REGULATION OF eIF2B BY PHOSPHORYLATION

A thesis submitted to The University of Manchester for the degree of Doctor of Philosophy in the Faculty of Life Sciences

2013

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Abstract

The ability to sense and respond to environmental cues is crucial for the survival of all organisms. This response is often manifested by exerting control at different levels of gene expression, i.e. transcription, translation and post translation levels. Global control of protein synthesis is frequently exercised at the initial step of translation initiation and is generally achieved by changes in the phosphorylation state of initiation factors or the regulators that interact with them. The formation of ternary complex (TC) is considered first step of translation initiation and depends on the recycling of inactive eIF2-GDP to active eIF2-GTP form. This nucleotide exchange reaction is catalyzed by the eukaryotic initiation factor-2B (eIF2B). eIF2B is composed of a regulatory sub-complex of αβδ subunits and a catalytic sub-complex of the γε subunits. The guanine nucleotide exchange activity of eIF2B is regulated by phosphorylation of eIF2α and additionally in mammalian cells, by direct phosphorylation of eIF2B at multiple sites in ε subunit, where most of the catalytic activity of eIF2B resides.

Recent unpublished studies in the Pavitt laboratory identified novel phosphorylation sites by Mass Spectrometry in γ and ε subunits of eIF2B catalytic sub-complex. In order to study the functional significance of these phospho-sites for translation initiation, Site Directed Mutagenesis (SDM) was performed to generate Ser to Ala mutants. All mutations are viable and have no significant growth defect on rich or minimal media; however the significance of these sites in yeast growth became apparent by growing yeast in different stress conditions (e.g. Rapamycin, Torin1, amino acid starvation and 1-butanol). Effects on the phosphorylation pattern at these sites were monitored by using custom generated phospho-specific antibodies. All phosphorylation events appear independent of the eIF2α kinase (Gcn2p in yeast). The phosphorylation of ε-S528 depends on the presence of ε-S525. This study finds that addition of rapamycin, Torin1, amino acid starvation and butanol, which each inhibits global translation initiation, alters the phosphorylation pattern at ε-S435, ε-S525 and ε-S528 sites. Linking growth to phosphorylation, it appears that phosphorylation at ε-S435 and ε-S525 is directly proportional to growth. Phosphorylation of ε-S435 is necessary for effect of eIF2α-Ser51 phosphorylation on protein synthesis while phosphorylation of ε-S528 seems to be a target of various mechanisms. This study also suggests that eIF2Bε may be a key player of the cell cycle progression and phosphorylation changes can serve as marker for the regulation of eIF2B activity. The kinases responsible for phosphorylation at these sites are not yet known in yeast. Further investigation is required to find the functional significance of alterations in phosphorylation pattern to definitively establish eIF2Bε phosphorylation as a mechanism for regulating eIF2B activity in yeast. Models are presented to account for the results obtained that show how phosphorylation of eIF2Bε at these sites may contribute to the control of protein synthesis.

Keywords: eIF2B, Translation initiation, Phosphorylation, Regulation, Cell cycle
Declaration

The University of Manchester

PhD Candidate Declaration

Candidate Name: Rehana KOUSAR
Faculty: Life Sciences
Thesis Title: Regulation of eIF2B in Phosphorylation

Declaration to be completed by the candidate:

I declare that no portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

Signed:

Date: 30th June 2013
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To

My Parents,

Teachers

And

Husband

For their support, love, affection and guidance!!!
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This thesis is a story of contrasts: the tallest vs the shortest, genius vs dumb, competent vs lazy, persistent vs quitter and punctual vs always late-comer, surprisingly with a happy ending. The tallest dared to take the shortest under his kind supervision and showed confidence and faith that he could make best out of her and on the other side, the shortest was amazingly fortunate to have the tallest as her research supervisor. I am extremely indebted to Professor Graham Pavitt, who was not only a source of inspiration but a source of positive energy for me who continually and convincingly conveyed a spirit of keep on trying. I have learnt a lot from him and because of him. I acknowledge with gratitude my debt of thanks to my advisor, Dr Sue Crosthwaite for her kind guidance and support during this research. Thanks to Internal and external examiners for their valuable comments on my thesis. Thanks to Ben Turk for his help on bioinformatics.

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---

1 Principal Investigator of Pavitt Lab at Faculty of Life Sciences, University of Manchester, Manchester, UK
## List of abbreviations

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>Ala</td>
<td>Alanine</td>
</tr>
<tr>
<td>A-site</td>
<td>Amino acyl site</td>
</tr>
<tr>
<td>AUG</td>
<td>Initiator codon</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BP</td>
<td>Binding protein</td>
</tr>
<tr>
<td>CACH</td>
<td>Childhood ataxia with central nervous system hypomyelination</td>
</tr>
<tr>
<td>CITE</td>
<td>cap-independent translation enhancers</td>
</tr>
<tr>
<td>CK</td>
<td>Casein kinases</td>
</tr>
<tr>
<td>CTD</td>
<td>Carboxy terminal domain</td>
</tr>
<tr>
<td>DB</td>
<td>Downstream box</td>
</tr>
<tr>
<td>DYRK</td>
<td>Dual specificity tyrosine phosphorylated and regulated kinase</td>
</tr>
<tr>
<td>EF</td>
<td>Elongation factor</td>
</tr>
<tr>
<td>EGO</td>
<td>escape from rapamycin induced growth arrest</td>
</tr>
<tr>
<td>eIF</td>
<td>Eukaryotic initiation factor</td>
</tr>
<tr>
<td>eIF2B</td>
<td>eukaryotic translation initiation factor-2B</td>
</tr>
<tr>
<td>fMet-tRNAi&lt;sup&gt;Met&lt;/sup&gt;</td>
<td>Initiator tRNA charged with formyl methionine</td>
</tr>
<tr>
<td>FOA</td>
<td>Fluoro-orotic acid</td>
</tr>
<tr>
<td>FRB</td>
<td>FKBP-rapamycin binding region</td>
</tr>
<tr>
<td>GAAC</td>
<td>General amino acid control</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase accelerating protein</td>
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<td>GCD</td>
<td>General control depressive</td>
</tr>
<tr>
<td>GCN</td>
<td>General control non-derepressible</td>
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<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
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<td>Luria bertani</td>
</tr>
<tr>
<td>LioAc</td>
<td>Lithium acetate</td>
</tr>
<tr>
<td>LSB</td>
<td>Loading sample buffer</td>
</tr>
<tr>
<td>LβH</td>
<td>left handed β-helix</td>
</tr>
<tr>
<td>Met-tRNAi&lt;sup&gt;Met&lt;/sup&gt;</td>
<td>Initiator transfer RNA for methionine</td>
</tr>
<tr>
<td>MFC</td>
<td>Multifactor complex</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
</tr>
<tr>
<td>NTD</td>
<td>Amino terminal domain</td>
</tr>
<tr>
<td>NTs</td>
<td>Nucleotidyl transferases</td>
</tr>
<tr>
<td>OB-fold</td>
<td>Oligonucleotides-binding fold</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>P-site</td>
<td>Peptidyl site in ribosomal subunit</td>
</tr>
<tr>
<td>PABP</td>
<td>Poly(A) binding protein</td>
</tr>
</tbody>
</table>
**List of abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PERK</td>
<td>PKR-like endoplasmic reticulum kinase</td>
</tr>
<tr>
<td>PIC</td>
<td>Preinitiation complex</td>
</tr>
<tr>
<td>PIKK</td>
<td>Phosphatidylinositol kinase-related kinase</td>
</tr>
<tr>
<td>PKR</td>
<td>Proetin kinase R</td>
</tr>
<tr>
<td>PLD</td>
<td>NTP-hexose sugar pyrophosphorylases domain</td>
</tr>
<tr>
<td>RF</td>
<td>Release factor</td>
</tr>
<tr>
<td>RISC</td>
<td>RNA induced silencing complex</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RRF</td>
<td>Ribosome release factor</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal RNA</td>
</tr>
<tr>
<td>S</td>
<td>Svedberg unit of sedimentation</td>
</tr>
<tr>
<td>SD</td>
<td>Minimal synthetic dextrose</td>
</tr>
<tr>
<td>SDM</td>
<td>Site Directed Mutageneis</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>Ser</td>
<td>Serine</td>
</tr>
<tr>
<td>PAGE</td>
<td>Poly acrylamide gel electrophoresis</td>
</tr>
<tr>
<td>ShD</td>
<td>Shine Dalgarno</td>
</tr>
<tr>
<td>SUI</td>
<td>Suppressors of initiation</td>
</tr>
<tr>
<td>SXL</td>
<td>Sex lethal</td>
</tr>
<tr>
<td>TC</td>
<td>Ternary complex</td>
</tr>
<tr>
<td>TOR</td>
<td>Target of rapamycin</td>
</tr>
<tr>
<td>TORC</td>
<td>Target of rapamycin complex</td>
</tr>
<tr>
<td>tRNA</td>
<td>Transfer RNA</td>
</tr>
<tr>
<td>uORF</td>
<td>Upstream open reading frame</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated regions</td>
</tr>
<tr>
<td>VWM</td>
<td>vanishing white matter</td>
</tr>
<tr>
<td>YPD</td>
<td>Yeast extract, Peptone, Dextrose</td>
</tr>
<tr>
<td>( \varepsilon^{\text{cat}} )</td>
<td>Catalytic domain in ( \varepsilon ) subunit</td>
</tr>
</tbody>
</table>
Communications


Chapter 1

Introduction
1 Introduction

Translation or protein synthesis refers to the decoding of genetic information in the form of mRNA to essential building blocks of life, i.e. proteins. Translation is a multi-step process that requires large investment of resources both in terms of energy and space. The parasitic bacterium *Mycoplasma genitalium* has the smallest known cellular genome and encodes for 480 proteins. Out of 480 proteins, 21% play a role in protein synthesis (Fraser et al. 1995). A large number of genes are involved in translation in eukaryotes which require more ribosomal proteins and initiation factors, i.e. cells invest extensive resources in the synthesis of ribosomes (2000 ribosomes/ min in fast growing yeast), tRNA, enzymes and translation factors required for protein synthesis (Warner 1999). It is not surprising that cells monitor and regulate such a highly demanding process. Translational control specifies gene expression by regulating the utilization of the particular set of translational factors involved in protein synthesis.

The process of translation can be divided into three main stages, initiation, elongation and termination. This chapter will highlight the main features of translation with emphasis on eukaryotic translation initiation. The process of translation in prokaryotes will be discussed briefly only for comparison to describe the relative simplicity of the process in prokaryotes. As this study is interested in the regulation of eIF2B under various environmental challenges, the emphasis will be on the translation initiation in eukaryotes. The role of eIF2B in translation initiation and different mechanisms for regulation of eIF2B catalytic activity and hence translation initiation are also discussed. The complexity of eukaryotic translation over prokaryotic translation provides additional levels of translation regulation are also discussed here. A sub-section of the chapter is dedicated to the role of kinases/ phosphatases in the regulation of eIF2B activity described in mammals. The role of eIF2B mutations in neurodegenerative disease such as leukoencephalopathy with vanishing white matter is described later in this chapter before introducing the current study.
1.1 Prokaryotic translation initiation

In cells, complex biochemical reactions are catalyzed by several proteins. Prokaryotes have developed a mechanism to ensure the co-ordinated expression of the set of proteins. Often, the genes coding for proteins involved in a single process are arranged together, such functional organization of genes in a set is called Operon and single promoter is used for these genes expression, e.g. lactose operon for the metabolism of lactose. As a result of this, mRNAs transcribed from these genes are often polycistronic. Translational apparatus recognizes specific start signals and initiate translation process at different locations in the same mRNA. In bacteria, translation is coupled to transcription as the ribosomes start translating the mRNA that is still being transcribed (Brenner 1997).

Translation initiation is same in prokaryotes and eukaryotes in a sense that it involves the formation of an initiation complex competent for elongation. The initiation complex of prokaryotes is simple and consists of ribosome, mRNA, and formylmethionine-tRNA\textsuperscript{0Met} (fMet-tRNA\textsuperscript{iMet}) complex. Only three main factors e.g. IF1, 2 and 3 are required for the prokaryotic translation initiation (Laursen et al. 2005, Simonetti et al. 2008). The prokaryotic IFs with their eukaryotic orthologs and their roles are summarized in Table 1.1. The composition of prokaryotic ribosomal subunits is also less complex requiring less ribosomal protein and rRNA. A simplified scheme of the main steps involved in the formation of 70S initiation complex in prokaryotes is outlined in Figure 1.1.

The 70S ribosome exists in equilibrium with 30S and 50S subunits and at physiological free Mg\textsuperscript{2+} concentration (5mM) they exist mostly in associated 70S particles form. The 30S subunit generated after cycle of translation exists as a complex with IF3. The association of IF3 with 30S prevents the formation of 70S complex (Dallas and Noller 2001). IF3-30S complex then binds to IF1 and IF2-GTP. The next step of translation initiation is not fully understood. It is not established which factors bind first to 30S, mRNA or fMet-tRNA\textsuperscript{iMet} (Gualerzi and Pon 1990, Marzi et al. 2003, Simonetti et al. 2008).
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Regulation of eIF2B by Phosphorylation

Figure 1.1. Prokaryotic translation initiation.

30S initiation complex is formed by the assembly of IF3-30S, mRNA, IF1, IF3 and fMet-tRNA<sub>Met</sub>. 70S complex is formed by the association of 50S subunit followed by hydrolysis of GTP into GDP.
Two unique and salient features of translation initiation in bacteria are: the use of fMet-tRNA_{fMet} as initiator tRNA and presence of a Shine Dalgarno (ShD) sequence in mRNA that is used as ribosome binding site. The formylation of initiator tRNA increases the affinity of tRNAi for IF2. The recognition between mRNA and initiator complex depends on the interaction between a purine rich ShD in 5'-untranslated region (5'-UTR). The ShD sequence is present ~10 nucleotides upstream of initiator AUG codon of the open reading frame (ORF). Anti-shine dalgarno (anti-ShD) sequence found in the 3'-end of the 16S rRNA of 30S ribosome recognizes and base pairs with complementary ShD sequence. The conserved ShD sequence is 5'UAAGGAGGU3' (underlined nucleotides are most frequently present), but the function of ShD sequence is not dependent on its length or sequence. In some cases of translation initiation ShD sequence is completely absent (Condon et al. 2001).

IF1 and IF3 dissociates from 30S complex on binding of initiation complex to mRNA. 50S ribosomal subunit joins the complex in a way that fMet-tRNA_{fMet} occupies the P-site of ribosome. Hydrolysis of IF2-GTP takes place and 70S complex forms which is competent for elongation (Laursen et al. 2005, Simonetti et al. 2008). About 90% of the bacterial mRNAs initiate protein translation at an AUG codon, while GUG and UUG are also used for translation initiation (Sussman et al. 1996).

Table 1.1. List of prokaryotic translation initiation factors, their eukaryotic orthologs and function

<table>
<thead>
<tr>
<th>Prokaryotic IFs</th>
<th>Eukaryotic Ortholog</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>IF1</td>
<td>eIF1A</td>
<td>Prevents initiator tRNAi binding to A site</td>
</tr>
<tr>
<td>IF2</td>
<td>eIF5B</td>
<td>Assists fMet-tRNA_{fMet} binding to 30S</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mediates subunit joining GTPase</td>
</tr>
<tr>
<td>IF3</td>
<td>eIF1</td>
<td>Initiation site selection</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Proof-reading activity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ribosome subunit anti-association</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ribosome recycling</td>
</tr>
</tbody>
</table>

(Kapp and Lorsch 2004)
1.2 Eukaryotic translation

In contrast to prokaryotes, in eukaryotes, functional genes are not arranged as operons and each mRNA typically corresponds to one gene. mRNAs are transcribed in nucleus and are transported to cytoplasm as mRNA-protein complexes. Eukaryotes mRNAs have evolved mRNA features such as a cap at 5’ end and 3’ polyadenylated tail. These structures provide additional stability to mRNA in cytosolic environment and save it from degradation by exo nucleases. Eukaryotes have developed complex mechanisms for the recruitment of ribosome to mRNA. This added structural complexity provides additional levels of translation regulation. Infact, many IF factors are phospho-proteins and their activities can be regulated by diverse environmental signals (Dever et al. 1992, Krishnamoorthy et al. 2001, Proud 2001, Wang and Proud 2008).

1.2.1 Translation initiation

Common to prokaryotes, translation initiation in eukaryotes involves the formation of mRNA, tRNA, and ribosome initiation complex competent for elongation. However, translation initiation is more complicated in eukaryotes as compared to bacteria, i.e. eukaryotic translation initiation is assisted by >30 polypeptides that make up eleven initiation factors (Sonenberg and Hinnebusch 2009). The core eukaryotic translation initiation factors and their roles during initiation are summarized in Table 1.2. Some studies show the additional roles of some auxiliary factors, which are summarised in Table 1.3. As translation machinery is different from prokaryotes, the molecular processes also differ for the assembly of initiation complex.

Two different mechanisms of translation initiation have been proposed in eukaryotes, 5’-cap dependent mechanism also known as Scanning Model and Internal Ribosome Entry Site mediated translation initiation mechanism (IRES) (Myasnikov et al. 2009, Jackson et al. 2010) Scanning or 5’-cap dependent mechanism of translation initiation is discussed in detail here, while other mechanisms of translation initiation used under different condition by sets of mRNAs are also discussed briefly.
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Table 1.2. Core eukaryotic translation initiation factors in yeast

<table>
<thead>
<tr>
<th>Initiation factor</th>
<th>Subunit</th>
<th>Yeast gene</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>eIF1</td>
<td>SUI1</td>
<td>Fidelity of Initiation codon, Stimulates binding of TC to 40S subunit, Promotes ribosomal scanning, Prevents premature hydrolysis of eIF2-GTP by eIF5</td>
<td></td>
</tr>
<tr>
<td>eIF1A</td>
<td>TIF11</td>
<td>Promotes binding of TC to 40S, promotes eIF1</td>
<td></td>
</tr>
<tr>
<td>eIF2</td>
<td>α SUI2</td>
<td>Inhibits eIF2B catalytic activity by phosphorylation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>β SUI3</td>
<td>Binds Met-tRNA^Met^, eIF5 and eIF2B</td>
<td></td>
</tr>
<tr>
<td></td>
<td>γ GCD11</td>
<td>Binds GTP, GTPase</td>
<td></td>
</tr>
<tr>
<td>eIF2B</td>
<td>α GCN3</td>
<td>GEF that promotes GDP-GTP exchange on eIF2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>β GCD7</td>
<td>Part of regulatory subunit of eIF2B complex, that recognizes the phosphorylation of eIF2α and down regulates the activity of catalytic subunit of eIF2B</td>
<td></td>
</tr>
<tr>
<td></td>
<td>γ GCD2</td>
<td>GEF activity</td>
<td></td>
</tr>
<tr>
<td></td>
<td>δ GCD1</td>
<td>GEF activity</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ε GCD6</td>
<td>Target of kinases for regulating eIF2B activity</td>
<td></td>
</tr>
<tr>
<td>eIF3</td>
<td>a RPG1</td>
<td>A large multimeric complex</td>
<td></td>
</tr>
<tr>
<td></td>
<td>b PRT1</td>
<td>Essential for translation initiation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>c NIP1</td>
<td>Promotes binding of TC 40S</td>
<td></td>
</tr>
<tr>
<td></td>
<td>g TIF35</td>
<td>Helps assembly of PIC, AUG recognition</td>
<td></td>
</tr>
<tr>
<td></td>
<td>i TIF34</td>
<td>Essential for translation, Ribosome scanning</td>
<td></td>
</tr>
<tr>
<td></td>
<td>j HCR1</td>
<td>Required for processing of 20S rRNA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>k CLUI</td>
<td>Unknown function</td>
<td></td>
</tr>
<tr>
<td>eIF4A</td>
<td>I TIF1</td>
<td>Part of cap recognition eIF4F complex</td>
<td></td>
</tr>
<tr>
<td></td>
<td>II TIF2</td>
<td>ATPase, RNA helicase</td>
<td></td>
</tr>
<tr>
<td>eIF4B</td>
<td>TIF3</td>
<td>Promotes eIF4A activity</td>
<td></td>
</tr>
<tr>
<td>eIF4E</td>
<td>CDC33</td>
<td>Part of eIF4F complex</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cap-binding factor</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Target of 4E-BP phosphorylation</td>
<td></td>
</tr>
<tr>
<td>eIF4G</td>
<td>I TIF4631</td>
<td>Scaffolding protein, part of eIF4F complex</td>
<td></td>
</tr>
<tr>
<td></td>
<td>II TIF4362</td>
<td>eIF4G-I is usually more abundant, Binds 4A, 4E, eIF3, PABP, and RNA,</td>
<td></td>
</tr>
<tr>
<td>eIF5</td>
<td>TIF5</td>
<td>Stimulates eIF2 GTPase activity, GDI activity</td>
<td></td>
</tr>
<tr>
<td>eIF5B</td>
<td>FUN12</td>
<td>GTPase, Promotes 80S formation</td>
<td></td>
</tr>
</tbody>
</table>
Table 1.3. Auxiliary factors for eukaryotic translation initiation

<table>
<thead>
<tr>
<th>Secondary factors</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHX29</td>
<td>Promotes ribosomal scanning of mRNAs with long structured 5'-UTRs</td>
</tr>
<tr>
<td>Ded1</td>
<td>NTPase and RNA-helicase</td>
</tr>
<tr>
<td>eIF6</td>
<td>Promotes 80S dissociation</td>
</tr>
<tr>
<td>p97</td>
<td>Promotes initiation in mRNA specific manner</td>
</tr>
<tr>
<td>PABP</td>
<td>Binds to Poly(A) tail, eIF4G, 5'-cap and helps in recycling of post termination 40S back to 5'-cap</td>
</tr>
</tbody>
</table>

The key events taking place at initiation stage can be divided into four phases, i. Formation of TC, ii. Formation of 43S pre-initiation complex, iii. 48S complex formation and iv. Scanning and 60S subunit joining. Each of these steps is discussed in turn. Figure 1.2 represents the main steps involved in the assembly of 80S eukaryotic translation initiation complex.

1.2.1.1 Formation of TC

The eIF2-GDP inactive complex is produced after each completed round of translation initiation. For the next round of translation initiation to begin eIF2-GDP must be recycled to active eIF2-GTP. eIF2 has hundred-fold higher affinity for GDP than for GTP and at the physiological concentration of Mg^{++} the rate constant for GDP release is low (Kapp and Lorsch 2004, Kapp and Lorsch 2004). The first step in the initiation pathway is the formation of the TC consisting of eIF2-GTP-Met-tRNA^{Met} (Figure 1.1 Phase-I).

eIF2 is a G-protein and requires the action of GEF. In case of eIF2-GDP complex this reaction is catalysed by eukaryotic translation initiation factor 2B (eIF2B). eIF2B is a heteromultimeric protein complex of five subunits (α-ε). The essential exchange reaction is performed by the carboxy terminal of eIF2Bε subunit while other subunits favour the binding of eIF2B to eIF2-GDP complex (Dever et al. 1992, Krishnamoorthy et al. 2001). This step is tightly controlled and the mechanisms by which this step is controlled is discussed later in section 1.5.
1.2.1.2 Formation of 43S pre-initiation complex (43S PIC)

The TC (eIF2-GTP-Met-tRNAi\textsuperscript{Met}) binds jointly to the 40S small ribosomal subunit and the recruitment is promoted by (at least) eIFs 1, 1A, eIF5 and eIF3 complex (Asano et al. 2000, Algire et al. 2002, Singh et al. 2006). The resulting complex is called the 43S PIC. It was found that the eIFs 1, 1A, and 3 were minimum factors required for the stable binding of TC to the 40S subunit in mammalian initiation system (Majumdar et al. 2003); whereas in a yeast-based reconstituted initiation system, only eIFs 1 and 1A were critical (Algire et al. 2002). A variety of experiments have shown the \textit{in vivo} existence of a stable “MultiFactor Complex” (MFC) consisting of Met-tRNAi\textsuperscript{Met} and eIFs 1, 2, 3, 5. The integrity of this complex is important for translation initiation (Valasek et al. 2002).

    eIF5 is a central component of the multifactor complex and it interacts with the TC in addition to eIFs1 and 3. eIF5, also a GTPase accelerating protein (GAP) for eIF2, is likely to stabilize TC binding to the 40S subunit (He et al. 2003). It is also involved in the regulation of eIF2B mediated GDP exchange reaction and acts as a GDP dissociation inhibitor (GDI) (Jennings and Pavitt 2010).

1.2.1.3 Formation of 48S initiation complex

The first step in loading of the mRNA onto the small ribosomal subunit is the recognition of the mRNA’s 5’-cap. \textit{In vitro} translation of capped mRNAs is improved three to thirty fold relative to the uncapped message in both mammalian and yeast extracts (Preiss and Hentze 1998, Michel et al. 2000).

    The role of the cap is to enhance binding of mRNA to the 43S PIC mRNA is activated by binding of 5’ cap to cap binding complex eIF4F consisting of eIF4E, eIF4A and eIF4G subunits and poly(A) binding protein (PABP) to the poly(A) tail (Morino et al. 2000, Hilbert et al. 2011, Ozes et al. 2011). The 43S PIC complex interacts with eIF4E and this interaction is enhanced by eIF3 and PABP bound to the 3’ poly(A) tail (Figure 1.1 Phase III).
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Figure 1.2. Simplified representation of the eukaryotic translation initiation pathway.

For simplicity, binding of 5'-cap and poly(A)tail leading to closed loop complex is omitted. Initiation factors are shown as blank ovals with numbers. Pink ovals in the 80S represent P and empty A site respectively. Methionine is represented as black bold hexagon. Translation initiation stage is divided into 4 phases labelled accordingly, see text for more details, the complete protein model is taken from (Reid et al. 2012). (Adapted from (Kapp and Lorsch 2004, Jackson et al. 2010).
The discovery that eIF4G and PABP bind to each other suggests that this interaction circularizes eukaryotic mRNAs. It was also shown that the eIF4E and 4G subunits of eIF4F together with PABP could circularize capped and polyadenylated nucleic acids *in vitro*. A homolog of the central region of eIF4G, called PABP-interacting protein-1 (PAIP1) that binds to eIF4A and PABP but not eIF4E, has been discovered in mammals (Craig et al. 1998). Over expression of PAIP1 stimulates translation, suggesting that this protein can enhance initiation of polyadenylated mRNAs without joining the 5' and 3'-ends. It has been shown in a study that eIF2/eIF3 depletion impaired mRNA binding to free 40S ribosomal subunit while depletion of eIF4G led to the accumulation of mRNA on the 40S subunits suggesting that eIF3 and eIF2 are more critically required than eIF4G for stable binding of mRNA to PIC (Jivotovskaya et al. 2006, Park et al. 2011).

### 1.2.1.4 Scanning and initiation codon recognition

The eIF4F complex assembled on 5'-cap of the mRNA unwinds secondary structures found in the 5' UTR using ATP-dependent action of eIF4A-helicase. The helicase activity of eIF4F is assisted by the RNA-binding protein eIF4B (in mammals eIF4H) (Jivotovskaya et al. 2006, Jackson et al. 2010). The 48S PIC begins scanning of mRNA in the 5' to 3' direction in search of an initiation AUG codon. eIF1 and eIF1A play a role in the scanning process. A role for eIF1 in initiation codon selection has also been demonstrated *in vitro*. eIF1 directly checks the anticodon-mRNA pairings and when three perfect base pairs are found, it reduces the energy of the resulting duplex complex enough to stop scanning and eIF5 mediated hydrolysis of GTP bound to eIF2 takes place. For most of the time in translation, all decoding happens in the A site and thus eIF1 may also be required in the P site to direct TC binding to P-site during initiation (Pestova and Kolupaeva 2002) (Figure 1.2 Phase-IV).

When the 48S complex encounters the first AUG codon, codon-anticodon base pairing takes place between the initiation codon and the initiator tRNA. Several initiation factors (eIFs 1, 1A, 2 and 3) occupy surface of the 40S subunit and these factors must be evacuated from the surface of 40S subunit to permit 60S subunit joining. Release of eIF1 switches 40S from open scanning mode to closed
mode allowing AUG recognition. GTP hydrolysis can happen during scanning, but Pi release at AUG recognition. GTP hydrolysis is facilitated by the GAP protein eIF5, which is followed by the release of eIF2 and possibly other initiation factors (Asano et al. 2000, Kapp and Lorsch 2004).

GTP hydrolysis by eIF2 serves as a checkpoint for proper identification of the mRNA start codon. It was supposed that eukaryotic translation initiation requires the hydrolysis of only a single GTP but now it has been shown that two GTP hydrolysis events are required, one catalyzed by eIF2 upon initiation codon recognition and another after 80S complex formation. After GTP hydrolysis by eIF2, eIF2-GDP releases the Met-tRNA\textsubscript{i}\textsuperscript{Met} into the P site of the 40S subunit and dissociates from the complex along with several other initiation factors including (eIFs 1, 1A, 3, and 5). eIF5B-GTP binds to the complex and facilitates the joining of the large (60S) ribosomal subunit to the 40S-Met-tRNA\textsubscript{i}\textsuperscript{Met}-mRNA complex. The GTPase activity of eIF5B is activated by the combination of 40S and 60S subunits, and eIF5B-GDP complex dissociates from the complex. The mRNA poly (A) tail and PABP have also been implicated in the subunit joining process in yeast cells and in mammalian cell free extracts. The formation of 80S ribosome, with acylated tRNA in the P-site of 60S ribosomal subunit and empty A site marks the end of initiation (Pestova and Kolupaeva 2002, Kapp and Lorsch 2004, Jackson et al. 2010). The 80S complex produced is competent for elongation

### 1.2.2 IRES mediated translation initiation

Viruses inhibit the translation of host mRNAs while ensuring the synthesis of viral proteins by hijacking host translation machinery. Viruses usually achieve this by switching off 5′-end dependent translation initiation. As viruses do not contain their own translational machinery and are dependent on host for their replication, they have evolved mechanisms to ensure the expression of viral proteins. This is achieved by using alternative means of translation initiation that are not dependent on 5′-end of mRNA. IRESs are RNA sequences in mRNA that recruit ribosome in a 5′-end independent manner (Figure 1.3). The role of IRESs in recruiting ribosome to initiate translation in a 5′-end independent manner was established using bicistronic mRNA assay (Jang et al. 1988).
Four different groups of IRES have been identified so far based on the interaction with eukaryotic translation initiation factors and or 40S subunit. The structural relationship between different groups of IRESs is very little and classification is usually based on the mode of interaction. Type I and 2 IRESs (picornaviruses) interact with p50 domain of eIF4G, without interacting the eIF4E (~450 nt found in polio and encephalomyocarditis virus respectively). Type 3 (HCV-like) directly interacts with eIF3 and 40S without requiring eIF4F, eIF4B, eIF1 and eIF1A (~300 nucleotides found in hepatitis C virus), type 4 (dicistrovirus intergenic region) interacts with 40S subunits and does not require Met-tRNAi^Met (~200 nt found in cricket paralysis virus) (Jackson et al. 2010). HIV-I and HIV-II viruses mRNA contain eukaryotic mRNA features, i.e. it has 5'-cap and 3'-poly (A) tail. The synthesised mRNA is spliced into 40 different species and they are transported to cytoplasm. IRESs are one of the many mechanisms adopted for the expression of these mRNA species (de Breyne et al. 2013). IRES mediated translation initiation is resistant to the regulatory mechanism used by eukaryotic cells, i.e. eIF2α(p) and eIF4E sequestration.

IRESs mediated translation sometimes requires IRES-trans acting factors (ITAFs), which stabilizes the three-dimensional conformation of IRESs (Jackson et al. 2010, Shatsky et al. 2010). IRESs-mRNAs can be translated with 5'-cap dependent mechanism, which shows the switch between different modes of translation initiation. The concentration of eIF4G and ITAFs can play a major role in the decision for preferred mode of translation initiation.

1.2.3 CITE assisted translation initiation

Some recent studies have shown that cap-dependent mRNAs that do not possess RNA structural elements, i.e. IRESs in their 5'-UTRS continue to translate under unfavourable conditions, which dissemble or inactivate eIF4F complex. Various environmental stresses operate by the inactivation of eIF4F complex, i.e. activated 4E-binding proteins (4E-BPs) displace eIF4E from eIF4F complex, or the cleavage of eIF4G by proteases during viral infections. The use of bicistronic mRNA expression assays and transfection of cells with capped and uncapped reporter mRNAs constructs showed that uncapped mRNAs also followed
scanning mechanism, which can be considered as a rediscovery of a mechanism reported earlier in 1990 in plants (Gunnery et al. 1997).

These mRNAs consist of specialized sequences in their 5' and 3' UTRs. As these sequences enhance translation initiation, they are known as Cap-Independent Translation Enhancers (CITE), which can interact with eIF4G. The mechanism for CITE located in UTRs is cap-independent but depends on the 5'-end of mRNA. The presence of CITE-like elements in the mRNAs can provide them additional advantage under unfavourable translation conditions, which inhibit the cap-dependent translation initiation. Finding of some other studies also provide evidence in the support of idea of CITE sequences in UTRs (Dreher and Miller 2006, Miller et al. 2007). Based on studies presented in this review, different modes suggested for translation initiation in eukaryotes are shown in Figure 1.3.

1.2.4 Translation elongation

Translation elongation is the actual synthesis of polypeptide and ensures the processive addition of amino acids to the growing polypeptide. Machinery used for translation elongation is highly conserved across the three kingdoms of life suggesting that the mechanisms underlying elongation are the same in eukaryotes as they are in bacteria and archaea (Winterneyer et al. 2001, Schmitt et al. 2002, Kapp and Lorsch 2004).

Peptide chain elongation begins in the P-site of ribosomal subunit, which is bound to peptidyl Met-tRNA\textsuperscript{Met} next to a vacant A site. Elongation TC consisting of aminoacyl tRNA-GTP and elongation factor 1A (eEF1A; EF-Tu in bacteria) is carried to the A site. Several steps involving codon-anticodon base pairing, conformational changes in the small ribosomal subunit and GTP hydrolysis by eEF1A/EF-Tu ensures that only the correct tRNA is selected for entry into the next stage of elongation (Rodnina and Winterneyer 2001). Codon-anticodon base pairing induces three bases in the small ribosomal subunit to come out and interact with the resulting mRNA-tRNA duplex activating eEF1A/EF-Tu GTPase activity. eEF1A-GDP releases the aminoacyl tRNA into the A site.
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Figure 1.3. Three different mechanisms of eukaryotic translation initiation. A. Classical scanning model dependent on interaction of 5'-cap with eIF4E. B. IRES dependent translation initiation. C. 3'-CITE mediated translation that requires CITE and is 5' end dependent but independent of 5'-methylated cap.
The ribosomal peptidyl transferase centre then catalyses the formation of a peptide bond between the incoming amino acid and the peptidyl tRNA (Ogle et al. 2001, Moore and Steitz 2003). The complex is then translocated such that the deacylated tRNA moves to the E site, the peptidyl tRNA is completely in the P site and the mRNA is moved by three nucleotides to expose the next codon into the A site. This translocation is assisted by the elongation factor 2 (EF-G in bacteria), which hydrolyzes GTP while translocation. This cycle is repeated until a stop codon is encountered (Kapp and Lorsch 2004).

1.2.5 Translation termination

Translation termination occurs in response to the presence of a stop codon in the ribosomal A site. The peptidyl transferase centre of the ribosome is believed to catalyze the hydrolysis reaction. After the hydrolysis of ester bond between the peptide and the tRNA in P site, a nascent peptide chain is released. Translation termination is assisted by release factors. Class 1 release factors decode stop codons presented in the A site while Class 2 release factors are GTPases that stimulate the activity of class 1 release factors. In bacteria RF3 is the class 2 release factor, which stimulate the activity of RF1 and RF2, and this is also required to dissociate the peptidyl tRNA from ribosome (Arkov et al. 1998, Arkov et al. 2002).

In contrast to prokaryotic factors, eukaryotes have only one class 1 release factor (eRF1), which can promote the hydrolysis of peptidyl-tRNA in response to any of the three stop codons UAA, UAG, or UGA (Dontsova et al. 2000). Eukaryotes also possess a single class 2 release factor, eRF3. The role of eRF3 has yet to be verified experimentally. RF3 is dispensable in bacteria while eRF3 is an essential protein in eukaryotes. eRF1 and eRF3 bind to each other in the absence of the ribosome. This binding interaction is required for the optimum efficiency of termination in S. cerevisiae (Zavialov et al. 2002).

Followed by translation termination, recycling of ribosomal subunits takes place so that they can be reused for another round of translation initiation. Significant information of this process is available only for bacteria. At the end of the termination stage the ribosome is left on the mRNA with a deacylated tRNA.
In bacteria, this complex is recognized and disassembled by ribosome release factor (RRF). The disassembly of this complex is assisted by EF-G-GTP and IF3. GTP hydrolysis by EF-G is used to facilitate this dissociation. The factors and mechanisms involved in eukaryotes and archaea for ribosome recycling are less understood. In vitro experiments have shown that eIF1A, eIF3 and eIF6 all have ribosome anti association activity (Karimi et al. 1999). eIF3 is the prime candidate of the factors proposed to be involved in ribosome recycling in eukaryotes (Kapp and Lorsch 2004).

The closed-loop model of eukaryotic mRNAs suggests that termination and recycling may not release the 40S subunit back into the cytoplasm and 40S subunit may be sent back to the 5'-end of the mRNA via the 5'- and 3'-end-associated factors to facilitate another round of translation (Craig et al. 1998). This proposal was verified by the finding that eRF3 and PABP interacts with each other connecting the termination apparatus to the poly (A) tail (Hoshino et al. 1999).

1.3 Targets of translational control

The complexities attained by eukaryotes on the course of evolution by development of membranous organelles have provided cells with additional levels of control. Gene expression, which mediates the formation of proteome in cytoplasm from genome stored in nucleus, is regulated at multiple levels. Cells invest a substantial amount of their energy and space in carrying out two biosynthetic processes important for gene expression, i.e. transcription and translation. Compared to transcriptional control, translational control of mRNA allows for the rapid change in the concentration of the proteins (Sonenberg and Hinnebusch 2009). As translation is a multistep sequential process, the regulation is exercised at many levels of translational steps. It is not surprising that such an energy and resource demanding process is regulated at its initiation stage. This observation is in agreement to the logical principle that it is more efficient to control a pathway at its beginning than to interrupt it in midstream, which may have several consequences to deal with the resultant recyclable components and the accumulation of intermediates as by-products. Translation initiation is usually
rate limiting, most but not all translation controls operate at this phase of protein synthesis. Regulation is exerted through multi-step mechanism that target structural features of the mRNA, in prokaryotes i.e. primary and secondary structures of mRNA, the initiation codon, location and strength of ShD sequence. In eukaryotes, structural elements are distributed along the entire length of an mRNA. Efficiently translated mRNAs possess a 5'-cap structure, a proximal initiation codon and a poly (A) tail. mRNA efficiency is reduced by secondary structure in the 5'-UTRs and by the presence of upstream AUGs or upstream open reading frames (uORFs). The 3'-UTR is rich in cis-acting elements that determine mRNA stability and localization in the cytoplasm and serves to regulate translation initiation by making closed loop complex. 3' UTR also plays a role in CITE mediated translation initiation (Kapp and Lorsch 2004, Shatsky et al. 2010).

The factors that control translation will be discussed under two groups, i.e. cis- and trans-acting. How the activities of trans-acting factors can be regulated in response to environmental cues will also be covered. The role of miRNAs in regulation of translation will be discussed as well.

1.3.1 Cis-acting elements and translation regulation

cis-elements are the sequences or regions in DNA/RNA structure that can regulate the expression of gene located on the same molecular DNA/RNA molecule.

Importance of cis-acting element present in close proximity to the initiator codon has also been shown in prokaryotes. This downstream box (DB) is complementary to a sequence in the 16S rRNA and is thought to enhance initiation by stabilising the ShD-anti-ShD interaction. DB cannot initiate translation initiation if the ShD sequence is absent (Etchegaray and Inouye 1999).

