucleotides 5620-6013) was amplified using primers ES2018 and ES2019 from the template pDH111. This PCR product was purified and digested with restriction enzyme *Bgl*II. This digested PCR fragment was purified and inserted into pHQ531 which was also digested with *Bgl*II. The plasmid was verified via PCR using the primer ES2020 and ES2019. The plasmid was sequenced to check for any PCR errors using primer ES2020 (for the forward direction) or ES2019 (for the reverse direction) (Appendix B).

Table 2.1: Yeast Strains used in this study

Yeast Strain	Genotype	Reference
H1511	<i>a leu2-3 leu2-112 trp-Δ63 ura3-52 GAL2</i>	Foiani <i>et al.</i> (1991)
H2556	<i>a gcn1Δ leu2-3 leu2-112 trp-Δ63 ura3-52 GAL2</i>	Vazquez de Aldana and Hinnebusch unpublished
H2557	<i>a gcn2Δ leu2-3 leu2-112 trp-Δ63 ura3-52 GAL2</i>	Vazquez de Aldana and Hinnebusch unpublished
H2558	<i>a gcn20Δ leu2-3 leu2-112 trp-Δ63 ura3-52 GAL2</i>	Vazquez de Aldana <i>et al.</i> (1995)
H1613	<i>α inol ura3-52 leu2-3, 112 GCN2^E-E532K-E1522K (HIS4-lacZ ura3-52)^p</i>	Ramirez <i>et al.</i> (1992)
TKY864	<i>α leu2-3 112 his 4-713 ura3-52 trp1 tef 2 Δ 2 tef 1 :: met2-1, untagged TEF1 on TRP plasmid</i>	Terri Kinzy unpublished
TKY865	<i>α leu2-3 112 his 4-713 ura3-52 trp1 tef 2 Δ 2 tef 1 :: met2-1, 6XHis TEF1 on TRP plasmid</i>	Terri Kinzy unpublished
ESY10101	<i>α leu2-3,112 his4-713 ura3-52 trp1Δ tef2 Δ 2 tef1::LEU2 met2-1 gcn2Δ::hisG, 6XHis TEF1 on TRP plasmid</i>	Evelyn Sattlegger unpublished

Table 2.2: Plasmids used in this study

Plasmid	Gene	Selectable marker	Vector	Source
Plasmid borne genes for expression in yeast (all Amp ^r)				
pJV01	Gcn1-myc	Trp1, CEN6 / ARSH4	pRS314	This study
pRS314		Trp1, CEN6 / ARSH4	pRS314	R.P. Hieter
p2367	Gcn1-myc	Ura3, CEN6 / ARSH4	pRS316	Marton <i>et al.</i> (1997)
Yeast GST fusions for expression in yeast (all Amp ^r)				
TKB705	GST-eEF3(1-1044)	Ura3, Leu2-d, 2μ	pEG (KT)	Anand <i>et al.</i> (2006)
TKB706	GST-eEF3(1-775)	Ura3, Leu2-d, 2μ	pEG (KT)	Anand <i>et al.</i> (2006)
TKB707	GST-eEF3(100-367)	Ura3, Leu2-d, 2μ	pEG (KT)	Anand <i>et al.</i> (2006)
TKB708	GST-eEF3(775-910)	Ura3, Leu2-d, 2μ	pEG (KT)	Anand <i>et al.</i> (2006)
TKB709	GST-eEF3(910-1044)	Ura3, Leu2-d, 2μ	pEG (KT)	Anand <i>et al.</i> (2006)
TKB710	GST-eEF3(775-1044)	Ura3, Leu2-d, 2μ	pEG (KT)	Anand <i>et al.</i> (2006)
B2219	GAL-GST	Ura3, Leu2-d, 2μ	pEG (KT)	M. Kobayashi
pES335-471 b1	GST-eEF3(1-910)	Ura3, Leu2-d, 2μ	pEG (KT)	Sattlegger (unpublished)
Bacterial gene fusions				
pB130	GST-Gcn2(1-598)	Amp ^r	pGEX-5X-1	Garcia-Barrio <i>et al.</i> (2000)
pHQ551	GST-Gcn2(568-998)	Amp ^r	pGEX-5X-1	Qiu <i>et al.</i> (1998)
pHQ530	GST-Gcn2(970-1497)	Amp ^r	pGEX-5X-1	Qiu, described in Garcia-Barrio <i>et al.</i> (2000)
pHQ531	GST-Gcn2(1498-1517)	Amp ^r	pGEX-5X-1	Qiu <i>et al.</i> (1998)
pGEX-5X-1	GST	Amp ^r		Pharmacia
pJV02	GST-Gcn2(1498-1517)-K1552L K1553I K1556I	Amp ^r	pGEX-5X-1	This study
pDH111	Gcn2-FL-K1552L K1553I K1556I	Amp ^r	pEMBLyex4	Dong <i>et al.</i> (2000)

2.3 Media

Media for yeast and bacterial culture were prepared using Milli-Q® water and sterilized by autoclaving at 121°C, 15 psi for 20 minutes or filtered through 0.22µm millipore express® membrane filters.

Carbon sources were autoclaved/ filter sterilised separately from the media and added prior to use.

Liquid media were cooled to room temperature before the addition of supplements and were stored at room temperature unless mentioned otherwise.

If solid medium was required, it was prepared by adding agar (Formedium) to a final concentration of 2% (w/v). Solid media were cooled to 55°C before addition of supplements and pouring into plates. Plates were stored at 4°C until use.

All general chemicals and salts were sourced from Ajax Finechem, Sigma, Formedium, BDH and Biorad.

2.3.1 Bacterial media

Luria-Bertani (LB) Medium

1% (w/v) Tryptone (Oxoid)
0.5% (w/v) NaCl (Ajax)
0.5% (w/v) Yeast Extract (Formedium)
pH 7

2.3.2 Yeast media

Yeast extract Peptone Dextrose (YPD)

1% (w/v) yeast extract (Formedium)
2% (w/v) Bacto-Peptone (Formedium)
2% (w/v) Glucose (Formedium)

Synthetic Dropout (SD)

0.145% (w/v) Yeast Nitrogen Base without amino acids (BD)
0.5% (w/v) Ammonium Sulfate (Ajax)
2% (w/v) Glucose (Formedium) or
2% or 10% (w/v) Galactose (Formedium) or
2% (w/v) Raffinose (Formedium)

2.3.3 Media supplements

Table 2.3: List of antibiotics used

Antibiotic	Solvent	Final concentration
Ampicillin	water	100µg/mL
G418	water	200µg/mL

Table 2.4: List of induction drugs used

Drugs	Solvent	Final concentration
IPTG	water	10 mM
3AT	water	15-150 mM
SM	DMSO	0.25-2 µg/mL

Table 2.5: List of amino acids used

Amino acid	Solvent	Final concentration
Histidine	water	20 mg/L
Isoleucine	water	30 mg/L
Leucine	water	100 mg/L
Lysine	water	30 mg/L
Methionine	water	20 mg/L
Tryptophan	water	20 mg/L
Uracil	water	20 mg/L
Valine	water	150 mg/L

2.4 Growth conditions

2.4.1 Bacterial growth conditions

Escherichia coli cultures were grown at 37°C in LB broth or LB agar plates supplemented with appropriate antibiotic (Table 2.3). When grown in liquid, *E. coli* cultures were shaken at 180 rpm.

2.4.2 Yeast growth conditions

Saccharomyces cerevisiae were routinely grown at 30°C on YPD or SD plates supplemented with appropriate amino acids (Table 2.5) for 1-2 days. Cultures were then maintained at 4°C. When grown in liquid, *S. cerevisiae* cultures were shaken at 120-180 rpm.

2.5 Glycerol stocks

Bacterial cultures were stored at -80°C in 66% (v/v) glycerol (Univar).

Yeast cultures were stored at -80°C in 30% (v/v) glycerol (Univar).

2.6 DNA isolation and purification

2.6.1 Plasmid DNA isolation and purification

2.6.1.1 Commercial Plasmid isolation kits

This procedure was chosen when highly clean DNA was required, e.g. for sequencing reactions. Kits were purchased from either Invitrogen or Qiagen, and the plasmid was extracted according to the manufacturers' protocol.

2.6.1.2 Plasmid DNA purification by alkaline lysis

E. coli overnight cultures were pelleted by centrifugation at 12,000 rpm for 2 min and resuspended in 100 µL of ice-cold Solution I. Cells were lysed by addition of 200 µL of Solution II

followed by incubation at room temperature for 5 min. To neutralise the suspension, 150 μ L of ice-cold Solution III was added, followed by incubation on ice for 10 min. Cellular debris was then pelleted by centrifugation at 12,000 rpm for 10 min at 4°C. The supernatant was extracted with equal volume of phenol-chloroform-isoamyl alcohol and the DNA was precipitated with an equal volume of isopropanol for 10 min and pelleted by centrifugation at 12,000 rpm for 10 min at 4°C. The DNA pellet was then washed with 70% ethanol and air dried. The pellet was resuspended in 20 μ L of TE-RNase and left at 65°C for 15 min after which the concentration of extracted DNA was determined.

Solution I

50mM Glucose
25mM Tris-HCl (pH 8.0)
10mM Na₂EDTA (pH 8.0)

Solution II

0.2 N NaOH
1% SDS

Solution III

3M potassium acetate
2M glacial acetate

Phenol-chloroform-isoamyl alcohol

Phenol 25 volumes
Chloroform 24 volumes
Isoamyl alcohol 1 volume

TE

10mM Tris-HCl (pH7.5)
1mM Na₂EDTA

2.6.2 Genomic DNA isolation

5 mL of yeast overnight culture was pelleted by centrifugation at 12,000 rpm for 2 min. The pellet was washed by resuspending in 1mL of sterile Milli-Q® water and pelleted. The pellet was resuspended in 400 µL of sterile Milli-Q® water. To the suspension 200 µL of Solution A, 200µl phenol-chloroform, 200 µL acid washed glass beads was added. The cells were lysed by vortexing for 2 min followed by the addition of 200 µL TE. The glass beads and cellular debris were pelleted by centrifugation at 12,000 rpm for 5 min. The DNA in the supernatant was precipitated with 1 mL ice cold absolute ethanol and centrifuged at 12,000 rpm for 2 min at 4°C. The DNA pellet was resuspended in 400 µL TE. RNA in the solution was removed by treatment with 2 µg RNase at 37°C for 30 min. The DNA was precipitated by adding 18 µL 3M sodium acetate (pH 5.2), 1 mL of ice cold absolute ethanol and was left at -20°C overnight. The DNA was pelleted by centrifugation at 12,000 rpm for 10 min at 4°C. The DNA pellet was washed with 70% ethanol and air dried. The pellet was then resuspended in 25 µL sterile Milli-Q® water and the concentration was determined.

Solution A

2% Triton X-100

1% SDS

100mM NaCl

10mM Tris (pH 8.0)

1mM EDTA

Phenol-chloroform

Phenol 1 volume

Chloroform 1 volume

TE

10mM Tris-HCl
(pH 8.0)

1mMNa₂EDTA

2.7 DNA quantification

DNA was quantified either by agarose gel electrophoresis (section 2.8) and comparing the brightness of the bands with the brightness of DNA bands of known concentrations or by using the Act Gene Asp 3700 nanodrop.

2.8 Agarose gel electrophoresis

Agarose gels of desired concentrations (1-2% w/v) were made in 1X TAE buffer containing 1 μ L/mL ethidium bromide. Samples were mixed with 1X DNA loading dye and separated on the agarose gel at a constant voltage of 80V until the dye front had migrated two thirds of the gel using BioRad PowerPac 3000 (Bio Rad Laboratories, Inc., USA). After electrophoresis the DNA was visualized on a UV transilluminator and gel images were captured using a Gel Doc imager (Bio Rad Laboratories, Inc., USA).

Tris-Acetate-EDTA (TAE) Buffer (50X)

2M Tris
1M Acetate
100mM EDTA
pH 8.1

DNA loading dye

0.25% Bromophenol blue
0.25% Xylene cyanol
50% Glycerol

2.9 Restriction endonuclease digestion

Plasmid DNA and PCR products were digested with restriction enzymes using appropriate buffer conditions and temperature as recommended by the manufacturer. The reaction mix was checked for completion of digestion by agarose gel electrophoresis.

2.10 Polymerase Chain Reaction

2.10.1 Standard PCR

Standard PCR reactions were carried out in an iCycler (Bio Rad Laboratories, Inc., USA). DNA fragments were amplified in PCR reactions by using primers annealing to specific sites as required. A PCR reaction was typically carried out in a 20-50 μ L reaction volume containing 1X PCR reaction buffer, 300 μ M dNTPs, 40 pmol of each primer, 0.5 U *Taq* polymerase (Fermentas) or 1.25 U *Pfu* polymerase (Fermentas) (when proof reading was essential) and 10-50 ng of template DNA. The thermocycle conditions used were typically: one cycle at 94°C for 5 min, 30-40 cycles at 94°C for

30s, 55°C for 30-45s and 72°C for 1 min (per kb) and one cycle of final extension at 72°C for 10 min.

2.10.2 Colony PCR

The PCR reaction mix was exactly the same as the standard PCR mix without template DNA. Instead of template DNA a small amount of freshly streaked bacterial/ yeast cells were transferred into the PCR mix with a yellow tip. The thermocycle conditions were as described in standard PCR conditions.

2.10.3 dNTPs

dNTPs (Fisher Biotech, Australia) were prepared as 25 mM stocks by mixing equal volumes of each dNTP (1M) (dATP, dCTP, dGTP, dTTP). The dNTP stocks were stored at -80°C.

2.10.4 Primers

Oligonucleotide primers were synthesized by Invitrogen or Sigma and resuspended in sterile Milli-Q® water to a stock concentration of 200 pmol/μL. The primer stocks were diluted to 20 pmol/μL for PCR and 2 pmol/μL for sequencing reactions. All primers were stored at -80°C. Primers mentioned in this thesis are listed in Table 2.6.

Table 2.6: List of primers used

Primer Name	Sequence (5'-3')	Purpose
ES48	CTGGCAAGCCACGTTTGGT	Sequencing primer to verify GST-eEF3 fragments
ES2006	TGCTCATTAGAAAAGAAAGCATAGCAATCTAATCTAAGTTTTAATTACAACAGCTGAAGCTTCGTACGC	Forward primer for Tef1 knockout cassette
ES2007	AACTAATAGATAAGATTTAAATATAAAAAGATATGCAACTAGAAAAGTCGCATAGGCCACTAGTGGATCTG	Reverse primer for Tef1 knockout cassette
ES2008	AAAAGAGACCGCCTCGTTTC	Forward primer for Tef1 knockout verification
ES2009	CCAAACGGTGACGTTAAGAG	Reverse primer for Tef1 knockout verification
ES2010	GGAGTACTTGTTTTAGAAATATACGGTCAACGAACTATAATTAACCTAAACCAGCTGAAGCTTCGTACGC	Forward primer for Tef2 knockout cassette
ES2011	ACATAAAAATAAGAGGTATATAAAAATATTATATGGAAGCAATAAGCATAGGCCACTAGTGGATCTG	Reverse primer for Tef2 knockout cassette
ES2012	TCGACTATGCTGGAGGCAG	Forward primer for Tef2 knockout verification
ES2013	GTATCTGTCTTCTCCTTCTCT	Reverse primer for Tef2 knockout verification
ES2018	GGTAATTTATGTTCCAAACATGG	Forward primer for Gcn2-CTDK3
ES2019	ATGCGTTATAGCGCCGCAC	Reverse primer for Gcn2-CTDK3
ES2020	GCCGGTAGTAGTCAAGAAG	Forward verification for Gcn2-CTDK3

2.11 DNA purification

2.11.1 Purification of PCR products using QIAquick PCR Purification Kit (Qiagen)

PCR products were purified from the PCR reaction mix using the QIAquick PCR Purification Kit according to the manufacturer's protocol.

2.11.2 Extraction and purification of DNA from agarose gels using QIAquick Gel Extraction Kit (Qiagen)

The desired DNA fragment was excised from the agarose gel under long wavelength UV light 254 nm. The gel slice was solubilised in 3 volumes of Buffer QG at 50°C for 10 min. The DNA fragment was then purified using the QIAquick Gel Extraction Kit according to the manufacturer's protocol.

2.12 DNA ligations

2.12.1 Dephosphorylation

The vector plasmids were dephosphorylated with 1 U Calf Intestinal alkaline Phosphatase (New England Biolabs® Inc.) during restriction digestion. 1 U of CIP was added to the restriction digestion reaction mix prior to digestion and subjected to digestion conditions as recommended by the manufacturer.

2.12.2 Ligations

Ligation reactions (10 µL) were performed at ratios of 1:3, 1:6 moles (vector to insert) and a vector alone control in 1X ligation buffer and 1U T4 DNA ligase (New England Biolabs® Inc.)

Reactions were cyclically incubated for 20 seconds at 30°C and 20 seconds at 10°C overnight. 3 µL of this mix was then used for transformation.

2.13 Transformation of *E. Coli*

2.13.1 Preparation of competent *E. coli* using CaCl_2

Overnight cultures of *E. coli* strain DH5 α or BL21 (used for protein expression) were inoculated in 200 mL of LB broth and grown to an optical density at 600 nm (OD_{600}) of 0.5-0.7. The cells were then harvested by centrifugation at 5,000 rpm for 10 min at 4°C. The cells were resuspended in 20 mL of ice cold calcium chloride solution. This suspension was incubated on ice for 30 min. The cells were then re-pelleted by centrifugation at 5,000 rpm for 10 min at 4°C. The pellet was then resuspended in 4 mL of chilled glycerol- calcium chloride solution. The cells were incubated for a minimum of 6 hrs but not more than 12 hrs on ice. The mixture was then aliquoted, frozen on dry ice and stored at -80°C.

Calcium chloride solution

50 mM CaCl_2
Filter sterilized

Glycerol- Calcium chloride solution

15% (v/v) Glycerol
50 mM CaCl_2
Filter sterilized

2.13.2 Transformation of *E. coli* by heat shock

The competent *E. coli* was thawed on ice. 1 μL of plasmid DNA or 3 μL of ligation mix was added to 100 μL of cells. This mixture was incubated on ice for 30-40 min. The cells were heat shocked at 42°C for 3 min and immediately cooled on ice for 10 min. 1 mL of LB was added to the transformed cells and incubated at 37°C with shaking at 180 rpm for 1 hr, prior to plating the cells on to selective media with appropriate antibiotics.

2.14 DNA sequencing

3.2 pmol primer and 300 ng template in a total volume of 15 μL was submitted to the Alan Wilson Centre Genome Service for commercial sequencing.

2.15 Yeast transformation

2.15.1 Making Yeast Competent

1 mL of an overnight *S. cerevisiae* culture was added to 50 mL YPD and incubated with shaking for 3 hours. The cells were pelleted by centrifugation at 4,200 rpm for 5 min and resuspended in 5 mL 0.1M lithium acetate. The cells were again pelleted by centrifugation at 4,200 rpm for 5 min and resuspended in 500 μ L of 0.1 M LiOAc. The cells were incubated with shaking for 30 min. The competent cells were stored at 4°C for use within 24hrs.

TE

10 mM Tris HCl pH 8
1 mM EDTA

Lithium acetate

0.1 M LiOAc in TE
Filter sterilised

2.15.2 Transformation

5 μ L of single stranded DNA boiled at 100°C for 10 minutes, 5 μ L of plasmid and 100 μ L of competent yeast cells were incubated at 30°C for 15-30 min. 600 μ L 40% PEG was added and incubated again at 30°C for 30-45 min. 70 μ L of DiMethylSulfOxide was added to the cells prior to heat shocking at 42°C for 15 min. The cells were then immediately transferred on to ice for 10 min. The cells were centrifuged at 4000 rpm for 3 min. The pellets were resuspended in 50 μ L SD and plated on appropriate SD plates and incubated at 30°C for 2-3 days.

Lithium acetate

0.1 M LiOAc in TE
Filter sterilised

Poly Ethylene Glycol (PEG)

40% (w/v) PEG in LiOAc
Filter sterilised

2.16 Preparation of Whole Cell Extract (WCE)

2.16.1 Standard WCE preparation

Overnight yeast cultures were inoculated into 50 mL appropriate media. The cells were harvested at OD₆₀₀=1 by centrifugation at 4,200 rpm for 5 min. The pellet was resuspended in 1 mL sterile Milli-Q® water and transferred to 2 mL tubes. The cells were re-pelleted by centrifugation at 12000 rpm for 2 min. The pellets were either frozen on dry ice, stored at -80°C until used or used right away for breaking. For

breaking cells 1 pellet volume (~100 μ L) of ice cold breaking buffer and 1 pellet volume (~100 μ L) of acid washed glass beads (Sigma) were added to the thawed pellet. The samples were vortexed for 30s alternating with 30s intervals on ice-water mix 10 times. The cellular debris and glass beads were pelleted by centrifugation at 12,000 rpm for 10 min at 4°C. The supernatant containing the whole cell extract was transferred to fresh tubes. The whole cell extract was stored at -80°C. The protein concentration of whole cell extract was determined by the Bradford estimation method (section 2.17).

Breaking Buffer

50 mM Tris HCl pH7.5
50 mM NaCl
0.1% Triton X-100
0.5 M EDTA
0.5 M DTT
100 mM PMSF
10 μ g/mL Pepstatin

2.16.2 Formaldehyde cross-linked WCE preparation

2.16.2.1 Cross-linking cells with formaldehyde

Overnight yeast cultures were inoculated into 300 mL appropriate media. The cells were grown shaking at 120 rpm at 30°C to exponential phase (OD_{600} 1). 75 g of ice chips, 8.1 mL formaldehyde (1% final) were taken in a 500 mL centrifuge bottle and the yeast culture was added to this. The cells were kept on ice for 1 hr; shaking every 15 min. 15 mL of 2.5 M glycine (0.1 M final) was added to stop the cross-linking reaction and then the cells were harvested by centrifugation at 4,200 rpm for 5 min at 4°C. The cells were transferred with 5 mL breaking buffer without inhibitors to round bottom tubes and the cells were re-pelleted by centrifugation at 4,200 rpm for 5 min at 4°C. The pellets were either frozen on dry ice, stored at -80°C until used or used right away for breaking.

2.16.2.2 Breaking Cells

The thawed cell pellets were resuspended in 200 μ L breaking buffer + inhibitor. 700 μ L of acid washed glass beads (Sigma) were added to the cell pellet to break the cells. The samples were vortexed for 30s with 30s intervals on ice-water mix 10

times. The cellular debris and glass beads were pelleted by centrifugation at 4,200 rpm for 5 min at 4°C and the supernatant was transferred to a 1.5 mL tube. The supernatant was further clarified by centrifugation at 9,000 rpm for 10 min at 4°C. The supernatant containing the whole cell extract was transferred to fresh tubes. The whole cell extract was stored at -80°C. The protein concentration of whole cell extract was determined by absorbance under UV (section 2.17).

Breaking Buffer for cross-linked cells

20 mM Tris pH 7.5
50 mM KCl
10 mM MgCl₂

Inhibitors

1 mM DTT
1 mM PMSF
5 mM NaF
10 µg/mL Pepstatin
1 µg/mL Aprotinin
1 µg/mL Leupeptin

2.17 Estimation of protein concentration

2.17.1 Estimation by Bradford method

Bovine Serum Albumin (BSA) standards of increasing protein concentrations (1 µg, 2 µg, 4 µg, 6 µg, 8 µg, 10 µg, 20 µg) were made by diluting a stock of 10 mg/mL and 1 µL extracted protein of unknown concentration were taken in duplicates in a 96 well clear micro-titre plate. 200 µL of Bradford solution was added to samples in the plate and incubated for 5 min. Absorbance of the samples was read at 595nm in a FLUOstar OPTIMA plate reader (BMG Labtech). A standard curve was plotted with the absorbance against protein concentration of the known standards. Protein amount in the unknown sample was read off the standard curve, and concentration was calculated.

Bradford solution

0.5 mg/mL Coomassie Blue G
25% methanol
42.5% H₃PO₄
0.05 N NaOH
Store in a dark bottle

2.17.2 Estimation by absorbance under UV

This method was usually followed for formaldehyde cross-linked samples. 1 μL of WCE was added to 999 μL of Milli-Q[®] water. Absorbance of samples was measured at 260nm in duplicates. The protein concentrations were expressed as A_{260} per μL of the protein sample.

2.18 Ribosome co-sedimentation

12mL 4.7-47% sucrose gradients prepared were layered with 30 A_{260} of each sample. The samples were spun at 39,000 rpm for 2 hr 15 min at 4°C in the Sorvall[®] Discovery 100SE ultracentrifuge with a Sorvall[®] TH-641 rotor. The gradients were fractionated at 3 mL/min into 1 mL samples with the BioLogic BioFrac fraction collector (Bio Rad Laboratories, Inc., USA). Fractions were collected while measuring A_{260} continuously to trace the 40S and 60S ribosomal subunits, 80S ribosomes and polyribosomes.

Sucrose gradients

4.7% or 47% Sucrose in breaking buffer
1 mM DTT

2.19 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS PAGE)

2.19.1 Gradient gel electrophoresis

SDS-PAGE was used to separate protein samples. 1% agarose (w/v) in 1.5 M Tris-HCl (pH 8.8) was used to seal the gaps between the spacers in between the glass plates. A 17 cm (height) by 14.5 cm (width) gel with 1.8 mm thickness was used. 20 mL of 4% premix was taken in one chamber of the gradient mixer and 20 mL of 17% premix in the other chamber. 16 μL N,N,N',N'-TetraMethylEthyleneDiamine (TEMED) and 200 μL 10% Ammonium Per Sulfate (APS) was added to each chamber and valve was opened between the chambers so that the stocks in the chambers mixed and a gradient of acrylamide was poured between the glass plates. The wells were washed with protein running buffer to remove any non-polymerised acrylamide before the gel was assembled on the gel electrophoresis unit. The gel

was covered with protein running buffer and protein samples that were mixed with Laemmli dye and denatured at 85°C for 10 min were loaded on the gel. The gel was run at 250V and 100 mA until the dye front reached the end of the gel.

4% Premix (20 mL)

2 mL 29:1 Acrylamide
5 mL 1.5M Tris-HCl pH
8.8
200 µL 10% SDS (w/v)
13 mL Milli-Q® water

17% Premix (20 mL)

8.5 mL 29:1
Acrylamide
5 mL 1.5M Tris-HCl pH
8.8
200µl 10% SDS (w/v)

6.5 mL Milli-Q® water

**40% Acrylamide
(29:1::acrylamide: bis-
acrylamide)**

Protein running buffer

25 mM Tris base pH 8.8
192 mM Glycine
1% SDS (w/v)

5X Laemmli dye

0.312 M Tris-HCl pH6.8
10% SDS (w/v)
25% β-mercaptoethanol (v/v)
0.05% Bromophenol blue (w/v)

2.19.2 Discontinuous gel electrophoresis

Mini discontinuous gels were cast using the Mini PROTEAN®3 System (Bio Rad Laboratories, Inc., USA). The resolving gel was poured between the glass plates and left to set for 20 min. 1 mL of Milli-Q® water was layered on top of the gel to avoid exposure to air and to get a smooth surface prior to the gel polymerising. The water layer was removed and the stacking gel was poured between the glass plates and also left to set for 20 min. The wells were washed with protein running buffer to remove any non-polymerised acrylamide before the gel was assembled on the gel electrophoresis unit. The gel was covered with protein running buffer and protein samples that were mixed with Laemmli dye and denatured at 85°C for 10 minutes were loaded on the gel. The gel was run at 80mA until the dye front reaches the end of the gel.

10% Resolving gel (10 mL)

3.3 mL 29:1 acrylamide
2.5 mL 1.5 M Tris-HCl pH 8.8
100 µL 10% SDS (w/v)
4 mL Milli-Q® water
100 µL 10% APS
4 µL TEMED

Stacking gel (4 mL)

67 µL 29:1 acrylamide
0.5 mL 2M Tris-HCl pH 6.8
40 µL 10% SDS (w/v)
2.7 mL Milli-Q® water
40 µL 10% APS
4 µL TEMED

2.20 Staining proteins in acrylamide gels

After electrophoresis the gels were stained with Coomassie brilliant blue (G-250) over night and destained in Milli-Q® water until protein bands were visible.

Coomassie brilliant blue stain

17% Ammonium sulfate (w/v)
3% Phosphoric acid (v/v)
34% Methanol (v/v)
0.1% Coomassie G-250

2.21 Western Blotting

2.21.1 Gel Transfer

SDS- polyacrylamide gels were transferred to immobilon-P PolyVinylidene DiFluoride (PVDF) membranes (MILLIPORE) pore size 0.45 µm that was pre-treated with methanol and then equilibrated in transfer buffer. The gel and membrane were immersed in transfer buffer in transfer units (Idea Scientific Company, USA) and the transfer was run for 2.5 hr at 24V and 1A.

Transfer Buffer

25 mM Tris base pH 8.3
192 M glycine
20% Methanol (v/v)

2.21.2 Staining proteins on membranes

After transfer the membranes were incubated with gentle agitation on a platform rocker with Ponceau S stain for ten minutes and then destained with 1% acetic acid (v/v) until the transferred protein bands were visible.

Ponceau S

0.1% Ponceau S (w/v)
1% acetic acid (v/v)

2.22 Immunological detection of proteins

Membranes were blocked at room temperature with 5% non-fat milk (w/v) (Basics/Pam's) in TBS-T with gentle agitation on a platform rocker to prevent non-specific protein-antibody interactions. Membranes were then incubated at room temperature, with gentle agitation on the platform rocker with the appropriate primary antibody (Table 2.7) diluted in 5% non-fat milk in TBS-T for 1 hr. The membrane was washed at room temperature for 5, 10, 5 min on the platform rocker with TBS-T. The membrane was then incubated at room temperature, with gentle agitation on the platform rocker with the appropriate secondary antibody (Table 2.8) with a horse radish peroxidase conjugate for 1 hr. The membrane was washed at room temperature for 5, 10, 10, 5 min on the platform rocker with TBS-T. Super Signal[®] West Pico Chemiluminescence (Pierce, Thermo Scientific) solution was made according to the manufacturer's recommendations and incubated with the membrane for 5 min. The chemiluminescence signal on the membrane was detected on the Luminescent Image Analyser LAS-4000 (Fujifilm).

Tris Buffered Saline -Tween (TBS-T)

1 M Tris-HCl pH 7.4

5 M NaCl

0.1% Tween 20

Densitometry analysis of the signals obtained was done using the software NIH Image J (<http://rsbweb.nih.gov/ij/>) or Multi Gauge V3.1 (Fujifilm).

Table 2.7: List of primary antibodies and dilutions used in this study

Antibody	Dilution	Source	Secondary antibody
Gcn1	1 in 1000	HL1405, Vazquez de Aldana <i>et al.</i> , 1995	anti-rabbit
Gcn2	1 in 1000	Beatriz A. Castilho (unpublished)	anti-guinea pig
Gcn20	1 in 1000	CV1317, Vazquez de Aldana <i>et al.</i> , 1995	anti-rabbit
Rps22	1 in 2000	Jan van't Ried	anti-rabbit
Rpl39	1 in 5000	Wilson <i>et al.</i> , 1994	anti-mouse
GST	1 in 2000	Santa Cruz	anti-rabbit
Myc	1 in 500	Roche	anti-mouse
eEF1A	1 in 5000	Terri Kinzy	anti-rabbit
eIF2-P/ Sui2-P	1 in 5000	Invitrogen	anti-rabbit
eIF2/ Sui2	1 in 2000	Dever <i>et al.</i> , 1992	anti-rabbit
Pgk-1	1 in 5000	Invitrogen	anti-mouse

Table 2.8: List of secondary antibodies and dilutions used in this study

Secondary antibody	Dilution	Source
anti-rabbit	1 : 100,000	Pierce
anti-mouse	1 : 50,000	Pierce
anti-guinea pig	1 : 5,000	Santa Cruz

2.23 Protein-Protein interaction assays

2.23.1 Glutathione S-Transferase mediated pull down

GST fusion proteins were expressed in *E.coli* strain BL21 and whole-cell extracts were prepared by incubating the cell pellet with lysozyme (1 mg/mL) and GST breaking buffer with inhibitors for 30 min at 4°C and then frozen over night. The cell pellet was thawed and 5 µg/mL of DNase as well as 10 µg/mL of RNase was added and incubated for 30 min at 4°C. The whole cell extract was clarified by centrifugation at 12,000rpm for 10 min at 4°C to remove cell debris. Equal amounts of GST fusion proteins (estimated by Coomassie blue staining) were immobilized on glutathione–sepharose beads (12 µL of 100% beads per reaction) (GE Healthcare) at 4°C on the wheel for 90 min, washed thrice with GST breaking buffer with inhibitors and subsequently incubated for 60 min at 4°C on the wheel with 100 µg yeast extract that had been pre-cleared. The yeast extract was pre-cleared by incubation with glutathione-sepharose beads (12 µL of 100% beads per reaction) for 30 min at

4°C. The beads were further washed after incubation, 6 times with GST breaking buffer with inhibitors. After the final wash the beads were resuspended in 30µl 2X Laemmli's loading buffer. The samples were resolved by SDS–PAGE and subjected to immunoblot analysis.

For GST-pull down assays performed directly from yeast, whole-cell extracts from yeast strains expressing the GST fusion proteins were prepared and pre-cleared as described earlier. Pre-cleared yeast extracts (1 mg in 200 µL) were added to glutathione–sepharose beads. After incubating on the wheel for 60 min at 4°C, the beads were washed and analyzed as described above.

GST Breaking buffer

30 mM HEPES pH 7.4
50 mM KCl
10% glycerol

Inhibitors

1 complete tablet with EDTA (Roche)
per 25 mL buffer
1 mM DTT
1 mM PMSF
10 µg/mL Pepstatin

2.23.2 Poly His pull down

Poly His pull down assays were performed directly from yeast. Whole-cell extracts from yeast strains expressing the His₆ fusion proteins were prepared and pre-cleared as described earlier. Pre-cleared yeast extracts (3mg per reaction) were added to 400µL of 50% Ni charged resin (Bio Rad Laboratories, Inc., USA). After incubating on the wheel for 60min at 4°C, the beads were subsequently washed with buffer containing increasing imidazole concentrations (10mM-250mM imidazole), resulting in a stepwise elution. Input, supernatant and equal amounts of the eluates were resolved by SDS–PAGE and subjected to immunoblot analysis.

His pull down breaking buffer

30 mM HEPES pH 7.4
50 mM KCl
10% glycerol

Inhibitors

1 complete tablet without EDTA
(Roche) per 25mL buffer
5mM β-mercaptoethanol
5mM Imidazole pH7
1mM PMSF

Chapter 3 Colony Westerns

In the process of generating strains that efficiently over-express a desired protein, the strain with the highest expression levels must be identified. This requires the screening of several over-expression plasmid constructs. Over-expressing protein is a huge burden on the cells and therefore mutagenic events that abolish the over-expression give cells a growth advantage over the rest of the population (Rood et al., 1980). As a consequence, the mutated cells outgrow the others and reduce the level of protein expressed. Thus it is necessary to monitor which clones are still over-expressing the desired protein efficiently. Strains over-expressing GST-eEF3 that were used for experiments described in chapter 4 had high rates of mutation. Furthermore, these strains lost their capability over time to efficiently over-express GST-eEF3 from a plasmid borne gene, even when the selection for the plasmid was maintained and the cells were stored under conditions that did not induce cells to over-express proteins. To ensure that the experiments were performed when GST-eEF3 was being over-expressed sufficiently, the strains had to be routinely checked for over-expression.

The commonly-used method to monitor protein over-expression involves growing yeast strains on media containing the inducing agent (e.g. galactose), harvesting the cells and generating whole cell extract. The whole cell extract is then resolved by SDS PAGE and transferred onto a membrane before detection by immunoblotting. This proves to be a very time-consuming and laborious process for routine monitoring of strains for over-expression. Hence there was a need to devise a simple technique that would allow the detection of over-expressed proteins without being very time intensive. Although methods for expression screening have been described for other yeast (yeasterns for *Pichia pastoris*), it still involves transfer of proteins from lysed cells onto membranes via electroblotting. The method developed here, referred to as “colony western”, in essence allows one to perform an immunodetection on a colony of cells without the time consuming electroblotting step. The method is similar to colony hybridization but has been modified to allow a protein of interest to be detected. It involves transferring cells onto a nitrocellulose membrane directly from plates and lysing the transferred cells

on the membrane. The membrane is subject to immunodetection to check for over-expressed proteins. Different conditions were tested like lysis buffers, incubation times and so on. The method was developed by Dr. Sattlegger and was further optimised for use as a semi quantitative technique here. The optimised protocol is outlined below.

3.1 Methodology

3.1.1 Growing cells

A single colony of each of the yeast clones to be analysed was picked and patched onto selective media (SD + amino acids). The plates were incubated overnight at 30 °C. The clones were replica plated onto fresh plates containing the inducing agent (2% galactose). The plates were incubated at 30 °C for 1-2 days.

3.1.2 Transferring cells

A nitrocellulose membrane, cut to the dimensions of the plate, was carefully placed onto the replicated plate. Gentle pressure was applied to bring the cells in contact with the nitrocellulose membrane. The membrane was incubated on the plate for 5 minutes. The membrane was carefully peeled off the plate and air dried with the transferred cells-side up.

3.1.3 Lysing cells

Two layers of Whatman paper were soaked in lysis buffer (250mM Tris, 200mM glycine, 10% w/v SDS) and placed carefully in a container with lysis buffer. Any air bubbles trapped between the layers of Whatman paper were removed. Any excess lysis buffer was discarded. The air dried nitrocellulose membrane was placed cells-side up on the Whatman paper soaked with lysis buffer. Special care was taken to make sure that there was no air bubbles between the nitrocellulose membrane and the wet Whatman paper. The nitrocellulose membrane was incubated on Whatman paper for 3 minutes. The nitrocellulose membrane was air dried cells-side up on a paper towel. The membrane was then washed in distilled water for 3 minutes. This wash was repeated two more times, followed by washing in TBS-T three times for 3 minutes.

3.1.4 Analysis

The nitrocellulose membrane was stained with Ponceau S (as described in chapter 2) to check the efficiency of cell lysis. The nitrocellulose membrane was then analysed for protein expression by immunoblotting (as described in chapter 2).

3.2 Protein expression detectable by colony western

The aim was to develop a quick method to allow for the detection of over-expressed proteins in yeast cells. This would allow screening for the efficient over-expression of proteins from a galactose inducible promoter. Yeast strains over-expressing GST tagged proteins or myc tagged proteins as shown in the schematic (Figure 3.1 A), were grown on solid medium containing glucose as carbon source and then replica plated on medium containing galactose as carbon source. After 1-2 days the cells were transferred onto a nitrocellulose membrane and lysed as detailed in section 3.1. The membrane was stained with Ponceau S to test the efficiency of lysis and was used as loading control (Figure 3.1 B). Thereafter the membrane was subjected to a regular immunoblotting procedure using anti GST and anti myc antibodies.

GST epitope was detected in all strains expressing GST-eEF3, GST-Yih1 and GST alone (left two lanes Figure 3.1 C). The GST-Yih1 clone was used as a positive control since the clone was already verified for over-expression. Note that the clones over-expressing myc tagged proteins did not produce a signal when probed with anti-GST antibodies. This confirms that the technique is reliable and specific.

The next aim was to check if other epitope tags can also be detected by this technique. For this the blots were probed using anti-myc antibodies. The myc epitope was also detected in strains expressing myc-Gcn1 (right two lanes Figure 3.1 D). There was no myc-Gcn1 detected in the strain harbouring vector only. No myc-Gcn1 signal was detected in the strain harbouring single copy plasmid either, hinting that the limitation of this technique may be an inability to detect proteins expressed from a single copy plasmid. The maximum signal intensity was observed when myc-Gcn1 was expressed from a galactose inducible promoter (right lanes, rows 5 & 6 Figure 3.1 D). However, when myc-Gcn1 was expressed from its own promoter, an

expected dose response corresponding to plasmid copy number was not observed as clearly (right lanes compare row 4 with row 3, Figure 3.1 D).

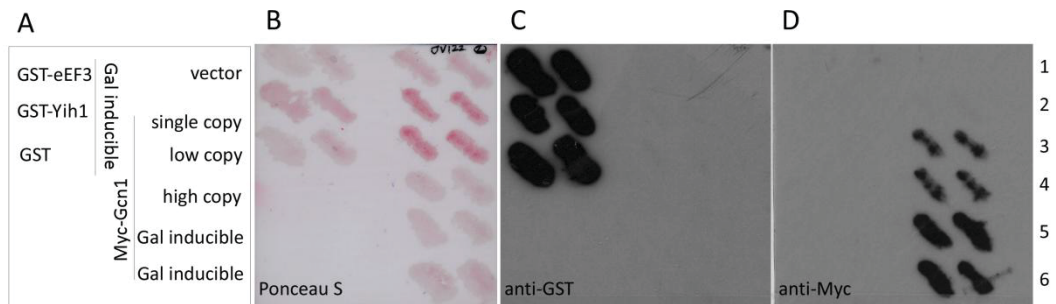


Figure 3.1: Protein expression detectable by colony western

Yeast strains over-expressing the indicated proteins from indicated promoters on a plasmid (A) were patched on solid medium and grown over night. This master plate was then replica plated onto an inducing plate containing 2% galactose and grown for 1-2 days. The cells were then transferred and lysed on nitrocellulose membrane as described in section 3.1. The lysis efficiency was checked by Ponceau S staining (B) before immunodetection using antibodies against the GST epitope (C) and myc epitope (D).

3.3 Colony western is a semi-quantitative technique

Since a difference in signal intensity was observed between galactose induced myc-Gcn1 and non-induced myc-Gcn1 strains in the previous section, there is a possibility to use this technique in a quantitative manner. To test if different levels of protein expression could be determined using this technique the *GCN1* allele under an endogenous promoter was expressed in wild type (H1511) and *gcn1Δ* (H2556) strains. Three plasmids were used: a low copy CEN plasmid, a high copy 2 micron plasmid and a high copy 2 micron plasmid with a galactose inducible promoter. The plasmid borne Gcn1 was myc-tagged at its C-terminus. Cells were subjected to the same experimental procedure as outlined in section 3.1, followed by immunoblotting using anti myc antibodies (Figure 3.2, bottom panel). As observed, Gcn1 expressed from a galactose inducible promoter gave the highest signal intensity (Figure 3.2 row 4 bottom panel). Gcn1 expressed from a high copy plasmid was also detected (Figure 3.2 row 3 bottom panel) albeit with slightly weaker signal intensity. Gcn1 expressed from a low copy plasmid was detectable as well (Figure 3.2 row 2 bottom panel), and the signal intensity was significantly weaker when compared to strains harbouring high copy or galactose inducible Gcn1. The assay is semi-quantitative since we can see a direct correlation of signal intensities and gene copy number of Gcn1.

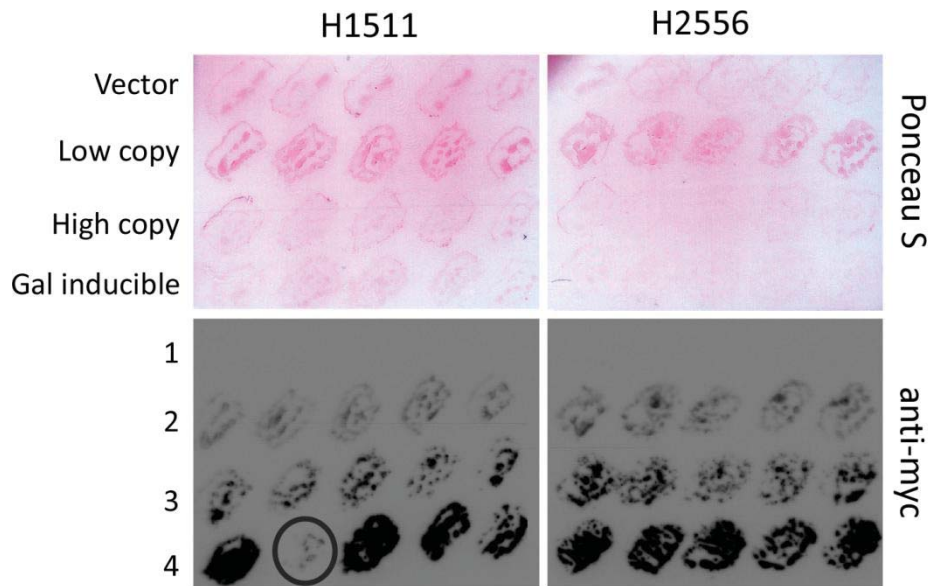


Figure 3.2: Colony western is a semi-quantitative assay

Wild type (H1511) and *gcn1Δ* (H2556) yeast strains expressing *myc-Gcn1* from an endogenous promoter on a low copy or high copy plasmid and from a galactose inducible promoter were used. The assay was performed as explained in Figure 3.1. The lysis efficiency was verified by Ponceau S staining (top panel) before immunodetection using antibodies against the *myc* epitope (bottom panel).

A H1511 clone indicated with a black circle in Figure 3.2 did not give a signal similar to that of its sister transformants. This suggests that it did not over-express *myc-Gcn1* as efficiently as its other sister transformants. Hence, the method allows for the identification of clones that are not able to over-express the protein.

3.4 Protein specific antibodies can be used for colony western

In the previous sections epitope tagged proteins were detected using antibodies against the epitope tag. To check if proteins without epitope tags could also be detected by this technique, antibody specific to the protein was used. The same strains used in section 3.3 were subjected to the experimental procedure outlined in section 3.1, followed by immunoblotting using antibodies against *Gcn1* instead of the epitope tag. The signal intensities were similar to that observed in section 3.3. *Gcn1* expressed from the galactose inducible promoter was readily detectable with highest signal intensity followed by *Gcn1* expressed from a high copy plasmid. The *Gcn1* expression levels from a low copy plasmid were weaker than those observed

in both high copy and galactose inducible Gcn1 strains. Some background signal was detected in *gcn1Δ* strains with vector alone, which may be caused by the polyclonal nature of the antibody used to detect Gcn1. Again, the clone that did not express myc-Gcn1 in Figure 3.2 did not give an apparent signal above the background when the membrane was probed for Gcn1. This indicates that the clone was not over-expressing myc-Gcn1; again pointing out that this assay is consistent in identifying clones that over-express desired proteins efficiently.

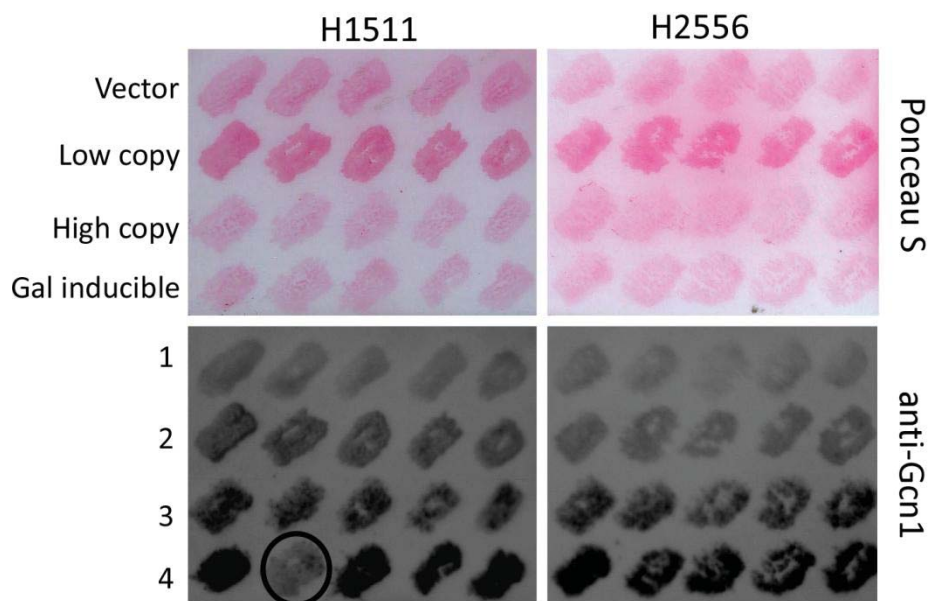


Figure 3.3: Untagged proteins can be detected by colony western

Wild type (H1511) and *gcn1Δ* (H2556) yeast strains expressing myc-Gcn1 from an endogenous promoter on a low copy or high copy plasmid and from a galactose inducible promoter were used. The assay was performed as explained in Figure 3.1. The lysis efficiency was verified by Ponceau S staining (top panel) before immunodetection using antibodies against Gcn1 (bottom panel).

3.5 Applications: optimising the concentration of inducing agents

Colony westerns can also be used to optimise the concentration of inducing agent sufficient for maximum protein over-expression. To do this, the method was modified as follows for galactose as the inducing agent. Plates containing an exact volume (25mL) of selective media were poured with increasing amounts of galactose. The final galactose concentrations ranged from 0.001%-10%. 5μl of saturated overnight cultures of yeast strains to be tested were spotted on the

above inducing plates. The plates were incubated at 30°C over night. A scalpel was used to excise the section of agar containing the grown cells and transferred onto a clean glass plate. The sections of agar from different galactose concentration plates were arranged next to each other on the glass plate in the order from low to high concentration of the inducing agent (galactose). The cells were then transferred onto a nitrocellulose membrane as described in section 3.1.2. The assay was performed as detailed in section 3.1. The blots were analysed using antibodies against myc and GST epitopes.

It was evident that GST-Yih1 could be detected from galactose concentrations of 0.005% onwards (Figure 3.4 bottom right panel). With increasing concentrations of galactose the signal intensity increased for strains over-expressing GST tagged proteins (Figure 3.4, bottom right panel), indicating that more protein was being over-expressed with increasing inducing agent (galactose) concentration.

However, such a gradient in signal intensity was less obvious in the strains with galactose inducible myc-Gcn1. Although there is a difference in signal intensities between cells grown on 0.001% and 0.1% galactose, there was no obvious difference in signal intensities between 0.1% and 10% galactose. The reason for this could be that the strain can express high levels of myc-Gcn1 when induced with low amounts of galactose and hence the level of expression does not increase with increasing concentration of galactose. The signal intensity of the strains bearing high copy plasmid was stronger than those bearing the low copy plasmid. In the case of low copy and high copy plasmid bearing strains, the concentration of galactose did not have an effect on the signal intensity since myc-Gcn1 was under an endogenous promoter.

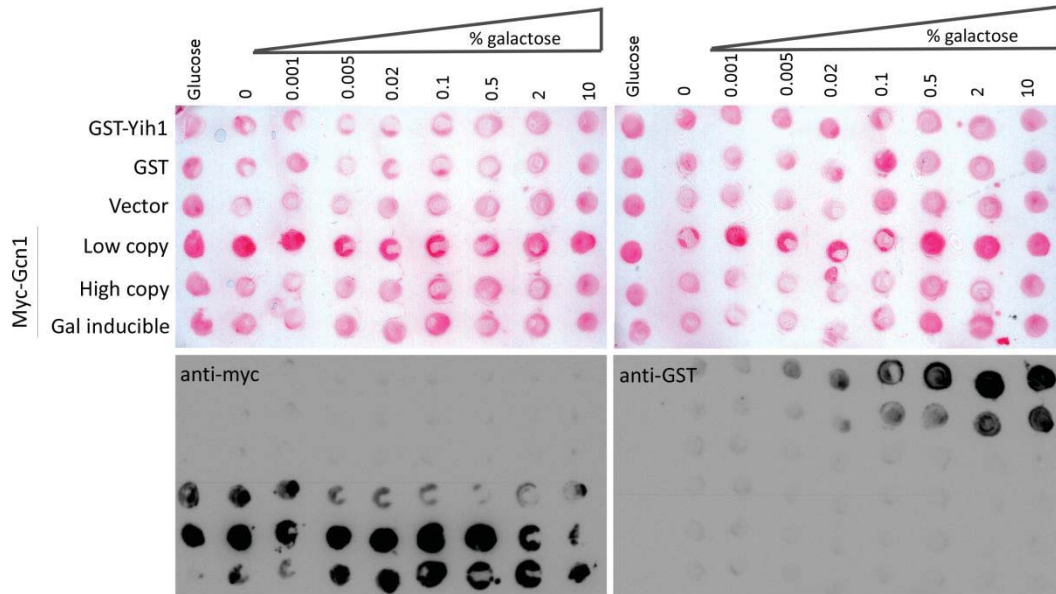


Figure 3.4: Colony westerns can be used to determine the optimal concentration of inducing agent for protein expression

5 μ l of yeast overnight cultures expressing the indicated proteins from a plasmid, were grown on plates with 2% raffinose and increasing concentrations of galactose as indicated, or on a plate with glucose alone. The cells were transferred onto a nitrocellulose membrane as described in section 3.1, stained with Ponceau S to determine the efficiency of cell breakage (top panel) and then subjected to immunoblotting (bottom panel) with antibodies against myc and GST epitopes.

From the above results it can be seen that every over-expression construct requires a unique concentration of inducing agent for optimal protein expression. While in strains expressing GST-Yih1 there was a noticeable correlation with signal intensities and amount of galactose, in strains expressing myc-Gcn1 the difference was not very obvious. Further, strains over-expressing GST-Yih1 could express protein at galactose concentrations of 0.005% while strains over-expressing myc-Gcn1 required lower galactose concentrations such as 0.001% galactose.

3.6 Discussion

Protein over-expression in living cells is required for many applications, such as protein production, purification and investigation of protein function. Strains expressing maximum amount of proteins are hence useful for these purposes. Thus, it is essential that these strains are monitored for maximum level of protein over-expression. Since the routine methods for monitoring protein over-expression are

laborious and time consuming, a simple and quick method described in this chapter was optimised for monitoring expression levels.

This method was used for scoring expression levels in yeast cells of either an untagged or an epitope tagged protein. The method was tested with three different proteins, each harbouring a different epitope tag. In all three cases the method helped to unambiguously identify the cells over-expressing the desired protein. Furthermore, the technique is sensitive and protein specific antibodies can also be used to detect protein expression. It is also semi-quantitative, exhibiting a direct correlation between signal intensity and gene copy number of the protein.

Some of the critical steps to be noted whilst performing the assay are: 1) after transferring the cells on to nitrocellulose membranes, care should be taken not to dry the membrane too long. The cells can flake off the membrane resulting in loss of cells and hence inefficient transfer. 2) During the lysis step, over saturation of the whatman paper with lysis buffer should be avoided. If there is too much lysis buffer, it can flood the membrane and may lead to smearing of the lysed cells. 3) Nitrocellulose membranes should be used; PVDF membranes do not work for this method.

Established protocols like whole cell extract generation or boiling small aliquots of yeast culture in Laemmli loading dye and resolving on a polyacrylamide gel followed by immunoblotting; have limitations on the number of clones that can be screened. Colony western allows one to screen a large number of colonies simultaneously without being as laborious as the other methods mentioned earlier. Moreover, colony western is time saving and cost-effective; the procedure does not involve SDS-PAGE electrophoresis and can be completed in less than a day. It is a sensitive and semi-quantitative technique allowing determination of protein expression levels. The method can also be used to adjust the concentration of inducing agents (e.g. galatose in yeast) for efficient protein expression.

Colony western was used routinely to monitor protein over-expression in the experiments described in chapter 4.

Chapter 4 eEF3 over-expression affects Gcn2 activation

Protein synthesis requires the concerted action of ribosomes and protein factors to decode the messenger RNA. There are three distinct phases of protein synthesis: initiation, elongation and termination. Translational elongation is well-conserved among eukaryotes, except in fungi, where an additional protein factor eEF3 is required. eEF3 is a fungal-specific ATPase that functions at the ribosomal E-site. It releases the deacylated tRNA from the E-site while stimulating the eEF1A-dependent delivery of amino acylated tRNA to the A-site (Trianaalonso et al., 1995). The eEF3 protein is comprised of different motifs, including HEAT repeats at the N terminus, two ABC cassettes (the second ABC cassette contains a chromodomain) and a basic C-terminus as discussed in section 1.9.

The middle portion of Gcn1 (1462-1650 aa) contains HEAT repeats that are homologous to the N-terminal HEAT repeat domain of eEF3 (85-332 aa). Gcn1 is known to form a sub-complex with Gcn20. Gcn20 is a member of the ABC family of proteins, with two ABC cassettes at the C-terminus (Dealdana et al., 1995). The Gcn20 C-terminus has homology to the eEF3 C-terminus, attributable to the ABC cassettes. So, the Gcn1/20 complex together contains structurally similar domains to eEF3, thus hinting that the Gcn1/20 complex could perform a function similar to that of eEF3 instead that it accesses the ribosomal A-site.

While in prokaryotes deacylated tRNA binding to the A-site leads to the activation of the ppGpp synthetase RelA and the stringent response (Cashel, 1987; Goldman and Jakubowski, 1990), in eukaryotes this leads to Gcn1-dependent Gcn2 stimulation and the activation of the general amino acid control (GAAC). It has been shown that in eukaryotes deacylated tRNA can enter the A-site in a codon specific manner (Murchie and Leader, 1978). Based on these findings the model for Gcn1-mediated Gcn2 activation under amino acid starvation conditions was proposed (Sattlegger and Hinnebusch, 2000). In this model it was put forward that Gcn1 mediates Gcn2 activation by facilitating transfer of deacylated tRNA to Gcn2. This allows us to speculate that the Gcn1/20 complex could be involved in delivering or releasing deacylated tRNA from the ribosomal A-site to Gcn2 for sensing amino acid starvation.

Supporting this idea, it was reported that over-expression of Gcn1 leads to hypersensitivity to the A-site binding drug paromomycin (Sattlegger and Hinnebusch, 2000). Gcn1 mediated paromomycin sensitivity was directly correlated with Gcn1-ribosome interaction, as deletion of Gcn1 leads to paromomycin resistance (Sattlegger and Hinnebusch, 2000). In addition, this sensitivity was independent of Gcn20, suggesting that the Gcn1 mediated paromomycin sensitivity is due to Gcn1 binding directly to the ribosome and affecting A-site function (Sattlegger and Hinnebusch, 2000).

Furthermore, Gcn1 over-expression results in a slow growth rate which is correlated with increased levels of Gcn1 associated with translating ribosomes (Sattlegger and Hinnebusch, 2000). Conversely, deletion of Gcn1 slightly increases the rate of cellular growth under replete conditions either due to a short lag phase or a slightly shorter doubling time (E. Sattlegger, unpublished observations), suggesting that Gcn1 affects an essential cellular process. In concert, the above findings suggest that Gcn1 binding to ribosomes may affect protein synthesis.

These observations raise the question of how both crucial processes - protein synthesis and monitoring amino acid availability - co-exist on translating ribosomes without interfering with each other. It is necessary to shed light on how the sensing complex can reside on the ribosome without significantly interfering with the constantly ongoing translational process.

The juxtaposition of the domains in the Gcn1/20 complex resembling an eEF3 molecule suggests that they may perform similar functions. The focus of this chapter will be to investigate whether over-expression of eEF3 affects Gcn1 function.

4.1 eEF3 over-expression causes sensitivity to amino acid analogue 3-amino-2, 4-triazole.

It is known that Gcn1 must bind to ribosomes for efficient activation of Gcn2 in response to amino acid starvation (Sattlegger and Hinnebusch, 2005). Gcn1 has homology to the N-terminal HEAT repeat domain of eEF3 (Marton et al., 1993)

which is known to be involved in ribosome binding (Andersen et al., 2006; Gontarek et al., 1998). This suggests that Gcn1 and eEF3 may bind similar or overlapping regions on the ribosome. These observations have led to the hypothesis that both Gcn1 and eEF3 cannot reside on the ribosome simultaneously. If this is true, then over-expression of eEF3 should in principle displace Gcn1 from the ribosome and thereby prevent Gcn2 activation. If this were the case, strains over-expressing eEF3 will not be able to overcome amino acid starvation. This is detectable as impaired growth on medium containing 3-amino-2,4-triazole (3AT), a drug causing histidine starvation by inhibiting the histidine biosynthetic enzyme encoded by *HIS3* (Klopotowski and Wiater, 1965). To test our prediction a semi quantitative growth assay was performed with strains over-expressing GST tagged eEF3 (GST-eEF3), or GST alone under a galactose inducible promoter.

In this study, eEF3 over-expression did not lead to any significant growth defect under replete conditions (Figure 4.1, compare rows 1 and 3, left panel). This is in agreement with the observations of Anand *et. al* (2003).

The *gcn1Δ* strain over-expressing GST alone could not grow on plates with 3AT (Figure 4.1, row 2, right panel) as it cannot overcome amino acid starvation, since *GCN1* is essential under amino acid starvation conditions. Strains over-expressing GST-eEF3 showed reduced growth on plates with 3AT (amino acid starvation medium) when compared to strains over-expressing GST alone (Figure 4.1, compare rows 1 and 3, right panel). This sensitivity to 3AT (3AT^S) may be because strains over-expressing GST-eEF3 may not be able to respond to amino acid starvation. This observation supports the idea that eEF3 competes with Gcn1 for ribosome binding. Over-expression of eEF3 renders Gcn1 non-functional and disrupts Gcn1 mediated Gcn2 activation. This finding is an independent reproduction of E. Sattlegger's unpublished data (Sattlegger and Hinnebusch, unpublished).