Special features are added to eukaryotic mRNA before transferring it to cytoplasm for translation. These include addition of methylated guanosine cap at 5'-end and addition of a string of polyadenylated nucleotide (200-250 nt) at 3’-end. These features not only facilitate the transport of mRNA from nucleus to cytoplasm and prevent it from the catalytic activity of cytoplasmic 5’ and 3’-exonucleases. Each one of these is discussed in turn.
1.3.1.1 The 5'-7-Methylguanosine cap

Translational fate of mRNA depends on the structural features of the mRNA. The important structural feature in eukaryotic mRNA includes the 5'-end modification of mRNA by a cap structure. 5'-cap is formed by binding of an inverted guanosine to the first nucleotide of an mRNA through a phosphodiester bond. The 5'-cap is heavily methylated in higher eukaryotes as compared to yeast.

In general three types of methylation pattern exist on the guanosine residue, Cap0, Cap1 and Cap2. Cap0 has only one methyl group added at position 7 of inverted guanosine nucleotide. Methylation of adjacent nucleotides can occur and give rise to cap1 and cap2 (Figure 1.4). Cap1 consists of one additional methyl group at first nucleotide while cap 2 has two additional methyl groups at 2-position of ribose sugar of nucleotide downstream of RNA molecules. Cap is characteristic of the mRNAs transcribed by RNA polymerase II. Rate of translation initiation can be controlled by the eIF4F, cap binding complex. Many viruses target the eIF4F complex (in particular eIF4E and eIF4G) to inhibit the translation initiation of host mRNAs. 4E-BPs also targets the cap binding complex to inhibit translation initiation. 5'-cap can act as both a negative and positive regulator of translation initiation. Its role is governed by the availability of initiation factors involved in canonical translation initiation (Gingras 2009, Mitchell et al. 2010).

1.3.1.2 The untranslated regions (UTRs)

The region between the initiation codon and the 5' end of mRNA can also regulate the translational efficiency of mRNA. Unstructured 5' UTR (of varying length) can enhance the translational efficiency of mRNA by recruiting more 40S ribosomal subunits. The presence of poly (G) or upstream open reading frames (uORFs) have been shown to inhibit the translation of mRNA. The presence of the secondary structures determines whether the ribosome is merely slowed down or completely blocked at this point.

Secondary structures, which are thermodynamically less stable (A-U rich) are removed by initiation factors functioning as helicases during the scanning mechanism (Koloteva et al. 1997).
Figure 1.4. Structural features of eukaryotic mRNA.

A. A typical RNA polymerase II transcribed eukaryotic mRNA has 5'-cap and 3' poly(A) tail added to its ends before being exported to cytoplasm. Some mRNAs also have IRES and CITE in their 5'- and 3'-UTRs respectively, latter being also found in 5'-UTR as well. These sequences also have ability to recruits 40S initiation complex. B. 5'-m\textsuperscript{7}G-cap is added to mRNA in inverted conformation through 5'-5' phosphodiester linkage. Additional methyl groups can be added to ribose sugars to give cap1 and cap2.
The role of IRES (present in 5'-UTR) and CITE (present in 5'- and 3'-UTR) that are structured RNA sequences have also been discussed in sections 1.2.2 and 1.2.3. These sequences have the ability to recruit 40S initiation complex and initiate translation when the 5'-cap dependent translation is inhibited. Insulin treatment increases the protein synthesis. One reason for this is the activation of 5'-terminal oligopyrimidine tract mRNAs (TOP mRNAs). TOP sequences are present in 5'-UTR. TOP sequences provide mRNA advantage under unfavourable translation conditions. For example when rapamycin treatment blocks the translation of most mRNAs, the response of TOP mRNAs varies (high sensitivity to complete resistance) (Patursky-Polischuk et al. 2009).

Most known regulatory sequences are found in the 3'-UTR although translation initiation begins at the 5' end of mRNA (Gebauer and Hentze 2004). During translation initiation, eIF4G interacts with the poly(A)-binding proteins (PABPs) and circularizes the mRNA. This closed loop model provides a framework in, which the 3'-UTR binding factors can regulate, the translation initiation.

Ceruloplasmin mRNA has special sequences known as GAIT element in its 3'-UTR. Upon interferon-γ- treatment GAIT complex (Glu-Pro-tRNA-synthetase, NS-associated protein1, GAPDH and L13a) form, which interacts with GAIT element and blocks the formation of 48S initiation complex resulting in the translation repression of ceruloplasmin mRNA (Kapasi et al. 2007, Sonenberg and Hinnebusch 2009). Sex lethal (SXL) protein that binds the 3'-UTR of male-specific lethal 2 (msl-2) regulates the translation by recruiting UNR that blocks the 43S PIC binding to 5'-end of mRNA. Scanning ribosome in 5'-UTR is also a target of SXL (Sonenberg and Hinnebusch 2009).

1.3.1.3 Kozak consensus

To ensure the fidelity of translation, initiation complex has the ability to discriminate and bind only the first triplet AUG codon in an optimum context. The sequence preceding the start codon is called Kozak consensus. The Kozak sequence used in vertebrates is −GCC(A/G)CCAUGG (start codon is underlined). Relative to the A of AUG codon, which is designated +1, a purine at -3 and G at
the +4 are conserved and are important for initiation codon selection. Mutation at these sites reduces translation initiation (Kozak 1991).

Approximately 13% of yeast genes and up to 50% of human genes encode mRNAs with a short upstream ORF preceding the main protein coding ORF (Calvo et al. 2009). Ribosomes follow the AUG priority rule and starts translation at the first encountered AUG codon. Less than 50% ribosome reinitiate scanning and translation of downstream ORF (Jackson et al. 2010). The quantity of eIF2-TC determines the initiation of downstream ORF. Usually the ORFs have inhibitory effects on the downstream ORF. Regulation of GCN4 in yeast and ATF4 in mammals is well understood examples of regulation of translation by upstream ORFs in response to environmental cues and will be discussed in detail later in section 1.5.5.

1.3.2 Trans-acting factors and translation regulation

Trans-acting factors are molecules usually proteins that interact with the cis-acting elements in DNA/RNA molecules located at site away from their site of synthesis to regulate the gene expression. The trans-acting factors regulate translation by two mechanisms, either by effecting either scanning mechanisms (eIFs) or by directly binding to mRNA (miRNAs). The roles of trans-acting proteins, i.e. eIFs have been discussed earlier in section 1.2.1. Their mechanism of action depends by interaction with the regulatory sequences located in 5'- or 3'-UTRs (see section 1.3.1.2 for details). The role of miRNA in regulating translation will be briefly described in the following section.

1.3.2.1 Translational control by miRNAs

miRNAs are short (~22 nucleotide) non-coding, oligonucleotides (RNAs), which regulate the translation at post-transcriptional level. The translation repression is achieved by two mechanisms, translation inhibition by blocking the access of eIFs to mRNA and degradation of mRNA through normal deadenylation pathway. The relative importance of these mechanisms varies among different mRNA and miRNA complexes (Filipowicz et al. 2008). The exact mechanism of translation repression is still controversial. There are ~1000 miRNAs in human genome and
each miRNA could control ~10 mRNAs, so it is estimated that half of the human genome is controlled by the miRNAs (Sonenberg and Hinnebusch 2009).

*lin*-4 and *let*-7 were the first miRNAs to be discovered, which regulates the developmental timings in *Caenorhabditis elegans* (Carrington and Ambros 2003). Processed miRNAs are loaded to the target mRNA as part of a RNA induced silencing complex (RISC) and hybridize to multiple sites in 3’-UTR. The inhibition at initiation stage has been reported while according to other studies, inhibition happened at post initiation (Jackson et al. 2010). *In vitro* experiments show that miRNAs inhibit only cap-dependent translation initiation (Mathonnet et al. 2007). Some studies have also shown the roles of miRNAs as translation activators rather than inhibitors in a cell-cycle dependent manner (Vasudevan et al. 2007).

### 1.4 Control of initiation factors activity by phosphorylation

Phosphorylation of the initiation factors is one of the best established mechanisms to regulate eIFs and hence all-scanning dependent translation events. Phosphorylation either activates (eIF2B and eIF4E) or inhibits (eIF2) the activity of protein. The two well-studied examples regulated by reversible phosphorylation are the availability of active eIF2 and eIF4F. The role of eIF2 phosphorylation in response to stress is discussed later in detail in Section 1.5.

Phosphorylation affects the intracellular levels of eIF4F complex. The concentration of eIF4F complex depends on phosphorylation of the members of family of translation repressors, i.e. eIF4E-binding proteins (4E-BPs). There are three homologues of 4E-BPs in mammals, (4E-BP1, 4E-BP2 and 4E-BP3) (Jackson et al. 2010). The binding of 4E-BP to eIF4E is regulated by the phosphorylation. Hypophosphorylated 4E-BP competes with eIF4G to bind eIF4E. This binary complex (eIF4E-4E-BP) blocks eIF4E association with eIF4G and repress cap-dependent translation initiation. mTOR phosphorylates 4E-BP at multiple sites and hyperphosphorylated 4E-BPs do not bind to eIF4E, which favours the formation of eIF4F. (Pyronnet et al. 1999, Morino et al. 2000, Svitkin et al. 2005). eIF4E is itself a phospho-protein. It is phosphorylated at Ser-209 by...
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MAP kinase interacting Ser/Thr kinase 1 and 2 (MNK1 and MNK2). MNK1 binds c-terminus of eIF4G and phosphorylates the adjacent eIF4E bound to it. Hyperphosphorylation of eIF4E can promote malignancy in mice (Wendel et al. 2007).

The phosphorylation of many other eIFs (eIF1, eIF4B, eIF4G, eIF5 and eIF5B) has also been observed and linked to translational activation. Three phosphorylation sites (Ser-1148, 1188 and 1232) have been identified in hinge region of eIF4G1, which are regulated by mTOR signalling in response to hormonal/ mitogenic stimulation. eIF4B has no catalytic activity but instead enhances the affinity of eIF4A for ATP and mRNA. eIF4B is required for the formation of 48S translation initiation complex and hyperphosphorylation of eIF4B in response to mitogens and esters results in increased rates of mRNAs translation.

eIF3 is a large multi-subunit complex consisting of at least 12 proteins in mammals. eIF3 was one of the first translation factors that were observed to be regulated by phosphorylation in response to insulin. Large scale phosphoproteomic studies have indicated that eIF3a, b, c, f, g and i subunits are phosphorylated under various conditions in vivo (Mayeur et al. 2003, Raught and Gringras 2007).

1.5 eIF2: function, structure and control

eIF2 is a member of G-family of proteins. It is functionally similar to the small GTPases and exchange factors of the RAS super family, but structurally it is complex. eIF2 consists of two smaller subunits α, β and a larger γ subunit, which in S. cerevisiae are encoded by SUI2, SUI3 (SUI-suppressors of initiation) and GCD11 (GCD-general control derepressed) respectively (Table 1.2).

1.5.1 Localization of eIF2

eIF2 co-localizes with its GEF (eIF2B) to a discrete cytoplasmic foci in cytoplasm in yeast. While eIF2B is a host of this focus, eIF2 shuttles at a very rapid rate between cytoplasm and the eIF2B concentrated foci (Campbell and Ashe 2006,
Taylor et al. 2010). In a previous study done in Hela cells, association was found between the translation machinery, eIF1, eIF2, eIF-4A, eIF-4B and cytoskeleton framework (Howe and Hershey 1984).

1.5.2 Role of eIF2

eIF2 functions to deliver Met-tRNA\textsubscript{Met} to 40S subunit in the form of TC (eIF2-GTP-Met-tRNA\textsubscript{Met}). Met-tRNA\textsubscript{Met} has greater affinity for eIF2-GTP than for eIF2-GDP. Methionine contributes thirteen fold to the binding of Met-tRNA\textsubscript{Met} to eIF2 (Kapp and Lorsch 2004, Shin et al. 2011). The high affinity of methionine for GTP and the role of unique sequence of acceptor stem, i.e. A1:U72 base pair (bp) in binding to eIF2 ensures the fidelity of initiation process and prevents the binding of elongator Met-tRNA to P-site of 40S complex. The binding of Met-tRNA\textsubscript{Met} to eIF2 has not been characterized as crystal or cryo-electron microscopy (cryo-EM) structure of eIF2 is unknown. The crystal structure of archael orthologs and various aIF2 sub-complexes bound to GDP or GTP analogs have been solved. The ribosome contacting surfaces for aIF2 have not been identified.

1.5.3 Structure of eIF2

eIF2 is a complex of three different subunits with a total molecular weight of ~125 kDa. The domain structure of three subunits of eIF2 is shown in Figure 1.5.

1.5.3.1 eIF2\textalpha

eIF2\textalpha is the smallest subunit of eIF2. This 36 kDa protein is critical for regulation of eIF2B activity in response to cellular cues and starvation. It is dispensable in yeast if eIF2\textbeta, \gamma and Met-tRNA\textsubscript{Met} are overexpressed (Erickson et al. 2001). The three dimensional structure for N-terminal portion of human and yeast eIF2\textalpha by x-ray diffraction has been solved. It shows the presence of two major domain, oligonucleotides-binding fold (OB-fold) and a helical domain (Nonato et al. 2002). The best characterized mechanism of translational control in eukaryotic cells involves phosphorylation of eIF2 in response to a wide variety of stresses. The recycling of inactive eIF2-GDP to active eIF2-GTP is catalyzed by eIF2B and this reaction is regulated by phosphorylation of eIF2\textalpha at Ser-51. Phosphorylation
of Ser-51 in eIF2α converts eIF2 from a substrate to a competitive inhibitor of its nucleotide exchange factor. eIF2α(p) has a ~150 fold higher affinity for eIF2B than in its unphosphorylated form and this increased affinity reduces the activity of nucleotide-exchange factor. As the amount of eIF2B is limiting compared to the amount of eIF2, phosphorylation of a small percentage of eIF2α(p) results in the increase formation of eIF2-eIF2B inactive complexes, which results in the inhibition of protein synthesis (Pavitt 2005, Smirnova et al. 2005).

1.5.3.2 eIF2β

eIF2β is a 38 kDa protein with three distinct domains. The amino terminal domain (NTD) consists of three lysine rich boxes known as the K-boxes. The NTD contributes to the binding of eIF2β to eIF5 and eIF2B (Asano et al. 1999). The central region interacts with eIF2γ, and the CTD contains a Cys2-Cys2 zinc finger motif and is required for RNA-binding activity. Several mutations in and around C-terminal zinc finger domain allow initiation at UUG codons suggesting its role in the start codon selection (Gutierrez et al. 2004). In the absence of eIF2β, eIF2α and γ cannot bind Met-tRNAiMet to form TC complex (Laurino et al. 1999). The affinity of eIF2γ for Met-tRNAiMet increases by ~ 100 fold in the presence of α and β subunits (Raught and Gringras 2007, Naveau et al. 2010).

1.5.3.3 eIF2γ

eIF2γ is the largest (52 kDa) and perhaps the best characterized subunit of eIF2. It was identified as a negative regulator of GCN4 mRNA. eIF2γ contains three domains, including a G domain, which is typical of the small RAS-like G protein family. A variety of biochemical and genetic experiments indicate that eIF2γ is the binding site for both GTP and Met-tRNAiMet. The γ subunit is homologous to the elongation factor EF-Tu, which is also a GTP-dependent carrier of aminoacylated tRNAs. EF-Tu binds to every aminoacylated elongator tRNA while eIF2γ is specific for binding to Met-tRNAi. There are tRNA identity elements that ensure the binding of initiator eIF2 not eEF1A. It has been shown that in addition to making contacts with eIF2α and β, this makes contacts with eIF2Be and eIF5 (Alone and Dever 2006, Shin et al. 2011).
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1.5.4 eIF2 kinases

Global and mRNA specific translation are the two principal modes of translational control. Many signalling pathways are now known by which these mechanisms operate. Phosphorylation of eIF2 represses global translation. Many stress signals regulate eIF2B activity by phosphorylating eIF2α at Ser-51. Several different protein kinases are activated in response to diverse stresses. Four eIF2α kinases have been characterized in mammals: (i) Heme Regulated Inhibitor of translation (HRI), (ii) Protein Kinase double stranded RNA dependent (PKR), (iii) PKR-like ER (endoplasmic reticulum) kinase (PERK), also called pancreatic eIF2 kinase (PEK) and finally (iv) General Control Non-Depressible 2 (GCN2), as all kinases phosphorylate eIF2α, it is termed integrated stress response (Donnelly et al. 2013, Trinh and Klann 2013). Yeast has only one kinase, i.e. Gcn2p. These kinases become active in response to stress and phosphorylate eIF2, resulting in reduction
of TC levels (Donnelly et al. 2013). Domain structure of eIF2α kinases and their mechanisms of activation are shown in Figure 1.6.

### 1.5.4.1 Heme regulated inhibitor of translation (HRI)

HRI kinase (EIF2K1) is the principal eIF2α kinase in erythroid cells and is activated under conditions of heme deprivation and stresses as diverse as proteasome inhibition and arsenite exposure. The primary function of HRI is to co-ordinate globin synthesis with available iron by inhibiting protein synthesis when heme is insufficient.

Mature HRI is a homodimer. Each monomer is composed of five domains. Its kinase activity is inhibited when it dimerizes through disulfide bonds between two monosubunits (Lu et al. 2001). Binding of heme to the dimerised HRI at the NTD inactivates HRI. It is reported that in the presence of heme, the kinase domain interacts with the amino terminal domain and this interaction is disrupted in conditions of heme deficiency that activate the kinase by releasing heme (Figure 1.6) (Yun et al. 2005). HRI is present in other cell types as well and has been reported to respond to other stress conditions including heat shock, osmotic stress, nitric oxide, aresnite and cadmium exposure. HRI is not activated by endoplasmic reticulum stress or nutrient starvation (McEwen et al. 2005). Activation of HRI inhibits global protein translation (reviewed in (Pavitt 2005).

### 1.5.4.2 Protein kinase double-stranded RNA-dependent (PKR)

PKR (or EIF2AK2) is thought to be present in all vertebrates. Expression of PKR is induced by interferon, oxidative stress, ER stress and dsRNA molecules. It is also involved in obesity and cancer (Donnelly et al. 2013).

With regard to structure and mechanism of activation, PKR is the best characterized eIF2α kinase. PKR enzyme has a molecular mass of about 68 kDa. PKR possesses two dsRNA-binding motifs (dsRBM1 & dsRBM2), which are linked to the C-terminal protein kinase domain by Ser/Thr spacer. It has been proposed that the dsRBMs of PKR prevent kinase activity in the absence of dsRNA and promote dimerization and kinase activation in the presence of dsRNA molecules. RNA binding to PKR results in the activation of PKR by autophosphorylation using ATP. Low concentrations of dsRNA activate PKR
while its activity is inhibited by higher concentrations of dsRNA molecules (Raught and Gringras 2007).

1.5.4.3 **PKR like endoplasmic reticulum (ER) kinase (PERK)**

PERK also called PEK (Pancreatic eIF2 Kinase) was discovered as a protein sharing sequence homology with the ER stress-responsive IRE1 kinase and as a pancreatic kinase. PERK is an ER transmembrane protein and consists of three domains: (i) ER luminal domain, (ii) a transmembrane domains and (iii) a cytoplasmic eIF2 kinase domain. Like IRE1, PERK is activated under ER stress conditions (higher concentration of unfolded proteins) limiting translation of ER-destined proteins while signals are sent to the nucleus for synthesis of ER chaperones e.g. BiP. In unstressed cells, PERK interacts with the ER chaperones (GRP78) and (GRP94) through its amino terminal regulatory region. Under the ER stress condition, unfolded proteins accumulate in the ER, and the chaperones dissociate from PERK, enabling kinase dimerization and activation (Bertolotti et al. 2000, Donnelly et al. 2013).

1.5.4.4 **General control of non-derepressible-2 (Gcn2p)**

Gcn2p (or EIF2AK4) is present in all eukaryotes including fungi, plants, invertebrates and vertebrates including mammals. Gcn2p, first identified in yeast is activated upon binding to uncharged tRNAs that accumulate in cells starved for any single amino acid. The activated Gcn2p phosphorylates eIF2α at Ser-51 (Hinnebusch 2005). Inactive eIF2-GDP complex produced by the hydrolysis of eIF2-GTP in TC during translation initiation is recycled to active eIF2-GTP by eIF2B. eIF2 is present in considerable excess of eIF2B, and in its phosphorylated state it binds to eIF2B with greater affinity and decreases the dissociation of eIF2B-eIF2(αP)-GDP complex. Small proportion of eIF2 is adequate to sequester all of the eIF2B in inactive complexes. It has been shown that eIF2α(p) has a greater association rate with eIF2B than does non-phosphorylated eIF2. This strong binding interferes with the recycling of non-phosphorylated eIF2. It is also found that the ratio of phosphorylated to non-phosphorylated eIF2 is more important than the absolute amount of eIF2α(p) in determining the extent of eIF2B inhibition (Pavitt 2005).
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Figure 1.6. Schematic representation of the domain structure of eIF2α kinases and their mechanism of activation.

i. PKR structure and mechanism
dsRNA binding motifs and a kinase domain. dsRNA binding induces conformational changes and dimerization, which leads to activation by autophosphorylation.

ii. HRI structure and mechanism
Heme binding domain and a catalytic domain. Inactive HRI is a dimer held by non-covalent interactions. Heme deficiency results in the autophosphorylation and activation of dimerized HRI.

iii. PERK structure and mechanism
In case of ER stress oligomerization of kinase domain takes place and PERK is activated by transautophosphorylation.

iv. Gcn2p structure and mechanism
Gcn2p has Yih1p, ψPK, PK, HisRS and CTD domains. Uncharged tRNAs bind Gcn2p and introduces conformational changes, which results in the activation of Gcn2p by autophosphorylation.
Gcn2p is thought to be a constitutive dimer. The Gcn2p structure is complex consisting of (i) N-terminal charged region, that binds Gcn1/Gcn20p complex, (ii) pseudokinase domain (iii) an eIF2 kinase domain; and (iv) a domain resembling histidyl tRNA synthetase (HisRS), and (v) a CTD that enhances tRNA binding upon dimerization and mediates ribosome binding in cell extracts (Dong et al. 2000).

It has been proposed that Gcn2p is maintained in an off state by inter-domain interactions in the absence of starvation condition. Under starvation conditions, uncharged tRNAs bind to HisRS (+CTD) regions, resulting in the release of protein kinase domain. Once the kinase domain is free, it undergoes a conformational change and Gcn2p is activated by auto-phosphorylation. This structural change allows Gcn2p to phosphorylate eIF2α at Ser-51 (Dong et al. 2000). Gcn2 can also be activated by dephosphorylation at Ser577 in response to rapamycin mediated TOR-signalling by TAP42, regulator of type 2A-related protein phosphatases (Cherkasova and Hinnebusch 2003).

Gcn2p is activated in yeast cells in response to different stresses e.g. amino acid starvation conditions, purine nucleotide limitation, high salinity, glucose limitation, rapamycin and methyl methane sulfonate (MMS, and in mammalian cells by ultraviolet (UV) light (Deng et al. 2002, Pavitt 2005).

Another role for Gcn2p has been reported related to food selection in rodents. Gcn2p is required in the anterior piriform cortex of the mouse brain to be aware of amino acid shortage in the diet and reduce intake of the deficient food. Thus, the same mechanism employed for sensing and adjusting to amino acid limitation in yeast cells functions in mammalian brain cells to guide food selection (Hinnebusch 2005). In the past few years, the role of Gcn2p in modulating life span in yeast and immune responses has been identified (Murguia and Serrano 2012). Substrates other than eIF2α have been identified that mediate Gcn2p impact on cell responses. The effects of DNA damaging reagents and ultraviolet radiation activates Gcn2 but independent of eIF2α(p) (Murguia and Serrano 2012).
Figure 1.7. Regulation of translation by eIF2α phosphorylation.
In response to diverse environmental signals, HRI, PKR, PERK and Gcn2p in mammals are activated, which cause phosphorylation of eIF2α at Ser-51. The consequences of this phosphorylation are low levels of TC due to reduced eIF2B GEF activity, which causes inhibition of general protein synthesis with simultaneous increase in the translation of transcriptional activator GCN4 in yeast and ATF4 in mammals. Green arrow denotes activation while Red blocked line indicates inhibition.
1.5.5 \textit{eIF2\(\alpha\)(p) and expression of GCN4}

The phosphorylation of eIF2\(\alpha\) by Gcn2p in response to diverse stress condition inhibits the global translation initiation but at the same time it up-regulates the translation of specific mRNAs. The two best characterized transcription factors, which are preferentially up-regulated in response to amino acid starvation are \textit{GCN4} (in yeast) and \textit{ATF4} (in mammals).

\textit{GCN4} encodes a transcriptional activator of almost all amino acid biosynthetic genes. \textit{GCN4} mRNA has four short upstream open reading frames that regulate the expression of the main gene downstream of these ORFs. Under optimum growth conditions (when there is surplus supply of TC), the scanning ribosome recognises the first ORF, translates it, continues scanning and scans all ORFs (1-4). Scanning complex dissociates from mRNAs without translating the main \textit{GCN4} gene. Insufficient supply of amino acids activates Gcn2, which reduces the recycling of eIF2-GDP complex by inhibiting a reaction catalysed by eIF2B. This results in the lower TC in cells. In this case, ribosomes scan first ORF, resume scanning, bypass the downstream ORFs (2-4) without any TC complex and reinitiate translation at downstream \textit{GCN4} ORF. If TC in acquired in the uORF4 to \textit{GCN4} interval, translation of \textit{GCN4} proceeds. (Figure 1.8). Starvation conditions increase the translation of \textit{GCN4} to \(\sim 10\) fold.

\textit{eIF2\(\alpha\)} phosphorylation in mammalian cells induces the translation of \textit{ATF4}-transcription factor. \textit{ATF4} is required for the induction of additional transcriptional factor, i.e. \textit{ATF3} and \textit{ATF5}. \textit{ATF4} is a homodimer but regulates the expression of target genes as heterodimer with NRF2 and \textit{ATF3}. The induction of \textit{ATF4} translation occurs by essentially the same reinitiation mechanism established for \textit{GCN4}. In addition to translational regulation, the level of \textit{ATF4} is regulated by exerting control at transcriptional and post-translational modification (Donnelly et al. 2013).

\textit{ATF4} has two uORFs in its mRNA leader sequence and the genetic studies show that \textit{ATF4} is translated by ribosomes that have earlier translated uORF1. Ribosomes resume scanning, bypass the latter uORF2 without any TC complex and reinitiate translation at downstream \textit{ATF4} ORF. The only significant
difference noted thus far is that elongation of *ATF4 uORF1* did not damage its positive function whereas such mutations inactivate *GCN4 uORF1* (Hinnebusch 2005). Numbers of translation initiation factors are identified by the effects of mutation they have on *GCN4* pathway. These are divided into two categories, Gcd’ (general control non derepressed, induction of *GCN4* even in the absence of starvation) and Gcn’ (General Control Non-Derepressible), cells fail to induce *GCN4* in starvation condition.

### 1.6 Target of Rapamycin pathway

Rapamycin was isolated from *Streptomyces hygroscopicus*, present in the soil sample collected from Easter island, Rapa-Nui in local language, in 1970's (Heitman et al. 1991). Rapamycin, a lipophilic macrocyclic lactone, is a natural secondary metabolite and was found to posses antifungal properties. Its use as an antifungal drug was discarded because of its side effects as immunosuppressant. Years later, it was rediscovered for the treatment of allograft rejection because of its inhibitory effects on mammalian T-cell proliferation. Rapamycin and its derivatives received clinical approval in 1999 (Loewith and Hall 2011).

The target of rapamycin, (TOR) is a central controller of cell growth and was identified by dominant mutations, *TOR1-1* and *TOR2-1*, which conferred resistant phenotype to rapamycin. Two isoforms were identified in *S. cerevisiae* (Heitman et al. 1991). Disruption of *TOR1* and *TOR2* results in the same growth defects as seen with rapamycin treatment suggesting that TOR is target of rapamycin-FKBP12 complex (Kunz et al. 1993).

TOR is conserved in all eukaryotes. Unlike yeast, which contains two *TOR* genes, all higher eukaryotes possess only one gene (Crespo and Hall 2002). Here the TOR complexes in yeast are discussed and evidences from mammalian are only included when it enhances the understanding of mechanism.
A. Structure of *GCN4*

![Structure of GCN4](image)

B. Non-starvation conditions—High levels of TC

![Model for the mechanism of GCN4 translational control](image)

C. Starvation conditions—Low levels of TC

![Model for the mechanism of GCN4 translational control](image)

**Figure 1.8. Model for the mechanism of *GCN4* translational control.**

A. *GCN4* mRNA open reading frame is preceded by 4 uORFs. B. Under non-starvation conditions, high levels of TC are available, the 40S subunit quickly rebinds the TC and reinitiates at uORFs 2, 3 and 4 leaving *GCN4* untranslated. C. TC levels are reduced in starved cells owing to phosphorylation of eIF2 by Gcn2p and the scanning 40S ribosome fail to rebind the TC until scanning past uORF4 and reinitiates at *GCN4* instead.
1.6.1 Structure of TOR

TOR1 and TOR2 are Ser/Thr specific kinases and members of Phosphatidylinositol kinase-related kinase (PIKK) family (Keith and Schreiber 1995). Although no member of PIKK family has lipid kinase activity but the catalytic domain of PIKK family members resembles the catalytic domain of lipid kinases, i.e. PI3Ks and PI4Ks (Wullschleger et al. 2006).

Eukaryote TORs are ~280 kDa proteins and 67% identical in their primary sequence. TOR is a central component of two distinct complexes in yeast TORC1 and TORC2) involved in two different signalling branches. All TORs contain similar domains. TOR consists of following domains arranged in order from N to C terminus, HEAT repeats, FAT domain, FRB domain, kinase domain and the FATC domain (Schmelzle and Hall 2000) (Figure 1.9).

Almost 20 tandem HEAT (Huntington, elongation factor 3, A subunit of PP2A and TOR1) repeats each composed of ~40 residues make the amino terminal half of TOR. HEAT repeats forms a pair of extended antiparallel α-helices and are the binding surface for protein-protein interaction (Wullschleger et al. 2005). The central FAT (FRAP, ATM, TTRAP) domain (~500) residues and the carboxy terminal FATC domain (~35 residues) are present in all members of PIKK family members and are always paired, this suggest that they may interact (Bosotti et al. 2000).

The FRB (FKBP-rapamycin binding region) domain consists of ~100 residues and the phenotypic resistant conferring mutations (Ser1972Arg/ Asn in TOR1 or Ser1975Ile in TOR2) to rapamycin are mapped in this region. FKBP12 is a cofactor that recruits rapamycin to FRB. FKBP12 is non-essential and is 54% identical to human FKBP12 (Heitman et al. 1991, Helliwell et al. 1994).

1.6.2 TOR complexes

Genetic and biochemical studies in yeast identified TOR as part of two distinct complexes and these show differential sensitivity to rapamycin (Loewith et al. 2002). TOR regulated processes can be divided into temporal and spatial control
of cell growth. Temporal control refers to TOR-regulated translation, ribosome biogenesis, autophagy, transcription and processes that determine the cell mass accumulation in response to change in nutrients conditions. Spatial response is cell cycle dependent regulation of the actin cytoskeleton, which establishes the cell polarity. The basics of functional diversity lie in the structural composition of TOR complexes. A brief description of the composition, location and functions of two complexes is discussed in next section.

1.6.3 Rapamycin sensitive TORC1

Gel filtration chromatography shows that whole TORC1 is dimeric like TORC2 (Wullschleger et al. 2005). Yeast TORC1 consists of KOG1, LST8, TSCO89 and either TOR1 or TOR2 (Figure 1.9). The structural components of TORC1 and their mammalian orthologs and salient features of proteins are summarized in Table 1.4.

Table 1.4. Components of TORC1 and TORC2 in yeast and human

<table>
<thead>
<tr>
<th>Complex</th>
<th>S. Cerevisiae</th>
<th>Mammals</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>TORC1</td>
<td>Tor1, Tor2 Lst8 Kog1 Tco89</td>
<td>mTOR mLST8/GβL Raptor</td>
<td>Protein kinase Stabilize kinase domain</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tor1 Tor2 Lst8 Vir7 Tco89 Kog1</td>
<td>Raptor mLST8/GβL</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tor2 Lst8</td>
<td>Raptor mLST8/GβL</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rictor/mAVO3</td>
<td>PRAS40/DEPTOR</td>
</tr>
<tr>
<td>TORC2</td>
<td>Avo1 Avo2 Avo3 Bit61 ---</td>
<td>mTOR mLST8/GβL mSIN1</td>
<td>Protein kinase Stabilize Kinase domain</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tor2 Lst8 Avo1 Avo2 Avo3 Bit61 ---</td>
<td>Rictor/mAVO3 PRR5/Protor</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tor2 Lst8 Avo1 Avo2 Avo3 Bit61 ---</td>
<td>Rictor/mAVO3 PRR5/Protor</td>
</tr>
</tbody>
</table>
1. Introduction

Regulation of eIF2B by Phosphorylation

A. TOR

![Conserved domain structure of TOR kinase and TOR complexes in yeast.](image)

B. TORC1

C. TORC2

Figure 1.9. Conserved domain structure of TOR kinase and TOR complexes in yeast.

A. Conserved domain in the TOR kinase. B. Composition of TORC1. TORC1 is ~2MDa in size and exists as a dimer of multimeric complex. C. Composition of TORC2. Dimer of a multimeric complexes consisting of TOR, AVo1, 2, 3 and Bit6
1.6.3.1 Localization of TORC1 in yeast

Immunogold electron microscopy, GFP tagging and cellular fractionation studies show that TORC1 is mainly found at the vacuolar membrane and this localization is constitutive (Loewith and Hall 2011). There are possibilities that a fraction of TORC1 can be present elsewhere in cells, i.e. TORC1 associates with the rDNA and regulates rRNA transcription (Li et al. 2006).

1.6.3.2 Upstream of TORC1

TORC1 couples environmental signals to the cell growth. The Rapamycin treatment and nutrient starvation induces the same affects in cell physiology. For example, inhibition of protein synthesis, activation of autophagy and cells enter in the G0 state (Barbet et al. 1996). Other factors, which can affect TORC1 are rapamycin, starvation of nitrogen, carbon, phosphate, high salt, low energy, redox stress, shift to high temp and caffeine (Wullschleger et al. 2006, Loewith and Hall 2011).

Screening of cells that could not recover from the G0 state led to the identification of escape from rapamycin induced growth arrest (EGO) complex that regulates TORC1 in yeast. EGO complex is composed of four proteins: Ego1, 3 and Gtr1, 2. Gtr1 and 2 are Ras-family GTPases while Ego1, 2 are homologs of vertebrae p18 and p14 and together work as regulator complex. The complex is present on the vacuolar membrane in complex with TORC1. Amino acid starvation leads to the destabilization of this complex leading to dephosphorylation of Sch9 (Binda et al. 2009). Upstream and downstream components of TORC1 signalling are shown in Figure 1.10.

1.6.3.3 Proximal effectors of TORC1

When growth conditions are optimal, TORC1 is active and regulates the cell growth. Two well known substrates are characterized in mammals S6K1 and 4E-BPs whose activation is regulated by mTORC1. The regulation of growth in yeast is achieved by the activation of cascade involving kinases and phosphatases. The direct proximal effectors (kinase and phosphatase) in yeast are discussed in brief here (Figure 1.10).
1.6.3.4 Sch9 kinase as TORC1 substrate

AGC family kinases are the best characterized substrates of TORC1 in yeast and metazoans. AGC family kinases consist of two conserved regulatory motifs, activation loop and hydrophobic motif in addition to turn motif located in the kinase domain and hydrophobic domain. The phosphorylation of these motifs activates the kinase domain. TORC1 mediates the phosphorylation of hydrophobic motif (Pearce et al. 2010). Sch9 analogous to the S6K for mTORC1 was found to be the TORC1 direct substrate (Powers 2007). TORC1 phosphorylated six sites in the C-terminus of Sch9. This phosphorylation is essential for the activation of Sch9 as Ser to Ala mutation at these sites lead to non-functional Sch9 (Powers 2007, Urban et al. 2007). The over simplified view of distal readouts of TORC1 is shown in Figure 1.11.

1.6.3.5 Tap42-PP2A phosphatase as TORC1 substrate

In addition to the Sch9 kinase, TORC1 also regulates PP2Ac (type 2A Phh21, Pph22 and Pph3) and 2A related phosphatases (Sit4, Ppg1). The role of these phosphatases as downstream targets of TORC1 was first described in the late 1990's (DiComo and Arndt 1996). These enzymes interact with Tap42 protein present in a complex with Rrd1 and Rrd2 in a TORC1 dependent manner. Both Rrd1 and Rrd2 confer phosphotyrosyl phosphatase activity to the catalytic phosphatase subunits. Active TORC1 phosphorylates Tap42. Phosphorylated Tap42 binds tightly to the Rrd- phosphatases complex. The exact mechanism of the phosphorylation of Tap42 is unclear (Jiang and Broach 1999). Tap42-PPase regulates the protein synthesis by the activation of Gcn2p. Rapamycin treatment causes dephosphorylation of Gcn2p at Ser-577 and this results in the phosphorylation of eIF2α, which causes inhibition of global protein synthesis (Cherkasova and Hinnebusch 2003).

Translation related factors (Sfp1, Eap1, eIF4G ) and many other proteins involved in diverse cell signalling networks have been identified in recent phosphoproteomics studies, which interact with TORC1 (Berset et al. 1998, Breitkreutz et al. 2010).
Figure 1.10. TORC1 signalling pathway in yeast.
Activation of TORC1 by nutrients results in the cell growth by stimulation of protein synthesis and inhibition of autophagy and several other pathways that inhibit growth on nitrogen poor sources. These processes are mostly regulated by Tap42-Sit4/PP2Ac or Sch9. Arrows and bars represent positive and negative interaction respectively.
1.6.4 Rapamycin insensitive TORC2

Yeast TORC2 is insensitive to rapamycin and exists as a dimer of a multimer complex consisting of Tor2, Avo1, Avo2, Avo3, Lst8 and Bit61 (Loewith et al. 2002). TORC2 is localized at the plasma membrane. Salient features of TORC2 are summarized in Table 1.4.

1.6.4.1 Localization of TORC2

Tor2p is component of both TORC1 and TORC2 complexes. The 90% of Tor2p is present in TORC2 as compared to TORC1 consisting of only 10%. The studies carried out to find the localization of Tor2p detect mostly TORC2. Sub-cellular fractionation, GFP-tagged TORC2 and immunogold electron microscopy studies indicate that TORC2 is present at plasma membrane domain termed MCT (membrane compartment containing TORC2) (Kunz et al. 2000, Aronova et al.
2007, Loewith and Hall 2011). Various ill-defined cellular locations, e.g. cytoplasm and cell interior with membrane tracks have also been reported.

1.6.4.2 Upstream of TORC2

Several studies indicate the regulation of TORC1 by nutrients, where as upstream and downstream effectors of TORC2 are poorly characterized. Reverse suppressor screen was done to identify upstream suppressors of TORC2 and Nip7, a ribosome maturation factor was found to be required for TORC2 activity. The role of Nip7 in TORC2 activation is poorly understood in yeast but studies in mammalian cells show that Nip7 activates mTORC2 indirectly (Zinzalla et al. 2011).

Some studies also suggest the inhibition of TORC2 by environmental stress. The mechanism of regulation of TORC2 under unfavourable conditions is poorly defined but suggests that it may require the role of Slm1 and calcineurin: a stress-activated phosphatase (Bultynck et al. 2006, Mulet et al. 2006).

1.6.4.3 Proximal effectors of TORC2

Some members of the AGC protein kinase family are reported to be the substrates of TORC2 in yeast and mammals. mTORC2 phosphorylates Akt/PKB at one out of two sites. Thr308 present in the activation loop is phosphorylated by PDK1 while Ser473 present in hydrophobic motif is the target site of mTORC2 kinase. Akt is upstream of mTORC1. Akt/PKB is involved in regulation of transcription, metabolism and cell proliferation (Sarbassov et al. 2005).