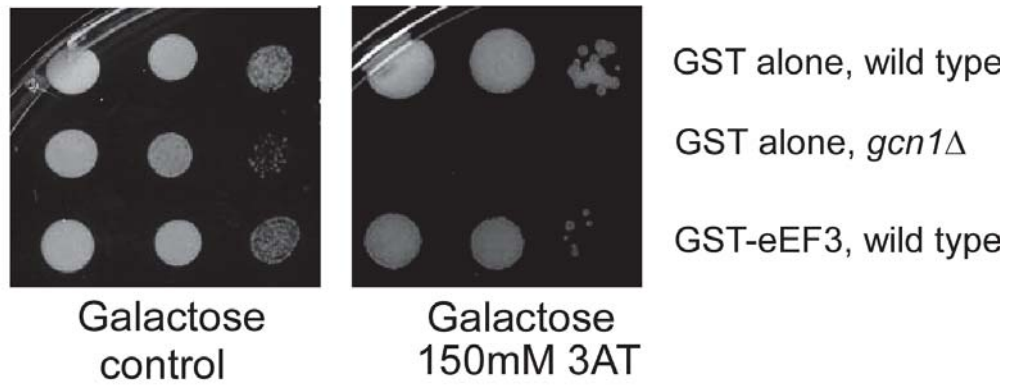


Figure 4.1: Over-expression of eEF3 causes sensitivity to amino acid analogue 3AT
H1511 strains (wild type) harboring plasmid borne alleles of GST-eEF3 (TKB705), GST alone (B2219) and H2556 (gcn1Δ) with GST alone (B2219) under galactose inducible promoter were grown to saturation. Saturated cultures were subjected to 10 fold serial dilutions and 5μl of undiluted culture and 5μl of each dilution was transferred to solid medium containing galactose (control) or galactose and 150mM amino acid analogue 3-amino-2, 4-triazole. Plates were incubated at 30°C until colonies were visible.

4.2 The slow growth phenotype (slg^-) associated with a constitutively active Gcn2 ($Gcn2^c$) can be reverted by over-expression of eEF3

The research in this section was carried out by Dr. Sattlegger (Sattlegger and Hinnebusch, unpublished); as it contributes towards understanding the effect of eEF3 over-expression on Gcn2 function it was included here.

It is known that for its activation Gcn2 must directly bind to Gcn1, and that Gcn1 must bind to the ribosome to promote full Gcn2 activation (Sattlegger and Hinnebusch, 2000, 2005). In order to test if the observed $3AT^S$ is indeed due to impaired Gcn2 activation, a $gcn2Δ$ strain expressing a plasmid borne constitutively active Gcn2 was used. Constitutively active Gcn2 ($Gcn2^c$ -E601K-E1591K, here abbreviated as $Gcn2^c$) is active even under replete conditions. This leads to increased basal eIF2α-P levels. Increased eIF2α-P levels result in a reduction of general protein synthesis. This can be observed in strains as impaired growth or as a slow growth phenotype (slg^-) (Hinnebusch, 2005). However, $Gcn2^c$ still requires Gcn1 for its activity. If eEF3 over-expression affects Gcn1 function then there should be diminished constitutive kinase activity of $Gcn2^c$ upon over-expression of eEF3. This can be observed as a reversion of the slg^- phenotype associated with $Gcn2^c$. To

test this, isogenic strains harbouring either *GCN2^c* gene or *GCN2* wild-type gene on its chromosome and plasmid borne GST-tagged eEF3, or GST alone under a galactose inducible promoter were streaked on plates containing glucose or galactose as carbon source. On plates containing glucose, i.e. when there is no over-expression of galactose regulated genes, the strains harbouring the *GCN2^c* allele showed reduced growth compared to the wild-type control (Figure 4.2 A). However, on galactose the *GCN2^c* strain over-expressing eEF3 showed improved growth as compared to the *GCN2^c* strain over-expressing GST alone (Figure 4.2 B). These results further support the hypothesis that over-expressed eEF3 reduces Gcn2 activity possibly by disrupting Gcn1 function on the ribosome.

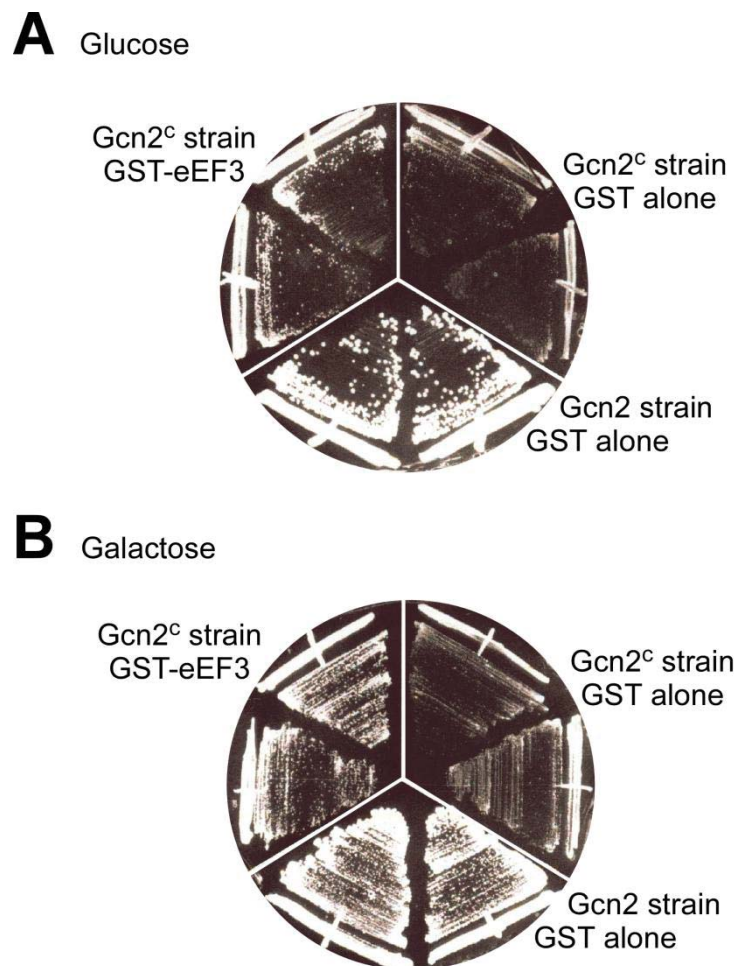


Figure 4.2: The *slg* phenotype associated with *GCN2^c* can be reverted by galactose induced over-expression of eEF3

H1613 strain (*GCN2^c* strain) harboring plasmid expressing GST-eEF3 (B2384) or GST alone (B2219) under a galactose inducible promoter and *H1511* harboring a plasmid expressing GST alone (B2219) under a galactose inducible promoter were streaked on solid medium with (A) glucose (control) or (B) galactose as the carbon source. Plates were incubated at 30°C until colonies were visible (Sattlegger and Hinnebusch, unpublished).

4.3 eEF3 over-expression impairs eIF2 α phosphorylation.

If over-expression of eEF3 affects Gcn1 function, Gcn2 activation should be hindered, indicated by diminished Gcn2 kinase activity. This can be detected by monitoring the level of eIF2 α phosphorylation since eIF2 α is the substrate of Gcn2. When Gcn2 activity is diminished there will be a reduction in the phosphorylation of eIF2 α . Therefore to determine the effect of eEF3 over-expression on eIF2 α phosphorylation levels under starvation conditions, strains harbouring galactose inducible GST-tagged TEF3 (eEF3 ORF) on a plasmid, or GST alone, were grown to exponential phase in media containing galactose as carbon source. Subsequently, 3AT was added to cause histidine starvation. The cells were starved for 20 minutes. The cells were then cross-linked with formaldehyde to prevent any further phosphorylation or de-phosphorylation events that may occur during harvesting or processing of the cells (B. Cao and E. Sattlegger, unpublished). Whole cell extracts were generated as described in chapter 2. The whole cell extract was then resolved by SDS-PAGE and transferred onto PVDF membrane as described in chapter 2. The membrane was then subjected to immunoblotting using antibodies against eIF2 α and specifically against the phosphorylated form of eIF2 α (eIF2 α -P).

It was observed that when GST-eEF3 was over-expressed it led to reduced eIF2 α -P levels, under both starved as well as replete conditions, when compared to over-expression of GST alone (Figure 4.3 A compare lanes 1, 2 with 3, 4). The amount of eIF2 α -P signal was quantified using the Multi Gauge V3.1 software and normalised against total eIF2. The normalised eIF2 α -P values are illustrated in the graph (Figure 4.3 B) as relative to unstarved strains over-expressing GST alone (wild type strain). A sample calculation from the raw data is included in Appendix A. Cells over-expressing GST alone had two times more phosphorylated eIF2 α than strains over-expressing GST-eEF3 (Figure 4.3 B). This result indicates that eEF3 over-expression inhibits Gcn2 activity, as the level of phosphorylation is decreased by 50%. This is in accordance with the observations from the 3AT sensitivity and *slg⁻* phenotype reversion.

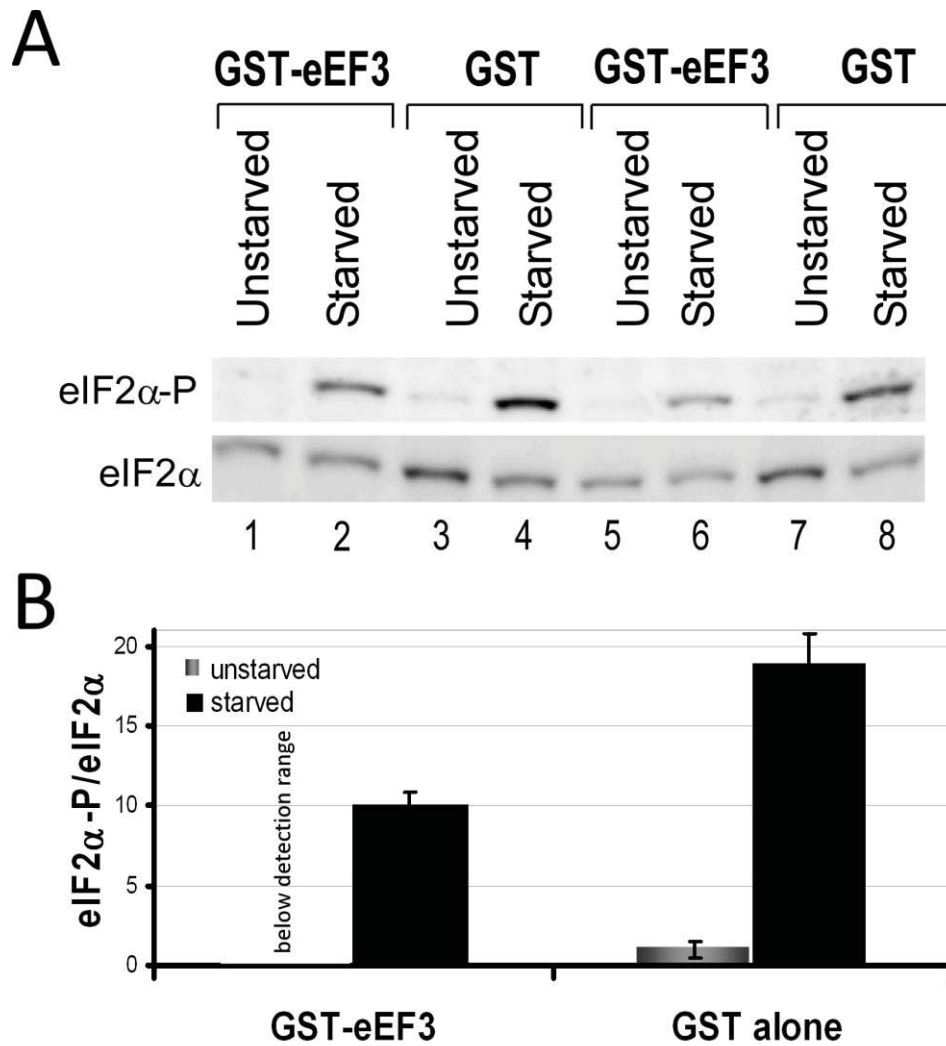


Figure 4.3: Over-expression of eEF3 reduces eIF2 phosphorylation by Gcn2 under starvation.

H1511 strains harboring plasmid borne alleles of *GST-eEF3* (TKB705), *GST* alone (B2219) under a galactose inducible promoter were grown to exponential phase on galactose as carbon source and subjected to amino acid starvation via 3-amino-2, 4-triazole (3AT) (10mM) 20 min before harvesting. An unstarved control was also grown where no 3AT was added. The whole cell extract was subjected to SDS-polyacrylamide gel electrophoreses and immunodetection using antibodies against the phosphorylated form of eIF2 α and total eIF2 α (A). The intensity of the signals was quantified using the Multi Gauge V3.1 software (Fuji Photo Film Co., Ltd.). The relative phosphorylation levels are illustrated on a graph (B). The relative levels of phosphorylation were determined by dividing the signal intensity of eIF2 α P by eIF2 α and setting the *GST* unstarved signal to 1, and illustrated in the graph. The error bars represent the standard error from replicates of two independent transformants.

4.4 Over-expression of eEF3 affects GAAC

The regulatory mechanism governing the amino acid biosynthesis (known as GAAC) will respond to any amino acid imbalance or starvation. The primary regulator for GAAC is Gcn4 which is known to stimulate the transcription of over 30 amino acid biosynthetic genes (Hinnebusch, 2005). Therefore, further support for the hypothesis could be obtained by verifying that the decreased eIF2 α -P levels

observed in section 4.3 were not unique to only one kind of amino acid starvation (histidine). To test this, the above experiment was repeated but instead of 3AT, sulfometuron (SM) was used. SM is a drug that causes starvation for branched-chain amino acids by inhibiting acetolactate synthase. Acetolactate synthase catalyses the first step in valine biosynthesis and the second step in leucine and isoleucine biosynthesis (LaRossa and Schloss, 1984).

Strains harbouring galactose inducible GST-tagged *YEF3* (gene coding for eEF3) on a plasmid, or GST alone, were grown to exponential phase in medium containing galactose as carbon source, and subsequently SM was added to cause branched amino acid starvation. The cells were starved for 15 and 60 minutes. The extracts were subjected to immunoblotting using antibodies against eIF2 α and specifically against the phosphorylated form of eIF2 α (eIF2 α -P). The amount of eIF2 α -P signal was quantified and normalized against eIF2 α . The normalized eIF2 α -P values are illustrated in the graph (Figure 4.4 B) as relative to unstarved wild type strain (strains over-expressing GST alone).

It was observed that when GST-eEF3 was over-expressed it resulted in reduced eIF2 α -P levels when compared with strains over-expressing GST under starvation conditions (Figure 4.4 A, compare lanes 2 and 5, and lane 3 and 6). Strains over-expressing GST alone had 3 times more phosphorylated eIF2 α -P than strains over-expressing GST-eEF3 upon starvation (Figure 4.4 B). These results suggest that eEF3 over-expression inhibits Gcn2 activation. Presumably, eEF3 over-expression prevents Gcn1 mediated Gcn2 activation on the ribosome, thus the strains are unable to overcome amino acid starvation (Gcn⁻ phenotype).

Interestingly, GST-eEF3 over-expression reduced eIF2 α -P levels under replete conditions as well (Figure 4.4 A, compare lanes 1 and 4). This observation indicates that the basal Gcn2 activity under replete conditions may also be mediated via Gcn1, and thus may require deacylated tRNAs. This suggests that the model proposed for Gcn2 activation by Sattlegger and Hinnebusch (2000) holds true even under replete conditions.

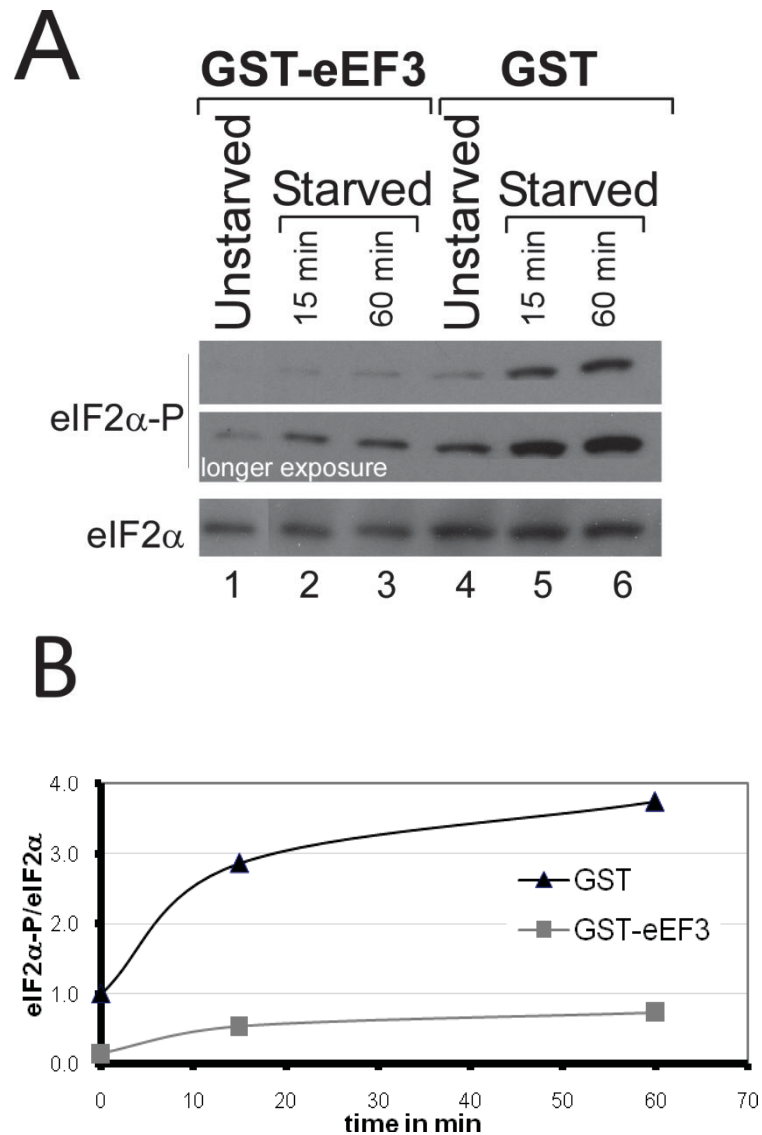


Figure 4.4: eEF3 over-expression impairs eIF2 phosphorylation by Gcn2 upon starvation.

Strains used in section 3.3 were grown to exponential phase on galactose as carbon source and subjected to amino acid starvation using sulfometuron (SM) (25 μ g) was added 15 min or 60 min before harvesting. An unstarved control was also grown where no SM was added. The whole cell extract was subjected to SDS-poly acryl amide gel electrophoreses and then immunodetection using antibodies against the phosphorylated form of eIF2 α and total eIF2 α (A). The intensity of the signals was quantified as described in Fig 3.3. The relative phosphorylation levels are plotted against time in the illustrated graph (B). The relative levels of phosphorylation were determined as described in Figure 4.3.

4.5 eEF3 over-expression barely affects Gcn1 polyribosomes association

Several lines of evidence indicate that eEF3 over-expression inhibited Gcn2 activation, presumably by hindering Gcn1-ribosome association. Since both eEF3 and Gcn1 have sequence homology and both bind the ribosome, over-expression of

eEF3 may hinder the function of Gcn1 or its ability to associate with the ribosome. To determine if eEF3 affects Gcn1-ribosome association, the effect of eEF3 over-expression on ribosome association of Gcn1 was investigated using a ribosome co-sedimentation assay. For this assay, strains bearing plasmids with either GST-tagged TEF3 (eEF3 ORF) or GST alone under a galactose inducible promoter, were grown to exponential phase in media containing galactose as the sole carbon source and then were cross-linked with formaldehyde before harvesting. Formaldehyde cross-linking stabilises protein-protein interactions, which is especially advantageous in this experiment, as ribosomal interactions are weak and hence may be lost during harvesting and processing of the samples. The whole cell extract obtained from these strains was subjected to velocity sedimentation through a sucrose gradient (4.5%-45%). Velocity sedimentation of cell extracts through a sucrose gradient allows the cellular components to resolve into the following fractions: light fractions, 40S and 60S ribosomal subunits, 80S ribosomes and polysomes. Polyribosomes or polysomes are elongating ribosomes. After velocity sedimentation, 1mL fractions were collected (using a fraction collector) while measuring A_{254} continuously to identify the positions of the separated light fractions, 40S and 60S ribosomal subunits; 80S ribosomes and polysomes. The proteins in the fractions were then precipitated with equal volumes of isopropanol and incubated at -80°C overnight. The precipitated proteins were then re-suspended in 2X protein loading dye and were resolved by SDS-PAGE and transferred onto PVDF membrane as described in chapter 2. Immunoblotting using antibodies against Gcn1, Gcn2, Rps22 (a ribosomal protein) and the GST epitope located at the eEF3 N-terminus were conducted to detect the presence and levels of the respective proteins.

In strains over-expressing GST alone, Gcn1 and Gcn2 were detected in fractions that corresponded to the 40S and 60S ribosomal subunits, 80S and polyribosomes, which were determined from the UV trace (Figure 4.5 A). The presence of ribosomal subunits or ribosomes in these fractions was confirmed by the presence of Rps22 the ribosomal small subunit protein that was detected by immunoblotting. Gcn1 co-sedimenting with ribosomes is indicative of Gcn1 binding to ribosomes (Figure 4.5 A). This is consistent with previous observations (Marton et al., 1993; Sattlegger and

Hinnebusch, 2000). GST did not associate with the ribosomes as it was detected mostly in the lighter fractions.

In eEF3 over-expression strains, GST-eEF3 was detected in fractions containing polyribosomes. Gcn1 and Gcn2 were detected in fractions that corresponded to the 40S and 60S ribosomal subunits; 80S ribosomes and polyribosomes. Rps22 was detected in fractions that had ribosomal subunits and ribosomes. (Figure 4.5 B).

The amount of Gcn1 co-sedimenting with the ribosomes was slightly reduced upon over-expression of eEF3 in comparison with strains over-expressing GST alone (Figure 4.5 A and B compare lanes 9, 10, 11, 12). There appears to be no change in the amount of Gcn2 co-sedimenting with the ribosomes in strains over-expressing eEF3 or GST alone (Figure 4.5 A and B compare lanes 9, 10, 11, 12).

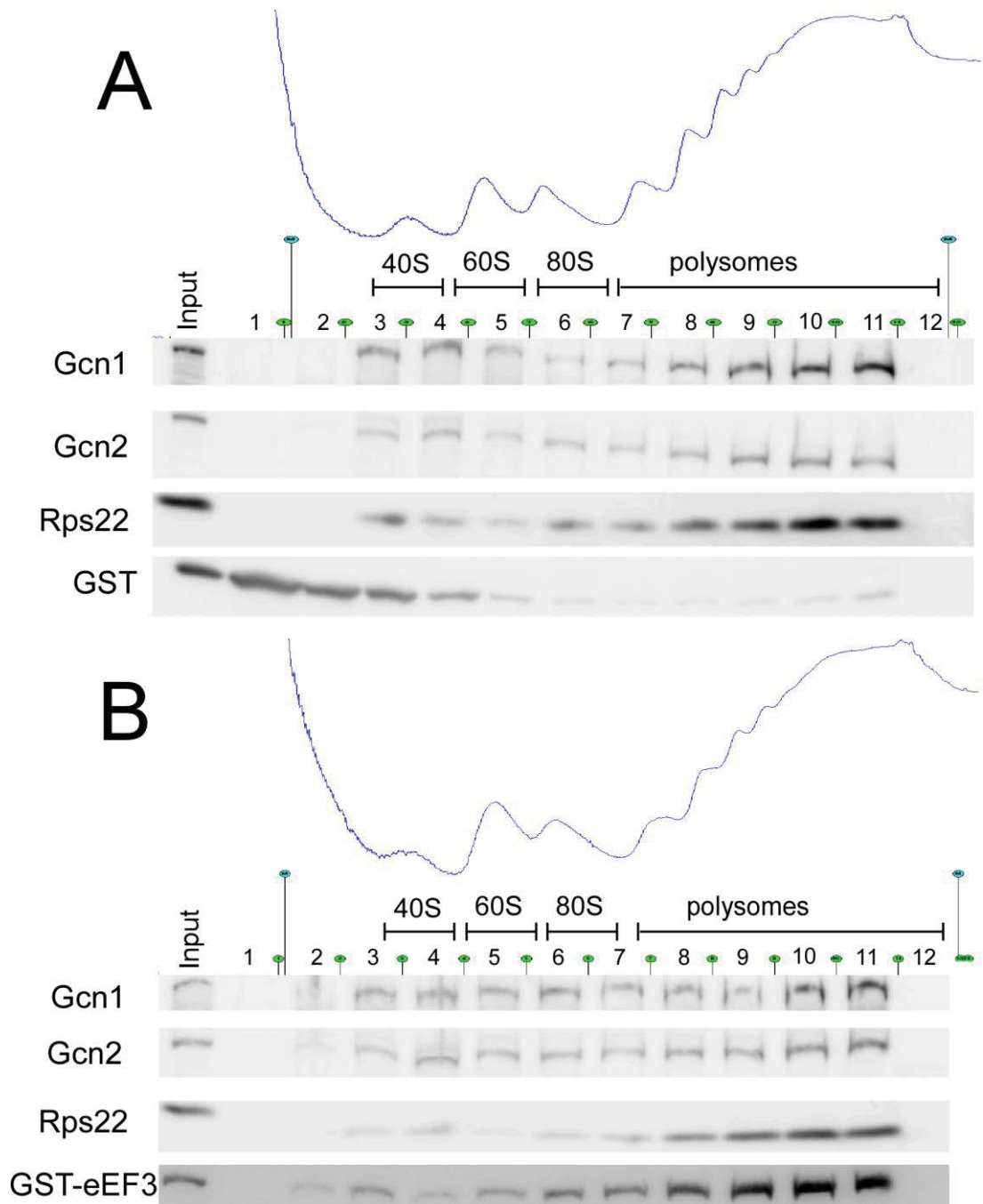
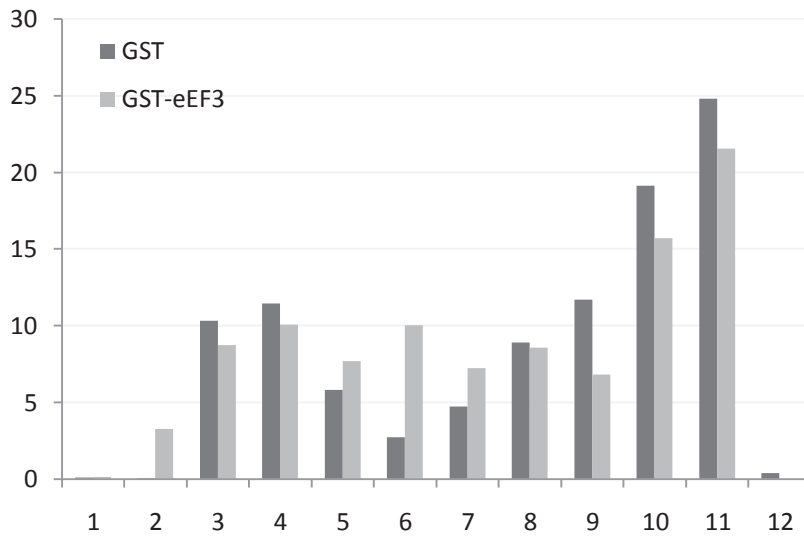


Figure 4.5: eEF3 over-expression barely displaces Gcn1 from the polyribosomes

H1511 strains over-expressing (B) GST-tagged eEF3 or (A) GST alone from a plasmid (TKB705, B2219 respectively), were grown to exponential phase to A_{600} and cross-linked with formaldehyde immediately before harvesting, and WCEs were prepared and resolved by velocity sedimentation through 4.5–45% sucrose gradients. Fractions were collected while measuring A_{254} continuously to identify the positions of polyribosomes, 80S ribosomes, and 40S and 60S ribosomal subunits. Equal proportions of the fractions were resolved by SDS-PAGE and subjected to immunoblot analysis using antibodies against Gcn1, Gcn2, RPS22, and the GST epitope located at the N terminus of eEF3.

A



B

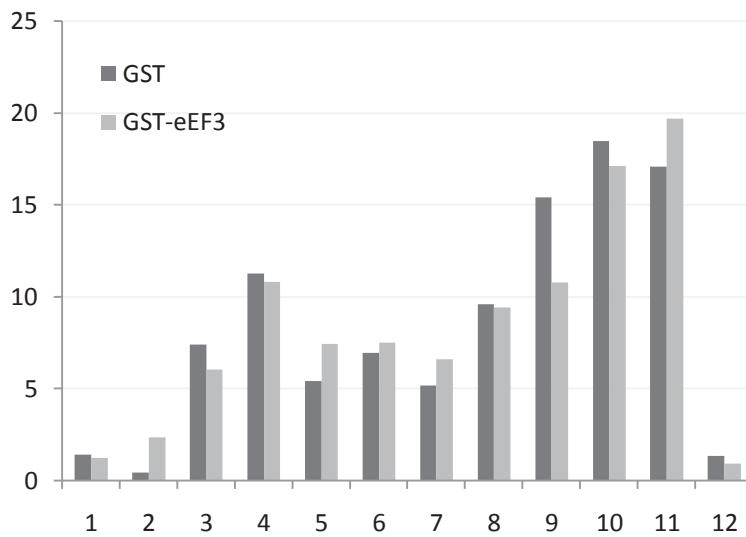


Figure 4.6: Percentage distribution of (A) Gcn1 and (B) Gcn2 in fractions collected in Figure 4.5

The amount of Gcn1 or Gcn2 in each fraction from Figure 4.5 was quantified using the Multi Gauge V3.1 software (Fuji Photo Film Co., Ltd.). Amount of Gcn1 or Gcn2 in each fraction is expressed as a percentage of the total Gcn1 or Gcn2 in all fractions.

Upon quantification of Gcn1-polyribosome association, there was a 17% reduction upon GST-eEF3 over-expression in comparison to over-expression of GST alone. This suggests that eEF3 over-expression barely affects Gcn1 ribosome binding. It should be noted that the amount of Gcn1 lost from the polysomes (~17%) was found associated to the monosomal fraction (~15%) in strains over-expressing eEF3 (Figure 4.5 B, fraction 6). This suggests that Gcn1 was displaced from polysomes to monosomes when eEF3 was over-expressed. Polysomes are elongating ribosomes where eEF3 is functional; hence the displacement of Gcn1 from the polysomes could be due to the association of the excess eEF3 to the polysomes.

When this co-sedimentation assay was repeated there was no observable reduction in the amount of Gcn1 associating with the polysomes when eEF3 was over-expressed (Appendix A). A reason for not observing a reduction could be that the displacement is dependent on the amount of eEF3 that is over-expressed and bound to the polyribosomes. In Figure 4.5 B it can be seen that eEF3 was over-expressed well and a lot of eEF3 associated with the polysomes. Hence there was an observable reduction in the amount of Gcn1 associating with polysomes. In the future, this experiment should be repeated, with eEF3 over-expressed and bound to the polysomes to the same extent as seen in Figure 4.5, to verify whether there is a similar reduction in the amount of Gcn1 associating with the polysomes.

The ribosome binding of Gcn1 was quantified from the replicates. The average percentage of Gcn1 found associating with the different ribosomal subunits in strains over-expressing GST or GST-eEF3 is illustrated in the graph below (Figure 4.7). The subtle reduction in Gcn1-polyribosome association was not found to be significant (P value 0.39 in a two tailed t-test).

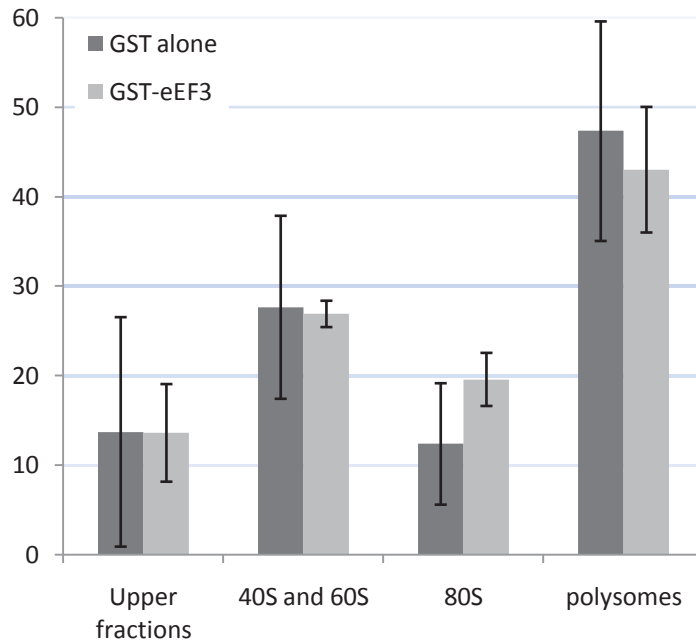


Figure 4.7: Percentage distribution of Gcn1 in the different fractions.

The amount of Gcn1 in each fraction from three independent replicates were quantified using the Multi Gauge V3.1 software (Fuji Photo Film Co., Ltd.). Amount of Gcn1 in each fraction is expressed as a percentage of the total Gcn1 in all fractions. The fractions were grouped as illustrated in the graph. The error bars represent standard error of three independent replicates. The reduction in the amount of Gcn1 found associating with the polysomes upon eEF3 over-expression was not found to be significant in a two tailed t-test (P value 0.39).

The ribosome binding of Gcn2 was also quantified from the replicates. The average percentage of Gcn2 found associating with the different ribosomal subunits in strains over-expressing GST or GST-eEF3 is illustrated in the graph below (Figure 4.8). There was no change in the amount of Gcn2-polyribosome association between strains over-expressing GST or GST-eEF3.

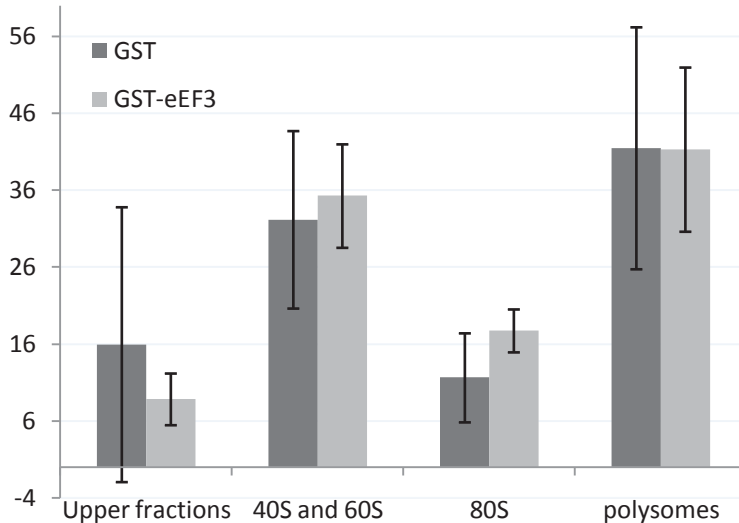


Figure 4.8: Percentage distribution of Gcn2 in the different fractions.

The amount of Gcn2 in each fraction from three independent replicates was quantified as explained in Figure 4.7. The fractions were grouped as illustrated in the graph. The error bars represent standard error of three independent replicates.

Even though it was not significant, there was a small reduction in Gcn1-polysome association when eEF3 was over-expressed. Since the reduction is subtle the experiment will have to be repeated several times to see if the subtle reduction is significant. However, if it was found to be significant, then this would suggest that over-expression of eEF3 affects only Gcn1 function but not Gcn2.

Contrary to our prediction, eEF3 over-expression only hindered Gcn1-ribosome association subtly. A possible explanation for this observation is that Gcn1 has a large ribosomal binding domain, hence has several ribosome binding sites. Many residues in the first 2052 amino acids are thought to bind the ribosome (Sattlegger and Hinnebusch, 2000, 2005). The middle portion of Gcn1 (1462-1650 aa) has sequence homology to the N-terminal domain of eEF3. Over-expressing eEF3 may hinder only one or a few ribosome contact points around the 1462-1650 amino acid residues on Gcn1. Hence Gcn1 is still able to bind the ribosome via other contact regions. Thus there was no significant reduction in Gcn1-ribosome association but instead only a weakened Gcn1-ribosome interaction was observed.

Although eEF3 over-expression did not impair Gcn1-ribosome association significantly, it somehow affects Gcn1 function. Hence the hypothesis that eEF3 and

Gcn1 compete for ribosome binding could now be modified to eEF3 over-expression negatively affects Gcn1 function.

4.6 The eEF3 C-terminus is sufficient and necessary for causing a Gcn⁻ phenotype.

If eEF3 shares ribosome binding sites with Gcn1, then over-expression of eEF3 fragments that are homologous to Gcn1 or harbouring ribosome binding domains should cause a Gcn⁻ phenotype to the same extent as the over-expression of full length eEF3. Hence the next aim was to map the region in eEF3 that was responsible for the Gcn⁻ phenotype. eEF3 has several ribosome binding sites (Andersen et al., 2006; Gontarek et al., 1998). These sites include N-terminal HEAT domain, the chromodomain which is situated within the ABC2 cassette in the C-terminus of eEF3, and a part of ABC2 N terminal to the chromodomain (Andersen et al., 2006). Of these the N-terminal HEAT domain and the chromodomain are most important for eEF3 to bind the ribosome. Gcn1 and eEF3 share homologies with each other in the eEF3 HEAT domain and the Gcn1 middle portion, which also has HEAT repeats (Marton et al., 1993). Furthermore, both eEF3 and Gcn1 have unique ribosome binding domains, which suggests that the binding sites on the ribosome may not be identical but overlapping. If eEF3 over-expression is affecting Gcn1 function on the ribosome, then over-expression of only ribosome binding fragments of eEF3 should cause a Gcn⁻ phenotype. The most obvious prediction would be that eEF3 affects Gcn1 function via the HEAT repeat domain. To test our prediction, eEF3 fragments, and full length eEF3 as a control (as shown in the schematic representation Figure 4.9 A), all fused to GST, were over-expressed in wild-type yeast cells and the cells were scored for 3AT sensitivity (3AT^S). As described earlier, impaired growth on 3AT is indicative of impaired GAAC presumably in this case by Gcn1 being rendered non-functional by the respective eEF3 fragment.

Of the fragments assayed, eEF3 fragments containing one or more ribosome binding domains did not cause (chromodomain) or barely caused (HEAT repeats, ABC cassette) a Gcn⁻ phenotype (Figure 4.9 A, rows 4, 5 & 6). To ensure that the reason for not observing a phenotype was not due to insufficient protein expression

the protein expression levels of the fragments were monitored (Figure 4.9 B). All fragments were expressed to similar or higher levels to that of full length eEF3 (Figure 4.9 C). This indicates that insufficient protein expression was not the reason for the strains to demonstrate no Gcn⁻ phenotype.

It was surprising that over-expression of the HEAT repeats fragment (which shares homology with Gcn1) did not cause a Gcn⁻ phenotype. The HEAT repeat fragment was expressed at lower levels than full length eEF3 (Figure 4.9 B, compare lanes 1 and 3 and 9 and 11). However the expression was only lower by ~8% (Figure 4.9 C), hence expression levels may not be the reason for not observing a Gcn⁻ phenotype when the HEAT repeat fragment was over-expressed.

The chromodomain (a major ribosome binding determinant) also did not cause a Gcn⁻ phenotype. It was expressed at levels comparable to that of full length eEF3 (Figure 4.9 B, compare lanes 1 and 4 and lanes 9 and 12), raising the possibility that eEF3 may require all ribosome binding sites to efficiently bind to the ribosome and hinder Gcn1 function.

Interestingly however, it was found that over-expression of the eEF3 C-terminal fragment caused a Gcn⁻ phenotype (Figure 4.9 A, rows 7 & 8). Considering that the eEF3 chromodomain was over-expressed to the same extent as the eEF3 C-terminal domain (Figure 4.9 B, compare lanes 4 and 5 and lanes 12 and 13; Figure 4.9 C) and the apparent lack of a Gcn⁻ phenotype in cells over-expressing GST alone at levels an order of magnitude greater than the GST-eEF3 fragments, indicates that the level of protein over-expression *per se* is not the cause of the Gcn⁻ phenotype.

Furthermore, a C-terminal fragment in addition to the chromodomain also caused a Gcn⁻ phenotype (Figure 4.9 A, row 8). Together these findings suggest the idea that the eEF3 C-terminal fragment may be responsible for regulating Gcn1 function on the ribosome and thereby Gcn2 function.

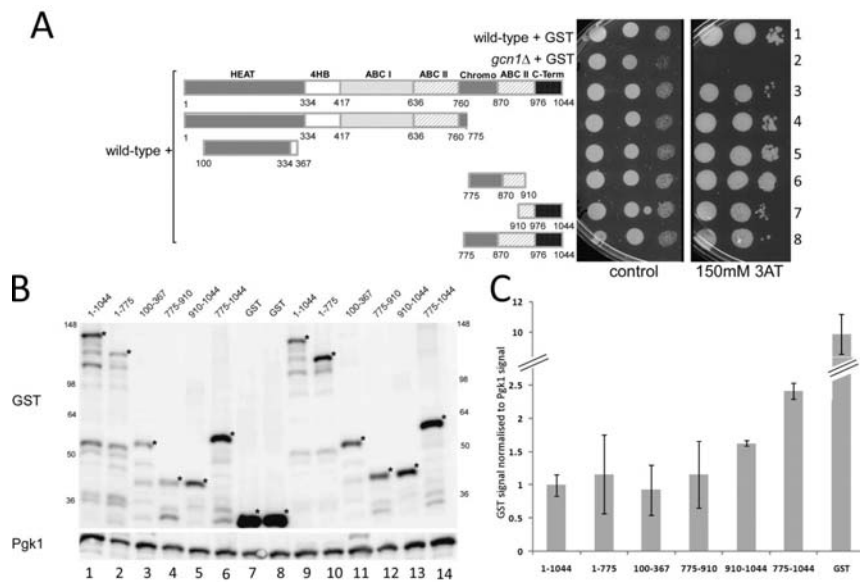


Figure 4.9: The eEF3 C-terminus is sufficient for causing a *Gcn⁻* phenotype.

(A) Saturated cultures of isogenic H2556 *gcn1Δ* strain and H1511 wild-type strain as indicated (*gcn1Δ*, and wild-type), harboring plasmid borne galactose inducible GST alone (B2219) or GST-eEF3 (TKB705), or eEF3 fragments as indicated (from top to bottom: TKB706, TKB707, TKB708, TKB709, TKB710), were subjected to 10 fold serial dilutions, and 5 μ L of undiluted culture and 5 μ L of each dilution was transferred to solid medium containing galactose (control) or galactose and 150mM amino acid analogue 3-amino-2,4-triazole. Plates were incubated at 30°C until colonies were visible. (B) H1511 harboring GST-eEF3 fragments as used A were grown to exponential phase and the WCE obtained was subjected to immunodetection with antibodies against GST epitope of the eEF3 fragments. The extract was also detected for Pgk1 for normalising the expression. (C) The expression levels of the different fragments were quantified using the Multi Gauge V3.1 software (Fuji Photo Film Co., Ltd.). The relative expression levels of each of the fragments with respect to full length are illustrated in the graph for comparison. The error bars indicate the standard error calculated for the expression levels of the proteins from two independent replicates.

Contrary to the expectations over-expression of known ribosome binding domains of eEF3 did not cause a Gcn⁻ phenotype. A possibility for this could be that eEF3 may require all ribosome binding sites to efficiently bind to the ribosome and hinder Gcn1 function. To investigate this, a GST-eEF3 fragment spanning all the known ribosome binding sites, excluding the C-terminal fragment (amino acid residues 1-910; referred to as eEF3₁₋₉₁₀), under a galactose inducible promoter was generated. A semi-quantitative growth assay was performed in which strains over-expressing full length eEF3, eEF3 C-terminal fragments and eEF3₁₋₉₁₀ fragment were scored for growth on media containing 3AT. Over-expression of eEF3₁₋₉₁₀ did not cause any growth impairment on 3AT plates, unlike over-expression of full length eEF3 or eEF3 C-terminal fragments (Figure 4.10 A compare row 6 with 3, 4 & 5). The expression level of eEF3₁₋₉₁₀ fragment and full length eEF3 were similar, although the eEF3₁₋₉₁₀ was expressed ~ 20% less in comparison to full length eEF3 (Figure 4.10 B, C). Therefore, protein expression levels may not have been the reason for not observing a Gcn⁻ phenotype. This demonstrates that the eEF3 C-terminal fragment is essential for causing the Gcn⁻ phenotype.

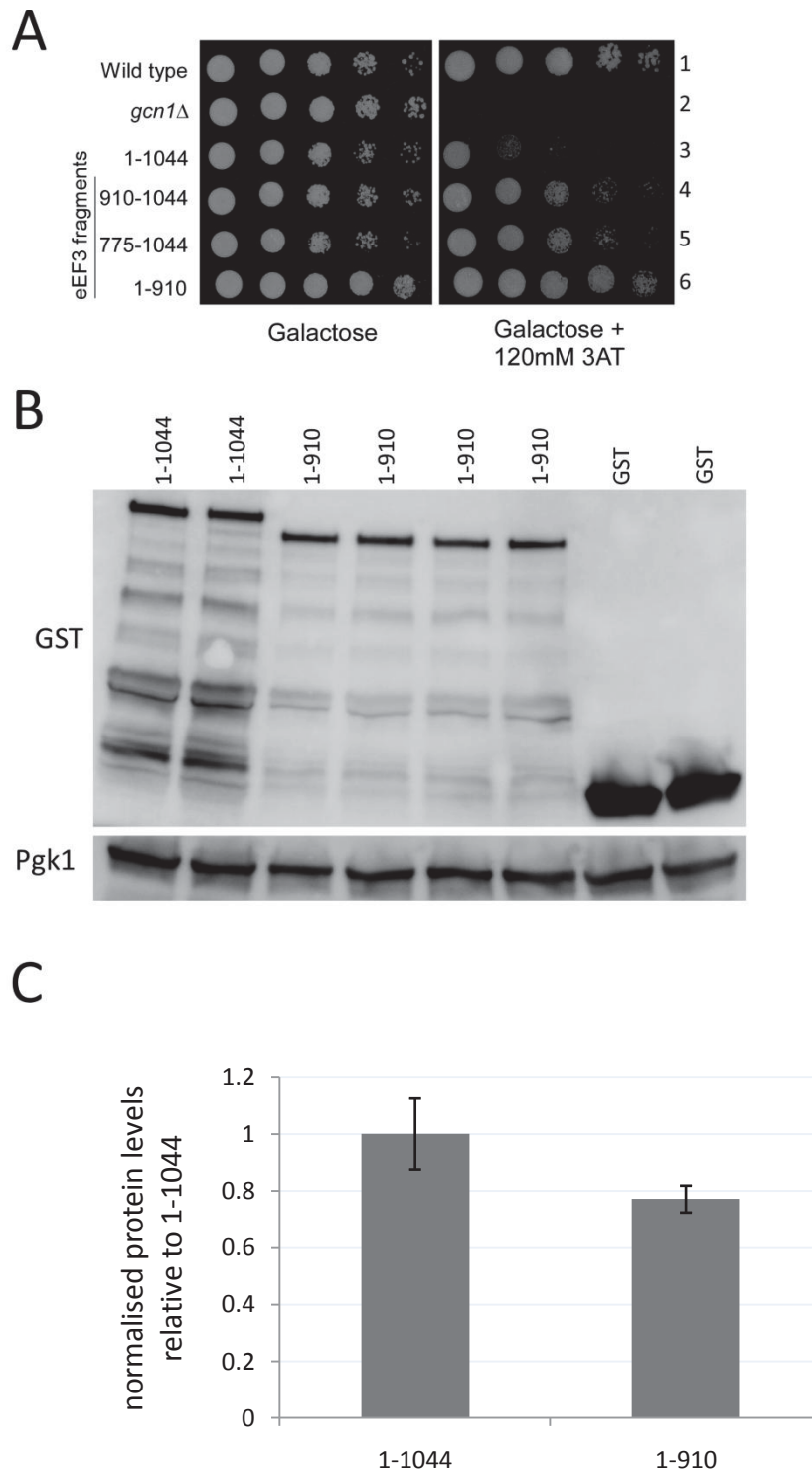


Figure 4.10: The eEF3 C-terminus is necessary for causing a Gcn⁻ phenotype

(A) Saturated cultures of isogenic H2556 strain and H1511 wild-type strain as indicated (gcn1Δ, and wild-type), harboring plasmid borne galactose inducible GST alone (B2219) or GST-eEF3 (TKB705), or eEF3 fragments as indicated were subjected to 10 fold serial dilutions, and 5μL of undiluted culture and 5μL of each dilution was transferred to solid medium containing galactose (control) or galactose and 120mM amino acid analogue 3-amino-2, 4-triazole. Plates were incubated at 30°C until colonies were visible (B) H1511 harboring GST-eEF3₁₋₉₁₀ fragment as used in (A) were grown to exponential phase and the WCE obtained was subjected to immunodetection with antibodies against GST epitope of the eEF3 fragments. The extract was also detected for Pgk1 for normalising the expression. (C) The expression levels of the fragment was quantified using the Multi Gauge V3.1 software (Fuji Photo Film Co., Ltd.). The relative expression level of the fragment with respect to full length is illustrated in the graph for comparison. The error bars indicate the standard error calculated for the expression levels of the proteins from two independent replicates for full length eEF3 and four independent replicates for eEF3₁₋₉₁₀.

4.7 The eEF3 C-terminus co-sediments with polysomes

The fact that over-expression of the ribosome binding fragments of eEF3 did not cause a Gcn⁻ phenotype, while a non-ribosome binding C-terminal fragment did, raises the possibility that the eEF3 C-terminal fragment is capable of binding the ribosomes and thereby affects Gcn1 function on the ribosome.

To investigate if the eEF3 C-terminus can bind ribosomes, ribosome co-sedimentation assays were performed using strains over-expressing eEF3 C-terminal fragments (amino acid residues 910-1044 and 775-1044 from section 4.6). The assay was performed as described in section 4.5. Upon immunoblot analysis, using antibodies against the GST epitope located at the eEF3 N-terminus, it was found that the eEF3 C-terminal fragment (amino acid residues 910-1044) co-migrates with polyribosomes (fractions 8-11, Figure 4.11 B).

This is the first observed evidence that the C-terminal fragment (amino acid residues 910-1044) of eEF3 binds the ribosomes, originally suggested as a possibility by Kambampati and Chakraborty (1997). However they suggested the C-terminal fragment of eEF3 (amino acid residues 775-1044) could bind the ribosome. The 775-1044 aa fragment contains the chromodomain, which was shown to be a major ribosome binding domain of eEF3 (Andersen et al., 2006). The cryo-electron microscopic study of the structure of eEF3 (Andersen et al., 2006) used an eEF3 (amino acid residues 1-980) lacking the C-terminal 64 amino acids. Hence ribosome binding was unlikely to have been observed in the cryo-EM study either.

Furthermore, the other fragment tested (amino acid residues 775-1044) contained chromodomain in addition to the C-terminal fragment. This fragment also co-migrated with the polysomes (fractions 8-11, Figure 4.11 C). This fragment interacted with the ribosomes more strongly than the fragment spanning the amino acid residues 910-1044 (compare fractions 8-11, Figure 4.11 B and C). This can be explained by the fact that the chromodomain is a major ribosome binding determinant in eEF3 (Andersen et al., 2006) and therefore interacts more strongly

with the ribosomes. This finding is in agreement with the findings from the eEF3 structure studies by Andersen *et. al* (2006).

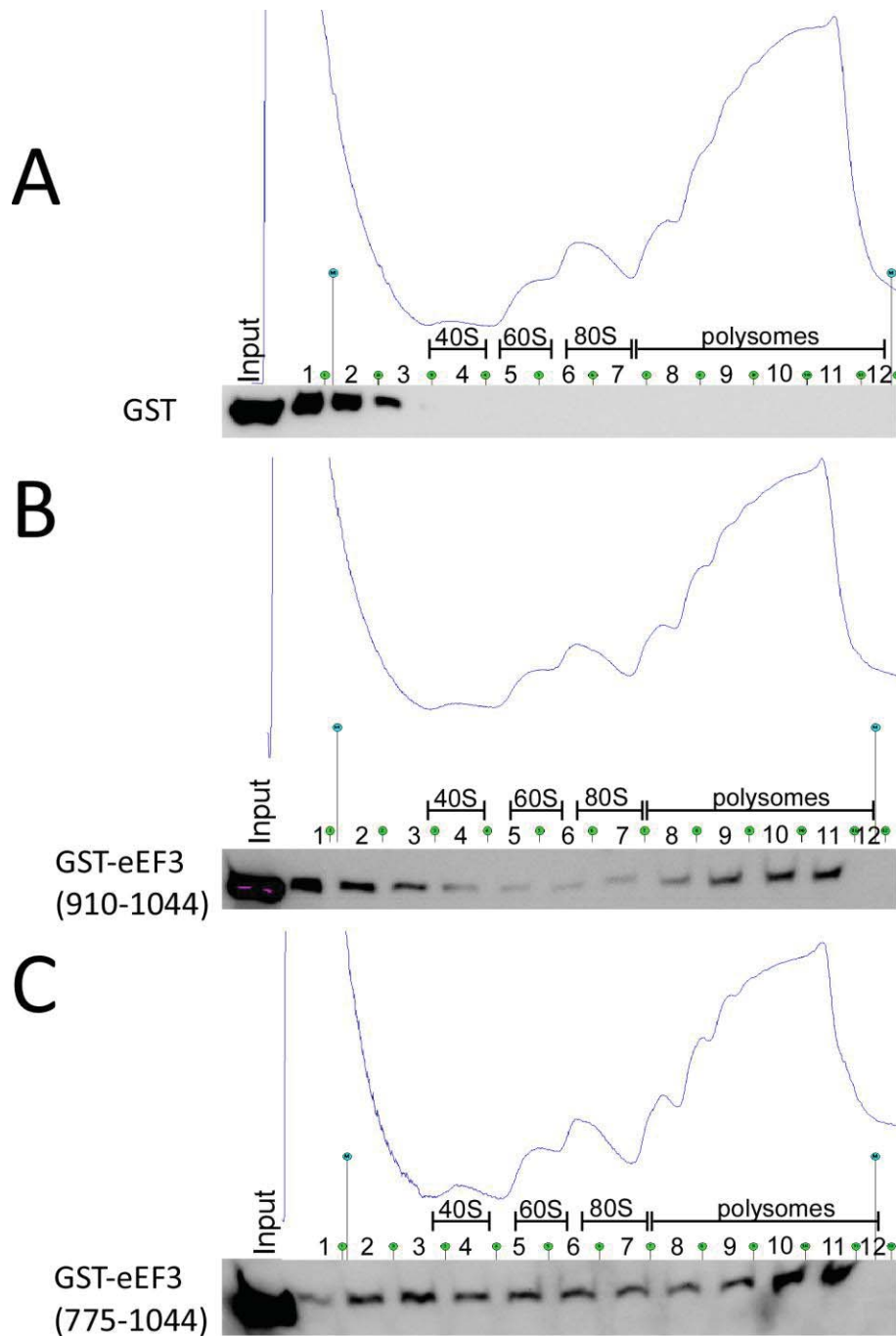


Figure 4.11: The eEF3 C-terminus co-migrates with polysomes

H1511 strains over-expressing GST-tagged eEF3 C-terminal fragments (amino acid residues 910-1044 (TKB709) or 775-1044(TKB710)) or GST alone (B2219) from a plasmid, were grown to exponential phase to 1 A₆₀₀ and cross-linked with formaldehyde immediately before harvesting, WCEs were prepared and resolved by velocity sedimentation through 4.5–45% sucrose gradients. Fractions were collected while measuring A₂₅₄ continuously to identify the positions of polyribosomes, 80S ribosomes, and 40S and 60S ribosomal subunits. Equal proportions of the fractions were resolved by SDS-PAGE and subjected to immunoblot analysis using antibodies against the GST epitope located at the N terminus of eEF3.

4.8 Discussion

Over-expression of eEF3 rendered cells incapable of responding to amino acid starvation (Gcn⁻ phenotype) by impairing Gcn2 activation as observed by a reduction in growth on medium containing 3AT and reduced eIF2 α phosphorylation upon starvation. Since eEF3 and Gcn1 are homologous proteins (Marton et al., 1993) and Gcn1 is Gcn2's effector protein, it only seemed logical that eEF3 may be affecting Gcn2 function via Gcn1. The sequence homology between eEF3 (amino acid residues 85-332) and Gcn1 is in the "middle" portion of Gcn1 (amino acid residues 1462-1650) (Marton et al., 1993), which is also known to be involved in ribosome binding (Sattlegger and Hinnebusch, 2000). This suggested that both Gcn1 and eEF3 may bind ribosomes at overlapping sites, hence competing for ribosome binding. However, polyribosome profiling indicated that over-expression of eEF3 barely affected Gcn1-ribosome association. This finding does not support the prediction that over-expression of one of the proteins hinders the ribosome association of the other protein. There could be two possible reasons for this observation. Firstly, since a grave reduction in the eIF2 α -P levels (~50%) was observed upon eEF3 over-expression it was expected that a proportional reduction in the amount of Gcn1 bound to the ribosome would be observed. However, only a subtle reduction in the amount of Gcn1 found associating with the ribosomes was observed. In any signal transduction cascade, there is amplification in the signal as the pathway progresses. Since eIF2 α -P is the penultimate signalling molecule in the GAAC pathway, the effect of eEF3 over-expression is drastic, but this may not have been the case upstream. Thus, only a subtle difference in Gcn1-ribosome association observed upon over-expression of eEF3 would be sufficient to cause a drastic reduction in phosphorylation of eIF2 α . Another possible explanation for the observed phenomenon could be based on the fact that Gcn1 is known to have many ribosomal contact points, spanning about 2052 amino acids, roughly two-thirds of the protein (Sattlegger and Hinnebusch, 2000). Over-expressing eEF3 may be competing with only one or a few ribosome binding domains in Gcn1. Hence, Gcn1 can still associate with the ribosome via its other contact points but has lost its ribosome contact points essential for its function. As a consequence, over-

expression of eEF3 renders Gcn1 non functional while its ribosome association is barely affected.

If it is true that over-expressing eEF3 only competes with a few ribosome binding sites in Gcn1, then over-expressing eEF3 in a strain with Gcn1 that has weakened ribosome binding should worsen the already weakened Gcn1 ribosome association. This would be manifested as an exacerbation of the Gcn⁻ phenotype and a more obvious reduction in polyribosome association of Gcn1 when eEF3 is over-expressed.

To further investigate these observations the domain in eEF3 that was responsible for affecting Gcn1 function was mapped. Since both eEF3 and Gcn1 have sequence homology to each other and bind the ribosomes, the logical expectation was that over-expression of the eEF3 fragments that are homologous to Gcn1 or fragments containing domains that bind the ribosome would cause the Gcn⁻ phenotype. However, this was not the case. eEF3 fragments containing one or more ribosome binding domains caused no (chromodomain) or only a slight (HEAT repeats, ABC cassette) Gcn⁻ phenotype. Instead, over-expression of the C-terminal fragment (910-1044 aa) of eEF3 caused a Gcn⁻ phenotype similar to that caused by full length eEF3. This was unexpected, as this C-terminal fragment was not previously shown but only suggested to have ribosome binding activity. This suggests that either the C-terminal fragment of eEF3 can bind the ribosomes and hinder Gcn1 function or that eEF3 affects Gcn1 function in a ribosome independent manner.

To investigate if the eEF3 C-terminus could bind ribosomes, ribosome co-sedimentation assay was performed. The C-terminal fragment (910-1044 amino acids) was found to co-migrate with the polyribosomes. The C-terminal domain of eEF3 (775-1044 amino acids) was speculated to bind ribosomes, specifically the 60S subunit of the ribosome (Kambampati and Chakraborty, 1997), while other additional ribosome binding domains were also suggested, for example the N-terminal 98-388 amino acids that binds 18S rRNA (Gontarek et al., 1998). When the structure of eEF3 was deduced via cryo EM it was found that the N-terminal HEAT domain, regions in ABCII and chromodomain (775-870 amino acids) bound the ribosomes (Andersen et al., 2006). However, the eEF3 used for the cryo EM study

contained only the first 980 amino acid residues. A previous study from Anand *et. al* (2006) had demonstrated that eEF3 lacking the last 64 amino acids was able to functionally replace the full length eEF3 in the cell in replete conditions. Hence, it was unclear if the last 64 amino acids of eEF3 could bind the ribosome, as this was never directly investigated. The co-migration of the C-terminal fragment of eEF3 with the polysomes observed in this study provides the first evidence that the C-terminal 134 amino acids may have a ribosome binding domain, albeit not a ribosome binding determinant crucial for eEF3 function. Thus, this study has revealed a novel ribosome binding domain in eEF3.

If the C-terminal fragment of eEF3 has a ribosome binding determinant, which is dispensable for eEF3 function then it raises the question as to what might the role of these C-terminal 64 amino acid residues be in the cell? Over-expression of the C-terminal eEF3 fragment affects GAAC. Taken together with the fact that eEF3 can also bind the ribosome via its C-terminal 134 amino acids, these observations suggest that the eEF3 C-terminus may be regulating Gcn1 function in order to accommodate ongoing translation elongation. eEF3 lacking the C-terminus may be able to function as full length eEF3, but it may not be able to influence Gcn1 function. Hence, a strain with eEF3 lacking the C-terminus would be predicted to have a Gcn⁻ phenotype as it would not be able to deal appropriately with amino acid shortages. It is worth noting that the strains with eEF3 lacking the C-terminal fragment as the only form of eEF3 have a slow growth phenotype (Anand *et al.*, 2006). This slow growth phenotype could be explained by the inability of these strains to regulate Gcn1 function on the ribosome, which in turn hinders protein synthesis by affecting eEF3 function and thus results in slow growth. Furthermore deletion of Gcn1 in such a strain should revert the slg⁻ phenotype.