The major known substrate of TORC2 in yeast is the protein kinase Ypk. Ypk1 and 2 are members of AGC kinase family. In vitro kinase assays with immunopurified TORC2 shows that Ypk2 is phosphorylated at two sites by TORC2. SGK1 and Gad8 mammalian are S. pombe orthologs of Ypk2 respectively and are also substrates of TORC2 (Kamada et al. 2005). In vivo and in vitro experiments show that Slm1 and 2 are substrates of TORC2 (Fadri et al. 2005). The TORC2 readouts are shown in Figure 1.12.
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Figure 1.12. Distal readouts of TORC2.
TORC2 phosphorylates the AGC kinases (Ypk1 and Ypk2) and Slm proteins. TORC2 controls spatial aspects of growth by organization of actin cytoskeleton. The role of TORC2 is endocytosis, sphingolipids biosynthesis and transcription of stress related genes is not fully understood.

1.7 eIF2B-GEF for eIF2

In the course of translation, eIF2-GTP in TC is hydrolyzed to GDP and eIF2-GDP complex is produced. To participate in next round of initiation, it must be recycled to active eIF2-GTP form. At physiological concentration of Mg$^{++}$, the rate of dissociation of GDP from eIF2 is very slow. This calls for a molecule that can catalyze this reaction. In eukaryotes this reaction is catalysed by guanine nucleotide exchange factor (GEF) eIF2B (Gomez and Pavitt 2000). This step is one of the key controlled steps in translation initiation that regulates protein synthesis. Other than eIF2α(p) in response to stress conditions, this reaction is also negatively regulated by eIF5, which acts as GDP dissociation inhibitor (GDI) (Jennings and Pavitt 2010). In the next sections structure, mechanism of action of eIF2B, regulation of eIF2B, kinases for eIF2B known in mammals and its role in CACH disease are discussed.
Table 1.5. eIF2B sub-unit composition with percentage identity to human EIF2B

<table>
<thead>
<tr>
<th>Sub-unit</th>
<th>Mol. weight Da</th>
<th>Yeast Gene</th>
<th>Human Gene</th>
<th>Sequence identity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>eIF2Bε</td>
<td>81, 160</td>
<td>GCD6</td>
<td>EIF2B5</td>
<td>30</td>
</tr>
<tr>
<td>eIF2Bγ</td>
<td>65, 699</td>
<td>GCD1</td>
<td>EIF2B3</td>
<td>25</td>
</tr>
<tr>
<td>eIF2Bδ</td>
<td>70, 852</td>
<td>GCD2</td>
<td>EIF2B4</td>
<td>36</td>
</tr>
<tr>
<td>eIF2Bβ</td>
<td>42, 569</td>
<td>GCD7</td>
<td>EIF2B2</td>
<td>36</td>
</tr>
<tr>
<td>eIF2Bα</td>
<td>34, 025</td>
<td>GCN3</td>
<td>EIF2B1</td>
<td>42</td>
</tr>
</tbody>
</table>

Modified and adapted from (Pavitt 2005).

1.7.1 Localization of eIF2B

According to the classical view of cytoplasmic GEFs involved in translation, the activity of GEFs is not related to the localization in cell and therefore they displayed different cytoplasmic localizations (Campbell and Ashe 2006). Recent studies in yeast have shown that pool of eIF2B occupies large cytoplasmic foci, termed 2B body. The dynamics and movement of this body is affected in response to environmental stresses, e.g. 1-butanol (Taylor et al. 2010).

1.7.2 Subunit structure of eIF2B

Initial biochemical and genetic studies on translational control of GCN4 identified yeast eIF2B genes (GCN3, GCD1, GCD2, GCD6 and GCD7). These studies indicated that these genes codes for the subunit proteins of eIF2B complex (Bushman et al. 1993, Hinnebusch 1993). eIF2B is a complex of five non-identical subunits α-ε that are well conserved form yeast to human (Price et al. 1996). Except GCN3, which encodes for α subunit, all are essential. The deletion of these genes is lethal although it has been shown that increased levels of initiator methonyl tRNA and the over expression of wild type eIF2 genes can replace eIF2B in vivo thus overcoming the lethal effects of eIF2B deletion. Although the yeast are very sick unless a mutant affecting GDP affinity is used (Erickson et al. 2001).

Compared to other GEFs, eIF2B is a relatively complex molecule with five subunits (Mohammad-Qureshi et al. 2007). This structural complexity may
reflect its mechanism of action, multiple regulatory inputs, or other roles in cell besides recycling eIF2, for example, cell cycle progression and integrating various stress responses to growth.

eIF2B complex can be divided into two functional sub-complexes i. the regulatory and, ii. the catalytic sub-complex. The division is based on the response to eIF2α(p). Subunits in the regulatory sub-complex sense the phosphorylation of eIF2B and regulate the GEF activity of catalytic complex (Pavitt et al. 1997). The domain structure of eIF2B complex is shown in Figure 1.13. Each of these sub-complexes is discussed in turn.

1.7.2.1 The regulatory sub-complex

As discussed earlier in section 1.5.5 that catalytic activity of eIF2B is regulated indirectly by phosphorylation of its substrate, eIF2. Gcn2p phosphorylates eIF2, leading to reduction in the TC formation, which induce translation of GCN4 mRNA, encoding a transcriptional activator of amino acid biosynthetic genes (Pavitt et al. 1997). By using both genetic and biochemical methods, it has been demonstrated that three subunits of eIF2B encoded by GCN3, GCD7 and GCD2 act as a complex to regulate the activity of eIF2B in response to eIF2 phosphorylation. The results of extensive genetic analysis provided insight for specific functions of eIF2B subunits (Pavitt et al. 1997).

Gcn3p (α), Gcd2p (δ) and Gcd7p (β) were first identified genetically by mutations that impair GCN4 translational control. By impairing the catalytic activity of eIF2B, Gcd mutated mutations lead to decreased concentrations of TC independently of eIF2α phosphorylation. GCN3-encoded subunit of eIF2B is non-essential. Inactivation of GCN3 affects only the ability to stimulate GCN4 translation under starvation conditions. Same Gcn− phenotype is observed upon inactivation of the protein kinase Gcn2p or the replacement of Ser-51 in eIF2α with non-phosphorylatable alanine.

On the basis of these findings it was proposed that Gcn3p is required to mediate the inhibitory effect of eIF2α(p) on eIF2B function and it is a regulatory subunit of eIF2B, which makes eIF2B more sensitive to the inhibition by eIF2α(p) (Pavitt et al. 1998).
GCN3 and GCD7 share sequence similarity over their entire lengths (Figure 1.13). The C-terminal half of GCD2 is similar in sequence to both GCD7 and GCN3. Together GCD7, GCD2 and GCN3 encoded subunits of eIF2B all have important roles in the regulation of eIF2B by eIF2α(p). These three subunits form one regulatory complex that senses the phosphorylation of eIF2α. It has been shown that the over expressing of three subunits of regulatory complex did not increase the level of functional eIF2B in the absence of the inhibitor eIF2α(p) (Yang and Hinnebusch 1996). eIF2B regulatory complex can sequester eIF2α(p) and prevent it from inhibiting eIF2B nucleotide exchange activity (Pavitt et al. 1997, Pavitt et al. 1998).

1.7.2.2 The catalytic sub-complex

The catalytic complex consists of γ and ε subunits and is necessary for accelerating the rate of nucleotide exchange. Gcd1p and Gcd6p show 47% sequence similarity over the entire length of Gcd1p (Figure 1.13). Gcd1p and Gcd6p have motifs which are present in other proteins families including a large family of enzymes called NTP-hexose sugar pyrophosphorylases (PLD) and a hexapeptide repeat (IGXXXX) common in acyltransferase called “I-patch”. I-patch assumes a left handed β-helix (LβH). The structure of NTD of eIF2Bε and γ is still not solved. The CTD domain of eIF2Bε is homologous both to the eIF4G and eIF5. CTD domain contains residues that can perform GEF activity of eIF2B although at a reduced rate compared to complete eIF2B complex (Mohammad-Qureshi et al. 2007).

eIF2Bγ forms a sub-complex with the ε subunit, which enhances the activity of the ε subunit both in yeast and mammals (Li et al. 2004). Overexpressed Gcd6p is capable of accelerating the nucleotide exchange to a lower rate than the wild type complex: however dissociation rate increases to up to ten-fold with co-over expression of Gcd1p and Gcd6p. Gcd1p and Gcd6p form a stable complex that binds to eIF2 in the absence of other three subunits. In a pull down experiment with extracts over expressing untagged Gcd1p and Gcd6p and purified His-tagged eIF2, it was shown that the level of binding of Gcd1p-Gcd6p sub-complex to eIF2 was independent of phosphorylation status of eIF2α. This
suggests that the catalytic subunit is unable to discriminate between the eIF2 and eIF2α(p) because it does not interact with α subunit of eIF2. (Pavitt et al. 1997, Pavitt et al. 1998). Gcd1p serves to stabilize the binding of eIF2B-eIF2 interaction, thus enhancing Gcd6p catalytic activity.

Figure 1.13. Domain structure of eIF2B Subunits.
eIF2B is a complex of five non-identical subunits. They can be divided into sub-categories. Regulatory sub-complex and catalytic sub-complex. Conserved regions are shown by same colour. N-terminal of Gcd6p consists of NTP-hexose sugar pyrophosphorylases (PLD) and a hexapeptide repeat (IGXXXX) common in acyltransferase which assumes a left handed β-helix (LβH region). The aromatic amino acid rich boxes in CTD of Gcd6p are shown as black bold bars.
1.7.2.3 The minimal catalytic domain of Gcd6p

Studies suggest that the C-terminal ~60 residues of eIF2Bε are important in binding to eIF2β (Asano et al. 1999, Wang et al. 2001). In vitro studies show that the C-terminal residues (518-712) contained a minimal functional unit that could interact with eIF2 and displace GDP. A genetic screen using conditional expression system was performed to examine the role of eIF2Bε in the guanine nucleotide exchange and inter subunit interactions. Random mutation identified that N-terminal is required for subunit interaction while C-terminal affects the GEF activity. T52I, S576N mutations impair the catalytic activity with no affect on the complex formation (Gomez and Pavitt 2000).

Series of N-terminal deletion identified that the catalytic activity resides in the 518-712 residues, and further deletions to 581 results in the loss of catalytic activity. This region was termed catalytic domain or $\varepsilon^{\text{cat}}$ (Gomez et al. 2002).

An atomic resolution structure for the catalytic domain of eIF2Bε (residues 524-702) revealed that it is formed from four stacked pairs of alpha-helices similar to HEAT repeats. In addition to the belt of acidic residues there is a basic patch of amino acids while the charges of other residues are randomly distributed over the surface. The overall negative charge on the ε subunit allows it to interact with the positively charged K-boxes on eIF2β (Boesen et al. 2004).

Site directed mutagenesis (SDM) approach used to characterize residues within the catalytic domain targeting conserved residues showed that E569 and W699 are critical for the catalytic activity as mutation of these residues is lethal in vivo. The C-terminal of eIF2Bε (652-712) and eIF5 has bipartite motifs rich in conserved acidic and aromatic amino acids. eIF2Bε residues 581-712 interact with eIF2, which confirmed that this region is sufficient for interaction with the eIF2. Additional residues 518-580 are required for GEF function. Mutations in the conserved amino acids in this region impair or eliminate the activity. The term 'catalytic centre' was used to describe its function (Mohammad-Qureshi et al. 2007).

eIF2B complex plays a critical role in the reactivation of eIF2 during protein synthesis. The translational control is exercised on holoenzyme, consisting
of all five subunits (α-ε), eIF2B. To better understand the domain organization through functional protein-protein interaction between catalytic sub-units of eIF2B two independent parallel studies using two different approaches were carried out in yeast and mammalian systems in two different laboratories. N-terminal of eIF2Bγ and ε shares sequence similarities with each other in PLD domain as well with a class of enzymes nucleotidyl transferases (NTs). It was shown that PLD and LβH domains of eIF2Bε and γ are required for binary complex formation and complete pentameric complex and both domains interact independent of each other. LβH of eIF2Bγ in humans is not critical for complete complex formation while in yeast it plays a key role in subunit assembly. These studies also demonstrate that that residues required for domain interaction have minimal affect on the catalytic activity of eIF2B (Reid et al. 2012, Wang et al. 2012). ε\textsuperscript{cat} is not required for complex formation demonstrating it is an independent domain.

1.7.3 Mechanisms of eIF2B catalysed reaction

As discussed earlier in section 1.2.1.2 eIF2 assembles 43S PIC complex by recruiting TC (eIF2-GTP-Met-tRNAi\textsuperscript{Met}) to 40S ribosomal subunit. In the course of initiation cycle, GTP is hydrolyzed and eIF2-GDP binary complex is produced. In common with other G protein, the function of eIF2 depends on the GTP bound state. eIF2-GDP is inactive and not participate in subsequent rounds of translation, so it is recycled to eIF2-GTP for renewed TC assembly. At the physiological concentrations of Mg\textsuperscript{++}, eIF2 has 100 fold higher affinities for GDP than for GTP. The reaction of dissociation of GDP from eIF2 is very slow and is accelerated by eIF2B. Two mechanisms have been proposed for the working model of eIF2B activity, each one is discussed in turn. Figure 1.14 is illustration of these proposed models.

1.7.3.1 Substituted enzyme mechanism

Standard substitute mechanism also known as ping-pong mechanism by which other GEFs act on their respective G proteins. This model applies to two substrate reactions. eIF2-GDP binds eIF2B and dissociates GDP from complex. eIF2-GTP
is produced by the binding of GTP followed by dissociation of eIF2B. Schematic illustration of this model is shown in Figure 1.14A.

### 1.7.3.2 Sequential mechanism

Sequential mechanism also known as TC mechanism involves formation of a complex (eIF2-GDP-eIF2B-GTP) containing both substrates bound at the same time (Nika et al. 2000). According to this model GTP binds before the release of GDP. Earlier studies carried out by Wahba's group support this mechanism of action of eIF2B mediated reaction (reviewed in (Price and Proud 1994), however a recent study done in the Pavitt laboratory does not support this mechanism of action. Although eIF2Bε has potential nucleotide binding regions required for this binding mechanism, mutations predicted to nucleotide binding had no observable effects on binding. A role for GTP binding to Gcd1p not yet excluded (Reid et al. 2012).

![Two possible mechanisms of eIF2B mediated reaction.](image)

**A. Standard substituted enzyme mechanism**

- eIF2-GDP
- eIF2B
- eIF2-GDP-eIF2B
- GDP
- eIF2-eIF2B
- GTP
- eIF2-GTP-eIF2B
- eIF2GTP

**B. Sequential mechanism**

- eIF2-GDP
- eIF2B
- eIF2B-GDP
- GTP
- eIF2B-GTP
- eIF2B-GTP-eIF2B
- GDP
- eIF2-B-GDP
- GTP
- eIF2-B-GTP

*Figure 1.14. Two possible mechanisms of eIF2B mediated reaction.*

**A.** Standard substituted enzyme mechanism according to which eIF2B does not have a nucleotide binding domain. **B.** Sequential or TC complex formation mechanism which requires the nucleotide binding site in eIF2B. Adapted and modified from (Price and Proud 1994).
1.7.4 Regulation of eIF2B activity

The activity of eIF2B plays an important role in regulating over all translation initiation, and eIF2B is regulated by several ways in response to environmental challenges. Regulation of eIF2B activity by eIF2α phosphorylation during amino acid starvation has already been discussed in details in section 1.5.5.

Another key player of regulatory mechanism under amino acid starvation is eIF5, a multifunctional protein that antagonise GEF activity of eIF2B. eIF5 is GAP for eIF2 and favours the ribosomal subunit joining during translation initiation. During this process, eIF2-GDP is released in complex with eIF5. To participate in other round of translation, it is recycled by a reaction catalyzed by eIF2B. eIF5 also posses GDI activity and eIF2/ eIF5 complex inhibits the GEF activity of eIF2B. It has been shown that mutations that impair the GAP activity do not affect the GDI activity of eIF5 suggesting that these two functions are independent of each other. Under optimal growth conditions where there is surplus supply of amino acids, and TC is high, the effect of GDI activity is not visible activity while in non starvation conditions, Gcn2p phosphorylates eIF2α at Ser-51, which the binds more tightly to eIF5 and antagonises eIF2B activity resulting in low TC level. Over expression of wt eIF5 in gcn2∆ cells can activate GCN4 pathway by avoiding eIF2α(p) resulting in Gcd− phenotype (Jennings and Pavitt 2010).

Fusel alcohols and also volatile anaesthetics affect the translation initiation and yeast growth (Palmer et al. 2005). Fusel alcohols are the amino acid metabolites of branched chain amino acids. Yeast fermentation results in the production of fusel alcohols under nitrogen-limiting conditions. If yeast is grown on the leucine as the sole source of nitrogen, a range of strain-specific morphological effects and these effects also appear if fusel alcohols are added to the yeast, for example, pseudohyphal growth. The addition of fusel alcohols such as butanol or isoamyl alcohol results in the inhibition of translation initiation in a strain specific manner by a mechanism involving eIF2B. The sensitivity was mapped to genes on chromosome XV and found GCD1 as a candidate gene for adding the sensitivity to butanol. The sensitivity is allele specific and it was
shown that eIF2Bγ (GCD1 Ser 180) is responsible for the sensitivity to butanol but the phosphorylation of Ser 180 is not required for the butanol dependent inhibition in butanol sensitive strains (Ashe et al. 2001).

Subsequent studies on other eIF2B subunits show that 1-butanol treatment affects at least four out of five subunits of eIF2B. GCN3 mutants proved to be resistant to butanol while GCD6 mutations that result in CACH disease are also sensitive to 1-butanol (Richardson et al. 2004, Taylor et al. 2010). It was also shown that 1-butanol affects the dynamics and movement of eIF2B body in cytoplasm.

Another mechanism for regulating eIF2B activity involves direct phosphorylation of eIF2Bε subunit. This mechanism has been reported to operate in mammals for regulation of translation initiation in response to environmental cues. Multiple phosphorylation sites were identified in mammalian eIF2Bε in vivo. Four different protein kinases are identified that phosphorylate eIF2Bε in vitro: CK1, CK2, DYRK and GSK3 (Five sites in the catalytic domain were identified that are targets of known protein kinases for eIF2Bε in vitro. Four of the sites lie in the catalytic region identified as catalytic region in yeast. The fifth site is in the central part outside the conserved mammalian domain. In order to identify the sites, which can be phosphorylated by these three kinases, rat eIF2Bε was expressed it in Escherichia coli as a GST fusion protein. It was found that GST-eIF2Bε can be phosphorylated efficiently by GSK3 and CK2, but weakly by CK1 (Wang et al. 2001, Woods et al. 2001).

1.7.5 Mammalian eIF2B kinases

eIF2Bε is phosphorylated in vitro by casein kinases 1 and 2 (CK1 and CK2), resulting in activation of eIF2B, while phosphorylation by glycogen synthase kinase3 (GSK3) results in the inhibition of eIF2B. Phosphorylation sites at the extreme C-terminus of eIF2Bε are required for binding and full activity of eIF2B. There are four known protein kinases that phosphorylate mammalian eIF2B in response to different conditions. Each one is discussed in turn.
1. Introduction

1.7.5.1 Glycogen synthase kinase 3 (GSK3)

GSK3 is responsible for phosphorylating eIF2Bε at Ser 535 (in rats). The phosphorylation of Ser 535 leads to inhibition of GEF activity of eIF2B. Insulin inactivates GSK3 causing dephosphorylation and subsequent activation eIF2Bε (Welsh et al. 1998).

GSK3 kinases are specific in the requirement of earlier phosphorylation at +4 position relative to GSK3 kinase site in their substrates i.e. phosphorylation at Ser 539, which recruit GSK3 to eIF2Bε. It was identified that members of the eukaryotic DYRK family act as primary kinase and they phosphorylate Ser 539, which facilitates the phosphorylation at Ser 535 and this priming phosphorylation event is the requirement for the action of GSK3 kinases. Mutation of Ser 539 to a non-phosphorylatable residue does indeed eliminate phosphorylation at Ser 535 (Woods et al. 2001).

1.7.5.2 Protein kinase CKI

CKI phosphorylate GST-eIF2Bε only weakly and it is supposed that CK1 phosphorylate eIF2Bε weakly at several sites that are better substrates for CK2/GSK3. In order to find candidate phosphorylation sites, vectors encoding eIF2Bε three mutant polypeptides containing S46A, S461A and S464A were transfected into cells, and it was shown that Ser464 is the true site of phosphorylation because of its sequence context i.e. with two acidic amino acids N-terminal to it, a requirement for the activity of CK1. This site and most of the residues in this region are conserved in known mammalian eIF2Bε.

To test the effect of point mutation on the interaction of eIF2Bε with eIF2, mutant proteins were expressed in HEK293 cells and immunoprecipitated from cells extracts using the anti-HA antibody. S535A, S539N, and mutations at residues 461 or 464 affected the binding of eIF2B with its substrate while S712/713GA mutants indicated the loss of CK2 sites and displayed reduced activity (~25% relative to wild type eIF2Bε). This region has been identified previously as playing a key role in binding to eIF2 (Wang et al. 2001).
1. Introduction

Regulation of eIF2B by Phosphorylation

Figure 1.15. Regulation of eIF2B activity. eIF2-GDP is recycled to eIF2-GTP by eIF2B to initiate another round of translation. This reaction is regulated by diverse mechanisms in eukaryotes. The eIF2 (shown as triangle with three different shades of pink (designating α, β, γ subunits. complexes with GDP (yellow) regulates eIF2B activity. In response to environmental signals, eIF2 kinases phosphorylates eIF2 (black circle represents PO4) and converts eIF2 from substrate to eIF2B inhibitor. eIF5 (shown as blue) posses GAP and GDI activity and antagonizes GEF activity of eIF2B. The eIF2B activity can be regulated directly by phosphorylation at ε subunit by CKI, CKII, GSK3 and DYRK kinases in mammals. Butanol is shown to inhibit the growth in yeast.
1.7.5.3 Protein kinase 2

Casein Kinase 2 (CKII) is an enzyme known to phosphorylate more than 300 different protein substrates including many protein translation factors, ribosomal proteins and mRNA binding proteins. Interestingly, both eIF2B (eIF2Bε subunit) and eIF5 have been recently shown to be CK2 substrates in vivo. This fact, together with the evidence that considerable levels of CK2 are present in ribosomal-enriched cellular sub-fractions suggest that CK2 might be important for maintaining some components of the machinery for protein translation in a functional conformation. Mutants of eIF2Bε lacking the extreme C-terminus are not substrates for CK2 in vitro. Two conserved sites (Ser 712/713) are phosphorylated by CK2 and they are required for the interaction of eIF2B epsilon with eIF2 (Wang et al. 2001, Llorens et al. 2003).

1.7.5.4 Dual-specificity tyrosine phosphorylated and regulated kinase (DYRK)

DYRK are kinases that exist in two different isoforms in cells, a cytoplasmic isoform DYRK2 and a nuclear isoform DYRK1. DYRK2 shows 45% identity to the catalytic domain of DYRK1. Using in vitro system it was shown that the phosphorylation of Ser535 by GSK3 requires the priming phosphorylation of Ser539 by DYRK2. Same results were produced when DYRK2 was replaced by DYRK1. Using tau protein, which is phosphorylated by GSK3 at Ser208, they showed that it is phosphorylated at this position only if it is phosphorylated earlier by DYRK at Ser212. This study provided conclusive evidence that phosphorylation by DYRK at position +4 relative to the GSK3 is the pre-requisite for GSK3 kinases (Woods et al. 2001).

1.7.6 eIF2B and Human Disease

The importance of the correct control of eIF2B for normal physiology is obvious by the involvement of the five genes that encode eIF2B subunits in the childhood ataxia with central nervous system hypomyelination (CACH)/leukoencephalopathy with vanishing white matter syndrome (VWM). This is an autosomal recessive disorder characterized by the occurrence of acute episodes of
deterioration during febrile illness or head trauma and symmetrical demyelination of the brain white matter, leading to progressive vanishing of the white matter, which is replaced by CSF (cerebrospinal fluid). So far at least 160 different mutations have been found in the genes encoding eIF2B subunits, 99 affecting the epsilon subunit. eIF2B is ubiquitously expressed it is surprising that mutation in this result in tissue specific disease (Pavitt 2005, Fogli and Boespflug-Tanguy 2006). Evidence that eIF2B mutations cause a failure in maturation of glial cells from their progenitor cells. A loss of astrocytes and increases number of immature oligodendrocytes are seen in VWM. Mutations impact on eIF2B in diverse ways either reducing ability to form complexes or reducing interactions with eIF2 or reducing 2B function and cause elevated stress response, for example, ATF4 activity.

1.8 Introduction to current study

eIF2B catalyse the first step of translation initiation and tight control is exercised on eIF2B in response to various environmental stress conditions. The best known mechanism already discussed earlier in this chapter described in detail the role of eIF2\(\alpha\) phosphorylation in regulating eIF2B activity in response to amino acid starvation. There are studies which show translational inhibition even with no increase in eIF2\(\alpha(p)\), e.g. insulin treatment in mammals. Also in yeast butanol and isoflurane affects the activity of eIF2B and yeast strains carrying mutations in four out of five subunits shows strain specific response to butanol, some mutants strains being sensitive while others show resistant phenotype. These evidences provide ideas for the search of mechanisms that directly affect the eIF2B activity rather than the well established eIF2\(\alpha(p)\) mechanism.

It is known that the activity of mammalian eIF2B is regulated phosphorylation in response to various stress conditions; however no detailed study has been conducted to monitor the changes in phosphorylation in yeast eIF2B. This study follow preliminary findings from the Pavitt laboratory where phosphorylation sites have been identified in Gcd6p and Gcd1p (\(\varepsilon\) and \(\gamma\)) subunits in yeast.
1.8.1 The aims and objective(s) of the research

The research reported in this thesis aims “to characterise the newly identified phospho-sites to determine their roles in the regulation of catalytic activity of eIF2B in yeast grown under different stress conditions”.

The following objectives were set to achieve the above stated aims:

- To optimize the working conditions for newly synthesized phospho-specific antibodies raised against the newly identified phospho-sites through MS in the catalytic sub-complex of eIF2B.
- To check the site and phospho-specificity of these antibodies
- To determine the role of newly identified eIF2B phosphorylation sites in translational control in yeast grown under different stress conditions.

1.8.2 Structure of thesis

The remaining thesis has been organised as follows:

Chapter 2 consists of materials (biological, chemical) and protocols used in this study to generate data presented in four results chapters (chapters 3-6).

Chapter 3 presents the initial characterization of novel phospho-specific antibodies, development of a strategy to monitor changes in phosphorylation and development of a method for fast harvesting of cells with post-harvesting minimal stress induction. Optimisation of an already developed method in laboratory used for the isolation of eIF2B complex from large scale is also discussed.

Chapter 4 presents a detail analysis of the data generated with TORC inhibitors i.e. rapamycin and Torin1.

Chapter 5 describes the affect of amino acid starvation by 3-AT addition on translation and eIF2B phosphorylation at selected sites.

Chapter 6 examines the effects of 1-butanol on yeast growth and phosphorylation of eIF2B.

Chapter 7 discuss results and provides a conclusion to this thesis. A summary of achievements and future research perspectives are also presented. This is followed by a supplementary appendix and list of references.
Chapter 2

Materials and Methods
2 MATERIALS AND METHODS

This chapter consists of all the materials and methods used to generate data that is presented in chapter 3, 4, 5 and appendices. First part of this chapter consists of biological and chemical materials and list of different software’s used for phosphorylation site with potential kinases/ phosphatases prediction Second part of the chapter is further divided into three sections. I. Genetic methods, II. Yeast phenotypic growth assays and III. Biochemical methods.

2.1 Yeast strains

To study the role of selected Ser residues on GCD6 function during growth under diverse stress conditions, various mutant strains were constructed. Site Directed Mutagenesis (SDM) was done to make plasmids (with LEU2 as selectable marker) carrying Ser to Ala/ or Ser to Asp/ Glu mutations and were maintained in Pavitt laboratory plasmid collection at -80°C. Plasmids were introduced by Lithium Acetate Aided Transformation procedure into parent yeast strain. GP 3750 deleted for the GCD6 chromosomal gene and carrying mutations in leu2 and ura3, which conferred growth requirements for leucine and uracil was used as parent strain. As GCD6 is essential for viability, in parent strain chromosomal deletion of gcd6∆ was complemented by wild type GCD6 gene on a single copy plasmid (URA3 marker). After transformation plasmids carrying mutated gcd6 gene were shuffled with the resident wild type plasmid using 5-fluoro-orotic acid (FOA). The yeast strains constructed for this study are listed in Table 2.1. Plasmids used for transformation are listed in Table 2.2.

2.1.1 S. cerevisiae strains

Several new yeast strains were constructed with desired mutations in GCD1 and GCD6. Table 2.1 contains the name and genotype of the S. cerevisiae strains used in this study.
Table 2.1. All yeast strains used in this study

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2.1.2 List of plasmids

The list of plasmids and their genotypes used to construct new yeast strains is presented in Table 2.2.

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<td>pAV 2115</td>
<td>gcd6-S525D LEU2 2μm</td>
</tr>
<tr>
<td>pAV 2129</td>
<td>gcd6-S525A LEU2 CEN4</td>
</tr>
<tr>
<td>pAV 2283</td>
<td>gcd6 I432L LEU2 CEN4</td>
</tr>
<tr>
<td>pAV 2285</td>
<td>gcd6 I432L, S435A LEU2 CEN4</td>
</tr>
</tbody>
</table>

2.2 Growth media

2.2.1 Yeast growth media

2.2.1.1 YPD medium

Yeast was usually grown at 30°C on the complete medium, Yeast extract, peptone, dextrose (YPD). YPD is a complex, enriched, non-selective medium with optimal proportions of nutrients required for growing most S. cerevisiae strains. Composition of YPD is described in Table 2.3. For growth phenotypic assays drugs/ chemicals (as listed in Table 2.6) were added to the medium to impose stress.
Regulation of eIF2B by Phosphorylation

Table 2.3. Complex Medium-YPD

<table>
<thead>
<tr>
<th>Medium</th>
<th>Componenta</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>YPD</td>
<td>1% Bacto-yeast extract</td>
<td>10 g</td>
</tr>
<tr>
<td>(For routine growth)</td>
<td>2% Bacto-peptone</td>
<td>20 g</td>
</tr>
<tr>
<td></td>
<td>Sterile water</td>
<td>1000 ml</td>
</tr>
<tr>
<td></td>
<td>*2% Glucose</td>
<td>20 g</td>
</tr>
<tr>
<td></td>
<td>**2% Agar</td>
<td>20 g</td>
</tr>
</tbody>
</table>

*aComponents were added in water and sterilised by autoclaving at standard sterilisation cycle (15 min at 121˚C). All the components are from Formedium™ unless specified.*

*40% (w/v) glucose stock solution was made separately and autoclaved at low temperature cycle (15 min at 115˚C) to avoid caramelization. 20 ml of glucose stock solution was added to the 400 ml medium (final conc. 2% glucose) before use.*

**To make solid medium, 20g Agar was added to 1000 ml YP solution lacking glucose and autoclaved. 50 ml of sterile glucose solution was added to the medium and 25 ml of medium was poured into a sterile Petri dish-100 mm in diameter (Fisher Scientific Ltd. UK). Plates can be stored at 4˚C for 3 months in a sealed plastic bag.

2.2.1.2 SD medium

Minimal or on synthetic dextrose (SD) medium is a blend of Bacto-Yeast Nitrogen Base without amino acids. The constituents are listed in Table 2.4. Growth phenotypic assays were done by adding drugs/chemicals (listed in Table 2.6) to the required concentration to the medium.

2.2.1.3 Selective SD medium

Amino acid supplements (Table 2.5) were added to the SD medium to make a Selective SD medium to meet the auxotrophic requirement of essential amino acids for mutant strains.

2.2.1.4 SCD medium

The synthetic complete medium (SCD) is the same as SD except that, in addition; it also contained 2002 mg of mixture of all 20 amino acids added to it.

2.2.1.5 SCD-drop out medium

The SCD-dropout medium was made just as SCD, except that the 20 amino acid mixture powder was replaced with the amino acid powder lacking one component that was covered by the selectable marker, i.e. LEU2.
**2. Materials and Methods**

**Regulation of eIF2B by Phosphorylation**

Table 2.4. Synthetic dextrose medium (SD)

<table>
<thead>
<tr>
<th>Medium</th>
<th>Component</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>^aMinimal SD (For transformants selection)</td>
<td>^0.67% Bacto-yeast nitrogen base (YNB) without amino acid</td>
<td>10 g</td>
</tr>
<tr>
<td></td>
<td>2% Glucose</td>
<td>20 g</td>
</tr>
<tr>
<td></td>
<td>*2% Agar</td>
<td>20 g</td>
</tr>
<tr>
<td></td>
<td>Sterile water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

| ^bSelective SD (For nutritional requirements) | SD medium | 1000 ml |
| | Required Amino acid | See Table 2.5 |

| ^cSynthetic complete (SC) medium | SD medium | 1000 ml |
| | 20 Amino acid mixture powder | 2002 mg |

| ^dSC drop out medium | SD medium | 1000 ml |
| | 20 Amino acid mixture lacking one or two a.a | 1926 mg |

^aSolution was sterilised by autoclaving using low temperature cycle (15 min at 115˚C).
^bTo make solid medium, 40 g Agar (4%) was added to 1000 ml water and autoclaved separately at standard sterilisation cycle (15 min at 121˚C). Equal volumes of 2X SD and 4% Agar solutions were mixed and 25 ml of medium was poured into a sterile Petri dish-100 mm in diameter (Fisher Scientific Ltd. UK)
^cFilter sterilised amino acid stock solution was added to SD medium.
^d20 amino acid powder was added to the SD media before autoclaving.
^dDrop-out mixture lacking Leu and His was added to SD medium.

Table 2.5. Amino acids stock and working solutions concentration

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>^aStock Concentration</th>
<th>^µl/ ml Medium</th>
<th>^µl/ ml of Medium</th>
<th>^Agar Plate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uracil</td>
<td>20 mM</td>
<td>10</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Leucine</td>
<td>100 mM</td>
<td>20</td>
<td>200</td>
<td></td>
</tr>
<tr>
<td>Isoleucine</td>
<td>50 mM</td>
<td>10</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>*Tryptophan</td>
<td>40 mM</td>
<td>10</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

^aAll amino acid stock solutions were either filter sterilized or autoclaved for 15 min at 121˚C.
^*Tryptophan is light sensitive, so stock solutions were stored at room temperature.
Table 2.6. Chemicals used to induce stress in *S. cerevisiae*

<table>
<thead>
<tr>
<th>Chemical</th>
<th><em>Stock Concentration</em></th>
<th>Working concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Rapamycin</em></td>
<td>100 μg/ ml</td>
<td>100 ng/ ml</td>
</tr>
<tr>
<td><em>Torin 1</em></td>
<td>40 μM</td>
<td>10 μM</td>
</tr>
<tr>
<td>3AT</td>
<td>1 M</td>
<td>10, 25, 50 mM</td>
</tr>
<tr>
<td>Butanol</td>
<td>99.9%</td>
<td>2%</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>10 M</td>
<td>0.5, 1, 2 mM</td>
</tr>
<tr>
<td>Methylglyoxal</td>
<td>6.5 M</td>
<td>5, 10 mM</td>
</tr>
</tbody>
</table>

*Stock solutions were made fresh on the day of use or no more than 4 days old.

* DMSO was used as drug carrier and solvent for rapamycin and Torin1. Stock solution was stored in eppendorf fully wrapped in foil and away from light at -20°C.

2.3 **List of *E. coli* strains**

In microbiology and molecular biology, term 'competence' is used to describe the ability of cells to take up extracellular DNA (plasmids) from the surrounding environment. Competence can be induced in cells in laboratory by treatment with CaCl₂ (induced competence). Table 2.6 contains the names, genotypes and sources of the competent *E. coli* strains used in this study.

Table 2.7. All *E. coli* strains used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>XL10-Gold®</td>
<td>Tet⁺, Δ(mcrA)Δ183, Δ(mcrCB-hsdSMR-mrr)Δ17, endA1, supE44, thi-1, recA1gyrA96, relA1 lac Hs, [F’ proAB lacIqZΔM15 Tn10 (Tet’)] Amy Cam’</td>
<td>Stratagene</td>
</tr>
<tr>
<td>dam⁻/ dcm⁺ competent <em>E. coli</em></td>
<td>ara-14, leuB6, fhuA31, lacY1, tsx78, glnV44, galK, galT22, mcrA, dcm-6, hisG4, rfbD, R(zgb210::Tn10) Tet⁺ endA1, rspl36 (Str⁺), dam13::Tn9 (Cam⁺), xylA-5, mtl⁻, thi-1, mcrB1, hsdR2</td>
<td>New England Biolabs inc</td>
</tr>
</tbody>
</table>

2.3.1 **Luria-Bertani medium for *E. coli***

Luria-Bertani (LB), a nutritionally enriched medium is used for the growth of *E. coli* cells. Appropriate antibiotic is added to LB to make selection of recombinant bacteria for maintaining and cultivating the plasmid carrying antibiotic resistance gene. The composition of LB is described in Table 2.7. To make a selective medium, antibiotic listed in Table 2.8 were added to LB medium.
Table 2.8. LB medium

<table>
<thead>
<tr>
<th>Medium</th>
<th>Component*</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luria-Bertani (LB)</td>
<td>Bacto-Tryptone</td>
<td>4 g</td>
</tr>
<tr>
<td></td>
<td>Bacto yeast extract</td>
<td>2 g</td>
</tr>
<tr>
<td></td>
<td>Nacl</td>
<td>2 g</td>
</tr>
<tr>
<td></td>
<td>* Agar</td>
<td>6 g</td>
</tr>
<tr>
<td></td>
<td>Distilled water</td>
<td>400 ml</td>
</tr>
</tbody>
</table>

*Components were added in water and sterilised by autoclaving at standard sterilisation cycle (15 min at 121°C).
* To make plates, agar was added to the medium prior to autoclave.

Table 2.9. Antibiotics used to make selective media

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>*Stock Concentration</th>
<th>Working concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>100 mg/ ml</td>
<td>100 µg/ ml</td>
</tr>
<tr>
<td>*Carbenicillin</td>
<td>100 mg/ ml</td>
<td>100 µg/ ml</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>34 mg/ ml</td>
<td>34 µg/ ml</td>
</tr>
</tbody>
</table>

Antibiotic stock solutions were stored at -20°C. Antibiotic was added to the LB medium prior to use and medium (liquid/ plates) containing antibiotic was stored at 4°C.
*Carbenicillin (semi-synthetic ampicillin analog) can be substituted for ampicillin as it tends to be more stable than ampicillin.

2.4 S. cerevisiae strains growth and maintenance

2.4.1 Strain maintenance

Yeast strains can be stored either for short or indefinite periods of time. Viability of strains differ ranging from few months to at least one year (Sherman 1991). For short term storage, strains growing on YPD/ SD medium on petri dishes were stored at 4°C for one week. To preserve the strains for indefinite periods of time, 800 µl of overnight grown culture was suspended in 400 µl of 50% glycerol in 2 ml sterile screw capped vials and stored at -80°C.

2.4.2 Reviving the strains from -80°C

Yeast cells (stored in 50% glycerol at -80°C) were revived by transferring a small portion of frozen sample with the help of a sterile wooden pick onto the appropriate solid medium surface, i.e. YPD-Agar/ SD-Agar plate. Plates were kept in the static incubator at 30°C for 3 days in upside down position.
2.5 DNA recombinant methods

2.5.1 Plasmid DNA isolation from *E. coli*

Plasmid isolation was done with small modification of the original method described by (Birnboim and Doly 1979). A single bacterial colony was picked with sterile loop and inoculated into 5 ml LB + Amp medium. *E. coli* was cultivated overnight at 37°C with vigorous shaking. Plasmid DNA isolation was carried out using a QIAprep spin miniprep kit as per the manufacturer's instructions (Qiagen Ltd. UK).