Unexpectedly, a Gcn⁻ phenotype was not observed when eEF3 fragments containing ribosome binding sites (other than the C-terminal fragment) were over-expressed. A possible reason for this is that all the known ribosome binding domains may not be present in one fragment; hence it could not bind ribosomes efficiently. To ensure that this was not the case, a fragment of eEF3 lacking the last 134 amino acids (eEF3₁₋₉₁₀) was over-expressed and checked for a Gcn⁻ phenotype. This fragment has

all the reported ribosome binding domains (except the one identified in this study), hence should be able to bind ribosomes efficiently and cause a Gcn⁻ phenotype. In contrast to this prediction, over-expressing eEF3₁₋₉₁₀ did not cause a Gcn⁻ phenotype as opposed to over-expressing the C-terminal fragment which did cause a Gcn⁻ phenotype. This further confirms that it is the C-terminal fragment (last 134 amino acids) that is sufficient and necessary for affecting GAAC.

One major caveat of this study is that it involves the over-expression of a protein. Since the observed effect is only apparent upon over-expression of a protein (eEF3), one cannot be sure that the observed effect is directly correlated to the over-expression of eEF3 and not some other indirect effect. One way to address this issue would be to intrinsically increase the amount of eEF3 associating with the ribosomes and use such a strain to check if Gcn1 is functional. A study claimed that when a yeast strain was deleted for Stm1 (a ribosome associated protein), it had elevated levels of eEF3 bound to the ribosome (Van Dyke et al., 2009). Assuming that deletion of *STM1* increases the amount of eEF3 associating with the ribosomes one can predict that a *stm1Δ* strain would have a Gcn⁻ phenotype (since the over-expression of eEF3 affects GAAC). However, this was not the case- the *stm1Δ* strain did not show a Gcn⁻ phenotype (see appendix A). However, one cannot rule out the possibility that the observations by Van Dyke *et al.* (2009) were strain background and condition dependent and hence a phenotype could not be observed in this study. Upon detailed review of the study by Van Dyke *et al.* (2009) it appeared that when *STM1* was deleted, the increased amounts of eEF3 was not found associated with translating ribosomes but initiating ribosomes. Supporting this, a very recent study suggested that Stm1 modulates translation after the 80S ribosome formation at a late initiation stage (Balagopal and Parker, 2011). Taken together, Stm1 may limit the ribosome association of eEF3 only on initiating ribosomes and hence it is very unlikely that a *stm1Δ* strain will provide insight on polysome association of Gcn1. This also potentially explains why the *stm1Δ* strain did not show a Gcn⁻ phenotype when investigated here.

Another way to verify the findings from this study without using over-expression would be to use a mutant eEF3 that could bind tighter to ribosomes. Such a strain

would be particularly useful, because the proteins will be expressed at native levels and no protein will be over-expressed. However, we would first have to test that such a strain does not have a pre-existing growth defect or other secondary effects. Unfortunately, this experiment could not be undertaken since published results yet describe mutations in eEF3 that cause eEF3 to bind the ribosome with greater affinity than wild type.

In summary, this chapter showed that over-expression of eEF3 causes a Gcn⁻ phenotype. This Gcn⁻ phenotype was probably due to eEF3 affecting Gcn1 function hence rendering Gcn2 inactive. Over-expression of Gcn1 causes a slg⁻ phenotype, presumably because surplus Gcn1 associates with the ribosomes, affecting protein synthesis and thus resulting in the slow growth (Sattlegger and Hinnebusch, 2000). Inversely, over-expression of eEF3 causes a Gcn⁻ phenotype because surplus eEF3 associates with ribosomes, thus presumably rendering Gcn1 inactive. Taken together, this suggests that both GAAC and translation cannot happen on the same ribosome at the same time. This would seem logical for the cell since both eEF3 and Gcn1 do not need to function on the same ribosome. Under replete conditions, eEF3 is required to release the deacylated tRNA from the ribosomal E-site, while under starvation Gcn1 is thought to be involved in releasing the deacylated tRNA from the ribosomal A-site, and delivering it to Gcn2 for detecting amino acid starvation.

Gcn1 was found to interact with Rps3 (a small ribosomal subunit protein) in a genome wide protein interaction screen of *S. cerevisiae* (Gavin et al., 2006). eEF3 was shown in complex ribosomal proteins, by cryo-electron microscopy, two of which are Rps18 and RpsX2 (thought to be Rps5)(Andersen et al., 2006). Based on the ribosome binding information available for eEF3, Gcn1 and the ribosomal structure information (Spahn et al., 2001; Taylor et al., 2009), the possible positions of eEF3 and Gcn1 are outlined on the small ribosomal subunit (Figure 4.12 A, B). It appears that eEF3 and Gcn1 bind close to each other on the small ribosomal subunit (Figure 4.12 C). This allows speculation that due to the close proximity between eEF3 and Gcn1, eEF3 may be able to influence Gcn1 function on the ribosome.

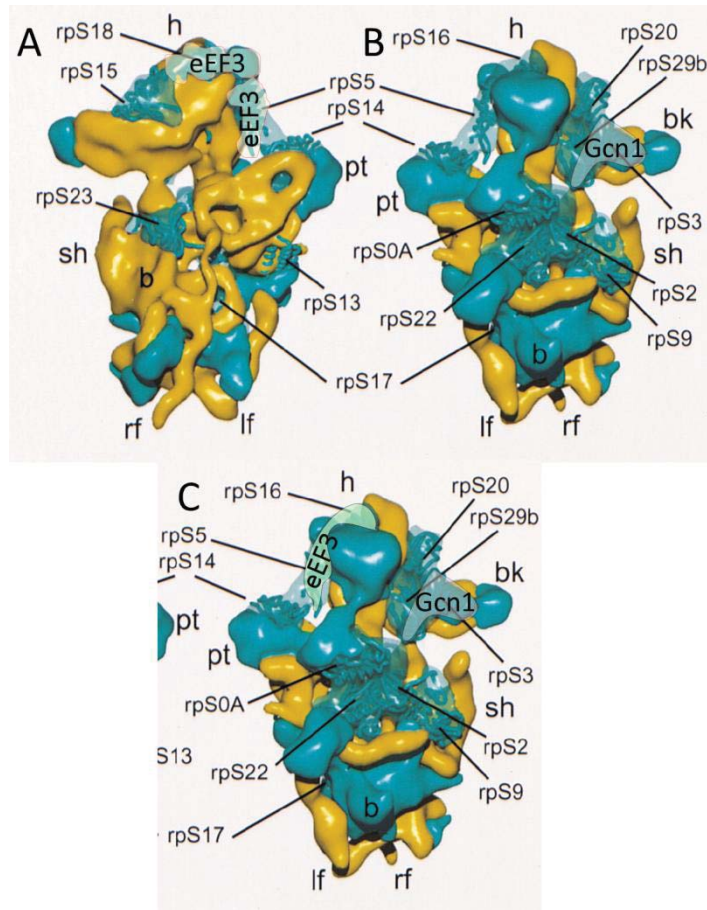


Figure 4.12: Positions of eEF3 and Gcn1 on the small ribosomal subunit

The 40S ribosomal subunit is shown from the inter-subunit side (A) and the solvent side (B, C). The RNA is coloured yellow and the protein in turquoise. The ribosomal proteins known to interact with eEF3 or Gcn1 are outlined and labeled eEF3 (A) or Gcn1 (B). Both eEF3 and Gcn1 have been outlined on the solvent side in C. Adapted from Spahn et al. (2001) with permission.

From the findings in this chapter, a model for exclusive ribosomal function of eEF3 and Gcn1 can be proposed (Figure 4.13). During normal translation, eEF3 is required to bind the ribosome to release the deacylated tRNA from the E-site to facilitate delivery of a charged tRNA at the A-site. However, when cells are starved there is an increase in the deacylated tRNA pool, and since the E-site is occupied a deacylated tRNA binds weakly to the A-site in a sequence specific manner (Nierhaus, 1990). Gcn1 can now become functional and release the deacylated tRNA at the A-site and deliver it to Gcn2 for the detection of amino acid starvation. Once the general amino acid control pathway is activated and the cell has recovered from amino acid starvation, Gcn1 no longer needs to be functional, the ribosomes can return to their translational state, and eEF3 can once again become functional for normal translation elongation.

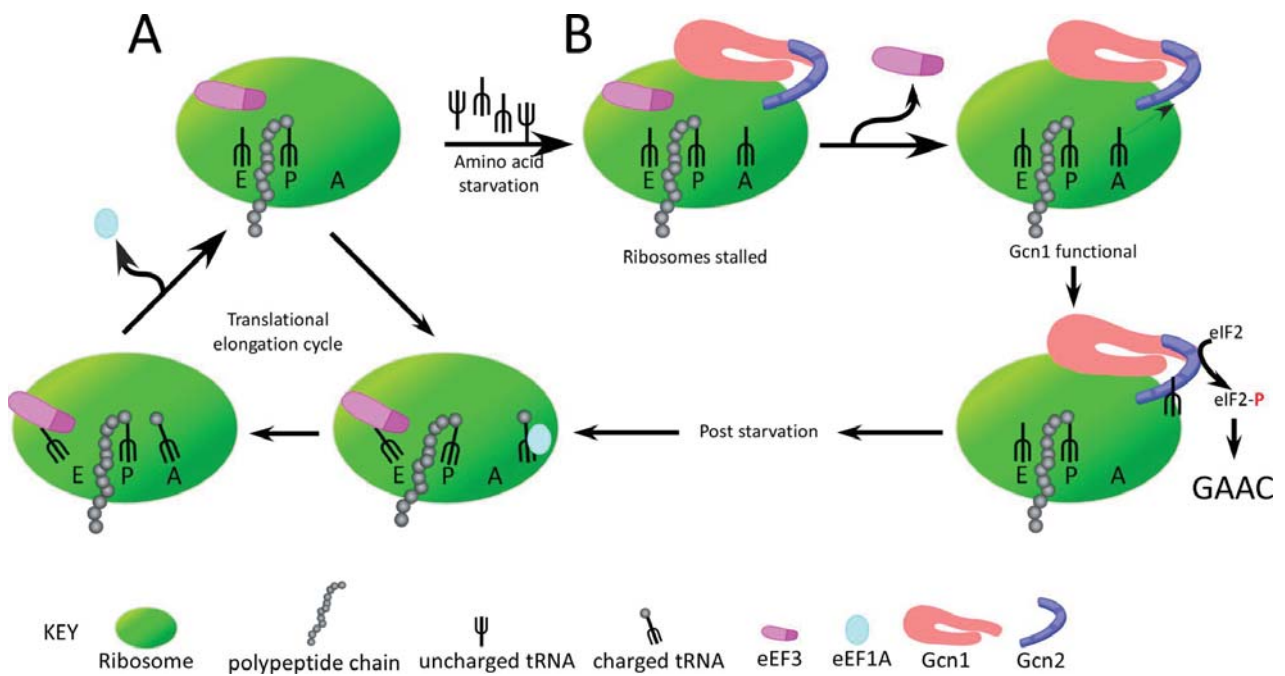


Figure 4.13: Model for exclusive ribosome function of eEF3 and Gcn1

(A) Under replete conditions eEF3 is functional on ribosomes where normal translation is underway. Gcn1 is also known to bind ribosomes in replete conditions but has not been depicted here for simplicity. (B) Upon amino acid starvation deacylated tRNAs accumulate in the cells. A deacylated tRNA binds the A-site weakly and the ribosomes are stalled. Gcn1 can now become functional and channels the deacylated tRNA to Gcn2 for detection and activation of GAAC. Once amino acid starvation is overcome normal elongation can resume. The representation of Gcn1 and Gcn2 has been adapted from Sattlegger and Hinnebusch (2000).

Chapter 5 eEF1A interacts with protein kinase Gcn2

Proteins are synthesised on the ribosome by sequential addition of amino acids to the growing peptide chain. Although the ribosome catalyses the peptide bond formation between amino acids, additional factors like translation elongation factors (EF) must associate with the ribosome in an orderly fashion for protein synthesis. One such factor, *eEF1A*, delivers the aminoacylated tRNA in a codon specific manner to the ribosomal acceptor site (A-site) (Carvalho Da da et al., 1984). The *eEF1A*-dependent binding of aminoacylated tRNA to the ribosomal A-site is stimulated by the release of deacylated tRNA at the ribosomal exit (E) site (Trianaalonso et al., 1995). The factor that assists with the release of deacylated tRNA from the ribosomal E-site is *eEF3*. However, little is known about the whereabouts of the deacylated tRNA exiting the ribosome.

As discussed in chapter 1, it is speculated that *Gcn1* is directly involved in the activation of *Gcn2* by delivering deacylated tRNAs to the A-site, transferring deacylated tRNAs from the A-site to *Gcn2*, and/or by acting as a scaffold protein for *Gcn2* and other proteins to allow them to access the A-site and the deacylated tRNAs in the A-site. Interestingly, it was shown that deacylated tRNA can enter the A-site in a sequence specific manner in eukaryotes (Murchie and Leader, 1978), however it is not known whether deacylated tRNA requires a factor, e.g. a protein, to gain access to the A-site.

According to the channelling hypothesis eukaryotic translation is thought to follow a pre-determined fate. Hence, the deacylated tRNAs should be bound to a factor or protein after it has been released from the ribosome. Considering the fact that *eEF1A* can bind deacylated tRNA (albeit with a 1000 fold lower affinity than acylated tRNA) it seems most likely that *eEF1A* shuttles deacylated tRNAs from the ribosome to the aminoacyl synthetases for recharging (Petrushenko et al., 2002). *eEF1A* then exchanges the deacylated tRNA with a recharged tRNA (Andersen et al., 2000) and delivers the aminoacylated tRNA to the ribosome for the next round of translation.

The fact that *eEF1A* binds deacylated tRNAs with a 1000 fold lower affinity than charged tRNAs (Petrushenko et al., 2002) may not support the channelling

hypothesis under replete conditions. However, upon amino acid starvation one would assume that the cell is no longer able to recharge the tRNAs causing a concomitant increase of deacylated tRNA bound to eEF1A. Thus tRNAs that cannot be charged due to amino acid starvation would remain bound to eEF1A and then have to be channelled to Gcn2 for detection. This would ensure that only deacylated tRNAs that have accumulated due to an amino acid starvation would lead to Gcn2 activation, but not the deacylated tRNAs occurring during the normal cycle of translation elongation.

The fact that eEF1A accesses the ribosomal A-site and the hypothesis that Gcn2 needs to detect deacylated tRNAs bound to the A-site in a Gcn1/Gcn2 complex raises the question if eEF1A has a role in GAAC. Gcn1 has been shown to affect the ribosomal A-site (Sattlegger and Hinnebusch, 2000). As mentioned previously, Gcn1 has homology to eEF3 (Marton et al., 1993), and eEF3 is known to interact with eEF1A (Anand et al., 2003), raising the possibility that eEF1A may interact with Gcn1. eEF1A has also been found in complex with aminoacyl tRNA synthetases (Petrushenko et al., 2002). Gcn2 has a HisRS domain, which resembles the histidyl tRNA synthetase like domain (as detailed in section 1.5); hence allowing one to speculate that eEF1A could also interact with Gcn2. The focus of this chapter is on research carried out to determine whether or not there is a link between eEF1A and GAAC.

5.1 His-eEF1A co elutes with Gcn2 but not Gcn1

According to the current model (as discussed in chapter 1), Gcn2 detects the amino acid starvation signal (deacylated tRNAs) on the ribosome whilst in a Gcn1/20/Gcn2 complex. eEF1A delivers amino acylated tRNAs to the ribosome during translation, and may also be the candidate that delivers the deacylated tRNAs to Gcn2 or Gcn1 (as discussed above). If this is the case then eEF1A and the Gcn proteins should be able to interact with each other, either directly or indirectly via the ribosome (i.e. coexist on the ribosome). This can be probed by *in vivo* protein-protein interaction assays. To test the prediction, a strain deleted for both genes encoding for eEF1A

(TEF1 and TEF2), harbouring His₆ tagged eEF1A (His₆-eEF1A) on a plasmid was obtained. The whole cell extract from exponentially growing His₆-eEF1A and untagged eEF1A strains were generated and incubated with Ni charged resin. This incubation captures the His₆-eEF1A onto the Ni charged resin. The resin was subsequently washed with buffers containing increasing concentrations of imidazole resulting in a stepwise elution of the His₆-eEF1A. When an excess of imidazole (in the buffer) is passed through the column it displaces the His-tag from nickel co-ordination, freeing the His₆-eEF1A, which can then be collected. The binding of His₆-eEF1A to the resin and subsequent elution of Gcn1, Gcn2 and eEF1A was investigated by resolving the washes by SDS PAGE and transferring onto a PVDF membrane as described in chapter 2. The elution profile of His₆-eEF1A could be seen by Ponceau S staining of the membrane. The membrane was then subjected to immunoblotting using antibodies against Gcn1, Gcn2 and Rps22.

It was found that both eEF1A and Gcn2 showed similar elution profiles (Figure 5.1). Maximum elution of Gcn2 coincided with that of eEF1A (at 50mM and 100mM imidazole), suggesting that Gcn2 interacts with eEF1A. This interaction was found to be specific, since the control strain harbouring untagged eEF1A did not show any eEF1A or Gcn2 being eluted.

Gcn1 was eluted in the first four washing steps. The amount of Gcn1 eluted was more or less uniform in all the four washes (Figure 5.1). If there was an interaction with eEF1A, then the washing step where maximum amount of Gcn1 eluted must coincide with the washing step where most His₆-eEF1A eluted. However, this was not the case. Hence, Gcn1 may not be interacting with eEF1A. However, one cannot rule out that there may be a weak interaction between eEF1A and Gcn1 which may have been disrupted with low imidazole concentrations. Another possibility could be that the Gcn1 that was found in the washes may have been associated with Gcn2 and low imidazole concentrations disrupted the Gcn1-Gcn2 interaction.

eEF1A is known to bind ribosomes, and as expected, the ribosomal protein Rps22 co-eluted with His₆-eEF1A, but not with untagged eEF1A. It is worth noting that Gcn2 also binds ribosomes. Since Gcn2 came down in the same elution steps as eEF1A and Rps22 and since Gcn2 is known to bind ribosomes, it raises the question

of whether the interaction between eEF1A and Gcn2 is bridged by the ribosome.

Together, the above observations suggest that Gcn2 and eEF1A are components of the same complex, and that Gcn1 may not be an integral part of this complex.

This finding is an independent reproduction of E. Sattlegger's unpublished data (Sattlegger and Hinnebusch, unpublished).

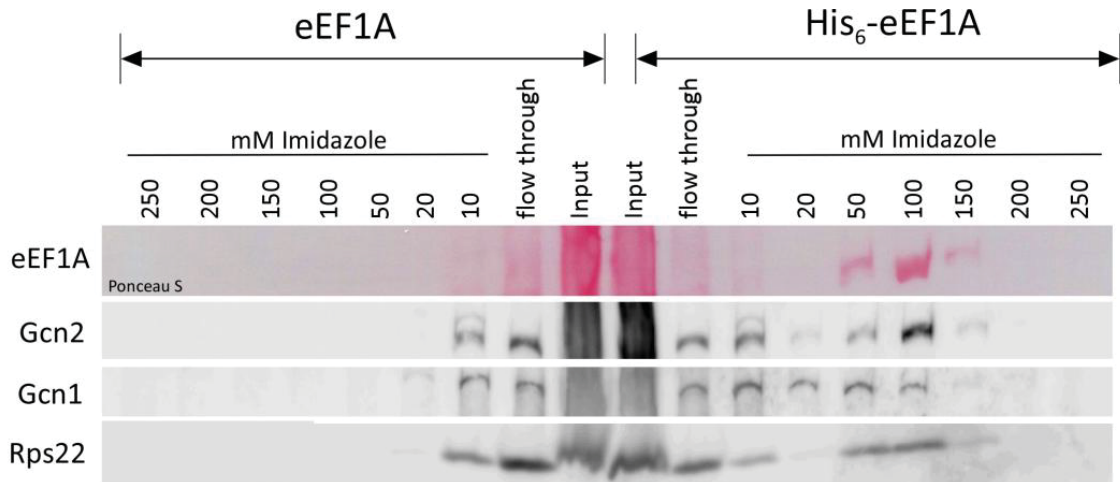


Figure 5.1: Gcn2 co-elutes with His₆-eEF1A

Strains harboring plasmid borne His₆-eEF1A or untagged eEF1A as the only form of eEF1A (TKY865 and TKY864 respectively) were grown to exponential phase. The whole cell extract generated from these strains was subjected to an *in vivo* protein-protein interaction assay, where the His₆-eEF1A was bound to a Ni NTA resin. A step wise elution with incremental imidazole concentrations was performed. Equal proportions of the flow through and eluates were resolved on a SDS-polyacrylamide gel and subjected to immunodetection using antibodies against Gcn1, Gcn2, and Rps22.

5.2 eEF1A co-immunoprecipitates Gcn2, but not Gcn1

The research in this section was carried out by Dr. Sattlegger (Sattlegger and Hinnebusch, unpublished); as it contributes towards investigating whether or not eEF1A and Gcn2 are part of the same complex it was included here.

In order to obtain independent evidence showing if eEF1A and Gcn2 exist in the same complex, another approach was sought in which neither of the proteins were tagged and both were expressed from the chromosomal gene, thus at native levels. A co-immunoprecipitation experiment using eEF1A-specific antibodies was performed. Since eEF1A is essential, it was not possible to use a strain deleted for eEF1A as a negative control, a no antibody control was used instead. Wild-type yeast strain was grown to exponential phase and harvested. Whole cell extract was generated as described in chapter 2. This whole cell extract was incubated with

sepharose beads coated with either eEF1A antibody or no antibody. The beads were washed to remove all unbound proteins. The immune complexes were resolved by SDS PAGE and transferred onto a PVDF membrane as described in chapter 2. The samples were subjected to immunoblotting analysis using antibodies against Gcn2 and Gcn1. Gcn2 specifically co-immunoprecipitated with eEF1A antibodies, in contrast to Gcn1 (Figure 5.2), implying that Gcn2 and eEF1A must reside in the same complex. Since eEF1A is known to directly associate with ribosomes, it is very likely that ribosomal proteins also co-precipitated in addition to Gcn2. However, since this assay does not allow for the distinction of whether Gcn2 binds directly to eEF1A or via ribosomes, probing for ribosomal proteins was not attempted.

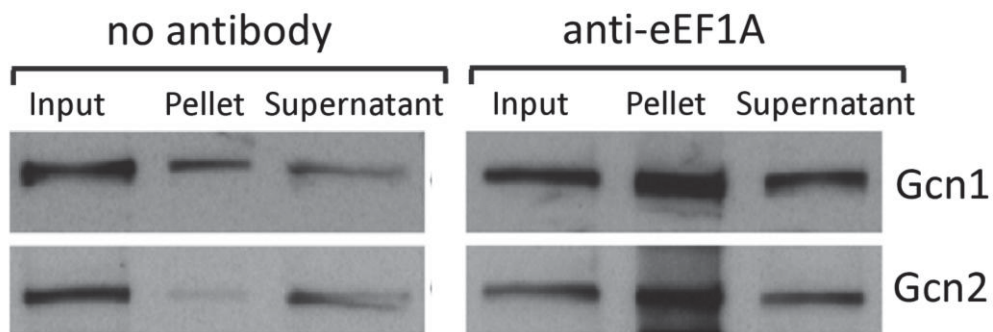


Figure 5.2: Gcn2 co-immunoprecipitates with eEF1A

Whole cell extracts from exponentially growing yeast strains (H1511) were generated and incubated with sepharose beads or sepharose beads coated with anti-eEF1A antibody. The immune complexes were resolved by SDS PAGE and analysed by immunoblotting with antibodies against Gcn1 and Gcn2 (Sattlegger and Hinnebusch, unpublished)

5.3 eEF1A interacts with the C-terminus of Gcn2

To further investigate the Gcn2-eEF1A interaction, identifying the domain in Gcn2 that interacts with eEF1A was undertaken. In order to map the region in Gcn2 necessary for eEF1A binding, various fragments spanning Gcn2 were tested for eEF1A interaction. For this, a GST tag was fused at the N-terminus of Gcn2 fragments, and these fragments were over-expressed and purified from *E. coli* BL-21. Each GST-Gcn2 fragment was immobilised on glutathione beads and then incubated with yeast extract derived from *gcn2Δ* strains. The precipitates were resolved by SDS PAGE and transferred onto a PVDF membrane as described in chapter 2. The samples were then subjected to immunoblotting assays using

antibodies against eEF1A, Gcn1, Rps22 and GST.

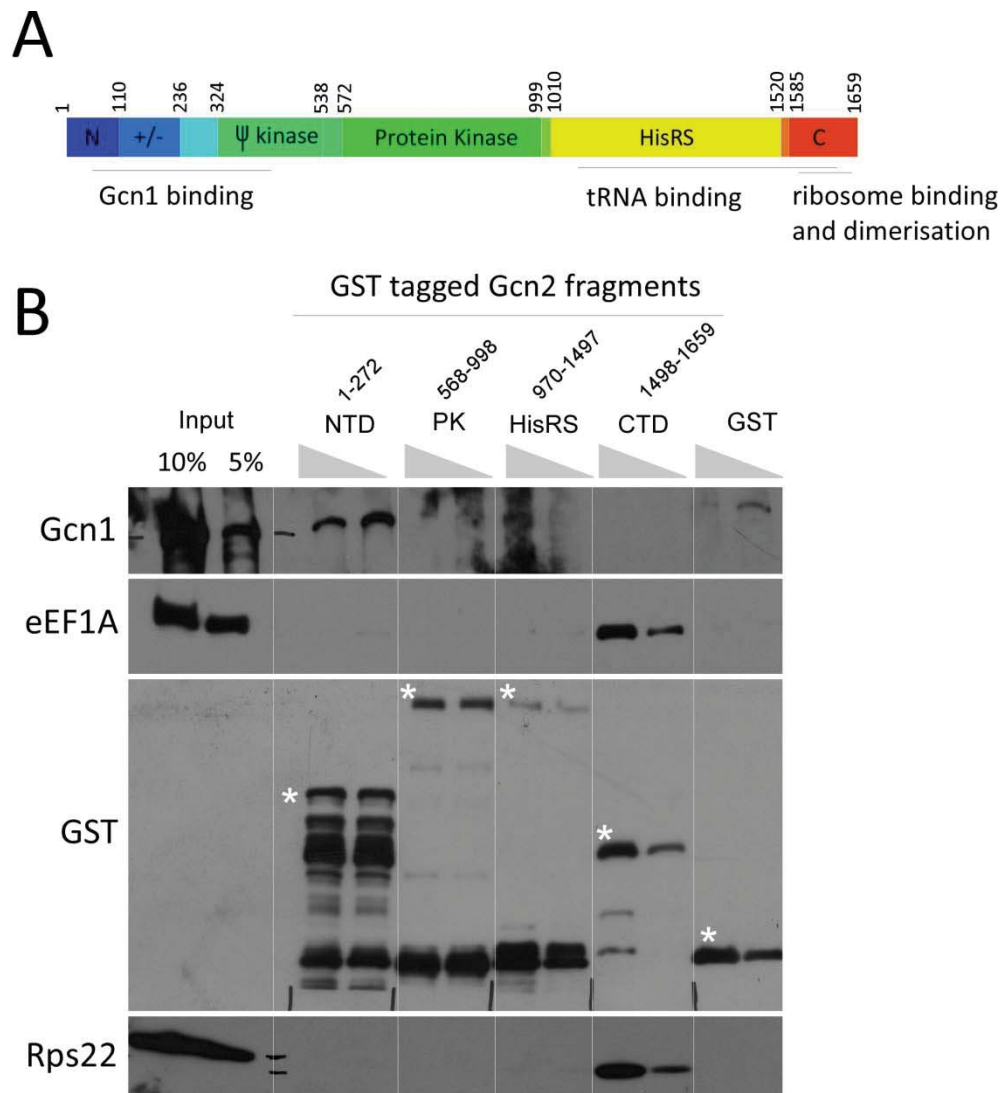


Figure 5.3: GST-Gcn2-C terminus interacts with eEF1A and ribosomes

(A) Schematic representation of the domains in Gcn2, as detailed in Chapter 1. (B) GST-Gcn2 fragments (NTD- N- terminal domain, PK- protein kinase domain, HisRS- Histidyl tRNA synthetase like domain, CTD- C-terminal domain) indicated with white star (the numbers represent amino acid residues) expressed and purified from *E. coli* were immobilized on glutathione beads and incubated with whole cell extract from strains deleted for Gcn2 (H2557). The proteins bound to the beads were then resolved on a SDS-polyacrylamide gel and subjected to immunodetection using antibodies against Gcn1, eEF1A, GST and Rps22

Among the fragments tested, the Gcn2-C-Terminal Domain (amino acid residues 1498-1659) (CTD) appeared to be the fragment that most strongly co-precipitated eEF1A (Figure 5.3 B), indicating that the Gcn2-CTD interacts with eEF1A. The Gcn2-CTD also harbours the ribosome binding site (Ramirez et al., 1991; Zhu and Wek, 1998), and as expected it co-precipitated the ribosomal protein Rps22. This suggests that Gcn2 binds eEF1A via the ribosome; i.e., the interaction between Gcn2 and

eEF1A is bridged by the ribosome and both eEF1A and Gcn2 can co-exist on the ribosomes. However, one cannot rule out that the interaction may be direct and that the Gcn2-CTD harbours the eEF1A binding site in addition to the ribosome binding activity.

In accord with the expectation, the Gcn2 N-terminal domain (containing the RWD domain) co-precipitated Gcn1. This observation is in corroboration of previous findings (Garcia-Barrio et al., 2000). However, it did not co-precipitate any eEF1A. The HisRS fragment did not precipitate eEF1A, however one cannot rule out that the reason for this could be due the partial degradation of the fragment when assayed. When the constructs used were verified, it was found that the N-terminal fragment only spanned 1-272 amino acids instead of 1-598 amino acids as previously assumed. A stretch of 296 amino acids of Gcn2 (272-598 amino acids) has therefore not been investigated for eEF1A binding which may harbour an additional eEF1A binding site. Due to time constraints this was not included in this study.

5.4 The interaction between eEF1A and Gcn2 is not bridged by the ribosome

Since the C-terminal domain of Gcn2 is involved in ribosome binding and it precipitated eEF1A, it was still unclear if their interaction was mediated by the ribosome. Hence it was interesting to investigate if the eEF1A-Gcn2 interaction is bridged by the ribosome. The Gcn2-CTD harbours residues K1552, K1553, and K1556, all of which are essential for ribosome association (Zhu and Wek, 1998) and required for tRNA binding (Dong et al., 2000). If the Gcn2-CTD-eEF1A interaction is bridged by ribosomes, then mutating these lysine residues should reduce or abolish ribosome binding and therefore reduce eEF1A precipitation.

In order to determine whether the observed eEF1A Gcn2-CTD interaction was direct or indirect via the ribosome, a GST tagged Gcn2-CTD containing K1552L, K1553I, and K1556I substitutions (Gcn2-CTD K3) was generated, expressed from *E. coli* BL21 cells and the above co-precipitation assay was repeated. As expected and observed in the previous section the Gcn2-CTD co-precipitated eEF1A; interestingly the Gcn2-

CTD *K3* also co-precipitated eEF1A (Figure 5.4), indicating that the eEF1A-Gcn2 interaction may not be bridged by the ribosome. The precipitates were probed for ribosomal proteins Rps22 and Rpl39 and as expected and previously observed both ribosomal proteins co-precipitated with the Gcn2-CTD. A small amount of ribosomal protein also co-precipitated with the Gcn2-CTD *K3* (Figure 5.4).

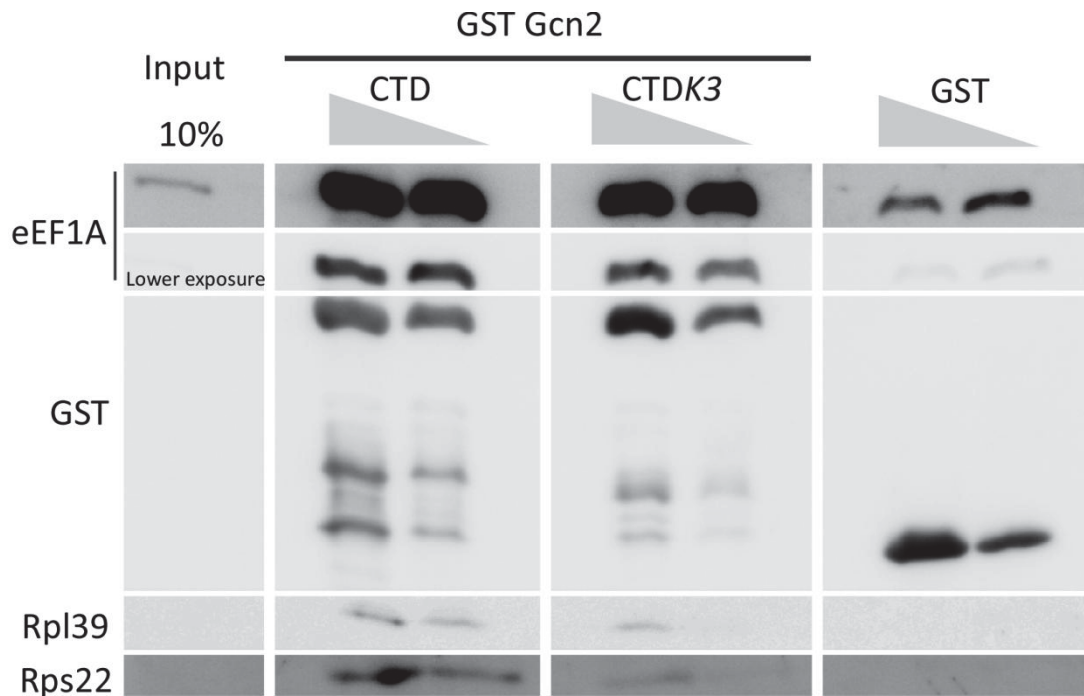


Figure 5.4: eEF1A-Gcn2 interaction is not mediated via the ribosome

Gcn2-CTD and *Gcn2*-CTD *K-3* fragments expressed and purified from *E. coli* were immobilized on glutathione beads and incubated with whole cell extract from *gcn2Δ* strains (H2557). The proteins bound to the beads were then resolved on a SDS-polyacrylamide gel and subjected to immunodetection using antibodies against eEF1A, GST, Rpl39 and Rps22

Quantification of the ribosomal signals showed ribosomal binding to be reduced by 78% (Rps22) and 60% (Rpl39) in *Gcn2*-CTD *K3* when compared to *Gcn2*-CTD. This result provides hints that there is some residual ribosome binding activity in the fragment with the C-terminal fragment of *Gcn2* containing the lysine substitutions mentioned earlier. Supporting this, in figure 4 of Zhu *et al.* (1998) the *K3* mutant *Gcn2* (called *Gcn2*-605 by Zhu *et al.*, 1998) shows some residual ribosome binding (see discussion for details). Furthermore, it indicates that the three lysine mutations do not completely abolish ribosome binding, as reported by Zhu *et al.* (1998), rather

they severely reduce it.

If the eEF1A-Gcn2 interaction was mediated via the ribosome then one would expect to find at least 80% or 60% reduction in eEF1A binding to Gcn2-CTD K3. However, when the eEF1A signal was quantified, this was found not to be the case. The amount of eEF1A binding to Gcn2-CTD K3 was reduced by only 11% when compared to Gcn2-CTD, suggesting that the interaction is not mediated by the ribosome. This is further supported by the fact that reduction in ribosome binding between Gcn2-CTD K3 and Gcn2-CTD was near significant (P value 0.06 for Rps22 and P value 0.04 for Rpl39 in a two tailed t-test) while the reduction in eEF1A binding was not significant (P value 0.57 in a two tailed t-test). These observations indicate that the eEF1A – Gcn2 interaction is not principally mediated via the ribosome and is not dependent on the ribosome. However, there was an 11% reduction in eEF1A binding to the Gcn2-CTD K3 in comparison with Gcn2-CTD and hence there is a possibility that the ribosomes may be stabilising the Gcn2-eEF1A interaction.

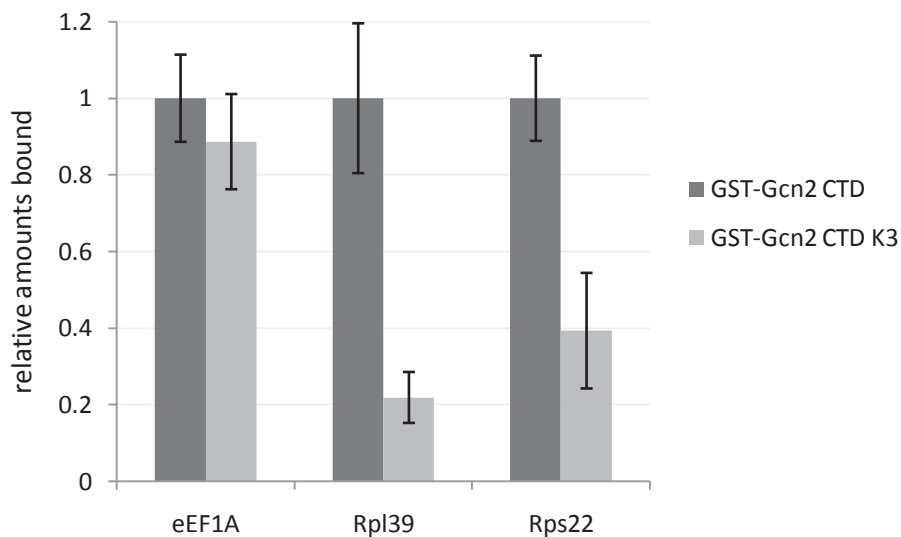


Figure 5.5: Comparison of the amount of eEF1A and ribosomal proteins bound to Gcn2 CTD and the Gcn2 CTD K3

The signals of GST, eEF1A, Rpl39 and Rps22 from two replicates were quantified using Multi Gauge V3.1 software (Fuji Photo Film Co., Ltd.). Amount of eEF1A, Rpl39, Rps22 were expressed relative to the amount of GST and the average was illustrated in the graph. The error bars represent standard error of the two replicates.

To ensure that the interaction observed was independent of the ribosomes, another approach was taken in which the yeast extract derived from *gcn2Δ* strains was first subjected to high speed centrifugation of 200,000g for 1 hr at 4°C to pellet out the ribosomes. The resulting post ribosomal supernatant (PRS) was collected and checked for any residual ribosomal proteins by resolving equal amounts (25µg) of both whole cell extract before and after centrifugation (PRS). The resolved proteins were then transferred onto a PVDF membrane as described in chapter 2. The membrane was probed for ribosomal protein Rpl39. Rpl39 was below the detection limit in the PRS sample (Figure 5.6 A), indicating that the post ribosomal supernatant contained negligible amounts of ribosomes.

The GST Gcn2-CTD and GST fragments were immobilised on glutathione beads and then incubated with equal amounts of yeast extract derived from *gcn2Δ* strains and the post ribosomal supernatant derived from this extract. The assay was performed as described in section 5.3. The samples were subjected to immunoblotting assays using antibodies against eEF1A, Rpl39 and GST.

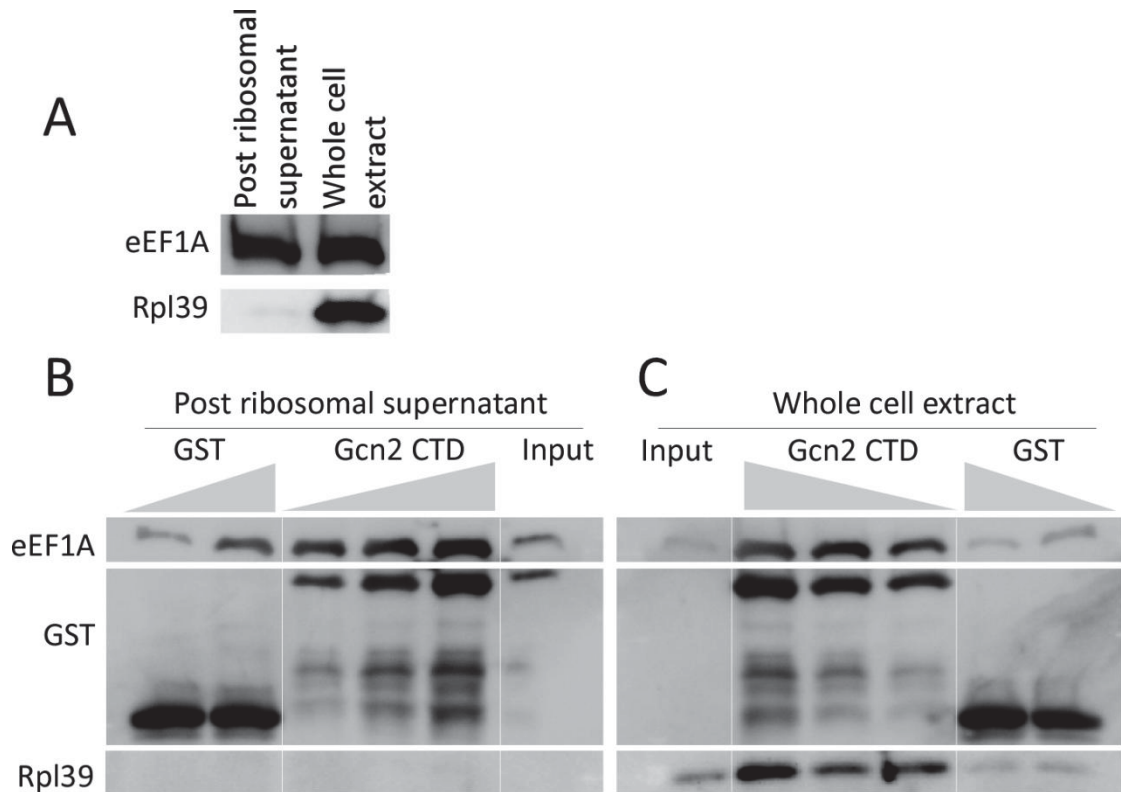


Figure 5.6: eEF1A-Gcn2 interaction is independent of the ribosome

(A) 25 μ g of post ribosomal supernatant and whole cell extract were probed for eEF1A and Rpl39 to check the reduction in the ribosomal proteins in PRS sample. Gcn2-CTD fragment expressed and purified from *E. coli* was immobilized on glutathione beads and incubated with post ribosomal supernatant (B) and whole cell extract (C), from *gcn2 Δ* strains (H2557). The proteins bound to the beads were then resolved on a SDS-polyacrylamide gel and subjected to immunodetection using antibodies against eEF1A, GST, Rpl39

In accordance with the observation in figure 5.4, eEF1A associated with the Gcn2-CTD fragment both in the presence and absence of ribosomal proteins (Figure 5.6 B and C). This result indicates that eEF1A and Gcn2-CTD interact independently of ribosomal proteins. No Rpl39 was detected in the samples incubated with PRS (Figure 5.6 compare B and C), further supporting the idea that eEF1A and Gcn2 interact independently of ribosomal proteins.

5.5 Direct interaction between eEF1A and Gcn2

The next approach used to ensure that the interaction is not bridged by any other molecule in the yeast whole cell extract was an *in vitro* binding assay with purified eEF1A. Purified eEF1A was supplied by Dr. Sattlegger. Briefly a *GCN2 Δ* yeast strain with His₆-eEF1A as the only form of eEF1A (ESY10101) was grown and the whole cell extract was prepared. The whole cell extract was then incubated with Ni charged

resin. The resin was subsequently washed to remove all unbound proteins. The His₆-eEF1A was then eluted with buffer containing 250mM imidazole. The eluate was resolved by SDS PAGE. The gel was stained using Coomassie brilliant blue stain to check for any contaminating proteins in the purified eEF1A protein; none were detectable (Figure 5.7).

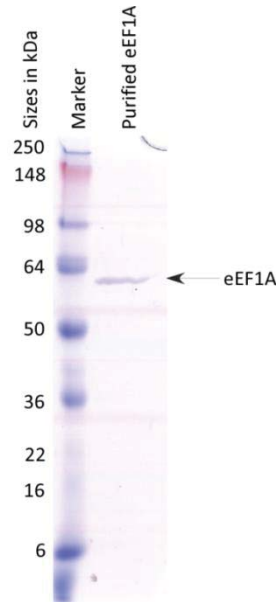


Figure 5.7: Coomassie stained gel with purified eEF1A

His₆-eEF1A was purified from WCE of ESY10101 by affinity binding to a Ni charged resin and eluted with 250mM imidazole. The purified His₆-eEF1A was resolved on a 10% polyacrylamide gel. The gel was stained with Coomassie Brilliant Blue stain for 30 min and destained until bands were clearly visible.

The GST-Gcn2-CTD fragment, GST-Gcn2-CTD K3 and GST alone were immobilised on glutathione beads followed by incubation with purified His₆-eEF1A. The precipitates were resolved by SDS PAGE and immunoblotting with antibodies against eEF1A and GST. eEF1A associated with both Gcn2-CTD and Gcn2-CTD K3 (lanes 2 and 3 Figure 5.8). However, Gcn2-CTD K3 precipitated less eEF1A in comparison to the Gcn2-CTD fragment. This provides further proof that the interaction between Gcn2 and eEF1A is not bridged by the ribosome or other proteins from the yeast extract.

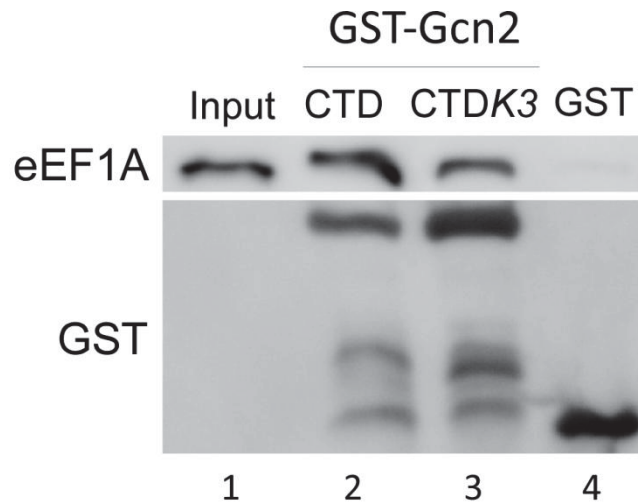


Figure 5.8: Direct interaction between eEF1A and Gcn2

GST tagged Gcn2-CTD and Gcn2-CTD K3 fragments, *GST* alone expressed and purified from *E. coli* were immobilized on glutathione beads and incubated with purified eEF1A. The proteins bound to the beads were then resolved on a SDS-polyacrylamide gel and subjected to immunodetection using antibodies against eEF1A and *GST*.

It is worth noting that the lysine substitutions mentioned in section 5.4 affected Gcn2 CTD's ability to bind eEF1A. Not only does Gcn2-CTD K3 bind eEF1A weaker than Gcn2-CTD (compare lanes 2 and 3 Figure 5.8), it also required about twice as much of Gcn2 CTD K3 when compared to Gcn2 CTD (compare lanes 3 with 2 bottom panel Figure 5.8). When the signal intensities were quantified and normalised for GST-Gcn2, it was found that the Gcn2-CTD K3 bound 60% less eEF1A in comparison to Gcn2-CTD (Figure 5.9). This indicates that the lysine substitutions in Gcn2-CTD reduced affinity for eEF1A.

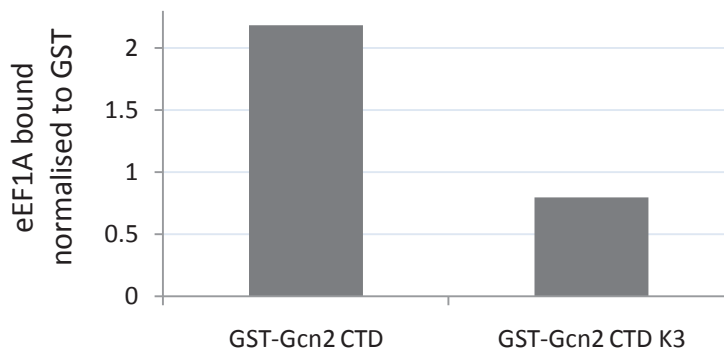


Figure 5.9: Amount of eEF1A bound to the Gcn2 fragments

The amount of eEF1A bound to *GST-Gcn2 CTD* or *GST-Gcn2 CTD K3* was quantified using Multi Gauge V3.1 software (Fuji Photo Film Co., Ltd.) and plotted normalised to *GST*.

Since eEF1A and Gcn2 are both known to bind tRNAs (Dong et al., 2000; Legocki et al., 1974), their interaction could possibly be mediated by RNA molecules. To ensure that the interaction was not bridged by RNA molecules, the *in vitro* binding assay carried out in Figure 5.8 was repeated with purified eEF1A and the Gcn2 fragments that were pre-treated with RNase A for 15 minutes on ice, as published previously (Marton et al., 1997). The efficiency of the RNase treatment was confirmed by digesting 1µg of yeast RNA at conditions identical to those in the assay. The treated and untreated RNA samples were resolved on a 1% agarose gel. No RNA could be detected after the RNase treatment (compare treated and untreated lanes Figure 5.10 A), indicating that the RNase treatment was efficient and complete.

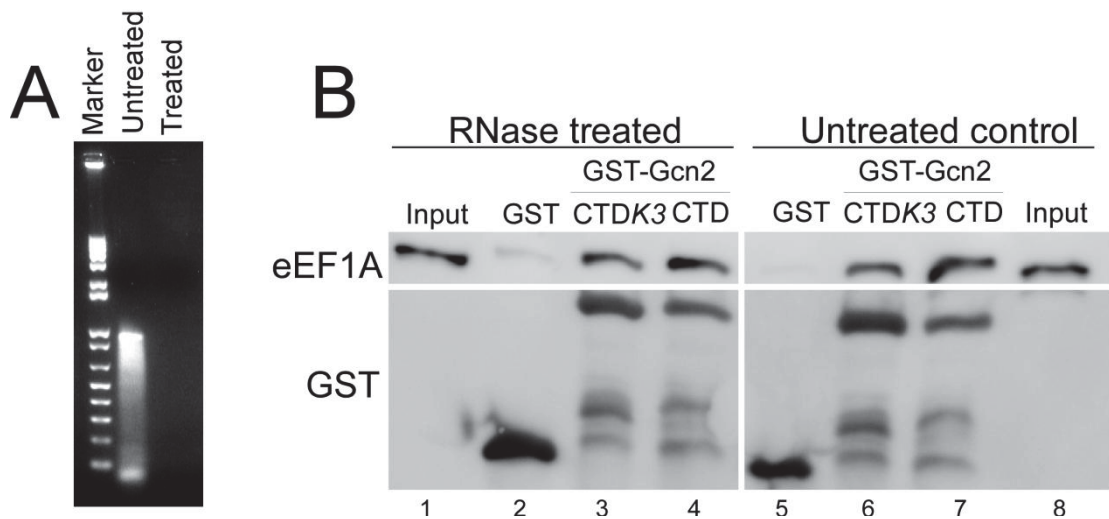


Figure 5.10: eEF1A-Gcn2 interaction is not bridged by RNA

(A) 1µg of yeast total RNA was digested with RNase A for 15 min on ice. The untreated control and treated samples were run on an agarose gel. The gel was stained with ethidium bromide and visualised under UV light. (B) RNase treated and untreated GST alone, GST tagged Gcn2-CTD and Gcn2-CTD K3 fragments that were expressed and purified from *E. coli* were immobilized on glutathione beads and incubated with RNase treated and untreated purified eEF1A. The proteins bound to the beads were then resolved in a SDS-polyacrylamide gel and subjected to immunodetection using antibodies against eEF1A and GST.

The RNase-digested GST-Gcn2-CTD fragment, Gcn2-CTD K3 and GST alone were immobilised on glutathione beads, followed by incubation with RNase digested purified eEF1A. The precipitates were resolved by SDS PAGE and analysed by immunoblotting with antibodies against eEF1A and GST. Similar amounts of eEF1A associated with Gcn2-CTD and Gcn2-CTD K3 under both RNase treated and untreated conditions (Figure 5.10 B, compare lanes 3 and 4, lanes 6 and 7). The

signal intensities of eEF1A precipitated by GST-Gcn2 CTD and GST-Gcn2 CTDK3 were quantified and normalised against GST-Gcn2 (CTD or CTDK3) and plotted in a graph (Figure 5.11). The RNase treatment did not seem to affect the amount of eEF1A bound by the Gcn2-CTD and Gcn2-CTD K3 fragments (compare lanes 4 and 7, lanes 3 and 6, Figure 5.11). This indicates that the Gcn2 and eEF1A interaction is not mediated by RNA molecules. Once again, the lysine mutations (K3) were shown to affect Gcn2's ability to bind eEF1A. It required twice as much of the Gcn2-CTD K3 to bind the same amount of eEF1A when compared to Gcn2-CTD (Figure 5.11).

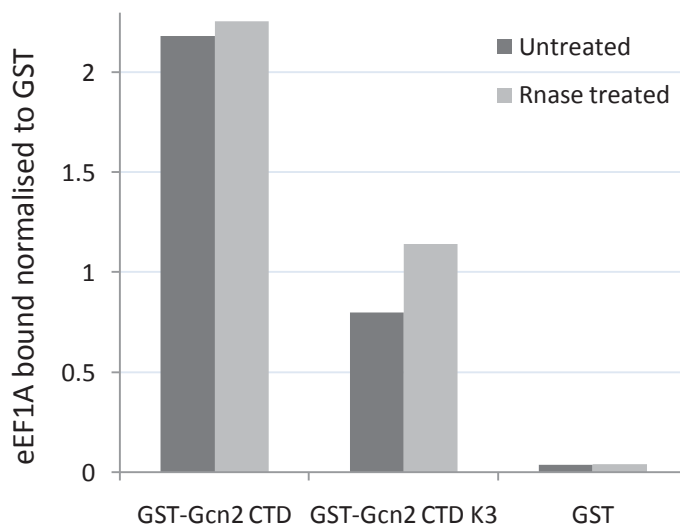


Figure 5.11: Similar amounts of eEF1A bound the Gcn2 fragments with or without RNase digestion

The amount of eEF1A bound to GST-Gcn2 CTD, GST-Gcn2 CTD K3 and GST from Figure 5.10 was quantified using Multi Gauge V3.1 software (Fuji Photo Film Co., Ltd.) in both treated and untreated samples. The amount of eEF1A bound was normalised to GST-Gcn2 and plotted.

With the above observations taken together, it can be concluded that the interaction between Gcn2 and eEF1A is not bridged by the ribosome, RNA or other proteins from the yeast extract.

5.6 eEF1A-Gcn2 interaction is not detectable during amino acid starvation

Observations from the previous sections have established that eEF1A interacts with Gcn2; however the biological significance of this interaction is unknown. In light of

the fact that eEF1A can bind deacylated tRNA (Petrushenko et al., 2002), eEF1A might channel the deacylated tRNA (the signal for amino acid starvation) to Gcn2 for detection of amino acid starvation. If eEF1A is channelling deacylated tRNA to Gcn2 under amino acid starvation conditions, then eEF1A and Gcn2 should interact with each other under amino acid starvation conditions too. One would therefore expect an increase in the amount of eEF1A interacting with Gcn2 upon amino acid starvation in comparison to the basal interaction under replete conditions. This can be tested by an *in vivo* pull down as done in section 5.1. If an increased interaction under amino acid starvation conditions is detected, it would provide support for the idea that eEF1A channels deacylated tRNA to Gcn2.

To test the above mentioned hypothesis, strains used in section 5.1 (TKY865) were grown to exponential phase and either not starved or starved for branched amino acids using the drug sulfometuron methyl (final concentration of 1 μ g/ μ l) for 30 min. The strains were harvested and whole cell extract was generated as described in chapter 2. An *in vivo* pull down was performed with the starved and unstarved extracts as described in section 5.1. The binding of His₆-eEF1A to the resin and subsequent elution of Gcn1, Gcn2 and eEF1A was investigated by resolving the washes by SDS PAGE and transferring onto a PVDF membrane as described in chapter 2. The elution profile of His₆-eEF1A was seen by Ponceau S staining of the membrane. The membrane was then subjected to immunoblotting using antibodies against Gcn1, Gcn2 and Rps22. The blots were also probed for the phosphorylated form of eIF2 α to confirm that the strains were starved for amino acids.

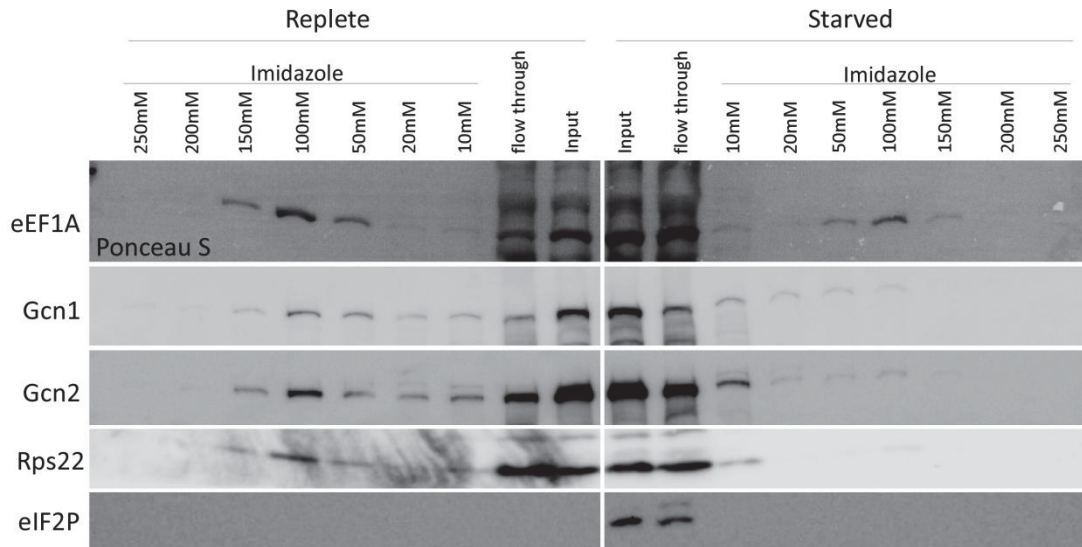


Figure 5.12: eEF1A-Gcn2 interaction not detectable upon amino acid starvation.

Strains harboring plasmid borne *His₆-eEF1A* as the only form of eEF1A (TKY865) were grown to exponential phase and starved for 20 min with SM. The whole cell extract generated from these strains was subjected to a His pull down assay, as described in section 5.1. Equal proportions of the flow through and eluates were resolved on a SDS-polyacrylamide gel and subjected to immunodetection using antibodies against Gcn1, Gcn2, Rps22 and eIF2 α P.

As observed previously, under replete conditions eEF1A and Gcn2 showed the same elution profile (Figure 5.12, left panel). Maximum elution of Gcn2 coincided with that of eEF1A, suggesting that Gcn2 interacts with eEF1A. The ribosomal protein Rps22 also had an elution profile similar to that of eEF1A and Gcn2 (Figure 5.12, left panel). Gcn1 was eluted in the first five washing steps (Figure 5.12, left panel), with little similarity to the pattern in which eEF1A eluted. This suggests that Gcn1 is not in a complex with eEF1A or that they may interact weakly and the interaction could be disrupted with low imidazole concentrations as observed before.

Under starvation conditions barely any Gcn2 co-eluted with eEF1A (Figure 5.12, right panel). This implies that the interaction found between eEF1A and Gcn2 under replete conditions may have been lost during amino acid starvation. There were no detectable ribosomal proteins in complex with eEF1A either (Figure 5.12, right panel). Since the cells are starving (as indicated by the eIF2 α -P signals in the input Figure 5.12, right panel), global protein synthesis will be reduced and hence eEF1A is not required to deliver aminoacylated tRNA to the ribosomes. This could be a reason for not detecting ribosomal proteins in complex with eEF1A upon starvation. In starved conditions the elution profile of Gcn1 did not match that of eEF1A (Figure

5.12, right panel) either, indicating that Gcn1 may not be in a complex with eEF1A.

An increased eEF1A interaction with Gcn2 was expected during amino acid starvation conditions over replete conditions; on the contrary little interaction between eEF1A and Gcn2 was detected (Figure 5.12, compare left and right panels). This suggests that either eEF1A does not channel deacylated tRNA to Gcn2 upon amino acid starvation or that the eEF1A-Gcn2 interaction is transient and was lost before it could be detected.

5.7 Amino acid starvation is detected instantly

The eEF1A-Gcn2 interaction that was detected under replete conditions was lost upon amino acid starvation. The interaction might have been lost after Gcn2 detected starvation (as there was an eEF1A-Gcn2 interaction detected prior to starvation). A reason for the loss of interaction could be that since Gcn2 undergoes a conformational change upon activation and eEF1A can no longer bind Gcn2 in its activated conformation. Hence it was of interest to find how long it would take Gcn2 to detect amino acid starvation. When Gcn2 is activated, it phosphorylates the initiation factor eIF2 α (Dever et al., 1992). This can be monitored by probing the whole cell extract for the phosphorylated form of eIF2 α , which would indicate that Gcn2 detected starvation.