2.5.1.1 Measurement of DNA concentration

Concentration of DNA was determined by measuring absorbance at 260 nm ($A_{260}$) using nanodrop-8000 spectrophotometer (Thermo Scientific). 1 unit of optical density at $A_{260}$ is equivalent to 50 μg/ ml DNA. Almost 20 µg of high copy plasmid DNA and 5-10 µg of single copy plasmid DNA were purified from 5 ml of overnight grown culture of *E. coli*.

2.5.2 Site directed mutagenesis

Site directed mutagenesis also known as oligonucleotide-directed mutagenesis is a technique used to make specific intentional changes in the DNA sequences. *In vitro* site directed mutagenesis is a very useful tool to understand and characterize the complex relationship between structure and function of protein and to introduce modifications in plasmids (Braman et al. 1996). The basic consists of a PCR cycling reaction using super-coiled dsDNA having an insert of interest and two synthetic primers containing desired mutations. Mutagenic primers hybridize with the DNA in gene of interest. New DNA strand is synthesized by extending the primer by DNA polymerase. QuickChange® Lightning Site-Directed Mutagenesis Kit (Stratagene) was used to carry out mutagenic primer-directed replication of vector as per manufacturer instructions. PCR reaction produced dsDNA molecules, consisting of an old template strand and a newly synthesized mutagenic strand with nicks in it, which are sealed by the components in the QuickChange Lightning enzyme blend. Two reactions were performed i.e. one with control primers and pWhitescript™ template and second with the mutagenic primers and dsDNA vector carrying insert of interest, i.e. *GCD6*. The
compositions of SDM reaction mixture is described in Table 2.10 and parameters of cycling reaction are outlined in Table 2.11.

<table>
<thead>
<tr>
<th>Table 2.10. Site-directed mutagenesis reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Component</strong></td>
</tr>
<tr>
<td>10x reaction buffer</td>
</tr>
<tr>
<td>pWhitescript™ vector (25 ng)</td>
</tr>
<tr>
<td>Control primer 1 (125 ng)</td>
</tr>
<tr>
<td>Control primer 2 (125 ng)</td>
</tr>
<tr>
<td>dNTP mixture</td>
</tr>
<tr>
<td>QuickSolution reagent</td>
</tr>
<tr>
<td>QuickChange® enzyme</td>
</tr>
<tr>
<td>dH₂O</td>
</tr>
</tbody>
</table>

*Forward primer: 5’ GATAGAACACAAAACATCTGCCAGTCCA 3’
*Reverse primer: 5’ TGGACTGGCAGATAGTTTTGTGTCTATC 3’

<table>
<thead>
<tr>
<th>Table 2.11. Cycling parameters of SDM reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Stage</strong></td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>3</td>
</tr>
</tbody>
</table>

* 30 sec/ kb of vector length

The PCR product was restriction digested with endonuclease DpnI at 37°C for 2 hours. DpnI, which is specific for methylated and hemimethylated DNA strands (target sequence: 5’-Gm⁶ATC-3’), so the parent template strands were digested and newly synthesized mutagenic strands remain unaffected by DpnI digestion. 5 µl of DpnI treated DNA was used for transformation of *E. coli*.

**2.5.3 Transformation of *E. coli***

*E. coli* cells are ideal for making plasmid DNA libraries. XL10-Gold® ultracompetent cells (Stratagene) were used for transformation because of the high transformation efficiency of ligated and large super coiled DNA molecules.
2. Materials and Methods

Regulation of eIF2B by Phosphorylation

Figure 2.1. Site directed mutagenesis.

PCR extends mutagenic primers that contain the mutated codon to generate multiple copies of mutated plasmid.

Competent cells (50 µl), stored at -80°C were thawed on ice. Cells were added to the pre-chilled 14-ml BD Falcon polypropylene round bottom tubes (BD Biosciences). 2 µl of β-Mercaptoethanol (Stratagene) was mixed into the cells and tubes were incubated on ice for further 10 minutes. 50-100 ng of plasmid DNA (5 µl of DpnI treated or 2 µl of ligation reaction) was added to cells and tubes were swirled gently and placed on ice for further 30 minutes. Cells were heat-shocked at 42°C for 30 seconds followed by two minutes incubation on ice. 400 µl of pre-warmed (42°C) LB was added and tubes were kept at 37°C for 1 hour in shaking incubator with 225-250 rpm. 200 µl of the transformation mixture was spread on LB agar plates containing an appropriate antibiotic. Plates were incubated overnight at 37°C in a static incubator. Next day, different colonies of the bacteria were picked up and inoculated into LB+Amp and grown overnight at 37°C.

Transformed bacteria (once confirmed through sequencing that they carried the plasmid with desired mutation) were grown overnight in 2.5 ml of LB+ selective antibiotic medium. 1 ml of overnight grown bacteria culture was added to 77 µl DMSO in 2 ml sterile screw capped vials and stored at -80°C.
2. Materials and Methods

2.5.4 Restriction digestion

Restriction enzymes or restriction endonucleases are bacterial proteins, which destroy bacteriophages or other viruses that transfect bacteria hence work as immune system in bacteria. These enzymes recognize a specific sequence and cut the DNA where this sequence is found.

Restriction digestion reaction was assembled as described in Table 2.12 and was incubated at 37°C for 2-4 hours to convert circular DNA plasmid to a linear DNA molecule.

<table>
<thead>
<tr>
<th>Table 2.12. Restriction digestion reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Component</strong></td>
</tr>
<tr>
<td>dsDNA Plasmid</td>
</tr>
<tr>
<td>*Enzyme</td>
</tr>
<tr>
<td>*NEB buffer (10x)</td>
</tr>
<tr>
<td>BSA (100x)</td>
</tr>
<tr>
<td>H2O</td>
</tr>
</tbody>
</table>

*a Enzymes with a unique target sequence in plasmid was selected. Volume of enzyme used depends on the quantity of dsDNA plasmid.
*b Buffer used was provided with the enzyme from NEB or otherwise specified.

2.5.5 Agarose gel electrophoresis

Agarose, a linear galactan polymer composed of D- and L-galactose residues, is used to make porous gel. Agarose gel is used to separate DNA fragments according to their sizes. When electric field is applied, DNA molecules move to anode because of the negative charge and the rate of migration depends on the size, conformation of DNA molecule, voltage applied and agarose gel percentage used. Agarose percentage ranges from ~0.7% (for large plasmids) to ~2% (for PCR products). The composition of agarose gel is described in Table 2.13. 10 µl of digested product (Section 2.5.4) was mixed with 1 µl of 5x DNA loading sample buffer (Bioline) and loaded into agarose gel (0.7%). For DNA fragment size measurement, a standard DNA marker i.e. Hyperladder-I (Bioline) was used. Electrophoresis was done for 30-40 minutes by applying 5 volts per cm to the gel.
Regulation of eIF2B by Phosphorylation

TABLE 2.13. Agarose gel composition

<table>
<thead>
<tr>
<th>Solution</th>
<th>Components</th>
<th>Quantity required</th>
</tr>
</thead>
<tbody>
<tr>
<td>aAgarose solution</td>
<td>Agarose</td>
<td>0.7 g</td>
</tr>
<tr>
<td></td>
<td>Tris</td>
<td>242 g</td>
</tr>
<tr>
<td></td>
<td>0.5 M Na₂EDTA</td>
<td>100 ml Na₂EDTA</td>
</tr>
<tr>
<td></td>
<td>Glacial acetic acid</td>
<td>57.1 ml / 1000ml</td>
</tr>
<tr>
<td>cSafeview</td>
<td></td>
<td>10 µl</td>
</tr>
</tbody>
</table>

aAgarose was solubilised in 1x TAE by boiling in a microwave.
b1x TAE was made by dilution of 50x TAE at the time of use.
cSafeview was added after dissolving and cooling the agarose solution and mixed by gently swirling the flask.

2.5.6 DNA sequencing

Once size of the plasmid was confirmed and no sign of contamination with bacterial DNA were seen, next step was to confirm that whether it carries the desired mutation introduced through SDM or not.

The "Dideoxy" sequencing method developed by Fredrick Sanger formed the basis of the sequencing reaction used today (Sanger et al. 1977). The first step of DNA sequencing consists of separating the DNA into two strands. The strand to be sequenced is copied using modified nucleotides, so called 2’,3’-dideoxy analogs. These analogs lack 3’-hydroxal group which is used for making phosphodiester bonds with the 5’ phosphate of adjacent nucleotide. Each time an analog is incorporated into growing DNA strand, strand elongation stops. As the concentration of analogs is limited, strand termination happens occasionally. With four different analogous nucleotides, numerous fragments corresponding to each nucleotide are synthesized. Fragments are put together to find the sequence of original DNA strand. The newly synthesized DNA fragments are marked; the sequence can be detected by excitation with photodiodes in a sequencing machine.

TABLE 2.14 contains the list of the primers and their sequences used for sequencing of GCD6 gene. The PCR samples involved in sequencing was assembled as described in Table 2.15 and amplification of sequence was done under the cycling parameters described in Table 2.16. After PCR reaction, 10 µl
of 3M NaAc (pH 5.2), 1 µl of Glycobluue and 250 µl of 100% EtOH was added to sample and samples were mixed by vortexing and left for 15 mins at RT to precipitate the DNA. Samples were centrifuged for 30 minutes at full speed in a table top centrifuge. After discarding the supernatant, pellet was washed with 300 µl of 70% EtOH for 5 mins. Pellet was dried at RT and was sent for sequencing to Manchester University Sequencing facility. The sequence received was analysed through BLAST (N) to confirm the sequence and desired mutation. The plasmid with desired mutation was used to transform yeast.

### Table 2.14. Primers used for GCD6 sequencing

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>GCD6 30F</td>
<td>5' TGGACTAGGCAACATGGA 3'</td>
</tr>
<tr>
<td>GCD6 1841</td>
<td>5' GGAGGATCCACGGCTACAAAGAAAAGAAGAGATG 3'</td>
</tr>
<tr>
<td>M13PUC</td>
<td>5’ CCCATACGACGGTTGAAAACG 3’</td>
</tr>
<tr>
<td>1870R</td>
<td>3’ TCTTGAACAACAGTCCCCACT 5'</td>
</tr>
<tr>
<td>1364-1381</td>
<td>5’ GGAACCCAATCCTCGTGATGGAAAGG 3'</td>
</tr>
<tr>
<td>pBluescript SK</td>
<td>5’ TCTAGAAAACACTGGGATTC 3’</td>
</tr>
</tbody>
</table>

### Table 2.15. Sequencing PCR reaction

<table>
<thead>
<tr>
<th>Solution</th>
<th>Vol required</th>
</tr>
</thead>
<tbody>
<tr>
<td>dsDNA (miniprep)</td>
<td>1 µl</td>
</tr>
<tr>
<td>Primer (10 nMol/ µl)</td>
<td>1 µl</td>
</tr>
<tr>
<td>Buffer (5x)</td>
<td>3.5 µl</td>
</tr>
<tr>
<td>Big Dye (V1.5)</td>
<td>1 µl</td>
</tr>
<tr>
<td>H2O</td>
<td>3.5 µl</td>
</tr>
</tbody>
</table>

### Table 2.16. Cycling parameters for sequencing PCR sequencing

<table>
<thead>
<tr>
<th>Stage</th>
<th>No. of cycles</th>
<th>Time</th>
<th>Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>1 min</td>
<td>96</td>
</tr>
<tr>
<td>2</td>
<td>25</td>
<td>10 sec</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5 sec</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>*4 min</td>
<td>60</td>
</tr>
</tbody>
</table>

#### 2.5.7 Lithium acetate aided yeast transformation

Parent strain (GP 3750) was revived from -80°C on YPD plate and 5 ml of starter culture was set for overnight growth at 30°C. Next day, cells were diluted with 50 ml fresh YPD to $A_{600}$ 0.5 and were grown for 5-6 hours to make sure that cells are exponential growth phase. Cells were harvested by centrifugation in centrifuge-415-K (Sigma) at 5100 rpm for 5 mins. Supernatant (medium) was discarded and
cell pellet was resuspended in 1 ml sterile water and sample was transferred into 1.5 ml microfuge tube. Cells were collected by centrifugation at 13,000 rpm for 2.0 mins, and were washed twice with 1 ml lithium acetate/ TE (LiAc/ TE). Finally for transformation, the cell pellet was resuspended in 0.5 ml LiAc/ TE. Two transformation reactions were carried out i.e. control (blank) and sample (transformation). The transformation reactions were set as described in Table 2.17.

### Table 2.17. Yeast transformation reaction

<table>
<thead>
<tr>
<th>Components</th>
<th>Control</th>
<th>Sample reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 µg Plasmid DNA</td>
<td>--</td>
<td>2-10 µl of miniprep</td>
</tr>
<tr>
<td>10 mg/ ml denatured ssDNA (salmon sperm)</td>
<td>5 µl</td>
<td>5 µl</td>
</tr>
<tr>
<td>Yeast competent cells (LiAc treated)</td>
<td>50 µl</td>
<td>50 µl</td>
</tr>
<tr>
<td>40% PEG/ 0.1 M LiAc</td>
<td>250 µl</td>
<td>250 µl</td>
</tr>
</tbody>
</table>

Samples were mixed by light vortexing or tapping the tubes with finger and were incubated at 30°C for 60 mins in a shaking incubator. Samples were given a 15 mins heat shock at 42°C in a water bath. Samples were centrifuges for ~5 sec in table top centrifuge, PEG was discarded and cells were resuspended in 200 µl SD medium. 100 µl of transformed cells was spread out on SD plate and were incubated at 30°C for 2-3 days in a static incubator. No growth on a plate with a sample from a blank tube and colonies on plates containing transformed cells indicated a successful transformation.

#### 2.5.8 Plasmid shuffling

5-Fluoro-orotic acid (5-FOA) medium is used to shuffle the plasmid (wild type gene-*URA3* as selectable marker) by co-transformation with another plasmid (mutant gene-different selectable marker). Cells deleted for the chromosomal gene that was complemented by the wild type allele on a plasmid with *URA3* marker were grown on 5-FOA. Cells with wild type *URA3* and *URA5* convert 5-FOA to a cytotoxic compound, fluorodeoxyuridine (FUMP), which inhibits the pyrimidine (uracil) synthesis. Thus the clones produced on FOA are without *URA3* plasmids (selects against *URA3*). Taking advantage of this, cells are transformed with mutant allele on a plasmid with a different selectable marker (*LEU2*). If the mutation is lethal, cells will not grow while in case of non-lethal mutations,
regional plasmid (selectable marker \textit{URA3}) will be replaced by mutant plasmid with a different marker (\textit{LEU2}) and clones will appear using the mutant gene from the replaced plasmid. Transformed cells were streaked out onto 5-FOA plates (Table 2.19) and were incubated for 3 days at 30˚C in a static incubator. Following the growth, colonies were re-streaked onto SD+Ura and SD plates. No growth on SD plates indicated that plasmid had been replaced with a plasmid containing \textit{LEU2} selection marker.

\textbf{Table 2.18. Solution used for yeast transformation}

<table>
<thead>
<tr>
<th>Solution</th>
<th>Quantity required</th>
</tr>
</thead>
<tbody>
<tr>
<td>* Tris-HCl 1 M (pH 7.5)</td>
<td>121.14 g Tris</td>
</tr>
<tr>
<td></td>
<td>HCl ~ 60 ml / 1000 ml</td>
</tr>
<tr>
<td>** Na$_2$EDTA 0.5 M (pH 8.0)</td>
<td>186.12 g Na$_2$EDTA</td>
</tr>
<tr>
<td></td>
<td>NaOH 20 g / 1000 ml</td>
</tr>
<tr>
<td>1x TE</td>
<td>10 ml 1 M Tris</td>
</tr>
<tr>
<td></td>
<td>2 ml 0.5 M Na$_2$EDTA/ 1 litre</td>
</tr>
<tr>
<td>1 M LiAc/ TE</td>
<td>10.2 g LiAc/ 100 ml TE</td>
</tr>
<tr>
<td>0.1 M LiAc/ TE</td>
<td>5 ml 1 M LiAc/ 45 ml TE</td>
</tr>
<tr>
<td>44% PEG/ 0.1 M LiAc</td>
<td>110 g PEG/ 155 ml TE</td>
</tr>
<tr>
<td>40% PEG/ 0.1 M LiAc</td>
<td>45 ml 44% PEG</td>
</tr>
<tr>
<td></td>
<td>5 ml 1 M LiAc/ TE</td>
</tr>
<tr>
<td>SD medium (Liquid and solid/ SD plates)</td>
<td>Section 2.2.3</td>
</tr>
</tbody>
</table>

* Solutions were filter sterilised except SD which was autoclaved.
* HCl was used to adjust pH. Solution was autoclaved and stored at RT.
** EDTA only dissolves if the pH of solution is adjusted to 8.0 by addition of NaOH pellet. Stock solution was stored at RT after sterilising by autoclaving at standard cycle.

\textbf{Table 2.19. 5-FOA plates}

<table>
<thead>
<tr>
<th>Solution</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-FOA</td>
<td>400 mg</td>
</tr>
<tr>
<td>2x SD</td>
<td>200 ml</td>
</tr>
<tr>
<td>Uracil (20 mM)</td>
<td>4 ml</td>
</tr>
<tr>
<td>*4% Agar</td>
<td>200 ml</td>
</tr>
</tbody>
</table>

* FOA and Uracil were dissolved in 2x SD and were sterilised by using an appropriate filter unit (pore size 0.22 µm).
* 4% autoclaved agar was added to the 5-FOA solution. Solution was poured into Petri plates. After drying 5-FOA plates were stored at 4˚C for extended periods of time.
2.6 Growth assays

Yeast growth in general, depends on translation factors and mutations in them can cause inhibition of overall translational leading to growth defects, i.e. slow growth phenotype, conditional and unconditional lethality, and sensitivity to a particular stress. These growth defects can be analyzed by different growth assays, which measures either the quantitative (section 2.6.1) or qualitative (section 2.6.2) aspects of yeast growth.

2.6.1 Yeast quantitative growth assays

The overall translation inhibition reduces the rate of yeast growth. The easy and simplest way to assay yeast growth is to measure the growth rate in liquid medium. The measurement of optical density is a very quick and easy way widely used to determine approximate number of cells in a culture. It is calculated as the logarithm of the ratio of the incident light to transmitted light. Optical density is equal to $log_{10} \frac{I_0}{I}$, where $I_0$ and $I$ are the intensity of the incident light and transmitted light respectively.

Optical density was measured by spectrophotometer (Eppendorf, Biophotometer Plus). Spectrophotometer is a machine that passes light of a known wavelength through cell suspension in a cuvette and measures the extent of unscattered light that passes through. Optical density in somewhat extent is relative to the number of cells. There is a direct linear relationship between yeast cell number and optical density ($A_{600}$) at low cell number. Above $A_{600} > 2$ the relationship between cell number and optical density departs from linearity and $A_{600}$ does not correspond to the true measure of cell number.

To measure the growth rate, single colony was picked with a sterile loop to inoculate 5 ml of liquid YPD/ SD medium in 30 ml sterile tube (International Scientific Suppliers). Culture was grown overnight at 30°C at 180 rpm in Innova®-44 shaking incubator (New Brunswick Scientific). In order to take an accurate measure of yeast density, 100 µl of overnight culture was diluted with 900 µl of medium in a 1.5 ml cuvette and light absorption was measured against 1 ml blank medium at $A_{600}$. Based on the absorbance value, overnight culture was diluted in 50 ml to $A_{600} 0.2$ in 250 ml round bottom flask. Cell density was
measured after every hour for the next 10-12 hours. Normal haploid strains used in laboratory doubles every 90 mins in YPD medium and approximately 140 min in SD medium (Sherman 1991).

In order to check the effect of environmental stress, i.e. physical (temperature shock) or chemical (drug/compound) on growth rate, cells were grown to $A_{600}$ 0.5-mid log phase. At this point culture was divided into two samples i.e. control and treated. In control samples, cells kept on growing under normal condition while in treated sample, cells were given chemical (i.e. rapamycin, butanol) or physical (temperature shock) treatment. Effect of drug on growth was monitored over the time course, i.e. after every hour for the next 10-12 hours using spectrophotometer. Growth rate was represented graphically by plotting $\log_{10} A_{600}$ at Y axis and linear scale with time in mins/ hours on X axis. Exponential curve fitting function was used to generate equation in the form of $Y = Ae^{Bx}$ where A and B are numbers. To calculate the doubling time (Td) ln2 (0.693) was divided by B, (number from the equation from the graph). The values in $A_{600}$ 0.2-1 range were considered as they represent the exponential phase of growth.

### 2.6.1.1 Spot Assays

A very informative and easy method to measure both quantitative and qualitative aspects of yeast growth is the "spot assays". In this assay, equal volumes (i.e. numbers) were spotted on the solid medium and then efficiency of plating of wild type and mutant strains was observed under normal and stress conditions.

Overnight grown culture from a single colony was diluted back to $A_{600}$ 0.2 as described in section 2.6.1 and grown for 2 more hours. 10-fold serial dilutions were made with fresh medium and 3 µl of each dilution was spotted on the solid medium and plates were incubated at 30°C for 3 days. Depending on the strain and growth medium, strains showed different efficiency of plating (EOP), i.e. strain not only grew slow but also form less/ small colonies. EOP provides information about growth rate measured by colony size and frequency of colony formation.
2. Materials and Methods

Alternative to this method, yeast growth can be evaluated qualitatively by streaking yeast strains on solid medium and observing colony size/number.

2.6.1.2 *Kirby-Bauer Disc Diffusion Susceptibility assay*

Another method to measure the sensitivity of yeast growth to a drug is through Kirby-Bauer Disc Diffusion susceptibility assay also known as halo assay. In this method, a disc soaked with a known concentration of a compound is placed on lawn of cells on an agar plate. Disc starts absorbing water from agar and the compound begins to diffuse from disc into the surrounding agar. As the rate of diffusion of drug is not very fast, this results in gradient distribution of drug i.e. highest concentration near the disk and logarithmic decrease along the distance from disk. The region of no cell growth known as zone of inhibition (diameter measured in mm) appears around a filter disc saturated with selected compound, which indicates the sensitivity of strain to that compound. The method described here is modified from the original protocol (Bauer et al. 1966).

Cells were collected from 10 ml of fresh overnight grown culture in YPD/SD to $A_{600}$ 0.5 by centrifugation at 3000 rpm in for 3 mins in centrifuge. Cells were resuspended in 1 ml fresh YPD/SD medium and 300 µl of this suspension was mixed with 0.6 % YPD/Agar (which was maintained liquid by incubating at 30°C. Tubes were gently swilled on rotor to mix cells with agar uniformly and this suspension was poured onto surface of pre-warmed YPD/agar plates. Plates were swirled gently to evenly cover the entire surface of the plate before the agar sets. Plates were incubated at 30°C for 4-5 hours. With sterilised tweezers, 4 sterilised discs of Whatmann 3MM filter paper (prepared by using paper hole punch)/plate (3 for the various concentrations of drug and one to be used for drug carrier i.e. DMSO) were placed in the lawn of cells (middle of four quarters of the plate). 5 µl of the drug dilution was added on the disc and plates were incubated overnight until lawn of cells appeared. Single Halo usually appeared around filter discs soaked with drug but leaving the plates for longer produced double halos consisting of a ring of no cell growth around the disk and an outer concentric ring with partial cell growth. Images were taken using BioRad Gel Doc XR and
Quantity1 software. Diameter (mm) of each halo around discs where no visible cell growth has occurred was measured.

Apparent sensitivity of a strain to a given drug was calculated by plotting halo diameter vs. drug concentration. To calculate the relative apparent sensitivity of a strain to a given drug/compound, halo diameter vs. log of drug concentration was plotted. Using the line equation, calculate the drug concentration needed to produce a halo of x diameter was calculated. "Relative apparent sensitivity" is calculated by dividing the drug concentration to produce a halo of x diameter on an experimental plate by the drug concentration required to produce a halo of the same x diameter by a wild-type strain (control plate).

2.6.1.3 Chemotolerance assays

Gradient plates supplemented with a stress reagent (i.e. Butanol and 3AT) were prepared according to the method as described previously by (Eaglestone et al. 1999, Richardson et al. 2004). 50 ml of YPD agar supplemented with a stress reagent was poured in Omni tray (Fischer Scientific UK Ltd). The plate was kept in a titled position and medium mixed with drug was poured into plate. The angle of plate was adjusted so as to distribute the drug maximum at one end and zero on other side of plate. 50 ml of YPD agar was added on the top and plate was kept level to settle the agar. Plates were dried at room temperature. Cultures were grown and back diluted to A_{600} 0.2. Three µl of this dilution was spotted across the gradient. Plates were sealed to avoid any evaporation and incubated for 3-5 days at 30°C.

2.7 Polysome profiling

Translation efficiency can be assessed in vivo by measuring abundance of polysome on sucrose density gradient sedimentation. Whole cell RNA extracts are prepared from growing yeast are layered onto a sucrose density gradient and are separated by ultracentrifugation. If the mutation or treatment of cells with any stress effects translation initiation step, then there are less polysomes (Polysome runoff) because of reduction of ribosomes translating a mRNA (due to defective ribosome loading), while defective elongation step results in increase of the
number of polysome on a mRNA (due to slow migration of translating ribosomes) (Lee et al. 2007). In cells ribosomes exist in two fractions, actively engaged in translation (polysome) and vacant (monosome) and their ratio changes according to the rate of translation in cells. Decrease in polysome fraction leads to increase in the monosomes fractions. Polysome abundance in whole cell extract is measured as polysome-to-monosome (P/M) ratio.

2.7.1 Whole cells extract preparation

Overnight grown starter culture of wild type and mutant yeast strains were diluted in 100 ml of appropriate medium (YPD/SD) at 30˚C. Once the culture reached to $A_{600}$ ~0.6, culture is paired into two flasks, 50 ml of the culture was transferred to a flask containing 500 µl of cycloheximide (Sigma) and cells were grown for 5 more mins at 30˚C. Cycloheximide was added to arrest and to preserve the polysomes in vivo during extract preparation. To left over portion of the culture (50 ml), cells were given treatment (physical/chemical) for a particular time. Five minutes before harvesting, 500 µl of cycloheximide was added and samples were incubated in 50 m falcon tubes (left overnight at 4˚C) in ice slurry for 30 mins. All the solutions and equipment used to make cell extract were left overnight at 4˚C.

Table 2.20. Cell lysis buffer for polysome analysis

<table>
<thead>
<tr>
<th>Solution</th>
<th>Component</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x lysis buffer</td>
<td>HEPES 1M</td>
<td>*200 mM, pH 7.4</td>
</tr>
<tr>
<td></td>
<td>MgAcetate 1M</td>
<td>20 mM</td>
</tr>
<tr>
<td></td>
<td>K Acetate 5M</td>
<td>1 M</td>
</tr>
<tr>
<td>1x lysis buffer</td>
<td>10x lysis buffer</td>
<td>20 ml</td>
</tr>
<tr>
<td></td>
<td>Cycloheximde 10mg/ml</td>
<td>2 ml</td>
</tr>
<tr>
<td></td>
<td>DTT</td>
<td>1 M 100 µl</td>
</tr>
<tr>
<td></td>
<td>DEPC water</td>
<td>upto 200 ml</td>
</tr>
</tbody>
</table>

* pH 7.4 was adjusted using KOH.

Cells were collected in ice cold 50 ml falcon tubes by centrifugation at 5100 rpm for 5 mins at 4˚C. Supernatant was discarded and cells were washed with 25 ml lysis buffer (Table 2.20). After decanting, cells were suspended in 800 µl lysis buffer and transferred to 1.5 ml eppendorf tube. Samples were centrifuged for ~30 sec at 5100 rpm and cells were re-suspended in 200 µl of lysis buffer by pipetting.
2. Materials and Methods

Chilled glass beads (~200 µl) were added and lysis was done by vortexing at 4°C for 6 consecutive cycles of 20-30 sec, with 40 sec intervals (ice slurry was used for quick chilling between cycles). Samples were centrifuged at 10,000 rpm for 10 mins, and clear supernatant was transferred to a fresh ice cold eppendorf. Process was repeated to obtain a clear supernatant. Total RNA concentration was measured at $A_{260}$ using nanodrop-8000 Spectrophotometer (Thermo Scientific) and 25 units of $A_{260}$ were loaded on sucrose gradients.

2.7.2 Sucrose density gradients preparation

Polysome profiling was done using 15-50% sucrose gradients. For this purpose 400 ml of 60% (w/v) sucrose solution was prepared. Sucrose solution was filter sterilised using 0.22 µm 500 ml filter sterilising unit. Five different concentrations of sucrose gradient were made by combing different volume of 60% sucrose solution with 10x polysome buffer and DEPC in 50 ml falcon tubes as described in Table 2.21. 2.25 ml of 50% solution of sucrose was poured into the polysome tubes and layers were frozen using liquid N$_2$. This process was repeated with 42%, 33.3%, 24% and 15% of sucrose solutions and between each stage liquid N$_2$ was added to the container to keep the layers frozen. Gradients were stored at -80°C.

**Table 2.21. Sucrose gradient solutions**

<table>
<thead>
<tr>
<th>Composition/ Sucrose conc</th>
<th>50%</th>
<th>42%</th>
<th>33.3%</th>
<th>24%</th>
<th>15%</th>
</tr>
</thead>
<tbody>
<tr>
<td>*10x polysome buffer</td>
<td>5.51</td>
<td>5.51</td>
<td>5.51</td>
<td>5.51</td>
<td>5.51</td>
</tr>
<tr>
<td>60% sucrose</td>
<td>45.93</td>
<td>38.59</td>
<td>30.63</td>
<td>22.05</td>
<td>13.78</td>
</tr>
<tr>
<td><strong>DEPC sterile distilled water</strong></td>
<td>3.67</td>
<td>11.03</td>
<td>18.99</td>
<td>27.56</td>
<td>35.83</td>
</tr>
</tbody>
</table>

*10x polysome buffer consists of 100 mM Tris Acetate pH 7.4, 700 mM Ammonium acetate and 40 mM Magnesium Acetate.

**100 µl of DEPC was added to 1 litre of water and mixed vigorously overnight by using magnetic stirrer. Next day solution was autoclaved to inactivate the remaining DEPC. DEPC water was stored at RT.

2.7.3 Procedure of sucrose gradient sedimentation

Sucrose gradients were thawed overnight at 4°C and were carefully loaded with 25 $A_{260}$ units of RNA extracts. Samples were centrifuged in a SW41 rotor at 40,000 rpm for 2.5 hours at 4°C in L-80 ultracentrifuge (Beckman).
60% sucrose was run through the UA-6 UV/ VIS detector (ISCO) to remove air bubbles and get a straight line on the chart. Sample was placed onto analyser and pump speed was set at 1 ml / 45 sec. The sucrose that came out of the tube was monitored and recorded using a filter for $A_{254}$ as the sample run into the optical unit. Signal is transmitted from optical unit to UA-6 detector, which records the polysome profile on the recorders chart paper. The parameters of the recorder were set according to the experimental requirements (Chart speed 1 cm/min, sensitivity at 0.5 and noise filter 5.0). The graphical representations of the polysome profiles obtained on chart were scanned using graphic software (Adobe Photoshop). Different settings for hue and saturation were used to obtain a sharp black and white image. Polysome to monosome ratio was calculated using appropriate graphic software ImageJ 1.46.

2.8 Biochemical methods

2.8.1 Cell lysis and protein extraction (small scale)

Cells were grown in 50 ml YPD in 250 ml round bottom flask with constant shaking at 30°C as described in section 2.5.7. Samples were moved to 50 ml sterile plastic falcon tubes and cells were collected by centrifugation at 5000 rpm for 5 mins at 30°C in centrifuge-415K (Sigma). The effect of different harvesting conditions on the phosphorylation of some of residues in eIF2Bε is discussed in detail in chapter 3. Medium was removed and cell pellet was washed twice with distilled water. Pellet was resuspended in 1 ml sterile water and cells were transferred to a screw capped 2 ml microfuge tube by re-suspending them in double volume of cell lysis buffer (Table 2.20). For cell lysis 2/3 volume of acid washed sterile glass beads were added in the sample and microfuge tubes were agitated at 6.0 m/sec for 30 sec in the Fastprep®-24 instrument twice with a five mins interval in which tubes were kept on ice slurry to chill the tubes (chilling inhibits the activation of proteases). Supernatant was transferred to another screw capped microfuge tube and was subject to centrifugation at 13000 rpm for 30 mins at 4°C to remove large cell debris. Supernatant was transferred to a fresh microfuge tube. Protein concentration was measured using Bradford assay.
Table 2.22. Composition of lysis buffer

<table>
<thead>
<tr>
<th>Solution</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 M Tris-HCl (pH 8.0)</td>
<td>100 mM</td>
</tr>
<tr>
<td>1 M KCl</td>
<td>500 mM</td>
</tr>
<tr>
<td>1 M MgCl₂</td>
<td>5 mM</td>
</tr>
<tr>
<td>*14.3 M β-Mercaptoethanol</td>
<td>7 mM</td>
</tr>
<tr>
<td>50% Glycerol</td>
<td>10%</td>
</tr>
<tr>
<td>10% TritonX-100</td>
<td>0.1%</td>
</tr>
<tr>
<td>1 M NaF</td>
<td>5 mM</td>
</tr>
<tr>
<td>*1 mg/ml Pepstatin</td>
<td>1 µg/ml</td>
</tr>
<tr>
<td>*1 mg/ml Leupeptin</td>
<td>1 µg/ml</td>
</tr>
<tr>
<td>*1 mg/ml Aprotinin</td>
<td>5 µg/ml</td>
</tr>
<tr>
<td>0.5 M PMSF</td>
<td>2.5 mM</td>
</tr>
<tr>
<td>*PhosStop tablet (Roche)</td>
<td>1x</td>
</tr>
<tr>
<td>*EDTA-Free protease inhibitor tablet</td>
<td>1x</td>
</tr>
</tbody>
</table>

* Added at the time of use. Lysis buffer was stored at 4°C.

2.8.2 Quantification of protein in whole cells extract-Bradford assay

Two different methods were used for the determination of total protein concentration in a sample. The choice of methods depends on the protein sample i.e. whole cell extract or purified protein.

Bradford assay based on the method developed by Bradford (Bradford 1976) was routinely used to estimate the protein concentration in whole cells extract using Bradford reagent (Bio-Rad). Protein sample was diluted and 10 µl of each dilution was added to 1 ml of 20% diluted Bradford reagent in a cuvette. The red dye turned blue upon binding to protein. The protein concentration was measured by $A_{600}$ in spectrophotometer. A standard curve was generated using known concentrations of Bovine Serum Albumin (BSA) ranging from 0.5 µg to 10 µg BSA. Absorbance against BSA concentration was plotted to make a standard linear graph. The comparison with the standard curve gave a relative measure of protein concentration. As $A_{600} = 2$ saturates and readings above 2 are not reliable, samples with $A_{600} > 2.00$ were diluted 10 times to obtain $A_{600} < 2.00$.

2.8.3 Purification of FLAG-tagged eIF2B

Yeast strains expressing FLAG-tagged GCD1 subunit of eIF2B were grown and harvested as described previously in section 2.5.7. Cell lysis and protein extraction was carried out as described above in section 2.8.1. The method used
for the purification of protein uses antiFLAG resins (Sigma) and is modified from the previously published method (Mohammad-Qureshi et al. 2007). 60 µl of 50% view red antiFLAG resins (Sigma) were washed twice with chilled lysis buffer kept on ice. Storage buffer was removed and resins were collected by centrifugation at 2000 rpm for 2 mins at 4°C. 1 ml of the whole cell extract with 1 mg of protein concentration was added to resins and samples were incubated for 2 hours at 4°C with constant rotation. Resins were collected by centrifugation at 4000 rpm for 5 mins at 4°C. In order to remove unbound and non-specifically bound proteins, resins were washed twice with ice-cold 250 µl wash buffer at 4000 rpm for 5 mins. Supernatant was discarded and protein was eluted by using 50 µl of elution buffer (1x antiFLAG peptide in wash buffer) for 30 min at 4°C with constant rotation. Supernatant was collected after spinning the sample for 3 mins at 13000 rpm at 4°C and stored at -20°C or kept on ice for further operational purposes.

### Table 2.23. Solutions required for FLAG tagged protein purification

<table>
<thead>
<tr>
<th>Solution</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wash buffer</td>
<td>Lysis buffer as detailed in Table 2.20 except KCl 100 mM</td>
</tr>
<tr>
<td>(^b)Elution buffer</td>
<td>same as wash buffer with 1x antiFLAG peptide</td>
</tr>
<tr>
<td>(^c)4x LSB</td>
<td>200 mM Tris-HCl (pH 6.8) 8% SDS 0.4% Bromopenol blue 50% Glycerol 5 mM β-Mercaptoethanol</td>
</tr>
</tbody>
</table>

\(^a\)Wash buffer is same as high salt lysis buffer (Table 2.20) except concentration of KCl is reduced to 100 mM. Wash buffer was stored at 4°C.  
\(^b\)Elution buffer was made by adding 1x antiFLAG peptide to wash buffer.  
\(^c\)1X LSB was made by diluting 4x LSB in lysis buffer.

#### 2.8.3.1 Quantification of purified protein using nanodrop

The concentration of FLAG purified protein sample was estimated by measuring absorbance at 280 nm \(A_{280}\) using nanodrop-8000 spectrophotometer (Thermo Scientific). A rough approximation for protein concentration is 1 unit of \(A_{280}\) proteins is almost equal to 1mg/ ml of protein.
2. Materials and Methods

Regulation of eIF2B by Phosphorylation

Figure 2.2. eIF2B purification using anti-FLAG M2 affinity resins.

Main steps of purifying FLAG-tagged eIF2B proteins from yeast whole cell extracts using anti-FLAG M2 affinity agarose gel are outlined in diagram (see section 2.8.3 for details).
1-5 μg of purified protein was added to 100 μl 1 x Laemmli Sample Buffer (LSB) and boiled at 95°C for 5 mins before loading onto SDS gel.

2.8.4 Dephosphorylation of purified protein
Proteins were purified as described above in previous section, but instead of boiling with 4x LSB, proteins were dephosphorylated while still bound to resins. 0.5-1.0 unit of λ phosphatase was added to the purified protein bound to the antiFLAG view red resins. 10 μl of each of 1x phosphatase buffer and MnCl₂/μg of the purified protein were added and samples were incubated for 45-60 mins at 30°C. Resins were collected by centrifugation at 2000 rpm for 2 mins at 4°C and washing was done twice with 200 μl of lysis buffer. Samples for gel loading were prepared by adding 60 μl of 4 x LSB to resins and boiling the beads at 95°C for 5 mins. Supernatant was collected by centrifugation at 5000 rpm for 1 min. Now samples were ready for loading onto the acrylamide gels.

2.9 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)
SDS-PAGE is used to separate proteins according to their molecular weight. LSB contains SDS which removes secondary structures in proteins and gives negative charge to proteins so the distance travelled by protein corresponds to the size of protein and hence molecular weight.

2.9.1 Sample Preparation for SDS-PAGE
Loading samples were prepared by mixing protein extract with 1x LSB (Table 2.21) to make a sample of ~100 μg/100 μl of protein concentration while 1-5 μg/100 μl of sample. Samples were boiled at 95°C for 5 mins. 15 μl of sample was loaded onto the gel. Standard known molecular weight markers (colour plus pre-stained markers- New England BioLabs) were used as size guide to measure size of protein.

2.9.2 SDS-PAGE
SDS-PAGE gel consists of two parts, i.e. stacking and resolving gel. 12.5% Resolving gel was prepared by combining the components described in Table 2.24.
and 6 ml of solution was poured in the gel cassette-1.00 mm (Invitrogen). A layer of 70% ethanol was added on the top of resolving gel to remove air bubbles and gel was left at room temperature for 5-10 mins to polymerise.

Stacking gel was prepared as by mixing components listed in Table 2.24 and was poured onto the top of the resolving gel after removing 70 % ethanol. A 12 well comb was inserted in the gel cassette and the gel was left to polymerise completely. Seals were removed from the gel cassettes and cassettes were loaded into the gel tank (Invitrogen). Inner chamber of the gel tank was filled with 1x running buffer and comb was removed. Sample were run at 120 V was for 2 hours.