To achieve this, an exponentially growing wild type yeast strain (H1511) was starved using 3AT (an amino acid analogue causing histidine starvation) for different durations of time (5, 2 and 1 minutes). As a negative control a *gcn2 Δ* yeast strain was also starved with 3AT for the maximum amount of time the wild type was starved (5 minutes). The cells were cross-linked with formaldehyde to ensure that no further phosphorylation or dephosphorylation events happened during the harvesting and processing of the cells. The whole cell extracts generated were resolved by SDS-PAGE and transferred onto a PVDF membrane as described in chapter 2. The membrane was then subjected to immunoblotting using antibodies that specifically recognise the phosphorylated form of eIF2 α (eIF2 α -P) and total eIF2 α .

It was found that at a growth temperature of 30°C, eIF2 α was phosphorylated within 1 minute after addition of 3AT, and the amount of phosphorylated eIF2 α increased with increasing starvation duration (top panel, Figure 5.13). As expected there was no phosphorylation of eIF2 α in a *gcn2* Δ strain. This time course of eIF2 phosphorylation at 30°C was done by B. Cao and E. Sattlegger.

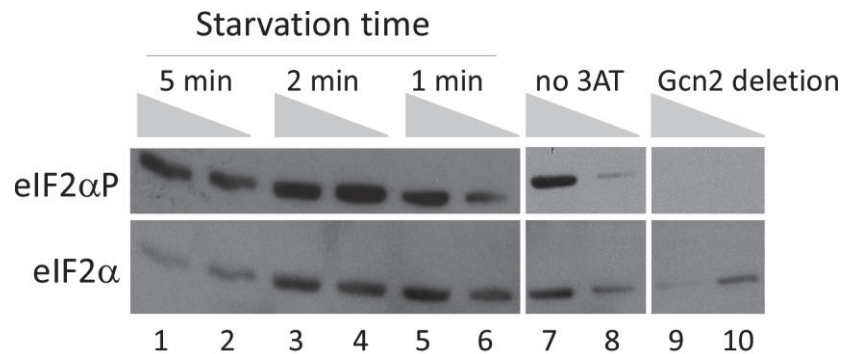


Figure 5.13: Histidine starvation is detected within 1 min after the addition of 3AT

H1511 strains were grown at 30°C to exponential phase and subjected to amino acid starvation by addition of 3AT (final conc. 10mM) for the time durations indicated. An unstarved control was also grown where no 3AT was added. A *gcn2* Δ strain was grown to exponential and starved with 10mM 3AT for 5 min. 50 μ g and 25 μ g (shown as grey triangle) of the whole cell extract was subjected to SDS-poly acryl amide gel electrophoreses and immunodetection using antibodies against the phosphorylated form of eIF2 α and total eIF2 α (B.Cao and E. Sattlegger).

In principle there should be no eIF2 α -P detected in strains not starved with 3AT. However, some eIF2 α -P was detected (Figure 5.13, lanes 7 and 8), representing the amount of basal eIF2 α phosphorylation occurring in the cell as observed by Dever *et al.* (1992). It should be noted, however that the amount of eIF2 α -P in samples treated with 3AT (starved) is far greater than in strains not treated with 3AT (unstarved).

Since the phosphorylation event occurred almost immediately after the addition of 3AT at a growth temperature of 30°C, it was not yet clear at what point Gcn2 detects amino acid starvation. One approach to determine this time frame would be to slow down the molecular events involved. At lower temperatures, metabolic processes are slower and thus sensing starvation should also take longer. Consequently, the experiment described above was repeated for cells grown at lower temperatures (25°C and 20°C).

It was found that even at lower temperatures eIF2 α was phosphorylated between 1 (25°C) to 2 minutes (20°C) (Figure 5.14), indicating that the process of sensing amino acid starvation by Gcn2 is extremely quick. It was observed again that with increased duration of starvation time the amount of eIF2 α -P increased.

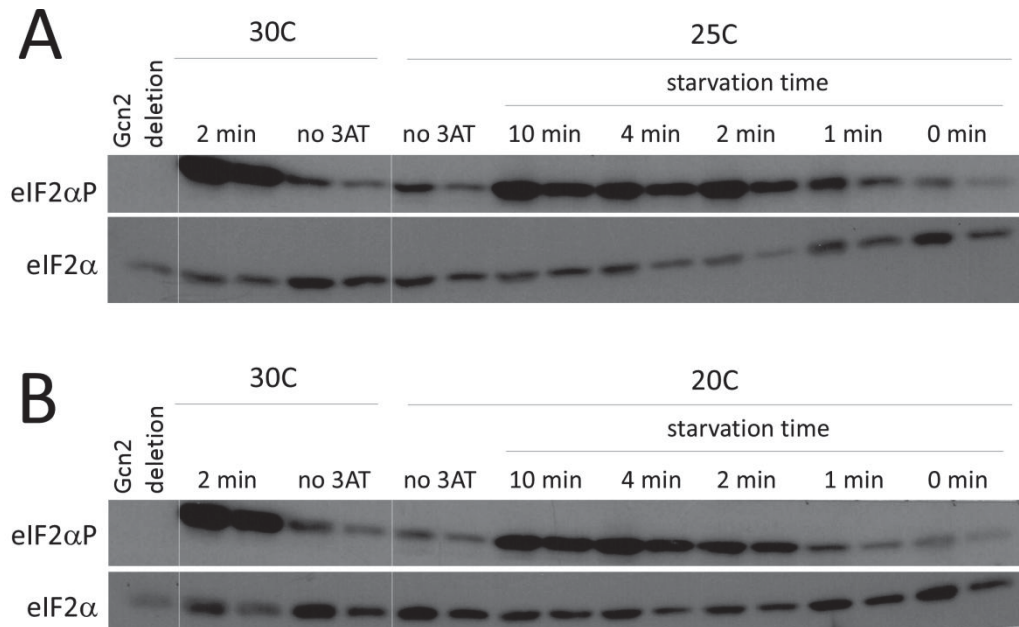


Figure 5.14: Reduction in growth temperature delays eIF2 α phosphorylation

H1511 strains were grown at (A) 25°C or (B) 20°C to exponential phase and subjected to amino acid starvation as explained in Figure 5.13. The *gcn2* Δ , a 2 min starvation sample and no 3AT controls were used as controls from Figure 5.13.

Quantification of the eIF2 α -P signal intensities appeared to indicate that the amount of eIF2 α -P increased with the duration of starvation. At growth temperatures of 25°C and 20°C, however, after 4 minutes of starvation the phosphorylation had reached a steady state and the level of eIF2 α -P remained constant (Figure 5.15). Since the strains grown at 30°C were not starved for longer than 5 minutes it cannot be concluded if the levels of eIF2 α -P had reached a steady state.

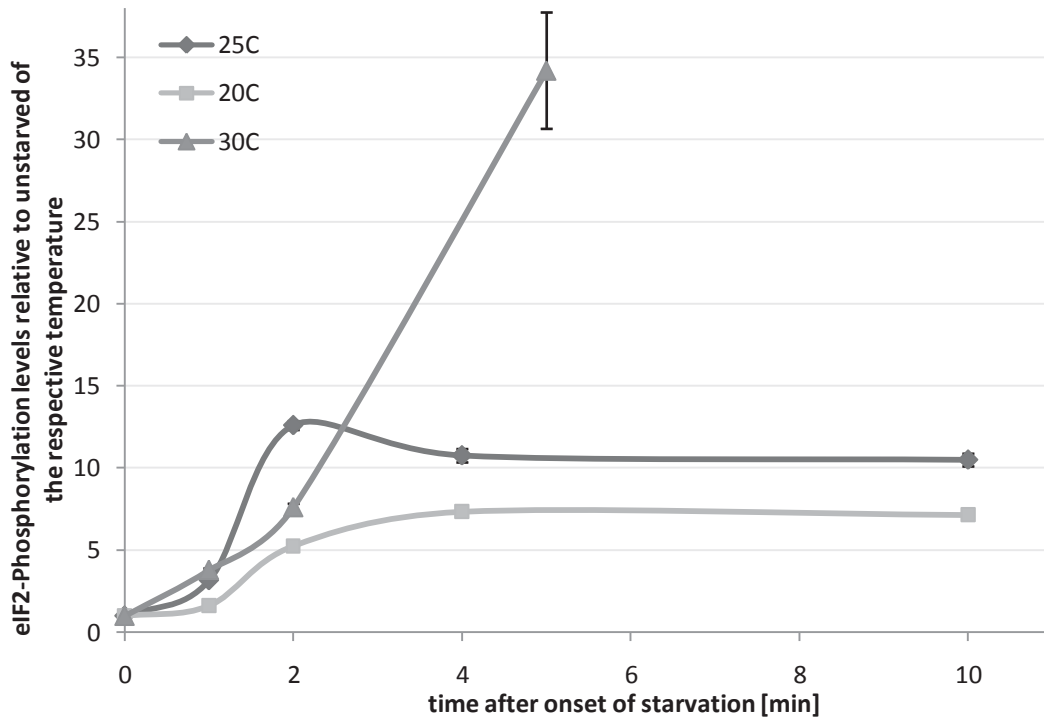


Figure 5.15: eIF2 α -P levels over a duration of different starvation times

The intensity of the signals from Figure 5.13 and Figure 5.14 were quantified using the Multi Gauge V3.1 software (Fuji Photo Film Co., Ltd.). The relative levels of phosphorylation were determined by dividing the signal intensity of eIF2 α -P by eIF2 α and setting the unstarved control signal of the respective growth temperature to 1 and were illustrated in the graph. The error bars represent the standard error from two independent loadings. The standard error in some cases was very small and hence error bars are not visible

Further investigation of the molecular events was carried out to determine the time lapse between sensing starvation and translational shut down. The aim of this was to see how long it would take for translation to reduce after sensing starvation. Translation shut down should ensure a loss of polysomes (actively translating ribosomes) indicated by reduced polysome peaks in the UV trace. The whole cell extracts from the above experiments were resolved on a sucrose gradient by high speed velocity sedimentation and fractionated while measuring absorbance at 260 nm. From the UV traces it can be seen that there was a reduction in polysomes as quickly as 2 minutes (at 30°C and 25°C) after the addition of 3AT (Figure 5.16 and Figure 5.17, respectively). This hints that translational shut down happens within a few minutes after sensing starvation. Reduced temperature slows molecular processes and hence it was expected that translational shut down would take longer when the growth temperature was reduced. At a growth temperature of

20°C it took 4 minutes after addition of 3AT, for reduction in polysomes (Figure 5.18), indicating a reduction in translation took longer at lower growth temperatures.

From these experiments it can be concluded that amino acid starvation is sensed within 2 minutes after the onset of starvation. This suggests that the eEF1A-Gcn2 interaction may have been lost within 2 minutes after the onset of amino acid starvation. It would be difficult to study the complex within this time frame since processing the cells would take longer than 2 minutes and the cells will be starving during the processing. To ensure that the cells are starved for 2 minutes or less one could use formaldehyde cross-linking. Formaldehyde cross-linking “freezes” the cellular complexes as they would occur in the cells instantly. This would ensure that the cells are not starving during processing. However, due to the close proximity of these proteins with the ribosome, cross-linking will not allow us to distinguish between eEF1A-ribosome-Gcn2 complexes (indirect interaction) and eEF1A-Gcn2-ribosome complexes (direct interaction). Hence it would be difficult to investigate if there was an eEF1A-Gcn2 interaction before Gcn2 sensed starvation without cross-linking the cells.

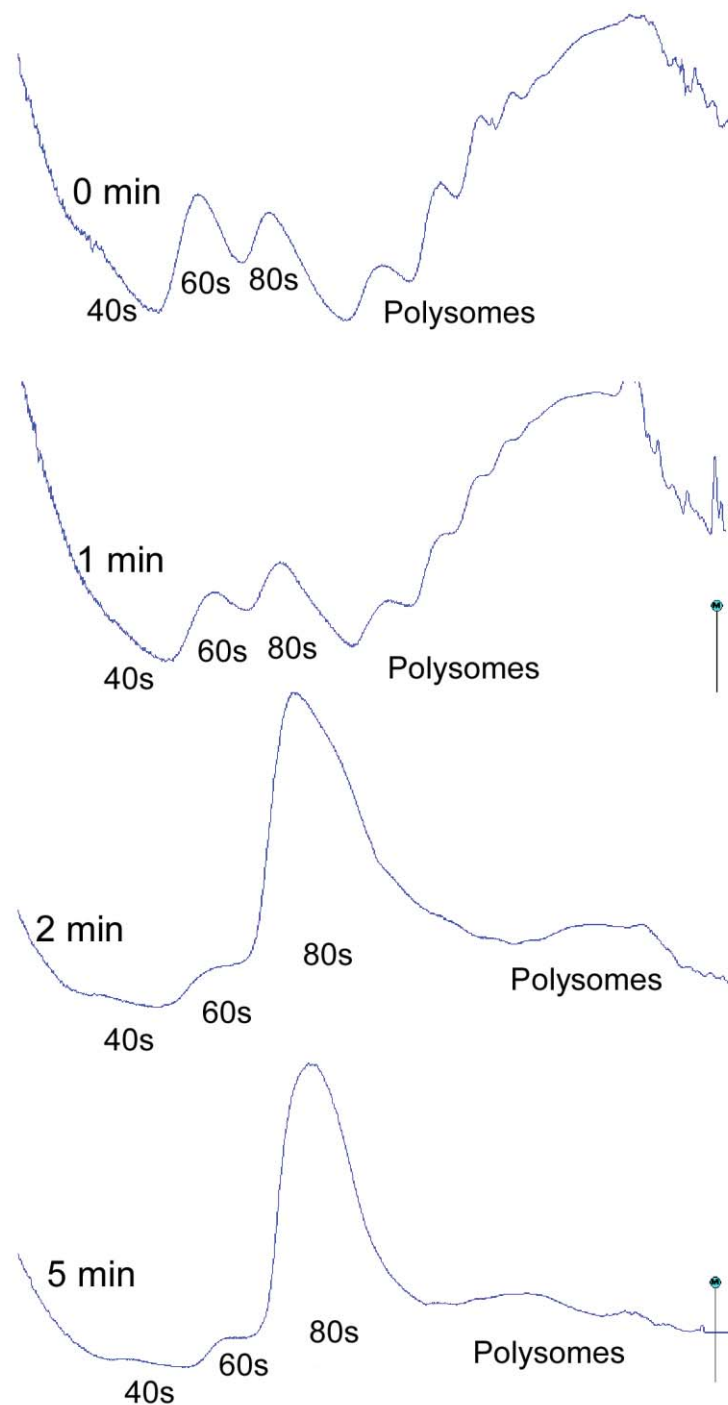


Figure 5.16: General protein synthesis is reduced within 2 minutes of adding 3AT at a growth temperature of 30°C

H1511 strains were grown to exponential phase at 30°C and starved with 10mM 3AT for time durations indicated and cross-linked with formaldehyde immediately before harvesting, and WCEs were prepared and resolved by velocity sedimentation through 4.5–45% sucrose gradients. Fractions were collected while measuring A_{254} continuously to identify the positions of polyribosomes, 80S ribosomes, and 40S and 60S ribosomal subunits and are indicated.

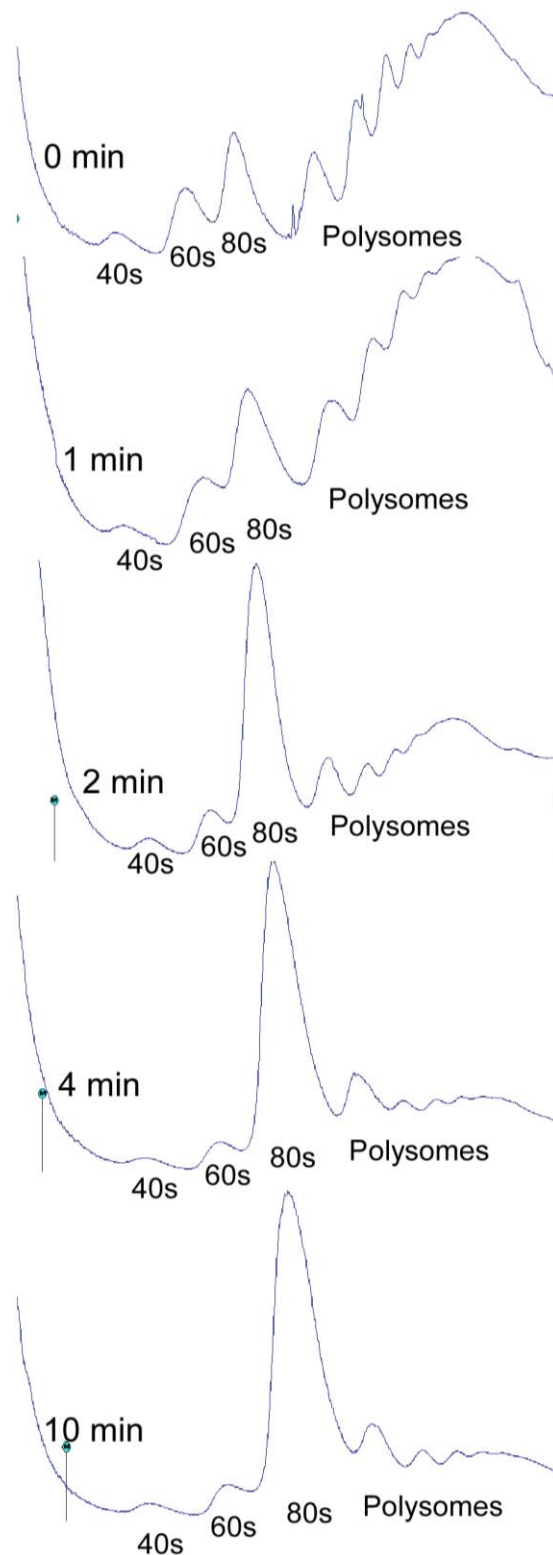


Figure 5.17: Reduction in translation takes 2 minutes after addition of 3AT at a growth temperature of 25°C

The H1511 strains were grown to exponential phase at 25°C and starved with 10mM 3AT for time durations indicated and cross-linked with formaldehyde immediately before harvesting, and WCEs were prepared and resolved by velocity sedimentation through 4.5–45% sucrose gradients. Fractions were collected while measuring A_{254} continuously to identify the positions of polyribosomes, 80S ribosomes, and 40S and 60S ribosomal subunits and are indicated

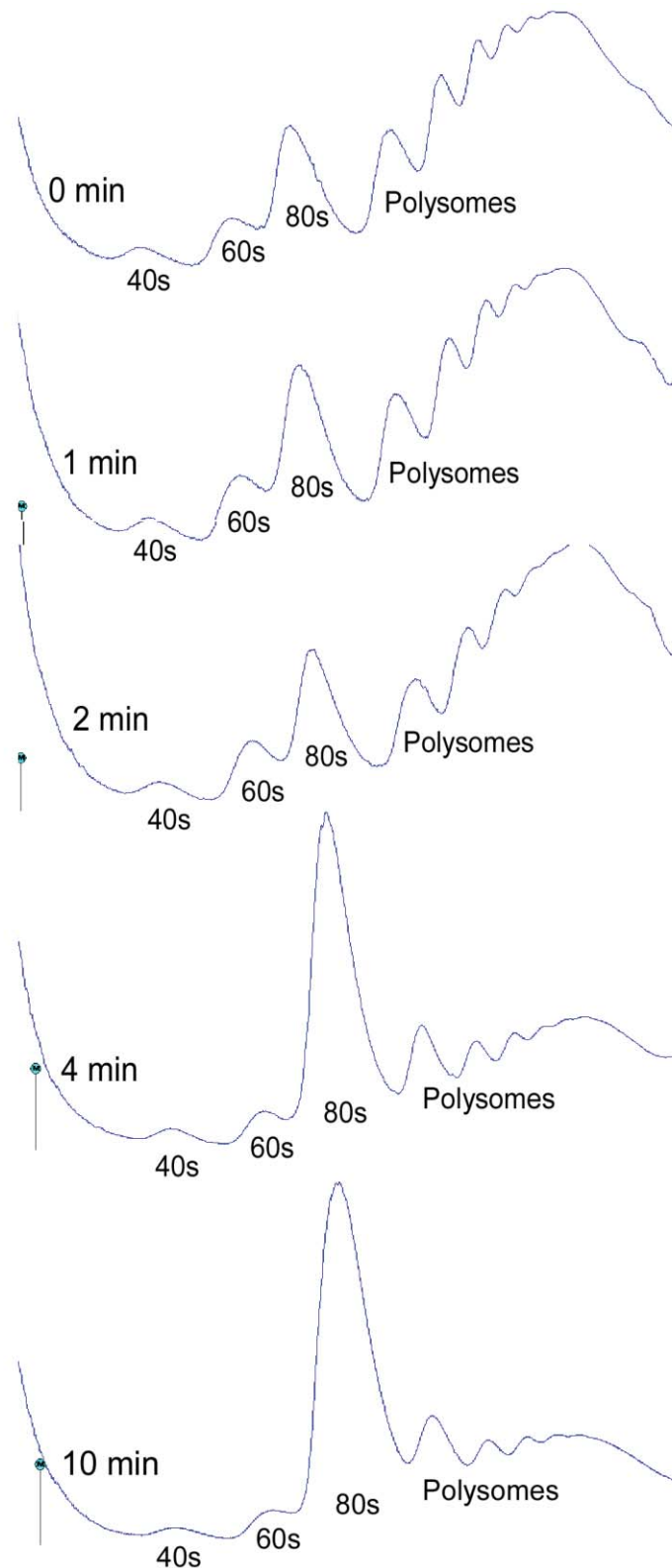


Figure 5.18: Reduction in translation takes 4 minutes after addition of 3AT at a growth temperature of 20°C

H1511 strains were grown to exponential phase at 20°C and starved with 10mM 3AT for time durations indicated and cross-linked with formaldehyde immediately before harvesting, and WCEs were prepared and resolved by velocity sedimentation through 4.5–45% sucrose gradients. Fractions were collected while measuring A_{254} continuously to identify the positions of polyribosomes, 80S ribosomes, and 40S and 60S ribosomal subunits and are indicated

Discussion

Gcn1 has sequence homology to *eEF3* (Marton et al., 1993), and as *eEF1A* and *eEF3* are known to interact (Anand et al., 2003), it was predicted that *eEF1A* would interact with *Gcn1*. However, this interaction was not detected. Instead, an interaction between the translational factor *eEF1A* and the kinase *Gcn2* was found. Since ribosomal proteins were also found in the *eEF1A-Gcn2* complex, it seemed likely that the *eEF1A* and *Gcn2* interaction is bridged by the ribosomes (particularly considering both *eEF1A* and *Gcn2* are known to bind the ribosomes).

eEF1A interacts with aminoacyl tRNA synthetases in order to recharge deacylated tRNA (Petrushenko et al., 2002). *Gcn2* has a region that has sequence homology to histidyl tRNA synthetase. If *eEF1A* interacts with *Gcn2* directly, then the domain in *Gcn2* that *eEF1A* would most likely interact with was predicted to be the HisRS domain. However, upon investigation it was found that the C-terminal domain (amino acid residues 1498-1659) of *Gcn2* interacts with *eEF1A*. It cannot be ruled out that HisRS domain may also interact with *eEF1A*, as the HisRS fragment purified in this study was partially degraded. Furthermore, the N-terminal stretch of *Gcn2* (amino acid residues 272-568) that was not included in this study may also harbour an additional *eEF1A* binding domain. The C-terminal domain of *Gcn2* is responsible for ribosome binding (Zhu and Wek, 1998). Since both *eEF1A* and the *Gcn2* C-terminus can bind the ribosomes it was thought that the *eEF1A-Gcn2* interaction was most likely bridged by the ribosomes.

To investigate if the interaction was bridged by the ribosomes several approaches were sought. It was verified that the *eEF1A-Gcn2* interaction was independent of the ribosomes by using whole cell extract devoid of ribosomes for the assay and by using a mutated *Gcn2* C-terminal fragment that is severely deficient in ribosome binding.

The lysine residues in the *Gcn2* C-terminal domain were reported to be essential for ribosome association (Zhu and Wek, 1998). However, in this study it was observed that the C-terminal domain with the lysine substitutions had residual ribosome binding activity. Upon detailed review of the study by Zhu and Wek (1998), it

appeared that the lysine substitutions did not completely abolish ribosome binding of Gcn2; rather they significantly reduced ribosome binding. Although the Gcn2-CTDK3 had residual ribosome binding, it was clear from the significant eEF1A-Gcn2-CTDK3 interaction that the eEF1A-Gcn2 interaction was not principally mediated by the ribosome.

Due to its abundance, electrostatic nature and RNA binding properties, eEF1A is often detected as a false positive in protein-protein interaction assays. However the eEF1A-Gcn2 interaction discovered in this study was demonstrated to be a direct interaction mediated by the C-terminus of Gcn2. The interaction was demonstrated to be a direct interaction by numerous methods for example in the absence of ribosomes, RNA and other proteins.

The Gcn2 C-terminal domain containing the mutated lysine residues could not bind eEF1A as efficiently as the wild-type Gcn2, indicating that these lysine residues may also be involved in eEF1A binding in addition to ribosome binding (Zhu and Wek, 1998) and tRNA binding *in vitro* (Dong et al., 2000). These lysine residues may be a part of a larger domain involved in eEF1A binding since mutating these residues reduced the amount of eEF1A the fragment precipitated when compared to the wild-type fragment.

The next aspect of the eEF1A-Gcn2 interaction investigated was its biological significance. eEF1A can be either a positive or a negative regulator of Gcn2 activation.

If eEF1A is a positive regulator of Gcn2 then it should aid Gcn2 activation for sensing starvation. So, as a positive regulator eEF1A could channel deacylated tRNA to Gcn2 for detection during starvation conditions. The direct interaction between eEF1A and Gcn2 could be taken as support for the channelling hypothesis. As mentioned earlier the channelling hypothesis suggests that translational components are governed by predetermined fates. However, little is known about the fate of deacylated tRNAs exiting the ribosome. It has been suggested that eEF1A binds these deacylated tRNAs and shuttles them for recharging to aminoacyl tRNA synthetases (Petrushenko et al., 2002). Supporting this idea eEF1A has been shown

to bind deacylated tRNAs, although with lower affinity when compared to aminoacylated tRNA (Petrushenko et al., 2002). Under amino acid starvation there should be an increase in the amount of deacylated tRNAs in the cell. It is unlikely that these deacylated tRNAs will be recharged immediately, therefore, they may remain bound to eEF1A. eEF1A could transfer the signal for amino acid starvation (deacylated tRNA) to Gcn2, which would then activate the general amino acid control pathway. While the dissociation constant (K_d) of eEF1A-GDP-deacylated tRNA is 20nM (Petrushenko et al., 2002), the K_d of Gcn2-deacylated tRNA is 60 fold higher (Dong et al., 2000). Although the channelling of deacylated tRNA from eEF1A to Gcn2 is not thermodynamically favourable under replete conditions, one cannot discount the possibility that channelling could happen under amino acid starvation conditions *in vivo*. However if this were the case then the mechanism of deacylated tRNA transfer from eEF1A to Gcn2 would need further investigation.

The most straightforward way to determine if eEF1A could channel deacylated tRNA to Gcn2 would be to investigate if eEF1A and Gcn2 interacted upon amino acid starvation. Furthermore, one would expect to see an increased eEF1A-Gcn2 interaction upon amino acid starvation when compared to replete conditions. An *in vivo* interaction assay was performed with strains harbouring His₆ tagged or untagged eEF1A as the only form of eEF1A, under both starved and replete conditions. Although an eEF1A-Gcn2 interaction was detected under replete conditions, the interaction could not be detected when the cells were starved for the amino acid histidine. This observation suggests that the eEF1A-Gcn2 interaction is lost upon amino acid starvation. This loss of interaction could be explained if the channelling of deacylated tRNA to Gcn2 had already activated the GAAC and the eEF1A interaction was lost before it could be studied.

Upon investigation it was found that Gcn2 can detect amino acid starvation in less than one minute of starvation. Hence, the possibility exists that upon amino acid starvation, eEF1A had already channelled the deacylated tRNA to Gcn2 and the interaction was lost before it could be captured. To disentangle this problem one can reduce the growth temperature of the strains; in this case, however, it would not help, since it only takes 2 minutes for Gcn2 to detect starvation, even at 20°C.

Also, there is no way to ensure that the starvation time is less than 2 minutes without cross-linking, since processing the cells takes longer and the cells will be starving for longer than intended. Formaldehyde cross-linking will not allow us to distinguish between eEF1A-ribosome-Gcn2 complex (indirect interaction) as opposed to eEF1A-Gcn2-ribosome complex (direct interaction) and hence will not be useful in this case. An alternative approach to this would be to use a strain with a mutant Gcn2 that is defective in sensing starvation. A mutation called the “m2” was reported by Wek *et al.* (1995) which renders Gcn2 incapable of binding tRNA (Wek *et al.*, 1995). To prolong the eEF1A-Gcn2 interaction under amino acid starvation conditions, a yeast strain with Gcn2 m2 in addition to His tagged eEF1A as the only forms of the respective proteins in the cell could be used. Such a strain could be starved and an *in vivo* pull down assay performed as described in section 5.1; this would answer the question raised above. This would assumedly prolong the interaction between eEF1A and Gcn2 since Gcn2 can no longer accept the deacylated tRNA thereby allowing the complex to be detected and studied. As discussed earlier it could be that an increase in the eEF1A-Gcn2 interaction upon amino acid starvation might ensue. However, this was not attempted in this study due to time constraints.

On the other hand, eEF1A might be acting as a negative regulator of Gcn2. If this were the case then by binding Gcn2, eEF1A may be preventing Gcn2 from activation. This idea is supported by the observation that an *in vivo* interaction is detected between eEF1A and Gcn2 only under replete conditions. This could also be the reason for the loss of interaction upon starvation, since Gcn2 needs to be activated in order to detect the amino acid starvation. To investigate this, kinase assays can be performed to investigate if Gcn2 can phosphorylate its substrate eIF2 α when in complex with eEF1A. If Gcn2 is inactive and cannot phosphorylate eIF2 α in complex with eEF1A then eEF1A is an inhibitor of Gcn2. However, if Gcn2 is active and can phosphorylate eIF2 α in complex with eEF1A then eEF1A may not be an inhibitor of Gcn2 and the eEF1A-Gcn2 interaction may be very brief and could not be captured in this study. These kinase assays were beyond the scope of this study.