**Table 2.24. 12.5% polyacrylamide gel reagents**

<table>
<thead>
<tr>
<th>Solution</th>
<th>Resolving Gel</th>
<th>Stacking Gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>aAcrylamide/ Bis-acrylamide</td>
<td>4 ml</td>
<td>1.7 ml</td>
</tr>
<tr>
<td>1.5 M Tris-HCl (pH 8.8)</td>
<td>2.5 ml</td>
<td>-</td>
</tr>
<tr>
<td>1 M Tris-HCl (pH 6.8)</td>
<td>-</td>
<td>1.3 ml</td>
</tr>
<tr>
<td>10% (w/ v) SDS</td>
<td>100 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td>10% (w/ v) Ammonium persulphate</td>
<td>100 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td>*TEMED</td>
<td>8 µl</td>
<td>4 µl</td>
</tr>
<tr>
<td>H2O</td>
<td>3.3 ml</td>
<td>3.5 ml</td>
</tr>
<tr>
<td>1x Running buffer</td>
<td>192 mM Glycine,</td>
<td></td>
</tr>
<tr>
<td></td>
<td>25 mM Tris,</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.1% SDS</td>
<td></td>
</tr>
</tbody>
</table>

aReadymade solution of Acrylamide/ Bis-acrylamide 30% / 0.8% w/v (Sigma-Aldrich) was used to make gel.

*TEMED (Sigma) is stored at 4°C and was added just prior to pouring.

### 2.9.3 Gel staining

Gel was removed from the gel cassette and stained with instant blue (Bio-Rad). The gel was stained until separate bands appear on the gel. Stained gel was washed with distilled water to remove excess stain.

### 2.10 Western blotting

Blotting refers to the transfer of DNA/ RNA or protein sample from a gel to a membrane followed by subsequent detection on the membrane. Western blotting (also known as immuno blotting because immobilized antigen is detected by a
specific antibody) technique introduced in 1979 by Towbin (Towbin et al. 1979) is now routinely used for protein analysis. This technique produces qualitative and semi quantitative data for the protein. It is a multi-step procedure consisting of separation, transfer and detection of proteins.

### 2.10.1 Western transfer

Following SDS electrophoresis, proteins were transferred to a nitrocellulose membrane. Electrophoretic transfer method because of its speed and transfer efficiency is used to transfer protein from gel to membrane. Four sponges and two sheets of 3 MM Chromatography filter paper (Whatman®) were soaked in chilled western transfer buffer. For making the sandwich, two sponges followed by a 3 MM filter paper and a sheet of Protran-Nitrocellulose membrane (Whatmann®) soaked in western transfer buffer (20% Methanol, 25 mM Tris, 192 mM Glycine) were placed and gel was layered on the top of nitrocellulose membrane soaked in transfer buffer. Air bubbles were removed and chromatography filter paper was placed onto the gel surface. Two more sponges were placed on the filter paper and this sandwich was assembled in an XCell II™ blot Module (Invitrogen) and was clamped in the gel tank between two electrodes submerged in a conducting solution (transfer buffer). The assembly of sandwich for western transfer is shown in Figure 2.2. Electric field (25 V for 2 hours) was applied, which forces the proteins to move out from the gel and attach tightly to the surface of membrane. Before proceeding with the western blot, membrane was stained. Ponceau Stain was used because it is easily removable and reversibly stains protein on a membrane. After staining, nitrocellulose membrane with same pattern of proteins that was observed in the PAGE gel was seen. PAGE gel was stained with Instant Blue (BioRad) to check the transfer efficiency.

### 2.10.2 Immunoblotting and protein detection

Western blotting consists of series of incubations with different solutions separated by washing steps. Once the transfer has taken place, membrane was washed for three times for 10 mins in 1x Tris buffered Saline Tween (TBST).
2. Materials and Methods

Figure 2.3. Western blotting

A. Assembly of sandwich for electrophoretic transfer of proteins from Polyacrylamide gel to membrane. B. Protein detection using enzyme conjugated secondary antibodies. (For details see section 2.10)
2. Materials and Methods

The 1 hour incubation in blocking milk at RT was followed by additional three washes each for 10 mins with 1x TBST. Blocked membrane was probed with primary antibody dilution (Table 2.26) that recognised specific protein or epitope for at least 1 hour at room temperature or overnight at 4°C. The membrane was washed three times for 10 mins in 1x TBST. Different methods are used for protein detection but enzyme conjugated antibodies are preferable because of the flexibility in detection and documentation methods. 1:5000 dilution of anti rabbit IgG coupled to horseradish peroxidase was prepared in 1x TBST and the membrane was incubated for 1 hour at RT with constant rotation. The mechanism for the detection of antigen is outlined in Figure 2.3.

### Table 2.25. Solutions used for western blotting

<table>
<thead>
<tr>
<th>Solution</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>aTBST (pH 8.0)</td>
<td>10 mM Tris</td>
</tr>
<tr>
<td></td>
<td>150 mM NaCl</td>
</tr>
<tr>
<td></td>
<td>1% Tween 20</td>
</tr>
<tr>
<td>Blocking solution</td>
<td>5% (w/ v) Non-fat dried semi skimmed milk (Marvel)</td>
</tr>
<tr>
<td></td>
<td>Alternatively 5% BSA and 2% semi skimmed milk</td>
</tr>
</tbody>
</table>

*pH was adjusted using HCl before the addition of Tween 20.

After incubation membrane was washed as before. Proteins were detected using the western lightning™-ECL detection kit (PerkinElmer). X-ray film is most sensitive method to detect signal so the signal was detected and documented onto Kodak BioMax MS autoradiography film which was developed in X-ray film processor, Mini medical series (AFP Imaging Corp).

2.11 Antibodies

Synthetic peptides (phosphorylated and non-phosphorylated versions of peptides) were designed by R. Tyler and custom antibodies were commercially synthesized by CRB Ltd. in Fmoc solid phase. Sequence of synthetic peptides with phosphorylation sites Gcd6p-pS435, Gcd6p-pS525, Gcd6p-pS528, Gcd6p-pS538, Gcd1p-pS296, Gcd1p-pS300 and Gcd1p-pS32/ pT33) are shown in Table 2.24 and Figure 3.4, 3.5 B, C).
2. Materials and Methods

Each synthetic peptide had the flanking 5-6 amino acids at both sides of the target residues. Five or six amino acid residues are considered to constitute an antigen epitope that can be recognized by an antibody molecule. In order to conjugate this peptide to a carrier protein, a Cys residue was attached at the amino-terminal side of the synthetic peptide. Blood from the rabbits was collected before immunization as control serum. Rabbits were immunized by injecting adjuvant/antigen (phospho-peptide) emulsion and booster injections were given after every four weeks. Blood was collected 10-14 days after booster injection. Crude antisera were purified by affinity chromatography by Cambridge Research Biochemicals (CRB) Ltd. For purification crude extract was passed down a Thiopropyl Sepharose 6B column derivatised with the non-phospho peptide twice to deplete any antibody that was not phospho-specific. The eluted liquid fraction was then applied to Thiopropyl Sepharose 6B column derivatised with the phosphorylated peptide. The glycine elution of phospho-column in PBS was supplied by Ltd.

Secondary antibodies coupled to horseradish peroxidase and His-antibodies were used for the detection of primary antibodies conjugated to specific sites in the eIF2B. Table 2.26 contains the list of all the customized and commercially available antibodies used in this study.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Antigen Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT Gcd6p (CM 003)</td>
<td>Polyclonal antibody-Lab source</td>
</tr>
<tr>
<td>Gcd6p pS525 (RTYL-1)</td>
<td>Acetyl-KRRTM-(pS)-VN-*Ahx-[C]-amide</td>
</tr>
<tr>
<td>Gcd6p pS435 (RTYL-2)</td>
<td>[C]-Ahx-TKISA-(pS)-PLKNA-amide</td>
</tr>
<tr>
<td>Gcd6p pS528 (RTYL-3)</td>
<td>Acetyl-VN-(pS)-ITYDR-Ahx-[C]-amide</td>
</tr>
<tr>
<td>Gcd6p pS538 (RTYL-4)</td>
<td>[C]Ahx-REEID-(pS)-EF-Ahx-amide</td>
</tr>
<tr>
<td>WT Gcd1p</td>
<td>Polyclonal antibody</td>
</tr>
<tr>
<td>Gcd1p pS296 (RTYL-5)</td>
<td>[C]-Ahx-GPQSM-(pS)-RQA-amide</td>
</tr>
<tr>
<td>Gcd1p pS300 (RTYL-6)</td>
<td>[C]-Ahx-RQA-(pS)-FKDPF-amide</td>
</tr>
<tr>
<td>Gcd1p pS32/T33 (RTYL-7)</td>
<td>[C]-Ahx-TQNKD-(pS)-(pT)-AATSG-amide</td>
</tr>
<tr>
<td>His</td>
<td>Commercial-Becton Dickinson Ltd</td>
</tr>
<tr>
<td>eIF2</td>
<td>Lab source</td>
</tr>
<tr>
<td>eIF2α (p)</td>
<td>Commercial antibody-Invitrogen</td>
</tr>
<tr>
<td>Secondary antibody</td>
<td>Anti-rabbit coupled to **HRP</td>
</tr>
<tr>
<td>Secondary antibody</td>
<td>Anti-mouse coupled to HRP</td>
</tr>
</tbody>
</table>

*Ahx= aminohexanoic acid

** HRP= Horseradish peroxidase
2.12 Bioinformatics

Protein sequence alignment was carried out using ClustalW2 (Larkin et al. 2007, EMBL-EBI 2011). The presence of SDM in plasmid was confirmed by comparing the plasmid sequence to the Genbank database sequence maintained by *Saccharomyces* Genome database (SGD) by using BLAST(N) (Altschul et al. 1997). Following phosphorylation sites prediction software were used to predict phosphorylation sites in Gcd1p and Gcd6p along with potential kinase/phosphatase i.e. NetPhosYeast 2.0 (Blom et al. 1999, Technical University of Denmark 2012), NetphosYeast 1.0 (Ingrell et al. 2007, Technical University of Denmark 2012), PhosphoGrid (Stark et al. 2010, Sadowski et al. 2012), PHOSIDA (Gnad et al. 2011, Gnad and Mann 2011), Phosphopep v20 (Bodenmiller et al. 2008, Bodenmiller and Campbell 2012) and KinasePhos 2.0 (Wong et al. 2007, Wong et al. 2012).
Chapter 3

Optimising conditions to evaluate the roles of multiple phosphorylation sites in eIF2B
3 Optimising conditions to evaluate the roles of multiple phosphorylation sites in eIF2B

Many proteins undergo post-translational modifications and one of the most common modifications is reversible phosphorylation, which occurs both in prokaryotes and eukaryotes. The term "phosphorylation" refers to the addition of a phosphate group to one of the amino acid residue side chain (e.g. Ser, Thr, His and Tyr). The phosphorylation/ dephosphorylation mechanism is the most common way to respond to changes in the environment used by a cell because it is rapid, reversible and does not require new proteins to be synthesized or degraded, e.g. the phosphorylation/ dephosphorylation of eIF2α and 4E-BP1 affects translation initiation in eukaryotes. Protein surfaces are designed for biological interactions with other molecules and addition of a phosphate (PO$_4^-$) molecule to a polar R group of an amino acid residue can turn a hydrophobic portion of protein into a polar and hydrophilic portion of molecule. Phosphorylation can lead to conformational changes in the structure of the phosphorylated protein due to changes in the interactions of hydrophobic and hydrophilic residues in the vicinity of the modified amino acid.

It is estimated that 30% of the proteins present in mammalian cells are phosphorylated (Manning et al. 2002). *S. cerevisiae* have 30 proteins with phosphatase activity (Arino 2002, Arino et al. 2007) and almost 160 proteins with kinase activity (Mok et al. 2010). The structural and functional homologues of these proteins are present in plants and animals, which indicate the extensive use of this control mechanism in regulating different aspects of cellular events. Phosphorylation activates/ deactivates while creating new binding sites in proteins to interact with other proteins/ substrates (Figure 3.1). Once activated, cellular phosphorylation pattern changes with many proteins being phosphorylated and dephosphorylated. Cells regulate a diverse set of processes, for example, cell signalling, cell cycle, cellular movement, reproduction, apoptotic pathways and metabolism through the use of phosphorylation cycles and cascades.
Figure 3.1. Reversible phosphorylation and regulation of protein activity.

Phosphorylation can either activate (Orange) or inactivate (green) a protein. Kinases themselves are phospho-proteins and their activity is regulated by phosphorylation. Kinases phosphorylate the proteins while phosphatase enzyme dephosphorylates the proteins. Phosphorylation/ dephosphorylation leads to conformational changes in the structure of the proteins creating new binding sites for substrates.
3. Optimization of working conditions

3.1 Background of current project

Several different phosphorylation sites are known for mammalian eIF2B with their cognate kinases, which regulate the activity of eIF2B in response to different signals (Singh et al. 1994, Wang et al. 2001, Wang and Proud 2008). The regulation of eIF2B activity by phosphorylation in response to diverse environmental conditions in mammals is discussed in detail in introduction section 1.7.5.

This study examines the role(s) of eIF2B phosphorylation in yeast. It continues the work initiated by Mohammad-Qureshi, S. and Tyler, R., two post doctoral members of Pavitt laboratory who laid the foundation for this study. Their main findings are summarised in this section. eIF2B complex was purified by use of antiFLAG affinity resins from cells over-expressing all five subunits (α-ε) of eIF2B. Phosphorylation of yeast eIF2B was confirmed by the treatment of purified eIF2B complex with λ phosphatase, which revealed multiple banding pattern shifts on SDS-PAGE for Gcd1p and Gcd6p subunits (Figure 3.2A). Further investigation of the phosphorylation status of purified catalytic subcomplex (Gcd1p/ Gcd6p) by the use of Phos-tag staining, which is specific to phosphorylated proteins also suggested modifications of the protein (Figure 3.2B and C). Phospho-sites in catalytic subunits eIF2Bγ and eIF2Bε were identified by using Tandem Mass Spectrometry (MS): a versatile and most comprehensive tool to identify phosphorylation sites in biological molecules (Figure 3.2 and other unpublished data). The positions of identified phospho-sites relative to surrounding sequences in whole gene are shown in Figure 3.4 and Figure 3.5. As it is clear from the Figure 3.4 that three out of four identified phospho-sites in eIF2Bε (ε-S525, ε-S528 and ε-S538) are located in the ε\textsuperscript{cat} domain while S435 is the only site that lies outside catalytic region in LβH domain. To see the conservation of these sites among different species multiple sequence alignment was done using Clustal Omega (Figure 3.3). These sites are not conserved among various organisms. S525 and S528 residues are present in a Ser rich sequence in S. cerevisiae whilst in Homo Sapiens phosphomimetic acid residues are present at these positions. These positions seem to be important from structural point of view and they might play an important role in the catalytic function of eIF2Bε.
Figure 3.2. Evidence that eIF2B subunits are phosphorylated.

A. Coomassie stained SDS Polyacrylamide gel of FLAG purified eIF2B. Dephosphorylation of proteins with λ phosphatase (Lane 2 and 4) contracts the doublets of Gcd6p and Gcd1p into single bands. 12.5% SDS Polyacrylamide gel stained with B. Comassie blue for total proteins and same gel stained with C. Phos-Tag specific for only phospho-proteins. M represents molecular marker of known protein sizes and 1, 2 and 3 denotes increasing order of protein loading (adapted from Mohammad-Qureshi, S.).
3. Optimization of working conditions

Regulation of eIF2B by Phosphorylation

Figure 3.3. The conservation of the selected phospho-ser residues of eIF2β.

Multiple sequence alignment of the catalytic domains of eIF2Bε was performed using Clustal Omega for the organisms listed and alignment file was processed in Jalview. * indicate the position of phospho-sites in Gcd6p. Red colour represents small and hydrophobic aromatic amino acids, blue acidic, pink basic, green hydroxyl and sulfhydryl amino acids.

To help uncover the functional occurrence and importance of phosphate at each of these sites, specific mutated genes were (Gcd6p-S435A, Gcd6p-S525A, Gcd6p-S528A, Gcd6p-S538A, Gcd1p-S32A/T33A, Gcd1p-S296A and Gcd1p-S300A) produced by site directed mutagenesis on GCD1 and GCD6 cloned genes in low and high copy vectors. The vectors with desired mutation were introduced into yeast strains deleted for the chromosomal copy of respective gene. Phospho-Ser specific synthetic peptides were designed and custom antibodies were commercially synthesized by CRB Ltd.
3. Optimization of working conditions

Regulation of eIF2B by Phosphorylation

Figure 3.4. Production of phospho-Ser specific eIF2Bε antibodies.

A. Phosphorylation sites were identified through MS. B. Sequence of Gcd6p with selected residues marked on it. C. Synthetic peptides with phosphorylation at selected Ser residues in eIF2Bε were synthesized. Phospho serine-specific antibodies were raised by injecting synthetic peptides into rabbits. Crude antiserum was purified by affinity chromatography by CRB Ltd.
3. Optimization of working conditions

Regulation of eIF2B by Phosphorylation

Figure 3.5. Production of phospho-Ser/ Thr specific eIF2Bγ antibodies.

A. Phosphorylation sites were identified through MS in Gcd1p. B. Sequence of eIF2Bγ with selected residues marked on it. C. Sequence of synthetic peptides with phosphorylation at selected Ser/ Thr residues in eIF2Bγ. Customised phospho Ser/ Thr specific antibodies were raised by injecting synthetic peptides into rabbits. Crude antiserum was purified by affinity chromatography by CRB Ltd.
3. Optimization of working conditions

3.2 Experimental strategy adopted to monitor growth phenotypes and changes in phosphorylation

Diverse signalling pathways regulate the translation in yeast in response to nutrient and environmental challenges. It is seen from the literature that the duration of the log (exponential) phase depends on the initial number of cells and environmental conditions such as temperature, pH, alcohol, oxygen, salt concentration and nutrients etc. Depending upon the earlier studies carried out by many research groups in eukaryotes, different conditions which are previously known to inhibit translation initiation and growth (with or without any change in the eIF2α phosphorylation) were selected (Table 3.1).

Stress conditions were selected depending on their effects on yeast growth (in particular on translation initiation), and the feasibility to carry out the experiments. An experimental strategy was designed for monitoring the stress responses in yeast. Different assays were decided, which differ in their sensitivity and measure various aspects of yeast growth. Main techniques used in this study are growth assays, halo assays, polysome analysis and western blotting. Schematic outline of this strategy is shown in Figure 3.6. Cells (wild type and mutants for selected phospho-Ser) were grown under both normal and stress conditions, growth phenotypes and changes in phosphorylation at selected residues were monitored by observing and quantifying different aspects of growth, i.e. slow growth or sensitive phenotype, effect on doubling time and mRNA translation initiation.

The customized antibodies used for monitoring phosphorylation status of eIF2B were not tested/ validated earlier so the working conditions were optimized and the phospho-Ser specificity was checked. This chapter is divided into three parts; first part describes the data obtained during optimization of Gcd6p phospho antibodies using strains over-expressing Gcd6p. Second part is related to troubleshooting for phospho-Gcd1p antibodies. Third part consists of a detailed description of protocols designed for eIF2B purification from strains with low copy plasmids and affects produced by harvesting conditions on the
phosphorylation of eIF2 and eIF2Bε at the selected residues while working with large culture (2 litres).

Table 3.1. Stress conditions known to affect the growth and translation initiation.

<table>
<thead>
<tr>
<th>Stress</th>
<th>Conc/ Time</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TOR signalling</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rapamycin</td>
<td>100 nM / ml, 45 mins</td>
<td>(Loewith et al. 2002)</td>
</tr>
<tr>
<td>Torin1</td>
<td>20 μM / ml, 60 min</td>
<td>(Thoreen et al. 2009)</td>
</tr>
<tr>
<td>Amino acid stress</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(3-AT)</td>
<td>25 mM, 60 mins</td>
<td>(Hinnebusch 2005)</td>
</tr>
<tr>
<td>Chemical shock</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Butanol</td>
<td>2%, 10-15mins</td>
<td>(Ashe et al. 2001)</td>
</tr>
</tbody>
</table>

Figure 3.6. Experimental strategy designed to monitor stress responses in yeast.

Growth phenotypes were determined by growth assays in liquid and solid medium with and without stress/ inhibitor. Polysome analysis was done to measure translational efficiency and phosphorylation status of Gcd1p and Gcd6p was measured by western blotting using customized phospho-specific antibodies.
3.3 Optimization of the working conditions

This chapter describes the optimization of working conditions for newly synthesized customized phospho-Ser/Thr specific Gcd6p and Gcd1p antibodies. The optimization of cell harvesting conditions for working at large scale (2 litres) with minimal stress was also done during the course of study. Impact of multiple stresses on the phosphorylation pattern of proteins (eIF2β and eIF2α) is also discussed at the end of this chapter. As these were newly synthesized antibodies, which were not commercially available, there was a need to validate the phospho-specificity and establishing optimal working conditions (blocking and antibody dilutions) for these antibodies. Initially the antibody concentration was optimized using strains over-expressing all eIF2B sub-units from plasmids. For the purpose of monitoring physiological changes in phosphorylation pattern, yeast strain GP3688 was used (Table 2.1) in which chromosomal GCD6 is the only source of protein and GCD1 is FLAG tagged. Whole eIF2B complex was purified using antiFLAG affinity resins (Sigma Aldrich) and used for immunoblotting with specific antibodies.

3.3.1 Confirmation of the GCD6 mutations through DNA sequencing

To study the consequences of the mutations of the conserved residues in eIF2B, on its activity in the cellular process of translation initiation, plasmids bearing mutations were generated. The sequencing reaction was carried out as described in materials and methods (section 2.5.6). Whole GCD6 gene was sequenced using primers listed in Table 2.14.

Results of the sequencing are shown in Figure 3.7. After BLAST the sequence was translated to the amino acid sequence by Translate Tool-ExPASY to confirm that the new codon generated with a single nucleotide change resulted in a Ser to Ala mutation in Gcd6p.
3. Optimization of working conditions

Figure 3.7. Confirmation of mutations by DNA sequencing.

The images shown here were viewed in Trace Viewer FinchTV 1.4. The shaded blue bars in images represent the codon sequence coding for Ala (mutated). Plasmids carrying these mutations were used for transformation of GP3750.
3.4 Impact of Ser to Ala mutations on yeast growth and morphology

3.4.1 Gcd6p-S435A, S525A, S528A and S538A mutations are non-lethal
High copy plasmids (with LEU2 marker) carrying Ser to Ala substitutions in Gcd6p at S435, S525, S528 and S538 sites were transformed into GP 3750. Plasmids were shuffled using 5-FOA medium. Substitutions of Ser with non-phosphorylatable Ala residues at 435, 525, 528 and 538 do not affect the growth of yeast strains under optimal growth conditions and on 5-FOA medium (Figure 3.8).

To investigate the impact of selected Ser to Ala mutations on yeast growth rate, time course experiment was done in liquid medium. No significant difference was observed in these eIF2Bε phospho mutants as compared to cells carrying wild type GCD6 (Figure 3.9). The growth assays showed that Ser to Ala substitutions at selected residues in eIF2Bε are non-lethal under optimal growth conditions.

3.4.2 Gcd6p- S435A, S528A and S538A mutations affect cell size
To investigate the impact of mutations on cell morphology, actively growing cells over-expressing wild type and mutant gcd6 (A595 0.5) were observed under light microscope. Cells looked the same and no remarkable variation was noticed, cell sizes were measured using Cellometer® Auto M10 (Nexcelom Biosciences). 20 μl of samples were pipette out in the counting chamber of the special slides (Nexcelom Bioscience) and data for cell sizes and cell number was obtained on excel sheets. The visual representation of the distribution of cells according to cell sizes in different samples is shown in BoxPlot graph (Figure 3.10).

Statistical analysis of the data showed that mutations at selected phospho-sites affect cell sizes but the affect is not uniform for all mutations. The S435A and S538A mutants are of larger cell size while a decrease in cell size of S528A mutants was observed. There was no significant affect of S525A mutation on yeast cell size. This experiment suggests that although mutations at these sites are non-lethal, but they play a role in cell morphology. These sites might play a role in cell physiology under stress conditions.
Figure 3.8. Construction of *GCD6* mutant strains using over-expressing subunits from plasmids.

Transformation was done using LiAc and 5-FOA medium was used for plasmid shuffling. Growth of over-expressing wild type and *gcd6* mutant strains on two different medium is shown, SD+Ura (bottom right) and 5-FOA medium (centre plate).
3. Optimization of working conditions

Regulation of eIF2B by Phosphorylation

**Figure 3.9.** Ser to Ala mutations at 435, 525, 528 and 538 in "ε" subunit do not affect growth under optimal conditions.

Cells over-expressing wild type and mutant gcd6 were grown in 50 ml YPD medium. Optical density was measured at 595 nm every 60 mins for 8 hours. Values are plotted as time against log10 A595. The results shown here are from three independent experiments. Different colours and markers are used for each strain.

**Figure 3.10.** Effects of Gcd6-S435A, S528A and S538A mutations on cell size.

Cells expressing wild type and mutant gcd6 on plasmids were grown to mid log phase in YPD medium. 20 μl of each sample was taken and cell sizes were measured using cellometer 5-95% of the data is shown in box plot graph with middle box represents 25 - 75% of the cell in that size range, middle line is median of the population. Upper and lower 5% values are shown as outliers. ***p < 0.001 from analysis of variance by Anova.
3. Optimization of working conditions

3.4.3 Ser to Ala substitutions of selected residues in eIF2Bε do not change the expression levels of Gcd6p

To study the effect of selected eIF2Bε Ser to Ala mutations on Gcd6p expression level, Gcd6p expression level was measured in all mutants by western blotting. Yeast strains expressing high copy plasmids were grown in YPD medium (50 ml), cell lysis and protein extraction was done as described in materials and methods (section 2.8.1). The western presented in Figure 3.11 shows that Ser to Ala substitutions in Gcd6p at selected sites do not affect the expression level of Gcd6p protein in cells.

![Western Blot](image)

Figure 3.11. Gcd6p expression level remains unaffected by Ser to Ala mutations at selected residues.

Whole cell extracts from wild type and mutant strains were prepared as described in material and methods (section 2.8.1). Protein concentration was determined using Bradford assay. Loading samples (1μg/μl) were prepared and 20 μl of whole cell extract was loaded onto 12.5% SDS-Polyacrylamide gel. Protein transfer was done for 2 hours using chilled transfer buffer at room temperature. Nitrocellulose membrane was incubated for 1 hour in primary antibody (Gcd6p 1:5,000, Gcd1p 1:1000). Gcd1p antibody is used as loading control.

3.5 Optimization of working conditions for Gcd6p antibodies

Blocking solutions with different combinations of BSA and non-fat dried semi skimmed milk were used. Antibody dilutions of various concentrations were assessed under different temperature (RT/4°C) and incubation time (2 hours/Overnight) to set optimal conditions in which these phospho-specific antibodies were most efficient. The conditions finally decided are shown in Table 3.2. Panel
of westerns done during optimization process are shown in Figure 3.12 A, B, C, D, E.

Table 3.2. Optimization of phospho-specific Gcd6p antibodies using hc plasmids expressing whole yeast cell extracts.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Blocking Solution/ time duration/ temperature</th>
<th>Primary incubation conditions</th>
<th>antibody Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gcd6p</td>
<td>5% (w/v) BSA + 2% semi skimmed milk/ overnight/ 4°C</td>
<td>1:10,000</td>
<td></td>
</tr>
<tr>
<td>RTYL-1 (Gcd6-pS525)</td>
<td>5% (w/v) BSA/ overnight/ 4°C</td>
<td>2-3 hours/ RT</td>
<td>1:1000</td>
</tr>
<tr>
<td>RTYL-2 Gcd6-pS435</td>
<td>5% (w/v) BSA/ overnight/ 4°C</td>
<td>overnight/ 4°C</td>
<td>1:2500</td>
</tr>
<tr>
<td>RTYL-3 Gcd6-pS528</td>
<td>5% (w/v) BSA/ overnight/ 4°C</td>
<td>overnight/ 4°C</td>
<td>1:1000</td>
</tr>
<tr>
<td>RTYL-4 Gcd6-pS538</td>
<td>5% (w/v) BSA/ 1 hour/ RT</td>
<td>overnight/ 4°C</td>
<td>1:500</td>
</tr>
</tbody>
</table>

3.6 Gcd6p-S525 primes S528 for phosphorylation

No previous study has examined the role of eIF2B phosphorylation in yeast under different stress conditions so far. In the first experiment we screened phosphorylation of these sites in the absence of stress using cells expressing wild type Gcd6p and cells bearing single S/A mutants as the sole source of Gcd6p. In each case as expected the Ala mutations prevented the recognition of specific site by the phospho-specific peptide antibody directed at that particular site. Unexpectedly however when ε-S525A cells were probed with ε-pS528 antibody, significantly reduced signal was obtained (Figure 3.13). Although S525 and S528 are close in sequence each peptide used to generate the antibodies only contained a single serine residue and they do not overlap (Figure 3.4A and C).

Two out of four phosphorylation sites identified through MS in eIF2Be in S. cerevisiae, exhibited interdependence, reminiscent of a process that is observed in mammals (Woods et al. 2001). S525A substitution inhibits the phosphorylation at S528 (Figure 3.13A, B Lane 4). This study suggests that S528 is only phosphorylated following prior phosphorylation at S525. This sequence matches the motif (pS-X-X-S, or a pS residue at -3), which is the substrate for CK1.

Regulation of eIF2B by Phosphorylation 123
3. Optimization of working conditions

SDM was performed to generate hc plasmids with Ser-Ala substitutions at sites identified through MS. Yeast strains expressing Ala mutated version of Gcd6p at 435, 525, 528 and 538 sites were grown in YPD medium and proteins were extracted as described earlier (section 2.8). 20 μg of whole cell extract was loaded onto the SDS-PAGE gel. Different combinations of blocking conditions and antibody dilutions were used to increase the phospho-specificity of antibodies (A) WT-Gcd6p, (B) ε-pS435, (C) ε-pS525, (D) ε-pS528 and (E) ε-pS538 antibodies.

Figure 3.12. Optimization of working conditions for Gcd6p antibodies.
3. Optimization of working conditions

3.6.1 GCN2 has no effect on Gcd6p phosphorylation

Ternary complex formation is the first step of translation initiation. The inactive eIF2-GDP complex generated by the hydrolysis of GTP to GDP during 60S ribosomal subunit joining is converted into active complex by a reaction mediated by eIF2B. eIF2-GDP complex is the substrate of eIF2B but phosphorylation of the eIF2α at Ser51 converts it from the substrate to the competitive inhibitor of the nucleotide exchange factor eIF2B. Many kinases are identified in eukaryotes which phosphorylate eIF2α and thus inhibit translation initiation (Dever 1999, Pavitt 2005).

Gcn2p is the only kinase in yeast that phosphorylates eIF2α Ser51. The activity of Gcn2p is regulated by phosphorylation and dephosphorylation (Cherkasova and Hinnebusch 2003). Activated Gcn2p inhibits global protein translation in an eIF2α phosphorylation mechanism involving eIF2B inhibition with corresponding activation of GCN4 mRNA, which controls the biosynthesis of many amino acids at the level of gene transcription (Dever et al. 1992, Yang and Hinnebusch 1996, Pavitt et al. 1997).

To test whether Gcn2p is involved in the phosphorylation of Gcd6p, different strains (GP5867, GP5868, GP5870, GP5872 and GP5874) over-expressing Ser to Ala gcd6 mutations were combined with chromosomal deletion of gcn2Δ (For plasmid genotype details see Table 2.2). In each case as expected the Ala mutations prevented the recognition of the specific site by the phospho-specific peptide antibody directed at that site. Comparison of results obtained with strains mutated for single Ser to Ala substitution combined with GCN2 (Figure 3.13A) and gcn2Δ background (Figure 3.13B) showed the same pattern of eIF2B phosphorylation at these Gcd6p sites. This data excluded the possibility of direct phosphorylation of eIF2Be by Gcn2p kinase during optimal growth condition (Figure 3.13).

3.6.2 Does substitution of S525 with acidic residues affect S528 phosphorylation?

Acidic residues are negatively charged like phospho-serine but carry a single negative charge as compared to phospho-Serine which carries double negative charge at physiological pH. The presence of acidic residues can cause
conformational changes in the secondary structure of proteins which affect protein interaction with its substrate and other proteins.

**Figure 3.13. Phosphorylation of eIF2Bε-S528 depends on the presence of S525 and role of Gcn2p in phosphorylation of eIF2Bε.**

**A.** Yeast strains over-expressing wild type eIF2Bε, ε-S435A, ε-S525A, ε-S528A and ε-pS538A in *hc* plasmids for in combination GCN2 with wild type were grown in YPD medium and protein was extracted using method described in section 2.8. 10µl of whole yeast cell extract was loaded in each well. Membranes were incubated in condition described in Table 3.1. WT eIF2B-ε (1:1000), ε-pS435 (1:2500), ε-pS525 (1:1000), ε-pS528 (1:1000) and ε-pS538 (1:500) antibody dilution were used for immune-blotting and protein detection. **B.** Role of Gcn2p in the eIF2B-ε phosphorylation. The procedure is same as in A except that different set of strains was used.

To investigate the effect of acidic residues on phosphorylation of S528, new strains with S525D and S528E substitutions in high copy plasmids were constructed using LiAc aided transformation and FOA-plasmid shuffling (described in section 2.4). The presence of an acidic residue three residues N-terminal to S528, has the same inhibition of S528 phosphorylation as the 525A (Figure 3.14). This suggests that the phosphorylation of S528 depends not only on the presence of Ser at -3 position i.e. S525. This mechanism has been observed in mammals where GSK3 kinases require priming phosphorylation of a residue (phospho-Ser) in the surrounding sequence at +4 position (Woods et al. 2001).
3. Optimization of working conditions

Figure 3.14. Effect of S525 on the phosphorylation of S528.

50 ml of culture was grown to A600 0.5 and cell lysis was done using acid washed glass beads in FastPrep®. 10 ug of yeast whole cell extracts were analyzed for their effects on eIF2Bε-S528 phosphorylation. Membranes were blocked with 5% BSA (w/v)/ TBST overnight at 4°C. 1:1000 dilutions were used for phospho-specific ε-pS525 and ε-pS528 to detect phosphorylation of ε-S528.

3.7 Gcd1p phosphorylation sites

The eIF2Bε and eIF2Bγ subunits form the catalytic sub-complex that is necessary for accelerating the rate of nucleotide exchange. 47% sequence similarity exists over the entire length of Gcd1p and Gcd6p (Bushman et al. 1993). In affinity chromatography experiment with extracts over-expressing untagged Gcd1p and Gcd6p and purified His-tagged eIF2, it was found that co-over expressed, Gcd1p and Gcd6p bound to eIF2 at four-to five fold higher levels than when each protein was over expressed singly. This showed that Gcd1p serves to stabilize the binding of eIF2B with eIF2 (Pavitt et al. 1998). Four phosphorylated sites were identified in Gcd1p through MS by Mohammad-Qureshi, S. (Unpublished data). The position of the phospho-residues in Gcd1p and the sequence of peptides designed to generate phospho-peptides for the synthesis of Gcd1p phospho-antibodies against these sites are shown in Figure 3.5.

3.7.1 Trouble shooting for working of Gcd1p phospho-specific antibodies

Gcd1p-pS296, pS300, pS32/ pT33p phospho-specific antibodies were synthesized for the four newly identified sites in Gcd1p. During optimization for the working
conditions for Gcd1p antibodies, different combinations of blocking and various concentrations of antibody dilutions were used but Gcd1p antibodies did not work properly and gave many non-specific bands. These antibodies proved not to work at all well with whole cell extracts, even when GCD1 was over expressed using high copy plasmids (data not shown). The strains used for optimization of antibodies contained three high copy plasmids expressing all the five subunits of eIF2B.

To increase the phospho-specific recognition using these newly synthesized antibodies, it was decided to use purified eIF2B instead of whole cell extract. New strains expressing FLAG-tag Gcd1p were constructed using 5-FOA medium. eIF2B was purified using anti-FLAG M2 affinity gel by a method established in laboratory, described earlier in detail in section 2.8.3 and Figure 3.15. Optimization of conditions was done by using different combinations of blocking solutions and antibody dilutions. The conditions in which these phospho-specific antibodies worked best are summarised in Table 3.3.

The phospho-specificity of γ-pS300 was validated afterwards. Proteins were dephosphorylated by treating them with calf intestinal phosphatase (CIPase) and λ phosphatase and immunoblotting was done as described earlier. These antibodies show non-phospho specific binding (Figure 3.16-3.17).

A decision was made at this point to focus on Gcd6p only as Gcd1p showed ambiguous results and were not reliable for purpose of monitoring changes in phosphorylation. From this point onward all the western were carried out only using Gcd6p antibodies.
3. Optimization of working conditions

Regulation of eIF2B by Phosphorylation

Figure 3.15. Purification of FLAG-tagged eIF2B.

Cells carrying eIF2B with and without FLAG-tag were grown in YPD medium and cell harvesting was done using acid washed glass beads. 10 μl of each reaction was loaded onto SDS-PAGE. Coomassie stained gel is shown. Broken line shows that wash 2 and 3 are not shown. ‘-’ and ‘+’ indicates presence of no FLAG and FLAG tagged respectively.

Table 3.3. Optimization of Gcd1p phospho-specific antibodies using FLAG-tagged Gcd1p purified eIF2B extracts.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Blocking Solution/ Time/ Temp</th>
<th>Primary antibody incubation time/ Temp</th>
<th>Antibody Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt Gcd1p</td>
<td>5% (w/v) BSA/ 1 hour/ RT</td>
<td>Overnight/ 4</td>
<td>1:1000</td>
</tr>
<tr>
<td>RTYL-5</td>
<td>5% (w/v) BSA/ 1 hour/ RT</td>
<td>Overnight/ 4</td>
<td>1:1000</td>
</tr>
<tr>
<td>Gcd1 pS296</td>
<td>5% (w/v) BSA/ 1 hour/ RT</td>
<td>Overnight/ 4</td>
<td>1:1000</td>
</tr>
<tr>
<td>RTYL-6</td>
<td>5% (w/v) BSA/ 1 hour/ RT</td>
<td>Overnight/ 4</td>
<td>1:1000</td>
</tr>
<tr>
<td>Gcd1 pS300</td>
<td>5% (w/v) BSA/ 1 hour/ RT</td>
<td>Overnight/ 4</td>
<td>1:1000</td>
</tr>
<tr>
<td>RTYL-7</td>
<td>5% (w/v) BSA/ 1 hour/ RT</td>
<td>Overnight/ 4</td>
<td>1:1000</td>
</tr>
<tr>
<td>Gcd1 pS32/T33</td>
<td>5% (w/v) BSA/ 1 hour/ RT</td>
<td>Overnight/ 4</td>
<td>1:1000</td>
</tr>
</tbody>
</table>
3. Optimization of working conditions

Regulation of eIF2B by Phosphorylation

Figure 3.16. Optimization of Gcd1p phospho-specific antibodies.
eIF2B was purified with anti-FLAG M2 affinity resins from whole cell yeast extract. 60 μl of LSB was added to resins and boiled for 5 mins at 95°C. 10 μl of supernatant was loaded and resolved by SDS-PAGE. Western analyses using different dilutions of polyclonal Gcd1p and phospho-specific monoclonal Gcd1p antibody was performed under different blocking conditions. A. GP3688 and GP5224 expressing FLAG tagged wt GCD1 in combination with GCN2 and gcn2Δ. B. GP5435 (FLAG-gcd1-S296A). C. GP5437 (FLAG-gcd1-S32A/ T33A), D. GP5441 (FLAG-gcd1-S300A).

<table>
<thead>
<tr>
<th>Gcd1p dilution</th>
<th>Blocking conditions</th>
<th>pS296 dilution</th>
<th>Blocking conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:200</td>
<td>5% (w/v) non-fat dried milk / 1hr / RT</td>
<td>1:500</td>
<td>5% (w/v) non-fat dried milk / 1hr / RT</td>
</tr>
<tr>
<td>1:500</td>
<td>5% (w/v) non-fat dried milk / overnight / 4°C</td>
<td>1:1000</td>
<td>5% (w/v) non-fat dried milk / overnight / 4°C</td>
</tr>
<tr>
<td>1:1000</td>
<td>5% (w/v) BSA / overnight / 4°C</td>
<td>1:1000</td>
<td>5% (w/v) BSA / overnight / 4°C</td>
</tr>
</tbody>
</table>

Figure 3.17. Phospho-specificity of Gcd1p-phospho-specific antibodies.
eIF2B was purified with anti-FLAG M2 affinity resins from whole cell yeast extract. Dephosphorylation of proteins were carried out whilst the purified proteins were still bound to the anti-FLAG M2 resins. 1 unit of CIPase and λ phosphatase was added and incubation was done for 1 hour at 30°C for 60 mins. ‘_‘ indicates no phosphatase treatment.
3.8 Optimization of cell harvesting conditions

Customized Gcd6p phospho-antibodies were optimized using over-expressed GCD6 but for the purpose of monitoring changes in eIF2B it was decided to use strain in which the sole source of Gcd6p is the chromosomal GCD6 gene. It was found bit difficult to work Gcd6p phospho-antibodies using whole cell extract. Phospho-specific antibodies did not work properly and no signals were detected in western blotting even when the membranes were left for longer exposures. The only way to increase the yield of Gcd6p was to use purified protein. Protein purification was done from large cultures often to > 2 litres as the yield of Gcd6p from 50 ml YPD grown culture (A_{600} 0.5) was not enough for six western blots. Handling of large scale cultures with stress conditions proved to be an important variable as it created the problem of speed, efficiency and reproducibility with which the cells can be harvested and lysed. The protocol described here shows the effect of harvesting conditions on phosphorylation pattern of Gcd6p. GP 3688 was grown in 2 litres YPD medium to A_{600} ∼ 0.6 in 5 litre flasks at 30°C with constant shaking. Cells (∼2 g wet weight) were harvested by centrifugation at 6500 rpm (Beckman-JLA8.1000) for 5 mins. Once cells were collected, two different strategies were adopted to do lysis of cells. According to one approach mentioned hereafter as M1, cell pellet was processed as described in (de Almeida et al. 2013) except water was replaced with ice-cold high salt cell lysis buffer for pellet washing for 5 mins.

Cells were resuspended in lysis buffer (2 ml/ g of cells) and beads were made by dripping this cell suspension solution to liquid N\textsubscript{2} drop by drop. There was another strategy hereafter mentioned as M2 (unpublished) according to which pellet was given a snap shot in liquid N\textsubscript{2}. In both approaches cell were lysed using a large cryogenic freezer mill (Spex Certiprep Ltd). In Approach M2 the lysis buffer was added to 2 ml/ g after the yeast has been ground and left on ice for 30 mins. All subsequent steps were performed at 4°C. Cells were thawed and cell debris were removed by centrifugation at 5000 rpm (Sigma 4K15) for 10 mins. Protein was purified and eluted as described in section 2.8.
To check the effect of harvesting conditions on phosphorylation pattern of Gcd6p, two different concentrations of purified proteins were analyzed by SDS-PAGE and immunoblotting with the indicated phospho-specific monoclonal Gcd6p antibodies. It seems that washing the pellet during harvesting with ice-cold lysis buffer activates the phosphatases that affect the phosphorylation of Gcd6p at S528 and eIF2α (Figure 3.18B). Cold lysis buffer was chilled and contained Tris-HCl, KCL and many other chemicals, whilst also lacking amino acids and glucose, so it seemed that there were multiple factors operating together for very short time that changed the phosphorylation pattern of Gcd6p.

3.8.1 Multiple stresses activated for short time effect phosphorylation pattern of Gcd6p

As discussed earlier, cell harvesting conditions affected the phosphorylation pattern of yeast proteins at multiple sites. In order to find the factor that was responsible for bringing the change different conditions were assessed one at a time to assign the role to one environmental condition. Cells were grown in 2 litre of YPD medium to A_600 0.5. Cells were harvested by centrifugation at 6500 rpm (Beckman JLA8.1000 rotor). Medium was removed and the pellet was processed in five different ways before proceeding to grinding (Figure 3.19A). 1. Pellet was frozen in liquid N_2 as big clump of cells, 2. The pellet was washed with cold lysis buffer (M1), 3. Pellet washed with 4% glucose and amino acid solution and 4. Pellet washed only with 4% glucose solution only.

Cells were treated identically from this part onward and were frozen in liquid Nitrogen. Grinding and further protein purification was carried out as described in Section 2.8. Two different amounts of proteins were analyzed by SDS-PAGE and immunoblotting with the phospho-specific Gcd6p antibodies. From the panel of westerns shown in Figure 3.19B it is clear that changes in phosphorylation pattern was an accumulative affect of the many variables operating for short term during post harvesting stage. From this point onwards, all the cell pellets were frozen in liquid N_2 prior to further processing in order to minimize the effect of post growth harvest factors.
Figure 3.18. Harvesting conditions effect the phosphorylation of Gcd6p.

**A.** Two different approaches taken for harvesting the cells. **B.** Panel of western blots. 10 μl of the purified protein was loaded on the gel and immunoblotting done as described earlier. This experiment was repeated twice technically with same results.
Figure 3.19. Impact of different cell harvesting conditions on Gcd6p phosphorylation.

A. Dissections of M1 method that change the phosphorylation pattern of Gcd6p in order to find the stress condition that activate the phosphatases. 1. Cell pellet was freeze in liquid N2, 2. Washed with cold lysis buffer, 3. Washed with 4% Glucose + amino acid solution, 4. Cell pellet washed with 4% glucose only. B. Effect on the phosphorylation of eIF2Bε. Proteins were purified using antiFLAG-tagged M2 resins and two different amounts of proteins were loaded onto SDS-PAGE. Odd represent 1x and even represent 2x concentration of protein.
3.9 Discussion

Several different phosphorylation sites are known for mammalian eIF2B with their cognate kinases which regulate the activity of eIF2B in response to different environmental stress conditions. Prior to this current study several residues were identified in the sub-units of the catalytic sub-complex of yeast eIF2B through MS. Except S435, all sites (S525, S528 and S538) lie in the catalytic domain. The crystal structure of C terminal domain of ε subunit from residues 544-704 is known. These sites lie adjacent to that sequence so these might possibly influence catalytic activity of eIF2Be. New plasmids expressing Ser to Ala mutations at the selected phospho residues in low and high copy plasmids were constructed. This study is an attempt to determine the role of newly identified eIF2B phosphorylation sites in yeast in translational control. Because eIF2B and protein synthesis are known to be controlled by environmental stress different environmental stress challenges were selected based on their effect on yeast growth and the translation initiation process. Key to this approach was the production of phospho-specific antibodies raised against these selected sites.

As these antibodies were newly synthesized and not validated previously, working conditions were optimized. This chapter described some preliminary work, (i) verification of the mutant strains (ii) optimization of working conditions for increasing identification using phospho-antibodies (iii) development of a rapid cell harvesting method with minimum stress induction and (iv) effects of various combinations of harvesting conditions on phosphorylation of eIF2α and eIF2B.

It was found that the substitution mutations at phospho-residues identified in subunits of catalytic sub-complex (γ and ε) are non lethal and do not confer any phenotype under optimal growth condition. Data obtained with growth in liquid medium also confirm this statement, as mutants grew indistinguishably from wild type cells and no difference in growth rate and doubling time was observed in YPD and SD medium (Figure 3.7-3.8). Cells divide when they attain a particular size, but if there is problem with cell cycle progression then cells continue to grow in size which can account for large sizes.
Three out of four mutations significantly affected the cell size profiles. ε-S435A and ε-S538A mutants were found with larger cell size population while strains bearing an ε-S528A mutation were of smaller size (Figure 3.10). Precise reasons for smaller cell size are unclear but may indicate subtle translation defects not clear from doubling time measurement alone.

Antibody working conditions were optimized using yeast strains over-expressing wild type and mutant Gcd6p on plasmids. Experiments with a variety of constructs containing different Ser to Ala substitutions in catalytic Gcd6p sub-unit suggested that various sites can be phosphorylated independently; only the phosphorylation of S528 seemed to depend on prior phosphorylation of S525 (-3 position). This is reminiscent of a mechanism observed in mammals. GSK3 has been reported to phosphorylate eIF2Bε S535 in mammals (rats) and insects and impairs GEF activity of eIF2B. When cells are treated with insulin and growth factors, GSK3 activity is inhibited which is associated with the dephosphorylation of eIF2Bε at S535 and increase in GEF activity of eIF2B (Woods et al. 2001). GSK3 shows unusual substrate behaviour in that its substrate is primed by phosphorylation at position +4 (S539) to the GSK3 site by another kinase. Two isoforms of DYRK (DYRK1 and 2) are identified in rats which phosphorylate eIF2Bε-S539. Comparison of the yeast and mouse eIF2Bε sub-units provides a link that a priming event exists in yeast that appears conserved in evolution.

Although the structure of phospho-Ser and Asp/ Glu residues differ quite significantly, in some cases the presence of acidic residues can mimic the effect of phospho-serine by contributing a negative charge to the peptide. To ensure that S528 phosphorylation is dependent on S525, mutant strains were constructed with S525A and S525D substitution mutations. Expression of non-phosphorylatable Ala or acidic residues prevented the S528 phosphorylation. In this case it can be concluded that the presence of acidic residues does not mimic phosphorylation at S525 (Figure 3.7). This finding indicates that S525 is essential for the phosphorylation of S528 and phosphorylation of S525 seems likely to create a docking site for the binding of same or another kinase at S528. Based on this observation, a hypothetical model (Figure 3. 20) can be predicted to explain this interdependence of phosphorylation of one site on earlier phosphorylation at
another site. The kinase/ or kinases involved in the phosphorylation of each of these sites (S525 and S528) in yeast remain to be identified.

A number of kinase prediction software are used in this study (Section 2.10). It suggests that following earlier phosphorylation at S525 the potential candidate kinase for S528 could be CK1 as the sequence of recognition motif for CK1 matches with the position of Ser528 (pS-X-X-S, or a pS residue at -3). The yeast CK1 isoforms are Yck1, Yck2, Yck3, and Hrr25. As there are multiple genes encoding CK1 and knock out strain are sick due to phosphorylation of many substrates affected. Simply knock out of these is not a sensible experimental approach to identify if CK1 phosphorylates Ser528.

In this study we queried whether the phosphorylation of eIF2B is dependent upon Gcn2p kinase which is known to phosphorylate eIF2 in yeast. To address this question gcnaΔ strains were made and checked for the effect on the phosphorylation of eIF2Be. No significant difference on the phosphorylation of Gcd6p-S525 and Gcd6p-S528 was observed in wild type GCN2 and gcnaΔ cells. The data demonstrated that Gcn2p is not required for the phosphorylation of eIF2Be suggesting the role of some unknown kinases in the phosphorylation of eIF2Be (Figure 3.13).

After establishing the working conditions for Gcd6p phospho-specific antibodies, conditions for Gcd1p newly synthesized antibodies were optimised. These antibodies did not prove promising and non-specific pattern were obtained. Only Gcd1-pS300 gave site specific signals but a later experiment suggested that they are Ser specific and not phospho-Ser specific in their behaviour. The initial results obtained were ambiguous so it was decided that these antibodies would not be taken further and the study would focus on eIF2Be phosphorylation.

Antibodies conditions were optimised using high copy plasmids and initial experiments were done using 50 ml of culture. It was a matter of concern that the continued use of cells overexpressing eIF2B would upset the ratio of eIF2B-eIF2 and also the kinase-substrate ratio so it was necessary to use cells not over expressing eIF2B. Therefore for the purpose of monitoring changes in phosphorylation, GP3688 strain was selected (Table 2.1).
The source of Gcd6p in this strain is chromosomal copy of gene while it also expresses FLAG-tagged version of Gcd1p which can be used to purify the protein using anti-FLAG M2 affinity resin. To increase the identification of protein by phospho-specific antibodies proteins, it provided means to purify from large scale cultures (2 litres). A protocol for the purification of eIF2B at larger scale was optimised and it was found that cell harvesting conditions can alter the phosphorylation pattern of these proteins in cells. Washing of cells with cold lysis buffer lead to dephosphorylation at S528 and eIF2α(p). Dissection of stress imposed by cold lysis buffer was done and an attempt was made to evaluate the stress causing this activation of phosphatases. Cell pellet was processed in four different ways, (i) freeze in N₂, (ii) washed with cold lysis buffer, (iii) washed with 4% glucose and amino acid solution and (iv) cell pellet washed with 4% glucose solution. Results were consistent for S528 dephosphorylation. It can be concluded that multiple stresses operating together can lead to activation of unknown phosphatases and ε-S528 dephosphorylation is one of the consequences of these dephosphorylation mediated events. A new method for cell harvesting with minimal stress induction was developed and it was decided that for further experiments, cell pellets were to be frozen in liquid N₂ as soon as they are harvested without washing prior to lysis as this would cause nutrient starvation or cold shock. It has been shown in a study that glucose depletion for 1 min can cause translation arrest (Ashe et al. 2000). Time for sample processing is also important. It is slower to harvest cells from large cultures (2 litres) than from small culture (50 ml). The optimisation of working conditions for phospho-specific anti-bodies and development of a fast cell harvesting method for large scale culture laid the basis of protocols used for experiments presented in chapter 4, 5 and 6.
Figure 3.20. Comparison of rat and yeast Gcd6p phosphorylation.
Phosphorylation of S535 by GSK3 depends on prior phosphorylation of S539 by DYRK observed in rats (right). It seems that phosphorylation at S528 depends on the earlier phosphorylation or presence of S525 in yeast (left). The crystal structure of C-terminal of ε subunit from residues 544-704 in yeast and 548-714 in mammals is known. The phospho-sites lie adjacent to this region.
Chapter 4

Regulation of eIF2B phosphorylation by TOR signalling
4 Regulation of eIF2Bε phosphorylation by TOR signalling

In eukaryotes ranging from yeast to mammals, TOR signalling pathways are global regulatory networks that integrate nutrient derived environmental signals to control cellular growth. The central components of the TOR pathways are the TOR protein kinases which were identified in yeast as the target of the antifungal drug rapamycin. Yeast has two TOR homologue proteins, Tor1p and Tor2p, which exist in two complexes named TORC1 and TORC2. It is generally accepted that each complex control a different aspect of growth in response to nutrients. Rapamycin has been a valuable tool throughout the history of TOR research and is widely used as a TORC1 inhibitor in yeast. TORC1 is sensitive to rapamycin and controls temporal aspects of yeast growth while TORC2 is insensitive to rapamycin and controls spatial aspects of growth, i.e. actin polarization. TOR signalling pathways and how they regulate protein translation and growth has been already discussed in detail in Section 1.6. In this case study rapamycin and a newly discovered compound "Torin1" were used to understand the effect of inhibition of TOR signalling pathway on yeast growth. The aim was to investigate the phosphorylation changes in Gcd6p in response to stress induced by these treatments and to establish whether Gcd6p is a substrate (direct or indirect) substrate of TOR kinases. The first section of this chapter describes the data generated using rapamycin while data with Torin1 is discussed in the second half.

4.1 ε-S435A mutation confers rapamycin sensitive phenotype

To investigate the effect of rapamycin on the growth of wild and Gcd6p phospho-mutant yeast, growth assays were performed. Dimethyl Sulfoxide (DMSO), which is a polar solvent was used as drug vehicle. DMSO itself possesses antifungal properties and can affect the results of the tested drug. The previous literature shows that higher concentrations of DMSO 10% (v/v) results in the growth inhibition of S. cerevisiae (Murata et al. 2003) while other studies carried out in Candida albicans (C. albicans) (Akram Randhawa 2008) and S. cerevisiae
(Napper et al. 2011) reported significant growth inhibition (~25%) with 1% or even less DMSO. Therefore, DMSO concentration was kept less than 0.2% during assays. As a second control, cells were also spotted on YPD plates with DMSO. Growth tests were done as described in chapter 2 (Section 2.5.2).

A very promising mutant was identified in growth assays i.e. ε-S435A which showed sensitive growth phenotype to rapamycin (Figure 4.1). ε-S525A (Figure 4.1 Lane 3) seemed resistant but on repeating this experiment again, this phenotype was not reproduced, so it was just because of the more number of cells plated here.

![Figure 4.1. Effect of rapamycin on yeast growth.](image)

GP 3750 strain (∆gcd6) was transformed with low copy plasmids expressing wt-ε, ε-S435A, ε-S525A, ε-S528A and ε-S538A. Transformants were grown overnight in YPD medium and 10-fold serially diluted as described in Section 2.5. 3μl aliquots of each dilution were spotted on each plate, i.e. YPD, YPD with DMSO, and YPD with 100 nM/ ml rapamycin. Pictures were taken on day 3 of incubation.

### 4.2 The role of GCN2 in growth under rapamycin stress

To examine the effects of rapamycin on yeast growth and doubling time, two experiments were conducted with different sets of strains that allowed assessment of the role of Gcn2p in rapamycin sensitivity. This is because previous work has shown that Gcn2p is a target of TOR kinases and it can regulate eIF2B via eIF2 phosphorylation (Dever et al. 1992, Krishnamoorthy et al. 2001). Rapamycin
activates Gcn2p activity by dephosphorylation at S577 by TAP42 (Cherkasova and Hinnebusch 2003). By comparing $\pm$ GCN2 strains we were able to assess GCN2 dependent and independent effects of rapamycin.

In the first set of experiment, wild type and $\varepsilon$-S435A mutant yeast strains in $gcn2\Delta$ background were used. Growth was determined by measuring absorbance at $A_{600}$ over a time course with and without treatment of the cultures with 100 nM/ml rapamycin (Figure 4.2 A). Same experiment was repeated with strains having wild type and mutant $\varepsilon$-S435A in combination with GCN2 (Figure 4.2 B). Doubling time was calculated using the equation generated on graph (Section 2.5.1).

Control culture without any treatment continued to grow normally, with doubling in cell number (measured by $A_{600}$) every ~140 min over the time course. Rapamycin growth inhibition effect was more severe in $\varepsilon$-S435A mutant where rapamycin treated cells took ~9-10 hours to complete one doubling (Figure 4.2C). Growth of yeast strains carrying wild type $\varepsilon$ was inhibited for first two hours followed by an adaptation response over time (Figure 4.2A, B). Thus, as expected rapamycin causes growth arrest. The presence of Gcn2p has a very small effect on growth and doubling time under rapamycin stress in YPD medium (Figure 4.2C).

### 4.3 Rapamycin treatment induces starvation

It has been shown in a study relating to the effects of rapamycin on cell morphology that treatment of yeast culture with 0.2 $\mu$g/ ml of rapamycin leads to changes in yeast cell size (Barbet et al. 1996).

The effect of rapamycin treatment on yeast cell size was examined during a time course treatment of phospho-mutants strains with rapamycin (Section 4.1.1). Along with measuring the growth rate of cells, cells undergoing rapamycin treatment were also analyzed under Eclipse-E600 microscope (Nikon) at 100x. When samples were analysed for cell size, biphasic size distribution was observed in samples treated with rapamycin. The major sub-population consists of cells of increased size while other cells remained small in size throughout the experiment. The small sized cells most likely correspond to starved daughter cells. No
significant morphological changes, i.e. pseudohyphae growth was found, but closer examination of cells showed that large vacuole (autophagosome) was

Figure 4.2. Yeast growth assays in liquid medium and doubling time under rapamycin stress.

A. Cells were grown in YPD to $A_{600}$ 0.2 and 100 nM/ ml of rapamycin was added to medium and cells were grown at 30C with constant rotation. Growth was measured by measuring absorbance at $A_{600}$ using spectrophotometer. Wild type (square) and mutant (triangle), (-) untreated and (+) treated, red arrow indicates the time point of rapamycin addition. B. Effect of Gcn2p on growth. Procedure for B was same as A. C. Bar graph representation of the effects on doubling time.
present in cells with larger cell size, indication of nutrient starvation (Granot and Snyder 1991) and this might account for the large size of cells as well (data not shown).

4.4 Finding the minimum inhibitory concentration of rapamycin to assess the sensitivity of ε-S435A

Kirby-Bauer disk diffusion susceptibility tests were performed to assess apparent sensitivity of ε-S435A to rapamycin concentrations (Figure 4.3). 6-mm filter paper disk soaked with known concentrations of rapamycin (100-250 nM) were placed on an agar plate seeded with a lawn of wild type and ε-S435A cells. The drug gets distributed into nearby agar medium by the process of diffusion. The rate of diffusion through the agar is slow as compared to the rate of extraction of the drug out of disk. This result in the highest concentration of drug closest to the disk and logarithmic decrease as the distance increased from the disk. When cell growth is inhibited it produces a 'halo' zone of inhibition. The measurements of the halos obtained showed that there is a linear relationship between sensitivity and \( \log_{10} \) of drug concentration.

ε-S435A was clearly more sensitive than cells carrying wild type eIF2Bε. The presence of GCN2 affects the response to rapamycin, a slight decrease in sensitivity was observed but the magnitude of the effect induced by the presence of GCN2 varied in wild and phospho-mutant ε-S435A. The effect was more obvious for wild type with small halo while for S435A the decrease in halo diameter was not much prominent. First this experiment was done with a higher concentration of rapamycin (100-250 nM). It was observed that 100 nM concentration of rapamycin was strong enough to arrest the growth of yeast strains with wild type ε, which masked the results of disk assay. Sensitivity over 100 nM does not increase in log scale in wild type yeast and 100 nM concentration was high enough to saturate the halo (Supplementary Figure A.1). Another observation was made during the course of experiments that activity of rapamycin solution decreases overtime even it was stored at -20°C away from light. It was decided that the rapamycin concentration can be reduced to lower levels and calculations for comparative sensitivity level determinations should be done at the lower
concentrations of rapamycin which could affect only ε-S435A phospho-mutant strain. The next step was to find the minimum inhibitory concentration for yeast strain with ε-S435A mutation.

Rapamycin treatment induces the starvation condition which could activate the GCN4 pathway via GCN2 (Cherkasova and Hinnebusch 2003), so the experiment was repeated with two different set of strains to measure the rapamycin sensitivity and to assess the impact of GCN2 under stress on area of zone of inhibition at the same time. Experiment was done using YPD medium with various concentrations of rapamycin. Zone of inhibition started appearing in yeast strains with wild type ε at higher concentrations (> 10 nM) but they were not very well defined and clear. According to the quantification of halo assay data ε-S435A proved to be a hypersensitive mutant to rapamycin with the zone of inhibition appearing even at 1 nM of rapamycin (Figure 4.3A). There was a direct relation between the area of zone of inhibition and concentration of rapamycin till 50 nM while beyond that point, increase in concentration of rapamycin did not cause a significant increase in the diameter, hence area of zone of inhibition. Apparent sensitivity was calculated by plotting a graph of drug concentration (log10) against area of zone of inhibition (mm²). Relative apparent sensitivity dividing the rapamycin concentration required to produce a halo of a particular area (x) for mutant ε-S435A strain by the drug concentration required to produce a halo of the same area with a wild-type strain (Figure 4.3B). ε-S435A more sensitive to rapamycin at lower concentration (≤ 50 nM) while the ratio of relative sensitivity decreases with higher concentrations of rapamycin (> 50 nM) (Figure 4.3B). One explanation for this could be that at higher concentrations of drug dose the growth of wild type strain is also affected visible as larger zone of inhibition which leads to decrease in the ratio of relative sensitivity.

As the experiment was carried out in rich medium (YPD) and GCN2 was plasmid borne and may be not selected for and might be lost during growth in rich medium (GCN2 is a non-essential gene). The data obtained from this experiment regarding the role of GCN2 was therefore inconclusive, so the experiment was repeated with minimal medium to assure the maintenance of GCN2.
4. Regulation of eIF2B phosphorylation by TOR signalling

Figure 4.3. Minimal rapamycin concentration in YPD medium to measure ε-S435A sensitivity.

A. Halo disk assay was performed as described in Section 2.6.1. 5 μl of the rapamycin dilution was added to the disk to get the following concentration of rapamycin 0= DMSO, 1 nM, 5 nM, 10 nM, 20 nM, 50 nM, 100nM, and 200nM. Plates were incubated for ~16 hours at 30°C. B. Diameter of the zone of inhibition was measured and the area was calculated using imageJ 1.46. Bar graph is plotted for drug dose against the area of zone of inhibition.
4.4.1 The role of \textit{GCN2} in rapamycin induced starvation is obvious in SD medium

To characterize the role of Gcn2p under rapamycin induced starvation and the effect of medium on (nutrient rich medium vs. nutrient poor medium) Gcn2p activity, halo disk assay was performed with SD medium. 1-200 nM rapamycin was used and assays were done as described previously (Section 4.4).

The role of Gcn2p becomes more apparent in ε-S435A mutants in minimal medium (SD). Quantification of the halo assays presented in Figure 4.4, shows 30 - 40% decrease in the diameter of the zone of inhibition in cells with \textit{GCN2}. A very interesting finding worth mentioning is that rapamycin induces starvation effect was more visible in rich medium (YPD) as compared to minimal medium (SD) as larger halos indicating more growth inhibition were obtained with the same drug concentrations on YPD plates compared to SD plates. Cells are more resistant on SD than YPD.

![Graph showing effect of rapamycin on area of zone of inhibition](image)

**Figure 4.4. Effect of rapamycin in minimal medium and role of \textit{GCN2}**: Halo disk assay was performed as described in previously in Figure 4.3. 1, 5, 10, 20, 50, 100 and 200 nM of rapamycin was used while DMSO (drug vehicle) was used as blank control. Plates were incubated for ~16 hours at 30°C. Diameter was measured in mm and area was calculated and corrected for by subtracting disk area. Bar graph was plotted to show the effect of \textit{GCN2} on area of zone of inhibition in SD medium.
One explanation could be that Gcn2p has more protective effect on SD for S435A in overcoming the starvation challenge in minimal medium. It could be that cells were already in minimal medium without any amino acids (semi-starvation condition) and the addition of rapamycin leads to the rapid activation of GCN4 pathway. This could be the survival strategy to cope with the starvation stress.

4.5 Rapamycin is a general inhibitor of translation initiation

Rapamycin blocks activation of translation. Translation efficiency was assessed *in vivo* by measuring polysome to monosome ratio using sucrose density gradient sedimentation. Polysome profile analysis gives information whether the rapamycin induced growth arrest was because of the inhibition of protein synthesis at initiation or elongation phase. 1 hr treatment time was decided based on the response observed during growth curves, where the difference in optical density was detectable after 60 mins of treatment (Figure 4.2).

Wild type and eIF2Bε mutants were incubated with 100 nM/ml of rapamycin at 30°C for 60 mins. Cell extracts were prepared followed by 10 min treatment with cycloheximide which is translation elongation inhibitor to prevent "run off" of any polysomes that are present. To check the effect of drug vehicle, wild type cells were also treated with DMSO alone as a second control. Polysome analysis was performed as described earlier in materials and methods (Section 2.6).

There was a general decrease in the polysomes and a parallel increase in 80S peak (Figure 4.5A). Due to defective ribosome loading the translation initiation was blocked in rapamycin treated samples. The polysome run off effect was significant for the ε-S435A mutant. The polysome profiles of other ε-phospho-Ser to Ala mutants resemble wild type profile in sensitivity to rapamycin. This data is in agreement with the earlier observations described in Section 4.2.1 that ε-S435A is more sensitive to rapamycin than other eIF2Bε phospho-mutants.
4. Regulation of eIF2B phosphorylation by TOR signalling

Figure 4.5. Rapamycin treatment blocks translation initiation.

A. Upper panel shows the polysome profiles of untreated wild-type and eIF2Bε-phospho mutants and lower panel represents the profiles of the samples treated with 100 nM/ml rapamycin for 60 mins. In both cases 500 µl of cycloheximide (10 mg/ml) was added 10 min before harvest to prevent polysome "run off." Cell extraction and all the subsequent steps were performed at 4°C as described in Section 2.7. 25 units of A260 were loaded onto 15-50% sucrose gradients. B. Quantification of the polysome profile was done using ImageJ 1.46 and polysome to monosome (P/M) ratio is shown indicating the effects of 60 mins rapamycin treatment on P/M ratio.
4. Regulation of eIF2B phosphorylation by TOR signalling

4.6 Is eIF2Bε phosphorylation altered by rapamycin?

Investigation was carried out to find the impact of rapamycin on eIF2Bε phosphorylation at selected Ser residues. Immunoblotting was performed with phospho-specific antibodies. Cells were grown in 2 litre YPD medium to mid log phase \((A_{600} \ 0.6)\) and were treated with rapamycin to a final concentration of 100 nM/ml for 1 hour. Treatment time was selected based on the growth assays in liquid medium where it became evident that rapamycin effects with in 1 hour to reduce the growth rate. Cell harvesting was done using the new fast harvesting method with minimum stress during harvesting and cell lysis was done in the large cryogenic liquid N\(_2\) grinding mill.

Two different amounts of the FLAG purified eIF2B proteins were loaded and immunoblotting was done according to the procedure and conditions described earlier in Section 3.2. The quantification of the westerns blots (Figure 4.6A) using ImageJ 1.46 shows that there was a slight increase in the phosphorylation at ε-S435, S525 and S538 while decrease at S525 following rapamycin treatment (Figure 4.6B).

Analysis of growth in liquid medium showed that wild type cells adapt to the rapamycin stress after ~2 hours of treatment. If eIF2Bε is a downstream target of TOR signalling, the adaptation could be because of the changes in phosphorylation pattern and activity of eIF2Bε. To further examine the changes in phosphorylation over time during rapamycin stress an experiment was performed in which cells were harvested prior to treatment and every hour following treatment for 4 hours. The protein was isolated using antiFLAG M2 affinity resins and blotted with specific antibodies Quantification of the data showed an increase in phosphorylation at all sites investigated, after 1 hour of treatment, followed by a decrease to pre-treatment levels over next 3 hours at S435, S525 and S528 while S538 remains continuous over phosphorylated (Figure 4.7B). It can be concluded that there is an increased phosphorylation at all sites after 1 hour of treatment is obvious (Figure 4.6 and 4.7) while the effect over time varies between various phospho-mutants.
Figure 4.6. Phosphorylation of eIF2Bε under rapamycin stress.

A. GP3688 was grown in YPD to $A_{600} = 0.6$ / 2 litres and then treated with 100 nM/ml rapamycin for 60 mins. Cells were harvested as described in Section 3.8. Grinding was done in liquid N2 large cryogenic grinding freezer mill. Proteins were purified using FLAG M2 affinity resins and two different amounts of eluted proteins were analyzed on SDS-PAGE and immunoblotting with phospho-specific antibodies mentioned. "−" represents untreated while "+" is for rapamycin addition. B. Quantification of the western shown in A. Signals were normalized against Gcd6p antibody. "−" represents untreated while "+" is for addition of rapamycin. Data is mean ± S.E.M (n=3).
4. Regulation of eIF2B phosphorylation by TOR signalling

A.

**Figure 4.7. Phosphorylation of eIF2Bε under rapamycin stress over the time course.**

**A.** Cells were grown and treated with rapamycin for 4 hours. Cells were harvested every hour and lysis was done as described in Figure 4.6A. Grinding was done in liquid N₂ using large cryogenic freezer mill. Proteins were purified using FLAG M2 affinity resins and two different amounts of eluted proteins were analyzed. **B.** Quantification of the panel of western blots shown in A. 0 represent time before treatment while 1, 2, 3, 4 represent treatment time in hours. Signal was normalized against Gcd6p. Data is shown as Mean ± S.E.M (n=2).
4. Regulation of eIF2B phosphorylation by TOR signalling

4.7 "Torin1" and its impacts on eIF2Bε phosphorylation

*In vitro* biochemical experiments showed that FKBP12-rapamycin complex can only bind to TORC1 but not to TORC2 so only TORC1 function is sensitive to rapamycin. TORC2 is insensitive to rapamycin as complex cannot bind to TORC2 (Rohde et al. 2008). In yeast rapamycin has been used over history as a convincing mimic of the inactivation of the TORC1. Although the structural information of the rapamycin-FKBP12 complex is available still it is not fully known how this complex prevents the phosphorylation of the substrates of TOR kinases. Different models have been proposed suggesting that as the complex is of large size it can block access to specific substrates. Now a day’s many different compounds have been identified that are structural analogs of rapamycin and can inhibit TORC (Nowak et al. 2009, Verheijen et al. 2009, Kaplan et al. 2010). It was found in one recent study that rapamycin does not inhibit TORC1 completely rather there are some aspects of TORC1 that are insensitive to rapamycin (Thoreen et al. 2009).

Through biochemical screen of the library of heterocyclic chemical compounds, a small molecule was discovered (Thoreen et al. 2009) which was further elaborated through medicinal chemistry efforts to produce a new highly selective inhibitor of TOR complex, "Torin1" (Liu et al. 2010). Torin1 is a member of the pyridinonequinoline class of kinase inhibitors (Thoreen et al. 2009).

*In vitro* kinase assays were carried out using purified mTORC1 or mTORC2 and it was shown that Torin1 inhibited both mTOR containing complexes without affecting the stability of the complexes. It works in an ATP-competitive manner and it was proved using the Ambit Biosciences KinomeScan platform that it has no indication of off-target effect. Initially it was assumed that it inhibits TORC2 but further investigations indicated that the effect induced by Torin1 were not due to inhibition of mTORC2 but it is because of the some functions of mTORC1 that were resistant to rapamycin (Thoreen et al. 2009).
Recently a genome wide analysis was carried out in *Schizosaccharomyces pombe* in Janni’s Petersen laboratory at The University of Manchester resulting in the identification of around 4000 phospho-sites whose intensity varied (up or down) in Torin1 treated cells. The phosphorylation at TIF225-S500 and T503 was down regulated following Torin1 treatment. Global alignment does not fit with the local alignment, but local alignment shows that these sites are equivalent to *S. cerevisiae* ε-S525 and ε-S528 sites (Figure 4.8) (personal communication with J. Peterson).

**Figure 4.8. Conservation of phospho-sites across two species of yeast.**

A. Global sequence alignment of segments of amino acids sequences between *S. pombe* and *S. cerevisiae*. Ser is conserved at S528 and S538 between two species. B. Local alignment of sequence, although it does not fit global alignment but shows site conservation at S525. *S. cerevisiae* residues equivalent to *S. pombe* residues observed to exhibit phosphorylation change are indicated by (*). The change in phosphorylation at S500 in *S. pombe* was significant with p< 0.05. Sequence alignment was done using ClustalW2 and alignment file was processed in Jalview. * indicate the position of phospho-sites in Gcd6p. Red colour represents small and hydrophobic aromatic amino acids, blue acidic, pink basic, green hydroxyl and sulfhydryl amino acids.

These two lines of evidence that Torin1 inhibits the TORC completely and change the phosphorylation pattern at some sites of TIF225 in *S. pombe* combined suggests that Torin1 can impair protein synthesis to a greater degree than...
rapamycin. This study was carried out as a step towards finding the impact of Torin1 on protein synthesis and eIF2Bε phosphorylation.

4.8 Torin1 impairs growth more than rapamycin

To check the phenotypic sensitivity of eIF2B mutants to Torin1, growth was monitored on rich (YPD) and minimal medium (SD) with and without Torin1. Mutants grew indistinguishably from wild type on YPD and SD medium (Figure 4.9A, B left panel) while eIF2B mutants carrying Gcd6-S435A, Gcd6-S525A and Gcd6-S528A strain showed sensitive phenotype to Torin1. Sensitivity to Torin1 varied in medium as mutants were insensitive to 5 μM/ ml Torin1 in YPD (data not shown) and showed sensitivity to 10 μM/ ml in YPD medium (Figure 4.9A) while in SD medium mutants were even sensitive to as low as 5uM/ ml Torin1 (Figure 4.9B). Leaving the plates for longer at 30°C, ε-S528A starts growing which can be explained either as the inactivation/ depletion of Torin1. ε-S525A and ε-S528A both are sensitive to Torin1 while resistant phenotype was observed against rapamycin. As ε-S528A phosphorylation is dependent on earlier phosphorylation event of εS525A (Section 3.6). Next step was to check the sensitivity level of both mutants.

4.8.1 Torin1 is a potent inhibitor of TOR signalling

To quantify the sensitivity level of ε-S435, S525A and S528A to Torin1 and overcoming the drug inactivation problem with time data, Kirby-Bauer (KB) test was performed. As the zone of inhibition appears within 16 hours, so problem of drug inactivation with time seemed to be sorted out. Wild type eIF2Bε and ε-S538A showed only slight sensitivity to Torin1 on higher concentration, i.e. 30 μM, ε-S435, ε-525A and ε-528A mutants made clear zone of inhibition even with 10 μM Torin1 (Figure 4.10). Diameter of the zone of growth inhibition conferred by various concentrations of Torin1 was measured and area of zone of inhibition was calculated. The diameter of zones of inhibition is same but in qualitative terms ε-S435A mutant forms clear zones of inhibition. Quantification of the data shows that ε-S435A, ε-S525A and ε-S528A expressed almost the same level of sensitivity.
Figure 4.9. Effect of Torin1 on growth.

Strain Δgcd6 was transformed with eIF2Bε, ε-S435A, ε-S525A, ε-S528A and ε-S538A on low copy plasmids. Transformants were grown overnight, and 10-fold serially diluted as described in Section 2.5.2. 3μl aliquots of each dilution were spotted on selected medium, A. YPD and YPD with 10 μM/ ml and 20 μM/ ml Torin1. B. SD+Ura+Leu and SD+Ura+Leu with 10 μM /ml and 20 μM/ ml Torin1. Plates were fully wrapped in tin foil to avoid exposure to light and were incubated at 30°C. Torin1 stock solution 4mM/ ml was made fresh in DMSO on the day of use and stored at -20°C for 20 days wrapped in tin foil. Plates were also wrapped in foil to avoid exposure to light. Pictures were taken on day 3 and day 4 of incubation.
A. Overnight grown culture was diluted to $A_{600}$ 0.3. 10 ml of the culture was centrifuged and cells were resuspended in 1ml of YPD. 300μl of cell suspension was added to 3 ml of 0.6% YPDA and poured onto YPD plates and left to settle. Disks soaked with 10, 20 and 30 μM Torin1 were placed on plates and diameter of zone of inhibition was measured after 16 hours of incubation. 0=DMSO, 1=10 μM, 2=20 μM, 3= 30 μM

B. Quantification of the KB disk assay. Drug dose is plotted against area of zone of inhibition.

**Figure 4.10. Kirby-Bauer Disk Diffusion Susceptibility Test.**

A. Overnight grown culture was diluted to $A_{600}$ 0.3. 10 ml of the culture was centrifuged and cells were resuspended in 1ml of YPD. 300μl of cell suspension was added to 3 ml of 0.6% YPDA and poured onto YPD plates and left to settle. Disks soaked with 10, 20 and 30 μM Torin1 were placed on plates and diameter of zone of inhibition was measured after 16 hours of incubation. 0=DMSO, 1=10 μM, 2=20 μM, 3= 30 μM B. Quantification of the KB disk assay. Drug dose is plotted against area of zone of inhibition.
4.9 Effects of Torin1 on growth inhibition of eIF2Bε phospho mutants

To monitor the effect of Torin1 on growth of wild type and phospho-mutants over time, growth rate was measured in liquid medium. Starter culture was split into two equal halves. Sample in one flask were labelled as untreated and used as control while experimental culture was treated with 20 μM/ml Torin1. It had already been shown on halo assays with rapamycin that DMSO has no affect on growth at a concentration of 0.25%; (used to dissolve the drug). $A_{600}$ was measured every hour and was plotted against time in hours (Figure 4.11). Yeast strain expressing wild type eIF2Bε and ε-S538A mutantions started responding to drug after two hours while the growth rate of ε-S435A reduced to almost half as compared to control samples within the first hour of Torin1 treatment. Only very slight unnoticeable difference was found in responses of ε-S525A and ε-S528A mutants to Torin1, ε-S525A being a little more sensitive.