The lysine substitutions in the Gcn2 C-terminus decreased the amount of eEF1A it bound. This further supports the idea that eEF1A could be an inhibitor of Gcn2. eEF1A can be inhibiting Gcn2 activation by sequestering the essential tRNA binding amino acid residues in Gcn2. Gcn2 needs to be bound to the ribosome and tRNA for activation and these lysine residues are required for tRNA binding *in vitro* (Dong et al., 2000) and binding ribosomes (Zhu and Wek, 1998). With these observations, a model can be proposed in which eEF1A acts as an inhibitor of Gcn2. When Gcn2 is in complex with eEF1A, Gcn2 cannot bind deacylated tRNA, as eEF1A has sequestered the essential residues for tRNA binding. Therefore, Gcn2 cannot be activated when in complex with eEF1A. When the cells are starving for amino acids, this eEF1A-Gcn2 interaction is lost, thus freeing up the amino acid residues in Gcn2 required for tRNA binding. Gcn2 can now bind tRNA, get activated and phosphorylate eIF2 α (Figure 5.19) to activate the GAAC pathway to overcome the amino acid starvation.

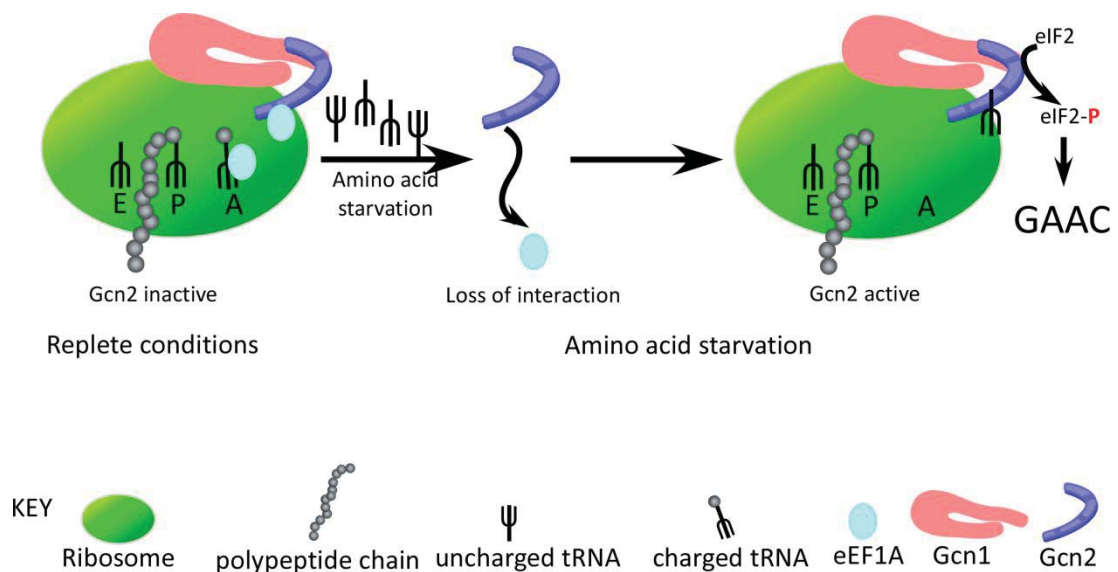


Figure 5.19: Model for eEF1A as an inhibitor of Gcn2

Under replete conditions eEF1A inhibits Gcn2 activation by sequestering the tRNA binding amino acid residues. Upon amino acid starvation when Gcn2 needs to be activated eEF1A dissociates from Gcn2 allowing the tRNA binding amino acid residues to be accessible and hence allowing Gcn2 activation. The representation of Gcn1 and Gcn2 has been adapted from Sattlegger and Hinnebusch (2000).

In summary, this chapter has revealed a novel interaction between the kinase Gcn2 and the elongation factor 1 A (eEF1A). The C-terminal domain in Gcn2 was found to be responsible for this interaction.

Chapter 6 Conclusions

This study has revealed a link between factors involved in translation elongation and translational control. Two factors involved in translation elongation, eEF3 and eEF1A may be regulating two key factors, Gcn1 and Gcn2 in the general amino acid control (GAAC) pathway.

eEF3 is a unique fungal elongation factor that is known to facilitate the release of deacylated tRNA from the ribosomal E-site. It is thought that eEF3 controls the accuracy of translation by stimulating the delivery of cognate but not non-cognate aminoacyl tRNA to the ribosomal A-site (Kamath and Chakraburty, 1989; Uritani and Miyazaki, 1988).

There is an allosteric linkage between the ribosomal E-site and A-site. When the E-site is occupied there is reduced affinity for the A-site and *vice versa*. A-site occupation induces low affinity at the E-site thus triggering the release of deacylated tRNA by eEF3 (Nierhaus, 1990). Hence, it has been suggested that eEF3 could be monitoring translational accuracy.

This study suggested that eEF3 negatively influences Gcn1 function and proposes that both eEF3 and Gcn1 cannot function on the same ribosome at the same time. Upon amino acid starvation, there is a scarcity of aminoacylated tRNA in the cell. If eEF3 is monitoring the accuracy of translation, then the ribosomes will most probably be stalled due to the unavailability of aminoacyl tRNA to be delivered to the A-site. In such stalled ribosomes, when the E-site is occupied, a deacylated tRNA (albeit cognate) can bind the A-site only weakly. Thus the deacylated tRNA remains bound weakly to the ribosomal A-site in such stalled ribosomes, allowing Gcn1 to facilitate the channelling of the weakly bound deacylated tRNA at the A-site to Gcn2, which upon sensing the deacylated tRNA activates the GAAC.

Supporting the above proposed model Belfield *et al.* (1995) conducted a tertiary structure analysis and found that regions in eEF3 and Gcn1 not only shared sequence homology but were predicted to have structural similarity with *E. coli* ribosomal protein S5 (Rps5). Rps5 along with two other factors (Rps 4 and Rps12) are known to coordinate the accuracy of translation in *E. coli*. The structural

homologies between Gcn1, eEF3 and S5 might suggest that a common mechanism exists for interacting with the tRNA by ribosome association.

eEF3 has been shown to interact with eEF1A (Anand et al., 2006; Anand et al., 2003). Various researchers have suggested that this interaction plays a role in eEF1A-mediated delivery of cognate aminoacylated tRNA to the ribosomal A-site (Anand et al., 2006; Nierhaus, 1990; Trianaalonso et al., 1995). Since eEF3 and Gcn1 are similar, our prediction was that Gcn1 may interact with eEF1A. However, when investigated in this study it was found that eEF1A did not interact with Gcn1. We discovered instead that eEF1A interacted with Gcn2.

eEF1A is a translational elongation factor that delivers aminoacylated tRNA to the ribosomal A-site. While this is its principal role, eEF1A has been found to participate in a number of other processes. This study suggests that eEF1A may also be acting as a regulator of Gcn2 function.

eEF1A is thought to have a role in nuclear export, in protein quality control and degradation, apoptosis, viral propagation and actin bundling (Mateyak and Kinzy, 2010). The most extensively studied non canonical role of eEF1A is the binding and bundling of actin. This actin binding and bundling function is mutually exclusive to the binding of aminoacyl tRNA *in vitro* (Liu et al., 1996). Further, the function of eEF1A in translation and actin bundling could be regulated by other binding factors like eEF1B α and some actin remodelling proteins (Mateyak and Kinzy, 2010).

Yih1 is one such actin binding protein that is thought to regulate the general amino acid control pathway by inhibiting Gcn2 activation. Yih1 is known to exist in a complex with monomeric actin *in vivo* (Sattlegger et al., 2004). According to the model suggested by Sattlegger *et al.*(2004), when Yih1 is released from actin it inhibits Gcn2. Actin patches are highly dynamic structures and contain many actin binding and regulatory factors that are constantly turned over. High concentrations of actin binding proteins involved in actin polymerisation may be able to displace Yih1 from monomeric actin. This would release Yih1, which can now inhibit Gcn2 (as discussed in (Sattlegger et al., 2004)). Taken together that eEF1A is involved in bundling actin and Yih1 released from actin can inhibit Gcn2 activation, it is

tempting to speculate that in the process of bundling actin eEF1A could regulate Gcn2 function by releasing Yih1 that was bound to actin.

As discussed in chapter 5, the interaction between eEF1A and Gcn2 may be a mechanism for inhibiting Gcn2 activation under replete conditions. eEF1A dissociates from Gcn2 under amino acid starvation conditions, thus allowing Gcn2 activation. Supporting this hypothesis, it was found that there was reduced phosphorylation of eIF2 α by Gcn2 in the presence of eEF1A, whilst its activation (auto phosphorylation) was not affected *in vitro* (Sebastien Lageix, Evelyn Sattlegger, Lara Izotova, Terri Kinzy and Alan Hinnebusch, personal communication). This suggests that eEF1A is a partial inhibitor of Gcn2, since it allows Gcn2 activation but inhibits substrate phosphorylation.

This raises the question as to how an incomplete inhibition mechanism would be efficient for the cell. For activation Gcn2 is required to be bound to Gcn1 (Sattlegger and Hinnebusch, 2000), and Gcn1 needs to be bound to the ribosome (Sattlegger and Hinnebusch, 2005). These findings, taken together with the finding from this study that eEF3 inhibits Gcn1's function, and the fact that eEF1A interacts with eEF3 (Anand et al., 2003), a model for the cross talk between translation elongation and translation control can be suggested (Figure 6.1). Under replete conditions, when general protein synthesis is maximal, the two translational elongation factors eEF1A and eEF3 act in concert to inhibit Gcn2 activation. eEF1A inhibits Gcn2 from phosphorylating its substrate eIF2 α , while eEF3 inhibits Gcn1 from transducing the starvation signal to Gcn2, thereby achieving complete inhibition of Gcn2.

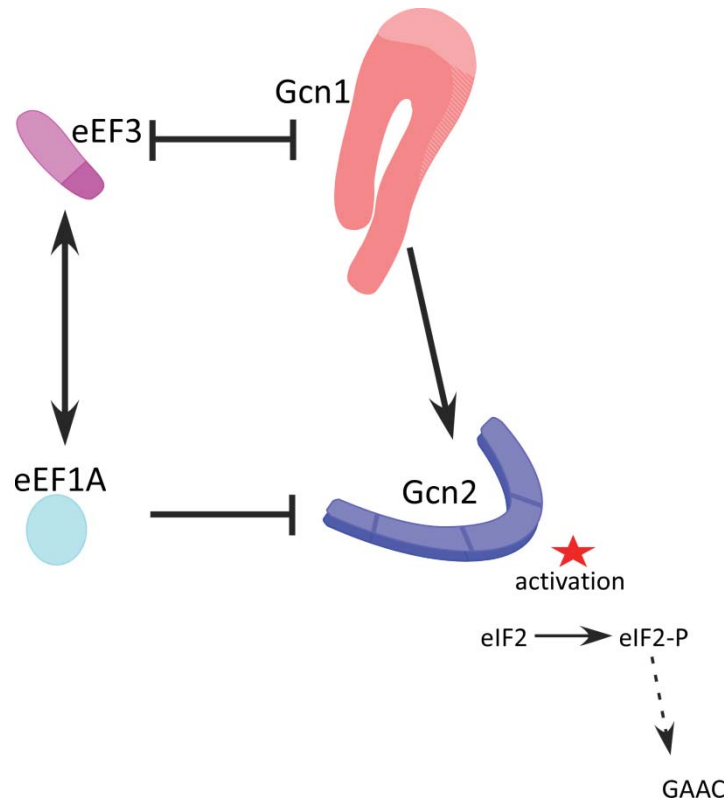


Figure 6.1: Model for translation elongation regulating translational control

Under replete conditions eEF3 and eEF1A act in concert to inhibit Gcn2 activation by inhibiting Gcn1 mediated activation and eIF2 α phosphorylation respectively. The representation of Gcn1 and Gcn2 has been adapted from Sattlegger and Hinnebusch (2000).

However, there are still a few questions that remain unanswered; for example how does eEF1A realise that Gcn2 no longer needs to be inhibited upon amino acid starvation? eEF3 is known to interact with eEF1A, and eEF3 and Gcn1 influence each other on the ribosome. Does Gcn1 recognise the deacylated tRNA in the A-site and relay the signal to eEF3, which in turn relays it to eEF1A which dissociates from Gcn2 to facilitate its activation?

The results from this study are beginning to shed light on new links between translational elongation and the general amino acid control pathway. The emerging secondary functions of many proteins are important links between such diverse cellular processes. These links emphasise the complex nature of the cross talks that exist between cellular pathways. Future discoveries of secondary functions of proteins are likely to play a central role in revealing the intricate network between cellular pathways and will change our approach towards studying cellular processes.

Appendix A

A.1 Verification of pJV01

The plasmid constructed for experiments in chapter four called pJV01 was verified by restriction digestion. *XhoI* in *myc-GCN1* was a diagnostic site to check for insertion of *myc-GCN1*. When digested with *XhoI* pJV01 was digested and released an insert of 3.7 Kb as expected (Figure A.1 lane 4).

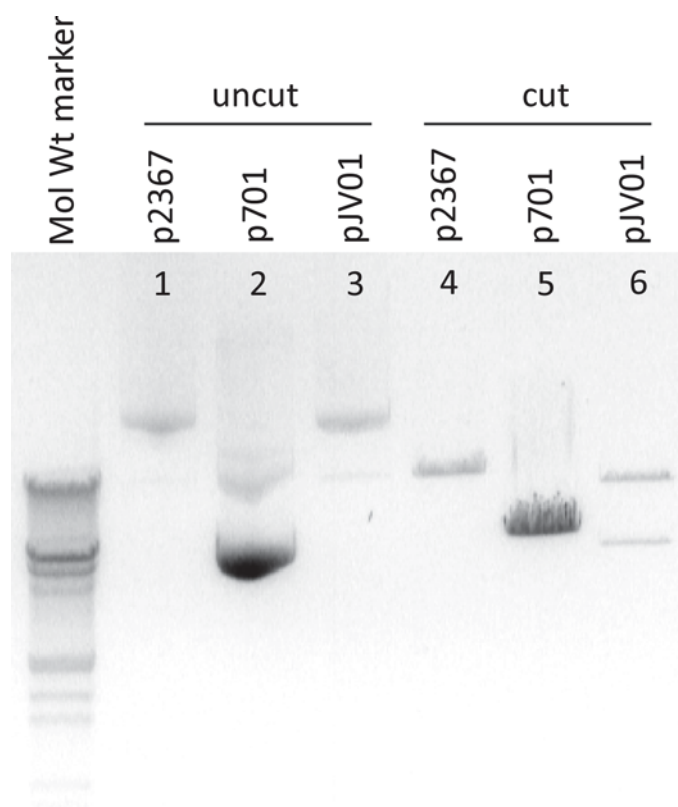
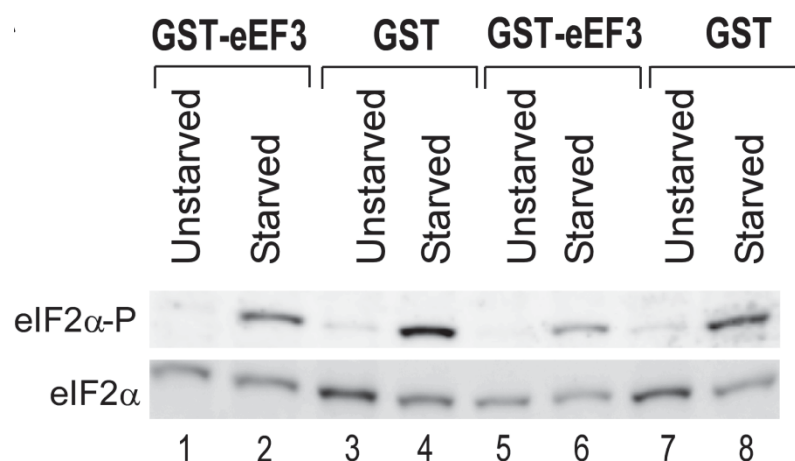


Figure A.1: Verification of pJV01 by restriction digestion

The pJV01 and indicated plasmid controls were digested with *XhoI*. After digestion the reactions were resolved on a 1% agarose gel, stained with EtBr and visualised in a transilluminator.

A.2 Calculation of relative levels of eIF2 α -P from raw data

Raw Data					
	Lane number	sample	signal intensities of the bands		normalised eIF2 α -P levels [#]
			eIF2 α -P	eIF2 α	
replicate 1	1	GST-eEF3 unstarved	BDR	1417455.6	BDR
	2	GST-eEF3 starved	788479.26	1516213.97	0.520031655
	3	GST unstarved	53897.19	2507274.3	0.021496328
	4	GST starved	1196219.43	1457507.22	0.820729677
replicate 2	5	GST-eEF3 unstarved	BDR	910218.14	BDR
	6	GST-eEF3 starved	376889.37	849750.04	0.443529688
	7	GST unstarved	156183.67	2090309.68	0.074717958
	8	GST starved	1213601.16	1214298.83	0.999425454

BDR: Below Detection Range

$$\text{\# normalised eIF2}\alpha\text{-P levels} = \frac{\text{signal intensity of eIF2}\alpha\text{-P}}{\text{signal intensity of eIF2}\alpha}$$

Example calculation for Lane 2:

$$\frac{\text{signal intensity of eIF2}\alpha\text{-P}}{\text{signal intensity of eIF2}\alpha} = \frac{788479.26}{1516213.97} = 0.520031655$$

$$\text{For Lane 6: } = \frac{376889.37}{849750.04} = 0.443529688$$

$$\text{Average of the replicates} = \frac{(0.520031655 + 0.443529688)}{2} = 0.481780671$$

Sample	Average of normalised eIF2 α -P	levels of eIF2 α -P relative to GST unstarved*
GST-eEF3 unstarved	BRD	BDR
GST-eEF3 starved	0.481780671	10.01474303
GST unstarved	0.048107143	1
GST starved	0.910077566	18.91772231

$$* \text{ relative levels of eIF2}\alpha\text{-P} = \frac{\text{signal ratio of sample}}{\text{signal ratio of GST unstarved}}$$

Example calculation for GST-eEF3 starved:

$$= \frac{0.481780671}{0.048107143} = 10.01474303$$

A.3 eEF3 over-expression barely affects Gcn1-polysome association

Results of the co-sedimentation assays from replicates were performed as described in section 4.5 and are shown below.

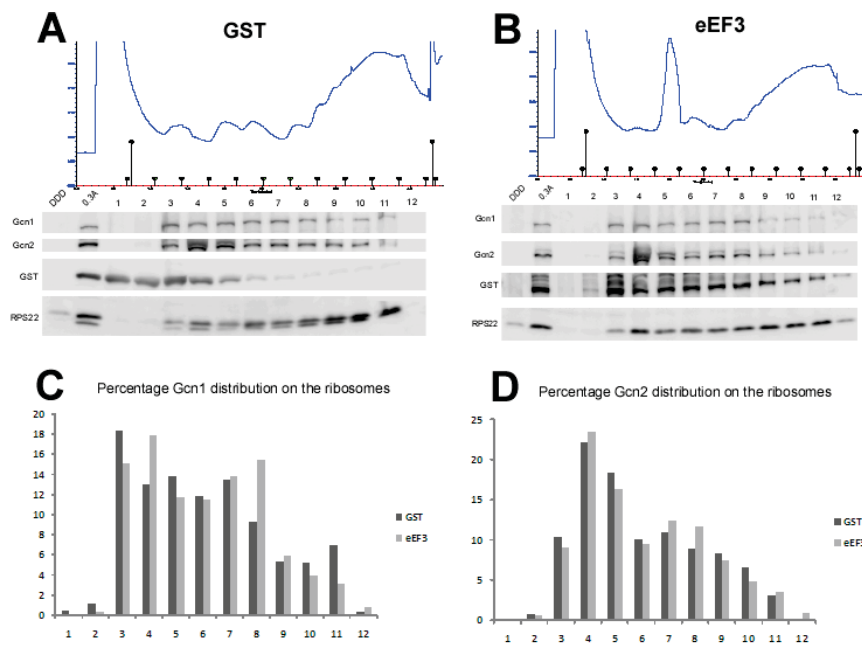


Figure A.2: eEF3 over-expression barley displaced Gcn1 from the polyribosomes

Ribosome co-sedimentation assays were performed as described in section 4.5 and analysed by immunoblotting using antibodies against Gcn1, Gcn2, RPS22, and the GST epitope located at the N-terminus of eEF3. The amount of Gcn1 or Gcn2 in each fraction from A and B was quantified using the Multi Gauge V3.1 software (Fuji Photo Film Co., Ltd.). Amount of Gcn1 (C) or Gcn2 (D) in each fraction is expressed as a percentage of the total Gcn1 or Gcn2 in all fractions.

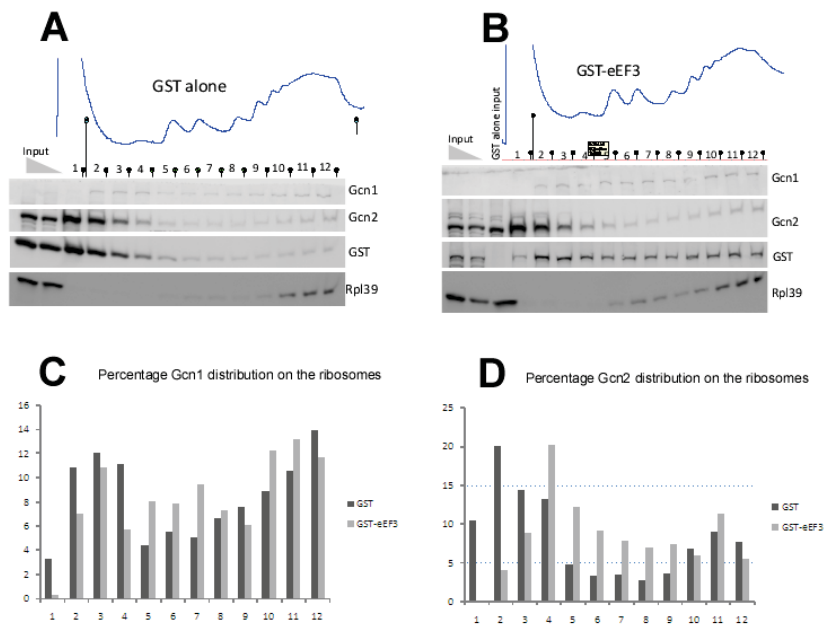


Figure A.3: eEF3 over-expression barley displaced Gcn1 from the polyribosomes

Ribosome co-sedimentation assays were performed as described in section 4.5 and analysed by immunoblotting using antibodies against Gcn1, Gcn2, RPS22, and the GST epitope located at the N-terminus of eEF3. The amount of Gcn1 or Gcn2 in each fraction from A and B was quantified using the Multi Gauge V3.1 software (Fuji Photo Film Co., Ltd.). Amount of Gcn1 (C) or Gcn2 (D) in each fraction is expressed as a percentage of the total Gcn1 or Gcn2 in all fractions.

A.4 *stm1Δ* strain is SM resistant

As discussed in chapter four a study claimed that when a yeast strain was deleted for *Stm1*, it had elevated levels of eEF3 bound to the ribosome. Such a strain would be predicted to have a *Gcn⁻* phenotype indicated by sensitivity to amino acid starvation drugs. However upon investigation this strain did not show a *Gcn⁻* phenotype.

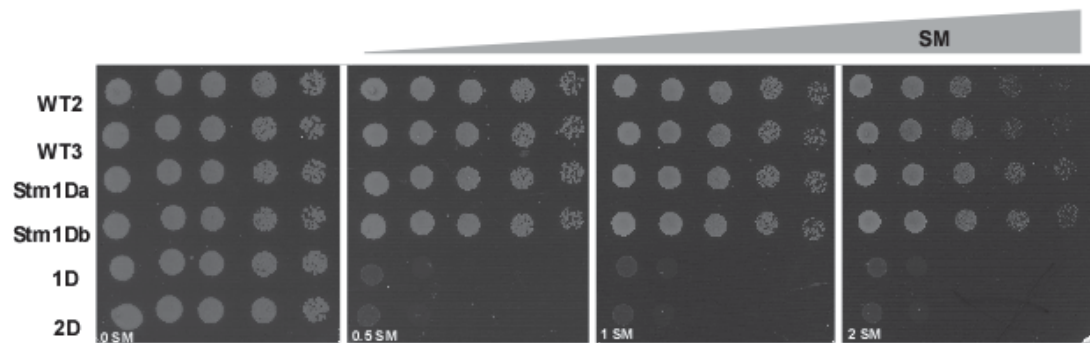


Figure A.4: *stm1Δ* strain is SM resistant

(A) Saturated cultures of isogenic *stm1Δ* strain, wild type strain, *gcn1Δ*, and *gcn2Δ* were subjected to 10 fold serial dilutions, and 5 μ L of undiluted culture and 5 μ L of each dilution was transferred to solid medium containing no SM (control) or increasing amounts of SM as indicated. Plates were incubated at 30°C until colonies were visible.

Appendix B

B.1 Verification of pJV02

The plasmid constructed for experiments in chapter five called pJV02 was verified by PCR and sequencing.

Clone L7-16 sequenced with primer ES2020

Sequence returned:

```

ARSWRWRKWKTTGTTGCTGGTTCGACTAATAATCAAAAAGGTAATTTATGTTCCAAACATGGCTACAAG
ATCTCTGATAGCTAATATAAGGGAAAAGTGGGTTTATGAGGATGCAGCCAGAAATTCCTCGAATATGA
TATTACACAATTTATCCAATGCACCAATTATCACTGTTGATGCCTTAAGAGATGAACTTTAGAAAATA
ATCTCAATTACTTCTTTGGCTCAGAAGGAAGAATGGCTGAGAAAAGTTTTTGGGTCAGGTAATAACTC
GACTCCTAGAAGCTTTGCCACGAGCATTATAATAACCTCTCCAAAGAGGCTCATAAAGGGAATAGGT
GGGCAATATTATACTGCCACAAAACCGGAAAATCATCTGTTATCGATTTACAGAGGTAGGCCTTTAAA
GATCCTCTACGCCGGACGCATCGTGGCCGGCATCACCGGCGCCACAGGTGCGGTTGCTGGCGCCTATA
TCGCCGACATCACCGATGGGGAAGATCGGGCTCGCCACTTTCGGGCTCATGAGCGCTTGTTCGGCGTG
GGTATGGTGGCAGGCCCGTGGCCGGGGACCGTTGGGCGCCATCTCCTTGATGCACCATTCTTGC
GGCGGCGCTATAACGCATCAGTTAAAGGTAAAGTATATACAATGTCTTAGATACTATATTTTAGGCTA
GCTGATTCATCTAATTCAYCGTTGTAAAGGGGGWRGGTCACWTTMCT

```

Six frame translation blasted in the SGD "TBLASTX"

```

Query: 13      VAGSTNNQKVIYVPMATRSLIANIREKWVYEDAARNSSNMILHNSNAPIITVDALRDE 192
               VAGSTNNQKVIYVPMATRS  AN REKWVYEDAARNSSNMILHNSNAPIITVDALRDE
Sbjct: 1025453 VAGSTNNQKVIYVPMATRSKKANKREKWVYEDAARNSSNMILHNSNAPIITVDALRDE 1025274

Query: 193     TLEIISITSLAQKEEWLKRVFGSGNNSTPRSFATSIYNNLSKEAHKGNRWAILYCHKTGK 372
               TLEIISITSLAQKEEWLKRVFGSGNNSTPRSFATSIYNNLSKEAHKGNRWAILYCHKTGK
Sbjct: 1025273 TLEIISITSLAQKEEWLKRVFGSGNNSTPRSFATSIYNNLSKEAHKGNRWAILYCHKTGK 1025094

Query: 373     SSVIDLQR*AFKD 411
               SSVIDLQR*AFKD
Sbjct: 1025093 SSVIDLQR*AFKD 1025055

```

The 3 highlighted amino acids are the intended K mutations!

B.2 Gcn2 co-elutes with eEF1A under replete conditions

The result from an *in vivo* pull down assay was performed as described in section 5.1 using a sister clone is shown below.

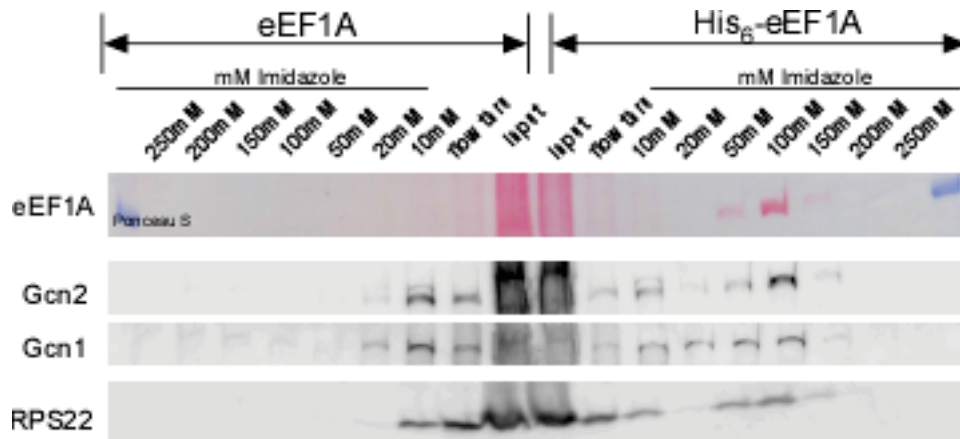


Figure B.1: Gcn2 co-elutes with eEF1A

In vivo pull down assays were performed as described in section 5.1. The precipitates were analysed by immunodetection using antibodies against Gcn1, Gcn2, and Rps22.

B.3 Gcn2-eEF1A interaction is lost upon amino acid starvation.

The result from an *in vivo* pull down assay was performed as described in section 5.6 using a sister clone is represented below.

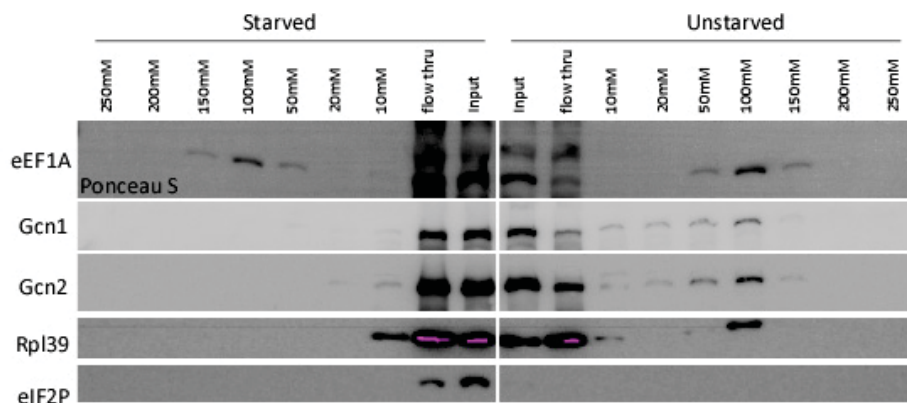


Figure B.2: Gcn2-eEF1A interaction lost upon amino acid starvation

In vivo pull down assays were performed as described in section 5.1. The precipitates were analysed by immunodetection using antibodies against Gcn1, Gcn2, and Rps22.

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