4.10 Torin1 differentially affects the cell sizes of ε-phospho mutants

Rapamycin is known to cause changes in the cell size in mammalian cells, and it has been shown earlier in this chapter that rapamycin cause changes in yeast cell size, wild type cells become bigger in size as their vacuoles get larger. To measure the effect of Torin1 on cell size, 20 μl of samples each from control and treated with Torin1 after 4 hour of treatment from wild type and phospho-mutant cultures were observed in Cellometer® Auto M10 (Nexcelom Biosciences). Data for cell numbers and cell sizes was obtained within 30 seconds. The visual representation of distribution of the cells according to cell sizes in different samples is shown in BoxPlot (Figure 4.12). 5 – 95% of the data is shown in box plot with middle box shows 25 - 75% of the cell in that size range with the middle line representing the median of population. Upper and lower 5% are shown as outliers. The data distribution showed that there are no significant outliers in this data that could negatively influence the results. Torin1 treatment affects the cell size of wild type and phospho mutants but the pattern was not uniform. It seems that Torin1 differentially affects different ε-phospho mutants.
Figure 4.11. Effect of Torin1 on growth of WT Gcd6p and phospho-mutants.

Black arrows represent the time point at which 20 μM/ml Torin1 was added to sample. Absorbance was measured every hour and log_{10} of it was plotted against time in hours. C represents control, and T represents treatment with Torin1. See Section 2.6 for details.
An independent-samples t-test was conducted to compare the cell sizes in control and treated conditions. Detailed results are presented in Table 4.1. These results show that Torin1 treatment also affects the cell sizes of wild type but the effect is not significant. The very surprising result that was observed showed that control samples of ε-S435A (7.6±1.93) are larger than treated (5.8±1.88) (t(1849) 20.882, p < 0.0001).

Same results were obtained with ε-S538A control (7.9±2.99) vs. 4 hours Torin1 treated sample (7.0±2.36) (t(1481)=7.134, p < 0.0001) suggesting that Torin1 treatment affects the cell size of mutants. These strains never behaved the same, ε-S435A being always sensitive to all the environmental stress conditions (Rapamycin, Torin1, 3-AT and cold shock) tested in this study while ε-S538A behaved like wild type and showed resistant phenotypes on growth assays. Torin1 treatment affected the sizes of ε-S525A and ε-S528A cells in the opposite way but the difference induced was significant. The reason underlying the unique response of each mutant to the same treatment is not yet clear.

4.11 Torin1 impairs translation initiation

As already discussed in Section 4.9-10 that Torin1 inhibits growth, next step was to determine the effect of Torin1 on protein synthesis, and whether the effect is on initiation step or elongation step. Polysome profile analysis was done to measure the protein synthesis efficiency. 20 μM/ ml Torin1 for 2 hours (one doubling time) treatment time was selected on the basis of information provided by growth curves. The graphical representation of the data shows that Torin1 is a general inhibitor of translation initiation (Figure 4.13B).

These results indicate the defective loading of ribosome onto mRNAs and affect of Torin1 mediated translation initiation inhibition is more pronounced in ε-S435A, ε-S525A, and ε-S528A strains with 83%, 69% and 74% decrease in the P/M ratio. The decrease in P/M ratio is equal in strains containing wild type ε and ε-S538A mutation. So the phospho-mutants vary in their sensitivity to Torin1.
4. Regulation of eIF2B phosphorylation by TOR signalling

Table 4.1. Student's t. test: two samples of unequal sizes assuming unequal variances

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(-) control, (+) treated with 20 μM/ml Torin1 for 4 hrs
Significance level to accept the hypothesis = p < 0.05

Figure 4.12. Effect of Torin1 on cell size.

Cells were grown in YPD to A600 0.5 and were treated with 20 μM/ml of Torin1 for 4 hours. Cells were counted and sizes were measured in cellometer. 5-95% of data distribution is shown. Boxplot Grapher was used to see the distribution of data. The bottom side of the box is 1\textsuperscript{st} quartile and the top side the 3\textsuperscript{rd} quartile. The vertical width of the central box is the inter-quartile distribution with middle horizontal line representing the median of a sample. Red dots represent outliers.
4. Regulation of eIF2B phosphorylation by TOR signalling

Figure 4.13. Torin1 inhibits translation initiation.
A. Cells were grown in YPD medium till OD<sub>600</sub> 0.5 and split into two equal halves. Treated samples were grown with Torin1 at a concentration of 20 μM/ ml for 2 hours at 30°C. RNA extraction and sucrose density gradient centrifugation was done as described in Section 2.6.1 to get the polysome profiles Upper panel showed profiles of control samples while the lower panel represents profiles of samples treated with 20 μM Torin1 / 2hours. B. Quantification of the ribosomal profiles using ImageJ 1.46. P/M ratio was calculated and shown as a bar graph.
4. Regulation of eIF2B phosphorylation by TOR signalling

4.12 Effect of Torin1 on eIF2Bε phosphorylation

To avoid any secondary effects and focus on direct translational output of TORC, cells were treated with Torin1 only for 2 hours. Cells were grown in YPD medium till mid log phase and were treated with Torin1 to a final concentration of 20 μM/ml for 2 hours. This experiment was done twice with two different cell harvesting conditions. Figure 4.14A showed the immunoblotting done with whole cell extract prepared with TCA method as described in Section 2.7. It is clear from the panel of westerns presented in Figure 4.14A, that there is a significant reduction in phosphorylation at ε-S435, ε-S525 and ε-S528, while there was no significant difference on phosphorylation at ε-S538. Increase in eIF2α phosphorylation was observed in cells treated with Torin1 (Figure 4.14A).

This result is in contradiction to the early findings where Torin1 treatment of mouse embryonic fibroblasts (MEFs) inhibited the mTORC1-dependent translation events without an increase in the phosphorylation of eIF2α (Thoreen et al. 2012).

The same experiment was repeated with proteins purified using antiFLAG M2 affinity resins, and two different amounts of eluted proteins were analyzed. The same pattern of down regulation of phosphorylation was observed (Figure 4.14B). Paired t-test was run to measure the effect of Torin1 treatment. Statistics analysis of the data shows that there is a significant difference in phosphorylation at ε-S435 (t = -6.080, p < 0.026), ε-S525 (t = -6.379, p < 0.0237) and ε-S528 (t = 8.718, p = 0.0129), while the effect on phosphorylation at ε-S538 is non-significant (t = 3.46, p = 0.0742). As the western presented in the panel B are of FLAG IP, so another thing that is in accordance with the known fact was observed i.e. the strong binding of eIF2α(p) with eIF2B. As more eIF2α was in eIF2α(p) form in cells treated with Torin1, and was tightly bound to eIF2B so during elution eIF2 was eluted more in samples gone through treatment with Torin1 showing that a large fraction of the eIF2α exist in eIF2α(p) form.

The effect of changes in phosphorylation on the activity of eIF2B was the next step in this series of experiment which could not be accomplished due to time constraints.
4. Regulation of eIF2B phosphorylation by TOR signalling

Figure 4.14: Effect of Torin1 on eIF2Bε phosphorylation.

A. GP3688 was grown in YPD to $A_{600} 0.6/50$ ml and then treated with 20 μM/ml Torin1 for 2 hours. Cells were harvested with TCA as described in Section 2.7.10 μl of the total extract was loaded on SDS-PAGE. Blocking was done for 1 hour at RT and membranes were incubated in 1:300 dilution of phospho-specific antibodies at 4°C overnight. B. 2 litres of the culture was grown to $A_{600} 0.6$ and Torin1 was added to a final concentration of 20 μM/ml. C. Quantification of the westerns presented in panel A and B. Error bar Mean ± SD in bar graph. Paired t-test was performed ($n = 3$, df = 2, * = $p < 0.05$).
4.13 Discussion

TOR kinases nucleates signalling pathways that couple cell growth to nutrients availability and functions as part of two distinct protein complexes TORC1 and TORC2. The sequences of TOR kinase and structure and function of complexes are conserved from yeast to man. TOR complexes regulate growth (spatial and temporal) by activating/ deactivating a myriad of translational factors (4E-BP) and kinases/ phosphatases. Rapamycin, an allosteric inhibitor of TORC1 (TORC2 is insensitive to rapamycin) has been used throughout the history of TOR research to examine TOR signalling pathways. Recently new ATP-competitive inhibitor molecules have been identified, which have the capability to inhibit both TORC1 and TORC2 in humans but their efficacy to inhibit TOR complexes in yeast is less due to drug uptake efflux and penetration problems. One of these compounds, Torin1 is a potent inhibitor of both mTORC1 and mTORC2.

Although the phenotypes observed with Torin1 treatment in mammalian cells were earlier associated with inhibition of mTORC2 but it was later found out that mTORC1 has some functions that are relatively rapamycin resistant so rapamycin is not a complete inhibitor of TORC1 signalling pathway. How rapamycin controls different aspects of growth is still not fully known and the list of TOR substrates and the pathways, which are regulated by TOR is increasing (Thoreen et al. 2009).

eIF2B plays a central role in the translation initiation in eukaryotes and it is known that rapamycin inhibits the growth by blocking translation initiation. This study is focused on the regulation of eIF2B activity by phosphorylation, so changes in phosphorylation in response to TOR inhibition were monitored. In fact recent technical advances in the field of phospho-proteomics have identified many new molecules as TORC substrates and Gcd6p is one of them (Breitkreutz et al. 2010). The consequences of inhibition of TOR kinase activity by Torin1 in comparison to rapamycin and their affects on eIF2Bε phosphorylation sites are also examined in this study. The assays used in this study to assess the consequences of TOR inhibition on growth recorded effects which can be categorized according to time duration of treatment, e.g. growth assays using...
liquid cultures recorded response from 1-16 hours (longer for plate growth, 3 days) while polysome and western blotting observed the short term response, i.e. 60 mins. The following conclusions can be made from the data presented in this chapter.

If Gcd6p is a substrate of TOR kinase, then the eIF2βε phospho-site mutants should exhibit an altered growth response to rapamycin treatment. To investigate the response of eIF2βε phospho-mutants to rapamycin, growth assays were done using gcd6Δ strains and expressing gcd6 gene with desired Ser to Ala mutations from low copy plasmids as a sole source of Gcd6p. Growth assays (Figure 4.1) show that rapamycin only inhibits growth of ε-S435A mutants. Growth time course experiments show that wild type cells adapt to rapamycin stress after 2 hours of treatment (growth curves) and then continue to grow at a slower rate. In contrast, the S435A mutant strain cannot overcome the inhibitory effects of rapamycin. This experiment could have been repeated using Ser to Asp or Ser to Glu mutant strains to see the growth response of phosphomimetic amino acids to rapamycin to better understand the eIF2βε function. The TOR pathway regulates growth by inhibiting catabolic processes, i.e. autophagy associated with starvation (Loewith and Hall 2011). To see effects on cell morphology, cells were observed under a microscope. Populations of two different sizes were observed. It was observed that rapamycin treatment induced starvation increased cell size owing to the presence of large vacuoles (autophagosomes) in cell. While a subpopulation of smaller cells might represent the growth arrest, newly budded yeast.

Halo assays were done to find the minimum inhibitory concentration of rapamycin to measure ε-S435A sensitivity to rapamycin. Halo assays showed that ε-S435A mutant is sensitive to small doses of rapamycin as low as 1 nM (Figure 4.3). Halo assays also showed that GCN2 play little role during rapamycin stress in YPD medium while its presence makes a significant contribution to survival when cells are treated with rapamycin in minimal medium (without any amino acids) (Figure 4.3-4.4). Another observation is that at higher concentrations of rapamycin (beyond 100 nM) the area of zone of inhibition does not follow the same trend (Supplementary Figure 1). It seems that at these high levels halos are saturated and higher concentrations do not cast a significant effect on the area of
zone of inhibition. The apparent discrepancy observed between the area of inhibition in YPD medium and SD medium may reflect the difference in sensitivities in two different media. During growth in minimal medium, cells have induced the basal level of \textit{GCN4} and upon treatment with rapamycin; cells can activate the pathways necessary for stress response to ensure survival.

Polysome analysis (Figure 4.5) shows that rapamycin inhibits yeast growth by blocking protein synthesis at translation initiation step as is seen by a marked increase in 80S peak and decay of polysome peaks. The decrease in yeast growth is due to decrease in protein translation initiation caused by rapamycin treatment.

To further elucidate the effects of rapamycin on eIF2B phosphorylation at selected phospho-sites in eIF2Bε, western analysis was done on protein extracts prepared from cells treated with and without rapamycin treatment (100 nM/ml for 60 mins in YPD medium). An increase in phosphorylation at S435, S528 and S538 was observed while the phosphorylation at S525 remains unaltered. In previous studies it was shown that translation inhibition is detectable after 120 mins (Barbet et al. 1996) and it was also observed during growth curves that cells adapt to rapamycin after two hours of incubation with rapamycin (Figure 4.2), so changes in phosphorylation patterns were monitored for four consecutive hours following rapamycin treatment. The results were surprising that after an increase in phosphorylation after one hour of treatment at S435, S528 was followed by subsequent dephosphorylation at these sites. This might be an indication that dephosphorylation at these sites is related to inhibition of protein translation initiation and less growth. Rapamycin treatment increases the phosphorylation at ε-S435 in the first hour which is not maintained over treatment time. In this experiment the effect of rapamycin on time course was followed only for samples treated with rapamycin. The changes in phosphorylation of control samples were not monitored which makes the results less reliable. Two concerns rose due to no control which needs to be addressed in future. Firstly there is a possibility that Ser to Ala substitution could have an effect on function of eIF2B which is not due to changes in phosphorylation but due to changes in the secondary structure of eIF2B. Secondly there could be a possibility that these changes could take place in the absence of rapamycin because control was not monitored for phosphorylation.
status at selected sites for the later time points in Fig. 4.7. The phosphorylation of these sites might change with different phases of cell cycle. Activities of kinases do not remain normal while varies with different stages of cell cycle, i.e. some are active at log phase while some are active during stationary phase. If phosphorylation of these sites is important, it needs to be monitored over time during all stages of cell cycle. These possibilities need to be excluded by performing this experiment with mutants carrying Ser to Glu/Asp mutations and monitoring phosphorylation status at the ε-S435, S528, and S538 sites by using phospho-specific antibodies at different time points of cell cycle.

Recent studies in mammalian systems have discovered analogs of rapamycin which have the ability to inhibit both TORC1 and TORC2 mediated signalling pathways (Thoreen et al. 2009, Liu et al. 2010). Torin1 is one of those compounds and was used in this study to monitor the effects on TOR signalling and eIF2B phosphorylation changes. Initial data showed that Torin1 is an inhibitor of yeast growth.

Growth assays showed that Torin1 used at a concentration of 10 and 20 μM was strong enough to inhibit the yeast growth. Interestingly it not only inhibited the growth of S435A cells, but also inhibited the growth of S525A and S528A to a considerable degree. This was further confirmed by halo assays and growth curves which showed that Torin1 is a potent inhibitor of yeast growth and it inhibited the growth of S435A, S525A and S528 of cells within first one hour of treatment while wild type and S538A strains responded to the drug after two hours of treatment. The following two reasons can account for the enhanced sensitivity of S435A and S528A cells observed with Torin1 growth assays. i. Rapamycin does not completely inhibit TORC1, ii. The sensitive phenotype of S525A and S528A with Torin1 treatment is due to inhibition of TORC1 and/or TORC2 and Gcd6p is a downstream effector of the TORC signalling network.

To elucidate the effects of Torin1 on translation, polysome analysis was done. It gave the information that whether translation inhibition was at initiation of elongation step. Polysome profiles of samples treated with 20 μM/ ml of Torin1 show that translation is inhibited at initiation step as is seen with a rise in the 80S
peak and decrease in polysomes. Translation was inhibited in all three strains that showed sensitive phenotype during growth assays in liquid and solid medium (Figure 4.13).

The effects observed with Torin1 on cell size are confusing and unclear. As it has already been discussed in chapter 3 that Ser to Ala substitutions at selected residues in ε subunit affected the cell sizes of mutants, S435A and S538A being of larger size while S525A and S528A mutations does not alter the cell size. Torin1 treatment appears to reduce the cell size of S435A and S538A while increases the cell size of S525A and S528A mutants, although the increase observed in case of S528A is not significant. The reason for this effect is currently unclear, but it may be speculated to be a consequence of Torin1 inhibition of TORC2 and subsequent affects on cytoskeleton organization (Kamada et al. 2005).

To examine the effects on eIF2Bε phosphorylation, western blotting was done. It was found that Torin1 changes the phosphorylation pattern at multiple sites in eIF2Bε (ε-S435, ε-S525 and ε-S528 being down regulated). The effects seen are more severe than rapamycin treatment. Rapamycin caused dephosphorylation following two hours of treatment, while Torin1 caused dephosphorylation within one hour of treatment. The level of eIF2α(p) increased in treated samples which is different from the affects observed with Torin1 treatment in mammalian cells. This indicates the Gcn2 activation which is a general nutrient stress response in yeast (Thoreen et al. 2012).

The data generated suggests that phosphorylation of Gcd6p is regulated by TOR or TOR regulated kinases. Although it is not confirmed that Gcd6p is a direct downstream target of TORC1 and/ or TORC2 as Torin1 inhibits both complexes through ATP-competitive mechanism. The role of eIF2 in cytoskeleton studies has been shown in an old study done in 1980's (Howe and Hershey 1984), this study suggests the role of eIF2B in cytoskeleton organisation and cell polarity. How these changes in phosphorylation pattern affect the protein synthesis rate and catalytic activity of eIF2B needs to be confirmed.
Based on kinase prediction software for cognitive phospho-sites based on their conserved sequence motifs (mentioned in material and methods, Section 2.27) and TOR signalling pathway from literature, the following tentative model can be made for the regulation of Gcd6 phosphorylation by TOR signalling (Figure 4.15).

Rapamycin treatment inhibits global translation initiation, which causes reduce translation of cyclin (Cln3). Low levels of Cln 3 causes G1 cell cycle arrest and increase cell size which might be cause of autophagosome (Barbet et al. 1996). Although the role of TORC1 is more characterized for G1 regulation of cell cycle, rapamycin also affects other phases of cell cycle. The localization of Cdc5 is regulated by TORC1. Cdc5 destabilizes Swe1 which regulates the activity of Cdc28. Inhibition of TORC1 by rapamycin prolonged G2/ M transition by inactivation of cdc28 (Nakashima et al. 2008, Loewith and Hall 2011). Ypk1 is the substrate for TORC2 and is involved in cell polarity in yeast. The position of S525 fits with the recognition sequence motif for Ypk1. If Cdc28 and Ypk1 are cognitive kinases for Gcd6p at these sites, then this study may suggests the new roles of Gcd6p in yeast growth by regulation of cell cycle progression in response to stress conditions and actin polarization.
Figure 4.15. Regulation of eIF2B phosphorylation by TOR signalling.

TORC1 regulates growth by cell cycle progression and translation initiation. Sequence motif of S435 site fits with the recognition sequence motif for Cdc28 kinase which is involved in cell cycle progression. Ypk1 is a substrate identified for TORC2, which is the member of AGC kinase family. Ypk1 may be the cognitive kinase for Gcd6 at S525 site and may play a role in the actin cytoskeleton organisation.
Chapter 5

Impact of environmental and genetic factors on eIF2B in response to amino acid starvation
5 Impact of environmental and genetic factors on eIF2B in response to amino acid starvation

Global and mRNA specific translational control are two modes of translational regulation in eukaryotes. Many pathways are known by which these two mechanisms regulate growth under different stress conditions. One of the very well known mechanisms of translational control during amino acid starvation is via phosphorylation of eIF2α at Ser51. Gcn2p kinase phosphorylates eIF2α and converts it from substrate to inhibitor of eIF2B. Lower eIF2B activity causes a reduction in the level of TC. The outcome of the whole series of events triggered by the phosphorylation of eIF2α(p) is the inhibition of global protein translation with simultaneous induction of GCN4 translation (Hinnebusch 1988, Hinnebusch 2005). The mechanism of activation of GCN4 translation by eIF2α(p) and the global inhibition of translation has been discussed already in detail in section 1.5.

Genome wide analysis conducted in yeast has shown that GCN4 induced expression of nearly one tenth of the yeast genome in amino acid starved cells. These include genes involved in the biosynthesis of amino acid, vitamins, purine, autophagy proteins and amino acid transporters. This response is known as CPC in Neurospora and GAAC in yeast (Hinnebusch 2005). Out of ~73 genes whose expression is elevated in GAAC response, >30 genes are involved in 12 different amino acid biosynthetic pathways and their expression can be induced by starving yeast for any one of the at least 10 amino acids. Genetic analyses have identified trans-acting protein which regulates the GAAC expression either positively or negatively. Inactivation of GCN4 and recessive mutations in the positive regulatory genes GCN1, GCN2 and GCN3 make yeast sensitive to multiple inhibitors of amino acid biosynthetic enzymes i.e, 3-aminotriazole (3-AT) by preventing induction of biosynthetic enzymes subject to GAAC pathway and produce the Gcn− phenotype while loss of function mutations in negative regulators factors of the GAAC pathway results in the constitutive expression of GAAC and produce Gcd− phenotype (Hinnebusch 2005). Even in the absence of additional amino acids in growth medium, yeast can maintain a basal expression level of amino acids genes that represses the GCN4 pathway. Provided yeast can
synthesize all 20 amino acids the GAAC pathway is not activated in yeast by growth in minimal medium (without any amino acid). The biosynthesis of proteins involved in amino acid synthesis can be inhibited by anti-metabolites or mutations in biosynthetic enzymes. The sixth step of the histidine biosynthetic pathway is catalysed by protein encoded by \textit{HIS3} gene: imidazoleglycerol-phosphate dehydratase. 3-AT, a heterocyclic compound is a competitive inhibitor of the product of \textit{HIS3} gene and is used to produce Histidine limitation (Hinnebusch 1988). Histidine starvation results in increased levels of phosphorylated eIF2α, which inhibits eIF2B activity.

The aim of this study is to investigate whether changes in eIF2B phosphorylation also accompany the response to 3-AT and if this contributes to the mechanism of protein translation regulation during amino acid starvation. Do Ser to Ala mutations at selected residues impart any growth phenotypes under amino acid starvation?. Different strategies were used to stress the cells (for the limited supply of amino acids) followed by measuring subsequent changes in the phosphorylation pattern of eIF2Bε by phospho-specific antibodies.

\textbf{5.1 ε-S435A mutation confers Gcn\textsuperscript{−} phenotype}

To examine the phenotypic effects of selected phospho-Ser residues mutations on growth in response to amino acid starvation, the growth responses of phospho-Ser to Ala mutants were assessed. 3-AT was used to impose histidine limitation (mechanism of action discussed above). Growth assays were done with yeast strain deleted for \textit{gcd6} and expressing ser to ala mutation versions of \textit{gcd6} at selected site on plasmids as the sole source of Gcd6p in \textit{GCN2} background. Growth assays with serial dilutions were done as described previously in materials and methods (section 2.6). All the eIF2Bε phospho-ser to ala mutants in \textit{GCN2} background grew indistinguishably from the wild type on minimal medium + 3-AT except ε-S435A, whose growth was inhibited (Figure 5.1A and B, Lane 2). ε-S435A mutation conferred Gcn\textsuperscript{−} phenotype (failure to grow on 3-AT in the presence of \textit{GCN2}).
5. eIF2B phosphorylation in response to amino acid starvation

Figure 5.1. The eIF2Bε-S435A mutant exhibits Gcn\(^{-}\) phenotype.

**A.** Overnight grown cultures were diluted to \(A_{600}\) 0.2 and were 10 fold serially diluted. 3 \(\mu\)l of each dilution was spotted on SD plates (left) and SD+25 mM 3-AT plates (right). **B.** Growth of an equal number of cells (2 \(\mu\)l of \(A_{600}\) 0.2) at every position on plates containing a linear gradient of 3-AT to a maximum concentration of 50 mM 3-AT. All the strains used for this section were \(gcn2\Delta\) and harboured a \(GCN2\) plasmid with \(URA3\) markers as a sole source of Gcn2p. Plates were incubated at 30°C. Pictures were taken on day 2, 3 and 4. All strains carry \(GCN2\) on \(URA3\) plasmid. SD agar ± 25 and 50mM mM 3-AT plates were made fresh on the day of use and were air dried.
5.1.1 The non-reproducibility of Gcn− phenotype of ε-S435A resulted in the identification of a suppressor mutation

To verify the Gcn− phenotype of S435A, growth assays were repeated under the same set of experimental conditions.

Unexpectedly, the 3-AT sensitive phenotype of ε-S435A mutant was not reproduced and results obtained were different from the earlier findings (Figure 5.1) with ε-S435A mutants exhibiting resistant phenotype (Figure 5.2). It appeared that ε-S435A strain has reverted back to wild type and is no longer sensitive to 3-AT in GCN2 background. The question arises that was there a suppressor mutation in the strain that was masking Gcn− phenotype or was this phenotype due to drug depletion from medium. This experiment was repeated twice with the same observation.

It was decided to follow up the reason for the revert of S435A Gcn− mutant to wild type phenotype response (resistant) to 3-AT. Plasmids were isolated from overnight grown yeast cultures in SD medium and whole gene was sequenced using primers listed in Table 2.14. The sequence received was analysed through BLAST (N) (Altschul et al. 1997) against yeast genome database. Three point mutations were found (Figure 5.3), and the resulting amino acid sequence was determined by nucleotide sequence translation Sixpack (EMBOSS). Other than the desired mutations, encoding for Ala at 435, mutant used in the second set of experiment (S435A strain with resistant phenotype), was found to have one extra A1296C substitution mutation (Figure 5.3).

The new nucleotide sequence (CTA) codes for Leu at 432 amino acid position (I432L mutation). There are 6 codons for Leu and usage of CTA for Leu is 14%. 7 out of 60 Leu residues in eIF2B are coded by CTA codon, indicating that it is not a rare codon. How does this mutation revert the Gcn− phenotype back to wild type is not known. The affect of I432L was investigated next.
5. eIF2B phosphorylation in response to amino acid starvation

Regulation of eIF2B by Phosphorylation

Figure 5.2. eIF2Be-S435A reverts back to 3-AT resistant phenotype.

SD, SD+Ura/ agar + 25 mM and 50 mM 3-AT plates were made on the day of use. Cells were grown overnight in SD and SD+Ura medium, and A_{600} 0.2 were tenfold serially diluted. 3 μl of each of the serial dilution was spotted on plates. Plates were incubated at 30°C in incubator. Top set of the strains are gcn2Δ background while lower set carries GCN2 plasmids with URA marker. Pictures were taken on day 3 and 4.

Figure 5.3. Sequencing of the plasmid isolated from e-S435A strain.

Cells were grown in SD medium and plasmid extraction was done using QIAprep spin miniprep kit (Qiagen). Sequencing was done in The University of Manchester and sequence was BLAST (N) against yeast genome database in NCBI. Black solid asterisks show substituted nucleotide. Sequences at the bottom represent the mutations.
5.2 Characterization of ε-I432L: the suppressor of ε-S435A

As it has already been shown in section 5.1.1 that S435A mutants show Gcn⁻ phenotype in GCN2 background and the strains carrying a suppressor mutation I432L in addition to S435A revert to wild type resistant phenotype. Next task was to address these questions; i. Does I432L mutation alone impart any phenotype and ii. Is I432L mutation suppresses 3-AT sensitive phenotype?

Mutagenic primers were designed and ordered from Sigma Aldrich. Site directed mutagenesis was done to produce plasmids with I432L mutations by the protocol described earlier in section 2.4.2. The newly synthesized mutagenic plasmid was transformed into E. coli and sequenced using GCD6 primers listed in Table 2.14. Once it was confirmed that the plasmid carries only one point mutation leading to Ile to Leu substitution mutation, plasmid was transformed into yeast parent strain (GP 3750) (by LiAc aided transformation, section 2.4.7). 5-FOA medium was used to replace the plasmid carrying wild type GCD6 (URA3 marker) with a plasmid carrying ε-I432L mutant (LEU2 marker). The series of steps done for construction of the ε-I432L mutant strain are outlined in Figure 5.4.

Serial dilution growth test confirmed the Gcn⁻ phenotype of the ε-S435A mutants (Figure 5.5, Lane 4). Yeast cells with ε-I432L mutation only are resistant to 3-AT in GCN2 background like WT-eIF2Bε cells, indicating that this is a non-lethal mutation as the cells grew normally under optimal growth conditions and no observable phenotype was detected under amino acid stress imposed by 3-AT. Whilst ε-S435A is sensitive to 3-AT, ε- I432L, S435A double mutants are resistant to varying concentrations of 3-AT tested (Figure 5.2). This shows that I432L is silent mutation, but it masks the Gcn⁻ phenotype of ε-S435A (Figure 5.4). The mechanism of this suppression of Gcn⁻ phenotype by this newly identified suppressor mutation is not known.
5. eIF2B phosphorylation in response to amino acid starvation

**Figure 5.4. Construction of the ε-I432L, GCN2 strain.**

Schematic illustration of the main steps for the construction of strain. (For details see section 2.4.7).
5.3 ε-S435A affects translation initiation under amino acid starvation

Amino acids starvation is known to cause temporary growth inhibition while cells adapt to nutrient limitation. Protein translation is one of the key processes involved in regulating growth response during stress conditions. Studies in eukaryotic cells have shown that tRNA acetylation is one of the mechanisms that regulate protein synthesis. Deacylated tRNA activates Gcn2p kinase via its Hist-RS-related domain (Hinnebusch 2005, Mascarenhas et al. 2008). Activated Gcn2p phosphorylates eIF2α which then sequesters eIF2B resulting in lower lowers TC levels (Pavitt et al. 1998). Hence shutdown of the global protein synthesis occurs while mRNA specific translation of GCN4 is activated (Hinnebusch 1997, Dong et al. 2000, Anthony et al. 2001).

Translational efficiency was analyzed by measuring the distribution of polysomes in response to the amino acid (His) starvation (imposed by the 3-AT addition). The role of Gcn2p was also assessed, and traces were obtained for
eIF2Bε-phospho mutant yeast strains both in gcn2Δ and GCN2 backgrounds (Figure 5.6, Figure 5.7). Extracts obtained from the untreated samples exhibited normal profiles for wild type and ε-phospho mutants in gcn2Δ and GCN2 background under normal growth conditions (Figure 5.6AI and Figure 5.7AI). The traces of gcn2Δ strains after treatment with 25 mM 3-AT for 40 mins do not show any signs of stress. As the profiles look normal with 40S, 60S, 80S and more polysome peaks, there was no inhibition of translation initiation assessed through polysome profiles (Figure 5.6 AII). A dramatic shift of ribosomes from polysomal to monosomal 80S peaks in eIF2Bε phospho-mutant strains in GCN2 background indicates that GCN2 mediates decrease of translation initiation in response to amino acid starvation (Figure 5.7 AII). Interestingly the inhibition of translation did not occur in ε-S435A and the profile of ε-S435A in GCN2 resembles more like gcn2Δ. No significant increase in 80S was observed. This suggests that ε-S435A fails to activate GCN4 and cells were not stressed while cells carrying an additional suppressor mutation responded like wild type and were able to activate GCN4 pathway. As GCN4 pathway was not activated in ε-S435A, this might have lead to Gcn− phenotype of ε-S435A strain.

In wild type and other eIF2Bε phospho-mutants (I432L, I432L/ S435A, S525A, S528A, S538A) GAAC pathway was activated via GCN4 leading to protein translation being carried out even during nutrients limiting conditions at a reduced level to ensure survival. The rise is monosome peaks (80S peak) shows that cells are under stress (Figure 5.6 AII). The treatment of strains with cycloheximide before harvesting, maintained the polysomes, so excluding the possibility of mRNA degradation. P/M ratio was calculated. These results show that amino acid limitation imposed by the addition of 25 mM 3-AT for 40 mins at a concentration for 25 mM is enough to induce GCN4 via Gcn2p. Amino acid starvation changes phosphorylation pattern at ε-S435 and ε-S528 that may affect eIF2B activity.
Figure 5.6. GCN4 is not activated in gcn2Δ strains.  
A. Cells carrying wild type and indicated mutations were grown in 100 ml SD+Ura medium to A₆₀₀ 0.5. Cultures were split into two flasks and to one half of the cultures 3-AT was added to a final concentration of 25 mM for 40 mins. Extracts were prepared after addition of 500 ul of 10 mg/ml of cycloheximide for 10 mins. Polysome profiles were analyzed as described in materials and methods (section 2.7). (-), (+) indicates no treatment and 3-AT addition respectively. Small ribosomal subunit (40S), large ribosomal subunit (60S), monosome (80S) and polysome peaks are labelled.  
B. Bar graph depicting the quantification of A using ImageJ 1.46. Data is Mean±S.E.M. Results shown here are repeats of two independent experiments.
Figure 5.7. \textit{GCN2} mediates translation initiation inhibition in response to amino acid starvation.

A, B. The sole source of \textit{GCN2} in all strains is plasmid with \textit{URA3} marker (Pav 1198). Same as for Figure 5.6.
To examine the effects of amino acid limitation on eIF2Bε phosphorylation immunoblotting was done on purified eIF2B. Proteins were extracted from cells grown under normal and amino acid starvation conditions. Two different approaches were taken to impose amino acid limitation, (i) addition of 3-AT, and (ii) transfer the cells from SCD medium (with all amino acids) to SD medium (lacking all amino acid).

In the first approach cells were grown in SD medium to mid log phase and were amino acid starved for 40 mins by the addition of 25 mM 3-AT. Amino acid starvation cause increased phosphorylation of eIF2α at S51 (Figure 5.8A). The pattern of phosphorylation for eIF2 is also like phosphorylated eIF2 because in these experiments purified eIF2B was used instead of whole cell extracts, so more eIF2 that is phosphorylated was bound to eIF2B and purified with the complex. Western blotting reveals significant changes in the phosphorylation pattern of eIF2B at two sites (S435 and S528). There was ~50% decrease and ~45% increase in the phosphorylation at S435 and S528 sites respectively (Figure 5.8B). It was observed during optimization steps that harvesting conditions can affect phosphorylation of eIF2Bε (section 3.8), so another method was used to impose amino acid starvation Cells were grown in rich medium; and cell harvesting was done at 30°C. Cells were washed twice with pre-warmed SD for 5 mins, and were grown for 30 mins in SD minimal medium. Approaches adopted to impose amino acid stress are outlined in Figure 5.9A. Cell harvesting and protein purification was done as described earlier and phosphorylation changes were monitored using phospho- antibodies (Figure 5.9B) Results were consistent with the first approach except that the affect was ~ 10% less as compared to the starvation induced by the addition of 3-AT (Figure 5.9C).
Figure 5.8. Amino acid stress affects phosphorylation of eIF2Bε

A. GP3688 was grown in 2 litres SD+Ura to mid log phase $A_{600} 0.6$. 3-AT was added to a final concentration of 25 mM for 40 mins. Cells were harvested as described in section 2.8. Grinding was done in liquid $N_2$ large cryogenic grinding freezer mill. Proteins were purified using antiFLAG M2 affinity resins and two different concentrations of eluted proteins were analyzed on SDS-PAGE and immunoblotting with the monoclonal phospho-specific antibodies mentioned. "−" represents untreated while "+" is for 3-AT addition. B. Quantification of the western shown in A. Signals were normalised using Gcd6p as a loading control. Error bars are Mean+SEM. Grey triangle represents multiple loadings. *p< 0.05 from t-test.
5. eIF2B phosphorylation in response to amino acid starvation

Regulation of eIF2B by Phosphorylation

Figure 5.9. Amino acid starvation affects Gcd6p phosphorylation at multiple sites.

A. Schematic outline of the procedure for amino stress starvation induction. Cells were grown in SCD-His medium to A_600 0.5 and then were split into three fractions and processed as follows, I. control, II. Washed with SD medium and grown in SD+Ura medium for 30 mins, III. 25 mM 3-AT was added for 40 mins. B. Two different amounts of eluted proteins were analyzed on SDS-PAGE and immunoblotting with the monoclonal phospho-specific antibodies. Grey triangle represents multiple loadings. C. Quantification of B. Signals was normalized with Gcd6p as a loading control. Error bars are Mean+SEM. *p< 0.05, **p< 0.001 from an analysis of variance (ANOVA).
5.4 Discussion

Yeast cells utilize multiple pathways to cope with diverse stress conditions. A mechanism for amino acid starvation induced translational control of GCN4, a key transcriptional activator of amino acid biosynthetic genes is well documented. This mechanism works through eIF2 phosphorylation and attendant inhibition of eIF2B activity. This study has focused on the changes in the phosphorylation pattern of eIF2B at selected residues in eIF2Bε. The following inferences can be made from the data presented in this study.

Yeast can synthesize all 20 amino acids by using amino acid biosynthetic pathways, so amino acid stress was imposed by the addition of an anti-metabolite, 3-AT that hampered the Histidine biosynthetic pathway. All Gcd6p Ser to Ala mutants are sensitive to 3-AT in a gcn2Δ background. The Gcn\(^{-}\) phenotype in the gcn2Δ cells is consistent with the idea that eIF2B function is not impaired significantly by the Ser/Ala mutations, otherwise Gcd\(^{-}\) phenotype would be evident. All strains are resistant to 3-AT in GCN2 background except S435A which exhibits a Gcn\(^{-}\) phenotype. This is the first time that mutation in the GCD6 subunit (S435A) results in Gcn\(^{-}\) phenotype. Previously Gcn\(^{-}\) mutations have been found in GCN3, GCD7 and GCD2 (α, β and δ) subunits of eIF2B (Pavitt et al. 1997) and in eIF2α (SUI2) (Vazquez de Aldana et al. 1993). This helped identify the subunit as "regulatory". The finding of a Gcn\(^{-}\) allele in Gcd6 is potentially very significant for understanding of how eIF2B is regulated by eIF2α(p).

A suppressor mutation was identified which on its own does not impart any phenotype under any stress condition assessed in this study, but it can suppress the Gcn\(^{-}\) phenotype of S435A. It is not known how this revert the sensitive cells back to wild type phenotype especially when the role of S435 is not fully established and the structure of NTD of Gcd6p is not solved. An interesting observation was made after incubating the plates further at room temperature. Colonies of all gcd6p mutants in gcn2Δ background strains also started appearing on day four on SD+10, 25 mM 3-AT medium except for S528A mutant. This indicates that all strains are viable and can resume growth once the drug is depleted from the medium, whilst 3-AT affects S528A more acutely.
To check the viability of S528A in medium with 3-AT, Wild type and S528A mutants were treated with 25 mM 3-AT and 500 μl of the culture was taken after 10, 24, 48 hours and plated onto SD plates. No significant decrease in the number of colonies obtained for wild type and S528A was observed (data not shown). This result shows that S528A was more sensitive to 3-AT in gcna2Δ background.

The existing model for the mechanism of eIFα(p) mediated by Gcn2p inhibiting the catalytic activity of eIF2B is based on observations that that Gcn2p activated by uncharged tRNA (under starvation condition) phosphorylates eIF2α at Ser51. The α subunit of the eIF2B regulatory sub-complex can sense this phosphorylation. eIF2α(p) inhibits the activity of eIF2B as eIF2α(p) binds more tightly to eIF2B. The strong binding of eIF2α(p)-eIF2B complex reduces the functional eIF2B quantity significantly resulting in low TC levels and general translation inhibition. This model is based on an allosteric interaction between the eIF2B regulatory sub-complex and eIF2α subunit followed by inactivation of eIF2B catalytic sub-complex. This inactivation happens in starved cells (Krishnamoorthy et al. 2001). Amino acid starvation imposed by the addition of 3-AT results in the translation inhibition in wild type and Gcd6p Ser to Ala mutants. Addition of 3-AT for 40 mins reduces the P/M ratio (90% less P/M ratio polysome) in all strains except for S435A mutants. The data obtained by polysome quantification shows that even at a period where eIF2α is phosphorylated cells are not able to activate GCN4 pathway. No decrease in the P/M ratio was observed for ε-S435A GCN2 mutants indicating that the GCN4 mechanism that ensures the survival under nutrient limitation was not activated. This might indicate an additional level of eIF2B translational control, by affecting the cross talk between eIF2B sub-complexes or preventing inhibition even after the interaction of eIF2B-eIF2α(p) (interaction is not fruitful in terms of regulation of eIF2B activity). The model presented in Figure 5.10 can be proposed from these observations. These hypothesis need to be confirmed by measuring eIF2α phosphorylation and interaction between eIF2α-eIF2Bε-S435A complex in non-starved and starved cells.
Two different approaches were adopted to impose amino acid starvation to monitor the changes in phosphorylation. Results were consistent and significant changes in phosphorylation pattern were observed. The addition of 3-AT proved to be a stronger inducer of amino acid starvation stress than transferring cells from rich to minimal medium. Amino acid starvation caused by the addition of 3-AT led to ~65% decrease in phosphorylation at S435 and ~35% increase in phosphorylation at S528 site. Although the transferring from rich to minimal medium also produced the same effect albeit at a lower magnitude (60% decrease and 28% increase at S435 and S528 sites respectively). Taking together polysome and phosphorylation data indicates that ε-S435 phosphorylation is inversely related to eIF2B catalytic activity, although to establish the role of phosphorylation of this site in eIF2B catalytic activity needs further confirmation through GEF assays.
Figure 5.10. Model for the regulation of translation by phosphorylation under amino acid stress.

A. Under non-starvation conditions, eIF2B catalyse the exchange reaction. B. Early starvation leads to reduce TC and no growth. C. Activation of GCN4 pathway due to low levels of TC leads to recovery from amino acid stress. D. S435A mutation affects the regulation of catalytic subunit by regulatory subunit, high levels of TC during starvation conditions confers Gcn⁻ phenotype.
Chapter 6

The effects of 1-butanol on eIF2Be phosphorylation
6 The effects of 1-butanol on eIF2Bε phosphorylation

Volatile anaesthetics are used in medicine and they affect all type of cells and tissues. Mammals are comparatively more sensitive to anaesthetics than yeast. The manner in which these compounds act as anaesthetics in mammals parallels their action as an inhibitor of yeast cell division. Addition of anaesthetics leads to rapid sharp dose response that is reversible both in yeast and mammals. The only non-essential gene of eIF2B complex, GCN3(α) is involved in the regulation of translation initiation during amino acid stress conditions. The yeast with zzz3-1 mutations in GCN3 are resistant to isoflurane, this indicates that eIF2B is a part of the mechanism involved in translation inhibition during volatile anaesthetic (Palmer et al. 2005). The ability of volatile anaesthetic to inhibit cell division in yeast depends on the availability of branched chain amino acids i.e. leucine and tryptophan (Palmer et al. 2002). Eukaryotes break down branched chain amino acid to produce components of the tricarboxylic acid cycle (TCA). As compared to other eukaryotes, the metabolism of branched amino acid is different in yeast. Under optimal growth conditions yeast do not rely on branch chain amino acid for sole source of carbon. However under nitrogen insufficiency, branched chain amino acids are used as nitrogen source with production of fusel alcohols (isoamyl alcohol and isobutyl alcohol) as the end products. Fusel alcohols affect the growth of both haploid and diploid strains of S. cerevisiae and induce hyphal growth when treated with isoamyl alcohol at a concentration of 0.5% (v/v) and pseudohyphae at a concentration of 0.25%. (Dickinson 1996, Martinez-Anaya et al. 2003).

Both Isoflurane and fusel alcohols have been shown to inhibit the translation initiation in yeast (Ashe et al. 2001). It was shown that two allelic versions of the W303-1A strain responded to the addition of 1-butanol differently, one being sensitive (BUTS) and other butanol resistant (BUTR). It was confirmed through the genetic mapping that the two isolate of W303-1A contains different alleles of GCD1, which codes for eIF2Bγ. W303-1A allele with γ-S180 shows butanol sensitive phenotype while strain with γ-P180 shows resistant phenotype.
Although deletion of GCN3 itself does not impact on the butanol sensitivity/resistance of strain but it was found that two semi dominant GCN3 R148K and T41K mutations confer butanol resistant phenotype to BUT^S strains (Taylor et al. 2010). Studies have been done to characterize the role of other eIF2B subunits in butanol response. eIF2Bβ-V341D confers butanol sensitive phenotype, while Gcd6p mutants response to butanol is not uniform. Gcd6p-C383W mutant is resistant while Gcd6p-I90F, R284H, and W618R mutants are sensitive to butanol.

The human analogous of these yeast mutations are involved in human VWM disease (Richardson et al. 2004). The involvement of four out of five subunits of eIF2B in mediating effects of butanol shows that this is a key player of the mechanism involved in translation regulation during fusel alcohols stress.

Fusel alcohols dephosphorylate eIF2α in a Sit4p-dependent manner but the inhibition of translation is not the consequence of eIF2α(p) dephosphorylation (Taylor et al. 2010). The inhibition of translation initiation by fusel alcohols and especially eIF2B subunits playing a role in translation inhibition mechanism provokes the idea of investigation of the effects of alcohols on eIF2Bε mutations of selected Ser residues for this study. This chapter describes the data obtained for the characterization of selected mutations using butanol (one of the fusel alcohols) as a translation inhibitor. Effect of butanol on different growth aspects of wild type and phospho-mutant yeasts were observed, followed by monitoring changes in phosphorylation at the selected phospho sites in eIF2Bε.

### 6.1 eIF2Bε phospho-mutants vary in their resistance to 1-butanol

Butanol effects yeast growth and induces morphological changes in a strain specific manner in \textit{S. cerevisiae}. For example butanol treatment leads to the growth of hyphae like extensions in W303-1A strain whilst having no effect in S288c strain. The difference in phenotype lies in gene mutation in \textit{FLO8} and some other unknown additional inputs that were not present in S288c. It has been shown previously that 1-butanol and isoamyl alcohols induce elongation of the haploid yeast cells, changes in the budding pattern and filamentous growth (Lorenz et al. 2000, Ashe et al. 2001).
Growth in nutrient poor medium such as minimal medium without any amino acids causes sublevel starvation stress and the subsequent addition of 1-butanol would result in two stresses operating together leading to the regulation of different sets of mRNAs (Smirnova et al. 2005). To avoid any side effects of the medium and to attribute the growth rate defects only to 1-butanol, growth assays were performed in YPD medium with and without +1-butanol at a concentration of 1% and 2% (Figure 6.1). Butanol is highly volatile so to avoid the problem of its evaporation from the medium; two different approaches were adopted while doing plating assays. 10-fold serial diluted cultures were spotted on plates containing 1% and 2% 1-butanol evenly distributed (Figure 6.1A). In the second approach the same concentration of cells was spotted on YPD plates varying in concentration of 1-butanol across the plate (Figure 6.1B).

Growth assays show that butanol sensitivity is mutation specific. ε-S528A is more sensitive to 1% and 2% butanol (Figure 6.1A, Lane 4). Despite consistent sensitivity of ε-S435A to all environmental stresses i.e. rapamycin, Torin1, and amino acid starvation (see chapters 4 and 5), it showed resistance phenotype to 1-butanol.

Growth assays in liquid medium were performed to measure the effects on doubling time (Figure 6.2). Data is in agreement to the results obtained on plates as S528A mutants took almost 5 hours to complete a single doubling while wild type and other phosphor-mutants took 4 hours to double in cell number. These initial growth assays identified two promising eIF2Bε phospho-mutants (ε-S5435A and ε-S528A). It can be said that 1-butanol inhibits growth in a strain specific manner.
A. Overnight cultures were back diluted and grown in fresh YPD medium to $A_{600}$ to 0.5. Cultures were 10 fold serially diluted. YPD+1-butanol (1% and 2%) plates were made fresh on the day and were dried on the bench with lids slightly tilted to avoid evaporation of butanol. 2 μl of each dilution were spotted on plates and incubated at 30°C. B. Gradient plates were made as described earlier in materials and methods (section 2.5.2). Gradient plates contain a gradient of butanol up to a maximum of 1% and 2% (v/v) and same concentration of cells (2 μl of the $A_{600}$ 0.3) were spotted across the plate at each position across the plate. YPD and YPD+1-2% butanol plates were made fresh on the day of use. Pictures were taken on day 2, 3 and 4.

Figure 6.1. eIF2Bε phospho-mutants vary in their sensitivity to 1-butanol.
6. The effects of 1-butanol on eIF2Bε phosphorylation

Regulation of eIF2B by Phosphorylation

Figure 6.2. Effects of 2% 1-butanol on doubling time of eIF2Bε phospho-mutants.

Cells were grown to \( A_{600} \) 0.6 and were split into two groups, control and treated. 2% (v/v) 1-butanol was added and growth was assessed by measuring absorbance at \( A_{600} \) every 1 hour for continuous 8 hours. Growth rates were plotted between \( \log_{10} A_{600} \) vs time and doubling time was calculated through equation that fits the data. Effect of 1-butanol on growth rate, measured as doubling time (hours) is shown in bar graph (Mean±SD is plotted, where \( n=3 \)).

6.2 1-butanol differentially inhibits the translation initiation of eIF2Bε phospho-mutants

To establish whether the inhibition of translation initiation by butanol was responsible for observed growth defects, polysome analysis was done to measure translational response to butanol. Cells were treated with 1-butanol 2% (v/v) in YPD for 10 mins. Cells were grown and harvested as described earlier in section 2.7. The protocol was used that was developed by Ashe laboratory (Ashe et al. 2001). Cell extracts were prepared following 10 mins cycloheximide treatment which is a translation elongation inhibitor. Butanol rapidly inhibits the growth, demonstrated as high 80S peaks within 10mins of treatment (Figure 6.3).
6. The effects of 1-butanol on eIF2Bε phosphorylation

Figure 6.3. 1-butanol differentially inhibits translation initiation in eIF2Bε-phospho mutants.

Polysome traces from eIF2Bε phospho mutant strains grown in YPD and incubated with 1-butanol at the concentrations of 2% (v/v) for 10 min at 30°C. Total cell extracts were prepared following 10 mins treatment with 500 μl of 10 mg/ml cycloheximide. 2.5 units of A_{260} extracts were layered on 15-50% sucrose gradients. Upper panel shows the profiles of control samples and lower panel represents the profile of treated samples. B. Quantification of the polysome profiles presented in Panel A Image J was used to calculate area under peaks and P/M ratio was calculated and shown as bar graphs. (-) is for control and (+) indicates the addition of butanol.
6. The effects of 1-butanol on eIF2Bε phosphorylation

1-butanol generally inhibits translation initiation of all strains within 10 mins of treatment, yet various eIF2Bε phospho Ser to Ala mutants differ in their susceptibility to 1-butanol. The polysome profiles of wild type S435, S525A and S538A look similar. Consistent with growth assays on plates (Figure 6.1, 6.2); a more effective inhibition of translation initiation was observed in ε-S528A strain (90% reduction in P/M ratio). However the mechanism underlying the differential behaviour of eIF2Bε phospho-mutants to 1-butanol cannot be predicted because of the limited knowledge of the structure and regulation of eIF2B activity especially when mutants with four out of five eIF2B subunits show phenotypes to butanol stress.

6.3 1-butanol affects the phosphorylation pattern of eIF2B

To see the effect of 1-butanol on eIF2B, the phosphorylation level of purified eIF2B was measured using Phos-tag stain. eIF2B was purified from untreated and cells treated with 2% (v/v) 1-butanol for 10 mins. Equal quantities of protein sample were loaded onto SDS-polyacrylamide gel and the gel was stained with Coomassie brilliant blue for total proteins and Phos-tag specific for phosphorylated proteins (Figure 6.4). The untreated sample (Figure 6.4A, Lane 1) shows phosphorylation of eIF2B subunits and that eIF2B ε and γ subunits phosphorylation diminishes following treatment with butanol (Figure 6.4A, Lane 2).

This observation cannot be attributed to unequal loading of samples purified from control and treated cells, as the same gel stained with commassie stain shows equal loading of the sample (Figure 6.4B, Lane 4). Overall this result shows that butanol treatment causes dephosphorylation of multiple subunits of eIF2B. To expand the analysis of the impact of 1-butanol on dephosphorylation of eIF2B complex; changes in phosphorylation pattern at selected phospho-sites were monitored through customized phospho specific antibodies. The turnout of the first attempt was very interesting and promising as changes in phosphorylation pattern of eIF2B at all selected sites were observed although the pattern varied with each site investigated.
The effects of 1-butanol on eIF2Bε phosphorylation

Regulation of eIF2B by Phosphorylation

Figure 6.4. Effect of 1-butanol on eIF2B phosphorylation.

A. GP 3688 was grown in YPD medium to mid log phase and eIF2B was purified from cells grown under control and cells treated with 2% (v/v) 1-butanol for 10 mins. The purified protein was run on SDS-PAGE and the gel was stained with Phos-tag stain. Gel was viewed under UV light and the image was taken using GelDoc. B. Same gel was later stained with coomassie blue stain (Bio-Rad) to see all proteins in the loaded sample.

The phosphorylation of some sites was up regulated i.e. ε-S435, S528 and S538, while S525 was down regulated in response to 10 mins 2% (v/v) butanol treatment. The more significant impact was on the phosphorylation at S528 and S538 where it went up to almost 100%. There was almost 30% decrease in the phosphorylation at S525 (Figure 6.5A, B). This result was not consistent with the observation made earlier with phos-tag staining (Figure 6.4B). Dephosphorylation of eIF2α was also observed. A number of unsuccessful attempts were made to set the reliability of these results. Repeated experiments showed that upregulation of ε-S528 and ε-S538 and dephosphorylation of eIF2α(p) were consistent and reproducible while the down regulation of S525 never happened again in all repeats done and same is true for up regulation of phosphorylation at ε-S435 (Figure 6.5C,D).
6. The effects of 1-butanol on eIF2Bε phosphorylation

Regulation of eIF2B by Phosphorylation

Figure 6.5. Butanol alters the phosphorylation pattern of eIF2Bε at multiple sites.

A. 2 litre of culture was grown in YPD and cells were harvested before and post treatment 2% (v/v) 1-butanol for 10 mins. Proteins were purified using FLAG M2 affinity resins. Protein extracts were blotted and probed with phospho-specific antibodies. C. Same as A except two different amounts of the eluted proteins were loaded and analysed through westerns blotting. B, D. Quantification of the panel of westerns presented in A and C. Data is Mean + S.E.M (n=3). Level of significance was measured by t-test. *p< 0.05.
6.4 Discussion

Eukaryotic cells regulate translation by various mechanisms developed in the course of evolution to sustain growth in various environmental stress conditions. Cells respond to various stresses via different mechanisms. During nitrogen starvation conditions, branched chain amino acids are catabolised and fusel alcohols are produced as by products. Fusel alcohols have been shown to affect range of cellular processes, membrane transport, cell cycle and translation initiation. Although the mechanism of translation initiation inhibition by fusel alcohols is not fully understood but studies show that eIF2B is a key component of translation inhibition by fusel alcohols. The function of four subunits of eIF2B has been shown to regulate translational response yeast (Ashe et al. 2001, Taylor et al. 2010).

In this study the growth response of eIF2Bε phospho-site mutants (S435A, S525A, S528A and S538A) to 1-butanol and its impact on the phosphorylation were examined. Growth responses were assessed by different growth assays which measured the short and long term impact on yeast growth ranging from 10 minutes to four days. Growth assays show that eIF2Bε mutants differ in their sensitivity to 1-butanol. Mutations produce different responses and make strains either more sensitive or resistant to 1-butanol than wild type cells; e.g. ε-S528A mutation confers butanol sensitivity while strain carrying S435A mutation is resistant to butanol. Affects on doubling time also agrees, as doubling time recorded for S528A is ~ 5 hours as compared to other strains (~ 4 hours doubling time). This response of S435A mutation is remarkably different from the other stress conditions tested and reported in chapter 4 and 5, e.g. rapamycin and 3-AT induced amino acid starvation as it exhibits resistance to 1-butanol in growth assays. S435A and S528A seem two promising candidates for future studies to study the mechanism of translation regulation in butanol response, S435A being resistant whilst S528A is sensitive to 1-butanol.

To assess the affect of butanol on translation initiation, polysome analysis was performed with all strains. Results show that 1-butanol differentially inhibits translation initiation in eIF2Bε phospho mutant strains. Inhibition of polysomes
was more pronounced in S528A mutants. Although the polysome profile of S435A shows less polysomes, but the level of inhibition was less as compared other strains (Figure 6.3).

A recent study in yeast has shown that butanol affects the dynamics and movement of eIF2B cytoplasmic body and causes dephosphorylation of eIF2(α)p (Taylor et al. 2010). If this is the case, 1-butanol may affect the phosphorylation pattern of eIF2B which could be the reason for the translation inhibition. To investigate the effect of butanol on eIF2B phosphorylation, eIF2B was purified from cells harvested without and post 10 mins treatment with 2% 1-butanol. Phos-tag staining was used (specific for phosphorylated proteins) to find the effect on phosphorylation of protein purified from treated samples. Figure 6.4 shows that protein purified from cells treated with 1-butanol seems dephosphorylated. It has been shown that during butanol treatment causes Sit4p dependent dephosphorylation of eIF2α(p). In terms of substrate specificity, phosphatases are less specific than kinases. The possibility that Sit4p causes dephosphorylation at multiple sites of eIF2B as observed with phos-tag staining could be investigated using mutant strains. This was not attempted due to time constraints.

To extend the analysis of 1-butanol affect on individual eIF2Bε phospho-sites selected for this study, western blotting with phospho-specific antibodies was done. The first attempt was very promising indicating changes at multiple sites in eIF2Bε. The results were not in accordance with the earlier observation made with the phos-tag staining pattern where butanol causes dephosphorylation of multiple subunits of eIF2B complex and S528 phosphorylation requiring priming phosphorylation of S525 sites. It was found that except for S525, where it went down phosphorylation was up regulated at all sites. To address this discrepancy, experiments were repeated. Phos-tag staining could not be repeated as PerkinElmer no longer market the Phos-tag stain product. The reason for withdrawal of this product is not known. Repeating the western blotting demonstrated that up regulation of S528 and S538 phosphorylation is consistent and reproducible while changes in phosphorylation at S435 and S525 were not reproduced.
Overall, this study indicates that 1-butanol inhibits the translation initiation in a strain specific manner. The growth inhibition of gcd6 mutants varied from the affects observed during amino acid starvation experiments. This shows that the inhibition of translation is not through the well established mechanism of translational regulation via eIF2α(p). Each site might be regulated by different kinase/phosphatase pairs under different condition. 1-butanol affects the phosphorylation pattern of eIF2B at multiple sites. Whether these changes affect the protein synthesis rate and eIF2B activity could be confirmed through \(^{35}\text{S}\) Methionine incorporation into yeast protein and GEF assays with proteins purified from untreated and cells treated with 1-butanol.
Chapter 7

Discussion
7 Discussion

Translation in eukaryotes is a complex multi-step process that involves many RNA-RNA and RNA-Protein interactions. In the course of evolution, eukaryotes have attained additional complexity that provided additional check points for translational regulation. The complexity of the stage is directly proportional to the translational control practiced at that stage. In eukaryotes, cells invest a substantial portion of their calorie intake in the synthesis of new proteins, for example, more than 30 polypeptides are required for translation initiation other than ribosomes and tRNAs. Translation initiation is under strict control. The translation factors not only facilitate translation but are also involved in the regulation of protein synthesis process.

eIF2B, a heteropentameric GEF protein, carries out the first step of translation initiation, i.e. recycling of eIF2-GDP to eIF2-GTP (Pavitt et al. 1998). Eukaryotic cells have evolved multiple pathways that target eIF2B to regulate translation initiation in response to various stress conditions (Pavitt 2005, Proud 2005). Two mechanisms are known to regulate eIF2B GEF activity which differs in their modes of action. First mechanism is indirect and regulates GEF activity of eIF2B via phosphorylation of eIF2α (Pavitt et al. 1997). Second mechanism directly affects eIF2B activity via phosphorylation of eIF2Bε subunit. The latter mechanism has been studied in detail in mammals, and different kinases have been identified for eIF2Bε which regulate eIF2B activity in response to various environmental conditions (Welsh et al. 1998, Woods et al. 2001, Proud 2005).

The role of phosphorylation in regulating the eIF2B activity in mammals is controversial as studies conducted by different groups have shown contradictory results. In this regard, one study suggested that phosphorylation of eIF2B by GSK3β inhibits GEF activity while phosphorylation by CK1 and CK2 did not affect eIF2B GEF activity (Oldfield and Proud 1992, Welsh et al. 1998). The inhibition of GEF activity of eIF2B by GSK3 phosphorylation was also demonstrated during another study investigating the role of GSK3 in apoptosis and cell survival (Pap and Cooper 2002). However, other studies reported that
phosphorylation by CK1 and CK2 kinase stimulated the catalytic activity of eIF2B and phosphorylation by GSK3 had no effect on the catalytic activity of eIF2B (Singh et al. 1996). This indicates the need to carry out further research to definitely establish the phosphorylation as a mechanism regulating the eIF2B activity. No study has been done so far in yeast regarding the phosphorylation as a regulatory mechanism of regulation of GEF activity of eIF2B.

Another less understood mechanism involving the translation inhibition in response to fusel alcohols has been shown to involve the role of eIF2B subunits. Although the mechanism of action is not known but four out of five subunits are shown to play a role in translational regulation by alcohols. Butanol treatment affects the localization of eIF2B body although it is not yet established that these do alter the GEF activity of eIF2B (Ashe et al. 2001, Taylor et al. 2010).

Yeast proteome is ~30% phosphorylated, but biochemical understanding of protein phosphorylation is extremely limited; kinases are known for less than 160 proteins while substrates are known only for half of the kinases (Ptacek et al. 2005). eIF2B is a phosphor-protein, eight different phosphorylation sites were identified in subunits of catalytic sub-complex of yeast through MS. This study was designed to monitor the changes in phosphorylation at these sites in response to different stress conditions. Multiple stress conditions were selected based on their inhibitory effects on translation initiation and yeast growth. The affects of changes in phosphorylation pattern at selected eIF2Bε residues on translation and yeast growth were assessed by growth assays, polysome analysis and western blotting using phospho-specific antibodies targeted against these residues. The data generated during this study is summarized in Table 7.1.

Based on changes in phosphorylation pattern observed at eIF2Bε selected sites and growth phenotypes, the following general conclusions can be made. The Ser to Ala mutations at these sites did not affect the Gcd6p expression level (see Figure 3.11) and GCN2 is not involved in the phosphorylation of these sites as shown by westerns presented in Figure 3.13.
Table 7.1. Effects of different stress conditions on various aspects of yeast growth

<table>
<thead>
<tr>
<th>Phospho-mutant</th>
<th>Phenotypea</th>
<th>Translationb inhibition</th>
<th>Phospho-specific western blottingc</th>
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<tbody>
<tr>
<td>WT-(\text{eIF2B}^\varepsilon)</td>
<td>*Sensitive</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>(\varepsilon)-S435A</td>
<td>Hyper-sensitive</td>
<td>Yes</td>
<td>up regulation for first one hour followed by down-regulation</td>
</tr>
<tr>
<td>(\varepsilon)-S525A</td>
<td>Resistant</td>
<td>Yes</td>
<td>up regulation for first one hour followed by down-regulation</td>
</tr>
<tr>
<td>(\varepsilon)-S528A</td>
<td>Resistant</td>
<td>Yes</td>
<td>Down-regulation</td>
</tr>
<tr>
<td>(\varepsilon)-S538A</td>
<td>Resistant</td>
<td>Yes</td>
<td>Up-regulation</td>
</tr>
<tr>
<td>Rapamycin (100 nM/ ml, 1-4 hours)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Torin1 (20 (\mu)M/ ml, 2 hours)</td>
<td></td>
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<td></td>
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<tr>
<td>Amino acid starvation (25 mM, 30-40 mins)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>1-Butanol (2%, 10 mins)</td>
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</table>

* Cells are sensitive for the initial period of stress (data from halo assays Figure 4.3, 4.10).

aData obtained from growth assays (Figure 4.1, 4.9, 5.1, 6.1)

bPolysome data shows translation inhibition (Figure 4.5, 4.13, 5.7, 6.3)

cWestern blotting was done using phospho-specific antibodies (Table 2.26) (Figure 4.6, 4.7, 4.14, 5.8, 5.9, 6.5)
Three out of four sites (S525, S528 and S538) are located in place equal to mammalian DYRK/GSK3 sites in a region adjacent to the catalytic domain so are ideally placed to moderate eIF2B GEF activity (see Figure 3.20).

\( eIF2B_{\varepsilon-S435} \) is the only phospho-site that lies outside catalytic region in \( L\beta H \) domain. Although S435A mutation does not affect the yeast growth under optimal condition assessed through growth assays on the plate and in liquid assays, but cell morphology is effected. Cells with S435A mutation are of larger size (Figure 3.9) and Torin1 treatment causes a decrease in cell size (see Figure 4.12). Linking growth data with western blotting data suggests that phosphorylation at S435 site affects growth, as dephosphorylation of pS435 under various stress conditions (rapamycin, Torin1 and amino acid starvation) accompanies translation inhibition and growth inhibition. This may be due to the inhibition of the associated kinase responsible for phosphorylation of the S435 residue. In case of fusel alcohol induced translation inhibition, \( \varepsilon-S435A \) strain showed resistant phenotype and no dephosphorylation at S435 was observed as well. This result suggests that S435 is essential for the proper functioning of eIF2B and different environmental signals target this site. Yeast strains with \( \varepsilon-S435A \) mutations fail to respond to eIF2\( \alpha(p) \) and activate \( GCN4 \) pathway during amino acid stress imposed by the 3-AT addition. The reason for this is unknown but it might be due to Ala at 435 position impairing interaction between eIF2B and eIF2\( \alpha(p) \) or interaction between regulatory and catalytic sub-complex not leading to activation of catalytic activity of eIF2B.

The optimization of antibodies using yeast strain expressing \( hc \) plasmids suggests that S525 primes the phosphorylation of an adjacent Ser located at +3 position S528 (Figure 3.13). \( eIF2B_{\varepsilon-S525A} \) mutation imparts no growth phenotype as assessed by growth assays on plates and in liquid medium in optimal growth conditions and stress conditions except with Torin1 which inhibits the growth of S525A mutant on plates and liquid cultures (Figure 4.9, 4.10 and 4.13). Torin1 significantly down regulates the phosphorylation of S525 residue and causes a significant increase in cell size of S525A mutants and growth inhibition.
The phosphorylation of S528 depends on the earlier phosphorylation of S525 (Figure 3.13). Although S528A mutation does not affect the cell morphology and growth in optimal growth conditions as assessed by growth assays on plates and in liquid cultures. The S528A mutation confers sensitive growth phenotypes in response to various stress conditions, i.e. Torin1, 1-butanol and amino acid (Figure 4.9, 4.10, 4.11, 4.13, 6.1 and Supplementary Figure 2). All phospho-Ser to Ala mutant strains (in gcn2 background) were able to resume growth on 3-AT medium except S528A mutants. The reason for this is not clear but it is not because of the viability of cells in 3-AT medium. Western blotting shows that selected stress conditions do alter the phosphorylation pattern at S528 site. However, the pattern of alteration in phosphorylation does not follow the same trend. In case of butanol stress, up regulation of phosphorylation at S528 was accompanied with sensitive phenotype while in case of Torin1 treatment down regulation of phosphorylation at S528 led to sensitive phenotype. This apparent discrepancy between phosphorylation pattern and sensitive growth phenotype shows that phosphorylation at S528 is targeted by more than one mechanism, which operate under various stress conditions.

eIF2Be-S538A mutations affect the morphology of cells as cell were of larger size as compared to cells expressing wild type eIF2Be. Although the effect on cell size is similar to the S435A mutation but the response of S538A cells to various stress conditions was not similar to S435A strain. Non-significant minor changes in phosphorylation pattern of S538 were observed which did not cause any growth defect. Based on the data following graphical model can be made to explain the relation between alteration of phosphorylation pattern at ε-S435, S525 and 528 sites and yeast growth (Figure 7.1).

7.1 Potential Kinases/ phosphatases for Gcd6p sites

Recent advances in the field of phospho-proteomics and MS have identified functional phospho-protein complexes in yeast. Bioinformatic analyses of these phospho-protein complexes have generated interaction maps which show the physical interactions of proteins. Various interactive maps have been generated for S. cerevisiae proteome with Gcd6p physically interacting with other
Figure 7.1. Effect of stress on yeast growth and eIF2B phosphorylation.

Amino acid starvation induced by the addition of 3-AT leads to the activation of Gcn4p pathway and cells can recover from stress and resume growth. Cells carrying e-S435A mutation fail to activate Gcn4p. TOR signalling pathway can be blocked by rapamycin or Torin1 which alters the phosphorylation pattern of eIF2B at multiple sites. Kinases/ phosphatases responsible for these changes are not known in yeast. Butanol inhibits eIF2B activity by an unknown mechanism but it causes dephosphorylation of eIF2α(p).
regulatory proteins, some of which are kinases/ phosphatases (Gavin et al. 2002, Ho et al. 2002, Breitkreutz et al. 2010). Based on the recognition sequence motifs for different kinases, following kinases can be considered as potential candidates for phosphorylation of selected residues in Gcd6p (Table 7.2).

Cyclin dependent kinase (Cdk1) is encoded by CDC28 in yeast and regulates the cell cycle progression. A study was conducted to identify sites that are down regulated by the inhibition of Cdks by using quantitative MS. Gcd6p was found to be a substrate of Cdc28. The consensus recognition sequence motif of Cdk1 indeed fits with the S435 position (Holt et al. 2009). Cdks bind with their cyclin partners to function in cell cycle progression. This binding interaction of Cdks with its cyclin partners is targeted by different cell cycle inhibitors, i.e. role of TOR signalling in cell cycle progression. The phosphorylation at S435 is altered in response to rapamycin and Torin1 treatment. This may be a consequence of the inhibition of Cdc28 activity by TORC1 inhibition. If Cdc28 is responsible for the phosphorylation of ε-S435, this suggests the role of Gcd6p in cell cycle progression.

ε-S525 lies in the sequence motif recognized by Ypk1, substrate for TORC2 in yeast (Kamada et al. 2005, Loewith and Hall 2011). Less is known about the regulation of TORC2 in yeast as it is insensitive to rapamycin, initially used to understand the mechanism of TOR signalling pathway. Although it could not be verified that TORC2 is responsible for S525 phosphorylation, if Ypk1 is responsible for S525 phosphorylation then this will lead to the identification of new roles of Gcd6p and TORC2. This can lead to the identification of the interaction of the cytoskeleton with translation machinery or either the role of TORC2 in translation initiation as well.

Products of isoforms of CK1 (YCK1 and YCK2) are peripheral plasma membrane proteins and essential for viability in yeast. During bud morphogenesis Yck2p concentrates at the site of polarized growth. The sequence of ε-S528 fits to the consensus sequence for Yck1p and Yck2p (Robinson et al. 1999). Phosphorylation of Gcd6p by CK1 in mammals is reported to increase the GEF activity of eIF2B (Wang et al. 2001). If CK1 is responsible for S528
phosphorylation, then this also links cell cycle progression with Gcd6p as speculated by S435 and S525 phosphorylation.

CK2 is highly conserved in eukaryotes. Whole enzyme is a tetramer composed of two catalytic active proteins Ck2α (encoded by isoforms of $CK2\alpha$ gene, $CKA1$ and $CKA2$) and two regulatory proteins Ck2β (encoded by two isoforms of $CK2\beta$ gene, $CKB1$ and $CKB2$). In mammals it is shown that phosphorylation of Gcd6p by CK2 stimulates the GEF activity. CK2 is essential for growth and play a role in cell cycle progression, cell polarity, cell survival and transcription (Glover 1998, Ackermann et al. 2001, Ahmed et al. 2002). According to kinase prediction based on the sequence motif, S538 can be the substrate for CK2. Although a change in phosphorylation at this site under various stress conditions seemed not to play a significant role but the functional significance of phosphorylation at this site is not yet clear.

Rapamycin treatment activates the Gcn2 by dephosphorylation at S577 via TAP42, a regulator of type 2A-related protein phosphatases (Cherkasova and Hinnebusch 2003). Sit4p is activated in response to butanol treatment which causes eIF2α(p) dephosphorylation (Taylor et al. 2010). As eIF2B is concentrated in cytoplasmic foci in the cytoplasm and eIF2 shuttles from this, it might be possible that phosphatases that affects the dynamics of 2B body and eIF2α(p) also affects eIF2B phosphorylation.

Table 7.2. Phosphorylation sites, recognition motifs and potential kinases/phosphatases

<table>
<thead>
<tr>
<th>Phosphorylation site</th>
<th>Recognition motif</th>
<th>Potential kinase/phosphatase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gcd6p-S435</td>
<td>S/T-P-X-K/R</td>
<td>Cdc28, Pho85</td>
</tr>
<tr>
<td>Gcd6p-S525</td>
<td>K-R-X-X-S</td>
<td>Ypk1, Ypk2, Sch9</td>
</tr>
<tr>
<td>Gcd6p-S528</td>
<td>pS-X-X-S</td>
<td>*CK1 (Yck1, Yck2, Yck3)</td>
</tr>
<tr>
<td>Gcd6p-S538</td>
<td>S/T-X-X-E</td>
<td>*CK2 (Cka1, Cka2)</td>
</tr>
</tbody>
</table>

*Isoforms of CK1 and CK2 in yeast are written in parentheses
Phosphatases are missing from the table as they are less specific and no potential site was identified in Gcd6p. But in response to butanol and rapamycin stress, Sit4p and Tap42-PPase are activated. They might directly or indirectly affect the eIF2B phosphorylation.
7.2 Conclusions

A lot of research has been carried out to elucidate the mechanism of protein synthesis. Despite this focus the knowledge of mechanisms employed by eukaryotes to regulate translation is still limited. Regulation of translation by phosphorylation in response to various environmental signals is relatively more studied in mammals than yeast. This is the first study conducted in *S. cerevisiae* to find the role of Gcd6p phosphorylation in the regulation of catalytic activity of eIF2B. The functional significance of the phospho-sites investigated in the course of this study became apparent in the regulation of translation initiation and yeast growth in response to rapamycin, Torin1 and amino acid starvation. Although due to time constraints the role of these sites in translation initiation could not be fully established but this study provides evidence for the regulation of translation by phosphorylation.

The growth phenotypes and changes in phosphorylation pattern observed with inhibition of TOR signalling pathway by rapamycin and Torin1 provides new targets of TOR regulated kinases. This not only provides information that Torin1 affects the growth of Gcd6p mutants (S435A, 525A and S528A) better than rapamycin but also provide insights into the new targets of TOR regulated kinases. The functional significance of these changes and affects on eIF2B GEF activity needs to be further validated.

The appearance of Gcn⁻ phenotype as a result of a Ser to Ala mutation (S435A) in Gcd subunit can provide new insight into the physical inter-subunit interactions of eIF2B and eIF2B-eIF2α(p) complex. The I432L silent mutation (identified during this study) overcomes the Gcn⁻ phenotype effect could not be explained due to limited information of the structure of Gcd6p. This finding suggests that it is important for the interaction of S435 site with the potential kinase responsible for phosphorylation or this site may help in the interaction between catalytic sub-complex and regulatory sub-complex, thus overcoming the interaction distorted by dephosphorylation of S435.

Effects of alcohols on yeast morphology and translation initiation are known for more than a decade but the mechanism of inhibition is still not
established. This study shows the role of Gcd6p phosphorylation in regulating growth, as increase in phosphorylation at S435 increased resistance to 1-butanol treatment, and dephosphorylation at S528 results in increased sensitivity to 1-butanol. This may suggest that butanol affects the translational control by regulating the phosphorylation of Gcd6p.

In conclusion this study shows the functional significance of eIF2Bε phospho-sites in yeast in various stress conditions. A new potential mechanism of regulation of eIF2B activity in yeast is identified that directly affects eIF2Bε phosphorylation rather than indirectly regulating eIF2B activity via eIF2α(p).

### 7.3 Future perspectives

The current study has identified changes in phosphorylation pattern in response to diverse environmental cues. These changes can serve as a marker for the regulation of eIF2B activity. The functional significance of these phosphorylation changes in regulating translation was only examined by polysome analysis. Further theoretical and experimental work needs to be done to establish the functional significance of this inhibitory affects on translation control. Following limitations of this study are identified. Firstly, as the exact structure of eIF2Bε is not known, effects of these phospho-sites on intra and inter-subunit interactions and eIF2B-eIF2 complex formation could not be established. Secondly there is a possibility that phosphorylation pattern at these Ser residues might not remain uniform and show variations during cell cycle. The phosphorylation at these sites needs to be monitored over different phases of cell cycle by doing time course experiment for the control sample. Thirdly, the effects of these changes on catalytic activity of eIF2B needs to be confirmed and guanine nucleotide exchange assays. Fourthly, the effect on protein synthesis rate needs to be measured through incorporation of \( ^{35} \)S Methionine into protein. This needs to be done to confirm the effect of butanol on protein synthesis rate. Fifthly, the exact target of Torin1 needs to be identified. To establish that the phenotypes observed with Torin1 treatment are either because of complete inhibition of TORC1 or TORC2 needs to be established. S525, S528 and S538 are located in the catalytic domain and are surrounded by multiple Ser residues in this region. It may be
worth making a S525A, S528A double and an S525E, S528E double or multiple phospho-site mutants to find the importance of this phospho-belt in the structure and regulation of the catalytic domain.

In vitro and in vivo kinase assays using potential candidate kinase and synthetic peptides can help in establishing the potential kinase for the phosphorylation of the eIF2Bε phospho-sites. This can identify new roles of Gcd6p in yeast growth. Future studies will provide additional structural information for the inter-subunits interactions required for the catalytic activity of eIF2B, new roles and upstream signalling for the regulation of eIF2B in yeast growth. This might contribute to in-depth understanding of the mechanism of regulation of translation initiation by eIF2B and its interaction with various kinases in response to diverse environmental challenges.
Appendix A

- Supplementary material
Appendix A – Supplementary material

Figure A.0.1 (supplementary). Sensitivity is not proportional to the concentration of rapamycin at higher concentration.

A. Cells corresponding to \( A_{600} \sim 1.5 \) was mixed with 0.6% melted YPD/Agar maintained at 40°C. 100 µM/ml of rapamycin stock solution were diluted to 20, 40 and 50 nM/ul with DMSO (drug vehicle). 5ul of each dilution was added to each disk and assay was performed as described in section 2.5.2. (0 = DMSO, 1 = 100 nM, 2 = 200 nM, 3 = 250 nM rapamycin). Diameter was measured using a ruler and area was calculated. B. Linear graphic representation of the quantification of A.
Figure A.0.2 (supplementary). Effect of 3-AT on phospho-mutants in \textit{gcn2}\textDelta \textit{background}.

Cells were grown in SD+Ura medium and 10 fold serial dilution were prepared. 3\(\mu\)l of each dilution was spotted on plates with 10 mM and 25 mM 3-AT. As cells are \textit{gcn2}\textDelta, \textit{GCN4} pathway is not activated and cells cannot overcome the amino acid starvation inhibitory effects imposed by 3-AT addition. However leaving the plates for longer at room temperature, all Gcd6p strain (wild type and phospho-mutants) resume growth except S528A (Lane 4).
References
References


Regulation of eIF2B by Phosphorylation


(eIF4G) HEAT domain interacts with eIF1 and eIF5 and is involved in stringent AUG selection." Mol Cell Biol 23(15): 5431-5445.


References


factor 2 (eIF2alpha) to the regulatory subunits of guanine nucleotide exchange factor eIF2B is required for inhibition of translation initiation. Mol Cell Biol 21(15): 5018-5030.


References


2B (eIF2B) catalytic domain and both eIF2beta and -2gamma mediate guanine nucleotide exchange." Mol Cell Biol 27(14): 5225-5234.


"Discovery of 4-morpholino-6-aryl-1H-pyrazolo[3,4-d]pyrimidines as highly potent and selective ATP-competitive inhibitors of the mammalian target of rapamycin (mTOR): optimization of the 6-aryl substituent." J Med Chem 52(24): 8010-8024.


